The extraction of resveratrol and other polyphenols from solid winery waste and an investigation into alternative resveratrol recovery techniques

by

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March 2020

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ABSTRACT

Resveratrol is a phenolic compound that is produced by several plant species such as grape (*Vitis vinifera*) as a protection mechanism against both biotic and abiotic stress. Resveratrol is currently of interest and under investigation as nutraceutical supplement, and there is a significant market value for the compound. Winemaking is one of the largest agricultural activities in the world and produces significant amount of solid biomass waste, which is often rich in resveratrol. The aim of this work was to investigate, through consecutive harvests to estimate variability, solid winery waste as a source of resveratrol to produce a high value antioxidant supplement. As well as to investigate aqueous two-phase systems and protein precipitation as resveratrol recovery methods and improve downstream purification processes.

In order to extract and recover the maximum amount of resveratrol, sample preparation and process conditions that could result in degradation were investigated. No resveratrol degradation was observed during biomass storage, drying and extraction. However, it was found that resveratrol is sensitive to changes in pH and will degrade under basic conditions.

In this study the different parts of solid winery waste from a 2018 and 2019 harvest was investigated as possible resveratrol sources. From the comparison of the different sources over time it was found that the 2019 Pinotage stems contained a maximum of 73 ± 4.3 µg/g resveratrol.

Maltodextrin (dextrose equivalence 16.5-19.5) and polyethylene glycol (PEG) 8000 aqueous two-phase systems (ATPS) were investigated to partition and concentrate extracted resveratrol into edible maltodextrin. It was found that for all the systems investigated most of the resveratrol remained in the PEG phase, indicating no concentrating effect to the desired phase. The use of proteins to recover resveratrol by forming a precipitate was investigated by determining the amount of resveratrol precipitated with ovalbumin, tryptone soy broth and yeast extract. For the systems investigated a maximum of 83 ± 2.1% resveratrol formed a recoverable precipitate with yeast extract, indicating a viable recovery method.

From the investigation of resveratrol degradation, it was concluded that the process conditions investigated can be used to quantify resveratrol in solid winery waste. Significantly variable resveratrol concentrations were noted between consecutive harvests, indicating a high variability in productivity. Further, resveratrol distribution throughout the plant was highly variable with negligible resveratrol extracted from the grape skins, seeds and leaves and up to 73 ± 4.3 µg/g resveratrol extracted from the canes. Nonetheless, it was concluded that Pinotage solid winery waste can be used as a possible source of resveratrol.

By evaluating protein precipitation as a resveratrol method, it was concluded that the selected proteins interacted with resveratrol to form a recoverable precipitate and could be used as a resveratrol recovery method. By comparing the recovery achieved with ATPS to protein precipitation, it was concluded that the amount of resveratrol recovered is too low with maltodextrin-PEG ATPS to be used as a feasible recovery method.

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TABLE OF CONTENTS

NOMENCLATURE

°C	Celsius
µg	Microgram
µg/g	Microgram per gram
µl	Microlitre
ATPS	Aqueous two- phase system
	Aqueous two- phase separation
B	Bottom phase
DE	Dextrose equivalence
FC	Folin Ciocalteu
g	Gram
g/L	Gram per litre
GAE	Gallic acid equivalence
h	Hour
HPLC	High performance liquid chromatography
Kp	Partition coefficient
L	Litre
LC-MS	Liquid chromatography–mass spectrometry
M	Molar
m	Mass
MD	Maltodextrin
mg	Milligram
mg/g	Milligram per gram
mg/L	Milligram per litre
mg/ml	Milligram per millilitre
min	Minute

mL	Millilitre
MW	Molecular weight
n	Mole
OIV	International Organisation of Vine and Wine
OVA	Ovalbumin
PEG	Polyethylene glycol
PES	Polyether sulfone
rpm	Revolutions per minute
SAWIS	South African wine industry statistics
STL	Slope of tie line
SU	Stellenbosch University
T	Top phase
TLL	Tie line length
TSB	Tryptone soy broth
UV	Ultraviolet
UV-vis	Ultraviolet–visible
v/v %	Volume percentage
w	Water
W	Watt
wt %	Weight percentage
wt/wt	Weight fraction
Υ	Recovery

1 INTRODUCTION

Resveratrol is a polyphenolic secondary plant metabolite produced by plant species such as *Vitis vinifera* and *Polygonum cuspidatum* as protection mechanism against extreme weather conditions, mechanical damage and fungal infections (Xiong *et al.*, 2014). Interest in resveratrol from *Vitis vinifera* originated from the 'French paradox', where the French population had a diet high in saturated fats along with red wine, while cardiovascular diseases were less than expected. While the Chinese knotweed (*Polygonum cuspidatum*) containing resveratrol has been used in traditional medicine to treat inflammation and cardiovascular diseases (Wang, Liu and Chen, 2013). However, insufficient human clinical trials have been successfully completed to use resveratrol in pharmaceutical products. Nonetheless, resveratrol is still of interest as nutraceutical supplement.

In 2018, South African wineries produced approximately 950 million litres of wine and South Africa is currently the 9th largest wine producing country (Roca, 2019) with the largest vineyard area in Stellenbosch (*SA wine industry 2018 statistics*, 2019). During the winemaking process some phenolic compounds like resveratrol solubilise into the wine, while some of the resveratrol remains in the skins and seeds making it a possible source of resveratrol to produce a valuable, saleable product from solid winery waste.

Aqueous two-phase systems (ATPS) is a low cost, low toxicity extraction and purification method used to separate and purify proteins, cell organelles and enzymes (Raja *et al.*, 2012). In order to extract or recover a specific molecule, aqueous solutions that can form two immiscible phases such as salt-polymer and polymer-polymer systems are used. Aqueous two-phase separation is a scalable system that can operate continuously with low cost and non-toxic chemicals such as polyethylene glycol, maltodextrin and tartrate. ATPS can also be used in a multistage extraction and recovery system to first extract and partition a specific molecule like resveratrol to one phase, followed by a subsequent ATPS to concentrate and recover that molecule (Raja *et al.*, 2012).

Polyphenols are multidentate ligands that can bind to proteins to form a polyphenol-protein precipitate if sufficiently mixed in solution. The addition of polyphenols to a protein solution is often used to recover proteins in solution (Papadopoulou and Frazier, 2004). Therefore, the recovery process can be reversed, and proteins can be used to precipitate polyphenols such as resveratrol. According to Ingham (1978) solutions like polyethylene glycol can be used to extract and recover proteins without interacting with or denaturing the protein.

Winemaking is one the largest agricultural activities in the world and produces significant amount of waste, in the form of grape skins and seeds, stems, canes and leaves. Some of the waste produced is valorised into products such as tartaric acid, xylitol and grape seed oil while the remaining waste is

incinerated, landfilled or composted (Devesa-Rey et al., 2011). To beneficiate the unused solid winery waste, resveratrol might be extracted to produce a high value product. The work in this thesis is based on the biorefinery concept to produce a high value product from waste biomass and moves towards the development of an alternative process to quantify, extract and recover resveratrol from grape skins, seeds, canes, leaves and stems from locally sourced solid winery waste. The research will build on work of Herbst (2019), investigating the use of a polyethylene glycol-tartrate two-phase system as a polyphenol extraction method but will focus on resveratrol recovery from polyethylene glycol, as well as quantifying the resveratrol in solid winery waste.

The aim of the research is to investigate a subsequent aqueous two-phase system with polyethylene glycol containing resveratrol and maltodextrin to concentrate resveratrol into maltodextrin that is often used in the food, beverage and pharmaceutical industry, as well as investigating the recovery of resveratrol from polyethylene glycol with different proteins to form a recoverable resveratrol-protein precipitate. Further, the study also aims to investigate solid winery waste as a source of resveratrol as well as to investigate factors that influence the resveratrol concentration.

The project aim and research questions that arose from the literature review will be more fully discussed in Chapter 3. In order to achieve the project aim, the literature research and experimental work will be divided into three main objectives. Firstly, in order to extract and recover resveratrol for a nutraceutical supplement, factors and processing conditions that can influence the resveratrol concentration should be investigated. Another objective is to investigate solid winery waste as a resveratrol source and the variability of resveratrol in winery waste. The final objective of the project is to investigate and compare the recovery of resveratrol from polyethylene glycol with a subsequent polymer-polymer ATPS and protein precipitation. To achieve the project aim, each objective will be investigated in the following chapters.

Chapter 2: A literature review will be conducted to identify gaps in the research field by investigating resveratrol production, current and new resveratrol extraction methods, ATPS and protein precipitation as recovery methods. As well as the feasibility of *Vitis vinifera* as resveratrol source.

Chapter 3: The project aim and objectives will be discussed in Chapter 3 as well as the key questions that arose from Chapter 2.

Chapter 4: In order to answer the research questions, experimental work to investigate resveratrol degradation, extraction and recovery will be conducted as discussed in the methodology.

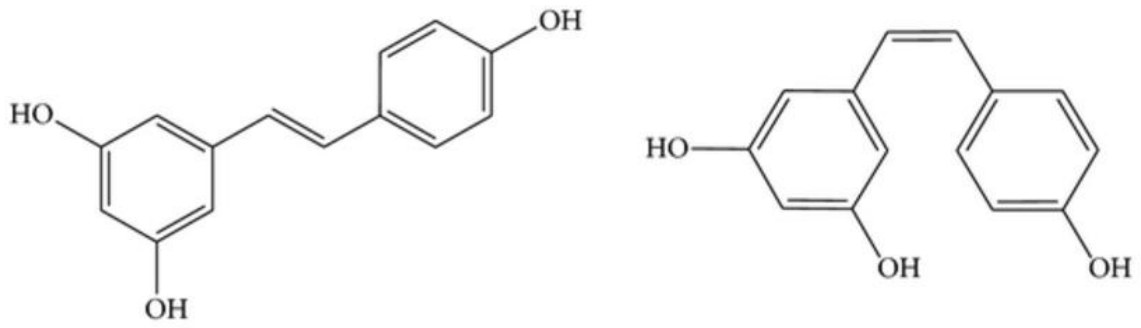
Chapter 5: The results achieved from the experimental work is summarised in Chapter 5 along with critical analysis of resveratrol degradation, the quantification of resveratrol in Pinotage from two consecutive harvests as well as the evaluation of ATPS and protein precipitation as resveratrol recovery methods.

Chapter 6 and 7: The project conclusions will be discussed in Chapter 6 followed by the recommendations for future work in Chapter 7.

2 LITERATURE REVIEW

2.1 Resveratrol

Resveratrol (3, 5, 4'- Trihydroxystilbene) is a polyphenolic molecule with a stilbene structure with two benzene rings connected by an ethane bridge, as seen in Figure 1. Resveratrol is a secondary metabolite produced by several plants species as mechanism to control environmental stress, such as UV radiation, fungal infections, mechanical damage or extreme weather conditions (Xiong *et al.*, 2014) and is classified as a phytoalexin.



(a) **(b)**

Figure 1. The molecular structure of (a) *trans*-resveratrol and (b) *cis*-resveratrol (Gambini *et al.*, 2015).

2.1.1 Importance of resveratrol

Interest in resveratrol from *Vitis vinifera* arose from the 'French paradox', where the French population had a diet high in saturated fats along with red wine, while cardiovascular diseases were less than expected. The health benefits of red wine were linked to resveratrol and led to the investigation of resveratrol as a nutraceutical.

Polygonum cuspidatum containing 0.524 mg/g resveratrol has been used in traditional Chinese medicine to treat cardiovascular diseases, inflammation and tumours (Wang, Liu and Chen, 2013). In order to prove the pharmacological activity of resveratrol, different medical studies are being conducted in animal models and humans. For instance, the ability of resveratrol to treat neurological diseases such as Alzheimer's and Parkinson's in rat models was investigated by Anekonda (2006). The study showed a decrease in the neurodegenerative toxins in rats with the treatment of resveratrol. Baur *et al.* (2006) investigated the effect of resveratrol ingestion to increase the lifespan of rat models with a high fat diet. It was found that rats fed resveratrol with a high fat diet had the same life span as rats with a low-fat controlled diet.

According to Gerogiannaki-Christopoulou *et al.* (2006) resveratrol can be chemo preventative agent that inhibits tumour initiation, promotion and progression by inhibiting free radical formation. Resveratrol can also potentially suppress oxidation of low-density lipoprotein, act as an estrogen receptor agonist and inhibits platelet aggregation. However, insufficient human clinical trials have been successfully completed to confirm that the response of animal models correlates to humans. Nonetheless, the molecule is of interest as a nutraceutical supplement with a significant market with current resveratrol supplements ranging between $0.15 to $2.76 per 100 mg (Skerrett, 2012). The current global *trans*-resveratrol market value is estimated as $97.7 million and it is predicted to grow 8.1% by 2028 (*An Incisive, In-depth Analysis on the Resveratrol Market*, 2019).

2.1.2 Chemical and physical properties

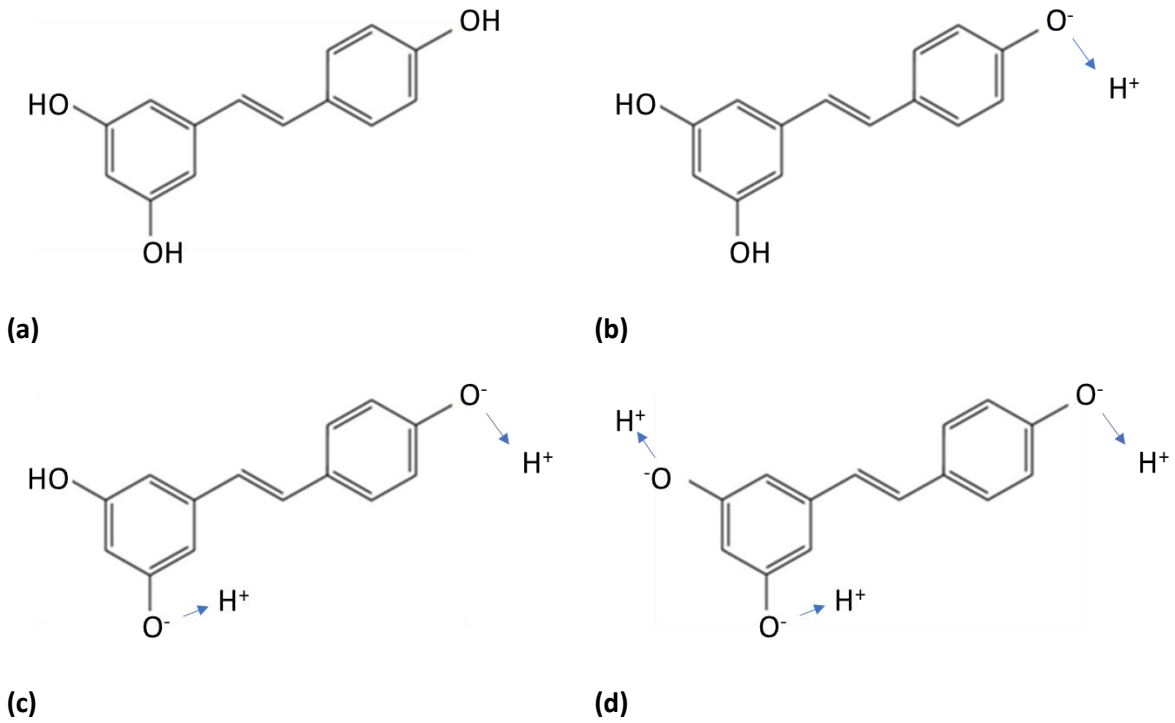


Figure 2. The molecular structure of **(a)** protonated *trans*-resveratrol and the deprotonation of resveratrol at each equivalence point at pK_{a1}=8.8 **(b)**, pK_{a2}=9.8 **(c)** and pK_{a3}=11.4 **(d)**.

As seen in Figure 2a, resveratrol is a non-polar, hydrophobic polyphenol with three hydroxyl groups. It is a weak acid with acidic dissociation constants of pK_{a1}=8.8, pK_{a2}=9.8 and pK_{a3}=11.4 (Robinson, Mock and Liang, 2015). As seen in Figure 2a-d, the polarity of resveratrol is influenced by the pH and as the pH increases above pK_{a1}=8.8, pK_{a2}=9.8 and pK_{a3}=11.4 resveratrol becomes more polar due to the charge of the oxygen atoms. Resveratrol is a crystalline solid with a molecular weight of 228.25 g/mol. According to Robinson, Mock and Liang (2015) resveratrol has a low water solubility of 0.05 mg/ml at 25°C but is soluble in solvents such as acetone, ethanol and methanol, as shown in Table 1.

Table 1. The solubility of resveratrol in methanol, ethanol, acetone and water at five different temperatures (Zhang *et al.*, 2018).

Temperature (°C)	Solubility (mg/ml)			
	Methanol	Ethanol	Acetone	Water
5	62.7	47.1	294	0.0106
15	68.9	55.6	294	0.0193
25	76.8	66.7	296	0.0388
35	84.9	77.5	293	0.0677
45	92.3	86.4	291	0.0936

As seen in Table 1, the solubility of resveratrol increases with an increase in temperature from 5°C to 45°C.

2.1.3 Why is resveratrol produced

Stilbenes are secondary plant metabolites produced by various plant species with several studies investigating the influence on plant disease resistance. According to Berman *et al.* (2017) the stilbene derivative, resveratrol, was found in elevated concentrations in infected and damaged leaves of *Veratrum grandiflorum*. This led to determining the concentration of resveratrol in other plant species experiencing environmental stress as a step to understanding the reason it produces resveratrol. According to Romero-Pérez *et al.* (2001) resveratrol is also produced as protection against plant pathogens such as *Botrytis cinerea* and *Plasmopara viticola* found on *Vitis vinifera*.

2.1.4 Sources of resveratrol

Resveratrol is found several plant species such as blueberries, peanuts, grapes and the Japanese herb *Polygonum cuspidatum*. According to Smoliga *et al.* (1997), *Polygonum cuspidatum* can contain *trans*-resveratrol concentrations up to 0.542 mg/g while also containing transpolydatin, which can be hydrolysed to *trans*-resveratrol (Wang, Liu and Chen, 2013). Smoliga *et al.* (1997) also asserted that grapes contain 3.54 µg/g resveratrol, while red wines can contain resveratrol up to 14 mg/l. The concentration of *trans*-resveratrol is too low in wine if consumed in moderation to have a therapeutic effect, while excessive consumption of wine can be disadvantageous (Vincenzi *et al.*, 2013). Xiong *et al.* (2014) states that peanut sprouts are the most economical source of resveratrol in terms of the isolation of the molecule.

2.1.4.1 Vitis vinifera as resveratrol source

As discussed in Section 2.1.5 resveratrol is produced by *Vitis vinifera* as a protection mechanism against plant pathogens such as *Botrytis cinerea* and *Plasmopara viticola*. The great interest in resveratrol has led to several studies investigating the resveratrol in *Vitis vinifera*. Langcake and Pryce (1976) first reported that resveratrol is only present is grapevine leaves that are infected, or UV irradiated. While several other studies investigated the resveratrol concentration in different grape varieties. Figure 3 summarizes the resveratrol concentration in different grape varieties of three different studies.

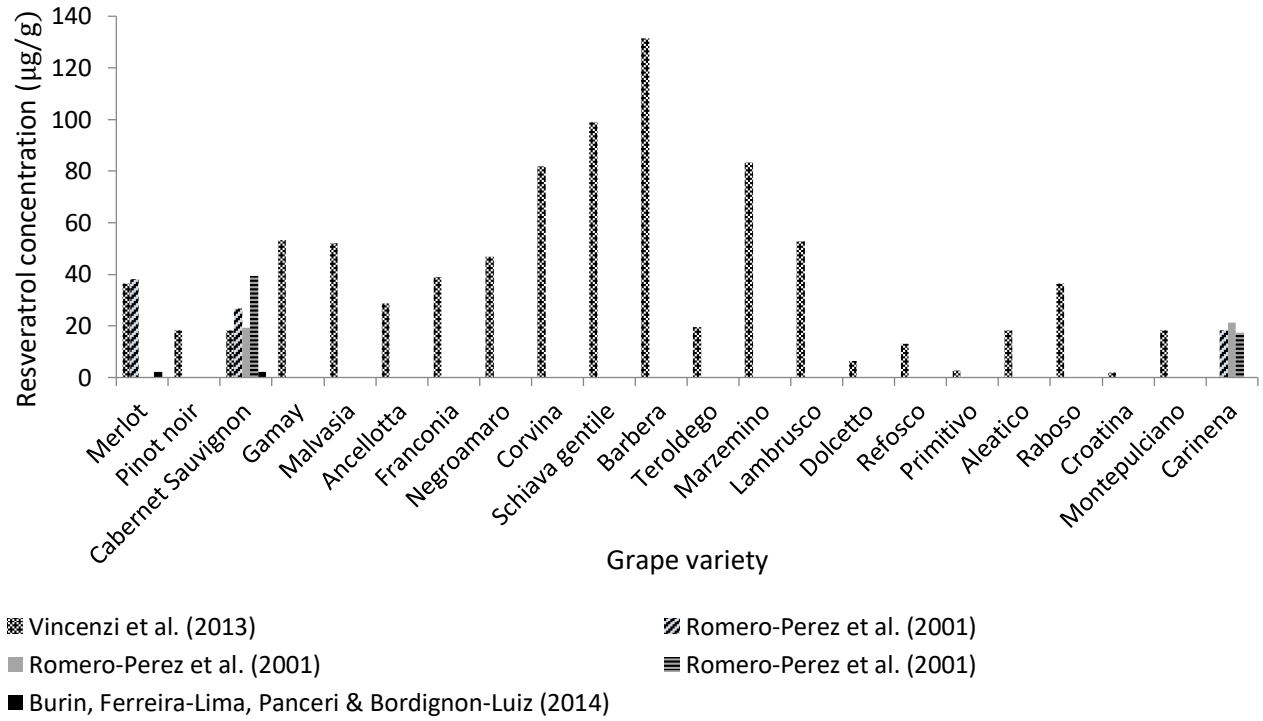


Figure 3. The difference in resveratrol concentration in different grape berries from various literature sources, indicating that resveratrol concentration within varietals vary (Romero-Pérez *et al.*, 2001; Burin *et al.*, 2014; Vincenzi *et al.*, 2013).

As seen in Figure 3, the resveratrol concentration is dependent on the grape variety but variation within a variety also occurs. Cho, Hong, Chun, Lee & Min (2006) also found that the grape vine stems contain up to 440 µg/g dry material. Resveratrol is produced in grape skins, seeds and stems resulting in resveratrol solubilizing in wine during fermentation from the skins and seeds. Due to the low solubility of *trans*-resveratrol in water the majority of resveratrol remain in the post fermentation skins and seeds making wine pomace a possible source of resveratrol and other polyphenols.

2.1.5 Factors influencing trans-resveratrol concentration in Vitis vinifera

2.1.5.1 Environmental factors

As seen in Section 2.1.4 the concentration of resveratrol in grapes and wine is dependent on the grape variety but also varies within varietals indicating that resveratrol production is also dependent on environmental factors and corresponds to the study of Feijóo, Moreno and Falqué (2008) that stated that resveratrol content in grapes vary with different grape varieties in different regions, as seen in Figure 4. The resveratrol concentration varies with geography due to different climatic conditions such as humidity, rainfall and UV radiation. According to Siemann and Creasy (1992), vineyards with a high humidity are more susceptible to fungal diseases and will produce more resveratrol. Geana *et al.* (2014) studied the resveratrol concentration in different varietals in the Southern Romania. It was found that the resveratrol contents varied within the three regions, as seen in Figure 4.

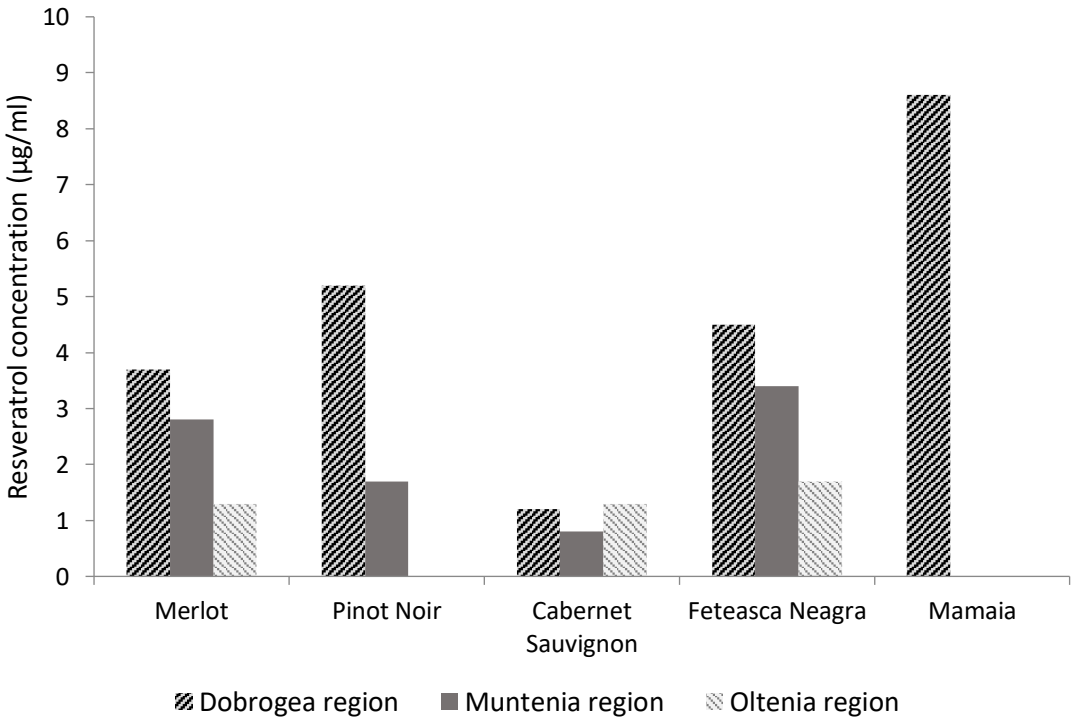


Figure 4. The measured resveratrol concentration in different red wines of the Dobrogea, Muntenia and Oltenia region in Romania, indicating that resveratrol concentration within varietals vary due to geography and wine making techniques.

This variation in resveratrol concentrations seen in Figure 4 may also be due to a difference in the vinification process. Factors such as maceration time, type of enzyme and yeast strain used as well as the malolactic fermentation process can influence the solubilization of resveratrol during wine making (Feijóo, Moreno and Falqué, 2008). According to Gerogiannaki-Christopoulou *et al.* (2006) *cis*-resveratrol

does not naturally occur but is derived from isomerization of *trans*-resveratrol. This can occur due to UV radiation during the vinification process (Vincenzi *et al.*, 2013). Zupančič *et al.* (2015) also found that UV radiation will result in isomerization, while other factors such as temperature and pH result in degradation.

2.1.5.2 Isomerisation

As seen in Figure 1 resveratrol has two stereoisomers, *trans*-resveratrol and *cis*-resveratrol. According to Gerogiannaki-Christopoulou *et al.* (2006) *cis*-resveratrol does not naturally occur due to the steric hindrance of the two aromatic rings but is derived from isomerisation of *trans*-resveratrol. This can occur due to UV radiation that will result isomerisation. Zupančič *et al.* (2015) also stated that *trans*-resveratrol will undergo light induced isomerisation since it is less stable than *cis*-resveratrol. Trela and Waterhouse (1996) stated that between 80% to 91% trans-resveratrol will isomerise to *cis*-resveratrol while Yokotsuka and Okuda (2011) stated that approximately 11% *trans*-resveratrol will isomerise to *cis*-resveratrol. The degree of resveratrol isomerisation is dependent on factors such as the irradiation time, wavelength and extraction conditions.

2.1.5.3 pH

Resveratrol is a weak acid and becomes ionized with an increase in pH resulting in degradation. It was found that at a pH of 8-9 it is the least stable with rapid degradation while it was the most stable below a pH of 6 (Robinson, Mock and Liang, 2016). Trela and Waterhouse (1996) stated that *trans*-resveratrol is stable in a system with a pH of 1, 3.5 and 7 up to 28 days. Zupančič *et al.* (2015) also studied the degradation rate of resveratrol with an increase in pH and found that *trans*-resveratrol is stable in an acidic medium of 1.2 for approximately 90 days while resveratrol degradation significantly increased in alkaline systems.

2.1.5.4 Thermal degradation

The effect of temperature on degradation was also investigated but it was found that pH had a more significant effect on degradation while an increase in temperature in basic conditions accelerated the degradation. In acidic conditions an increase in temperature from 4°C to 37°C did not accelerate the degradation (Zupančič *et al.*, 2015).

2.2 Resveratrol extraction

In order to extract resveratrol and other polyphenols from a biomass source, different extraction methods were investigated. Extraction methods that are currently used to extract resveratrol are

discussed below in Section 2.2.1 and aqueous two-phase extraction to extract resveratrol is discussed in Section 2.2.2.

2.2.1 Solvent extraction

Solid-liquid extraction techniques using organic solvents are usually used for polyphenol extractions from plant material. These solvents usually include ethyl acetate, ethanol, methanol and acetone (Geana *et al.*, 2015). Different solvent extraction techniques that have been used to extract resveratrol from different sources are summarised in Table 2.

Table 2. Different resveratrol extraction and recovery techniques from grape biomass

Resveratrol source	Extraction	Recovery	Reference
Grape stems	Ethanol-water extraction	Diethyl ether precipitation and polyamide column chromatography	Aaviksaar *et al.* (2003)
Grape leaves	Ultrasound assisted ethanol	Mesoporous carbon adsorption	Sun *et al.* (2018)
Grape canes	Acetone solvent extraction, methanol solvent extraction, fluidized-bed extraction with acetone or methanol, accelerated solvent extraction with methanol, microwave-assisted extraction with methanol and Soxhlet extraction with methanol	-	Soural *et al.* (2015)
Grape skins	Ethanol-water extraction	-	Averilla *et al.* (2019)
Grape skins	Solvent extraction with ethanol, methanol, acetone and ethyl acetate	-	Romero-Pérez *et al.* (2001)

The different solvent extraction techniques summarised in Table 2 have been successfully used to extract resveratrol from different sources of grape biomass but solvent extraction can be a time-consuming method as well as using large quantities of solvents (Solyom *et al.*, 2014).

2.2.2 ATPS

Aqueous two-phase systems or aqueous two-phase separation (ATPS) is an extraction and purification method used for biological products such as proteins, enzymes and cell organelles (Raja *et al.*, 2012). ATPS is based on two immiscible phases and several factors such as molecule size, bio-specific affinity, electrochemical interactions and hydrophobicity (Grilo, Aires-Barros and Azevedo, 2016). ATPS can either be polymer-polymer, polymer-salt, salt-alcohol, polymer-surfactants and ionic liquids (Grilo, Aires-Barros and Azevedo, 2016).

ATPS is an alternative extraction method if the desired molecule is sensitive to organic solvents or high temperature and pressure conditions. ATPS is also easy to scale-up, can operate continuously and uses low cost and toxicity chemicals. Since ATPS is a safe and low-cost extraction and recovery method, edible two-phase systems could be used to partition resveratrol.

2.2.2.1 Factors influencing extraction

The recovery and partitioning is dependent on several factors such as the polymer type and molecular weight, the polymer or salt concentration, pH and temperature (Grilo, Aires-Barros and Azevedo, 2016).

In polymer-salt ATPS phosphates and sulphates are generally used, since an ATPS with phosphate or sulphate as the salt phase will have a large biphasic region. However, it can produce large quantities of waste if used industrially (Xavier *et al.*, 2014). For the extraction of resveratrol for pharmaceutical purposes edible, bio-degradable and non-toxic salt such as citrate and tartrate are preferred (Raja *et al.*, 2012). Partitioning in a polymer-salt ATPS is influenced by the salt concentration used. An increase in the salt concentration will result in an increase in the system ionic strength.

In a polymer-salt two phase system, partitioning is also influenced by a difference in hydrophobicity. Both components are hydrophilic and a hydrophobic, non-polar molecule such as resveratrol will partition to the less hydrophilic phase.

According to Iqbal *et al.* (2016) partitioning is also dependent on the polymer molecular weight used. High molecular weight polymers are used in a low concentration. Partitioning can be improved by using low molecular weight polymers to decrease the interfacial tension between the two phases and increasing the hydrophobic area (Yang *et al.*, 2013). However, for low molecular weight polymers a higher concentration should be used. A decrease in molecular weight of polyethylene glycol (PEG) will result in a decrease in the hydrophobicity of the system by decreasing the amount of ethylene oxide groups

available for each PEG molecule. For systems using polyethylene glycol (PEG) as one phase, a molecular weight between 4000 g/mol to 20000 g/mol should be used (Walter and Johansson, 1994).

The pH of an ATPS can influence the extraction and partitioning of a molecule by changing the electrochemical interactions in the system by changing the charge of the solution or molecule. According to Raja *et al.* (2012), pH can also change the two-phase area of the system. Raja *et al.* (2012) also states that the two-phase area of a system is temperature dependent by affecting the density and viscosity of the two-phases. While Xavier *et al.* (2014) found that temperature does not influence the effectiveness of an ATPS.

2.2.2.2 Binodal curve

Each ATPS is characterized by phase diagrams known as binodal curves. These binodal curves are used to predict the biphasic area required for partitioning. Binodal curves are also used to determine each phase volume and composition. These phase diagrams also predict the phase's immiscibility. A decrease in the biphasic area indicates a decrease in the immiscibility. Figure 5 represents an example binodal curve.

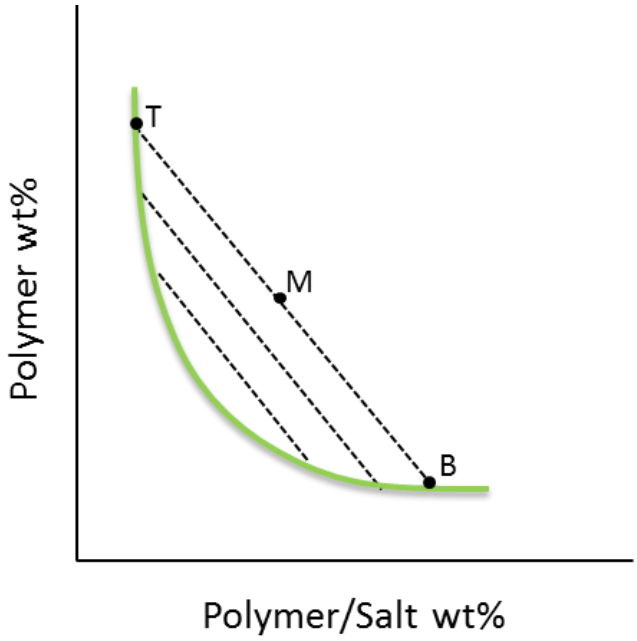


Figure 5. An example binodal curve with an example mixing point (M) and top (T) and bottom (B) phases. The right-hand side of the curve is the biphasic area where two phases will form to partition molecules and to the left is where a homogenous phase will form. The dotted lines represent tie lines to determine equilibrium phase compositions. The y-axis represents the polymer mass fraction in the system while the x-axis represents the second polymer or salt mass fraction in the system.

The tie lines are used to determine the equilibrium phase composition. Any mixing point on the same tie line will have the same top phase equilibrium composition and bottom phase equilibrium composition.

The tie line length (TLL) is calculated with mass of each component in the top and bottom phase as shown in Equation 1. Where B and T represent the bottom and top phases, respectively. While the mass of each component in the two phases are represented by x and y (Raja *et al.*, 2012).

$$TLL = \sqrt{\left[B_y - T_y\right]^2 + \left[T_x - B_x\right]^2} \quad [1]$$

Each tie line can also be used to predict the phase volume, since the tie lines are related to the mass of a system as shown in Equation 2. The volume (V) of the top (T) and bottom (B) phase can be determined with the phase density (ρ) and the segment length from the mixing point to the top phase (MT) and to the bottom phase (MB).

$$\frac{V_T \rho_T}{V_B \rho_B} = \frac{MT}{MB} \quad [2]$$

Binodal curve tie lines are parallel and the tie line slope can be used to construct more tie lines. Equation 3 can be used to determine the slope of the tie line (STL), with the mass fraction of each component in each phase.

$$STL = \frac{Y_T - Y_B}{X_T - X_B} \quad [3]$$

2.2.2.3 Partition coefficient

The efficiency of an ATPS can be evaluated in terms of the partition coefficient and the recovery. The partition coefficient, as calculated with Equation 4 indicates partitioning of a specific molecule to the top phase in an ATPS. C_T and C_B are the concentration of a specific molecule in the top and bottom phase, respectively. Where V_T and V_B represent the top and bottom phase volumes.

$$K_P = \frac{C_T V_T}{C_B V_B} \quad [4]$$

A partition coefficient greater than one indicates partitioning to the top phase. A large top phase partition coefficient indicates preferential partitioning of a molecule to the top phase, successfully concentrating the molecule. The greater the top phase partition coefficient, the more effective the two-phase system is in partitioning a specific molecule to the desired phase.

2.2.2.4 Recovery

The recovery of a specific molecule cab also be calculated to evaluate the partitioning to a specific phase and the top and bottom phase recovery can be calculated with Equation 5 and Equation 6 respectively.

C_T and C_B are the concentration of a specific molecule in the top and bottom phase, respectively. Where V_T and V_B represent the top and bottom phase volumes.

$$\Upsilon_T = \frac{V_T C_T}{V_{Total} C_{total}} \times 100 \quad [5]$$

$$\Upsilon_B = \frac{V_B C_B}{V_{Total} C_{total}} \times 100 \quad [6]$$

2.2.2.5 Polymer-Salt systems

Due to their low cost and low toxicity, salt-polymer systems are preferred rather than polymer-polymer systems. PEG is generally used as the polymer phase of a polymer-salt system since it is edible and due to its low cost. PEG-salt two phase separations have been used for protein separation and purification as well as polyphenol extraction. According to Xavier *et al.* (2014), different polyphenols can be extracted from a sodium citrate and PEG (2000 g/mol) system with a polyphenol partition coefficient in PEG of 117, indicating successful polyphenol partitioning to the PEG phase.

2.3 Recovery

2.3.1 ATPS

As discussed in Section 2.2.2, ATPS can also be formed using two structurally different polymers and according to Grilo, Aires-Barros and Azevedo (2016) polymer-polymer ATPS can be used for the purification of biological products but the polymers used should not damage the bio-molecule. PEG and dextran are usually used for ATPS. However, for industrial scale applications the use of a PEG-dextran system would not be economically feasible due to the high cost of dextran (Ramyadevi, Subathira and Saravanan, 2012). Other polymers like dextran such as maltodextrin can be used. Maltodextrin is not only a cheaper alternative; it is produced from starch and can thus be used in nutraceutical supplements.

Maltodextrin is a complex carbohydrate produced by the partial hydrolysis of starch from corn or wheat. The starch can be hydrolysed with hydrochloric acid, α-amylase enzyme or a combination. The degree of hydrolysis is measured in terms of dextrose equivalence ranging 3 to 20. The greater the degree of hydrolysis the higher the dextrose equivalence. The dextrose equivalence influences certain characteristics such as viscosity, flavour and biding power (Hofman, van Buul and Brouns, 2016).

Maltodextrin is often used in the food and beverage and pharmaceutical industry due to its low cost, high water solubility and easy digestibility. It is used in the food and beverage industry as bulking agent, sweetness reducing agent, confectionary coatings, energy source, stabilizer and food thickener. Maltodextrin is also an excipient and is used in the pharmaceutical industry in to inhibit crystallisation, act as a binder or diluent.

Even though maltodextrin is less expensive than dextran, it requires a greater concentration of maltodextrin to form two-phases with PEG (Da Silva and Meirelles, 2000) and according to Amid, Manap and Zohdi (2014) polymer-polymer systems are difficult to recycle. Even though maltodextrin and PEG are non-toxic and can be used in pharmaceuticals, the maximum amount of each should be removed and recycled to be economically feasible. As discussed in Section 2.2.2.1, two-phase systems are affected by polymer type and molecular weight, the polymer concentration, pH and temperature (Grilo, Aires-Barros and Azevedo, 2016).

The efficiency of a maltodextrin- PEG recovery system is evaluated in terms of the partition coefficient and recovery to the maltodextrin bottom phase. The partition coefficient, as calculated with Equation 7 indicates partitioning of a specific molecule to the bottom phase. Where C_T and C_B are the concentration of a specific molecule in the top and bottom phase, respectively. Where V_T and V_B represent the top and bottom phase volumes.

$$K_P = \frac{C_B V_B}{C_T V_A} \quad [7]$$

For successful partitioning the partition coefficient should be greater than 1. The recovery of a specific molecule to the bottom phase can be calculated with Equation 7.

2.3.2 Protein precipitation

Polyphenols are multidentate ligands that can bind to multiple points on the protein surface (Papadopoulou and Frazier, 2004). If the proteins and polyphenols are in solution with sufficient mixing time the polyphenol will form a hydrophobic layer around the protein and start to flocculate or will form a less hydrophilic mono-layer around the protein, depending on the protein concentration (Baxter *et al.*, 1997). Polyphenols and other phenols both want to bind to available sites on the protein surface and can influence the protein complex formed. As shown in Figure 6, the ratio of polyphenols and proteins influence the precipitation mechanism. A system with a low polyphenol and low protein concentration will form a saturated protein-polyphenol chain that will precipitate. If the protein concentration is too high in comparison to the polyphenol concentration, a partially saturated chain will form. By increasing the polyphenol concentration, a polyphenol-protein aggregate can then form. Other factors such as pH, protein and polyphenol structure and size, mixing time, temperature and type of solvents also influence the polyphenol-protein complex formed (Spencer *et al.*, 1988). Since the type of solvent can influence the protein-polyphenol complex, a solvent that will not denature proteins should be used. According to Ingham (1978) PEG can be used to extract and recover proteins without interacting with or denaturing the protein. Proteins can thus be used in a PEG solution to possibly form a precipitate with resveratrol since the protein properties will not change or interact with the PEG.

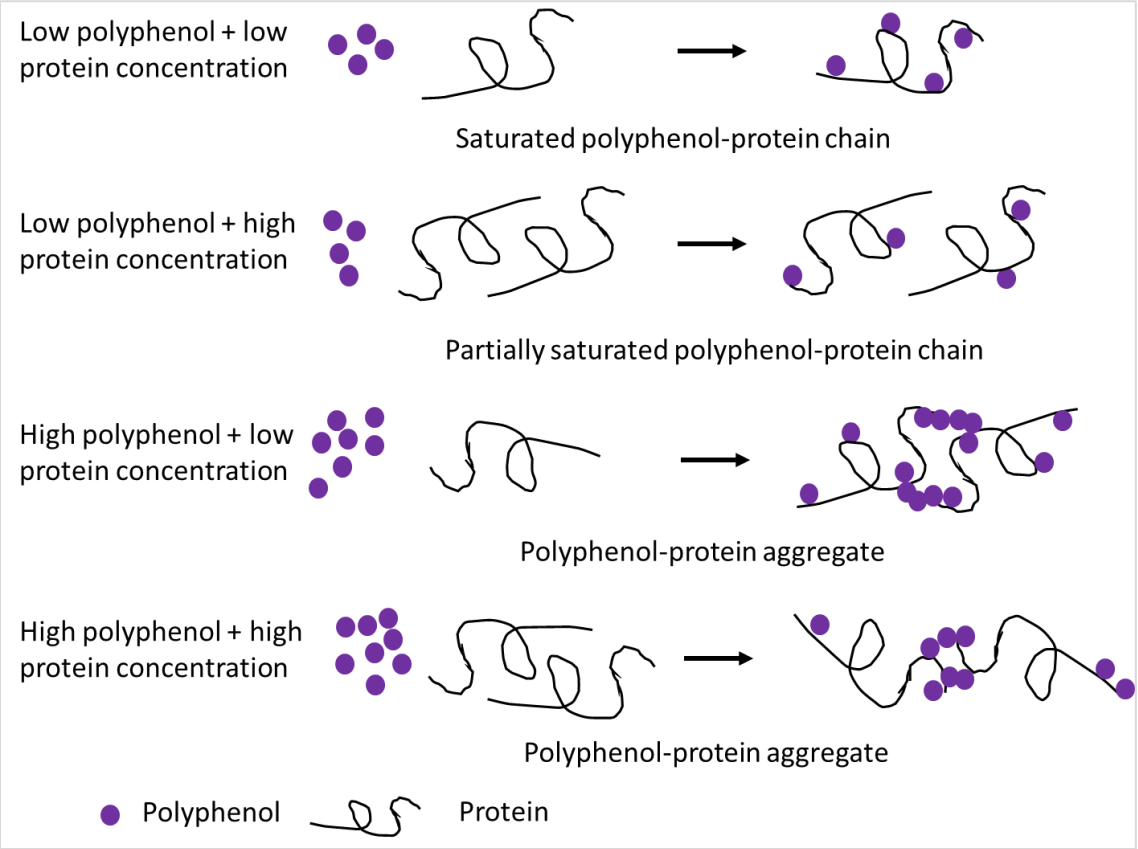


Figure 6. Different precipitates formed with different protein and polyphenol concentrations. Information adapted from (Jöbstl *et al.*, 2004; Bandyopadhyay, Ghosh and Ghosh, 2012).

Equation 8 can be used as a simplified method to determine the efficiency of a protein-polyphenol precipitation reaction by determining the decrease in polyphenol concentration after precipitation.

$$\Upsilon_{pp} = \left[1 - \left(\frac{C_f}{C_i}\right)\right] \times 100 \qquad [8]$$

Where C_f is the concentration of the polyphenol remaining in the supernatant after precipitation and C_i the initial polyphenol concentration to be recovered.

2.4 *Vitis vinifera* feasibility for resveratrol production

Grape crops are one of the largest agricultural activities in the world, with most of the grapes produced used for wine production (Roca, 2019). According to the International Organisation of Vine and Wine (OIV) 292 million hectolitres of wine was produced with a trade value of 31bn Euro for 108 million hectolitres. South Africa is currently the 9th largest wine producing country that produced approximately 950 million litres wine in 2018 (Roca, 2019). During the winemaking process significant quantities of solid waste are produced and Figure 7 illustrates the different components of a grape vine and the amount of solid waste of these components are discussed below.

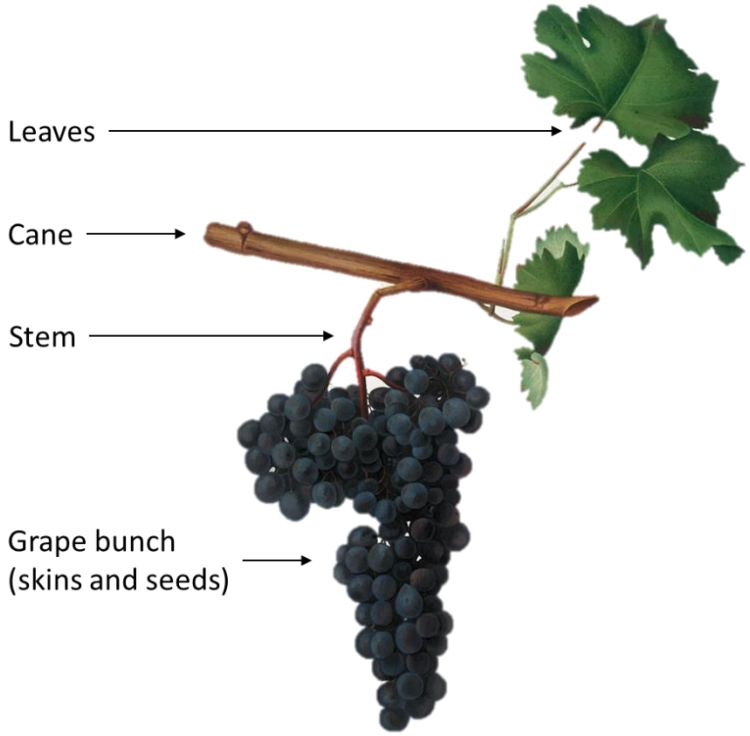


Figure 7. Illustration of a typical grape vine, indicating the parts that are harvested and pruned (Adapted from Gallesio, 2018)

According to (*SA wine industry 2018 statistics*, 2019) and Strever (2018) 960.2 million litres wine were produced in South Africa in 2018 producing approximately 432.0 kilotons of grape skins and seeds and 225.9 kilotons of stems. The amount of canes pruned were estimated as 305.7 kilotons and 16.09 kilotons leaves. Supplying enough biomass to be used as a possible resveratrol source.

South Africa has ten major wine regions producing still wine, fortified wine and sparkling wine with an approximate vineyard area of 93000 hectares (*SA wine industry 2018 statistics*, 2019). Figure 8 summarises the vineyard area distribution between the different regions.

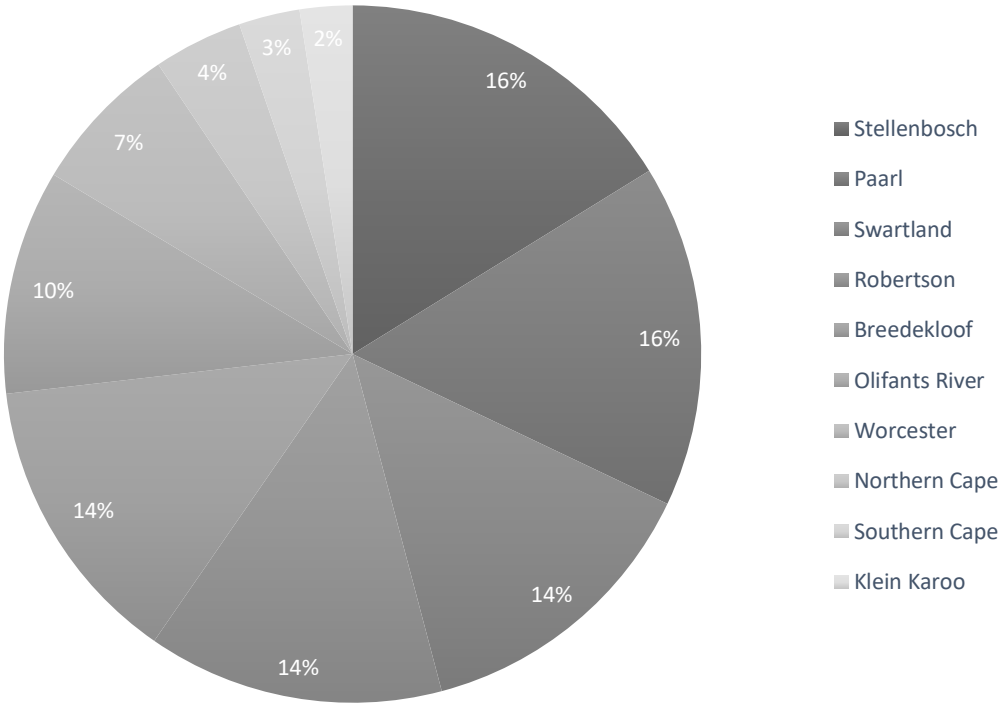


Figure 8. 2018 grape vineyard area distribution of South African wine regions (*SA wine industry 2018 statistics*, 2019).

As seen in Figure 8 Stellenbosch has one of the largest vineyard areas of 15000 hectares (16%) (*SA wine industry 2018 statistics*, 2019). The distribution of the grape varieties in Stellenbosch is summarised in Figure 9.

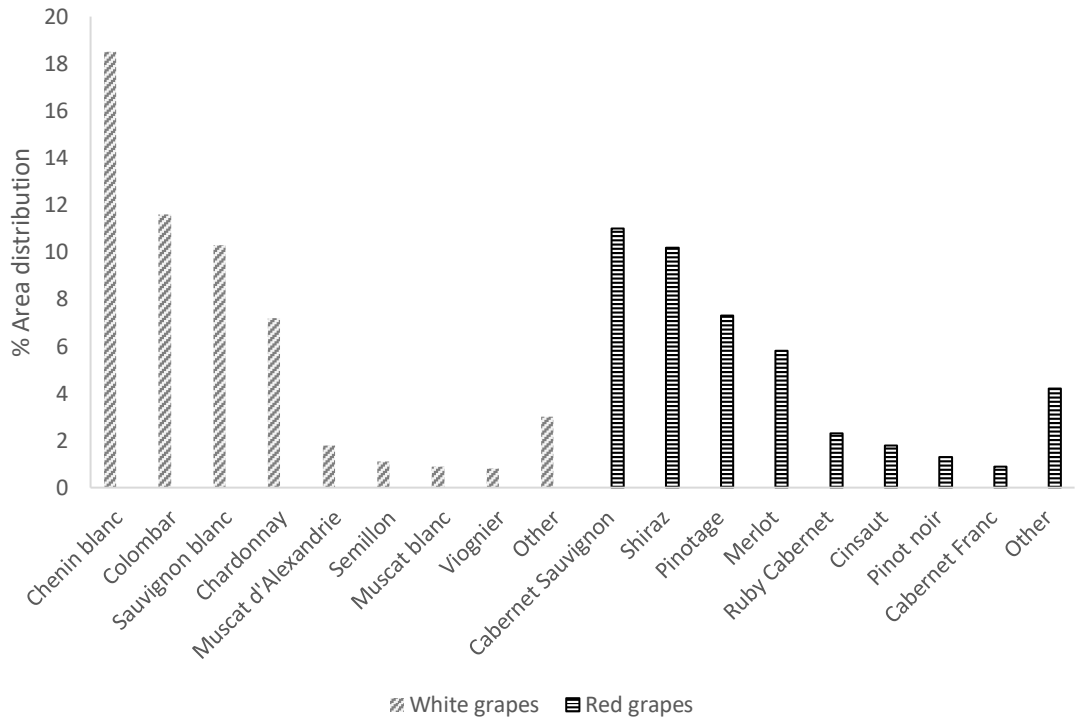


Figure 9. Percentage area distribution of white (diagonal) and red (horizontal lines) grape variety distribution of 2018 in Stellenbosch (*SA wine industry 2018 statistics*, 2019).

Pinotage is a red grape variety that originated in Stellenbosch that is a cross between Cinsaut and Pinot noir. The cross was created at Stellenbosch University's Welgevallen Experimental farm to create a grape variety with the taste of Pinot noir but with the growth characteristics of the robust Cinsaut. Pinotage is generally resistant to powdery mildew and can yield between 10 to 15 tonnes of berries per hectare. Even though Pinotage was commercialised in 1961 and 4.7 million litres were produced in 2018, little information about the phenolic content and bioactive properties is available.

2.4.1 Wine making procedure

The red wine making process consists of 6 basic steps as illustrated in Figure 10. During each processing step, different components of solid waste are removed, as shown in Figure 11, that can be used as a possible source of resveratrol.

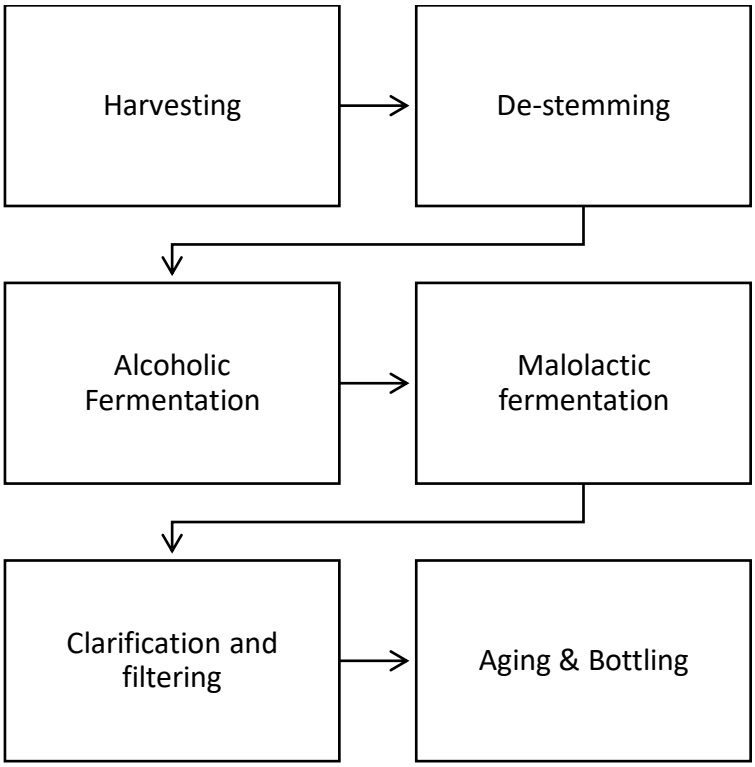


Figure 10. Basic illustration of a typical red wine making procedure.

The harvesting time depends on the wine varietal, as the harvesting time influences the grape acidity and sweetness. According to Strever (2018), the weather also affects the harvesting time, but in the Stellenbosch region harvesting typically start in February and end mid-March. While canes and leaf rest only occur from May to June. If all the solid waste components are used as resveratrol source, the canes and leaf rest that occur later is advantageous since it will improve the distribution of biomass as feed stream.

As seen in Figure 10 the red wine making process starts by harvesting approximately 1kg of grapes per bottle of wine produced. After harvesting, the grapes are sorted, and damaged and unripe grapes are removed. The number of stems removed from the grape bunch depends on the desired taste and tannin concentration. The grapes are then de-stemmed, usually mechanically, where they are partially crushed. During this crushing must is produced. Must is the grape juice still containing grape skins and seeds. To create the red colour of the wine, the anthocyanins are extracted by fermenting the grape must with the skin. During the first fermentation step the sugar in the grapes is fermented by the added or wild yeast to produce alcohol in one to two weeks.

To produce red wine, the must is contacted with the skins during fermentation typically for 10 days. Fermentation can occur with the wild yeasts or with commercial yeast. After the first fermentation step, the skins and seeds, now called the pomace, is pressed to recover more wine and the pomace is removed. To improve the taste, malic acid is converted to lactic acid by the addition of bacteria during the

malolactic fermentation process step. To remove the remaining solid residue the wine is settled, clarified and filtered. The final step is the aging process, where the wine is matured for a few weeks to years. After each processing step up to malolactic fermentation different solid waste streams possibly containing resveratrol are removed. These waste streams include stems, skins and seeds as well as canes and leaves from pruning.

2.4.2 Waste handling

During the winemaking process different waste streams are produced. To beneficiate some of waste that is landfilled, incinerated and discharged into wastewater different the solid winery waste streams and current methods of valorisation were investigated and summarised below.

2.4.2.1 Pomace

After the first fermentation step the solids that are removed are called pomace. Pomace includes the pulp, skins, seeds and some stems. During the fermentation process some of the phenolic compounds solubilises into the wine. However, the fermentation process does not change the characteristics of the bioactive compounds. The pomace contains potassium, nitrogen and calcium and can therefore be used as a fertilizer. The pomace that is not valorised is landfilled and can produce acetic acid and contaminate the soil or the groundwater (Devesa-Rey *et al.*, 2011). To reduce the amount of waste some pomace is valorised into different products such as bioethanol, compost and grape seed oil as summarised in Figure 11.

2.4.2.2 Lees

During the clarification and filtering step, the lees produced during fermentation is removed. During fermentation sugar is converted to alcohol with the addition of yeast. When all the nutrients are depleted the yeast cells will die and produce yeast autolysate or lees. Lees are often used in the sur lie production technique of white and sparkling wine to improve the complexity of the wine. The lees that are removed after fermentation do not have a good nutrient value but can be used as animal feed or nutrients for *Lactobacillus* and *Debaryomyces hansenii* (Beres *et al.*, 2017), as shown in Figure 11.

2.4.2.3 Canes

To prune the vines for the next harvest, the canes or shoots are trimmed. The canes are often used as mulch to improve the soil moisture and temperature. Some canes are burnt as a waste removal technique. The canes can also be used to produce high value products such as ruminal feed, xylitol and biosurfactants as summarised in Figure 11.

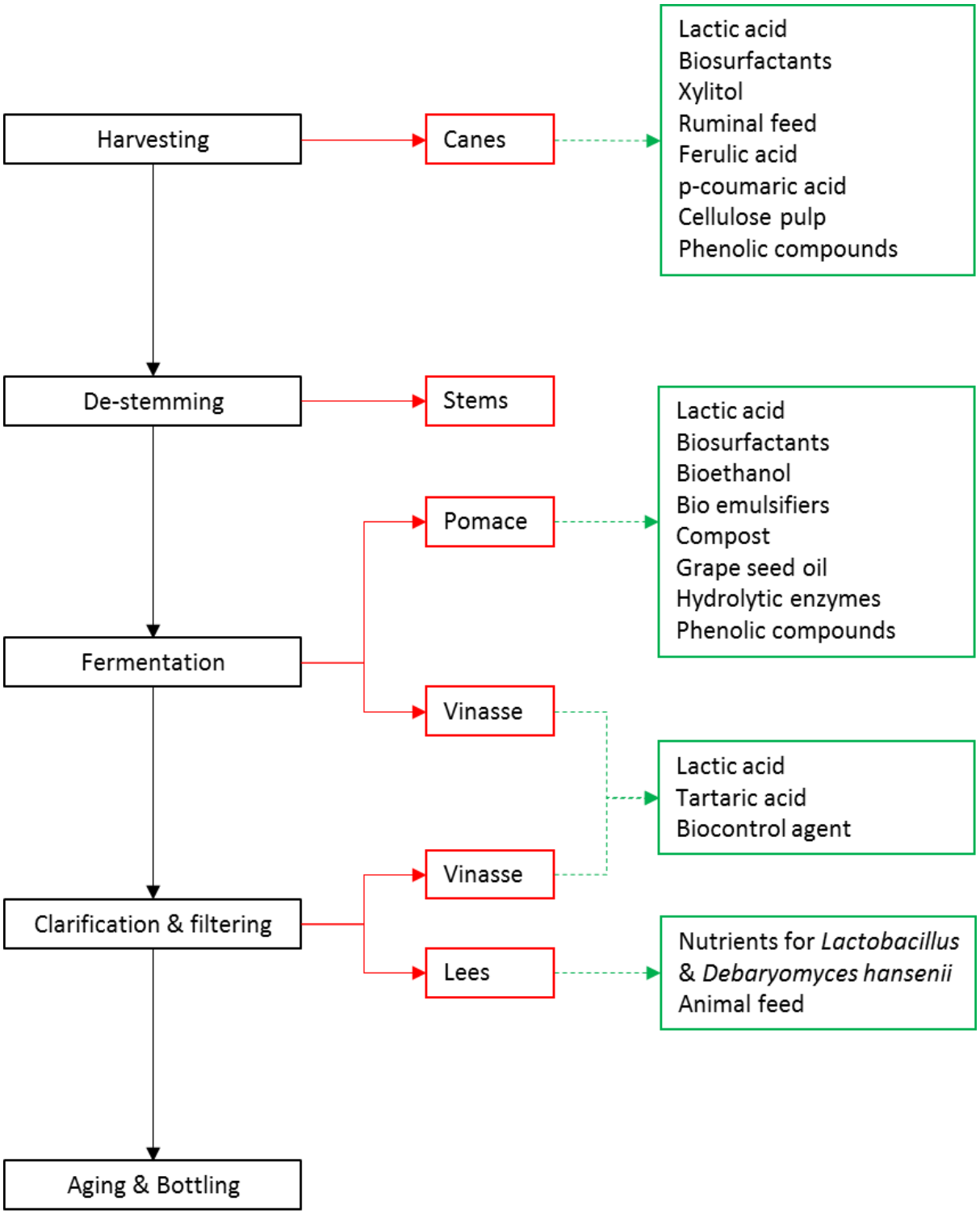


Figure 11. Basic illustration of a wine making procedure with the different waste streams (red) and valorised products (green), as summarised from Devesa-Rey *et al.* (2011) and Beres *et al.* (2017).

2.5 Conclusions

Several studies stated that resveratrol is produced in different grape varieties and can be extracted to produce a high value product (Skerrett, 2012). Since resveratrol is present in grape and the South African wine industry produces large quantities of solid waste (*SA wine industry 2018 statistics*, 2019), the solid winery waste could be used as a resveratrol source. It was observed from literature that factors that influence resveratrol production is fairly under researched field and the distribution of resveratrol in different parts of the vine and the variability of resveratrol over time is unknown.

ATPS have been widely used to extract and recover biological products and although ATPS has been used to partition polyphenols very little information is available on the extraction and recovery of polyphenols, specifically resveratrol (Xavier *et al.*, 2014). From the literature it was found that polyphenols and proteins will interact to form a precipitate and the literature suggests that proteins could be used to form a recoverable precipitate with resveratrol. These observations led to the formulation of the project aim and research questions, further discussed in Chapter 3.

3 AIMS AND OBJECTIVES

3.1 Aims

The primary aim of the project was to investigate the extraction of resveratrol and other polyphenolic compounds from solid winery waste. Another aim of this project was to investigate downstream recovery of resveratrol from PEG to create an edible resveratrol concentrate.

3.2 Objectives

In order to achieve the project aim, the following main objectives were specified:

1. *In order to extract the maximum amount of resveratrol, factors that influence isomerisation or degradation should be investigated.*
2. *A methodology to determine the resveratrol content in different components of wine waste was needed, in order to quantify the amount of resveratrol and other polyphenols in the sourced solid waste.*
3. *To recover extracted resveratrol from PEG to an edible concentrate, a subsequent ATPS and protein precipitation should be investigated. In order to determine if a subsequent maltodextrin-PEG ATPS can be used to recover resveratrol, factors that influence partitioning should be investigated. To develop a safe resveratrol recovery method from PEG, protein-polyphenol precipitation and factors that influence precipitation should be investigated.*

3.3 Key questions

From the investigation of previous research, the following key questions have arisen and should be answered to achieve the project aim.

I. Does resveratrol degradation occur during drying?
II. Is resveratrol degradation pH dependent?
III. Will resveratrol degrade or isomerise during extraction?
IV. Which component of solid wine waste has the highest resveratrol concentration?
V. Is there a correlation between total phenolic content and resveratrol concentration?
VI. Will environmental conditions influence resveratrol production?
VII. Can a tartrate-PEG ATPS be used to extract resveratrol and how does it compare to solvent extraction methods?
VIII. Can a maltodextrin-PEG ATPS system be used to recover resveratrol?
    a. Is the recovery affected by the resveratrol concentration and phase composition?
    b. Will an increase in the system pH improve resveratrol partitioning?
IX. Will the addition of proteins to polyphenols form a precipitate to be used as a recovery method?

a. Can protein precipitation be used as a resveratrol recovery technique?

b. Is it the precipitation affected by the polyphenol concentration?

4 METHODOLOGY

In order to achieve the specified objectives, the required experimental work was completed. The experimental work performed was planned to answer the key questions of the work as well as to identify process conditions that should be investigated for future work.

4.1 Materials and Resource requirements

4.1.1 Solid winery waste

To determine the amount of resveratrol in solid winery waste and to investigate factors that influence isomerisation and degradation, fresh grape biomass samples had to be collected during harvesting.

For the preliminary experimental work, various grape variety samples were provided by Thelema Mountain Vineyards in Stellenbosch. The different samples were collected after harvesting and stored at -18°C.

Grape berry clusters, canes and leaves of the Pinotage grape variety were collected during the Pinotage harvesting period from Welgevallen Experimental farm. The samples were collected during March 2018 and March 2019. The berries, stems, canes and leaves were harvested from the same vine in 2018 and again from the same vine in 2019.

4.1.2 Organic solvents

In order to determine the resveratrol concentration of various samples extracted using different solvents. Absolute ethanol (> 99.5%), pure acetone and ethyl acetate (≥ 98.0%) were purchased from Kimix chemical and lab supplies. 99.6% Methanol was purchased from Sigma Aldrich.

4.1.3 Aqueous two-phase system

To investigate ATPS as an extraction and recovery method, a PEG-tartrate system and PEG-maltodextrin system were used. Polyethylene glycol 8000 and Maltodextrin (Dextrose equivalence 16.5-19.5) were purchased from Sigma Aldrich. Potassium sodium tartrate tetrahydrate (≥ 99.5%) was from Sigma Aldrich was used.

4.1.4 Protein precipitation

Yeast extract from Sigma Aldrich, tryptone soy broth from Merck and ovalbumin from Acros organics were selected as protein sources for precipitation.

4.1.5 Resveratrol and polyphenol analysis

To quantify the resveratrol in each sample, *trans*-resveratrol standard (≥ 99%) from Sigma Aldrich was used for all high-performance liquid chromatography (HPLC) and liquid chromatography–mass spectrometry (LC-MS) analysis. Anhydrous sodium carbonate (≥ 99.5%), 2 Molar Folin Ciocalteu reagent, gallic acid and methanol (≥99.6%) were purchased from Sigma Aldrich for the determination of the total phenolic content.

4.2 Experimental procedure

4.2.1 Analytical methods

4.2.1.1 HPLC

The resveratrol in each solvent sample was quantified with RP-HPLC on a Dionex Ultimate 3000 system with UV detection at 306 nm. 100 µL samples were analysed on a Phenomenex Jupiter C18 column (4.6 x 250 mm) at 30°C. The column was eluted with water and 5 mM trifluoroacetic acid as the mobile phase A and acetonitrile with 5mM trifluoroacetic acid as mobile phase B over a 20% - 100% acetonitrile gradient. While the resveratrol samples in PEG and MD were quantified with a Dionex Ultimate 3000 system with a Polysep GFC column (7.8 x 300 mm).

4.2.1.2 FC UV-vis spectroscopy

Folin- Ciocalteu is a colorimetric assay that was used to determine the polyphenolic content of the different biomass samples. Each of the samples were diluted to make up a volume of 100 µl. The 100 µl sample was mixed with 200 µl 10 v/v% Folin- Ciocalteu using a vortex mixer. 800 µl 0.7 M Na_2CO_3 solution was then added and vortexed again. The samples were stored for 2 hours at ambient temperature (Ainsworth and Gillespie, 2007). 200 µl of each sample were transferred to a 96- well microplate to be analysed using a BioTek Elx800 spectrophotometer at 750 nm. The absorbance of each sample was converted to concentration in terms of gallic acid equivalence with a gallic acid standard curve. The constructed FC standard curve can be found in Appendix A (Figure 29).

4.2.1.3 LC-MS

The two isomers of resveratrol have an elemental composition of $C_{14}H_{11}O_3$ ($[M-H]^-$) with m/z 227.07 and were quantified with LC-MS on an Acquity liquid chromatograph (2.1 x 100 mm) with a Waters Synapt G2 mass spectrometer with electrospray ionization (negative ion mode) and with a cone voltage of 15V. Water with 0.1% formic acid was used as mobile phase A and acetonitrile with 0.1% formic acid was used as mobile phase B, over a 0-100% gradient.

4.2.2 Resveratrol degradation

4.2.2.1 Thermal degradation

100 g whole grape skins were first dried in a drying oven for 24 hours at 80°C to determine the water content. The dried grape skins were weighed, and Equation 9 was used to determine the average water content. The effect of thermal degradation was investigated with by determining the total phenolic content and the resveratrol concentration in dried grape skin samples. Nine Shiraz grape skins samples were dried at 40°C, 50° and 60°C for 24 hours. The nine dried samples and three fresh biomass samples were mixed with absolute ethanol and demineralised water to achieve a 1:10 solid to solvent ratio with 80 v/v% ethanol-water solution. The samples were mixed for 24 hours in 50ml falcon tubes at 50 rpm with a rotary sample mixer. The extracted samples were centrifuged at $14.5x10^3$ rpm for 5 minutes and the supernatants were filtered using 0.2µm polyethersulfone filters (PES) for further analyses. The sample containing 100 µL Shiraz extract and 900 µL 80 v/v% ethanol-water solution was analysed with HPLC to determine resveratrol concentration. The same extract samples were then diluted in the same ratio and 100 µL samples were analysed using the FC method to determine the total phenolic content. The resveratrol concentration and total phenolic content in each of extracts were compared to determine if an increase in drying temperature will result in a decrease in resveratrol concentration.

4.2.2.2 Effect of pH

The effect of pH degradation was investigated by measuring the resveratrol concentration of a pure resveratrol solution exposed to a pH over time. Four 30 mg/L resveratrol samples were dissolved in an 80:20 v/v % ethanol-water mixture with a pH above and below each acidic dissociation constant of resveratrol. The resveratrol- ethanol solutions were mixed in 50ml falcon tubes at 50 rpm with a rotary sample mixer for 24 hours. The samples were filtered using 0.2 µm PES filters and the undiluted samples were analysed with LC-MS analysis to determine the change in resveratrol concentration.

Resveratrol degradation in ATPS was investigated by determining the change in resveratrol concentration in a 35 wt% maltodextrin (DE 16.5-19.5) and 7.5 wt% PEG 8000 two phase system exposed to a pH over time. Four 0.5 g/L resveratrol samples were mixed with maltodextrin and PEG with a pH above and below each acidic dissociation constant of resveratrol. The two-phase systems were mixed in 50ml falcon tubes at 50 rpm with a rotary sample mixer for 24 hours. The neutralized PEG top phase and maltodextrin bottom phase were filtered using 0.2 µm PES filters. A 200 µL sample of each phase was diluted with 800 µL water and analysed with HPLC analysis to determine the total resveratrol concentration.

4.2.2.3 Isomerisation

To determine if the sourced biomass will isomerise from *trans*-resveratrol to *cis*-resveratrol under the extraction conditions, dried Grenache grape skins samples were extracted with an 80 v/v% ethanol-water solution in a solid to solvent ratio of 1:10 at 50 rpm and ambient temperature. Samples were removed after 1, 4, 7- and 24-hours extraction. The samples were centrifuged at $14.5x10^3$ rpm for 5 minutes. The samples were with filtered 0.2 µm PES filters and 100 µL of the Grenache extracts were diluted with 1 ml 80 v/v% ethanol-water solution. The diluted samples were analysed using liquid chromatography-mass spectrometry (LC-MS) to determine if the *trans*-resveratrol isomerised to *cis*-resveratrol over time.

LC-MS analysis was also used to investigate if degradation of samples will occur if it is stored at -18°C. Three dried grenache grape skin samples were extracted with an 80 v/v% ethanol-water solution in a solid to solvent ratio of 1:10 at 50 rpm and ambient temperature. Two samples were centrifuged at $14.5x10^3$ rpm for 5 minutes. The filtered and diluted supernatants were stored at -18°C for 30 days or 24 hours and then analysed with LC-MS. While the one sample was centrifuged at $14.5x10^3$ rpm for 5 minutes, filtered and diluted supernatant was analysed within a few hours with LC-MS.

4.2.3 Resveratrol extraction

4.2.3.1 Solvent selection

To determine the amount of resveratrol present in the sourced wine waste, different solvents and dilutions that were investigated by Romero-Pérez *et al.* (2001) were also investigated to confirm the use of 80:20 v/v% ethanol-water mixture to extract resveratrol.

Dried grenache skins were mixed at 50 rpm in a solid to solvent ratio of 1:10 with ethanol-water (80:20 v/v %), absolute ethanol, ethyl acetate-methanol (50:50 v/v %), acetone-water (75:25 v/v %) and pure acetone for 24 hours at ambient temperature. Samples of each system were removed after 1, 4, 7- and 24-hours extraction. The extracted samples were centrifuged at $14.5x10^3$ rpm for 5 minutes and the supernatants were filtered using 0.2 µm PES filters for further HPLC analyses.

4.2.3.2 Quantification of resveratrol and other polyphenols in solid winery waste

To account for the environmental factors that could influence resveratrol production, grape clusters, canes and leaves were collected from the same vine for two years. After each harvest, the grapes were fermented for 10 days using wild yeast. After the first fermentation step the skins and seeds, now called the pomace, were pressed and removed. The skins and seeds were separated, dried and homogenised for 2 minutes using a 1700 W Nutribullet. The pruned canes were dried and milled using a hammer mill. The milled canes were then homogenised for 2 minutes to a fine powder using a 1700 W Nutribullet. The sourced leaves and stems were dried until all the moisture was removed and homogenised for 2 minutes.

The dried pre-fermentation skins and seeds, post-fermentation skins and seeds, canes, stems and leaves were each mixed in a 1:10 solid to 80:20 v/v% ethanol-water ratio and mixed for 24 hours at 50 rpm in 50 ml falcon tubes. Samples of each component were removed after 1, 4, 7 and 24 hours. The extracted samples were filtered with 0.2 µm PES syringe filters. 300 µL of each of the 2018 Pinotage samples were diluted with 700 µL 80:20 v/v% ethanol-water solution for HPLC analysis while the samples used for FC analysis were diluted in a 1:1 ratio. The sample preparation and extraction process were repeated with the 2019 Pinotage biomass samples, but the samples used for HPLC analysis were diluted with 300 µL extract and 1 ml diluent. From the HPLC analysis the resveratrol concentration in each sample was determined, while the FC method using UV-vis spectroscopy was used to determine the total phenolic content in each sample.

4.2.3.3 Salt-polymer ATPS extraction

To evaluate the extraction of resveratrol in ATPS, a tartrate and PEG 8000 system was investigated. From the work of Herbst (2019), an equal volume system of 35% PEG 8000 and 35% potassium sodium tartrate tetrahydrate was used for the extraction of the Pinotage biomass. 30 ml of aqueous two-phase systems were continuously mixed at 50 rpm with 3 g dried canes and stems for 24 hours at ambient temperature. After sufficient mixing, the different systems were centrifuged for 10 minutes to separate the PEG top phase and the tartrate bottom phase. The volume of the top phase and bottom phase was noted, removed, filtered and analysed to determine the amount of resveratrol that was extracted in both phases. The resveratrol concentration and partitioning were determined by HPLC analysis.

4.2.4 Resveratrol recovery

In order to investigate resveratrol recovery from PEG-tartrate ATPS and to minimise resveratrol variability, a known concentration pure resveratrol in PEG was used. A subsequent ATPS with the resveratrol rich PEG phase was used as the top phase in a PEG-maltodextrin ATPS to concentrate resveratrol into edible maltodextrin. While the recovery of resveratrol with protein precipitation was investigated by determining the amount of resveratrol that formed a recoverable precipitate in PEG with ovalbumin, tryptone soy broth and yeast extract.

4.2.4.1 Aqueous two-phase recovery

A subsequent ATPS with maltodextrin and PEG was investigated as a resveratrol recovery method from PEG. The PEG-tartrate ATPS was used to extract resveratrol from the sourced biomass as discussed in section 2.2.2.5. The PEG top phase containing the extracted resveratrol is used as the PEG top phase for the subsequent ATPS. Maltodextrin (DE 16.5-19.5) was added to the PEG phases containing the resveratrol from the stems and canes to from a polymer-polymer ATPS of 35 w/w% maltodextrin and 5

w/w% PEG. The two systems were continuously mixed at ambient temperature for 24 hours with a rotary sample mixer. After sufficient mixing, the mixture was centrifuged for 10 minutes at $14.5x10^3$ rpm to separate the two phases. After clear phase separation, the top and bottom phase volumes were noted. Sample of each phase and system were filtered with 0.2 µm PES filters and analysed for resveratrol with HPLC.

4.2.4.2 Resveratrol partitioning

To investigate factors that influence partitioning pure resveratrol with a known concentration was used. Different maltodextrin-PEG systems were investigated by varying the maltodextrin and PEG concentration. The different systems investigated are summarised in Table 3 below.

Table 3. Maltodextrin and PEG weight percentages investigated as recovery ATPS.

System	Maltodextrin concentration (w/w %)	PEG concentration (w/w %)
1	35 %	5 %
2	35 %	10 %
3	35 %	20 %
4	30 %	15 %
5	25 %	20 %
6	25 %	25 %

After 24 hours continuous mixing, the top and both phases were separated with centrifugation ($14.5x10^3$ rpm). The phase volumes of each system were noted. 200 µL samples of each phase and system were diluted with 1 ml deionised water and were analysed for resveratrol with HPLC analysis. The resveratrol concentration and phase volumes were used to the calculate partitioning to the maltodextrin bottom phase.

The effect of resveratrol concentration on partitioning was investigated by determining the resveratrol partitioning in 35 wt% maltodextrin and 5 wt%, 7.5 wt% and 10 wt% PEG systems, each with a resveratrol concentration of 0.22 g/L and 2.7 g/L. After 24 hours continuous mixing, the top and both phases were separated with centrifugation ($14.5x10^3$ rpm). The phase volumes of each system were noted. 200 µL samples of each phase and system were diluted with 1 ml deionised water and were analysed for resveratrol with HPLC analysis.

To evaluate the effect of the system hydrophobicity on resveratrol partitioning in ATPS, four systems with 35 wt% maltodextrin and 7.5 wt% PEG were constructed. The pH of each system was changed to above and below the resveratrol acidic dissociation constants. The pH adjusted systems were mixed for 24 hours before the phases were separated and the phase volumes measured. After centrifugation 200 µL of the PEG top phase and 200 µL of the maltodextrin bottom phase were both diluted with 800 µL deionised water and analysed to evaluate the resveratrol recovery achieved.

4.2.4.3 Protein precipitation

Ovalbumin, tryptone soy broth and yeast extract were selected as protein sources to form a recoverable and edible protein-polyphenol precipitate. To determine if the selected proteins will bind to the polyphenols, protein solutions were added to the extracted Pinotage polyphenols. Ovalbumin, tryptone soy broth and yeast extract solutions ranging from 0.032 to 0.75 g/L were added to the extracted polyphenols from the Pinotage leaves. The polyphenol-protein solutions were mixed for 24 hours. The solutions were centrifuged at $14.5x10^3$ rpm for 10 minutes to separate the precipitate and the supernatant. The supernatant of each sample was filtered using 0.2 µm PES filters and diluted 5 times for further FC analysis.

After testing the precipitation reaction with polyphenols, the experiment was repeated with pure resveratrol with a known concentration. A 35 % PEG stock solution containing 700 mg/L resveratrol was used. Ovalbumin, tryptone soy broth and yeast extract solutions with concentrations ranging from 30 mg/L to 750 mg/L were added to the PEG solution. Each of the samples were mixed for 24 hours to allow flocculation to occur to form a precipitate. The solutions were centrifuged at $14.5x10^3$ rpm for 10 minutes to separate the precipitate and the PEG supernatant. The PEG solutions were removed and filtered. 10 µL of the PEG solutions were diluted with 1 ml deionised water for HPLC analysis.

To determine if the precipitation reaction is influenced by the resveratrol concentration, the experiment was repeated with 1600 mg/L and 77 mg/L resveratrol solution.

After testing the selected proteins, the precipitation of polyphenols, including resveratrol, from the sourced canes and stems were tested. The PEG-tartrate ATPS was used to extract resveratrol from the sourced biomass as discussed in section 4.2.3.3. The PEG top phase containing the extracted resveratrol was used as the PEG solution containing the extracted resveratrol. Solutions of ovalbumin, tryptone soy broth and yeast extract were mixed for 24 hours with the PEG solution. After sufficient mixing, the samples were centrifuged to remove the precipitate. The PEG solutions were analysed with HPLC for resveratrol to determine if a resveratrol-protein precipitate formed.

5 RESULTS AND DISCUSSION

In order to achieve the aim of the project, the experimental work and results were divided into three main sections namely degradation, extraction and recovery. Factors such as biomass drying, system pH, storage and extraction time that could result in resveratrol degradation were investigated. From the investigation of resveratrol degradation and biomass preparation results, Pinotage was investigated as a possible source of resveratrol and other polyphenols. Solvent extraction was investigated as method to quantify and compare the resveratrol and phenolic content in different parts of a Pinotage grape vine over time as well as to compare the resveratrol extraction achieved with a tartrate-PEG ATPS. In the final recovery section, polyphenol-protein precipitation and a subsequent polymer-polymer ATPS were investigated and compared as alternative resveratrol recovery techniques to concentrate the extracted resveratrol into a saleable and edible form.

5.1 Resveratrol degradation and isomerisation

5.1.1 Drying temperature

After the wine making procedure, vine pruning and leaf fall, the pomace, canes and leaves contain up to 30% moisture (Appendix B, Table 5). In order to achieve maximum resveratrol extraction, the solid winery waste particle size must be reduced by pulverising each component. However, to decrease the particle size of the sourced waste to a powder the moisture should first be removed by drying each component without degrading the extractable resveratrol and other polyphenols. Figure 12 below shows the resveratrol concentration of the samples dried at 40°C to 60°C.

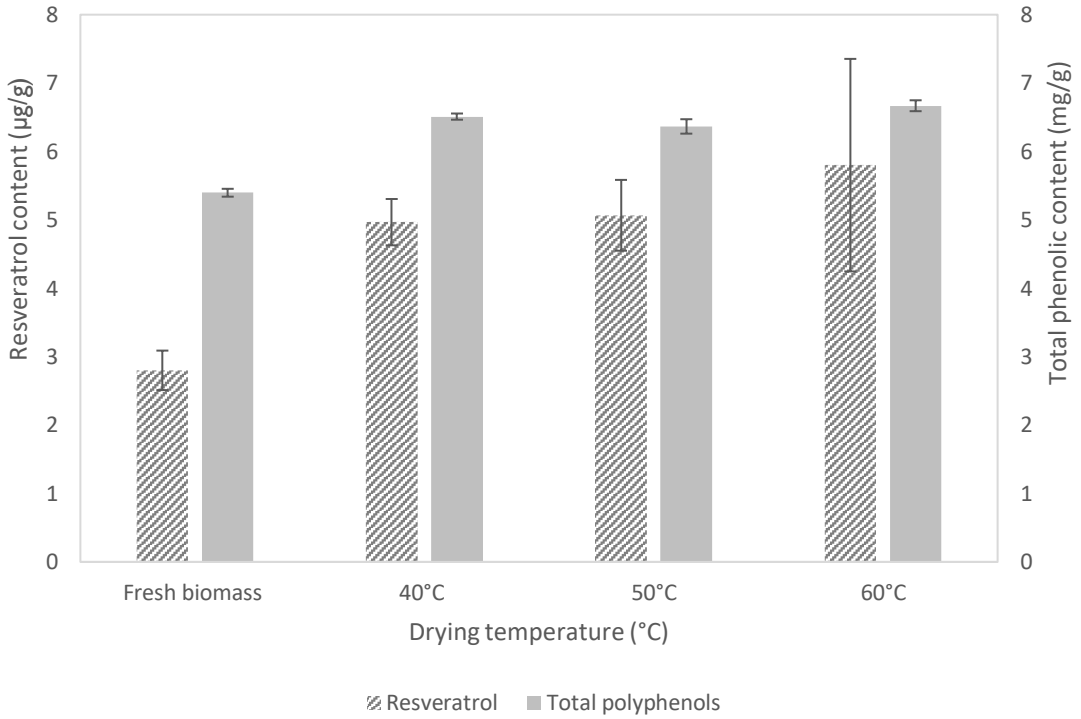


Figure 12. Mean resveratrol content (diagonal) and total phenolic content (grey) of triplicate samples (µg resveratrol/ g dried grape skins ± standard error) extracted from Shiraz skins dried at 40°C, 50°C and 60°C for 24 hours and fresh Shiraz skins that were extracted for 24 hours with 80:20 v/v% ethanol- water mixture under ambient conditions.

As seen in Figure 12, the resveratrol concentration of the different dried samples ranged from 5.0 ± 0.34 µg/g to 5.8 ± 0.52 µg/g resveratrol, showing no change in the concentration resveratrol with a change in drying temperature. The resveratrol concentration of the grape skin extract did not decrease with an increase in drying temperature. The extract of the samples dried at 40°C, 50°C and 60°C were compared to the extract of the fresh biomass to determine if any thermal degradation occurred. During the homogenisation of the fresh biomass it was observed that the particle size of the grape skins did not significantly decreased. The fresh biomass extract contained 2.8 ± 0.29 µg/g resveratrol that is lower than the dried grape skin extracts due to an increase in the fresh biomass particle size. According to Romero-Pérez *et al.* (2001) resveratrol is a thermolabile compound and degradation is accelerated with an increase in extraction temperature over time and will degrade after 45 minutes of extraction at 60°C. However, it can be concluded that resveratrol in the different samples did not degrade during the drying process step. The extracted Shiraz samples were also analysed to determine if the total phenolic content decreased with an increase in drying temperature. In order to extract the maximum amount of

resveratrol and other valuable polyphenols the biomass should be prepared in a way that will not only minimise resveratrol degradation but also the total phenolic content degradation.

The total phenolic content ranged from 6.5 ± 0.05 mg/g to 6.7 ± 0.08 mg/g. It was observed that an increase in the oven drying temperature did not result in a decrease in the amount polyphenolic compounds extracted from the Shiraz grape skins. Even though no decrease in the phenolic content was observed with the FC analysis method, the antioxidant activity was not measured and could decrease with an increase in drying temperature. According to Larrauri, Rupérez and Saura-Calixto (1997), the amount of extractable polyphenols and antioxidant activity will decrease with an increase in drying temperature when compared to freeze dried samples. However, when comparing the total phenolic content and antioxidant activity of samples dried at 60°C to freeze dried samples, no difference was observed (Larrauri, Rupérez and Saura-Calixto, 1997). While a decrease of 33% and 50% for the total phenolic content and antioxidant activity was observed at a drying temperature of 140°C (Larrauri, Rupérez and Saura-Calixto, 1997). From the experimental work it was concluded that an increase in drying temperature up to 60°C will not degrade the biomass sample. All biomass samples used in further experiments were dried at 50°C until all the moisture was removed.

Drying is an energy intensive and high cost processing step and according to Parikh (2014) between 12-20% of the overall energy consumption is from a solids drying step. By increasing the drying temperature, the drying rate is accelerated to decrease the total drying time. An increase in the drying temperature will also increase the energy requirements. To minimise the energy consumption of the drying process step, an efficient ratio between the drying time and temperature should be calculated in terms of an economic analysis. Further investigation into different drying processes units are required for process scale-up or possible integration of resveratrol into existing products and process plants. However, for the lab scale drying the energy consumption was not minimised and all biomass samples were dried at 50°C.

5.1.2 Effect of pH

In order to determine if resveratrol degradation is related to the degree of dissociation, the pH of four 30 mg/L resveratrol samples were adjusted to a pH above and below each acidic dissociation constant of resveratrol. The concentration of *trans*-resveratrol in each sample was determined with LC-MS to determine if degradation occurred as shown in Figure 13.

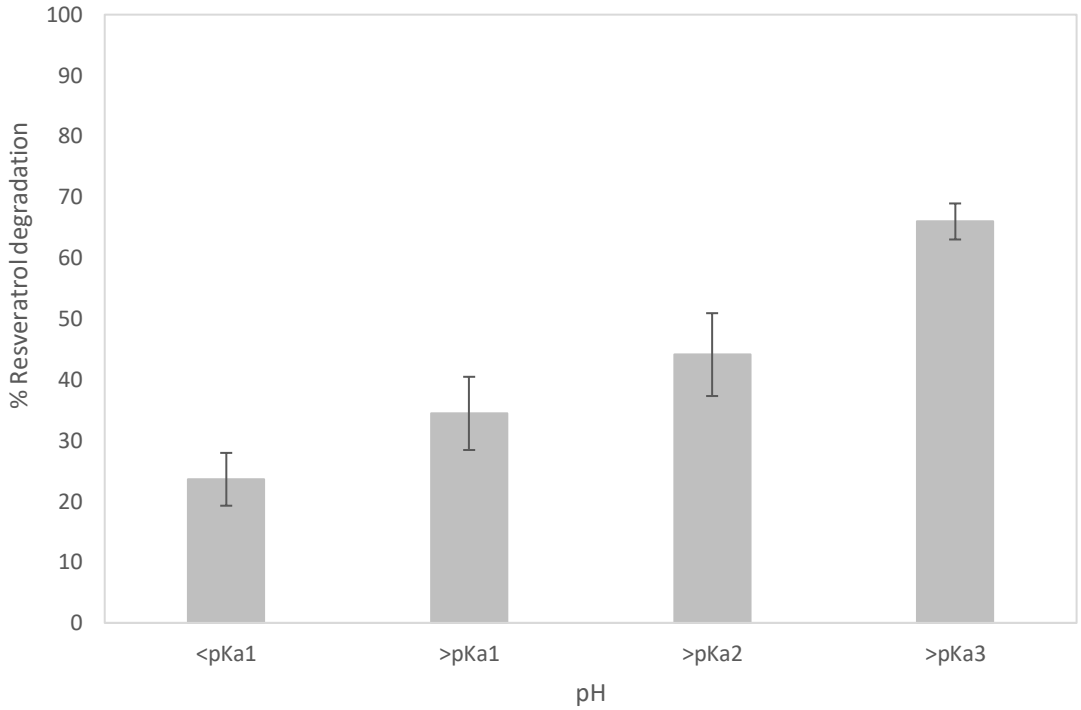


Figure 13. Mean percentage *trans*-resveratrol degradation of triplicate samples (% degradation ± standard error) in 80:20 v/v% ethanol-water solutions with a system pH above and below pK_{a1}=8.8, pK_{a2}=9.8 and pK_{a3}=11.4.

As seen in Figure 13, resveratrol degradation increased with an increase in system pH. When evaluating the degradation of resveratrol at the different pH levels with one-way analysis of variance, assuming an alpha value of 0.05, the p-value was calculated as 0.000769. The results suggest that there is strong evidence to reject the null hypothesis and the difference between the samples is statistically significant.

The data suggests that the resveratrol degradation mechanism is related to the degree of dissociation. As seen in Figure 2, as the pH is increased above pK_{a1}=8.8, pK_{a2}=9.8 and pK_{a3}=11.4 (Robinson, Mock and Liang, 2015) resveratrol is deprotonated. The data suggests that the deprotonated resveratrol structure is more susceptible to oxidation and possibly resulted in the formation of degradation products. By comparing the results summarised in Figure 34 (Appendix B) and Figure 13 it was concluded that resveratrol degradation is pH dependent and will degrade in an alkaline system and the pH of alkaline samples should be neutralised to minimise degradation.

5.1.3 Isomerisation

Resveratrol is found in several plant species as *trans*-resveratrol while the undesired, *cis*-resveratrol is derived from t*rans*-resveratrol (Gerogiannaki-Christopoulou *et al.*, 2006). To determine if the sourced

biomass will isomerise from the more biologically active *trans*-resveratrol to *cis*-resveratrol under the extraction conditions, dried Grenache grape skins samples were extracted and is summarised in Figure 14.

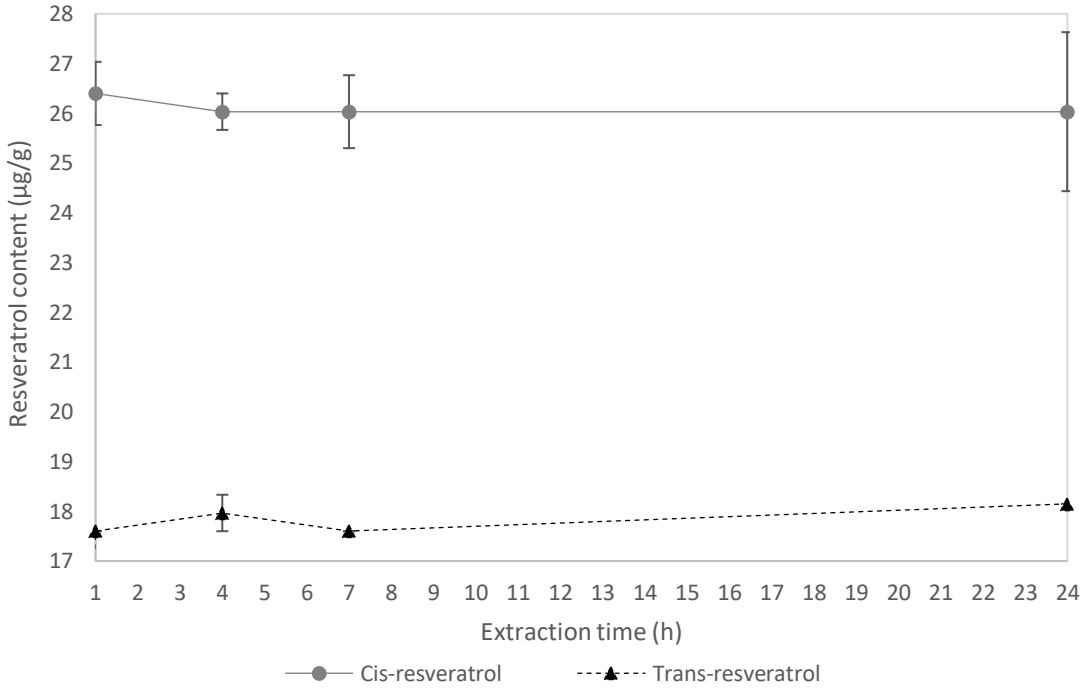


Figure 14. Mean *trans*-resveratrol (▲) and *cis*-resveratrol (●) content of triplicate samples (µg resveratrol/ g dried grape skins ± standard error) extracted with 80:20 v/v% ethanol-water mixture from Grenache skins under ambient conditions over 24 hours and analysed with LC-MS.

Using LC-MS, the two different peaks were measured to differentiate between *cis*- and *trans*-resveratrol in the same sample, as seen in the LC-MS chromatograms (Figure 32, Appendix A). As seen in Figure 14 between 26 ± 0.64 µg/g to 26.4 ± 1.6 µg/g *cis*-resveratrol was extracted, remaining constant over a 24-hour extraction time. While the *trans*-resveratrol concentration was significantly lower, ranging between 17.6 ± 0 µg/g to 18.2 ± 0.55 µg/g. Both *cis*-resveratrol and *trans*-resveratrol concentrations remained constant over time. The amount of *cis*-resveratrol did not increase during the extraction, indicating that the degree of isomerization is not influenced by the solvent extraction conditions. It was also observed that no degradation of resveratrol occurred during extraction. According to Gerogiannaki-Christopoulou *et al.* (2006) *cis*-resveratrol does not naturally occur but is derived from isomerization of *trans*-resveratrol. To minimize possible isomerization, the biomass was collected and stored at - 18°C immediately after harvesting.

During the LC-MS results discussion conducted on 11 September 2018, Mr. Malcom Taylor stated that as expected other compounds including piceid isomers are present in the extract sample. From the LC-MS analysis it was assumed that the amount of resveratrol isomerization will remain constant if extracted under similar process conditions and HPLC can be used to determine resveratrol concentration for all further experiments.

From the results summarized in Figure 14 isomerize during extraction. The isomerization response to the extraction conditions is desired for process scale-up by not requiring adjustments to standard pharmaceutical or food and beverage manufacturing conditions. However, isomerization still occurred, possibly during harvesting. According to Trela and Waterhouse (1996) *trans*-resveratrol is very sensitive to UV radiation and will easily isomerize.

5.2 Quantification of resveratrol and other polyphenols in solid winery waste

5.2.1 Solvent selection

Different organic solvents, as discussed in Section 4.2.3.1, were investigated to quantify and compare the amount of resveratrol and polyphenols in different grape biomass samples. From the evaluation of the different solvents and ratios investigated, it was determined that a mixture of 80:20 v/v% ethanol-water resulted in the maximum *trans*-resveratrol extraction. The *trans*-resveratrol extraction achieved with the different solvents are summarised in Figure 33 in Appendix B. From the comparison of the extraction achieved with the different solvents it was concluded that an 80:20 v/v% ethanol-water solution should be used to accurately quantify resveratrol in the different parts of the vine.

5.2.2 Resveratrol quantification

Resveratrol is a phytoalexin produced by *Vitis vinifera* as a response to biotic and abiotic stress. Several studies have reported that the resveratrol concentration is dependent on factors such as geography, grape variety, the part of the vine, vinification process, and fungal disease susceptibility (Romero-Pérez *et al.*, 2001; Vincenzi *et al.*, 2013). To investigate the effect of environmental conditions and the resveratrol distribution in different parts of the vine, samples from the same rootstock were collected and analysed during the 2018 and 2019 harvest.

As mentioned in Section 2.4, the Pinotage cross was created at Stellenbosch University's Welgevallen Experimental farm to produce a robust variety with a high growth yield. During 2018 4.7 million liters Pinotage were produced in South Africa and produced approximately 2.1 kilotons grape pomace, 1.1 kilotons stems, 1.5 kilotons canes and 79 tons leaves (*SA wine industry 2018 statistics*, 2019). Limited research about the phenolic content in Pinotage is available and thus it was selected as the biomass

source and analysed for resveratrol. Pinotage berries, stems, leaves and cane trimmings were collected from the exact same rootstock from Welgevallen experimental farm during the Pinotage harvest in 2018 and 2019 to eliminate any variation in resveratrol that could have occurred due to factors such as the amount of rainfall and UV radiation and to be able to compare the resveratrol concentration in the different parts of the vine, since resveratrol distribution in Pinotage vines were still unknown. The different biomass samples were processed as discussed in Section 4.2.3.2, extracted with 80:20 v/v% ethanol-water mixture and analysed. The resveratrol concentration in the post fermentation skins and seeds, stems, canes and leaves were determined and is shown in Figure 15 and Figure 16, below.

5.2.2.1 2018 Pinotage

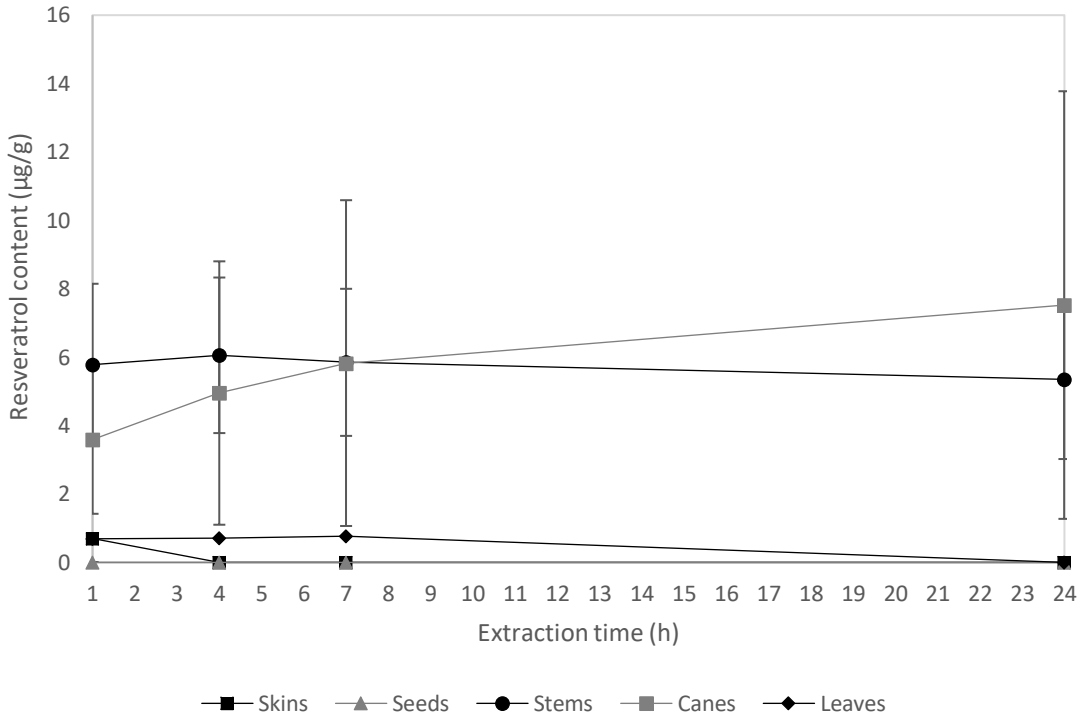


Figure 15. Mean resveratrol content of triplicate samples (µg resveratrol/ g dried biomass ± standard error) extracted from the 2018 Pinotage canes (■) and stems (●) over 24 hours with 80% ethanol under ambient conditions. Mean resveratrol concentration of duplicate samples (µg resveratrol/ g dried biomass) of the 2018 Pinotage post fermentation skins (■), post fermentation seeds (▲) and leaves (♦) extracted with 80% ethanol.

As seen in Figure 15, the resveratrol concentration varies within the different parts of the vine with the canes and stems having the highest resveratrol concentration of 7.5 ± 3.6 µg/g and 5.4 ± 2.3 µg/g, respectively. A maximum of 0.8 µg/g and 0.7 µg/g resveratrol was extracted from the leaves and post fermentation skins. While no resveratrol was extracted from the post fermentation seeds. If the

maximum resveratrol is recovered and 100% recovery is assumed, only 7.5 ± 3.6 mg resveratrol is produced per kg of dried grape canes, which is comparable to the concentration of resveratrol in red wine as mentioned in Section 2.1.5.1 (Geana *et al.*, 2014).

According to Langcake and Pryce (1976) *Vitis vinifera* leaves can contain between 50 µg/g to 400 µg/g resveratrol. When comparing the amount of resveratrol extracted from the leaves to the study of Langcake and Pryce (1976) it was found that the resveratrol concentration is significantly lower, confirming that it is dependent on factors such as geography and grape variety. Even though the resveratrol concentration is lower than expected, the results correspond to the work of Lachman *et al.* (2016). According to Lachman *et al.* (2016), grape canes have the highest resveratrol concentration of 9.26 ± 1.53 µg/g, followed by the stems and leaves each containing 1.76 ± 0.51 µg/g and 0.51 ± 0.33 µg/g resveratrol.

In order to determine if resveratrol concentration will vary between harvests, grape berries, stems, leaves and canes from the exact same rootstock were collected and analysed to investigate possible resveratrol variation.

5.2.2.2 2019 Pinotage

The resveratrol concentration in the 2019 skins, seeds, stems, canes and leaves is shown in Figure 16, below.

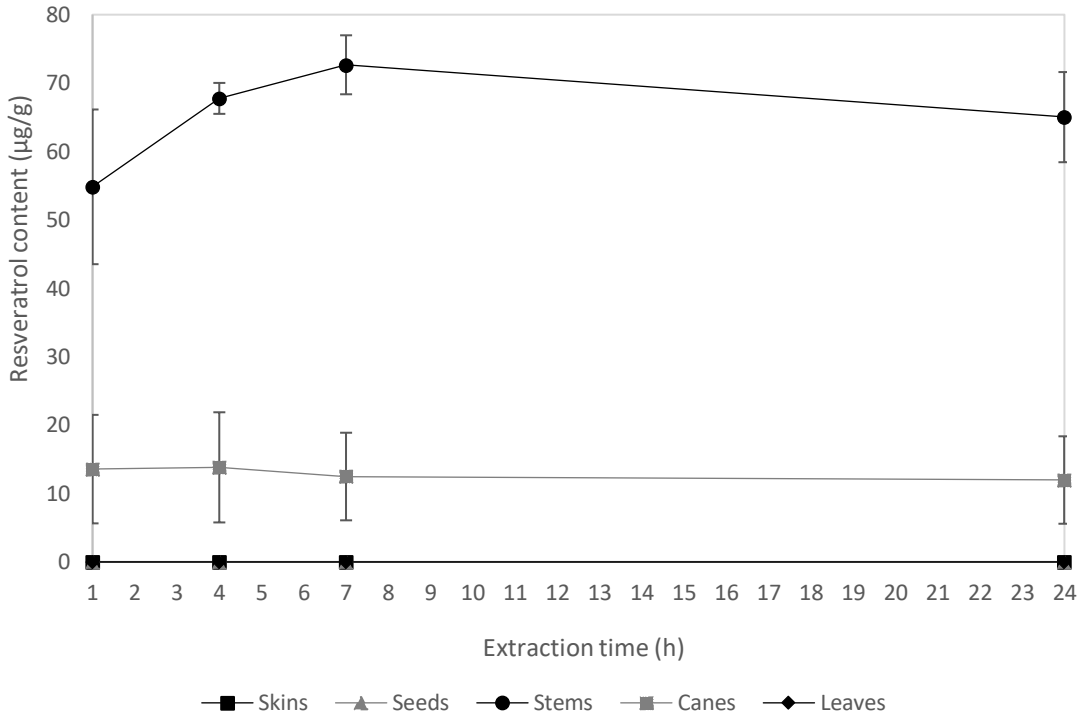


Figure 16. Mean resveratrol content of triplicate samples (µg resveratrol/ g dried biomass ± standard error) extracted from the 2019 Pinotage canes (■), stems (●), post fermentation skins (■) and seeds (▲) and leaves (♦) over 24 hours with 80% ethanol under ambient conditions.

As seen in Figure 16, the resveratrol content varies within the 2019 Pinotage harvest. From resveratrol HPLC analysis it was found that the maximum amount of 73 ± 4.3 µg/g resveratrol was extracted from the stems. The resveratrol extracted from the canes varied from 12 ± 6.4 µg/g to 14 ± 8.1 µg/g. No resveratrol was extracted from the 2019 harvest leaves, post fermentation skins and seeds. From the comparison of the resveratrol content in the leaves, skins and seeds from the 2018 and 2019 harvest, the data suggest that more resveratrol solubilized during the 2019 fermentation. To determine the amount resveratrol that solubilized during fermentation, the pre fermentation skins and seeds were extracted and analyzed. From the analysis of the pre fermentation skins and seeds extracts, it was found that no resveratrol is present. It was concluded that the Pinotage skins and seeds contained no resveratrol. However, from the investigation of resveratrol degradation and isomerization as discussed in Section 5.1 it was found that both the Shiraz and Grenache skins from Stellenbosch contained resveratrol, indicating that resveratrol can be present in the grape berry but is variety dependent. This corresponds to findings from literature, summarized in Figure 3 that resveratrol variation not only occurs within different regions but also between different varieties. By comparing the extracted resveratrol content from the canes and stems, it was found that the resveratrol content varies over time even in the same rootstock.

5.2.2.3 2018- 2019 Resveratrol comparison

It should be noted that the Pinotage rootstock used in 2018 and 2019 was not infected with *Botrytis cinerea* or *Plasmopara viticola*. It was assumed that biotic stress did not influence the resveratrol content.

The average Stellenbosch weather conditions from pruning to harvesting are summarised in Table 4 below to compare the 2018 and 2019 harvest growth conditions, possibly influencing the resveratrol concentration (*World Weather Online*, 2019).

Table 4. Comparison of the average climatic conditions during the 2018 and 2019 growth and harvest period in Stellenbosch.

	Rainfall (mm)	Temperature (°C)	Humidity (%)	UV index
2018 Harvest	463	15.8	67.7	4.67
2019 Harvest	685	17.8	66.4	5.08

As seen in Table 4, the average humidity is similar and since the rootstocks were not infected with *Botrytis cinerea* or *Plasmopara viticola* it did not have an effect on the resveratrol production. During the 2017 growth period Stellenbosch experienced a drought with an approximate total rainfall up to the 2018 harvest of 463 mm. The average rainfall during the 2019 harvest significantly increased as seen in Table 4. The average ambient temperature and UV index also increased from the 2018 to 2019 harvest while the average humidity remained constant over the two years. If assumed that the climatic conditions are the only factors that changed and by comparing the 2018 and 2019 resveratrol content, it was concluded that it has a significant impact and the large variability should be taken into consideration if scaled up.

Even with the variation, the canes and stems contained more resveratrol compared to the skins, seeds and leaves and should be used as a resveratrol source. According to Devesa-Rey *et al.* (2011), cane trimmings are being valorized into several products, summarized in Figure 11, but is mostly used as mulch in South African wineries, while the stems are not used and are currently being composted or incinerated. The stems and the canes could be used as a resveratrol source to beneficiate the biomass landfilled or incinerated by producing a high value product from the waste material.

The significant difference in resveratrol concentration between the consecutive harvests indicates that the process would have to be adjusted for each harvest due to the variable resveratrol feedstock. As seen in Figure 15 and Figure 16, some of the biomass contained no resveratrol or have a very low concentration and would not be a feasible resveratrol source. From the results summarized in Figure 15 and Figure 16 it was noted that a long term study with different grape varieties is required to more accurately

determine the average resveratrol concentration in the different parts of the vine in order to determine the feasibility of solid winery waste as a resveratrol source.

5.2.3 Total phenolic content

Polyphenols are phytochemicals found in many food sources including grapes. Some of the phenolic compounds include anthocyanins, flavonoids, phenolic acids and stilbenes (Xia *et al.*, 2013). To determine if there is correlation between the resveratrol concentration and the phenolic content, the total phenolic content of the skins, seeds, stems, canes and leaves from the 2018 and 2019 were determined in terms of gallic acid equivalence and compared in Figure 17 and Figure 18.

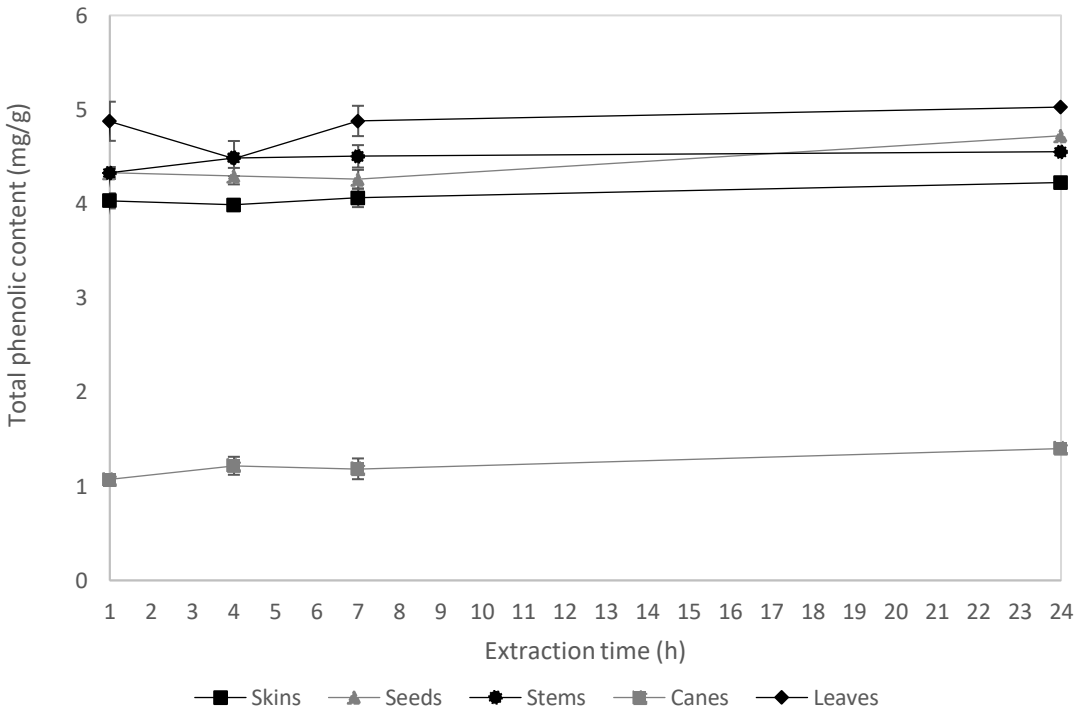


Figure 17. Mean total phenolic content of triplicate samples (µg resveratrol/ g dried biomass ± standard error) extracted from the 2018 Pinotage canes (■), stems (●), post fermentation skins (■) and seeds (▲) and leaves (♦) over 24 hours with 80% ethanol under ambient conditions.

From the comparison of the total phenolic content in the different waste samples it was found that the leaves had the highest overall total phenolic content with a maximum of 5.02 ± 0.16 mg/g. While the stems and seeds had a similar phenolic content. The phenolic content in the post fermentation skins ranged from 3.98 ± 0.081 mg/g to 4.22 ± 0.10 mg/g and from 1.07 ± 0.013 mg/g to 1.4 ± 0.11 mg/g in the cane trimmings. By comparing the resveratrol content to the total phenolic content of the 2018 Pinotage it was concluded that there is no clear correlation. The canes had the highest resveratrol concentration

but the lowest total phenolic content while the skins, leaves and seeds had the minimum resveratrol concentration but contains other polyphenols.

5.2.3.1 2019 Pinotage

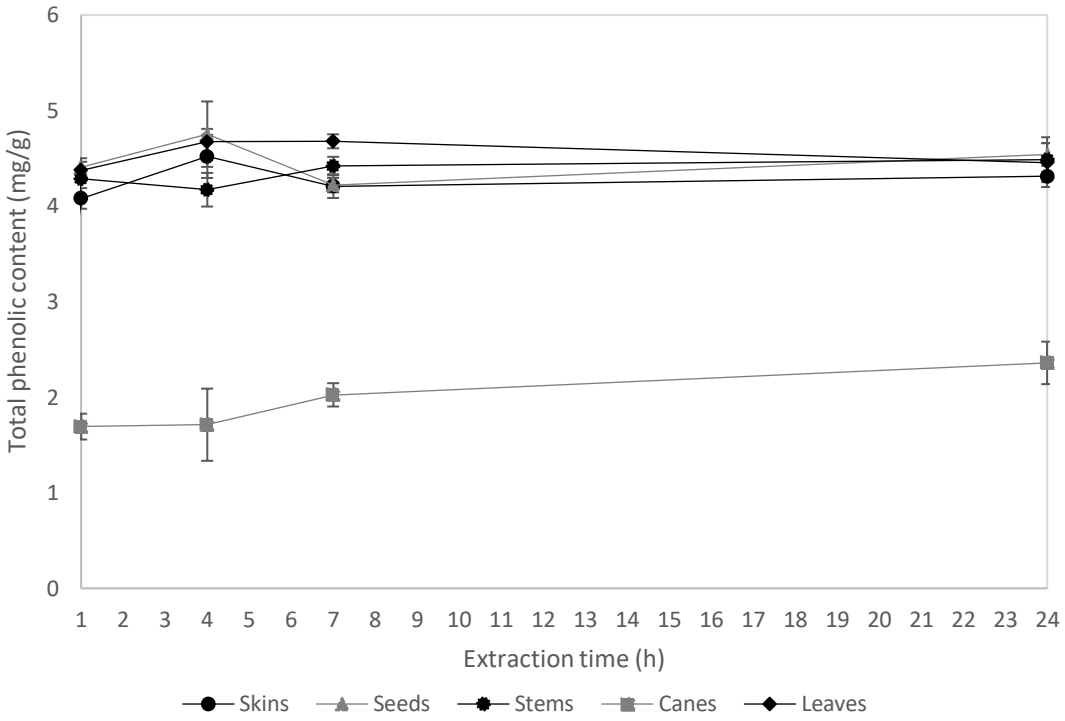


Figure 18. Mean total phenolic content of triplicate samples (µg resveratrol/ g dried biomass ± standard error) extracted from the 2019 Pinotage canes (■), stems (●), post fermentation skins (■) and seeds (▲) and leaves (♦) over 24 hours with 80% ethanol under ambient conditions.

The 2019 Pinotage extracts were also analysed with UV-vis spectroscopy to determine the total polyphenolic content. The total phenolic content of the stems, leaves, post fermentation skins and seeds were within the same range within the error margins. The amount of polyphenols extracted from the canes increased over time, with a maximum of 2.36 ± 0.22 mg/g after 24 hours extraction.

5.2.3.2 2018- 2019 Polyphenol comparison

The total phenolic content of the different samples were compared, to determine if the total phenolic content changed from the 2018 to the 2019 harvest and if the change in resveratrol concentration over time will affect the total phenolic content. It was observed that from 2018 to 2019 the total polyphenol content remained between 4 – 5 mg gallic acid equivalence per gram of dried skins, seeds, stems and leaves. While the average phenolic content in the cane trimmings increased from 1.22 ± 0.06 mg/g to 1.95 ± 0.22 mg/g from 2018 to 2019. By comparing the polyphenol and resveratrol content it was

concluded that there is no correlation between the total phenolic content and resveratrol. The distribution of the phenolic compounds is dependent on the part of the vine. The main polyphenols present in the different parts of the vine, according to the study by Xia *et al.* (2013), is summarised in Table 6 in Appendix C.

During the fermentation some of the polyphenols solubilize from the skins and seeds into the must making wine rich in polyphenols. To determine the amount of solubilisation, the total phenolic content in the pre- and post-fermentation Pinotage skins and seeds were determined and compared. Approximately 610 mg and 130 mg polyphenols solubilised per kilogram of dried grape skins and seeds respectively. Since very little information is available about resveratrol and other polyphenols in Pinotage, the polyphenolic content was compared to a study of the polyphenols present in South African Pinotage wine by De Beer (2002). According to De Beer (2002), the average polyphenolic content in Pinotage is 625 mg/kg assuming 1 kg of grapes are used per 750 ml wine. By comparing the amount of polyphenols that solubilized during the fermentation step to the total phenolic content in Pinotage wine as determined by De Beer (2002) it was found that the polyphenol concentration is similar. It was concluded that even over a longer period the total phenolic content in Pinotage remained constant.

From the comparison of the average resveratrol and polyphenol concentration from 2018 it was observed that the stems had the second highest resveratrol and polyphenol concentration. While the stems from the 2019 harvest had the overall highest resveratrol concentration and third highest average polyphenol concentration. As mentioned in Section 2.4.2 the stems are not currently being valorized and could be used as a source of resveratrol and other polyphenols for a possible resveratrol-polyphenol supplement.

5.2.4 ATPS extraction

To produce a resveratrol nutraceutical supplement from a waste source, the chemicals and processes used should not only be safe for consumption but should utilise low toxicity chemicals, making ATPS a possible extraction process.

A polyethylene glycol 8000 and potassium sodium tartrate tetrahydrate system was investigated as an alternative extraction method. The investigation of the PEG 8000 and tartrate ATPS to extract resveratrol and other polyphenols from solid winery waste builds on a previous project by Herbst (2019). One of the systems investigated by Herbst (2019) with equal volumes of 35 w/v% PEG and 35 w/v% tartrate was repeated to investigate the extraction of resveratrol and other polyphenols from the 2019 Pinotage stems. The PEG top phase was analysed to determine the resveratrol and total polyphenol concentration. If assuming the extraction achieved with ethanol solvent extraction is the maximum extractable polyphenols and resveratrol, the ATPS extraction yield could be calculated. 20.7% and 96.3% resveratrol

and polyphenols were recovered from the 2019 Pinotage stems respectively. The partitioning of resveratrol and other polyphenols to the PEG top phase corresponds to work of Xavier *et al.* (2014), which successfully extracted phenolic compounds from eucalyptus wood waste. According to Xavier *et al.* (2014), phenolic compounds tend to partition to the PEG phase in a PEG-salt two-phase system, predominantly due the hydrophobic interactions between molecules and the two phases. Hydrophobic molecules will selectively dissolve and partition to the PEG top phase when extracted in a PEG-tartrate system. Factors such as phase composition, pH and temperature influence the partitioning in an ATPS. The effect of these factors should be investigated to improve the resveratrol extraction as a possible industrial extraction method. However, for this study it was only important to determine if resveratrol will partition to the PEG phase.

5.3 Recovery

If a PEG – tartrate two phase system is used to extract the resveratrol, the extracted resveratrol should be further concentrated into an edible form. After the removal of the resveratrol-rich PEG top phase from the extraction two- phase system, the PEG phase is processed to concentrate the extracted resveratrol. A subsequent polymer-polymer ATPS and protein-polyphenol precipitation technique was investigated to decrease the high cost and improve the bottleneck of downstream purification processes such as chromatography. The two recovery methods were investigated to determine if resveratrol recovery is possible with these alternative techniques.

5.3.1 ATPS recovery

A two stage ATPS, the polymer- salt system was used to partition resveratrol to the PEG phase, as well as to eliminate other contaminants extracted. The resveratrol rich PEG phase was then reused as the PEG top-phase in a maltodextrin- PEG recovery system to concentrate the extracted resveratrol as shown in Figure 19.

As shown in Figure 19, the pulverised winery waste is added to the PEG-tartrate two-phase system were the resveratrol is extracted and partitioned to the PEG top phase. The PEG phase is filtered, and maltodextrin is added as the second polymer phase to possibly concentrate the extracted resveratrol into the starch derived, edible maltodextrin.

The PEG- maltodextrin recovery method was investigated by determining the resveratrol partitioning to the maltodextrin bottom phase in different systems with PEG 8000 and maltodextrin (DE 16.5-19.5). Different factors such as the PEG and maltodextrin concentration, the effect of resveratrol concentration and system pH were investigated.

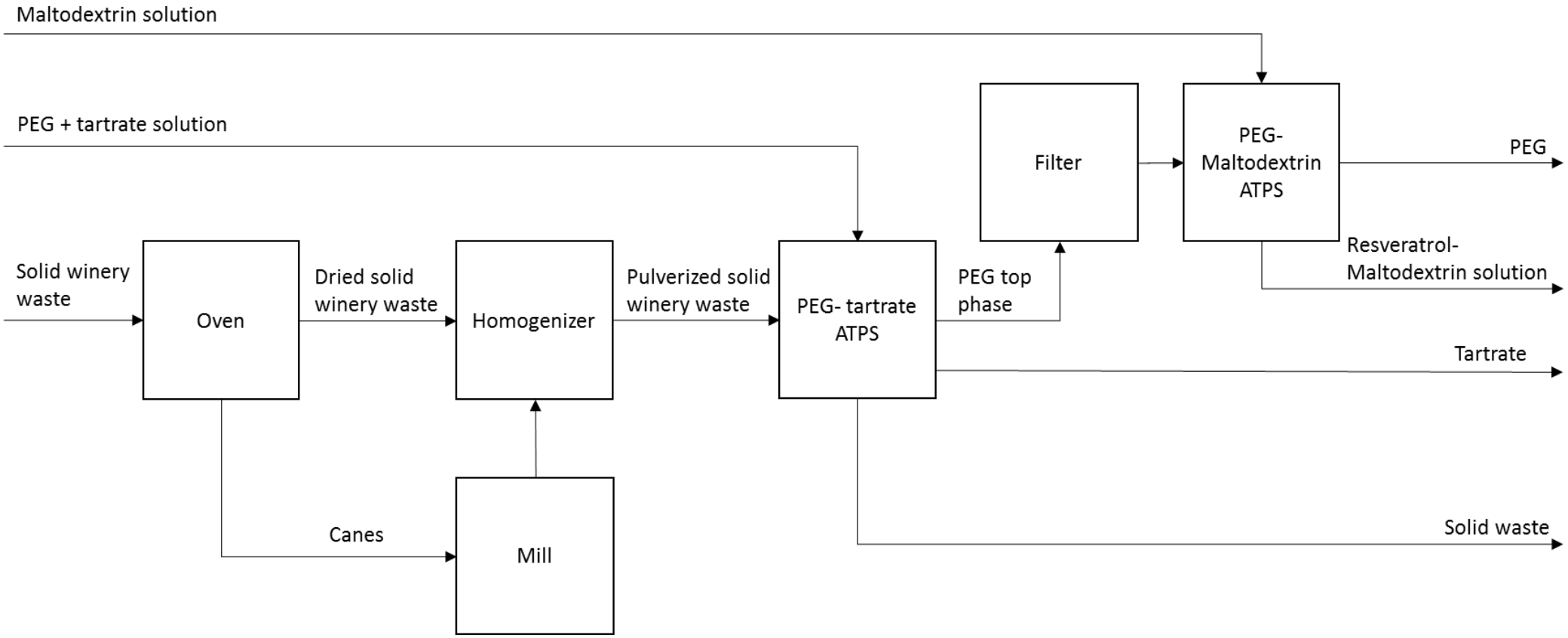


Figure 19. Proposed process diagram for resveratrol extraction from solid winery waste and recovery with a subsequent PEG-maltodextrin two-phase system.

5.3.1.1 Effect of phase composition

The partitioning of pure resveratrol in different PEG-Maltodextrin systems were investigated to determine if resveratrol will partition into the maltodextrin phase to produce an edible resveratrol concentrate.

Different mixing points were selected from the PEG 8000 – Maltodextrin (DE 16.5-19.5) binodal curve to determine the effect of phase composition on resveratrol partitioning. The resveratrol partitioning to the maltodextrin phase was investigated for six different systems with an average resveratrol concentration of 1.31 g/L and was evaluated in terms of the resveratrol partition coefficient in maltodextrin. The partition coefficient, as discussed in Section 2.2.2.3, is the concentration ratio of resveratrol in the maltodextrin phase to the PEG phase. A partition coefficient of one indicates no selectivity and a resveratrol partition coefficient greater than one indicates selective resveratrol partitioning to the specific phase. The partition coefficient of resveratrol in maltodextrin in each system is summarised in Figure 20.

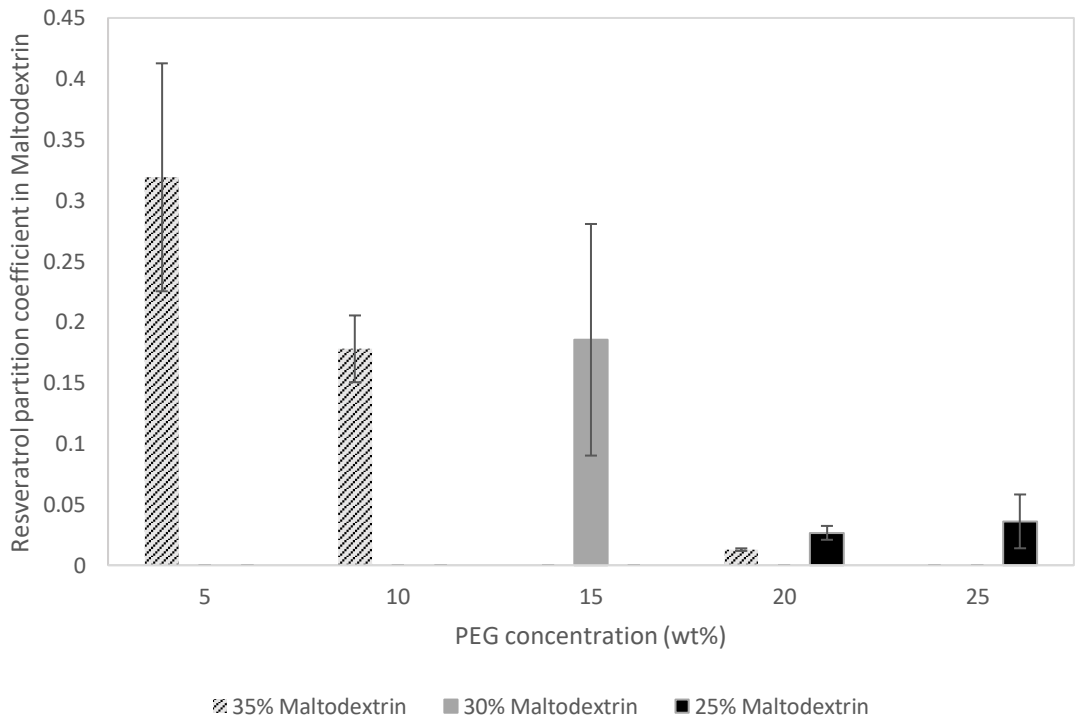


Figure 20. Pure resveratrol partition coefficient of triplicate samples with standard error to the maltodextrin bottom phase in different PEG-maltodextrin ATPS. The system PEG concentration (wt%) is represented on the x-axis while the maltodextrin concentration (wt%) is represented by the different

columns as diagonal bars (35% maltodextrin), a solid grey bar (30% maltodextrin) as well as black bars (25%).

As seen in Figure 20, the partition coefficient of resveratrol in maltodextrin is less than one for all six systems, indicating no concentrating effect to the maltodextrin phase, but rather to the PEG phase. More resveratrol remained in the PEG top phase and did not successfully partition to the desired maltodextrin bottom phase. As discussed in Section 2.2.2, partitioning is dependent on the difference in hydrophobicity of the two phases. Non-polar resveratrol will selectively partition to the less hydrophilic PEG top phase than to the more hydrophilic maltodextrin bottom phase. A system closer to the phase envelope is more sensitive to slight changes in the phase composition and can result in no two phase formation to partition a specific molecule (Walter and Johansson, 1994). The bottom phase partitioning increased with an increase in the maltodextrin concentration and a decrease in the PEG concentration. An increase in the maltodextrin concentration decreases the hydrophilicity of the bottom phase, resulting in improved partitioning of the non-polar resveratrol. By decreasing the PEG concentration, the hydrophobicity of the PEG phase also decreases and will improve resveratrol partitioning to the maltodextrin bottom phase. Partitioning in ATPS is also influenced by the volume exclusion effect. By increasing the polymer concentration, in this case PEG, the available volume for resveratrol decreases, resulting in resveratrol molecules moving to the bottom phase. According to Walter and Johansson (1994) several factors can influence partitioning and depending on the system certain factors will have a greater effect on the partitioning.

Even though an increase in maltodextrin concentration resulted in higher resveratrol partitioning, the increase in the concentration will also increase the specific phase volume resulting in a less concentrated resveratrol sample. The increase in maltodextrin concentration will not only deliver a less concentrated product but will also increase the recovery operating cost.

5.3.1.2 Effect of resveratrol concentration

The effect of resveratrol concentration on the partitioning in a PEG-Maltodextrin system was investigated over a PEG concentration range of 5-10%. The partition coefficients of the different systems were determined and is shown in Figure 21.

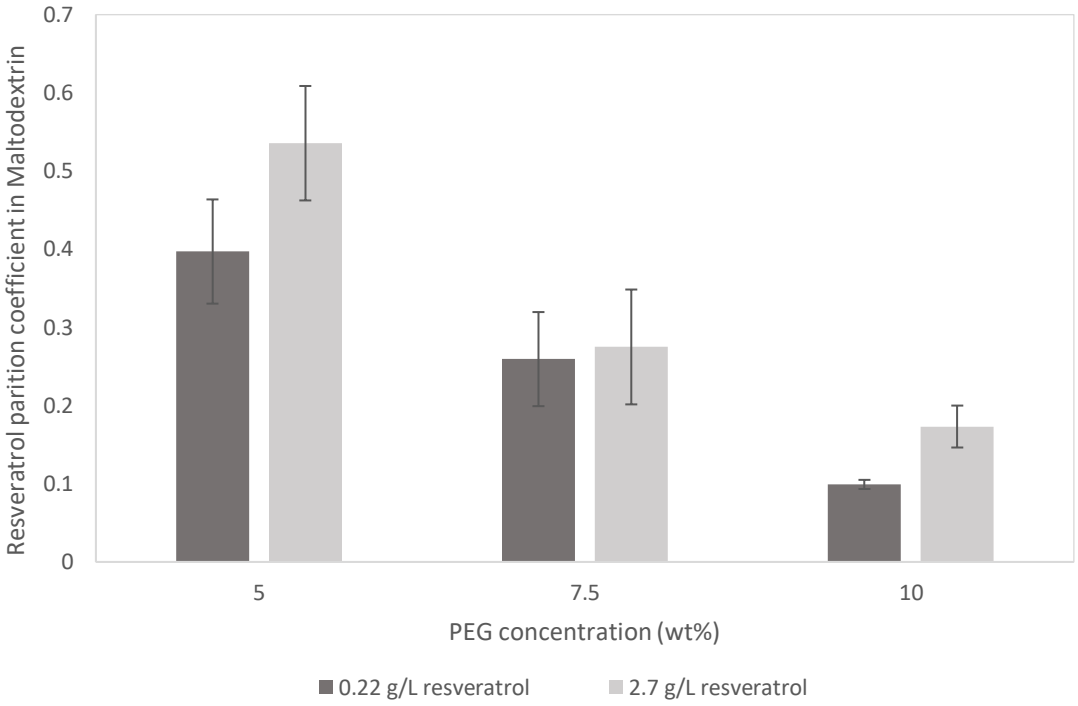


Figure 21. Pure resveratrol partition coefficient of triplicate samples with standard error to the 35% maltodextrin bottom phase in different PEG-maltodextrin ATPS to show the effect of resveratrol concentration and PEG concentration on partitioning. The system PEG concentration (wt %) is represented on the x-axis while the effect of the resveratrol concentration is represented by the dark grey bars (0.22 g/L resveratrol) and the light grey bars (2.7 g/L resveratrol).

A maximum resveratrol partitioning of 0.54 ± 0.073 and 0.40 ± 0.067 was achieved for a 35% maltodextrin and 5% PEG ATPS with a resveratrol concentration of 2.7 g/L and 0.22 g/L, respectively. While the lowest resveratrol partitioning of 0.17 ± 0.027 and 0.10 ± 0.0058 was achieved for a system of 35% maltodextrin and 10% PEG with a resveratrol concentration of 2.7 g/L and 0.22 g/L, respectively. By comparing the results summarised in Figure 20 and Figure 21 it was concluded that the decrease in the PEG concentration will result in improved partitioning by decreasing the PEG hydrophobicity as well as minimising the effect of volume exclusion. It was also observed that by increasing the resveratrol concentration in the two-phase system greater partitioning to the maltodextrin phase was achieved. By increasing the resveratrol in the ATPS the PEG phase becomes saturated with resveratrol since only a specific phase volume is available. The data suggests that the remaining resveratrol will then partition to the maltodextrin phase. If the PEG phase concentration is then increased a larger volume is available for resveratrol and will result in lower bottom phase partitioning. A lower PEG concentration is desired if scaled up since it will decrease the operating cost, but the resveratrol concentration cannot be adjusted

since it is dependent on the PEG-tartrate extraction system. It was also noted that even with the increase in resveratrol partitioning, the resveratrol partition coefficient in maltodextrin is still below 1 indicating unsuccessful partitioning. In order to successfully use the subsequent maltodextrin- PEG two phase system as a recovery method to concentrate resveratrol into the edible maltodextrin phase, the partition coefficient of resveratrol in maltodextrin should be greater than one. The greater the resveratrol partition coefficient in maltodextrin the more cost effective the recovery is and should be maximised to deliver a concentrate of the high value resveratrol.

5.3.1.3 Effect of pH in partitioning

The effect of a change in system pH was investigated to determine if increasing the pH will improve resveratrol partitioning to the more hydrophilic maltodextrin phase. The system pH was adjusted above and below the acidic dissociation constants of resveratrol. The resveratrol partition coefficient in maltodextrin was determined and is represented by Figure 22.

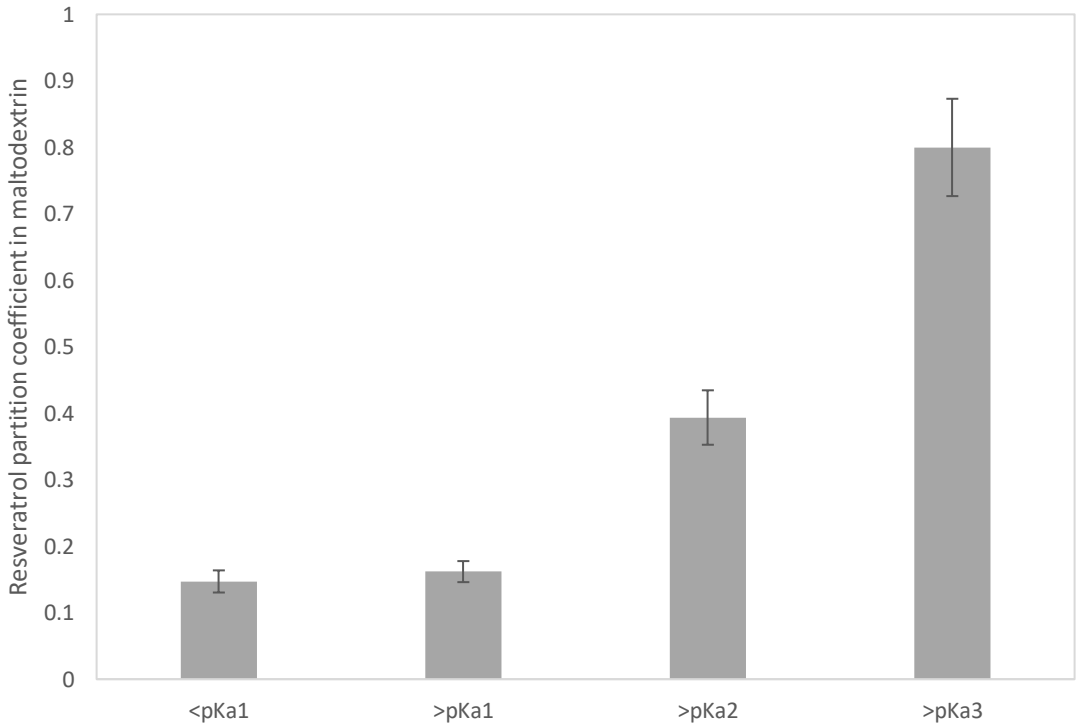


Figure 22. Pure resveratrol partition coefficient of triplicate samples (± standard error) to the 35% maltodextrin bottom phase in different PEG-maltodextrin ATPS to show the effect of the system pH (above and below each acidic dissociation constant of resveratrol) on the resveratrol partitioning. The system pH is represented on the x-axis and is above and below pK_{a1}= 8.8, pK_{a2} = 9.8 and pK_{a3} = 11.4.

The system pH of a 35% Maltodextrin – 7.5% PEG system was adjusted to a pH above 8.8, 9.8 and 11.4 and was compared to a system with no pH adjustment (below 8.8). As seen in Figure 22, the resveratrol partitioning to the maltodextrin bottom phase increased with an increase in pH above each dissociation constant. By increasing the system pH above 11.4 the resveratrol partition coefficient in maltodextrin increased from 0.15 ± 0.02 to 0.80 ± 0.07. By adjusting the system pH, the protonation of resveratrol will change as seen in Figure 2. As the system pH increase, the polarity of resveratrol changes and will become less hydrophobic. The more polar resveratrol partitioned more easily to the more hydrophilic maltodextrin phase, due to a decrease in the hydrophobic interactions between the two phases. However, as discussed in Section 4.2.2.2, resveratrol degradation is pH dependent and will degrade under alkaline conditions. In order to compare the increase in resveratrol partitioning to the maltodextrin phase with an increase in system pH to the increase in resveratrol degradation, the change in resveratrol concentration in PEG 8000 and maltodextrin (DE 16.5-19.5) were determined as shown in Figure 23.

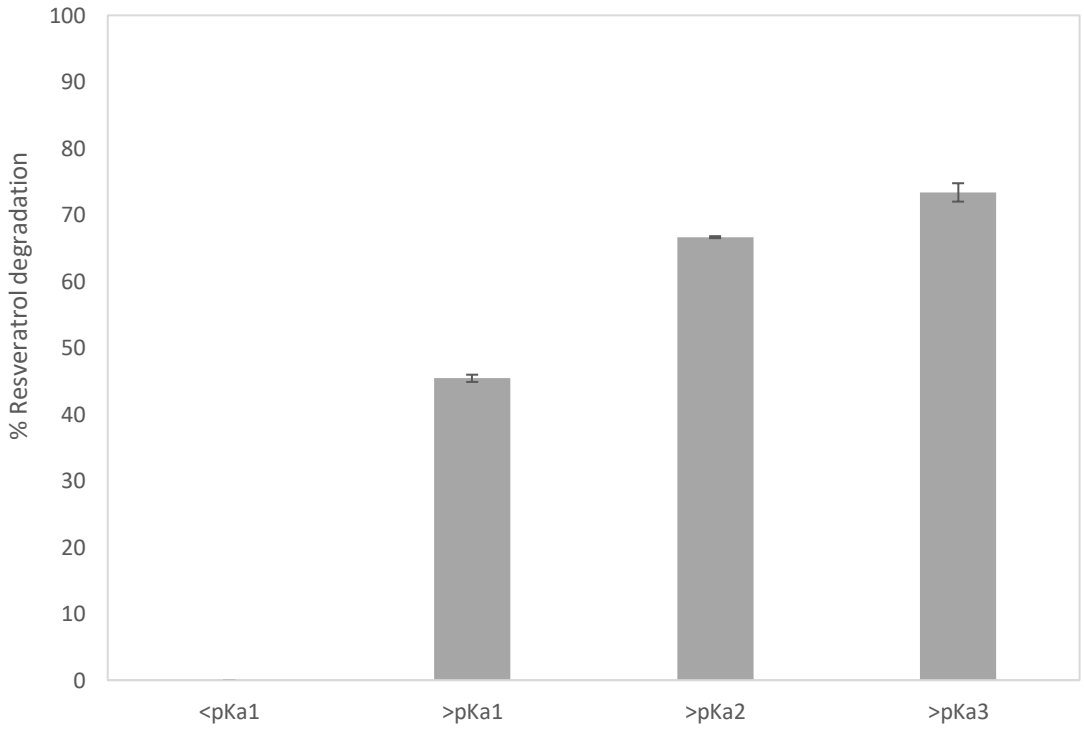


Figure 23. Mean percentage *trans*-resveratrol degradation of triplicate samples (% degradation± standard error) in 35 w/w% maltodextrin and 7.5 w/w% PEG two phase system with a system pH above and below pK_{a1}=8.8, pK_{a2}=9.8 and pK_{a3}=11.4.

As seen in Figure 23, resveratrol degradation increased with an increase in system pH. As discussed in Section 4.2.2.2, resveratrol degradation is related to the degree of dissociation and the degradation will increase as the pH is increased above pK_{a1}=8.8, pK_{a2}=9.8 and pK_{a3}=11.4. If it is assumed that no resveratrol

degradation occurred in the unadjusted PEG-maltodextrin sample, up to 73 ± 1.4% resveratrol degraded with a system pH above 11.4. From the evaluation of the results summarised in Figure 22 and Figure 23 it was observed that an increase in the system pH will improve partitioning to the hydrophilic maltodextrin phase but will result in significant degradation. It can be concluded that a change in system pH cannot be used to improve resveratrol recovery in a PEG-maltodextrin.

5.3.1.4 Resveratrol recovery from grape stems with ATPS

A PEG 8000 and potassium sodium tartrate tetrahydrate system was investigated to extract resveratrol from Pinotage stems. To recover the extracted resveratrol from the PEG phase, a subsequent PEG-maltodextrin ATPS was investigated to concentrate the extracted resveratrol into the maltodextrin phase.

By adding maltodextrin to the resveratrol rich phase, a second two-phase system with 35 w/w% maltodextrin (DE 16.5-19.5) and 5 w/w% PEG 8000 formed. Both the PEG phase and maltodextrin phase were analysed to determine the resveratrol partition coefficient to the maltodextrin phase. From the analysis of resveratrol in both phases it was found that all the extracted resveratrol remained in the PEG top phase. These results and the results discussed in Section 5.3.1.1 to 5.3.1.3 indicate that resveratrol extracted from solid winery waste cannot be concentrated from the PEG phase into a maltodextrin-resveratrol supplement. These results confirm that the use of a subsequent maltodextrin-PEG is not a feasible technique to recover resveratrol from solid winery waste.

5.3.2 Protein precipitation

An alternative method to recover extracted resveratrol is to precipitate the resveratrol out of the PEG solution. As shown in Figure 24, in the proposed process the pulverised winery waste is added to the PEG-tartrate two-phase system were the resveratrol is extracted and partitioned to the PEG top phase. The PEG phase is removed and filtered and a protein solution of ovalbumin, tryptone soy broth or yeast extract is added and sufficiently mixed to form an edible, solid form resveratrol-protein precipitate.

The use of proteins to precipitate resveratrol and other polyphenols were investigated as a possible recovery method by first investigating the precipitation of the extracted polyphenols, followed by the investigation of pure resveratrol precipitation from PEG and the investigation of resveratrol precipitation extracted with ATPS from 2019 Pinotage stems.

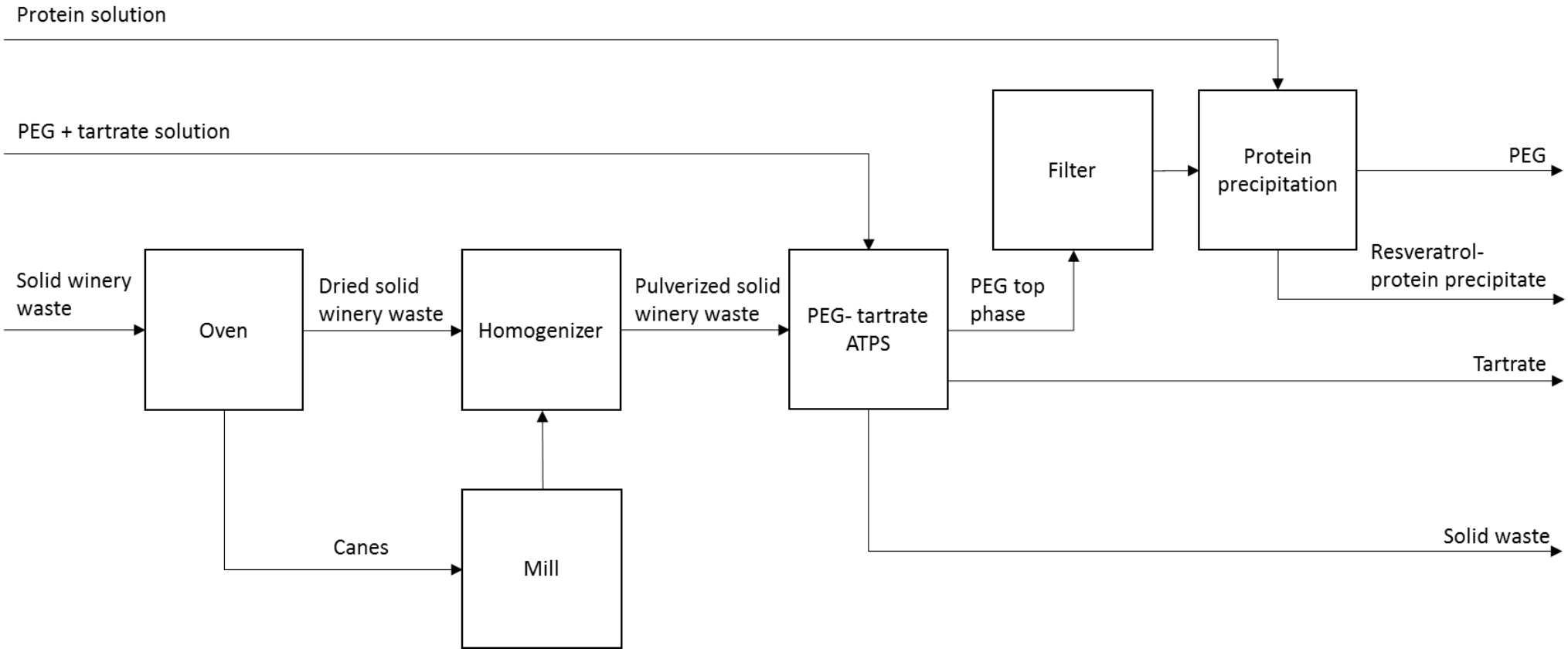


Figure 24. Proposed process diagram for resveratrol extraction from solid winery waste and recovery with resveratrol-protein precipitation.

5.3.3 Polyphenol precipitation

Tryptone soy broth and yeast extract contain casein peptone, soy and peptides that are used in protein supplements while ovalbumin (protein from egg whites) is an inexpensive source of protein. Tryptone soy broth, yeast extract and ovalbumin were selected as protein sources and were added to the polyphenol extract and mixed for 24 hours and centrifuged to form a recoverable precipitate. Protein solutions with concentrations ranging from 0.047 g/L to 0.73 g/L were added to the polyphenol extract from the 2019 Pinotage stems and the percentage polyphenol recovery achieved is summarised in Figure 25.

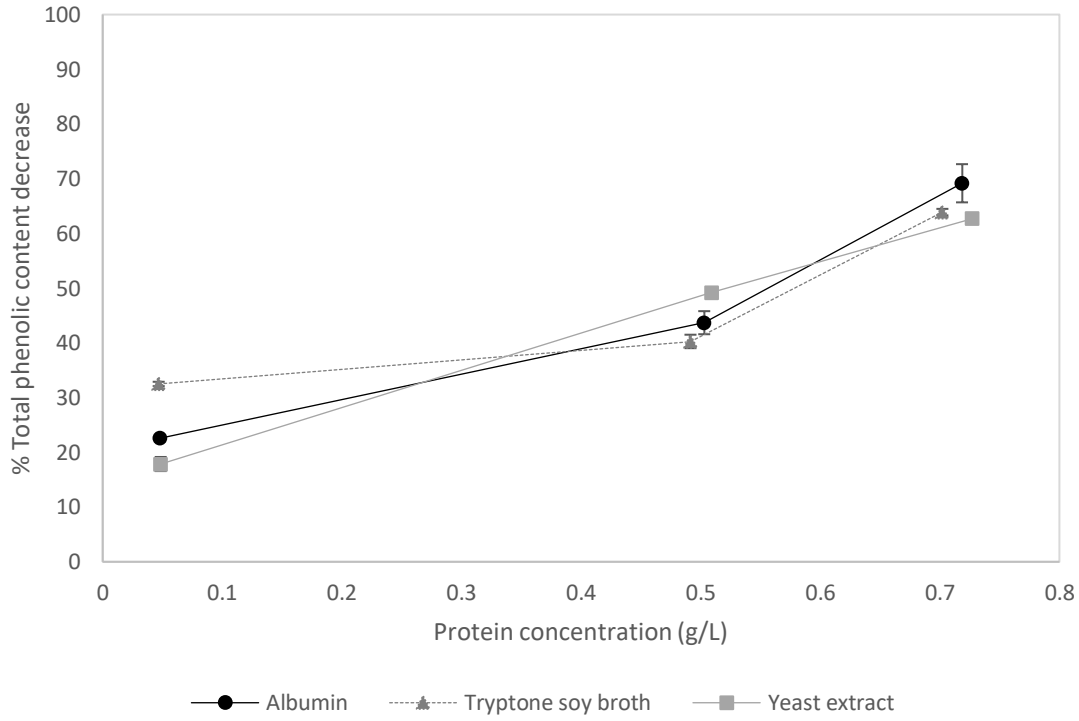


Figure 25. The mean percentage of triplicate total phenolic content (± standard error) recovered from the 2019 Pinotage leaf extract with albumin (●), tryptone soy broth (▲) and yeast extract (■) with concentrations up to 0.73 g/L.

As seen in Figure 25, the different proteins in solution with the polyphenol extract interacted to form a precipitate and the amount of polyphenol precipitation increased with an increase in the protein concentration of all three protein solutions. Between 22.6 ± 0.56% to 69.2 ±3.49 polyphenol precipitation was achieved with the ovalbumin concentrations investigated. While a maximum precipitation of 63.9 ± 0.60% and 62.7 ± 0.80% was achieved with 0.70 g/L tryptone soy broth and 0.73 g/L yeast extract solutions. As seen in Figure 25 a polyphenol protein precipitate formed with ovalbumin, tryptone soy broth and yeast extract and it can be concluded that it can be used to precipitate polyphenols and should be further investigated as a possible resveratrol recovery method.

5.3.3.1 Resveratrol in PEG precipitation

From the investigation of the polyphenol-protein precipitation it was concluded that the selected proteins formed a precipitate with the polyphenol and can be used to possibly precipitate resveratrol out of the extracted PEG phase, as an alternative to adsorption and chromatography as resveratrol recovery method. Ovalbumin, tryptone soy broth and yeast extract were solutions ranging from 0.032 g/L to 0.75 g/L were each added to PEG solution with an average resveratrol concentration of 0.82 g/L to investigate and compare the precipitation achieved with the same protein-resveratrol ratio as the protein-polyphenol ratio. The percentage resveratrol precipitation achieved is summarised in Figure 26.

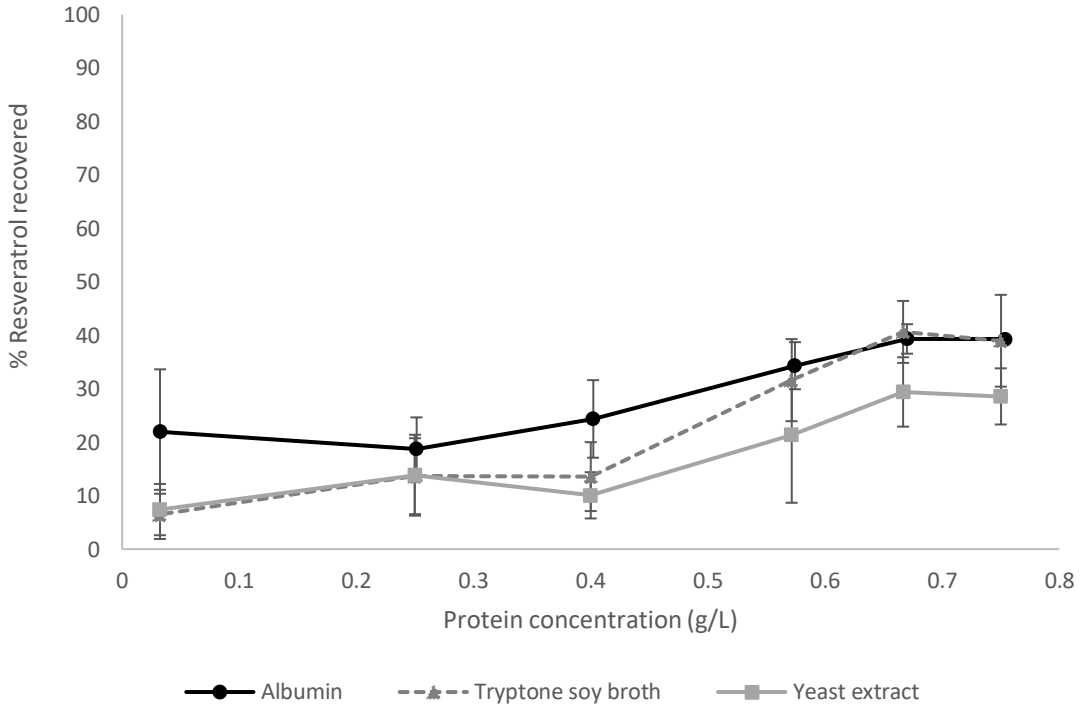


Figure 26. The mean percentage of triplicate pure resveratrol samples (± standard error) recovered from a 33 wt% PEG solution with albumin (●), tryptone soy broth (▲) and yeast extract (■) with concentrations up to 0.75 g/L.

As seen in Figure 26, the different proteins in solution with resveratrol interacted to form a precipitate. The recovery achieved was calculated as the percentage resveratrol recovered. The amount of resveratrol that precipitated out of the PEG solution increased with an increase in the protein concentration. Between 18.7 ± 5.93% to 39.3 ± 2.76% resveratrol was recovered with ovalbumin. While a maximum 40.6 ± 5.79 % and 29.4 ± 6.49 % resveratrol precipitated with tryptone soy broth and yeast extract. The addition of all three proteins achieved statistically the same resveratrol precipitation, considering the error. It was also observed that the amount of precipitate that formed did not increase with an increase in protein concentration from 0.67 g/L to 0.75 g/L for all three solutions. From the comparison of the

polyphenol precipitation to the resveratrol precipitation achieved it was observed that a lower percentage resveratrol was recovered with the same proteins and concentration.

Although the results indicate that resveratrol can be recovered from PEG with protein precipitation, the recovery achieved is still significantly lower than current recovery methods. Up to 93.3% resveratrol can be recovered with chromatography (Bai *et al.*, 2014) while Xiong *et al.* (2014) stated that 88.3% resveratrol can be recovered with microporous adsorption. From the comparison of the recovery achieved to current resveratrol recovery techniques, it is evident that factors that can improve recovery should be investigated.

Several factors can influence how the precipitate will form and some factors like system pH, temperature and ionic strength can be adjusted to change the covalent interactions between the polyphenol and protein to improve precipitation. However, several sources have stated that non-covalent bonds have a greater effect on the precipitate formed (Bandyopadhyay, Ghosh and Ghosh, 2012). Hydrogen bonds can form between the nitrogen or oxygen, from the amino or hydroxyl groups from the protein, and the hydrogen from the polyphenol hydroxyl groups to bind together. Hydrophobic interactions also partake in precipitation. The non-polar part of resveratrol (aromatic rings) will interact with the non-polar part of the protein. The precipitation achieved is usually a combination of these bonds. Other factors such as the polyphenol structure, protein size, molecular weight and protein to polyphenol ratio can also influence the precipitation reaction and should be investigated to achieve the maximum resveratrol recovery.

5.3.3.2 Resveratrol - protein ratio

As discussed in Section 2.3.2 the ratio of proteins to polyphenols influence the precipitate formed. To determine if the resveratrol concentration will affect the precipitation reaction, two PEG solutions with 0.077 g/L and 1.63 g/L resveratrol were mixed with ovalbumin, tryptone soy broth and yeast extract. The percentage resveratrol recovered from the two PEG systems is represented by Figure 27 and Figure 28.

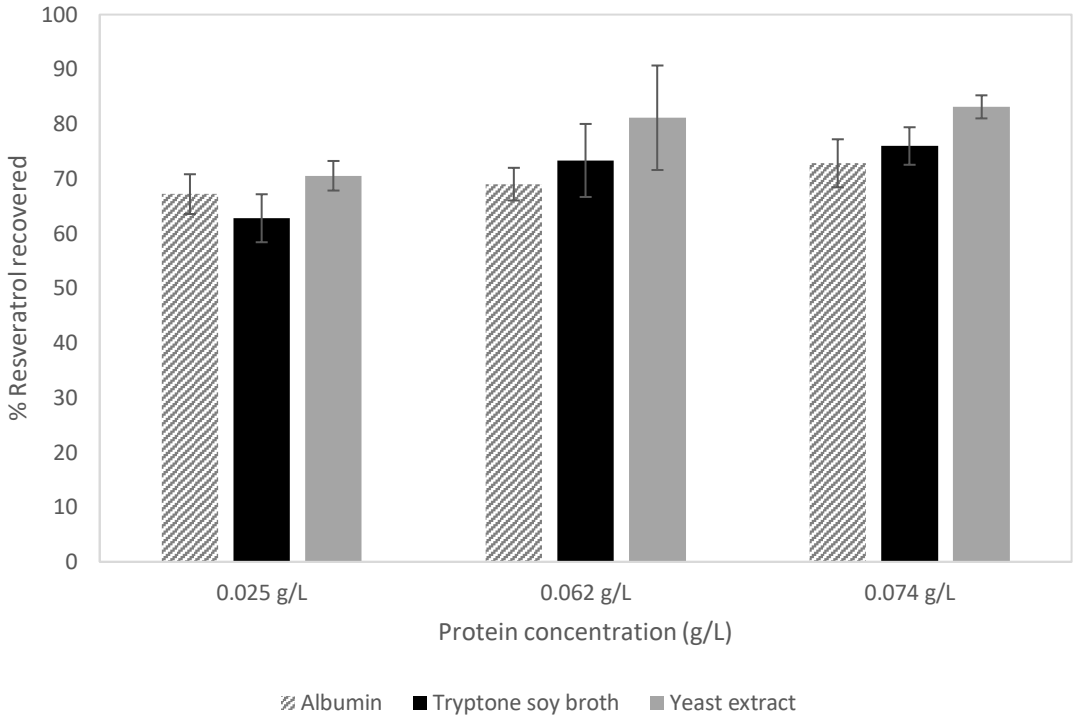


Figure 27. The mean percentage of triplicate pure 0.077 g/L resveratrol samples (± standard error) recovered from a 33 wt% PEG solution with ovalbumin (diagonal), tryptone soy broth (black) and yeast extract (grey) with concentrations ranging from 0.025 g/L to 0.074 g/L.

The low resveratrol concentration sample was mixed with the three protein solutions with concentrations ranging from 0.025 g/L to 0.074 g/L. A minimum and maximum of 62 ± 4.4% and 83 ± 2.1 % resveratrol was precipitated from the PEG phase, with all three protein solutions resulting in comparable recoveries within the standard error margins. By comparing the resveratrol recovery achieved in Section 5.3.3.1 to the resveratrol recovery in Figure 27, a significant difference was observed. A decrease in the resveratrol and protein concentrations resulted in improved precipitation. From the comparison of the resveratrol precipitation achieved to the percentage polyphenols recovered it was concluded that a high percentage resveratrol recovery similar to the total phenolic content recovery can be achieved but is influenced by the resveratrol concentration. The resveratrol-protein precipitation was repeated with a higher concentration resveratrol to determine it will affect the recovery achieved and the results are summarised in Figure 28.

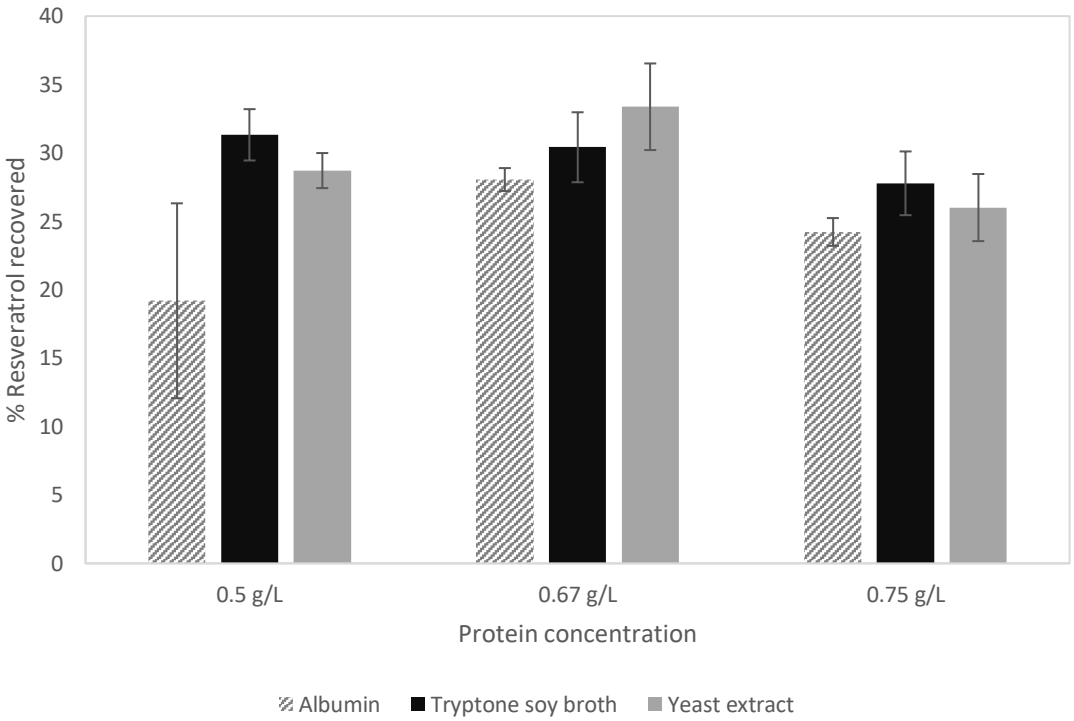


Figure 28. The mean percentage of triplicate pure 1.63 g/L resveratrol samples (± standard error) recovered from a 33 wt% PEG solution with ovalbumin (diagonal), tryptone soy broth (black) and yeast extract (grey) with concentrations ranging from 0.05 g/L to 0.75 g/L.

The 1.63 g/L resveratrol-PEG solution was mixed with the three different protein solutions with concentrations ranging from 0.05 g/L to 0.75 g/L. A minimum and maximum of 19.2± 7.12 % and 28.2 ± 0.85 % resveratrol was precipitated from the PEG phase with ovalbumin. While a minimum and maximum of 26.0± 2.45 % and 33.4 ± 3.16 % resveratrol was recovered with yeast extract and between 27.8± 2.33% and 31.3± 1.88 % resveratrol formed a precipitate with tryptone soy broth. As seen Figure 28 the percentage resveratrol recovered did not increase with an increase in protein concentration. The resveratrol recovered from the high concentration system was significantly lower than the low concentration system. This confirms that the polyphenol-protein ratio influences the interaction and thus the precipitate formed. The protein-polyphenol interaction mechanism for the different protein-polyphenol ratios is illustrated in Figure 6 in Section 2.3.2.

A system with a low polyphenol and low protein concentration will form a saturated protein-polyphenol chain that will precipitate. If the polyphenol concentration is too low compared to the protein concentration, a partially saturated chain will form. To form an aggregate the protein ratio should be decreased to recover the polyphenol. By comparing the recovery achieved, protein and polyphenol concentration to the precipitation mechanisms, it was concluded that the proteins and polyphenols were

in the correct ratio to form a saturated chain that will precipitate out of solution. The low protein and polyphenol concentrations are desired since the amount of resveratrol in the solid winery waste is also very low. This will also decrease the protein requirement and cost.

From the quantification of resveratrol in different parts of the vine during consecutive harvests it was found that the resveratrol concentration is highly variable and would result in a variable resveratrol feed stream. The change in resveratrol concentration in the biomass feed will result in a change in the recovery process. As seen in Figure 27 and Figure 28, the resveratrol recovery achieved is dependent on the resveratrol concentration and the resveratrol-protein ratio. As the resveratrol feed stream changes, the protein concentration should be adjusted accordingly. This will introduce more variability into the process in terms of process conditions and operating cost and will result in a change in the recovered product.

Even though resveratrol recovery was achieved indicating that protein precipitation can be used to recover resveratrol, several other factors such a system pH, temperature, ionic strength and different proteins and concentrations should be investigated and improved in terms of efficiency and process economics in order to be used as a competitive resveratrol recovery method.

5.3.3.3 Resveratrol precipitation from grape stems

To recover the extracted resveratrol from the PEG-tartrate system, the resveratrol rich PEG phase was mixed with solutions of ovalbumin, tryptone soy broth and yeast extract to form a precipitate to recover the resveratrol. Again, if assuming the maximum extractable resveratrol is the same as the extraction achieved using 80:20 v/v% ethanol-water, the resveratrol recovery with protein precipitation was calculated. 57% Resveratrol was recovered with 0.035 g/L ovalbumin and 67% of the extracted resveratrol was recovered with 0.034 g/L tryptone soy broth and yeast extract from the 2019 Pinotage stems. Even though the average overall resveratrol recovery from the Pinotage stems is approximately 13.2% (9.6 µg/g), the results confirmed that resveratrol can be recovered from grape stems with protein precipitation. However, several parameters should be investigated and adjusted to achieve maximum resveratrol recovery to be a feasible recovery technique.

6 CONCLUSIONS

The aim of the project was to investigate the extraction of resveratrol from solid winery waste to beneficiate the waste before being landfilled or incinerated. Another aim was to investigate alternative resveratrol recovery techniques that can be used to produce a nutraceutical supplement and to decrease the high cost and improve the bottleneck of downstream purification processes such as chromatography.

To date, no study has determined the resveratrol content in all the different components of solid winery waste and how the resveratrol concentration in the same vine can vary between two consecutive harvests. A PEG-MD two phase system has also not been used or investigated as a method to concentrate resveratrol from PEG. As well as the use of proteins to precipitate and recover resveratrol. The work in this project was presented at the 9th IWA Specialized conference on sustainable viticulture, winery wastes and agricultural wastewater management. The work in this study also contributed to a patent submitted about the method for separating resveratrol from biomass and resulting products (P3661ZA00). In order to achieve the project aim, each key question had to be answered. The conclusions relating to each objective are discussed below.

6.1 Resveratrol degradation and isomerisation

In order to extract and recover the maximum amount of resveratrol, sample preparation and process conditions that could result in degradation or isomerisation of *trans*-resveratrol to *cis*-resveratrol were investigated. The effect of drying grape skins between 40°C and 60°C on resveratrol concentration and total polyphenol content was investigated to determine if any thermal degradation occurred. No change in the resveratrol and polyphenol concentration was observed suggesting that no degradation occurred during the drying process step and the solid winery waste can be dried at up to 60° for 24 hours. However, drying time and temperature should be adjusted to minimise the energy requirements and reduce the operating cost of a large-scale process.

The effect of system pH on the resveratrol concentration was also investigated to determine if resveratrol will degradation will increase in basic conditions. By increasing the system pH above pK_{a1}=8.8, pK_{a2}=9.8 and pK_{a3}=11.4, resveratrol degradation increased and up to 66 ± 3.0% of the resveratrol degraded. It was concluded that resveratrol degradation is pH dependent and alkaline samples should be neutralised to minimise resveratrol degradation.

Resveratrol isomerisation during extraction investigated. The *trans*-resveratrol extracted remained between 17.6 ± 0 µg/g to 18.2 ± 0.55 µg/g over a 24-hour extraction. While the *cis*-resveratrol concentration ranged from 26 ± 0.64 µg/g to 26.4 ± 1.6 µg/g. From the comparison of *trans*-resveratrol and *cis*-resveratrol extracted it was concluded that while the trans-resveratrol concentration was significantly lower it was still present in the extract and did not completely isomerize. It was concluded

that the 80:20 v/v% ethanol-water extraction did not result in degradation or isomerization and could be used as a method of resveratrol quantification in solid winery waste.

From the evaluation of findings, it was concluded that the objective to determine factors that could result in resveratrol degradation and isomerisation was met and provided useful information on the preparatory process of solid winery waste as a resveratrol source.

6.2 Quantification of resveratrol and other polyphenols in solid winery waste

Several questions regarding *Vitis vinifera* as a resveratrol source arose from literature and the work in this study aimed to answer those specific questions by quantifying the amount of resveratrol and other polyphenols in Pinotage solid winery waste, as a model grape strain. Pinotage skins, seeds, stems, canes and leaves that were collected from the exact same rootstock during the 2018 and 2019 harvest were extracted with an 80:20 v/v% ethanol-water mixture. From the resveratrol extracted from the different parts of the 2018 Pinotage waste it found that the stems and canes had the highest resveratrol concentration of 5.4 ± 2.3 µg/g and 7.5 ± 3.6 µg/g, respectively. It was observed that the resveratrol extracted from the same rootstock during the 2019 harvest significantly increased to 73 ± 4.3 µg resveratrol per gram of dried stems and 14 ± 8.1 µg resveratrol per gram of dried canes. It was concluded that the stems and canes from the consecutive harvests had the highest resveratrol concentration. While no or negligible amounts of resveratrol were extracted from the skins, seeds and leaves. However, it could not be concluded that grape pomace cannot be used as a resveratrol source since the Shiraz and Grenache skins also tested contained up to 5.8 µg/g and 44 µg/g resveratrol, respectively. These results indicate that resveratrol concentration does not only vary within the different parts of the vine or the variety but also change over time depending on the environmental conditions during the growth period, indicating a high variability in resveratrol productivity that will influence the biomass feed stream and thus the large scale production process.

Due to the high resveratrol concentration variability, the total phenolic content of the different parts of the waste of the consecutive harvests were extracted to determine if there is a correlation between the total phenolic content and resveratrol. From the polyphenols extracted from the different parts of the 2018 Pinotage waste the leaves had the highest total phenolic content of 5.02 ± 0.16 mg/g in terms of gallic acid equivalence as determined with the FC method. The cane trimmings had the lowest total phenolic content of 1.4 ± 0.11 mg/g. It was observed that the total phenolic content of the leaves, stems, skins and seeds from the 2019 harvest remained the same while the total phenolic content of the canes increased to 1.95 ± 0.22 mg/g. It was confirmed that there is no correlation between resveratrol concentration and total phenolic content, but it was noted that the stems from both harvests had a high

polyphenol and resveratrol concentration and can be used as a source of resveratrol and other polyphenols.

Once the resveratrol and polyphenol concentration were determined a two-phase system of tartrate and PEG 8000 was investigated as an extraction method. The stems from the 2019 Pinotage harvest was used a polyphenol and resveratrol source. Up to 96.3% polyphenols and 20.7% resveratrol were extracted, confirming that ATPS can be used as an extraction method.

From the evaluation of the results, it was concluded that each of the key questions were answered and the objective to determine the resveratrol concentration and total phenolic content in different parts of solid winery waste was met. The study also provided new information about Pinotage characteristics, the variability of resveratrol and the use of solid winery waste as a resveratrol source.

6.3 Resveratrol recovery

Since resveratrol can be extracted from solid winery waste with a tartrate-PEG 8000 ATPS and partitioned to the PEG top phase, a method to recover resveratrol from the PEG phase was investigated. A subsequent ATPS with maltodextrin (DE 16.5-19.5) and PEG 8000 containing resveratrol was investigated to concentrate resveratrol into maltodextrin. From the investigation of resveratrol partitioning to the desired maltodextrin phase in different systems ranging from 5wt%-25wt% PEG 8000 and 25wt%-35wt% maltodextrin (DE 16.5-19.5) it was found that the resveratrol partition coefficient in maltodextrin was less than one, indicating no concentrating effect to the maltodextrin. It was confirmed that some resveratrol will partition to the maltodextrin phase. However, to concentrate the resveratrol into the edible maltodextrin, the partition coefficient should be greater than one. It was observed that by increasing the maltodextrin concentration to 35wt% the phase hydrophilicity decreased and decreasing the PEG concentration to 5wt% the PEG hydrophobicity decreased and the hydrophobic resveratrol more easily partitioned to the bottom phase but will increase the amount of maltodextrin used and thus the cost as well as deliver a less concentrated resveratrol product.

It was also determined that resveratrol concentration in the system will affect the partitioning achieved by comparing that same systems with different resveratrol concentrations. By decreasing the resveratrol concentration from 2.7 g/L to 0.22 g/L the resveratrol partition coefficient increased from 0.40 ± 0.067 to 0.54 ± 0.073 and the data suggested that partitioning in a two-phase system was also affected by the volume exclusion effect.

By increasing the system pH above each acidic dissociation constant of resveratrol, it was confirmed that resveratrol became more polar and more easily partitioned to the more polar maltodextrin phase. This resulted in an increase in resveratrol partitioning to the maltodextrin phase, confirming that partitioning in a two-phase system is pH dependent. However, an increase in pH in the two-phase systems resulted

in up to 73 ± 1.4% of the resveratrol degrading. It was thus concluded that an increase in pH in a PEG-maltodextrin will not improve resveratrol recovery.

Protein precipitation was also investigated as a method to recover resveratrol from PEG by forming a protein-resveratrol precipitate. Ovalbumin, tryptone soy broth and yeast extract solutions containing proteins were selected. The protein solutions were mixed with the polyphenols extracted from the 2019 Pinotage leaves to form a protein-polyphenol precipitate. Up to 69.2 ± 3.5%, 63.9 ± 0.6% and 62.7 ± 0.8% of the polyphenols were recovered with ovalbumin, tryptone soy broth and yeast extract, confirming that the selected proteins will form a precipitate. The precipitation of resveratrol from PEG 8000 was also investigated and compared to the polyphenol precipitation. Up to 39.3 ± 2.76%, 40.6 ± 5.79% and 29.4 ± 6.49% resveratrol was recovered with ovalbumin, tryptone soy broth and yeast extract, confirming that resveratrol will form a recoverable precipitate. It was also observed that precipitation is affected by the resveratrol concentration and by decreasing the resveratrol concentration 10-fold and the protein concentration accordingly, between 62 ± 4.4% and 83 ± 2.1% resveratrol was recovered. A PEG system higher in resveratrol was also investigated and a maximum of 28 ± 0.8%, 31.3± 1.9% and 33.4 ± 3.2% resveratrol was recovered with ovalbumin, tryptone soy broth and yeast extract. It was concluded that the recovery achieved is dependent on the precipitation mechanism and thus the polyphenol concentration and should be taken into consideration when used in a large-scale process to achieve maximum resveratrol recovery.

From the evaluation of ATPS as a resveratrol method, it was concluded that the objective was met, and resveratrol can be recovered with a subsequent maltodextrin-PEG system. However, the recovery to the edible maltodextrin system is too low to be used as a feasible recovery method. From the evaluation of protein precipitation as a recovery method it was concluded that the objective was met, and proteins can be used to form a recoverable resveratrol precipitate that can be used as a possible industrial recovery technique. This study provided new information on resveratrol recovery and future work that is discussed in the recommendations below.

7 RECOMMENDATIONS

After a thorough analysis of the results, the following recommendations for future work relating to each objective are made and discussed below.

7.1 Resveratrol degradation and isomerisation

Although some factors that can result in resveratrol degradation were investigated, more research about the solid waste storage and processing is required. Since the overall aim of this work is to use solid winery waste as a resveratrol and polyphenol source, more information about the waste disposal conditions and how it can affect resveratrol is required. An investigation into the effect of UV radiation and long-term heat exposure on resveratrol degradation and isomerisation is required to determine if the solid winery waste should be extracted immediately or if there is a buffer period to decrease the initial biomass feed and improve the process efficiency.

7.2 Quantification of resveratrol and other polyphenols in solid winery waste

The data from this study suggest that significant variance in resveratrol extracted from the different parts of solid winery waste exists and is dependent on the grape variety, geography and climatic conditions. A long-term study on the change in resveratrol concentration in a specific vine is required to improve the process variability by confirming that Pinotage stems and canes have the highest resveratrol concentration and should be used as a resveratrol source. It is also recommended to investigate and compare other varietals from the same and different regions to determine how the environmental conditions will affect resveratrol production. Given that large variability in resveratrol concentration exists, further investigation into the process design and economics are required to determine if recovering resveratrol from the solid winery waste is a feasible solution to beneficiate the waste.

7.3 Resveratrol recovery

Given that a very low amount of resveratrol partitioned to maltodextrin with the systems investigated, different systems of PEG and maltodextrin and system conditions should be investigated to determine if ATPS could be a feasible recovery method.

Although the work in this study confirmed that protein precipitation can be used as a resveratrol recovery method, the process should still be optimised. Factors such as system pH, temperature and mixing time should be investigated to increase the amount of resveratrol recovered. Other factors that also influence the resveratrol-protein precipitate such as the type of protein and protein to polyphenol ratio should also be investigated and optimised to recover the maximum amount of resveratrol.

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APPENDIX A – SAMPLE ANALYSIS

The FC method was used to determine the total phenolic content in different samples, as discussed in Section 4.2.1.2. The constructed standard curve to convert from absorbance to concentration is represented by Figure 29.

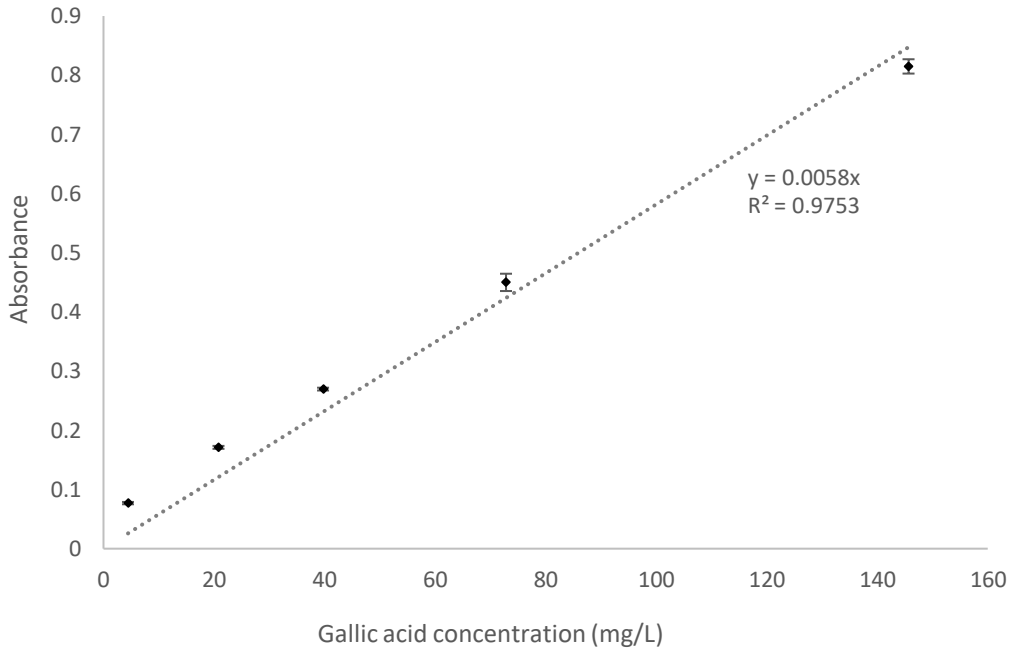

Figure 29. Total phenolic content standard curve with the absorbance of triplicate gallic acid samples (mean ± standard error).

HPLC was used to determine the resveratrol concentration in different biomass extracts and pure resveratrol samples, as discussed in Section 4.2.1.1. Figure 30 represents a HPLC chromatogram of resveratrol extracted from grape biomass. A HPLC chromatogram of a pure resveratrol sample is represented by Figure 31.

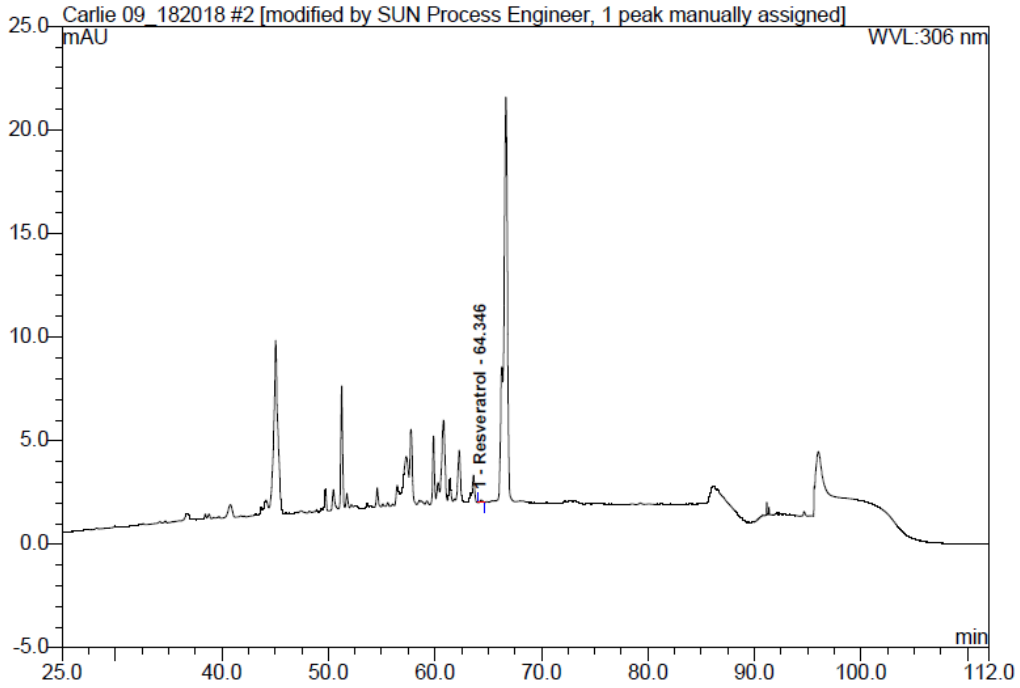

Figure 30. An example HPLC chromatogram of a Shiraz skin extract sample analysed with a Phenomenex column with a resveratrol concentration of 0.023 mg/L as quantified on a Dionex Ultimate 3000 system with a UV detection at 306 nm.

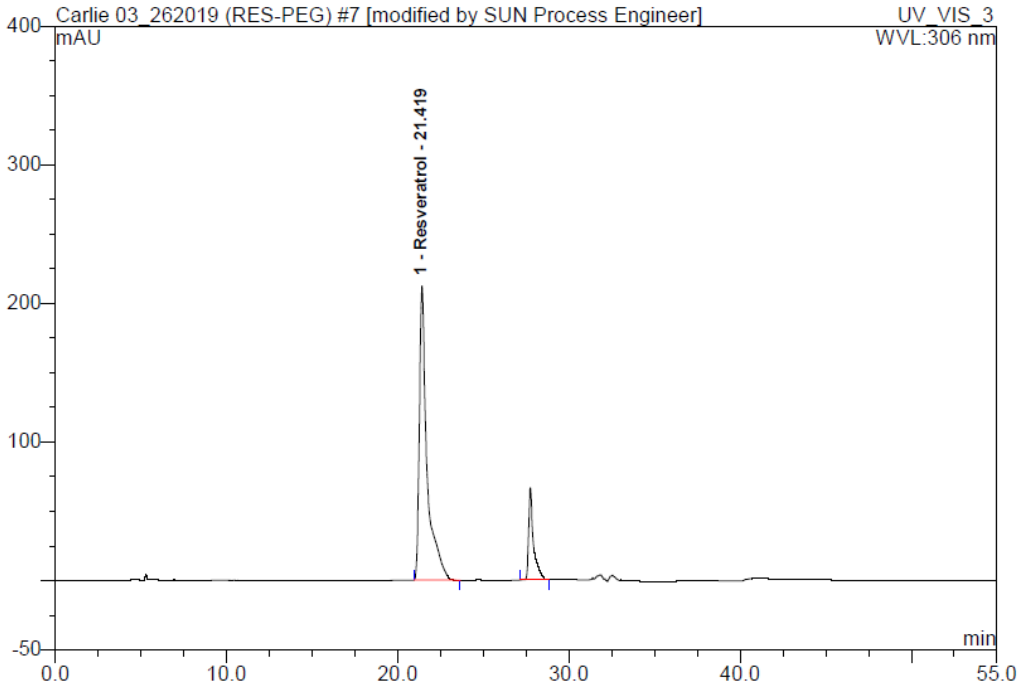

Figure 31. An example HPLC chromatogram of a pure resveratrol sample in PEG 8000 analysed with a Polysep GFC column with a resveratrol concentration of 5.589 mg/L as quantified on a Dionex Ultimate 3000 system with a UV detection at 306 nm.

LC-MS analysis was used to differentiate between *cis*-resveratrol and *trans*-resveratrol, as discussed in Section 4.2.1.3. Figure 32 represents a LC-MS chromatogram of *cis*-resveratrol and *trans*-resveratrol extracted from grape biomass.

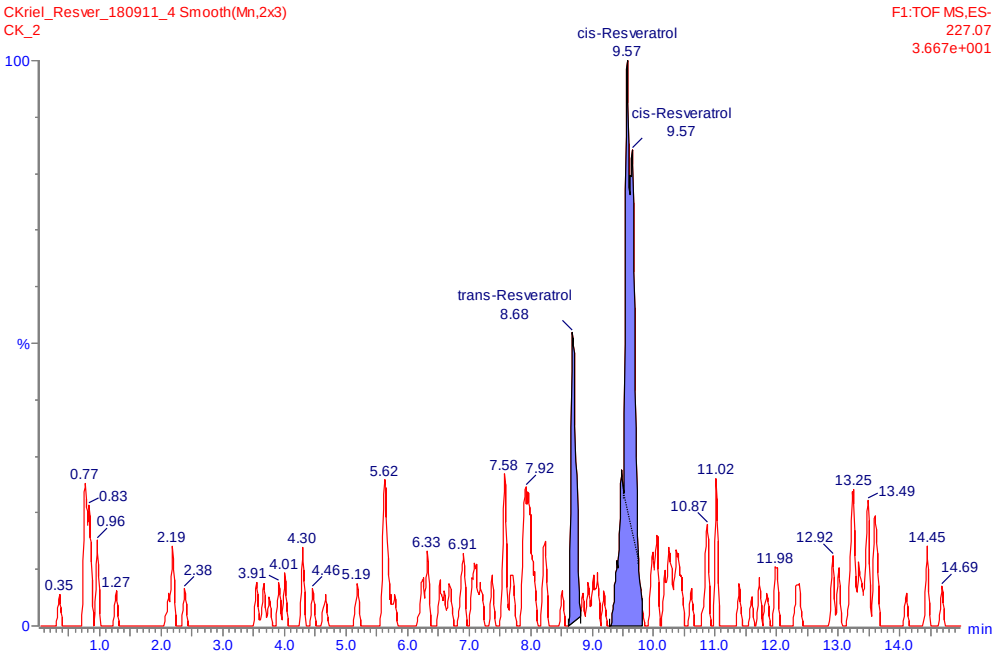

Figure 32. An example LC-MS chromatogram of a Grenache skin extract sample with a cis-resveratrol concentration of 0.24 mg/L and trans-resveratrol concentration of 0.17 mg/L as quantified with an Acquity liquid chromatograph with a Waters Synapt G2 mass spectrometer.

APPENDIX B – ADDITIONAL EXPERIMENTS

The water content in each component of the solid winery waste were determined and the results are summarised in Table 5.

Table 5. Water content (wt %) in each component of the solid wine waste.

Sample	Water content (%)
Skins	30.6
Seeds	27.2
Stems	21.5
Canes	11.2
Leaves	15.3

Different solvents were investigated to extract resveratrol in order to quantify the resveratrol in different samples. The resveratrol extraction achieved with the different solvents are summarised in Figure 33.

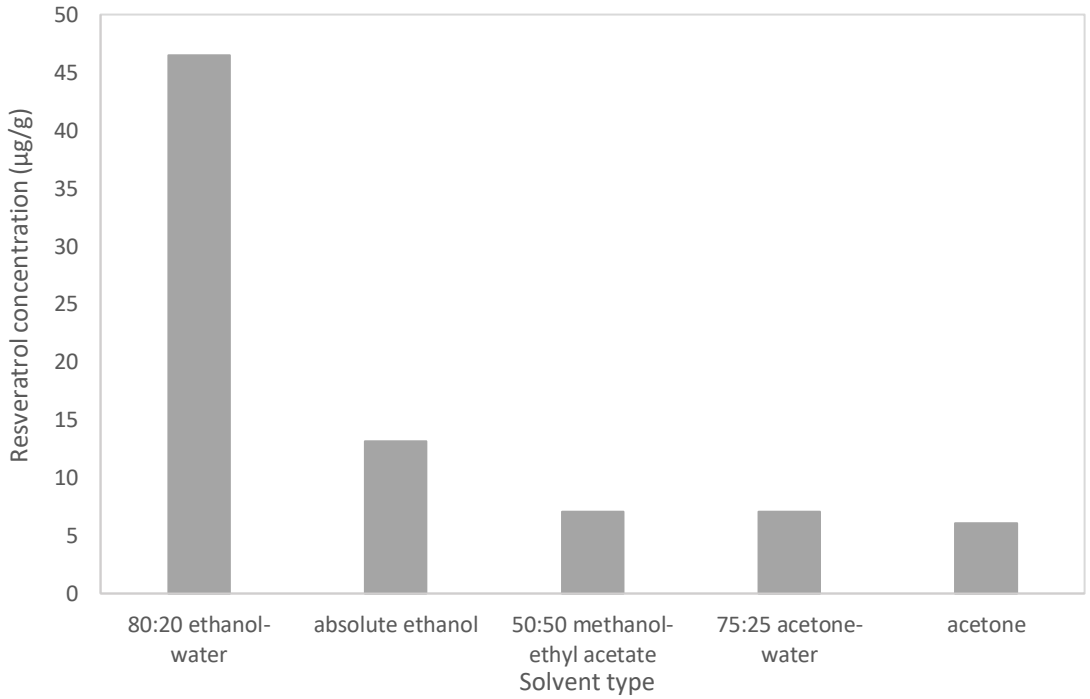

Figure 33. Amount of resveratrol extracted (µg/g) from Grenache grape skins with different solvents, represented on the x-axis, in a 1:10 solid to solvent ratio.

As discussed in Section 2.1.5.3 several sources stated that resveratrol degradation is influenced by the system pH and degradation can be prevented or minimised in acidic mediums (Trela and Waterhouse, 1996). In order to determine if resveratrol degradation is pH dependent, four samples of 85 mg/L *trans*-

resveratrol in 80:20 v/v% ethanol-water mixture with a pH of 2, 4, 6 and 8 were mixed for 24 hours. The percentage resveratrol degradation as determined with HPLC analysis is summarised in Figure 34, below.

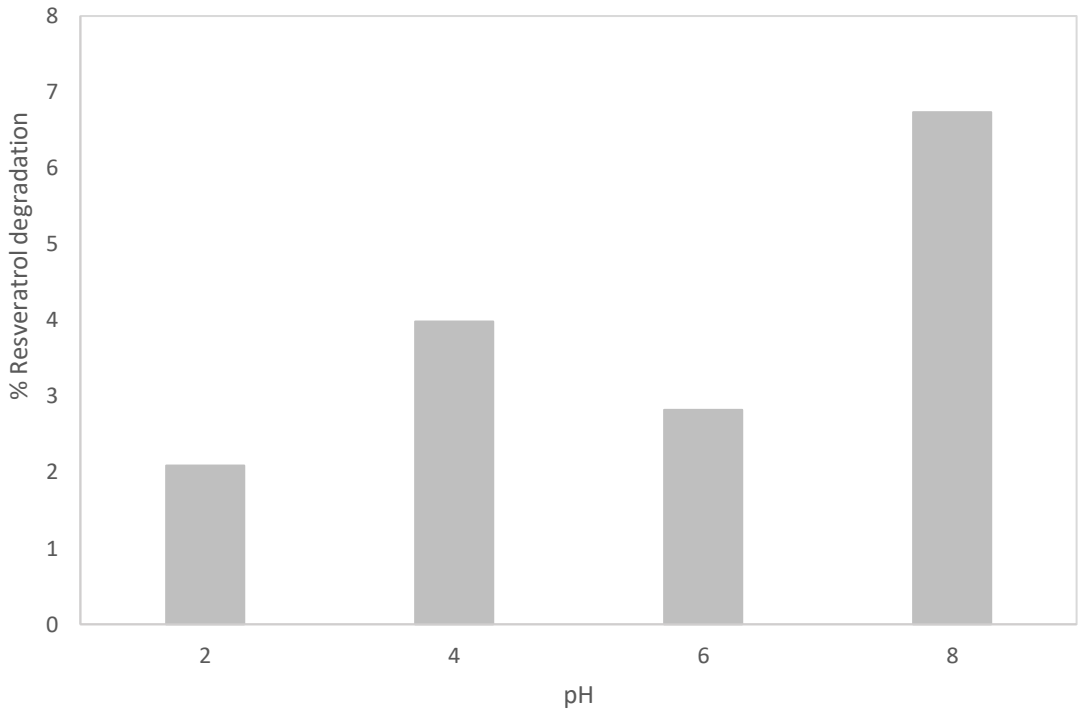

Figure 34. The percentage degradation of an 85 mg/L *trans*-resveratrol in an 80:20 v/v% ethanol-water solution with a system pH ranging from 2 to 8.

As seen in Figure 34, between 2.0 % to 6.7 % resveratrol degraded in the ethanol solution after 24 hours. It was observed at a system pH of 8, the resveratrol degradation increased. According to (Zupančič, Lavrič and Kristl, 2015) resveratrol degradation will increase in alkaline systems and degradation will accelerate with an increase in pH. It was observed that the *trans*-resveratrol absorbance peak of 306 nm decreased and shifted to 265 nm, indicating the formation of degradation compounds.

To determine if storage time will result in *trans*-resveratrol isomerisation, one of the three Grenache extracts were analyzed immediately for *cis*- and *trans*-resveratrol while the other two extract samples were stored for 1 day and 30 days, respectively at -18°C before being analyzed for *cis*- and *trans*-resveratrol. The concentration of *cis*- and *trans*-resveratrol of the 3 different samples are shown in Figure 35.

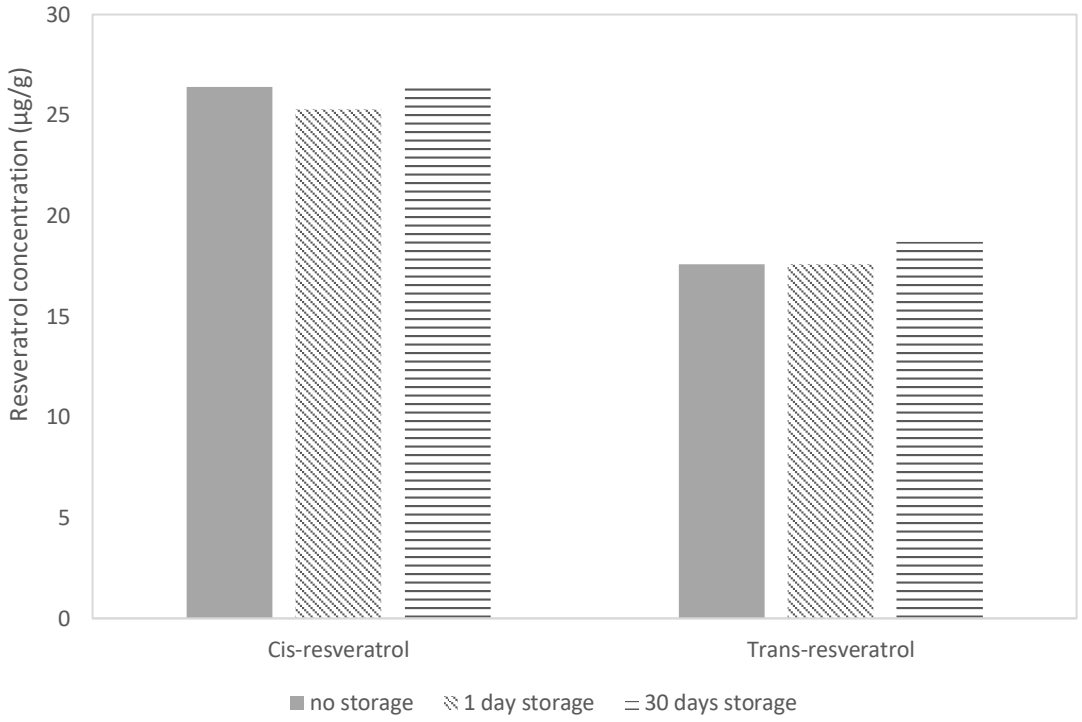

Figure 35. *Cis*-resveratrol and *trans*-resveratrol concentration (µg resveratrol/ g dried Grenache skins) of three samples that were analysed immediately (solid), stored for 24 hours (diagonal) or stored for 30 days (horizontal lines) to show the effect of storage time on resveratrol isomerisation.

As seen in Figure 35 the *cis*-resveratrol concentration did not increase with an increase in storage time, indicating that isomerization was not affected by the storage time when stored at – 18°C. It was also observed that the *trans*-resveratrol concentration did not decrease with an increase in storage time. It was concluded that the *trans*-resveratrol in the extract did not degrade.

APPENDIX C – LITERATURE DATA

A PEG-tartrate binodal curve, represented by Figure 36 from Herbst and Pott (2019), were used to construct a PEG-tartrate ATPS.

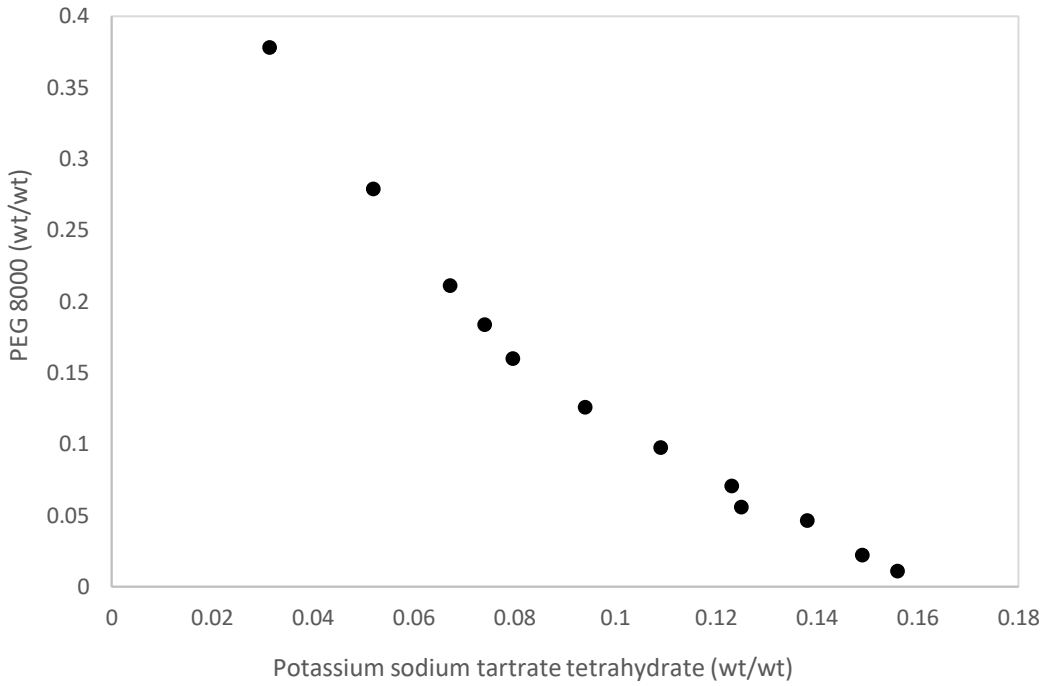

Figure 36. An adapted PEG 8000 and potassium sodium tartrate tetrahydrate binodal curve constructed at ambient temperature (Herbst and Pott, 2019). The right-hand side of the curve is the biphasic area where two phases will form to partition molecules and to the left is where a homogenous phase will form.

A PEG-maltodextrin binodal curve, represented by Figure 37 from Da Silva and Meirelles (2000), were used to construct different PEG-maltodextrin two phase systems.

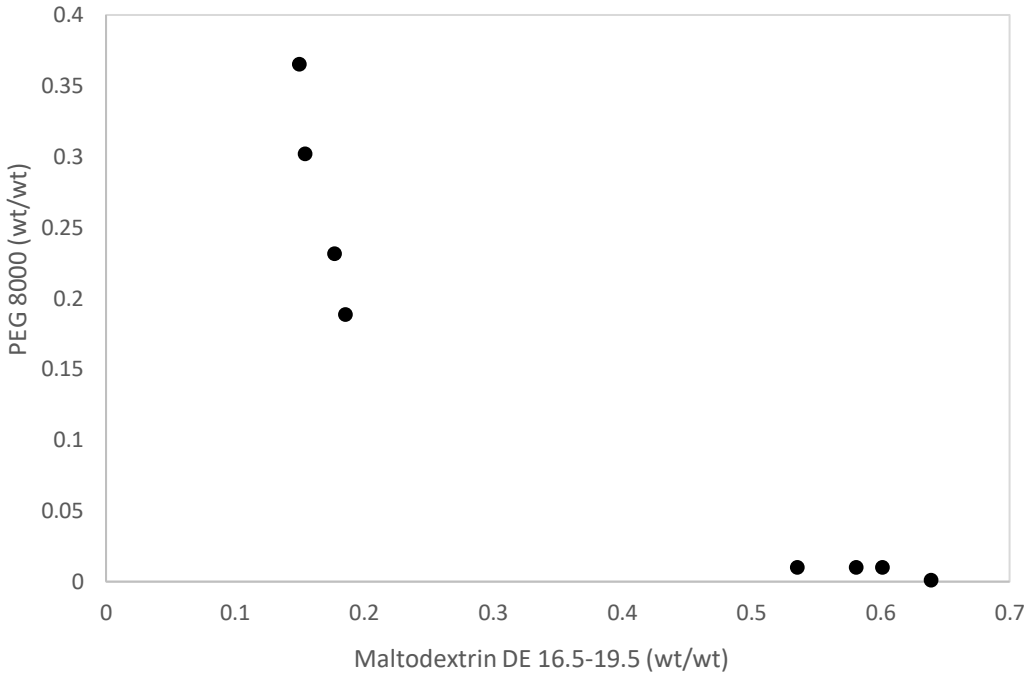

Figure 37. An adapted PEG 8000 and maltodextrin (DE 16.5-19.5) binodal curve constructed at ambient temperature (Da Silva and Meirelles, 2000). The right-hand side of the curve is the biphasic area where two phases will form to partition molecules and to the left is where a homogenous phase will form.

Xia *et al.* (2013) stated that the types of polyphenols found in different parts of the vine varies and some of the types of polyphenols found in each component is summarised in Table 6.

Table 6. Summary of some polyphenols found in different part of a grape vine (Xia *et al.*, 2013).

Source	**Type of polyphenols**
Seeds	Gallic acid, proanthocyanidin dimers, epicatechin, catechin and proanthocyanidins
Skins	Proanthocyanidins, quercetin, myricetin, ellagic acid, kaempferol and resveratrol
Leaves	Quercetin, myricetin, ellagic acid, kaempferol, gallic acid and resveratrol
Stems	Quercetin 3-O-glucuronide, rutin, astilbin and resveratrol

APPENDIX D – SAMPLE CALCULATIONS

a) Water content

In order to decrease the particle size of the solid winery waste, the moisture of the samples was removed by drying each component. The water content of the different samples as calculated with Equation 9 are summarised in Table 5. The average moisture content of grape stems is calculated below as an example.

$$\%\ \text{Moisture} = [\frac{m_i - m_f}{m_i}] \times 100 \quad [9]$$

$$\%\ \text{Moisture} = [\frac{106.6 - 83.7}{106.6}] \times 100$$

$$\%\ \text{Moisture} = 21.5\%$$

b) Resveratrol content

The resveratrol concentration of different biomass samples was determined with HPLC and LC-MS analysis and were quantified in terms of mg/L. In order to evaluate the fraction of resveratrol in the solid waste, concentration was converted to content with Equation 10.

The resveratrol content of a 1 g Shiraz skin sample extracted with 10 ml 80:20 v/v% ethanol-water solution is calculated below as an example.

$$\text{Resveratrol content} = \text{Resveratrol concentration} \times \frac{\text{sample volume}}{\text{biomass mass}} \quad [10]$$

$$\text{Resveratrol content} = 0.23 \frac{\text{mg}}{\text{L}} \times \frac{10\ \text{ml}}{1\ \text{g}} \times \frac{1\ \text{L}}{1000\ \text{ml}} \times \frac{1000\ \mu\text{g}}{1\ \text{mg}}$$

$$\text{Resveratrol content} = 2.3 \frac{\mu\text{g}}{\text{g}}$$

c) Total phenolic content

To calculate the total phenolic content of a sample, the standard curve (Figure 29) was used to convert from measured absorbance to concentration. The average total phenolic content of the 2019 Pinotage stems extracted for 24 hours is calculated below.

$$\text{Y} = 0.0058\text{X} \quad [12]$$

$$\text{Absorbance} = 0.0058\text{concentration}$$

$$\frac{\text{Absorbance}}{0.0058} \times \text{Dilution factor} = \text{concentration}\ (\frac{\text{mg}}{\text{L}})$$

$$\text{concentration}\ (\frac{\text{mg}}{\text{L}}) = \frac{0.65}{0.0058} \times 4$$

$$\text{concentration} = 448 \frac{\text{mg}}{\text{L}}$$

$$\text{concentration} = 4.5\frac{\text{mg}}{\text{g}}$$

d) Resveratrol degradation

In order to determine the effect of pH on resveratrol degradation, Equation 8 was adapted to determine the percentage degradation. Where C_f is the concentration of the resveratrol remaining in the sample after an extraction at a specific pH and C_i the initial resveratrol concentration in the sample before the pH adjustment.

% Resveratrol degradation of the average resveratrol degradation above pKa_1 (Determined with LC-MS analysis):

$$\%\ \text{degradation} = \left[1 - \left(\frac{C_f}{C_i}\right)\right] \times 100 \qquad [11]$$

$$\%\ \text{degradation} = \left[1 - \left(\frac{19.67\ \text{mg/L}}{30\ \text{mg/L}}\right)\right] \times 100$$

$$\%\ \text{degradation} = 34\%$$

e) Resveratrol extracted with PEG-tartrate ATPS

The extraction of resveratrol to the PEG phase was calculated to evaluate the extraction achieved with ATPS in comparison to solvent extraction. The percentage resveratrol extraction was calculated with equation 5. C_T is the concentration of resveratrol in the PEG phase and V_T the volume of the PEG phase.

$$\Upsilon_T = \frac{V_T C_T}{V_{Total} C_{total}} \times 100$$

$$\Upsilon_T = \frac{C_T\left(\frac{\mu g}{g}\right)}{C_{total}\left(\frac{\mu g}{g}\right)} \times 100$$

$$\Upsilon_T = \frac{13.5\left(\frac{\mu g}{g}\right)}{65.0\left(\frac{\mu g}{g}\right)} \times 100$$

$$\Upsilon_T = 20.7\%$$

f) Resveratrol recovered to maltodextrin

The efficiency of a maltodextrin- PEG recovery system was evaluated in terms of the partition coefficient of resveratrol to the maltodextrin bottom phase. The resveratrol partition coefficient was calculated with Equation 7. Where C_T and C_B are the concentration of a specific molecule in the PEG top and maltodextrin

bottom phase, respectively. Where V_T and V_B represent the PEG top and maltodextrin bottom phase volumes.

$$K_P = \frac{C_B V_B}{C_T V_A} \quad [7]$$

$$K_P = \frac{0.28\left(\frac{mg}{L}\right) \times 14\ (ml)}{1.34\ \left(\frac{mg}{L}\right) \times 17\ (ml)}$$

$$K_P = 0.17 \text{ resveratrol partitioning to maltodextrin}$$

g) Resveratrol recovered with precipitation

Equation 8 was used to determine the efficiency of a protein-resveratrol precipitation reaction. Where C_f is the concentration of resveratrol remaining in the supernatant after precipitation and C_i the initial resveratrol concentration to be recovered.

$$\Upsilon_{pp} = \left[1 - \left(\frac{C_f}{C_i}\right)\right] \times 100 \quad [8]$$

$$\Upsilon_{pp} = \left[1 - \left(\frac{462\ \left(\frac{mg}{L}\right)}{667\ (\frac{mg}{L})}\right)\right] \times 100$$

$$\Upsilon_{pp} = 40\% \text{ resveratrol precipitation with tryptone soy broth}$$

APPENDIX E – RAW DATA

The data summarised in **Table 7** to **Table 18** represent the measured experimental results used in Section 5.

Table 7. The undiluted resveratrol concentration and the measured absorbance of Shiraz skins dried at 40°C to 60°C for 24 hours and fresh Shiraz biomass extracted for 24 hours with 80:20 v/v% ethanol-water mixture under ambient conditions.

Sample	Run	Resveratrol concentration (mg/L)	Total phenolic content (Absorbance)
Fresh biomass	1	0.23	-
	2	0.33	0.310
	3	0.28	0.316
40°C	1	0.54	0.381
	2	0.43	0.372
	3	0.52	0.380
50°C	1	0.45	0.378
	2	0.61	0.357
	3	0.46	0.373
60°C	1	0.88	0.386
	2	0.5	0.379
	3	0.36	0.395

Table 8. The concentration of pure resveratrol samples (with an initial concentration of 30 mg/L) in 80:20 v/v% ethanol-water mixture with a pH above and below each resveratrol acidic dissociation constant.

Sample	Run	Resveratrol concentration (mg/L)
No adjustment	1	26.9
	2	21.3
	3	18.6
	4	23.5
	5	21.1
	6	26.1
>pKa1	1	23
	2	19.2
	3	16.8
>pKa2	1	12.8
	2	17.9
	3	19.6
>pKa3	1	11.9
	2	8.9
	3	9.8

Table 9. The undiluted *trans*-resveratrol and *cis*-resveratrol concentration of Grenache skins extracted with 80:20 v/v% ethanol-water mixture over 24 hours.

Time (h)	**Resveratrol concentration (mg/L)**					
	Run 1		Run 2		Run 3	
	Trans-resveratrol	*Cis*-resveratrol	*Trans*-resveratrol	*Cis*-resveratrol	*Trans*-resveratrol	*Cis*-resveratrol
1	1.76	2.53	1.76	2.75	-	2.64
4	1.87	2.64	1.76	2.53	1.76	2.64
7	-	2.53	1.76	2.53	1.76	2.75
24	1.76	2.86	-	2.31	1.87	2.64

Table 10. The undiluted resveratrol concentration of different parts of the same Pinotage vine over two consecutive harvests extracted with an 80:20 v/v% ethanol-water mixture over 24 hours.

Resveratrol concentration (mg/L)							
Extraction time (h)	Sample	2018 Pinotage			2019 Pinotage		
		Run 1	Run 2	Run 3	Run 1	Run 2	Run 3
1	Skins	0.00	0.14	-	0.00	0.00	0.00
	Seeds	0.00	0.00	-	0.00	0.00	0.00
	Stems	0.64	0.95	0.14	3.28	6.12	7.04
	Canes	0.23	0.78	0.07	2.94	0.59	0.54
	Leaves	0.00	0.14	-	0.00	0.00	0.00
4	Skins	0.00	0.00	-	0.00	0.00	0.00
	Seeds	0.00	0.00	-	0.00	0.00	0.00
	Stems	0.71	0.94	0.17	6.43	7.20	6.70
	Canes	0.16	1.26	0.06	2.99	0.71	0.45
	Leaves	0.00	0.14	-	0.00	0.00	0.00
7	Skins	0.00	0.00	-	0.00	0.00	0.00
	Seeds	0.00	0.00	-	0.00	0.00	0.00
	Stems	0.80	0.80	0.16	6.91	8.13	6.76
	Canes	0.16	1.53	0.06	2.53	0.68	0.54
	Leaves	0.00	0.15	-	0.00	0.00	0.00
24	Skins	0.00	0.00	-	0.00	0.00	0.00
	Seeds	0.00	0.00	-	0.00	0.00	0.00
	Stems	0.51	0.95	0.15	7.79	5.61	6.11
	Canes	0.20	2.00	0.06	2.18	0.00	1.41
	Leaves	0.00	0.00	-	0.00	0.00	0.00

Table 11. The measured absorbance used to calculate the total phenolic content of different parts of the same Pinotage vine over two consecutive harvests extracted with an 80:20 v/v% ethanol-water mixture over 24 hours.

Total phenolic content (Absorbance)							
Extraction time (h)	Sample	2018 Pinotage			2019 Pinotage		
		Run 1	Run 2	Run 3	Run 1	Run 2	Run 3
1	Skins	0.59	0.58	0.59	0.56	0.62	0.60
	Seeds	0.62	0.62	0.64	0.66	0.65	0.61
	Stems	0.61	0.62	0.66	0.62	0.63	0.62
	Canes	0.15	0.16	0.15	0.27	0.26	0.21
	Leaves	0.70	0.71	0.72	0.61	0.66	0.63
4	Skins	0.59	0.58	0.56	0.62	-	0.69
	Seeds	0.62	0.62	0.63	0.79	0.66	0.62
	Stems	0.63	0.65	0.67	0.62	0.56	0.64
	Canes	0.17	0.18	0.18	0.29	0.32	0.14
	Leaves	0.61	0.63	0.71	0.67	0.65	0.71
7	Skins	0.60	0.58	0.59	0.65	0.59	0.60
	Seeds	0.62	0.60	0.64	0.61	0.63	0.60
	Stems	0.64	0.66	0.65	0.64	0.67	0.62
	Canes	0.15	0.17	0.20	0.33	0.29	0.26
	Leaves	0.72	0.66	0.74	0.70	0.67	0.66
24	Skins	0.64	0.60	0.60	0.59	0.64	0.64
	Seeds	0.68	0.71	0.66	0.62	0.65	0.71
	Stems	0.66	0.63	0.69	0.60	0.66	0.69
	Canes	0.19	0.19	0.24	0.40	0.34	0.29
	Leaves	0.68	0.74	0.76	0.67	0.59	0.69

Table 12. The undiluted concentration of pure resveratrol and maltodextrin (DE 16.5-19.5) and PEG 8000 phase volumes used to calculate the partitioning of resveratrol in different maltodextrin-PEG two phase systems.

Run	Sample	Phase composition (wt%)	Resveratrol concentration (g/L)	Phase volume (ml)
1	1	35% PEG	1.27	17
		10% MD	0.26	14
	2	35% PEG	1.18	14
		5% MD	0.46	15
	3	30% PEG	1.23	15
		15% MD	0.46	15
	4	25% PEG	1.09	24
		20% MD	0.10	8
	5	35% PEG	0.00	24
		20% MD	0.02	13
	6	25% PEG	1.24	26
		25% MD	0.04	8
2	1	35% PEG	1.60	17
		10% MD	0.29	14
	2	35% PEG	1.67	14
		5% MD	0.24	15
	3	30% PEG	1.40	15
		15% MD	0.12	15
	4	25% PEG	1.30	24
		20% MD	0.14	8
	5	35% PEG	1.00	24
		20% MD	0.03	13
	6	25% PEG	0.83	26
		25% MD	0.05	8
3	1	35% PEG	1.16	17
		10% MD	0.31	14
	2	35% PEG	0.00	14
		5% MD	0.00	15
	3	30% PEG	-	15
		15% MD	0.23	15
	4	25% PEG	0.99	24
		20% MD	0.05	8
	5	35% PEG	1.14	24
		20% MD	0.02	13
	6	25% PEG	1.02	26
		25% MD	0.27	8

Table 13. The undiluted concentration of pure resveratrol and maltodextrin (DE 16.5-19.5) and PEG 8000 phase volumes used to calculate the partitioning of resveratrol in different maltodextrin-PEG two phase systems with an average total resveratrol concentration of 0.22 g/L and 2.7 g/L.

Run	Sample	Phase composition (wt%)	Resveratrol concentration (g/L)		Phase volume (ml)
			Low resveratrol concentration system	High resveratrol concentration system	
1	1	5% PEG	0.23	1.41	7.0
		35% MD	0.08	0.52	7.5
	2	7.5% PEG	0.15	3.48	7.5
		35% MD	0.02	0.82	7.5
	3	10% PEG	0.17	3.73	8.5
		35% MD	0.02	0.62	7.0
2	1	5% PEG	0.11	1.29	7.0
		35% MD	0.05	0.77	7.5
	2	7.5% PEG	0.14	1.84	7.5
		35% MD	0.03	0.32	7.5
	3	10% PEG	0.24	2.90	8.5
		35% MD	0.03	0.55	7.0
3	1	5% PEG	0.23	0.57	7.0
		35% MD	0.06	0.30	7.5
	2	7.5% PEG	0.13	1.06	7.5
		35% MD	0.05	0.44	7.5
	3	10% PEG	0.17	2.93	8.5
		35% MD	0.02	0.80	7.0

Table 14. The undiluted concentration of pure resveratrol samples in 7.5 wt% PEG 8000 and 35 wt% maltodextrin (DE 16.5-19.5) two phase systems with a pH above and below each resveratrol acidic dissociation constant.

Run	Sample	Phase composition (wt%)	Resveratrol concentration (g/L)
1	<pKa1	7.5% PEG	0.43
		35% MdX	0.08
	>pKa1	7.5% PEG	0.23
		35% MdX	0.04
	>pKa2	7.5% PEG	0.12
		35% MdX	0.05
	>pKa3	7.5% PEG	0.03
		35% MdX	0.07
2	<pKa1	7.5% PEG	0.43
		35% MdX	0.05
	>pKa1	7.5% PEG	0.23
		35% MdX	0.03
	>pKa2	7.5% PEG	0.12
		35% MdX	0.04
	>pKa3	7.5% PEG	0.08
		35% MdX	0.07
3	<pKa1	7.5% PEG	0.42
		35% MdX	0.06
	>pKa1	7.5% PEG	0.23
		35% MdX	0.03
	>pKa2	7.5% PEG	0.00
		35% MdX	0.05
	>pKa3	7.5% PEG	0.08
		35% MdX	0.06

Table 15. The measured absorbance used to calculate the amount of polyphenols from Pinotage stems that were recovered with albumin, tryptone soy broth and yeast extract.

Run	Sample	Total phenolic content (Absorbance)		
		Albumin	Tryptone soy broth	Yeast extract
1	1	0.96	0.83	1.02
	2	0.65	0.77	0.65
	3	0.32	0.46	0.48
2	1	0.96	0.84	1.05
	2	0.72	0.74	0.63
	3	0.47	0.44	0.46
3	1	0.98	0.85	1.00
	2	0.73	0.72	0.62
	3	0.37	0.46	0.45

Table 16. The undiluted resveratrol concentration (with an initial concentration of 0.82 g/L in PEG 8000) used to calculate the resveratrol recovery achieved with different concentrations of albumin, tryptone soy broth and yeast.

Run	Sample	Protein concentration (g/L)	Resveratrol concentration (g/L)		
			Albumin	Tryptone soy broth	Yeast extract
1	1	0.03	0.63	0.67	0.65
	2	0.25	0.61	0.57	0.80
	3	0.40	0.53	0.59	0.62
	4	0.57	0.40	0.52	0.56
	5	0.67	0.43	0.42	0.42
2	1	0.03	0.42	0.65	0.64
	2	0.25	0.53	0.58	0.57
	3	0.40	0.47	0.58	0.63
	4	0.57	0.47	0.58	0.42
	5	0.67	0.48	0.52	0.50
	6	0.75	0.47	0.47	0.55
3	1	0.03	0.88	1.12	1.04
	2	0.25	0.85	1.14	0.84
	3	0.40	0.86	0.97	0.97
	4	0.57	0.74	0.53	0.98
	5	0.67	0.55	0.48	0.83
	6	0.75	0.61	0.43	0.81

Table 17. The undiluted resveratrol concentration (with an initial concentration of 1.63 g/L in PEG 8000) used to calculate the resveratrol recovery achieved with different concentrations of albumin, tryptone soy broth and yeast.

Run	Sample	Protein concentration (g/L)	Resveratrol concentration (g/L)		
			Albumin	Tryptone soy broth	Yeast extract
1	1	0.5	1.50	1.11	1.19
	2	0.67	1.18	1.09	1.00
	3	0.75	1.23	1.10	1.23
2	1	0.5	1.34	1.07	1.12
	2	0.67	1.18	1.09	1.06
	3	0.75	1.20	1.22	1.12
3	1	0.5	1.10	1.17	1.16
	2	0.67	1.14	1.21	1.18
	3	0.75	1.26	1.21	1.25

Table 18. The undiluted resveratrol concentration (with an initial concentration of 0.077 g/L in PEG 8000) used to calculate the resveratrol recovery achieved with different concentrations of albumin, tryptone soy broth and yeast.

Run	Sample	Protein concentration (g/L)	Resveratrol concentration (g/L)		
			Albumin	Tryptone soy broth	Yeast extract
1	1	0.025	0.027	0.029	0.027
	2	0.062	0.023	0.029	-
	3	0.074	0.023	0.017	0.010
2	1	0.025	0.017	0.033	0.020
	2	0.062	0.015	0.012	0.024
	3	0.074	0.011	0.013	0.015
3	1	0.025	0.018	0.021	0.021
	2	0.062	0.019	0.017	0.020
	3	0.074	0.013	0.022	0.015