Aqueous two-phase systems for the extraction of polyphenols from wine solid waste

by

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December 2019

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ABSTRACT

The South African wine industry produces large amounts of solid waste, which is left over after the wine making process is complete. This solid waste accounts for approximately 25% of the fresh grape mass used for wine making and is called pomace. The pomace includes the parts of the grape not converted to wine: the skins, seeds and stems. These contain valuable compounds which could be used, for instance, for nutraceuticals, thus allowing for the valorisation of the wine solid waste. Included in these compounds are polyphenols. Polyphenols are compounds containing aromatic rings with hydroxyl groups, many of which have interesting or useful properties, such as being strong antioxidant molecules, and are therefore sought after for therapeutics and cosmetics.

Polyphenols have been extracted from various plant sources using conventional extraction methods, such as solvent extraction using either methanol or ethanol or supercritical CO_2 extraction. These solvents or processes are often expensive, and the high volumes needed drives up the processing costs. Alternative methods are needed to provide more cost-effective extraction processes, while also a Green Chemistry approach to extraction by taking into consideration the environmental impacts of the process and reducing harmful solvent use.

One such alternative extraction is the use of Aqueous Two-Phase Systems (ATPS), which have been used as a process alternative for biomolecule extractions, including polyphenols. ATPS are composed of two immiscible aqueous solutions, often created with polyethylene glycol (PEG) and salt. Two phases form when these components are within specific concentration bounds. The biomolecules are extracted from the plant material using the ATPS and then concentrated to one phase of the ATPS. Many different PEG and salt combinations exist and have been studied in the literature, looking at the phase behaviour as well as the ability of the ATPS to extract and concentrate the biomolecules.

In this study, ATPS with PEG 6000, PEG 8000 and PEG 10 000 with potassium sodium tartrate were studied. In the first set of experimentation, the phase behaviours were looked at, at different temperatures by constructing phase diagrams which included binodal curves and tie-line information (which define the two-phase region). The binodal data were fitted with a non-linear model, called the Merchuck equation, and the tie-line data were validated using the Othmer-Tobias and Bancroft equations. Phase diagrams were constructed to visualise these effects and indicated that higher temperatures and larger PEG molecular weights favoured

phase formation, producing ATPS with a wider range of PEG/salt compositions. These results have been published in The Journal of Chemical and Engineering Data.

In the next set of experimentation, these ATPS were evaluated for their ability to extract and concentrate polyphenols from wine solid waste, compared to a solvent extraction using ethanol/water (80:20 v/v). Various parameters were investigated including PEG M_w, salt type, TLL, extraction temperature, extraction time, pH, biomass loading and phase separation temperature. It was found that temperature, PEG composition (TLL) and biomass loading were the biggest drivers in improving the extraction and concentrating ability of the ATPS. The ATPS ability was judged using yield and partitioning coefficient (K) of the polyphenols by gallic acid equivalents (GAE). Yields upwards of 85% were achieved, with the K varying between 2-4, the highest K of 7.2 achieved by only one ATPS, which had the biggest fraction of PEG in the total ATPS composition. The results show that ATPS can be successfully used as an extraction method for polyphenols from wine solid waste.

OPSOMMING

Die Suid-Afrikaanse wynindustrie produseer groot hoeveelhede vastestofafval, wat oorbly nadat die wynmaakproses voltooi is. Hierdie vastestofafval geld vir omtrent 25% van die vars druiwe massa wat gebruik word om wyn te maak, en word pulp genoem. Die pulp sluit die dele van die druif in wat nie na wyn omgeskakel kan word nie: die dop, saad en stingels. Hierdie bevat waardevolle samestellings wat gebruik kan word vir byvoorbeeld neutraseutiese middels, en dus die valorisasie van die wynvastestofafval toelaat. Hierdie samestellings bevat polifenole. Polifenole is samestellings wat aromatiese ringe bevat met hidroksielgroepe, waarvan daar baie met interessante en bruikbare eienskappe is, soos om sterk anti-oksidant molekules te wees, en word daarom vir terapeutiese en kosmetiese doeleindes gesog.

Polifenole is deur verskeie plantbronne geëkstraheer deur konvensionele ekstraksiemetodes te gebruik, soos ’n oplosmiddelekstraksie wat of metanol-, etanol- of superkritiese CO2-ekstraksie gebruik. Hierdie oplosmiddels of prosesse is gereeld duur, en die hoë volumes wat benodig word, stoot die prosesseringkostes op. Alternatiewe metodes word dus benodig om meer koste-effektiewe ekstraksieprosesse te verskaf, terwyl ’n Groen Chemie-benadering op ekstraksie terselfdertyd geneem moet word deur ook die omgewingsimpak van die proses in ag te neem en die skadelike oplosmiddels wat gebruik word, te verminder.

Een so ’n alternatiewe ekstraksie is die gebruik van Waterige Twee-fase Sisteme (ATPS), wat gebruik is as ’n alternatiewe proses vir biomolekulêre ekstrakies, insluitend polifenole. ATPS word deur twee onmengbare waterige oplossings saamgestel, dikwels uit poliëtileenglikol (PEG) en sout geskep. Twee fases vorm wanneer hierdie komponente binne spesifieke konsentrasies bind. Die biomolekules word geëkstraheer vanuit die plantmateriaal deur die ATPS te gebruik, en dan gekonsentreer na een fase van die ATPS. Baie verskillende PEG en sout kombinasies bestaan en is bestudeer in die literatuur, waar gekyk word na die fase gedrag sowel as die vermoë van die ATPS om die biomolekules te ekstraheer en konsentreer.

In hierdie studie, is ATPS met PEG 6000, PEG 8000 en PEG 10 000 met kaliumnatriumtartraat bestudeer. In die eerste stel eksperimente, is daar na die fase gedrag gekyk, by verskillende temperature deur fase diagramme te konstrueer wat binodale kurwes en bindlyninformasie (wat die twee-fase streek definieer), bevat. Die binodale data is gepas met ’n nie-liniêre model, wat

die Merchuck-vergelyking genoem word, en die bindlyndata is gevalideer deur die Othmer-Tobias en Bancroft vergelykings. Fase diagramme is konstrueer om hierdie effekte te visualiseer en het aangedui dat hoër temperature en groter PEG-molekulêre massas die fase formasie bevoordeel, wat ATPS met ’n wyer bestek PEG/sout-samestellings produseer. Hierdie resultate is in The Journal of Chemical Engineering Data gepubliseer.

In die volgende stel eksperimente is hierdie ATPS geëvalueer vir hulle vermoë om polifenole uit wynvastestofafval te ekstraheer en konsentreer, in vergelyking met ’n oplosmiddelekstraksie wat etanol/water (80:20 v/v) gebruik. Verskillende parameters is ondersoek, insluitend PEG Mw sout tipe, TLL, ekstraksietemperatuur, ekstraksietyd, pH, biomassa-lading en fase verdeling-temperatuur. Dis gevind dat temperatuur, PEG-samestelling (TLL) en biomassa-lading die grootste drywers was om die ekstraksie- en konsentrasievermoë van die ATPS te verbeter. Die ATPS-vermoë is beoordeel deur opbrengs en verdelingskoëffisiënt (K) van die polifenole by gallussuurekwivalente (GAE) te gebruik. Opbrengs opwaarts van 85% is bereik, met die K wat tussen 2 en 4 varieer, die hoogste K van 7.2 is deur slegs een ATPS bereik, wat die grootste fraksie van PEG in die totale samestelling gehad het. Die resultate wys dat ATPS suksesvol gebruik kan word as ’n ekstraksiemetode vir polifenole uit wynvastestofafval.

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Winetech for providing funding to make this project possible.

Dr Pott for believing in me and giving me the opportunity to enter the engineering field, and for his endless patience when things took a bit too long.

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My boyfriend Thomas for his continuous support, cups of coffee, and interest in my work.

My friends for their comradery and love during our studies.

Table of contents

Nomenclature

ATPE	Aqueous two-phase extractions
ATPS	Aqueous two-phase systems
GAE	Gallic acid equivalent
K	Partition coefficient
M_w	Molecular weight
PEG	Polyethylene glycol
SAWIS	South African Wine Industry Information and Systems
STL	Tie-line slope
TLL	Tie-line length
TPC	Total polyphenol content
w/w	Mass fraction weight/weight
Y	Yield

List of tables

List of figures

Arising publications

Jacqueline Herbst & Robert W.M. Pott. The effect of temperature on different aqueous two-phase diagrams of polyethylene glycol (PEG 6000, PEG 8000, and PEG 10 000) + potassium sodium tartrate + water. The Journal of Chemical and Engineering Data, 2019, 64(7), pp. 3036-3043. DOI: 10.1021/acs.jced.9b00133.

Chapter 1 Introduction

1.1 Background

The market for natural pharmaceuticals (nutraceuticals) is ever increasing. Polyphenols are a popular chemical group used in nutraceuticals. A big driver in this market is the phenomenon known as "The French Paradox". A study done in 1993 looked at the populations of 40 countries with regards to average saturated fat and cholesterol intake as well as the death rate due to coronary heart disease (CHD) of the population (Artaud-Wild et al., 1993). While the majority of countries displayed a positive correlation between cholesterol and saturated fat intake, and CHD death rate, the French population was an outlier, seen in Figure 1 (Artaud-Wild et al., 1993). It seemed that although the French had a relatively high intake of cholesterol and saturated fat, their diet rich in antioxidants (found in vegetables) and unsaturated fats 'saved' them from the same fate as the other countries with high cholesterol and saturated fat diets. Ferrières (2004) reports on further correlation studies that observed that the French population have a moderate intake of red wine consistently throughout the week, while other countries may have a tendency for 'binge' drinking, as was found in Northern Ireland. This led to the believe that red wine, and more specifically the polyphenol resveratrol, found in red wine, may partly contribute to the French Paradox.

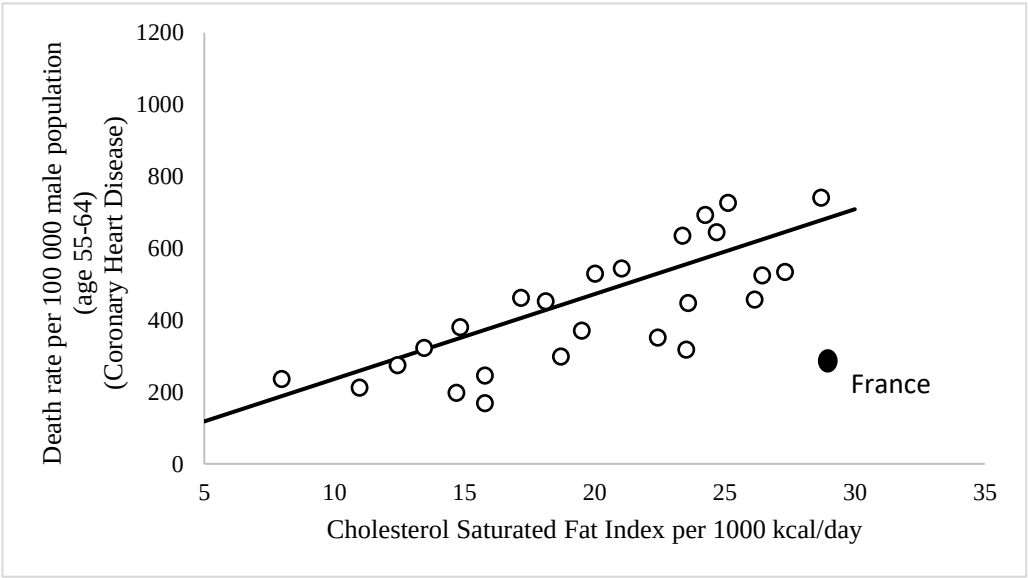


Figure 1 Positive correlation of Coronary Heart Disease mortality with cholesterol and saturated fat daily intake of populations from different countries. French population shown at • (Redrawn approximately from Artaud-Wild et al. 1993)

Wine production is a prominent player in the South African, and specifically the Western Cape province, economy. While the finished product, wine, is the main constituent of this economy, selling not only to local consumers but to international consumers as well, there are still untapped resources which can be utilised to a fuller extent. One such resource is the spent grape pomace. The pomace consists of the skins, seeds and occasional stems that are left over after the juice has been extracted from the grapes. While the main ingredient for wine, the juice of the grape, is depleted the pomace contains many other valuable components, including the polyphenols made popular by the French paradox.

Many processes have been developed to extract these polyphenols form the wine solid waste. Theses extraction methods, including solvent extraction, prove to be costly, and alternative extraction processes need to be developed. Aqueous two-phase extractions (ATPE) have been used for the extraction of many different biomolecules and has the potential to be successfully used for the extraction of polyphenols from the wine solid waste.

In this study, the main aim is to develop an appropriate aqueous two-phase system (ATPS) using polyethylene glycol (PEG) and salt to extract polyphenols from wine waste, allowing for the valorization of grape pomace which would otherwise be discarded. Chapter 2 is a literature review which details the polyphenols to be extracted from the wine solid waste as well as extraction methods used conventionally. ATPS is discussed in detail, explaining what ATPS consists of and how different factors affect an ATPS. The application of ATPS is then discussed, looking at different studies which have used the technology for polyphenol extraction from different plant materials. Chapter 3 is an experimental study of different ATPS made with PEG 6000, PEG 8000 and PEG 10 000 with potassium sodium tartrate at different temperatures. This chapter looks at how these ATPS form and the implications of different PEG M_w and temperatures. Chapter 4 then looks at using these ATPS for the extraction of polyphenols from grape pomace. Different factors are varied including PEG M_w, salt type, TLL, extraction temperature, extraction time, pH, biomass loading and phase separation temperature. The extraction ability of these ATPS are evaluated using yield of polyphenols and partitioning coefficient (K) in the ATPS.

1.2 Objectives

To construct phase diagrams that have not previously been published in the literature, using potassium sodium tartrate with PEG 6000, PEG 8000 and PEG 10 000, each at three different temperatures of 10°C, 25°C, 45°C.

To evaluated how the PEG molecular weight and temperature impacts the phase formation and behaviour of these ATPS.

To carry out a solvent extraction of polyphenols from the grape pomace to find a 'maximum' level of polyphenols available for extraction.

To evaluate the effect that PEG molecular weight and phase formation temperature has on the extraction of TPC from grape pomace, by looking at the yield in comparison to the solvent extraction, and the partitioning of the polyphenols in the ATPS

Directive experiments of different variables to pinpoint best possible conditions for extraction of polyphenols

1.3 Research questions

1. How does PEG molecular weight and temperature affect the formation of phases in an aqueous two-phase system?
2. Can aqueous two-phase systems be used as an alternative method for the extraction of polyphenols from grape waste?
3. How do the different factors relating to aqueous two-phase systems affect the extraction and purification of polyphenols from grape waste? These factors include PEG molecular weight, salt type, tie-line length, ATPS phase formation temperature, extraction time, extraction temperature, biomass loading, and pH.

1.4 Project scope and limitations

The scope of this project is limited to bench scale experiments of the ATPS setups in the laboratory. The temperatures studied were limited to the temperature regulating equipment available in the laboratory. The grape biomass used for polyphenol extractions were limited to what was available for procurement in the local area (Stellenbosch, South Africa) at the time of Harvest for the years 2017 and 2018.

Chapter 2 Literature Review

2.1 Valorisation of spent grape pomace from the South African wine industry

In 2018, South Africa ranked 9th globally in wine production according to the International Organisation of Vine and Wine's April 2019 report. South African Wine Industry Information and Systems (SAWIS) release yearly statistics on the previous year's harvest and reported in June 2019 that the 2018 South African harvest saw 1.24 million tons of grapes crushed (with an average of 1.38 million tons in 2015-2018), producing 824 million liters of wine (averaging 902.25 million liters in 2015-2018). These grapes were harvested from vines spanning 93021 hectares of land, 15062 hectares being in the Stellenbosch region. About 25% of the total grape mass is left over after the vinification (wine making) process as grape pomace, consisting of the grape skins, seeds and stems (Gonzalez-Centeno, 2010). While the pomace is often used for fertiliser or feed (Burg et al., 2014; Guerra-Rivas et al., 2016), finding an alternate use for the pomace will add value to the whole process of harvesting and vinification of the grapes.

The pomace as a whole and individual components (skins, seeds, stems) as well as the lees (spent yeast) left over after vinification have been valorized in various ways to obtain secondary products. Zacharof (2017) summarised some of these in a review, an abridged version shown in Table 1. Various processes were used to derive these products. Secondary fermentations using various microbiota including *T. viride*, *L. pentosus* and *D. hansenii* preceded by hydrolysis was used to produce bioethanol, biosurfactants, biocontrol agent and lactic acid, yeast induced fermentations were used to produce proteins, and tartaric acid was precipitated out (Zacharof (2017) citing Arvanitoyannis et al. (2006) & Devesa-Rey et al. (2011)).

Table 1 Biomolecules extracted from different components ofwine waste (adapted from Zacharof, 2017)

Component	Product
Lees	Proteins
	Nutritional supplements
Pomace	Hydrolytic enzymes
	Polyphenols
	Bioethanol
	Lactic acid
	Tartaric acid
	Biocontrol agent
Seeds	Oil
	Biosurfactants
Stems	Lactic acid
	Biosurfactants
	Xylitol

The grape skins are complex and made up of many components which are shown in Figure 2 which was adapted from Mendes et al. (2013). While many of these components are structural, some of these such as the tannins have other biochemical functions in the grape skins. Tannins are phenolic compounds and are typically found in bigger abundance in red wines, due to the elongated skin contact time during the red wine making process. Phenolics are a broad class of molecules, the simplest structure characterised by a benzene ring and one or more hydroxyl groups, with variations to allow for a larger group of unique phenolics. Polyphenols are found in many food sources other than grapes including berries, tea and coffee (Manach et al., 2004). The phenolics found in wine can be categorised into flavonoids (flavonols, falvan-3-ols and anthocyanins) and non-flavonoids (hydroxycinnamates, hydroxybenzoates, and stilbenes) and each of these categories consists of many different molecules (Waterhouse, 2002). Some of

these phenolics found in wine grapes are outlined in Table 2, along with alternate food sources for the same phenolics. These polyphenols have various roles in the grape structure. Flavonoids are responsible for the colour, aroma and taste of the berry (Panche et al., 2016). These can be seen in the red colour of certain grapes (Cabernet Sauvignon, Pinotage and Shiraz amongst others) and is responsible for the dry mouth feeling that is often associated with red wines. Resveratrol is an antifungal phytoalexin and is produced in response to infection of the grapes by *Botrytis cinerea* (Jeandet et al., 2002).

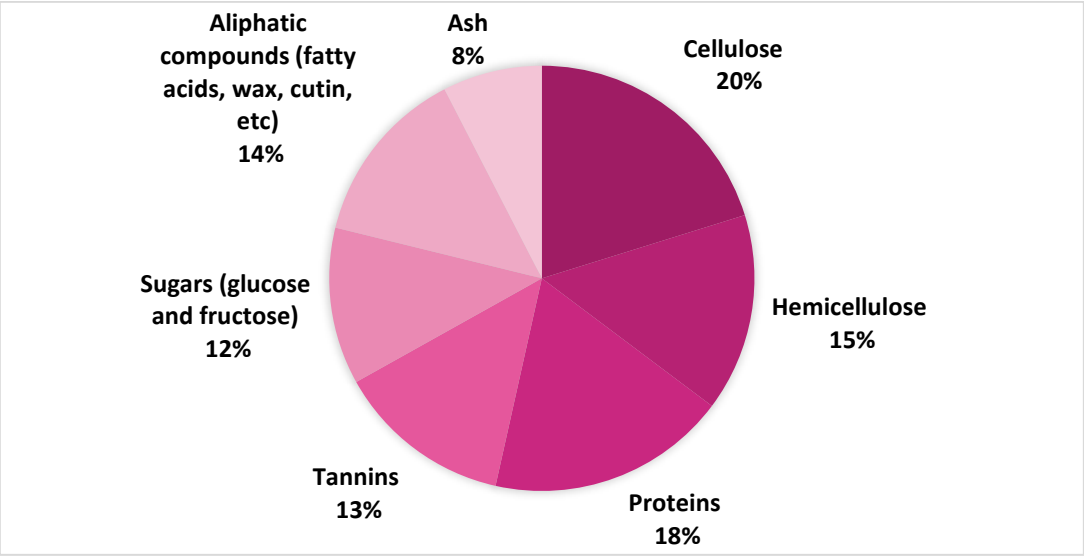


Figure 2 Chemical composition of macromolecular components of grape skins (adapted from Mendes et al., 2013)

Table 2 Polyphenols found in wine grapes divided into groups and subgroups adapted from Lorraine et al. (2013) and drawn with ChemSpace (chem-space.com), along with food sources of polyphenols (Manach et al., 2004 & Sanders et al., 2000)

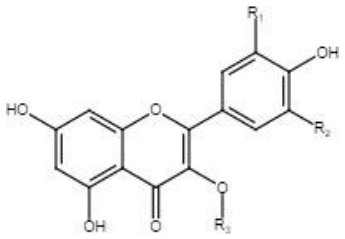
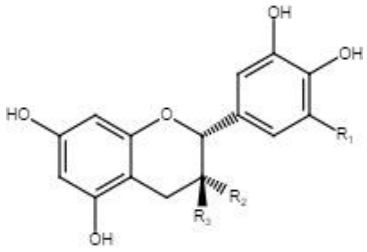
Polyphenols		Food source (>100mg/kg fresh weight)
Flavonoids		
Flavonols	Kaempferol	Yellow onion
	R_1=H, R_2=H, R_3=H	Leeks
	Quercetol	Curly Kale
	R_1=OH, R_2= H, R_3=H	Cherry tomatoes
	Myricetol	Broccoli
	R_1=OH, R_2=OH, R_3=H	Blueberry
	Isorhamnetol	
	R_1=OCH_3, R_2=H, R_3=H	
Flavan-3-ols	(+)-catechin	Chocolate
	R_1 =H, R_2 =H, R_3 =OH	Beans
	(-)-epicatechin	Green tea
	R_1=H, R_2 =OH, R_3=H	Black tea
	(+)-gallocatechin	Apricot
	R_1 =OH, R_2 =H, R_3 =OH	Cherry
	(-)-epigallocatechin	
	R_1 =OH, R_2 =OH, R_3 =H	

Table 2 **cont.**

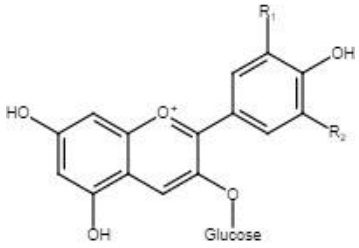
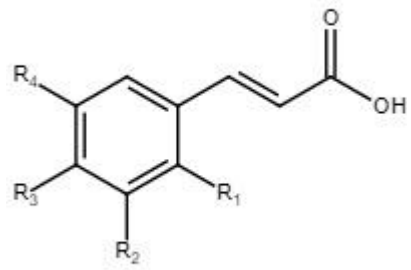
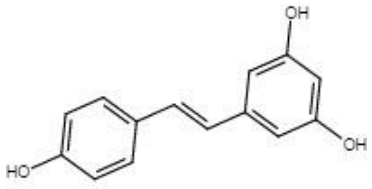
Polyphenols		Food source (>100mg/kg fresh weight)
Anthocyanins 	Cyanidin-3-O-gluc R_1=OH, R_2=H delphindin-3-O-gluc R_1 =OH, R_2 =OH paeonidin-3-O-gluc R_1 =OCH_3, R_2 =H petunidin-3-O-gluc R_1=OCH_3, R_2=OH malvidin-3-O-gluc R_1=OCH_3, R_2=OCH_3	Aubergine Rhubarb Black currant Red cabbage Cherry
Non-flavonoids		
Hydroxycinnamates 	*p*-coumaric acid R_1=H, R_2=H, R_3=OH, R_4=H Caffeic acid R_1=H, R_2=OH, R_3=OH, R_4=H Ferulic acid R_1=H, R_2 =OCH_3, R_3 =OH, R_4=H Sinapic acid R_1=H, R_2=OCH_3, R_3=OH, R_4=OCH_3	Kiwi Blueberry Coffee Potato Cornflour Cider

Table 2 **cont.**

Polyphenols		Food source (>100mg/kg fresh weight)
Hydroxybenzoates 	p-hydroxybenzoic acid R_1 =H, R_2 =H, R_3 =OH, R_4 =H Protocathecuic acid R_1 =H, R_2 =OH, R_3 =OH, R_4=H Vannilic acid R_1=H, R_2=OCH_3, R_3=OH, R_4 =H Gallic acid R_1=H, R_2=OH, R_3=OH, R_4 =OH Syringic acid R_1 =H, R_2 =OCH_3, R_3=OH, R_4 =OCH_3	Blackberry Raspberry Black current
Stilbenes 	Resveratrol	Peanuts

Free radicals are involved in many disease and aging mechanisms in the human body and are present due to many factors ranging from normal metabolism reactions to pollution or UV exposure (Phaniendra et al., 2014). The hydroxyl groups associated with polyphenols are highly reactive, with the ability to scavenge free radicals. The mechanism is shown in Equation 1, adapted from Nimse and Pal (2015), where RO^- is the free radical with a reactive oxygen, inactivated by a hydrogen taken from the polyphenol hydroxyl group. This property makes polyphenols a good antioxidant, making it an attractive source for nutraceuticals, which are naturally derived medicines.

$$RO^- + (polyphenol - OH) \rightarrow ROH + (polyphenol - O^-) \qquad \text{Equation 1}$$

Table 3 serves as a sample of some possible applications of polyphenols in fighting diseases or aging. Many studies have been done *in vitro* and *in vivo* on the effectiveness of different polyphenols in fighting an array of diseases, including cardiovascular diseases, cancer and neurodegenerative disease amongst others. Much more research needs to be done though to advance the understanding and implementation of these polyphenols in fighting diseases (Scalbert et al., 2005). Resveratrol has been shown a great interest in both the nutraceutical and aesthetic industries. Many supplements marketed as containing resveratrol and claiming to have anti-aging effects can be found on the market, including from the well-known brand Solal priced at ZAR 340 (USD 22.16) for 60 capsules in 2019 (wellnesswarehouse.com). Skin care products containing resveratrol are aimed at protecting skin from pollution and thus providing anti-aging properties, one such product being The Ordinary with 3% resveratrol and 3% ferulic acid selling for ZAR 103/30ml (USD 6.71) in 2019 (ASOS.com). The total resveratrol market value was estimated at USD $50 million in 2012 as reported by Frost and Sullivan (Evolva.com). The prospect of entering this huge market by utilising these naturally available polyphenols for new products has seen many a process developed to extract these molecules from plants. In the next section, some of these extraction processes will be discussed.

Table 3 Potential application of polyphenols in disease prevention and cure tested in different models

Potential application	Polyphenols	Model	
Cardiovascular			
Inhibition of lipoprotein oxidation	Tea catechin	Human	Nakagawa et al. (1999)
Inhibition of platelet aggregation (ie antithrombotic effect)	Non-specific	Rat	Ruf et al. (1995)
Inhibition of plaque build-up in arteries	Resveratrol	Human	Agarwal et al. (2013)
Cancer			
Enhancement of prostate cancer cell radiation sensitivity	Resveratrol	In vitro	Fang et al. (2012)
Stimulation of DNA repair	Quercetin glycoside	Rat	Webster et al. (1996)
Induce apoptosis in tumor cells	Quercetin	In vitro	Ishikawa & Kitamura (2000)
Neurodegenerative diseases			
Improve memory impairment	Catechin and epicathechin	Mouse	Matsuoka et al. (1995)
Protection of neuronal cells against oxidative damage	Epigallocatechin gallate	In vitro	Levites et al. (2002)
Protection of learning and memory performance	Ferulic acid	Mouse	Yan et al. (2001)
Anti-aging			
Stimulate increase in collagen in skin cells	Resveratrol	Human	Ratz-Łyko & Arct (2018)

2.2 Upstream and downstream processing of polyphenols

Biomolecules that are of interest to manufacture for selling on the market are produced either through upstream processing or downstream processing. In upstream processes, biomolecules are synthesised *de novo*. This can be through chemical reactions or through manipulating synthesis in biological systems. Downstream processing utilises existing biological producers of the biomolecule to extract from.

Resveratrol is produced naturally in plants through the expression of the stilbene synthase gene (STS), amongst other genes. The STS gene can be constitutive (ie transcribed in the grape genome regardless of other factors), but the expression is often induced by abiotic stresses or biotic stresses such as *Botrytis cinerea* as mentioned in the previous section (Jeandet et al., 2002). Upstream processing of resveratrol has been achieved by transforming *Saccharomyces cerevisiae* yeast cells as well as human HEK293 kidney cells with these plant genes (Beekwilder et al., 2006 & Zhang et al., 2006).

Upstream processing, such as the production of resveratrol through fermentation by transformed yeasts done by the company Evolva, is useful for producing purer grade resveratrol. Downstream processing, however, is useful when the biomolecule to be produced can be extracted from bio-waste, utilising resources that would have otherwise gone to waste, this is known as valorisation.

Downstream processing of bioproducts to be used in pharmaceuticals or other consumer products occurs in four stages; recovery, isolation, purification and polishing. The final required purity of the bioproducts depends on the application of the product, with therapeutics needing to be near 100% pure while industrial enzymes have a bigger purity scope. Getting the bioproducts 'market ready' can be time consuming, taking into consideration the product development stage, production stage (in which the extraction or production needs to be scaled up) and finally the different phases of testing (from animal testing to clinical trials) (Harrison et al., 2003).

As means of downstream processing, resveratrol is commonly extracted from biomass using solvent extraction. Other extraction methods have been used, such as the extraction of resveratrol from peanuts using microporous adsorption resins (Xiong et al., 2014), extraction from grape skins using HPLC (Liu et al., 2013) and solid-phase extraction of resveratrol from peanut meal (Schwarz et al., 2016). These are however not suitable for large scale extractions; thus solvent extraction is preferred.

In studies investigating the properties of resveratrol, a common solvent used in solvent extraction is methanol (Pezet et al., 1994 & Jeandet et al., 1995). This involves homogenising the biomass with either pure methanol or methanol together with water at different ratios. While methanol may be effective in the extraction of resveratrol, it is toxic in high amounts. Safer, less toxic methods would be desirable for the extraction of compounds that are to be consumed by humans. Resveratrol has been extracted using ethanol as a solvent. Using a ethanol/water

(80:20 v/v) at 60°C for 30 minutes, resveratrol yield was just under 250 µg/g dry grape skin (Romero-Pérez et al., 2001).

Lafka et al. (2007) found that a mixture of ethanol and water (1:1) performed better when extracting polyphenols (as total polyphenol content) compared to using undiluted solvents of methanol, ethanol or acetone, as the addition of water increases the polarity of the solvent, allowing for better extraction of the benzoic and cinnamic acid polyphenols which are very polar (Mojzer et al., 2016). Labarbe et al. (1999) were able to dissolve different proantocyanidins sequentially by changing the ratio of methanol in a solvent mixture, the size of the polymer along with other characteristics of the molecules changing the solubility of the polyphenols allowing for higher purity of the distinct proantocyanidins to be separated. The final step in solvent extraction is the evaporation of the solvent to concentrate the polyphenols. The temperature at which the solvent is evaporated has been shown to affect the antioxidant activity of the polyphenols, Larrauri et al. (1998) found that a temperature of 60°C did not affect the activity, while higher temperatures led to decreased antioxidant activity of the polyphenols. Some studies have made use of ultrasonication (Cho et al., 2006 & Magwaza et al., 2015) or microwaving (Lovrić et al., 2017) to assist the solvent extraction process. It is unclear whether these techniques provide a clear advantage to the extraction ability compared to conventional heating techniques and may contribute to a higher cost of the process, diminishing the perceived advantage it provides.

Outlined in Figure 3 is a solvent extraction process adapted from the various literature cited above. Parameters such as solvent type and concentration, biomass loading, temperature and extraction time can vary depending on the plant source and polyphenols to be extracted as well as the resources available at any specific time for polyphenol extraction, thus the process outlined is non-specific and serves as a suggestion for a process to be followed.

The pre-treatment step of the process can also vary. This may be as simple as homogenisation of the pomace by drying and milling followed by immediate freezing (Čepo et al., 2017), or adding an additional step whereby the lipid content of the grape seeds is first extracted by acidification of the grape pomace followed by an extraction with *n*-hexane (Lafka et al., 2007). In the pre-treatment stage, care should be taken to avoid high light exposure to the pomace as far as possible to prevent degradation of the polyphenols, as it has been seen that elongated UV exposure leads to degradation of certain polyphenols in grape skins (Volf et al., 2014). Resveratrol changes from the *trans* isomer into the *cis* isomer when exposed to light for a long

period of time, as illustrated in Figure 4 (Yokotsuka and Okuda, 2011). The bioavailability of *cis*-resveratrol has not been investigated thoroughly, and as a result is not available commercially, thus making it a less desirable molecule to be extracted than *trans*-resveratrol (Orallo, 2006).

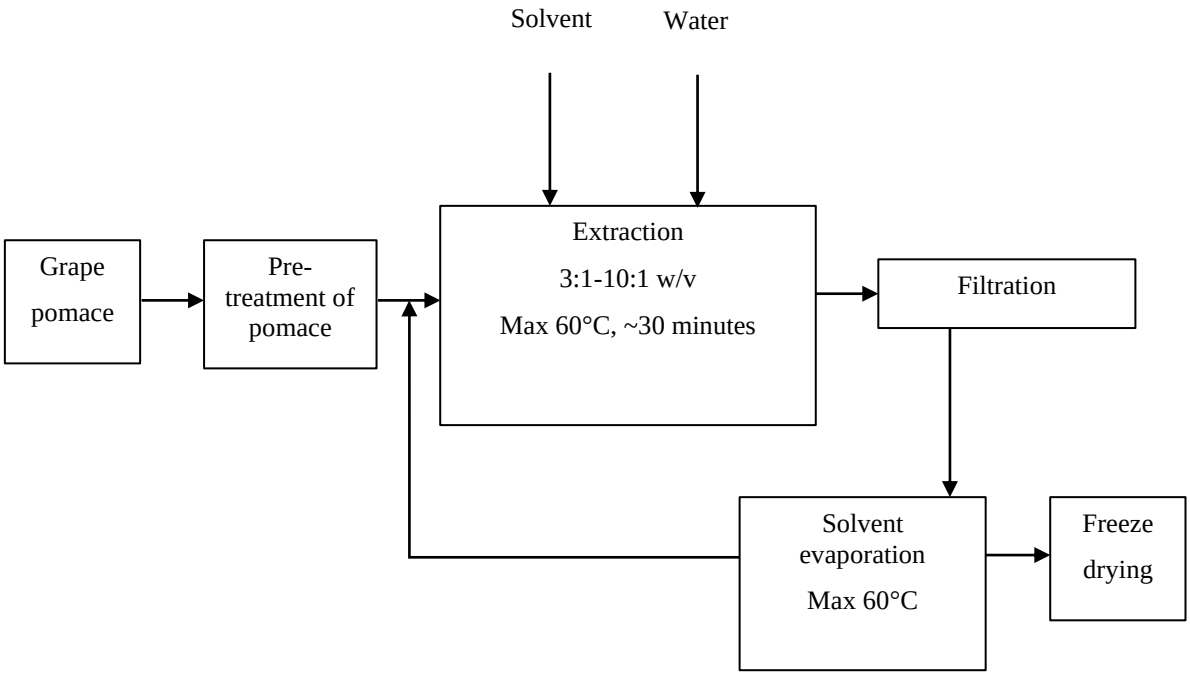


Figure 3 Solvent extraction of polyphenols process adapted from several literature sources

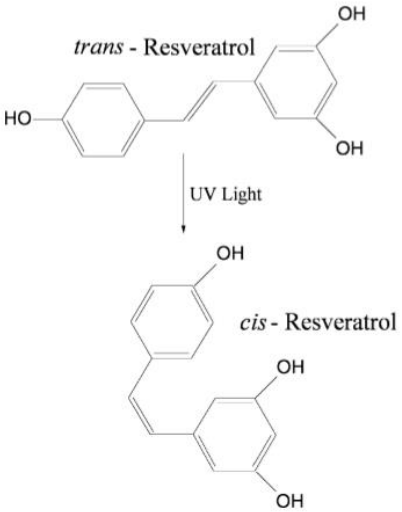


Figure 4 Isomerisation of resveratrol from a trans configuration to a cis configuration due to UV exposure

While these solvent extractions are effective, the economic impact needs to be considered. A 1 kL batch ethanol/water (80:20 v/v) solution would cost ZAR 11 342 (USD 756), referencing the list of chemical prices in Appendix 1. While this solution can be recycled back into the process, it would require further processing and thus funds. The use of supercritical fluids for extraction have been suggested as an alternative method of extraction, due to a decrease in solvents needed while increasing the rate of the extraction process. At a high pressure and low temperature, supercritical carbon dioxide (SC-CO_2) together with ethanol as a co-solvent (5%) has a higher recovery rate for resveratrol than conventional solvent extraction using methanol and HCl (Casas et al., 2010). Venturi et al. (2017) found that different plant material (in their case chillies and tomatoes) required different pressure and temperature parameters when extracting polyphenols with SC-CO_2. This same study found that SC-CO_2 alone was a poor solvent, and ethanol had to be used as a co-solvent.

The scope of this project will only extend to the extraction of polyphenols from the grape pomace, but it is important to keep in mind how this extract will further be used and processed. The final overarching goal is to develop a product delivered to the pharmaceutical or food processing companies and ultimately to the consumer in a fashion which is safe for the consumer and of a high integrity. Novel delivery systems for resveratrol has been reported in literature. Chitosan-zinc-pectinate-polyethylene glycol nanoparticles are hardy in that they are resistant to breakage caused by stomach enzymes, while also having an affinity to bind with other biomolecules such as resveratrol (Andishmand, 2017). In the study, the resveratrol was dissolved first in a PEG solution before being added to the chitosan, zinc and pectin components. Another novel drug delivery system including PEG was developed (Li & Niu, 2018). In this system, a model drug was carried by a block polymer poly(ethylene-glycol)-poly(propylene-carbonate)-poly(ethylene-glycol) (PEG-PPC-PEG).

Using solvent extraction for polyphenols is effective, but a more cost effective and potentially greener method should be explored. Aqueous two-phase extraction is an established method that could possibly be applied to polyphenol extraction from wine waste. This method will be investigated in the following sections.

2.3 Aqueous two-phase extraction as a downstream process

Aqueous two-phase extraction (ATPE), first described by Albertsson (1970), is the extraction of biomolecules in a system that consists of two immiscible aqueous liquids. When the

separation forcing components are above a certain concentration in combination, two phases form, reaching heterogenous equilibrium. ATPE has been successfully used in the downstream processing of biomolecules such as proteins, nucleic acids, cell organelles and viruses (Raja et al., 2012 & Harrison et al., 2018). A typical ATPS experimental process is shown in Figure 5, where two components (discussed in the next section) are added together in one systems to form an aqueous two-phase system (ATPS) which will then act as an extractant and a biomolecule separation technique.

The formation of the two phases (separation or 'demixing') is dependent on the physical properties of the phase components, such as density, viscosity and interfacial tension between the phases (Taylor et al., 2013). These properties are changed and affected by different phase components properties such as concentration, molecular weight or salt types and different conditions of the ATPS such as pH or temperature.

Different compounds will have different partitioning behaviours in a specific ATPS. The chemical properties of polyphenols will influence which parameters of a system would be optimal. The focus in this section is on the formation of ATPS, and the factors affecting it.

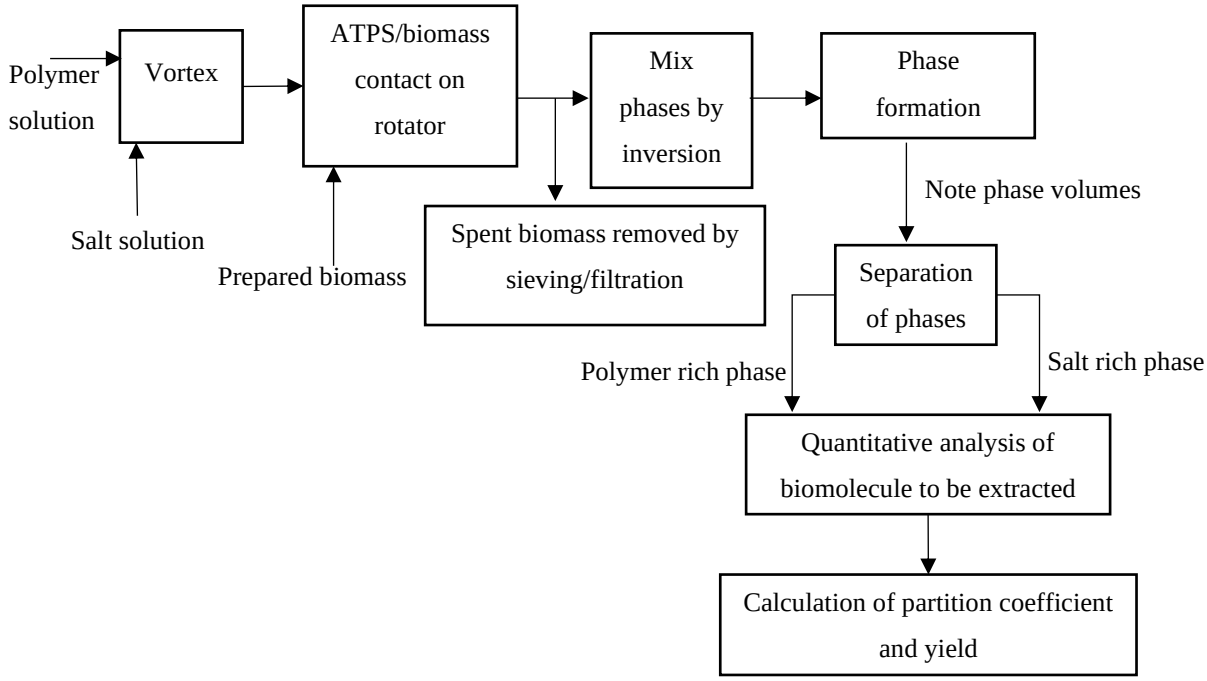


Figure 5 Typical Aqueous two-phase extraction process using polymer and salt

2.3.1 Phase forming components

The most common aqueous two-phase systems consist either of two polymers (polymer/polymer) or a polymer and a salt (polymer/salt) (Harrison et al., 2003). Polymer/polymer systems, specifically poly(ethylene glycol) (PEG)/dextran systems are well characterised, and are used extensively in the separation of proteins from solutions (Grilo et al., 2016). Dextran, however, is expensive and cost-effective alternative polymers have been investigated. PEG/maltodextrin systems have been used successfully in protein purification and described as having similar properties to PEG/dextran systems, while being cheaper (Ramyadevi et al., 2012). Other polymer/polymer combinations include PEG/ficoll, dextran/ficoll, dextran/hydroxypropyl dextran and PEG/hydroxypropyl dextran (Albertsson, 1970). Cheaper systems would be more cost effective in scale ups for industrial use.

Polymer/salt systems have been investigated as even cheaper alternatives to polymer/polymer systems. Polymer/salt systems have a higher difference in density between the phases, lower viscosity and greater selectivity ability (Raja et al., 2012). This gives polymer/salt systems the advantage of being adaptable to a wider variety of types of biomolecules. The most common salts used in polymer/salt systems include sulfate (Xavier et al., 2014 & Murari et al., 2015), phosphate (Sé and Aznar, 2002b) and citrate (Raja and Murty, 2012, Carneiro-da-cunha et al., 2014 & Xavier et al.,s 2015).

Short-chain alcohol/salt systems have been considered for aqueous two-phase systems (Grilo et al., 2016). Compared to polymer systems, it is easier to recover the biomolecules from the alcohol phases (Wang et al., 2008). Ethanol/salt systems have been successfully utilised in the extraction of biomolecules from solutions (Wu et al., 2014). These systems provide an alternative for use in extraction of biomolecules which may be more soluble in the short-chain alcohols, making for more efficient extraction.

The type of system used will depend largely on the molecules to be extracted, the upscale potential of the system in the industry of application and the budget of the project as some of the components are more expensive than others. The environmental impact of the systems should also be considered.

2.3.2 The phase diagram

In order to design the best suited two-phase system for a specific requirement, phase diagrams are utilised to represent that phase behaviour, and then using these diagrams process decisions can be made. The phase diagram is a visual representation of specific components and the compositions there-of, a generic example is shown in Figure 6. The diagram consists of a binodal curve, which represents the 'division' between component compositions which form two phases and compositions in which the system remains monophasic (Raja et al., 2012). Together with the binodal curve, the phase diagram has tie-lines, which are representative of two-phase systems with different component compositions and volume ratios. Systems that lie on the same tie-line had different component compositions initially, but the equilibrium concentrations of the components in the top and bottom phases respectively are the same. The tie-line length (TLL) is a parameter which describes the difference between the top and bottom phases in terms of thermodynamic functions (Chakraborty and Sen, 2016). Both the binodal curve and the tie-lines are functions of the mass fractions (w/w) of the bottom phase (salt) on the x-axis and the top phase (polymer) on the y-axis (Raja et al., 2012 & Chakraborty and Sen, 2016). These different components of the phase diagram will be discussed in more detail in Chapter 3 of this thesis.

Phase diagrams of aqueous two-phase systems provide insight into the phase separation behaviour of specific systems. The diagrams are also useful in understanding how the different component compositions of the system and environmental factors such as temperature or pH will affect the phase formation of the systems. Table 4 outlines some of the different types of conditions for PEG/salt ATPS studied and published as phase diagrams in the literature. There are many different combinations of PEG molecular weights (M_w), ranging from PEG 400 to PEG 35 000, and salt types including tartrates, citrates, phosphates, sulfates and carbonates. Table 4 highlights that not only are different PEG/salt combinations of these ATPS being studied, but different conditions as well, these including temperature, pH and the addition of NaCl. Wysoczanska & Macedo (2016) studied the effect on phase behaviour of different PEG M_w with citrate salt, while Sé and Aznar (2002a) constructed phase diagrams spanning different PEG M_w as well as different salt types. A few studies have looked at the effect that temperature has on the phase behaviour of different ATPS combinations, the specific studies outlined in the table. While there are a good number of studies done on the phase behaviour of different ATPS, there is a need to expand the knowledge of phase behaviour of different combinations of PEG

molecular weights and salt types, therefore opening up the possibilities for systems to be used in extraction of biomolecules from biomass. The ATPS combinations to be covered in this study are annotated with ‘c’ in Table 4, and will be discussed in more detail in Chapter 3.

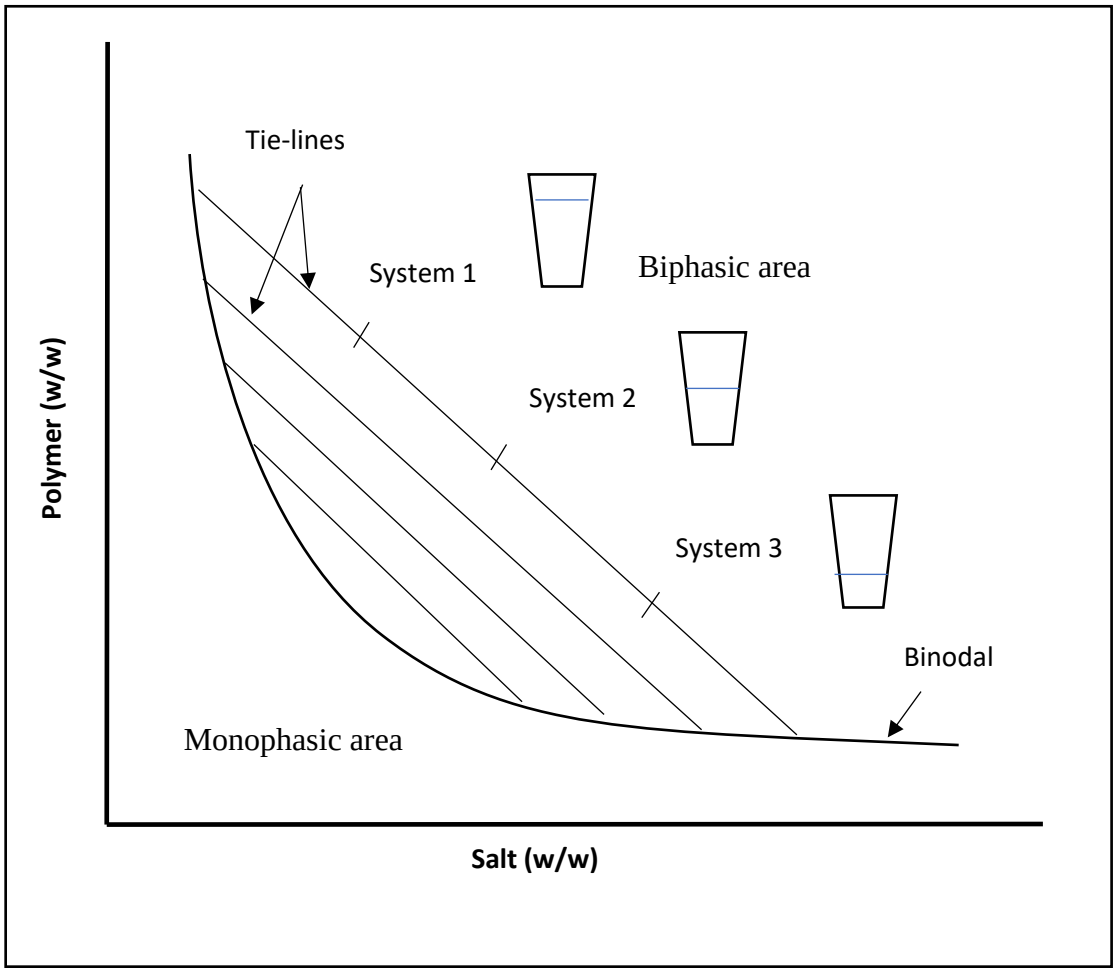


Figure 6 Generic representation of a polymer/salt aqueous two-phase system phase diagram indicating the binodal curve between the monophasic and biphasic areas, and the tie-lines which describe the phase components

Table 4 Existing phase diagrams of different salt types and PEG molecular weights with annotations of literature published

Salt type	PEG Mw	400	600	1000	1450	1500	2000	3350	4000	6000	8000	10000	35000
	Temperature						V						
Sulfate	*No Variables*			V				V			V		
	Temperature	II					VIII		VIII	VIII		II	II
Phosphate	*No Variables*			V					V		V		
	NaCl Addition								XIV				
	Temperature								XI[a]	XIII[b]			
Citrate	*No Variables*		IV	IV	IV				X	X	X		
	pH							IV					
	Temperature	I				VI	VII		VI	XII	IV		
Tartrate	*No Variables*		III[a]				III		X	III, X	X		
	NaCl Addition			III					III		III		
	Temperature								IX	c	c	c	

[a] Phase diagrams includes either only binodal curve or tie-lines, [b] Cation-type varied, [c] This study
I) Hamzehzadeh & Zafarani-Moattar 2015, II) Murari et al. 2015, III) Malpiedi, et al. 2008, IV) Tubio et al. 2009, V) Sé & Aznar 2002a, VI) De Oliveira, et al. 2008, VII) Perumalsamy & Murugesan 2006, VIII) Sosa et al. 2017, IX) Zafarani-Moattar & Tolouei 2008, X) Wysoczanska & Macedo 2016, XI) Sé & Aznar 2002b, XII) Zafarani-Moattar et al. 2004, XIII) Zafarani-Moattar & Sadeghi 2002 , XIV) Merchuk, et al. 1998

2.3.3 Partitioning of biomolecules in ATPS system

The objective of an aqueous two-phase system is for the partitioning of specific molecules to occur between the phases. The partitioning of the molecules is characterised and quantified by the partition coefficient (K). The partition coefficient is dependent on the size of the molecules as well as the ionic composition of the phases (Albertsson 1970). The driving forces of these

factors contribute to the partition coefficient in different ways, the final partition coefficient of a molecule in a specific system being a product of the contributions of these different driving forces.

These driving forces, as mentioned before, exist as a result either of the molecules partitioned or the environment of the system. The molecule size or conformation may cause the molecules to partition towards a specific phase. Interaction of the molecules with the phases also contribute as driving forces. The molecules may have hydrophobic interactions with either of the phases, one phase may consist of a component that has an affinity with the molecule to be partitioned or charge differences in the phases may drive a charged particle towards the phase with the opposite charge (Grilo et al., 2016). While the driving forces of molecule partitioning in the systems can be speculated on, the mechanisms are still not fully understood. The driving forces contribute to the partition coefficient in different combinations and capacities depending on the system composition and environment. Partitioning in one system will not have the same characteristics as partitioning in another system. It is therefore important to realise that systems are unique, and the driving forces needed would depend on the molecule to be partitioned.

2.3.3.1 Factors affecting phase separation and biomolecule partitioning

2.3.3.1.1 Polymer molecular weight and concentrations

Being the component that is common to most ATPE systems, the polymer composition and its effect on phase separation and biomolecule partitioning has to be taken into consideration when designing an ATPS. The polymer M_w and concentration plays a big role in the system and subsequently the extraction of biomolecules. Due to the excluded volume effect, whereby the occupation of a polymer in a solution takes up any potential space which other molecules may occupy (Ramyadevi et al., 2012), higher PEG M_w would result in a lower partition of biomolecules to the top phase (Yang et al., 2013). The volume exclusion effect has been demonstrated in a study which investigated the partition of proteases produced by *C. perfringens* in a PEG/citrate ATPS, whereby higher partition coefficients were found when a lower PEG M_w was used with a higher salt concentration, compared to systems which had higher PEG M_w but lower salt concentrations (Porto et al., 2008).

An increase in PEG concentration causes the phase density to increase (Regupathi et al., 2009). The density differences between the phases change along with changing PEG M_w and PEG

concentrations, whereby an increase in PEG concentration results in an increase in density differences, while an increase in PEG M_w results in a decrease in phase density differences (Taylor et al., 2013). These differences in densities of the phases allows phase separation to occur by means of one phase 'floating' on top of the other phase.

As the polymer concentration increases, the solution properties becomes more like the polymer and less like water (Grilo et al., 2016). This will affect molecule partitioning differently in each case, depending on the hydrophobicity of the molecules, as well as possible affinity between the polymer and the molecules.

2.3.3.1.2 Salt, pH and charge

The pH of a system influences the phase formation, with the two-phase area expanding with an increase in pH. The charge of the biomolecules to be extracted is affected by the pH of the system, thus a biomolecule may have different partition behaviours at different pH levels (Raja et al., 2012).

It has been seen that the partition coefficient of enzymes such as glucose-6-phosphate and hexokinase (extracted from baker's yeast) increased with an increase in pH of the ATPS system in a study done by Da Silva et al. (2002). This trend has also been observed in the extraction of α-amylase, with the pH of the system having a positive correlation with the K of α-amylase (Asenjo et al., 1994). This means that at a higher pH, the biomolecules are more concentrated in the top phase of the system. At a higher pH, resveratrol becomes deprotonated (López-Nicolás & Garciá-Carmona, 2008). This deprotonated state may be repulsed by the anions in the salt phase, allowing it to partition towards to polymer phase, serving as an example of how polyphenols may be affected by a pH change in an ATPS.

The salt type used in a system affects phase separation. Some salts are less compatible with PEG, which would result in better phase separation. The electrolyte solution interacts differently with the PEG depending on which cations and anions are present. When comparing the cations Mg^{2+}, Zn^{2+}, NH_4^+ and Na^+ it is clear that Na^+ salts result in better phase separation; this is due to the fact that Mg^{2+} and Zn^{2+} cations interact with ether oxygens of PEG (Hey et al., 2005).

Repulsion between anions and anion-like groups on the polymer may drive phase formation, especially when the cation is nonbonding such as K^+, Na^+ or NH_4^+ (Sadeghi and Jamehbozorg,

2008). When comparing five different sodium salts, different degrees of incompatibility with PEG is detected. Tri-sodium phosphate showed to be the least compatible with PEG, indicating it to be a good choice for ATPS (Hey et al., 2005). There is a vast amount of different salts that can be studied in terms of phase separation in ATPS.

Biomolecules interact with salts differently depending on the ions present. Small, multivalent ions are more effective in salting out biomolecules (Hey et al., 2005). Commonly used polymer/salt combinations for ATPS are PEG/potassium phosphate and PEG/magnesium sulfate. These create effluents high in phosphate and sulfate however, which is damaging to the environment. Citrate systems have been proposed as a less toxic and biodegradable alternative (Porto et al., 2008). Environmentally benign ATPS have been developed using PEG and citrate salts to extract proteins from tannery waste water (Raja and Murty, 2012).

2.3.3.1.3 Temperature and interfacial tension

An increase in the temperature of the system results in a decrease in density of the phases (Regupathi et al., 2009). In polymer/polymer systems, phase formation is best at lower temperatures, while higher temperatures are favoured for phase separation in polymer/salt systems (Grilo et al., 2016). The interaction of polymer with water is affected by temperature. Increasing temperatures result in lower attraction between the polymer and water molecules. This drives water towards the salt phase, causing an increase in the salt phase volume and consequently decreasing in the salt concentration in the bottom (salt component) phase. It follows that the polymer in the top phase becomes more concentrated due to volume decrease of the top (polymer component) phase (Sadeghi and Jamehbozorg, 2008).

The interfacial tension of a system affects the degree to which a molecule can be partitioned. Lower interfacial tensions make for easier partitioning, thus the partition coefficient increases (Raja et al., 2012). The tie-line length of a system is related to the interfacial tension of the system, thus factors that affect the tie-line length of the system will also influence the interfacial tension. In systems which have the same component compositions, interfacial tensions increase with decreasing temperatures, while systems that do not have the same components but are similar in tie-line length, interfacial tensions are directly proportional to temperature increase (Grilo et al., 2016). Lower PEG M_w in the polymer phase lowers the interfacial tension of PEG/salt systems (Raja et al., 2012).

2.3.4 Aqueous two-phase extraction of phenolics

Many different ATPS have been developed for the extraction of different biomolecules, outlined in Figure 7. One research group optimised an ATPS for the extraction of phenols from *Eucalyptus* wood veneer trimmings (Xavier et al., 2014 & Xavier et al., 2015). The two optimised systems were both polymer/salt systems: PEG 2000/ ammonium sulfate (Xavier et al., 2014) and PEG 2000/sodium citrate (Xavier et al., 2015) respectively. These systems were optimised by way of experimentally determining the best variable for different parameters. For each study, three systems were used based on phase composition, which was determined using previously published phase diagrams for the specific phase combinations. Other parameters optimised were extraction temperature, extraction time, settlement time and solid-liquid ratio.

The two studies showed similar trends for the optimisation of the different parameters. Both polymer/salt systems showed a preferential partitioning of the phenols to the top phase of the system (the polymer phase). Different compositions of phases in each type of ATPS did not display any significant increase in phenols concentrations in the top phases. Phenol concentrations in the top phases increased with higher temperatures, but with this, partition coefficient in each system decreased. A similar trend was noted for extraction time, except there was no significant increases of phenol yields with time, but K did decrease with longer extraction time. It was also noted that a longer settling time did not have any significant influence, and partition occurred relatively quickly (Xavier et al., 2014 & Xavier et al., 2015). The first study determined that a lower solid-liquid ratio was more beneficial to higher yields as a greater percentage recovery from the solids was achieved (Xavier et al. 2014).

The work done by Xavier et al. (2014 & 2015) has shown that polymer/salt ATPS systems are feasible for the extraction of polyphenols from biomass. The same principles used in the previous studies can be used to study the extraction of polyphenols from grape biomass.

Along with the polymer/salt ATPSs, short-chain alcohol/inorganic salt systems have also been used in the extraction of phenols. The extraction of anthocyanins from purple sweet potatoes was done in an ethanol/ammonium sulfate system. Higher yields of the anthocyanins were observed when the ethanol concentration was increased (Liu et al., 2013). A similar study, extracting anthocyanins from grape juice using an ethanol/monosodium phosphate, also found that more of the anthocyanins partition towards the top phase with increasing ethanol concentration, with 90% of the anthocyanins concentrated in the top phase at optimal

conditions (Wu et al., 2014). When using an ultrasonic-assisted ethanol/ammonium sulfate ATPS for the extraction of curcumin, Xu et al. (2017) also found that increasing ethanol concentrations lead to higher yields of the phenol, but only up to a certain point, after which it levels out. The ultrasonication increases the rate of extraction, thus it is possible that all the anthocyanin is extracted after a shorter period. It should thus not be the goal to get the highest possible concentration of ethanol, but rather to evaluate which concentration works best for the phenol in question while optimising the amount of solvent used in experiments to minimise waste.

The ultrasonic-assisted ATPS was compared to conventional extraction methods (ultrasonic only extraction and stirring extraction) and found to extract higher yields of phenols while using less solvent (Xu et al., 2017). These results were however not tested for significance, thus further studies would be needed to draw a sound conclusion on the efficiency of assisted ethanol/salt ATPS. Another study compared the extraction of resveratrol, piceid and emodin using microwave-assisted ethanol/ammonium sulfate ATPS with microwave-assisted solvent extraction and heat reflux extraction (Wang and Dong, 2008). While the study also claims to show higher yields in the microwave-assisted ATPS with no significance testing, it makes a valid point that the ATPS would use less ethanol as the ethanol would be concentrated in the top layer, needing a lower volume of ethanol compared to the same ‘stock solution’ of ethanol in a solvent extraction which would need larger volumes. The study also mentions that the ethanol layer in the ATPS is clearer, resulting in purer purification (Wang and Dong, 2008).

The different studies agree that these ethanol/salt systems have the potential for industrialisation with larger scale extractions (Wu et al., 2014 & Xu et al., 2017). It would be beneficial to compare polymer/salt ATPS and ethanol/salt ATPS of the same phenol in parallel in order to see whether one is more efficient than the other. Should the systems show similar results, ethanol/salt systems may be a more cost-effective alternative to using large amounts of polymer.

Using these previous studies as a foundation, the extraction of polyphenols from wine waste using an ATPS can be investigated in a similar manner. It is evident that many possible systems could be used for the extraction of polyphenols. While several systems may technically be used, some systems will be more efficient for the extraction of polyphenols. The efficiency of extraction is evaluated by the yield of polyphenols obtained and the partition coefficient of polyphenols in a system. Optimal extraction will depend on the interaction of the polyphenols

with the phase components and the effects that system factors have on the partitioning. In the current study, PEG/salt systems will be looked at, taking into consideration the higher solubility of polyphenols in PEG compared to alcohol (Robinson et al., 2015).

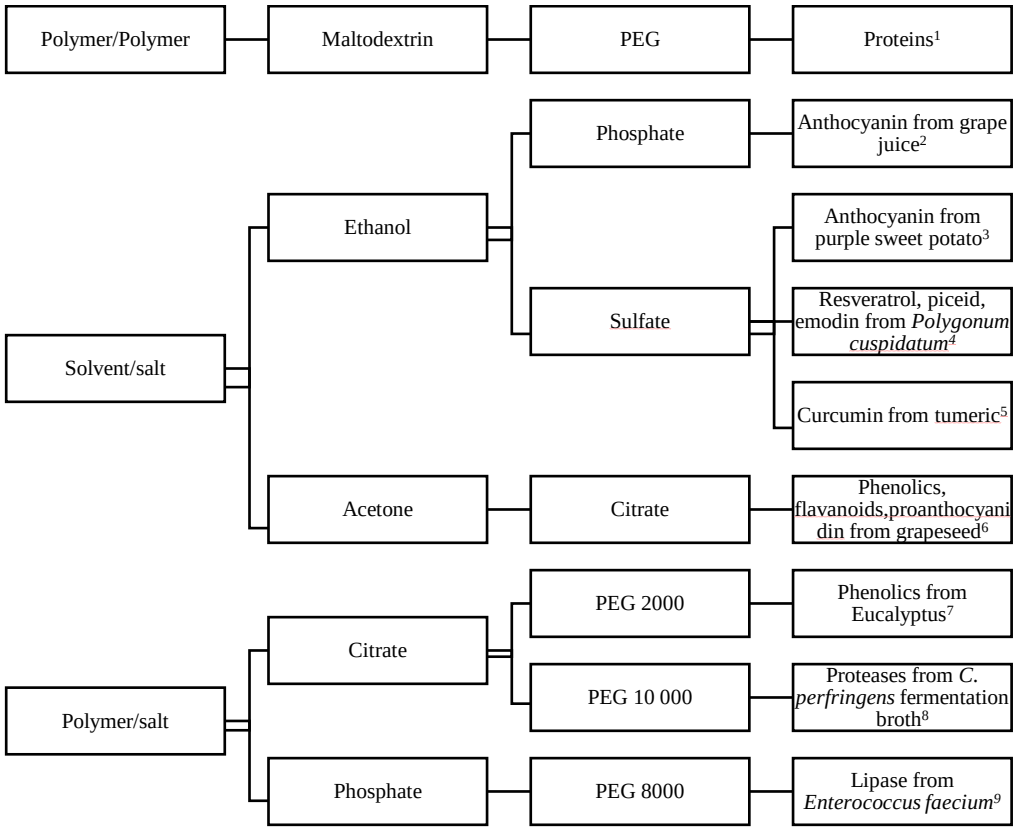


Figure 7 Different ATPS types used for the extraction of different molecules from various sources. ATPS types differ by different combinations of PEG, salt and solvents. ([1]Ramyadevi et al., 2012, [2]Wu et al, 2014, [3]Liu et al., 2013, [4]Wang et al, 2008, [5]Xu et al 2017, [6]Dang et al, 2013, [7]Xavier et al., 2015, [8]Porto et al., 2008, [9]Ramakrishnan et al., 2016)

2.3.5 Cost and environmental consideration of ATPS

Figure 8 serves as an input and output scheme for the resources of the ATPS process. The process preceding the dotted line is the primary process of the plant matter used, in this case the grapes being used for wine making. The process past that point is where the valorisation of the grape pomace starts, and thus where the cost and environmental considerations come into play for the purposes of this study. The cost of the grape pomace would depend on the source, as each wine producer may ask a different price at their discretion. In 2017 fresh grapes cost

an average of R4055 per tonne in South Africa (SAWIS); it is assumed that the spent grape pomace would cost less than this rate. The cost for the process would include capital cost, reagents and electricity. The capital would include infrastructure, tanks, mixers, refrigeration equipment and heating equipment. The electricity costs will be determined by the rate of the city or town in which the operations run, and capacity at which the process is running at any given time. The energy input of the process includes the energy used during the pre-treatment stage in the form of drying, milling and refrigeration should the pomace need to be stored, the energy used for the actual processing which would include heating and mixing, and the energy in the post process stage which could include heating or running of an additional separation technology. Waste outputs of the process include the spent pomace once the polyphenols are extracted and the used PEG and tartrate solutions. To minimise waste the solutions should be recycled back into the system to be used again, and the spent pomace sent to be used as fertiliser. The reagent costs would depend on the supplier and the ATPS being implemented, taking into consideration the PEG molecular weight, salt type and compositions of each of these, referring again to Appendix 1 for the costs. The cost put into the process needs to be minimised in order to gain a profit at the end of the day. A company supplying different polyphenols of HPLC grade (99.9%) quotes a range of ZAR 1712-3624 per 10mg depending on the specific polyphenol (Polyphenols AS, Norway), while a supplement with a blend of plant extracts (grape skins and seeds, apple and pomegranate amongst others) with lower concentrations of polyphenols sells for ZAR 600 (USD 40) per 53g of plant extract (120 capsules) (Bulletproof, USA). The quality of the product aimed for production should then be considered when looking at the cost invested into the process, as higher-grade polyphenols would need more equipment (eg HPLC for separation) but would yield a higher income.

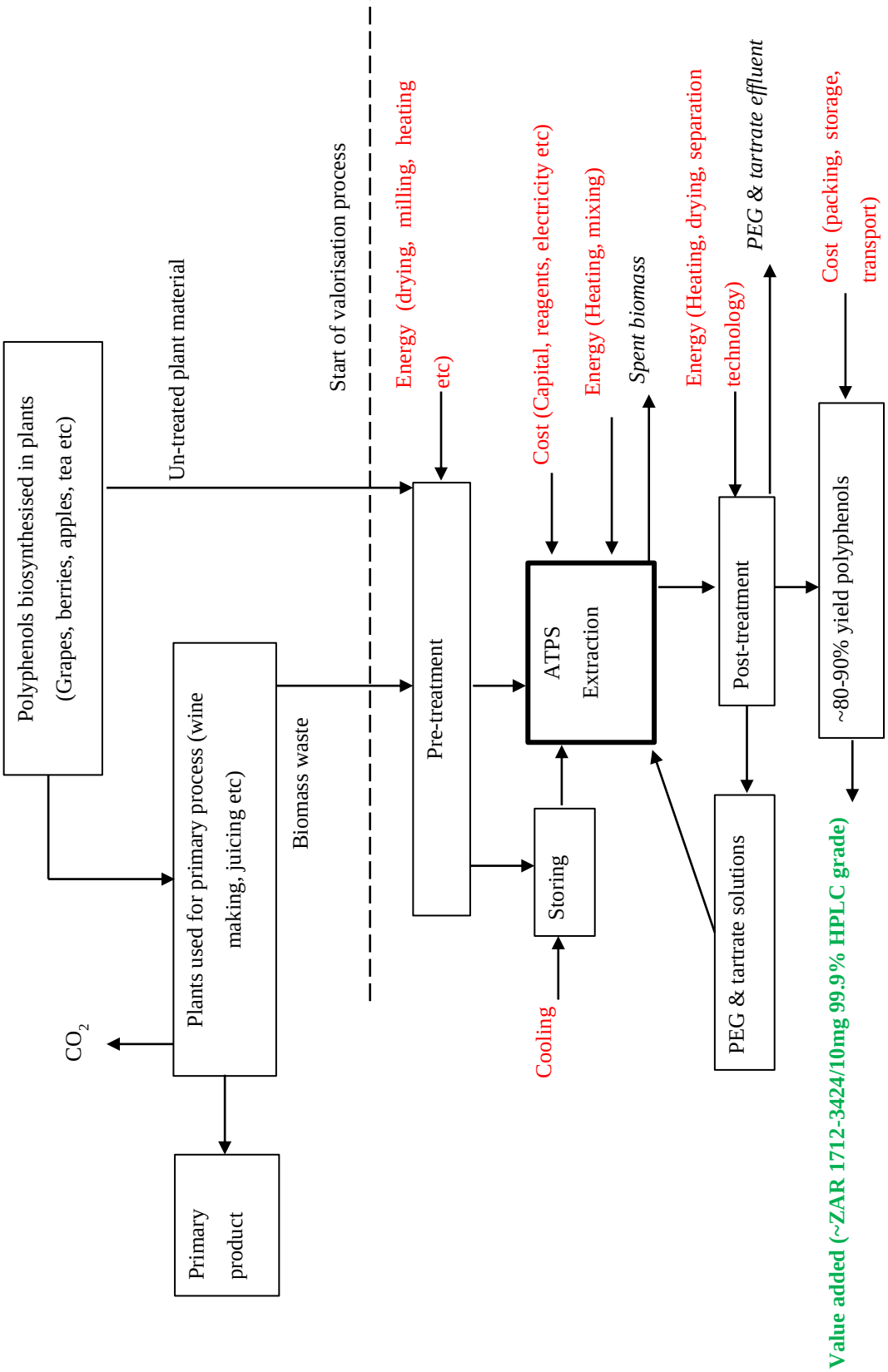


Figure 8 Cost and energy input for ATPS process

2.4 Concluding remarks

The use of different polyphenols for natural therapeutics has grown in popularity. While the investigation on its usability and scope of effectiveness is ongoing, the way in which it is extracted and prepared for further use has the potential to grow as well. Conventional extraction methods are employed, but are not necessarily the most cost effective or the most environmentally friendly. In the pursuit of a Green Chemistry centered extraction method, ATPS are a promising alternative. While the use of ATPS has been proved to be effective, there is still a gap in the knowledge of how it can be used for many different types of biomolecules. This work will then aim to close that gap by investigating the use of ATPS for extraction of polyphenols from wine solid waste, which is produced in huge quantities in South Africa, specifically in the Western Cape province. In doing that, a gap will also be closed in the characterisation of the ATPS systems employed, in terms of phase formation of different ATPS. This information can then be used in future studies as well as industry, allowing the gap of information to become smaller and smaller until there is a thorough understanding of the use of ATPS for extraction of biomolecules

Chapter 3 Phase formation and behaviour of PEG 6000, 8000 and 10 000 with potassium sodium tartrate aqueous two-phase systems at different temperatures

3.1 Introduction

A gap in the literature was found when looking for suitable ATPS for the context of this study. It was desirable to use an ATPS that uses environmentally friendly and consumer safe components while extracting polyphenols from grape pomace. To fill this gap, new phase diagrams were constructed using components that were identified as suitable. The construction of phase diagrams for ATPS are two-fold. The first component in the binodal curve, which indicates at which ratios of the two components, (i.e. the PEG and the salt) two phases will form. The second component is the tie-lines, which are straight lines which illustrate the phase behaviour at different conditions how the volume and composition of the phases change. These separate components are constructed individually using separate methods and analysis. In this current study new phase diagrams were constructed; the ATPS of interest were PEG with salt, using potassium sodium tartrate with different PEG M_w, at different temperatures. Potassium sodium tartrate was chosen for its biodegradability and use in food products (Vickers et al., 2007), as well as the fact that it is naturally present in grapes, which will come into play in the next chapter titled 'Aqueous two-phase systems for the extraction of polyphenols from wine solid waste'.

These phase diagrams produced were used to study the effect that temperature and PEG M_w have on the formation of the ATPS. The primary use of these phase diagrams however is to use the information for the next section, in which these ATPS will be utilized and modified for the extraction of polyphenols from grape skins, as discussed in the objections of the main study.

The data produced in this chapter has been published in an article titled 'The effect of temperature on different aqueous two-phase diagrams of polyethylene glycol (PEG 6000, PEG 8000, and PEG 10 000) + potassium sodium tartrate + water' in the Journal of Chemical and Engineering Data in June 2019 (DOI: 10.1021/acs.jced.9b00133).

3.2 Materials and Methodology

3.2.1 Materials

Poly(ethylene-glycol) of the following average molecular weights, 6000 $g.mol^{-1}$ (avg 5400-6600 $g.mol^{-1}$) and 10000 $g.mol^{-1}$ (avg 8500-11500 $g.mol^{-1}$) were purchased from Sigma Aldrich and 8000 $g.mol^{-1}$ (avg 7000-9000 $g.mol^{-1}$) was purchased from Kimix and used without further purification. Potassium sodium tartrate tetrathydrate was obtained from Sigma Aldrich and Kimix and used without further purification. Chemicals used for pH adjustments, ie HCl and NaOH were of analytical grade. Deionised water was used for solutions.

3.2.2 Parameters and variables

For this study, the two factors varied were PEG molecular weight and temperature. The salt component as well as the system pH was kept constant due to time constraint. The salt employed in this study was potassium sodium tartrate tetrahydrate. Each factor had three variables. The PEG molecular weight identified having a gap in the literature was PEG 6000, PEG 8000 and PEG 10000. Each PEG M_w was studied at three different temperatures; 10°C, 25°C and 45°C.

The phase diagram construction process was done in two parts. The first part collecting binodal data and constructing the binodals, and the second part collecting the phase composition data and constructing the tie-lines.

3.2.3 Methodology

3.2.3.1 Binodal curve construction

The binodal curve data was obtained by employing the cloud point method (Ananthapadmanabhan and Goddard, 1987). Stock solutions of PEGs of different molecular weights (PEG 6000, PEG 8000 and PEG 10000) and potassium sodium tartrate tetrahydrate were made up to mass fractions of 50% (w/v) using volumetric flasks. The pH of each solution was adjusted to 7 using small amounts of either hydrochloric acid or sodium hydroxide. In previously weighed and dried graduated falcon tubes, small aliquots of different volumes (0.1ml to 1.9ml) of the PEG solutions and tartrate solutions were added to obtain a total volume

of 2ml of phase forming systems. Each component was weighed on a mass balance. The two-phase systems were vortexed in order to mix the phase components, resulting in a turbid system, indicating that the individual systems were in the biphasic form. In a controlled thermostatic bath (with a fluctuation of ±1°C), demineralised water was added to each system dropwise while shaking until the previously turbid systems became clear. At this point, the total system mass was noted. This data was fitted to the semi-empirical Merchuck model from Merchuck et al. (1998) and Alvarenga et al. (2014) using non-linear regression in Graphpad Prism 7 to produce the binodal curves.

3.2.3.2 Experimental data collection for tie-lines

As done previously for the binodal curves, stock solutions of the PEGs and potassium sodium tartrate were made up in volumetric flasks to get 35% (w/v) solutions. The pH of each solution was adjusted to 7 using small amounts of either hydrochloric acid or sodium hydroxide. To make up three different ratios of systems, different volumes of PEG solution and potassium sodium tartrate solution were aliquoted into glass graduated measuring cylinders. The systems were mixed to turbidity and left at three different temperatures to settle for up to 24 hours to ensure equilibrium has been reached. Complete separation of phases was characterised by two clear phases with a clear interface. The separated top and bottom phase volumes were observed visually against the graduations on the cylinders and noted. The top phase was transferred to a clean test tube using a 1ml pipette, leaving behind a small volume above the interphase. The bottom phase was reached and transferred to a new test tube using a syringe with a hypodermic needle, ensuring no mixing with the remaining top phase. The separated phases were diluted appropriately for further analyses. The compositional data of the top and bottom phases as well as the total systems, collected as outlined in section 3.2.3.3 and 3.2.3.4, were used to fit straight lines to form the tie-lines. These tie-lines were added to the binodal curve graphs to get an overall picture of the phase diagrams.

3.2.3.3 Tartrate quantification

The bottom phases of the systems were quantitatively analysed for tartrate. In order to get a total tartrate concentration for the three different ratios, the 35% (w/v) potassium sodium tartrate solution was diluted with water to get the correct concentration. These were analysed in the same way as the samples. An end point method using an Enzytec™ Color Tartaric Acid

kit was employed and executed on a Thermo Scientific Arena™ 20XT Analyzer. The kit included a decolourant, buffer, Chromogen and calibrator. The samples were centrifuged in 1.5ml Eppendorf tubes and transferred to the instrument cartridge. The sample information as well as dilution factor to be applied by the instrument was programmed into the instrument computer, after which the instrument performed the analyses. Equal volumes of the diluted sample and decolourant (20µl each) were mixed and incubated for 2 minutes. The buffer was added (150µl) and the sample incubated for a further 2 minutes, after which the first absorbance reading (A1) was taken. The absorbance was measured at a primary wavelength of 505nm and a secondary wavelength of 700nm. The reaction was initiated by adding 15µl Chromogen to the sample and incubating at 37°C for 10 minutes. A second absorbance measurement (A2) was taken at 505 and 700nm. The first absorbance was subtracted from the second absorbance (A2-A1), and the difference was used to extrapolate the concentration in g/L from the calibration curve. The final result given has already taken into account the dilution factor applied by the computer.

3.2.3.4 PEG quantification

The PEG concentration was determined using a Dionex 3000 Ultimate HPLC system with an RI detector (Thermo Scientific), equipped with a Polysep GFC 3000 column sized 300x7.8mm (Phenomenex) using water as an isocratic eluent. The concentration of PEG in the top phase as well as the total system was determined, from which the bottom phase PEG concentration was calculated.

3.2.3.5 Analysis

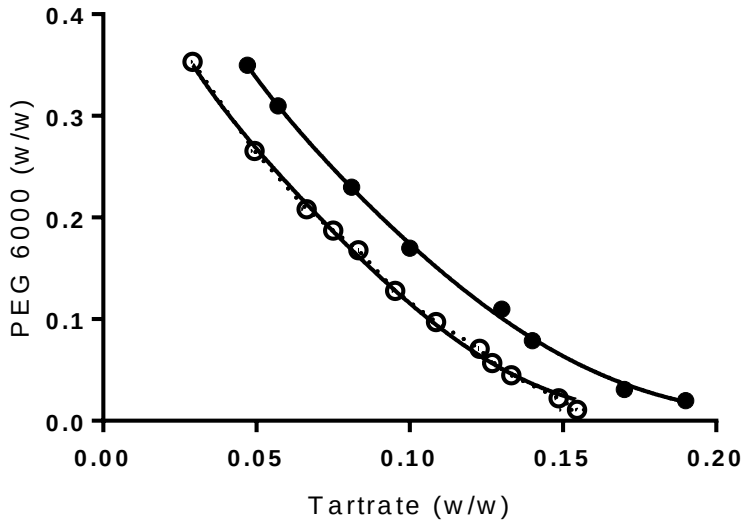
The tie-line data were used to analyse the effects of the PEG M_w and temperature on the phase formation of the different ATPS. The data were used to calculate the tie-line slopes (STL), tie-line length (TLL) and the tartrate partition coefficient (K). The data were also validated using the Othmer-Tobias and Bancroft equations as used in similar studies (Malpiedi et al., 2008 & de Andrade et al., 2011).

3.3 Results and discussion

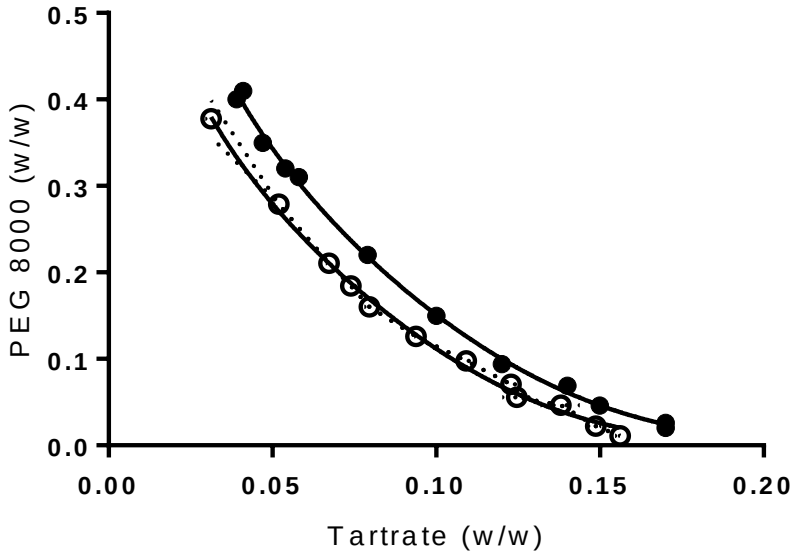
3.3.1 Method validation

The method used for the construction of the binodal element of the phase diagrams was adapted from other studies (Ananthapadmanabhan and Goddard, 1986 & Murari et al., 2015). Two previously published diagrams were repeated, to be used as a comparison to validate the method used for this study. The systems previously identified that were of interest were PEG 6000, PEG 8000 and PEG 10000 with potassium sodium tartrate. Of these, PEG 6000 and PEG 8000 (at 25°C) were already constructed in a previous study (Malpiedi et al., 2008). An adaptation of the cloud point method (as outlined in the Methodology section) was used in this study and compared to the results obtained using a turbidimetric titration method used in the literature. The data of the previous study was taken as presented in the literature and fit to the same model to be used in this study, the Merchuck equation (Merchuck et al., 1998; Alvarenga et al., 2014). The data collected using the method employed for the current study was converted to mass fraction (w/w) and also fit using the Merchuck equation.

The binodals produced in this study and those produced in the previous study are shown to be similar in Figure 9. The shape of the binodals follow the same shape and trend. The envelope size difference is small, the current study's method producing slightly bigger two-phase regions on the graph. These differences may be attributed to minor differences in experimental temperatures or pH of the system components, as the study used to compare the current data to prepared systems at a pH of 4.90. These differences are slight though, and as such the method chosen for this study can be confidently used for the production of further unpublished phase diagrams.



a



b

Figure 9 Equilibrium data (w/w) of systems formed using potassium sodium tartrate + water with: a) PEG 6000 gmol^{-1} and potassium sodium tartrate tetrahydrate and (b) PEG 8000 gmol-1 and potassium sodium tartrate tetrahydrate; (○) this work (pH 7) with standard deviation (···), (•) previous study (pH 4.90) (Malpiedi et al., 2008)

3.3.2 Construction of new phase diagrams

3.3.2.1 Binodals

Having tested the cloud point method, it was then employed to produce additional phase diagrams. To examine what the effect of PEG M_w is on the phase formation of these ATPS, a phase diagram of a system of PEG 10000 with potassium sodium tartrate at 25°C was produced. The temperature effects on the phase formation was then also looked at, and each ATPS (PEG 6000, PEG 8000 and PEG 10000 with potassium sodium tartrate) were reproduced at two additional temperatures, 10°C and 45°C. Figure 10 below outlines all the different systems produced, with diagrams previously published (Malpiedi et al., 2008) highlighted in grey.

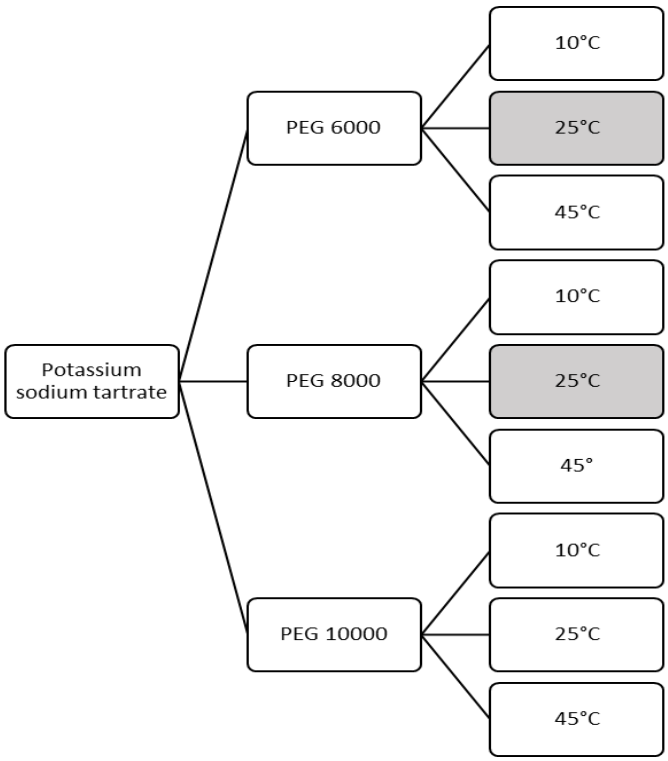


Figure 10 Outline of phase diagrams produced in this study. Factor one is constant, using potassium sodium tartrate. Factor 2 varies the PEG M_w. Factor 3 varies the temperature (°C)

The data point collected using the cloud point method were fit to a curve using non-linear regression, by employing a mathematical model known as the Merchuck equation shown in Equation 2,

$$w_P = a \exp(b{w_S}^{0.5} - c{w_S}^3)$$ Equation 2

where w_P and w_S are the mass fractions of PEG and potassium sodium tartrate (the experimental data) respectively and are used as coordinates for the binodal curves. The fit parameters of the equation are expressed as *a*, *b* and *c*, and were determined using non-linear regression. This empirical equation was proposed by Kaul et al. (1995) in collaboration with Merchuck et al. (1998) through a series of fluid dynamic studies of PEG/salt ATPS taking into account phase formation of different compositions of PEG and salt, and has subsequently been used successfully in phase characterization studies of PEG/salt ATPS (Alvarenga et al., 2014 & Zafarani-Moattar and Tolouei, 2008). The fit parameters for the three different PEG molecular weights studied at three different temperatures, along with the root mean square error (RMSE) and the coefficient of determination (R^2) values of the binodals produced are summarized in Table 5. These parameters effectively describe the shape and boundaries of the envelope, allowing us to accurately calculate either PEG or tartrate quantity if the other is known in areas on the envelope not covered by experimental data. This method does not act as a predictive model, as done previously for ATPS characterisation using NRTL modeling (Roosta et al., 2016).

Table 5 Values of fit parameters a, b, and c fit to the Merchuck equation for potassium sodium tartrate + PEG + Water Binodals at different temperatures, PEG Mw and pH 7

	Parameters				
Temp (°C)	**a**	**b**	**c**	**RMSE**	**R^2**
PEG 6000					
10	0.53	-2.84	-488.80	1.15E-04	0.999
25	0.71	-4.10	-525.90	2.51E-04	0.998
45	0.79	-4.63	-770.20	4.57E-04	0.996
PEG 8000					
10	0.64	-3.54	-458.60	2.65E-04	0.998
25	1.05	-5.66	-447.80	4.51E-04	0.997
45	0.67	-3.77	-1234.00	1.64E-03	0.986
PEG 10000					
10	1.14	-6.22	-334.90	2.78E-03	0.973
25	0.67	-4.30	-697.70	3.26E-04	0.996
45	0.89	-5.42	-1002.00	3.21E-04	0.997

RMSE, Root Mean Square Error, defined RMSE $= \sqrt{\frac{\sum(\text{residual}^2)}{\text{n-1}}}$

The RMSE values of the curves were low, while the R^2 values neared 1 (the lowest being R^2=0.973) indicating that the curves were fit well around the data points using the non-linear regression method. The impact of molecular weight of the PEG and system temperature on the phase formation will be discussed below, which will be important for industrial processing application.

3.3.2.1.1 Effect of temperature

When evaluating the effect that temperature has on the binodal curve of the phase diagrams, the envelope is the biggest consideration. The envelope is visualised on the phase diagram and quantified as the ratio of PEG and salt concentrations needed to form phases, effectively describing the extent of the biphasic region of the system. The envelope may get bigger or smaller depending on the conditions applied to the system. Any composition ratios above the envelope will produce an ATPS with two defined phases and may thus be used industrially for biomolecule extraction or purification. The temperature needed to form an ATPS will also have an effect on the industrial application of these ATPS. Higher temperature would need more energy input and thus be more costly. The temperature will also have to be considered when a specific molecule is used in application, as different molecules degrade at different temperatures. This will be looked at in more detail in the next chapter titled 'Aqueous two-phase systems for the extraction of polyphenols from wine solid waste'. The effect of temperature on the phase formation for three different PEG molecular weights was examined. The binodal curves for PEG 6000, PEG 8000 and PEG 10000 with potassium sodium tartrate at three different temperatures are reported in mass fraction (w/w) in Figure 11. Higher temperatures produced bigger envelopes regardless of the PEG M_w used. The increasing temperature had a direct effect on the density of the solutions, causing an increase in the density differences of the PEG and salt phases which drives the phase formation to be clearer (Barani et al., 2018). For all three systems, 45°C would then create a wider range of PEG-salt ratio at which two phases will successfully form. These findings are in agreement with previous phase behaviour studies in other polymer-salt systems which considered the effect of temperature on phase formation (Barani et al., 2018 & Grilo et al., 2016). The systems at 45°C appear to diverge the most from the other temperatures, having a bigger gap in the envelope size compared to the lower temperatures, especially for the systems of PEG 6000 and PEG 8000. This suggests that lowering the temperature below 25°C (or around room temperature) does

not have a big impact on phase formation and would be redundant. For future application of these systems, it would mean that at higher temperatures a lower concentration of components would be needed, saving costs on reagents. The energy required to raise and maintain the temperature for a time would have to be considered though to weight against the benefit of saving on reagents. The biomolecules to be used in the application of the ATPS would also have to be considered, as these may lose stability at different temperatures.

3.3.2.1.2 Effect of molecular weight

Together with temperature, the molecular weight of the PEG used in the system also has an effect on the phase formation of the ATPS. As with previous studies Barani (2018), it was found that higher PEG molecular weights create a bigger biphasic area when mixed with similar concentrations of salt (Figure 11d). PEGs with higher molecular weights are more hydrophobic, thus allowing for better phase formation compared to lower molecular weight PEGs Barani (2018). In the systems produced in the current study, the highest PEG M_w (PEG 10000) had a noticeably bigger envelope, compared to the envelopes of PEG 6000 and PEG 8000 which were practically similar. The application of this would mean that less reagent would be needed if using PEG 10000 in the systems compared to using either PEG 6000 or PEG 8000. This again should be evaluated in relation to cost of the different PEG M_w, as well as the biomolecules to be separated, as these may have different affinities to different PEG M_w.

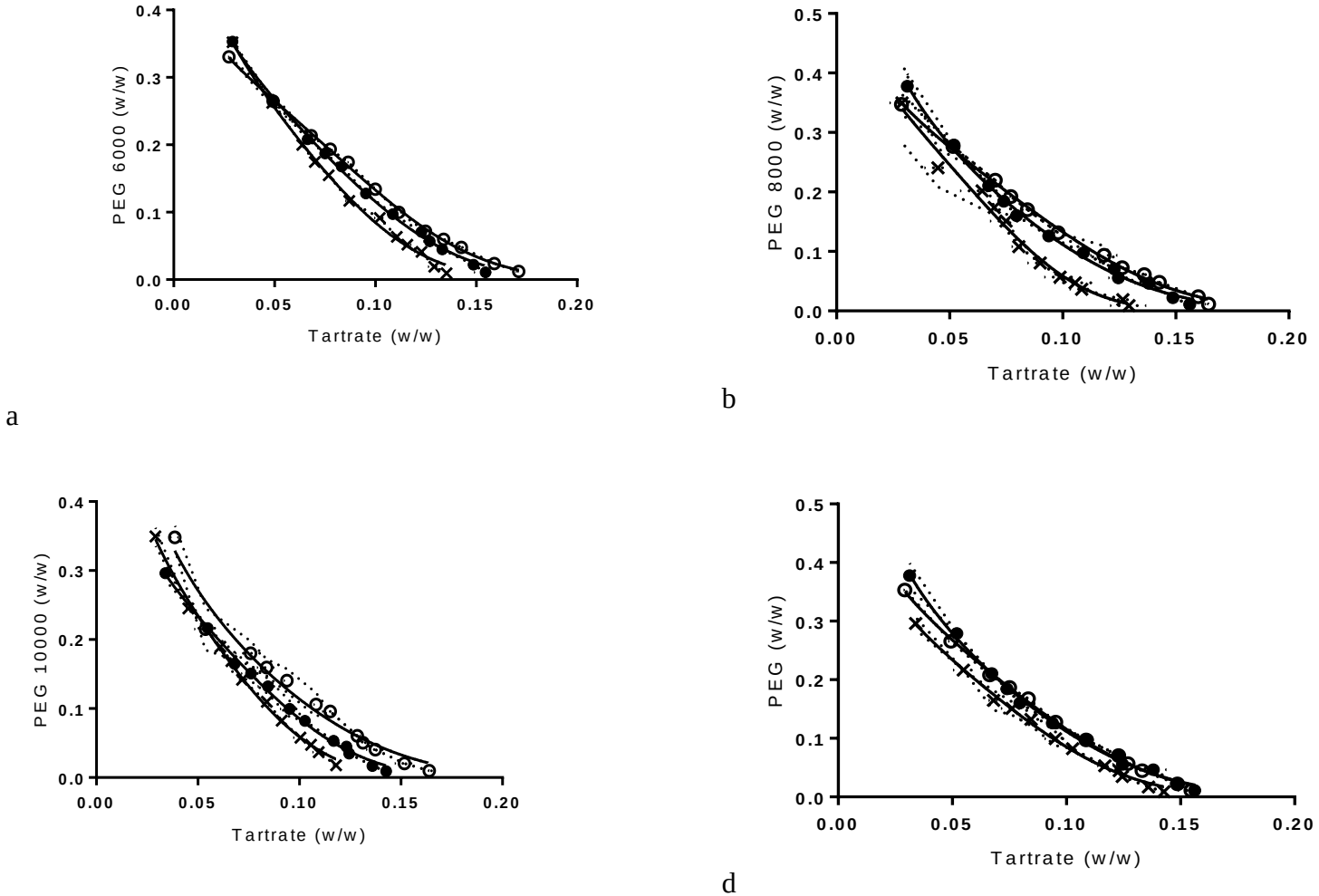


Figure 11 Equilibrium data (w/w) of systems formed using potassium sodium tartrate + water with: a) PEG 6 000 gmol-1, b) PEG 8000 gmol-1, c) PEG 10000 gmol-1 at pH 7 and temperatures (○) 10°C, (•) 25°C and (x) 45°C respectively; d) effect of PEG molecular weight on phase formation using potassium sodium tartrate tetrahydrate + water and (○) PEG 6000, (•) PEG 8000 and (x) PEG 10000 at 25°C and pH 7. Standard deviations are shown (···).

3.3.2.2 Liquid-liquid equilibrium study (Tie-lines)

The liquid-liquid equilibrium (LLE) part of the study centres around the phase compositions once a biphasic ATPS has been achieved. The LLE tells us how the phases separate, the components of each phase being dependent on the conditions applied to the ATPS. The LLE information is obtained through experimental studies and the data manipulated into tie-lines, allowing for easy visualisisationof what is happening in the separated phases of the ATPS. Figure 12 serves as an illustration of how these data are represented in a tie-line.

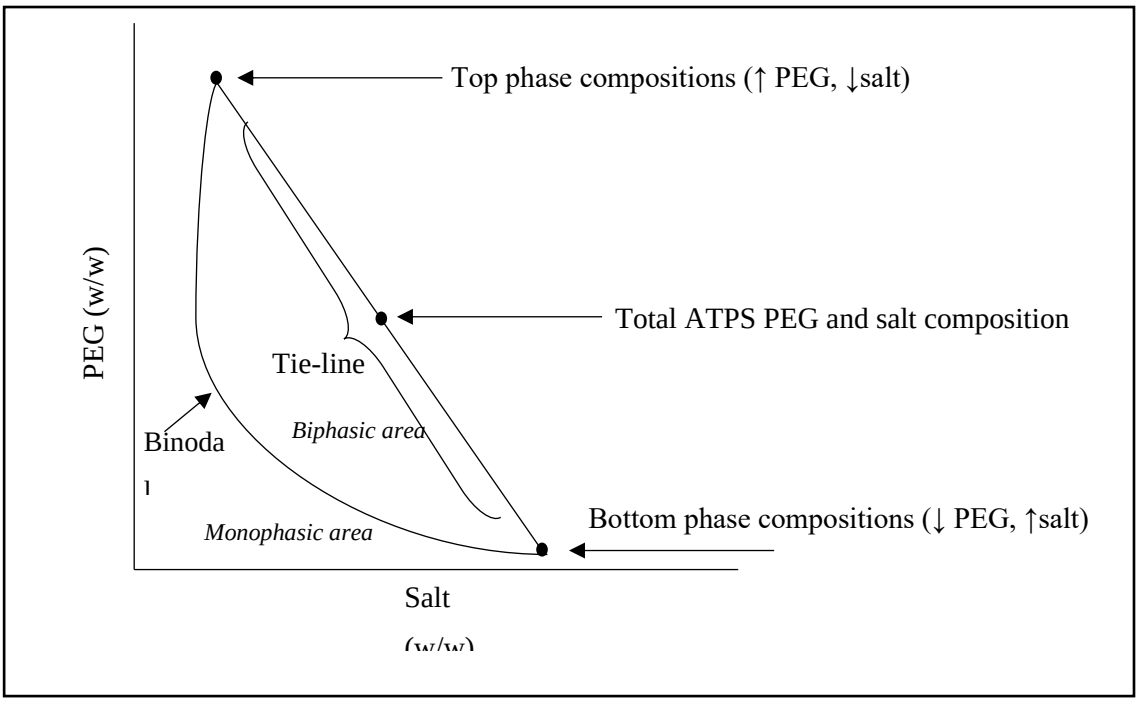


Figure 12 Depiction of tie-line components, including top phase, bottom phase and total compositions

Through the experimentation outlined in section 3.2.3, the composition data for ATPS separated phases was obtained. These ATPS were put under the exact same conditions as the previous section in which the binodals were obtained. Thus, tie-lines for PEG 6000, PEG 8000 and PEG 10000 each with potassium sodium tartrate and at 10°C, 25°C and 45°C each were studied. The data obtained from these experiments and presented in Table 6 were the volume of the separated phases, the concentration of the PEG in each phase, and the concentration of the tartrate in each phase. Along with the composition of the total systems and the top and

bottom phases, the R^2 values of the tie-lines formed using these data points is given. These R^2 values were mostly above 0.9, indicating that the tie-lines were fit well onto the LLE data. A theoretical R^2 of 1 would mean a perfect mass balance. In reality uncertainties arise from different factors including environment where certain things were beyond the control of the researcher such as shared oven space. The analysis, in this case HPLC for PEG quantification and an Enzyme kit for tartrate analysis would also contribute to uncertainties, the Enzyme kit contributing the most uncertainty. This was managed by analysing the samples all together with the same kit, with the help of a trained technician. Uncertainties which may arise from volume estimation was managed by using glass volumetric flasks, meaning no or less volume expansion taking place due to heat application on the vessel, and the same flasks were used over again.

It was clear that the PEG and the tartrate separated into two distinct phases. The top phases of all systems regardless of the system total components consisted mainly of the PEG component, nearing a concentration of 30% (w/w) of PEG and less than 10% (w/w) of tartrate. The bottom phases were tartrate heavy, ranging in concentrations between 11-13% (w/w) of tartrate and the PEG concentration being less than 1% (w/w) for all systems. The top phases would then be the organic, hydrophobic phase, with the bottom phase being the aqueous phase. It is these differences in hydrophilicities that will later drive the biomolecules to be separated from one phase to the other. The high separation ability of the PEG and salt also make potential recycling of the solutions easy, as there would be no need for an extra purification step. The fact that the top and bottom phase compositions were similar for ATPS with relatively different total compositions means that the reagent can be optimised for cost effectiveness. Considering Appendix 1, PEG 8000 is 9x more expensive than potassium sodium tartrate, thus optimising the reagents to use as little PEG as feasibly possible would cut down costs of the process. Similar phase compositions are seen in a previous study where PEG 6000 and PEG 8000 were paired with tartrate at 25°C, despite the pH differences of the two studies (pH 7 for this study and pH 4.90 for the previous study) (Wysoczanska & Macedo, 2016). This data was used for several calculations which help us to better grasp the behaviour of the systems.

ANOVA tests were performed to ascertain whether the STLs were significantly different for the different PEG M_w at different temperatures (at 95% confidence). For the majority of the systems, it was indicated that the STL were not significantly different. This means that using

the known STL, top and bottom phase compositions of a system with known total compositions can be calculated.

The LLE data produced in this study for PEG 10000 follows this same trend at all three temperatures. This similarity suggests that the phase compositions of different PEG molecular weights with potassium sodium tartrate systems can be estimated at the very least, allowing for quick and economical set up of different systems for practical use.

By looking at the partition coefficient (K) of tartrate calculated using Equation 3 in the systems, a quick view of the separation efficiency of the systems can be seen.

$$K = \frac{[tartrate]_{bottom}}{[tartrate]_{top}} \qquad \text{Equation 3}$$

A K value above 1 is desirable and would signify that the tartrate has separated well from the PEG component, producing ATPS with two defined phases. High K values were obtained, ranging between 1.63-5.55 shown in Table 6, signifying good phase separation and confirming that the top phase of the ATPS consists mainly of the PEG component and the bottom phase the tartrate component.

A more comprehensive view of the system is given by the slope of the tie-lines (STL) in Equation 4.

$$\text{STL} = \frac{\Delta\text{PEG}}{\Delta\text{Salt}} \qquad \text{Equation 4}$$

The STL indicates the magnitude of the mutual solubility of the PEG and salt solutions, an increase in STL depicting a decrease in the mutual solubility. A higher STL value would therefore mean that a system has a bigger difference in PEG concentration between the two phases at a given difference in salt concentration (Malpiedi et al., 2008), providing a more defined two phase system with the top phase being more hydrophobic making the driving force between the phases bigger.

The tie-line lengths (TLL) were calculated using Equation 5,

$$TLL = [(w_P^t - w_P^b)^2 + (w_S^t - w_S^b)^2]^{0.5} \qquad \text{Equation 5}$$

where w^t and w^b are the top and bottom mass fractions of PEG (P) and potassium sodium tartrate (S) respectively.

Table 6 Phase compositions in mass fraction (w/w) for Potassium sodium tartrate + PEG + water biphasic systems at different temperatures and pH 7

Total compositions		Top compositions		Bottom compositions						
Salt	**PEG**	**Salt**	**PEG**	**Salt**	**PEG**	**R^2**	**Sy.x**	**STL**	**100TLL**	**K**
PEG 6000										
10°C										
0.0982	0.140	0.0642	0.296	0.137	0.0116	0.991	0.0190	-3.90	29.4	2.13
0.0845	0.174	0.0653	0.291	0.133	0.00967	0.978	0.0299	-3.99	29.0	2.04
0.0722	0.210	0.0740	0.270	0.121	0.0339	0.924	0.0481	-4.28	24.1	1.63
25°C										
0.0982	0.140	0.0625	0.347	0.129	0.00757	0.993	0.0197	-5.15	34.6	2.06
0.0845	0.154	0.0631	0.323	0.124	0.00356	0.959	0.0458	-5.08	32.6	1.96
0.0722	0.210	0.0622	0.298	0.126	0.0273	0.968	0.0345	-3.95	27.8	2.03
45°C										
0.0982	0.140	0.0554	0.393	0.130	0.000	0.939	0.0587	-4.33	32.6	2.35
0.0845	0.154	0.0437	0.379	0.123	0.0102	0.987	0.0296	-4.64	37.7	2.82
0.0722	0.210	0.0547	0.357	0.111	0.0160	0.982	0.0329	-5.89	34.6	2.03
PEG 8000										
10°C										
0.098	0.121	0.050	0.219	0.140	0.0596	0.995	0.00834	-1.78	18.3	2.82
0.084	0.148	0.065	0.247	0.131	0.0591	0.958	0.0272	-2.73	19.9	2.00
0.072	0.173	0.071	0.196	0.126	0.173	0.768	0.00774	-0.322	5.90	1.76
25°C										
0.098	0.121	0.038	0.289	0.145	0.0382	0.999	0.00162	-2.54	27.2	3.78
0.084	0.148	0.054	0.230	0.133	0.0646	0.991	0.0110	-2.07	18.3	2.46
0.072	0.173	0.060	0.215	0.125	0.111	0.981	0.0104	-1.53	12.3	2.08
45°C										
0.098	0.121	0.034	0.302	0.127	0.0458	0.999	0.00379	-2.76	27.2	3.69
0.084	0.148	0.042	0.280	0.122	0.0543	0.995	0.0112	-2.83	23.9	2.89
0.072	0.173	0.047	0.258	0.115	0.0820	0.983	0.0164	-2.54	18.9	2.47
PEG 10000										
10°C										
0.0982	0.145	0.0469	0.296	0.144	0.0376	0.996	0.0121	-2.66	27.6	3.07
0.0839	0.177	0.0560	0.291	0.135	0.0391	0.987	0.0205	-3.12	26.5	2.42
0.0722	0.200	0.0601	0.270	0.136	0.0274	0.982	0.0238	-3.04	25.5	2.27
25°C										
0.0982	0.145	0.0275	0.347	0.146	0.0204	0.999	0.00574	-2.76	34.7	5.31
0.0845	0.178	0.0518	0.323	0.131	0.0222	0.994	0.0164	-3.79	31.1	2.52
0.0722	0.200	0.0550	0.298	0.123	0.0217	0.988	0.0217	-3.93	28.4	2.24
45°C										
0.0949	0.141	0.0237	0.393	0.132	0.0445	0.995	0.0180	-3.27	36.5	5.55
0.0845	0.178	0.0361	0.379	0.129	0.0248	0.997	0.0127	-3.83	36.6	3.57
0.0722	0.200	0.0483	0.357	0.122	0.0000	0.983	0.0328	-4.74	36.5	2.52

3.3.2.2.1 Effect of temperature

As with the binodals, the effect that temperature has on the formation of the ATPS was looked at in the context of the LLE data. To achieve this in a way that is easily comprehensible, the correlation between the three temperatures studied were correlated to the three factors calculated using the LLE data.

The first and most simple factor, the K of the tartrate, showed a moderate positive correlation across all three PEG M_w systems as can be seen in Table 7. This shows that when the system temperature is increased, the fraction of potassium sodium tartrate in the bottom phases of the systems increases. Better phase separation is thus achieved at higher temperatures. Sé & Aznar (2002) came to the same conclusion, stating that the mutual solubility of a PEG 4000 solution and potassium phosphate solution in an ATPS decreases with increasing temperatures, meaning that the phase separation ability increases.

These conclusions were corroborated with the STL data. Increasing temperatures saw steeper slopes of the tie-lines, with moderate to strong correlations for all three PEG M_w systems. While the correlations reported are negative numbers in Table 7, this is because of the negative slope of the tie-lines, and the correlation is interpreted as being positive. This proves again that the systems have a higher separation resolution at higher temperatures. These findings are in accordance with previous LLE studies in which it was shown that the separation process of the ATPS is entropy driven (Murari et al., 2015).

The TLL of the LLE data for the systems of all the PEG M_w had strong correlations to both temperature and lnK. The strong correlation between TLL and ln(K) indicates that longer tie lines are indicative of better phase separation. When interpreting LLE data for further implication in studies or industries, it would then be useful to use the TLL as a quick indicator of which systems would have higher separation qualities. Energy would be needed for heating to the appropriate temperatures when applying these ATPS to an industrial scale. This energy usage will increase costs as well as decrease the ‘green’ effect of these ATPS as fuel would need to be used (in the form of electricity supplied or fuel burned on site).

Table 7 Correlation of TLL, lnK, STL and temperature for the ATP systems

	Temperature	TLL
PEG 6000		
lnK	0.645	0.770
STL	-0.559	-0.617
TLL	0.771	-
PEG 8000		
lnK	0.523	0.886
STL	-0.587	-0.902
TLL	0.543	-
PEG 10 000		
lnK	0.441	0.654
STL	-0.648	-0.437
TLL	0.931	-

3.3.2.2.2 Effect of PEG molecular weight

The effect that the PEG M_w has on the phase separations was studied as well. Each factor was correlated again with the PEG M_W at each different temperature in

Table 8. From these results, it seems that the PEG M_w has an impact on the phase separations. PEG M_w was positively correlated to the K of tartrate, indicating that a higher PEG M_w excludes more tartrate from the top phase. PEG with higher M_w increases the hydrophobicity of the solution, which would explain why a higher PEG M_w leads to better phase separation from the aqueous phase (salt rich phase) of the ATPS and has been exhibited in other phase formation studies (Wysoczanska & Macedo, 2016). The STL correlation is interpreted as being negative (due to the STL data being negative). The moderate negative correlation of STL to

PEG M_w suggests that the PEG M_w has an inverse impact on the STL, meaning that the differences in PEG and salt concentrations between the phases becomes more equal.

The mechanisms of these interactions are complex and are influenced by many competing factors. The outtake of these data is that increasing the PEG M_w in an ATPS will lead to better phase formation. The range of PEG M_w looked at in this study is relatively narrow, thus these differences may be exaggerated with a wider selection of PEG M_w. For application to an industrial scale, these results would indicate that a higher PEG M_w would be beneficial for a clearer ATPS in terms of phase separation. Higher PEG M_w does have a higher viscosity though (Gonzalez-Tello et al., 1994), thus the workability of the PEG should also be taken into account considering the equipment available.

Table 8 Correlation of TLL, lnK, STL and PEG Mw for the ATP systems

	PEG M_w	**TLL**
10°C		
lnK	0,607	0,317
STL	0,393	-0,878
TLL	-0,057	-
25°C		
lnK	0,553	0,270
STL	0,418	-0,767
TLL	-0,012	-
45°C		
lnK	0,623	0,189
STL	0,388	-0,699
TLL	0,100	-

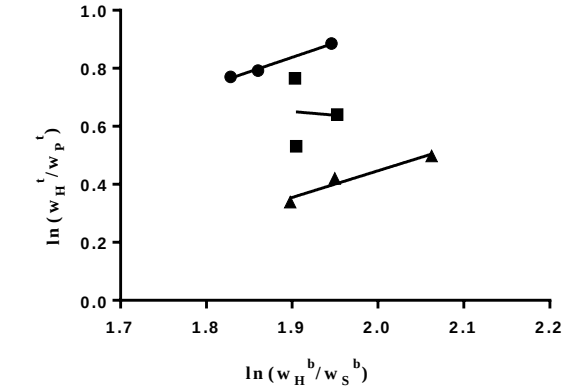
3.3.2.2.3 Tie-line Validation

The LLE data were validated using two empirical correlations, the Othmer-Tobias in Equation 6 and Bancroft in Equation 7 empirical equations which have been used in previous phase separation studies to validate the data is thermodynamically sound (Murari et al., 2015 & Hamzehzadeh & Zafarani-Moattar, 2015),

$$\left(\frac{w_H^b}{w_S^b}\right) = k\left(\frac{w_H^t}{w_P^t}\right)^r \qquad \text{Equation 6}$$

$$\left(\frac{1-w_P^t}{w_P^t}\right) = k_1\left(\frac{1-w_S^b}{w_S^b}\right)^n \qquad \text{Equation 7}$$

where w^b and w^t indicates the bottom and top weight fractions (w/w) of water (H), PEG (P) and potassium sodium tartrate (S) respectively and k, k_1, r and n are fit parameters. Linearisation of these equations indicate that the LLE data is valid and, in most cases, produced acceptable consistency in Figure 13. The inconsistencies seen in Figure 13.a and Figure 13.c could be due to possible discrepancies in measurement or temperature fluctuations during experimental setup. The experiments were repeated and while there was some larger variation with larger error, these errors and R^2 values were within what is generally considered an acceptable range. Taking into consideration the low errors (Sy.x) and R^2 which near 1 as presented in Table 6, these inconsistencies prove not to be significant enough to disregard the data. It is possible that another factor had an impact on the inconsistencies. The fit parameters, together with the standard deviations of the residuals were determined and are presented in Table 9.



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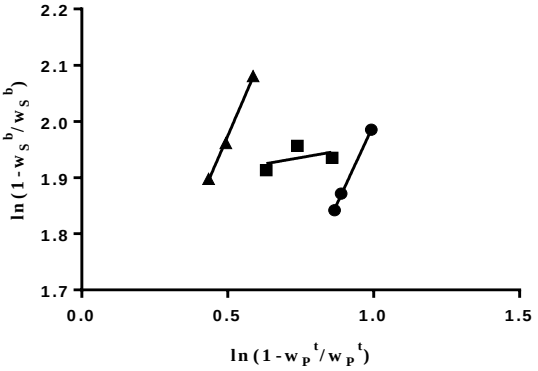

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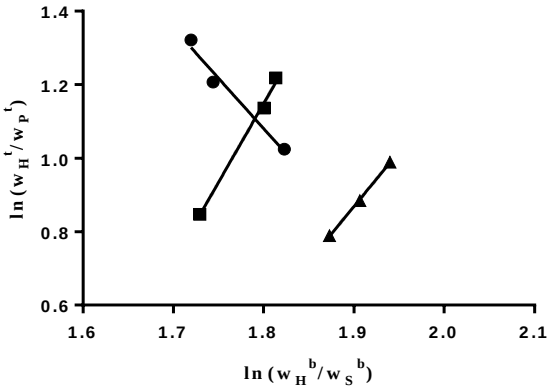

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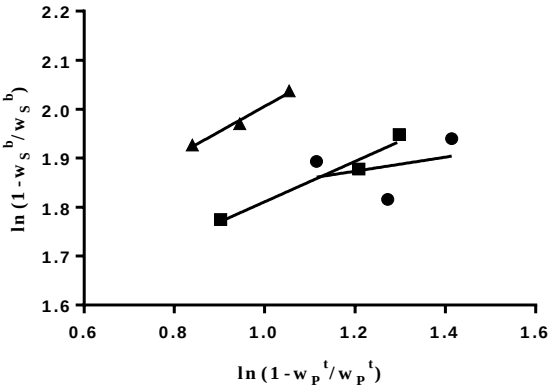

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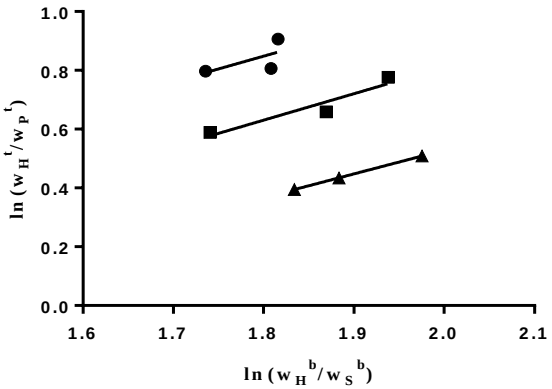

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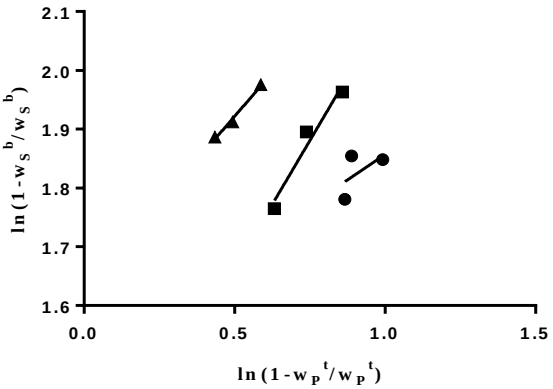

f

Figure 13 Linearisation of thermodynamic consistency models- Othmer-Tobias equation (a) PEG 6000, (c) PEG 8000, (e) PEG 10000 and Bancroft equations (b) PEG 6000, (d) PEG 8000, (f) PEG 10000 ; •10°C;■ 25°C ; ▲45°C

Table 9 Values of the Parameters of the Othmer-Tobias and Bancroft equations, k, r, k1 and n for potassium sodium tartrate +PEG+ water systems at pH 7 and different temperatures

Temperature	k	r	Sy.x	K_1	n	Sy.x
PEG 6000						
10°C	0.342	1.01	0.0177	2.39	1.12	0.0224
25°C	4.18	-0.406	0.316	6.48	0.0886	0.187
45°C	0.254	0.909	0.0377	3.93	1.21	0.0417
PEG 8000						
10°C	455	-2.80	0.118	5.42	0.154	0.538
25°C	0.00131	4.33	0.0616	4.02	0.418	0.164
45°C	0.00796	3.00	0.0116	4.43	0.519	0.0607
PEG 10 000						
10°C	0.466	0.896	0.156	4.57	0.338	0.292
25°C	0.352	0.929	0.0842	3.47	0.849	0.196
45°C	0.338	0.807	0.000984	5.08	0.595	0.0481

Sy.x, standard deviation of the residuals, defined $\text{Sy.x} = \sqrt{\frac{\sum(\text{residual}^2)}{\text{n-K}}}$, where K=number of parameters fit

3.4 Conclusion and implications for further study

Phase diagrams for ATPS systems of PEG 6000, PEG 8000 and PEG 10000 with potassium sodium tartrate at 10°C, 25°C and 45°C were produced and presented. A cloud point method was evaluated and deemed viable to use in the current study by comparison of results from a previous study. The current study produced a total of seven new phase diagrams, contributing to greater data base of ATPS phase formation studies. In addition to producing these new phase diagrams, the diagrams were used to investigate the effect that temperature as well as PEG M_w weight has on the phase formation of these ATPS. It was found that higher temperatures and bigger PEG M_w were favourable to phase formation.

Clear phase separations were seen where the top phases of the ATPS consisted mainly of PEG and the bottom phases were mainly consisting of tartrate. The equilibrium studies showed that temperature had a positive effect on the LLE, in that higher temperatures led to a definite increase in phase separation clarity. The effect of PEG M_w was not so clear concerning the LLE,.This could be due to the narrow range of PEG molecular weights employed. . These phase diagrams can now be used for application in extraction of biomolecules from plant matter. The next Chapter of this study does just that, applying these systems to the extraction and separation of polyphenols from grape skins.

Chapter 4 Aqueous two-phase systems for the extraction of polyphenols from wine solid waste

4.1 Introduction

The wine industry produces relatively large volumes of waste in terms of natural resources. While there are many facets of potential wastage ranging from water usage for irrigation or cleaning to underutilising CO_2 produced during the fermentation process, this study looks at utilising the grape pomace left over after it has been used for its primary application in the wine making process. The spent grape pomace is used again in an agricultural sense for fertiliser or sheep feed (Burg et al., 2014 & Guerra-Rivas et al., 2016) , but it has the potential to be utilised further in a biotechnological sense by extracting useful molecules that are present in the grape skins, seeds and stems.

In this section, the primary goal was to examine the use of PEG/salt ATPS for the extraction of phenolics from the spent grape pomace for future application in supplements or cosmetics as antioxidants. This was done by a series of experiments in which the factors such as PEG M_w, salt type, TLL, temperature, time, pH and biomass loading, which have an influence on ATPS formation as well as phenolic extraction were varied. The phase diagrams produced in Chapter 3 were used to inform the first set of experiments in which PEG MW and phase formation temperature were the factors studied, from where the other factors were then varied. Ultimately, an ATPS suitable for extraction of TPC from grape pomace on a large scale was suggested based off the results from these experiments, taking into consideration the yield of total phenolic content extracted and the concentration ability of the ATPS.

4.2 Materials and Methodology

4.2.1 Grape preparation

Spent wine grape skins were collected from an undisclosed wine farm in the Stellenbosch winelands region. The skins were dried in an oven at 55°C to increase homogeneity of the grapes, eliminating the possibility of the water content of the grapes influencing the mechanism

of the ATPS through dilution. The grapes were then milled for 5 minutes in batches of 250ml in a kitchen blender (Nutribullet, 600 watt) until it had a consistency of powder. The particle size distribution is shown in Appendix 2.. The kitchen blender is akin to an industrial mill which would be used for bigger scale processes. The powdered grapes were kept in the dark in a closed container. The same grapes were used for all the experiments, minimising any heterogenous effect that natural biomass has on experiments of this nature. Variations in sample characteristics were avoided by storing the milled grapes in one container, shaking the container before each sample extraction, avoiding sample variation in so far possible.

4.2.2 Solvent extraction

Dried and milled grapes skins prepared as detailed in section 4.2.1 were used for solvent extraction of polyphenols in order to quantify TPC available for extraction from the grapes using a traditional extraction method. Ethanol (99% v/v) was diluted with demineralised water to a ratio of 80:20 (v/v). A biomass loading of 1:10 of solid to liquid ratio was used and three different extraction times compared. The extraction took place at room temperature (~20°C) on a rotator (50 rpm). Once the respective extraction times (one hour, four hours and 24 hours) were over, 10ml of the solutions with the biomass were centrifuged for 10 minutes at 4000 rpm (Eppendorf) and the supernatant drawn off from the biomass with a syringe for analysis. Tripilicate extractions were done to get an average result.

4.2.3 Aqueous two-phase system set up

For the experiments outlined below, the same setup was used, varying different factors. For the sake of brevity, the setup will be explained and outlined once and only the variables discussed further. The extractions were performed as per the process shown in Figure 14. The PEG solution (50% w/v) and salt solutions (50% w/v) were added together to a total volume of 10ml in a 50ml falcon tube and vortexed to ensure the system was in fact in its biphasic region, which was confirmed if the liquid was turbid. The previously prepared grape skins were added to the system and set on a rotator for a period of time (a variable outlined below). Once the contact time was over, the falcon tubes were centrifuged for 10 minutes at 4000 rpm (Eppendorf) and the supernatant (being the ATPS) extracted using a 1 ml plastic syringe and transferred to a graduated glass volumetric flask. Any residue left over (ie the biomass with some left-over liquid from the ATPS) was filtered through a muslin cloth. The volumetric flasks were inverted

by hand for 30 seconds to mix the phases, and then the ATPS's were left at specific temperatures (another factor outlined below) to separate into two phases. The top and bottom phase volumes were then observed visually using the graduation on the volumetric flasks and noted. The top phase was drawn off using a clean 1ml plastic syringe and transferred into a clean tube, leaving a small volume above the interphase area to avoid phase mixing. The bottom phase was transferred using a clean 1ml plastic syringe with a hypodermic needle in order to 'pierce' through the remaining top phase and avoid mixing of phases. The separated phases were then frozen at -18°C for further analysis. This was done in triplicate for each variable.

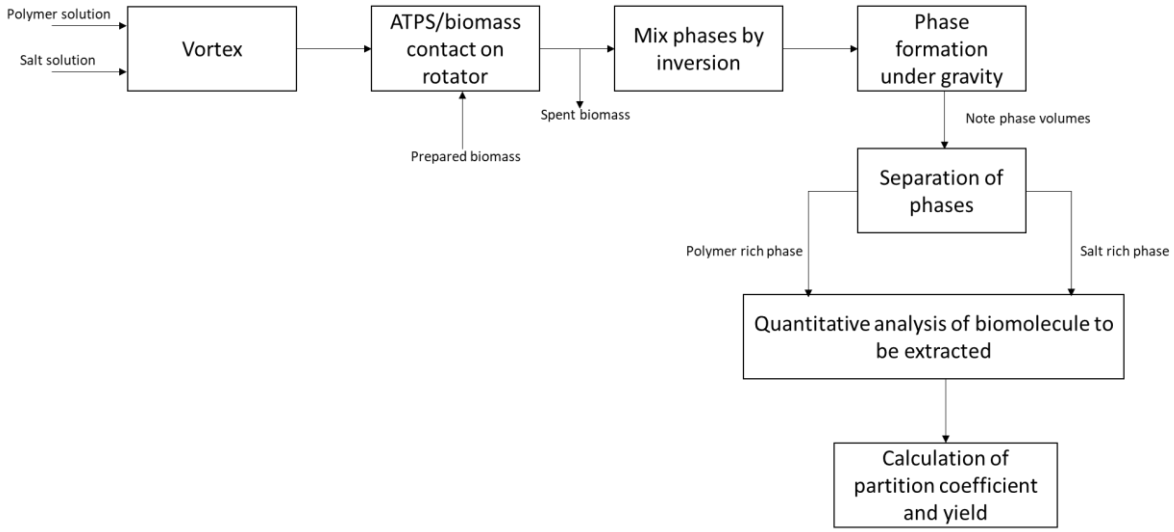

Figure 14 Flow diagram of typical ATPS experimental process

4.2.3.1 Response surface of PEG M_w and settling temperature

Response surface methodology was used to study the effect of PEG M_w and settling temperature on the yield and K of TPC. Using the phase diagrams produced in Chapter 3, three variables of each factor were looked at, using PEG 6000, PEG 8000 and PEG 10000 as the different molecular weights and studying each PEG M_w at 10°C, 25°C and 45°C. The salt was kept as a constant, using potassium sodium tartrate. Other factors kept constant were biomass loading at 10% (w/v), Tie-line length (18.3%), extraction temperature (room temperature), a system pH of 7 and an extraction time of 4 hours.

4.2.3.2 Independent Variables

Using a references system from the above experiments, a few other contributing factors were varied to see how the yield and K were affected. The factors varied included the tie-line length, extraction time, extraction temperature, salt type, pH, and biomass loading. Outlined in Table 10 below are the different variables for each factor. A one factor at a time approach was used as a way to get a scope of which factors are important to consider when choosing an ATPS for application.

Table 10 Six factors with three variables each studied independently, creating different ATPS for TPC extraction from dried grapes and partitioning between phases

Factor	**Variable**		
Salt type	Tartrate	Citrate	Phosphate
pH	4	7	12
Tie-line length	12.3	18.3	27.2
Extraction temperature	Room temp	30°C	45°C
Biomass loading	3%	7%	10%
Extraction time	1 hr	4 hrs	7 hrs

4.2.3.3 Polyphenol quantification

The polyphenol content of the system phases was quantified using the Folin-Ciocalteu method (Blainski et al., 2013). The protocol used in this study was taken from Ainsworth and Gillespie (2007). The samples were diluted and a volume of 100µl mixed with 200µl 10% (v/v) Folin-Ciocalteu reagent by vortex. A Na_2CO_3 solution of 700mM was made, and 800µl added to each sample. The samples were then left in a dark cupboard at room temperature (~20°C) for 2 hours. The samples were then transferred to a clear 96-well microplate, adding 200µl of each

sample to each well. The absorbance of each sample was read at 750nm using an absorbance microplate reader (BioTek ELx800). An ethanol blank (80:20 v/v) processed in the same manner as the samples was used as a control.

A standard curve was constructed using different known concentrations of gallic acid (0.0860-2.15 g/l) and following the above protocol. A curve of known gallic acid concentration (g/l) against the absorbance was set up using the average of three repeats. Using the equation produced, the concentration of polyphenols of the samples were extrapolated and reported as gallic acid equivalent (GAE), a phenolic used as a 'standard' when quantifying polyphenols as a whole, since it is more costly and a lengthier process to quantify each individual polyphenol.

4.2.4 Data analysis

The polyphenol quantification data was used to calculate the yield of phenolic for the grape pomace (as compared to the phenolic levels obtained using solvent extraction) using Equation 8, as well as the partition coefficient of phenolics using Equation 9.

$$Total\ Polyphenol\ Yield\ (\%) = \frac{Total\ mg\ GAE_{ATPS}}{Total\ mg\ GAE_{\ solvent\ extraction}} \times 100 \qquad \text{Equation 8}$$

$$K = \frac{mg\ GAE_{top\ phase}}{mg\ GAE_{bottom\ phase}} \qquad \text{Equation 9}$$

This data was then used to run an ANOVA analysis, along with paired t-tests to ascertain whether the different ATPS conformations performed significantly different in terms of polyphenol extraction, and then partitioning to one phase within in the systems. Using this information, a decision was made on which systems would be the best for phenolic extraction from dried grape pomace.

4.3 Results and discussion

4.3.1 Calculation of total phenol content (TPC)

The standard curve for the absorbance of known gallic acid concentration at 750nm wavelength was produced in Figure 15. The line fitted to the data points had an R^2=0.999, indicating the data can be confidently used to calculate the total phenol content (TPC) levels in the samples

using the absorbances. Equation 10 was used to calculate the gallic acid equivalent (GAE) in the samples in g/l.

$$g/l = \frac{Absorbance - 0.0602}{0.4802} \qquad \text{Equation 10}$$

From this and with the phase volumes noted of the ATPS, the mg GAE in each phase was calculated. This was then used to calculate the total mg GAE in the systems, from which the yield and the K were calculated.

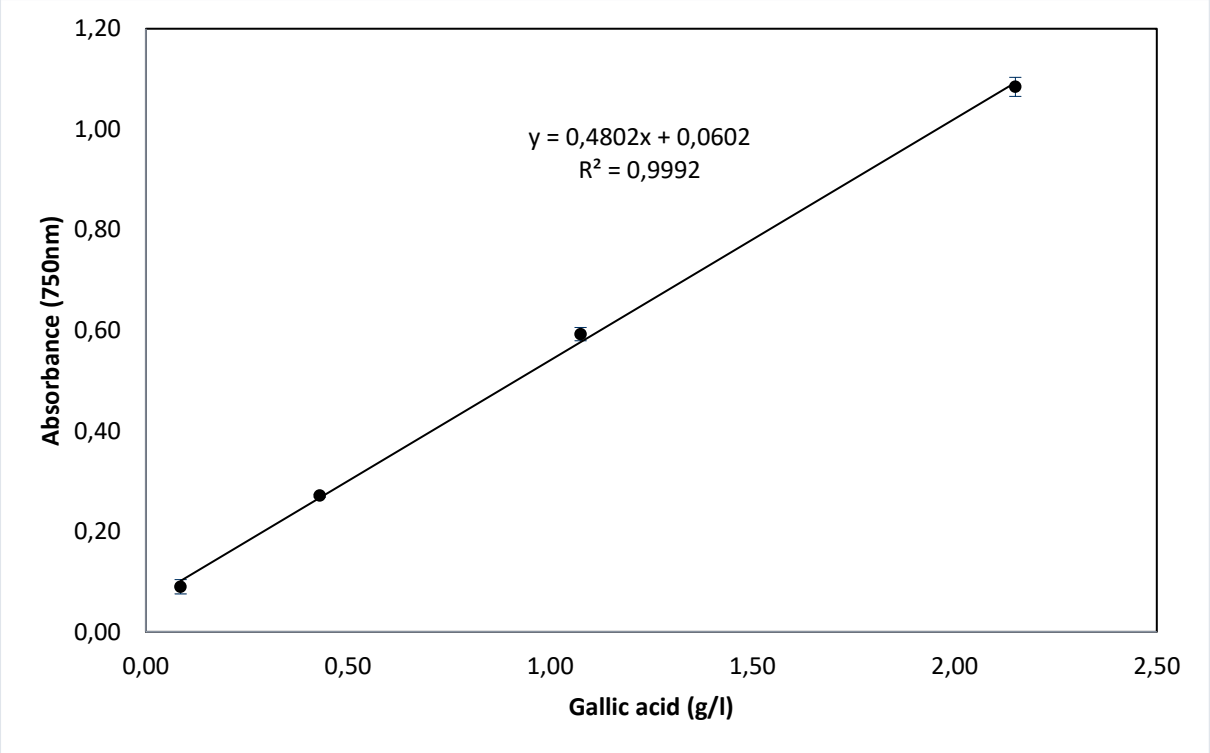

Figure 15 Standard curve produced for absorbances at 750nm of known gallic acid concentrations (g/l) in Ethanol solutions; n=3 with standard deviations

4.3.2 Solvent extraction

Solvent extraction of the dried grape skins was done to get a base of the total phenolic content (TPC) levels in the dried grapes. There was no significant difference in TPC extracted from one hour to 24 hours as can be seen in Figure 16, the levels rising only slightly after 24 hours. As evident in the data presented, the TPC levels quantified at 24 hours had a bigger variation

(calculated as standard deviation) than the other time points. It is possible that degradation of the TPC started taking place after several hours of solvent contact, with some exposure to light. Since these levels are statistically the same, the average of the three data sets was used as the base level of TPC in the grape skins. This came to 27,2 mg GAE/1g DM and was used as a marker to determine how much TPC is yielded by the different ATPS further on in the study. These levels were comparable to previous solvent extractions done, where a maximum level of 28.06mg/g dry weight was achieved using solvent extraction (Librán et al., 2013). This indicates that the assumed amount of TPC used in this study was accurate.

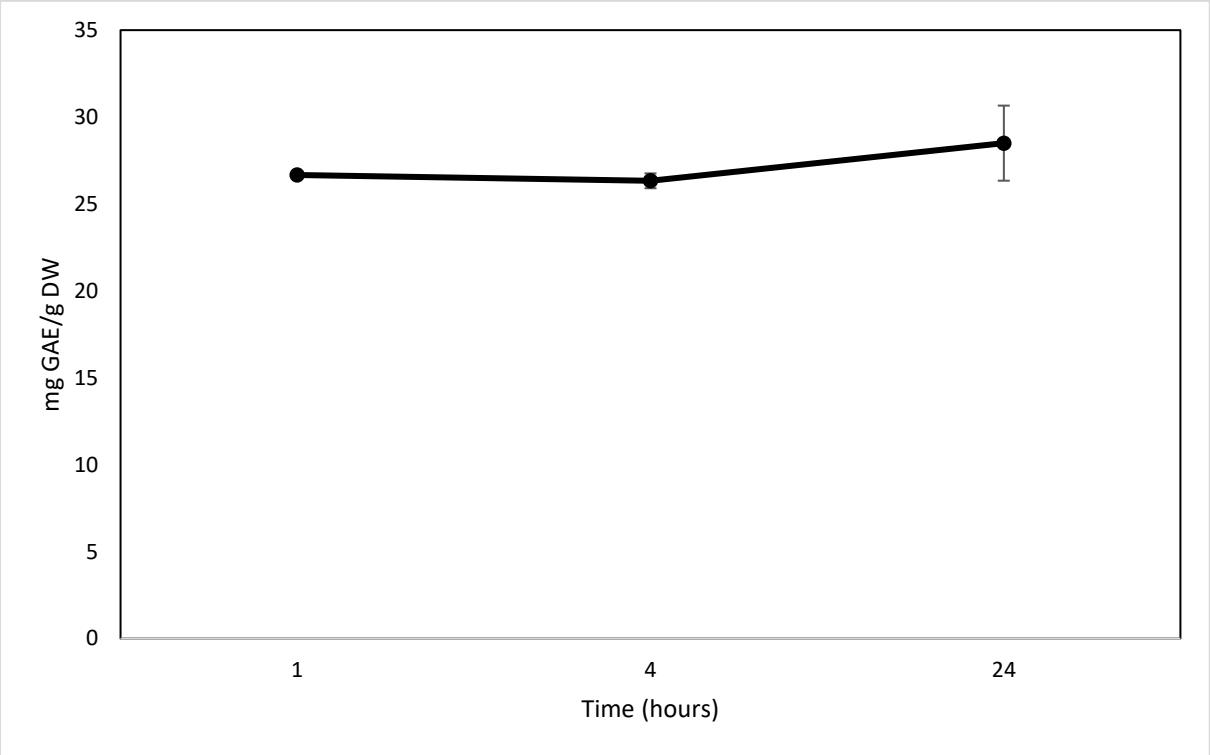

Figure 16 Total polyphenol content (given as gallic acid equivalent) extracted from dried grape pomace using ethanol/water solution (80:20 v/v) at different time points. n=3 with standard deviations.

4.3.3 PEG molecular weight and settling temperature combined effects

Full factorial experimentswere done to see the effect that PEG M_w together with settling temperature has on the yield and K of TPC from the grape pomace. PEG M_w and settling temperature were the factors looked at in the previous section where phase diagrams were constructed of different PEG M_w and potassium sodium tartrate at different temperatures. These factors are important drivers in the phase formation of ATPS based on the previous

chapter's findings and may have competing effects on both phase formation and biomolecule partitioning, thus it would be beneficial to assess how the polyphenols are extracted and concentrated in different conditions of these parameters. Systems of PEG 6000, PEG 8000 and PEG 10000 with potassium sodium tartrate were set up, and each PEG M_w done at 10°C, 25°C and 45°C. All systems were adjusted to pH 7, and the TPC extracted at room temperature for four hours.

4.3.3.1 TPC yield

A relatively wide yield of TPC was achieved, Figure 17 showing the range from 77.3% to 90.0%. Higher PEG M_w favoured higher yields, at each settling temperature. This could be due to the volume exclusion effect which increases with increasing PEG MW (Barani et al., 2018), where longer chain PEGs are more hydrophobic, allowing for more water to be freely available for the polyphenols to dissolve in. At 25°C the PEG 6000 ATPS yielded significantly lower ($p<0.05$) TPC than the PEG 10000 ATPS, and at 45°C significantly lower ($p<0.05$) than both the PEG 8000 and PEG 10000 ATPS. There were no significant differences between the yields of the PEG 8000 and PEG 10000 systems at any of the temperatures, but in absolute terms the PEG 10000 systems yielded marginally more TPC than the PEG 8000 systems (the biggest difference being 2.48% at 10°C). The opposite trend was seen by Mohammadi et al. (2008) when extracting a recombinant phenolic molecule phenylalanine dehydrogenase, in ATPS with different PEG M_w, where the ATPS with PEG 6000 yielded higher levels than the ATPS with PEG 10000. This suggests again that the yield of a molecule cannot be predicted based on the components of an ATPS since each molecule will interact with the chemicals in the ATPS differently depending on its chemical structure, which may even vary between similar molecules like phenolics.

In each PEG M_w system, lower settling temperatures favoured higher yields. While the trends were obvious in absolute numbers, the only significant difference was in the PEG 6000 ATPS, where a settling temperature of 45°C yielded significantly lower ($p<0.05$) levels of TPC compared to the PEG 6000 ATPS at 10°C and 25°C. The settling temperature has no active effect on the extraction of the polyphenols has it is applied after the extraction, but the longevity of the polyphenols could depend on it. While the temperatures applied here were still below the 60°C believed to be the threshold where polyphenols start degrading, it is possible that the higher temperatures had an effect on the stability of the polyphenols, giving us better yields at

lower temperatures. Moldovan et al. (2016) saw that polyphenols had higher degradation rates at higher storage temperatures.

No significant interaction effects between PEG M_w and phase formation temperature were seen when these data were analysed using two-way ANOVA, even though each factor had an effect in its own capacity4.3.3.2 TPC partitioning

Section 4.3.3.1 established that phenolics can be extracted from grape pomace using ATPS, the next step is to determine whether these phenolics can be concentrated in the ATPS. After extraction, any biomass still present settled to the bottom phases of the ATPS, while the phenolics were concentrated in the top phases, indicating that the phenolics were not only extracted from the grape pomace but concentrated away from the biomass as well. This was confirmed by calculating the partition coefficients (K) of the TPC in the ATPS. While good partitioning was seen across all systems in Figure 18, ranging between a K of 2.30-4.34, there was no clear trend in the effect that PEG M_w had on the K of TPC in the different systems. The partitioning achieved here was comparable to previous PEG/tartrate ATPS extractions of biomolecules, which saw partition coefficients of 2.51 for proteases (Lario et al., 2016). In the systems at 10°C, the PEG 6000 ATPS had a significantly higher ($p<0.05$) K than the other PEG M_w, while at 25°C the PEG 8000 ATPS had a significantly higher ($p<0.05$) K for the TPC. There were no significant differences between the PEG M_w at 45°C. Similar results were seen by Yucekan & Onal (2011) when invertase was extracted from tomatoes using sodium sulfate systems with different PEG M_w. The K of the invertase increased from PEG 1000 to PEG 3000 and decreased from there to PEG 8000. The same was seen for the effect that temperature had on the K of TPC, whereby no clear-cut trend could be found. This can be explained by the fact that shorter PEG chains (ie lower M_W PEG) have more hydroxyl groups available for interaction. Thus, more polyphenol groups would interact with the PEG phase, increasing the K (Glyk et al., 2017). A settling temperature of 10°C did produce a significantly higher ($p<0.05$) K in both the PEG 6000 and PEG 10000 systems, and a settling temperature of 45°C had a significantly lower ($p<0.05$) K in the PEG 8000 ATPS, but the middle temperature of 25°C threw off the trend in all three PEG M_w ATPS. Similar results were seen in a study where wheat-esterase was concentrated using ATPS, where K of proteins and enzymes fluctuated and had no defined trend with differing temperatures (Jiang et al., 2015). A two-way ANOVA showed that while both PEG M_w and phase formation temperature had significant effects on

the partitioning of the TPC, there were no significant interaction effects, with a p value of 0.074 being produced.

Taking into consideration both the yield and K of the TPC in these different systems, it would seem that PEG M_w has little effect on the extraction and partitioning of TPC from the grape pomace. Different biomolecules may be affected differently by PEG M_w. The molecular weight of the target molecule may influence how it dissolves in the PEG solutions, for example the partitioning of larger proteins is more susceptible to PEG M_w changes (Grilo et al., 2016). It is then entirely possible that due to the relatively small molecular size of the polyphenols that the PEG M_w would not have a big impact on the partitioning behaviour of the polyphenols as a conglomerate, but could have an impact if differently sized polyphenols were looked at individually. This gives the freedom in choice when designing a system for industrial use, allowing for leniency in choosing a PEG M_w suitable for the equipment, the budget available or even the PEG available in the market at any time. It would cost less as the purity of the PEG in terms of M_w would not be of such importance, and 'mixtures' of PEG M_w tend to cost less due to less processing required during production. These results are based off a relatively small range of PEG M_w and smaller (~400-100 $g.mol^{-1}$ for example) or bigger (~30 000-40 000 $g.mol^{-1}$ for example) molecular weights may behave completely differently. The temperature would be a bigger consideration when designing an extraction plant. Since lower temperatures (10°C) are favourable in the phase separation stage, some energy for refrigeration would have to be used for cooling the ATPS, raising the cost of the process. Since these ATPS had different PEG M_w and settling temperatures, it should not be overlooked that the TLL would be different as well, and could possibly have an effect on the yield and K. The effect of TLL will be discussed later on as an independent variable.

These results show that a PEG/salt system is effective at extracting the polyphenols from the grape skins and partitioning to the top phase. While the exact mechanism pertaining to polyphenols, and especially a conglomerate of polyphenols, has not been explained explicitly, it can be conjectured that the salt extracts the polyphenols from the grape cells through osmosis, attracting the water and water soluble polyphenols from the grape cells into the high salt solution. The PEG then binds with polyphenols through hydrogen bonds, separating it from the salt solution and causing a partitioning within the ATPS.

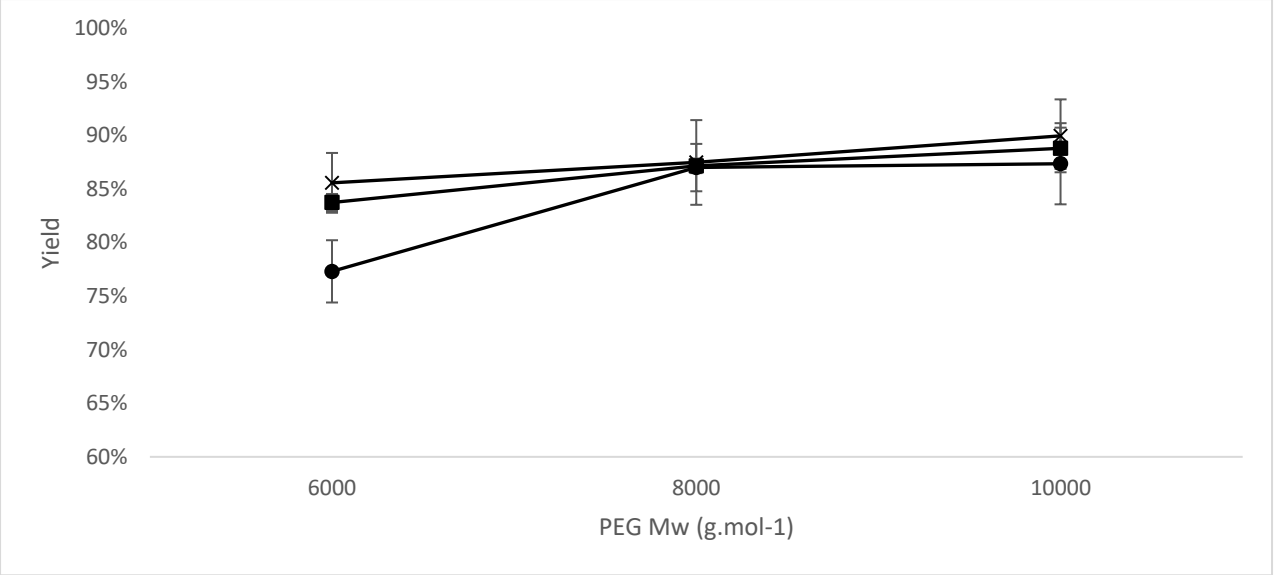

Figure 17 TPC yield (%) from dried grape pomace using ATPS of different PEG M_w (PEG 6000, PEG 8000 and PEG 10 000) with potassium sodium tartrate at different settling temperatures (x 10°C, ▪25°C and •45°C), with constant extraction time of 4 hours, extraction temperature of 25°C, biomass loading of 10% (m/v) and pH 7; n=3

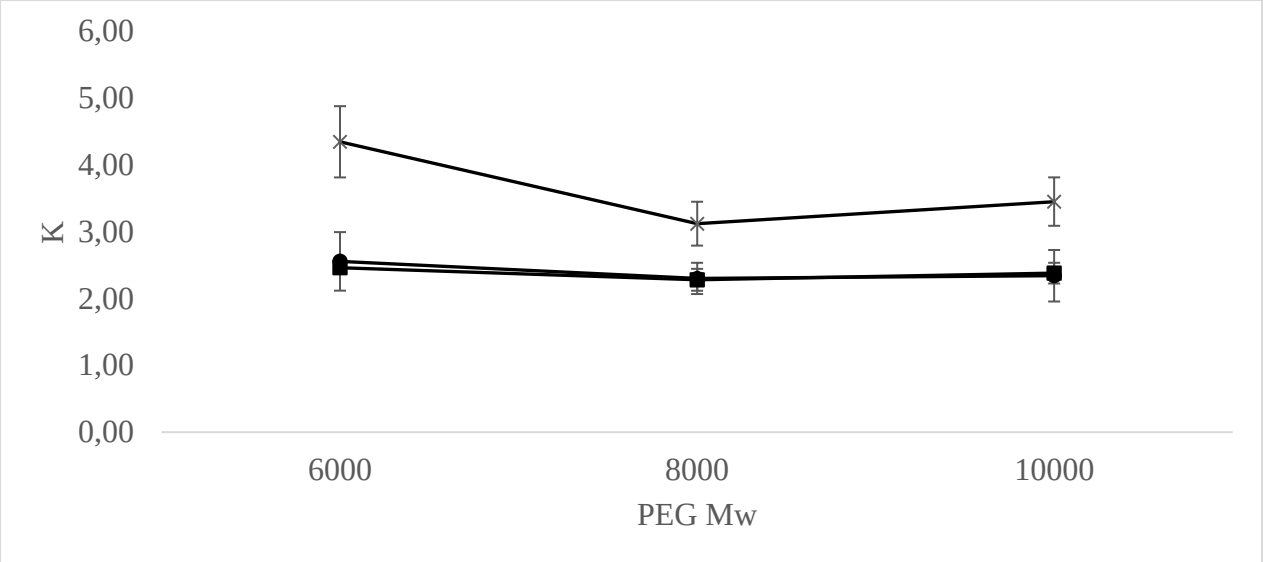

Figure 18 TPC partitioning between top and bottom phases (K) in ATPS of different PEG M_w (PEG 6000, PEG 8000 and PEG 10 000) with potassium sodium tartrate at different settling temperatures (x 10°C, ▪25°C and •45°C), with constant extraction time of 4 hours, extraction temperature of 25°C, biomass loading of 10% (m/v) and pH 7; n=3

4.3.4 Variables investigated

The system with PEG 8000 and potassium sodium tartrate with TLL of 18.3 at pH 7, extraction done at room temperature for 4 hours and settled at 25°C was used as the reference system in the comparative experiments to follow below. Each level in the variables studied were compared to the reference system to see if that variable had any effect on the yield of TPC extracted from the grape pomace and the K which tells us the concentrating ability of the ATPS.

4.3.4.1 Effect of TLL

Using the ATPS equilibrium data produced in the previous chapter of this study (Phase formation and behaviour of PEG 6000, 8000 and 10 000 with potassium sodium tartrate aqueous two-phase systems at different temperatures), different tie-lines were used to evaluate the effect that changing the compositions of PEG and salt has on the extraction and purification of the phenols from the grape skins. In this experimental setup, the three TLL determined for ATPS of PEG 8000 and tartrate at 25°C were compared, thus a system with TLL 27.2% (9.8% w/w tartrate and 12.1% w/w PEG 8000), one with TLL 18.3% (8.4% w/w tartrate and 14.8% w/w PEG 8000) and one with TLL 12.3% (7.2% w/w tartrate and 17.3% PEG 8000) . The phase composition information for these systems is found in the previous chapter, but as a reminder, a bigger TLL meant that the top phase of the ATPS had a bigger fraction of the overall PEG available in the ATPS, even though the overall PEG in the ATPS was lower. The bigger TLL had better phase separation.

The system with TLL of 27.2% yielded significantly lower ($p<0.05$) TPC than those with TLL of 18.3% and 12.3%, yielding less than 80% polyphenols from the grape skins. Both the ATPS with TLL 18.3% and 12.3% yielded over 80% and were not significantly different from each other as shown in Figure 19.

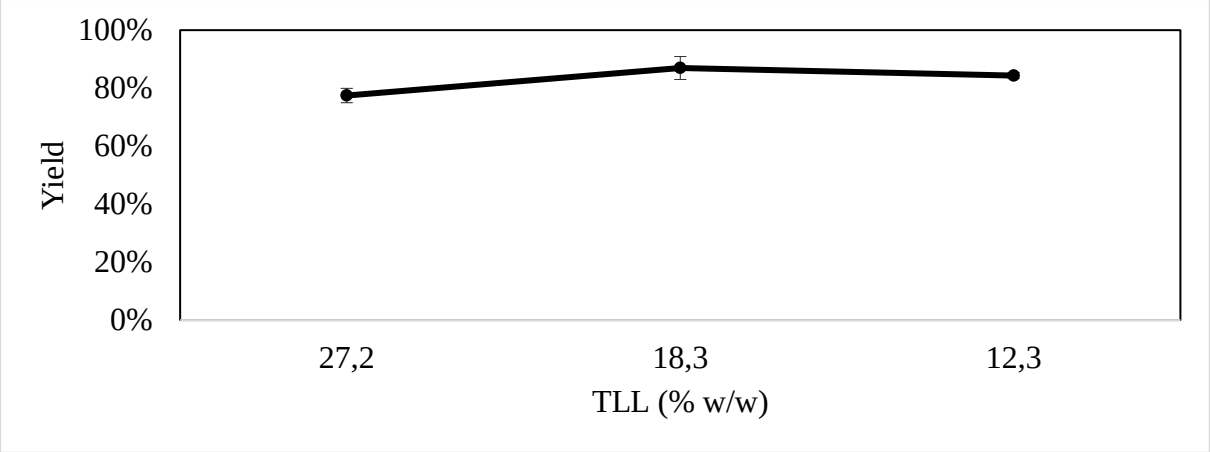

Figure 19 Average Yield (%) of TPC from dried grape pomace using PEG 8000 and potassium sodium tartrate ATPS with varying TLL, with constant extraction time of 4 hours, extraction temperature of 25°C, biomass loading of 10% (m/v), settling temperature of 25°C and pH 7; n=3 with standard deviation

The same trend is carried over into the partition coefficients of the systems shown in Figure 20, whereby the ATPS with TLL of 27.2% had a significantly lower ($p<0.05$) K. This means that less TPC partitioned to the top phase of the system in relation to the TPC extracted into the system. This system has an average K of 1.35, which could be interpreted as the TPC being distributed somewhat equally between the top and bottom phases. While this ATPS (TLL 27.2%) does extract polyphenols from the grape skins, albeit significantly less than the other ATPS with smaller TLL, the concentration ability is not very good, leading the system to be deemed not viable for the purposes of this study. The other ATPS both had good K values. The ATPS with TLL 12.3% had more than double the K than that of the ATPS with TLL 18.3%. The ATPS with TLL 12.3% had more PEG making up the total system, resulting in a bigger top phase (ie PEG rich) volume. Taking into consideration that the total yield for the two systems did not differ significantly (TLL 12.3% yielding in absolute numbers a few % lower than TLL 18.3), the ATPS with a TLL of 12.3% seems to be the better system, concentrating the polyphenols to a better extent.

It is clear from these different systems that the phenolics have an affinity for the PEG component of the ATPS as the phenolics are found in the phase where PEG is the dominant component. Similar conclusions were drawn by Carneiro-da-Cunha et al. (2014) when extracting clavulanic acid in ATPS of PEG (20 000 $g.mol^{-1}$) and citrate, obtaining K values ranging from 1.26 to 5.92, indicating that the biomolecule has an affinity for the PEG rich

phase. Clavulanic acid, while not a phenolic chemical, does have both a hydroxyl and a carboxyl functional group, both of which are present in gallic acid, and would then behave similarly to phenolics (which are characterised by a hydroxyl group) in the ATPS. Knowles et al. (2015) found that PEG interacts favourably with aromatic carbon groups, hydroxyl O group and carboxylic acid O groups, thereby increasing the solubility of compounds with these components, explaining why these polyphenolic molecules would be more easily associated with the PEG phase. It seems that the difference in phase components (ie phase clarity) matters less for the partitioning of the molecules than the total amount of PEG present.

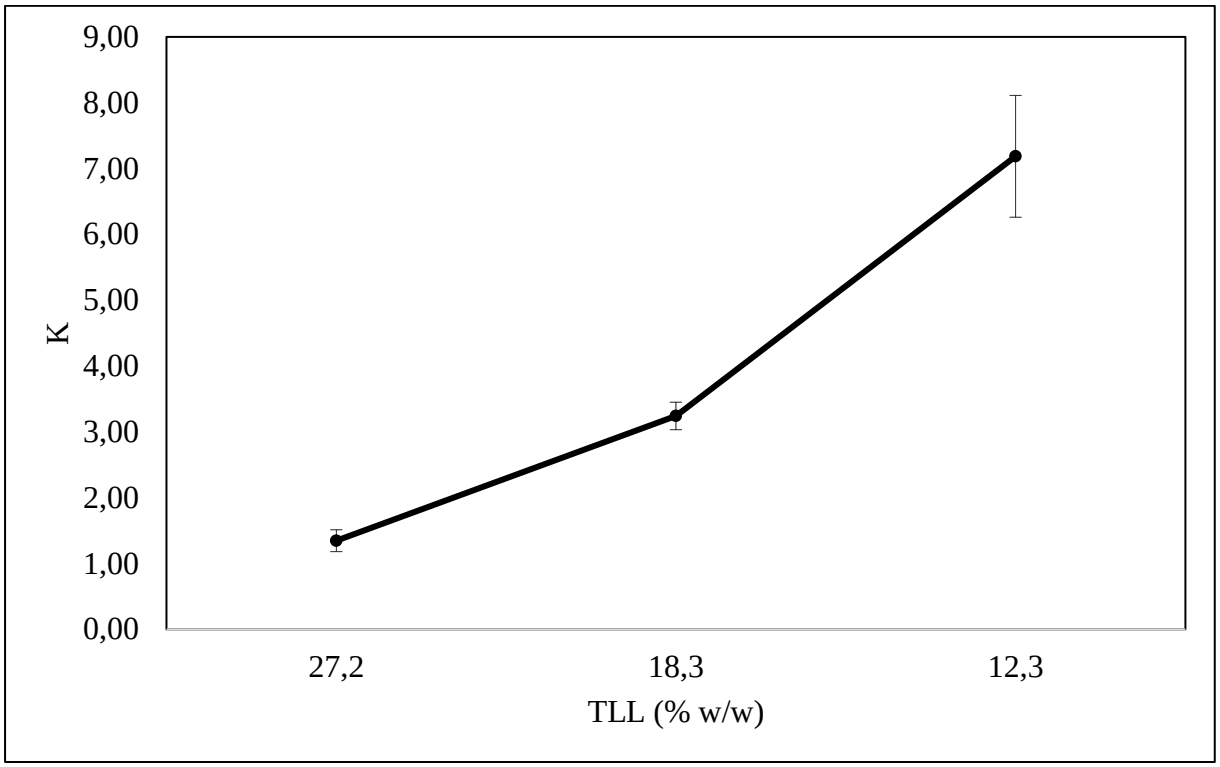

Figure 20 Average K of TPC in PEG 8000 and potassium sodium tartrate ATPS with varying TLL, with constant extraction time of 4 hours, extraction temperature of 25°C, biomass loading of 10% (m/v), settling temperature of 25°C and pH 7; n=3 with standard deviation

It is recommended then that should the extraction be applied to industrial scale, ATPS with a bigger overall fraction of PEG should be used in terms of w/w%, where the PEG nears 20% (w/w) of the ATPS and the tartrate makes up about 7% (w/w) of the ATPS. The cost of the process would then be driven up due to the high price of PEG compared to potassium sodium tartrate, according to the prices in Appendix 1. Since PEG is very viscous, the plant components

should be designed to handle this, and any piping present should have a big enough diameter to avoid blockage.

4.3.4.2 Effect of Extraction time

The effect of extraction time was looked at to determine if there would be a benefit to having a longer contact time of biomass to the ATPS, or whether the time could be cut shorter in order to have a faster production time. For PEG 8000/potassium sodium tartrate systems with a TLL of 18.3, the total yield of TPC over three different time periods of one hour, four hours and seven hours in Figure 21 did not differ significantly, each contact time yielding practically the same amount of TPC at 87%. This effectively means that the ATPS extracted the TPC to its full ability after 1 hour of contact time. A shorter period of time should be investigated to ascertain the minimum time needed to extract all the TPC, which will allow for optimum time usage when applying the process to an industrial scale.

The partition of the TPC to the top phase (K) was significantly higher ($p<0.05$) in the system that had an extraction time of four hours, compared to the systems left for one hour and seven hours, seen in Figure 22. The partition for all times were good, the systems left for one hour and seven hours had above 2.5 times the TPC in the top phases compared to the bottom phases, while the system left for four hours had over 3 times the TPC levels in the top phase compared to the bottom phase. The decreasing K at 7 hours may indicate possible polyphenol degradation. Considering the yields of the three different ATPS are not significantly different, this difference in K is not convincingly different enough to claim that the ATPS of 4-hour contact time is better than a shorter contact period and would therefore not be taken as a consideration for increasing the contact period

Taking all this into account, it is clear that a contact time of one hour is beneficial, allowing sufficient time for extraction, yet short enough to prevent loss of TPC due to degradation. A study extracting phenols (specifically catechin) from green tea leaves using a solvent extraction method showed the same tendency, whereby a longer extraction period leads to degradation of the molecule (Perva-Uzunalic et al., 2006). This study suggested that if a longer contact period is employed, lower temperatures should be used. A different trend was seen when an ATPS was used for extracting phenols from wood, where a longer extraction time lead to more phenols (GAE) present in the top phase (Xavier et al., 2013). These differences convey that many factors play a role in extraction time effect, such as the source, molecule type, extraction

temperature or the extraction method. These results bode well for application of this study's purpose on an industrial scale, since a shorter contact period would mean a quicker turnover or production time when extracting these polyphenols.

A study looking at the degradation of *trans*-resveratrol due to light exposure determined that UV-Vis light degraded the molecule at a kinetic rate of 6.63 x 10^{-1} min^{-1}, which translates to a rapid conversion rate. These experiments were however done at a light intensity of 50 mW/cm^2 (Silva et al, 2013), while the current study was carried out under normal room lighting conditions, thus a degradation experiment would be useful to understand the time period constraints for future applications.

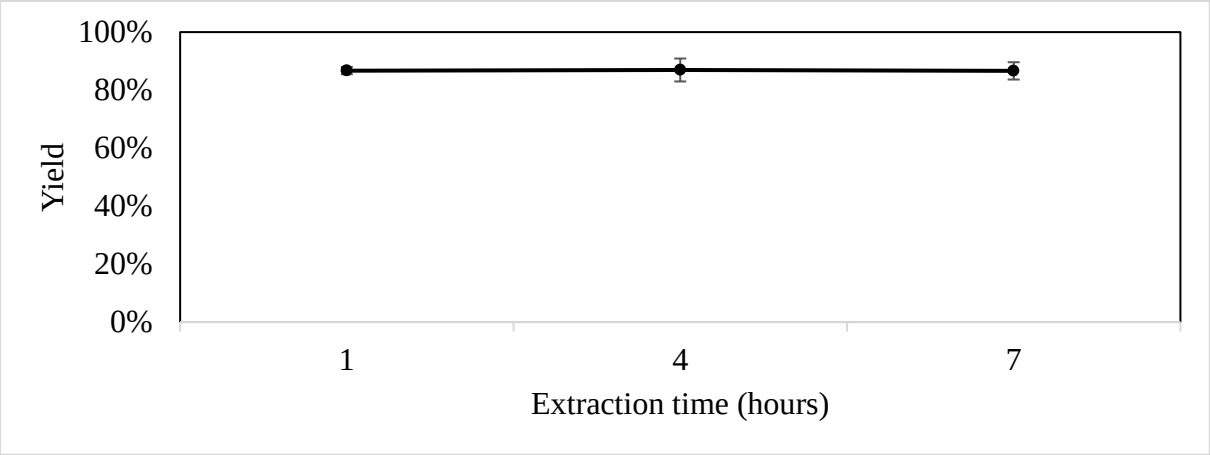

Figure 21 Average Yield (%) of TPC from dried grape pomace using PEG 8000 and potassium sodium tartrate ATPS with varying extraction times (hours), with constant TLL of 18.3, extraction temperature of 25°C, biomass loading of 10% (m/v), settling temperature of 25°C and pH 7; n=3 with standard deviation

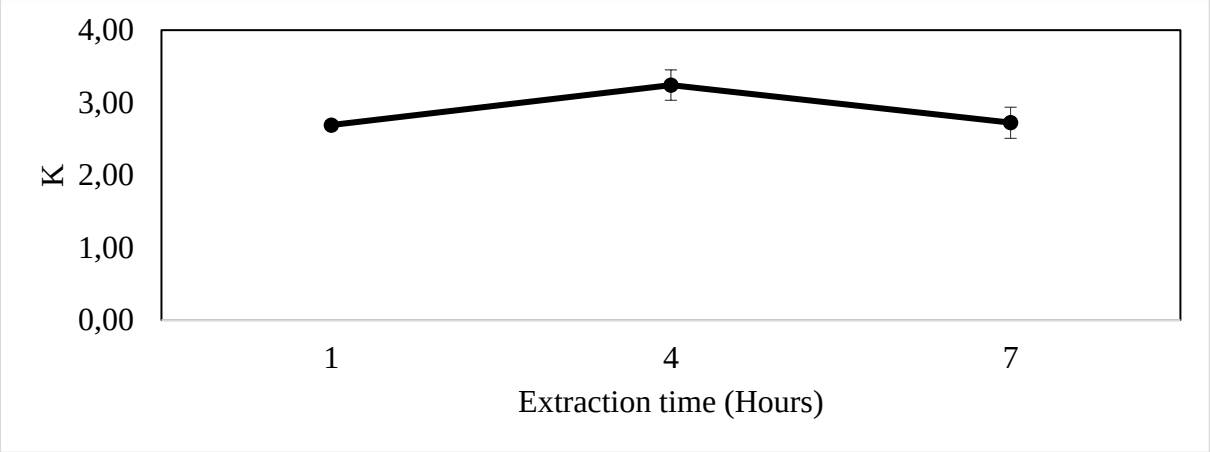

Figure 22 Average K of TPC in PEG 8000 and potassium sodium tartrate ATPS with varying extraction times (hours), with constant TLL of 18.3, extraction temperature of 25°C, biomass loading of 10% (m/v), settling temperature of 25°C and pH 7; n=3 with standard deviation

4.3.4.3 Effect of extraction temperature

A secondary temperature experiment was done to see whether the polyphenol extraction was affected by differing temperatures in PEG 8000/potassium sodium tartrate systems with a TLL of 18.3. This takes into account the temperature at which the systems are during the biomass contact time and is thus a different factor to the temperature which is taken into account during the ATPS phase separation stage. Temperature has had an effect on the extraction of polyphenols in other extraction methods. When using supercritical CO_2 for the extraction of resveratrol, Casas et al. (2009) found a lower temperature yielded higher levels of the compound. In a study which extracted polyphenols from winery waste using solvent extraction, it was found that temperatures over 60°C (at drying stages post extraction) significantly lowered the yield as well as the antioxidant activity of the polyphenols (Lafka et al., 2007).

Relatively low temperatures were used in this study, the goal being to determine whether it would be beneficial to raise the temperature above room level, or if room temperature would be sufficient for polyphenol extraction, thus saving energy on heating. Three temperatures were thus chosen for experimentation, room temperature (~20°C), 30°C and 45°C. The extraction done at room temperature had a bigger yield in absolute terms, at 86.9% compared to 78.0% and 81.1% respectively for the higher temperatures but as can be seen in Figure 23, was not

significantly different to the higher temperatures. It could be that the polyphenols started to degrade or oxidize at these higher temperatures, despite being lower than 60°C.

The extraction done at room temperature had a significantly higher ($p<0.05$) K than the higher temperatures, at 3.24, shown in Figure 24. The higher temperatures had K values 2.52 at 30°C and 2.29 at 45°C. The system at 45°C has only just over double the TPC in the top phase compared to the bottom phase. This could indicate again that the phenolics are degrading at higher temperatures, or possibly dissociating from the PEG component at higher temperatures. These possible degradation products will differ depending on the polyphenols present and the mechanism of degradation, which would also then determine where these degradation products will be found in the ATPS. One possible degradation product includes chalcones which are formed through hydrolytic reactions of anthocyanins (Diaconeasa, 2018).

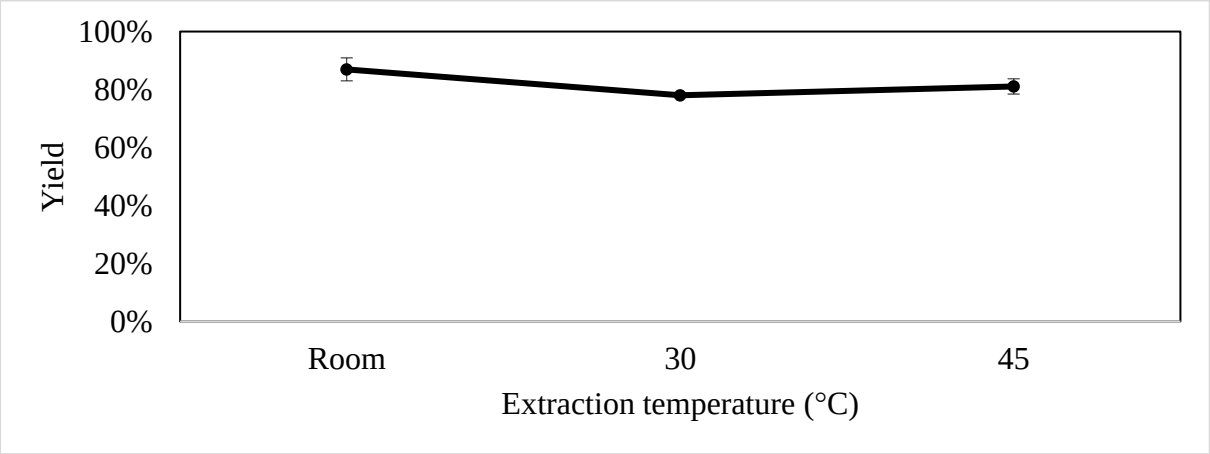

Figure 23 Average Yield (%) of TPC from dried grape pomace using PEG 8000 and potassium sodium tartrate ATPS with varying extraction temperatures (°C), with constant extraction time of 4 hours, TLL of 18.3, biomass loading of 10% (m/v), settling temperature of 25°C and pH 7; n=3 with standard deviation

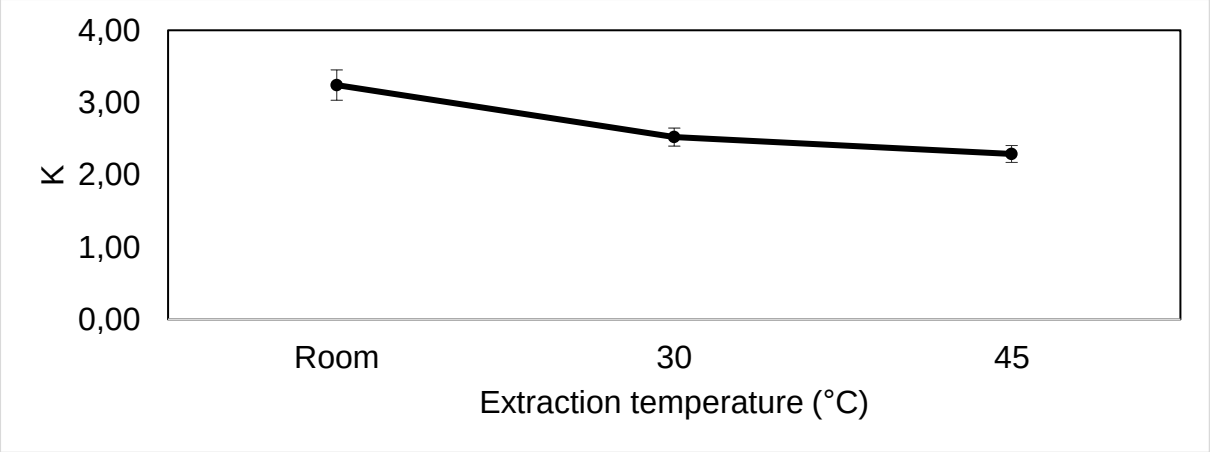

Figure 24 Average K of TPC in PEG 8000 and potassium sodium tartrate ATPS with varying extraction temperatures (°C), with constant extraction times of 4 hours, TLL of 18.3, biomass loading of 10% (m/v), settling temperature of 25°C and pH 7; n=3 with standard deviation

It is clear that during the contact period of grape skins to the ATPS, a system at room temperature is preferable. This could be economically beneficial should the systems be applied at a bigger scale. It would also be time efficient, as the process can be instigated as soon as the grape skins come into contact with the ATPS requiring no warmup time.

4.3.3.4 Effect of salt type (anion)

Salt types were compared to see whether the ATPS would perform differently with different salts in PEG 8000 systems. It was decided to use one salt that is widely used in ATPS extractions (phosphate), and two that are better for the environment as well as human consumption (tartrate and citrate). The anions were the varied factor and the specific salts used (in their crystalised form) were potassium sodium tartrate tetrahydrate, sodium citrate dihydrate and potassium phosphate dibasic trihydrate. The cations were similar in the different salt, being either potassium or sodium, yet the ratios were different and could possibly have an effect on the phase separation as well as the partitioning of the phenolics. The pH of the systems were kept constant at a pH of 7. There were no significant differences in the yields of the TPC between the different salt ATPS. In absolute terms however, the tartrate ATPS was more efficient giving a higher yield of TPC at 86.9% compared to 83.3% for the citrate ATPS and 80.7% for the phosphate ATPS shown in Figure 25. This may be attributed to the fact that grapes are a natural source of tartrate thus some of this tartrate may have influenced the ATPS

composition by addition of the grape tartrate to the tartrate initially present in the ATPS. This could potentially cut down costs on the salt component of the ATPS when the systems are used industrially together with grape skins. The phosphate ATPS had the lowest yield of TPC in absolute terms and was only marginally similar to the tartrate systems at a P value of 0.060. ATPS with phosphate salt are better suited to extractions of proteins, as is the case where c-phycocyanin, a colour protein, was extracted using a PEG1450/phosphate ATPS, yielding 98% (w/w) from *Spirulina maxima* (Benavides and Rito-Palomares, 2005).

Figure 26 shows that the phosphate ATPS also had a significantly lower ($P<0.05$) K value than the other salts at 2.73. The tartrate and citrate systems had similar K values of 3.24 and 3.27 respectively. This can be attributed to the salting out ability of the salts, as according to the Hofmeister series, tartrate has the biggest salting out ability, with phosphate having the lowest salting out ability meaning that the biomolecules will be more repelled by the tartrate and citrate salts in respect to the phosphate salt (Hyde et al., 2017). When applying these systems to industry, phosphate ATPS would then be inefficient for the purposes of this specific case, as more reagent would be needed to extract and concentrate the same amount of TPC. While citrate ATPS have been previously shown by Xavier et al. (2015) to work well for phenolic extractions, tartrate ATPS do not appear so prevalently in the literature for phenolics extractions but have been shown to extract lipases well in conjunction with PEG 4000 by Dobreva et al. (2019). The results in the current study could then mean that tartrate ATPS can be introduced to industry as a viable means to extract phenolics from plant material.

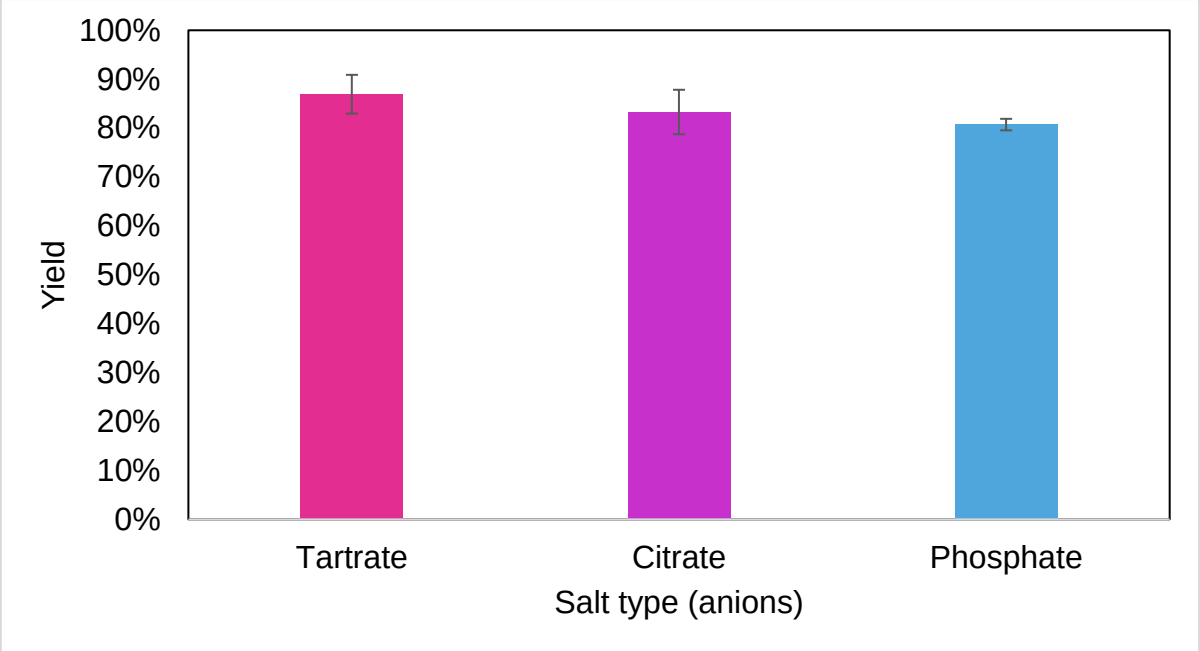

Figure 25 Average yields (%) of TPC from dried grape pomace using ATPS of PEG 8000 with varying salt types with constant extraction time of 4 hours, extraction temperature of 25°C, biomass loading of 10% (m/v), settling temperature of 25°C and pH 7; n=3 with standard deviation

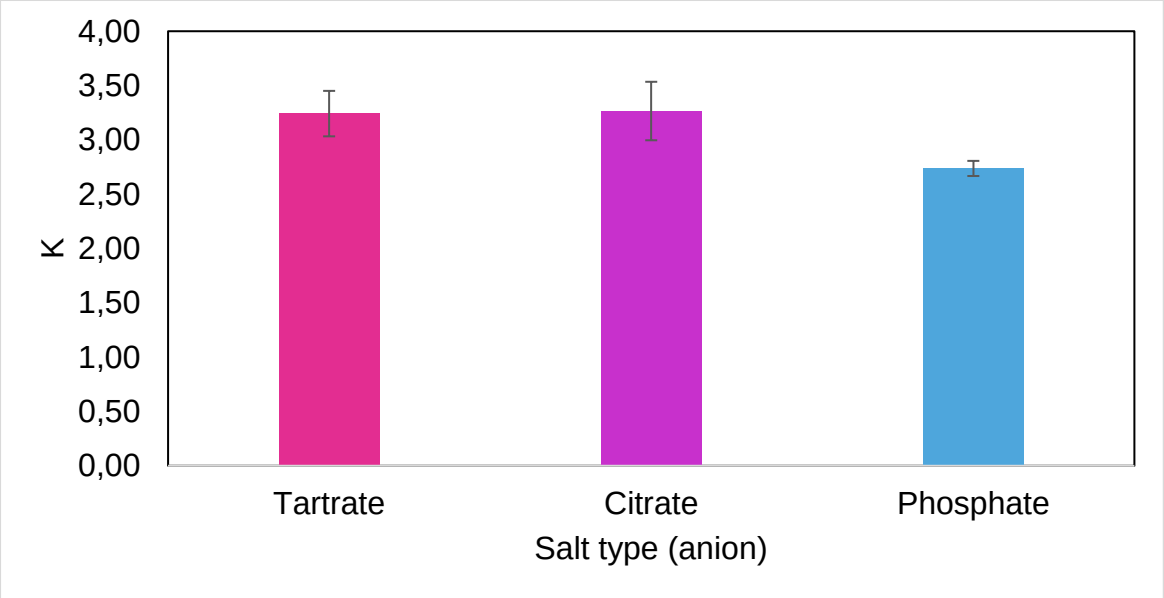

Figure 26 Average K of TPC from dried grape pomace using ATPS of PEG 8000 with varying salt types with constant extraction time of 4 hours, extraction temperature of 25°C, biomass loading of 10% (m/v), settling temperature of 25°C and pH 7; n=3 with standard deviation

4.3.3.5 Effect of system pH

The pH of the ATPS may influence how the molecules are partitioned between the two phases of the ATPS. Pereira da Silva et al. (2002) found that higher pH levels were favourable for partitioning of Glucose-6-phosphate dehydrogenase (G6PDH) and hexokinase (HK) to the PEG phase in PEG/phosphate ATPS. The pH of the extraction system influences the stability and solubility of the biomolecule extracted as the different functional groups of the biomolecules would interact with the Hydrogen ions differently, and so these results would naturally be different for different biomolecules extracted and is therefore necessary to investigate for the current study's goals. It was intended that three pH values would be investigated, basic (pH 4), neutral (pH 7) and acidic (pH 12) to see how the pH of the solution affect the chemistry of the phenolics in PEG 8000/potassium sodium tartrate systems of TLL 18.3. An acidic pH of the ATPS proved to be unviable since the tartrate in the systems precipitated once the pH was dropped below 7, thus only pH 7 and pH 12 were investigated for ATPS efficiency. This poses a significant limitation for industrial processing, as care would need to be taken to ensure all solutions are above this limiting pH, and possibly treating the pomace beforehand. The pomace pH will differ depending on many factors that are at play during the wine making process. The ATPS at both pH 7 and 12 yielded similar quantities of TPC and was deemed the same significantly, yielding 86.9% at pH 7 and 84.2% at pH 12 seen in Figure 27.

The partition coefficient of TPC in the system at pH 7 shown in Figure 28 was significantly higher ($p<0.05$), meaning more of the TPC was located in the top layer compared to the system at pH 12. This indicates that the pH could have an effect on the phenol structure, perhaps changing the biochemistry making it affiliate with the bottom phase rather than the top phase. The molecular structure of resveratrol can be used as a model to show this phenomenon. With increasing pH, resveratrol becomes more and more deprotonated losing a hydrogen as the pH of the molecule rises through the three pKa values of resveratrol (Lopez-Nicolaz and Garcia-Carmona, 2008), as a result losing stability at pH levels above 7 (Robinson et al., 2015). Since the extract in this study is TPC, each molecule will have its own deprotonation pKa values and pinpointing the molecular change and effect on partitioning is difficult. At the time of writing, there seem to be no studies touching on the effect of pH specifically in ATPS where phenolics are extracted, perhaps due to the wide variation in pKa for different phenolics.

In these experiments, the pH of the system components was adjusted, and not the pH of the grape skins. Thus, should the ATPS be applied to a bigger scale, the system need only be adjusted to pH 7 and not the biomass, allowing for easier control as the biomass may be heterogenous and thus unpredictable when adjusting the pH. Should the ATPS be used for extraction of any other biomolecule the pH for that molecule specifically should be studied. It could also be worth looking at whether changing the pH progressively could mean extracting different specific polyphenols systematically, depending on their pKa values.

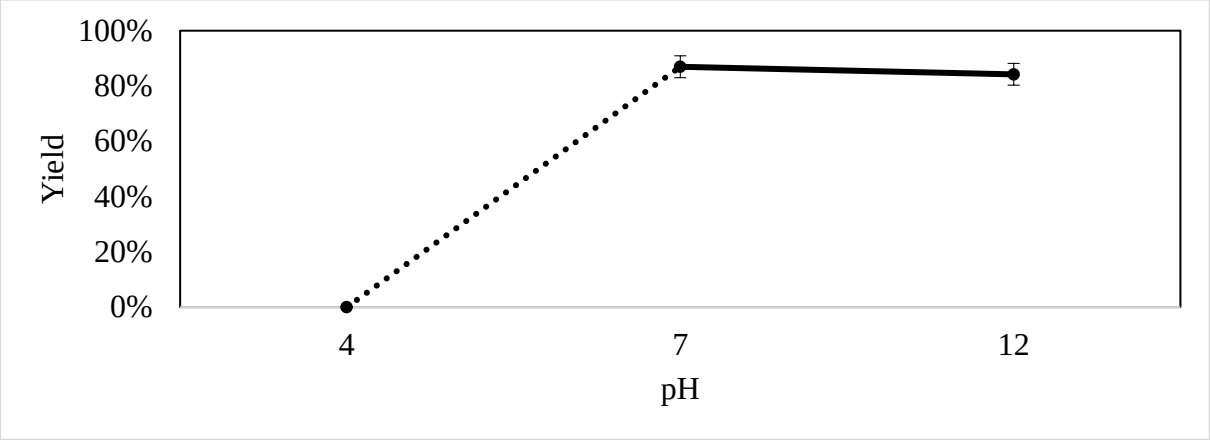

Figure 27 Average Yield (%) of TPC from dried grape pomace using PEG 8000 with potassium sodium tartrate ATPS with varying pH, with constant extraction time of 4 hours, extraction temperature of 25°C, TLL of 18.3, biomass loading of 10% (m/v) and settling temperature of 25°C; n=3 with standard deviation. The dotted line (···) indicates the pH at which ATPS were unviable due to tartrate precipitation

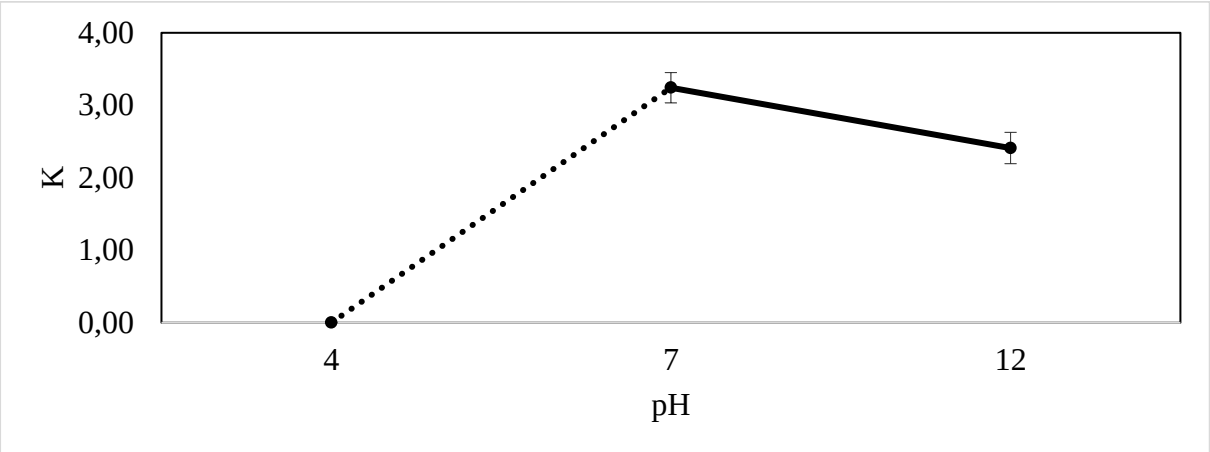

Figure 28 Average K of TPC from dried grape pomace using PEG 8000 with potassium sodium tartrate ATPS with varying pH, with constant extraction time of 4 hours, extraction temperature of 25°C, TLL of 18.3, biomass loading of 10% (m/v) and settling temperature of 25°C; n=3 with standard deviation. The dotted line (···) indicates the pH at which ATPS were unviable due to tartrate precipitation

4.3.3.6 Effect of biomass loading (% solids/volume ATPS)

The effectiveness of the ATPS could be affected by the biomass loading of the system, as the kinetics of the molecules extracted could be changed depending on biomass contact with the ATPS components. In an ATPS of PEG 2000 and a sulphate salt for lipase partitioning from *Rhizopus microspores*, an increase in the crude material load to 30% (w/w) lead to a higher yield as well as better partitioning, but a load bigger than the 30% (w/w) lead to saturation of the extractant phase resulting in the yield and partitioning decreasing again (Anyari, 2015). The biomass loading (%w/v) was kept relatively low in this study. Figure 29 shows that decreasing the biomass loading below 10% (w/v), yielded TPC levels above 100%. This is because the yields were calculated on the assumed maximum of TPC extracted using the solvent extraction, which was done with 10% (w/v) loading. This could indicate that at lower biomass loadings, the ATPS may be more effective at extracting polyphenols than the traditional solvent extraction. When looking at the absolute amount of TPC extracted in terms of mg GAE, there are no big differences between 3% loading, 7% loading and 10% loading. However, when normalising the data to mg GAE per 1g of dry mass as shown in Figure 30, a big jump is seen in the lower loadings. It would seem that the ATPS has a saturation point for polyphenols, and a smaller biomass loading would allow for more polyphenol to be extracted per gram before this saturation point is reached.This would mean that more ATPS would be needed, if the biomass was to be split into smaller batches. This would take double the time, yet more than triple the amount of TPC would be extracted, offsetting the time ‘loss’. The K of the 10% loading ATPS is significantly higher ($p<0.05$) than both the lower loadings seen in Figure 31, indicating that this variable has no effect on the phase formation or concentrating ability of the ATPS.

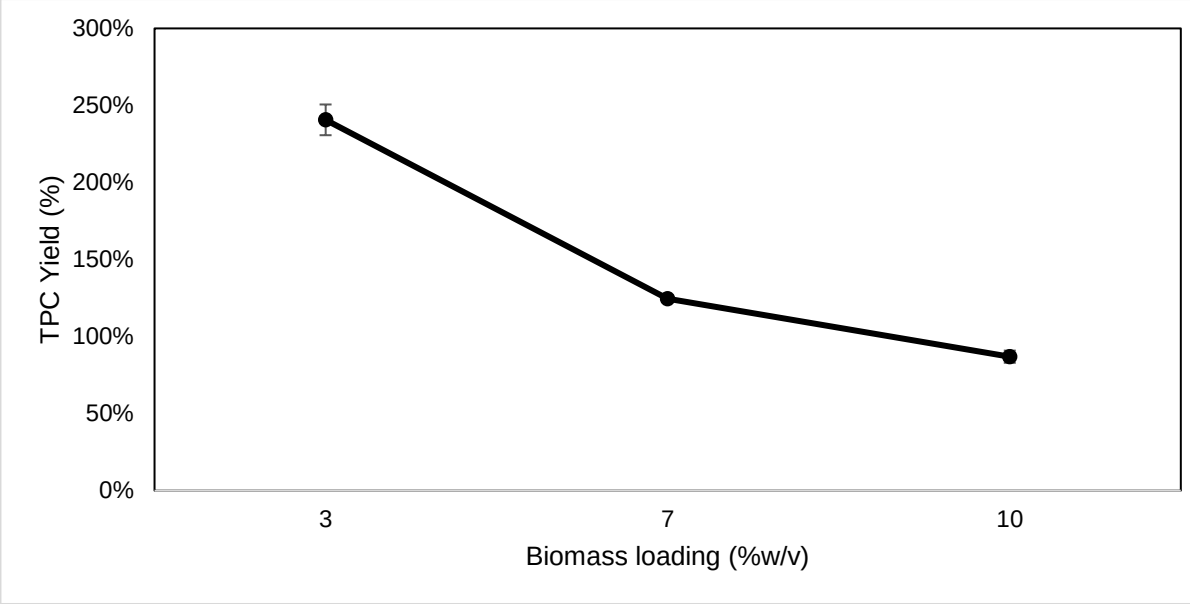

Figure 29 Average Yield (%) of TPC from dried grape pomace using PEG 8000 with potassium sodium tartrate ATPS with varying biomass loadings, with constant extraction time of 4 hours, extraction temperature of 25°C, TLL of 18.3, pH 7 and settling temperature of 25°C; n=3 with standard deviation.

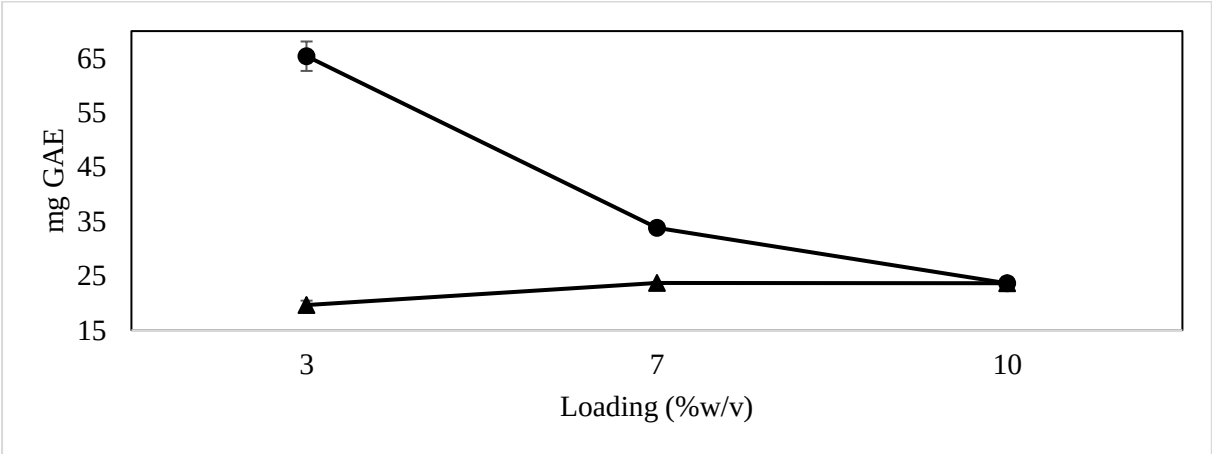

Figure 30 Average levels of TPC extracted from dried grape pomace using PEG 8000 with potassium sodium tartrate ATPS with varying biomass loadings and constant TLL of 18.3, extraction time of 4 hours, extraction temperature of 25°C, settling temperature of 25°C and pH 7. n=3 with standard deviation. (▲) mg GAE equivalent of TPC extracted in each system and (•) TPC normalised to mg GAE/1g DM

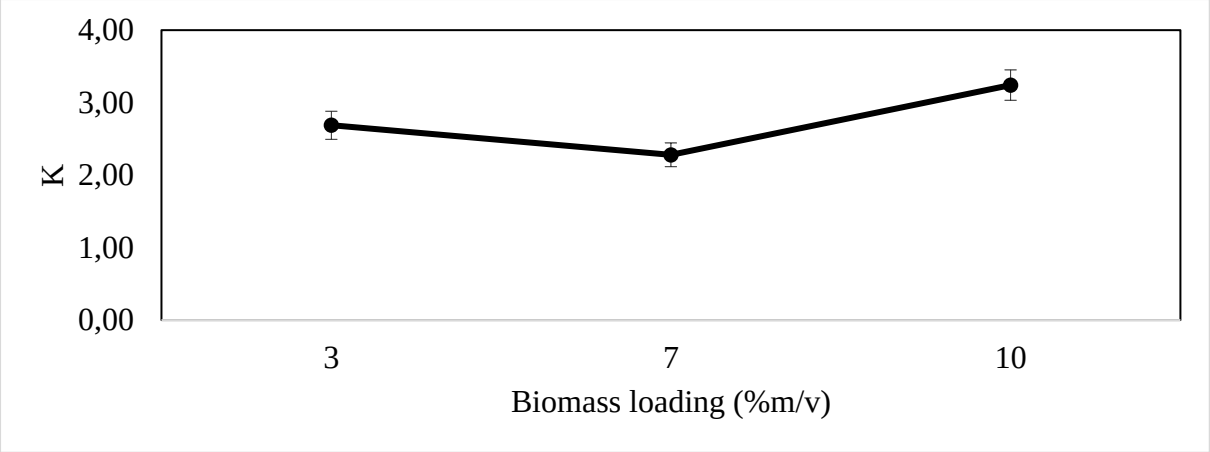

Figure 31 Average K of TPC from dried grape pomace using PEG 8000 with potassium sodium tartrate ATPS with varying biomass loadings (% m/v), with constant extraction time of 4 hours, extraction temperature of 25°C, TLL of 18.3, settling temperature of 25°C and pH 7; n=3 with standard deviation

4.3.4 Summary and suggested ATPS

Below in Table 11 is the summary of a total of 20 ATPS that were studied, with Figure 32 showing the average TPC extracted in terms of mg GAE/1g DM of these systems as well as the TPC extracted using solvent extraction, and Figure 33 showing the average K of all these systems. System 5 was used as the primary reference ATPS in the comparative study above. This system, PEG 8000/potassium sodium tartrate with a TLL of 18.3% and pH 7, with an room temperature extraction for 4 hours and a settling temperature of 25°C, extracted 23.62 mg GAE/g DM, which translates to a yield of 86.9% compared to the solvent extraction done on the same grape skins. The partition coefficient of this system was 3.24, meaning the TPC concentrated well to the top PEG rich phase of the ATPS. A study comparing different ratios of ethanol to water as a solvent at different temperatures extracted a range between 800-2080mg GAE/100g DM from Cabernet Sauvignon grape pomace (Nayak et al., 2018), thus the ATPS employed in this study performed very well when comparing the TPC extracted. Higher yields, reaching over 80mg/g DM, were extracted using an ATPS of acetone and citrate to extract polyphenols from grape seeds (Dang et al., 2013). Whether this is due to the different composition of ATPS or using only the seeds as a polyphenol source would need to be determined.

When compared to system 5, seven other systems (4,6,7,8,9,19,20) yielded more TPC when compared to the solvent extraction, with system 19 yielding the most. This system was composed of PEG 8000 with potassium sodium tartrate with a TLL of 18.3% at pH 7, extracted at room temperature for 4 hours and phases separated at 25°C. This system had the lowest biomass loading of 3% (w/v). The lowest yield, 77.3%, was achieved by system 3 which was PEG 6000 with potassium sodium tartrate with a TLL of 37.7 at pH 7, extracted at room temperature for 4 hours, with a 45°C settling temperature.

Four systems (1,7,12,18) achieved higher K than system 5. The highest K by far was achieved by system 18, consisting of PEG 8000 and potassium sodium tartrate with a TLL of 12.3 at pH 7, extraction done at room temperature for 4 hours and settled at 25°C. The lowest K was achieved by System 17, which had the same conditions as system 18, except with a TLL of 27.2.

While looking at the effect of each variable gave insight into how these systems operate and how they can be manipulated, from the summary below it would seem that biomass loading had the biggest effect on increasing extraction ability, while the PEG and salt concentrations (TLL) had the biggest effect on the partitioning (or concentrating) of the polyphenols within the ATPS. Considering all the outcomes, a system can then be chosen as the 'best' for this case. An optimal system can be developed by widening the parameters. Taking into consideration the yield and K, it would be suggested that system 18 should be used for any further extractions, but adjusting the biomass loading to 3% (w/v) and the extraction time to 1 hour. This lends itself to concentrating the TPC extracted from the grape skins 1.66-fold more than the next best system, which was system 1 with a K of 4.34, and 2.22-fold better than the reference system 5 with a K of 3.24. A better concentrating ability is preferable since it would mean less steps in the extraction process, and ultimately a timelier and more cost-effective process should there be tons of grape skins to be processed. The schematic below in Figure 34 is then the suggested process for extraction of the phenolics from spent grape skins. This scheme is a suggestion for bigger quantities, taking into consideration that precise temperatures and equipment used will be different to that achievable at a smaller bench scale. It eliminates the step taken in these experiments where the biomass was first filtered off. The process here is a batch process, but a continuous process could also be developed. Indicated in this process is the recycling of the bottom phases for further use in additional ATPS cycles. If this is to be done, the polyphenols would need to be removed first. Villegas et al. (2016) reviewed an array of techniques which

could be employed. An attractive technique noted was non-destructive distillation, where the polyphenols can be retrieved. Other techniques mentioned included enzymatic treatment, adsorption and Fenton treatment amongst others. These would however mean degrading and thus losing the valuable polyphenols. The effect on the tartrate composition would also need to be evaluable before being employed.

Taking into consideration the prices of the reagents listed in Appendix 1, the suggested system reagents would cost roughly ZAR 5000 (USD 332) per metric tonne ATPS (when working in w/w terms and considering the PEG component is 17.3% w/w, tartrate component is 7.2% w/w and water is 75.5% w/w of the ATPS). A kL of ethanol/water solution (80:20 v/v) would cost ZAR 11 342 (USD 756), making the ATPS less than half the price at a similar scale when considering only the reagents and water needed.

Table 11 Summary of ATPS systems investigated in this study. Factors varied include PEG Mw, settling temperature, TLL, extraction time, extraction temperature, salt type, pH and biomass loading. Factor varied from reference system 5 indicated by *

System	PEG M_w	Settling temperature (°C)	Salt type	100TLL	pH	Extraction temperature (°C)	Extraction time (hrs)	Biomass loading (%w/v)
1	6000*	10*	Tartrate	29,0	7	Room	4	10
2	6000*	25	Tartrate	32,6	7	Room	4	10
3	6000*	45*	Tartrate	37,7	7	Room	4	10
4	8000*	10*	Tartrate	19,9	7	Room	4	10
5	8000	25	Tartrate	18,3	7	Room	4	10
6	8000	45*	Tartrate	23,9	7	Room	4	10
7	10000*	10*	Tartrate	26,5	7	Room	4	10
8	10000*	25	Tartrate	31,1	7	Room	4	10
9	10000*	45*	Tartrate	36,6	7	Room	4	10
10	8000	25	Tartrate	18,3	7	Room	1*	10
11	8000	25	Tartrate	18,3	7	Room	7*	10
12	8000	25	Citrate*	18,3	7	Room	4	10
13	8000	25	Phosphate*	18,3	7	Room	4	10
14	8000	25	Tartrate	18,3	7	30*	4	10
15	8000	25	Tartrate	18,3	7	45*	4	10
16	8000	25	Tartrate	18,3	12*	Room	4	10
17	8000	25	Tartrate	27,2*	7	Room	4	10
18	8000	25	Tartrate	12,3*	7	Room	4	10
19	8000	25	Tartrate	18.3	7	Room	4	3*
20	8000	25	Tartrate	18.3	7	Room	4	7*

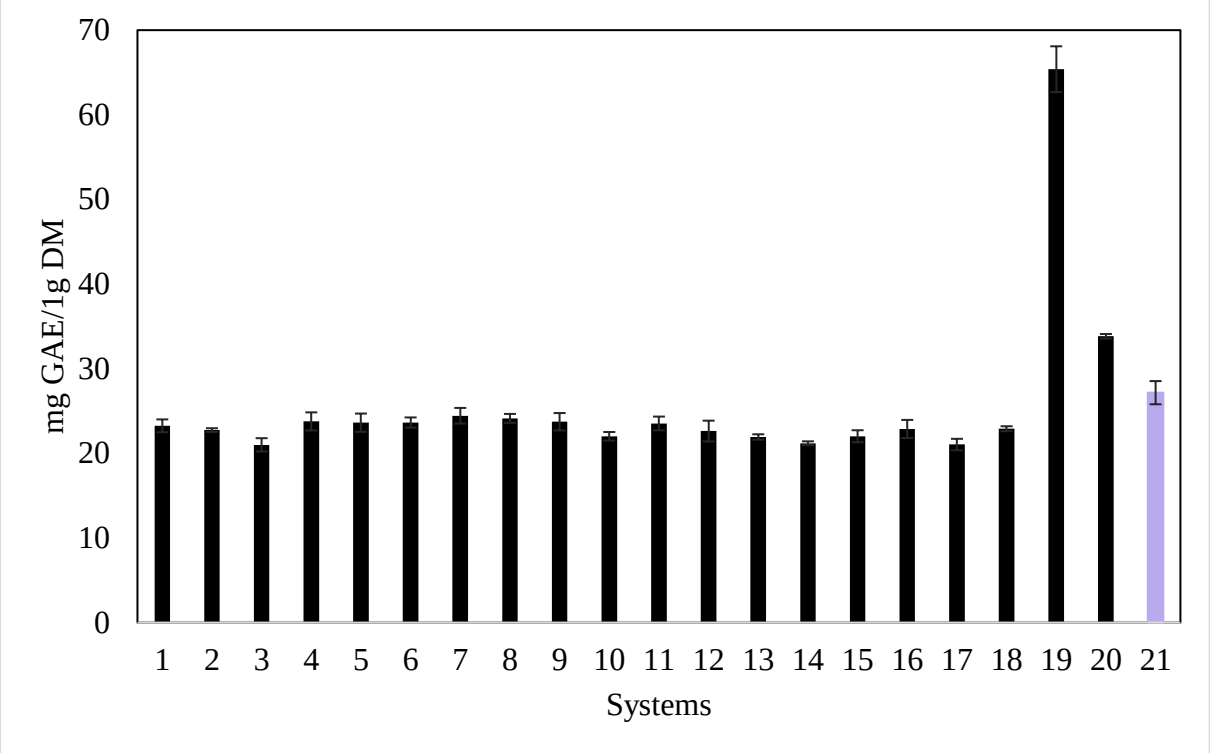

Figure 32 Average TPC (in mg GAE/1g DM) extracted from dried grape pomace using the different ATPS (1-20) as outlined in Table 11. System 21 (blue column) is the solvent extraction used to get an assumed total TPC available in the dried grape pomace per 1g dry mass; n=3 with standard deviation

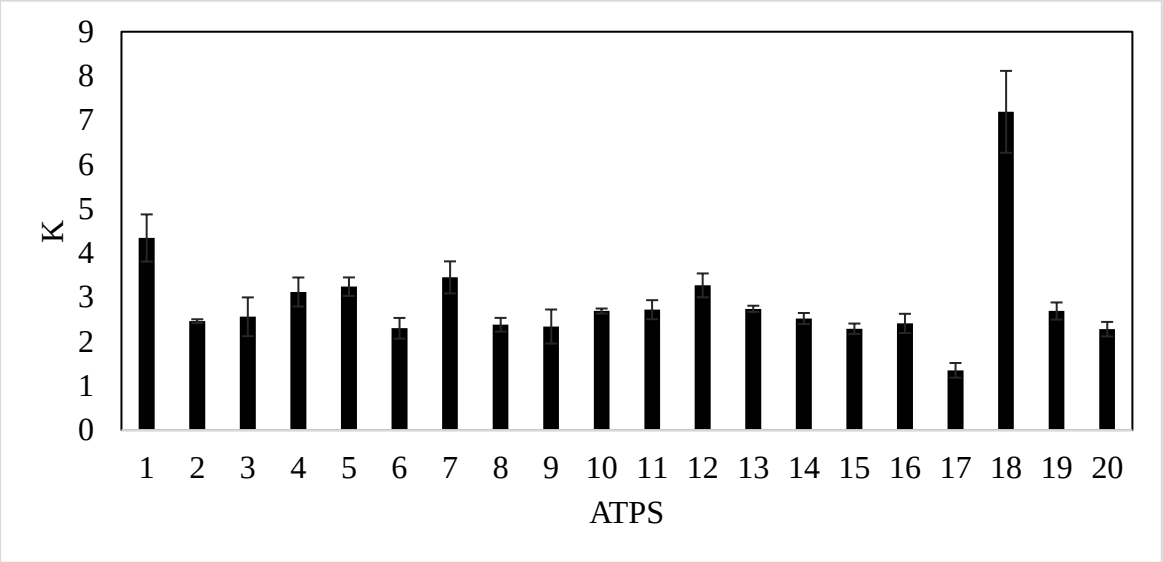

Figure 33 Average K of TPC extracted from dried grape pomace using the different ATPS (1-20) as outlined in Table 11; n=3 with standard deviation

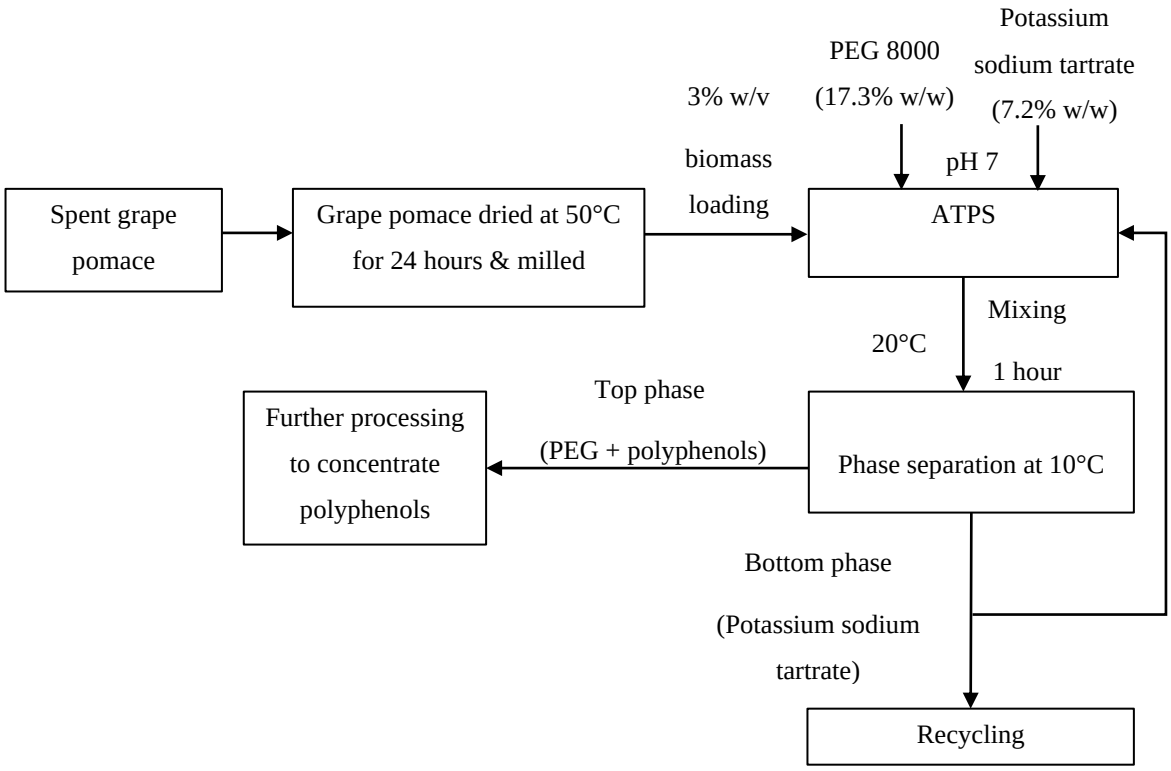

Figure 34 Suggested ATPS setup for TPC extraction from grape pomace

4.4 Conclusion

The total phenolic content (TPC) of dried grape pomace was determined using a solvent extraction method. The TPC levels extracted was similar to those extracted in previous studies. Using a reference system from the previous Chapter, several variables were studied to see the effect that these have on the yield as well as the partitioning of TPC from the dried grape pomace. that the influence of PEG M_w and settling time on the yield and K of the TPC was examined and no interacting effects were found. It was concluded that the settling temperature had a bigger impact and should have a heavier consideration when designing a plant for TPC extraction. From there, six more variable were studied independently. These were the extraction time, TLL, salt type, extraction temperature, pH and biomass loading. Each variable had some significant differences in TPC yield and K, with a lower biomass loading increasing the yield significantly and a higher PEG concentration increasing the K of the TPC significantly. Ultimately, an ideal ATPS was suggested for TPC extraction from grape pomace.

Chapter 5 Conclusion

The grape pomace produced by the wine making industry is rich in usable biomolecules. Polyphenols are desirable for the application of nutraceuticals and cosmetics, and so extracting these polyphenols from wine solid waste would add value to the grape pomace while supplying a natural chemical to the pharmaceutics and cosmetics industry. While solvent extractions are used for extracting polyphenols from wine waste, a new method can provide a cheaper alternative. In this study potential aqueous two-phase systems (ATPS) were studied, looking at the behaviour of the ATPS in terms of phase formation as well as the ability of these ATPS to extract polyphenols from grape pomace.

Phase diagrams of ATPS of PEG 6000, PEG 8000 and PRG 10 000 with potassium sodium tartrate at 10°C, 25°C and 45°C were constructed using experimental data fitted with the Merchuck equation (non-linear regression) to a curve for binodals, and tie-lines validated using the Othmer-Tobias and Bancroft equations. Using these phase diagrams, it was found that higher temperatures and bigger PEG M_w were favourable for phase formation, meaning a bigger range of PEG/tartrate concentrations led to phase formations at these conditions.

The ATPS in this study were made of PEG 6000, PEG 8000 and PEG 10 000 with potassium sodium tartrate. Numerous conditions were varied to study how these conditions impact the extraction and separation of polyphenols from the grape pomace. ATPS has been successfully used in this study to extract polyphenols from wine solid waste. The ATPS also provided good separation of the polyphenols from the biomass, concentrating the polyphenols in the PEG phase of the ATPS.

The factors that showed to have the biggest significant effects on polyphenol yield were phase formation temperature, TLL and biomass loading. The yields ranged from 21mg GAE/1g DM to 65mg GAE/1g DM, the highest being achieved by lowering the biomass loading to 3% w/v. The partitioning of the polyphenols between the phases, and effectively separation from the biomass and concentrating the polyphenols, were affected to a bigger extent by these factors, phase formation temperature, TLL, extraction temperature, salt type, pH and biomass loading all having significant effect on the partitioning coefficient of the polyphenols. The biggest partition coefficient of 7.2 was achieved by the ATPS with the smaller TLL, or in other word the ATPS which had the highest concentration of PEG.

The results obtained in this study indicate that ATPS of PEG and potassium sodium tartrate can be used successfully as a process for the valorisation of wine solid waste. The polyphenols extracted from the solid waste, or the grape pomace, add value to the grapes, and can be used in further medical studies, or processed into nutraceuticals or cosmetics. The relatively high concentration of polyphenols obtained using the ATPS process outlined in this study will lead to a higher quality end product. Considering this, the current study's objectives were successfully met, fulfilling the aim of the study.

Chapter 6 Recommendations for future work

The results produced in this study were broadly defined, and thus it is recommended that the following refinements be included:

- Scaling up the process from bench scale to industrial scale, using equipment that would be typically used in industry to evaluate potential issues that the ATPS components may cause in the industrial equipment.
- Investigating a continuous process, as opposed to a batch process as done in this study. This could improve on the reagent use and time span of the process.
- It would also be beneficial to conduct a precise economic and energy usage evaluation on the process for industrial scale to determine the economic viability of the process.
- The recyclability of the PEG and tartrate phases should be evaluated to minimise to waste
- On an experimental level, some parameters studied could be narrowed down on or expanded,
    - Smaller PEG M_w can be investigated to see whether similar yields of TPC can be achieved, allowing a less viscous solution to be used
    - A shorter extraction time should be investigated to streamline the process further
    - Investigate interaction effects of temperature, PEG and salt concentration and biomass loading
- The pre-treatment process of the grape pomace should be refined, evaluating the effect that temperature and time may have on the polyphenols
- A final purification step should be investigated to separate the polyphenols from the PEG phase, and the antioxidant activity of the polyphenols after this final step should be tested.

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Appendix 1

Table 12 Current* prices in ZAR and USD of chemicals used in polyphenol extraction processes.

Product	ZAR	USD	Source
Polyethylene glycol (6000 g/mol^{-1} & 8000 g/mol^{-1})	27372/metric ton	1800/metric ton	Zibo Jiashitai Chemical Technology, China
Potassium sodium tartrate	3041/ metric ton	200/ metric ton	Richem, South Africa
Water (industrial zoning)	31.41/kL	2.04/kL	City of Cape Town, South Africa
Ethanol (99.9%)	14 170/kL	920/kL	Enterprise Ethanol, South Africa
Methanol (99.9%)	7307/kL	600/kL	Duvalier group, South Africa

** August 2019*

1 USD = 15 ZAR (August 2019)

Appendix 2

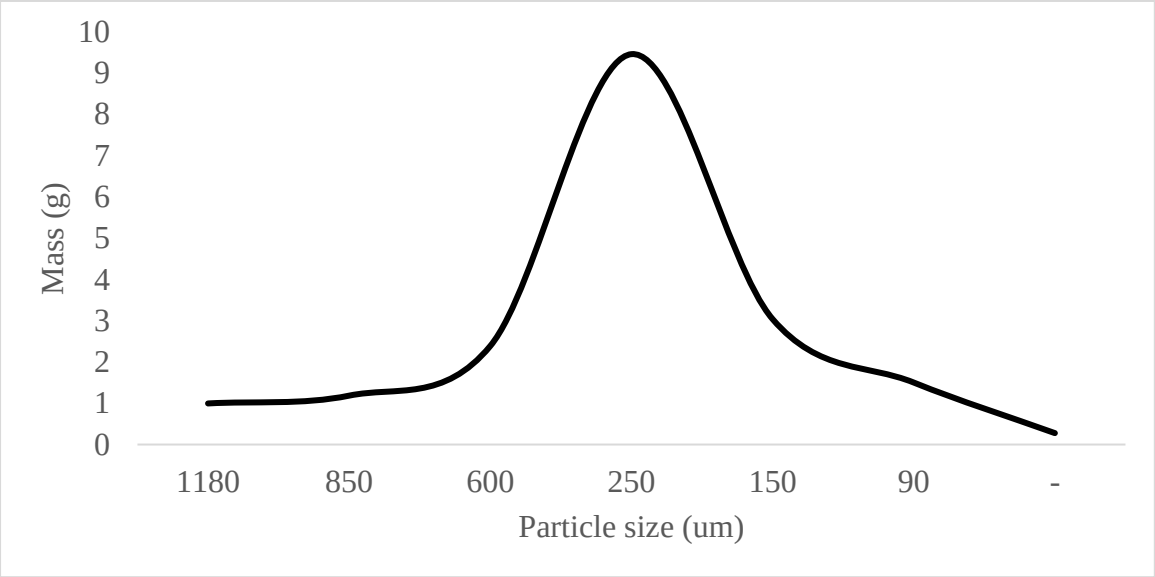

Figure 35 Particle size distribution (µm) of dried and milled grape skins