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An Investigation into the Production of Polyphenol Antioxidants from Grape Marc

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***This thesis was completed by the Grace of God
and with the help and support of my family and friends.***

Abstract

The objective of this research was to determine the feasibility of extracting polyphenols from red grape marc for use in the nutraceutical and food industry. The health benefits of polyphenols are associated with the prevention of cardiovascular and neurodegenerative diseases, because of their free radical scavenging ability. Wines contain large concentrations of polyphenols, some of which are extracted from grapes during the vinification process. However, a significant proportion of the polyphenols remain in the pressed grape skins and seeds (grape marc). Approximately 250 000 tons of grape marc are produced per annum in South Africa as a by-product of the wine industry and this represents a vast potential source of natural polyphenols. The aims of this research were to establish an extraction process on a laboratory scale, identify key variables and optimum operating conditions. Detailed experiments were conducted to investigate the effect of solvent type, solvent concentration and extraction temperature. Polyphenols were extracted from grape marc and characterised using HPLC and UV spectrophotometry for quantification of total polyphenols, total flavanols and total anthocyanins. The antioxidant capacity of polyphenol extracts was determined using a 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay for *in vitro* analysis. The free radical scavenging ability of polyphenol extracts was measured to be 65 ± 5 mg phenols/g DPPH. The total polyphenol yield obtained from a two-stage extraction process was calculated to be 0.3% (w/w) using 50% ethanol at 50°C. The results from these investigations were used in a preliminary design for an industrial scale polyphenol process. This process was designed to produce 2 900 kg of total polyphenols per year from 940 tons of grape marc. The proposed extraction process included a cold storage facility and downstream process unit. The product sales, total capital costs and production costs were used to investigate the process feasibility. The results from the feasibility study, projected over 15 years, showed a discounted break-even period of 7 years and a return on investment of 31.7% before tax. Therefore, the proposed process was shown to be profitable at the preliminary design stage. Pilot scale investigations and further extract purification research are recommended to expand on the work conducted in this study, to maximise the benefits of the proposed process.

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ABBREVIATIONS

ABTS	2,2'-azinobis-(3-ethylbenzthiazoline)-6-sulfonic acid
AC	anthocyanins
AE	antiradical efficiency
AMPS	All Media Product Survey
CF	cash flow
CHD	coronary heart disease
DPPH	2,2-diphenyl-1-picrylhydrazyl
F-C	Folin-Ciocalteu
GAE	gallic acid equivalents
HPLC	high performance liquid chromatography
LDL	low density lipoprotein
NPV	net present value
PA	proanthocyanidins
PD	positive displacement pump
PLE	pressurised liquid extraction
RP	rotary pump
ROS	reactive oxygen species
SAE	stirring-assisted extraction
SFE	supercritical fluid extraction
TPC	total polyphenol concentration
UAE	ultrasound-assisted extraction
WHO	World Health Organisation

SYMBOLS

C_s	instantaneous polyphenol concentration in solution
C_g	polyphenol concentration in grape marc
C^*	maximum polyphenol concentration in solution
t	extraction time
T	extraction temperature
T_r	room temperature
k	mass transfer coefficient
θ	temperature coefficient
ϵ	absorption coefficient
Q	heat
U	heat transfer coefficient
A	condenser surface area
dT_m	log mean temperature
C_p	heat capacity
H	enthalpy
D_t	tank diameter
D_i	turbine diameter
rpm	revolutions per minute

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Chapter 1: Introduction

1.1 Project background

The majority of vineyards in South Africa are situated in the mountainous Western Cape Province. Grapes vines grown in this region benefit from a Mediterranean climate suited to the cultivation of grapes, which are harvested at the end of summer for the production of wine. In 2004, 1 016 million litres of wine were bottled in South Africa [www.sawis.co.za], resulting in an estimated 250 000 tons of grape marc (grape seeds and skins) produced as a waste product.

Processed grape marc from the wine industry is traditionally used in the production of high quality spirits using batch distillation processes. These spirits are produced in Europe and South Africa, although the demand for high quality spirits in Europe has decreased due to increased food quality standards [Silva *et al.*, 1996]. As an alternative to the production of high quality spirits, grape marc is used for agricultural purposes as a soil conditioner or compost and is also mixed with animal feed [Negro *et al.*, 2003].

Grape seeds and grape skins have recently become popular sources of natural antioxidants used in health supplements which help prevent and repair the damage to cells caused by free radicals. The abundant supply of grape marc, its high polyphenol content, and the lack of suitable disposal methods, suggest the need for further research to investigate the extraction of polyphenols from grape marc. This research is of potential value to i) wine manufacturers, who will benefit from reduced environmental impacts, ii) medical researchers because of the associated health benefits of polyphenols, and iii) the public, who may benefit from the use of natural antioxidant products.

1.2 Problem Statement

The waste products from the wine industry, the winery waste-water and grape marc, can cause pollution to the ecosystems surrounding wine farms. Polyphenol compounds, which occur in grape waste at high concentrations, have a negative impact on the environment because of their antimicrobial activities. In addition to this problem, drainage from the grape marc and organic residues leads to soil and water contamination due to high polyphenol concentrations and high acidity levels.

1.3 Research objectives

The research objectives were formulated to enable the design of a process that produces polyphenol extracts. This required determination of which process variables and extraction conditions would result in the maximum amount of polyphenols recovered from grape marc, using a stirring-assisted extraction method, and also which solvent would produce polyphenol extracts with the highest free radical scavenging ability. A process feasibility assessment was also conducted to determine the return on investment of the proposed process.

1.4 Hypotheses

The hypotheses proposed for this thesis are:

- Polyphenol compounds can be extracted from grape marc through a batch process using a stirring-assisted solvent extraction method. It is further hypothesised that the production process of antioxidants, designed on the results obtained in this study, would be profitable.
- Polyphenol extraction in well-mixed systems can be efficiently modelled using first order mass transfer kinetics, with the major assumption that the rate limiting step is the polyphenol mass transfer from the grape skins into the solvent.

1.5 Research outline

For the purpose of this project, grape marc was investigated as a possible source of polyphenols. Experiments were conducted in the laboratory to investigate the following process stages: i) grape marc storage, ii) particle size reduction, iii) polyphenol extraction, and iv) solvent evaporation. Each investigation involved the extraction of polyphenols from grape marc using magnetic or mechanical agitation methods. Parameters affecting the extraction stage were investigated in more detail than the other stages. These parameters (solvent type, solvent concentration, extraction temperature, extraction time, and extraction atmosphere) were evaluated by measuring the polyphenol concentration and free radical scavenging ability of the extracts. The proposed process was then designed, based on these laboratory results, for large scale production of natural polyphenol extracts to be used in the nutraceutical or food industry.

1.6 Structure of thesis

This thesis comprises of six chapters. Chapter 1 (Introduction) describes the project background and the research approach used in this study. Chapter 2 (Literature Review) outlines the important information about grape marc and polyphenols, uses of grape polyphenols, and a review of analytical procedures found in literature. Chapter 3 (Materials and Methods) describes extraction methods for obtaining polyphenol extracts and analysis procedures used in the research. Chapter 4 (Results and Discussion) presents the results of the investigations performed in the laboratory. The experimental findings are discussed and compared with results found in literature. Chapter 5 (Proposed extraction process) shows the process design and the economic evaluation of the proposed extraction process. Lastly, the conclusions of this study are presented in Chapter 6, outlining the important findings and objectives that were achieved.

Chapter 2: Literature Review

2.1 Introduction

This section summarises information from literature about the flavonoid characteristics, polyphenol health benefits, polyphenol content of grape marc, the measurement of polyphenol concentrations, the role of antioxidants, and the properties of free radicals. Also mentioned in this literature review are the differences between traditional and modern methods of extracting polyphenols, and the use of solid phase extraction for the downstream processing of polyphenol extracts. There are many reports published about grape polyphenols, covering studies on a range of different grape sources, including fresh grape skins, grape marc, grape pomace (grape marc with stalks), grape seeds or wines. However, only a few reports were specific to grape marc studies, such as Negro *et al.* [2003] and Bonilla *et al.* [1999].

2.2 Properties of flavonoids and polyphenols

Polyphenols are among the non-essential components of the diet, and are found in most fruits and vegetables. They are one of the major classes of plant secondary metabolites and consist of aromatic ring(s) with substituted hydroxyl groups. A more precise definition is that polyphenols are substances “derived from the shikimate pathway and phenylpropanoid metabolism” [Robards *et al.*, 1999, p. 401]. Polyphenol types found in plants include flavonoids and phenolic acids as well as lignans, coumarins, stilbenes, curcuminoids, and quinones [Cai *et al.*, 2005]. The main structural difference between flavonoids and phenolic acids is that flavonoids have a diphenyl structure, while phenolic acids only have one aromatic ring. Flavonoids and phenolic acids are found abundantly in grape marc, and consequently are discussed in more detail below.

2.2.1 Phenolic acid compounds

Phenolic acids are divided into three types, namely hydroxybenzoic acids, hydroxyphenylacetic acids, and hydroxycinnamic acids. Examples of hydroxybenzoic acids commonly found in grape are shown in Figure 2-1. The antioxidant activity of gallic acid, of 3.01 mM Trolox, was shown to be higher than the activity of caffeic and protocatechuic acid, of 1.26 mM and 1.19 mM Trolox respectively, [Rice-Evans *et al.*, 1996]. Trolox is an antioxidant standard used in the ABTS assay (see Section 2.5.4).

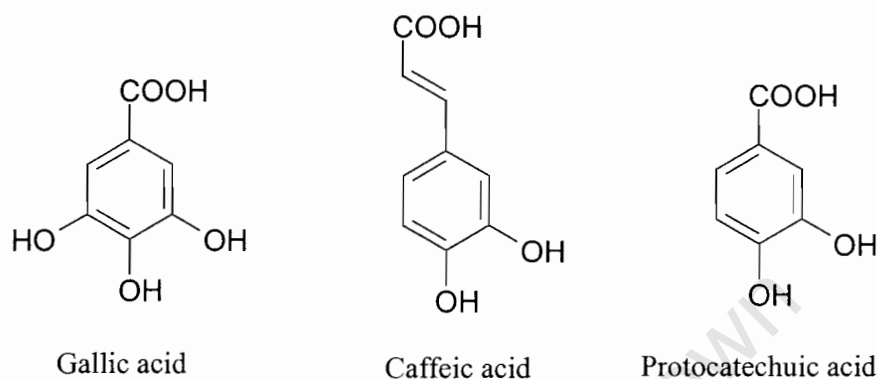
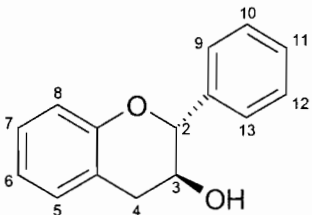
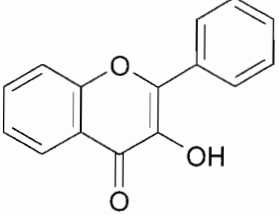
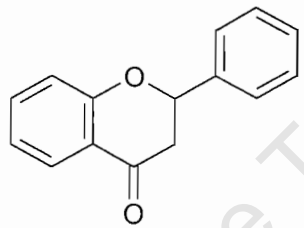
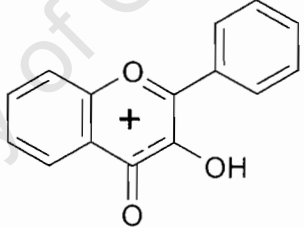
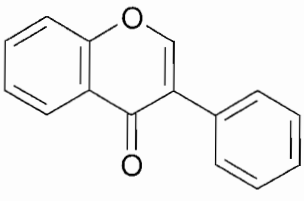


Figure 2-1: Structures of phenolic (hydroxybenzoic) acids found in grape marc.

2.2.2 Classes of flavonoids

Flavonoids have a diphenylpropane ($C_6-C_3-C_6$) structure and are sub-divided into classes which include the flavanols, flavanones, anthocyanidins, flavones and flavonols. The basic flavonoid structure is a combination of two fused aromatic rings, A and C, and an attached polyphenol ring B at the 2-position on the C ring. Table 2-1, adapted from Lien *et al.* [1999], shows the differences between the flavonoid classes with respect to the position and number of hydroxyl groups. The positive charge is unique to the anthocyanidin class where the C ring has two double bonds. Reports in literature show that increased numbers of hydroxyl groups are related to higher antioxidant activity of polyphenol compounds [Lien *et al.*, 1999; Rice-Evans *et al.*, 1996]. The results from free radical scavenging activity assays, expressed in Trolox equivalent antioxidant capacity (TEAC), showed that tannins have the highest average activity, of 7.58 mM, followed by flavanols (4.21 mM) and flavonols (1.83 mM) [Cai *et al.*, 2005].

Table 2-1: The classes of different flavonoids showing structures and hydroxyl positions [Lien *et al.*, 1999].

Flavonoid classes	Basic ring structure	OH position
Flavanol		
1. Epicatechin gallate		5, 7, 10, 11
2. Epigallocatechin gallate		5, 7, 10, 11, 12
3. Epigallocatechin		3, 5, 7, 10, 11, 12
4. Epicatechin		3, 5, 7, 10, 11
5. Catechin		3, 5, 7, 10, 11
Flavonol		
6. Quercetin		3, 5, 7, 10, 11
7. Myricetin		3, 5, 7, 10, 11, 12
8. Morin		3, 5, 7, 9, 11
9. Rutin		3, 5, 10, 11
10. Kaempferol		3, 5, 7, 11
Flavanone		
11. Naringenin		5, 7, 11
12. Hesperetin		5, 7, 10
13. Hesperidin		5, 10
14. Narirutin		5, 11
15. Dihydrokaempferol		3, 5, 7, 10, 11
16. Eriodictyol		3, 5, 7, 11
Anthocyanidin		
17. Delphinidin		3, 5, 7, 10, 11, 12
18. Cyanidin		3, 5, 7, 10, 11
19. Apigenidin		5, 7, 11
20. Peonidin		3, 5, 7, 11
21. Malvidin		3, 5, 7, 11
22. Pelargonidin		3, 5, 7, 11
23. Keracyanin		5, 7, 10, 11
24. Ideain		5, 7, 10, 11
25. Oenin		5, 7, 11
Isoflavone		
26. Genistein		5, 7, 11
27. Genistin		5, 11
28. Daidzein		7, 11
29. Daidzin		11
30. Biochanin		5, 7
31. Formonetin		7
32. Ononin		-

2.2.2.1 Proanthocyanidin structures

There are two chemically and biologically different groups of tannins found in fruits [Seeram *et al.*, 2004]. The first group of tannins are hydrolysable tannins, known as ellagitannins and gallotannins, which are found in strawberries, raspberries and pomegranates. The second group, the condensed tannins or proanthocyanidins, are present in tea, cranberries, and also in grapes, where significantly high concentrations have been recorded in red grape seeds and skins [Peyrot des Gachons & Kennedy, 2003]. Proanthocyanidins are polymeric structures that consist of two or more monomeric flavanol compounds. In grapes, proanthocyanidins have up to 30 monomeric units and the number of monomeric units in seed tannins varies from 2 to 16. Proanthocyanidin B2 is shown below as an example of a two unit polymer.

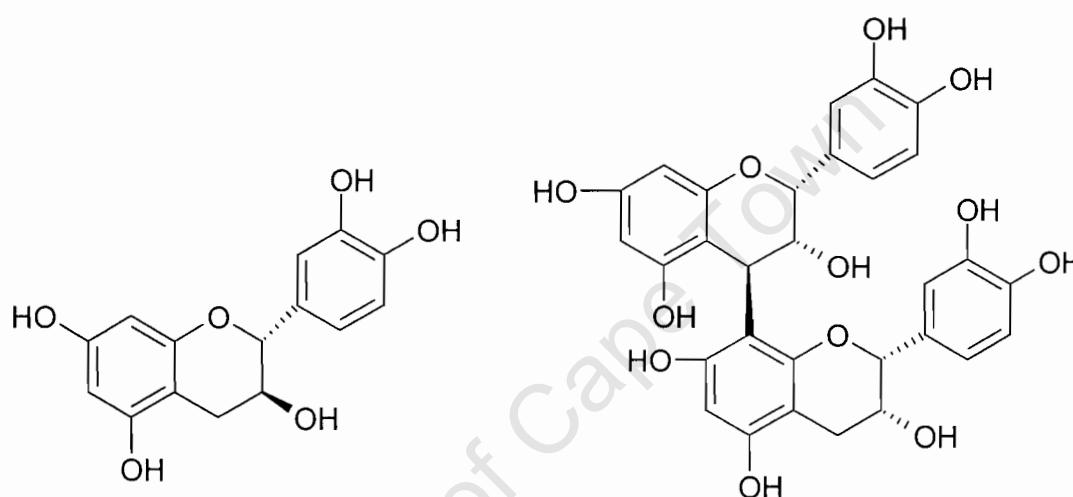


Figure 2-2: Catechin (left) and proanthocyanidin B2 (right) structures [Shoji *et al.*, 2006].

2.2.2.2 Properties of anthocyanins

Anthocyanins are the polymeric forms of anthocyanidins and their names are derived from the Greek words anthos (flower) and kyanos (blue). Anthocyanins are electron-deficient compounds and are highly reactive with reactive oxygen species [Galvano *et al.*, 2004]. Anthocyanins are known to possess phytoalexin (growth inhibition of parasites) and antibacterial properties which assist in protecting plants; for example: cyanidin 3-glucoside (Figure 2-3) protects cotton leaves against tobacco budworm.

According to Kong *et al.* [2003], more than 400 anthocyanins have been identified in nature, and the most widespread of these anthocyanins is cyanidin 3-glycoside. The differences between types of anthocyanins depend on the nature, number and position of hydroxyl groups, sugar residues, and aliphatic or aromatic acid units attached to the anthocyanin molecule. The six most common types of anthocyanidins are cyanidin, malvidin, petunidin, delphinidin, peonidin and pelargonidin.

Anthocyanins are polar compounds commonly extracted from plant material using polar organic solvents such as methanol and acetone. Because of their chromatic properties, they are responsible for the dark red, purple and blue colours in fruits, vegetables, plants and flowers. Their colour is pH dependent. At low pH anthocyanins have a red/pink colour due to the flavylum cation form, and at high pH they become colourless. The use of anthocyanins in the food and beverage industry as natural colorant is becoming increasingly important [Spagna *et al.*, 2003; Shrikhande, 2000; Bonilla *et al.*, 1999].

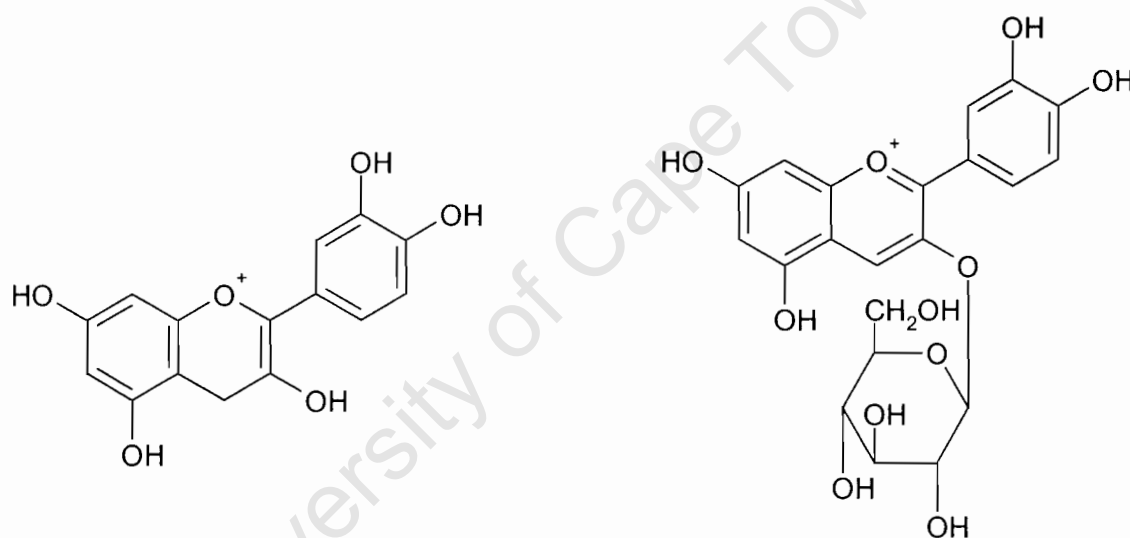


Figure 2-3: Anthocyanidin (left) and cyanidin-3-glucoside, the corresponding anthocyanin (right).

2.2.3 Health benefits of grape polyphenol compounds

Applications of antioxidants extend throughout the food, cosmetic and nutraceutical industries, where they are used as natural food preservatives and colourants, in anti-aging skincare products, in sun protection products and in health supplements. The use of natural compounds, such as polyphenols from fruits and vegetables is gaining considerable public favour, in preference to the synthetic compounds that are commonly found in drinks and foods. The perceived advantages of natural antioxidants have attracted worldwide interest, especially in the medical and health research fields. This has been the driving force in recent studies investigating *in vitro* and *in vivo* biological activities of polyphenol compounds. The antioxidant behaviour of polyphenols is related to their free radical scavenging ability, inhibition of lipoxygenases and metal chelating properties [Rice-Evans *et al.*, 1996]. This is discussed further in Section 2.5.

Lipid auto-oxidation occurs frequently *in vivo*, resulting in cardiovascular disease. Low density lipoprotein (LDL) oxidation causes higher levels of cholesterol accumulation in the arteries leading to coronary heart disease (CHD) and atherosclerosis [Fragopoulou *et al.*, 2003]. Studies by the World Health Organisation (WHO) have shown that the French population has proportionally fewer cases of CHD than the American population, although the French have high saturated fat consumption and comparable plasma cholesterol levels to American populations [Shrikhande, 2000]. This apparent discrepancy, known as the French Paradox, has consequently led to a surge of research into the health benefits of wine and wine by-products. Related studies show that drinking moderate amounts of wine, as the French do, results in lower risk of cardiovascular diseases. There is also growing evidence that grape polyphenols decrease the peroxidation of LDL [Frankel *et al.*, 1995].

Medical research has shown that there is a strong indication that antioxidants from wine may also help in the prevention of neurodegenerative diseases, such as Parkinson's and Alzheimer's disease [Esposito *et al.*, 2002]. In these aged-related diseases, oxidative damage and mitochondrial dysfunction have been found to be promoters for the

progression of these disorders. Since the brain requires large amounts of oxygen and has high lipid concentrations, it is susceptible to oxidative damage. Oxidative damage is controlled by factors that include substrate (fatty acid) composition, pH, oxygen concentration and concentrations of prooxidants, such as reactive oxygen species, transition metals and enzymes [Decker, 1997]. Free radicals, such as hydroxyl ($\text{OH}^\bullet$) and superoxide radicals ($\text{O}_2^{\bullet-}$), are known as reactive oxygen species (ROS). Oxidative stress occurs when the antioxidant defence systems of cells are not effective against high concentrations of ROS. Polyphenol products from grape and wine are adsorbed by the body and are efficient scavengers of ROS [Tedesco *et al.*, 2000]. Various biological roles of polyphenols, summarised in Table 2-2, suggest that the polyphenols in red wine and wine by-products may reduce the incidence of atherosclerosis. Thus, research into the extraction of polyphenols from grape marc is contributing to developing natural antioxidant products that could be used for the prevention of frequently occurring illnesses such as neurodegenerative and cardiovascular diseases.

Table 2-2: Summary of reported biological effects of polyphenol components in wine and wine by-products [Shrikhande, 2000].

Effect	Product
LDL oxidation inhibition (<i>in vitro</i>)	Red wine
Lipid adsorption (<i>in vivo</i>)	Seed extract
Inhibition of platelet activity (<i>in vivo</i>)	Grape juice/wine
Antioxidation capacity of plasma (<i>in vivo</i>)	Red wine phenolics
Absorption of flavonoids in plasma	Red wine
LDL oxidation inhibition (<i>in vivo</i>)	Grape seed proanthocyanidins

2.3 Polyphenol content of grape marc

The polyphenol content remaining in grape marc after pressing depends on the length of time the grape marc is in contact with the grape juice. Red wine is fermented on skins for three to ten days, and the grape marc is separated later from the wine during the pressing stage. White wine has a contact time with the skins of less than 12 hours, which results in lower polyphenol concentrations and lighter colour than red wine. It has been shown that white wines have lower polyphenol concentration than red wines. According to Manzocco *et al.* [1999] the chain-breaking and oxygen scavenging properties of red and white wine were reported to be similar, but conflicting results were reported by Shrikhande [2000], where red wine showed 37 to 65% LDL inhibition and white wine only 3 to 7% inhibition.

Table 2-3: Polyphenol compositions of grape marc, seed and skin extracts obtained from red grapes using 80% ethanol [Negro *et al.*, 2003].

Grape fraction	Total polyphenols (GA) g/100 g DM	Total anthocyanins (ME) g/100 g DM	Condensed tannins (CT) g/100 g DM
Marc	4.19	0.98	2.23
Seed	8.58	0	6.41
skin	3.33	1.92	0.86

Assay standards: GA = gallic acid, CT = catechin, ME = malvin.
DM = dry marc.

2.3.1 Grape seed composition

Grape seeds from *Vitis vinifera* are made up of 40% fibre, 16% oil, 11% protein and 7% polyphenols, in addition to sugars and mineral salts. Grape seed oil contains mono- and polyunsaturated fatty acids, 70% of which consists of linolenic acid [Kim *et al.*, 2006]. According to Guendez *et al.* [2005] 3.8 g of polyphenols are extractable per kg of crushed fresh grape seeds using ethyl acetate and three extractions. Negro *et al.* [2003]

reported that the polyphenol content of grape seeds showed high concentrations of condensed tannins and no anthocyanins. The polyphenol fraction consists mainly of catechins, proanthocyanidins and small amounts of gallic acid, as shown in Table 2-4. In addition to these compounds, procyanidins B3 and B4 have also been identified in fresh grape seeds by Gonzalez-Manzano *et al.* [2004].

Table 2-4: Weight % of various polyphenols extracted from fresh grape seed using ethyl acetate [Guendez *et al.*, 2005].

Polyphenol	GA	CT	ECT	ECG	EGCG	EGC	B1	B2
% (wt)	1.3	49.8	26.0	9.3	1.9	0.8	5.8	5.1

GA = gallic acid, CT = Catechin, ECT = epicatechin, ECG = epicatechin gallate, EGCG = epigallocatechin gallate, EGC = epigallocatechin, B1 = procyanidins B1 and B2 = procyanidins B2.

2.3.2 Grape skin composition

The cell wall material of dried grape skins contains approximately 30-35% of neutral polysaccharides, 20% acidic pectins and more than 15% insoluble proanthocyanidins [Lecas & Brillouet, 2001]. Identified polyphenol classes in grape skins include flavanols, anthocyanins, proanthocyanidins and phenolic acids. Anthocyanins are found in the intra-lamellar region of grape skins [Spagna *et al.*, 2003]. Several anthocyanidin-glucoside complexes (e.g. delphinidin-, cyanidin-, petunidin-, peonidin- and malvidin-glucosides) have been identified in grape skins [Revilla *et al.*, 1998; Heredia *et al.*, 1998] as well as oligomeric anthocyanins [Vidal *et al.*, 2004]. Bonilla *et al.* [1999] identified a wide range of polyphenol compounds extracted from crushed grape marc using ethyl acetate and water. These compounds included vanillic acid, protocatechuic acid, catechin, epicatechin, procyanidins (B1, B2, B3, B4), flavonols (quercetin, kampherol) and anthocyanin compounds.

2.4 UV spectrophotometric methods for polyphenol extract analysis

The chemical properties of polyphenols determine their maximum absorption wavelengths, which are used to detect and quantify polyphenols by spectrophotometric analysis. Merken and Beecher [2000] summarised information about the different absorption wavelength bands for various flavonoid classes (Table 2-5).

Table 2-5: Wavelengths of maximum absorption (λ) for different flavonoid classes [Merken & Beecher, 2000].

Flavonoid class	Example	Absorption λ_{max} (nm)
Anthocyanidin	Delphinidin	210, 525
Flavanol	Epicatechin	200
Flavanone	Hesperetin	200, 285
Flavone	Luteolin	210, 350
Flavonol	Quercetin	200, 370
Isoflavone	Genistein	200, 260

Robards [2003] reported that polyphenol extracts can be characterised on the basis of comparison with one standard polyphenol compound. For example, catechin can be used as a model compound to quantify the flavanol concentrations and cyanidin can be used to quantify the anthocyanin concentrations. Common spectrophotometric methods and model compounds, used as standards, are shown in Table 2-6.

Table 2-6: Model compounds used to quantify different flavonoids.

Model compound	Assay	λ	Reference
Gallic acid	Total polyphenol	765 nm	Singleton & Rossi [1965]
Quercetin	Total flavonoid	376 nm	Luximon-Ramma <i>et al.</i> [2005]
Catechin	Flavan-3-ol	500 nm	Gonzalez-Rodriguez <i>et al.</i> [2002]
Cyanidin-3-o-glucoside	Anthocyanin	525 nm 700 nm	Wrolstad & Giusti [2000]

2.4.1 The Folin Ciocalteau method for determination of total polyphenol concentrations

The Folin Ciocalteau (F-C) method was developed by Singleton and Rossi [1965] and is an accurate method for the determination of total polyphenol concentrations (TPC) of organic samples. In this assay, polyphenol compounds oxidise yellow polyphosphomolybdate-tungstate anions to form a dark green colour. This method is also used to determine the total polyphenol concentration in commercial antioxidant products [Shrikhande, 2000].

2.4.2 Determination of flavanol concentration

The quantification method for flavanol compounds was derived from the analysis of red and white wines using catechin as a model compound [Gonzalez-Rodriguez *et al.*, 2002]. This assay is also known to detect proanthocyanins and dihydrochalcones as well as monomer flavanol compounds [Katalinic *et al.*, 2004]. The method is based on a chemical reaction of vanillin with catechin to form a quinoid product whose colour ranges from pale to bright pink-red, depending on the concentration of HCl used.

2.4.3 Determination of total anthocyanin concentration

This method of total anthocyanin quantification by Ciusti and Wrolstad [2000] is a pH difference method. Samples are diluted using acidified potassium chloride (pH 1.0) and sodium acetate buffer (pH 4.5). The difference between the absorbances at these pH values is then used to calculate the anthocyanin concentration. Higher absorbance readings are expected in the acidic sample, since anthocyanins change to a red colour in acidic solutions and remain colourless in neutral pH solutions. Equations for calculating the anthocyanin sample absorbance and concentration are shown in Section 3.3.3.

2.5 Reaction mechanisms of free radicals

2.5.1 Production of free radicals in the body

Free radicals are produced by biological processes occurring in the body. They play important roles, such as assisting the immune system in destroying bacteria and viruses. However, in high concentrations they are harmful and cause tissue and cell damage. When extremely high concentrations of free radicals occur in the body, they can alter the genetic material of cells. This initiates mutations that may lead to various types of cancer. Free radicals are also believed to be the cause of age-related and neurodegenerative diseases [Rock *et al.*, 1996]. External factors that also result in the production of free radicals occur when people are exposed to poor quality air (pollution, smoke) and high radiation levels [Tedesco *et al.*, 2000].

2.5.2 The reaction mechanism of lipid auto-oxidation

In mammalian cells, (non-enzymatic) free radical oxidation processes lead to destructive chain reactions, via a number of lipid oxidation pathways. The auto-oxidation process consists of four stages, namely the initiation, propagation, branching

and termination stage (Table 2-7). In the initiation stage, lipid alkyl radicals ($L^\bullet$) are produced when lipid molecules react with free radicals ($R^\bullet$). In the propagation step, the lipid alkyl radical reacts with oxygen to form a peroxy radical ($LOO^\bullet$), which is further oxidised by a lipid molecule to form a lipid hydroperoxide ($LOOH$) and alkyl radical. In the branching step, the lipid hydroperoxide breaks down into more lipid radicals, which react with each other to produce non-radical compounds in the termination step. The termination reaction brings an end to the chain reaction via radical-radical reactions. This forms undesirable lipid compounds that are believed to cause the diseases that were discussed previously (see Section 2.2.3).

Table 2-7: The free radical mediated lipid auto-oxidation mechanism [Robards, 2002].

Initiation	$R^\bullet + LH \longrightarrow L^\bullet + RH$
Propagation	$L^\bullet + O_2 \longrightarrow LOO^\bullet$
	$LOO^\bullet + LH \longrightarrow LOOH + L^\bullet$
Branching	$LOOH \longrightarrow LO^\bullet + HO^\bullet$
	$2LOOH \longrightarrow LOO^\bullet + LO^\bullet + H_2O^\bullet$
Termination	$LOO^\bullet + LOO^\bullet \longrightarrow LOOL + O_2$
	$LO^\bullet + LO^\bullet \longrightarrow LOOL$

2.5.3 Polyphenol quenching of free radicals

There are two requirements for polyphenols to be defined as antioxidants [Rice-Evans *et al.*, 1995]. Firstly, polyphenols must prevent auto-oxidation or free radical-mediated oxidation when in low concentrations relative to free radicals. Secondly, radicals that are formed after free radical scavenging processes must be stable. Another similar definition for antioxidants, reported by Antolovich *et al.* [2002], stated that antioxidants were substances that inhibited or prevented the oxidation of a substrate, when the antioxidants were present at low concentrations relative to the substrate.

There are two types of antioxidants, chain breaking (primary) antioxidants and preventative (secondary) antioxidants. Secondary antioxidants remove the substrate or singlet oxygen, and therefore inhibit the rate of oxidation. Primary antioxidants interrupt the chain reaction mechanism (described in Table 2-7) by reacting with lipid radicals and peroxy radicals formed in the initiation and propagation steps. The food industry uses synthetic primary antioxidants, such as BHT and BHA, to preserve lipid foods from auto-oxidation.

The potential for polyphenol antioxidants to scavenge free radicals depends on the chemical properties and structure of each particular polyphenol; the structures of individual compounds affect their ability to donate hydrogen or electrons and thus play an important role in determining the activity of polyphenols. During free radical scavenging reactions, hydrogen radicals from polyphenols are donated to free radicals, or the polyphenols accept unpaired electrons which are stabilised by delocalisation. The greater the degree of delocalisation possible, the more stable the polyphenol-free radical, and hence the greater the ability of the polyphenol to act as an antioxidant.

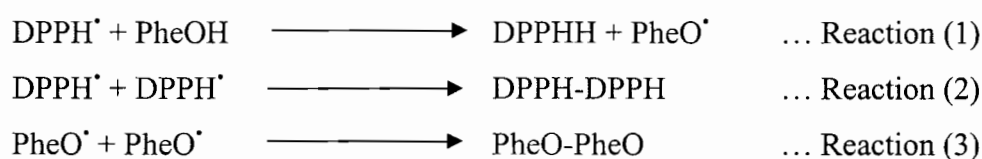
2.5.4 DPPH and ABTS free radical scavenging assays

The DPPH and ABTS assays are two commonly reported free radical scavenging assay methods used for the determination of the antioxidant activity of polyphenol compounds. These *in vitro* assays use 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azinobis-(3-ethylbenzthiazoline)-6-sulfonic acid (ABTS) as model free radical compounds. Both assays involve decolourisation, where the polyphenol sample is mixed with the free radical solution, resulting in a decrease in colour of the solution. The extent of decolourisation indicates the degree of free radical quenching.

The ABTS assay involves an enzymatic system with peroxidase enzyme, hydrogen peroxide and ABTS. These components are mixed together to form ABTS^{•+}. After the

resulting radical is formed, the reaction of ABTS^{++} (2 mL) with the sample (100 μL) is observed at absorption of 414 nm for 15 min at 37°C.

Reaction (1) is the fundamental reaction that occurs during the DPPH assay when free radicals are scavenged by polyphenol compounds [Sanchez-Moreno *et al.*, 1998]. Reaction (1) is terminated when DPPH radicals reacts with each other or when polyphenol radicals neutralise each other, as shown in reactions (2) and (3).



The absorbance readings are monitored at 515 nm until the quenching reaction ceases, showing a hyperbolic decrease of DPPH concentration with time. Information from the assay can be used to determine the steady state times and % DPPH remaining values, from which the EC_{50} concentration and antiradical efficiency (AE) are calculated (see Sections 3.3.4 and 4.2.1 for definitions and equations).

Antioxidant activity measurements of wines comparing the ABTS and DPPH assays lead to similar conclusions, and showed that the flavanols and anthocyanins accounted for the majority of the antioxidant activity, followed by phenolic acids and flavonol compounds [Fernandez-Pachon *et al.*, 2004].

2.6 Polyphenol extraction methods and parameters

There are a number of different extraction techniques available for the extraction of antioxidants from grape marc, most of which are adaptations of traditional stirring-assisted extraction (SAE) methods. Some of the commonly used modern techniques are ultrasound-assisted extraction (UAE), pressurised liquid extraction (PLE) and supercritical fluid extraction (SFE). These extraction techniques are not specific to

antioxidants and can also be used for the extraction of other organic compounds. Pre-treatment methods for batch extractions involve the size reduction and drying of grape marc, and common extraction variables include solvent choice, solvent concentration, extraction time and solvent temperature.

2.6.1 Pressurised liquid extraction (PLE)

The samples are subjected to variable temperatures up to 200°C and pressures of 100 atm. Once the set temperature is reached, the sample is held for a given time for the extraction process to take place. A pressure of 100 atm was used to keep the solvent in the liquid state. The advantages of using PLE are due to the elevated temperatures and pressures, which allow quicker diffusion of solvent into the grape marc matrix, lower solvent viscosity, and higher polyphenol solubility. The disadvantage of PLE is that it is an energy intensive extraction method and scaled-up designs would therefore be expensive.

2.6.2 Supercritical fluid extraction (SFE)

Supercritical fluids have properties of gases and liquids. The most important property of solvents used for antioxidant extractions is the transport capability. This characteristic enables a supercritical fluid to act as a more effective extractor in terms of extraction rates and phase separation than other extraction solvents. A supercritical fluid has only one phase and cannot be liquefied if it is above its critical temperature. However, it is possible to change the supercritical fluid from a liquid to a gas without inducing a phase change by going through the supercritical fluid region, as indicated with arrows in Figure 2-4.

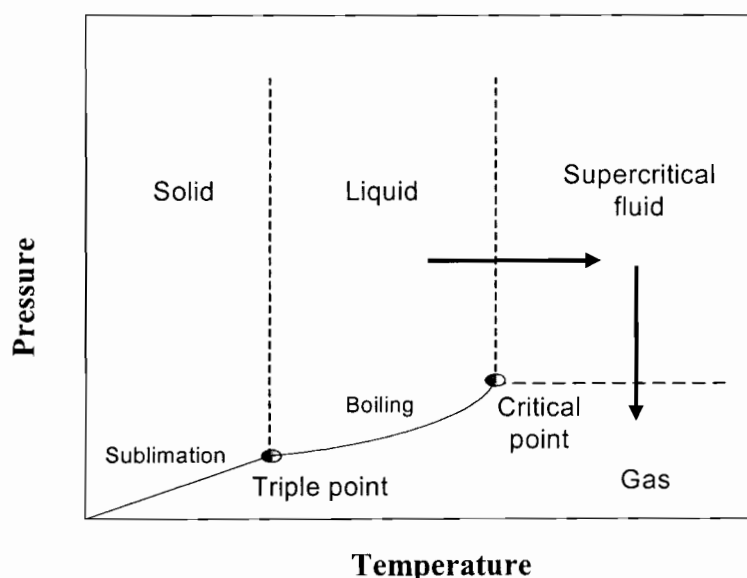


Figure 2-4: Pressure-temperature diagram illustrating the different phases for supercritical fluids [Starmans & Nijhuis, 1996].

To further increase the solubility of solutes in supercritical fluids, co-solvents can be added. Co-solvents (methanol, ethanol) have solute-fluid properties and small amounts can change the critical properties of the supercritical fluid. They are used to enhance the extraction selectivity of certain solutes such as antioxidants. The most influential variables for SFE are supercritical fluid density, co-solvent type and temperature.

2.6.3 Ultrasound-assisted extraction (UAE)

UAE is similar to stirring-assisted extraction but differs in the use of ultrasonic vibrations to agitate the extraction system. In this extraction technique, conventional line voltage is converted into high frequency electrical energy through the ultrasound instrument. Transducers then change the electrical energy into mechanical vibrations, which cause the probe tips to vibrate. This action results in the formation and disappearance of microscopic vapour bubbles and shockwaves that keep the solvent in an agitated state.

2.6.4 **Stirring-assisted extraction (SAE)**

In SAE systems, the grape marc and solvent slurries can be agitated using magnetic stirrers, impellers or orbital shakers (see Table 2-10). The extraction times commonly vary from 30 minutes to several hours. This extraction method is typically performed at atmospheric pressure and room temperature, and thus is an energy-conservative method compared to PLE and SFE extraction methods. Multiple extractions are also common for SAE to increase the recovery of antioxidants.

2.6.5 **Comparisons of methods for extraction of grape polyphenols from grape seeds**

The recovery of catechin using SAE, UAE and SFE methods from crushed grape seeds is shown in Table 2-8. Catechin represents the majority of the polyphenols extracted from grape seeds or grape marc, and is therefore a good indicator for polyphenol recovery. Aqueous methanol was used for the SAE and UAE. The SFE used the following extraction conditions: liquid CO₂ flow rate of 1 mL/min, 20 min extraction time, 0.25 mL of organic modifier and an extraction temperature of 50°C. The results from this study showed that the SFE method had a slightly higher catechin recovery than the SAE method.

Table 2-8: Recovery comparisons of catechin using SAE, UAE and SFE methods [Palma *et al.*, 1999].

Extraction method	Recovery mg CT/100 g seeds
SAE	65.6
UAE	63.0
SFE	77.6

CT = catechin.

Additional comparisons between the different extraction methods are shown in Table 2-9. Results from Pineiro *et al.* [2004], showed the recoveries of catechin and epicatechin obtained from grape seeds extracted using methanol. The extraction temperatures used for PLE, UAE and SAE were 100°C, 60°C and 60°C respectively. The PLE method shows a higher catechin recovery than either the UAE or SAE method.

Table 2-9: Recoveries of catechin and epicatechin using different extraction technologies [Pineiro *et al.*, 2004].

Extraction methods	Catechin mg/g	Epicatechin mg/g
PLE	1.90	0.72
UAE	0.93	0.46
SAE	0.85	0.44

2.6.6 Solvent extraction choice

The results from the literature survey were used to select the extraction method for this study. Based on the results shown in Tables 2-8 and 2-9, stirring-assisted extraction was the method selected. The reason for this is simply that the more advanced extraction methods (especially PLE and SFE) would need to produce significantly larger quantities of polyphenols than the SAE method for these methods to be considered viable, but this was not the case. PLE and SFE equipment is expensive and high operating conditions (temperature and pressure) will result in greater manufacturing costs than SAE equipment. Also, SAE is a simple extraction method that is easier to scale-up than PLE and SFE methods. Various stirring-assisted extraction methods reported in the literature are shown in Table 2-10.

Table 2-10: Conditions used to produce polyphenol extracts from grape marc, skins and seeds using solvent extraction.

Sample	Pre-treatment	Solvent	Extraction Method	Purification	Product	Reference
Red grape marc	Dry, crushed	80% Ethanol 0.5% 0.1N HCl	Mixed 1:30 w/v ratio, centrifuge at 2000 g			Negro <i>et al.</i> , 2003
Red grape skin		40% Ethanol		Toyopearl gel column, eluted with 2% HCl aq.	Anthocyanins (malvidin- 3-glucoside)	Pissarra <i>et al.</i> , 2004
Red grape marc	Crushed, uncrushed	Ethyl acetate water	250 g, 500 mL ethyl acetate, 500 mL water	Liquid-liquid separation of ethyl acetate and H ₂ O	Anthocyanins separated from other phenols	Bonilla <i>et al.</i> , 1999
Fresh grape seeds		Acetone/water (7:3)	50 g seeds extracted twice in solvent for 12 h under N ₂ with stirring	Liquid-liquid separation, evap. and extracts mixed	Monomers, oligomers, proanthocyanidin	Saucier <i>et al.</i> , 2001
Skins from frozen grape berries	Skins frozen in N ₂ (l), crushed using blender	Ethanol/water (75/25), 2% acetic acid	Extracted at 4°C overnight. 7 L solvent, 600 g skins	Concentrated with rotary evap. Toyopearl column, 3 step wash sequence		Vidal <i>et al.</i> , 2004
Grape skins and grape seeds	Dried, ground seeds. Seeds de- oiled n-hexane	Ethanol/water, acetone/water	1:10 (w/v) ratio, sonication, shaking and centrifugation	Supernatants filtered through glass wool	Total phenol content	Yilmaz & Toledo, 2006
Red grape skins		Methanol with 5% 1N HCl	Maceration at 4°C, methanol extraction, vacuum, 30°C	N-hexane (remove lipo- solublites), packed column	3-monoglucoside antho- cyanin derivatives	Heredia <i>et al.</i> , 1998

For alternative extraction methods, see also Revilla *et al.*, 1998: “Comparison of several procedures used for the extraction of anthocyanins from red grapes”; Spigno and Faveri, 2006: “Antioxidants from grape stalks and marc: Influence of extraction procedure on yield, purity and antioxidant power of the extracts”.

2.7 Solvent polarity

Solvent extraction is a commonly used unit operation in the chemical industry. The degree of polarity of organic solvents plays an important role in selecting the solvent for an extraction process. The polarity of the solvent depends on three criteria: hydrogen-bonding (H-bonding), dipole moment and dielectric constant. A range of solvents listed in order of increasing polarity is: fluoroalkanes, hexane, toluene, chloroform, ethyl acetate, acetone, isopropanol, ethanol, acetic acid, methanol, acetonitrile, water.

Physical properties of common solvents used for the extraction of polyphenols from grape marc are included in Table 2-11. The data from this table shows that ethyl acetate has the lowest relative polarity, resulting in ethyl acetate/water mixtures forming two liquid layers. This property is useful for separating ethyl acetate/water extracts that contain hydrophobic and hydrophilic compounds. For recycle considerations, the boiling points are used to determine which solvent requires the least energy for evaporation and recycle. Ethanol and ethyl acetate have similar boiling temperatures, and energy requirements to evaporate and recycle these solvents would be similar. However, provided methanol or acetone are used as extraction solvents less energy would be needed for recycle, because these solvents have lower boiling points than ethanol or ethyl acetate.

Table 2-11: Physical property data for organic solvents and water
[<http://virtual.yosemite.cc.ca.us>].

Solvent	Boiling point (°C)	Density (g/mL)	Relative Polarity	Solubility in H ₂ O [#] (g/100g)
Acetone	56.2	0.786	0.355	M
Ethanol	78.5	0.789	0.654	M
Ethyl acetate	77.0	0.894	0.228	8.7
Methanol	64.6	0.791	0.762	M
Water	100.0	0.998	1.000	M

[#] M stands for miscible.

In grape marc, grape pomace or grape seed extractions, organic solvents are used in preference to aqueous solvents because of their higher extraction rates and selectivity for polar polyphenol compounds. Solvents such as n-hexane and dimethyl ether are not good solvents for polyphenol extraction because they extract non-polar organic compounds present in grape marc [Pansera *et al.*, 2004]. Previous research has shown that acetone and ethanol are the most effective solvents for the extraction of polyphenols from grape marc and grape pomace [Pinelo *et al.*, 2005; Peng *et al.*, 2001]. Ethanol is a non-toxic solvent and is currently used by nutraceutical companies to extract polyphenols for use in health supplements [www.polyphenolics.com].

2.8 Preparation of grape marc for solvent extraction

The amount of polyphenols that can be extracted from grape marc depends on the feed preparation. To maximise the amount of polyphenols extracted from grape marc, size reduction methods are used. Additional preparation methods are summarised below and include the removal of moisture and unwanted compounds, such as lipids and sugars, from grape extracts [Longo *et al.*, 2005; Jayaprakasha *et al.*, 2001; Heredia *et al.*, 1998; Jaworski & Lee, 1987].

2.8.1 Size reduction of grape marc

It has been shown that crushed grape marc yields more polyphenols than uncrushed grape marc [Bonilla *et al.*, 1999]. Grape marc can be crushed in cutting machines or pulverised in grinding machines. The degree of size reduction is reported qualitatively where the machine type is reported [Spigno & De Faveri, 2006; Vidal *et al.*, 2004; Jaworski & Lee, 1987], or quantitatively, where the particle size of crushed grape marc is given [Cho *et al.*, 2005; Mantell *et al.*, 2002].

2.8.2 Solvent extraction of undesired components

After grape marc is extracted with a polar solvent, it can be mixed with a non-polar solvent, such as n-hexane or petroleum ether, to separate the polyphenols from undesired components [Baydar *et al.*, 2004; Jayaprakasha *et al.*, 2001]. The polar solvent and n-hexane form two liquid layers and allow for easy separation of the undesired lipid substances. However, trace amounts of n-hexane that dissolve in the polar solvent would need to be removed in order for application of polyphenol extracts in the food, cosmetic or nutraceutical industry. The grape marc extract can then be purified using a fractionation column, where sugar and other polar (non-polyphenol) substances are eluted first from the packing using water, and polyphenols would be eluted afterwards using organic solvents [Longo *et al.*, 2005; Heredia *et al.*, 1998].

2.8.3 Removing moisture from grape marc

Drying grape marc may prove useful as part of the feed pre-treatment, provided it does not adversely affect the recovery or activity of the extract. Grape marc can be dried in electrical ovens at 50°C [Negro *et al.*, 2003] or in a gas-fired oven which blows hot air at 93°C, to remove the moisture [Yilmaz & Toledo, 2006].

2.9 Separation of polyphenol compounds

Extracts from grapes and grape by-products may contain polyphenols, lipids, polysaccharides and sugars. Therefore, it may be necessary to separate the polyphenols from the non-polyphenol compounds in grape extracts to obtain purified products which could then be sold to industries manufacturing nutraceutical products.

One of the most common means of separating crude polyphenol samples is by using solid phase extraction methods (for example, C-18 Sep Pak[®] cartridges). These methods utilise the adsorption properties of polyphenol compounds that allow them to bind to the

resin of the cartridge. After polyphenols adsorb onto the resin, they can be eluted using lower polarity solvents (alcohols). To obtain different polyphenol fractions, solvents of increasing polarity can be used to elute the adsorbed polyphenol compounds. The procedures of two methods reported in literature are outlined below.

In the first method, developed by Jaworski and Lee [1987], polyphenol samples were obtained from grapes. These samples were fractionated to produce acidic and neutral polyphenol samples. The acidic fractions yielded tartrate derivatives, and the neutral fraction contained catechin, epicatechin, procyanidin B2 and B3.

In the second fractionation method [Longo *et al.*, 2005], samples were taken from grape berries containing high concentrations of anthocyanins. An aqueous berry extract was passed through a C-18 Sep Pak[®] cartridge that was preconditioned with acidified methanol. The three eluting steps performed to remove the undesired components first and then the anthocyanins, are shown below:

- Sugars, acids and water soluble compounds were first eluted using 0.01% HCl.
- Ethyl acetate was then used to remove the less polar polyphenols.
- Anthocyanins were eluted with acidified methanol.

2.10 Product composition and safety concentration limits of polyphenols and solvents

A product guideline for grape seed extracts was given by the Canandaigua Wine Company [Shrikhande, 2000], stating that a quality product from a grape seed extract should contain more than 90% total polyphenols, more than 65% oligomers (2-7 catechin units), less than 15% polymers, and more than 10% monomers. The motivation for this product composition was that monomers and oligomers are better absorbed by the body than the polymeric compounds. It was previously believed that the sugar residues prevented polyphenol glycosides from being secreted through the intestine wall

into the bloodstream, but recent studies show that polyphenol glycosides are also absorbed into the bloodstream without the sugar residues being cleaved [Youdim *et al.*, 2000]. This shows that polyphenols with attached sugar molecules should enter the bloodstream and can therefore be included in polyphenol extracts for use in health supplements.

Regarding the safety concentrations of polyphenols, a study was conducted by Product Safety Laboratories to determine whether high concentrations of polyphenols are physiologically safe [Shrikhande, 2000]. The findings, from *in vivo* analyses, were that the safety limit for polyphenol consumption was 3 g of polyphenols per kg body weight. Above this concentration, the polyphenols exhibited negative health effects. At extremely high concentrations, antioxidants are known to change their characteristic property from antioxidant to prooxidant [Decker, 1997].

Ethyl acetate and ethanol are food grade solvents. Ethyl acetate is commonly used in fermented drinks and concentrations up to 100 mg/L are permissible [Bonilla *et al.*, 1999]. Ethanol has been part of the human diet for a long time and is a major ingredient in alcoholic beverages. The alcohol concentration in beer is 4 to 5%, in wines 12 to 15% and in high quality spirits 40%. Therefore, small concentrations of ethyl acetate or ethanol in dried polyphenol extracts will not cause any harm to consumers.

2.11 Literature Review summary

The information summarised in this literature review shows that grape marc has significant concentrations of polyphenols, which can be extracted using traditional or advanced solvent extraction methods. However, a stirring-assisted extraction method was chosen for this study because it is less energy intensive while producing similar polyphenol recoveries to advanced extraction methods (PLE, SFE). Simple sample analysis methods have been found involving colourimetric assays to quantify the polyphenol concentrations of grape marc extracts. The polyphenol extraction yields can be determined in terms of gallic acid equivalents and the free radical scavenging

abilities of extracts can be measured using the DPPH assay. According to the literature, crushing grape marc and extracting at above room temperatures results in increased total polyphenol yields, which were also dependent on solvent concentration. In this study, the effect of solvent type and solvent concentration was investigated to select the most suitable solvent for the proposed process. In addition, model equations relating extracting time to TPC and extraction temperature to TPC were used to determine the maximum polyphenol yield obtainable from grape marc using stirring-assisted extraction equipment. This yield, which relates the amount of extractable polyphenol to the grape marc, was then used in the design of the proposed industrial scale process.

University of Cape Town

Chapter 3: Materials and Methods

3.1 Reagents

AR reagent grade acetone, ethanol absolute, ethyl acetate, hydrochloric acid, glacial acetic acid, HPLC and AR grade methanol were obtained from uniLAB (Saarchem, Merck, South Africa). Distilled water was generated in the laboratory using Millipore equipment. Model polyphenolic compounds used as standards included gallic acid, (+)-catechin, vanillin, cyanidin-3-glucoside (Extrasynthese, France), and DPPH (Aldrich, 95% purity). General chemicals used for analysis purposes included potassium chloride, sodium acetate and sodium carbonate.

3.2 Stock solutions

The stock solutions listed below were made in preparation for the analysis of grape marc extracts using UV-vis spectrophotometry: gallic acid and sodium carbonate solution (total polyphenol assay); catechin and vanillin solutions (total flavanol assay); cyanidin-3-glucoside solution, potassium chloride buffer, sodium acetate buffer (total anthocyanin assay); DPPH solution (free radical scavenging assay).

Sodium carbonate (Na_2CO_3) solution

Sodium carbonate solution is used in the total polyphenol assay. 100 mg (10% w/v) of Na_2CO_3 was weighed and dissolved in 1 litre of distilled water.

Gallic acid solution

A solution of 100 mg/L standard gallic acid was made using 20 mg gallic acid dissolved in 200 mL distilled water. To prevent oxidation, the Schott bottle containing the standard solution was covered in foil and stored at 4°C.

Vanillin solution

A 1% vanillin solution was prepared in absolute ethanol. 2 g of vanillin was dissolved in 200 mL ethanol. The bottle was covered in foil and stored at 4°C.

Catechin solution

A 250 mg/L standard solution of catechin was prepared in absolute ethanol. 50 mg of catechin was weighed and dissolved using magnetic agitation in 200 mL of ethanol. The standard solution was placed in a 250 mL Schott bottle, covered in foil and stored at 4°C.

Cyanidin-3-glucoside solution

5 mg of cyanidin-3-glucoside was dissolved in 125 mL of absolute ethanol to prepare a standard concentration of 40 mg/L. The bottled solution was covered in foil and stored at 4°C.

DPPH stock solution

12.5 mg of DPPH was added to 500 mL of methanol in a Schott bottle to make a standard solution of 25 mg/L [Fernandez-Pachon *et al.*, 2004; Sanchez-Moreno *et al.*, 1998]. The bottle was covered in foil and stored at 4°C.

Potassium chloride buffer, 0.025 M, pH 1.0

0.93 g KCl was added to 490 mL of distilled water and the pH was adjusted to 1.0 using concentrated HCl before dilution with distilled water to 500 mL.

Sodium acetate buffer, 0.4 M, pH 4.5

27.2 g $\text{CH}_3\text{CO}_2\text{Na} \cdot 3\text{H}_2\text{O}$ was added to 480 mL of distilled water and the pH was adjusted to 4.5 using concentrated HCl before diluting with distilled water to 500 mL.

3.3 Analysis methods of grape marc extracts

All spectrophotometric quantification and activity assays were conducted using a UNICAM UV-vis spectrophotometer (Helios α , England). Colorimetric assays included the total polyphenol assay [Cheung *et al.*, 2003], total flavanol assay [Gonzalez-Rodriguez *et al.*, 2002], total monomer anthocyanin assay [Ciusti & Wrolstad, 2000] and the DPPH free radical scavenging assay [Sanchez-Moreno *et al.*, 1998]. Where necessary, samples were diluted with the same solvent that was used to extract, in each case [Kim *et al.*, 2005].

3.3.1 Assay for determining total polyphenol concentrations

The Folin-Ciocalteu (F-C) method was adapted from Cheung *et al.* [2003], although the original method was developed by Singleton and Rossi [1965]. Gallic acid stock solution was made up in distilled water at 100 mg/L. Concentrations of 25, 50, 75 and 100 mg/L of gallic acid were used to produce a standard curve. The slope of the standard curve (108 mg cm⁻², see Appendix E-1) relating concentration to absorbance represents the absorption coefficient (ϵ). Total polyphenol concentrations (TPC) of samples were expressed in units of mg gallic acid equivalents (GAE) per litre or per kg (GAE/L or GAE/kg). The calculation for determining the TPC of samples is shown below, where DF is the dilution factor.

$$\text{Sample concentration (mg GAE / L)} = \text{Absorbance sample} * \epsilon * DF$$

... Equation 1

$$\text{Sample concentration (mg GAE / kg)} = \text{Sample concentration (mg GAE / L)} * \frac{\text{solvent L}}{\text{marc kg}}$$

... Equation 2

The assay procedure involved the following steps: 400 μL gallic acid standard was added to 400 μL Folin-Ciocalteu reagent in a 4 mL cuvette. After 3 min, 400 μL 10% Na_2CO_3 was added to the mixture, together with 2.8 mL distilled water. The sample colour changed from orange to green after the Na_2CO_3 was added. The sample blank contained the same components except that 400 μL distilled water was used instead of gallic acid solution. The solutions were left in a dark place for 90 min. Cuvettes were covered with parafilm and gently agitated to dislodge air bubbles in the cuvettes before being placed in the spectrophotometer. Triplicate samples were prepared and the absorbances were read at 765 nm. The sample blank was used to zero the baseline.

3.3.2 Assay for determining total flavanol concentrations

The assay method used in this study was adapted from Gonzalez-Rodriguez *et al.* [2002], although the original method was developed by Swain and Hillis [1959]. The quantification of total flavanols includes both monomeric flavanols (catechin, epicatechin) and polymeric flavanols (proanthocyanidins). Standard 1% vanillin solution and 250 mg/L catechin solution were made up in absolute ethanol. In preparation of standard samples, different concentrations of catechin solution, diluted with ethanol, were added to vanillin solution and hydrochloric acid (Table 3-1). Standard samples and extracts were prepared in 7 mL glass test tubes and transferred to cuvettes for analysis. The blank solutions used to zero the baseline contained HCl, vanillin, and ethanol (test tube 1). The catechin-vanillin solution changed from colourless to pink on addition of 37% hydrochloric acid. The absorbance of each sample was measured at 500 nm, 20 min after mixing the reagents, and the concentrations were expressed as catechin equivalents (CT):

$$\text{Total flavanol concentration (mg CT / L)} = (\text{Abs sample} - \text{Abs reference}) * \epsilon * DF$$

... Equation 3

Table 3-1: Volumes (mL) of solutions used for the total flavanol standard curve [adapted from the method of Gonzalez-Rodriguez *et al.*, 2002].

Solution	Test tube 1 (blank)	Test tube 2	Test tube 3	Test tube 4	Test tube 5
Ethanol	2	1.8	1.6	1	0
Catechin (250mg/L)	0	0.2	0.4	1	2
37% HCl	2	2	2	2	2
1% Vanillin	1	1	1	1	1

For analysis of grape marc extracts, samples were diluted to ensure that the absorbance readings were <1.2 absorbance units. Due to the red colour in grape marc samples, the absorbance readings were corrected for by subtraction of the reference absorbance from the sample absorbance. Each extract had its own reference sample since the colour intensity of samples varied.

Table 3-2: Reagent volumes (mL) used for duplicate sample analysis of flavanols.

Reagents	Reference	Sample 1	Sample 1
Ethanol	1	0	0
Diluted sample	2	2	2
37% HCl	2	2	2
1% Vanillin	0	1	1

3.3.3 Assay for determining total anthocyanin concentrations

This method was conducted according to Current Protocols in Food Analytical Chemistry [Ciusti & Wrolstad, 2000]. Two dilutions of each sample were prepared with potassium chloride solution (0.025 M) and sodium acetate buffer (0.4 M) at pH 1.0 and pH 4.5 respectively. The absorbance readings of diluted samples were taken at 520 nm and 700 nm, and the spectrophotometer was zeroed with distilled water. The anthocyanin concentration was expressed on the basis of cyanidin-3-glucoside (CY), molecular weight (MW) of 484.8 g mol⁻¹. Grape marc sample absorbance and anthocyanin concentration were calculated with the following equations:

$$\text{Sample absorbance} = (Abs_{520} - Abs_{700})_{pH 1.0} - (Abs_{520} - Abs_{700})_{pH 4.5}$$

... Equation 4

$$\text{Total anthocyanin concentration (mg CY/L)} = Abs_{\text{sample}} * MW * DF * 1000 / \epsilon$$

... Equation 5

An absorption coefficient (ϵ) of 27 579 cm² mol⁻¹ was calculated from the standard curve of cyanidin-3-glucoside chloride dissolved in 50% ethanol (see Appendix E-3). This absorption value agrees closely with that reported by Rapisarda *et al.* [2000], whose value for 53% water/ethanol mixture was 27 876 cm² mol⁻¹.

3.3.4 Free radical scavenging activity assay for efficacy determination of polyphenol extracts

Free radical scavenging activity assays were conducted according to the methods of Brand Williams *et al.* [1995] and Sanchez-Moreno *et al.* [1998]. Both methods used DPPH as a model free radical compound, dissolved in methanol. EC₅₀ concentration results obtained from activity analyses were expressed in mg phenol/g DPPH (phenol refers to total polyphenols). For the standard curve, 25 mg/L DPPH stock solution was diluted using methanol to 5, 10, 15 and 20 mg/L and measured immediately at 515 nm (see Appendix E-4). Pure methanol was used as a blank solution.

The assay procedure to analyse the free radical scavenging activity of extract samples involved the following steps: Firstly, a serial dilution of the sample was performed to obtain 4 or 5 diluted samples. Typically these concentrations would vary from 25-150 mg GAE/L for grape marc samples. An aliquot of 0.1 mL of sample was added to 3.9 mL of DPPH (25 mg/L) in a 4 mL cuvette. The percentage DPPH remaining was determined by reading the solution absorbance over time. The first reading at $t = 0$ was taken before the extract was added to obtain the 100% DPPH absorbance. As the DPPH

was neutralised, the solution colour changed from purple to light yellow and absorbance readings decreased until the scavenging reaction reached steady state (1-3 h).

3.3.5 Analysis of grape marc extract using High Performance Liquid Chromatography (HPLC)

Samples were analysed using HPLC system manager (Merck, Hatachi) using D 7000 data software. Samples were filtered to remove solid particles using filter paper (Whatman No.1, England). Methanol was used to wash the reverse phase, 5 μ m column (C-18 Waters, 250 mm x 4.6 mm). The mobile phase, consisting of distilled H₂O/MeOH/glacial acetic acid, 80:20:2.5 [Ting, 2004], was degassed using a Millipore vacuum pump prior to sample analysis. Isocratic elution was performed and the mobile phase flow rate was maintained at 1 mL/min for 45 min. Monitoring was effected by measuring UV absorbance at 280 nm.

3.4 Grape marc collection and storage

The grape marc was collected in March 2004, which was at the end of the wine making season in South Africa, from the cellar of the J.H. Neethling building at the Stellenbosch Institute of Wine and Biotechnology, with permission of Dr. Pierre van Rensburg. Four 28 L containers of fermented red and white marc were collected. All the containers used were lined with clear, industrial plastic bags and tightly sealed. The wet grape marc was transported to the Bioprocess Engineering Research Unit at UCT and stored at -20°C. The grape marc has a high moisture content (~74%) and is thus referred to in this study as wet grape marc.

3.5 Methods used in size reduction of grape marc

The three conditions of marc tested, in order of increasing degree of size reduction, were uncrushed, crushed and pulverised wet grape marc. In this study, grape marc was crushed using small scale blending machines or pulverised using a Sieb mill (The Ferguson Industrial Equipment Group, JHB). The Sieb mill was used with permission from the Mineral Processing Research Unit (UCT).

3.5.1 Grape marc size reduction using blending machines

The two blending machines used to crush grape marc were a Waring (USA) and a Pineware (RSA) blender. Both blending machines have a 350 W power requirement and 1 L capacity. The grape marc was crushed or diced by rotating blades made of stainless steel. The Waring blender was made of stainless steel and the Pineware blender of plastic. Blending wet marc without water resulted in the marc being partly crushed. The wet marc was crushed for 15 sec on low speed and this method of crushing marc without water was sufficient for magnetically agitated extractions using 40 g of crushed marc. Larger quantities (300 g) of wet marc were crushed in the presence of water (300 mL) to ensure that the marc was evenly crushed. This crushing technique with water was used to prepare crush marc for larger (mechanically agitated) extraction systems.

3.5.2 Grape marc size reduction using the pulveriser

Grape marc was pulverised in preparation for magnetically-stirred extractions. The pulverising mechanism consists of circular metal plates (rings). This machine is normally used for the crushing of ore material into fine powder. Grape marc placed between the metal plates was completely homogenised by the centrifugal force of the spinning plates. Approximately 250 g of wet marc was pulverised for 1 minute. After removing the pulverised marc it was noted that the grape skins were completely pulverised to form a purple paste, but the seeds were left uncrushed. The pulverised marc was placed in air-tight plastic bag, inside a container, and stored at -20°C until needed for extractions.

3.6 Fractionation of grape marc polyphenols using solid phase extraction

3.6.1 Fractionation procedure of grape marc extracts

From the two solid phase extraction (SPE) methods mentioned in the Literature Review (see Section 2.5.1), the fractionation procedure of Jaworski and Lee [1987] was selected for this investigation. This decision was made because the alternative fractionation method, by Longo *et al.* [2005], used acidified solvent which is known to lead to overestimated anthocyanin concentrations, since it has been suggested that flavanol and proanthocyanidins compounds change to form anthocyanins at low pH conditions [Revilla *et al.*, 1998]. A schematic representation of a Sep-Pak[®] Cartridge (Waters Corporation, USA) is shown in Figure 3-1.

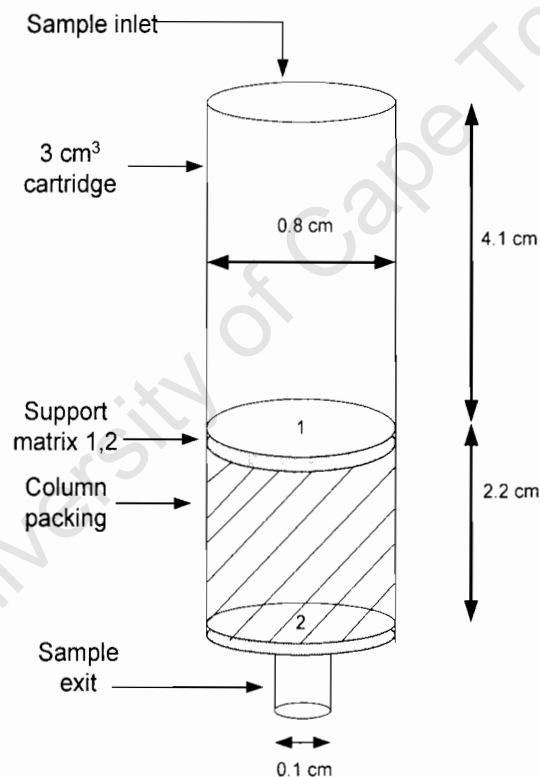


Figure 3-1: C-18 Sep-Pak[®] Cartridge was used for the fractionation of polyphenol compounds (not to scale).

Two 500 mg Waters C-18 Sep-Pak[®] Cartridges were used for each fractionation experiment. Neutral cartridges were pre-conditioned consecutively with 2.0 mL AR grade methanol and 2.0 mL of distilled water, and acidic cartridges were washed with 0.01 M HCl instead of distilled water. In each run, a sample aliquot (16 mL) was taken from the extract (grape marc filtrate) and adjusted to pH 7.0 by dropwise addition of 1.0 M sodium hydroxide before being loaded onto the neutral cartridge. The eluent was acidified using 1.0 M hydrochloric acid, to pH 2.5, before being passed through the acidic cartridge (see Figure 3.2).

A polyphenol mass balance was conducted over both the neutral and acidic cartridges by measuring the difference between $TPC_{initial}$ and $TPC_{exiting}$, which showed the amount of total polyphenols adsorbed ($TPC_{adsorbed}$) on both cartridges. The adsorbed polyphenol compounds in the cartridges were eluted with methanol, and monomeric anthocyanins were characterised by the pH difference method [Ciusti & Wrolstad, 2000] (see Section 3.3.3). This analysis allowed a comparison between the anthocyanin results from the fractionated extracts obtained from the methanol and acetone extracts.

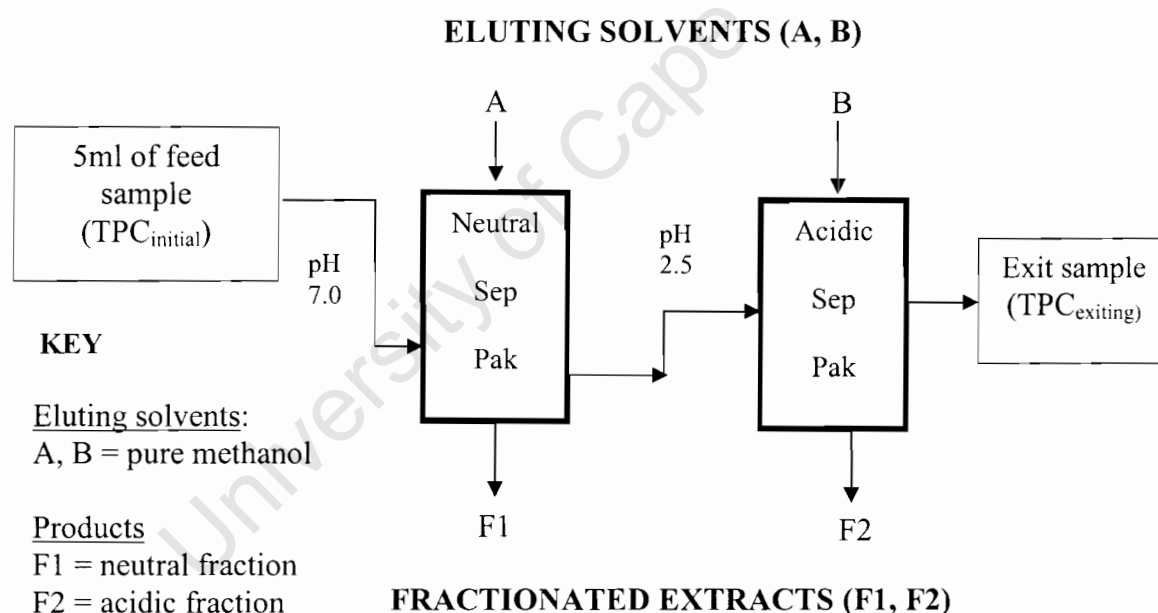


Figure 3-2: Experimental procedure for use of Waters Sep-Pak[®] Cartridges, showing the fractionation and elution of grape marc extracts.

3.6.2 Sample preparation method for SPE

The grape marc was taken from cold storage, and 15 g of uncrushed marc was agitated with magnetic stirrers using 45 mL of solvent for 30 min and then filtered afterwards with Whatman No. 1 filter paper. Methanol or acetone was used as extracting solvent to determine the anthocyanins recovered. Aliquots of filtrate samples from each extraction were placed in 20 mL McCartney bottles and stored at 4°C to be used later in fractionation experiments.

3.6.2.1 Fractionation of methanol grape marc extracts using different wait times

Three 15 g samples of uncrushed grape marc were extracted using 45 mL of methanol, and the resulting extracts were fractionated. Varying wait times of 1, 6 and 24 h were recorded for each of the samples during the pre-condition stage of the cartridges. For the purpose of this experiment, wait times are defined as the time period between pre-conditioning the cartridges and loading the polyphenol samples. Polyphenol compounds binding to the cartridge packing were eluted, collected and stored in 2 mL screw-cap eppendorf tubes at 4°C. Each sample mass of anthocyanins recovered was measured.

3.6.2.2 Testing the consistency of the fractionation procedure using acetone/water grape marc extracts

The grape marc extracts, obtained from 15 g of uncrushed grape marc using 45 mL of acetone/water (2:1, v/v), were evaporated and re-dissolved in methanol. Grape marc extracts were placed on the rotary evaporator at 43°C for 25 min until a small volume remained. The concentrated samples were diluted to 16 mL volume with methanol. The fractionation procedure for the re-dissolved extracts used a 1 h wait time, and the fractionated polyphenols were collected in screw-cap eppendorf tubes and stored at 4°C prior to anthocyanin analysis. This experiment was repeated under the same conditions to test the consistency of the fractionation method by measuring anthocyanin content.

3.7 Extraction and filtration setup for magnetically agitated systems

Magnetic stirrer plates (630 W, Heidolph 2002, Germany) in the laboratory have a maximum temperature of 350°C and magnetic agitation speeds of up to 1250 rpm. The stirrer bars used were ordinary laboratory magnetic stirrers coated in white plastic and sizes varied from 2 to 3 cm in length. The working volumes of magnetically extracted systems (marc and solvent) varied between 60 to 160 mL. Extraction vessels used for agitating grape marc and solvent were placed on magnetic stirrer plates. Typical extraction vessels were 500 mL Schott glass beakers or 250 mL Schott bottles.

The method of extraction used was according to the method of Lu and co-workers [Lu & Foo, 1999; Revilla *et al.*, 1998]. This extraction method used a wet marc to solvent ratio of 1:3 (w/v) to obtain high polyphenol extraction yields. The agitation speeds used in these investigations varied from 400 to 600 rpm, depending on the working volume of the system. Agitation speeds were selected to ensure that the system was well mixed and that the grape marc remained suspended in the solvent. Performing extractions in well mixed systems allowed for the assumption that there were negligible polyphenol concentration gradients in the solvent.

The filtration procedure for magnetically stirred systems was conducted after every extraction stage, to separate the solvent extract from the grape marc. The filter material was supported by a funnel which allowed the filtrate to be collected in a separate container. First cheese cloth was used, followed by a second filtration step using Whatman No. 1 filter paper. Filtrate samples were labelled and stored at 4°C for analysis and the filter paper, cheese cloth and grape marc were discarded. In some experiments, the filtrates were evaporated to determine the extract weight after freeze drying. These extracts were re-dissolved using the same extraction solvent and stored at 4°C. The flow sheet below shows the laboratory process used to obtain polyphenol extracts. Note, either the filtrate or the polyphenol extract can be used for analysis.

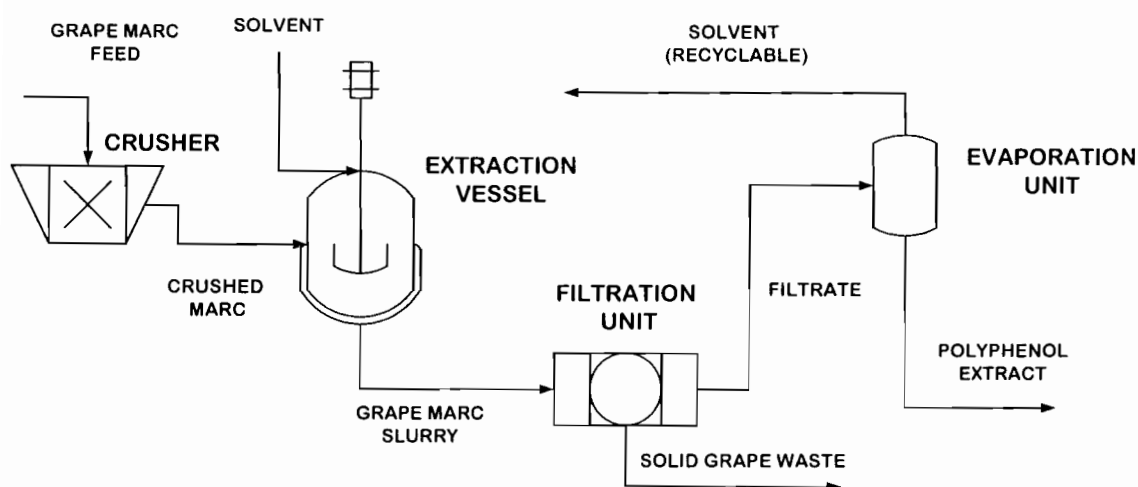


Figure 3-3: A basic flow sheet showing the different stages of the polyphenol extraction process used in the laboratory.

3.7.1 Investigation of effects of pre-treatment on the extraction of grape marc polyphenols

3.7.1.1 Polyphenol degradation during storage under ambient conditions

Wet grape marc was crushed and placed in a plastic container, sealed with foil and stored at room temperature (25°C) for several weeks. 25 g batches of the marc were extracted at 0, 2 and 5 weeks from the plastic container. Grape marc was magnetically agitated in 50 mL of pure ethyl acetate for 10 min at room temperature. Extraction mixtures were filtered using filter paper and the filtrates were stored at 4°C, and later analysed using the total polyphenol assay.

3.7.1.2 Comparison of polyphenol concentrations of dried and wet grape marc extracts

Grape marc (wet marc) was taken from the freezer, pulverised and then dried at 80°C for 3 h. The moisture content of grape marc was determined at 74%, which is within the reported range of 70 to 80% [Shrikhande, 2000]. 10 g of the dried marc was mixed with 3x50 mL of acetone in three consecutive extractions. After each extraction, the slurry was filtered and 50 mL of clean acetone was added to the grape marc. Each of the three filtrates were analysed separately to determine the TPC for each extraction stage. The same extraction and filtration procedure was repeated using 10 g portions of wet marc, and the total polyphenol assay was conducted.

3.7.2 Characterisation of polyphenols extracted from crushed grape marc

3.7.2.1 UV-vis spectrophotometric characterisation of grape marc extracts

Extractions using 40 g of crushed grape marc and 120 mL of 50% ethanol were conducted in beakers using magnetic agitation. After 30 min agitation, the filtrate from the slurry (extract) was rotary evaporated at 42°C for 2 hours. The resulting dark red/purple extract, containing a small amount of water, was frozen in liquid nitrogen, and the frozen extract was dried on the freeze drier (Instruvac, Vertex VD-2003, South Africa) for 20 h. Net weights of dried polyphenol extracts were measured. The dried extracts were then re-suspended using half the filtrate volume. The extracts were analysed using the UV-vis spectrophotometer for total polyphenol, total flavanol and total anthocyanin concentrations.

3.7.2.2 HPLC characterisation of grape marc extracts

HPLC analyses of grape marc extracts obtained using ethanol, methanol and ethyl acetate as solvents were conducted. These extracts were obtained by agitating 50 g of grape marc with 100 mL of organic solvent for 30 min. Aliquots from these prepared polyphenol extracts were placed in 1 mL HPLC vials. Reverse phase HPLC was operated isocratically at 280 nm for 40 min at a flow rate of 1 mL/min. The mobile phase used consisted of water/methanol/acetic acid (80:20:2.5). The model compounds used for identification of polyphenols were resveratrol, gallic acid, catechin, caffeic acid and epicatechin.

3.7.3 Measurement of the free radical scavenging activity of model compounds

The method used in these experiments was taken from Sanchez-Moreno *et al.* [1998]. A stock solution of gallic acid at 100 mg/L was diluted to 5, 12.5 and 25 mg/L using distilled water; a cyanidin-3-glucoside solution was diluted to 10, 20, 30 and 40 mg/L; and a catechin solution was diluted to concentrations of 40, 80 and 150 mg/L. The assay was performed by adding 50 μ L of these diluted samples to 1.95 mL of DPPH at 25 mg/L in 2 mL cuvettes. Absorbance readings were recorded after 0, 0.5, 1, 1.5, 2, 10, 15, 30, 60 min at 515 nm, using the UV-vis spectrophotometer. The percentage DPPH remaining was used to find the EC₅₀ concentration and steady state reaction times (T_{EC50}), which in turn were used to calculate the antiradical efficiency (see Section 4.2.1).

3.8 Investigations using magnetically agitated extraction systems

3.8.1 Investigation of the effect of solvent polarity on efficiency of polyphenol extraction

The extraction system consisted of 15 g of uncrushed red grape marc and 45 mL of solvent. The solvents tested were acetone, 50% acetone/water, ethanol, 50% ethanol/water, methanol, 50% methanol/water, ethyl acetate and water. Extraction mixtures were agitated for 1 h under ambient conditions, then filtered and stored at 4°C, until analysed. Extraction times of 1 h were used to ensure that the extraction systems were as concentrated as possible. After extraction, the samples were filtered and analysed for TPC and free radical scavenging activity.

3.8.2 Effect of solvent concentration on polyphenol extraction

Crushed grape marc extracts were used for this experiment. 33 g of crushed marc and 100 mL of solvent (acetone or ethanol) was mixed at room temperature for 15 min using magnetic stirring at 500 rpm, with acetone and ethanol concentrations of 0, 25, 50, 75, 100% (v/v). Solvent-marc slurries were filtered and filtrates were analysed for total polyphenol concentrations.

3.8.3 Effect of temperature on the extraction of polyphenols using ethanol and acetone

Crushed and uncrushed grape marc were extracted using acetone/water (2:1, v/v) and ethanol/water (2:1, v/v). The extraction system consisted of 15 g of grape marc (crushed and uncrushed), 45 mL of solvent in a 200 mL Schott bottle on a magnetic stirrer, fitted

with a thermometer. Extraction mixtures were agitated at atmospheric pressure for 20 min at 250 rpm at set temperatures that varied from 25 to 50°C. Higher extraction temperatures were avoided since catechin and epicatechin compounds degrade up to 36% in boiling solvents [Palma *et al.*, 2001]. All extraction temperatures tested were below the boiling points of the extraction solvents. After the extraction, the samples were filtered and analysed for TPC and DPPH activity.

3.8.4 Investigation of polyphenol mass transfer kinetics during grape marc extraction

Two separate batches of 33 g crushed marc were mixed in 250 mL beakers with 100 mL of acetone/water and 100 mL ethanol/water at room temperature. 1 mL aliquots of extracts were manually collected at eight designated times: 0, 0.5, 2, 5, 10, 15, 20 and 25 min. This same procedure was repeated for uncrushed grape marc. An assumption was made that the polyphenol concentrations were equal everywhere in the extraction system, after ensuring that the extraction system was well mixed. This manual sampling method produced consistent TPC results over repeated extractions.

3.8.5 Effect of nitrogen headspace on antioxidant activity of polyphenols obtained using solvent extraction

Two samples of grape marc, crushed using the Waring blender, were extracted at room temperature with ethanol/water (2:1, v/v) and acetone/water (2:1, v/v) under nitrogen atmospheres by flushing nitrogen through the head space to displace the air. Control samples were obtained using the same extraction conditions in closed Schott bottles, except having air in contact with the solvent-marc slurry. Mixtures of 15 g crushed marc and 45 mL of solvent were agitated for 20 min at 250 rpm. Schott bottles of 250 mL capacity were flushed with nitrogen (Air Liquide, 99.9%) after the marc, solvent and magnetic stirrers were placed inside. Nitrogen was flushed through the head space ($\pm$ 200 mL) for 6 min at a cylinder pressure reading of 7 mPa. The gas inlet and outlet tubes were pinched closed with metal clips to seal the nitrogen inside the Schott bottle

during the extraction time. After the extraction, the samples were filtered and analysed for TPC and DPPH activity.

3.8.6 The effect of the rotary evaporator on the free radical scavenging activity of polyphenol extracts

The Waring blender was used to crush 20 g of wet grape marc, which was extracted with 60 mL absolute ethanol. Absolute ethanol was used because it evaporates completely, leaving a dried extract as opposed to ethanol/water (2:1, v/v) extracts which contain small amount of water. The extraction conditions were: room temperature (25°C), 750 rpm stirring and 30 min extraction time. The marc-solvent slurry was filtered using cheese cloth. 30 mL of the filtrate was dried using a rotary evaporator, under vacuum (-0.85 bar) at 45°C, weighed and re-dissolved in 30 mL absolute ethanol.

A similar procedure was carried out using ethyl acetate as an extraction solvent. 30 g of grape marc, crushed using the Waring blender, was mixed with 90 mL of pure ethyl acetate for 1 h. The slurry was then filtered using cheese cloth. The solvent was evaporated off using the rotary evaporator at 36°C, and the dry extract was weighed and re-dissolved in 30 mL of solvent. Aliquots from re-dissolved ethanol and ethyl acetate extracts were diluted to concentrations of 20, 50 and 85 mg/L and reacted with DPPH. The time needed for the absorbance of the free radical scavenging reaction to reach steady state was found to be 2, 3 and 4 hours respectively.

3.9 Mechanically agitated extraction using crushed and uncrushed grape marc

Overhead mechanical stirrers (105 W, Heidloph, Germany) are used routinely for large scale laboratory systems. They can operate at two varying rotational speed ranges from 45 to 300 rpm and 300 to 2000 rpm. The extraction vessels are made of glass, have a 1.2 L maximum working volume, and a H:D (height to diameter) ratio of 2:1.

3.9.1 Extraction procedure for mechanically agitated systems

The extraction setup was similar to the magnetic extraction system, except that the extraction volume was 10 times greater. The 1.2 L working volume consisted of 300 g wet marc, 450 mL absolute ethanol and 450 mL water. Before addition of marc to the vessel, the Pineware blender was used to crush the marc-water mixture for 30 sec on power 1. The marc-water slurry was then placed in the extraction vessel with 450 mL of absolute ethanol. The system was agitated for 30 min using an agitation speed of 650 rpm, and the TPC of the filtrate was measured.

Further investigations were conducted where 300 g of uncrushed marc was agitated with 900 mL of ethanol/water (50%) for 90 min in two consecutive extractions. Once the extraction and filtration of the first stage had finished, 900 mL of fresh ethanol/water (50%) was added to the marc for the second extraction stage. In this uncrushed marc system, solvent samples of 1 mL were taken from the sample port to measure TPC at fixed time intervals of 0.5, 1, 3, 8, 14, 20, 30 and 45 min.

3.9.2 Filtration method for mechanically agitated systems

Large quantities of marc and solvent used in mechanically stirred systems were not efficiently separated using cheese cloth. Consequently in mechanically stirred systems, chicken wire was used to separate the solvent extract from the grape marc as a first filtration step followed by the use of cheese cloth. The use of wire mesh proved effective in separating the majority of marc from solvent and also reduced the total filtration time. The filtrates from the cheese cloth were collected and stored at 4°C.

Chapter 4: Results and Discussion

4.1 Introduction

This chapter describes the results of investigations into the characteristics of polyphenols in grape marc extracts. The grape marc used in this study was donated by the Institute of Wine and Biotechnology, Stellenbosch University. The grapes (*Vitis vinifera*) had been processed over a 5 to 7 day vinification period to make red wine, and the red grape marc used in this study was a by-product from this wine-making process. The grape marc extracts produced in this study were analysed by determining the total polyphenol content, the total flavanol content, the total anthocyanin content and the free radical scavenging capacity. A set of model compounds, namely gallic acid, catechin, cyanidin-3-glucoside and DPPH, were used as standards in the assays.

The aims of the investigations were to determine and optimise the process variables affecting the recovery and antioxidant efficacy of the grape marc extracts. A stirring-assisted extraction method was used in these investigations because it has low energy costs and scale-up designs using this method were more feasible than other extraction methods, such as pressurised liquid extraction and supercritical fluid extraction. A set of preliminary investigations were conducted using model antioxidant compounds, grape marc extracts and fractionated polyphenol extracts (Section 4.2). After preliminary investigations, the process variables investigated and optimised included grape marc storage, size reduction (crushing, pulverising), grape marc drying, extraction solvent, solvent concentration, extraction time, extraction temperature and extraction atmosphere (Section 4.3, Section 4.4). In the next section (Section 4.5), the suitability of rotary evaporation as a solvent separation and recovery method was tested. In Section 4.6, a bench scale extraction system using a pitched-bladed impeller was tested to determine the total polyphenol yield, the extract characterisation using UV-vis spectrophotometry, and the free radical scavenging activities of grape marc extracts. Then, the antioxidant efficacies of grape marc extracts and nutraceutical products were compared.

4.2 Preliminary investigations of model antioxidant compounds and grape marc extracts

The preliminary investigations consisted of three separate experiments. The first experiment was performed to establish the antioxidant activity methods used to determine the EC₅₀ concentrations and antiradical efficiencies of model compounds. In the second experiment, individual polyphenol compounds present in grape marc extracts were identified through HPLC analysis. In the third experiment, polyphenol extracts were fractionated using solid phase extraction and the anthocyanin yields of the fractionated extracts were measured.

4.2.1 Measurement of antioxidant capacity of model compounds

Free radical scavenging activity assays were first used to measure the antioxidant activities of model antioxidant compounds gallic acid, catechin and cyanidin-3-glucoside by using graphical methods to determine their EC₅₀ concentrations and antiradical efficiencies. This antioxidant activity analysis was performed to compare the antiradical efficiencies of the model compounds, using the analysis procedure from Sanchez-Moreno *et al.* [1998]. Results obtained using gallic acid illustrate this analysis procedure (Figures 4.2-1, 4.2-2, 4.2-3). Similar figures for the determination of the EC₅₀ concentrations of catechin and cyanidin-3-glucoside compounds are shown in Appendix C.

A serial dilution of gallic acid was performed to obtain sample concentrations 5, 12.5 and 25 mg/L. Aliquots of these samples were added to DPPH in separate cuvettes and free radical scavenging reactions were monitored spectrophotometrically at 515 nm (see Section 3.3.4). Decreases in the sample absorbance correlating to decreasing DPPH

concentrations (scavenged DPPH free radicals) are shown in Figure 4.2-1. The percentage DPPH remaining was calculated using Equation 6.

$$\% \text{ DPPH remaining} = \frac{[DPPH]_{t=t}}{[DPPH]_{t=0}} \times 100$$

... Equation 6.

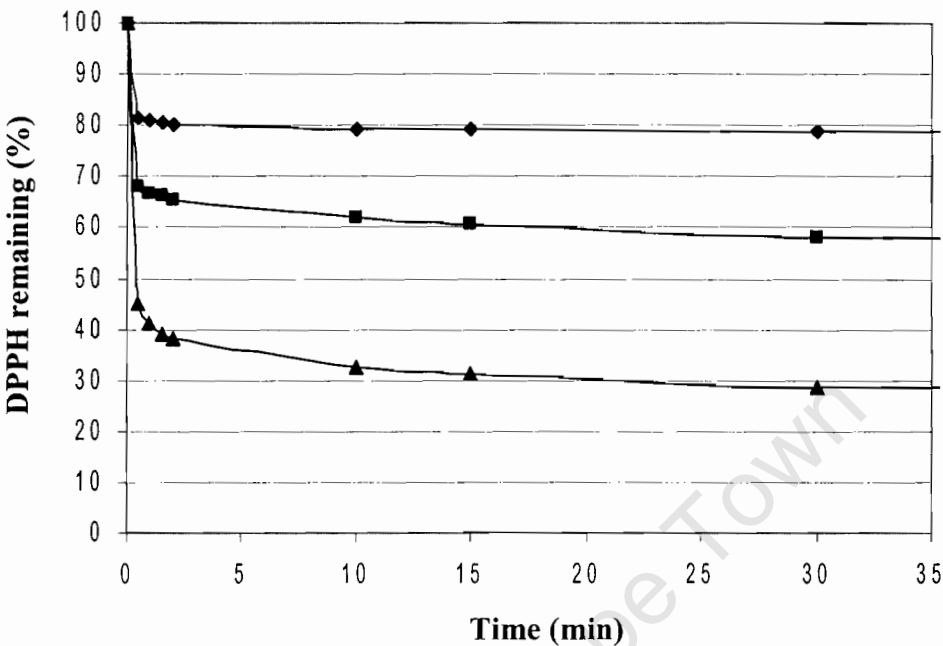


Figure 4.2-1: % DPPH remaining over time of three separate samples containing 5 ●, 12.5 ■ and 25 ▲ mg/L gallic acid added to 25 mg/L DPPH.

A graph was drawn to relate the concentration of DPPH remaining (%) to the mass fraction of polyphenols added to DPPH (see Figure 4.2-2). As expected, the sample with the lowest gallic acid concentration (5 mg/L) showed the highest amount of DPPH remaining (79%), and hence indicated the least amount of the free radicals scavenged. Conversely, the sample with the highest gallic acid concentration (25 mg/L) scavenged the most amount of DPPH, leaving 28% DPPH remaining at steady state. The EC₅₀ concentration (determined graphically from Figure 4.2-2) is a standard method for measuring the free radical scavenging capacity of polyphenol samples. The EC₅₀ concentration is defined as the amount of polyphenols required to quench by 50%, the initial DPPH concentration, expressed in units of mg phenol/g DPPH.

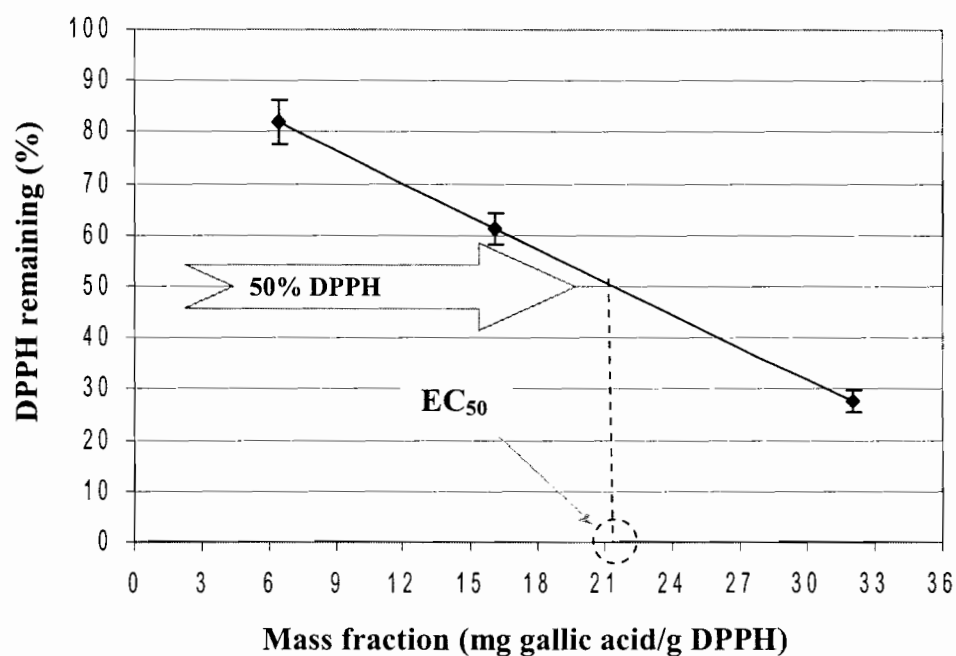


Figure 4.2-2: Experimental results showing the relationship between % DPPH remaining at steady state and the mass fraction concentrations, which was used to determine the EC₅₀ value of 21.1 mg phenols/g DPPH for gallic acid.

The EC₅₀ concentration was used to determine the steady state reaction time (T_{EC50}) of 25.0 min, as shown in Figure 4.2-3. The steady state time was the time at which the DPPH concentration reached a constant value, after being quenched by the grape marc polyphenols. The T_{EC50} and EC₅₀ concentrations were then used to calculate antiradical efficiency with the following equation, where the higher the antiradical efficiency value the higher the antioxidant capacity of the polyphenol sample:

$$AE = \frac{1}{EC_{50} \times T_{EC50}}$$

... Equation 7

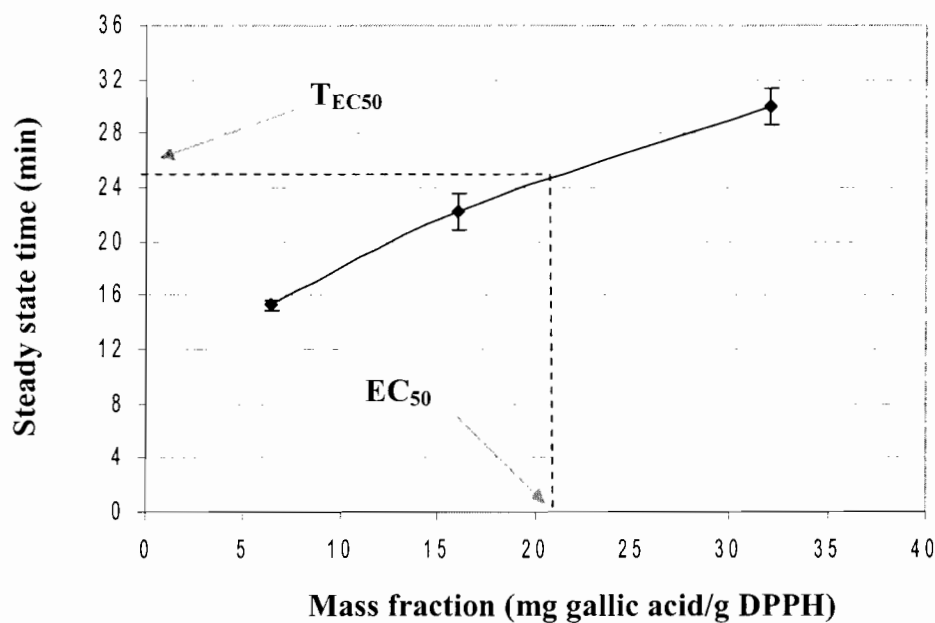


Figure 4.2-3: Experimental results showing the time (T_{EC50}) required to quench 50% of the DPPH at the EC_{50} concentration of 21.1 mg/g DPPH for gallic acid.

The results of the free radical scavenging assays for catechin and cyanidin-3-glucoside are shown together with the results of gallic acid in Table 4.2-1. The EC_{50} concentration for gallic acid of 21.1 mg/g DPPH compare well with the 26.0 mg/g DPPH determined by Sanchez-Moreno *et al.* [1998], which confirms the accuracy of this method. The antiradical efficiencies of cyanidin-3-glucoside and catechin (0.36×10^{-3} and 0.33×10^{-3} respectively) were both lower than that of gallic acid (2.06×10^{-3}), because of slow quenching rates shown by their higher T_{EC50} times. This means that of the three model compounds tested, gallic acid is the most effective free radical scavenger because it has a higher antiradical efficiency than either cyanidin-3-glucoside or catechin.

Table 4.2-1: Analysis of DPPH quenching activity for model compounds gallic acid, catechin and cyanidin-3-glucoside.

Model compounds	EC₅₀ (mg/g DPPH)	T_{EC50} (min)	Antiradical efficiency (x10⁻³)
Gallic acid [#]	26.0 ± 1	14.7 ± 1	2.62
Gallic acid [*]	21.1 ± 1	23.0 ± 5	2.06
Catechin [*]	74.0 ± 1	41.5 ± 1	0.33
Cyanidin-3-glucoside [*]	13.4 ± 1	210.0 ± 1	0.36

[#] Values obtained by Sanchez-Moreno *et al.* [1998].

^{*} Standard deviations for EC₅₀ and T_{EC50} values and were obtained from duplicate experiments.

4.2.2 HPLC characterisation of polyphenols extracted using ethanol, methanol and ethyl acetate

HPLC analysis was used to characterise the major components of three concentrated polyphenol extracts obtained from grape marc using ethyl acetate, ethanol and methanol respectively. This analysis was performed at 280 nm because all aromatic (polyphenolic) compounds absorb at 280 nm, whereas at this wavelength sugars and organic acids do not absorb. The extracts were separated from the grape marc residue by filtration and concentrated in the rotary evaporator.

The HPLC chromatograms (Figures 4.2-4 a, b and c) showed that the ethyl acetate, ethanol and methanol extracts had 2, 3 and 5 major peaks respectively. Based on the peak heights and total area under the graph, ethyl acetate did not extract high concentrations of polyphenols. Likely reasons for the differences in the chromatographic profile of the ethyl acetate extract are: firstly, ethyl acetate extracts negligible amounts of anthocyanins [Bonilla *et al.*, 1999], unlike ethanol and methanol; secondly, its relative polarity (0.228) is considerably lower than that of ethanol or methanol and hence ethyl acetate does not extract the same type and quantity of polar polyphenol compounds. The solvents ethanol and methanol have similar polarity values relative to water of 0.654 and 0.762 respectively [<http://virtual.yosemite.cc.ca.us>], and

they have been reported to extract the same polyphenol compounds from grapes [Romero-Perez *et al.*, 2001]. Therefore it is understandable that the ethanol and methanol HPLC chromatograms are similar.

Some of the peaks in the chromatograms were identified on the basis of equivalent retention times, by comparing with the retention times of model compounds. The respective retention times of these polyphenol compounds resveratrol, gallic acid, catechin, caffeic acid and epicatechin, reported by Ting [2004], were: 2.95 min; 4.16 min; 7.38 min; 12.90 min; 14.53 min. The major peaks closely matching these retention times are numbered and shown in Figures 4.2-3 a, b, and c. Major peaks are peaks that have peak areas above 10% of the total peak area of the chromatogram. Complete characterisation of these samples can be confirmed by spiking, but since the final product consisted of a natural extract as opposed to separated polyphenol compounds, the identification of compounds by spiking was out of the project scope.

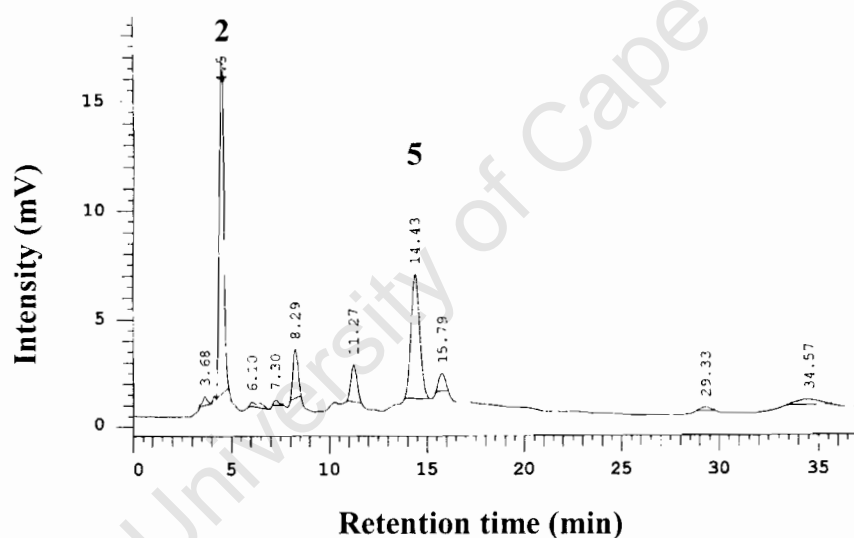


Figure 4.2-4a: HPLC chromatogram of grape marc extract obtained using ethyl acetate. The two major peaks with retention times 4.50 and 14.43 min correspond to gallic acid (2) and epicatechin (5).

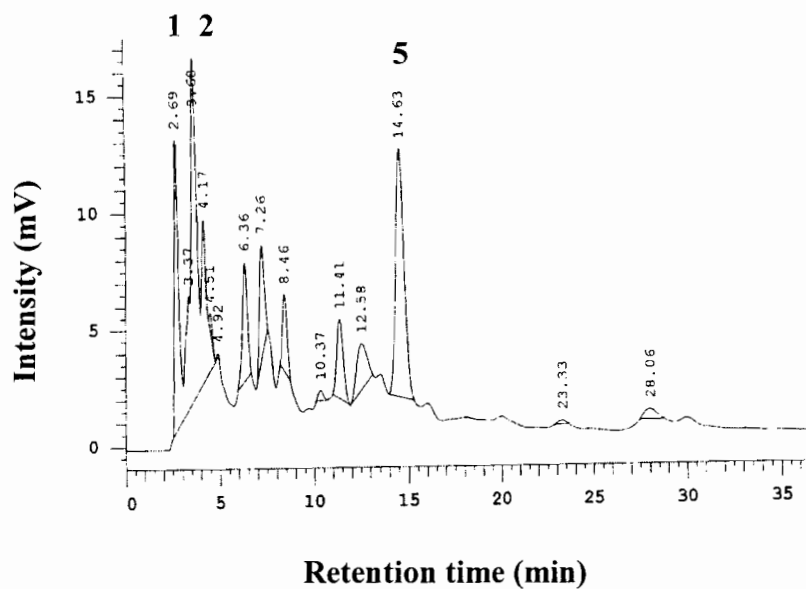


Figure 4.2-4b: HPLC chromatogram of grape marc extract obtained using ethanol. The three major peaks with retention times 2.69, 3.68 and 14.63 min correspond to resveratrol (1), gallic acid (2) and epicatechin (5).

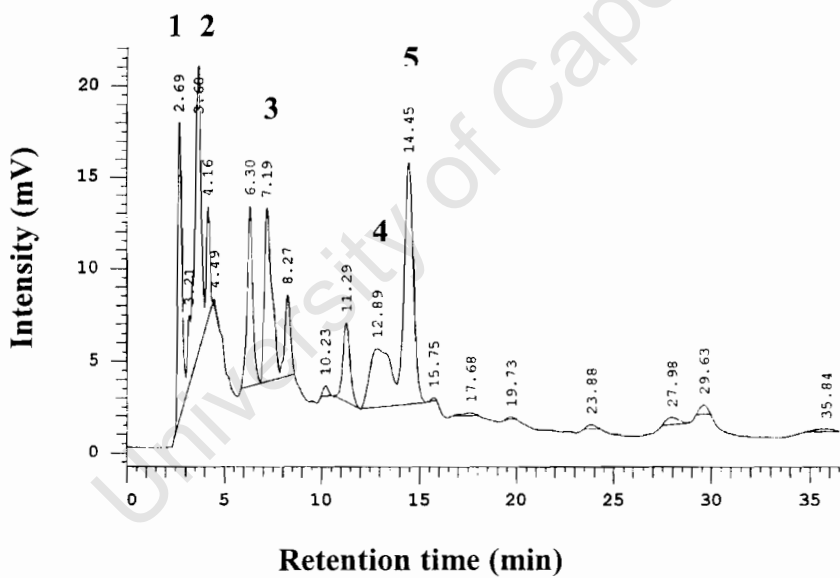


Figure 4.2-4c: HPLC chromatogram of grape marc extract obtained using methanol. The five major peaks with retention times 2.69, 3.68, 7.19, 12.89 and 14.45 min correspond to resveratrol (1), gallic acid (2), catechin (3), caffeic acid (4) and epicatechin (5).

4.2.3 Investigations into the fractionation of grape marc extracts using Solid Phase Extraction

Solid Phase Extraction (SPE) is an efficient and common fractionation method for polyphenol compounds found in grapes [Jaworski & Lee, 1987] and other fruit [Scordino *et al.*, 2005; Du *et al.*, 2004; Trappey *et al.*, 2005]. Polyphenol extracts are passed through packed columns, which adsorb the polar polyphenols (e.g. anthocyanins, flavanols, phenolic acids). These polyphenols are then eluted using specific organic solvents to produce fractionated products. Isolated polyphenol compounds have high market values and therefore, targeting production of specific groups of polyphenol classes (e.g. flavanol, anthocyanidins) would make the process more profitable than producing a crude extract with mixed polyphenol and non-polyphenol compounds. The purpose of the investigation was to determine the quantities of anthocyanins that it was possible to separate from methanol and acetone extracts using SPE. Two different SPE experiments were performed, where grape marc extracts of methanol and acetone were fractionated into neutral and acidic polyphenol fractions. According to the method of Jaworski and Lee [1987], fractionated extracts were analysed for catechin, epicatechin, proanthocyanidins B2 and B3 content. However, in this study the fractionated extracts were analysed for anthocyanin content, since anthocyanins are high value compounds with potential use as food colourants.

4.2.3.1 Fractionation of grape marc extracts obtained using methanol

The aim of the first investigation was to determine the amounts of total polyphenol and anthocyanins eluted from the cartridges. During this experiment it was noted that these results were affected by different wait times (times between pre-conditioning and loading of polyphenol extracts on SPE cartridges). Three extracts were made from separate batches of uncrushed grape marc, magnetically agitated with methanol at room temperature, using a 1:3 (w/v) ratio.

Table 4.2-2: TPC and anthocyanin yields of fractionated samples from uncrushed grape marc extracts obtained using methanol (1:3, w/v).

Sample	Wait time (h)	TPC of extract (mg/kg marc)	Anthocyanin yield (mg/kg marc)
1	24	489.4	18.6
2	6-7	405.8	33.0
3	1	421.7	39.6

Comparable yields of neutral and acidic anthocyanin fractions were noted in the eluted products (Figure 4.2-4). The highest anthocyanin yield (39.6 mg/kg marc) was obtained from the third sample which had the shortest wait time (1 h) and, correspondingly, the first sample with the longest wait time (24 h) gave the smallest anthocyanin yield of 18.6 mg/kg marc. It was suspected that long wait times caused the pre-conditioned cartridges to dry out, and thus affected the amount of anthocyanins recovered. Therefore, in the following fractionation experiment, short wait times were used (< 1h).

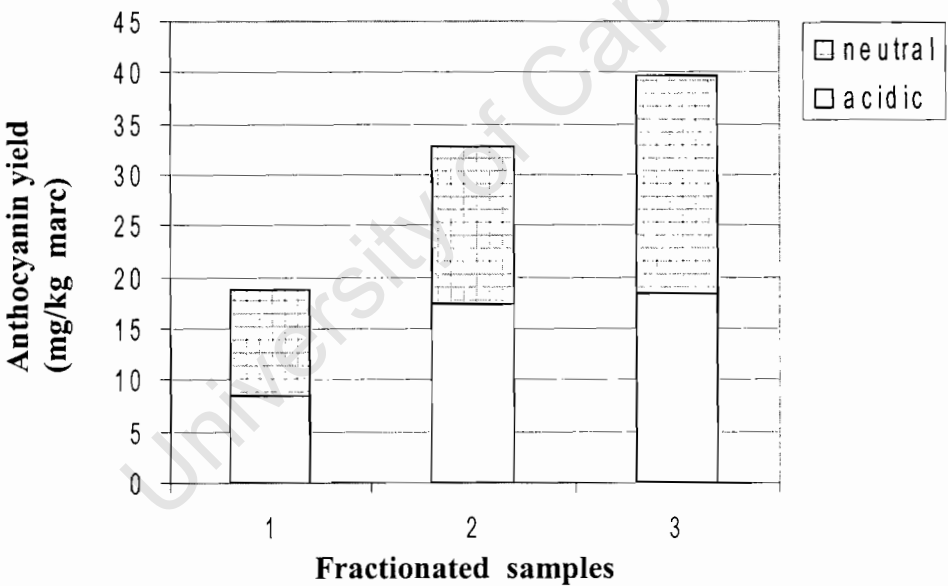


Figure 4.2-5: Neutral and acid anthocyanin yields of fractionated samples eluted from neutral and acidic SPE cartridges.

4.2.3.2 Fractionation of concentrated acetone extracts

The aim of this investigation was to determine the effect of using acetone instead of methanol, as an extracting solvent, on the anthocyanin concentration of the eluted products by SPE. Duplicate grape marc extracts were obtained by extracting 2x15 g of uncrushed grape marc with 67% acetone (1:3, w/v). These extracts were then evaporated using a rotary evaporator and re-dissolved in 16 mL acetone/water to obtain concentrated extracts. The two extracts were loaded immediately after the neutral and acidic cartridges were pre-conditioned. The neutral and acidic concentrations of anthocyanidins in Table 4.2-3 shows that the total amounts of anthocyanins fractionated from the extracts were comparable, at 157 and 188 mg/kg marc anthocyanins.

Table 4.2-3: Anthocyanidin yields of fractionated samples from acetone extracts.

Eluted fraction	Anthocyanidin yields of elutant (mg/kg marc)	
	Sample 1	Sample 2
Neutral	119	143
Acidic	38	45
	157	188

The anthocyanin yields obtained from the concentrated acetone/water extracts were more than four times higher than those obtained from methanol extracts, as expected since acetone extracts greater amounts of polyphenols than methanol (as shown later in Section 4.4.2.1). The advantages of using acetone were also recorded by Garcia-Viguera *et al.* [1998] who stated that the extraction results produced by acetone were more efficient and reproducible than those from methanol.

In order to further improve on the SPE method reported in this study, solvents with different polarities should be tested to improve the separation of polyphenol compounds. Such a fractionation method, reported by Fernandez-Panchon *et al.* [2004], uses SPE for the separation of phenolic acids, anthocyanins, flavanols and flavonols, by eluting the cartridges with different solvents. This method should be tested later for the purpose of obtaining separated polyphenol fractions, because of the limited time frame.

4.3 Investigations into the effects of grape marc pre-treatment on polyphenol extraction

Two separate investigations were performed to determine which pre-treatment methods of storage and drying would result in the highest recovery of total polyphenols. The first investigation showed the effect of storage at ambient temperature on the efficiency of extraction of polyphenols. The second investigation examined the effect of extracting polyphenols from dried grape marc.

4.3.1 Grape marc polyphenol degradation during storage under ambient conditions

Refrigerated storage is necessary for all fruits, vegetables and foods, in general, to prevent them from spoiling. This experiment was conducted to show that refrigerated storage is necessary for obtaining high polyphenol recoveries, by determining the rate of polyphenol degradation of wet grape marc stored at ambient conditions. Refrigerated storage is expensive, and hence the results from this experiment have an important impact on the profitability of the proposed process.

Extractions were conducted using three separate samples of crushed wet marc, stored for varying periods, and then magnetically agitated with organic solvent (1:3, w/v), and filtered. The graph (Figure 4.3-1) shows that as a result of two and five weeks of storage under ambient conditions in a closed container prior to extraction, the total polyphenols recovered decreased to 74% and 28% respectively, relative to the initial TPC. The total polyphenol degradation rate in red grape marc stored at room temperature (25°C) was estimated from Figure 4.3-1 to be approximately 620 mg GAE/week per kg of crushed marc.

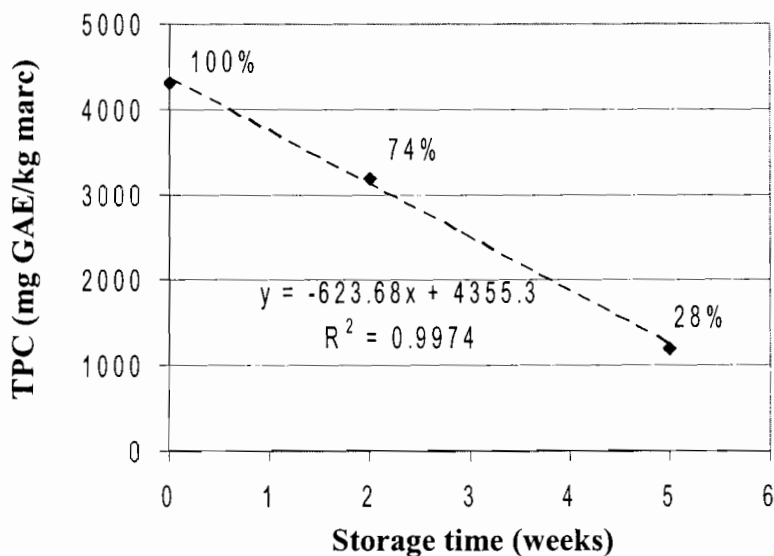


Figure 4.3-1: Total polyphenol concentrations of crushed marc extracts stored under ambient conditions over a five week period.

In this study negligible total polyphenol degradation was observed from grape marc stored at -20°C . High polyphenol extraction yields were obtained by Negro *et al.* [2003], who also used red grape marc stored at -20°C . Thus, all subsequent experiments used grape marc stored at -20°C in order to prevent polyphenol degradation. Similarly, grape marc feed should also be placed in refrigerated storage for the proposed process, provided it is economically feasible. However, -20°C is very low and it is likely that storage at temperatures closer to 0°C will be feasible. Therefore, for further research it is suggested that additional temperatures between 0°C and -20°C be investigated to determine how low a temperature is necessary for the process.

4.3.2 Comparison of polyphenol concentrations in extracts obtained using dried and wet grape marc

In this investigation, the maximum amount of polyphenols extractable from pulverised grape marc was determined using multiple-stage extractions, where the same sample of grape marc was repeatedly filtered and extracted with fresh solvent. The aim of this multiple-stage extraction experiment was to compare the recovery of polyphenols from

wet marc with that from an equivalent mass of dried marc. The motivation behind this experiment was to determine whether storing wet or dry grape marc resulted in higher total recoveries of polyphenols, since storing wet marc requires a refrigeration system with high capital costs (see Chapter 5).

Wet grape marc samples were consecutively extracted using acetone, and the total flavanols of each filtrate were quantified. This procedure was repeated using the same amount of wet grape marc in a sample which was dried prior to extraction. The extracts obtained from the wet marc showed that after three sequential extractions the cumulative concentration of flavanols was 3 559 mg CT/kg, as opposed to 794 mg CT/kg extracted from the dry marc. These results showed that the total recovery of flavanols from dried marc was only 22.3% of the flavanols recovered from wet grape marc, and hence 77.7% of the flavanols remained unextracted in the dry grape marc. Therefore, drying of the grape skins negatively affected the extraction of polyphenol compounds. High drying temperatures of 80°C may have decreased the amount of extractable polyphenol compounds through heat degradation, or drying may have changed cellular components of the grape marc so that the extraction of polyphenols was more transfer-limited after drying. The increased recovery from wet marc potentially justifies the cost of cold storage for the proposed process, because the greater the amount of polyphenols extracted the greater the sales revenue generated for the proposed process (see Chapter 5).

Table 4.3-1: Total flavanol concentrations from consecutive extractions of pulverised dry and wet grape marc extracted using acetone.

Extraction stage	Dry marc extract (mg CT/kg marc)	Wet marc extract (mg CT/kg marc)
1	545	2 413
2	136	1 031
3	113	115
Total	794	3 559

CT (catechin) is the flavanol standard.
GAE (gallic acid equivalents) is the total polyphenol standard.

Further investigations into the effect of drying grape marc by testing various drying methods, temperatures and times was not performed due to the time constraints of this project, but are worth considering in a separate study. Reasons for further investigations include the search for a drying method that does not negatively affect the polyphenol recovery from grape marc, thus eliminating the need for cold storage.

Using a curve-fitting approach, the effect of additional extraction stages was predicted to have negligible effect on the recovery of total polyphenols. This is shown in Figure 4.3-2, where an exponential decrease of total polyphenols is observed with increasing number of extractions. A fitted exponential curve illustrates that the use of four or five extraction stages would result in low additional polyphenol recoveries. Therefore, using more than three extraction stages for the extraction of polyphenols in batch mode would not be efficient for the proposed process.

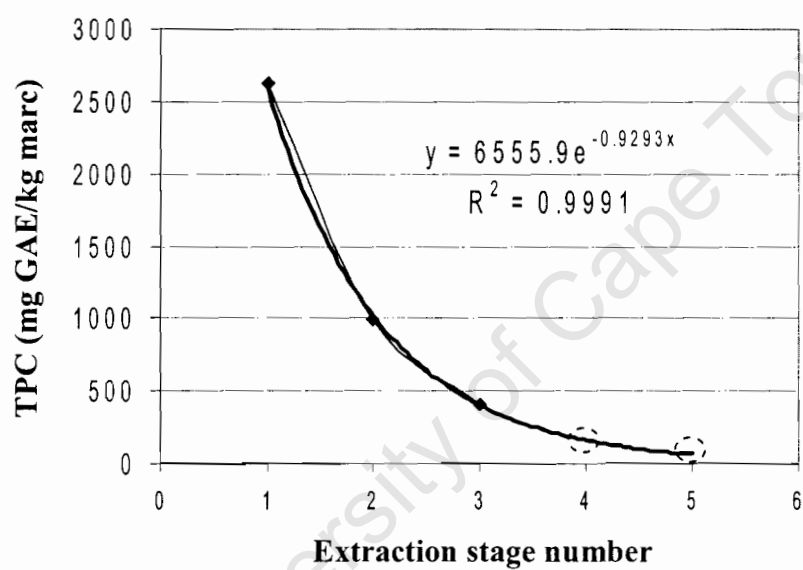


Figure 4.3-2: The exponential decrease in TPC during the sequential extraction of polyphenols from wet grape marc using acetone, showing the predicted effect of extraction stages 4 and 5.

Cumulatively, the first three stages extracted 4 030 mg/kg of total polyphenols from wet marc using acetone, 3 559 mg/kg of which were quantified as flavanols. Therefore, as an additional result, approximately 88% of the grape marc polyphenols extracted using acetone were quantified as flavanols. Flavanols are also known as proanthocyanidins,

which are the active ingredient in supplements derived from grapes. These supplement typically contain 70 mg of proanthocyanidin (see Section 4.6.3). This indicates that the majority of the polyphenols present in these extract represent a substantial amount of the polyphenols needed for the proposed process, which would be able to produce grape marc extracts for use in the nutraceutical industry.

4.4 Investigation of the effects of extraction variables on total polyphenol extract concentrations

In this section, extraction variables were examined to determine in more detail the optimal conditions to be used in the proposed process for the production of polyphenols. The extraction variables tested were solvent type, solvent concentration, extraction temperature, extraction time and the use of nitrogen headspace during the extraction stage. Only wet grape marc and single stage extractions were tested in this part of the study. The extraction variables were optimised to determine which conditions resulted in the highest TPC recoveries and which solvents produced extracts with the highest antioxidant efficacies. In the investigation of extraction time and extraction temperature, model equations were also developed to predict the extraction rate of total polyphenols.

4.4.1 Ranges for extraction variables and experimental conditions for the extraction of grape marc

It was necessary to conduct experiments to determine the optimal extraction conditions for the grape marc obtained from Stellenbosch, although these extraction variables have already been studied [Bonilla *et al.*, 1999; Negro *et al.*, 2003; Lapornik *et al.*, 2005; Louli *et al.*, 2003]. This is because the variety of grape and the conditions to which the grape marc are exposed during the wine-making process, affect the concentration and composition of the polyphenol extract. The ranges selected for testing the extraction variables were based on guidelines from the literature reports on the extraction of grape

polyphenols. In this study, solvents (acetone, ethanol, methanol, ethyl acetate, water) and solvent (in water) concentration ranges (25, 50, 75, 100%) were investigated first, followed by various extraction times (0-30 min), the use of a nitrogen headspace and lastly extraction temperatures (25-50°C). Several temperatures were selected for each solvent-marc system, to determine a total polyphenol concentration-temperature relationship. Although size reduction of grape marc is a pre-treatment parameter rather than an extraction variable, it was included in this section and not with the pre-treatment investigations because of the strong influence of grape marc size reduction on the TPC of extracts during the extraction time and temperature investigations. The extraction variables and experimental conditions used in this study are shown in Table 4.4-1.

Table 4.4-1: Summary of the conditions used to investigate extraction variables in magnetically stirred extraction systems.

Section	4.4.2	4.4.3	4.4.4	4.4.5	4.4.6
Extraction variable	Solvent type	% solvent	Extraction time	Nitrogen	Temperature
Solvent types	A, E, M, EA, W	A/W E/W	A/W E/W	A/W E/W	A/W E/W
Solvent concentrations	100% 50%	0, 25, 50, 75, 100%	67%	67%	67%
Grape marc (g)	15	33	33	15	15
Pre-treatment of wet marc	Uncrushed	Crushed	Crushed, uncrushed	Crushed	Crushed, uncrushed
Solvent (mL)	45	100	100	45	45
Extraction time (min)	60	15	0-30	20	20
Temperature	T _r	T _r	T _r	T _r	25-50°C
Atmosphere	air	air	air	N ₂	air
Agitation speed (rpm)	250	500	500	250	250
Extraction vessel (mL)	200	250	250	250	200
Analysis	TPC, DPPH	TPC	TPC	TPC, DPPH	TPC, DPPH

A, E, M, EA, W refer to acetone, ethanol, methanol, ethyl acetate and water respectively. T_r = 25°C.

4.4.2 Investigation of the effect of solvent type on the extraction recovery of grape marc polyphenols

4.4.2.1 Total polyphenol concentrations of different solvent extracts

The TPC of extracts obtained using acetone, acetone/water, ethanol, ethanol/water, methanol, methanol/water, ethyl acetate and water were compared to determine the effect of solvent polarity on the amount of total polyphenols recovered using the Folin Ciocalteu method. Marc/solvent mixtures were magnetically agitated in open beakers at 25°C. After extracting the marc for 1 h, the mixtures were filtered and the extracts were stored at 4°C.

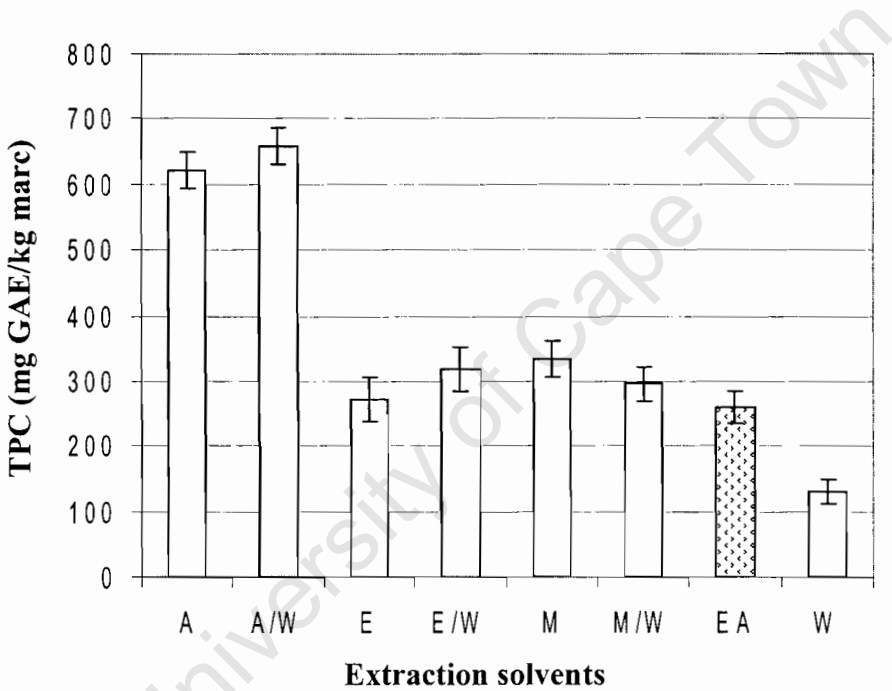


Figure 4.4-1: Total polyphenol concentrations of the polyphenol extracts obtained from uncrushed marc using: acetone (A), 50% acetone (A/W), ethanol (E), 50% ethanol (E/W), methanol (M), 50% methanol (M/W), ethyl acetate (EA), water (W).

In these experiments, the TPC from extracts obtained using different organic solvents varied from 250 to 650 mg GAE/kg, whereas the TPC of water extracts was the lowest, at 130 mg GAE/kg. Figure 4.4-1 shows that the acetone extract contained approximately twice the TPC of ethanol or methanol extracts. Similar results, obtained from grape seeds, showed that acetone extracted 1.5 times more total polyphenols than ethanol or methanol [Yilmaz & Toledo, 2006]. The recoveries of total polyphenols from ethanol and methanol extracts were found to be 2 to 3 times higher than those from pure water extracts. These results are also in agreement with the findings reported by Lapornik *et al.* [2005], obtained by extracting grape seeds for 1 h at room temperature, where the TPC of ethanol and methanol extracts were also higher (4 to 5 times) than the TPC of water extracts.

4.4.2.2 Solvent choice for polyphenol extraction process

In order to select an extraction solvent for the proposed process producing non-toxic extracts, free radical scavenging investigations were conducted, focussing on ethanol and ethyl acetate as possible extraction solvents, while acetone extracts were used as a bench mark for the maximum antioxidant capacity of natural grape marc extracts. Although acetone has shown to extract the highest amount of total polyphenols (Figure 4.4-1), for toxicological reasons it would not be a good solvent choice for a process that produces extracts for human consumption [www.terressentials.com]. However, acetone would be a good solvent choice for a process producing non-consumable extracts to be used in products or materials requiring protection against oxidation. Such investigations of acetone extracts are outside of the scope of this study and were consequently not researched.

The antioxidant activity of extracts (produced from either ethanol or ethyl acetate) was then considered in order to select the extraction solvent for the proposed process. Crushed grape marc was extracted at room temperature, then filtered and analysed using the Folin Ciocalteu and DPPH assays. The results were analysed to give the EC_{50} concentrations of the grape marc extracts. As expected, the acetone extract showed the highest antioxidant capacity corresponding to the lowest EC_{50} concentration of 36 mg

phenol/g DPPH, while ethanol and ethyl acetate extracts exhibited EC_{50} concentrations of 65 and 99 mg phenol/g DPPH respectively (Figure 4.4-2). Therefore, approximately 65 mg of polyphenols from the ethanol extract would be required to quench 0.5 g (50% of 1 g) of DPPH, and similarly 99 mg of polyphenols from the ethyl acetate extract would be required to quench 0.5 g of DPPH. The reason why less polyphenols from the ethanol extract are required is that ethanol extracts different polyphenol compounds than ethyl acetate. Differences in polyphenol compounds extracted (shown by HPLC in Section 4.2.2) are mainly due to the polarity difference between the solvents, where ethanol is more polar than ethyl acetate, and therefore extracts more polar polyphenol compounds. Hence ethanol was selected as the extraction solvent for the process producing food grade extracts, based on the results that the EC_{50} concentration of ethanol extract was lower than ethyl acetate extract.

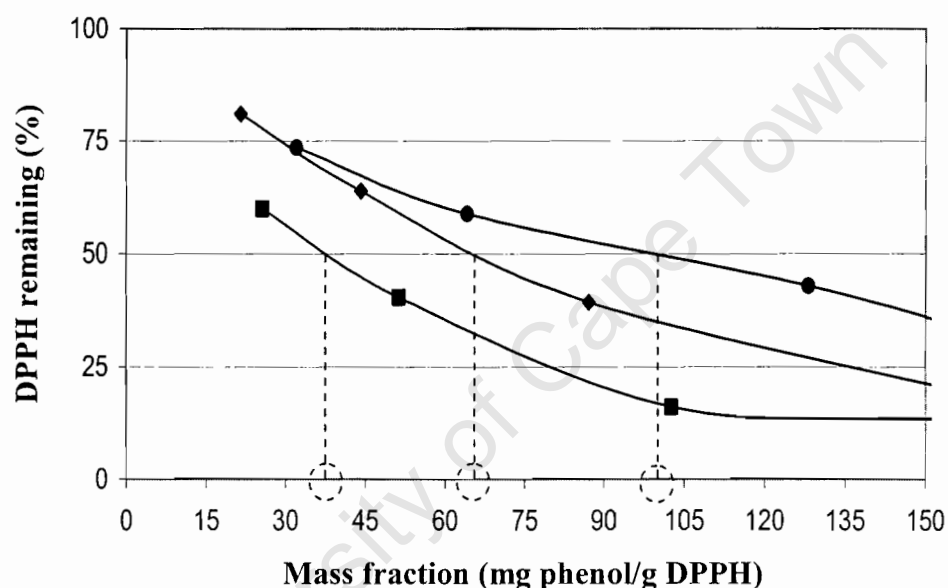


Figure 4.4-2: The EC_{50} concentrations obtained from grape marc extracted at 25°C using ethyl acetate ●, ethanol ◆ and acetone ■ solvents.

In support of the selection of ethanol as an extraction solvent for the proposed process, recent literature has stated that ethanol was used to extract polyphenols from grape pomace and grape seeds based on its efficient polyphenol extracting abilities [Lu & Foo, 1999; Peng *et al.*, 2001]. In another study, ethanol/water (80%) was selected as a more efficient extracting solvent than either acetone/water (75%) or ethyl

acetate/methanol (50%) for the extraction of resveratrol [Romero-Perez *et al.*, 2001]. Furthermore, ethanol present in wines (12-14% alc.) is also the natural solvent for polyphenol compounds.

4.4.3 Effect of varying acetone and ethanol concentrations on TPC in extraction of grape marc

Crushed grape marc was extracted using varying solvent concentrations of acetone and ethanol in water. This investigation was conducted to identify the best conditions for polyphenol recoveries at room temperature, by using varying acetone and ethanol concentrations of 0, 25, 50, 75, 100 % (v/v). After the extraction stage, the grape marc/solvent mixtures were filtered and the extracts were stored at 4°C until analysed.

Experimental results showed that acetone/water extracts and ethanol/water extracts obtained using 50% (v/v) solvents gave higher TPC than extracts obtained using 0%, 25%, 75% or 100% solvent. The TPC of 50% acetone and 50% ethanol extracts were 1700 ± 54 mg/kg and 760 ± 60 mg/kg marc respectively, and extracts obtained using pure water had the lowest TPC of 415 ± 25 mg/kg marc. The results from this investigation, using *Vitis vinifera* grape marc, were compared with the results by Yilmaz and Toledo [2006], who used grape seed extracts from the *Vitis rotundifolia* (Muscadine) grape species to determine the TPC-solvent concentration trends. The results of Yilmaz and Toledo [2006] showed close similarity with maximum TPC also at 50% solvent concentrations (see Figure 4.4-3). They further mentioned that single component solvent systems (pure acetone or ethanol) were inefficient for polyphenol extraction.

In a second study, a suggested explanation for the higher TPC of solvent/water extracts, as opposed to pure solvent extracts, was that water increases the permeability of grape marc and therefore increases the mass transport of polyphenols by diffusion

[Jayaprakasha *et al.*, 2001]. In a third study, it was also suggested that most of the water-soluble polyphenols had already been extracted from the grape marc during the wine making process, where crushed grapes ferment in weak ethanolic solutions of 0 to 12% alcohol. On this basis, more concentrated alcohols (70% ethanol and 70% methanol) were used by Lapornik *et al.* [2005] to extract polyphenols. These findings [Yilmaz & Toledo, 2006; Lapornik *et al.*, 2005; Jayaprakasha *et al.*, 2001] support the TPC-solvent concentration trends shown in Figure 4.4-3. Thus, the experimental results obtained in this study, supported by recent literature, shows that pure water or pure solvents (100% ethanol or 100% acetone) are inefficient solvents for extraction of grape marc polyphenols. More importantly, the findings in Figure 4.4-3 show that in order to maximise the amount of total polyphenols extracted, under the conditions used in this investigation, 50% solvent concentrations of acetone or ethanol should be used.

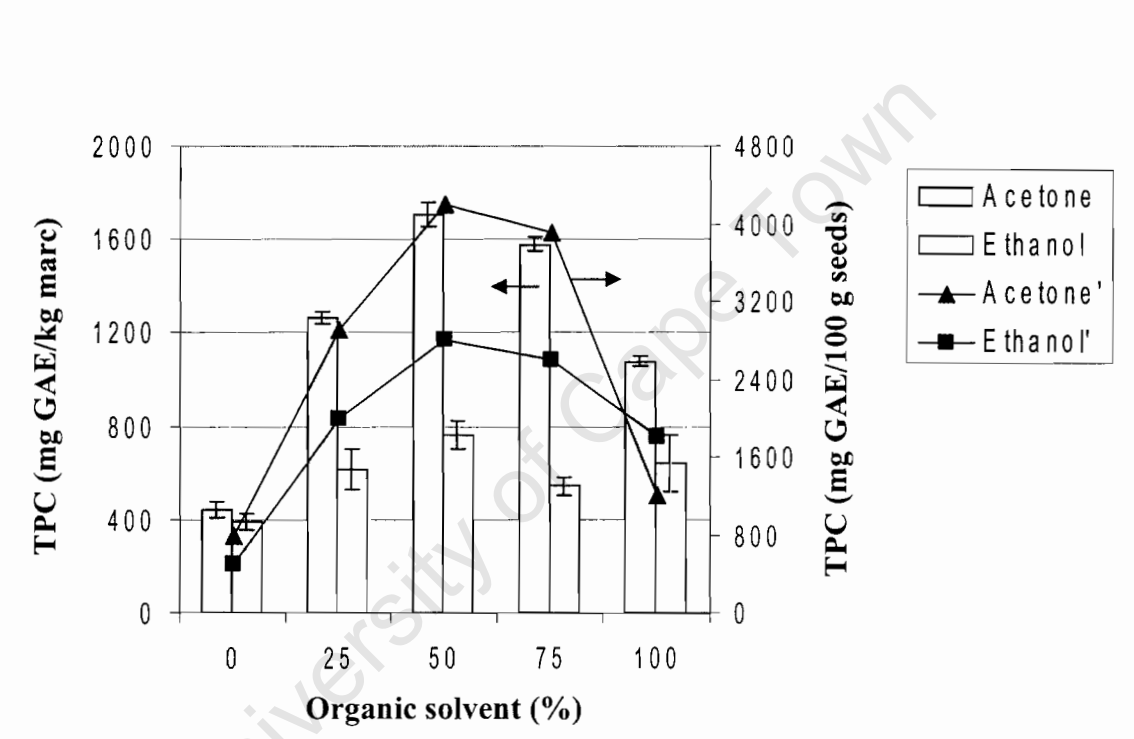


Figure 4.4-3: TPC profiles of acetone and ethanol extracts (primary axis) obtained experimentally from crushed grape marc, using 0, 25, 50, 75 and 100 % solvent. The TPC profiles of acetone' and ethanol' extracts obtained by Yilmaz and Toledo [2006] were included on the secondary axis.

4.4.4 Investigation of polyphenol mass transfer kinetics during grape marc extraction

4.4.4.1 Background for the mass transfer of polyphenols

In this investigation, a mass transfer model was developed to predict the extraction of total polyphenols over time in agitated grape marc/solvent systems. According to Mantell *et al.* [2002], there are two useful models describing the extraction process from a solid substrate, namely the penetration model and the film model. The penetration model provides a more representative and realistic description of the extraction process. This model has good correlations with experimental results, at high flow rates, from data obtained using packed beds operating in semi-batch mode conditions. In contrast, the film model is more suited to batch processes where the solvent has adequate time to diffuse into the material. Since extractions in this study were performed in batch mode, the film model was used to determine the mass transfer constants for polyphenols.

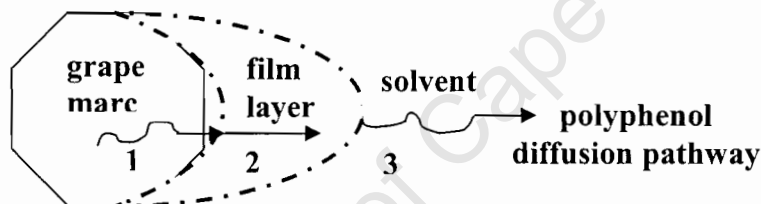


Figure 4.4-4: Schematic representation of polyphenol mass transfer steps using the film model in non-agitated systems [Thomson, 2000].

The film model (Figure 4.4-4) describes the transfer of polyphenols in three steps, namely the diffusion of the polyphenols in the marc to the outer skin layer (step 1), the mass transfer through the liquid film (step 2) and the diffusion into the bulk liquid phase (step 3). The transfer step that slows the extraction rate of polyphenols most is called the limiting step. The limiting step for a static mixture of marc and solvent would be the mass transfer through the film layer (step 2), which is the liquid film surrounding the grape skin. However, as the marc-solvent slurry is agitated, this film layer becomes

negligible and the transfer of polyphenols can be modelled using the concentration difference between the grape skin and bulk solvent. The polyphenol concentration in the grape skin decreases over time, while the polyphenol concentration in the solvent increases due to the mass transfer of polyphenols (Figure 4.4-5). Hence, the limiting step for the agitated marc systems in this study was hypothesised to be the transfer of polyphenols from the grape skin into the surrounding solvent.

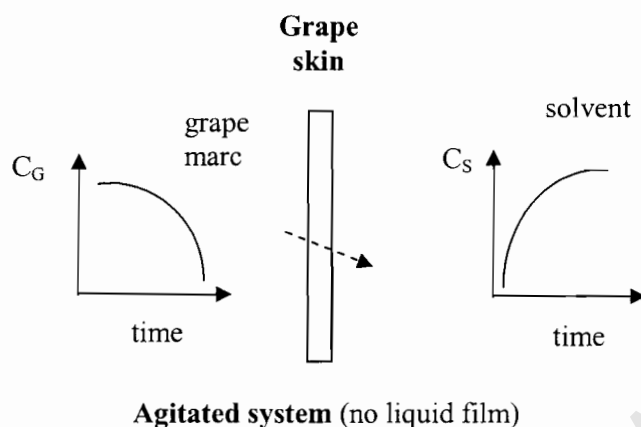


Figure 4.4-5: A simplified illustration of an agitated extraction system showing the resistance from grape skin to the mass transfer of polyphenols.

4.4.4.2 Modelling the mass transfer of polyphenols

In this study, mass transfer model equations describe the extraction of polyphenols from the grape marc into the solvent. Samples were taken during the extraction process to compare the experimental TPC data with the predicted TPC results using first order mass transfer kinetics. The mass transfer coefficient (k , min^{-1}) is a constant that relates the rate of polyphenol extraction to the polyphenol concentration gradient ($C^* - C_S$), the difference between the maximum polyphenol concentration and the instantaneous polyphenol concentration. Mass transfer coefficients are a function of the solvent type, grape marc particle size and temperature. Equation 8 describes the rate of change of polyphenol concentration in the solvent over time, and can be integrated to produce Equation 9 (see Appendix A-2 for derivation).

$$\frac{dC_s}{dt} = k(C^* - C_s)^n \quad \dots \text{Equation 8}$$

$$C_s = C^* (1 - \exp^{-kt}) \quad \dots \text{Equation 9}$$

In this model it was assumed that extraction systems were well mixed so that the solvent surrounding the marc had the same polyphenol concentration as the bulk solvent. On the assumption that there were no polyphenol concentration gradients in the solvent, samples taken at time intervals from the bulk solvent were taken to be representative of the solvent surrounding the marc. It was also assumed that the maximum polyphenol concentration in the solvent (C^*) did not vary during grape marc extractions, provided the extraction conditions (temperature, agitation speed) were constant.

4.4.4.3 Testing the effect of grape marc size reduction using the mass transfer model

Grape marc size reduction was investigated since it has been shown to have an important effect on the extraction rate of polyphenols [Bonilla *et al.*, 1999; Pinelo *et al.*, 2005]. The aim of this investigation was to compare the mass transfer coefficients of polyphenols in crushed and uncrushed marc using the mass transfer model described by Equation 9.

During the extraction of polyphenols, solvent samples were collected and analysed for TPC. In Figure 4.4-6, the TPC-time profiles of four extraction systems, using uncrushed and crushed marc extracted with acetone/water and ethanol/water, are shown. The steep initial concentration-time slope found for the crushed marc system extracted using acetone/water indicated that the mass transfer coefficient was higher in this case than the other three extraction systems. An approximate twofold increase in TPC is shown by comparison of the crushed and uncrushed grape marc extracts obtained from ethanol

or acetone. Similar findings were recorded by Bonilla *et al.* [1999], where a two- to three-fold increase of polyphenols resulted from using crushed grape marc.

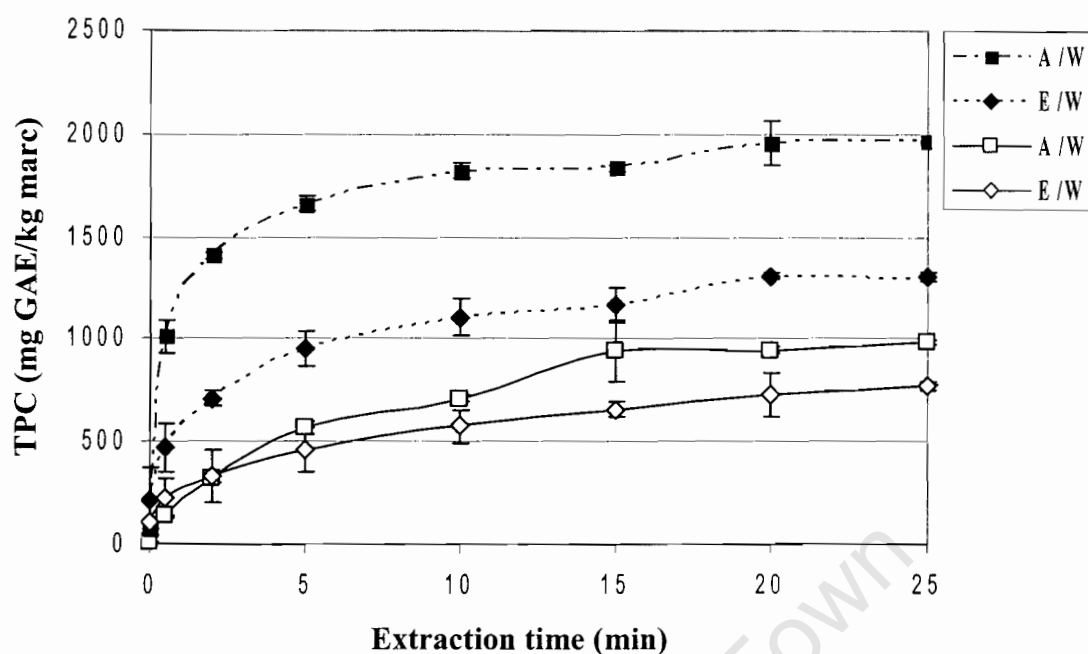


Figure 4.4-6: Measured TPC in samples of acetone/water (A/W) and ethanol/water (E/W) extracts obtained at 25°C using crushed (dotted lines) and uncrushed grape marc (solid lines).

Figures 4.4-7 and 4.4-8 were obtained from Figure 4.4-6. High R^2 values indicate that the proposed first order mass transfer model provides an accurate prediction of TPC in solution during small scale extractions. The procedure used to plot the model graphs shown in Figures 4.4-7 and 4.4-8 is briefly outlined: Equation 9 calculated the predicted TPC (C_s) by using the final TPC obtained from the experimental data as C^* and an estimated k value as input values for Solver (Excel). The instantaneous polyphenol concentrations (C_s) were then calculated for every sample time. Then, the total sum of squares of the difference between the experimental and predicted data, at each sample time, was minimised using Solver, by varying the C^* and k values.

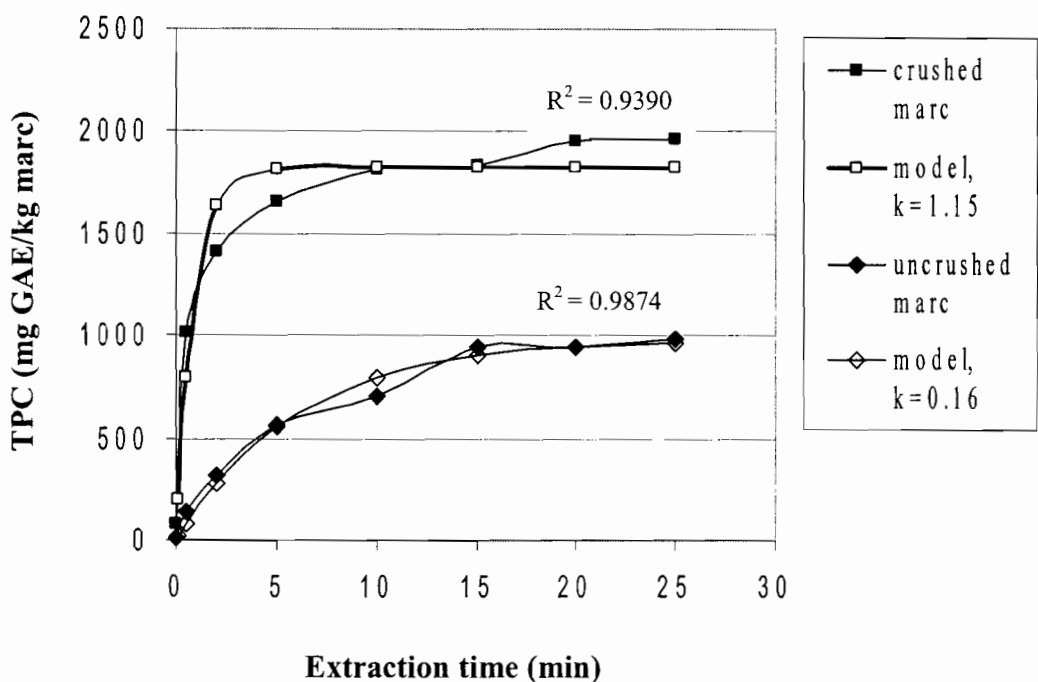


Figure 4.4-7: Acetone/water extractions graphs showing modelled and experimental total polyphenol concentrations obtained using crushed and uncrushed grape marc. $TPC = C_s$.

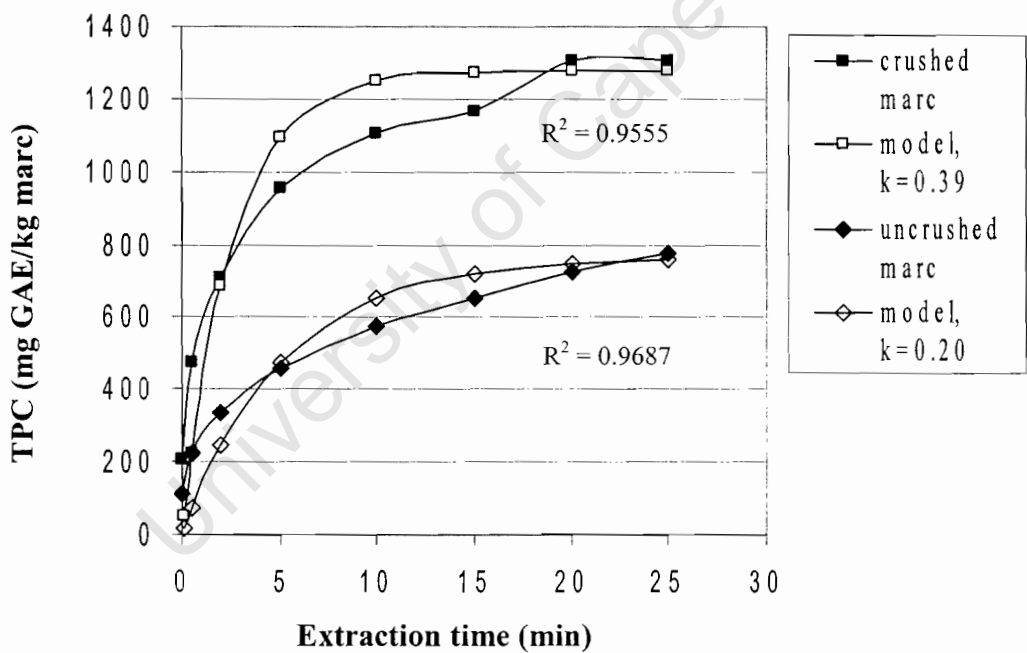


Figure 4.4-8: Ethanol/water extraction graphs showing predicted and experimental total polyphenol concentrations obtained using crushed and uncrushed marc. $TPC = C_s$.

The maximum polyphenol concentrations and mass transfer coefficients from Figures 4.4-7 and 4.4-8 are shown in Table 4.4-2. From this table differences in the mass transfer coefficients are clearly evident. For the mass transfer coefficients of the ethanol extracts, the extraction of crushed marc gave a twofold higher mass transfer coefficient (0.39 min^{-1}) than the extraction of uncrushed marc (0.20 min^{-1}). Similarly, the mass transfer coefficients for acetone extracts show a seven fold increase for the crushed marc over the uncrushed grape marc extract (1.15 and 0.16 min^{-1} respectively). These results show that the overall mass transfer coefficients for extracts depend significantly on the degree of grape marc crushing and the extraction solvent selected. Results from both ethanol and acetone extracts also reconfirms literature findings, which indicate that crushed marc produced extracts with higher total polyphenol concentrations than uncrushed marc [Bonilla *et al.*, 1999; Pinelo *et al.*, 2005]. A possible reason for this is that the polyphenols in crushed grape marc have less mass transfer resistance, because the damaged cell walls allow more efficient polyphenol extraction. Hence larger quantities of polyphenols were extracted at faster rates than uncrushed grape marc. Thus the use of blending or cutting machines is recommended for the process design.

Table 4.4-2: Calculated saturation concentrations and mass transfer coefficients for polyphenols obtained at 25°C from acetone and ethanol extracts.

Mass transfer constants	Units	Crushed marc		Uncrushed marc	
		A/W	E/W	A/W	E/W
C*	mg GAE/kg	1820	1275	985	760
k	min ⁻¹	1.15	0.39	0.16	0.20

Polyphenol saturation concentration or steady state TPC (C*), polyphenol mass transfer coefficient (k).
A/W = acetone/water, E/W = ethanol/water.

Comparable mass transfer coefficients were reported by Cho *et al.* [2005] who monitored the extraction of resveratrol from grape stems using ultrasonicated-assisted extraction, where values of 0.78 and 0.48 min^{-1} were reported for Campbell and Gerbong grape cultivars respectively. The differences between mass transfer

coefficients determined in the present study and those by Cho *et al.* [2005] can be attributed a number of factors, namely differences in the grape variety, grape material (grape marc versus grape stems), the extraction method (stirred-assisted extraction versus ultrasonicated-assisted extraction), the method of polyphenol analysis (UV-vis spectrophotometer versus HPLC), and the model compounds used as the basis for analysis (gallic acid versus resveratrol).

4.4.5 Effect of nitrogen headspace on antioxidant activity of polyphenols obtained using solvent extraction

Nitrogen is an inert gas and would therefore prevent, at least partially, extracted polyphenols from oxidising in solution. Different extraction methods, such as pressurised liquid extraction and supercritical fluid extraction, involve the use of inert atmospheres to prevent polyphenol degradation [Palma *et al.*, 2002; Palma & Talyor, 1999].

The purpose of this experiment was to investigate the effect of extracting polyphenols under a nitrogen atmosphere from crushed grape marc. It was expected that extracts obtained under nitrogen would retain greater antioxidant efficacies than extracts extracted under an air atmosphere. The results from the DPPH assay for acetone/water and ethanol/water extracts are shown in Figures 4.4-9 and 4.4-10. The EC_{50} concentrations for acetone/water extracts extracted under nitrogen or air headspace, were 44 and 37 mg phenol/g DPPH respectively. These results were different from the expected results, since the extract produced under nitrogen showed less free radical scavenging capacity than that produced in air. For ethanol/water extracts, the EC_{50} concentrations for extracts produced in air and nitrogen were identical at 64 mg phenol/g DPPH, although the extract produced under nitrogen showed a slightly better efficacy, with less than 35% DPPH remaining (see Figure 4.4-10).

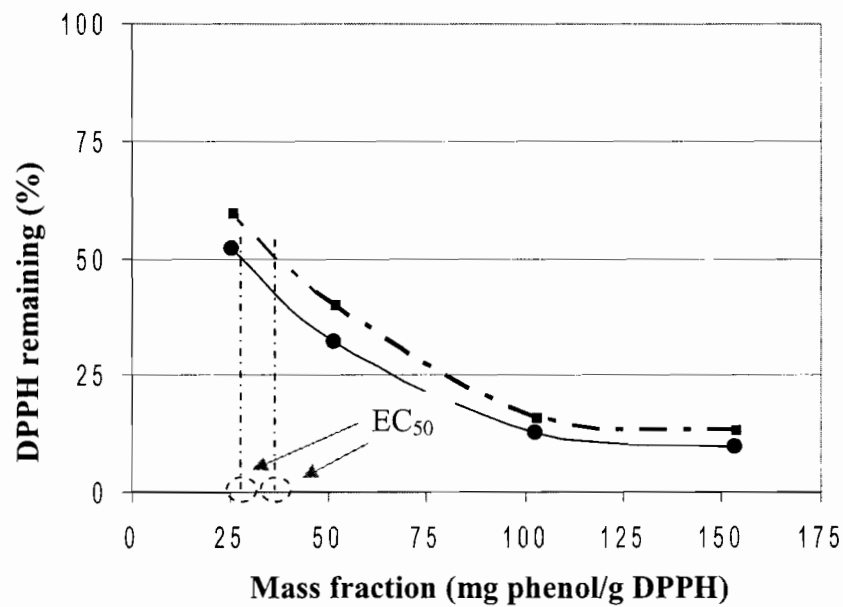


Figure 4.4-9: Free radical scavenging activity of acetone/water extracts obtained under nitrogen- and air-atmosphere.

Nitrogen ----- Air —————

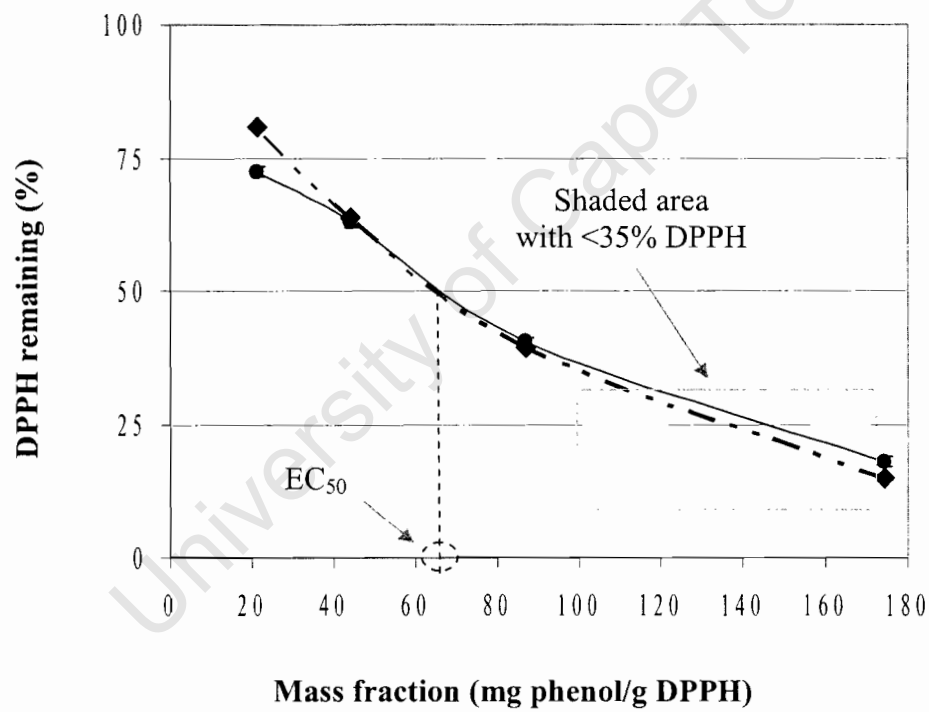


Figure 4.4-10: Free radical scavenging activity of ethanol/water extracts obtained under nitrogen- and air-atmosphere.

Nitrogen ----- Air —————

These results showed that there was no advantage in using a nitrogen atmosphere during stirred-assisted extraction of crushed marc using ethanol/water or acetone/water solvent, since they do not produce extracts with enhanced antioxidant activity. Therefore, the use of nitrogen was not recommended during the extraction stage of the proposed process.

4.4.6 Effect of temperature on the extraction of polyphenols using ethanol and acetone

The extraction capabilities of heated solvents were tested by measuring TPC and free radical scavenging activity of extracts at higher temperatures than the room temperatures previously used in this study. Higher extraction temperatures would be expected to increase the amount of polyphenol compounds extracted, because of increased polyphenol diffusivities [Pinelo *et al.*, 2005]. Acetone/water and ethanol/water solvents were heated in Schott bottles to desired temperatures before the grape marc was added. These extraction systems were agitated with grape marc for 20 min and then filtered. From Figure 4.4-11, first order relationships were observed in the four linear graphs relating the TPC to extraction temperature (T) described by Equation 10.

$$C_s = \Theta * T + \text{constant}$$

... Equation 10

The equations fitted to the graphs allow for the TPC (C_s) to be predicted, provided that the extraction temperatures are specified. The gradients of the lines from this data are polyphenol temperature coefficients (Θ), expressed in units of mg/kg marc per °C. The values of temperature coefficients are specific to the conditions of this investigation, and are thus only used to compare the data depicted in Figure 4.4-11.

4.4.6.1 Total polyphenol analysis of extracts obtained at various extraction temperatures

Four separate batch extractions were performed on crushed and uncrushed marc using acetone/water and ethanol/water. These results show that for each of the four extraction systems, agitated for the same amount of time of 20 min, higher solvent temperatures resulted in more total polyphenols being extracted. Extracting with crushed marc (as opposed to uncrushed marc) also allowed more polyphenols to be extracted using either acetone or ethanol. Linear trend lines, fitted to the data points in Figure 4.4-11, showed a good correlation to the data with R^2 values greater than 0.9660.

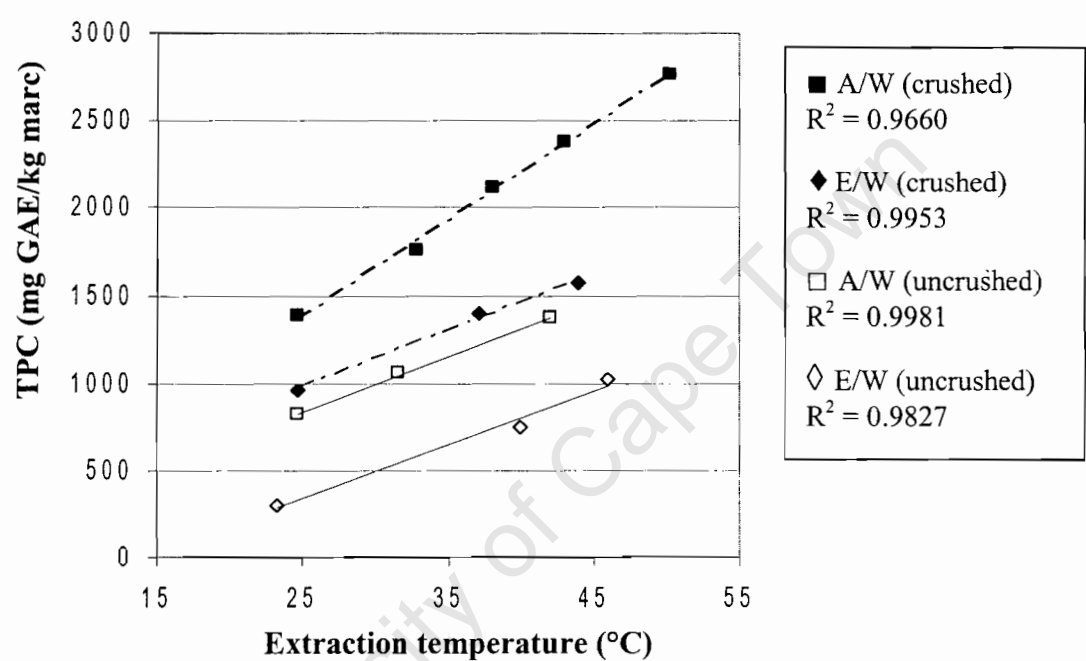


Figure 4.4-11: TPC measured for extraction of crushed (dotted line) and uncrushed grape marc (solid line) using acetone/water (A/W) and ethanol/water (E/W). $TPC = C_S$.

For the ethanol/water systems, the lines fitted for the crushed and uncrushed marc systems showed similar temperature coefficients (31.9 and 30.3 mg/kg per °C respectively), which means that the particle size of grape marc did not significantly affect the temperature coefficients (Θ) for extraction with ethanol/water. However, with

use of acetone/water, a significant difference in temperature coefficients was observed (55.0 and 31.5 mg/kg per °C respectively), showing that the temperature coefficients of acetone/water system was influenced by grape marc size reduction. Acetone, which had previously been identified as the solvent with the highest polyphenol extracting capacity (Sections 4.4.2), also showed that its thermodynamic properties as a solvent are superior to those of ethanol, based on the higher polyphenol temperature coefficients observed.

Table 4.4-3: Equations and polyphenol temperature coefficients fitted to data for correlation of TPC with extraction temperature.

Solvent	Marc type	TPC equations (mg GAE/kg)	Θ (mg/kg per°C)
A/W	Crushed	$y = 55.0 \cdot T + 0.2$	55.0
E/W		$y = 31.9 \cdot T + 181.0$	31.9
A/W	Uncrushed	$y = 31.5 \cdot T + 48.2$	31.5
E/W		$y = 30.3 \cdot T - 413.3$	30.3

Equations in this table model the TPC of various extracts taken between 25 and 50°C using straight line graphs. $y = \Theta \cdot T + \text{constant}$, where Θ is the polyphenol temperature coefficient and T is the extraction temperature in °C.

4.4.6.2 The effect of extraction temperature on DPPH quenching ability of extracts

The free radical scavenging activities of acetone and ethanol extracts obtained at three different temperatures were determined using the DPPH assay. A short quenching time of 8 min was observed, resulting from the high polyphenol sample concentrations in the extracts used. The results from this experiment, shown in Table 4.4-4, were comparable because the extracts were obtained using the same amounts of grape marc and solvent (1:3, w/v). As expected, the data shows that the % DPPH remaining in experiments using acetone extracts was found to be slightly lower than ethanol extracts for each extract concentration tested. This indicates that the acetone extracts quench more free radicals and thus have higher antioxidant efficacies, as previously shown in Sections

4.4.2.2 and 4.4.5. The DPPH activity results also showed that the increase in temperature during extraction (up to 44°C) did not significantly affect the free radical scavenging activity of ethanol or acetone extract.

Table 4.4-4: % DPPH remaining after 8 min, in DPPH assays of acetone and ethanol extracts obtained at varying extracting temperatures.

Extract concentrations (mg GAE/L)	Extraction temperature (°C)	% DPPH remaining	
		Ethanol extract	Acetone extract
320	25	16.25	8.56
464	38	13.20	8.18
524	44	15.03	11.29

Although extraction temperatures up to 50°C were used in this study (Figure 4.4-11), and by Pinelo *et al.* [2006], higher temperatures have been reported in similar studies. An extraction temperature of 60°C was reported Romero-Perez *et al.* [2001] as an optimal temperature for resveratrol extraction from grape stems using 80% ethanol. However, caution must be taken using high extracting temperatures for long time periods. This was suggested by Romero-Perez *et al.* [2001], who reported that extractions at 25°C for 2 h showed higher resveratrol recoveries than recoveries at 60°C for 2 h, because the higher extraction temperature (60°C) caused phenols to degrade. The results from this study and from literature indicate that an operating temperature between 44°C and 60°C needs to be explored further in a later study, using short extraction times (<2 h) to avoid phenol degradation and hence the possible loss of free radical scavenging activity.

4.5 Investigating the effect of evaporation of solvent

The aim of this stage of investigation was to test the activity of polyphenol extracts before and after removal of the solvent using the rotary evaporator, to determine the effect of solvent evaporation on the activity of the extracts. Since the rotary evaporator was operated under conditions of reduced pressure, lower evaporation temperatures of 45°C and 36°C were expected to have negligible effect on the free radical scavenging activity of evaporated ethanol and ethyl acetate extracts respectively [Louli *et al.*, 2003].

Ethanol and ethyl acetate grape marc extracts were obtained after extraction and filtration of solvent-marc systems. Once the solvents were evaporated, the extracts were subsequently re-dissolved for analysis (see Section 3.8.6). The results of the DPPH quenching activity measurements were used to construct the EC₅₀ concentration graphs (Figure 4.5-1, Figure 4.5-2). The EC₅₀ concentration for the aliquots of grape marc extracts obtained using ethanol was 56 mg phenol/g DPPH for the filtered extracts before evaporation, and 64 mg phenol/g DPPH for the dried extract. This suggests that there was a small degradation effect on polyphenol compounds due to the evaporation of ethanol. The EC₅₀ concentrations for ethyl acetate extracts were found to be approximately 93 mg phenol/g DPPH before and after the rotary evaporation stage. Thus, the effect of solvent evaporation at 36°C had no obvious effect on the efficacy of ethyl acetate extract.

The lower EC₅₀ concentrations of ethanol extracts observed here also verifies the selection of ethanol as an extraction solvent for the polyphenol production for food grade additives (see Section 4.4.2.2). Despite the small amount of degradation on the ethanol extract, rotary evaporation is a good and effective method of solvent separation for the proposed process, where the evaporated solvent can be recycled back to the extraction stage. This solvent separation stage is likely to be a highly energy intensive operation for the proposed process.

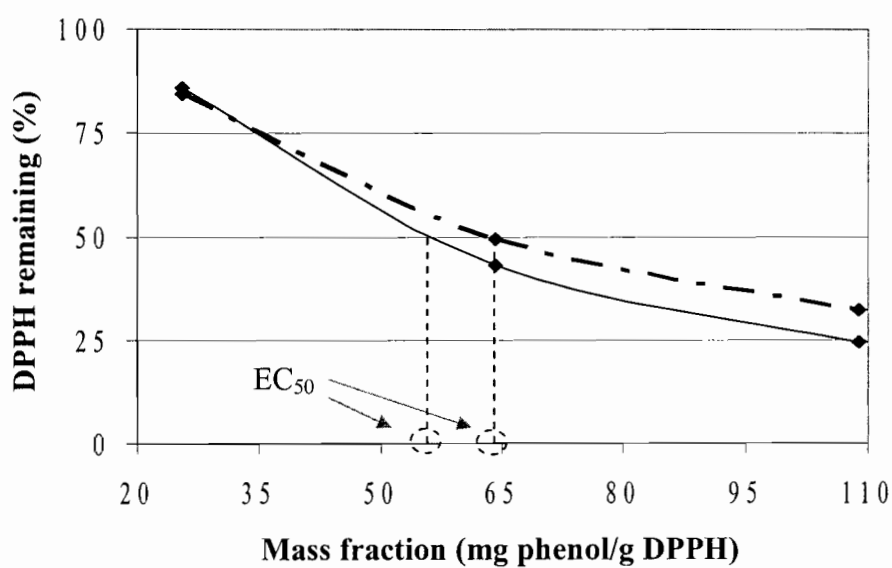


Figure 4.5-1: Graphs showing the EC_{50} concentration for DPPH activity of ethanol extracts before and after solvent evaporation.

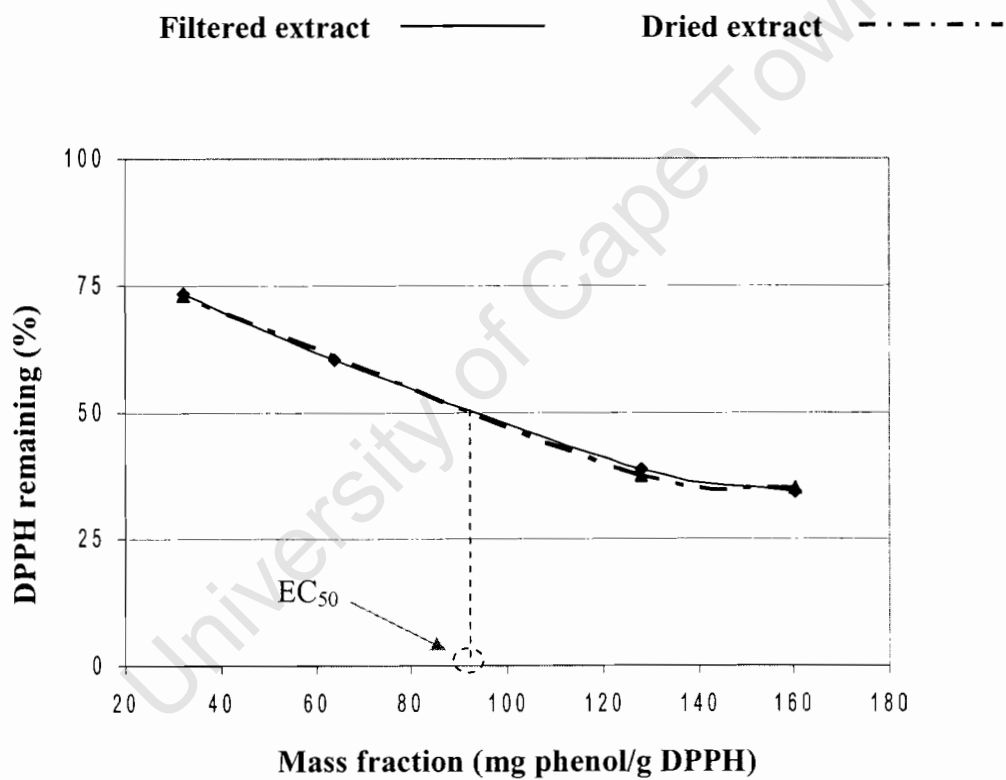


Figure 4.5-2: Graphs showing the EC_{50} concentration for DPPH activity of ethyl acetate extracts before and after solvent evaporation.

4.6 Investigations of scaled-up extraction and characterisation of ethanol/grape marc extracts

4.6.1 Scaled-up mechanically stirred extractions from grape marc using 50% ethanol

Extractions were performed using an axial-flow turbine in a 1.2 L cylindrical, glass extraction vessel. An axial flow turbine was selected because it has a higher solid suspension efficiency than a radial flow turbine [Ditl & Reiger, 2006], and also because the magnetic stirring equipment available would have been ineffective at agitating the 1.2 L extraction volume. The use of the axial flow turbine simulated the extraction method intended for use in the design of a large-scale extraction process (see Section 5.5.1). The TPC yields obtained from these experiments would be used as a basis for determining the total amount of polyphenols that could be produced in scaled-up process operations. 50% ethanol was used in these experiments, because among the ethanol concentrations tested, 50% ethanol had previously shown the highest recoveries of TPC. Ethanol proved to be the most acceptable solvent for use in the production of consumable polyphenol products, as opposed to acetone which could not be used for toxicological reasons (see Section 4.4.3).

4.6.1.1 Uncrushed grape marc extracts obtained at 1.2 L scale using mechanical agitation

The working volume of the 1.2 L vessel, ten times the working volume of magnetically agitated systems described previously, contained 300 g of uncrushed grape marc and 900 mL of 50% ethanol (1:3, w/v). The grape marc used in the first extraction stage was filtered and used again in a second extraction stage. Samples were taken through the sample port at various time intervals during both extraction stages and the TPC of the samples were measured.

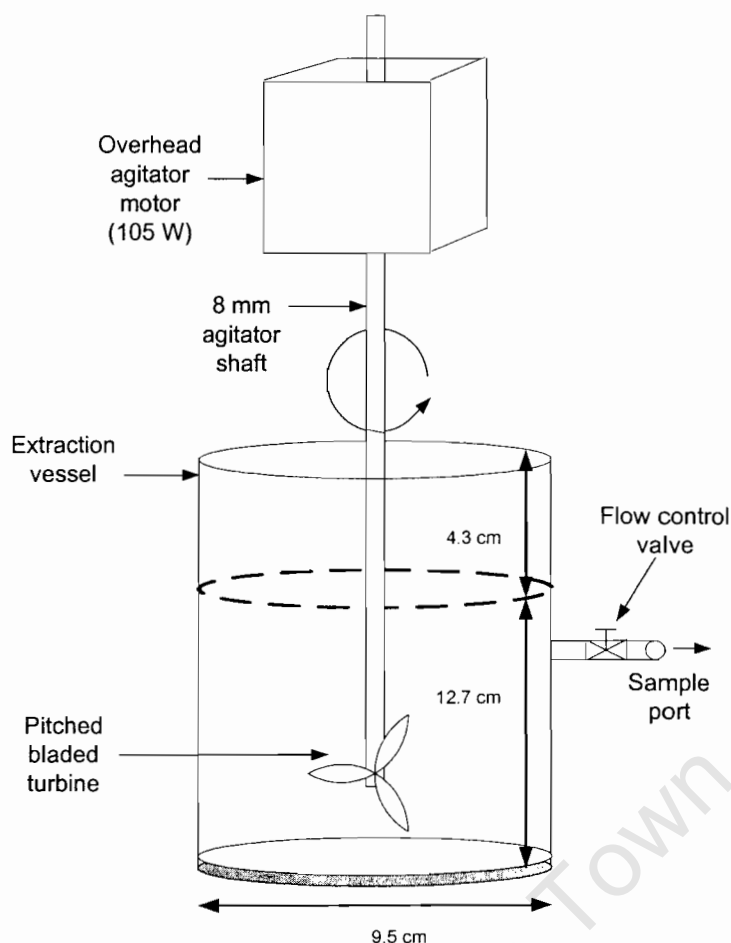


Figure 4.6-1: A schematic diagram of the mechanically agitated extraction vessel (not to scale).

The polyphenol mass transfer coefficients (k) and polyphenol saturation concentrations (C^*) achieved in stages 1 and 2 are shown in Figure 4.6-2. The data points were obtained experimentally and the fitted curves (solid and dashed lines) represent the predicted TPC obtained using the first order mass transfer model (see Section 4.4.4). The final TPC (measured values) of the samples taken in stages 1 and 2 differed by 70%, from 480 mg GAE/kg in stage 1 to 336 mg GAE/kg in stage 2. It was expected that the TPC would be lower in stage 2, since lower amounts of polyphenols remained after the first stage extraction, for extraction in the second stage. The results from the mass transfer model show that the mass transfer coefficients increased from stage 1 to stage 2, which implies that the polyphenols were being transferred faster in stage 2 than stage 1.

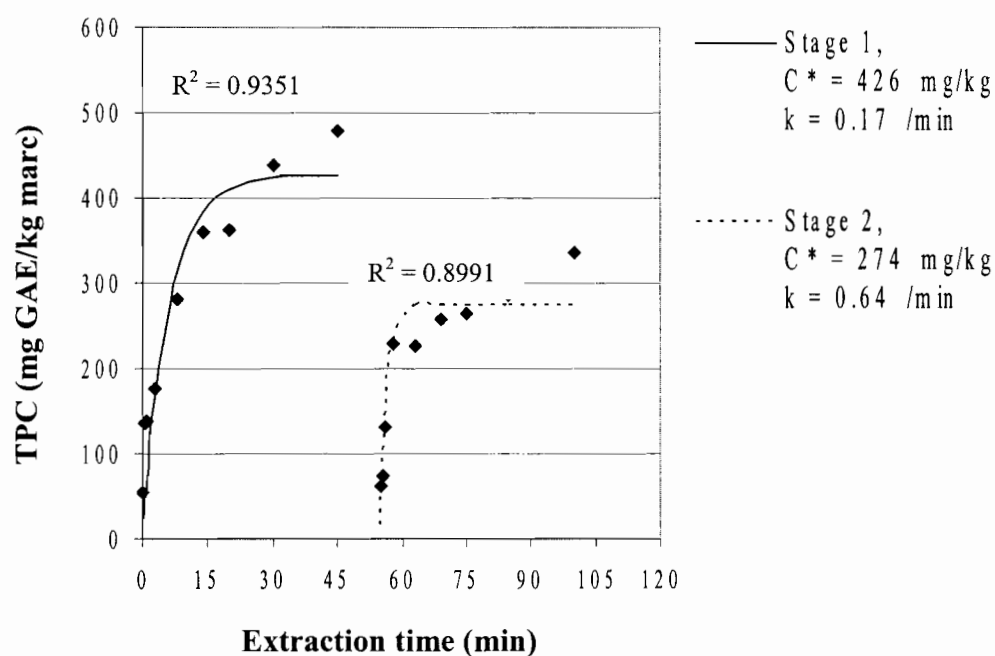


Figure 4.6-2: Total polyphenol concentrations achieved in extraction stage 1 (solid line) and stage 2 (dotted line) using wet uncrushed marc agitated with 50% ethanol in a 1.2 L mechanically stirred extraction vessel.

The saturated TPC and mass transfer coefficient from smaller extraction volumes previously obtained using magnetic agitation under similar extraction conditions (25 min extraction time, room temperature, 1:3 w/v ratio, uncrushed marc) were 760 mg GAE/kg and 0.20 min^{-1} respectively (see Section 4.4.4.3). In comparing these results (shown in Table 4.6-1), a noticeably lower polyphenol saturation concentration was obtained using uncrushed marc in the larger scale (mechanical agitation) setup, possibly because the TPC in the extraction system shown in Figure 4.6-2 had not reached steady state. Therefore, the 1.2 L scaled-up extraction system should have longer extraction times, greater than the 45 min used in this experiment, to allow for polyphenol concentrations to reach the saturated TPC.

Table 4.6-1: Polyphenol saturation concentrations and mass transfer coefficients obtained from the first extraction stage using uncrushed grape marc and 50% ethanol.

Extraction size	Small-scale	Large-scale
Reference	Figure 4.4-8	Figure 4.6-2
C* mg GAE/kg	760	426
k /min	0.20	0.17
R ²	0.9687	0.9351

4.6.1.2 Crushed grape marc extracts obtained by extraction with mechanical stirring at 1.2 L scale

In a second experiment, using the 1.2 L mechanical extraction vessel, crushed marc was extracted once using 50% ethanol for 30 min and the slurry was filtered. Sampling over time was not possible for the crushed marc system, because the sample port was frequently blocked with small pieces of crushed grape marc, and instead only the polyphenol concentration of the filtrate was measured. The filtrate TPC was found to be 1 040 mg GAE/kg, which was lower than the TPC of 1 275 mg GAE/kg obtained under similar conditions (crushed marc, ethanol/water, 1:3 ratio (w/v), 25 min), at smaller scale and using magnetic agitation (see Figure 4.4-6). It is possible that more time was needed for the large scale extraction to reach steady state conditions, and therefore longer extraction times of 1.5 h were suggested for the proposed process.

Analysis of the TPC of the extracts from crushed and uncrushed marc, in the larger scale (mechanically agitated) extraction systems, indicates that the total amount of polyphenols obtained in 30 min from a single extraction from crushed marc (1 048 mg GAE/kg) was greater than the combined results from two consecutive extractions using uncrushed marc (816 mg GAE/kg) over 90 min. These results confirm the benefit of using crushed marc to maximise the amount of polyphenols extracted per kg of grape marc. Although more energy would be required to crush large amounts of grape marc for the proposed process, the advantages are that less grape marc is required to produce

the same amount of polyphenols from a crushed marc system than from an uncrushed grape marc system. Using less grape marc also implies that less solvent would also be needed and hence less energy would be used in evaporating the solvent for recycle.

4.6.1.3 Determination of the TPC yield of ethanol extracts obtained from crushed grape marc

The TPC yield is a percentage that represents the total amount of polyphenols that is extractable per mass of grape marc. Based on the results obtained in Section 4.6.1.2, for a single extraction at room temperature, using 50% ethanol, approximately 1 000 mg (1 g) of polyphenols is extractable per kg of grape marc. Therefore, the TPC yield under these conditions was 0.10%, calculated using Equation 11.

$$\text{Yield (\%)} = \frac{\text{polyphenol mass}}{\text{grape marc mass}} * 100 \quad \dots \text{Equation 11}$$

Using the TPC-temperature equation $y = 31.9 * T + 181$ (Table 4.4-5), 1 776 mg GAE/kg would be extractable at 50°C. Figure 4.6-2 shows that about 70% of the TPC extracted in the first stage is obtainable in the second stage extraction stage, which would result in an additional 1 243 mg GAE/kg of polyphenols extracted in the second stage. Hence, the total amount of polyphenols recovered over two extraction stages would be 3 019 mg GAE/kg (~3 g GAE/kg or 0.30 g GAE/100 g marc), which is equivalent a TPC yield of 0.30%. The TPC yield for crushed marc was used later in mass balance calculations to estimate the amount of grape marc required to produce the desired amount of polyphenols (see Chapter 5). The TPC yield obtained in this study proved higher, comparable and lower than TPC yields reported by Bonilla *et al.* [1999], Spigno and De Faveri [2006] and Negro *et al.* [2003] respectively (see Table 4.6-2). Negro *et al.* [2003] used the same marc to solvent ratio as used in this study, producing a higher TPC yield of 0.42%. But since the extraction times were not given, it is not known the rate at which those yields were achieved, and thus it is not clear whether the method used in this study or by Negro *et al.* [2003] is the more efficient extraction method.

Table 4.6-2: Comparison of experimentally determined TPC yield with reported TPC yields obtained at different extraction conditions from red grape marc.

Extraction procedure	Solvent	TPC yield (% GAE, w/w)	Reference
300 g crushed marc, 900 mL, 50 °C, 2 stage extraction, 1 h*.	50% EtOH	0.30	Current study, 2006
250 g marc, 5-30 min, 1 L solvent, T NR.	EA:water (1:1)	0.11	Bonilla <i>et al.</i> , 1999
50 g marc powder, at 28/60°C, for 5/24 h, 200 mL solvent.	EtOH or EA:water (9:1)	0.15-0.33	Spigno & De Faveri, 2006
1 g marc/3 mL, T and time NR.	80% EtOH	0.42	Negro <i>et al.</i> , 2003

T = temperature, NR = not reported, EtOH = ethanol, EA = ethyl acetate, MetOH= methanol.

* The TPC yield 0.30% was determined using two 30 min extractions.

4.6.2 Analysis of grape marc extracts obtained using 50% ethanol

The characterisation of ethanol grape marc extracts was performed to determine the amount of polyphenols, the composition of the polyphenol fraction, and the amounts of sugars and proteins in the grape marc extract using the spectrophotometer. The standards used for the sugar and protein assays were glucose and bovine serum albumin respectively. This analysis is important because it determines the purity of the extract and its polyphenol composition, both providing information necessary for natural grape marc extracts to be sold as potential ingredients to the nutraceutical industry.

In these experiments, 120 mL of 50% ethanol was used to extract 40 g of crushed marc. After filtration, the extract was evaporated under vacuum to produce a concentrated liquid extract. This extract was frozen in liquid nitrogen and freeze dried to remove water. The net weight of the dried marc extract was measured to be 772.8 mg (19.3 mg

extract/g marc). The dry extract was then re-dissolved in 60 mL of 50% ethanol to obtain samples for characterisation. The combined masses of total polyphenols, sugars and proteins quantified accounted for 630.8 mg (81.6%) of the dried marc extract. The unidentified residual mass of extract (18.4%) was attributed to cellular material such as polysaccharides. The combined mass content of flavanols and anthocyanins (77.5% and 18.9%) was 96.4% of the total mass of extracted polyphenols (Table 4.6-3). The remaining 3.6% of polyphenol compounds would consist of other flavonoid groups, e.g. phenolic acids and flavonols, which have previously been identified in red grape marc [Bonilla *et al.*, 1999]. Therefore, under the defined extraction conditions used (50% ethanol, 1:3 (w/v) ratio, 25°C extraction temperature) 40 g of crushed marc produced 772.8 mg dry extract mass consisting of 43.7 mg of total polyphenols in a single extraction. From these results, the purity of polyphenols in the dry extract was determined to be 5.7 g total polyphenols/100 g extract (5.7%), which is lower than the purity levels of 6.0-22.3% shown by Spigno and De Faveri [2006]. This is because Spigno and De Faveri [2006] used hexane and petroleum ether, to remove non-polar compounds, before and after solvent extraction, resulting in increased polyphenol purity of the extracts.

Table 4.6-3: Concentrations of polyphenol components in extracts of crushed grape marc obtained from single extractions using 50% ethanol.

Extract component	Component concentration (mg/kg marc)	Extract mass (mg)	Dry extract content (wt%)	Polyphenol content (wt%)
Total polyphenols	1 092 ± 45	43.7 ± 1.8	5.7	
Total flavanols	846 ± 180	33.8 ± 7.2	4.4	77.5
Total anthocyanins	206 ± 13	8.3 ± 0.5	1.1	18.9
Reducing sugars	14 697 ± 696	587 ± 27	76.1	
Proteins	3.1 ± 0.5	0.1 ± 0.0	0.0	
Total		630.8*	81.8	96.4

*The total quantified mass was 630.8 mg, but the total measured mass was 772.8 mg of dried extract. Concentrations and masses are averages ± standard deviations of duplicate extractions.

4.6.3 Free radical scavenging activity of commercial products compared to grape marc extracts

Commercial grape antioxidant products are common health supplements available at local pharmacies. In order to assess the potential value of the extracts obtained in this study, analyses were performed using antioxidant health supplements (Procydin[®], Oxi-Provin[®]) obtained from local health product outlets. The capsules of health supplements contained antioxidant mixture of proanthocyanidins (70 mg), bioflavonoids (30 mg), ascorbic acid (30 mg) and Vitamin E (15 mg).

Sample capsules of each product were weighed and dissolved in methanol. After the total polyphenol assay was performed, serial dilutions were prepared for both products and EC₅₀ concentrations were determined. The EC₅₀ concentrations (6 and 11 mg phenol/g DPPH) of these health supplements were considerably lower than the grape marc extract values determined in this study, which means that these commercial products have a superior antioxidant efficacy on a gram for gram basis. However, an important factor in causing the large difference in efficacies is that the supplements contain a mixture of other antioxidants, such as Vitamin E and ascorbic acid. The synergistic effect of these antioxidant compounds added to the free radical scavenging ability of antioxidant supplements.

Table 4.6-4: TPC and antioxidant activity results of health supplements compared with a grape marc extract obtained using 50% ethanol.

Analysis	Units	Procydin [®]	Oxi-Provin [®]	Grape marc extract
Mass of polyphenols ^{*1}	g GAE	0.033	0.025	0.043
Tablet or extract mass	g	0.211	0.239	0.772
Percentage polyphenol	%	15.0	10.0	5.7
EC ₅₀ concentration	mg/g DPPH	11.0	6.0	65.0

^{*1} Determined by F-C method.

^{*2} 50% ethanol extract.

Based on these comparisons, an average of 8.4 ± 2.5 grams more polyphenols extracted using 50% ethanol, than current commercial antioxidant products Procydin[®] and Oxi-Provin[®], would be required in order for the grape marc extracts to have equivalent EC₅₀ concentrations as commercial grape antioxidant products. Additional research is recommended, for a later study, for the separation of polyphenols from non-polyphenol compounds, such as lipids, sugars and polysaccharides, which would increase the purity of the extracts, and possibly also increase their antioxidant efficacies as well. Although to obtain grape marc extracts with comparable antioxidant efficacies is desirable, it should be noted that extracts produced in this study were 100% natural extracts, and these extracts were obtained from grape marc, a waste product of the wine industry.

4.7 Results and Discussion summary

Important process variables, identified in this chapter, are summarised below and compared with the findings reported by other researchers.

The results from the pre-treatment experiments showed three findings. Firstly, that wet marc produced approximately 4 fold higher total polyphenol concentrations than dried marc. It was suggested that drying the marc hardened the grape skin and therefore inhibited the mass transfer of polyphenols from the grape marc into the solvent. Secondly, cold storage was proved necessary, as polyphenols in wet grape marc exposed to ambient conditions degraded rapidly at 620 mg GAE/kg per week. Thirdly, the highest concentration of polyphenols resulted from wet crushed marc, as opposed to wet uncrushed marc. This latter point was confirmed by Bonilla *et al.* [1999] who reported a two to three fold increase in the extraction of polyphenols from crushed red grape marc.

When uncrushed grape marc was extracted and consequently fractionated using SPE, the anthocyanin yields of the fractions obtained from methanol and acetone extracts were between 18-39 mg/kg marc and 157-187 mg/kg marc respectively. Therefore

acetone proved to be a more effective extraction solvent than methanol to produce samples for the fractionation of anthocyanins. Furthermore, it is suggested that to improve these low yields, crushed grape marc should be extracted to obtain higher anthocyanin yields after fractionation.

Investigations showed that extraction using acetone produces the highest polyphenol recoveries compared to extracts obtained using ethanol, methanol, ethyl acetate or water. By modelling the extraction of polyphenols using first order mass transfer kinetics, it was found that acetone gave higher mass transfer coefficients and steady state TPC than ethanol. As a result of its strong polyphenol extracting capabilities, acetone was used to determine the total extractable amount or yield of polyphenols of 0.40% w/w (4.0 g GAE/kg). This total yield is comparable to the polyphenol yield of 0.42% w/w [Negro *et al.*, 2003] obtained from red grape marc using 80% ethanol and the same marc to solvent ratio of 1:3 (w/v).

Extracts obtained using varying ethanol and acetone concentrations in water were tested for TPC. The results showed that 50% concentrations of ethanol or acetone produced the highest polyphenol concentrations in extracts, which confirms the findings reported by Yilmaz and Toledo [2006]. Raised extraction temperatures proved to increase the polyphenol recoveries considerably. Extracting from crushed marc using ethanol at 44°C resulted in a 150% increase in the TPC of extracts, compared to the TPC of extracts obtained at 25°C. Hence, extraction temperature was found to be one of the most influential process variables, together with grape marc particle size, solvent choice and solvent concentration.

The proposed process was designed to produce a food grade extract, and therefore acetone was not considered a suitable extraction solvent, and further investigations were conducted using ethanol and ethyl acetate. Ethanol extracts were found to give higher polyphenol recoveries as well as higher free radical scavenging efficacies (65 mg phenol/g DPPH), than ethyl acetate extracts (99 mg phenol/g DPPH) obtained under the same conditions. Therefore, ethanol was chosen as an extraction solvent for the proposed process.

Free radical scavenging analyses were conducted by using the DPPH assay to test the effect of extraction temperature, nitrogen headspace and rotary (vacuum) evaporation. Testing extraction temperatures up to 50°C showed no significant negative effect on the free radical scavenging ability of acetone and ethanol extracts. Extractions under a nitrogen atmosphere showed no improved antioxidant activity compared to ethanol or acetone extractions exposed to air. Removing the solvent by rotary evaporation at low temperatures (<45°C) had no significant degrading effect on the antioxidant activity of the extracts. Therefore, it was suggested that grape marc is extracted at temperatures of 50°C without the use of nitrogen, and the rotary evaporation could be used as a method of solvent separation for the proposed scaled-up process.

The total polyphenol yield of 0.30% w/w obtained from larger scale ethanol/water extractions (crushed grape marc, at 50°C, 1.2 L extractor, two stage extraction), was comparable to recent TPC yields of 0.11, 0.15-0.33 and 0.42% w/w reported by Bonilla *et al.* [1999], Spigno and De Faveri [2006] and Negro *et al.* [2003] respectively. The polyphenol yield determined using ethanol/water (0.3% w/w) was later used in process design calculations.

Chapter 5: Proposed extraction process

5.1 Introduction

This chapter describes the investigation conducted to determine the feasibility of a polyphenol extraction process using a stirring-assisted solvent extraction method. A batch process operation was used for laboratory scale experiments, therefore the scaled-up process was based on this model (To design, construct and operate a continuous extraction process in the laboratory was not possible considering the time-frame given for this project.). This antioxidant capacity of polyphenols did not show significant decreases in activity after being stored in the freezer in the laboratory. Thus, to produce a consistent supply of polyphenols the proposed process was also designed to include a refrigeration system.

Two dominant polyphenol classes, flavanols (proanthocyanidins) and anthocyanins, were identified in the polyphenol extract and the possibility of selling one purified polyphenol extract (containing mainly proanthocyanidins) to the nutraceutical industry, or two separate polyphenol extracts to the nutraceutical and beverage industries, was considered. This proposed design includes the process stages of refrigeration, crushing, extraction, filtration, solvent separation and product purification.

The challenges facing the proposed industrial scale process is that the energy input relative to the size of the extraction vessel would not be as high as the laboratory scale extraction system. As a result of the lower energy input, lower mass transfer rates of polyphenols can be expected for the industrial scale process. This would impact on the extraction efficiency, causing longer extraction times to achieve equivalent polyphenol yields as obtained in laboratory scale experiments. Table 5-1 summarises the key parameters as determined from results reported in Chapter 4. These results were used as the basis for the proposed process located in the Stellenbosch Wineland district.

Table 5-1: Key process parameters and optimal conditions.

Process parameter	Optimal conditions
Grape marc (red)	Crushed
Solvent concentration ^{#1}	50%
Extraction solvent, temperature	Ethanol/water, 50°C
Solvent evaporation	Vacuum, 45°C
TPC yield of grape marc ^{#2}	0.30% (w/w)
TPC composition of extract ^{#3}	
Proanthocyanidins	77.5%
Anthocyanidins	18.9%

^{#1} Figure 4.4-3.
^{#2} Two-stage extraction using crushed grape marc (Section 4.6.1.3).
^{#3} Composition of 50% ethanol extract (Table 4.6-3).

A block flow diagram showing the mass and volumetric flows of the process is illustrated in Figure 5-1 for a single batch extraction (1:3 w/v marc to solvent ratio). The spreadsheet for the process mass balance and assumptions is shown in Appendix B-4.

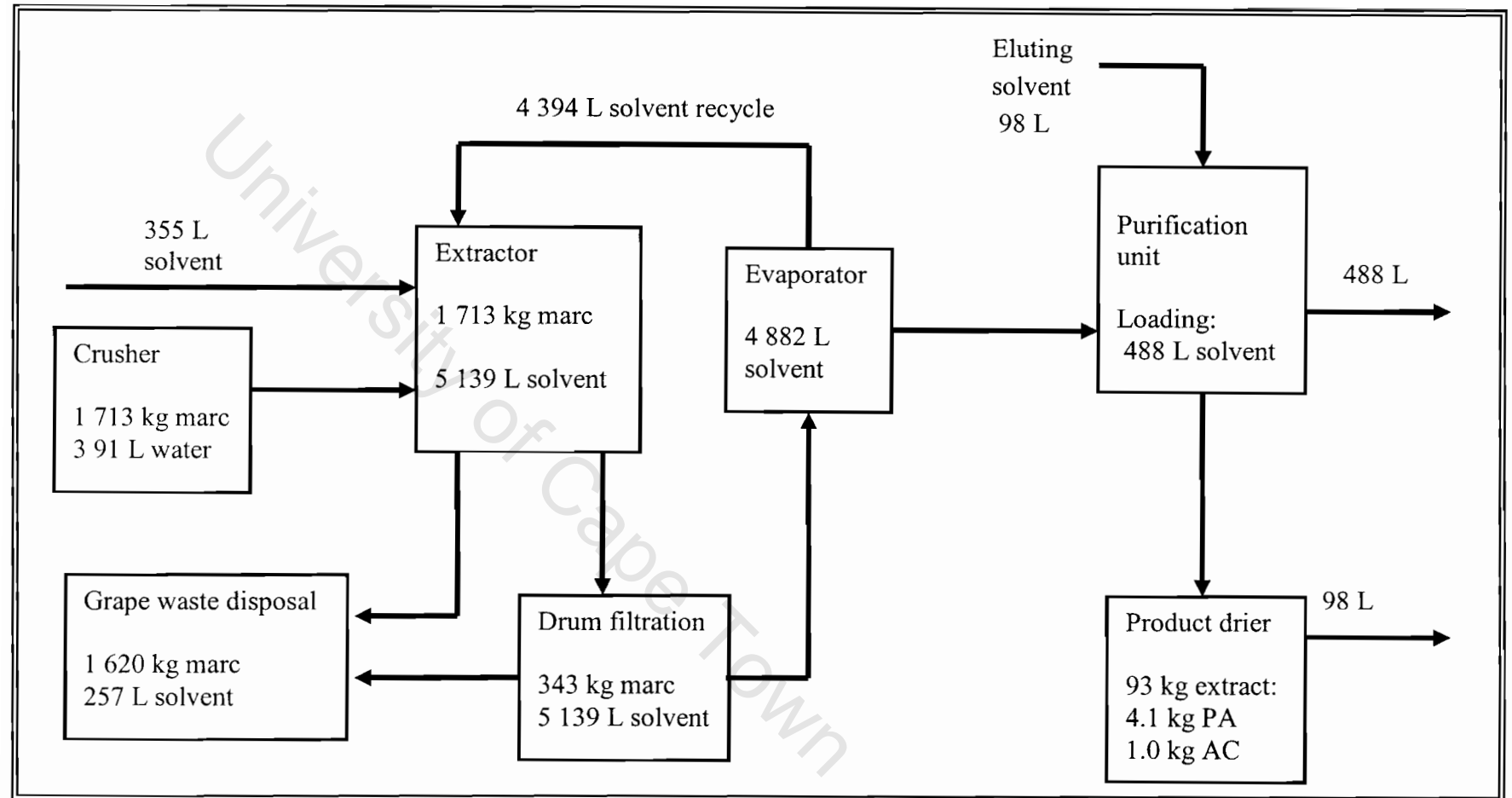


Figure 5-1: Block flow diagram of the proposed process showing the quantities of grape marc and solvent used per extraction (PA = proanthocyanidins, AC = anthocyanidins). The amount of grape marc feed was determined from the total polyphenol yield (0.3% w/w) and the production rate of polyphenols (see Appendix A-1).

5.2 Polyphenol markets

Data on the multi-vitamin product market was used to determine the potential demand for antioxidant supplements. Although the possible consumer market for antioxidant supplements in South Africa included all major cities, Cape Town was initially selected as the target market. Provided the process is feasible, increased production can then be investigated in a separate study to include other cities such as Johannesburg, Pretoria and Durban. AMPS [All Media Product Survey, 2005] database estimated that 8% of the middle to upper class population Cape Town (153 400 people) bought multi-vitamin products and consumed tablets/capsules 7 days per week. Traditionally, marketing strategies use less than 50% of an existing target market to avoid over-estimated sale predictions. Therefore, as a basis for this process design, 40% of this population group of 153 400 people was targeted as potential customers who would be expected to buy antioxidant supplements containing this new polyphenol extract. Hence, the proposed process was designed to produce a daily supply of antioxidant capsules for 61 400 people.

Table 5-2: Marketing data and targeted customers of antioxidant products.

Description	People
Cape Town population	4 645 000 ^{#1}
Middle-upper class population >16 years	1 917 500 ^{#2}
Daily consumers of multi-vitamins	153 400 ^{#3}
Antioxidant product customers	61 400 ^{#4}

^{#1} Population statistics obtained from www.statssa.gov.za.

^{#2,3} Obtained from AMPS database (2005).

^{#4} Estimated 40% of multi-vitamin market.

5.2.1 Daily production

Current grape antioxidant products contain 70 mg of proanthocyanidins and therefore a product containing 100 mg of proanthocyanidins was decided on as a marketing strategy to increase product sales. A basis of 274 working days per year was used to

determine the daily production rate of proanthocyanidins. This basis was determined by working six days per week, less a shut-down period from 17 December to the start of wine making season (1 February).

The daily consumption of 100 mg (1 capsule) per day per person was used to arrive at a production rate of 8.167 kg/working day of proanthocyanidins required to support the Cape Town demand (Appendix A-1). The daily production of 8.167 kg of proanthocyanidin is equivalent to a grape marc feed requirement of 3 422 kg/day (Appendix A-1). Considering that Stellenbosch produced 10 700 tons of red grape marc in 2005 (estimated from www.sawis.co.za), this feed requirement of 3.422 tons/day or 940 tons pa could easily be supplied by the surrounding wine farms. The extraction process was designed to extract two batches of grape marc daily, and therefore each batch of grape marc feed would weigh 1 711 kg.

5.2.2 Production value

Locally available Procydin[®] costs varied between R 50 and R 60 per bottle, therefore an average price of R 55 was used per bottle containing 60 capsules, 70 mg proanthocyanidin per capsule. The retail price was thus calculated to be R 13 300 per kg proanthocyanidin and the industrial value of proanthocyanidin produced was estimated at 30% of the retail price (R 3 980 per kg). Therefore the annual production of 2 240 kg was equivalent to R 8.91 million (US\$ 1.49 million) in sales. The proposed polyphenol extraction process also produced 546 kg of anthocyanins per year. It was assumed that the industrial value of anthocyanins produced was three times that of proanthocyanidins, that is R 11 940 per kg (US\$ 1 999 per kg). This was a fair assumption to make considering that average market value of β -carotene (natural food colourant) was US\$ 700 per kg, and other similar fermented products varied from US\$ 1 000 to 2 000 per kg [Business Communications Co., Inc., 2000]. Therefore, the revenue from anthocyanins sales was determined to be R 6.52 million (US\$ 1.09 million). These annual sales figures were used later to determine the feasibility of the

first process producing proanthocyanidin only and then the second process which would produce proanthocyanidin and anthocyanins separately. The first process has two purification units for the separation of polyphenols from non-polyphenol compounds, such as sugars and lipids. The second process has an additional purification unit, which was used for the separation of proanthocyanidin from anthocyanidins using eluting solvents with different polarity.

Table 5-3: Estimated values and product sales of polyphenol extracts.

Polyphenol products	Estimated values (R/kg)		Annual production (kg)	Annual sales [#] x10 ⁶	
	Retail	Industrial		(R)	(US\$)
PA	13 300	3 980	2 240	8.91	1.49
AC	-	11 940	546	6.52	1.09

PA = proanthocyanidin, AC = anthocyanin.
#Annual sales determined using estimate industrial values for PA and AC.

5.3 Process overview

5.3.1 Collection and storage of grape marc

The process overview describes the proposed cycle from grape marc to dry antioxidant powders. Red grape marc, used as the solid feed material for the process, is taken directly from Stellenbosch wine cellars and delivered to the refrigeration facility. Fresh grape marc is processed from February to April, during the wine making season. During these months, grape marc is also frozen and stored in the refrigerators for use after April. Approximately 8 900 kg/day of grape marc is frozen in individual containers. The frozen marc would then be removed the following day and transferred using another conveyor belt to one of the refrigeration units. When the fresh supply of grape marc ends, frozen grape marc is defrosted the day before processing using heat energy from the refrigeration unit.

5.3.2 Processing grape marc

Grape marc is manually loaded onto a conveyor belt and transferred to the cutting unit. The cutting unit is positioned 3 m above ground level and therefore the conveyor belt is necessary. Grape marc is reduced in size to increase the efficiency of the polyphenol extraction process. The crushed grape marc is then removed from the crushing unit using wash water, down the slide loader (operated using gravity) and into the extraction vessel. The distillate recycle stream entering the extractor is above 50% ethanol concentration, therefore the wash water dilutes this recycle stream to the desired extraction concentration of 50% ethanol. The extraction system, a closed system operating in batch mode at atmospheric pressure, uses a grape marc to solvent ratio of 1:3 (w/v). Solvent that is evaporated during the extraction stage would need to be condensed and recycled back.

The extraction vessel design includes a metal cage fitted inside, so that when the solvent is pumped out of the extraction vessel, the majority of the grape marc (~80%) remains behind. Small pieces of grape skins and grape seeds that exit with the polyphenol extract (~20% of the marc feed) are separated using vacuum filtration. The extraction system is operated over two stages. Therefore after the first stage the polyphenol extract is pumped out and the grape marc remains in the extraction vessel for the second stage where fresh extraction solvent is added. After the removal of the polyphenol extract from the second extraction, the grape marc is then taken from the metal cage and pressed to remove excess solvent. (The grape marc from the roll press is dried using refrigeration heat, and reused as an energy fuel for the process.) Vacuum filtration, using rotary drum filters, is best suited for slurry systems. An estimated 5% of the solvent entering the filtration unit is lost and exits with the grape waste. Two rotary drum filters will be used in parallel to ensure that the filtration time is kept to a minimum.

5.3.3 Downstream processing of grape marc extract

The evaporation stage is operated in batch mode, at 45°C under reduced pressure to prevent polyphenol degradation. It was assumed that the entering stream was 50% ethanol, and that 90% of the solvent entering the unit is evaporated and recycled back to the extraction unit. The bottom stream contains mostly water (10% ethanol), dissolved antioxidant extract and non-polyphenol compounds (polysaccharides, sugars). This concentrated polyphenol extract undergoes further downstream processing to purify the extract. The first purification column is used to separate the polyphenols from non-polyphenol compounds. The polyphenols that are adsorbed onto the packing of the first column are eluted using absolute ethanol. This elutant is loaded onto a second column which is used for the fractionation of flavanols (or proanthocyanidins) and anthocyanins. Once the purified antioxidant extracts are collected, they are dried to powder form using a small (100 L) batch distillation unit that is operated under reduced pressure at 45°C. The dried products are mainly proanthocyanidins and anthocyanins. Proanthocyanidins will be sold to nutraceutical industries producing grape extract health products and anthocyanins are sold to the beverage industry as natural antioxidant colourants.

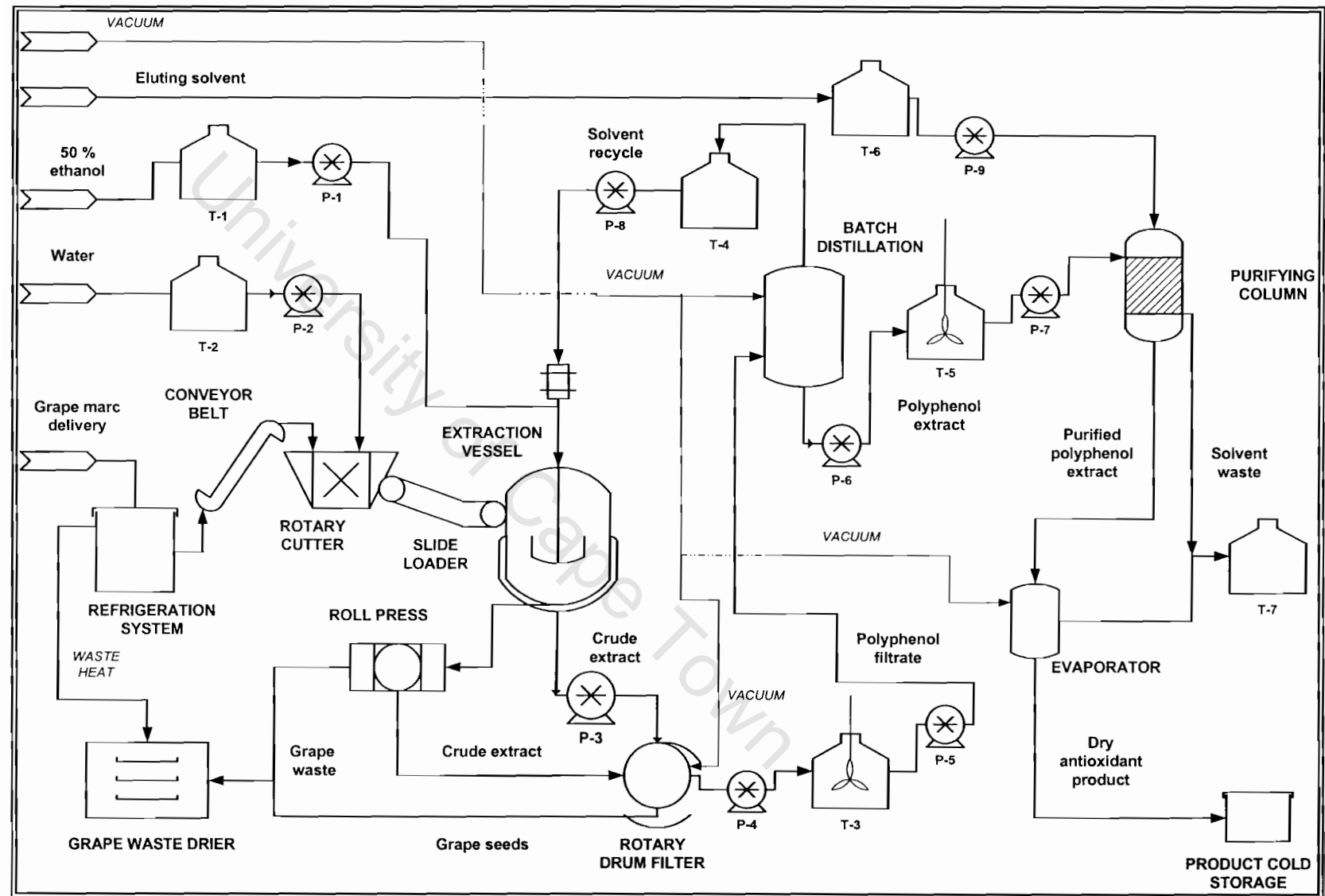


Figure 5-2: Process flow diagram for the proposed antioxidant process, showing equipment (upper case) and stream labels (lower case).

5.3.4 Time management and labour costs

Two batches of grape marc are extracted per day, and each batch is extracted twice. The operating times for the upstream processes are from 5:20 am to 13:20 pm for the early shift and from 9:50 am to 17:50 pm for the late shift (Table 5-4). The downstream processes start after the evaporator, where the polyphenol extract is then stored overnight in an agitated tank (T-5) before being purified and dried the following day. The purification and drying units operate without effecting the time management of the upstream processes. Two groups of ten employees (eight workers, one foreman and one engineer) work one early and one late shift per day.

Internationally based labour cost are commonly approximated at 10% of the total capital costs, but in South Africa the labour cost is expected to be less than in America and Europe (<10%). Therefore, a more accurate estimate is obtained, using hourly rates of R 20, R 40 and R 60 for workers, foremen and engineers respectively. These hourly rates are used to determine the annual labour cost at R 1 141 000, which is 5% of the total capital costs.

Table 5-4: Operation times for early and late working shifts.

Unit operation	<u>Early shift</u>		<u>Late shift</u>	
	Extraction 1	Extraction 2	Extraction 1	Extraction 2
Conveyor belt	5:20 – 5:30		9:50 – 10:00	
Cutting	5:40 – 6:20		10:10 – 10:50	
Extraction	6:35 – 8:05	8:20 - 9:50	11:05 – 12:35	12:50 – 14:20
Filtration	8:20 – 8:50	10:05 - 10:35	12:50 – 13:20	14:35 – 15:05
Evaporation	9:05 – 11:05	11:20 - 13:20	13:35 - 15:35	15:20 – 17:20
Clean-up				17:20-17:50
Time per shift	8 hours		8 hours	

5.4 Design of a refrigeration facility

5.4.1 Background

A vapour compression refrigeration system was used to design the refrigeration facility for freezing and storing grape marc. The refrigeration system comprises one freezer unit and two refrigeration units used to store the grape marc at -8°C . (Although -8°C was used in this design, temperatures between -8°C and 0°C may also be acceptable). Assuming that the process will be situated on a wine estate in Stellenbosch, approximately 12.3 tons/day of grape marc is transferred from the wine cellars to the refrigeration facility. During the wine making season, from February to April, 3.4 tons/day of fresh grape marc was processed while an additional 8.9 tons/day was frozen and stored in the refrigeration units. It was estimated that the maximum temperature of the grape marc would be 25°C before it is frozen. The frozen grape marc supplied a feed for the process from May to 17 December (198 working days, 6 days per week).

Ammonia was used as a refrigerant for the refrigeration system. It is used industrially and has negligible ozone depletion potential and global warming potential [McQuay, Application Guide AG 31 007, 2002]. The advantages of ammonia are that it has a low specific volume and high latent heat of evaporation, which favours the use of small equipment [Pita, 1984]. Ammonia is also a cheap refrigerant compared to other hydrocarbon based refrigerants. The mass flow rates of ammonia for the freezer unit and both refrigeration units were calculated to be 2.65 kg/min and 0.60 kg/min respectively.

The conditions at points A and D in Figure 5-3 were chosen in order to satisfy the conditions for an economical condenser design. The temperature of the refrigerant exiting the condenser at point A (40°C) was chosen to be 10°C above typically hot summer temperatures. After the temperature at point A was set (assuming that the refrigerant is a saturated liquid), the conditions for the other points were determined for an ideal refrigeration system by using the pressure-enthalpy diagram for ammonia (Appendix A-3). The assumptions for this system were that from points A to B the expansion valve operates under constant enthalpy, from points B to C the evaporators

operate under constant pressure (temperature), from C to D the compressors operate under constant entropy, and lastly from D to A the condenser operates at constant pressure.

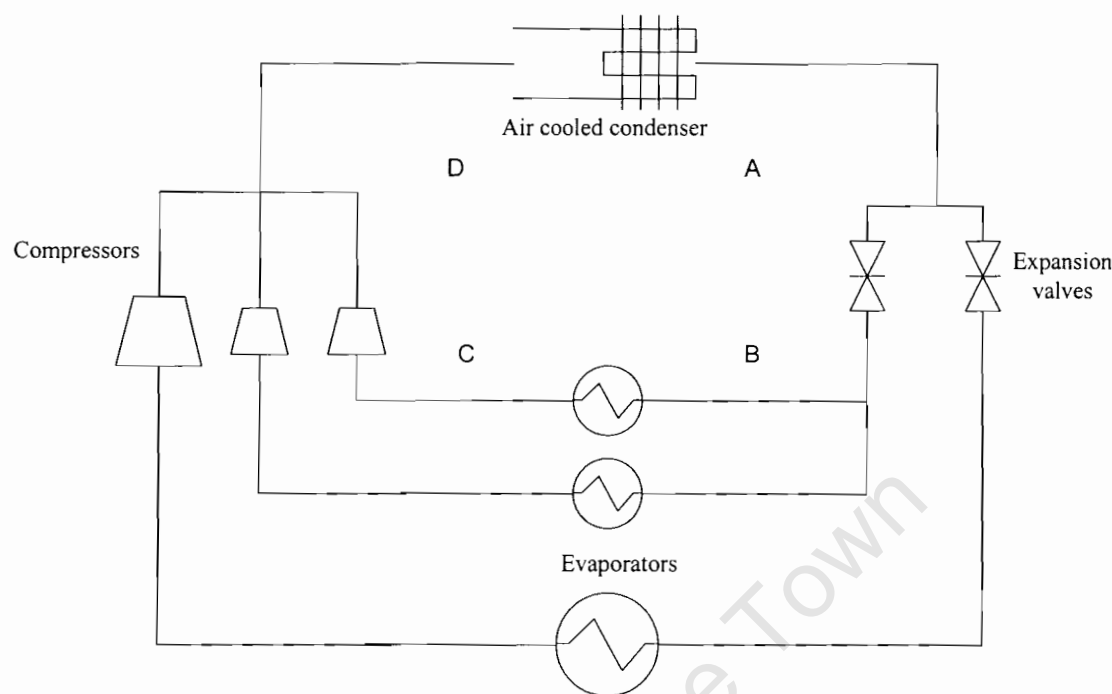


Figure 5-3: Refrigeration cycle showing the flow of ammonia through the expansion valve (A-B), evaporator (B-C), compressor (C-D) and condenser (D-A).

Table 5-5: Information and conditions relating to the refrigeration cycle.

Parameter	Unit	A	B	C	D
Temperature	K	313	270	270	383
Pressure	MPa	1.5	0.4	0.4	1.5
Enthalpy	kJ/kg	-582	-582	496	680
Phase		L	L/V	V	V'

L = saturated liquid, V = saturated vapour, V' = superheated vapour.

5.4.2 Heat exchanger design for the condenser

An air-cooled condenser was designed to make use of the waste heat exiting the refrigeration system. The use of air-cooled exchangers is a valid choice for process temperatures above 65°C [Sinnott, 1983], and hence for this process also since the refrigerant temperature entering the condenser is 110°C. The air inlet and outlet temperatures were 25°C and 60°C respectively. The hot air will be used to dry the processed grape waste so that it can be burnt to supply energy to the process evaporators and the extractor’s heating jacket.

The design of the condenser involved determining the surface area (A) of the exchanger from Equation 12, where Q is the heat to be removed from the refrigerant, U is the heat transfer coefficient and dTm is the log mean temperature:

$$Q = U \cdot A \cdot dT_m$$

... Equation 12

U was taken to be 230 W/m² °C from air-cooled exchanges, using ammonia at 15 bar (1.5 MPa) [Ulrich, 1984]. The amount of refrigerant heat to be removed was obtained from the ammonia pressure-enthalpy graph to be 4 332 kJ/min. This was calculated using the enthalpy difference from points D to A (1 262 kJ/kg) multiplied by the total mass flow rate of ammonia of 3.43 kg/min (Appendix A-1). The condenser cost was determined after calculating the required exchanger area of 12.1 m², using a unit cost of US\$ 12/ft² [estimated from Perry, 1997], to be US\$ 5 500.

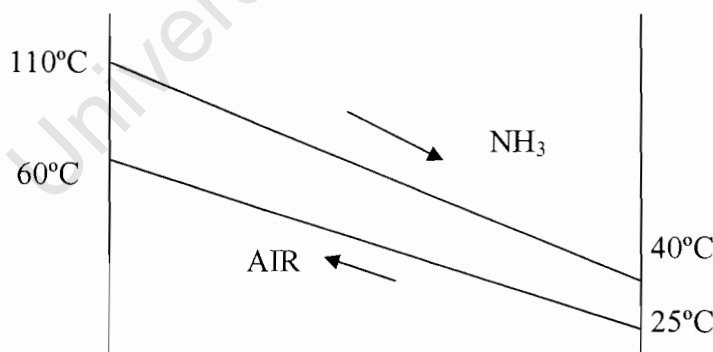


Figure 5-4: Temperature profiles for the counter-current air-cooled condenser.

5.4.3 Freezer capacity, capital and operating cost calculations

The amount of grape marc to be frozen in February, March and April, was 684 tons, which is equivalent to 8.9 tons/day or 9.1 m³/day. The freezer was designed to a capacity of 11.4 m³ and included 25% air space. The freezer dimensions, of 1.25 m by 1.45x6.30 m² base area, were used to calculate the total surface area of 28.5 m², which was used to determine the heat transmission through the freezer walls. The freezer is to be placed in a room with a maximum ambient temperature of 25°C during warm summer days. The grape marc enters the room via a conveyor belt, is packed into freezer containers and manually loaded into the freezers. The grape marc should be frozen in small batches (30 to 35 L) to allow for efficient freezing times. (Freezing the grape marc in small quantities would also result in faster defrosting times). The frozen grape marc is then removed the following day, and transferred to the adjacent refrigeration units in a closed conveyor belt for cold storage at -8°C.

The freezer load was calculated taking into account the heat removal required to freeze the grape waste from a maximum temperature of 25°C to -8°C (see Equation 13). The heat capacity of water and ice were used for grape marc since it contained 74% water. Other sources of heat energy were calculated and were found to have a small effect (see Table 5-6), such as the heat transmission through the walls of the freezers, the heat generated from the workers and lights.

$$\text{Heat removal} = C_{p,w} * \Delta T + \Delta H_{\text{fusion}} + C_{p,\text{ice}} * \Delta T \quad \dots \text{Equation 13}$$

A value of 72 Btu/ft² per 24 h [Pita, 1984] was used to determine the heat transmission, assuming 4 inches of molded polystyrene was used to insulate the freezer walls. This heat transmission, as well as the heat generated by people and lights, contributed to 4% of the total power requirement of 47.7 kJ/s.

Table 5-6: Calculated heat loads and freezer unit costs.

Energy, cost	Calculated value	Unit
Heat removal from grape marc	2 993 800 ^{*1}	kJ per 24 h
Heat transmission through walls	23 300	kJ per 24 h
Heat generated from people	48 100	kJ per 24 h
Heat generated from lights	25 800	kJ per 24 h
Total freezer heat load	3 091 000	kJ per 24 h
	47.7 ^{*2}	kJ/s
Freezer cost, 1982 (excluding heat exchanger)	45 000 ^{*3}	US\$
Freezer cost, 2006	79 900	US\$

^{*1} Calculated from Equation 13.

^{*2} 18 h basis per day.

^{*3} Ulrich, 1984.

5.4.4 Refrigeration capacity and cost calculations

The refrigeration capacity was designed for a maximum capacity of 684 tons of grape marc (approximately 700 m³). Refrigeration units are typically designed with 60% extra space to allow for air circulation and space for workers to move. Therefore the refrigeration volume was approximately 1 120 m³ for both units or 560 m³ for each unit.

Table 5-7: The dimensions and maximum capacity per refrigeration unit.

Parameter	Value	Unit
Total volume	560	m ³
Grape marc volume	350	m ³
Air volume	210	m ³
Height	2.4	m
Base area (square)	233	m ²
Surface area (walls, ceiling)	380	m ²

The total refrigeration heat load was the sum of the heat transmission, heat infiltration and heat gains from internal sources (people, lights, motors). Similar to the freeze design, the refrigeration units were also insulated with 4 inches of molded polystyrene, and were constructed indoors with a maximum ambient temperature of 25°C. This information was used to obtain a heat transmission gain of 72 Btu/ft² per 24 h and together with the surface area of 380 m² was used to determine a heat transmission through the walls and ceiling of the units. This contributed most towards the total refrigeration heat load. The heat gain resulting from air infiltration through the refrigeration doors was calculated based on 5 air changes (179 m³ air) per 24 h, and 50% humidity. Heat produced from internal sources (people and lights) was determined to have a minor contribution. The refrigeration units were also design to account for possible heat gains in transferring the frozen grape marc using the conveyor belt, from the freezers to the refrigeration units.

Table 5-8: The total refrigeration unit costs.

Parameter		Value	Unit
Heat transmission through walls		310 700	kJ/day
Heat generated from air infiltration		102 900	kJ/day
Heat gain from conveyor belt transfer		21 800	kJ/day
Heat generated from lights, people		17 300	kJ/day
Total refrigeration heat load	(1 unit)	452 700	kJ per 24 h
		7.0 ^{*1}	kJ/s
Refrigerator cost, 1982	(1 unit)	7 000 ^{*2}	US\$
Refrigerator cost, 2006	(1 unit)	12 400 ^{*3}	US\$
Total refrigerator cost, 2006	(2 units)	24 800	US\$

^{*1} Provided the compressor operates for 18 h per day.

^{*2} Ulrich, 1984.

^{*3} Calculated using cost indices (see Section 5.6.1).

5.4.5 Refrigeration system energy balance

The evaporator added energy to the refrigerant, causing it to change from a saturated liquid/vapour mixture to a saturated vapour. In order to remove this energy, the refrigerant was passed through a compressor where work is done on the refrigerant, resulting in a superheated vapour of increased enthalpy. This vapour was then cooled in an air-cooled condenser to a saturated liquid. The energy balance for this system is shown below.

$$Q_{evaporator} + W_{compressor} = Q_{condenser}$$

... Equation 14

Positive displacement compressors are commonly used in industry together with ammonia, and were thus also used in this design. The minimum power required to drive the compressor in an ideal cycle for any given conditions was determined by the change in enthalpy over the compressor (184 kJ/kg) multiplied by the flow rate of refrigerant. This was the minimum power requirement to drive the compressor. The capital costs were calculated from the power requirements using Ulrich [1984] and Perry [1997] for an ideal cycle refrigeration system.

Table 5-9: Annual refrigeration system capital and operating costs.

Energy parameter		Number of units	Power required (kJ/s)	Capital cost (US\$)	Operating cost (US\$) ^{*4}
Evaporator:	Freezer	1	47.7	79 900 ^{*1}	
	Fridge	2	14.0	24 800 ^{*2}	
Compressor:	Freezer	1	8.14	-	} 1 310
	Fridge	2	2.38	-	
Condenser		1	72.2	5 500 ^{*3}	8 960
Pump		1	-	-	520
Total				110 200	10 790

*1 *2 Ulrich, 1984.

*3 Perry, 1997.

*4 Obtained using the original costs and cost index data from Table 5-12.

5.5 Equipment design and energy requirements

Preliminary designs are the first phase of designs which use ratio factors to estimate the total production costs and total capital investment based on the total equipment cost. For the proposed process, a preliminary design was performed on an industrial scale to determine whether the process is feasible. The preliminary design includes sizing and costing the equipment, determining the production costs, net profit and the expected return on investment. The factors used for cost estimation in this study were specific to solid-liquid processes, since this process contained both grape marc and solvents. The maximum error associated with these factors was $\pm 30\%$ [Ulrich, 1984].

5.5.1 Extraction vessel design

The extraction vessel was cylindrical in design, with a closed top to prevent evaporated solvent from escaping into the atmosphere. The tank bottom was rounded to avoid of poor circulation. A pitch-blade (axial flow) turbine with 45° blades was selected because it produced good grape marc suspension and overall circulation. Good agitation also ensured efficient heat transfer from the heating jacket to the solvent/marc mixture. The extraction vessel was jacketed to allow for extraction temperatures of 50°C . Dried, processed grape marc was burnt to produce energy to heat the water circulating in the heating jacket. The shell of the vessel and the heating jacket was made of carbon steel.

The design measurements of the extraction vessel include the slurry height (H'), vessel diameter (D_t), turbine diameter (d_t), and turbine off-set height (E). Typical proportions for standard turbine design were used in order to size the extraction vessel. These proportions were: $H'/D_t = 1$, $D_t/d_t = 1/3$, and $E/D_t = 1/3$ [McCabe *et al.*, 2001]. The head space (the height of free space between the top level of the marc-solvent slurry and the maximum height of the vessel) was taken to be 25% of the slurry height (H').

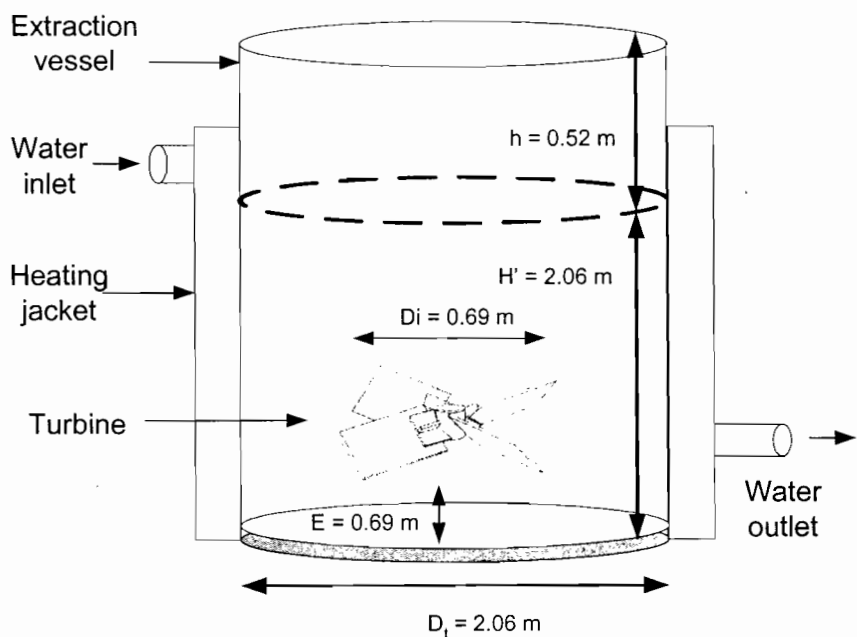


Figure 5-5: Schematic diagram of the extraction vessel (not to scale) with a pitched-blade turbine [McCabe *et al.*, 2001].

The extraction vessel was designed with a working volume of 6.89 m^3 , to contain 1.76 m^3 of grape marc and 5.13 m^3 of 50% ethanol. The vessel diameter (D_t) of 2.06 m was calculated using the volume equation of a cylinder Equation 15. The sizes and dimensions of the extraction vessel are shown in Table 5-10.

$$D_t = \{V'/(0.25 * \pi * H')\}^{(1/3)}$$

... Equation 15

Table 5-10: Extraction vessel size and dimensions.

Parameter	Symbol	Unit	Lab scale [#]	Industrial scale
Working volume	V'	m ³	0.90×10^{-3}	6.89
Vessel diameter	D_t	m	0.095	2.06
Slurry height	H'	m	0.127	2.06
Vessel volume	V	m ³	1.2×10^{-3}	8.61
Vessel height	H	m	0.170	2.58
Head space	h	m	0.043	0.52
Turbine diameter	D_i	m	0.043	0.69
Turbine off-set	E	m	0.030	0.69

[#] Note: The laboratory scale dimension had different proportions due to equipment availability.

5.5.2 Purification column design

A downstream process unit was needed after the evaporation unit to purify crude polyphenol extracts. Experimental results showed that unpurified dried extracts obtained using 50% ethanol contained approximately 5.7% (w/w) polyphenols (Table 4.6-1). Therefore the removal of sugars and lipids was expected to increase the purity of the extracts. Individual antioxidant fractions can be separated using solid phase extraction methods. Purified extracts and isolated grape polyphenol compounds are highly valuable and would therefore generate substantial revenues for the process. The packed column specifications used to fractionate and purify the crude polyphenol extract were based on laboratory scale experiments, where 16 mL of sample was fractionated using 0.5 g cartridges with a packing volume of 1.11 cm³ (see Section 3.6). Therefore, to separate polyphenols in 487 L of extract, 15 kg of packing and a column size of 33.7 L were approximated. In the laboratory, semi-preparative columns have high height to diameter ratios of 20:1, but a much lower ratio was chosen in this design for the industrial scale column of 2:1 to decrease the pressure drop over the column. Sephadex LH-20 gel has size exclusion and adsorption properties making it superior than G-50 gel, which only has size exclusion properties. Properties of sephadex LH-20 enable it to be used for the separation of natural products, such as vitamins, steroids, and lipids, and it was therefore chosen for the fractionation of grape marc polyphenols. Further research in a later study is required to determine the optimal eluting solvent(s) of proanthocyanidins and anthocyanins. However, for the purposes of determining the heating requirements for the evaporator, ethanol was used to elute the polyphenols from the columns.

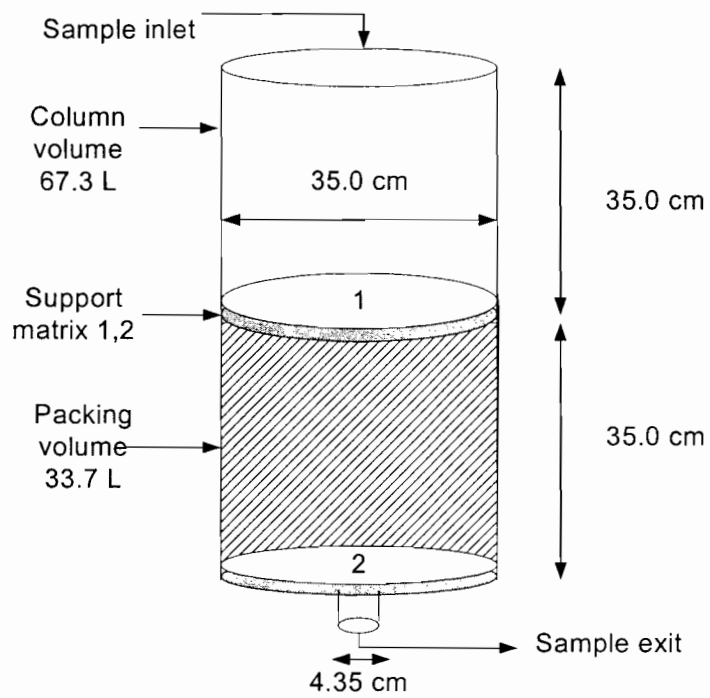


Figure 5-6: Diagram showing the purification column specifications used to fractionate extracted polyphenols.

5.5.3 Cost of energy requirements for process units

The heat capacities (C_p) of water and ethanol are 4.18 kJ/kg/C° and 2.44 kJ/kg/C°, and the heats of vaporisation (ΔH_{vap}) are 2 257 kJ/kg and 854 kJ/kg respectively [Ebbing, 1993]. These values were used in Equation 16 to determine the energy requirements for the extractor, distillation unit and evaporator per extraction. For details of these calculations see Appendix A-1. In the equation below, m stands for the mass of solvent and ΔT for temperature difference:

$$Q = m * C_p * \Delta T + m * \Delta H_{\text{vap}} \quad \dots \text{Equation 16}$$

In 2005, the wholesale electricity price during the high demand season was 13.53c per kWh [www.eskom.co.za], however in this study 15c per kWh (or 4.17c per MJ) was used. To save energy costs, processed grape marc (once dried) was used as fuel to

generate energy for the process. Only 26% of the marc entering the process constitutes the dry marc, since the fresh grape marc contains 74% moisture. The energy value for dry grape marc, 17.2 MJ/kg, was taken as the average lower heating value of dry biomass [Jekayinfa & Omisakin, 2005]. This biomass energy supply accounted for 60% of energy requirements for the process, and is shown as the potential energy saving in Table 5-11. The total annual cost of energy for the process, including the refrigeration costs and energy saved, was calculated to be US\$ 19 600.

Table 5-11: Summary of the energy requirement and costs for the process.

Unit operation	Energy requirement (MJ)		
	Per extraction	Per day	Per year
Refrigeration system	1 300	5 280	1 545 800
Rotary cutter	90	340	93 800
Extractor	1 400	5 600	1 537 200
Distillation	1 840	7 400	2 023 400
Evaporator	70	280	77 600
Agitated tanks ^{#1}	220	860	237 000
Pump ^{#2}	340	1 400	373 200
Energy/heat loss ^{#3}	1 000	4 100	1 119 600
Total energy requirement	6 300	25 200	7 007 600
Potential energy saving	3 800	15 300	4 194 200
Reduced energy requirement	2 500	9 900	2 813 400
Total cost US\$	17	69	19 600

#1 Two agitated tanks (T-3, T-5) both had 10 kW agitators.

#2 Assumed at 10% of the total energy required.

#3 Assumed at 30% of the total energy required.

Notes: Four extractions per day and 274 working days per year.

5.6 Total capital and production costs

For the financial evaluation of this proposed process, it was necessary to calculate the expected sales, production costs, total fixed capital costs and net profit. The ratio of net profit over total capital costs gave the return on investment which was used to decide whether the proposed process was feasible. For the purpose of this part of the study, the evaluation period for the process was 15 years, although the actual life-time of the process depends on factors controlling the market demand for grape antioxidant products, such as the public perception of antioxidants and local competition between antioxidant producing companies. Cost estimates for the proposed process were reported in US\$, because most cost data from reference material was expressed in US\$. Currency conversions were ZAR-Euro 7.369, ZAR-US\$ 5.971 and ZAR-GBP 10.868 [www.xe.com, January 2006].

5.6.1 Process equipment costs

The equipment costs shown in this preliminary design included: 5 holding tanks, 2 blending tanks, 9 pumps, 3 conveyor belt, 1 slide loader, 2 refrigeration units, 1 freezer, 1 cutting unit, 1 extractor, 2 vacuum filters, 1 press filter, 1 batch distillation unit, 2 purifying columns and 1 drying unit. The sizes for the processing units were estimated using the flow rates from the mass balance (Figure 5-1). The purchasing costs for process equipment were obtained from Sinnott [1983], Ulrich [1984], George [1988], and Peters and Timmerhaus [1991]. Present purchase costs were determined using all industry cost indices, as shown in Equation 17.

$$\text{Present cost} = \text{original cost} * \frac{\text{cost index}_{\text{present}}}{\text{cost index}_{\text{original}}} \quad \dots \text{Equation 17}$$

Table 5-12: All industry cost indices used for purchase cost estimations for equipment [Peters and Timmerhaus, 1991].

Year	Cost index
1970	438
1979	647
1982	717
1986	797
1990	904
2006	1273*

*The cost index for 2006 was estimated by extrapolating the indices.

Table 5-13 shows the process equipment of the proposed design, including specifications and purchase costs. The costs have been converted to 2006 costs prices using Equation 17.

Table 5-13: Process equipment descriptions, specifications and 2006 costs obtained from Ulrich (1984), unless otherwise specified.

Number of units	Description	Size	Details	Purchasing costs (US\$)
1	Rotary cutter	1.4 m ³	~3 kg/s	48 000
1	Agitated extractor	8.63 m ³	Carbon steel	54 500 ^{#1}
2	Rotary drum filter	10 ft ²	Vacuum filtration	42 600
1	Roll press	0.1 kg/s	Carbon steel	64 000
1	Distillation unit	4.88 m ³	Carbon steel	93 800 ^{#2}
1	Evaporator	0.1 m ³	Carbon steel	7 400
2	Purifying column	66.1 L	LH-20gel	151 200 ^{#3}
1	Refrigeration	950 m ³	-8°C, NH ₃	110 200 ^{#4}
3	Conveyor belt	5 m x 0.25 m	Bucket type	16 500 ^{#5}
1	Slide loader	5 x 0.3 x 0.3 m	Carbon steel	2 000
Total				590 000

^{#1, #5} Sinnott, 1983.

^{#2} See Section 5.6.1.1.

^{#3} See Section 5.6.1.2.

^{#4} Cost includes 2 refrigerators, 1 freezer, 1 air-cooled condenser (see Table 5-9).

5.6.1.1 Purchase cost of a batch distillation unit

Estimating the price for a 4.88 m³ distillation unit was achieved using an equation relating cost as a function of capacity [Ulrich, 1984]. A purchase cost ($C_{p,u}$) of R 500 000 for a 1 m³ stainless steel distillation unit (including boiler, condenser) was obtained from the internet [www.nedlac.org.za]. Using Equation 18 below, where V is 4.88 m³ and U is 1.0 m³, the purchase price for a 4.88 m³ evaporator was initially estimated at US\$ 234 700 in current prices. According to Sinnott [1983], the price of stainless steel is 2.5 times the price of carbon steel. Therefore, a final cost estimate for the distillation unit made of carbon steel, of US\$ 93 800, was used in this design. The same equation was also used to estimate the cost of a 100 L evaporator used to dry the purified polyphenol fractions, which amounted to US\$ 7 400.

$$C_{p,v} = C_{p,u} * \left(\frac{V}{U} \right)^{0.65} \quad \dots \text{Equation 18}$$

5.6.1.2 Purchase cost of purification unit

The purification column was designed to use Sephadex LH-20 gel, since this gel is commonly used for separations of lipids, vitamins and other small bio-molecules. The gel is also compatible with organic solvents and aqueous solvent mixtures, which make it suitable for this process since the feed streams to the column contained ethanol and water. Since the gel was expected to be replaced every five years, 40 kg was expected to last each column the 15 years projected operation time. The bulk price was US\$ 75 600 for 40 kg of LH-20 gel [www5.amershambiosciences.com], and the cost of glass, used as the materials of construction of the purifying column, was assumed to be negligible. Therefore the cost for two columns for the purification and separation of proanthocyanidins was estimated at US\$ 151 200.

5.6.2 Costing of pumps and storage tanks

Rotary pumps used, to control low flow rates and ethanol, are preferred to centrifugal pumps which are designed for larger flow rates (>100 gpm). Positive displacement pumps were used for pumping polyphenol extracts containing antioxidants to avoid possible shear effects on the extracted antioxidants. The material of construction for all pumps was cast iron.

Table 5-14: Pump descriptions and costs [Peters & Timmerhaus, 1991].

Pump * number	Description	Actual flow [#]	Capacity (gpm)	Pump type	Purchasing cost (US\$)
P-1	Pump for 50% from T-1 to extractor	6.2	10	RP	1 000
P-2	Pump water from T-2 to cutter	10.3	20	RP	1 500
P-3	Pump solvent out of extractor to filtration unit	61.1	70	PD	6 200
P-4	Pump extract from filter to T-3	43.0	60	PD	6 200
P-5	Pump extract from T-3 to evaporator	43.0	60	PD	6 200
P-6	Pump aqueous extract from evaporator to T-5	8.6	10	PD	2 000
P-7	Pump aqueous extract from T-5 to purify unit	1.7	20	RP	1 500
P-8	Pump solvent from T-4 to extractor	58	60	RP	2 200
P-9	Pump eluting solvent from T-6 to purify unit	1.7	20	RP	1 500
US\$ ₁₉₉₀					28 300
US\$ ₂₀₀₆					39 900

PD = positive displacement pump, RP = rotary pump, gpm = gallons per min.

* See Figure 5-2 for pump numbers.

T-1 was designed to store solvent for 6 days, as a weekly supply of 50% ethanol was expected. Since water is readily available, only a 2 day storage time was used for T-2. Two of the storage tanks, T-3 and T-5, were designed with 5 kW agitators to prevent the extracted polyphenols from settling at the bottom of the tanks. Tanks T-3, T-4 and T-5

were designed at double the capacity required for each extraction. The formula for determining the tank costs (C_e) are shown below, where C (500) is the cost constant (from the year 1979), V is the tank volume, n (0.59) is the equipment index for tanks [Sinnott, 1983]. The tanks were designed using carbon steel.

$$C_e = C * V^n$$

... Equation 19

Table 5-15: Storage and mixing tank specifications and costs [Sinnott, 1983].

Tank number	Description	Size (m ³)	Storage (day)	Purchasing costs (US\$)
T-1	Storage tank for 50% ethanol	8.5	6	3 400
T-2	Storage tank for water	3.1	2	1 900
T-3	Mixing tank for filtrate	9.8	1	3 700
	Agitator			2 400*
T-4	Solvent recycle tank	8.8	1	3 500
T-5	Mixing tank for extract	1.0	1	900
	Agitator			2 400*
T-6	Storage tank for eluting solvent	2.3	6	1 600
T-7	Storage tank for solvent waste	7.0	3	3 100
US\$ ₍₁₉₇₉₎				23 100
US\$ ₍₂₀₀₆₎				45 500

* 5 kW carbon steel agitators.

The costs of the process equipment, storage tanks and pumps were added together to determine the total equipment cost of US\$ 675 000. Additional equipment costs, such as installation, instrumentation, piping and electrical costs were all based on the total equipment cost using ratio factors applicable to solid-fluid processes. This method of estimation is called the percent of deliverable-equipment costs [Ulrich, 1984]. For a process selling anthocyanins separately from proanthocyanidins, an additional

purification column is needed, resulting in increased equipment cost of US\$ 75 600. The estimates used to determine the total investment are applicable to this process, because the total costs in Table 5-16 were between US\$ 1 to 20 million range specified by Peters and Timmerhaus [1991].

Table 5-16: Fixed costs used to estimate the total investment [Peters & Timmerhaus, 1991].

Cost category	PA US\$ x 1000	PA+AC US\$ x 1000	Estimate
Equipment	675	751	E
Installation	263	293	39%*E
Instrumentation	88	98	13%*E
Piping	209	233	31%*E
Electrical	68	75	10%*E
Land	101	113	15%*E
Building	149	165	22%*E
Yard improvement	68	75	10%*E
Service facility	371	413	55%*E
Total direct costs	1 992	2 215	
Supervision	216	240	32%*E
Construction	230	255	34%*E
Total direct + indirect costs	2 438	2 711	D+I
Contingency	244	271	10%*(D+I)
Constructor's fee	122	136	5%*(D+I)
Total capital costs	2 804	3 118	TCC
Start up costs	140	156	5%*TCC
Working capital	1 402	1 559	50%*TCC
Total investment	4 346	4 833	

PA = proanthocyanidin, AC = anthocyanin.

Cost estimates for administration and overheads, depreciation, contingency, insurance and taxes were obtained from Ratledge and Kristiansen [2001]. The number of working hours was calculated based on 10 workers working in two 8 hour shifts per day, 274 days per year. A 10% labour cost increase was made for the process selling proanthocyanidin and anthocyanin, to account for an extra person working in the purification unit.

Table 5-17: Costs of annual polyphenol production [Ratledge & Kristiansen, 2001].

Cost category	PA US\$ x 1000	PA + AC US\$ x 1000	Estimate
Raw material	-	-	free
Transportation	19	19	#1
Utilities (including refrigeration)	20	20	#2
Waste treatment	11	11	#3
Labour	191	210	
Admin, overheads	76	84	40%*labour
Depreciation	199	222	10%*TDC
Contingency	56	62	2%*TCC
Insurance	28	31	1%*TCC
Taxes	350	390	12.5%*TCC
Total production	951	1 049	

#¹ Estimated at US\$ 250 per day for 77 working days, delivering 8 tons per day.

#² Estimated from Table 5-11.

#³ Estimated at US\$1,000 per month.

PA = proanthocyanidin, AC = anthocyanin.

TDC = total direct costs, TCC = total capital costs.

5.7 Process feasibility study

Moderate risks are encountered in most industrial projects, and as an approximate guide, the minimum pretax return on investments for viable projects is 20 to 30% [Peters & Timmerhaus, 1991]. Feasibility studies with low associated errors only need returns of up to 10%, but designs with large cost errors are high risk investments and therefore need large returns to attract investors. The return on investment is an index of performance of the money invested and is expressed as a ratio of net profit over total investment.

$$\text{Return on investment} = \frac{\text{Net profit}}{\text{Total capital investment}} \quad \dots \text{Equation 20}$$

An additional method for comparing the economic performance of projects is achieved by calculating the discounted cash flow or net present value (NPV). The NPV takes into account the depreciation of money over time. NPV was calculated using Equation 21, where the discounted rate (i) was 10%, CF is the cash flow, and n is the number of years:

$$\text{NPV} = \frac{\text{CF}}{(1+i)^n} \quad \dots \text{Equation 21}$$

The return on investment was also calculated in Excel using Goal Seek, setting the sum of the discounted cash flow to zero by varying i . The resulting value of i gave the return on investment for a zero NPV after 15 years, and was more accurate than the return on investment calculated using the ratio of net profit to investment since it took into account varying revenue of annual sales (the first year sales were at 70%). The process designed to sell proanthocyanidins and anthocyanins showed an annual net profit of US\$ 1,074,000 and a return on investment of 31.7% before tax, compared to the process selling proanthocyanidin only, which had significantly lower annual net profit and pretax return of US\$ 379 000 and 12.4% respectively, as shown with other financial indicators in Table 5-18.

Table 5-18: Expected return on investment for the process selling proanthocyanidins only or proanthocyanidins and anthocyanins.

Financial indicator	Selling PA US\$ x 1000	Selling PA and AC US\$ x 1000
Annual sales (income)	1 492	2 584
Production costs	951	1 049
Gross profit	541	1 535
Taxes (30%)	162	460
Net profit	379	1 074
Returns on investment:		
Before tax	12.4%	31.7%
After tax	8.7%	22.2%

PA = proanthocyanidin, AC = anthocyanin.

Figures 5-7 and 5-8 show the cash flows for the proposed process selling proanthocyanidin and anthocyanin products, or proanthocyanidin, respectively. After 15 years, the operating time for the proposed process, the cost of the land and the working capital were written off, as shown by the noticeable increase in the graphs at this time. To accommodate the production of anthocyanins, an additional purification column was added to the downstream process and the labour cost was increased by 10%. Thus, after 15 years the effect of selling anthocyanins resulted in a relatively smaller increase in annual production costs of US\$ 98 000 compared to the increase in sales of US\$ 1 092 000. From Figure 5-7, the time required to pay off the capital was read off the non-discounted cash flow graph gave the pay back period of 5 years and the discounted graph gave the discounted break-even period of 7 years. There results from Table 5-18 and the Figures 5-7 and 5-8 clearly show that selling anthocyanidin as an additional product changes the process from a poor investment to an attractive investment with high returns on investment of 31.7 % before tax or 22.2% after tax.

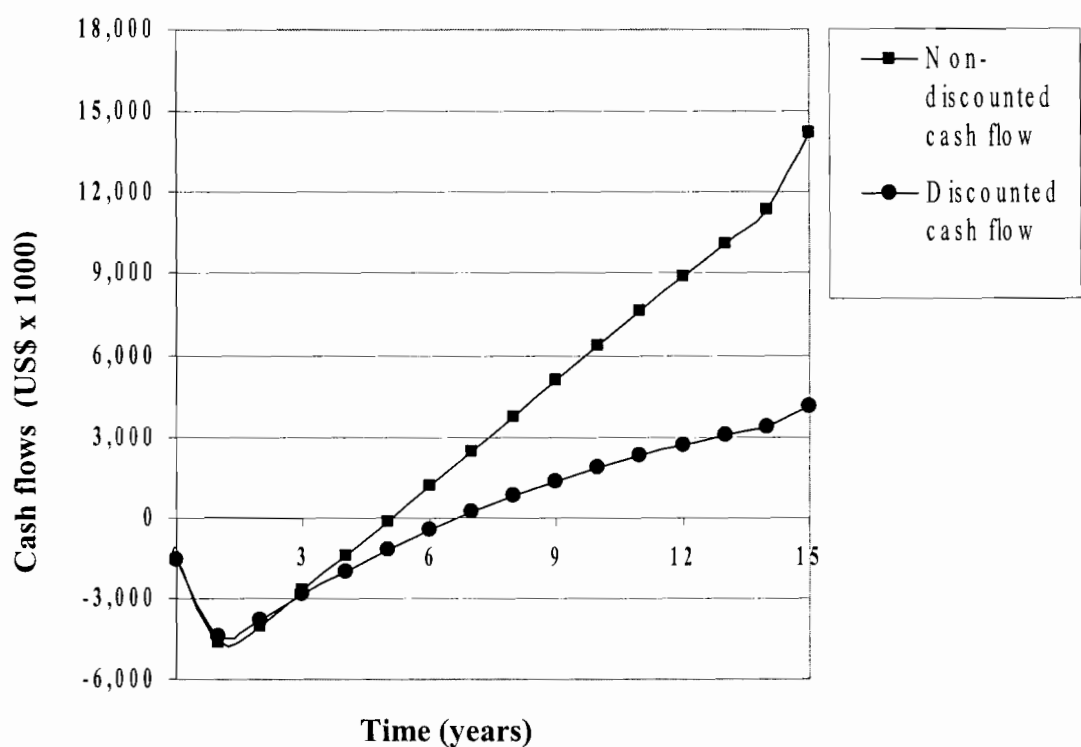


Figure 5-7: Discounted and non-discounted projected cash flows for the proposed process design, selling both proanthocyanidin and anthocyanin products ($i=10\%$).

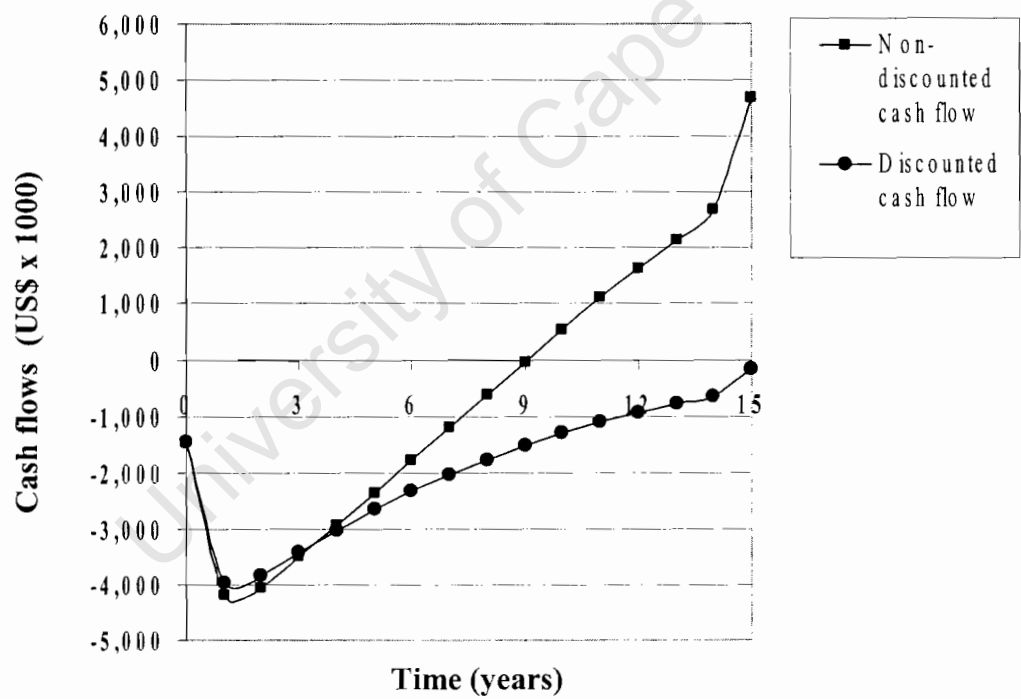


Figure 5-8: Projected cash flows (discounted and non-discounted) for the proposed process design, selling proanthocyanidin product only ($i=10\%$).

5.8 Process design summary

The fixed capital and production cost estimates determined in this preliminary process design were based mainly on the total equipment costs. Although cost indices were used to obtain current equipment costs, recent costing data would prove useful in obtaining more accurate estimates for equipment cost. The income of the process was obtained in Rands, and converted to US\$, whereas the total equipment costs were obtained in US\$ from readily available textbooks. Therefore the income is dependent on the ZAR-US\$ exchange rate while the total capital investment is constant. A weaker ZAR-US\$ exchange rate would thus decrease the income, resulting in a lower return on investment. This problem can be avoided provided equipment prices are accessible in local currency (ZAR), and then fluctuations in the exchange rate will not affect the feasibility study calculations.

This feasibility study showed that a process selling proanthocyanidin and anthocyanin products had a good pretax return of 31.7%. This return on investment is above the 20 to 30% range required for potentially viable processes and therefore the process is currently feasible. Additional sources of revenue from potential by-products, such as grape seed oil and tartaric acid present in the filter cakes, were not considered at this stage of the design.

This process was designed to minimise energy requirements, since South Africa has experienced recent power shortages. Therefore, waste heat from the refrigeration system will be used to dry the processed grape marc. It was estimated that by burning the dried marc, 60% of the process energy requirements would be accounted for. In addition to the energy savings, negligible effluent is also expected, since most of the ethanol is recycled for use in the extraction stage. Stellenbosch is one of the largest wine producing districts in the country and provides more grape marc (10 times) than required for the proposed process. Other important factors that would affect the economic performance of the proposed process include safety, environmental effects, location of customers, and company experience in the antioxidant product field.

Chapter 6: Conclusions

The investigations conducted in this study were designed to determine which factors affected the recovery of polyphenols in the solvent extraction process. The optimal process conditions for storing, crushing and extracting grape marc were examined on a laboratory scale. These conditions were then used in developing the design for an industrial scale process. This design included a feasibility study for the extraction of grape marc polyphenols to produce natural extracts for use in the nutraceutical and food industries. The major findings from this study are outlined below.

6.1 Major findings

- i) Refrigeration of grape marc is necessary to prevent polyphenol degradation, because it was observed that the polyphenols in grape marc exposed to ambient conditions degraded rapidly. The cost of the refrigeration facility proved affordable for the process producing proanthocyanidin and anthocyanin products, with the refrigeration fixed costs amounting to 14.6% of the total equipment costs and the refrigeration energy was estimated at 22% of the total energy requirements of the process.
- ii) The advantage of using crushed over uncrushed grape marc was shown by a twofold increase in total polyphenol recovery using ethanol or acetone as extracting solvents. These results concur with results showing a two- to three-fold increase of polyphenols by Bonilla *et al.* [1999]. The smaller the particle size of grape marc the greater the quantity of polyphenols extracted, which is in accordance with mass transfer theory. Therefore, to maximise the amount of polyphenols extracted, crushed grape marc was used in the proposed process.

- iii) A first order mass transfer equation effectively modelled the extraction of polyphenols from grape marc into solution. High R^2 values illustrated that model equations provided a good fit to the experimental data and that this model can be used to predict the TPC of extractions systems. Mass transfer coefficients, obtained from this model, were shown to be higher for crushed marc than for uncrushed marc. The type of solvent used also affected mass transfer, where polyphenols extracted with acetone/water showed higher mass transfer coefficients than those extracted using ethanol/water (1.15 and 0.39 min^{-1} for crushed marc respectively).
- iv) During investigations with varying acetone or ethanol dilutions, extracts containing the highest amount of total polyphenols were obtained using 50% acetone/water or 50% ethanol/water. Results reported in the recent literature confirm that grape extracts obtained using 50% concentration of ethanol or acetone showed the highest concentrations of total polyphenols [Yilmaz & Toledo, 2006].
- v) The use of higher temperatures for extraction resulted in greater amounts of total polyphenols being extracted from both crushed and uncrushed grape marc. Extraction temperatures up to 50°C resulted in no negative effect on the free radical scavenging activity of the extracts and thus were used in the proposed process.
- vi) The results from DPPH assays using ethanol and ethyl acetate showed that the EC_{50} concentrations were 65 and 99 mg phenol/g DPPH respectively. These results indicate that ethanol extracts have higher free radical scavenging activity than ethyl acetate extracts, and therefore ethanol was selected as the extraction solvent for the proposed process. Because ethanol is a more polar solvent than ethyl acetate, it extracts more polar polyphenols from grape marc which result in higher efficacies found in ethanol extracts. Although acetone extracts have shown the highest free radical scavenging activity, acetone was not selected for the process because it is toxic.
- vii) In terms of antioxidant capacity, the effect of solvent evaporation under vacuum had no significant effect on the efficacy of ethyl acetate extracts or ethanol extracts, at

temperatures <45°C. Thus, the method of rotary (vacuum) evaporation was used in the proposed process.

- viii) In a comparative investigation, commercial grape antioxidant products Procydin[®] and Oxi-Provin[®] were tested with grape marc extracts for free radical scavenging activity. The EC₅₀ concentrations for the antioxidant products of 11 and 6 mg phenol/g DPPH were significantly lower than those obtained from grape marc extracts (65 mg phenol/g DPPH). This indicated that the higher antioxidant capacities, obtained by the grape antioxidant products, were caused by the synergistic effects of Vitamin E, ascorbic acid, proanthocyanidins and bioflavonoids contained in the tablets. However, the advantage of grape marc extracts is that they are 100% natural extracts, whereas commercial antioxidant products contain grape extracts as well as synthetic ingredients.
- ix) A consecutive two-stage extraction was tested using a bench-scale extractor and a mechanically driven impeller. The polyphenol composition of this ethanol/water extract was measured to be 77.5% flavanols and 18.9% anthocyanins. The extraction yield of 0.30% mass total polyphenols per mass crushed grape marc was obtained at 50°C using 50% ethanol. This yield concurs with the polyphenol yields of 0.11% [Bonilla *et al.*, 1999] and 0.42% [Negro *et al.*, 2003], which were obtained using similar solvent extraction methods using red grape marc.
- x) A feasibility study was conducted on the proposed extraction process, which included an ammonia refrigeration system. This study showed that a process selling separated proanthocyanidin and anthocyanin polyphenols had a good return on investment of 31.7% before tax and the time required to pay off the capital was 7 years. Proposed industrial processes require a minimum pre-tax return on investment of 20 to 30%, and therefore this proposed polyphenol extraction process would be attractive to investors at this stage of the investigation. For the design proposal to be improved, local equipment costs must be obtained. Pilot scale extraction conditions and fractionation of polyphenol extracts, are mentioned below in the recommendations, as areas requiring further study.

6.2 Recommendations

It is recommended that a pilot plant be designed and constructed to verify the results obtained in this study, since it is possible that some parameters are dependent on the equipment size. Key parameters to verify for the process optimisation include the:

- extraction concentration of ethanol of 50%
- extraction temperature of 50°C,
- extraction time of 1.5 h,
- two-stage extraction yield of total polyphenols of 0.30%.

Further downstream processing research is necessary to develop a purification method of polyphenol extracts for the removal of sugars and carbohydrates. Removing the sugars and carbohydrates would increase the purity of the polyphenol extract, thus resulting in a higher quality product. Investigations are also required to determine which solvents are the best to elute proanthocyanidins and anthocyanins separately from Sephadex LH-20. Commercial values of the proanthocyanidin and anthocyanidin extracts need to be obtained from manufacturers to achieve accurate product sale estimates, since retail based estimates were used in the present study. An additional recommendation is that the economic assessment be extended to include the effect of production scale on capital costing.

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Appendix A-1

Calculations used in Chapter 5

Marketing calculations

Market size (Ms) of Cape Town

$$\begin{aligned} Ms &= 1\,918\,000 \text{ antioxidant buyers} * 8\% \text{ weekly consumers} * 40\% \text{ selected potential buyers} \\ &= 61\,376 \\ &\sim 61\,400 \text{ weekly antioxidant consumers} \end{aligned}$$

Antioxidant production (Ap)

$$\begin{aligned} Ap &= 61\,376 \text{ consumers} * 100 \text{ mg RDA} * \frac{1\text{g}}{1000\text{mg}} * \frac{1\text{kg}}{1000\text{g}} \\ &= 6.1376 \frac{\text{kg}}{\text{day}} * \frac{365 \text{ days}}{274 \text{ production days}} \\ &= 8.167 \text{ kg / production day} \end{aligned}$$

Grape marc daily feed requirement

PA = proanthocyanidins, TP = total polyphenols.

$$\begin{aligned} \text{Feed} &= Ap / (PA \text{ composition} * TP \text{ yield}) \\ &= 8.167 \text{ kg PA} * \frac{TP \text{ kg}}{0.77 \text{ PA kg}} * \frac{100 \text{ kg grape marc}}{0.31 \text{ kg TP}} \\ &= 3\,422 \text{ kg grape marc / day or } 1\,711 \text{ kg / batch} \end{aligned}$$

Freezer calculations:

$$\begin{aligned} \text{Total heat removal} &= Q_{gm} + Q_t + Q_p + Q_l \\ &= 2\,993\,800 + 23\,300 + 48\,100 + 25\,800 \\ &= 3\,091\,000 \text{ kJ / day} \\ &= 3\,091\,000 \frac{\text{kJ}}{\text{day}} * \frac{\text{day}}{18 \text{ h}} * \frac{\text{h}}{3600 \text{ s}} \\ &= 47.70 \text{ kJ / s} \end{aligned}$$

Heat removal from grape marc

$$\begin{aligned}
 Q_{gm} &= C_{p,w} * \Delta T + \Delta H_{fusion} + C_{p,ice} * \Delta T \\
 &= \left\{ 4.18 \frac{kJ}{kg \cdot K} * (25 - 0) \cdot K + 333.9 \frac{kJ}{kg} + 2.24 \frac{kJ}{kg \cdot K} * (0 - (-8)) \cdot K \right\} * (74\% * 8866 \frac{kg \text{ marc}}{day}) \\
 &= 2 \, 993 \, 842 \, kJ / day
 \end{aligned}$$

Heat transmission (Q_t) through the freezer walls and ceiling

$$\begin{aligned}
 Q_t \left(\frac{kJ}{24h} \right) &= wall \, area \, (ft^2) * 34 \frac{Btu}{ft^2 \, 24h} * 1.055 \frac{kJ}{Btu} \\
 &= 28.5 m^2 * 10.7636 \frac{ft^2}{m^2} * 72 \frac{Btu}{ft^2 \, day} * 1.055 \frac{kJ}{Btu} \\
 &= 23 \, 300 \, kJ / day
 \end{aligned}$$

Heat generated by people (Q_p)

$$\begin{aligned}
 Q_p \left(\frac{kJ}{24h} \right) &= 4 \, people * 950 \frac{Btu}{person \, h} * 12 \frac{h}{day} * 1.055 \frac{kJ}{Btu} \\
 &= 48 \, 100 \, kJ / day
 \end{aligned}$$

Heat generated by lights (Q_l)

$$\begin{aligned}
 Q_l \left(\frac{kJ}{24h} \right) &= floor \, area \, (ft^2) * 1.5 \frac{W}{ft^2} * 3600 \frac{s}{h} * \frac{1kJ}{1000J} * 12 \frac{h}{day} \\
 &= 398 ft^2 * 1.5 \frac{W}{ft^2} * \frac{1kJ}{1000J} * 3600 \frac{s}{h} * 12 \frac{h}{day} \\
 &= 25 \, 790 \, kJ / day
 \end{aligned}$$

Refrigeration calculations per unit (1 of 2):

$$\begin{aligned}
 Total \, heat \, removal &= Q_t + Q_{air} + Q_p + Q_l + Q_c \\
 &= 310 \, 700 + 102 \, 900 + 6 \, 650 + 10 \, 460 + 22 \, 000 \\
 &= 452 \, 700 \, kJ / day \\
 &= 452 \, 700 \frac{kJ}{day} * \frac{day}{18 \, h} * \frac{1h}{3600 \, s} \\
 &= 7.0 \, kJ / s
 \end{aligned}$$

Heat transmission (Qt) through the frig walls and ceiling

$$\begin{aligned}
 Q_t \left(\frac{kJ}{24h} \right) &= \text{wall area } (ft^2) * 34 \frac{Btu}{ft^2 \ 24h} * 1.055 \frac{kJ}{Btu} \\
 &= 380m^2 * 10.7636 \frac{ft^2}{m^2} * 72 \frac{Btu}{ft^2 \ day} * 1.055 \frac{kJ}{Btu} \\
 &= 310 \ 700 \ kJ / day
 \end{aligned}$$

Heat generated by air infiltration (doors)

$$\begin{aligned}
 Q_{air} \left(\frac{kJ}{24h} \right) &= 211 \ m^3 * 35.313 \frac{ft^3}{m^3} * 5 \frac{\text{volume air}}{day} * 2.62 \frac{Btu}{ft^3} * 1.055 \frac{kJ}{Btu} \\
 &= 102 \ 900 \ kJ / day
 \end{aligned}$$

Heat generated by people (Qp)

$$\begin{aligned}
 Q_p \left(\frac{kJ}{24h} \right) &= 2 \ \text{people} * 1050 \frac{Btu}{\text{people} \ h} * 3 \frac{h}{day} * 1.055 \frac{kJ}{Btu} \\
 &= 6 \ 650 \ kJ / day
 \end{aligned}$$

Heat generated by lights (Ql)

$$\begin{aligned}
 Q_l \left(\frac{kJ}{24h} \right) &= 15 \ m^2 \ \text{floor area} * 10.7636 \frac{ft^2}{m^2} * 1.5 \frac{W}{ft^2} * 3600 \frac{s}{h} * \frac{1kJ}{1000J} * 12 \frac{h}{day} \\
 &= 10 \ 460 \ kJ / day
 \end{aligned}$$

Heat gain with conveyer belt

$$\begin{aligned}
 Q_c &= m * C_p * \Delta T \\
 &= 74\% * 8,866 \frac{kg}{day} * \frac{1}{2 \ units} * 2.24 \frac{kJ}{kg \ K} * ((-5) - (-8))K \\
 &= 22 \ 000 \ kJ / day
 \end{aligned}$$

Refrigerant flow rates of ammonia

Freezer unit (Mf)

$$\begin{aligned} M_f &= \text{Total heat removal} * \{1 / \Delta H_{\text{vap}} \text{ NH}_3 (270\text{K}, 0.4\text{MPa})\} \\ &= 47.7 \frac{\text{kJ}}{\text{s}} * \frac{60\text{s}}{\text{min}} * \frac{1 \text{ kg}}{(496 - (-582)) \text{ kJ}} \\ &= 2.65 \text{ kg NH}_3 / \text{min} \end{aligned}$$

Refrigeration unit (Mr)

$$\begin{aligned} M_r &= \text{Total heat removal} * \{1 / \Delta H_{\text{vap}} \text{ NH}_3 (270\text{K}, 0.4\text{MPa})\} \\ &= 7.0 \frac{\text{kJ}}{\text{s}} * \frac{60\text{s}}{\text{min}} * \frac{1 \text{ kg}}{(496 - (-582)) \text{ kJ}} \\ &= 0.39 \text{ kg NH}_3 / \text{min} \end{aligned}$$

Total refrigeration flow rate

$$\begin{aligned} M_{\text{refrig}} &= M_f + 2 * M_r \\ &= 2.65 + 2 * 0.39 \\ &= 3.43 \text{ kg NH}_3 / \text{min} \end{aligned}$$

Compressor power requirement for refrigeration

$$\begin{aligned} \text{Comp}(\text{power}) &= M_{\text{refrig}} * \frac{\text{min}}{60\text{s}} * \Delta H_{\text{comp}} \\ &= 3.43 \frac{\text{kg}}{\text{min}} * \frac{\text{min}}{60\text{s}} * (680 - 496) \frac{\text{kJ}}{\text{kg}} \\ &= 10.52 \text{ kW} \end{aligned}$$

Condenser area (A), where U = 230 J/s m² K

$$\begin{aligned} \Delta T_{lm} &= \frac{((T_1 - t_2) - (T_2 - t_1))}{\ln \frac{(T_1 - t_2)}{(T_2 - t_1)}} = \frac{(110 - 60) - (40 - 25)}{\ln \frac{110 - 60}{40 - 25}} \\ &= 29.07 \end{aligned}$$

R and S were calculated to find the correction factor (Ft).

$$\Delta T_m = \Delta T_{lm} * F_t$$

$$= 29.07 * 0.89$$

$$= 25.87 \text{ K}$$

$$Q_{cond} = M_{refrig} * \Delta H_{cond}$$

$$= 3.43 \frac{\text{kg}}{\text{min}} * \frac{\text{min}}{60 \text{ s}} 1262 \frac{\text{kJ}}{\text{kg}} * 1000 \frac{\text{J}}{\text{kJ}}$$

$$= 72,144 \text{ J / s}$$

$$Q_{cond} = U * A * \Delta T_m$$

$$A = 72,144 \frac{\text{J}}{\text{s}} * \frac{\text{s m}^2 \text{ K}}{230 \text{ J}} * \frac{1}{25.87 \text{ K}}$$

$$= 12.12 \text{ m}^2$$

$$\text{Condenser price} = 12 \frac{\text{US\$}}{\text{ft}^2} * 10.764 \frac{\text{ft}^2}{\text{m}^2} * 12.12 \text{ m}^2 * 1.2 * \frac{1273}{438}$$

$$= \text{US\$ } 5\,460$$

Pump refrigeration power requirements (e = 80%)

$$\text{Pump}(\text{power}) = \frac{dP * Q}{e} * 100$$

$$= 343.7 \frac{\text{kN}}{\text{m}^2} * 9.79 \text{e}^{-5} \frac{\text{m}^3}{\text{s}} / 0.8$$

$$= 4.2 \text{ kJ / s}$$

$$dP = \rho * g * h$$

$$= 584 \frac{\text{kg}}{\text{m}^3} * 9.81 \frac{\text{m}}{\text{s}^2} * 60 \text{ m}$$

$$= 343.7 \text{ kN / m}^2$$

$$Q = 3.43 \frac{kg \text{ } NH^3}{min} * \frac{min}{60 \text{ } s} * \frac{m^3}{584 \text{ } kg}$$

$$= 9.79e^{-5} \text{ } m^3 / s$$

Equipment cost calculations

Extraction vessel [Sinnott, 1983]

$$Jacketed \text{ extractor (cost,1979)} = 6000 * V(m^3)^{0.4} * 1.95 \frac{US\$}{GBP}$$

$$= 6000 * 6.89^{0.4} * 1.95$$

$$= US\$ 27 \text{ } 700$$

$$cost,2006 = 27 \text{ } 700 * \frac{1273}{647}$$

$$= US\$ 54 \text{ } 500$$

Conveyor belt [Sinnott, 1983]

$$Convey \text{ belt (cost,2006)} = 500 * 5(m)^{0.65} * 1.95 \frac{US\$}{GBP} * \frac{1273}{647}$$

$$= US\$ 5 \text{ } 462$$

Distillation unit

$$Distillation(cost) = R500 \text{ } 000 * \frac{US\$}{R5.97} * (\frac{4.876 \text{ } m^3}{1 \text{ } m^3})^{0.65}$$

$$= US\$ 234 \text{ } 500 \text{ (M.O.C = stainless steel)}$$

$$= US\$ 234 \text{ } 500 * \frac{1}{2.5} \text{ (M.O.C = carbon steel)}$$

$$= US\$ 93 \text{ } 800$$

Energy calculations

Rotary cutter.

Half of 1,711 kg cut in 20 min [Equation from Ulrich, 1984].

$$= 100 * \frac{1711}{2} \text{ } kg$$

$$= 85.6MJ$$

Extractor heating requirements

$$Q_{\text{extractor}} = Q_{\text{water}} + Q_{\text{ethanol}} + Q_{\text{grape marc}}$$

$$\text{Where: } Q = m * C_p * \Delta T + m * \Delta H_{\text{vap}}$$

$$Q_{\text{water}} = 2566 \text{ kg} * 4.18 \text{ kJ/kg/K} * (50 - 20)K + 10\% 2566 \text{ kg} * 2257 \text{ kJ/kg} \\ = 901 \text{ MJ}$$

$$Q_{\text{ethanol}} = 2566 \text{ L} * 0.797 \frac{\text{kg}}{\text{L}} * 2.24 \text{ kJ/kg/K} * (50 - 20)K + 10\% (2566 * 0.797 * 854) \text{ kJ/kg} \\ = 312 \text{ MJ}$$

$$Q_{\text{grape marc}} = 74\% * 1711 \text{ kg marc} * 4.18 \text{ kJ/kg/K} * (50 - 15)K \\ = 185 \text{ MJ}$$

Therefore

$$Q_{\text{extractor}} = 901 + 312 + 185 = 1,398 \text{ MJ per extraction}$$

$$= 1,398 * 4 = 5,593 \text{ MJ per day}$$

$$= 5,593 * 274 = 1,534,173 \text{ MJ per year}$$

Similar calculations follow for the distillation unit and evaporator.

Grape marc energy saving

$$Q_{\text{grape marc fuel}} = 3,422 \frac{\text{kg marc}}{\text{day}} * (1 - 74\%) * 17.2 \frac{\text{MJ}}{\text{kg}} \\ = 15,300 \text{ MJ/day}$$

$$= 15,300 \frac{\text{MJ}}{\text{day}} * 274 \frac{\text{day}}{\text{year}} = 4,194,000 \text{ MJ/year}$$

Appendix A-2

Derivations: Mass Transfer Equations

$$\frac{dM}{dt} = \frac{k' A (C^* - C_s)}{b} \quad \dots \text{equation 1}$$

- M - mass of polyphenols transferred
- t - time
- A - area of solid-liquid interface
- b - effective thickness of the liquid film surrounding the particles
- C* - maximum concentration of polyphenols in solution
- Cs - concentration of polyphenols at time t in the bulk solution
- k' - diffusion coefficient

The equation 2 is substituted in equation 1 where the solvent volume V is constant during the extraction, and using k (overall mass transfer coefficient) to simplify equation 1 further results in equation 4.

$$dM = V dC_s \quad \dots \text{equation 2}$$

$$k = \frac{k' A}{b V} \quad \dots \text{equation 3}$$

$$\frac{dC_s}{dt} = k (C^* - C_s) \quad \dots \text{equation 4}$$

Equation 4 can be integrated to produce equation 6, using the concentration limits of C_{S0} initially (t=0) and C_S at time t. C_{S0} equals zero assuming pure solvent is used. Since the physical characteristics of grape marc is unaltered during the extraction (assumption, provided they are well crushed and that the extractor impeller does not

cause further size reduction of the crushed grape marc), A, b and V are constant and hence k is also constant.

$$\int_{C_0}^C \frac{dC_s}{C^* - C_s} = \int_0^t k dt \quad \dots \text{equation 5}$$

$$\ln(C^* - C_s) - \ln(C^* - C_{s0}) = -kt$$

$$\ln \frac{(C^* - C_s)}{C^*} = -kt$$

$$C^* - C_s = C^* (\exp^{-kt})$$

$$C_s = C^* (1 - \exp^{-kt}) \quad \dots \text{equation 6}$$

Appendix A-3

Thermodynamic data – ethanol/water.

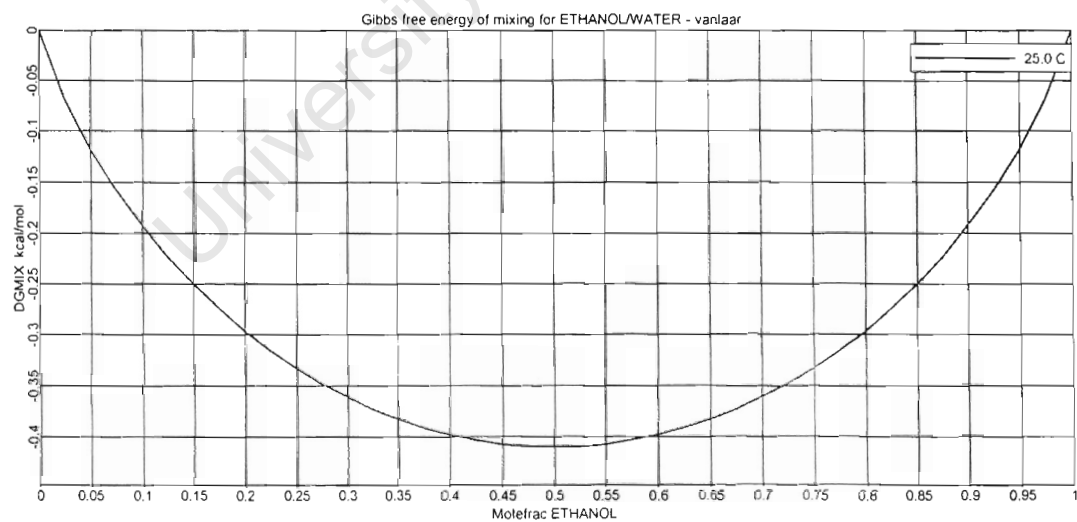
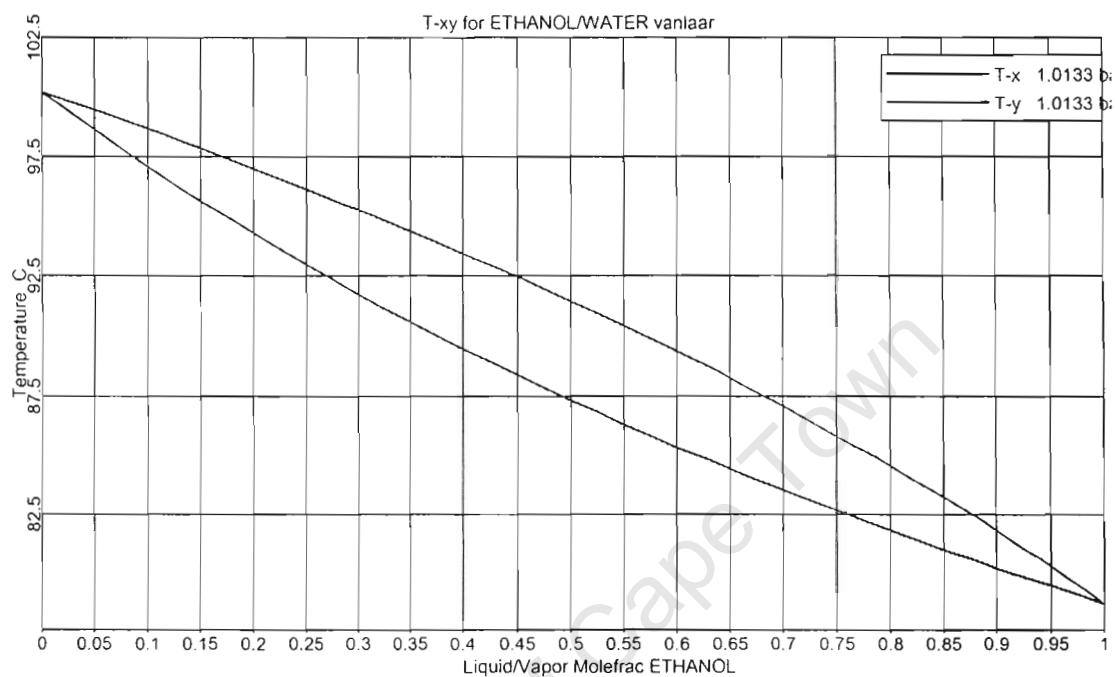
Ammonia: Pressure enthalpy data – table and graph.

Refrigerant properties (Application Guide AG 31-007).

Food colourant prizes (β -carotene).

University of Cape Town

Ethanol/water mixtures



Temp K	Pressure MPa	Volume Vapor m ³ /kg	Density Liquid kg/m ³	Enthalpy		Entropy		Temp K	Pressure MPa	Volume Vapor m ³ /kg	Density Liquid kg/m ³	Enthalpy		Entropy	
				Liquid kJ/kg	Vapor kJ/kg	Liquid kJ/kg·K	Vapor kJ/kg·K					Liquid kJ/kg	Vapor kJ/kg	Liquid kJ/kg·K	Vapor kJ/kg·K
195.48	0.006075	15.648	733.86	-1110.11	380.09	4.2032	11.8265	280	0.55077	0.22831	629.22	-730.90	505.99	5.8107	10.2284
200	0.008646	11.237	728.85	-1088.77	388.51	4.3111	11.6976	282	0.59083	0.21350	626.43	-721.54	507.87	5.8438	10.2036
205	0.012512	7.9469	723.25	-1066.17	397.68	4.4228	11.5635	284	0.63372	0.19984	623.61	-712.15	509.68	5.8768	10.1792
210	0.017746	5.7290	717.54	-1044.12	406.68	4.5290	11.4375	286	0.67771	0.18721	620.77	-702.73	511.42	5.9095	10.1550
215	0.024706	4.2037	711.72	-1022.33	415.50	4.6315	11.3190	288	0.72469	0.17553	617.91	-693.28	513.08	5.9422	10.1311
220	0.033811	3.1351	705.80	-1000.59	424.12	4.7314	11.2072	290	0.77413	0.16472	615.02	-683.81	514.68	5.9747	10.1076
222	0.038159	2.8000	703.41	-991.89	427.50	4.7707	11.1643	292	0.82613	0.15470	612.11	-674.30	516.19	6.0071	10.0843
224	0.042959	2.5065	701.00	-983.18	430.85	4.8097	11.1223	294	0.88077	0.14540	609.17	-664.76	517.64	6.0393	10.0612
226	0.048248	2.2488	698.58	-974.45	434.16	4.8485	11.0812	296	0.93813	0.13676	606.20	-655.19	519.00	6.0715	10.0384
228	0.054061	2.0220	696.16	-965.71	437.43	4.8870	11.0410	298	0.99830	0.12873	603.21	-645.59	520.28	6.1035	10.0158
230	0.060439	1.8219	693.72	-956.95	440.66	4.9252	11.0017	300	1.0614	0.12126	600.19	-635.95	521.47	6.1354	9.9935
232	0.067420	1.6450	691.27	-948.17	443.85	4.9631	10.9632	305	1.2324	0.10472	592.50	-611.70	524.08	6.2145	9.9384
234	0.075048	1.4882	688.82	-939.38	447.00	5.0009	10.9256	310	1.4235	0.09079	584.63	-587.23	526.10	6.2931	9.8845
236	0.083366	1.3490	686.36	-930.56	450.11	5.0383	10.8887	315	1.6362	0.07898	576.55	-562.51	527.51	6.3710	9.8314
238	0.092420	1.2251	683.89	-921.72	453.18	5.0756	10.8525	320	1.8721	0.06893	568.24	-537.53	528.24	6.4484	9.7789
239.82	0.101325	1.1241	681.64	-913.67	455.92	5.1092	10.8203	325	2.1327	0.06033	559.70	-512.25	528.25	6.5253	9.7268
240	0.10226	1.1145	681.41	-912.86	456.20	5.1126	10.8171	330	2.4196	0.05293	550.89	-486.65	527.48	6.6019	9.6750
242	0.11293	1.0157	678.92	-903.98	459.17	5.1494	10.7823	335	2.7344	0.04653	541.79	-460.68	525.86	6.6783	9.6232
244	0.12448	0.92726	676.43	-895.07	462.10	5.1859	10.7483	340	3.0789	0.04099	532.36	-434.30	523.31	6.7546	9.5711
246	0.13696	0.84790	673.92	-886.15	464.99	5.2223	10.7149	345	3.4549	0.03615	522.56	-407.44	519.75	6.8309	9.5184
248	0.15044	0.77657	671.40	-877.20	467.82	5.2584	10.6821	350	3.8641	0.03191	512.34	-380.02	515.07	6.9075	9.4650
250	0.16496	0.71234	668.88	-868.23	470.61	5.2944	10.6499	355	4.3085	0.02819	501.62	-351.97	509.13	6.9846	9.4103
252	0.18058	0.65441	666.34	-859.24	473.35	5.3301	10.6184	360	4.7902	0.02489	490.33	-323.15	501.79	7.0625	9.3541
254	0.19736	0.60206	663.79	-850.23	476.04	5.3656	10.5874	365	5.3112	0.02197	478.35	-293.42	492.81	7.1416	9.2957
256	0.21536	0.55468	661.23	-841.20	478.68	5.4009	10.5569	370	5.8740	0.01936	465.54	-262.58	481.93	7.2222	9.2345
258	0.23465	0.51174	658.65	-823.14	481.27	5.4361	10.5270	375	6.4811	0.01701	451.69	-230.38	468.76	7.3051	9.1696
260	0.25529	0.47274	656.06	-823.06	483.80	5.4710	10.4976	380	7.1352	0.01489	436.52	-196.46	452.74	7.3911	9.0996
262	0.27733	0.43728	653.46	-813.95	486.28	5.5058	10.4687	385	7.8395	0.01294	419.59	-160.25	433.01	7.4814	9.0224
264	0.30086	0.40498	650.84	-804.82	488.71	5.5403	10.4403	390	8.5977	0.01113	400.21	-120.88	408.14	7.5783	8.9348
266	0.32593	0.37553	648.20	-795.67	491.07	5.5747	10.4124	395	9.4144	0.009410	377.04	-76.642	375.49	7.6856	8.8303
268	0.35262	0.34863	645.55	-786.50	493.39	5.6089	10.3849	400	10.2956	0.007689	346.94	-23.456	328.97	7.8133	8.6943
270	0.38100	0.32402	642.88	-777.29	495.64	5.6430	10.3578	405.4	11.304	0.00426	235.	142.7	142.7	8.216	8.216
272	0.41113	0.30148	640.19	-768.07	497.84	5.6768	10.3312								
274	0.44310	0.28081	637.48	-758.81	499.97	5.7106	10.3049								
276	0.47698	0.26183	634.75	-749.54	502.04	5.7441	10.2791								
278	0.51284	0.24438	632.00	-740.23	504.05	5.7775	10.2536								

**Triple point
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**Critical point

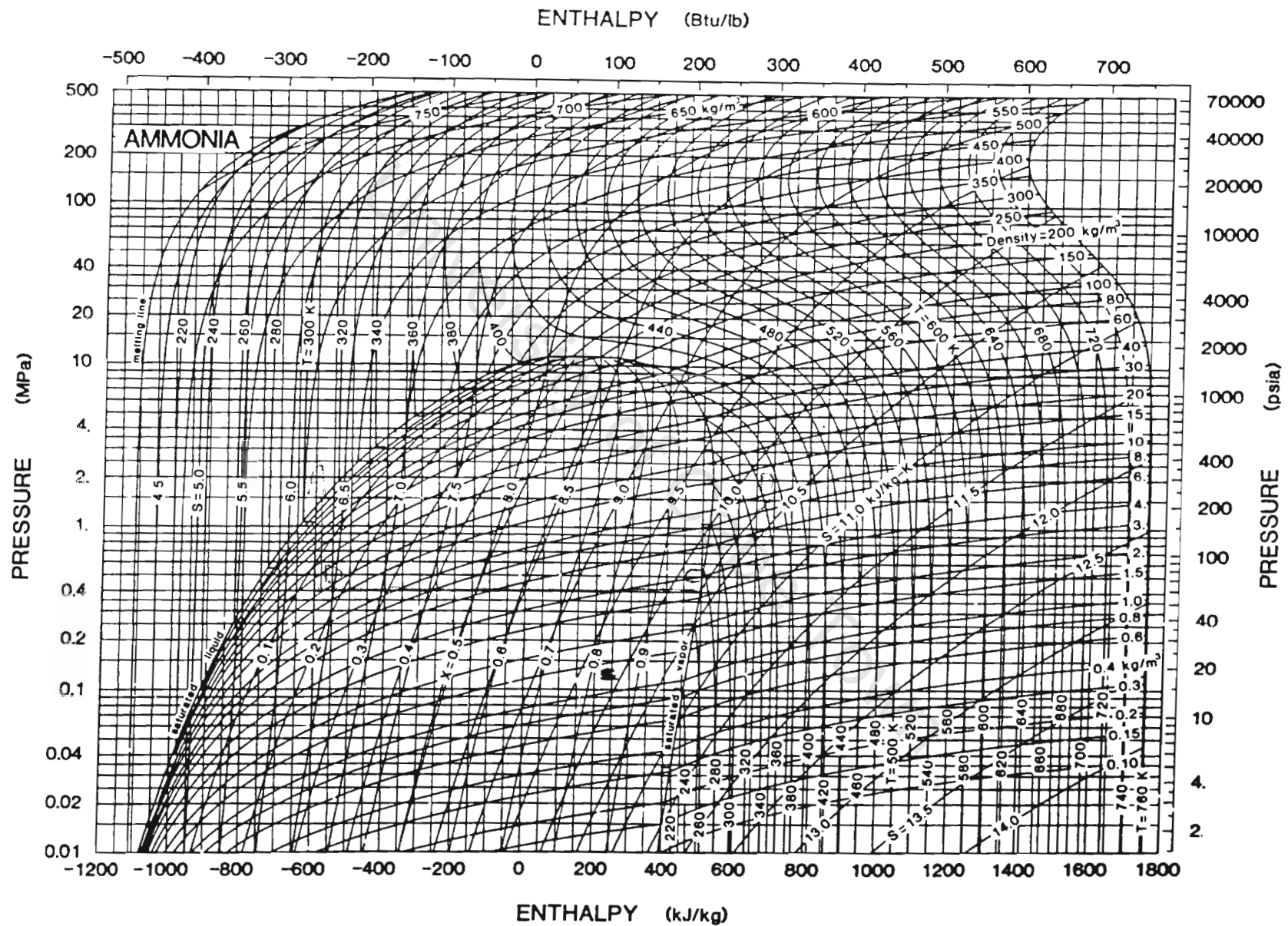


FIGURE A.8 Pressure-enthalpy diagram for Ammonia (SI units). (Reprinted with permission from the 1981 Fundamentals ASHRAE Handbook & Directory)

Table 1 - Refrigerant Properties

General Information

Refrigerant Number ^a	Chemical Name ^a	Chemical Formula ^a	Molecular Mass ^a	Safety Group ^a	Atmospheric Lifetime ^b (Yrs)	ODP ^c	GWP
11	trichlorofluoromethane	CCl ₃ F	137.4	A1	50	1	3800
12	dichlorodifluoromethane	CCl ₂ F ₂	120.9	A1	102	1	8100
22	chlorodifluoromethane	CHClF ₂	86.5	A1	12.1	.055	1500
32	difluoromethane	CH ₂ F ₂	52	A2	5.6	0	650
123	2,2-dichloro-1,1,1-trifluoroethane	CHCl ₂ CF ₃	153	B1	1.4	.02	90
125	Pentafluoroethane	CHF ₂ CF ₃	120	A1	32.6	0	2800
134a	1,1,1,2-tetrafluoroethane	CF ₃ CH ₂ F	102	A1	14.6	0	1300
245fa	1,1,2,2,3-Pentafluoropropane	CHF ₂ CH ₂ CF ₃	134.05	B1	8.8	0	820
290	Propane	CH ₃ CH ₂ CH ₃	44	A3	<1 ^h	0	-0
404a	R-125/143a/134a (44/52/4)			A1			3260 ^h
407C	R-32/125/134a (23/25/52)			A1		0	1530
410A	R-32/125 (50/50)			A1		0	1730
500	R-12/152a (73.8/26.2)			A1		.74	6010
507a	R-125/143a (50/50)			A1			
600	Butane	CH ₃ CH ₂ CH ₂ CH ₃	58.1	A3	<1 ^h	0	-0
717	Ammonia	NH ₃	17	B2	N/A	0	0
718	Water	H ₂ O	18	A1	N/A	0	<1
744	Carbon dioxide	CO ₂	44	A1	N/A	0	1

Operational Information

Refrigerant Number ^a	Normal Boiling Point (°F) ^a	Velocity of Sound (ft/s) @ 40 °F	Critical Point ^g		Bubble ^g (°F) @ psi	Dew ^g (°F) @ psi	Glide (°F)	Viscosity Lb _m /ft ² h ^g @ 40 °F Liq.	Specific Heat at Btu/lb·°R @ 40°F Liq.	Thermal Cond. ^g Btu/h·ft ² ·°F @ 40°F Liq.
			Temp (°F)	Press. (psi)						
11	74.67	443	388.33	639.27				1.304	.2059	.0548
12	-21.55	448	233.55	599.89				.574	.2253	.0429
22	-41.46	535	205.06	723.74				.503	.2825	.0537
32	-60.97	688	172.59	838.61				.361	.3106	.0872
123	82.08	414	362.63	531.1				1.292	.2379	.0476
125	-54.64	409	150.83	526.34				.457	.3044	.0397
134a	-14.93	482	213.91	588.75				.620	.2194	.0521
245fa	58.82	436.2	309.2	527.1				1.296	.3121	.0506
290	-43.75	723	206.06	616.07				.291	.6077	.0600
404a	-51.66b	473	162.5	548.18	38.8 @ 100	39.8 @ 100	1.0	.405	.3349	.0438
407C	-46.82b	519	186.9	672.2	37.0 @ 90	47.8 @ 90	10.8	.479	.3403	.0582
410A	-60.83b	553	158.4	694.87	42.9 @ 140	43.2 @ 140	0.3	.380	.3652	.0652
500	-28.31	490	222.0	641.9				.557	.2579	.0480
507a	-52.79	457	159.34	538.79				.401	.3331	.0432
600	31.04	659	305.62	550.56				.469	.5588	.0665
717	-27.99	1319	270.05	1643.71				.392	1.1094	.3155
718	211.95	1352	705.1	3200.1				3.738	1.0555	.3293
744	-109 ^f	687	87.76	1069.99				.222	.6460	.0607

^a ASHRAE, 1997. *ANSI/ASHRAE Standard 34-1997, Designation and Safety Classification of Refrigerants*. Atlanta, Ga.: ASHRAE

^b 1995 IPCC Report: HFCs Table 2.9; CFCs and HCFCs Table 2.2 (Houghton et al. 1996).

^c Ozone Secretariat UNEP (1996).

^h ARTI Refrigerant Database, based on WMO and IPCC assessments, August 1998. GWP shown are for 100 year integrated time horizon.

^g NIST *Standard Reference Database* 23, Version 6.01 (NIST 1996).

^e 2001 ASHRAE Fundamentals Handbook chapter 20.

^f Sublimes.

TABLE 51
STRUCTURE OF THE β -CAROTENE MARKET BY REGION AND APPLICATION, 1999
(Tons of Food)

Region	Food	Feed	Pharma	Total
Europe	90.0	15.0	15.0	120.0
U.S.	44.5	0.8	70.0	115.3
Japan	6.5	0.5	7.0	14.0
Asia (excl. Japan)	16.0	1.0	5.0	22.0
Rest of the World	18.0	1.0	5.0	24.0
Total	175.0	18.0	102.0	295.0

Source: BCC, Inc.

Price developments of β -carotene do not show a clear trend. For many years, prices for the synthetic product have been very stable. However, price differences for various formulations are significant, and also price differences between the synthetic product and the natural ones are very high.

TABLE 52
OVERVIEW ON β -CAROTENE PRICES BY TYPE OF FORMULATION AND ORIGIN, 1999
(\$/kg)

Origin	Formulation	Price	Price of Crystalline Equivalent
Synthesis	Crystalline, 96%	450.000	469.000
	10% powder	75.000	750.000
	1% powder	19.000	1.900
	30% oil	145.000	483.000
Fermentation	Crystalline, 96%	900.000	937.000
	30% oil	310.000	1.035
Algae	1% powder	27.000	2.700
	30% oil	283.000	945.000
Palm oil	2.5% powder	59.000	2.380

Source: BCC, Inc.

Synthetic product shows an average market value of approximately \$700/kg, considering the various formulations. Fermentative products are in a price range of \$1.000/kg, algae products in the range of \$1.200/kg and palm oil products show prices of exceeding \$2.000/kg.

Appendix A-4

Miscellaneous data

Table 8-1: Number of grape products in America [Shrikhande, 2000].

Grape seed	22
Grape skin	5
Red wine powder	7
Anthocyanin extract	5
Other (grape extract)	4

Table 8-2: Percentage weight, water, and phenolics found in grapes and wines [Shrikhande, 2000].

	Weight (%)	Water (%)	Phenolics (%)
Stems	2-6	60-80	1-4
Berry	95-97		
Skin	5-12	70-80	1-2
Pulp	85-87	60-85	
Seeds	0-5	30-40	5-8
Red wine			0.1-0.3
White wine			0.01-0.03

Table 8-3: Critical temperature and pressures of extraction solvents.

Fluid	Critical Temperature (K)	Critical Pressure (bar)
Carbon dioxide	304.1	73.8
Ethane	305.4	48.8
Ethylene	282.4	50.4
Propane	369.8	42.5
Propylene	364.9	46.0
Trifluoromethane (Fluoroform)	299.3	48.6
Chlorotrifluoromethane	302.0	38.7
Trichlorofluoromethane	471.2	44.1
Ammonia	405.5	113.5
Water	647.3	221.2
Cyclohexane	553.5	40.7
n-Pentane	469.7	33.7
Toluene	591.8	41.0

Appendix B

Appendix B-1: Refrigeration design spreadsheets

Appendix B-2: Heat removal calculations for refrigeration

Appendix B-3: Feasibility study spreadsheets

Appendix B-4: Process mass balance and assumptions

University of Cape Town

APPENDIX B-1

REFRIGERATION SYSTEM COSTING DESIGN

Calculated HEAT REMOVAL (kJ/s) of freezing for grape marc

Weekly delivery c	20556 kg	Ref: General Chemistry, Ebbing 5th ed
Cp water	4.18 kJ/kg/C	
Cp ice	2.24 kJ/kg/C	
dHvap W	2257 kJ/kg	
dHvap, EtOH	854 kJ/kg	
dHfreeze W	333.9 kJ/kg	
dH,freeze E	104.0 kJ/kg	

Specific energy req'ed to freeze

Qwater	105 kJ/kg
dHfreeze	334 kJ/kg
Qice	18 kJ/kg
Total	456 kJ/kg

	C	F
Ti	25.0	77
Tfreeze	0.0	32
To	-8.0	17.6

	3600 3.6MJ=15c
1MJ =	4.17 cents

Assumptions

Grape marc freshly available Feb. March, April
Grape marc frozen in April and stored for May-mid December

Amount frozen per daily req'ment:	
grape marc	3426 kg/day
moisture content	74%
	2535 kg/day
	7 months
	28 weeks
	6 days/week
Amount of days	200 days of production
from May-December 20	

	water	marc	units
Total amount frozen p.a.	506135	683,966	kg per year
		97709	kg per month
Time to feeze total amount in	77		days
Feb, March and April	3		months
Freezing rate	168712		kg/month
	6561	8866	kg/day
Freezer size	6561	9.1	m3/day
25% air space		11.4	m3/day
Specific heat of grape marc (25 to -8C)		456	kJ/kg

FREEZER DESIGN		1 FREEZER	units
		11.39	m3
Freezer size	Dimensions	11.42	m3
	height	1.25	m
	length	6.30	
	breadth	1.45	m2
	wall area	28.5	m2
		307	ft2
1) Heat removal from grape marc water		76,984,650	kJ/month
		2,993,847	kJ/day
	Hours per day	18	h/day
2) Heat transmission walls (negl)			
	P304		
	4 inch, k=0.2, dT=24F		
		72.0	Btu/ft2 per 24h
		23,312	kJ per 24h
3) People	4 worker, 12 h per day	48,111	kJ per 24h
4) Lights	est area = 4*(3x3)m2	25,785	kJ per 24h
HEAT LOAD per unit		3,091,056	kJ per 24h
HEAT LOAD		171,725	kJ per h
HEAT LOAD		47.7	kJ/s

R.E. (refrigeration effect)	too much NH3	248.5	kJ/kg
actual (at 0.4 MPa)		1078	kJ/kg
Ammonia for freezer		2.654	kg/min
Compressor power req'ment		184.0	kJ/kg
		8.14	kJ/s
Assume the heat loss is 15% of ideal power			
Operating freezer cost (ANNUAL)		12,659,379	kJ per month
		40,690,861	kJ per year
		40,691	MJ per year
		1,695	R per year
Capital costs			
Freezer (unit) cost, 1982 US\$		45,000	for 47.5 kW
Total cost (2006 US\$)		79,895	

Refrigeration (unit) cost, 1982 US\$				
Refrigeration size for 1 unit		683,966 kg marc		
	density	973 kg/m3		
	approx capacity	703 m3 marc	351	
60% extra volume of		1124 m3 storage space	562	
air circulation	approx	1120	560	
Storage dimensions				
	cold storage unit	1	2	units
	Height	2.4	2.4	m
	Area	467	233	m2
	square	22	15	m
		233	164	ft2
	Storage volume	1120	560	m3 storage
	Total capacity (marc)	703	351	per unit
	air volume	417	209	m3
	area walls + roof	674	380	m2
		7255	4090	ft2

For two refrigeration unit design		2
Refrigeration load = 1+2+3+4		
area walls + roof	4090 ft2	
1. heat transmission gains		
4 inch, molded polystyrene	72	Btu/ft2 per 24 h
dT (F) = 60		
assume no sun!		
Heat gain	294473	Btu per 24h
	310,691	kJ per 24h
2. Air infiltration		
Volume of room air	211	m3
	7,445	ft3
air changes per 24h	5.0	table 14.5, P292)
refrigeration cycle time	18.0	h
air flow rate exiting evaporator	59	m3/h
table 14.6, 50% humidity	2.62	Btu/ft3
Heat load	97525	Btu per 24h
	102,896	kJ per 24h
3. Freezer loads (separate)		
(ASSUME 3 DEGREE TEMPERATURE	21,803	J per day (24 h)
LOSS ON CONVEYOR BELT	3,401,228	kJ per year
4. Lights, motor effect		
people effect, 2 workers, 3 h	2100	Btu/h
	6647	kJ per 24h
1.5 W per ft2	247	W
P 319, P 300	10654	kJ per 24h
	17,301	kJ per 24h

Total HEAT LOAD	452,692	kJ per 24h	
Total heat load, refrigeration (2 units)	7.0	kJ/s	
Refrigeration (unit) cost, 1982 US\$	7,000	kJ/s heat removal	
2006 US\$	12,428	24,856	
Condenser cost (1982)	2,022	11.5 m2 size	Ulrich
2006 US\$	3,590		
	1881	est \$12/ft2	Perry 1970
	5,465	2006 US\$	
Total cost of 2xfrig + cond	30,322	2006 US\$	

R.E. (refrigeration effect)	1078	kJ/kg (chart)
Solver Ammonia for frig		
dH(compressor) P-ENTHALPY CHART	184	kJ/kg
Compressor functions	18	h per day
mass refrigerant, M	0.389	kg/min
Compressor power	1.19	kJ/s
Compressor power	21,199,532	kJ per year
MAXimum Compressor energy	21200	MJ per year
Operating comp cost	1767	R per year
2 comp energy	42,399	MJ per year

Q condenser = m*Dh =	4332	kJ/min
for both refrigerator and freezer	259912	kJ/h
Cp air [T = 25C]	1.010	kJ/kg/C
density [T = 25C]	1.200	kg/m3
air flow rate for condenser	7353	kg/h
	6127	m3/h
Q=U*A*dTm	72198	J/s
dTm	25.9	C
U	230	J/m2 s C
A	12.1	m2
	130.6	ft2
Actual area	243	m2
Condenser power	0.15	kW/m2
	1.82	kW
	32,363	MJ per year

ENERGY BALANCE			
	<i>Eevap</i>	+ <i>Ecomp</i>	= <i>Sum</i> <i>Econdenser</i>
kJ/s	47.70	8.14	72.20
	13.97	2.38	
	61.67	10.52	
MJ per year	1,096,737	187,153	1,283,890
Rands per year		7798	53495

APPENDIX B-2

Calculated the HEAT REMOVAL (kJ/s) of freezing for grape marc
and the pump costs for the evaporator

Assuming water is 74% of grape marc
Assuming the heat removal from -5 to -8C EVERY month

Specific energy to be removed because of heat gained over conveyor
belt transferring grape marc from freezer to refrigerator

Cp ice	2.24	kJ/kg/C
Temp,C	-5	-8
Enthalpy	6.72	kJ/kg

24 days per month
18 h per day

1) Max, heat load released by grape marc (H, gm, max)
H, gm, max 47,239 kJ/day 1 refrigerator
H, gm, max 23,620 kJ/day 2 refrigerator

Total HEAT LOAD	452,692 kJ per 24h
	419 kJ/min
Amount of NH3	
1 refrig UNIT =	1.82 ton per 24 h
	1.687 kg/min
2 refrig UNIT =	0.91 tons NH3
	817 kg NH3

Month	kg grape marc	Refrigerator kJ/month
January		
February	227,989	1,133,743
March	455,978	1,133,743
April	683,966	1,133,743
May	683,966	
June	586,257	
July	488,547	
August	390,838	
Sept	293,128	
Oct	195,419	
November	97,709	
kJ per year		3,401,228
MJ per year		3,401

Evaporator

Q = U A dTm
U 100 [W/m2 C] P514, Sinnott, 1983
230 [J/m2 s K] P165, Ulrich, 1984

Temp	C	K	P530, Sinnott, 1983
dTm	25.87	25.87	
dTlm	29.07	29.07	
T1	110	383	
T2	40	313	
t2	60	333	
t1	25	298	
Ft	0.89	0.89	

PUMP ENERGY COSTS		P380 Sinnott
Power = dP Q / e * 100		
	m3/h	head (m)
Rotary (gear) pump	0.05-500	60-200
Assume	efficiency (e)	80%
	mass flow rate	3.4 kg/min
		205.9 kg/h
	density at 1.53 Mpa	584 kg/m3
		0.35 m3/h
	Q	9.79E-05 m3/s
	dP	rho*h*g
		343742.4 N/m2
	Pump power, P	4208 J/s
		4.2 kJ/s
		74,718,915 kJ per year
		74719 MJ per year
	Operating cost	3,113 Rands p.a.

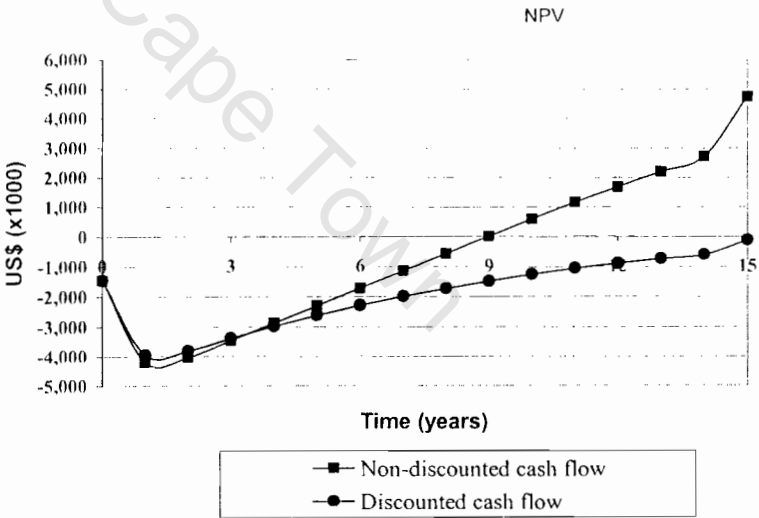
APPENDIX B-3

Feasiblility study

Process selling proanthocyanidin only

Years	Land	TDC	Cstartup	Cwc	SR	COM	GE	D	T	CF	DCF	Total DCF	Total CF
0	101	1,351								-1,453	-1,453	-1,453	-1,453
1		1,351		1,402						-2,753	-2,503	-3,956	-4,206
2			140		1,045	676	76	199	-14	166	138	-3,818	-4,040
3					1,492	676	76	199	162	578	434	-3,384	-3,462
4					1,492	676	76	199	162	578	395	-2,989	-2,884
5					1,492	676	76	199	162	578	359	-2,630	-2,306
6					1,492	676	76	199	162	578	326	-2,304	-1,728
7					1,492	676	76	199	162	578	297	-2,008	-1,150
8					1,492	676	76	199	162	578	270	-1,738	-572
9					1,492	676	76	199	162	578	245	-1,493	6
10					1,492	676	76	199	162	578	223	-1,270	583
11					1,492	676	76	199	162	578	203	-1,068	1,161
12					1,492	676	76		222	518	165	-903	1,679
13					1,492	676	76		222	518	150	-753	2,198
14					1,492	676	76		222	518	136	-616	2,716
15	-101			-1402	1,492	676	76		222	2,021	484	-132	4,737

TDC	Total depreciable capital (equipment + install + land + building)
Cstartup	start up costs
Cwc	working capital
SR	Sales revenue
COM	Cost of manufacture
D	linear depreciation (10% over ten years)
T	tax = (SR-Cstartup-TPC-GE-D) for that year
	r = 30%
CF	cash flow = SR-land-TDC-Cstartup-CWC-TPC-GE-T)
DCF	discounted cash flow = CF/(1+i)^n
	i required Discounted Rate of return =
	n number of years
NPV	net present value = sum of DCF for all years



Feasibility study

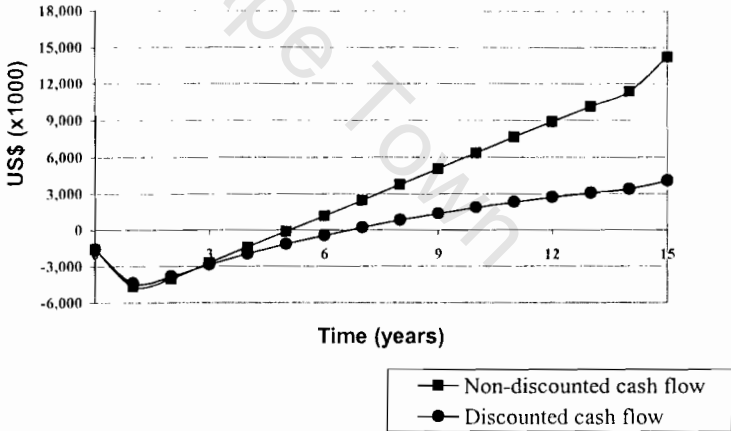
All figure in US\$ x 1000

Total capital invest 4,833
(i.e., \$ 7.398 million for total capital investment)

Process selling proanthocyanidin and anthocyanin

Years	Land	TDC	Cstartup	Cwc	SR	COM	GE	D	T	CF	DCF	Total DCF	Total CF
0	113	1,503									-1,615	-1,615	-1,615
1		1,503		1,559							-3,061	-2,783	-4,398
2			156		1,809	743	84	222	181	644	532	-3,866	-4,032
3					2,584	743	84	222	460	1,296	974	-2,892	-2,736
4					2,584	743	84	222	460	1,296	885	-2,007	-1,441
5					2,584	743	84	222	460	1,296	805	-1,202	-145
6					2,584	743	84	222	460	1,296	732	-471	1,151
7					2,584	743	84	222	460	1,296	665	194	2,447
8					2,584	743	84	222	460	1,296	605	799	3,743
9					2,584	743	84	222	460	1,296	550	1,348	5,039
10					2,584	743	84	222	460	1,296	500	1,848	6,335
11					2,584	743	84	222	460	1,296	454	2,302	7,631
12					2,584	743	84		527	1,229	392	2,694	8,861
13					2,584	743	84		527	1,229	356	3,050	10,090
14					2,584	743	84		527	1,229	324	3,374	11,320
15	-113			-1,559	2,584	743	84		527	2,901	694	4,068	14,221

- TDC
- Total depreciable capital (equipment + install + land + building
- Cstartup
- start up costs
- Cwc
- working capital
- SR
- Sales revenue
- COM
- Cost of manufacture
- D
- linear depreciation (10% over ten years)
- T
- tax = (SR-Cstartup-TPC-GE-D) for that year
- r = 30%
- CF
- cash flow = SR-land-TDC-Cstartup-CWC-TPC-GE-T)
- DCF
- discounted cash flow = CF/(1+i)^n
- i
- required Discounted Rate of return =
- n
- number of years
- NPV
- net present value = sum of DCF for all years



Appendix B - 4: Process mass balance and assumptions

BASIS: ONE WORKING DAY - 2 REPEATED SHIFTS (AM, PM)

EXTRACTIONS PER SHIFT MARC 1,711 kg (wet) marc
SOLVENT 5,132 L

THIS MASS BALANCE IS FOR A SINGLE EXTRACTION

- ASSUMPTIONS
- ANTIOXIDANTS IN SOLUTION HAVE NEGLIGIBLE MASS
 - 90% OF SOLVENT REMAINS WITH MARC AFTER EXTRACTION, LEAVES IN STREAM 7a
 - 20% OF MARC (SEEDS) REMAINS WITH SOLVENT, LEAVES IN STREAM 6
 - 5% OF ENTERING SOLVENT LOST DURING FILTRATION, AND ALL SOLIDS REMOVED IN FILTER CAKE
 - 10% OF SOLVENT ENTERING EVAPORATOR 90% (WATER) IS NOT RECYCLED
 - THEREFORE 90% IS RECYCLED.
 - 20% ELUTE WITH 10% SOLVENT VOLUME OF STREAM 9
 - 50% OF SOLVENT IN STREAMS 5, 6, 7A, 7B, 8
 - 5.41% DRY EXTRACT YIELD over 2 stage!
 - 5.7% TPC CONTENT OF DRY EXTRACT
 - 77.5% PROANTHOCYANIDIN CONTENT OF TPC
 - 18.9% ANTHOCYANIN CONTENT OF TPC

1 L = 0.264173 US gallons

MB CHECK

Extractor	IN	OUT
solvent	5132	5132

MB CHECK black box

	IN	OUT
solvent	842	842
water	567	567
ethanol	177	177
eluting solven	98	98
	842	842
grape marc	1711	1711

Second stage mass balance (marc extracted for the second time, and is subsequently removed).

OPERATION UNITS	UNITS	STORAGE	1	2	CRUSHING	3	RECYCLE	4	5	EXTRACTION	6a	7a	6b	7b	FILTRATION	8	EVAPORATION	9	10	PURIFICATION	11	12	DRYING	13
STREAM NUMBER																								
TEMPERATURE	°C		-15			25			25	50						25	45			25		7		
LOST SOLVENT (see assumptions)							90%		50%	90%	50%			5%		50%	10%		20%					
										20%	90%						90%							
GRAPE MARC	KG	1711			1711					342	1276			342									92.62	
EXTRACT	kg																					4.09		
proanthocyanins	kg																					1.00		
anthocyanins	kg																							
SOLVENT																			98	98	98			
ELUTING SOLVENT	L																					439		
WATER	L		390		390		1999	177	2310	257	2566	128		2438		439						49		
ETHANOL	L						2389	177	2310	257	2566	128		2438		49						585		
TOTAL	L		390		390		4388	354	4619	513	5132	257		4876		488			98	98	585			
SOLVENT																								
WATER	US gallons		103		103		528	47	610	68					644		116					116		
ETHANOL							631	47	610	68					644		13					13		
TOTAL			103		103		1159	94	1220	136				68	1288		129		26	26	155			
OPERATION TIMES					20				90							30	120	120						
ESTIMATED TIMES FOR PUMPS	min		10				20	15		20						30.00	15.00	77.00	15					
FLOW RATES	GPM		10.3				58.0	6.2		61.0						42.9	8.6	1.67	1.67					
			P-2				P-8	P-1	P-3						P-4, P-5	P-6	P-7	P-9	4.1	L/min				
tank numbers	m3		T-2				T-4	T-1								T-3		T-5		T-6		T-7		
Tank sizes	gallons		3.12				8.78	8.50								9.75		0.98		2.34		7.02		
stoorage time	days		824				2318	2245								2576		258		618		1855		
extractions	per day		2				1	6								1		1		6		3		
			4				2	4								2		2		4		4		

Appendix C

EC50 data and graphs for:

- Catechin
- Cyanidin-3-glucoside

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APPENDIX C- 1

Catechin DPPH analysis

Experiment date

Date	day	month	year
26	Friday	August	2005

Sample preparation

Solvent dissovled in	100% ethanol
Stock solution concentration	250 mg/L

DPPH std curve

conc	mg/L	5	10	15	20	25
abs	nm	0.134	0.238	0.352	0.477	0.602

DPPH std curve

equation y = 42.488x - 0.3211 R 2 0.9985

DPPH Absorbance results

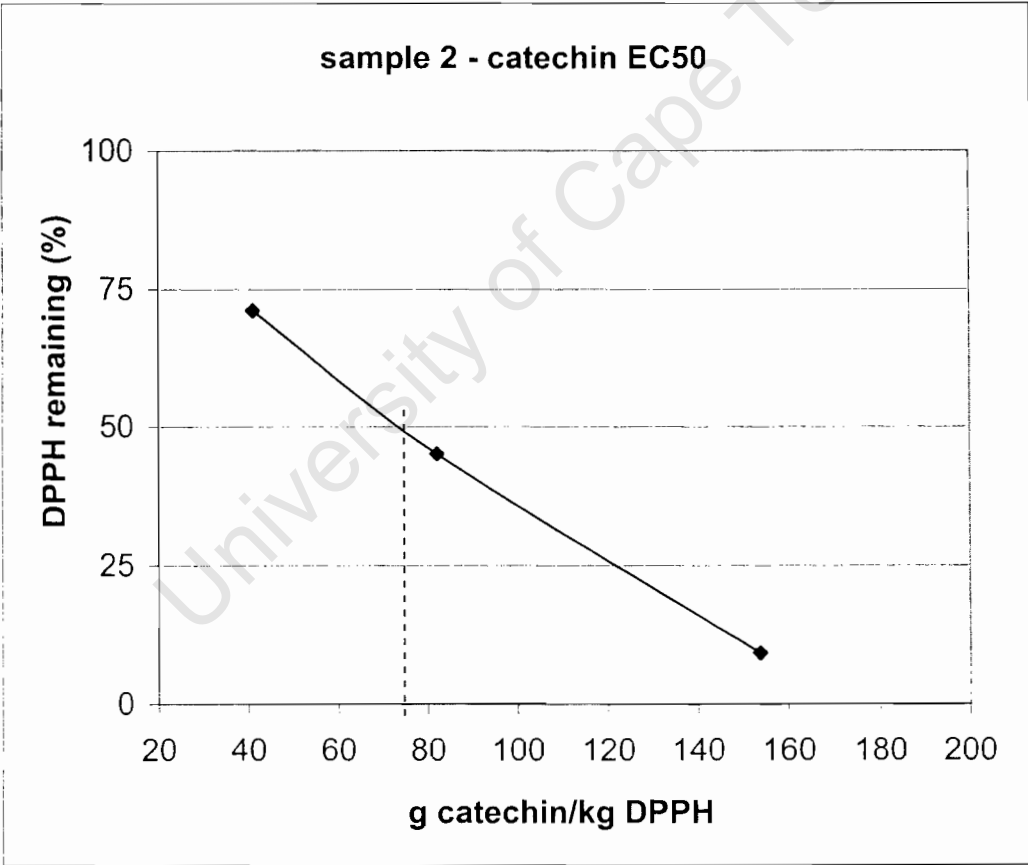
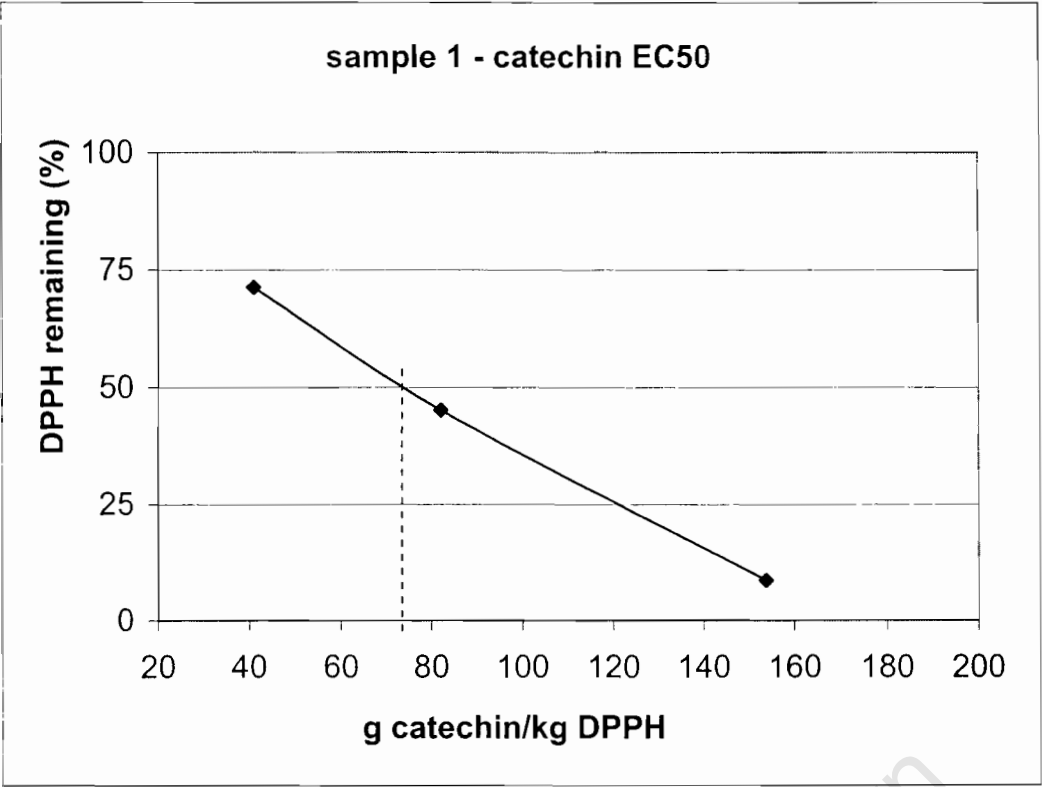
Conc.mg/L	40		80		150	
Sample	1	2	1	2	1	2
Time						
0	0.608	0.608	0.629	0.605	0.607	0.609
1	0.504	0.495	0.416	0.411	0.348	0.298
3	0.493	0.495	0.409	0.409	0.265	0.254
10	0.475	0.475	0.372	0.366	0.192	0.173
30	0.433	0.436	0.293	0.284	0.062	0.056
70	0.427	0.431	0.276	0.268	0.054	0.052
90	0.425	0.425	0.269	0.262	0.057	0.056

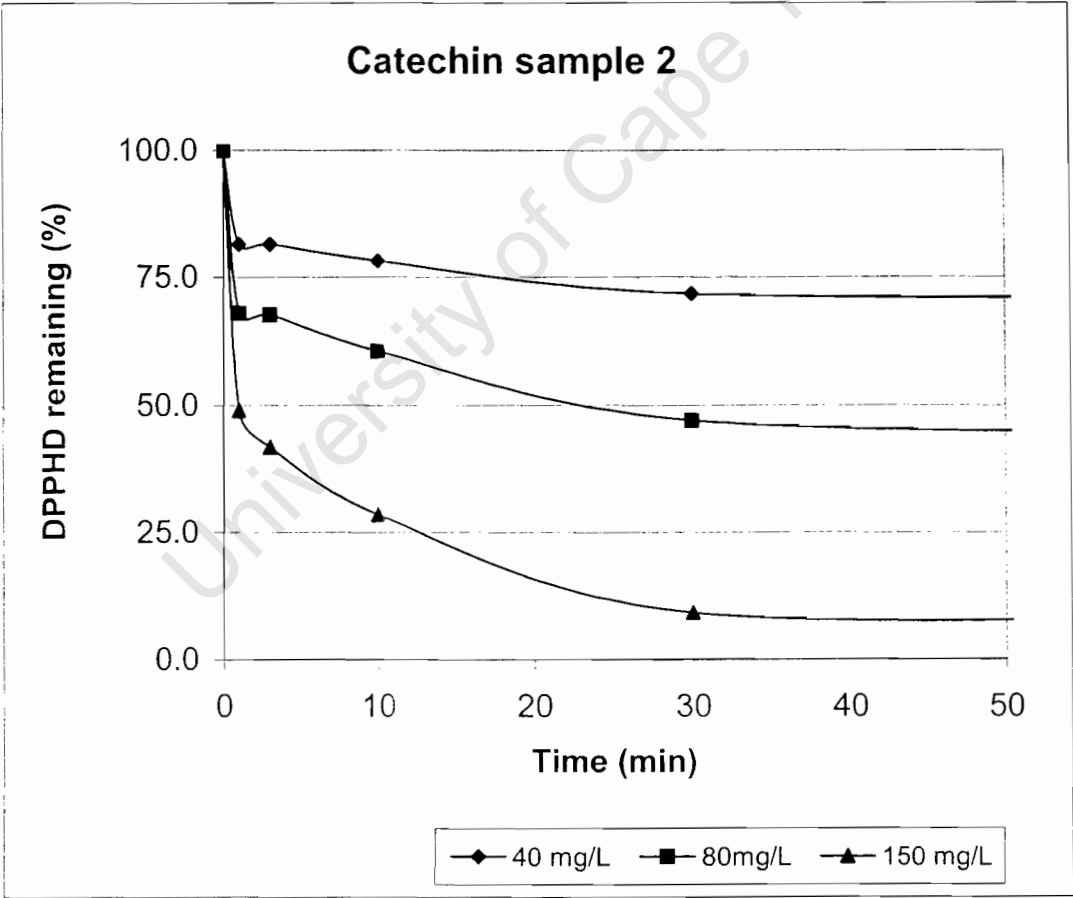
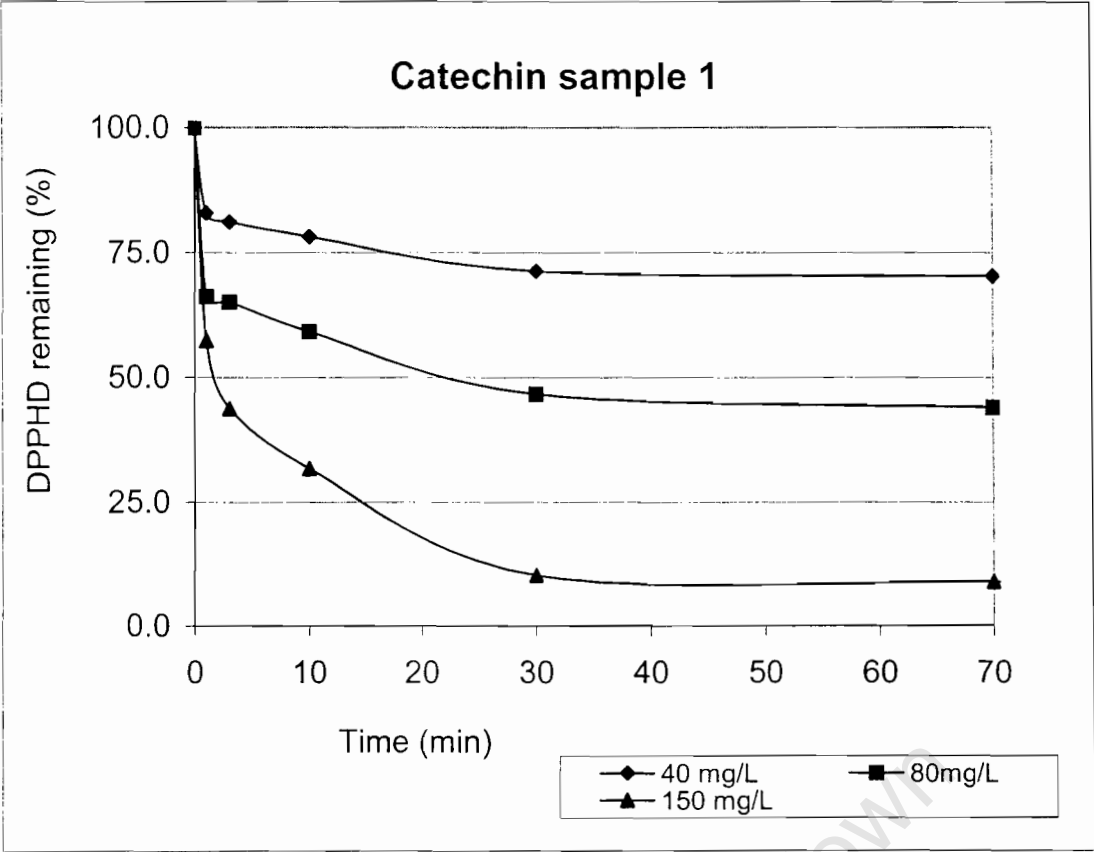
DPPH % remaining results

Conc.mg/L	40		80		150	
Sample	1	2	1	2	1	2
Time						
0	100.0	100.0	100.0	100.0	100.0	100.0
1	82.9	81.4	66.1	67.9	57.3	48.9
3	81.1	81.4	65.0	67.6	43.7	41.7
10	78.1	78.1	59.1	60.5	31.6	28.4
30	71.2	71.7	46.6	46.9	10.2	9.2
70	70.2	70.9	43.9	44.3	8.9	8.5
90	69.9	70.4	42.8	43.3	9.4	9.2

Table EC₅₀ and steady state time

Concentration		Sample 2		Sample 1	
mg/l	g/kg DPPH	time	% dpph	time	% dpph
150	154	35	9.2	38	8.6
80	82	44	45	42	45.1
40	41	30	71.1	30	71.2





APPENDIX C- 2

Cyanidin-3-glucoside DPPH analysis

Experiment date

Date	day	month	year
22	Friday	November	2005

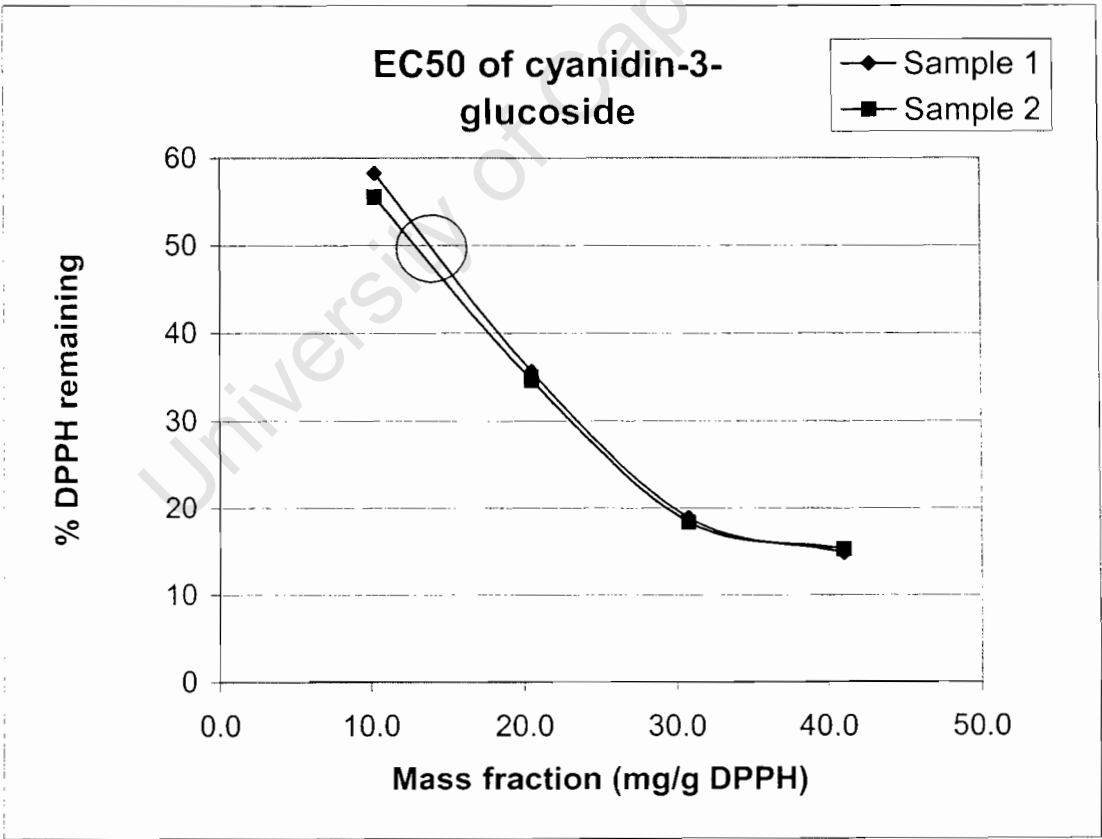
conc mg/L	Mass fraction mg/g DPPH	DPPH remaining (%)	
		Sample 1	Sample 2
10	10.3	58.2	55.5
20	20.5	35.6	34.6
30	30.8	18.9	18.4
40	41.0	14.8	15.3

	Sample 1	Sample 2
EC50	13.8	12.9

Average EC₅₀ 13.4 ± 0.45

T_{EC50} 210.0 min

Antiradical efficiency (AE) 0.0003567
0.36 x10⁻³



Raw data for cyanidin-3-glucoside DPPH assay

Sample 1

DPPH Absorbance results

Conc.	20		30		40	
Sample	1	2	1	2	1	2
Time						
0	0.629	0.633	0.651	0.653	0.649	0.649
1			0.621	0.613	0.625	0.610
3	0.533	0.537	0.537	0.531	0.531	0.522
10	0.406	0.412	0.396	0.397	0.363	0.361
60	0.285	0.288	0.204	0.205	0.171	0.170
120	0.245	0.243	0.147	0.147	0.120	0.123
150	0.234	0.232	0.138	0.131	0.110	0.111
180	0.228	0.222	0.125	0.123	0.101	0.104
210	0.224	0.219	0.123	0.120	0.096	0.099

Sample 2

DPPH % remaining results

Conc.	20		30		40	
Sample	1	2	1	2	1	2
Time						
0	100.0	100.0	100.0	100.0	100.0	100.0
1			95.4	93.9	96.3	94.0
3	84.7	84.8	82.5	81.3	81.8	80.4
10	64.5	65.1	60.8	60.8	55.9	55.6
60	45.3	45.5	31.3	31.4	26.3	26.2
120	39.0	38.4	22.6	22.5	18.5	19.0
150	37.2	36.7	21.2	20.1	16.9	17.1
180	36.2	35.1	19.2	18.8	15.6	16.0
210	35.6	34.6	18.9	18.4	14.8	15.3

Appendix D

HPLC chromatograms of grape marc extracts obtained using:

- **Ethanol**
- **Methanol**
- **Ethyl acetate**

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D-7000 HPLC System Manager Report

Analyzed: 02/15/05 03:08 PM

Reported: 02/15/05 03:54 PM

Processed: 02/15/05 03:54 PM

Data Path: C:\Win32App\HSM\Jason\DATA\0009\

Processing Method: 80MeOH-20water

System(acquisition): Sys 1

Series:0009

Application: Jason

Vial Number: 1

Sample Name: ethanol

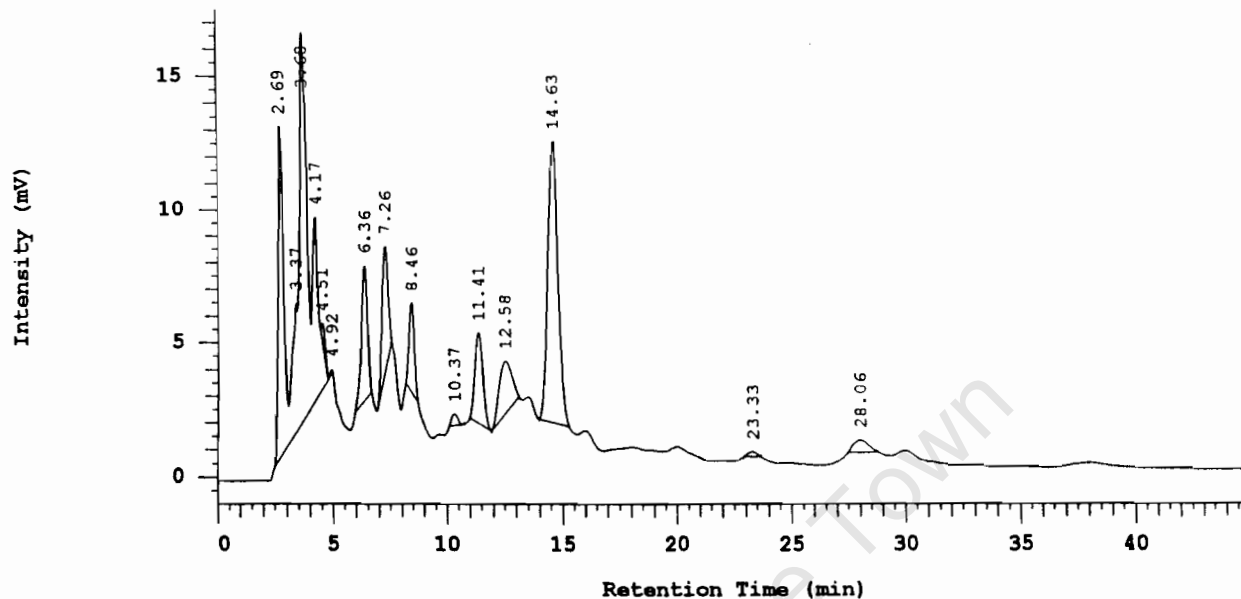
Vial Type: UNK

Injection from this vial: 1 of 1

Volume: 10.0 ul

Sample Description:

Chrom Type: HPLC Channel : 1



Acquisition Method: 80MeOH-20water

Column Type:

Developed by: Jason

Pump A Type: L-7100

Solvent A:

Method Description: 2.5% aq acetic acid +20% methanol, 80% water

Chrom Type: HPLC Channel : 1

Peak Quantitation: AREA

Calculation Method: AREA%

No.	RT	Area	Conc 1	BC
1.	2.69	186330	12.969	BV
2	3.37	69311	4.824	VV
3	3.68	300781	20.935	VV
4	4.17	140892	9.806	VV
5	4.51	9197	0.640	TBI
6	4.92	1095	0.076	BB
7	6.36	88554	6.164	BB
8	7.26	85260	5.934	BB
9	8.46	52424	3.649	BB
10	10.37	7536	0.525	BB
11	11.41	78343	5.453	BB
12	12.58	74374	5.177	BB
13	14.63	317521	22.100	BB

D-7000 HSM: Jason		Series: 0009	Report: original	System: Sys 1	
14	23.33	5049	0.351	B	
15	28.06	20050	1.396	B	
1436717			100.000		

Peak rejection level: 0

University of Cape Town

D-7000 HPLC System Manager Report

Analyzed: 02/15/05 03:54 PM

Reported: 02/15/05 04:40 PM

Processed: 02/15/05 04:40 PM

Data Path: C:\Win32App\HSM\Jason\DATA\0009\

Processing Method: 80MeOH-20water

System(acquisition): Sys 1

Series:0009

Application: Jason

Vial Number: 2

Sample Name: methanol

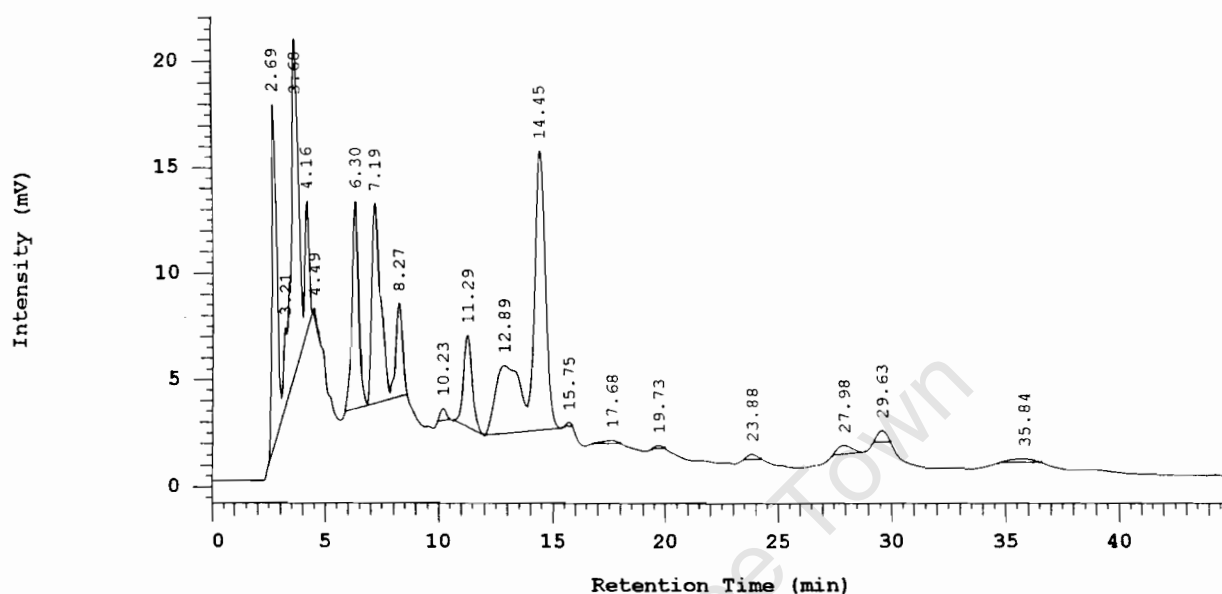
Vial Type: UNK

Injection from this vial: 1 of 1

Volume: 10.0 ul

Sample Description:

Chrom Type: HPLC Channel : 1



Acquisition Method: 80MeOH-20water

Column Type:

Developed by: Jason

Pump A Type: L-7100

Solvent A:

Method Description: 2.5% aq acetic acid +20% methanol, 80% water

Chrom Type: HPLC Channel : 1

Peak Quantitation: AREA

Calculation Method: AREA%

No.	RT	Area	Conc 1	F
1	2.69	225249	11.003	F
2	3.21	35756	1.747	\
3	3.68	342680	16.739	\
4	4.16	76096	3.717	\
5	4.49	4033	0.197	F
6	6.30	190113	9.287	F
7	7.19	248784	12.152	F
8	8.27	88167	4.307	\
9	10.23	9684	0.473	F
10	11.29	110065	5.376	F
11	12.89	230966	11.282	F
12	14.45	422298	20.628	\
13	15.75	2446	0.119	F

D-7000 HSM: Jason		Series: 0009	Report: original	System: Sys 1	
14	17.68	6405	0.313		B
15	19.73	3325	0.162		B
16	23.88	6236	0.305		B
17	27.98	17485	0.854		B
18	29.63	14642	0.715		B
19	35.84	12763	0.623		B
2047193			100.000		

Peak rejection level: 0

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D-7000 HPLC System Manager Report

Analyzed: 02/15/05 04:40 PM

Reported: 02/15/05 05:25 PM

Processed: 02/15/05 05:25 PM

Data Path: C:\Win32App\HSM\Jason\DATA\0009\

Processing Method: 80MeOH-20water

System(acquisition): Sys 1

Series:0009

Application: Jason

Vial Number: 3

Sample Name: ethyl acetate

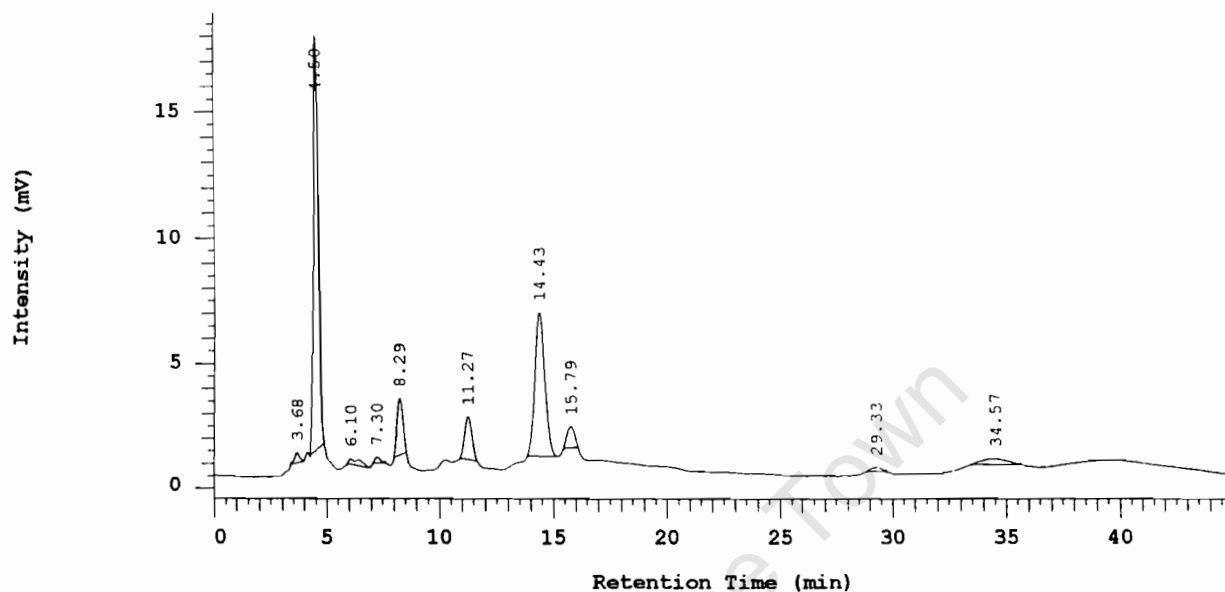
Vial Type: UNK

Injection from this vial: 1 of 1

Volume: 10.0 ul

Sample Description:

Chrom Type: HPLC Channel : 1



Acquisition Method: 80MeOH-20water

Column Type:

Developed by: Jason

Pump A Type: L-7100

Solvent A:

Method Description: 2.5% aq acetic acid +20% methanol, 80% water

Chrom Type: HPLC Channel : 1

Peak Quantitation: AREA

Calculation Method: AREA%

No.	RT	Area	Conc 1	I
1	3.68	6762	1.224	I
2	4.50	241548	43.720	I
3	6.10	8903	1.612	I
4	7.30	3527	0.638	I
5	8.29	39522	7.154	I
6	11.27	36661	6.636	I
7	14.43	173070	31.326	I
8	15.79	19132	3.463	I
9	29.33	5112	0.925	I
10	34.57	18245	3.302	I
		552482	100.000	

Appendix E

Standard curves and UV wavelength scans for:

- Gallic acid
- Catechin
- Cyanidin-3-glucoside
- DPPH

University of Cape Town

APPENDIX E-1

Gallic acid standard curve

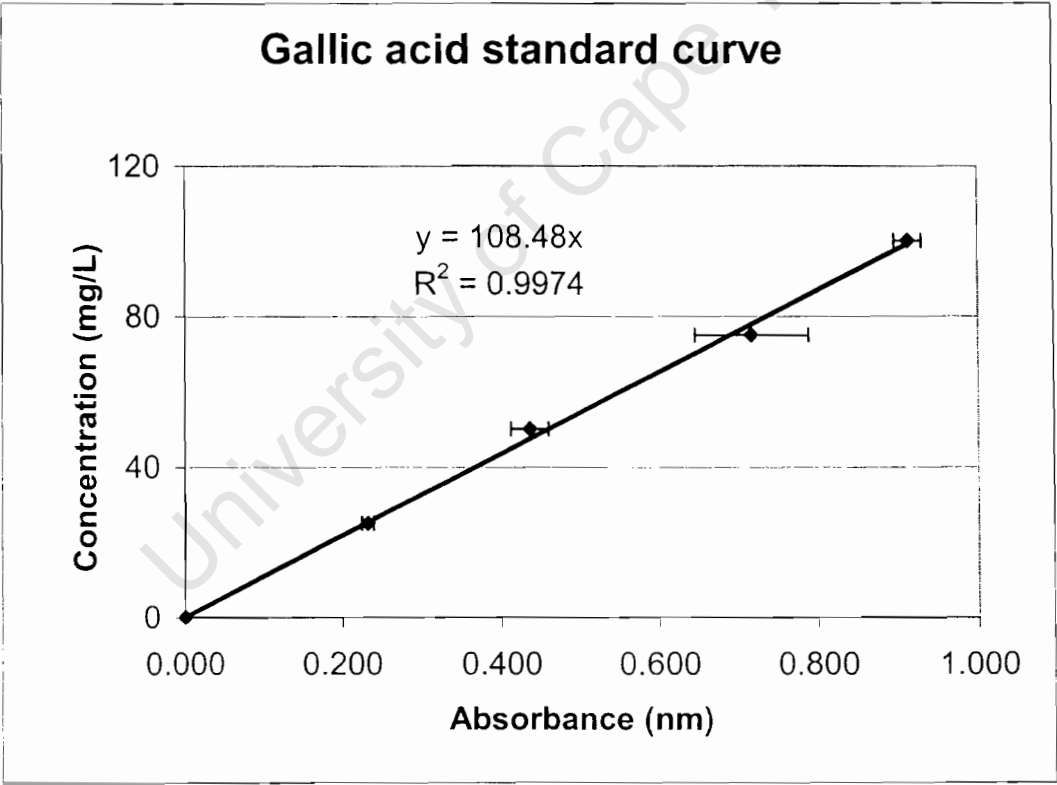
Experiment date

Date	day	month	year
22	Friday	November	2005

Standard preparation

Gallic acid	0.4	mL
Folin Ciolateau	0.4	mL
Sodium carbonate	0.4	mL
water	2.8	mL

Gallic acid conc (mg/L)	0	25	50	75	100
Average absorbance (nm)	0.001	0.233	0.436	0.717	0.913
Sample 1	0.000	0.227	0.419	0.768	0.901
Sample 2	0.001	0.238	0.453	0.666	0.925
Standard deviation	0.00071	0.00778	0.02404	0.07212	0.01697



APPENDIX E-2

Catechin standard curve

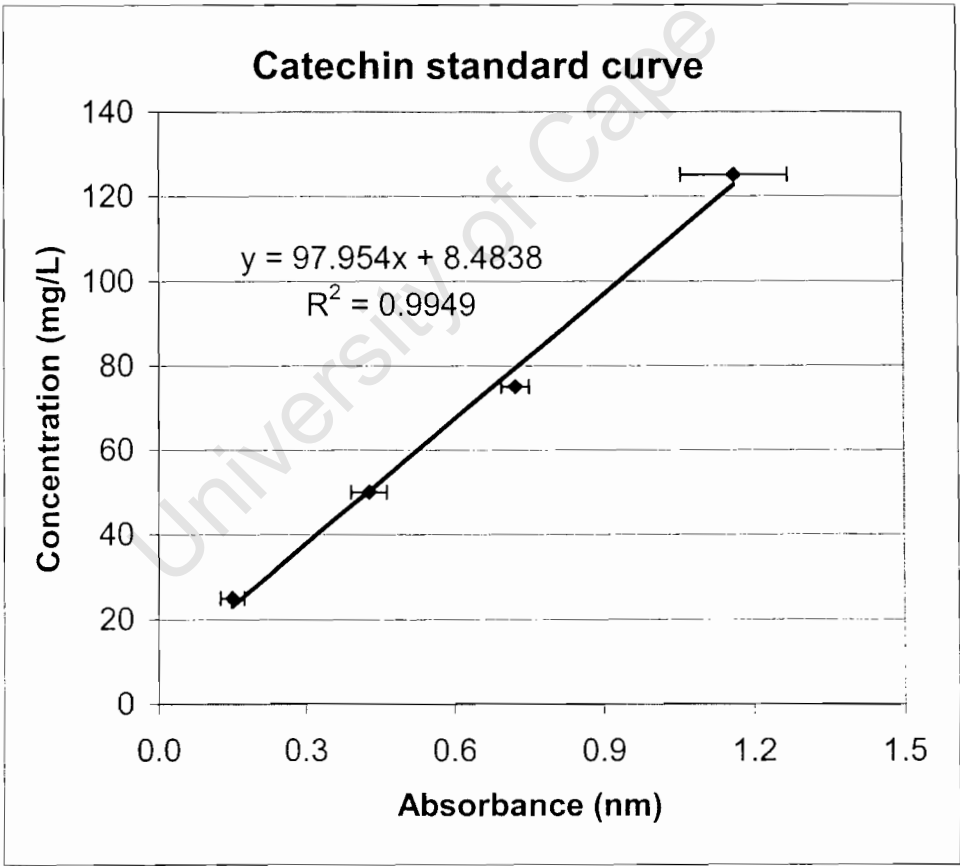
Experiment date:

time	date	day	month	year
16:00	19	Thursday	May	2005

Standard preparation

1% vaillin in ethanol = 2.5g in 250ml
1 % (g/100ml)
250mg/L catechin stock = 0.0125g in 50ml 250 mg/L

Catechin	mL	1	2	3	5
Concentration	mg/L	25	50	75	125
Abs. average	nm	0.1485	0.4255	0.7225	1.1645
Sample 1		0.1310	0.4000	0.7030	1.089
Sample 2		0.1660	0.4510	0.7420	1.240
Standard deviation		0.0247	0.0361	0.0276	0.1068



APPENDIX E- 3

Cyanidin-3-glucoside standard curve

Experiment date

Date	day	month	year
18	Monday	July	2005

Sample preparation:

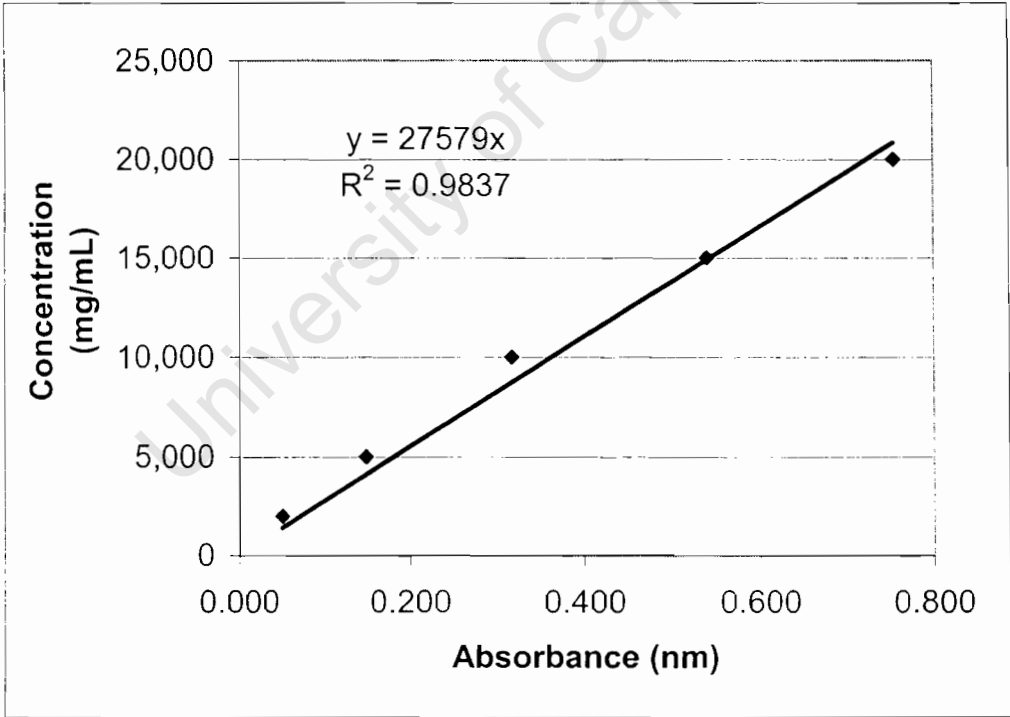
Cyanidin-3-glucoside dissolved in 100% ethanol
stock concentration 40 mg/L

Concentration

mg/L	2	5	10	15	20
mg/mL	2,000	5,000	10,000	15,000	20,000

Absorbance readings at 540 nm

run 1	0.052	0.143	0.315	0.54	0.761
run 2	0.05	0.159	0.322	0.543	0.758
run 3	0.049	0.145	0.313	0.536	0.747
average	0.050	0.149	0.317	0.540	0.755
stdev	0.002	0.009	0.005	0.004	0.007

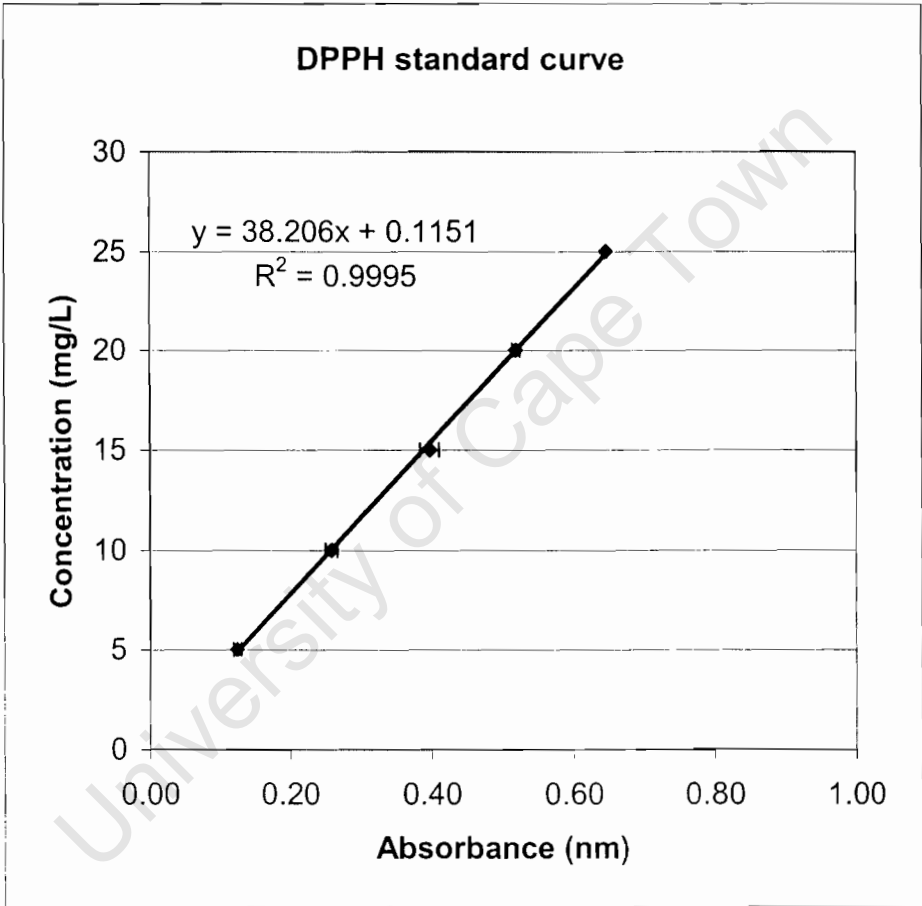


APPENDIX E-4

DPPH standard curve

Standard preparation:
Dissolve 12.5 mg in 500 mL of methanol
(25 mg/L stock solution)

Concentration	mg/L	5	10	15	20	25
Abs. average	nm	0.124	0.259	0.398	0.520	0.648
Sample 1		0.128	0.253	0.407	0.516	0.647
Sample 2		0.120	0.265	0.388	0.524	0.648
Standard deviation		0.006	0.008	0.013	0.006	0.001



WAVELENGTH SCAN (400-600nm)

TOTAL POLYPHENOLS ASSAY

GALLIC ACID

100 mg/L

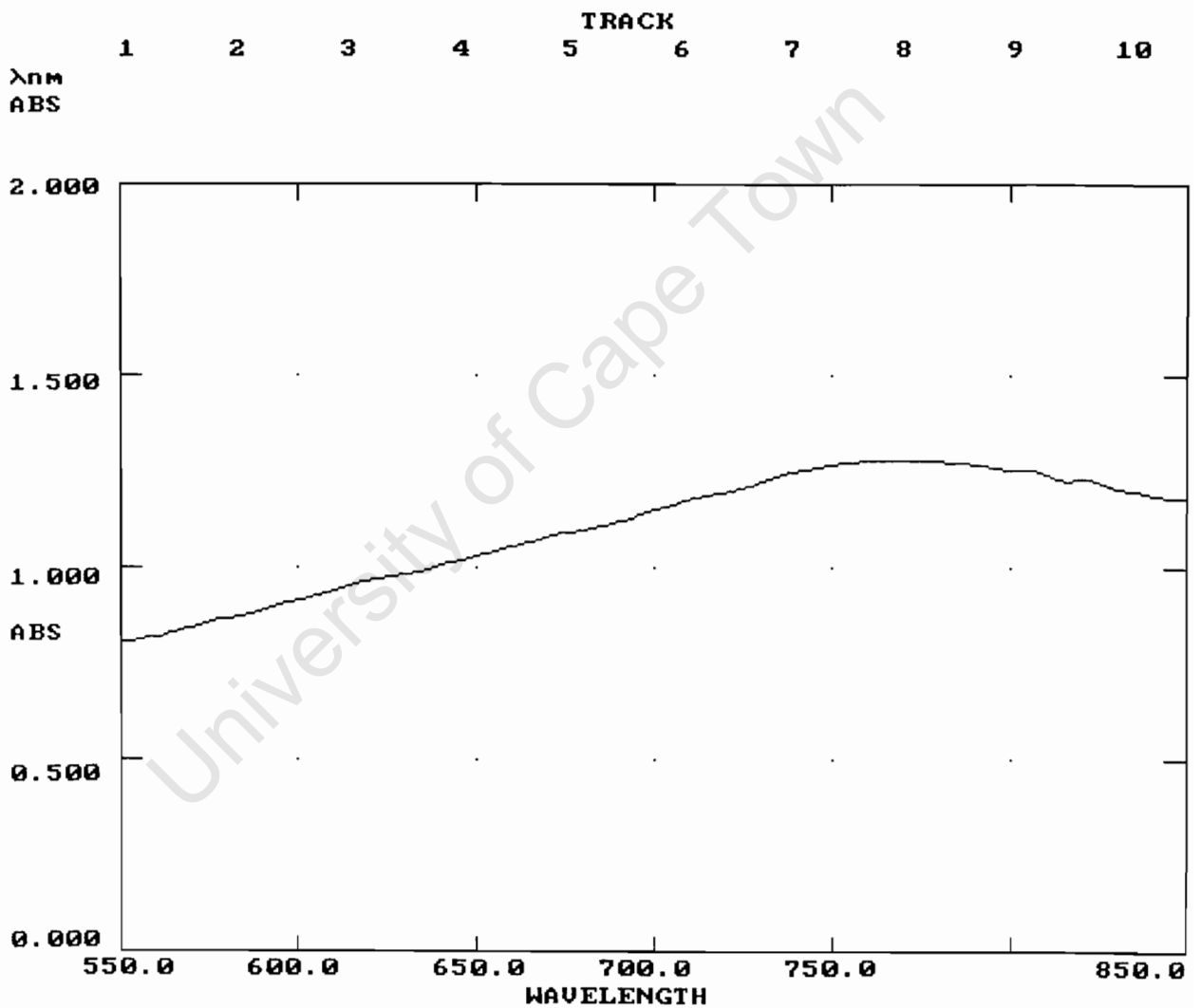
$\lambda_{\text{MAX}} = 765 \text{ nm}$

HEXIOS α UV-VISIBLE SPECTROPHOTOMETER v4.04 PAGE 1

DATE :19/07/05 SERIAL No:074910 ID :PHENOL
TIME :12:56:18 OPERATOR :

SCAN TYPE:INTELLISCAN SPEED:NORMAL DATA INT:1.0nm
BASELINE:DEFAULT BANDWIDTH:2.0nm LAMP CHANGE:325nm

SIPPER MODE:SIP&RUN VOLUME:2.000ml AIR GAP:10cm
SAMPLE:WASTE TUBING CAL:1.000 LOW VOL:ON
CELL PROG CELLS:7 CELL PROG CYCLES:1
REF. MODE:OFF CELL PROG MODE:MANUAL



WAVELENGTH SCAN (400-600nm)

CATECHIN

250 mg/L

$\lambda_{\text{MAX}} = 500 \text{ nm}$

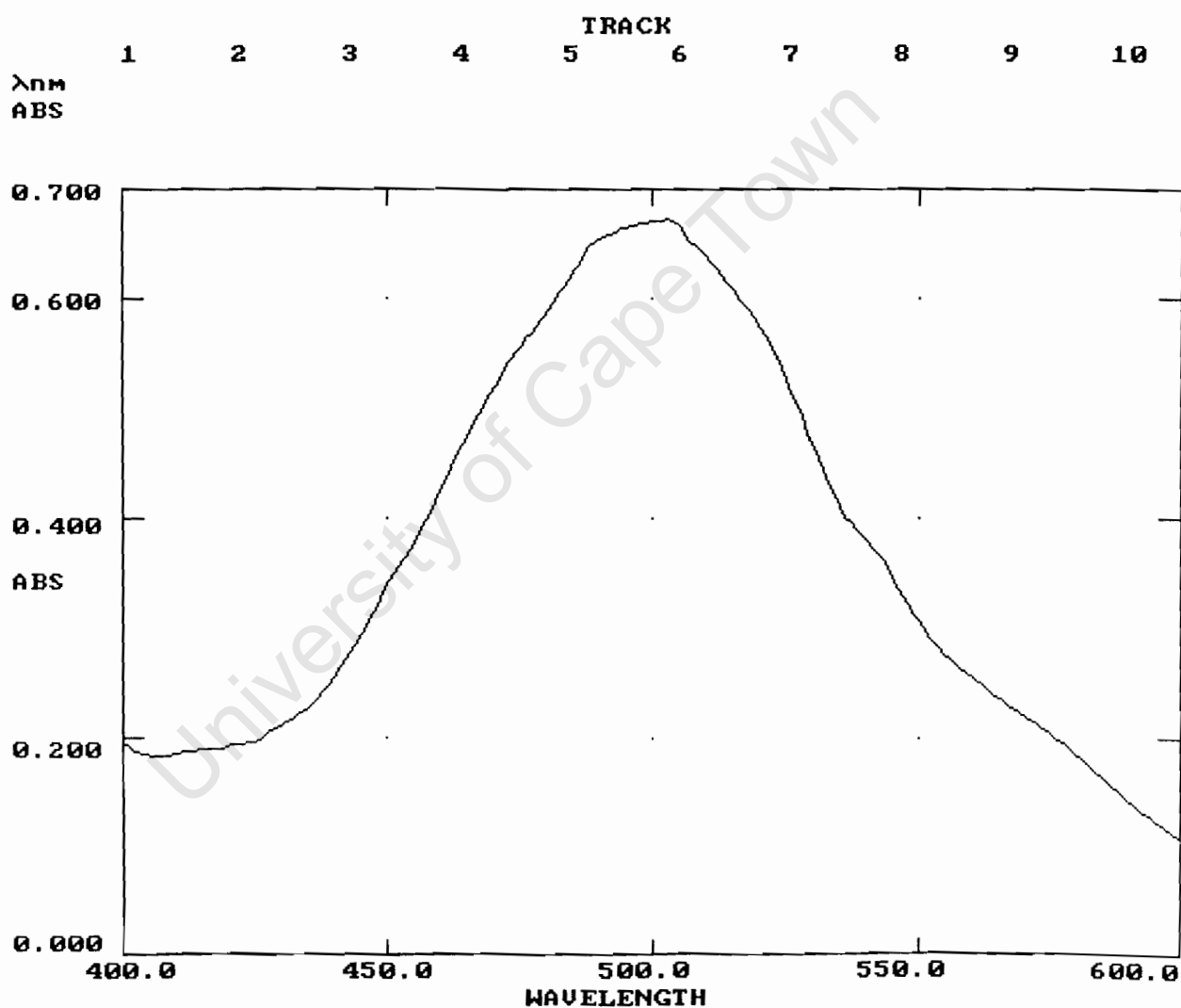
HEXIOS α UV-VISIBLE SPECTROPHOTOMETER v4.04 PAGE 1

DATE :14/07/05 SERIAL No:074910 ID :

TIME :14:45:12 OPERATOR :

SCAN TYPE:INTELLISCAN SPEED:NORMAL DATA INT:1.0nm
BASELINE:DEFAULT BANDWIDTH:2.0nm LAMP CHANGE:325nm

SIPPER MODE:SIP&RUN VOLUME:2.000ml AIR GAP:10cm
SAMPLE:WASTE TUBING CAL:1.000 LOW VOL:ON
CELL PROG CELLS:5 CELL PROG CYCLES:1
REF. MODE:OFF CELL PROG MODE:MANUAL



WAVELENGTH SCAN (400-600nm)

CYANIDIN-3-GLUCOSIDE

40 mg/L

$\lambda_{\text{MAX}} = 540 \text{ nm}$

HEXIOS α UV-VISIBLE SPECTROPHOTOMETER v4.04 PAGE 1

DATE : 11/07/05 SERIAL No: 074910 ID :
TIME : 15:25:34 OPERATOR :

SCAN TYPE: INTELLISCAN SPEED: NORMAL DATA INT: 1.0nm
BASELINE: DEFAULT BANDWIDTH: 2.0nm LAMP CHANGE: 325nm

SIPPER MODE: SIP&RUN VOLUME: 2.000ml AIR GAP: 10cm
SAMPLE: WASTE TUBING CAL: 1.000 LOW VOL: ON

