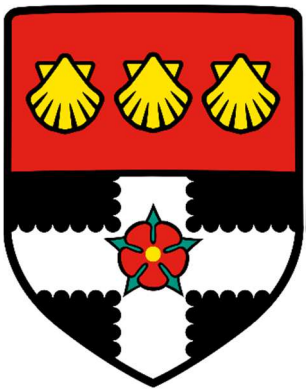

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RECOVERY OF POLYPHENOLS FROM RED GRAPE (*Vitis Vinifera*) POMACE EXTRACTS USING COLLOIDAL GAS APHRONS (CGA)

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DOCTOR OF PHILOSOPHY

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Recovery of Polyphenols from Red Grape (*Vitis Vinifera*) Pomace Extracts using Colloidal Gas Aphrons (CGA)

A thesis submitted by

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as a partial fulfilment for the Degree of Doctor of Philosophy

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Read in the name of your Lord Who created. He created man from a clot. Read and your Lord is Most Honorable, Who taught (to write) with the pen. Taught man what he knew not.

(Surah Al Alaq 96:1-5)

Declaration

I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.'

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NURMAHANI MOHD MAIDIN

Reading, 2017

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ANCORA IMPARO

Yet, I am still learning.

ABSTRACT

Polyphenols from grape pomace are of high interest because of their great sources of natural antioxidants, their potential benefits to human health and other functionalities related to their antioxidant capacity. However, the extraction process with organic solvents can lead to toxicity, so there is a need to remove the solvents before inclusion to food or non-food applications. Therefore, an integrated, cost-effective and environmental friendly separation process is necessary. In the present study, the recovery of polyphenols from grape pomace extracts was investigated using Colloidal Gas Aphrons (CGA). CGAs are defined as stabilised surfactant micro-bubbles generated by intense stirring of a surfactant solution with unique properties; making them an attractive alternative bio-separation process.

Hydro-alcoholic (EE) and hot water (HWE) extracts of grape pomace was applied to the CGA generated from TWEEN20 (non-ionic). Similar trend of recovery was obtained with both extracts, with some selectivity in relation to sugar, particularly in EE at lower volumetric ratio (volume of CGA to the volume of extract). An increase in volumetric ratio led to an increase in the polyphenols recovery but a decrease after ratio 16. The opposite trend was noted with drainage time; anincrease in drainage time led to a decrease in the polyphenols recovery.

The EE and HWE along with their CGA fractions were tested for inhibitory activity against skin relevant enzymes (collagenase and elastase) which were responsible for skin aging. The highest inhibition was found in the HWE. Further separation with CGA from both EE and HWE led to more potent fractions, particularly against collagenase. This effect might be related to the ability of TWEEN20 to solubilise the polyphenols and in this way facilitated the interaction of these polyphenols with the enzymes. When individual polyphenols permeability was tested across pig skin,a higher penetration and diffusion was obtained with the polyphenols in TWEEN20 than in water. Moreover, the highest stability of anthocyanins was obtained in CGA fractions that contained 8.58mM TWEEN20 when compared against crude extract and extract with sorbic acid. This suggested the role of surfactant at a given concentration in protecting the anthocyanins from oxidation.

As a final conclusion, CGA is an interesting, novel and promising recovery-formulation technique especially with TWEEN20 as the surfactant since its removal is no longer needed. CGA can contribute to the development of “greener” processes by

reducing the use of solvents and hence addressing the environmental concerns, making the CGA very attractive to be applied in cosmetics and food applications/formulations.

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Name	Explanation	Units
AAAPVN	N-Succ-Ala-Ala-Ala-p-nitroanilide	mM
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt	
AOP	Antioxidant power	
AP	Aphron phase	
BCA	Bicinchoninic acid	
BSA	Bovine serum albumin	
CGA	Colloidal Gas Aphrons	
ChC	*C. hystoliticum* collagenase Type IA	U/mL
CMC	Critical Micellar Concentration	M
CTAB	Hexadecyl Trimethyl Ammonium Bromide	
DPPH	2,2-diphenyl-1-picrylhydrazyl	
EE	Ethanolic extract	
FALGPA	N-[3—(2-furyl)acryloyl]-Leu-Gly-Pro-Ala	mM
GAE	Gallic acid equivalent	
HWE	Hot water extract	
HTAB	Hexadecyl Trimethyl Ammonium Bromide	
LP	Liquid phase	
ME	Malvidin-3-o-glucoside equivalent	Mg/L
n	Sample size	
PPE	porcine pancreatic elastase	U/mL
ROS	Reactive oxygen species	
RPM	Rate per minute	
SDBS	Sodium Dodecyl Benzene Sulphonate	
SDS	Sodium Deodecyl Sulfate	
SF	Separation factor	
TE	Trolox equivalent	µM
TP	Total phenolic	Mg/L
TPI_{280}	Total phenolic index (measured at 280nm)	
H_0	initial height of the dispersion	
H_{10}	height of initial volume of liquid	
V_{CGA}	Volume of CGA	mL
V_{feed}	Volume of feed	mL
V_g	Volume of gas	mL
$V_{surfactant}$	Volume of surfactant	mL
V_0	Volume of dispersion	mL
V_{10}	Volume of the initial	mL
$[y]_{aphron}$	concentration of compounds (y) in the aphron phase	Mg/L
$[y]_{liquid}$	concentration of compounds (y) in the liquid phase	Mg/L
Ɛ	Gas hold-up	
$T_{1/2}$	Half-life	Sec
$t_{1/2}$	Half-life	day

Chapter 1: Introduction and Literature Review

1.1 Introduction

Grapes are one of the largest and most important fruit crops being cultivated in the world. They may be processed into juice, jams, raisins and wines. Eighty percent of grape production goes into wine-making resulting in a massive amount of grape pomace (Kammerer, Kammerer, Valet, & Carle, 2014).An estimation of about 5 to 15 million tons of grape pomace is produced solely from Europe, with 80% of the pomace being disposed as soil conditioner or compost. Grape pomace may still contain a large number of polyphenols which can be beneficial as natural antioxidants (Spigno & De Faveri, 2007)and with additional activities such as, anti-diabetic(Sri Harsha, Lavelli, & Scarafoni, 2014), anti-collagenase and anti-elastase (Wittenauer, Mäckle, Sußmann, Schweiggert-weisz, & Carle, 2015). Moreover, red grape pomace is also rich in anthocyanins. Anthocyanins are natural colorants that can be further used to replace synthetic colourants that also have beneficial health effects (Castañeda-Ovando, Pacheco-Hernández, Páez-Hernández, Rodríguez, & Galán-Vidal, 2009). These residues which are high in polyphenols and organic matter with low pH can cause phytotoxic effects if applied to crops and wetlands (Lavelli, Harsha, Laureati, & Pagliarini, 2017), thus extracting and exploiting this pomace is advantageous and may also signify economic gains and decrease environmental problems caused by grape pomace accumulation.

Solvents extraction has been used in recovering these polyphenols because it is considered the most efficient method and for its simplicity (Syed, Brazinha, Crespo, & Ricardo-da-Silva, 2017). However, the choice of solvents is vital because some solvents could be toxic to the environment and unsuitable for human consumption/application,

thus they may need to be removed at a later stage of the extraction. Moreover, the use of solvents at large-scale could be limited due to their high costs. Therefore, in addressing this issue, a surfactant-based separation technique has been developed. Surfactants unique structural characteristic consists of a group that has little tendency for the water, called hydrophobic, and a group that has strong attraction for the water called the hydrophilic group (Rosen, 2004). The hydrophobic group is usually a long chain carbon residue whereas the hydrophilic group can be non-ionic, ionic (cationic-anionic) or zwitterionic.

CGA are surfactant based microfoams which have been used as a separation (flotation) method for waste water treatment (Hashim & Sen Gupta, 1998), removal of dyes (Basu & Malpani, 2001; Huang, Wang, & Dai, 2002), recovery of proteins (Fuda, Bhatia, Pyle, & Jauregi, 2005; Santos-Ebinuma, Teixeira, Pessoa, & Jauregi, 2016), carotenoids (Alves, Ulson De Souza, Ulson De Souza, & Jauregi, 2006; Dermiki, Bourquin, & Jauregi, 2010; Dermiki, Gordon, & Jauregi, 2009), natural colourant (Santos-Ebinuma et al., 2016) and polyphenols (Spigno, Dermiki, Pastori, Casanova, & Jauregi, 2010; Spigno, Amendola, Dahmoune, & Jauregi, 2015; Spigno & Jauregi, 2005). CGA have also been used remediation of contaminated soil (Hashim, Mukhopadhyay, Gupta, & Sahu, 2012; Mukhopadhyay, Mukherjee, Hashim, & Sen Gupta, 2015),

Based on the applications of CGA listed above, it is clear that CGA application shows some promise in the separation and recovery technique. However most studies focus on CGA as a separation method in chemical waste treatments applications and the studies on applications of CGA in food and cosmetics remain limited.

Apart from the unique characteristics that CGA possess, having surfactant could be advantageous. Surfactants are often used in cosmetic formulations in the form of

emulsions in order to help water insoluble compounds/active ingredients remain intact in the emulsions. With regard to topical application of polyphenols onto skin which need to pass the great barrier of stratum corneum, surfactant has the ability to alter the composition of stratum corneum by denaturing the secondary structure of proteins in it. This eventually will result in an increase in permeability into the skin; thus facilitating delivery of the polyphenols to the target areas. Moreover, surfactant has a strong influence on the stability of polyphenols (Lin, Wang, Qin, & Bergenståhl, 2007). This could be related to the ability of surfactants to solubilise, thus, encapsulating these polyphenols into micelles, resulting in better stability of polyphenols against oxidation during storage. With the advantages above, we therefore believe that CGA separation leads to extracts in a surfactant rich solution which may be an optimum medium for formulation of polyphenols; thus CGA shows great promise and has a place in the food and cosmetic industry.

1.2 Literature Review

1.2.1 Polyphenols of grape pomace

Over the past decades, there is a growing attention to the study of polyphenols. These are mainly driven by the recognition of the antioxidant properties of polyphenols, their great abundance in our diet, and their probable role in the prevention of various diseases associated with oxidative stress and age-related diseases, such as cancer and cardiovascular and neurodegenerative diseases (Lurton, 2003).Haslam & Cai (1994) briefly described the meaning of polyphenol by water-soluble plant phenolic compounds, having molecular masses ranging from 500-4000 Da and possessing 12-16 phenolic hydroxyl groups on five to seven aromatic rings per 1000 on relative molecular mass.

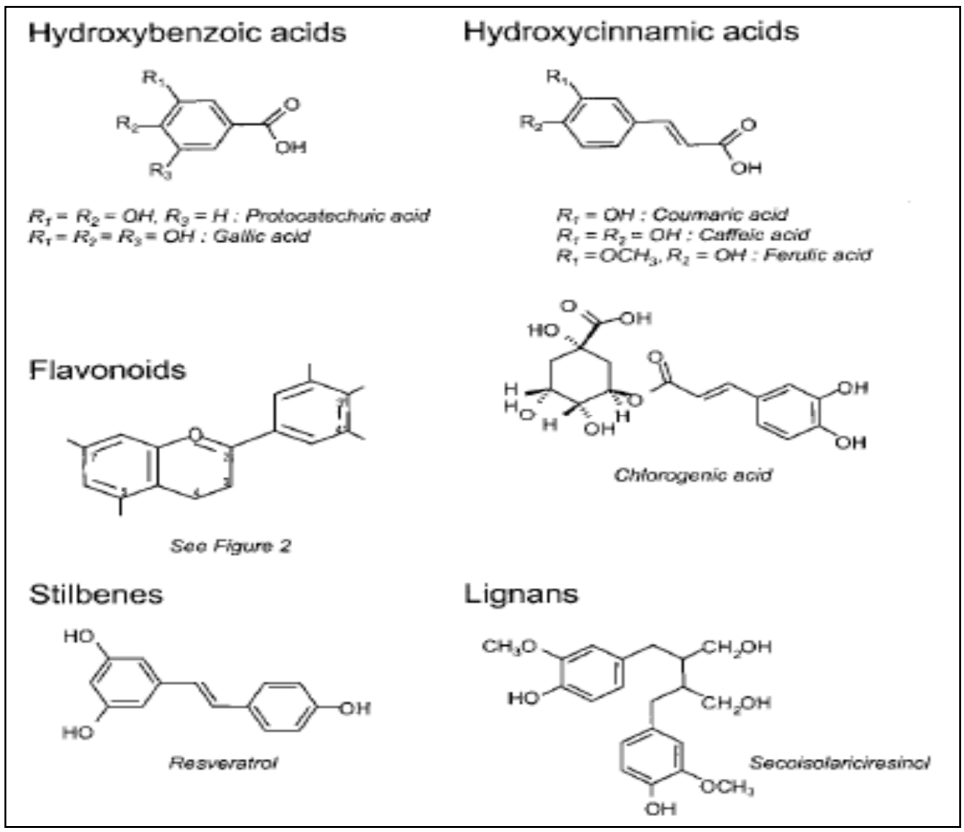


Figure 1.1Classof polyphenols (Manach, 2004).

Polyphenols can be classified into four main classes: i) phenolic acids, ii) flavonoids, iii) stilbenes and iv) lignans (Figure 1.1) (Manach, 2004). These classes are made based on a function of the phenol rings number they contain and of the structural elements that bind these rings together or to one another. Flavonoid is the most abundant type of polyphenol that exists in fruits and vegetables, and hence is further divided into six other classes (Figure 1.2).

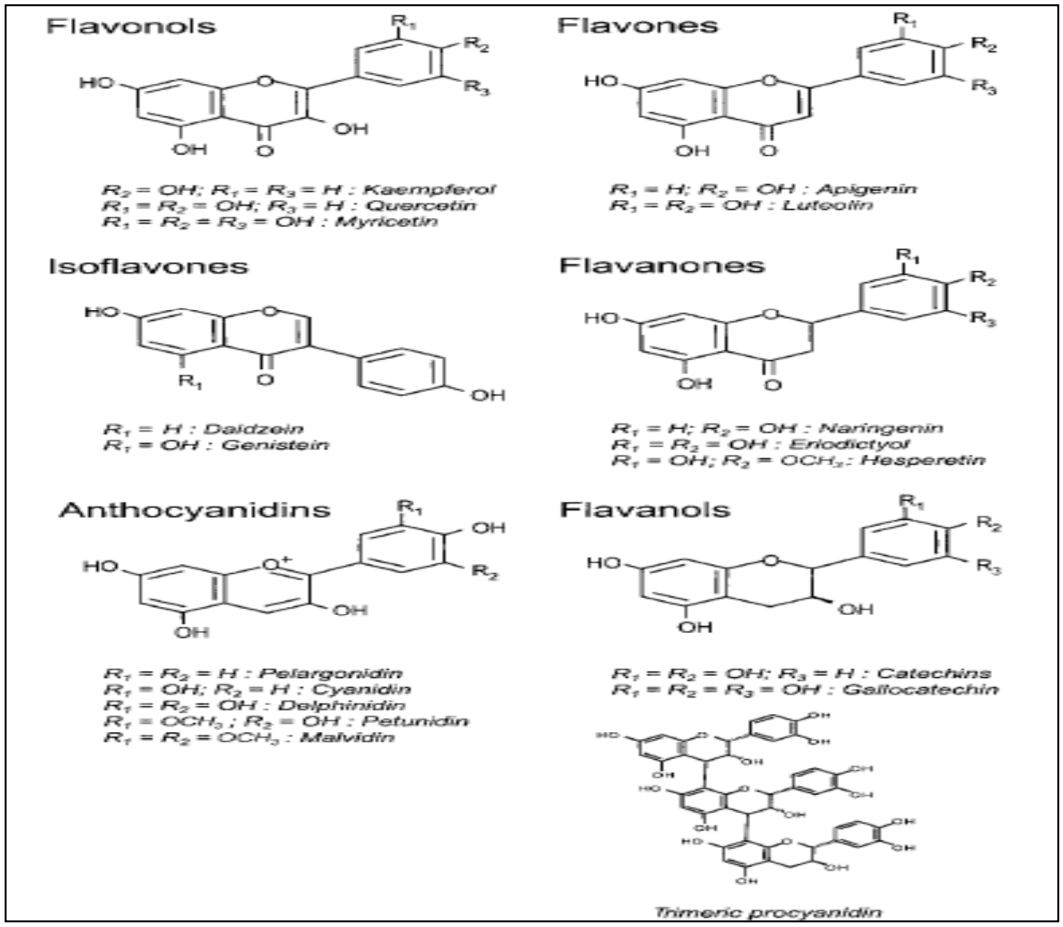


Figure 1.2 Chemical structure of flavonoids (Manach, 2004).

Wine contains different kinds of polyphenols with desirable biological properties. The most abundant types of polyphenols found in wine are the flavonoids, phenolic acids and proanthocyanidins (Medjakovic et al., 2008). Anthocyanins are the major phenolics exclusively present in red grape skin. They are monoglucosides of five anthocyanidins called delphinidin, cyanidin, petunidin, peonidin and malvidin. These compounds are responsible for the blue and red colour of grapes, but the use in processed foods are limited because of the colour instability to degradation (Wrolstad, Durst, & Lee, 2005). Their stability is affected by several factors such as pH, storage temperature, chemical structure, concentration, light, oxygen, solvents, and the presence of enzymes, flavonoids, proteins and metallic ions(Castañeda-Ovando et al., 2009).

The distribution of different polyphenols presence in grapes is tabulated in Table 1.1. Apart from the growing conditions such as soil, sun exposure and climate, the wine-making technology is the most influential factor to the quantity and quality of polyphenol in the final product. During wine-making, most polyphenols are extracted out; but due to incomplete fermentation, considerable high amount of polyphenols remained in the pomace. Approximately, 20% of the grapes used in wine-making are of pomace after the fermentation, which is a huge amount considering that wine production is one of the leading beverage production in the world.

Previously, these by-products are used as animal feed or soil compost, leaving them in wetland. However, in recent years, the environmental management aforementioned these by-products could pose serious environmental concerns because their residues have a low pH, high organic matter content and may exert phytotoxic effects if applied to crops or wetlands (Lavelli et al., 2017). Therefore, the by-product recovery and conversion into value added products is considered as a unique strategy to overcome the cost of not recycling, including waste disposal and decontamination of affected areas (Devesa-Rey et al., 2011).

Table 1.1 Distribution of main phenols in grape (Pinelo, Arnous, & Meyer, 2006).

Compound	**Grape pomace**	**Skin**	**Seed**	**Stem**
Gallic acid	0.03-0.11	0.03	0.10-0.11	-
Coutaric acid	0-1.23	0.03-1.23	-	-
Caftaric acid	0-6.97	0.11-6.97	-	0.04
Phenolic acids	0.03-8.31	0.17-8.23	0.10-0.11	0-0.04
Catechin	0-0.18	0-0.16	2.14-2.15	0.06
Epicatechin	0-0.16	0-0.13	0.88-0.91	0.28
Epigalocatechin	0-0.05	Traces	0.05	0.01
Epigallocatechin 3-gallate	0-0.07	-	0.06-0.07	-
Epicatechin 3-galate	0-0.03	0.04	0.25-0.31	0.07
B1	0.11-0.6	0.11-0.6	0.14-0.16	-

B2	0.01-0.84	0.01-0.84	0.04-0.18	-
Tannins	0.22-2.32	1.61	2.32	0.22-0.39
Total flavan-3-ols	0.34-4.25	0.12-3.38	3.56-6.15	0.22-0.89
Delphinidin 3-glc	0.44-1.11	0.44-1.11	-	-
Cyanidin 3-glc	1.51-3.81	1.51-3.81	-	-
Petunidin 3-glc	0.53-1.34	0.53-1.34	-	-
Peonidin 3-glc	0.99-2.49	0.99-2.49	-	-
Malvidin 3-glc	4.12-10.19	4.12-10.19	-	-
Delphinidin 3-acglc	0.08-0.19	0.08-0.19	-	-
Cyanidin 3-acglc	0.11-0.28	0.11-0.28	-	-
Peonidin 3-acglc	0.62-1.74	0.27-0.30	-	-
Malvidin 3-acglc	0.07-0.22	0.62-1.74	-	-
Total anthocyanis content	11.47-29.82	11.47-29.82	-	-
Quercetin 3-glucoside	0.01-0.2	0.15-0.2	0.01-0.02	0.02
Myrecetin 3-glucoside	Traces	-	-	Traces
Quercetin 3-glucoronide	0.01-0.29	0.22-0.29	0.01-0.02	0.2
Kaempferol 3-glucoside	0.01-0.14	0.11-0.14	0.01	Traces
Myrecetin 3-glucoronide	Traces	-	-	Traces
Total flavonols	0.03-0.63	0.48-0.63	0.02-0.05	0-0.22

- All values are in mg/g
- glc = glucoside
- aglc = acetyl glucoside

1.2.2 Activities and applications of grape pomace

1.2.2.1 Antioxidant Activity

Grape pomace is known as one of the cheapest source of antioxidant. In a study conducted by Rockenbach et al., (2011) on selected red wine pomace from Brazil revealed that there was a significant different in total phenolics and total anthocyanins among all the variety. This study focused on measuring the antioxidant activity of the grape pomace extracted with acidified methanol using four different techniques, namely the ABTS, DPPH, FRAP and linoleic acid model system. The study also observed that

the inhibition of oxidation measured by β-carotene/linoleic acid method tends to increase with the increase in anthocyanins content. Quantification of individual phenolic compounds from grape pomace extract observed the presence of anthocyanins, quercetin, kaempferol, catechin and epicatechin, t-resveratrol and gallic acid with catechin (150.16 mg/100g) being the most abundant, non-anthocyanin compounds in the grape pomace extract.

Vergara-Salinas et al., (2015) conducted a characterisation of grape pomace extracted with pressurised hot water in terms of its chemical and biological antioxidant property. The study revealed that a high temperature extract (200°C) hada higher antioxidant activity of 15mg AAE/g dry weight as measured by FRAP assay and Maillard reaction products (MRPs), but with a lower polyphenol content as opposed to extract of 100°C. The polarity of water decreased with the increasing of temperature due to the breakage of hydrogen bonds, hence this could possibly explain the low recovery of polyphenol in 200°C extract. Furthermore, its antioxidant activity was evaluated using cell culture approach in which it showed that the grape pomace extract hadexhibited a protective effect of cells under oxidation state (Vergara-Salinas et al., 2015).

Murthy, Singh & Jayaprakasha, (2002)screened different fractions of grape pomace extracts, extracted from different types of solvents for its antioxidative capacity in different models. The results showed that the methanol extract of grape pomace gave the highest antioxidant activity when measured using DPPH assay. The extract was then tested further and the results revealed 71.7, 73.6and 91.2% inhibition respectively using the thiobarbituric acid method, hydroxyl radical scavenging activity, and LDL oxidation at 200mg/L. Treatment of albino rats of the Wistar strained with a single dose of carbon tetrachloride (CCl_4) at 1.25 mL/kg of body weight decreased the activities of catalase, superoxide dismutase (SOD), and peroxidase by 81, 49, and 89%, respectively, whereas

the lipid peroxidation value increased nearly 3-fold. Owing to this property, this study gave an indication for its possible application on food preservation as well as health supplement and nutraceutical.

Spigno & De Faveri (2007) aimed to optimise the extraction of phenolic compounds from grape marc by investigating the extraction time, temperature and solvent concentration on the phenols concentration and antioxidant power. Phenols yield increased for ethanol concentration from 70-90% and remained constant from 40-70%, while phenols concentration of extracts decreased when ethanol concentration was above 50%. Antioxidant power by means of ABTS method was found to be closely correlated to total phenols concentration, and was not influenced by water content of ethanol, suggesting that this variable only influenced the amount but not the nature of the extracted compounds. Furthermore, the optimisation of grape pomace solvent extraction was also conducted by Libran et al., (2013). The optimisation parameters include pH, time, and concentration of solvent used during solid-liquid extraction by analysing total polyphenol content, flavanols, flavonols, phenolic acids and anthocyanins. Antioxidant activity of the extracts was also determined using DPPH method. Results showed that the optimum conditions were 2 hours of extraction in 75% ethanol liquid mixture at pH 2.

Moreover, polyphenols obtained from white grape pomace by aqueous extraction were analysed for their antioxidant power. The results showed that smaller particle size influenced the recovery of the polyphenols in the extract(Sánchez-Alonso, Jimínez-Escrig, Saura-Calixto, & Borderías, 2006). The antioxidant activity of the polyphenols fractions was similar to catechin and procyanidin B2 but was lower than that of gallic acid.

1.2.2.2 Antimicrobial activities of grape pomace polyphenols

In addition to antioxidant activities, polyphenols extracted from grape pomace showed antimicrobial activity against specific strains of bacteria such as *Streptococcus mutans, Staphylococcus aureus, Candida albicans, Escherichia coli* and *Listeria innocua* (Daglia et al., 2007; Deng & Zhao, 2011).These studies showed that grape seed polyphenols functioned as bactericidal, which caused disruption of the bacterial cell wall. In another study, grape pomace extract-based incorporated in a protective film showed antibacterial activity against both *E.coli* and *L. innocua* with the reduction of 1.7-5log (Deng and Zhao, 2011). Furthermore, the spoilage microorganisms including yeast and moulds were also inhibited by 5-10% in beef patties (Sagdic, Ozturk, Yilmaz, & Yetim, 2011). Resveratrol which is rich in grape skin is one of the polyphenols found in grapes that has been reported to have strong antimicrobial activity. The study of Paulo, Ferreira, Gallardo, Queiroz, & Domingues, (2010) verified the antibacterial activity of resveratrol against Gram positive bacteria and found that the antibacterial effects of resveratrol were attributed to bacteriostatic action. Therefore, polyphenols extracted from grape pomace have the potential to be used as food preservative to suppress the growth of pathogenic bacteria.

1.2.2.3 Application of grape pomace in food

In recent years, there is a growing attention in incorporating fruit processing wastes as functional food ingredients since it is rich in dietary fibre and also bioactive compounds that would benefit human health. The idea of incorporating antioxidant dietary fibre into flour to make high dietary fibre bakery goods, whereby the polyphenols can greatly improve the colour, aroma and taste of the products has been conducted by

many researchers and food industry. For instance, grape pomace was mixed with sourdough for rye bread (Mildner-Szkudlarz et al., 2011) and grape seed flour was incorporated in cereal bars, pancakes and noodles (Rosales Soto, Brown, & Ross, 2012).

Apart from promoting human health, grape pomace plays a vital role as antioxidants and antimicrobial agents to extend the shelf life of a product. Several studies showed that the addition of grape pomace powder into minced fish and chicken breasts have delayed the process of lipid oxidation (Sánchez-Alonso et al., 2006; Sáyago-Ayerdi, Brenes, Viveros, & Goñi, 2009). Furthermore, study done by Sagdic, Ozturk, Yilmaz, & Yetim, (2011) revealed that the grape pomace has antimicrobial activity against foodborne pathogens including the spoilage microorganisms by inhibition of 10% throughout the storage period when added into beef patties. Furthermore, Garrido, Auqui, Martí & Linares (2011) studied the effect of incorporating grape pomace extracts in pork burgers quality (pH, microbial spoilage, lipid oxidation and colour coordinates) packed under aerobic conditions over a period of six days. The study revealed that grape pomace extracted with high-low instantaneous pressure at 0.06g/100g of polyphenols final product gave the highest colour stability, lipid oxidation inhibition and the best global acceptability after six days storage. These results might be useful for the industry to replace synthetic preservatives with natural ones to prevent microbial spoilage in meat and fish industries.

Tseng & Zhao (2013)studied the applicability of wine grape pomace as the source of antioxidant dietary fibre in enhancing nutritional value and improving the storability of yoghurt and salad dressing. This study revealed that the wine grape pomace might be utilized as an alternative source of antioxidant dietary fibre to fortify yoghurt and salad dressing, as the presence of grape pomace not only increased the total phenolic content and dietary fibre but also delayed the lipid oxidation samples during refrigeration storage.

The physicochemical properties of products fortified with the pomace extracts was almost similar to the control (no pomace added), but those fortified with dried pomace powder possessed the highest dietary fibre. However, the total phenolic content and DPPH scavenging activity decreased throughout the storage period with more reduction was observed in fortified yoghurt as opposed to salad dressing. The authors explained this observation could possibly due to the interactions between proteins in yoghurt and phenolic compounds in the pomace (Tseng & Zhao, 2013).

A study on evaluating the effects of chitosan films properties with the incorporation of grape pomace extracted from hot water, chloroform and hexane was conducted by Ferreira, Nunes, Castro, Ferreira & Coimbra (2014). The evaluation revealed that the film extracted with water had the most homogenous and smooth morphology whilst those with chloroform and hexane films showed some degree of stiffness. However, the chitosan-based films with chloroform and hexane extract exhibited highest antioxidant capacity measured by ABTS and DPPH assays whilst the water extraction chitosan-film based showed an improvement in FRAP and reducing power assays. A summary of the applications and biological activity of grape pomace is tabulated in Table 1.2.

Table 1.2 Summary of grape pomace and its application

Variety	Extraction method	Biological activity	Applications	Reference
Carbenet Sauvignon Merlot Bordeaux Isabel	0.1% HCl acidified MeOH	193-485 µMol TE/g (ABTS) 188-505 µMol/g (DPPH) 117-249 µMol/g (FRAP)	-	(Rockenbach et al., 2011)
Pinot Noir	70% acetone	37.46 mg AAE/g (DPPH)	Yoghurt and salad dressing	Tseng and Zhao (2013)
-	MeOH	DPPH	-	(Murthy, Singh & Jayaprakasha, 2002)
-	Water	DPPH	Fermented milk	(Aliakbarian et al., 2015)
Barbera Pinot Noir	60% EtOH	(ABTS)	-	Spigno et al., (2006)
White grape	-	466 µMol TE/g (FRAP) 284 µMol TE/g (ABTS)	Minced fish Chicken breast	Sanchez et al., (2008)
-	MeOH + High-Low Instantaneous Pressure	Lipid oxidation	Food packaging (pork patties)	(Garrido et al., 2011)
Turkey variety	Defatted, 95% EtOH	Antimicrobial property	Beef patties	(Sagdic et al., 2011)
Turkey variety	Defatted, 95% EtOH	105-480 µg/g (IC_{50} DPPH)	Orange/Apple juice	(Sagdic et al., 2011)
Pinot Noir Merlot	-	Antimicrobial property	Food packaging	Zhao and Tseng (2012)
Merlot Carbenet Sauvignon	-	9-12 µMol TE/g (DPPH)	Flour (for cereal bars, noodles)	(Rosales Soto et al., 2012)
Merlot, Syrah	Supercritical fluids	Antimicrobial property	-	(Oliveira et al., 2013)
-	Hot water Chlroform Hexane	(ABTS) (DPPH) (FRAP)	Food packaging (incorporate with chitosan formulation)	(Ferreira et al., 2014)
Verdejo	Enzyme	4238nmol TE/g(ORAC)	-	Rodriguez-Rodriguez et al., (2012)

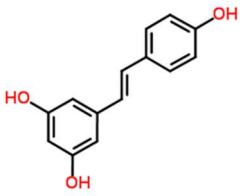
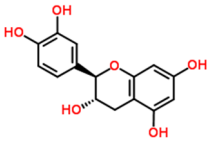
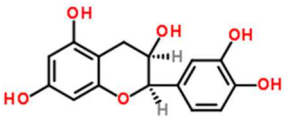
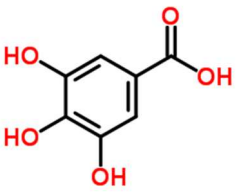
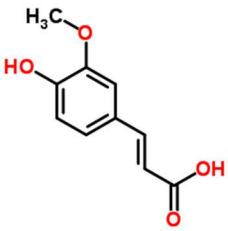
Carbenet Sauvignon	Pressurized Hot water	10-15 mg AAE/g (FRAP)	-	Vergara-Salinas et al., (2015)
White grape	-	62-524 g GAE/Kg (Total phenolic content)	Flour (for cookies)	(Acun & Gül, 2014)
Merlot Carbenet	-	DPPH	Bars, Pancakes, Noodles	(Rosales Soto et al., 2012)
Chardonnay	Grape skins fibre	FRAP	Puree	(Lavelli, Sri Harsha, Torri, & Zeppa, 2014)
-	Ultra sound assist extraction (water)	518 ± 7.4µMol TE/g (ABTS)	Pasta	(Marinelli, Padalino, Nardiello, Del Nobile, & Conte, 2015)
Grape pomace	-	DPPH	Sausages emulsion	(Riazi, Zeynali, Hoseini, Behmadi, & Savadkoohi, 2016)

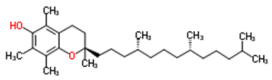
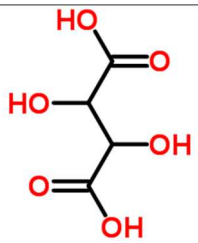
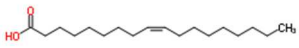
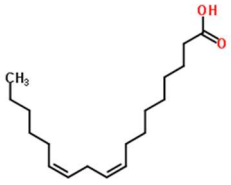
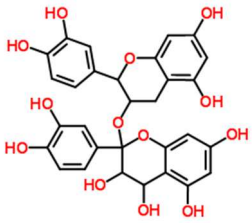
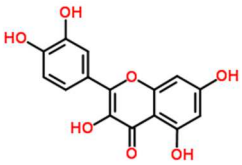
1.2.2.4 Application of grape pomace in cosmetics

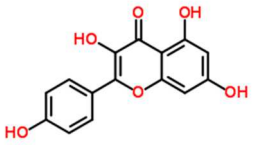
As described in the previous sections, many polyphenols can be extracted from grape pomace, but studies on the incorporation into cosmetic formulations or pharmaceutical products are limited. Safety issues, skin permeation and efficacy delivery are concerns in the development of new cosmetic products using agro-industrial by-products (Nunes, Rodrigues, & Oliveira, 2017). However, several cell-based in vitro models, predicting the safety and toxicity of cosmetic ingredient were allowed by the European Centre for the Validation of Alternative Methods (ECVAM) (Nunes et al., 2017). Assessment of skin irritant is considered a primary procedure in evaluating hazard and classifying a substance. Therefore, the application of grape pomace as active ingredients in cosmetic formulations remains challenging, although some studies showed

the potential applications of grape pomace as anti-aging agents (Wittenauer et al., 2015). Table 1.3 summarises the most important compounds present in grape pomace and their potential functions in topical formulations.

Table 1.3 Individual compound reported in grape pomace with cosmetic interest

Compounds	Chemical structure[c]	Function[a,b]
Resveratrol		Anti-aging
Catechin		Anti-aging
Epicatechin		Anti-aging
Gallic acid		Anti-aging UV protector
Ferulic acid		UV protector

Vitamin E		Anti-aging
Tartaric acid		Exfoliating Depigmenting
Oleic acid		Anti-aging Moisturising agent
Linoleic acid		Anti-aging
Proanthocyanidins		Anti-inflammatory
Quercetin		Anti-aging

Kaempferol		Anti-aging

[a]Nunes, Rodrigues, & Oliveira, (2017)
[b]Wittenauer et al., (2015)
[c]Royal Society of Chemistry, (2015)

1.2.3.1 Antioxidant and Anti-aging properties

Skin aging is a complex, progressive and inevitable biological process that can be influenced by several factors such as genetics, environmental changes, lifestyle and metabolic processes (Pimple & Badole, 2013). In normal/unstressed cells, reactive oxygen species (ROS) are being produced by the functions of mitochondria in everyday cells metabolism. These ROS have been kept in balance by the action of antioxidant enzymes found inside cells such as superoxide dismutase (SOD), catalase and peroxides (Menaa, Menaa, & Tréton, 2013). When cells become stress, this balance is interrupted. Skin is the major organ that is directly exposed to the sunlight, hence deterioration due to environments damage is inevitable. Exposure of skin to UV radiation and environmental pollutions are examples of cells stress-induced extrinsic factor which could overwhelm the ROS leading to a change in the skin normal behaviours (Menaa et al., 2013).

Several polyphenols present in the grape pomace extract were identified as the key active ingredients against collagenase and elastase enzymes, responsible for skin aging. Wittenauer et al., (2015)found that gallic acid showed considerable inhibition values against elastase but quercetin 3-o-glucoside and quercetin 3-o-glucoronide had more pronounced effect on the collagenase. However, resveratrol itself was found to exhibit

very low inhibitory effects against both enzymes. The study also showed that the crude grape pomace extract had a higher inhibitory activity in both enzymes suggesting synergistic interactions between polyphenols, and the enzyme could play an important role regarding the inhibition mechanism. The same observation has also been noted by Ghimeray et al., (2015) by combining several superfruit extracts which showed that the inhibition of collagenase was notably high.

Naturally occurring quercetin and kaempferol have been found to exhibit strong inhibition against collagenase in comparison to other compounds from different groups, suggesting the hydroxylation patter in the B-ring is an important determinant to the inhibitory activity (Sin & Kim, 2005). In addition, epicatechin that may be found in the grape stem also exhibited strong anti-collagenase activity by possibly binding to the Zn^{2+} ion within the enzyme, thus preventing it from binding with the substrate (Wahab, Rahman, Ismail, Mustafa, & Hashim, 2014).

Despite the above studies, the application of grape pomace in cosmetic is rather scarce. It is important to bear in mind that the topical effectiveness of grape polyphenols in human skin implies some considerations during formulations development. Polyphenols (or antioxidants) may be oxidised and thus inactivated before reaching the target site and permeability across the skin remained important. Formulations cream-based, lipid soluble, or organic solvent-based can be studied to enhance the permeation of polyphenols into the skin(Nichols & Katiyar, 2010). Abla & Banga, (2013)studied the permeation of three polyphenols (catechin, resveratrol and curcumin) and a vitamin (retinol) into porcine ear skin using propylene glycol as a vehicle. It was demonstrated that 10% of the retinol was retained in the stratum corneum whereas 90%penetrated into the underlying skin. The amount of total catechin in the stratum corneum was higher than

the resveratrol one, but similar amount of polyphenols was found in the underlying skin (Abla & Banga, 2013).

1.2.3.2 Protection against UV damages

Skin is exposed daily to environmental stress. For instance, UV radiation has the ability to penetrate deeply into the skin's epidermis and dermis layers, causing skin injuries, including the generation of ROS which after chronic exposure, can cause skin photo aging, hyper-pigmentation and also skin cancer (Soto, Falqué, & Domínguez, 2015). However, these can be prevented if a proper UV protection is used. Natural polyphenols have been proposed as an active ingredients in cosmetic formulations as sunscreens ingredients due to their similar structure with chemical UV filters, presenting the same mechanism of action (Nunes et al., 2017).

A number of studies also described the effects of polyphenols extracts on human cells and UV-irradiated cells. For example, the pre-treatment of HaCat keratinocytes with chlorogenic acid and rosemarinic acid leads to a decreased intercellular ROS formation, induced by UVB or hydrogen peroxide (Cha et al., 2014; Pérez-Sánchez et al., 2014). Resveratrol found in grape skin and/or seed is a promising natural compound with its ability to scavenge free radicals. Topical application of resveratrol to SKH-1 hairless mice before exposure to UVB radiation resulted in the inhibition of UVB-mediates skin edema and significantly inhibited the UVB-mediated induction of cyclooxygenase enzyme activity and protein expression of ornithine decarboxylase, a well-established markers for tumor promotion (Afaq, Adhami, & Ahmad, 2003).

1.2.3 Conventional and alternative extraction technologies for polyphenols recovery

Several methods have been proposed and developed to enhance the extraction and isolation of polyphenols from different plants. In this section, solvent extraction is regarded as a conventional technology as it is heavily used for polyphenols extractions. Meanwhile, microwave-assisted, pulsed-electric field, supercritical fluid and enzyme-assisted extractions are discussed in view of the alternative technologies.

1.2.3.1 Solvent extraction

Solvent extraction (SE) is the most common used technique to recover certain compounds from different types of materials including soil, microorganism and more frequently plants residues. In the SE process, a solid-liquid extraction is typically applied, where the raw material is dried for size reduction before being exposed to different solvents for extraction process to take place. SE can be repeated several times to the same solid residues to ensure complete extraction of desired polyphenols. The obtained extracts are generally submitted to centrifugation or filtration to remove the solid residues. The extracts which are rich in polyphenols can be further used for various purposes such as additives, food supplements or can be encapsulated for functional food or pharmaceutical purposes (Lavelli et al., 2014).

One of the main advantages of using certain organic solvents is their ability to recover both polar and non-polar molecules as alkaloids, phenols, fatty acids and for its simplicity (Li, Smith, & Hossain, 2006). The main disadvantage is the high toxicity for the human health and dangerousness for the environment. Furthermore the solvent has to be removed from the extract by evaporation or concentration, increasing overall process costs. Therefore, the choice of solvents is very important to tackle the toxicity and overall

processing cost problem. The use of hydro-ethanol solvents in extracting polyphenols from grape pomace has been seen advantageous as they are biocompatible and have the GRAS status (Syed et al., 2017). Additionally, in order to minimise the risk of thermos degradation of polyphenols, extraction at milder temperatures and longer extracting time could be carried out. In fact, long extraction time lead to an increase in total phenolic content in grape extracts (Pinelo et al., 2006).

1.2.3.2 Ultrasound-assisted extraction

Ultrasound-assisted extraction (UAE) has been widely proposed as an alternative technology to the organic solvent extraction. UAE method can result in cavitational effect that improves heat and mass transfer through plant cell wall disruption (Barba, Zhu, Koubaa, Sant'Ana, & Orlien, 2016). UAE also offers advantages such as greater penetration of the solvent into cellular material, shorter processing time, higher product yields, and greener and cheaper processing technique (Galanakis, 2012). Da Porto, Porretto, & Decorti, (2013) studied the recovery of grape seed oil by UAE at 30mins and soxhlet extraction running for 6 hours. The results showed that the same oil yield was obtained in both techniques. Therefore, the authors concluded that the same recovery could be achieved with significant decrease of extraction time and hence cheaper processing technique. Nevertheless, a study conducted by Cho, Hong, Chun, Lee, & Min (2006) found a significant increase in resveratrol yield from grape (up to 28%) and a decrease of extraction time as compared to a conventional solvent extraction at 60°C for 30mins.

1.2.3.3 Pulsed Electric Fields (PEF)

Pulsed electric fields (PEF) has been tested for polyphenols extraction from various by-products (Galanakis, 2012). PEF treatment is applied to a material placed between two electrodes, normally conducted at ambient temperature of a voltage from 20-80Kv/cm. When plant cells are exposed to electric fields, cell membranes are being damaged and subsequently created reversible or/and irreversible pores, a phenomenon called “electroporation”. Polyphenols or antioxidants are then expected to diffuse the membrane by using the principles of mass transfer. PEF has been acquired by numerous food industries for its scalability. However, the strength of the electric field is one of the most important factors that influence the concentration of the polyphenols. A recent study on evaluating PEF on the selective recovery of polyphenols from fermented grape pomace showed that PEF treatment was more selective for the recovery of anthocyanins with the increment of recovery up to 55% as compared to ultrasounds(Barba, Brianceau, Turk, Boussetta, & Vorobiev, 2015). Therefore, the results varied depending on the food matrices and also the PEF treatment conditions applied (Barba et al., 2016).

1.2.3.4 Supercritical fluid extraction

The most frequently used fluid for supercritical extraction process is CO_2because it has low critical point (31.1°C), no toxicity and is safe to use. In addition to these, the process using CO_2 occurred in the absence of light and air, thus minimising the oxidation reactions. Nevertheless, due to the non-polar characteristic of CO_2, it is not preferentially used alone to extract polar polyphenols. Therefore, it has often mixed with organic co-solvents (eg: ethanol, methanol or acetone). These solvents increase the solvating power of CO_2 ;thus, increasing the solubility and extractability of polar polyphenols.

Supercritical fluid extraction has been widely investigated to recover polyphenols from grape pomace, in particular the recovery of resveratrol (Barba et al., 2016). A study carried out by Pascual-Martı, Salvador, Chafer, & Berna, (2001) used ethanol as a co-solvent managed to recover 100% of resveratrol from grape skin of *V. vinifera*. On the other hand, Murga, Ruiz, Beltran, & Cabezas, (2000) evaluated the potential mixture of carbon dioxide and alcohol under supercritical conditions to selectively extract polyphenols from grape seed. The authors found that there was a significant increase in solvent capacity and the pressure and amount of alcohol were increased; hence it was concluded that the solvent capacity could be used to selectively extract individual polyphenol from the grape seed. Despite all these advantages of supercritical fluid extraction, the capital cost of these techniques is expensive and its scalability remains challenging.

1.2.3.5 Enzyme-assisted extraction

Enzyme-assisted extraction was developed in a small and large scale to recover various bioactive compounds including polyphenols (Maier, Göppert, Kammerer, Schieber, & Carle, 2008). Most studies used it in conjunction to another extraction method, typically solvent extraction. Enzymatic-assisted reaction is considered as safe, environmental friendly and some enzymes can be recovered at the end of the process, making the application interesting and scalable (Landbo & Meyer, 2001). Polyphenols are typically bound to the cell wall of the plant, making the extraction of these polyphenols difficult. Therefore, enzymes are used to enhance the release of these polyphenols by disrupting the cell walls. Pre-extraction of the grape pomace before enzymatic digestion with pectinolytic and cellulolytic preparations significantly improved extraction yields. After pre-extraction, 70.1% of phenolic acids, 75.2% of non-

anthocyanin flavonoids, and 1.7% of anthocyanins were recovered. After enzymatic treatment, total contents of phenolics obtained in this 2-stage extraction process amounted to 98.1% (phenolic acids), 96.8% (non-anthocyanin flavonoids and stilbenes), and 2.9% (anthocyanins) (Kammerer, Claus, Schieber, & Carle, 2005). However, these enzymes could be expensive and thus will contribute to the overall head cost.

1.2.3.6 Surfactants and polyphenols interaction

Surfactants (*surface-active-agents*) are one of the most versatile products of the chemical industry, appearing in such diverse products including motor oils in automobiles, the pharmaceuticals and the detergents we use in cleaning our laundry and homes and the flotation agents used in beneficiation of ores (Dermiki, 2009). A surfactant can be defined as a substance that, when present at low concentration in a system, has the property of adsorbing onto the surfaces, usually act to reduce interfacial free energy (Rosen, 1989). Surfactants are usually organic compounds and have amphiphatic structure, meaning that they contain both hydrophobic groups (tails) and hydrophilic groups (heads). Therefore, a surfactant has the property of being water soluble and/or oil soluble. In highly polar solvents like water, the hydrophobe group is usually a hydrocarbon equivalent of an 8 to 18 carbon, whereas in less polar solvent, the hydrophobe group could be fluorocarbon. In brief, the hydrophilic group is an ionic or highly polar group while the hydrophobic group is typically a long-chain hydrocarbon, and is less often a halogenated one. One of the important surfactants properties is the ability to form micelles above their Critical Micelles Concentration (CMC). The micelles are composed by hydrophilic pointing out toward the external surface and hydrophobic pointing in to the internal core. This particular structure allows the micelles to establish

chemical and physical interactions with both hydrophilic and hydrophobic molecules (Hosseinzadeh, Khorsandi, & Hemmaty, 2013).

1.2.3.7 Surfactant classification

The type of surfactant is dependent on the nature of the hydrophilic group, and therefore surfactants are classified as:

1) ***Anionic*** **-**The surface-active portion of the molecule bears a negative charge. Examples are soap and alkylbenzene sulfonate.
2) ***Cationic*** **-**The surface-active portion of the molecule bears a positive charge. An example is quaternary ammonium chloride.
3) ***Zwitterionic*** **-**Both positive and negative charges may present in the surface-active portion. Examples are long-chain amino acid and sulfobetaine.
4) ***Non-ionic*** **-** The surface-active portion bears no apparent ionic charge. An example is monoglyceride of long-chain fatty acid.

Table 1.4 Types of surfactants and their properties (adapted from Rosen, 1989)

Types	Advantage	Disadvantage
Anionic	• Easily prepared by neutralization of free fatty acids in simple equipment • Can be made in situ (for use of emulsifying agent)	• Form water-insoluble soaps with divalent/trivalent metallic ions • Insolubilized readily by electrolyte (NaCl) • Unstable at pH below 7, yielding water-insoluble free fatty acid
Cationic	• Compatible with non-ionics and zwiterionics • Surface active is positively charge, thus adsorb strongly onto most surface (which are typically negative charged), and can impart special characterisation to the substrate	• Non-compatible with anionics • More expensive than anionics/non-ionics • Show poor detergency
Zwitterionic	• Compatible with all other types of surfactants • Less irritating to skin and eyes • May be adsorbed onto negative or positive charged surfaces without forming hydrophobic film	• Often insoluble in most organic solvents, including ethanol
Non-ionic	• Compatible with other types of surfactants • Soluble in water and organic solvents • Generally 100% active material free of electrolyte	• Products are liquid or pastes • Poor foamers (could be advantage sometimes) • No electrical effects

As briefly mentioned above, ability to form micelles under certain conditions is an important and fundamental property of surfactants (see Fig. 1.3). Micelles are aggregates of surfactants in a liquid medium which are formed when the surfactant concentration exceeds the critical micelle concentration (CMC). These aggregates are only formed in polar solvents (like water) that have two or more potential hydrogen bonding centres and thus are capable of forming three-dimensional hydrogen-bonded

network (Rosen, 2004). Micelle formation is very important because it is related to detergency, solubilisation and also interfacial tension reduction. However it is worth mentioning that this definition is only for normal micelles; for the case of reversed micelles it is not necessary to have a critical micelle concentration (CMC).

CMC is a concentration of surfactants of which micelle form and all the surfactants added go to micelle. This phenomenon is described by the sharp break reduction in the conductivity of the solution, indicating a sharp increase in the mass per unit charge of the material in solution. In other words, at a given temperature, the minimal detergent concentration at which micelles are observed is called the critical micellisation concentration. Below this concentration, only detergent monomers are observed in the solution; above the CMC, detergent monomers are in equilibrium with the detergent micelles and further increasing concentration of detergents, a nonmicellar phases exists which are typically insoluble with water(Tadros, 2013). These phases could be hexagonal, reverse hexagonal or lamellar in structure and they are usually liquid-crystalline in nature.

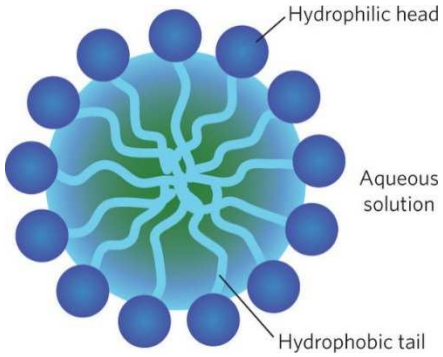


Figure 1.3 Micelle formation (image retrieved from https://www.quora.com/Why-is-it that-fatty-acids-form-micelles-but-phospholipids-form-a-bilayer)

1.2.3.8 Surfactant-based separations

Due to the ability of surfactants of forming micelles, the accumulate at the interfaces of air-water form foams at the interface of oil and water forms emulsions, at the interface of solid and liquid improves the dispersion and wetting of particles, hence they find applications in almost every chemical industry, including detergents, paints, cosmetics, pharmaceuticals, agrochemicals, fibres, plastics and food (Myers, 1999).

Surfactant-based separations have the general advantages of using a separating agent which can be green (biodegradable and nontoxic), often having low energy requirements and being capable of treating easily degraded materials, such as, biochemical, making this technique an attractive and promising separation technique (Scamehorn & Harwell, 1999). Their applications among others include remediation of contaminated soils, waste water treatment and paper recycling using micellar-enhanced ultrafiltration (Scamehorn & Harwell, 1999). The principle behind this method is that organic solute and multivalent ions will solubilise, and counter-ions bind respectively to micelles. The micelles are then ultrafiltered from solution.

1.2.4 Colloidal Gas Aphron (CGA)

1.2.4.1 Definition and characterisation of CGA

Colloidal gas aphrons (CGAs) are a system of microbubbles mostly above 25μm diameter and classified as “ball foams”, first described by (Sebba, 1972). CGAs can be generated by high speed stirring of the surfactant solution (>8000 rpm), whereby air is entrapped and microbubbles are formed. The structure of CGA was postulated to by Sebba, (1972) and later by Jauregi, Mitchell, & Varley(2000) that CGA are built based on multi-layers of surfactants as depicted in Figure 1.5. The layer which is in between the

inner and outer surface can be treated as different phase from bulk water because surfactant molecules at this surface have hydrophilic ends pointing inward and hydrophobic pointing outward (Chapalkar, Valsaraj, & Roy, 1993).

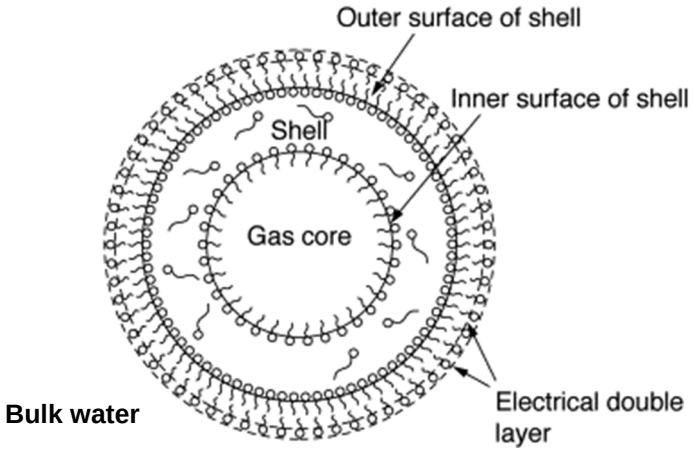


Figure 1.4 Proposed structure of colloidal gas aphrons by Sebba (1987) (Jauregi & Varley, 1999).

The CGA are suitable for process applications due to their ability to adsorb particles at microbubble interfaces, their large interfacial area and their stability during transport for enhanced mass transfer. Their properties are described in detail as below:

1) **Small size of bubbles** resulted in larger interfacial area per unit volume so the adsorption of molecules on the surface of the microbubbles is enhanced. However, it must be noted that the size of the bubbles depends on the concentration and the type of surfactant used and the presence of other molecule or particles in the system.
2) **Low viscosity** of the system lead to stability to pumping. The CGA has similar flow properties to water. Consequently, CGA has sufficient stability to pumping without collapse from one location to another. Moreover, due to the presence of air, they are considered to be compressible, and for this reason they can easily be pumped using a peristaltic pump.

3) The buoyancy of the encapsulated gas leads to **easy separation** of the aphron phase from the bulk liquid phase. Therefore, no centrifugation process is needed to separate the two phases.

CGA are being characterised for their stability, gas hold-up, bubble size and rheological properties. The stability is typically measured by the drainage of the CGA dispersion, as a function of time (Amiri& Woodburn, 1990). Ionic surfactants are generally more stable than the non-ionic surfactants (where electrostatic interactions are present); this is due to the presence of polar groups in the surfactant molecules at the gas-liquid interface of the adjacent aphrons, repelling with each other causing delayed in the coalescence of the aphrons (Jauregi & Varley, 1999). In the case of the non-ionic surfactants, where the electrostatic interactions are absent, other forces like steric forces must play a role in stabilising the dispersions. In many studies, the stability has been expressed as half-life, which is defined as the time taken for half of the initial liquid to drain, once the stirring has stopped (Jauregi and Varley, 1999).

The gas hold-up of CGA is defined as the amount of air incorporated into the dispersion before it collapsed (ε). The ratio of the gas volume (V_g) in the dispersion to the dispersion volume (V_0) in a container is calculated and is represented by the below equation.

$$\varepsilon = ((v_o - v_{10})/v_o) = ((H_o - H_{10})/H_o) \quad \text{Equation 1. 1}$$

Where V_{10} is the initial volume of liquid, $H_0$is the initial height of the dispersion and H_{10} is the height of initial volume of liquid.

There are several factors that could affect the stability of CGA including ionic strength and concentration of surfactant. Generally, high gas-hold up and small bubble sizes are generally desired to maximise the interfacial area (Jauregi and Varley, 1999). Several studies showed that stability tends to increase with the increase in surfactant concentration, with the optimum concentration being near to the cmc of the surfactant (Matsushita et al., 1992 and Chapalkar et al., 1993). Chapalkar et al., (1993) found that increasing surfactant concentration in a range below or approximately equal to the cmc of the surfactant resulted in a smaller bubble sizes. On the contrary, Jauregi et al., (1997) and Save & Pangarkar (1994) found out that increasing surfactant concentration above the cmc resulted in a larger bubble size. The addition of 0.002-0.05 mM NaCl has increased the stability of CGA and the concentration of NaCl beyond that resulted in decrease in CGA stability (Chapalkar et al., 1993).

1.2.4.2 Applications of CGA

CGA has a number of potential applications which are mostly separation-based. The main application being described over the past few decades is the flotation for the removal of biological and non-biological products. The conventional flotation process is carried out by sparging air into a solution containing compounds that needs to be removed (Jauregi and Varley, 1999). Adsorption takes place into the gas bubbles and being removed by flotation. In the improvement of this process, CGA has been sparged in replacing of conventional air bubbles. This technique has been widely used in the removal of metals (Amiri and Woodburn, 1990), dyes (Chapalkar et al., 1993) and for the flotation of algae (Wallis et al., 1985). Table 1.5 summarised some of the applications of CGA found in the literature.

Ciriello et al., (1982) have successfully removed copper, zinc and iron from waste water with the percentage of more than 90% when using CGAs made from cationic surfactant. In addition, Cabalerro et al., (1989) reported the successful removal of Cu(II) ions from CGAs generated from a cationic surfactant, CTAB and iron hydroxide as a coprecipitant. They also noted the advantage of using flotation column using CGAs as opposed to conventional flotation using bubbles in which the kinetics of the process improved with CGAs because there was no induction time needed for the formation of precipitation.

In another study on the recovery of dyes from waste water using CGA in flotation, the percentage of dyes recovered ranging from 90-98% with methylene blue and methyl orange respectively using CGA generated from CTAB, which further support the theory that CGA could act as ion-exchanger (Basu & Malpani, 2001). They have also observed that when the surfactant and dye had similar charge, 40% of recovery was achieved. This study concluded that the percentage of recovery of dyes from waste water was greatly affected by the increased of flow rate, smaller aphron diameter, increased in gas hold up and increased in residence time in the flotation column. However, no significant evidence was recorded with the increased of surfactant concentration above cmc. Huang et al. (2002) later observed that the flotation of organic dyes principally followed four mechanisms: ion coupling between the surfactant forming the CGAs and the organic dye having opposite charges; reactions between CGA and the organic dye; ion–dye complex adsorbed on the surface of CGA; and hydrophilic or hydrophobic characteristics of the organic dyes.

CGA have been widely used for soil washing. Roy et al. (1992) were the first to utilise CGA for the removal and mobilization of 2,4-dichlorophenoxy acetic acid (2,4-D), a syntheticauxin hormone used widely as a herbicide from contaminated soil. When

the performance of CGA was compared with conventional surfactant flushing, no significant difference was observed in the performance of the two processes. Nevertheless, CGAs flushing required a much lower volume of surfactant per gram of recovered2,4-D than with conventional washing. Due to low flow rates and lower pressure drops across the soil column, flushing with CGA was found to be advantageous over the use of surfactant solutions. In a recent study, CGA was generated from saponin, a biodegradable surfactant produced from soapnut fruit (*Sapindusmukorossi*) and SDS used to remove low level of arsenic from an iron rich soil (Mukhopadhyay et al., 2015). The results obtained showed that soapnut CGA removed up to 70% of arsenic while SDS CGA removed up to 55% arsenic from the soil. The soapnut wash solution could be recovered after the removal of arsenic by precipitation and that it caused a negligible amount of soil corrosion as confirmed by scanning electron microscope (SEM). This study has proven that CGA offers a good recovery in soil flushing and has become a promising technology particularly for the agricultural industry.

A study was conducted by Jarudilokkul, Rungphetcharat, & Boonamnuayvitaya, (2004) on the recovery of protein from wastewater discharged from food processing plants using non-ionic surfactant, TWEEN20. Various parameters such as surfactant concentration, speed of generating CGA, stirring time, pH, initial concentration of protein in the feed and concentration of NaCl had been tested for the maximum recovery of protein, particularly lysozyme and β-casein. The outcome of the study revealed that non-ionic surfactant was suitable for recovery of protein with initial protein concentration for lysozyme was 0.1mg/mL, the volumetric ratio of protein to CGA to be 1:3 with the pH of 4.0. For β-casein, the initial protein concentration was halved to 0.5mg/mL with volumetric ratio and pH remained the same as lysozyme. The percentage of protein recovery for each parameter ranged from 50.2-57.7% which was in theory with the usage

of non-ionic surfactant. This has then lead to a conclusion that the separation was driven by hydrophobic interaction and that electrostatic interaction between protein molecules and CGA played an important role in the separation.

Table 1.5 Recent applications of CGA

Application	**Surfactant**	**References**
Astaxanthin	CTAB	(Dermiki et al., 2009)
Norbixin	CTAB	(Alves et al., 2006)
Natural colourants from fungi	CTAB SDS TWEEN20	(Santos-Ebinuma et al., 2016)
Gallic acid	CTAB	(Spigno et al., 2010)
Polyphenols	CTAB TWEEN20	(Spigno, Amendola, Dahmoune, & Jauregi, 2015)
β-lactoglobulin	CTAB	(Fuda et al., 2005)
Whey Proteins	CTAB, AOT	(Fuda & Jauregi, 2006)
Lactoferrin, lactoperoxidase	AOT	(Fuda, Jauregi, & Pyle, 2004)
Glucoamylase	TTAB	(Zidehsaraei, Moshkelani, & Amiri, 2009)
Lipase immobilisation	SDS	(O'connell and Varley, 2001)
Removal of fine particles	HTAB TWEEN-20 SDBS	(Mansur, Wang, & Dai, 2006)
Removal of pulp fibre	Natural surfactant saponin	(Mukherjee et al., 2015)
Removal of arsenic	Natural surfactant saponin	(Mukhopadhyay et al., 2015)
Synthesizing copper oxide nanoparticles	SDS	(Banifatemi, Mohammadifard, & Amiri, 2016)

For the past recent years, application of CGA to food processing by-products has been investigated for example, whey protein, which is a by-product of cheese making (Fuda et al., 2005). This study focused on protein separation by CGA focusing on the mechanism of protein separation by CGA generated from ionic surfactants. The results obtained confirmed that the separation of protein by CGA generated from ionic surfactant

was driven by electrostatic interaction, whereby promoting hydrophobic interaction resulted in poor recoveries. Furthermore, the strength of interactions between protein and surfactant molecules was dependant on the conformational features of the protein which subsequently resulted in the selectivity of the separation. Thus, this finding showed that the recovered fractions could be manipulated by changing the conformation of some proteins (Fuda & Jauregi, 2006).

Furthermore, Dermiki, Gordon, & Jauregi, (2009) had successfully recovered astaxanthin using different combination of surfactants and varying the pH, volumetric ratios of astaxanthin to CGA and under different astaxanthin suspension: 1) astaxanthin in aqueous solution, 2) astaxanthin dispersed in ethanol/aqueous solution. The maximum recovery of astaxanthin dispersed in aqueous solution favoured the operating condition under strong alkaline conditions, resulting in overall negative charge of the molecule. The main driving force for the separation was the electrostatic interactions which was in agreement with the hypothesis that CGA (generated from CTAB) work as ion-exchangers (Dermiki et al., 2009).In the case of astaxanthin dispersed in ethanol/aqueous solution, the highest recovery was achieved using non-ionic surfactant which indicated the separation was mainly driven by hydrophobic interactions since ethanol hindered the effect of electrostatic interactions. However, the authors noted that increasing the amount of ethanol in the dispersion did not necessarily increase the recovery of astaxanthin. This was possibly due to the surface charge of astaxanthin that did not decrease with ethanol.

At the same time, the application of CGA was investigated in waste generated from agricultural industry. Alves et al., (2006) investigated the recovery of norbixin with CGA from an alkaline extract of annatto seeds. The study revealed high recovery of more than 94% was achieved using cationic surfactant whereas low recovery (40%) was achieved using anionic surfactant. Interestingly, this study concluded that molar ratio of

around 3-4 gave the highest recovery of norbixin, although increasing the molar ratio further resulted in decreased of recovery. They also concluded that the main driving force for the recovery was the electrostatic interactions.

Furthermore, the CGA application has also been extended to the recovery of gallic acid generated from CTAB (Spigno & Jauregi, 2005). The results revealed that the main driving force for the separation was electrostatic interactions between the gallic acid and the cationic surfactant molecules. However, this interaction was greatly influenced by several factors including pH. Working pH higher than the Pka of gallic acid lead to dissociation of gallic acid and promoted the oxidation of gallic acid, hence it was recovered without the antioxidant property (Spigno, Dermiki, Pastori, Casanova, & Jauregi, 2010). The study continued to investigate other factors including volumetric ratio, molarity ratio, contact time and drainage time using flotation column for scaling up the process. The outcome of the study revealed that the gallic acid recovery was mainly affected by pH, ionic strength, surfactant/gallic acid molar ratio, mixing conditions and contact time. The flotation column experiment resulted in higher recovery and also reproducibility (Spigno et al., 2009).

Recently, CGA have also been applied to real crude extracts obtained with aqueous ethanolic extract from red grape pomace (Spigno et al., 2015). These trials were conducted in a batch flotation column, investigating the influence of surfactant type (CTAB vs TWEEN20) and also the extract concentration. The results showed some stabilisation effect on the CGA, leading to high recovery. Anthocyanins in particular showed high affinity for the CGA phase. Lowering the pH of CTAB led to slight reduction in anthocyanins recovery, confirming that the separation was driven by electrostatic and hydrophobic interactions. When the separation was carried out using TWEEN20, the recovery increased with the volumetric ratio and at high extract

concentration. Interestingly, these fractions had higher antioxidant capacity as compared to those with CTAB.

1.3 Aim and Objectives of the study

This study expands from previous work described above on the application of CGA to crude grape pomace extracts. The aim of the present study is to investigate the application of CGA to crude grape pomace extracts for the recovery and formulation of polyphenols by taking advantage of the particular surfactant rich media in the CGA fractions. The potential application of the CGA extracts in cosmetics is investigated here for the first time. In addition, the potential of these extracts in the food colourant industry is also considered. The structure of the thesis with their specific objectives of this study are as follow:

- ***Introduction and literature review*** **Chapter 1:** A brief introduction related to the recent situations of grape pomace abundancy and the need to extract the polyphenols was given. Several applications of CGA in recovering and separating various compounds from bio-products were reviewed where the research gap was identified.
- ***Characterisation of crudegrape pomace extracts and further extraction of polyphenols with colloidal gas aphrons (CGA)*** **Chapter 2:** Initially, the **crude** grape pomace extracts obtained by hydroalcaholic extraction were characterised for their composition. First CGA were applied to a mixture of methylene blue and methyl orange dyes using anionic and non-ionic surfactants in order to develop an understanding on the effect of type of surfactant, mainly charge, on the selectivity of separation, CGA were applied to the recovery of polyphenols using a flotation column where different type of surfactants, the effect of different volumetric ratios of CGA to grape pomace extracts and

the effect of drainage time were investigated leading to optimised conditions of CGA separation using a non-ionic surfactant, TWEEN20. This chapter served as an important basis to establish main CGA separation conditions which were then applied in subsequent studies described in the next three chapters.

- ***Polyphenols extracted from grape pomace extracts by CGA show anti-collagenase and anti-elastase activity*** **Chapter 3:** In this chapter, selected separation parameters were applied to produce CGA fractions which were tested *in vitro* for their inhibitory activities against enzymes relevant to skin ageing, collagenase and elastase.
- ***The effect of individual polyphenols, crude grape extract and its CGA fraction on skin permeability*** **Chapter 4:** In this chapter, individual polyphenols, ethanolic extract and its CGA fraction were investigated for their permeability through membranes and pig skin. The effect of TWEEN20 on skin permeability was investigated.
- ***Stability of polyphenols extracted from grape pomace by colloidal gas aphrons (CGA)*** **Chapter 5:** In this chapter, the study focus is on the storage and stability of the CGA fractions as applied in the food industry. The stability and shelf-life of the polyphenols, particularly anthocyanins were evaluated and compared against the sorbic acid as the most widely used food preservative.
- ***General conclusions and future work recommendations*** **Chapter 6:** In this chapter the main outcomes from this study are described. Recommendations for future work are also given.

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CHAPTER 2: Characterisation of Grape Pomace Extracts and its Separation with Colloidal Gas Aphrons (CGA)

Abstract

The aim of this study was to investigate the effects of surfactant, volumetric ratio and drainage time to the recovery of polyphenols using colloidal gas aphrons (CGA). Earlier, the grape pomace extracts were chemically characterized and the principles of CGA separation were demonstrated using organic dyes. Results showed that polyphenols recovered with TWEEN20 showed greater antioxidant activity. An optimum recovery of polyphenols was achieved with volumetric ratio of 16 and drainage time of 5 mins.

2.1 Introduction

Grapes are one of the most cultivated fruit crops throughout the world and about 80% of the harvest is used by the winemaking industry (Drosou, Kyriakopoulou, Bimpilas, Tsimogiannis, & Krokida, 2015). After fermentation, millions tonnes of grape pomace are generated, leading to a waste management issue, both ecologically and economically (Fontana, Antoniolli, & Bottini, 2013). The application of grape pomace as soil conditioner or to make fertilizers is not appropriate due to high levels presence of phenolic compounds that might cause germination problems (Kammerer, Kammerer, Valet, & Carle, 2014).Thus, these polyphenols need to be recovered and removed.

The polyphenols present in grapes and their bioavailability are well documented in the literature (Kammerer, Claus, Carle, & Schieber, 2004; Rockenbach et al., 2011; Tseng & Zhao, 2013; Wittenauer, Mäckle, Sußmann, Schweiggert-weisz, & Carle, 2015). The polyphenols mainly include anthocyanins (eg: malvidin), flavan-3-ols (eg: catechin), flavonols (eg: quercetin), stilbenes (eg: resveratrol) and phenolic acids (eg: gallic acid) (Ribeiro et al., 2015; Sagdic et al., 2011). These polyphenols have antioxidant properties

(Chidambara Murthy, Singh, & Jayaprakasha, 2002; Rockenbach et al., 2011), antihyperglycemic effect (Lavelli, Harsha, Laureati, & Pagliarini, 2017), cardioprotective effect (Zhu, Du, Zheng, & Li, 2015) as well as anti-inflammatory effect (Trikas, Melidou, Papi, Zachariadis, & Kyriakidis, 2016).

Grape pomace extracts may be produced by novel extraction technologies such as microwave solid extraction, supercritical fluid extraction, ultrasound assisted extraction, pulsed electric field extraction (Azmir et al., 2013; Barba, Zhu, Koubaa, Sant'Ana, & Orlien, 2016) and membranes processing (Syed, Brazinha, Crespo, & Ricardo-da-Silva, 2017). Nevertheless, solvent extraction is still the most widely used method of extraction due to its simplicity. However, the choice of solvents is very important because there are some limitations to this technique, particularly in large scale applications, their cost and impact on the environment, and because of the fact that some organic solvents may lead to irreversible product degradation. Moreover, solvents need to be removed at the later stage before formulation. Recently, there is an emerging interest on the application of surfactants to separation processes. Surfactants possess unique characteristics, such as, a tendency to adsorb onto surfaces associate in solution to form micelles which dissolve non-polar solutes (Roy, Kommalapati, Valsaraj, & Constant, 1995),and because most of them are non-toxic and biodegradable (Williams & Barry, 2004). Therefore, surfactant based separation processes are considered as promising separation techniques and technologies (Galanakis, 2012).

One application of surfactant based separations is the use of Colloidal Gas Aphrons (CGA). These were first described by Sebba (1972)as surfactant-stabilised microbubbles (10-100) generated by intense stirring of a surfactant solution at high speeds (>8000rpm). Research studies have been conducted in order to determine the structure of CGA (Jauregi, Mitchell, & Varley, 2000) and it was postulated that they possess a surfactant

multilayer structure, and for this reason they have different dispersion characteristics compared to conventional foams. Depending on the surfactant used to produce CGA, e.g.: cationic, anionic, or non-ionic, the outer surface of the microbubble may be positively, negatively or non-charged to which the opposite or non-charged molecules will adsorb resulting in their effective separation from bulk liquid (Spigno & Jauregi, 2005), therefore, the selectivity of adsorption can be adjusted (Fuda & Jauregi, 2006).

CGA exhibits unique characteristics, including high interfacial area, high stability compared to conventional foams, ability to be pumped and can be separated easily from the liquid phase without mechanical aid, thus reducing the number of steps/operations needed for recovery and making them a cost effective separation technique as compared to other methods such as centrifugation and supercritical fluid extraction. Furthermore, the use of biodegradable surfactants can result in environmental friendly processes. While the final product can be safe for human consumption, it would not be necessary to remove the surfactant after the recovery. Moreover, the presence of surfactant can be beneficial in formulating the final product for human consumption/usage.

Several applications of CGA have been documented in the literature including the flotation yeast cell (Hashim & Sen Gupta, 1998; Hashim, SenGupta, & Subramaniam, 1995), the removal of toxic wastes from soil (Hashim, Mukhopadhyay, Gupta, & Sahu, 2012; Mukhopadhyay, Mukherjee, Hashim, & Sen Gupta, 2015) and waste waters (Basu & Malpani, 2001) and the removal of fine particles (Mansur, Wang, & Dai, 2006) among others. More recently, CGA have been used as an alternative method for the recovery of a variety of bio-products from complex systems, including proteins (Fuda & Jauregi, 2006; Fuda, Bhatia, Pyle, & Jauregi, 2005; Fuda, Jauregi, & Pyle, 2004; Jarudilokkul, Rungphetcharat, & Boonamnuayvitaya, 2004), polyphenols such as gallic acid (Spigno & Jauregi, 2005), carotenoids such as norbixin(Alves, Ulson De Souza, Ulson De Souza,

& Jauregi, 2006) and astaxanthin (Dermiki, Bourquin, & Jauregi, 2010; Dermiki, Gordon, & Jauregi, 2009).

The recovery of proteins, gallic acid and carotenoids showed that CGA act as ion exchangers and that the recovery of products was optimum under conditions that favour electrostatic interactions, thus the use of ionic surfactants. Ionic surfactants can be harsh and irritating when applied to human (Williams & Barry, 2004).Thus, there is a need to use non-ionic surfactants which tend to be regarded as safe. Moreover, the use of non-ionic surfactant covers about 45% of the overall industrial production (Salaguer, 2002).

Therefore, the purpose of the current study was to gain understanding of the principles of CGA by applying them to chemical dyes. The recovery of polyphenols from grape pomace using CGA generated from a non-ionic surfactant (TWEEN20) under different conditions (volumetric ratio and drainage time) was also investigated. The parameters used in this study were based on the findings from Spigno, Amendola, Dahmoune, & Jauregi, (2015) on the recovery of polyphenols from ethanolic extract of grape pomace. However, in the present study we took an extra step by investigating the CGA separation in water and ethanolic extracts of grape pomace, of which both solvents were considered as green solvents. The effect of different surfactants was studied to give an overview of the recovery pattern. Finally, the different ratio of CGA to the extract and the different drainage time were investigated in both ethanolic and water extracts of grape pomace.

2.2 Materials and Methods

Plant Materials

Grape pomace sample was kindly provided by a winery in Northern Italy. Skins were separated from the seeds and dried in an oven at 65°C for 2 days until the residual moisture of 7% was obtained. The dried skins were grounded and sieved at a particle size of≤ 2mm.

Materials

The materials needed were the laboratory mixer (SL2T) fitted with a four bladed impeller (D=30mm) with a digital readout of the impeller speed in rpm supplied by Silverson (Waterside, Bucks, UK), shaking incubator (Grant OLS200), quartz cuvette, plastic cuvette and a spectrophotometer Ultrospec 1100 Pro, purchased from Amersham Pharmacia Biotech (Biochrom, Cambridge, UK).

Chemicals

The chemicals used were ethanol (>98%), gallic acid (>98%), sodium carbonate, sodium acetate, potassium persulfate, ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)), TWEEN20, sodium dodecyl sulphate (SDS), hydrochloric acid, sulphuric acid, phenol, bovine serum albumin (BSA), copper^{2+} sulphate reagent and bichicnoninc acid (BCA) reagent. These chemicals were from Sigma Aldrich,Folin-Ciocalteau Reagent (Merck, Germany). Potassium chloride, sodium bicarbonate and methyl orange were from Fisher Scientific (Loughborough, UK). Methylene blue was from BDH Chem Ltd.

2.2.1 Characterisation of grape pomace

2.2.1.1 Ethanolic and hot water extraction of grape pomace

The use of hydro-ethanolic solvents as extractants is advantageous for grape pomace as they are biocompatible, have the GRAS status and are easily accessible. Thus, they were used throughout this study. The extraction of the grape pomace sample was done according to Amendola, De Faveri, & Spigno, (2010) in 1:8 ratios, with a slight modification. 125g of dried powder of grape pomace was extracted with 1 L of 60% aqueous ethanol and they were mixed using a shaking incubator for 2 hours at 60°C with arotating speed (circular motion) of 100rpm. For hot water extraction, the boiling temperature of 100°C was used and for 1 hour. The extracts were respectively filtered with vacuum filtered using Whatman No.1 filter paper and the filtrates were adjusted to the same volume and stored at -20°C prior analysis. The extraction was done in triplicate (n = 3).

2.2.1.2 Total Phenolic Content (Direct Measurement)

This method is based on the measurement of a number of aromatic benzene rings in gallic acid at 280nm (Amendola et al., 2010). A calibration curve of gallic acid (0-100mg/L) was constructed and acted as a standard curve. The total phenol of grape pomace extract was measured at 280nm and calculated based on the gallic acid calibration curve expressed as mg/L gallic acid equivalent (GAE_{280}).The analysis was done in triplicate (n = 3).

2.2.1.3 Total Phenol Content (Folin-Ciocalteau Method)

This method was adopted from Singleton & Rossi, (1965).The FC reagent contains phosphomolybdic/ phosphotungstic acid complexes. The method relies on the transfer of electrons in alkaline medium from phenolic compounds to form a blue chromophore constituted by a phosphotungstic/ phosphomolybdenum complex. A calibration curve of gallic acid was constructed by adding 0.2mL of gallic acid (concentration ranging from 0-1000mg/L) to 6.0mL of distilled water and 0.5mL Folin reagent in a 10mL volumetric flask. After 1 min and before 6 mins, 1.5mL of 20% of sodium carbonate was added and the volume was adjusted accordingly to the 10mL volumetric flask and left to stand for 2hours.The total phenol of grape pomace extract was measured against 760nm and calculated based on the gallic acid calibration curve expressed as mg/L gallic acid equivalent (GAE_{760}).The analysis was done in triplicate (n = 3).

2.2.1.4 Total Anthocyanin

This method is based on the anthocyanins structural transformation that occurs with a change in pH. This method has been adopted as an Official First Action by AOAC(Lee, Durst, & Wrolstad, 2005). Two buffers were prepared.

1) pH 1.0 buffer was prepared using potassium chloride; and
2) pH 4.5 buffer was prepared using 0.4M sodium acetate.

The dilutions were carried out in 50mL volumetric flasks. The test portion added should be 1:5 (maximum of 10mL of extract and 40mL of buffer). The extract was diluted with pH 1.0 buffer until the absorbance was between 0.2-1.4 at A520nm. Then, the sample was diluted with both buffers and was left to stand for 20-50 mins. Both buffers

were measured at A520nm and A700nm. The analysis was done in triplicate (n = 3). The calculation of the total monomeric anthocyanin was as follow:

$$Total\ Anthocyanins\ \left(\frac{mg}{L}, ME\right) = \frac{A*MW*DF*10^3}{\varepsilon*1} \qquad \text{Equation 2.1}$$

where A = $(A_{520nm}\text{-}A_{700nm})_{pH1.0}$ – $(A_{520nm}\text{-}A_{700nm})_{pH4.5}$; MW (molecular weight of malvidin-3-glucoside = 493.43g/mol; DF = dilution factor; 1 = pathlength in cm; = Ɛ 28000 molar extinction coefficient and 10^3 = factor for conversion from g to mg and cm.

2.2.1.5 Antioxidant activity using ABTS assay

This method is based on the generation of ABTS radical directly from ABTS chromophore and potassium persulfate(Re et al., 1999). The addition of antioxidants to the pre-formed radical cation reduces the ABTS to an extent and on a time-scale, depending on the antioxidant activity, the concentration of the antioxidants and the duration of the reaction. This method is applicable to study both water and lipid soluble antioxidants, pure compounds and food extracts.

The radical solution was prepared by mixing 5mL of 7mM ABTS with 80µl of 150mM Potassium persulfate and left to stand for 12-16 hours. Prior working, the absorbance of the radical was adjusted to be around 0.70 ± 0.02 at A734nm with pure ethanol. The radical was then left to equilibrate at 30°C until stability was achieved.

For the analysis, 2mL of ABTS radical was mixed with 20µL of sample/standard/blank. In this case, the blank was the ethanol (dilution of ABTS radical

was by ethanol). The samples were from extract, aphron and liquid phase. The absorbance of the mixture was taken after 6 mins. The antioxidant power was expressed as antioxidant power and the calculation was as follow:

$$Antioxidant\ power\ (\%\ inhibition) = \frac{A\ Blank_{\ t=6} - A\ samples\ or\ standards_{\ t=6}}{A\ ABTS_{t=0}} * 100$$

(Equation 2.2)

Since the analysis was carried out on different dilutions of the samples, the results have been reported as trend of AOP as a function of total phenolic content (TPI_{280}) in the sample. The analysis was done in triplicate (n = 3).

2.2.1.6 Total protein determination

The total amount of protein present was determined by the bicinchoninic acid (BCA) method (Smith et al., 1985). A calibration curve was produced using bovine serum albumin as the protein standard solution. The principle of this method lies upon the fact that when protein is in alkaline system containing Cu^{2+}, then a complex is formed between peptide bonds and the Cu^{2+} and the Cu^{2+} is reduced to Cu^{1+}. $Cu^{1+}$is further detected by the reaction with BCA. BCA forms a 2:1 complex with Cu^{1+}resulting in a stable deep purple chromophore with maximum absorbance at 562nm. The amount of reduction is proportional to the protein present in solution.

Briefly, 100µl of standard or sample was mixed with 2 mL of the BCA working reagent (copper sulphate solution:BCA solution at a ratio of 1:50). The mixture was allowed to stand at 37°C for 30 minsand then allowed to cool at room temperature for 5 mins. Finally, the absorbance was read for each sample/standard, at 562nm within 8 minswith water as a blank.The results were expressed as mg/L BSA equivalent.

2.2.1.7 Total sugar determination

The total sugar content present was determined by adopting a method from Dubois, Gilles, Hamilton, Rebers, & Smith, (1956). A calibration curve using glucose standard solution was produced. This method used the phenol in the presence of sulfuric acid to quantitate the amount of sugars and their methyl derivatives, oligosaccharides and polysaccharides. Simple sugars, oligosaccharides, polysaccharides, and their derivatives, including the methyl ethers with free or potentially free reducing groups, gave an orange yellow colour when treated with phenol and concentrated sulfuric acid. This method offers advantages because it is simple, rapid and sensitive and gives reproducible results. The reagents used were inexpensive and stable, and was useful in determining sugars separated with volatile solvent/water environment such as ethanol/water.

Briefly, 0.4mL of glucose standard/sample was added in test tubes,followed by 0.2mL 5% of phenol solution. Subsequently, 1mL of sulphuric acid was pipetted directly to the solution and vortexed. The mixture was allowed to stand and cool off for 20-30mins.The mixture absorbance was read at 485nm.The results were expressed as mg/L glucose equivalent.

2.2.2 Characterisation of Colloidal Gas Aphron (CGA) using TWEEN20 surfactant

2.2.2.1 CGA generation

This section described the steps taken to generate CGA from surfactant that was used throughout this study. The solution of 10mM TWEEN20 was prepared in distilled water. This concentration was chosen based on the previous work done by (Spigno, Amendola, Dahmoune, & Jauregi, 2015). Briefly, 10mM of TWEEN20 solution was subjected to high intense stirring using the Silverson homogenisor (8000rpm) for 5 mins

at room temperature. Then, the CGA were pumped into the flotation column (i.d 5cm, height: 50cm) from the bottom which had already contained the extract. The CGA were allowed to pass through the extract until it filled up the top of the column and allowed to drain for 5 mins before the collapsed phase (liquid phase) was collected. The aphron phase was left to collapse and then collected. Both phases were weighed and kept at 4°C for further analysis.

2.2.2.2 Determination of CGA's stability

CGAs stability plays an important role in achieving maximum recoveries. A less stable system does not permit bond formation hence phenols will remain in the liquid phase. In this experiment, the CGA stability was studied at three different concentrations of TWEEN20; 1mM, 10mM and 20mM. The CGA generation was described in section 2.2.1. The stability of CGA was evaluated by measuring the half-life of the dispersion. The half-life (Ƭ) was defined as the time required for half the initial volume liquid ($V_{surfactant}/2$) to drain (Fuda et al., 2005). The analysis was done in triplicate (n = 3).

2.2.2.3 Determination of CGA's Gas Hold-Up

The gas hold-up ε, is defined as the ratio of gas volume (V_g) to the dispersion volume (V_{CGA}) (Fuda, Bhatia, Pyle, & Jauregi, 2005). The gas hold-up was determined following the method described by Amiri and Woodburn (1990). After the generation, the CGA were poured into a 1000mL measuring cylinder, and the volume of the clear liquid below the CGA dispersion was measured at one minute intervals. After the dispersion had collapsed, the volume of the liquid was measured. This corresponded to the initial volume of the surfactant solution ($V_{surfactant}$) in the CGA dispersion.

$$\varepsilon = \frac{(V_{CGA} - V_{surfactant})}{V_{CGA}} x\ 100\ (\%) \qquad \text{(Equation 2.3)}$$

The V_{CGA} is the volume of CGA after the intense stirring for 5 mins and the $V_{surfactant}$ is the volume of surfactant before CGA was generated. The analysis was done in triplicate (n = 3).

2.2.3 Separation of dyes using CGAs generated from SDS and TWEEN20 surfactant

According to literatures, the mechanisms of CGA separation are mainly driven by electrostatic and hydrophobic interactions depending on the type of surfactant used (Dermiki et al., 2009; Fuda & Jauregi, 2006). In this experiment, methylene blue and methyl orange dyes which carries different nett charges, along with different type surfactant were used to visually understand the separation mechanism of CGA. Two types of surfactant were chosen to generate CGA – sodium dodecyl sulphate (SDS) and TWEEN20 due to the former being anionic and the latter being non-ionic. Methylene blue has a nett positive charge while methyl orange has a negative nett charge. Therefore it is essential to conduct this experiment with an ionic surfactant (in this case, the surfactant is SDS) since the recovery of dye is known to be driven by electrostatic interactions (Basu & Malpani, 2001). This experiment will provide fundamental understanding on how CGA work with dyes because they are very visual before they are being subjected to the real sample, which is more complex.

The CGA were generated using 400mL of each surfactant using Silverson Homogenizer, rotating at 8000rpm for 5 mins. Upon the generation, 40mL of CGA were quickly transferred to beakers containing 5mL (25mg/L) of individual dye. The mixtures were allowed to mix using magnetic stirrer for 2 mins and the mixtures were then allowed

to separate for 5 mins. The liquid phase (at the bottom of the beaker) was pipetted out and weighed. The aphron phase was left to completely collapsed and weighed. The concentration of methylene blue and methyl orange were determined spectrophotometrically, at 660nm and 490nm respectively. The experiments were conducted in triplicate.

The recovery of the dyes in the aphron phase was calculated using the equations below, assuming that there were no other compounds being recovered. The analysis was done in triplicate (n = 3).

$$\text{Mass of dye(feed)} = \text{Mass of dye (aphron)} + \text{Mass of dye (liquid)} \quad \text{(Equation 2.4)}$$

$$Recovery\ (\%) = \frac{Mass\ of\ dye(aphron)}{Mass\ of\ dye\ (feed)} x\ 100 \quad \text{(Equation 2.5)}$$

2.2.4 The effect of surfactant on the recovery of polyphenols from grape pomace extracts in a flotation column

In this experiment, the effect of different types of surfactants was studied on the recovery of polyphenols from grape pomace extracts. Aqueous solutions of the non-ionic surfactant TWEEN20 (10mM) and the cationic surfactant CTAB (1mM) were used. Volumetric ratio of 16 (V_{CGA}/V_{feed} =16) with a drainage time of 5 mins was chosen for this experiment. The CGA generation were done similar to the procedure as described in section 2.2.2.1. All collected phases were tested for the experiments described in section 2.2.1.2-2.2.1.6. The recovery (%) of compounds (*y*) in the aphron phase was defined as:

$$Recovery, y\ (\%) = \frac{Mass\ of\ y\ (aphron)}{Mass\ of\ y\ (feed)} x\ 100(\%) \quad \text{(Equation 2.6)}$$

where mass of (*y*) (aphron) was the mass of *y* (mg) in the aphron phase and mass of (*y*) (feed) was the mass of *y* (mg) in the initial solution.

The separation factor (SF) was defined as the ratio of the concentration of compounds (*y*) in the aphron phase $[y]_{aphron}$ to the liquid phase $[y]_{liquid}$.

$$Separation\ factor\ (SF) = \frac{[y]_{aphron}}{[y]_{liquid}} \quad \text{(Equation 2.7)}$$

2.2.5 The effect of volumetric ratio on the recovery of polyphenols from grape pomace extracts using CGA in a flotation column

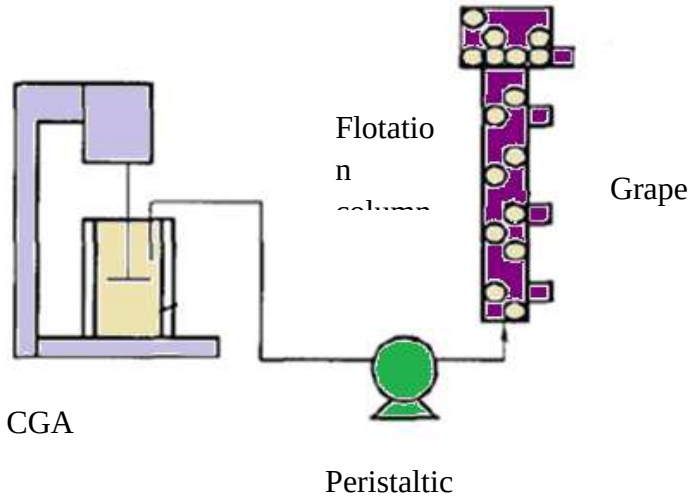

Figure 2.1 An overview of the process of separation of grape pomace extract using flotation column

In this experiment, a preliminary trial was conducted with the flotation column to recover the polyphenols from grape pomace extract using CGA generated from TWEEN20. The setup of this experiment is described in Figure 2.1. Initially, the grape pomace extract was introduced inside the column at a given volume. Then CGA were introduced at different flow rates, changing the contact time between the extract and the CGA. The drainage time remained at 5 mins. The drained liquid phase and the aphron

phase were collected separately. The weighs of liquid and aphron phase were recorded before further analyses of 2.2.1.2-2.2.1.7 were carried out. The tested parameters were tabulated in Table 2.1 below.

Table 2.1 The parameters used for the effect of varying volumetric ratio to the recovery of polyphenols

Experiment	A	B	C	D
V_{CGA}/V_{feed}	4	8	12	16
Flow rate (mL/min)	160	178	185	188
Initial volume of feed (mL)	200	111	77	60
Estimated contact time (min)	5	4.5	5	4.5
Drainage time (min)	5	5	5	5

2.2.6 The effect of drainage time on the recovery of polyphenols from grape pomace extracts using CGA in a flotation column

In this experiment, the effect of varying the drainage time was investigated. Drainage time is defined as the time taken by the CGA to drain and become liquid. The volume of CGA to the volume of feed was kept constant at 16:1 while varying the drainage time. Similarly, in the previous experiment, the 60mL of the extract (feed) was introduced into the column. CGA then was pumped into the column using the peristaltic pump from the bottom until the column was fully filled. Time of residence is defined as the time of the CGA and the feed was in contact. The drainage times were allowed for 3, 5, 8, 10, 15 and 20 mins respectively before the collapsed aphron phase was collected. The weighs of liquid and aphron phase were recorded before further analyses of 2.1.2-2.1.7 were carried out. The summary of the different parameters used in this experiment was as in Table 2.2.

Table 2.2 The parameters used for the effect of varying drainage time to the recovery of polyphenols

Experiment	A	B	C	D	E	F
V_{CGA}/V_{feed}	16	16	16	16	16	16
Flowrate (mL/min)	188	188	188	188	188	188
Volume of feed	60	60	60	60	60	60
Drainage time (min)	3	5	8	10	15	20
Time of residence (min)	4.3	4.2	4.3	4.3	4.4	4.2

2.2.7 Statistical analysis

Data were subjected to analysis of variance using IBM® SPSS® Statistics21 software programme where statistical differences were noted. Differences among different treatments were determined by using the Tukey test. The significance level was defined at $p<0.05$.

2.3 Results and Discussions

2.3.1 Characterisation of Colloidal Gas Aphron (CGA) using TWEEN20 surfactant

Table 2.3 Evaluation of CGAs stability (as gas hold up and $t_{1/2}$) as a function of different TWEEN20 concentration.

TWEEN20 (mM)	Gas Hold Up (%)	$t_{1/2}$ (sec)
1	48.36 ± 1.47^{a}	247.50 ± 10.61^{a}
10	61.28 ± 0.57^{b}	605.50 ± 7.78^{b}
20	62.56 ± 0.27^{c}	684.34 ± 22.37^{c}

Results are expressed as mean ± SD (n=3).
Same superscript letters within the same column indicates means are not statistically different according to ANOVA $p<0.05$.

Based on the results obtained in Table 2.3, it was observed that 20mM TWEEN20 had the highest stability, followed by 10mM and finally 1mM. This was evident by the half-life values in 20mM TWEEN20, and longer time (685s) was taken for the CGAs to drain into half of the initial volume of the surfactant. Furthermore, 20mM TWEEN20 also has the highest gas hold up of about 63% as compared to 10mM (61%) and 1mM (48%). The result obtained in this experiment was slightly higher than those obtained by Dermiki, Gordon, & Jauregi, (2009)who used TWEEN60 as the surfactant. Similar gas hold-up was obtained for 20mM TWEEN60 as for 10mM TWEEN20. This was probably due to the different structure of TWEEN20 and TWEEN60. The increase in the average carbon chain has been associated with greater hydrophobic interactions between surfactant molecules leading to suppression of the formation of CGA (Jarudilokkul et al., 2004). In the case of the cationic surfactant, an increase in the alkyl chain leads to the formation of CGA with higher stability (Save and Pangarkar, 1994).

The gas hold-up and consequently the stability of CGA increase with the increasing concentration of TWEEN20. The same observation was noted by Jarudilokkul et al., (2004) when they studied the effect of non-ionic surfactants on the stability of CGA. This phenomenon could be ascribed to the increasing elasticity at higher concentration of non-ionic surfactants which further delays the coalescence of the adjacent aphrons (Rosen, 2004). Moreover, the repulsive and stabilising force between the aphrons are more likely to increase with the increasing concentration thus leading to a more stable dispersion (Fuda et al., 2005). Additionally, the surface elasticity and the surface viscosity can be increased by packing high concentrations of surfactants or particles in the surface causing high adhesive or cohesive bonding (Yan et al, 2005), hence leading to a more stable aphrons.

According to Dermiki et al., (2009), further increase in surfactant concentration did not result in significant increase in gas hold-up or half-life. Therefore, higher TWEEN20 concentration was not tested since it did not influence the gas hold up and half-life of CGA. The possible reason for this was that the adsorption of surfactant molecules at the air-liquid interface has reached a saturation limit, as it was also noted by Fuda, Bhatia, Pyle, & Jauregi, (2005).

Sebba,(1972) stated that higher concentrations of non-ionic surfactants are needed to produce more stable CGA as opposed to those of ionic surfactants although it was noted that the critical micellar concentration (CMC) of the non-ionic was typically lower than the CMC of anionic or cationic. For instance, a cationic surfactant CTAB solutions reached its maximum stability at 2mM and its CMC was 0.9mM whilst in the case of TWEEN20 the CMC was0.06mM but the concentration for maximum stability was significantly higher (10mM).This could be attributed to the absence of charged surface films by the non-ionic surfactant as charged surfaces delay/hinder the coalescence of the bubbles due to repulsive electrostatic interactions between them (Rosen, 2004).

The results are in agreement with the findings by Jarudilokkul et al., (2004)who pointed out that the concentration of the surfactant (TWEEN) above the CMC was vital in order to generate CGAs. This could also be due to the fact that above the CMC, micelles were spontaneously formed. Below this concentration, surfactants merely partition into membranes without solubilising membrane proteins. The same observation was also noted by Dermiki, Gordon, & Jauregi, (2009), where stability of CGA produced by TWEEN60 was optimum above its CMC. From the results obtained, 10mM TWEEN20 was chosen as the concentration used throughout this study since it has a considerably high gas hold-up with sufficient stability. Furthermore, this study was aimed

to minimise the concentration of surfactant so that it could be integrated into the food system without having to remove it.

2.3.2 Separation of dyes using CGAs generated from SDS and TWEEN20 surfactant

Figure 2.2 showed the recovery of individual dyes (methyl orange and methylene blue) and the mixture of both dyes using three different concentration of SDS. The recovery of methylene blue dye was the highest with 10mM SDS (98.03%) and 1mM of SDS (91.94%), but much lower with 20mM SDS (22.90%). The trend wasslightly different for the recovery of methyl orange dye where the highest recovery was with 10mM SDS (5.91%), followed by 20mM SDS (4.80%) and 1mM SDS (2.56%). In the mixture of dyes solution, the recovery of methylene blue dye in the aphron phase by 10mM SDS (52.46%) was slightly higher than those with 1mM SDS (50.27%). However at 20mM SDS, there was no separation of phases observed.

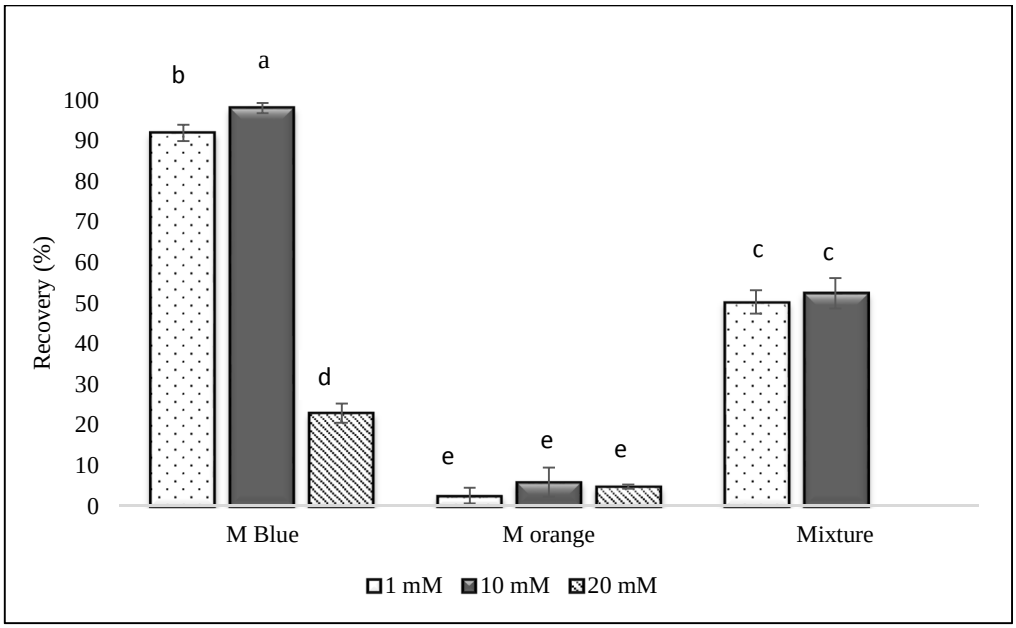

Figure 2.2The recovery of individual dyes from single solutions and mixtures of dyes using different concentration of SDS surfactant. Error bars indicate means± SD. Differences were considered to be significant at $p<0.05$.

These results were in agreement with the initial hypothesis. The SDS surfactant is an anionic surfactant which carries negative charge. In theory, methylene blue which is positively charged will interact by attractive electrostatic interactions with the surfactant in the CGA and will preferentially partition into the aphron phase, whilst the methyl orange which is negatively charged will have repulsive electrostatic interactions with the surfactant on the CGA and thus will remain in the liquid phase.

The recovery of methylene blue was high with 10mM and 1mM SDS, but interestingly there was a rapid decline of the methylene blue recovery with CGA generated with20mM SDS which suggested that there was a competition between micelles and CGA. The micelles formed in the bulk of the liquid at surfactant concentrations above the Critical Micellar Concentration (CMC), which in the case of SDS is 8mM. The micelles which are smaller in size (~0.01µm) may compete with the CGA which are much larger in size (~10µm) resulted in a less effective separation of the CGA, whilst the micelles remained in the liquid phase, leading to a low recovery of the methylene blue dyes in the aphron phase.

Basu & Malpani, (2001) found that 90-98% of methyl orange was recovered when using CGAs generated from CTAB, a cationic surfactant. They concluded that the oppositely charged dye interacted strongly with the surfactant and this resulted in 98% recovery. On the other hand, when the charges of the surfactant and the dye were similar, 40% of dye removal was observed. This was in agreement with the results described above. When the anionic (negatively charged) surfactant was used, 98% of methylene blue was recovered. Therefore, it can be concluded that the main driving force for the recovery of the dye was the electrostatic interactions between the dye and the surfactant at the CGA interface, as noted by Basu & Malpani, (2001). Additionally, Huang et al., (2002) found the same observation (92%) when they recovered methyl orange using

hexadecyltrimethyl ammonium bromide (HTAB), a cationic surfactant, whilst there was almost no recovery of methyl orange when using SDBS. This result was in agreement with the result obtained in this experiment, using SDS with minimum recovery of methyl orange dye between 2.5-5.9%.

A lower recovery (40%) of methylene blue dye was achieved using 0.13mM sodium dodecyl benzene sulfonate (SDBS), an anionic surfactant (Huang et al., 2002) as compared to the result obtained in this experiment. Huang et al., (2002) however observed that better separation performance was recorded when CGA were generated with surfactant of opposite charge to the dye's. In this case, the recovery of methylene blue dye with SDBS (40%) was higher than those recovered from CGA generated from HTAB, a cationic surfactant (30%). The reason for the lower recovery could possibly be due to the concentration of surfactant used to produce CGA. Caballero et al., (1989) reported that the concentration of surfactant could influence the volume of surfactant added into a flotation column and also the stability of CGAs. This is a very important factor as stability of CGA is related to the contact time between dye and CGAs.

For the mixture of dyes, the separation took place with CGA generated with 1mM and 10mM of SDS surfactant showed 50.3% and 52.46% recovery of methylene blue dye, respectively. The methylene blue dye was visually observed in the apron phase while the methyl orange dye was in the liquid phase. It was interesting to note that, despite the high recovery of methylene blue individually with CGA generated with 1mM and 10mM SDS, the recovery from the mixture was low. The possible explanation was due to the charges of the dyes (Figure 2.3). Since both dyes had opposite charges, possibly they interacted by attractive electrostatic interactions and this rendered the separation rather difficult. When CGA were generated with 20mM of SDS, there was no separation of dyes

observed. This clearly confirmed that at this high concentration of surfactant the dyes partitioned into the micelles of the liquid phase rather than the CGA.

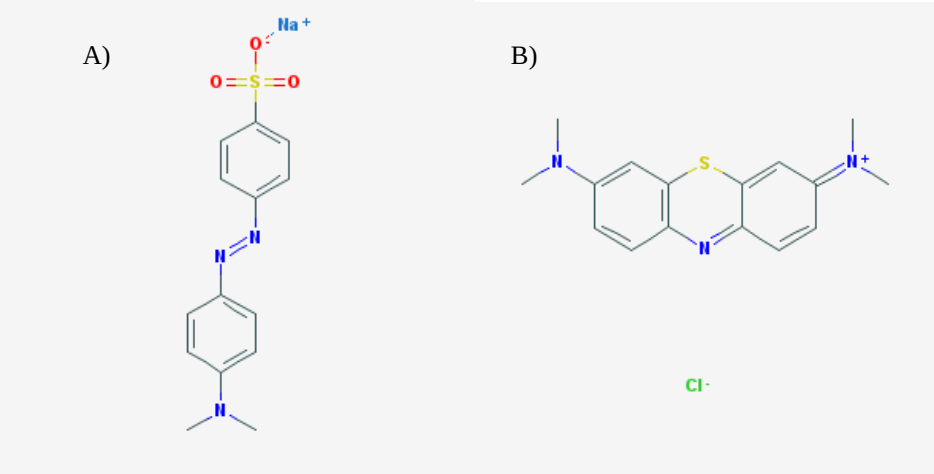

Figure 2.3a) Chemical structure of methyl orange; b) Chemical structure of methylene blue

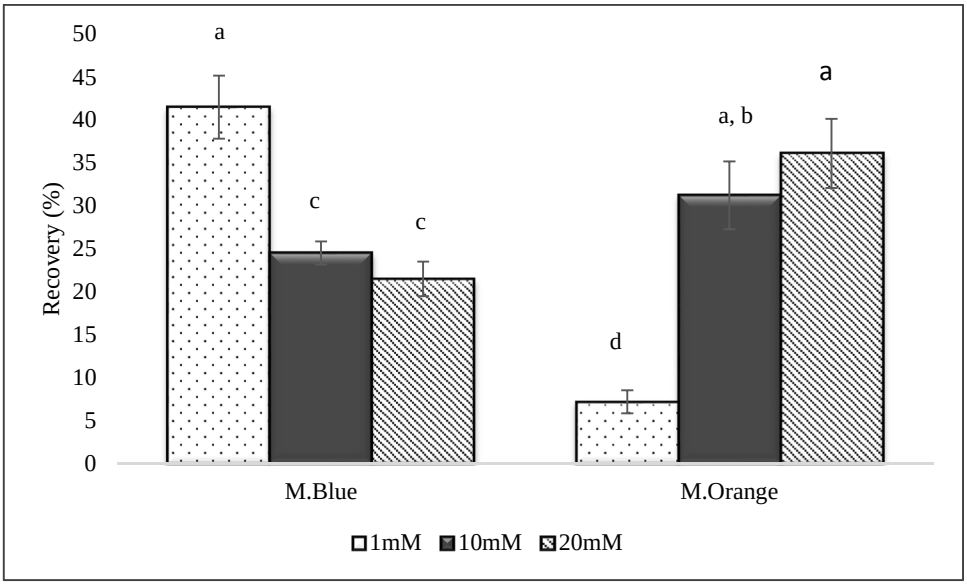

Figure 2.4The recovery of individual dyes from single solutions and mixtures of dyes using different concentration of TWEEN20 surfactant. Error bars indicate means± SD. Differences were considered to be significant at $p<0.05$.

Figure 2.4 showed the percent of dyes recovered in aphron phase separated by CGA generated from different TWEEN20 concentration. The recovery for methylene blue dye was in reverse with the trend shown in methyl orange. To be specific, the recovery for methylene blue dye decreased with the increasing of TWEEN20 concentration. Meanwhile for methyl orange, the recovery increased with the increasing of TWEEN20 concentration. At 1mM TWEEN20, the recovered methylene blue dye achieved was 41.48%. In the case of a mixture solution of both methylene blue and methyl orange dyes, there were no separation of dyes occurred in all three different concentration of TWEEN20. This further supported the principle of CGAs generated from ionic surfactants worked as ion exchangers as observed by Fuda & Jauregi, (2006), where in the case of TWEEN20, ions, hence electrostatic interactions were absent.

Huang et al., (2002) found about 20% removal of reactive brilliant blue dye when using CGAs generated from TWEEN20. This result was slightly higher than the removal percentage of SDBS which was about 10%. According to Huang et al., (2002), this reactive brilliant blue dye was hydrophobic with a functional group of $-SO_3Na$. Therefore, this separation of dye using TWEEN20 being better than SDBS could possibly due to its hydrophobic interaction between the dye and the surfactant.

From this experiment, it can be concluded that the recovery of dyes with ionic surfactant was better than the non-ionic. This was mainly because the separation was driven by electrostatic interactions between the surfactant used to generate CGA and the dyes. In a non-ionic surfactant, this effect was absent, thus suggesting the recovery could be due to hydrophobic interactions. However, in a more complex system (like in a mixture of dyes) the recovery efficiency reduced greatly thus suggesting the recovery may be influenced by the structure of the dye.

2.3.3 Characterisation of grape pomace extracts

Table 2.4 Chemical characterisation of grape pomace extracts.

Analysis/ Sample	Experimental Result		Literature	
	EE	HWE	EE	HWE
Total Phenol Index (280nm) (mg GAE/g)	41.80 ± 0.42	12.56± 1.22	43.44 [a]	NA
Total Phenol Folin-Ciocalteau (760nm) (mg GAE/g)	54.85 ± 0.96	28.80 ± 2.11	69.92 [a]	0.42 ± 0.04 [c]
Total Anthocyanin (g ME/100g)	0.96± 0.15	0.23 ± 0.15	0.98[b]	NA
Antioxidant Activity ABTS method (µMolTrolox eq./g)	333.33 ± 3.72	168.44 ± 3.72	485.42[b]	43.80 ± 4.90[c]
Total protein (mg BSA eq./g)	0.39 ± 0.01	0.24 ± 0.01	84.9[d]	NA
Total glucose (mg glucose eq./g)	88.32 ± 3.47	75.88 ± 5.70	79.5[d]	NA

[a]Spigno, Dermiki, Pastori, Casanova, & Jauregi, (2010)
[b] Rockenbach et al., (2011)
[c] Beres et al., (2016)
[d]Sousa et al., (2014)
NA – not available

Table 2.4 showed the experimental values of EE and HWE obtained in this study on different analyses. In general, the experimental values in EE were higher than those in HWE in all analyses. However, the results obtained were within the values obtained in the literature on grape pomace (Amendola et al., 2010; Tseng & Zhao, 2013). Total phenolic content measured by Folin-Ciocalteau method gave higher values than the direct measurement method. This is probably explained by the principles behind each method. Total phenol index measured the number of aromatic ring in the sample (as in gallic acid structure) at UV light, whereas in Folin-Ciocalteau method, relies on the transfer of

electrons in alkaline medium from phenolic compounds to form a blue chromophore constituted by a phosphotungstic/phosphomolybdenum complex. Although this method is commonly considered for polyphenol analysis, it indeed determines all compounds in the sample with antioxidant capacity and not only polyphenols (Pérez-Jiménez, Neveu, Vos, & Scalbert, 2010). Therefore, this method could possibly measure phenols together with other compounds involved in the reduction process, which explained the higher estimation of total phenol in grape pomace sample by Spigno, Amendola, Dahmoune, & Jauregi, (2015) as compared to the direct measurement method.

Furthermore, results vary with many different factors. These values are first determined by the grape variety because fruits of different varieties are known to significantly differ in their profiling (Kammerer, Kammerer, Valet, & Carle, 2014). These are further affected by the maturity of the grape and wine-making process such as vinification which greatly affects the yields of phenolic compounds. Among others includes skin maceration, fermentation temperature and pressing parameters. Secondly, type of solvents for extraction is always highly significant. Spigno and Faveri (2007) reported that high purity was achieved by using ethylacetate because the use of methanol, ethanol, acetone and their mixtures with water in different ratio generally yielded a significant co-extraction of concomitant substance, making the procedure of extract purification more difficult and decreased the yield of targeted antioxidants (Pekić, Kovač, Alonso, & Revilla, 1998).

The analysis of total proteins and sugar in our grape pomace revealed that EE had higher amount in both analyses as compared to HWE (Table 2.4). However, in comparison to the work by Sousa et al., (2014), EE and HWE had significantly low amount of proteins and sugars. Their grape pomace extracted with different solvents

including methanol, ethanol, acetone and n-hexane resulted in 84.9mg/g total protein and 79.5mg/g total glucose.

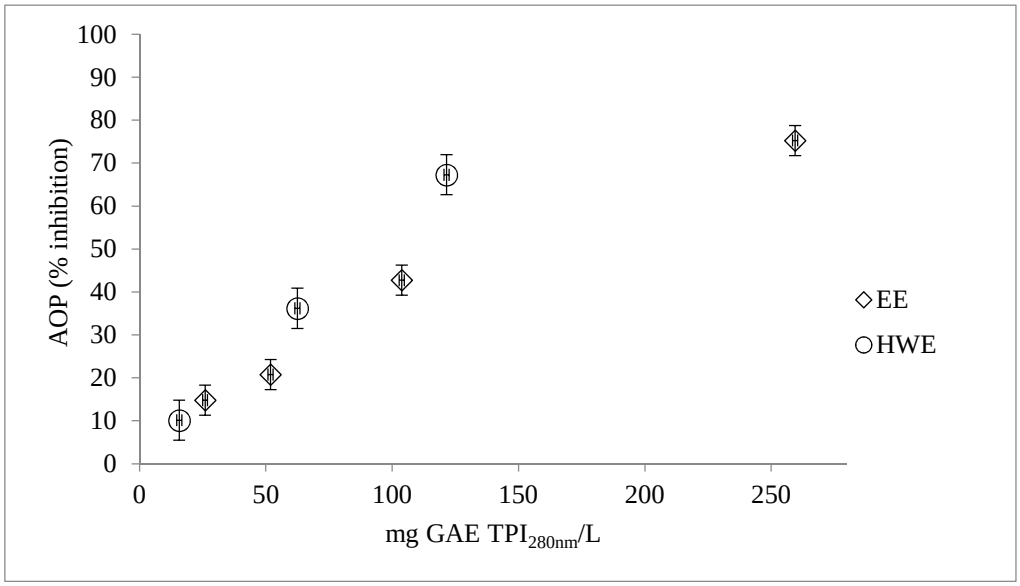

Figure 2.5 The antioxidant activity of grape pomace extract as measured by ABTS method. Results are expressed as percent of inhibition as a function of total phenolic content (mg GAE TPI_{280nm}/L).

Total phenolics content can have a strong association with the antioxidant activity observed within a system. Figure 2.5shows the antioxidant power (% inhibition) as opposed to concentration of phenols (mg GAE TPI_{280nm}/L) as measured by direct measurement at 280nm. From this graph, it was apparent that the antioxidant power increased linearly in correspondence to total phenolic content in both EE and HWE. For instance, 51.89mg GAE TPI_{280nm}/L of EE was able to inhibit ABTS radical at 20.77%. In addition, 62.5mg GAE TPI_{280nm}/L of HWE has 36.19% of antioxidant power.Futhermore strong positive correlation (R^2 = 0.9784 for EE and R^2 = 0.9997 for HWE) was observed between antioxidant acitivity and total phenolics content as also observed by other authors (Spigno & De Faveri, 2007).

In this study, the antioxidant activity in EE was 333.33µMol Trolox/g, which was almost two times higher than HWE (168.44µMol Trolox/g) (Table 2.3). Since a linear

relationship between the antioxidant power and total phenolic contents was established, the specific antioxidant power (AOP/mg GAE_{TPI}/L) can be calculated. The specific antioxidant power for HWE was 0.583 while EE had 0.289, indicating the potency of these two extracts. Interestingly, the inhibitory potency of HWE was higher than EE. This could possibly be attributed to other non-phenolic compounds that have the ability to donate a hydrogen atom or transfer an electron, such as proteins. The other possible reasons for this could be due to the different polyphenolic composition in both extracts. Specifically, antioxidant activity generally increased with increasing number of phenolic rings and cinnamic acid derivatives generally showed greater antioxidant activity than benzoic acid derivatives. The substitution of sugars into flavonoids resulted in impaired antioxidant activity (speculated to be due to steric hindrance), and antioxidant activity of flavonoids increased in a linear manner with an increase in free OH groups around the flavonoid frame (Kim & Lee, 2004).Therefore, the high potency of HWE could possibly be due to the different major polyphenols obtained in the extract.

Although many studies have shown strong correlations between total phenolic content and antioxidant activity, the mode of action of antioxidants is complex and may be dependent upon many factors within a system. In sources in which a strong correlation is observed, it is typically concluded that phenolics are largely responsible for the antioxidant activities seen within the samples; while in sources in which strong correlations are not observed, it is commonly concluded that there are significant amounts of antioxidants other than the measured phenolics present in the system, or that the specific phenolic species present in the system cannot be quantified properly through the total phenol assay (Craft, Kerrihard, Amarowicz, & Pegg, 2012).

In comparison to the literature, Spigno and Faveri (2007) found that antioxidant activity in grape stalk extract was slightly higher as compared to grape marc extract. This

can be observed with the bleaching percentage of β-carotene where 75% inhibition was associated with 150mg GAE/L for grape marc extract and around 100mg GAE/L of grape stalk. Ruberto et al, (2007) also reported a range of 14.45-38.93 mg/L extract of different needed to quench 50% of the initial radical as measured by DPPH. The antioxidant activity of our grape pomace was within the range obtained by Rockenbach et al., (2011) and was higher than the white grape (284µMol TE/g) as obtained by Sánchez, Franco, Sineiro, Magariños, & Núñez, (2009). The differences could be attributed to the different solvents used during extraction that could influence the total extractable compounds, hence its antioxidant activity. Rockenbach et al., (2011) used acidified methanol which has higher polarity than ethanol could possible give a better total extracable phenolic compounds. Moreover, the acidic condition in their study could possibly ruptured the matrix structure of the powder pomace, thus increased the accessability of the solvents to the compounds (Amorin-Carrilho et al., 2014). In addition to that, Jara-Palacios et al., (2014) found antioxidant acitivity of white grape pomace ranging from 225.0-594.2 µM TE/g using ABTS method. The variation could possibly be due to the different variety of the grapes and the solvents/methods used in the extraction that could affect the antioxidant activity of the extract (Chidambara Murthy et al., 2002).

2.3.4 The effect of surfactant type on the recovery polyphenols from grape pomace extracts in a flotation column

In this experiment, 10mM TWEEN20 and 1mM CTAB were used to investigate the effect of surfactants on the recovery of polyphenols from grape pomace extracts. The recovery of polyphenols was almost similar in both EE and HWE by TWEEN20 and CTAB as measured by direct measurement (Figure 2.6 A). Slight higher recovery of polyphenols was achieved under CTAB separation as compared to TWEEN20 when

using Folin-Ciolcalteau method in both EE and HWE. For anthocyanins, the recovery was higher with CTAB in EE, and was also higher with TWEEN20 in HWE. The initial result agreed with the findings from Spigno et al., (2015) with EE. In this study, the pH of the grape pomace ranged from 3.4-4.3, therefore anthocyanins were not charged since the flavilium ion were only ionised at $pH \leq 2$. In the case of proteins, higher recovery was achieved under separation of CGA by TWEEN20 in both extracts suggesting that they were drained with the liquid phase. This further supported that the recovery of polyphenols and proteins driven by electrostatic and hydrophobic interactions for CTAB and only hydrophobic interactions with TWEEN20, suggesting that hydrophobic interactions were stronger than the electrostatic interactions, or simply due to the different fold/structure of the proteins. In the recovery of sugar, there was no significant difference ($P<0.05$) noted between EE and HWE (71% and 68% respectively) when separated with CGA generated with TWEEN20. The same trend was further noted with sugars recovered wit CTAB where the recovery percentage was 65% in EE and 61% in HWE, respectively.

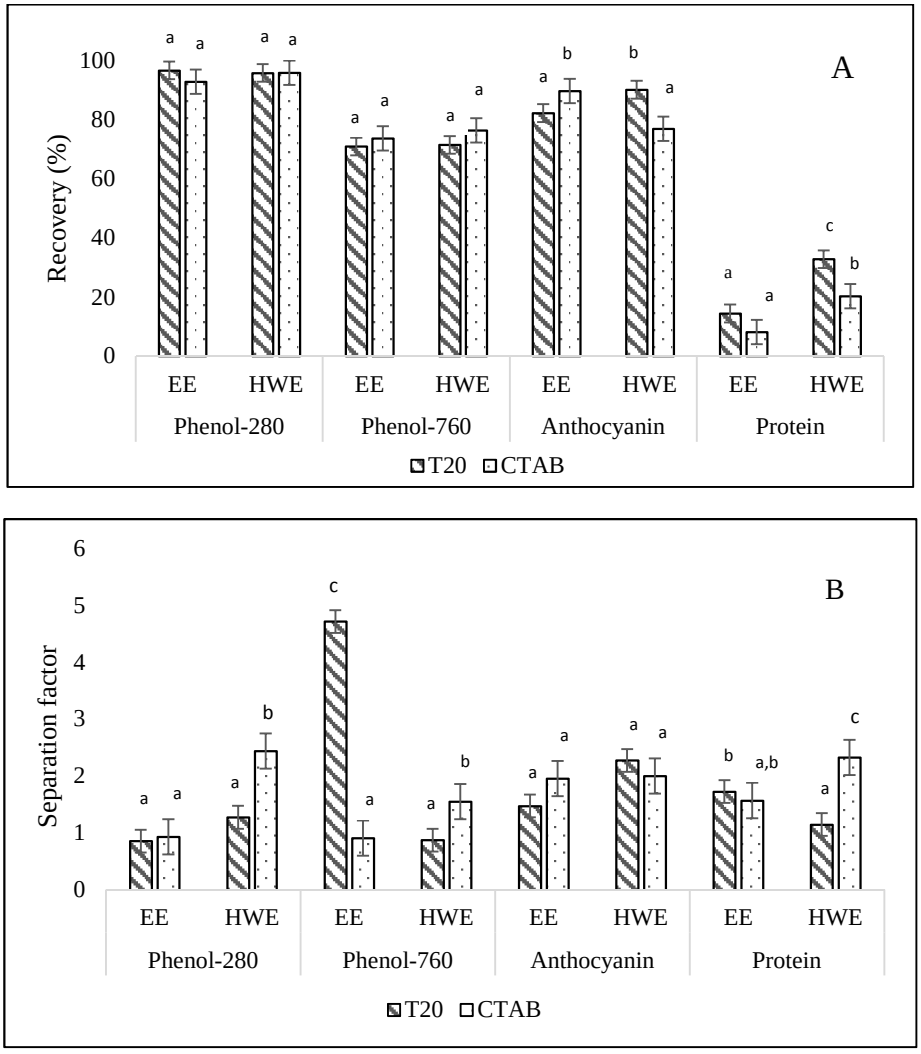

Figure2. 6 The recovery of (A) and separation factor (B) polyphenols and protein from ethanolic and hot water extracts from grape pomace at V_{CGA}/V_{feed} = 16 using different surfactants, n = 3. Error bars indicate means ± SD. Same superscript letters within the same column indicates means are not statistically different according to ANOVA $p<0.05$. Differences were considered to be significant at $p<0.05$.

The selectivity of compounds was generally higher in the CTAB than in TWEEN20 (Figure 2.6 B). However, in phenol-760 analysis of EE, selectivity was significantly higher, $p\leq0.05$ in TWEEN20 than in CTAB. It was interesting to highlight that the recovery of compounds analysed by phenol-760 was not significantly different from CTAB. However, when the analysis of total phenolic content was done by Folin-Ciocalteu method caution must be exercised in interpreting the data as the assay also

measured other readily oxidised substances such as proteins(Huang, Boxin, & Prior, 2005). Finally, the selectivity of protein in EE and anthocyanins of HWE were also higher with CTAB than in TWEEN20, although they were not significantly ($p \leq 0.05$) different from each other. In the case of sugar, the selectivity was similar between both extracts EE and HWE (0.26 and 0.24, respectively) separated with CGA generated from TWEEN20 and CTAB.

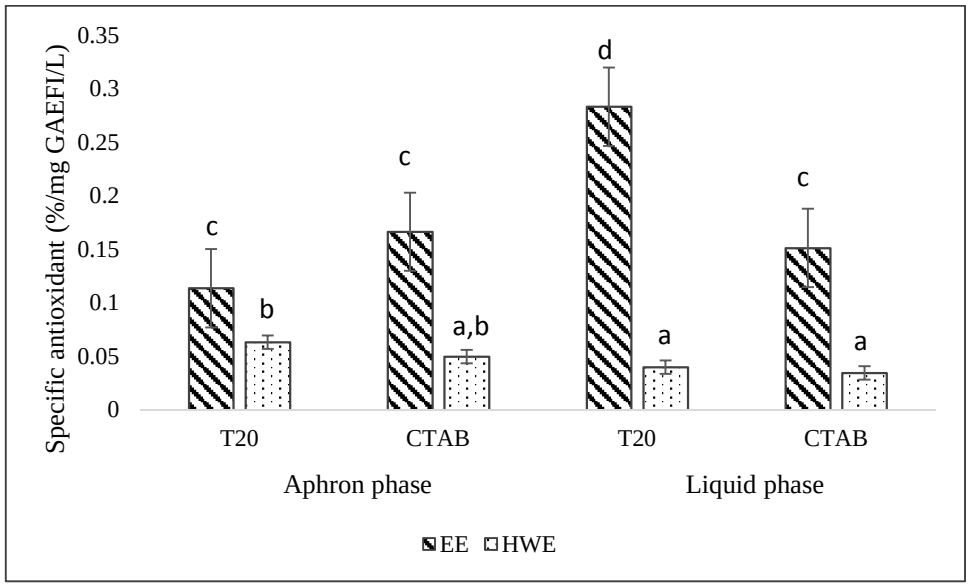

Figure 2.7 Specific antioxidant activity power of aphron and liquid phase from EE and HWE separated with different surfactants. Error bars indicate means ± SD. Differences were considered to be significant at $p<0.05$.

The analysis of specific antioxidant power of aphron and liquid phases from EE and HWE separated from CGA generated from TWEEN20 and CTAB showed a protecting effect by TWEEN20 on the polyphenols (Figure 2.7). Although in the aphron phase of EE, those separated from TWEEN20 and CTAB had similar specific antioxidant power, since they were not significantly different. The same trend was observed in the HWE, with their specific antioxidant power was much lower than in EE. Interestingly, the specific antioxidant power in the liquid phase of EE separated with TWEEN20 was

substantially high as opposed to HWE and the trend extended to the separation by CTAB. These results were in agreement with those obtained by Spigno et al., (2015) suggesting minimal oxidation of polyphenols in the fractions separated by TWEEN20. Moreover, a study by Lin, Wang, Qin, & Bergenståhl, (2007) suggested that micelles could protect the polyphenols from oxidation.

From the results obtained here, the recovery of polyphenols in particular with CGA generated from TWEEN20 and CTAB was almost similar in both EE and HWE. However, the antioxidant activity of CGA fractions separated with TWEEN20 had higher activity, particularly in EE. In the next experiments, the CGA separation is going to focus on TWEEN20 since no difference in polyphenols recovery was noted between the two surfactants. Moreover, this study aimed to assess the potential applications of these fractions for consumption or topical application; thus TWEEN20 was seen as the best surfactant for its non-toxicity/less irritant properties.

2.3.5 The effect of volumetric ratio on the recovery of polyphenols from grape pomace extracts in a flotation column

In this experiment, the different ratio of CGA to the feed (extract of grape pomace) was investigated. Figure 2.8 shows the recovery percentage of polyphenols, protein and sugar from ethanolic extract (A) (EE) and hot water extract (B) (HWE) with CGA generated from 10mM TWEEN20. The recovery of compounds increased with volumetric ratio for both EE and HWE and decreased at ratio 20. In EE, with the exception of proteins, highest recovery was achieved at ratio 16. Around 74.64% and 79.40% recovery of polyphenols was achieved by direct measurement (Phenol-280nm) and Folin-Ciocalteau method (Phenol-760nm). In the case of anthocyanins and sugars,

the recovery was 84.99% and 34.16%, respectively. It was interesting to note that the recovery of sugar was low at ratio 4.

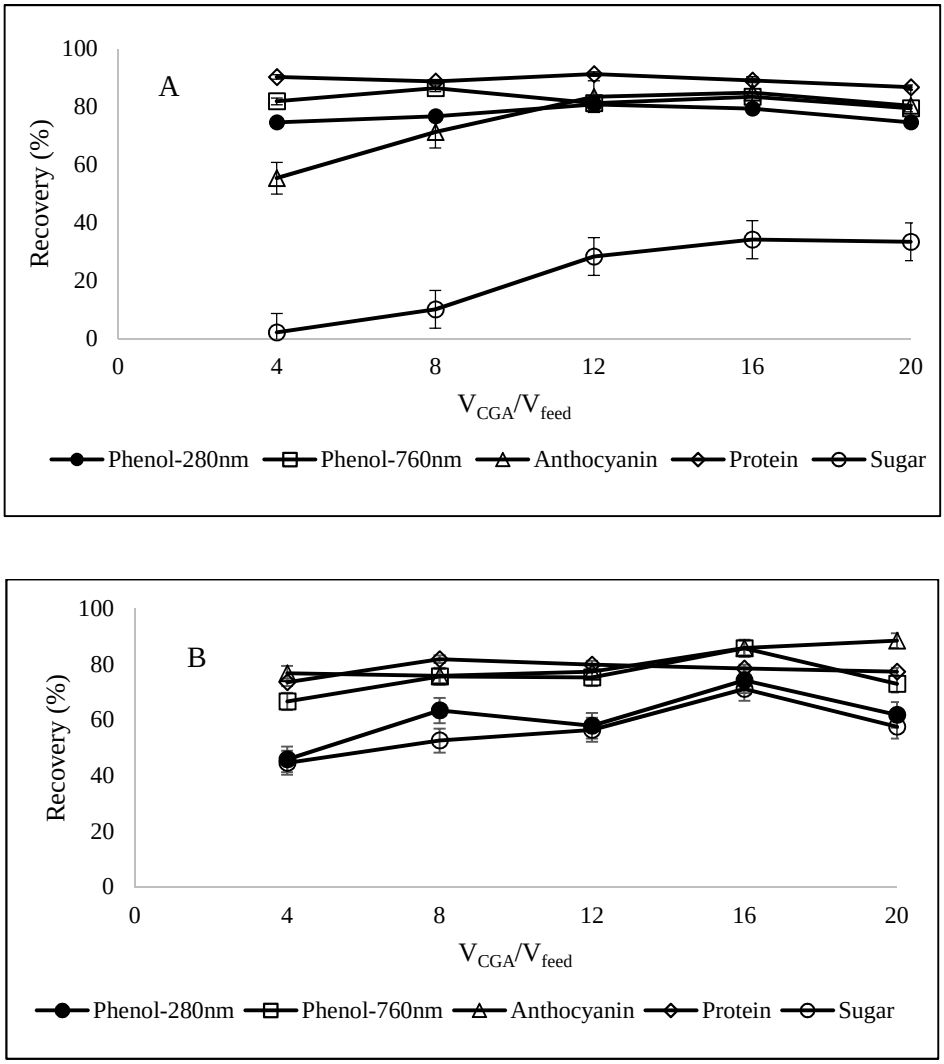

Figure 2.8The recovery of polyphenols, sugars and proteins from ethanolic extract (A) and hot water extract (B) from grape pomace at different volumetric ratio, n = 3. Error bars indicate means± SD. Differences were considered to be significant at $p<0.05$.

Similar trend was also observed in the recovery of compounds from HWE. In general, the recovery of compounds increased with the ratio; however polyphenols (measured by both methods) and sugar decreased at ratio 20. In this case, the ratio 16 gave considerably high recovery of all compounds including polyphenols and

anthocyanins. The recovery of polyphenols was 74.26% and85.74% by direct measurement (Phenol-280nm) and Folin-Ciocalteau method (Phenol-760nm), respectively while the recovery of anthocyanins was 85.87%.

The increase of recovery with the volumetric ratio was also noted by Spigno et al., (2015) . The low recovery at low volumetric ratio of TWEEN20 could possibly be due to the low effective gas hold up. For instance, at volumetric ratio 4, TWEEN20 has a low gas hold-up which coincides with low recovery of polyphenol (Spigno et al., 2015). The reduction in the gas hold-up has led to a reduction in interfacial area hence lower recovery of polyphenol. This has been also observed in the recovery of astaxanthin (Dermiki et al., 2009).It is also important to highlight that some aggregates were observed in EE which did not completely solubilise during analysis; hence this would probably lead to underestimation of the net recovery. This could be attributed to the role of ethanol on the micelle formation, as it can replace the water molecules around the micelles and participate in the aggregation process (Li, Han, Zhang, & Wang, 2005).

It can be seen that the recoveries of polyphenols increased with the decreasing of volume of initial feed leading to high volumetric ratio. This could probably be due to the fact that CGA could be saturated with phenolic compounds (Dermiki et al., 2009). Moreover, when high volume of feed was introduced, the CGA dispersion may not have providedenough surface area for adsorption (Hashim et al., 1995).

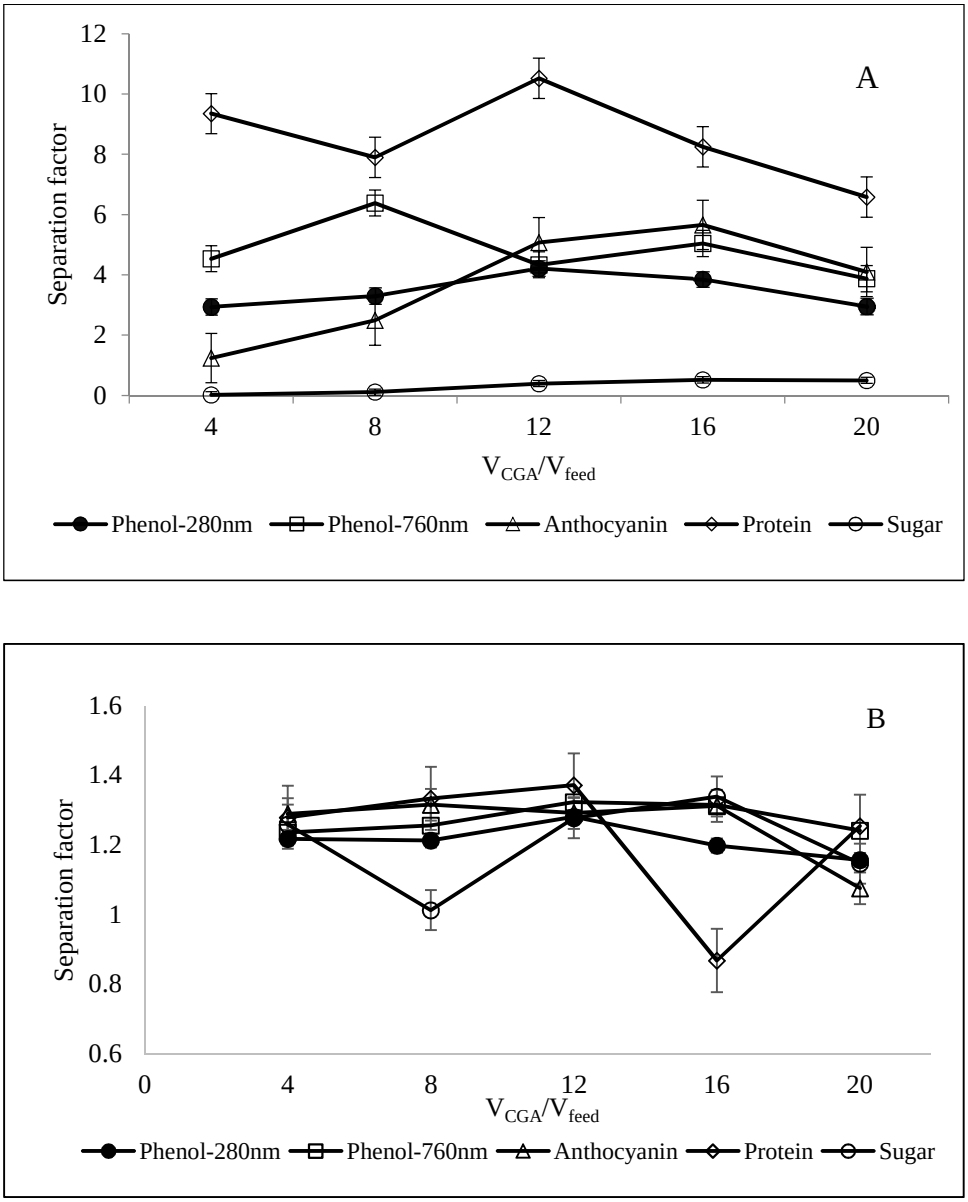

Figure 2.9 The separation factor of polyphenols, sugars and proteins from ethanolic extract (A) and hot water extract (B) from grape pomace at different volumetric ratio, n = 3. Error bars indicate means ± SD. Differences were considered to be significant at $p<0.05$.

When the separation factor was calculated, a more distinctive pattern was observed between EE and HWE (Figure 2.9). The separation was better in EE than in HWE. In EE, the SF of all compounds increased with the volumetric ratio and decreased after ratio 16. For total phenolics contents and anthocyanins, high SF was achieved with ratios 12 and 16.However, it must be stressed that ratio 12 also gave high SF ratio for proteins which showed high affinity of proteins for the aphron phase. In the separation of

sugars, the SF increased and reached its highest at ratio 16 and plateau after that. In HWE, the selectivity pattern for all compounds generally varies between 1.0-1.3 which indicated similar partitioning of compounds between the aphron and liquid phase. A similar trend was observed in the volume of liquid and aphron phase drained (Figure 2.10), which suggested the affinity of total phenolic contents and anthocyanins as well as sugar towards the aphron phase, while proteins drained together with the liquid phase. Stabilisation of CGA was also noted in HWE than in EE as the volumetric ratio increased.

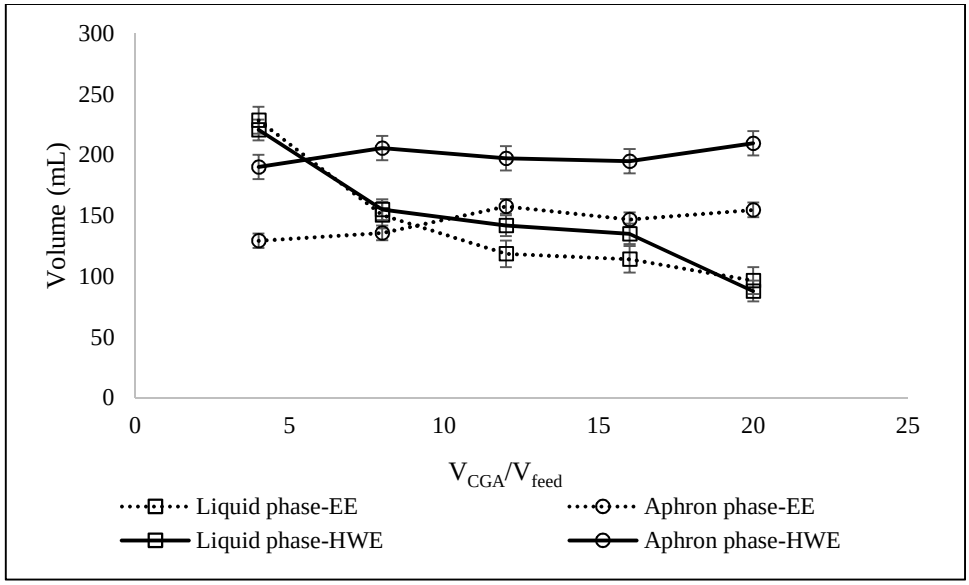

Figure 2.10 Volume of liquid and aphron phase drained as a function of volumetric ratio. Error bars indicate means ± SD.

Furthermore, the antioxidant activity of total polyphenolic recovered in the aphron phase reduced as compared to total polyphenolic drained in the liquid phase for both EE and HWE (Figure 2.11). This result was also observed by Spigno et al., (2015) which could possibly be caused by the oxidation of the polyphenols during the recovery. However, it is interesting to note that in HWE, there was a strong correlation between the

antioxidant activity and total phenolic content (R^2 = 0.9875) in the aphron phase and (R^2 = 0.8176) in the liquid phase.

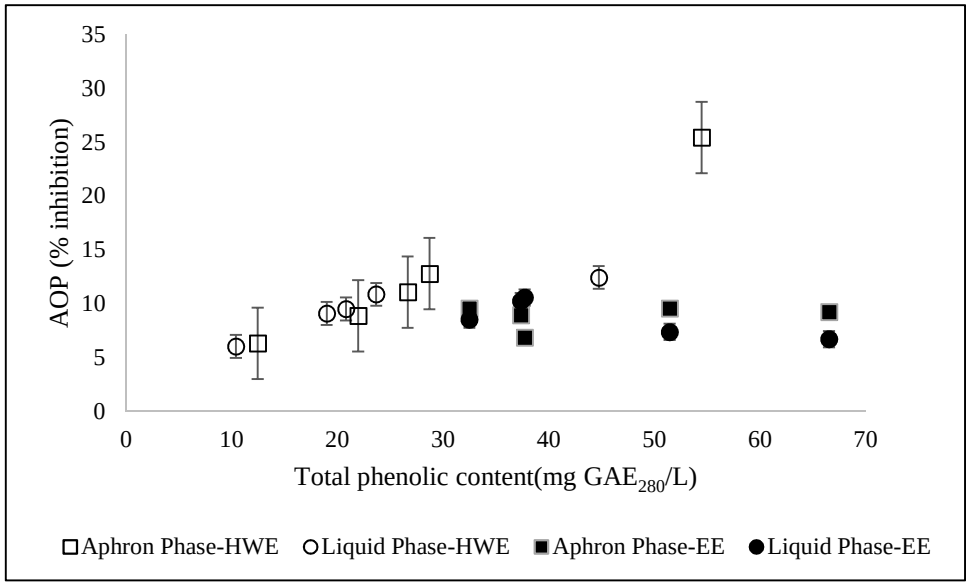

Figure 2.11 Antioxidant power as a function of total phenolic content (phenol-280) for the recovered liquid and aphron phase from EE and HWE. Error bars indicate means ± SD. Differences were considered to be significant at $p<0.05$.

It can be recalled that in section 2.3.3, the antioxidant activity per total phenolic content of EE and HWE also showed strong linear relationship (Figure 2.11). Interestingly in HWE, the specific antioxidant activity in the aphron phase was consistent throughout the CGA separation. The specific antioxidant activity in the liquid phase however decreased substantially throughout the separation process.

This was not the case for EE, where poor correlations were observed in both phases of EE (R^2 = 0.0871 aphron phase and R^2 = 0.6189 liquid phase), which demonstrated minimal antioxidant specificity in relation to total phenolic content. Although this result was not in agreement with Spigno et al., (2015) in their trial with Pinot noir pomace extract, the possible reason for this result could be caused by the aggregation of some polyphenols in both phases which lead to underestimation of antioxidant activity.

2.3.6 The effect of drainage time on the recovery of polyphenols from grape pomace extracts

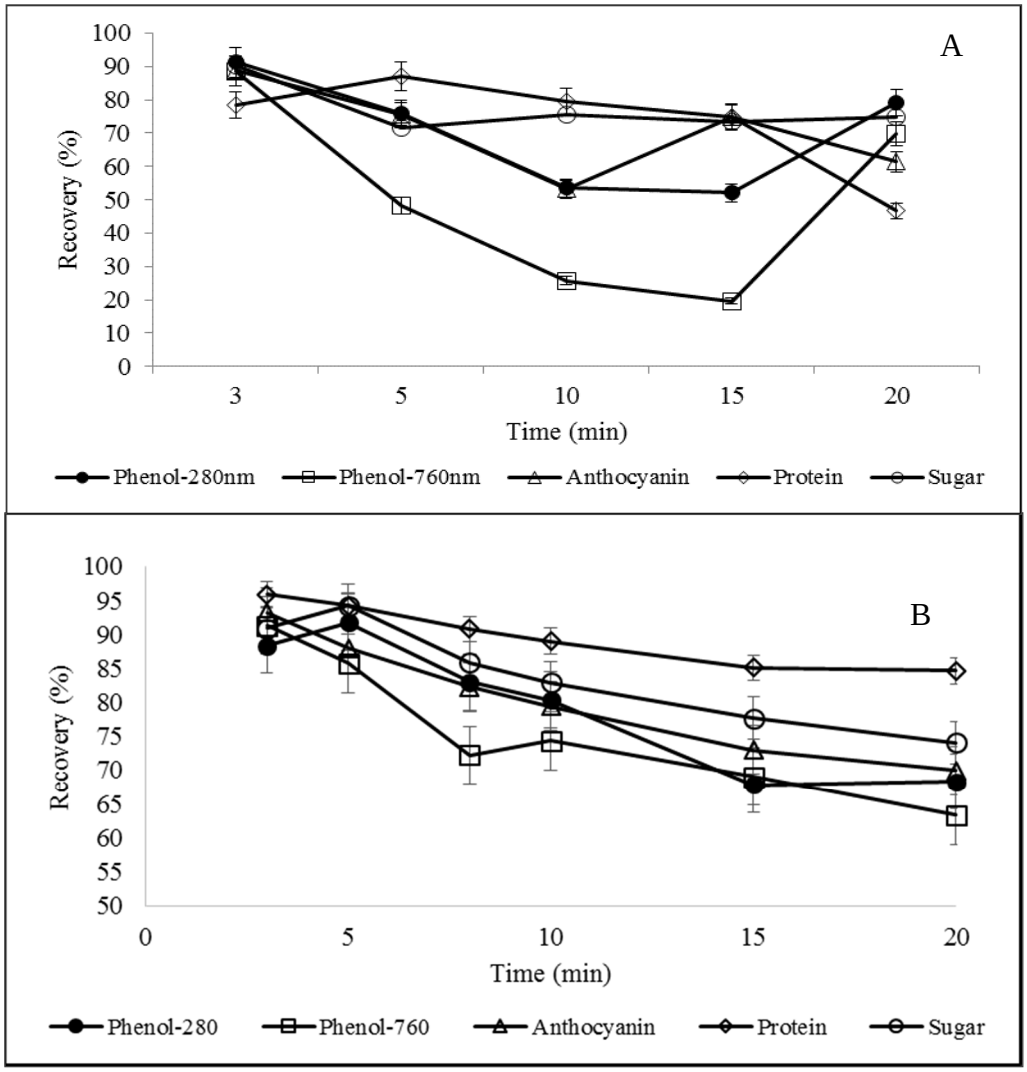

Figure 2.12 The recovery of polyphenols, sugars and proteins from ethanolic extract (A) and hot water extract (B) from grape pomace at different drainage time, n = 3. Error bars indicate means ± SD. Differences were considered to be significant at $p<0.05$.

Further separation trials at constant volumetric ratio 16 and varied drainage time were conducted in order to see if increased drainage led to an increase concentration of total phenolics and/or anthocyanins in the aphron phase as observed with asthaxantin (Dermiki et al., 2009). The recovery results from ethanolic extract (A) and hot water extract (B) of grape pomace at different drainage timeare shown in Figure 2.12. In EE,

the recovery decreased with drainage time but increased at time 20 mins. In HWE, a clearer trend was observed, with the recovery decreased with time for all compounds. These results were in agreement to those obtained by Spigno et al., (2015)using 2mM of CTAB suggesting that phenolic compounds may drained together in the liquid phase.

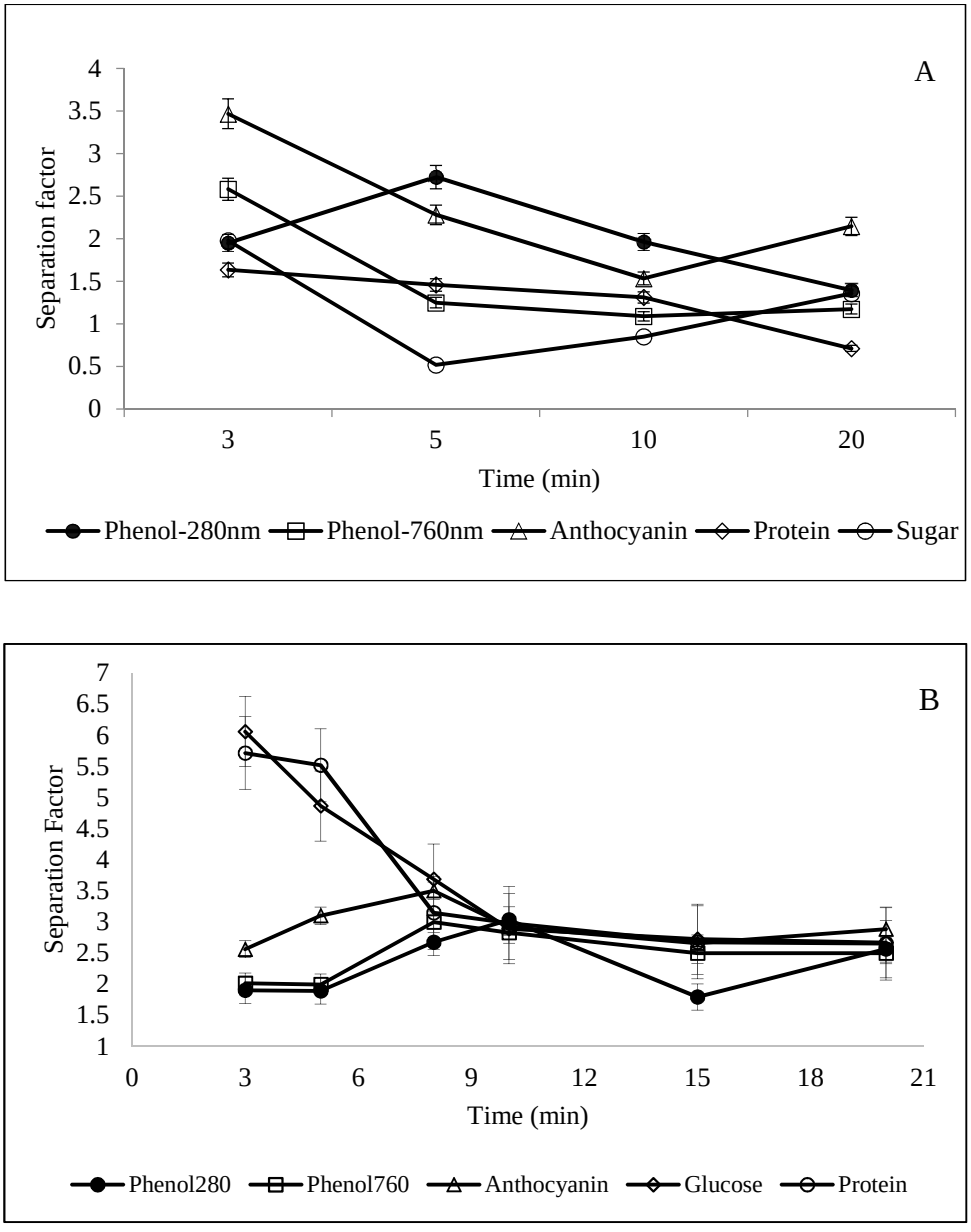

Figure 2.13 Separation factor of A) EE and B) HWE as function of drainage time. Error bars indicate means ± SD. Differences were considered to be significant at $p<0.05$.

There were no clear trends on the selectivity in both extracts (Figure 2.13). However, it can be said that in EE, the separation factor decreased with drainage time for all compounds. In the case of HWE, selectivity decreased with time for proteins and sugar but increased steadily for phenolics and anthocyanins until they reached a plateau after minute 10. A quite similar trend was followed by the ratio of drained liquid phase to the volume of collapsed aphron phase (Figure 2.14). The higher ratio of liquid phase to aphron phase in EE as compared to that in HWE suggests that the destabilisation effects of the ethanol towards the CGA, as evident by lower volume of aphron phase.

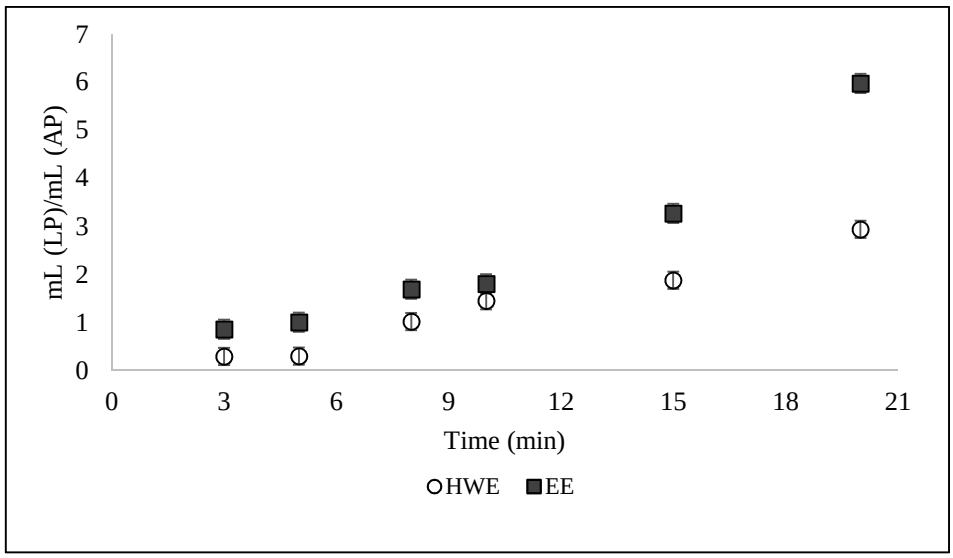

Figure 2.14 The volumetric ratio of drained liquid phase to drained aphron phase as a function of drainage time. Error bars indicate means± SD.

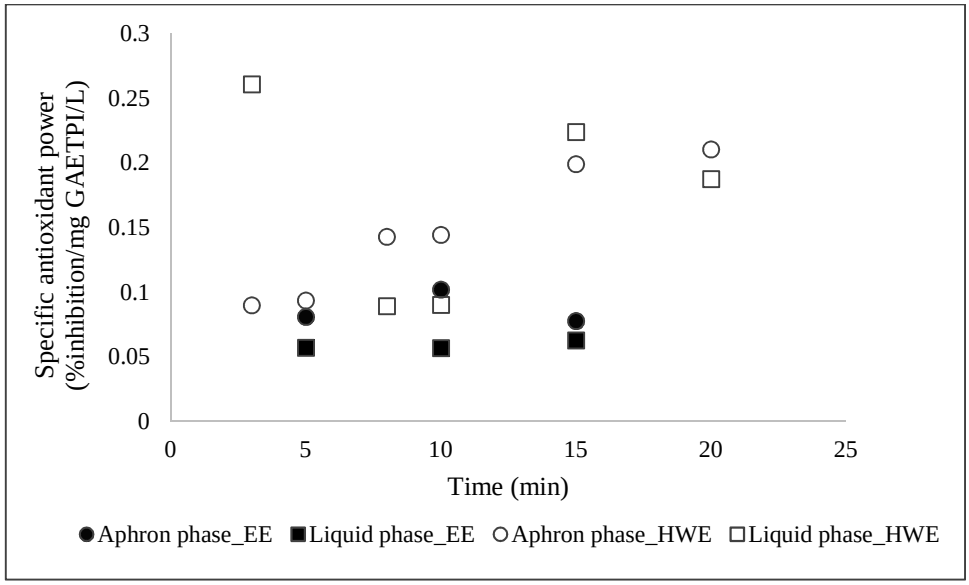

Figure 2.15 Specific antioxidant activity power of aphron and liquid phase from EE and HWE as a function of drainage time (min). Error bars indicate means± SD. Differences were considered to be significant at $p<0.05$.

When the specific antioxidant power was calculated as a function of time as plotted in Figure 2.15, the clearest trend observed in EE was that the aphron phase had higher specificity than the liquid phase. The specificity in each phase decreased over drainage time; this could be attributed to the high total phenolic content and lower volume of aphron phase in EE (refer Fig 2.12 and 2.14). However, this was not the case in HWE. Interestingly, the specific antioxidant power was higher in liquid phase as compared to aphron phase, although the specificity increased over time in the aphron phase. This could possibly suggest a strong affinity of particular compounds to the aphron phase which could got concentrated and hence lead to the increase in antioxidant activity.

2.4 Conclusions

In this chapter, the ethanolic (EE) and hot water extracts (HWE) of grape pomace were characterised for their main composition. In general, EE had higher amount of phenolics and anthocyanins than HWE, while HWE had slightly higher amount of proteins and sugars than EE; this result was expected. The antioxidant activity of EE was found to be stronger than HWE and there was a strong correlation between total phenolic content and antioxidant activity.

Preliminary work on the recovery of individual dyes from a mixture solution was conducted to understand the principles of CGA separation. Throughout this study, the TWEEN20 was chosen for its high polyphenols recovery and also for the stability of polyphenols against oxidation, as compared to CTAB. Furthermore, the effect of varying volume of CGA (generated from TWEEN20) to the volume of feed was studied for both EE and HWE. The results revealed that ratio 16 gave highest recovery of polyphenols and lower recovery of proteins and sugars. This result was in agreement with that obtained by Spigno et al., (2015). In addition to that, the effect of prolonged drainage time was also studied. Results showed that polyphenols recovery decreased with increase of time suggesting these compounds were drained with the liquid phase during the separation. From all of these results, the main outcomes were:

1. EE had 41.8mg GAE_{TPI}/g of dry weight pomace and0.96 g ME/g of dry weight pomace total monomeric anthocyanin content while HWE had 12.5 mg GAE/g total phenolic content 0.23g ME/g total monomeric anthocyanin. The antioxidant activity of EE was 333.3µMol Trolox equivalent whilst HWE had 168.4µMolTrolox equivalent but HWE had higher potency of antioxidant activity in relation to total phenolic content.

2. The recovery of polyphenols with TWEEN20 was almost similar to that of CTAB but higher antioxidant activity was observed in TWEEN20 compared to CTAB.
3. In general, the recovery of polyphenols in EE and HWE increased with volumetric ratio and maximum recovery was obtained at ratio 16 but decreased afterwards.
4. Polyphenols recovery decreased with drainage time for both EE and HWE.
5. In EE, anthocyanins had lower affinity to the aphron phase as compared to total phenolic content in the low volumetric ratios but had become similar as the volumetric ratio increased. In HWE, the affinity of both total phenolics and anthocyanins were very similar and consistent with the increase of volumetric ratio.

Overall, this chapter has shown a fundamental work on the important processing parameters for the recovery of polyphenols using CGA generated from TWEEN20. TWEEN20 could be used in food, cosmetic and pharmaceutical formulations and therefore it would not need to be removed. Having said all the above points, it must be stressed that this work will form the basis for further work described in the next chapters where the potential application of CGA in cosmetic and food industry is investigated.

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CHAPTER 3: Polyphenols extracted from red grape pomace by a surfactant based method show enhanced collagenase and elastase inhibitory activity*

BACKGROUND: The aim of this study is to separate polyphenols from grape pomace using a surfactant-based separation, Colloidal Gas Aphrons (CGA) and to investigate their inhibitory activity against skin relevant enzymes, collagenase and elastase. Ethanolic (EE) and hot water crude extracts (HWE) were produced first and then the CGA generated using TWEEN20 were applied resulting in polyphenols enriched fractions (CGA-EE and CGA-HWE, ethanol and hot water extracts derived fractions respectively).

RESULTS: Both crude extracts inhibited the enzymes in a dose-dependent manner however, further extraction by CGA led to fractions with higher inhibitory efficiency against collagenase. Although gallic acid was the main component of the CGA-HWE, others such as kaempferol must have contributed to its potency which was over six times more than gallic acid's. The CGA-EE was found to be about four times more efficient than its crude extract and over six times more efficient than gallic acid in collagenase's inhibition; quercetin was the major polyphenol in this fraction.

CONCLUSION: It is evident that ethanol and hot water extraction processes led to different polyphenols composition and thus different inhibitory activity against collagenase and elastase. Further separation with CGA increased the inhibitory potency

of both extracts against collagenase. Overall the results here showed the potential application of the CGA fractions from grape extracts in cosmetics.

Keywords: colloidal gas aphrons, grape pomace, polyphenols, collagenase, elastase

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3.1 Introduction

Over the past two decades, research on the use of natural products, particularly polyphenols, in beauty products has been active but remains challenging (Menaa, Menaa, & Tréton, 2013). Polyphenols with a hydroxyl group (-OH) attached to an aromatic benzene ring (C_6H_5-) naturally occur in plants and are therefore abundant in our diet (eg: vegetables, fruits, nuts, seeds and flowers), and have been extensively studied for their protective health effects against cardiovascular diseases and cancers (Haslam & Cai, 1994). Moreover, they have been proven to exhibit significant antioxidant activity, as well as a UV protection effect which are very crucial for skin care products formulation (Perona, Cabello-Moruno, & Ruiz-Gutierrez, 2006).

Green tea is the most widely studied plant for its cosmetic applications. Green tea polyphenols extract incorporated in derma gels were found to display significant antioxidant activity and prevent adverse effects of UV radiation by improving the elasticity of the skin (Chiu et al., 2005; Türkoğlu, Uğurlu, Gedik, Yılmaz, & Süha Yalçin, 2010). Catechins and epigalocatechingallate from green tea and cocoa beans extracts were found to possibly contribute to this effect (Hong, Jung, Noh, & Suh, 2014; Türkoğlu et al., 2010; Wahab, Rahman, Ismail, Mustafa, & Hashim, 2014). In addition, catechin

could stabilise the structure of collagen suggesting the involvement of hydrogen bonding and hydrophobic interactions as major forces in its stabilisation (Madhan, Krishnamoorthy, Rao, & Nair, 2007). Moreover Sin & Kim (2005) found that the flavonols, particularly quercetin and kaempferol exhibited higher inhibitory activity against collagenase than flavones/isoflavones. In a recent study, Wittenauer et al. (2015) found that free phenolic acids, particularly gallic acid extracted from grape had the most potent inhibitory activity against both collagenase and elastase. However, it is worth mentioning that the inhibitory concentration of polyphenols varies between studies and samples (268µM – 1000µM); this is partly due to the variations in polyphenols composition. Also the size of polyphenols restricts their permeation into the epidermal and corium layers (Zillich, Schweiggert-Weisz, Hasenkopf, Eisner, & Kerscher, 2013)which could hinder their application in cosmetics.

Surfactants are often used in cosmetics products to address the problem with the permeation of the desired molecules. Surfactants in their micellar form can help in the solubilisation of compounds (Löf, Schillén, & Nilsson, 2011) hence increasing the permeation through the skin and promoting absorption by lowering the interfacial tension at the skin surface. The delivery of resveratrol and curcumin has been improved by the presence of surfactants in pig skin (Yutani, Morita, Teraoka, & Kitagawa, 2012), and the acceleration of hydrocortisone and lidocaine has been observed on hairless mouse skin by using TWEEN80 (Williams & Barry, 2004). Therefore, using a surfactant based extraction method is an advantages the product is extracted in a media (surfactant solution) that is suitable and possibly optimum for its formulation, which can lead to the process of simplification and formulation.

In our group, we investigated a surfactant based extraction method, Colloidal Gas Aphrons (CGA), for the separation of polyphenols from grape (Spigno, Amendola,

Dahmoune, & Jauregi, 2015). CGA are microbubbles (10-100µm) generated by intense stirring (>8000rpm) of a surfactant solution above its critical micelle concentration. CGA are composed of an inner core gas surrounded by a thin layer film. The type of surfactant used to generate CGA determines the charge of the outer surface of the layer, which could be positive, negative or non-charged and oppositely or non-charged molecules will adsorb resulting in their effective separation (Spigno & Jauregi, 2005).

In the present study the aim was to determine if the extraction of polyphenols by the CGA method led to enhanced *in vitro* inhibitory activity against *Clostridium histolyticum* collagenase (ChC) and porcine pancreatic elastase (PPE) enzymes. The relationship between polyphenol composition of the raw and the CGA extracts, and their inhibitory activity were also investigated in order to identify the key polyphenols responsible for these activities. To the best of our knowledge, this is the first study report on the potential inhibitory activity of red grape pomace extracts and their CGA fractions against ChC and PPE.

3.2 Materials and Methods

Grape pomace (Barbera) provided by wineries in Nothern Italy was oven dried at 60°C until residual moisture content was <5% and milled into particle size of <2mm. The phenolic extracts were obtained by ethanol-aqueous extraction using 60% (v/v) and hot water extraction at 60°C and 100°C in shaking water bath (100rpm) in circular motion, for 2 hours and 1 hour, respectively (See Figure 1 for both extraction process). For both extractions, the ratio of solute to solvents used were 1:8 according to Amendola, De Faveri, & Spigno, (2010). Extracts were kept in -20°C freezer until further use. Extractions were done in triplicate.

C. hystoliticum collagenase type IA (ChC), N-[3-(2-furyl)acryloyl]-Leu-Gly-Pro-Ala (FALGPA), porcine pancreatic elastase (PPE) type III, N-Succ-Ala-Ala-p-nitroanilide (AAAPVN), phenol crystals and BCA reagents were obtained from Sigma (St. Louis, MO). Polyphenols standards used for HPLC analysis and inhibition studies were gallic acid (≥95%), caffeic acid (≥95%), epicatechin (≥95%), p-coumaric acid (≥95%), benzoic acid (≥99.5%), *trans*-resveratrol (≥95%), quercetin (≥95%), malvidin-3-o-glucoside (≥95%), cyanidin-3-o-glucoside (≥95%), petunidin-3-o-glucoside (≥95%) and delphinidin-3-o-glucoside (≥95%) from Sigma (St. Louis, MO): procyanidin (B2 ≥90%), from Fluka (Buchs, Switzerland). All solvents were of HPLC grade or LC-MS grade.

3.2.1 Characterisation of grape pomace

3.2.1.1 Phenolic compounds

The grape pomace extracts, liquid and CGA fractions recovered were characterised for its total phenolics and anthocyanins. **Total phenolics** were measured by: (i) direct measurement based on the absorbance reading at 280nm. Results were expressed by gallic acid equivalents (GAE_{280nm}) by means of calibration curve with standard gallic acid ranging from 0-150mg/L (Amendola et al., 2010). (ii) Folin-Ciocalteu method. The results were expressed as gallic acid equivalents (GAE_{760nm}) (Singleton & Rossi, 1965).

Total anthocyanins content was determined by applying the method from AOAC (Lee, Rennaker, & Wrolstad, 2008). This method is based on the anthocyanins structural transformation that occurs with a change in pH and commonly referred as pH differential method. The results were expressed as mg/L malvidin-3-glucoside equivalents (ME).

3.2.1.2 Non-phenolic compounds

Total proteins were quantified according to the bicinchoninic acid assay (BCA) (Smith et al., 1985). Briefly, 100µl of standards or samples were mixed with 2 mL of the BCA working reagent (copper sulphate solution:BCA solution at a ratio of 1:50). The mixture was allowed to stand at 37°C for 30 mins, and then allowed to cool at room temperature for 5 mins. Finally, the absorbance for each sample/standard was read at 562nm within 8 mins with water as a blank. Bovine serum albumin (0-1.0mg/L) was used as a standard for protein quantification.

Total sugar content was performed adopting the method from Dubois, Gilles, Hamilton, Rebers, & Smith, (1956). In test tubes, 0.4mL of glucose standard/sample was added followed by 0.2mL or5% phenol solution. Subsequently, 1mL of sulphuric acid was pipetted direct to the solution and vortexed. The mixture was allowed to stand for 20-30mins to cool off. The absorbance of the mixture was read at 490nm and a calibration curve was constructed with different concentrations (10-100mg/L) of glucose standard. The results were expressed as mg/L glucose equivalent.

3.2.2 Determination of polyphenols composition by HPLC

Separation of the polyphenols was performed using an Agilent HPLC 1100 series system equipped with a degasser, a quaternary pump and a photodiode array detector (Agilent, Waldbronn, Germany) with Chemstation software. The column used was a C18 HiChrom column (150 mm x 4.6 mm i.d; 5µm particle size and 100 Å pore size; part no.EXL-121-1546U) operated at 25°C.

The mobile phase consisted of 2% formic acid (v/v) and 5% acetonitrile (v/v) in water (mobile phase A) and 2% formic acid (v/v) in acetonitrile (mobile phase B) using

the following gradient: 5-15% B (15 mins), 15-30% B (15 mins), 30-50% B (10 mins), 50-95% B (5 mins) and 95-5% B (5 mins), at a flow rate of 1mL/min. The total run was 50 mins. The pre time of 10 mins was allowed for re-equilibrating. The injection volume was 20µL for pure standards and 100µL for grape extracts. The polyphenols were monitored simultaneously at 280nm (hyroxybenzoic acids and flavanols), 320nm (hydroxycinnamic acids and stilbenes), 365nm (flavonols) and 520nm (anthocyanins).

3.2.3 Identification of polyphenols by LC-MS

The LC-MS analyses were carried out using a Thermo Scientific Accela HPLC with PDA UV/Vis detector interfaced to a Thermo Scientific LTQ Orbitrap XL with ESI source. Chromatographic separation was carried out using an Ace-5 C18 column; 150 x 2.1 mm, 5µm particle, 300 Å pore (part no. 221.1502). All samples were analysed without dilution and in 10 fold dilution. Dilutions were done in mobile phase A2 buffer (0.1% formic acid (v/v) in LC-MS water). Mobile phase B2 buffer was 0.1% formic acid (v/v) in acetonitrile. Injections volumes were 20µL. The following gradient was used: 0 min 5% B2; 5-15% B2 (15mins), 15-30% B2 (15mins), 30-50% B2 (5mins),50-95% B2 (5mins), 95-5% B2 (5mins) and 5% B2 (10mins), at a flow rate of 200µL/min.

The MS parameters were as follow: a standard of caffeic acid was infused into the MS source alongside the HPLC flow at 20% mobile phase B; using a T-piece the source and transmission settings were optimised for both positive and negative ion modes. The salient settings were as follows: sheath gas flow at 45, aux gas at 10, sweep gas at 0 and the capillary temperature was at 300°C. For the positive mode, the source voltage was 5Kv, capillary voltage was 31v and tube lens was 125v. For the negative mode, the source voltage was 5Kv, capillary voltage was -35v and tube lens was -90v.

The MS was operated using a Data-dependent acquisition (DDA) method. In brief, an MS1 scan was performed using the Orbitrap detector scanning from 85 to 1000 m/z at a resolution of 30,000 storing data in profile. Phthalate (413.266230 m/z) was used as lock-mass. Then, MS2 (fragmentation event) was triggered on the most dominant ion found in the MS1 scan. This MS2 was performed in the ion trap, using collision-induced dissociation (CID) and the data was stored as centroid.

Data was analysed using Qual Browser (Xcalibur 2.1) Thermo Scientific. Theoretically, m/z was calculated for both the protonated (positive ion mode) and deprotonated (negative ion mode) for each compound. Extracted ion chromatograms (EICs) for these m/z (5ppm mass tolerance) as well as the UV chromatograms were generated at 280nm, 320nm and 520nm. The retention time of the standards from the MS1 scans and the MS2 fragmentation spectra from the standards were compared to the samples (unit resolution mass tolerance).

When the retention time, parent mass and fragmentation matched the standard, a confident match was determined. In some instances, due to the nature of DDA experiments, the ion of interest was not fragmented in which case only the retention time and parent mass could be used and a less confident match was determined. In the case of phenolics, when there were no standards and hence no retention time available, the fragmentation spectra were referred solely on the match of fragmentation spectra reported in Kammerer, Claus, Carle, & Schieber, (2004).

3.2.4 Separation with Colloidal Gas Aphrons (CGA) using 10mM TWEEN20

In the previous work by our group, it was found that high recovery of polyphenols from grape ethanolic extracts could be obtained by CGA generated with the cationic

surfactant Cetyltrimethylammonium bromide (CTAB) and the non-ionic TWEEN20 (Spigno, Amendola, Dahmoune, & Jauregi, 2015). In the present work, ethanolic and hot water extracts were first obtained from grape pomace (see Figure 3.1 for full separation process). Hot water extract (HWE) was applied to the CGA for the first time. CGA generated from 10mM TWEEN20 were then applied to each extract based on the optimum conditions found in our previous work eg: the ratio of extract to the CGA was kept constant at 16:1 and the drainage time was kept at 5min. CGA separations of grape pomace extracts were carried out in a flotation glass column (i.d 5cm, height: 50cm). The CGA were pumped by a peristaltic pump (Watson Marlow) from the CGA generating container into the column which contained 60mL of ethanolic extract of grape pomace. The volume of collapsed CGA and drained liquid phase were measured. The initial extracts of EE and HWE contained 2624 mg GAE_{TPI}/L and 1562 mg GAE_{TPI}/L respectively. Both fractions were diluted at an appropriate dilution with deionized water for all the tests.

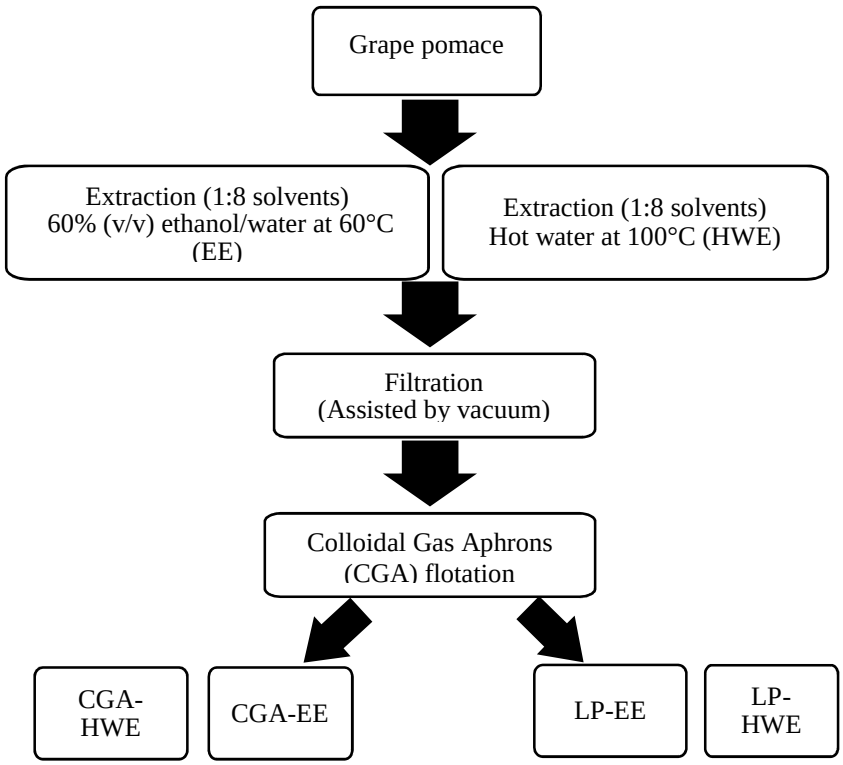

Figure 3.1 Schematic representation of the extraction of polyphenolic compounds present in grape pomace. The whole procedure was performed in triplicate (n = 3). EE, ethanol extract, HWE, hot water extract, CGA-EE; Aphron phase of EE, CGA-HWE; Aphron phase of HWE, LP-EE, liquid phase of EE and LP-HWE; liquid phase of HWE

The percentage recovery of a specific compound (*y)* in the CGA phase (R*y*) was calculated based on the differences between the total amount of added *y* in the feed ($M_{y/\text{feed}}$) and the amount of *y* measured in the separated liquid phase ($M_{y/\text{liq}}$). For some experiments, the amount of *y* in the CGA phase was also calculated and the mass balance deviation was within 10%.The separation factor (SF) was also calculated based on the concentrations of compound y in the CGA phase ($[y]_{\text{CGA}}$) and in the liquid phase ($[y]_{\text{LP}}$) as described inEq.3.1:

$$SF = \frac{[y]_{CGA}}{[y]_{LP}} \qquad \text{(Equation 3. 1)}$$

3.2.5 Collagenase and elastase inhibitory activity of crude extracts and CGA fractions

The inhibitory activity of gallic acid, grape pomace crude extracts and the CGA fractions against *C. histolyticum* collagenase (ChC) and porcine pancreatic elastase (PPE) were measured spectrophotometrically according to the method used by Wittenauer et al., (2015)by using a multi-mode Tecan GENios microplate reader equipped with analysis software Xfluor4 version 4.51 (Salzburg, Austria). Both enzymes were incubated with the extracts and their CGA fractions with relevant substrates, as described below. The inhibitory potential of the grape pomace extracts were examined in dilutions so as to establish a dose-dependent curve in order to calculate the half-maximal inhibitory concentrations (IC_{50}). Due to the high concentration of polyphenols in the grape pomace extracts, the dilutions of 1:50 to 1:200 with total polyphenolic contents ranging from 52.5 to 7.8 mg GAE/L were applied before being incubated with collagenase and elastase.

Collagenase (ChC) assay: In this assay the enzymatic reaction rate was measured based on the consumption of the substrate peptide FALGPA. Therefore, the slopes of the reaction rates decreased with the increased in extract (inhibitor) concentration. Briefly, ChC (0.16 U/mL) and FALGPA (3mM) were dissolved in 0.05M tricine buffer containing 0.4M NaCl and 0.01M $CaCl_2$; the pH was adjusted to 7.5 with 1M NaOH. The inhibitory activity of the following samples were measured:

a) Dilutions of ethanolic and hot water grape pomace extracts with water at concentration of (1:50), (1:100) and (1:200) (extract:water).
b) CGA and liquid fractions derived from CGA separations generated from TWEEN20 surfactant.
c) Aqueous solution of gallic acid (43 mg/L, 85 mg/L, 128 mg/L and 170 mg/L).

Briefly, 30 µl of the samples (a-c) were incubated with 10 µl of ChC solution and 60 µl of tricine buffer for 20 mins at 37°C, after which, 20 µl of FALGPA solution was added to initiate the reaction. The reaction rate was measured over 20mins by measuring the decreased in the absorbance of FALGPA at 340nm. Initial velocities were determined and a dose-dependent curve was established. The concentration to inhibit 50% of the enzyme activity, IC_{50} values were then determined from the curves. The inhibition activity (%) was calculated according to Eq.3.2.

$$ChC\ inhibition\ (\%) = \frac{Initial\ velocity_{\ control} - Initial\ velocity_{\ sample}}{Initial\ velocity_{\ control}} * 100$$

(Equation 3. 2)

Elastase (PPE) assay: porcine pancreatic elastase (PPE) inhibition of the individual samples (a-c) was determined spectrophotometrically by using the AAAPVN as the substrate and by monitoring the production of p-nitroaniline at 405nm to determine the reaction rate. Briefly, 10 µl was taken and loaded into wells together with 100 µl of Tris buffer and 30 µl of samples. The mixture was incubated for 20mins at 25°C. Subsequently, 40 µl of the AAAPVN (dissolved in 2mM Tris buffer at 0.25mg/mL) was added. Since the PPE was performed with AAAPVN as the substrate peptide, the enzyme activity can be calculated from the released of p-nitroaniline as a product, leading to the increased in absorption values. The absorbance was monitored for 20mins after the addition of AAAPVN and the initial velocities, the inhibitory effect and IC_{50}. The values were calculated as in Eq.3.2.

3.2.6 Statistical analysis

All the experiments were performed in triplicate. The data were subjected to the analysis of variance using IBM® SPSS® Statistics21 software programme where statistical differences were noted. Differences among different treatments were determined using Tukey test. The significance level was defined at $p<0.05$. The results were reported as means ± SD.

3.3 Results and Discussions

3.3.1 Ethanolic and hot water extraction

Table 3. 1 Chemical characterisations of grape pomace extracts and CGA fractions.

Analysis /Fraction (mg/g dry weight grape pomace)	Total Phenolic Content 280nm	Total Phenolic Content 760nm	Total Anthocyanin	Total Protein	Total Sugar
EE	21.0 ± 0.1^{b}	22.0 ± 0.2^{b}	6.6 ± 0.6^{b}	0.40 ± 0.2^{b}	75.9 ± 5.7^{a}
HWE	12.5 ± 0.1^{a}	17.0 ± 0.2^{a}	2.3 ± 0.7^{a}	0.20 ± 0.5^{a}	88.3 ± 3.5^{b}

Different letter in the same column indicates a significant difference using Tukey's test (p = 0.05). EE: ethanol extract; HWE: hot water extract.

The chemical composition of grape pomace extracts was initially determined and the results were as in Table 3.1. In general, the composition of phenolic compounds, anthocyanin and protein was higher in EE except for sugar which was higher in HWE. Total phenolic content in EE was 21.0 ± 0.1mg GAE/g of pomace. This value was almost two times higher than in HWE (12.5 ± 0.1 mg GAE/g pomace). A similar result was obtained with total phenolic contents determination by Folin-Ciolcateau method where EE had 22.0 ± 0.2mg GAE/g while HWE had 17.0 ± 0.2mg GAE/g dry weight pomace. These results closely followed the values obtained in the literature on grape pomace as

published by Amendola et al., (2010) and Tseng & Zhao, (2013) and even higher than from the Brazilian grape extract as reported by Beres et al., (2016). Total monomeric anthocyanin extracted in EE was 6.6± 0.6mg ME/g, almost three times higher than in HWE (2.3 ± 0.7mg ME/g dry weight). Low levels of protein were recovered in both extracts (0.4and 0.2mg BSA equivalent/g dry weight of grape pomace) and a slightly higher sugar was extracted in HWE than in EE.

3.3.2 Polyphenol composition of crude grape pomace extracts

The main composition of the EE and HWE analysed by HPLC is shown in Table 3.2. Qualitative analysis with LC-MS was also conducted to confirm the identification and/or identify the individual polyphenols in EE and HWE. It must be noted that minor amounts of phenolics may escape from the extraction due to the interaction with dietary fibres, proteins and other polymerised structures (Kammerer et al., 2004). In this analysis, fourteen standards of phenols and anthocyanins were analysed against both extracts as not all standards were commercially available. Retention time of standards, MS1 spectra and MS2 fragmentation spectra of the standards were compared to samples'. If the retention time, MS1 and MS2 matched, a confident assignment was given. If only the retention time and MS1 matched, a semi-confident assignment was given. The results of the mass spectrometry data in both positive-ion mode (anthocyanins) and negative-ion mode (phenolic acids, anthoxanthins, stilbenes, flavonols and flavanols) of compounds in the extracts are shown in Table 3.3.

Table 3. 2 Polyphenols contents (mg/L) of grape pomace extracts and CGA fractions.

Concentration (mg/L)						
Compound/Sample	**EE**	**CGA**	**LP**	**HWE**	**CGA**	**LP**
Phenolic acids						
Gallic acid	32.3 ± 2.8	4.1 ± 0.1	2.2 ± 0.3	74.5 ± 6.8	24.9 ± 1.4	12.2 ± 2.5
Caffeic acid	17.5 ± 1.4	ND	ND	12.4 ± 0.3	ND	ND
Syringic acid	24.5 ± 0.9	2.5 ± 0.1	2.0 ± 0.1	17.6 ± 0.4	10.2 ± 3.9	7.4 ± 1.8
Chlorogenic acid	7.1 ± 0.4	ND	ND	21.2 ± 2.3	ND	3.5 ± 2.5
4-hydroxy benzoic acid	ND	ND	ND	6.9 ± 0.1	ND	ND
Total phenol acids	81.3 ± 5.3	6.6 ± 0.1	4.2 ± 0.1	132.6 ± 9.1	35.1 ± 5.3	23.1 ± 6.8
Flavonols						
Resveratrol	36.80 ± 3.90	ND	ND	ND	ND	ND
Quercetin	108.40 ± 5.10	31.1 ± 0.5	17.4 ± 0.7	29.6 ± 0.3	ND	ND
Kaempferol	16.10 ± 1.50	6.5 ± 0.2	3.9 ± 0.7	67.5 ± 0.9	23.5 ± 0.1	24.4 ± 0.1
Total flavonols	161.30 ± 10.50	37.6 ± 0.7	21.3 ± 1.4	97.1 ± 1.1	23.5 ± 0.1	24.4 ± 0.1
		Flavanols				
Catechin	3.1 ± 0.2	ND	ND	30.9 ± 0.1	9.5 ± 0.3	ND
Epicatechin	28.7 ± 4.0	ND	ND	18.3 ± 0.6	ND	ND
Total flavanols	31.8 ± 4.2	ND	ND	49.2 ± 0.6	9.5 ± 0.3	ND
Anthocyanins						
Delphinidin 3-o-glucoside	72.2 ± 15.5	36.0 ± 6.3	17.6 ± 7.8	29.7 ± 0.2	1.9 ± 0.6	1.0 ± 0.9
Petunidin 3-o-glucoside	33.5 ± 16.3	17.4 ± 7.5	9.4 ± 0.3	11.6 ± 0.4	1.1 ± 0.4	0.7 ± 0.5
Cyanidin 3-o-glucoside	13.8 ± 2.1	8.2 ± 0.3	4.0 ± 0.2	4.9 ± 0.1	0.2 ± 0.3	0.1 ± 0.2
Malvidin 3-o-glucoside	85.0 ± 17.2	41.2 ± 0.1	23.9 ± 0.3	30.2 ± 0.1	3.7 ± 1.5	2.4 ± 1.3
Total anthocyanins	204.5 ± 51.1	102.8 ± 13.9	54.9 ± 7.9	76.5 ± 0.5	7.0 ± 2.6	4.2 ± 2.8
Total	478.9 ± 71.0	147.0 ± 14.8	80.44 ± 9.33	355.2 ± 11.4	75.0 ± 8.0	51.7 ± 9.6

ND: not detected; EE: ethanol extract; HWE: hot water extract; CGA: CGA phase; LP: liquid phase.

Values represent mean ± standard deviation (n = 3).

Qualitative analysis with LC-MS was conducted to confirm the identification and/or identify the individual polyphenols in EE and HWE. It must be noted that minor amounts of phenolics may escape from the extraction due to the interaction with dietary

fibers, proteins and other polymerised structures (Kammerer et al., 2004). In this analysis, fourteen standards of phenols and anthocyanins were analysed against both extracts as not all standards were commercially available. Retention time of standards, MS1 spectra and MS2 fragmentation spectra of the standards were compared to samples'. If the retention time, MS1 and MS2 matched, a confident assignment was given. If only the retention time and MS1 matched, a semi-confident assignment was given. The results of the mass spectrometry data in both positive-ion mode (anthocyanins) and negative-ion mode (phenolic acids, anthoxanthins, stilbenes, flavonols and flavanols) of compounds in the extracts are shown in Table 3.3.

Table 3. 3LC-MS data of phenolic compounds extracted from grape pomace

No.	*Compound*	*Retention time (min)*	*m/z*	*MS/MS fragments m/z*	*EE*	*HWE*
			Phenolic acids [M-H]-			
1	* Gallic acid	3.2	169.0142	125	√	√
2	Caftaric acid	6.5	311.0409	179/135		√
3	*p-hydroxybenzoic acid	7.3	137.0244	93	√	√
4	* Caffeic acid	10.6	179.0350	135	√	√
5	* Ferulic acid	10.8	193.0506	134	√	√
6	* Fertaric acid	10.9	325.0565	193	√	√
7	Syringic acid	11.4	197.0455	153/182	√	√
			Anthoxanthins and Stilbenes [M-H]-			
8	Procyanidin B1	7.4	577.1351	407/425	√	√
9	* Catechin	8.9	289.0718	245	√	√
10	* Procyanidin B2	10.1	577.1351	407/425	√	√
11	* Epicatechin	13.1	289.0718	245	√	√
12	* Epicatechingallate	19.6	441.0827	289		√
13	* *trans*-resveratrol	23.1	227.0714	185	√	√
14	* Quercetin	20.4	301.0354	151/179	√	√
15	Kaempferol	27.9	285.0405	257	√	√
16	Quercetin3-o-galactoside	19.8	463.0882	301	√	√
17	Quercetin 3-o-glucoside	20.4	463.0882	301	√	√
			Anthocyanins			

			[M]+			
18	*Delphinidin 3-o-glucoside	8.52	465.1028	303	√	√
19	*Cyanidin 3-o-glucoside	10.9	449.1078	287	√	√
20	*Petunidin 3-o-glucoside	12.4	479.1184	317	√	√
21	*Malvidin 3-o-glucoside	14.1	493.1341	331	√	√
22	Peonidin 3-o-glucoside	14.7	463.1235	301	√	√
23	Delphinidin 3-o-acetylglucoside	16.3	507.1133	303	√	√
24	Cyanidin 3-o-acetylglucoside	18.7	491.1184	287	√	√
25	Malvidin 3-o-acetylglucoside	21.5	535.1446	331	√	√
26	Peonidin 3-o-acetylglucoside	21.6	505.1341	301	√	√
27	Cyanidin 3-o-p-coumaroylglucoside	23.1	595.1446	287	√	√
28	Petunidin 3-o-p-coumaroylglucoside	23.9	625.1552	317	√	√
29	Peonidin 3-o-p-coumaroylglucoside	25.1	609.1603	301	√	√
30	Malvidin 3-o-p-coumaroylglucoside	25.6	639.1708	331	√	√

All compounds were confirmed with Kammerer et al., (2004).
*compounds were confirmed with pure standards.
EE: ethanol extract; HWE: hot water extract.

A total of 30 phenolic compounds were present in both extracts. Among these, 7 phenolic acids, 10anthoxanthins and stilbenes and 13 anthocyanins were detected in both extracts. All anthocyanins detected were of monoglucoside (glu), acetyl and p-coumaroyl derivatives of delphinidin (DEL), cyanidin (CYA), petunidin (PET), peonidin (PEO) and malvidin (MAL). Out of these 30 compounds, 15 were given confident assignment as theretention times, MS1 and MS2 matched with the standards. These compounds were gallic acid, p-hydroxybenzoic acid, caffeic acid, ferulic acid, fertaric acid, catechin, procyanidinB2, epicatechin, epicatechingallate, trans-resveratrol, quercetin, delphinidin 3-o-glucoside, cyanidin 3-o-glucoside, petunidin 3-o-glucoside and malvidin 3-o-

glucoside. The MS2 mode was used to provide information on the aglycone and its corresponding sugar due to the observed m/z fragmentation values (303 for DEL; 287 for CYA; 317 for PET; 301 for PEO; and 331 for MAL) which were matched to those reported by Kammerer et al., (2004). In this analysis, quercetin 3-o-glucoside and quercetin 3-o-galactoside have the same MS1 and MS2, therefore their retention times are the same; thus, differentiation of these polyphenols cannot be made. In the case of anthocyanins, all anthocyanins and derivatives were present in both EE and HWE. However, differences were noted in the composition of phenolic acids and anthoxanthins/stilbenes between both extracts where the EE was lacking the presence of caftaric acid and epicatechingallate.

In general, both extracts had the same type of compounds present but interestingly they differed in their composition. This is particularly clear when the mass percentage of groups of polyphenols (eg: phenolic acids) is calculated from data in Table 1. For example, phenolic acids were present at higher proportion in the HWE (37%) than in the EE (17%); in both extracts gallic acid was the predominant phenolic acid. Similarly flavanols where at higher proportion in HWE (14%) than in EE (7%). However the composition of flavonols was similar in both extracts, (34% and 27% in EE and HWE respectively) but quercetin was predominant in EE and kaempferol in HWE. The anthocyanins composition was higher in EE (43%), than in HWE (22%). However, with regards to the collagenase and elastase inhibitory activities and their relationship to polyphenols composition (see below) phenolic acids, flavonols and flavanols were the most relevant as anthocyanins have not been related to these activities.

3.3.3 Separation of crude grape extracts by CGA

Table 3.4 shows the recovery (%) and separation factor (SF) of the CGA separation from EE and HWE. Very similar recoveries of phenols and anthocyanins were obtained from both extracts. Generally, the recovery of compounds was higher in EE than in HWE. A separation factor higher than one indicated higher affinity of the compound for the CGA phase than the liquid phase. This was the case for all compounds in both extracts although higher SF's were obtained for EE. The selectivity of the separation in relation to both protein and sugar was low as these were also preferentially separated into the CGA phase although the SF of sugar from HWE was lower than one. The low ratio value of V_{LP}/V_{CGA} (ie: low volume of liquid drained in relation to volume of CGA) was an indication of a stable CGA which might be due to the presence of other compounds (glucose and proteins) as also found by Spigno et al., (2015) and (Dermiki, Gordon, & Jauregi, 2009) which could increase the viscosity of the liquid in the continuous phase and hence increased the stability of the CGA. It is also important to highlight that some aggregates were observed in the CGA phase which did not completely solubilise during analysis, hence this would probably lead to an underestimation of the net recovery. Overall, the recovery results were in agreement with our previous work.

Table 3. 4 Recovery efficiency (%) and separation factor (SF) by CGA separations of EE and HWE

Extract	**EE**	**HWE**
V_{CGA}/V_{feed}	16	16
$V_{LP}/V_{CGA\text{-}phase}$	0.50	0.55
Recovery (%)		
GAE_{FI}	83.45^{bc}	85.87^{c}
GAE_{TPI}	79.40^{b}	71.39^{ab}
ME	84.99^{c}	77.39^{b}
Glucose	71.74^{a}	68.91^{a}
Protein	85.86^{c}	66.45^{a}
SF		
GAE_{FI}	4.71^{c}	1.31^{b}
GAE_{TPI}	1.89^{b}	1.20^{b}
ME	1.47^{a}	1.31^{b}
Glucose	1.65^{a}	1.34^{b}
Protein	1.72^{ab}	0.87^{a}

GAE_{FI}, Gallic acid equivalent (Folin-Cioulcateau index; $GAE_{TPI,}$ Gallic acid equivalent (total phenol index); ME, Malvidin glucoside equivalent;$V_{LP}/V_{CGA\text{-}phase}$, ratio of volume of liquid phase to the volume of CGA phase. Same superscript letters in the same column (for each recovery and SF) indicates means were not statically different ($p>0.05$) according to ANOVA (n=3).

3.3.4 Collagenase and elastase inhibitory activity

The ethanolic (EE) and hot water extracts (HWE) of grape pomace were tested for their inhibitory effects against ChC and PPE. Collagen, which occupies around 70-80% of the skin weight is known to provide structural integrity (Hong et al., 2014). Due to skin ageing, collagen is rapidly degraded by the action of collagenase. As shown in Figures 3.2(A) and (B), the grape pomace extracts showed a linear inhibitory dose-dependent relationship with inhibitory activities. From these dose-dependent relationships, IC_{50} values were calculated to be 35.4mgGAE_{FI}/L (HWE), 78.8mgGAE_{FI}/L (EE) and 130mgGAE_{FI}/L(gallic acid).The maximum inhibitory activity measured for EE was 34%, therefore above this activity (up to 50%) a linear relationship with concentration was assumed in order to determine the IC_{50}.

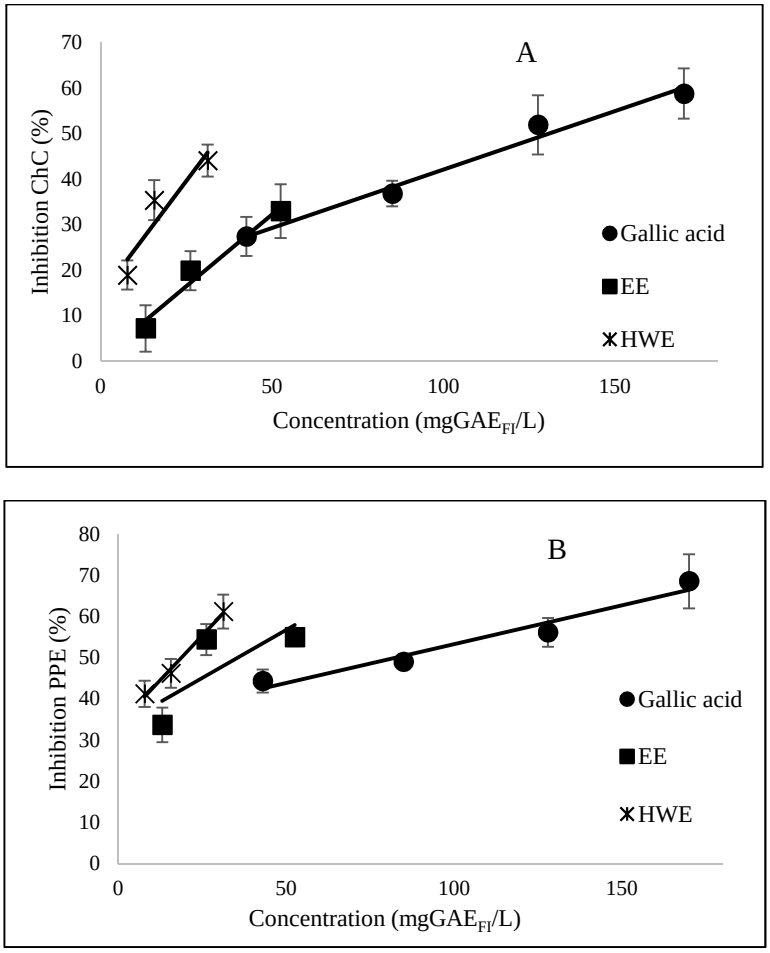

Figure 3. 2 Dose dependent inhibition of collagenase (A) and elastase (B) activity by gallic acid, ethanol extract (EE) and hot water extract (HWE) (n =3)

The same trend was observed in PPE. Elastin is an insoluble fibrous protein which occupies only 2-4% of the skin dermis weight but plays a vital role ensuring the elasticity of the skin (Hong et al., 2014). Based on the IC_{50} results, HWE had the highest potency as compared to EE and galic acid. The potency of HWE was almost similar to that obtained by Wittenauer et al., (2015)for a methanolic extract of grape pomace (14.7mg/L) which may suggest comparable polyphenol compositions.

The higher inhibitory activity of HWE than EE against collagenase and elastase can be explained by the differences in polyphenols composition. The phenolic acids such as gallic acid and chlorogenic acid in HWE which account for 37% of total polyphenols

could have a pronounced effect on the inhibitory activities. Gallic acid, a low molecular weight hydrophilic compound could play an important part in the observed activity by accessing the active centre site of the elastase and blocking the binding of substrates to this site (Wittenauer et al., 2015). However, given that the potency of the extract was superior to that of the gallic acid alone, it is clear that other components also may contribute to the activity, perhaps in a synergistic manner. Chlorogenic acid, for example, which is a derivative of cinnamic acid, could also contribute as it is well known for its potent antioxidant and anti-inflammatory activities (Farah, Monteiro, Donangelo, & Lafay, 2008). Moreover, the catechin and epicatechin which were present at high proportion in HWE (14%) could interact with the elastase by hydrophobic interactions, causing conformational changes of elastase and thus increasing the inhibitory activity (Wahab et al., 2014). On the other hand, EE had high composition of flavonols, particularly quercetin and resveratrol but they are larger molecules with lower solubility in water than the phenolic acids which could possibly limit their activity.

3.3.5 Collagenase and elastase inhibitory activity of CGA fractions in relation to polyphenolic profile

In order to determine the most active fractions after separation by CGA, CGA and liquid phases from both EE and HWE were tested for ChC and PPE. The inhibitory activities against ChC ad PPE are shown in Figures 3.3(A) and (B), respectively. Contrary to the crude extracts, EE fractions demonstrated higher activity than HWE fractions, CGA-EE had 67% collagenase inhibitory activity and CGA-HWE 55%; the liquid phases had 60% and 46% activity, respectively. This small difference in activity between the

liquid and CGA phases can be explained based on their polyphenol composition (Table 3.2).

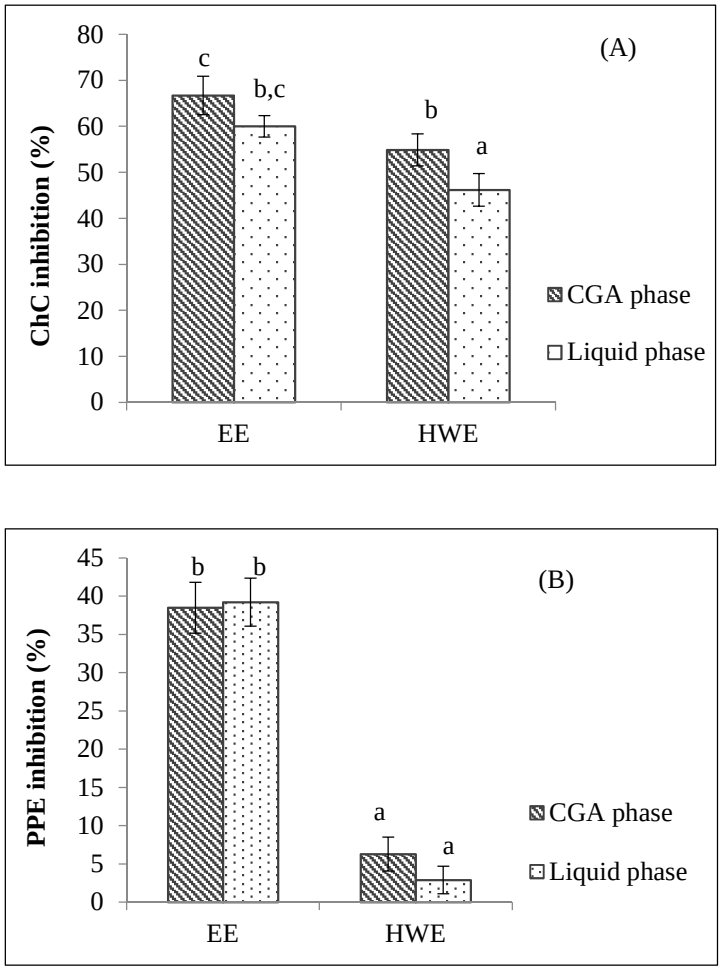

Figure 3.3 Anti-collagenase (A) and anti-elastase (B) activity of CGA fractions from EE and HWE. Bars are means ± standard deviation of three determinations (n = 3). Same superscript letters indicates means with no significant difference ($p>0.05$) according to ANOVA (n=3).

For example, the composition of phenolic acids in CGA-HWE and LP-HWE were almost the same (mass percentages of phenolic acids over total phenols were 47% and 45% respectively) and for flavonols composition was higher in the liquid phase (31% in CGA and 47% in liquid phase). The same trend was noted in CGA-EE and LP-EE where phenolic acids and flavonols composition was very similar in both fractions (4.5 and 5.2% phenolic acids in CGA and LP respectively and 26% flavonols in both fractions). This

similarity in composition supports the insignificant differences in inhibitory activities of these fractions against both enzymes. Kaempferol was found at high concentration in both CGA-HWE and LP-HWE (23.5 and 24.4 mg/L respectively) but in the case of CGA-EE, the most predominant flavonol was quercetin (31.1mg/L). These compounds could possibly be the main contributors to the inhibitory activities observed whereby the hydroxyl group in C-3 might played a role in conferring the inhibitory activity (Sin & Kim, 2005). Moreover, the high content of gallic acid in CGA-HWE (24.9mg/L) and in LP-HWE (12.2mg/L) could also be important for the ChC inhibitory activity. The hydroxyl group from gallic acid could act as a hydrogen bond acceptor/donors with the hydroxyl, amino or carboxyl groups of the collagenase's side chain functional groups which can alter its structure, while the benzene rings of the polyphenols can form hydrophobic interactions with collagenase (Madhan et al., 2007; Wittenauer et al., 2015).

The differences in inhibitory activity against PPE between the LP and CGA fractions (Fig 3.3B) could be explained based on the differences in composition (see above). On the other hand the much higher activity in the EE fractions than in the HWE fractions could not be clearly explained in terms of differences in composition of groups of polyphenols but individual polyphenols. For example, quercetin was predominantly present in the CGA-EE whilst none was detected in the CGA-HWE. This suggests that quercetin is a key compound responsible for PPE inhibition. Quercetin could possibly alter the specificity of the elastase substrate by interacting with subsite of MMP-9 active site (Saragusti et al., 2010).

Table 3. 5 Inhibitory efficiency (%/mg $GAE_{FI}L^{-1}$) of crude extracts and CGA fractions

Extract/Activity	EE	CGA-EE	LP-EE	HWE	CGA-HWE	LP-HWE	Gallic acid
ChC	0.63	2.43	2.18	1.41	2.45	0.37	0.38
PPE	1.41	1.40	1.43	2.67	0.28	0.02	0.61

In order to assess if any of the fractions had been preferentially enriched with the most active polyphenols the activity potency had to be determined. However, these fractions showed poor dose-dependency relationship (data not shown) and the IC_{50} could not be determined. Therefore, the inhibitory potency of CGA fractions was expressed as inhibitory efficiency which is the activity in relation to the total phenols content (% / mg $GAE_{FI}L^{-1}$) (Table 3.5). The CGA-EE fraction was found to be about four times more efficient than its crude extract and over six times more efficient than gallic acid in relation to ChC inhibitory activity. However the efficiency of both CGA and LP fractions was almost the same which is in agreement with results in Fig 3. Interestingly the efficiency in CGA-HWE was seven times higher than in LP and almost double that in the raw extract (HWE). Moreover the efficiency of the CGA fractions of both raw extracts was six times higher than gallic acid's which suggests that the inhibition of these enzymes could be the result of synergistic activity of different polyphenols. This has been observed in a formulation of four combined super fruits extract (*Ginkgo biloba*, *Punica granatum*, *Ficuscarica*, and *Morus alba*) against collagenase (Ghimeray et al., 2015).

In the case of PPE inhibitory activity, no increase in efficiency was noted for the CGA/LP fractions of EE and the efficiency of the HWE decreased after CGA separation. The inhibitory efficiencies of the raw extracts were superior to that of pure gallic acid.

From results above it could be hypothesised that TWEEN20 might play a role in facilitating the delivery of the polyphenols to the target site of the collagenase. This explained why the efficiency of the CGA-EE increased substantially as compared to the crude extract's and it was comparable to that of CGA-HWE. It is also worth mentioning that the surfactant did not inhibit or activate both ChC and PPE (data not shown) hence, the inhibitory activities were solely due to the action of polyphenols in the fractions. Non-ionic surfactants were known to cause the least irritating effect to skin compared to anionic surfactants hence they were preferred for inclusion in many skin care products (Zatz & Lee, 1997). Moreover, surfactants in general are known to alter the skin permeation by forming non-specific hydrophobic interactions involving the alkyl chains of the surfactant and the hydrophobic regions of the keratin in stratum corneum (Abraham, 1997). Most studies about non-ionic surfactants and biological activities revealed that the C12 alkyl chain was the most important character in terms of perturbation of the membrane which explained the surfactant solubility and partitioning (French, Pouton, & Steele, 1993). Although most studies revealed that their interactions with non-ionic surfactants did not alter skin permeation to a significant level, enhancement has been noted in some studies whereby penetration of lidocaine (a type of drug)significantly increased through hairless mouse skin with TWEEN20 and TWEEN60 (Zatz & Lee, 1997).

3.4 Conclusions

The extraction of polyphenols from grape pomace by ethanol and hot water led to crude extracts with different polyphenol compositions and this also resulted in differences in collagenase and elastase inhibitory activity. Phenolic acids were present at higher proportion in the HWE (37%) than in the EE (17%) which suggested their important role

in the inhibition. HWE was the most efficient at inhibiting both collagenase and elastase and both EE and HWE were superior to gallic acid. Further extraction by CGA led to higher inhibitory efficiency against collagenase although there was no difference in efficiency between the separated phases for EE but there was for HWE. Gallic acid was the main component of the CGA-HWE but other polyphenols (eg: kaempferol) must have contributed also to its potency as this fraction inhibited collagenase over six times more efficiently than gallic acid. The CGA-EE fraction was found to be about four times more efficient than its crude extract and over six times more efficient than gallic acid in collagenase's inhibition; quercetin was found to be the major polyphenol in this fraction. These results suggested that although quercetin was highly insoluble in water and had high molecular weight, TWEEN20 helped to improve its solubility and therefore facilitated its delivery to the enzyme. Therefore, CGA separation led to fractions enriched in active polyphenols with enhanced collagenase inhibitory activity in both CGA and liquid phases. Although the polyphenols composition in CGA and liquid phases in both extracts were very similar, and hence their inhibitory activities, it must be stressed that further separation with CGA led to CGA fractions with less sugar and protein (and ethanol when applied to the ethanolic extract) which can be an advantage in terms of formulation. It should be noted that the concentration of these polyphenols in the CGA fractions were topically relevant (generally between 25-100µM). Moreover the surfactant in these fractions could act as a carrier and solubilising agent to enhance the permeation of polyphenols across the skin. Therefore, the surfactant rich solution may provide an optimum media that could facilitate the permeation of the polyphenols through the skin. This research shows the potential of CGA to revalorise the grape marc and to obtain an extract with potential in cosmetics applications.

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CHAPTER 4: In vitro skin permeation of polyphenols extracted from grape pomace by colloidal gas aphrons (CGA)

Abstract

Model polyphenol solutions consisting of either gallic acid or resveratrol solubilised in water and 10mM TWEEN20 were tested for their permeability through artificial membranes and porcine skins. Results showed higher diffusion of both polyphenols solubilised in TWEEN20 across the artificial membrane and skins as compared to those solubilised in water; suggesting a penetration enhancement by the surfactant. The ethanoliccrude extract from the grape pomace and its colloidal gas aphrons (CGA) fraction were tested for permeability through the artificial membrane and limited diffusion was observed. This could be due to the complexity of the extract and CGA fraction that hindered the diffusion of polyphenols across the membrane.

Keywords: Skin permeability, gallic acid, resveratrol, grape pomace, colloidal gas aphrons

4.1 Introduction

Ageing is an unavoidable process which is primarily visible in the skin. Genetics, environmental exposure, metabolic process and hormonal changes influence the skin aging process. As described in Chapter 3, the primary ageing accelerator is the oxidative stress; cell damages resulting from an excess production of reactive oxygen species (ROS) in the tissue. Polyphenols are widely distributed in plants and they comprise of a large range of bioactive properties such as free-radical scavenging, antimicrobial, wound healing and chemopreventive activities (Fontana, Antoniolli, & Bottini, 2013). The delivery of polyphenols via the skin to protect the skin from oxidative stress, premature

ageing or skin disease has become very attractive recently. Polyphenols extracts are reported to possess anti-collagenase, anti-elastase and anti-hyaluronidase activities (Azmi, Hashim, Hashim, Halimoon, & Majid, 2014; Makimura et al., 1993; Wittenauer, Mäckle, Sußmann, Schweiggert-weisz, & Carle, 2015).

Drug permeation across the skin is a complex process. The main phases involved are the diffusion of the polyphenols within a topical formulation, the release from it, the penetration into the skin and the diffusion through it (Zillich, Schweiggert-Weisz, Hasenkopf, Eisner, & Kerscher, 2013). The skin is built by three main layers; the superficial skin layer stratum corneum (10-20µm thick) predominantly responsible for the barrier properties of human skin and limits delivery across the skin; the viable epidermis (50-100µm thick)containing various cell types responsible for skins generation; and the dermis (3-5mm thick) composed of a network of collagen and elastin in an environment similar to a hydrogel, embedded with appendages comprised of nerve endings, sweat glands and hair follicles (Williams, 2013).

For in vitro testing of drug permeation through skin, Franz type vertical diffusion cells are commonly used(Williams, 2013). The excised human or animal skin or artificial membrane is placed between the donor and receptor chambers; then a drug containing solution is applied on the top and the drug permeated through the membrane or skin into the receptor solution is determined over time. In most studies, pig skin is often used for the in vitro testing because of its similarity to human skins' structure and its availability (Casagrande et al., 2007).Due to the complexity of the skin structure, individual polyphenols are used to study the permeation instead of crude extracts.

As briefly described above, the stratum corneum acts as the main barrier for topical application. Besides sufficient stability of active polyphenols, the permeation of potent

polyphenols through the epidermis into the dermis is a prerequisite for an effective anti-ageing activity. Thus, having the polyphenols in a surfactant rich solution could potentially increase their permeability. Moreover, TWEEN20, a non-ionic surfactant has the least irritant effect to skins than the ionic surfactants, therefore are typically used in cosmetic formulations for its ability to solubilise lipophilic active ingredients and also lipids within the stratum corneum. As a continuation to the study described in Chapter 3 where it was found that further separation of polyphenols from grape pomace extract with CGA led to high inhibitory potency against skin relevant enzymes, here the permeation of these polyphenols through the skin is investigated. Therefore, this experiment aimed at investigating the in vitro skin permeation behaviour of individual polyphenols, ethanolic extract of grape pomace and also its CGA fraction, using a dialysis membrane and pig skin in Franz-type diffusion cells.

4.2 Material and Methods

Gallic acid (>98%), resveratrol (>99%), TWEEN20 (PhEur, JPE, NF)and phosphate buffered saline (PBS) were purchased from Sigma (USA).

4.2.1 Preparation of individual polyphenols and ethanolic extract of grape pomace

500mg/L gallic acid and 200mg/L resveratrol dissolved in deionised water and 10mM TWEEN20 respectively were prepared using magnetic stirrer and stirred for 30mins at room temperature to ensure complete solubilisation. The preparation of crude ethanolic extract was as described in Chapter 2, section 2.2.1.1. This extract contained 60% of ethanol with 2080mg GAE/L of the total phenolic content.

4.2.2 CGA separation using 10mM TWEEN20

The separation of CGA fraction was as described in Chapter 2, section 2.2.5. Based on the study on the inhibitory activity against skin relevant enzymes in Chapter 3, the same CGA fraction (V_{CGA}/V_{feed} = 16, drainage time = 5min) was selected for this experiment. The estimated concentration of TWEEN20 in this fraction was 8.58mM which was above the CMC of TWEEN20 (0.06mM). The total phenolic content of this fraction was 292mg GAE/L, with ethanol content of 8.3%.

4.2.3 Membrane and skin preparations

In this experiment, a dialysis membrane with pore size of 12-14000 Daltons was used in the permeability study of individual polyphenols, ethanolic extract and CGA fraction. Prior to the experiment, the dialysis membrane with exposed surface of 4cm^2 was soaked in PBS buffer.

Studies have reported that porcine skin has similar permeation characteristics to human skin; thus porcine ear skin was used for this study. Fresh porcine ears were obtained from a local slaughter house (P.C Turner, Farnborough, Hampshire).The outer region of the skin was separated from the cartilage using a scalpel. The subcutaneous fat layer was also removed and the skin was stored in -20°C until further use.

4.2.4 In vitro cells diffusion

This experiment involved employing a two-chamber diffusion cell as shown in Figure 4.1 with the chambers being separated by a membrane/skin. The skin permeation of polyphenols in the ethanolic grape pomace extract and its CGA fractions was determined using static, vertical glass Franz diffusion cells (Logan Instruments Corp.,

NJ, USA). The exposed membrane surface was 4.0cm^2 and the receptor volume was 22mL. The receptor solution was PBS buffer, at pH 7.4 and was continuously stirred with the help of magnetic stirrer. Frozen ear pig skin was thawed before use and examined visually for punctures or defects before being mounted into the Franz cell. Porcine skin was clamped between the donor and the receptor compartment with stratum corneum facing the donor compartment. The skin was then equilibrated for 10min at 35°C. In the donor compartment, 21mL of formulations were applied. Baseline samples (2 mL) were taken from the receptor chamber before the start of the experiment as controls. PBS (21 mL) was removed from the donor chamber and replaced with (21 mL) of the formulation to be studied (pre-equilibrated to 35°C). Magnetic stirrer bars were used in each receptor chamber for continuous mixing of the receptor fluid. The cell was occluded with Parafilm^{TM}. Samples were withdrawn at 0, 10, 30mins, 1,2, 3, 4, 5, 6, 23 and 24 hour. At each sampling time, 2.0mL of the receptor solution was removed and replaced by the same amount of fresh buffer to avoid diffusing effect. The samples were filtered using0.45µm microfilter and were stored at 4°C before being analysed by HPLC.

In the present study, gallic acid and resveratrol in water and in 10mM TWEEN20 were used as model polyphenols to study the permeability behaviour of polyphenols. Gallic acid was chosen for its high solubility in water and low molecular weight. Resveratrol was studied for its low solubility in water. Furthermore, gallic acid and resveratrol were two of the major polyphenols present in the ethanolic grape pomace extract, other than quercetin as identified in Chapter 3, Table 3.2.

4.2.5 HPLC analysis

Each sample with different time points was analysed using HPLC. The method of analysis was described earlier in Chapter 3, section 3.2.4.

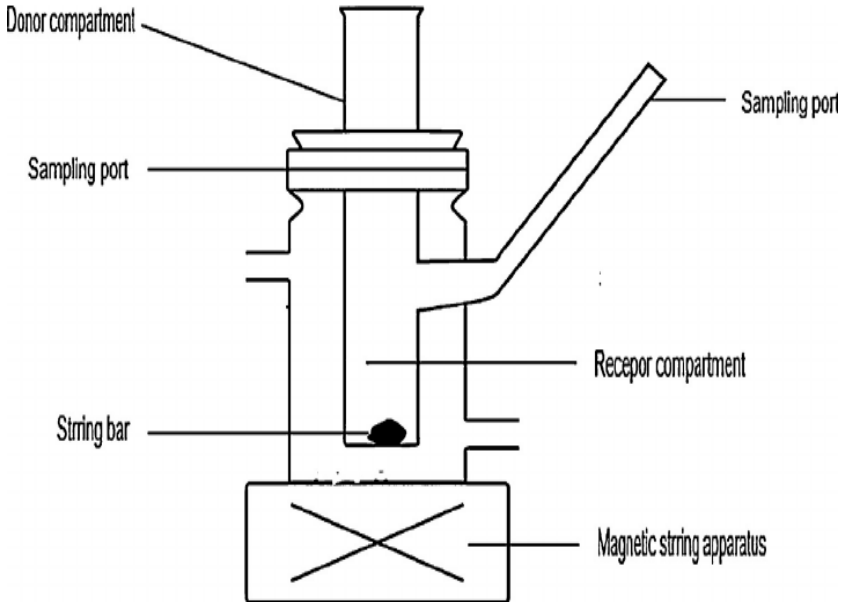

Figure 4. 1In vitro cells set up using Franz cell(Wang, Wang, & Liu, 2015).

The accumulative polyphenols concentration (mg/L) was calculated taking into account the dilution factor, the volume of the receptor and the concentration of the polyphenols in the receptor and donor solutions, before diffusion rate of polyphenols can be calculated. The diffusion percentage (%) equation was defined as the concentration of *y* in the receptor solution after 24hours over the concentration of *y* in the donor solution after 24hours which can be calculated using the equation below:

$$Diffusion\ (\%) = \left(\frac{[y]receptor}{[y]donor}\right) * 100 \qquad \text{(Equation 4. 1)}$$

4.3 Results and Discussion

4.3.1 In vitro permeability of "model polyphenols", ethanolic extract and CGA processed fraction

The gallic acid and resveratrol were studied as model polyphenols to determine their permeability through membranes. In theory, the dialysis membrane should allow the diffusion of both polyphenols across the membrane to the receptor chamber. Unlike skins, membranes do not provide such a barrier that will hinder diffusion of polyphenols. In this particular experiment, the gallic acid and resveratrol solutions were expected to diffuse across the membrane into the receptor solution, as explained by Fick's Law. Fick's law described that the molar flux due to diffusion was proportional to the concentration gradient. Therefore, the diffusion of polyphenols across the membrane was expected due to the difference in concentration of polyphenols between the donor and receptor chambers.

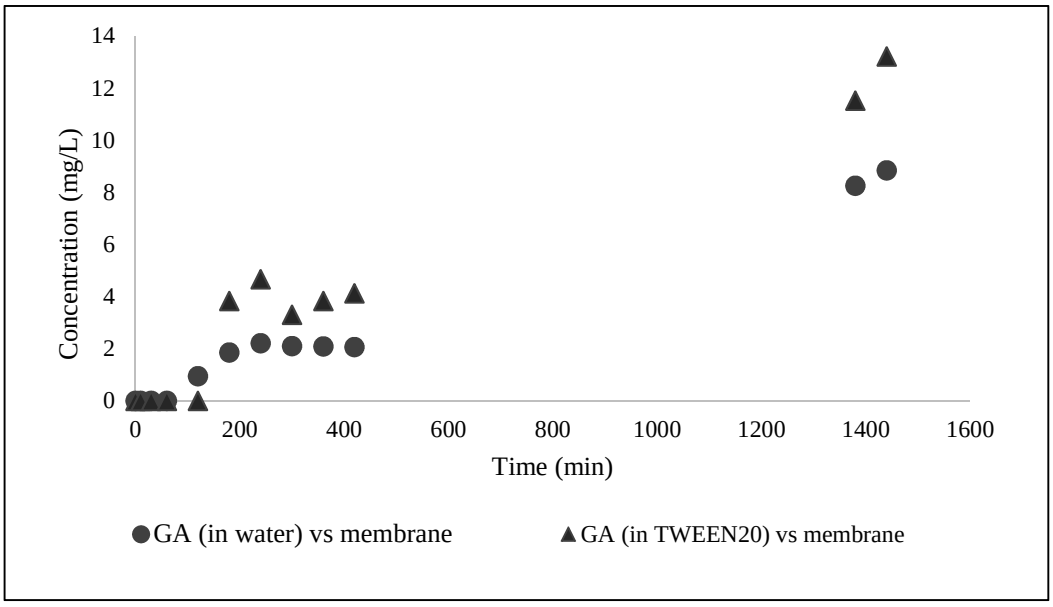

Figure 4. 2 Gallic acid in water and TWEEN20 against dialysis membrane and skin

The profile of gallic acid solubilised in water and TWEEN20 through the dialysis membrane is shown in Figure 4.2. From the results obtained, it was clear that there was diffusion of gallic acid across the membrane. However, the actual concentration of gallic acid solubilised in TWEEN20 was higher (13.2mg/L) than those solubilised in water (8.8mg/L) over 24 hours observation. On the other hand, the lag phase of gallic acid solubilised in water was shorter (120min) as compared to gallic acid solubilised in TWEEN20 (180min). This could be attributed to the different composition of water/TWEEN20 used in the gallic acid formulations. The same observation was noted by Zillich et al., (2013) where high water content in a formulation leads to shorter time of lag phase. Moreover, gallic acid solubilised in water could possibly degrade by the end of the experiment at a temperature of 35°C; thus lowering the concentration gradient in the donor chamber and leading to slower diffusion. Meanwhile, the oxidation of gallic acid solubilised in the TWEEN20 formulation was perhaps minimal. This could be due to the ability of micelles to protect the polyphenols from oxidation(Lin, Wang, Qin, & Bergenståhl, 2007).Thus, from these observations, it can be concluded that gallic acid was able to diffuse across the membrane, as anticipated and described by Fick’s law.

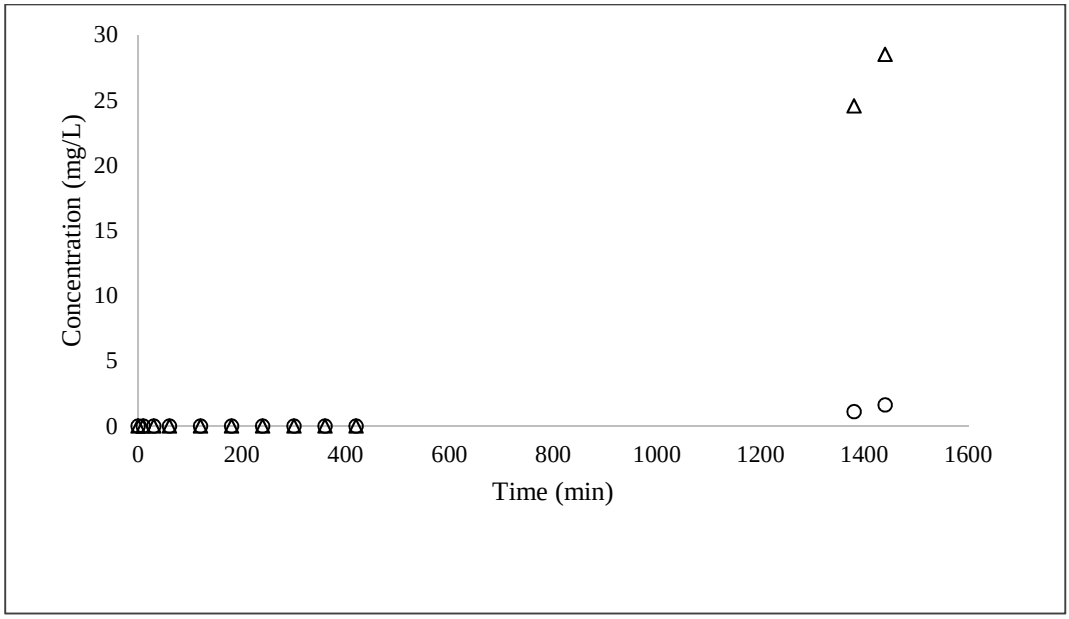

Figure 4. 3 Gallic acid in water and TWEEN20 against pig skins

The same formulations that contained gallic acid dissolved in water and TWEEN20, respectively, were applied to pig skin (Figure 4.3). In this experiment, the diffusion of polyphenols was expected to be greater in the TWEEN20 than in water formulation. From the results obtained, we could see clearly that gallic acid in both formulations diffused across the skin. The accumulative gallic acid concentration solubilised in water was 2.6mg/L while those solubilised in TWEEN20 had 28.4mg/L, more than 10 times higher than those solubilised in water after 24 hours exposure. The lag phase in both formulations was the same (approximately 420min). In this case, the exact point of diffusion could not be determined accurately as experiments were running over night. Interestingly, the accumulative concentration of gallic acid solubilised in TWEEN20 was distinctively higher than those solubilised in water. Despite the stratum corneum barrier of the skin, the formulation of gallic acid in TWEEN20 was able to show clear penetration and diffusion pattern over 24 hours observation. This suggests the ability of TWEEN20 to alter the permeability of the skin itself. Moreover, gallic acid was

unlikely to be solubilised in the micelles due to the hydrophilic nature of the compound; hence they were more likely to solubilise in the TWEEN20 solution. This again suggests that the permeability of gallic acid was due to the ability of TWEEN20 to solubilise lipids on the stratum corneum.

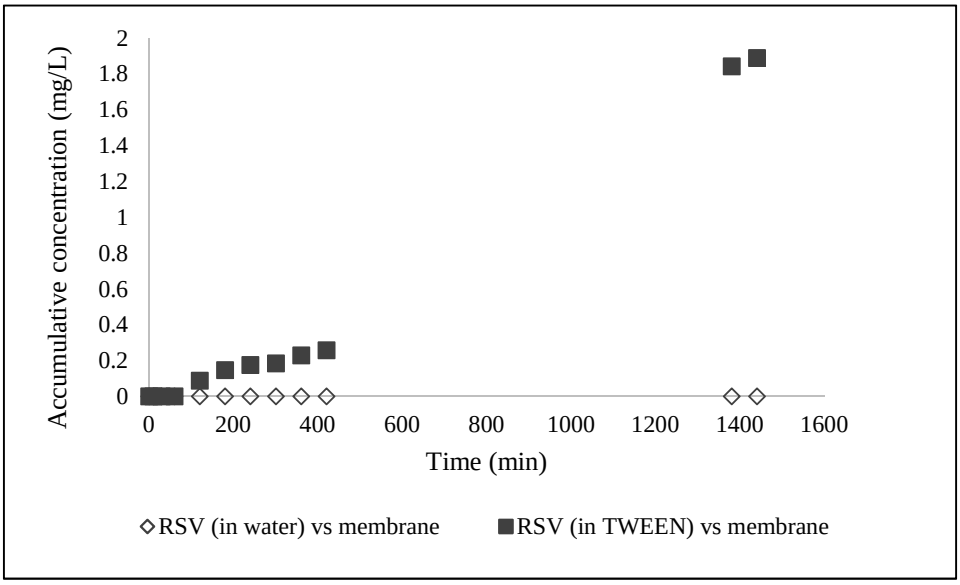

Figure 4. 4 Resveratrol in water and TWEEN20 against dialysis membrane

The same experiment was repeated with 200mg/L resveratrolthough a dialysis membrane (Figure 4.4). Resveratrol has low solubility in water (30mg/L). The results obtained showed that resveratrol solubilised in water did not diffuse through the membrane after 24 hours exposure. This could possibly be due to the high concentration of resveratrol used in this experiment (200mg/L) which was above the solubility concentration in water. However, resveratrol solubilised in TWEEN20 showed diffusion across the membrane at 120min after exposure. The accumulative concentration increased and reached 1.8mg/L by the end of 24 hours. Therefore, these results suggest

that the TWEEN20 enhanced the solubilisation of resveratrol, thus allowingthe molecules to pass through the membrane.

Table 4. 1 The physicochemical properties of polyphenols tested *

	Log P	**Molecular weight (g/mol)**	**Solubility in water**
Gallic acid	0.7	170.1	1.5g/100mL
Resveratrol	3.0	228.2	3mg/100mL

*National Center for Biotechnology Information (2017)

From these observations, it can be said that diffusion of individual polyphenols differed considerably. Two major factors have been described as the most important ones affecting this: molecular weight and log $P_{octanol\text{-}water}$ which is a measure of hydrophobicity (if log P >1then the compound is hydrophobic) (Table 4.1).Compounds with small molecular weight will generally have high diffusion coefficient, thus increasing the chemical potential gradient (Williams, 2013; Zillich et al., 2013). Gallic acid is a low molecular weight hydrophilic compound (with log P < 1), therefore its high penetration into the skin in this study could be due to its high hydrophilicity. Meanwhile, resveratrol which is a hydrophobic molecule with log P > 1showed low penetration to the membrane.

Table 4.2 Diffusion % of individual polyphenols, ethanol extract and CGA fraction of grape pomace across skins/membrane

Compound	*Solubility*	*Skin/Membrane*	*% diffusion **
Gallic acid	Water	Skin	1.6
Gallic acid	Tween20	Skin	16.6
Gallic acid	Water	Membrane	5.6
Gallic acid	Tween20	Membrane	8.3
Resveratrol	Water	Membrane	-
Resveratrol	Tween20	Membrane	0.6
Resveratrol	Water	Skin	-
Resveratrol	Tween20	Skin	-
Ethanol extract	-	Membrane	3.7**
V16 CGA fraction	-	Membrane	0.9**

* calculated based on the concentration of compound after 24 hours exposure
** calculated based on the total peak area (analysed with HPLC) after 24 hours exposure

The diffusion percentage of each formulation across the membrane/skin was calculated and results were tabulated in Table 4.2. The diffusion (%) was calculated according to Equation 4.1.Resveratrol solubilised in TWEEN20 diffused across the membrane after 24 hours of exposure by 0.6%, but resveratrol solubilised in water did not diffuse at all after 24 hours exposure. In addition, when the resveratrol solubilised in water and TWEEN20 was tested against skin, no diffusion was noted after 24 hours. In the case of gallic acid, 1.6% gallic acid solubilised in water diffused across the skin while 16.6% of gallic acid solubilised in TWEEN20 diffused across the skin. In the case of membrane, gallic acid formulated with TWEEN20 had a diffusion of 8.3% whilst formulation in water noted a diffusion of 5.6%. From these results, it can be concluded that the permeability of polyphenols increased with the presence of TWEEN20 in the formulation.

When the crude ethanol extract of grape pomace was tested for its permeability against the dialysis membrane, 3.7% of total polyphenols diffused through the membrane (Table 4.2). After CGA separation, the diffusion of total polyphenols reduced to 0.9%.

This possibly suggests the retaining of polyphenols in micelles. Since the micelles size is 10nm (Jauregi, Coutte, Catiau, Lecouturier, & Jacques, 2013), this hindered the diffusion of the majority of polyphenols across the membrane, resulting in lower diffusion rate. Moreover, the small percentage of diffusion observed could be due to the diffusion of polyphenols by the TWEEN20 that did not form micelles.

Furthermore, the concentration of polyphenols in the crude extract and the CGA fraction is also important in order for the polyphenols to diffuse across the membrane/skin. Maximum thermodynamic activity and thus the greatest flux is achieved when the polyphenol is concentrated or saturated (Williams, 2013). In the present study, the permeability of crude ethanol extract and its CGA fraction was expected to be lower than the permeability of individual polyphenols mainly because of the complexity of the crude extract composition which included other larger compounds such as sugar. Therefore, this could explain the lower penetration and diffusion of the extract and CGA fraction as compared to the pure polyphenols.

Suitable drugs/polyphenols molecules are also important in topical delivery. In theory, large hydrophilic molecules are poor candidates to deliver across the intact skin. Ideally, a drug should be moderately lipophilic (log P = 1-4), relatively low molecular weight (<500 Da) and effective in low doses (10mg/day for transdermal delivery). Due to the complexity of the crude grape extract, this could be limited without semi-purification/purification steps. Semi-purification steps could involve the use of solid phase extraction (SPE), fractionating the polyphenols into several fractions, depending on the type of cartridge and also eluents(Barbosa-Pereira, Pocheville, Angulo, Paseiro-Losada, & Cruz, 2013). Also, it must be pointed out that these polyphenols were quantified only in the receptor fluid. The skin surface, stratum corneum, and

epidermis/dermis could also be analysed in order to have a better understanding of the penetration and diffusion of polyphenols across the skin.

4.4 Conclusion

In this study, the skin permeation of “model polyphenols” such as, gallic acid and resveratrol dissolved in water and TWEEN20 were investigated. Overall, TWEEN20 increased the permeability of these model polyphenols across the membrane and skin.Good correlation was found between molecular weight and hydrophilicity of each compound and its permeability. Hydrophilic compounds with low molecular weight (gallic acid) diffused faster than hydrophobic ones (resveratrol). The enhanced diffusion of gallic acid in TWEEN20 across the skin demonstrated the effect of surfactants as a penetration enhancer. Resveratrol which is highly insoluble in water showed improvement in penetration and diffusion across the membrane when solubilised in TWEEN20, although none was noted when tested against skin. The diffusion of a complex polyphenol mixture such as the crude ethanolic and its CGA fraction was very limited, suggesting complexing of the active polyphenols with other large molecules. In future, the formulations could focus on increasing the chemical potential gradient by concentrating the polyphenols in the extracts; extracts could be semi-purified in order to increase the permeability of the polyphenols across the membrane/skins. In addition, the skins could be analysed in different parts including the stratum corneum and epidermis/dermis so that a better insight on the polyphenols’ behaviour could be obtained. Finally, this study showed that the presence of surfactant could enhance the solubility of polyphenols and diffusion across skin and/or penetration.

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CHAPTER 5: Stability of polyphenols extracted from grape pomace by colloidal gas aphrons (CGA) during storage*

Abstract

Grape pomace, a by-product from wine-making industry rich in polyphenols can be further used in food applications. However, polyphenols particularly anthocyanins are unstable during processing and storage. This is the first study showing the use of colloidal gas aphrons (CGA) as a separation and formulation technique in grape pomace extract during storage. The results show that the anthocyanins in CGA sample showed higher stability in half-life ($t_{1/2}$ 55 d) than the crude grape extract ($t_{1/2}$ 43 d). The stability of anthocyanins in CGA fractions is proportional to the concentration of TWEEN20 in the CGA fraction (6.07-8.58mM). The overall anthocyanins loss (by degradation) in the CGA sample was 34.90%, comparable to the crude ethanolic extract with sorbic acid (EE-SA) (31.53%) and significantly less than in the crude extract (44%). The colour changes in the CGA fractions, as well as the crude ethanolic extract (EE) and the EE-SA showed relation to the stability of the individual anthocyanins.

Industrial Relevance: An innovative, integrated and environmentally friendly separation method is important in the conversion of this by-product into value-added products to overcome the cost of waste disposal and decontamination. The CGA separation method offers the advantage of the integration of the separation and formulation steps targeted specifically to the anthocyanins in the grape pomace, which are often unstable after extraction. CGA processed samples represent tailored-made samples rich in anthocyanins and surfactant which can find applications for example, as food colourants. Results obtained here showed the stabilisation effect of that the surfactant in the CGA on the anthocyanins during storage as shown by slower degradation kinetics of anthocyanins

and less colour degradation, as compared to raw extracts of the pomace and raw extract with added sorbic acid (a typical food additive used in food industry). Overall, this study demonstrated that CGA is a promising separation technique that can also facilitates the formulation of the anthocyanins, protecting them from oxidation during storage.

Keywords: Grape pomace, polyphenols, anthocyanins stability, colloidal gas aphrons, storage

*submitted to Innovative Food Science and Emerging Technologies

5.1 Introduction

Grapes are one of the most important fruit crop cultivated across the world, whereby80% of the grape productions are used in wine-making industry(Fontana, Antoniolli, & Bottini, 2013). Wine production is considered as one of the most important agricultural activities, generating a huge amount of by-products including grape skins, stems and seeds(Yu & Ahmedna, 2013).At the end of the fermentation, these residues are left with high amount of polyphenols. Anthocyanins, catechins, flavonol glycosides, phenolic acids and stilbenes are among the major constituents of this by-product (Kammerer, Kammerer, Valet, & Carle, 2014). This isseen by the management authorities as a serious environmental threat because they are low in pH and high in organic matter that may cause phytotoxic effects if applied to crops or wetlands (D. Kammerer, Claus, Carle, & Schieber, 2004; Lavelli, Harsha, Laureati, & Pagliarini, 2017). Therefore, converting and utilising this by-product to another useful product is required. For instance, the anthocyanins from this pomace can be used as natural food colourants(Thakur & Arya, 1989). Anthocyanins are sensitive to thermal degradation making the processing steps rather difficult and complex, but they are in demand due to their wide applications in foodas well as in pharmaceuticals and cosmetics. Thus, various extraction techniques have been studied and used, including acidified alcohol, sub- and

supercritical fluid and high pressure processing and the yield varies from 787-936mg/L malvidin-3-o-glucoside equivalent(Barba, Zhu, Koubaa, Sant'Ana, & Orlien, 2016; Drosou, Kyriakopoulou, Bimpilas, Tsimogiannis, & Krokida, 2015; Lozovskaya, Brenner Weiss, Franzreb, & Nusser, 2012).

Extraction is the first step in the commercial isolation of anthocyanins. The extraction process of anthocyanins can be very delicate due to their instability and the necessity to use water as an extracting solvent. Food processing generally involves thermal processing prior to consumption and this process has a great influence on the anthocyanins content in the final product. Thermal processing involves high temperatures ranging from 50°C to 150°C, depending on the pH and the desired shelf life of the product. Anthocyanins are expected to degrade over time. However, the storage temperature has been found to be an important factor that is affecting anthocyanins' shelf life. Degradation of anthocyanins, that is, their half-life ($t_{1/2}$) are greatly affected by the type of anthocyanin, the origin of the samples and the storage temperature (Hellström, Mattila, & Karjalainen, 2013). The thermal degradation of anthocyanins in extracts and model systems are reported to follow the first-order reaction kinetics (Presilski, Presilska, & Tomovska, 2016).

The stability of anthocyanins can be improved by increasing anthocyanins concentration by self-association, removal of oxygen and inactivation of enzymes (Hellström et al., 2013). In food industry, the sensitivity of bioactive compounds is addressed by the incorporation of edible coatings as a structural matrix. It has been used widely to create a barrier from oxygen, moisture and solute movement (Falguera, Quintero, Jiménez, Muñoz, & Ibarz, 2011). Encapsulating is also considered as edible coatings by using different types of encapsulating systems such as spray drying/spray chilling or liposome. The former requires liquid droplets or small particles being

incorporated within a continuous edible coating, thus it requires an emulsifier. Liposomes are microscopic spherical particles consisting of one or more lipid bilayers that can encapsulate or bind a variety of molecules. Therefore, particularly in food applications, food grade surfactant such as the TWEEN20has been used as an emulsifying agent to fit this purpose (Quirós-Sauceda, Ayala-Zavala, Olivas, & González-Aguilar, 2014). Moreover, TWEEN20 has been found to have a protective effect on five different polyphenols, by slowing down the auto-oxidation process at pH 4.5(Lin, Wang, Qin, & Bergenståhl, 2007).

A surfactant-based separation technique, colloidal gas aphrons (CGA) has been previously studied in our group to recover various valuable bioactive compounds from different feedstock such as astaxanthin (Dermiki, Bourquin, & Jauregi, 2010; Dermiki, Gordon, & Jauregi, 2009), proteins (Fuda & Jauregi, 2006; Fuda, Bhatia, Pyle, & Jauregi, 2005) and polyphenols (Spigno, Dermiki, Pastori, Casanova, & Jauregi, 2010; Spigno, Amendola, Dahmoune, & Jauregi, 2015). The type of surfactant (i.e cationic, anionic and non-ionic) determines the outer charge of the CGA, where molecules with the opposite charge will attract to the CGA resulting in their effective separation into the CGA phase. Our previous work (in Chapter 3) showed that 70% of the anthocyanins could be recovered from the ethanolic extract of grape pomace using CGA generated from TWEEN20. Considering the high recovery of anthocyanins, this study specifically aims to assess the stability of polyphenols (particularly anthocyanins) in the surfactant rich solution ie, CGA fraction, as compared to their stability in the crude ethanolic extract (EE) (before the CGA separation) and the crude ethanolic extract with addition of sorbic acid (EE-SA) during storage.

5.2 Materials and methods

5.2.1 Materials

The grape pomace was obtained from the winery at northern Italy. All the solvents (95% and above) used in this project were obtained from Sigma-Aldrich Company Ltd., Dorset, UK.

5.2.2 Extract preparation

The grape pomace (Barbera variety) was kindly provided by a winery located in Nothern Italy. At the winery, the fermented pomace was recovered and oven dried at 60°C until the residual moisture content is <5%. The pomace powder was sieved with a 5-mm sieve to separate the skins from the seeds and milled into fine powders with particles size of <2mm. They were stored in vacuum bags, frozen at -20°C.

The phenolic extracts were obtained by ethanol-aqueous extraction using 60% (v/v)at 60°C in shaking water bath (Grant OLS 200) at 100rpm in circular motion for 2 hours. 250g of grape pomace were extracted with 2L of the ethanol-aqueous solvent. The ratio of solute to solvent used was 1:8 according to Amendola, De Faveri, & Spigno, (2010).Then, the extract was filtered using a Buchner filter, Buchner flask and Whatman No. 4 filter paper. Approximately, 300mL of the filtrate was labeled as ethanol extract (EE) and another 300mL was mixed with 100mg/L of sorbic acid (>99%) (Sigma, USA) and labelled as (EE-SA). In this sample, sorbic acid was chosen because of its wide application as preservative in the food industry, thus making it closer to the formulation of most low pH food products. Sorbic acid is mostlyused because its effectiveness in weak acid pH and neutral taste (Troller & Olsen, 1967).Both EE and EE-SA were selected as controlled samples.The remaining filtrate of 700mL was kept aside for CGA separation as described in Chapter 2, section 2.3. In this study, the chemical and physical properties

of these extracts (controls) will be compared to the CGA processed samples, which contain TWEEN20 up to a concentration of 10mM.

5.2.3 CGA separation using 10mM TWEEN20

The separation of polyphenols from the crude ethanolic extract was carried out at different ratios of the CGA to feed (V_{CGA}/V_{feed}). The ratios selected were 4, 8 and 16. The separations were individually carried out in a flotation glass column (i.d 5cm, height: 50cm). Briefly, the CGA were generated using 10mM TWEEN20 surfactant solution at 8000rpm for 5 minutes and were pumped into the column which contained the crude ethanol extract by peristaltic pump (Watson Marlow) from the bottom. Once the column was filled up, the mixture was left standing for 5 minutes before pumping out the separated liquid phase (at the bottom) and CGA (on top) of the column. The volume of collapsed CGA and drained liquid phase were measured. In this study, different V_{CGA}/V_{feed} was used which corresponded to the volume of CGA to the initial volume of the feed. The increase in volumetric ratio was proportional to the concentration of TWEEN20 in the solution. The concentration of TWEEN20 in V4 was 6.07mM, V8 was7.56mMand V16 was 8.56mM of the TWEEN20 and the CGA separation was done in triplicate.

5.2.4 Determination of degradation of chemical and physical properties over time

Briefly, the EE, EE-SA and CGA fractions were divided in equal volumes and kept in sterilized containers. These were then stored under room temperature 20°C (SD 1°C) which was regularly monitored using a thermometer for 32 days. The total phenolic content, total anthocyanin and antioxidant activity were determined as described in

Chapter 2, sections 2.5-2.9. The total anthocyanins, individual anthocyanins, antioxidant capacity and the colour degradation over time (32 days; every day for the first 7 days and subsequently 5 days intervals) were determined, as describe below.

The kinetics of total anthocyanins and individual anthocyanins degradation were assessed; the natural logarithms of these were plotted against time in order to test for first-order kineticsas described by the equation 5.1below:

$$-ln\left(\frac{A_t}{A_0}\right) = k * t \qquad \text{(Equation 5.2)}$$

Where A_0 is the initial anthocyanin content, A is the anthocyanin content at time *t*, *t* is the storage time and *k* is the rate constant. The degradation rate constant (*k*) was determined from the slope of the straight line between *Ln* and *t*. From the equation above, the time taken for the anthocyanin content to halve can be derived as the half-life ($t_{1/2}$), as described in equation 5.2 below:

$$t\frac{1}{2} = \frac{Ln(2)}{k} \qquad \text{(Equation 5.3)}$$

5.2.5 Total phenolic content

Folin Ciocalteu (FC) colorimetry method (Singleton & Rossi, 1965) was employed to determine the total phenolic content of the EE and EE-SA controlled samples and also in all of the CGA processed samples. This method involves the oxidation of phenols using a molybdotungstate reagent to yield a coloured product which can be measured at 760nm. Briefly, 0.2mL extract/standard was added to 6.0mL of distilled water in 10mL volumetric flasks after which 0.5mL Folin-Ciocalteu reagent (Sigma-Aldrich, Germany)

was added and mixed. After 1min and before 8mins, 1.5mL of 20% sodium carbonate (Fisher Scientific, UK) solution was added and the volume was adjusted with water to 10mL. The colour generated after 2 hours was read spectrophotometrically (Ultrospec 1100 pro UV spectrophotometer). Gallic acid (Sigma-Aldrich, Germany) standards with concentrations ranging from 0-1000mg/L were used to generate standard plots and an equation for the calculation of the total phenolic concentration in each extract. The analysis was done in triplicate. The total phenolic content in the CGA processed samples were compared to the controlled samples over time.

5.2.6 Evaluation of in vitro antioxidant activity

The antioxidant activity of the controlled samples (EE and EE-SA) along with the CGA processed samples were evaluated according to Re et al., (1999) using ABTS assay. This method assesses the ability of the antioxidants to scavenge the radical (ABTS) by decreasing its absorbance at 734nm. Different concentrations of Trolox standard were used to construct a calibration curve. The antioxidant activity of the CGA processed samples was compared to the controlled samples, expressed as µM Trolox equivalent. The ratio of percentage of the inhibition to the total phenolic content of all samples, termed as specific antioxidant power, was calculated.

5.2.7 Total anthocyanins content

The total monomeric anthocyanins of controlled samples, EE and EE-SA along with the CGA processed samples were determined over time using the pH differential method approved by AOAC (Lee, Rennaker, & Wrolstad, 2008). This method is based

on the anthocyanins structural transformation that occurs with a change in pH. Briefly, the extract was mixed individually with pH 1.0 and 4.5 buffer solutions in a ratio of 1:5 and left for 20 minutes. The absorbance of the test portions at both pHs were determined spectrophotometrically (Biotech Ultrospec 1100 pro UV spectrophotometer) at a wavelength of 520nm and 700nm.The results of the anthocyanin pigment were expressed as malvidin-3-glucoside equivalents (ME) according to equation 5.3.

$$Total\ Anthocyanins\ \left(ME, \frac{mg}{L}\right) = \frac{A*MW*DF*10^3}{\varepsilon*1} \quad \text{(Equation 5.3)}$$

Where A = $(A_{520nm}\text{-}A_{700nm})_{pH1.0} - (A_{520nm}\text{-}A_{700nm})_{pH4.5}$; MW (molecular weight of malvidin-3-glucoside =493.43g/mol; DF = dilution factor; 1 = path length in cm; Ɛ=28000 molar extinction coefficient and 10^3 = factor for conversion from g to mg and cm.

5.2.8 Quantification of anthocyanins

The separation of the polyphenols was performed using an Agilent HPLC 1100 series equipped with a degasser, a quaternary pump and a photodiode array detector model (Agilent, Waldbronn, Germany) with Chemstation software. The column used was a C18 HiChrom (150 mm x 4.6 mm i.d; 5µm particle size and 100 Å pore size; part no.EXL-121-1546U) operated at 30°C. The mobile phase consisted of 2% formic acid (v/v) and 5% acetonitrile (v/v) in water (mobile phase A), and 2% formic acid (v/v) in acetonitrile (mobile phase B) using the following gradient 5-15% B (15 mins), 15-30% B (15 mins), 30-50% B (10 mins), 50-95% B (5 mins) and 95-5% B (5 mins) at a flow rate of 0.8mL/min. The total run was 50 mins. The pre time of 10 mins was allowed for

re-equilibrating. The injection volume was from 20 µL for pure standards and 100µL for grape extracts. The polyphenols were monitored at 280nm and the UV/Vis spectra were recorded in the range of 200 to 760nm. The main anthocyanins were detected at 520nm and identified based on the retention times and by comparing the spectra with an external standard (Extrasynthese, Paris, France) of delphinidin-3-o-glucoside (>99%), cyanidin-3-o-glucoside (>98%), petunidin-3-o-glucoside (>98%) and malvidin-3-o-glucoside (>99%).

5.2.9 Determination of CIELab colour parameters and pH

The changes of colour in the EE, EE-SA and the CGA processed samples were measured using a CT-1100 ColorQuest HunterLab by taking the measurements in transmittance mode. Standard black plates were used for standardisation. L*, a* and b* measurements were obtained and used to calculate chroma and hue angles based on Equations 5.4. Delta E (ΔE) was calculated based on the changes of the values of L*, a* and b* at a given time, in comparison to these values at day 0. In other words, bigger ΔE imposed bigger changes in colour and vice versa.

$$Hue\ angle\ (°) = \left[180 * \left(\frac{ATAN\left(\frac{b}{a}\right)}{\pi}\right)\right] + \left(\frac{b}{a}\right)$$

$$Chroma = \sqrt{a^2 + b^2}$$

$$\Delta E = [(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2]^{\frac{1}{2}} \text{(Equations 5.4)}$$

The hue angle and chroma may be used on a CIE 1979 L*a*b* colorimetric system diagram to identify colour and monitor changes. The changes of colour in all the CGA processed samples over time were compared to EE and EE-SA as the controlled samples. The pH of all samples was checked regularly with a pH meter (Mettier-Toledo SevenEasy), and calibrated by using pH 4.0 and 7.0 buffer solutions (Sigma, USA).

5.2.10 Statistical analysis

All the experiments were performed in duplicate. The data were subjected to the analysis of variance using IBM® SPSS® Statistics21 software programme where statistical differences were noted. Differences among the different treatments were determined by using the Tukey test. The significance level was defined at $p<0.05$. The results were reported as means ± SD.

5.3 Results and Discussion

5.3.1 Total phenolic content

Initial values for total phenolic content, monomeric anthocyanins and antioxidants measured in controlled samples (EE and EE-SA) and three of the CGA processed samples were taken at day 0. The total phenolic content for all samples were from 285 to 2080 mg GAE/L. The TP content for EE-SA was higher than EE, which was explained by the presence of sorbic acid. The total monomeric anthocyanin was found between 99.1-422.9 mg ME/L. The antioxidant activity ranged from 2299-14469 µM Trolox equivalent.

Over a storage period of 32days, the losses in the TP content were minimal in all the samples (Fig.5.1). The maximum degradation observed in EE-SA and EE was not more than 10%, over time with the pH ranging from 3.5 to 3.8. Among all the CGA

processed samples, the lowest losses of the TP content was in V16 AP (4.91%), followed by V8 AP (5.44%) and finally V4 AP (6.42%), although they were not significantly different ($p = 0.062$). The hydroxyl group of the phenolic compounds is highly reactive; hence changes were expected during storage which was further explored in total antioxidant capacity and total anthocyanins.

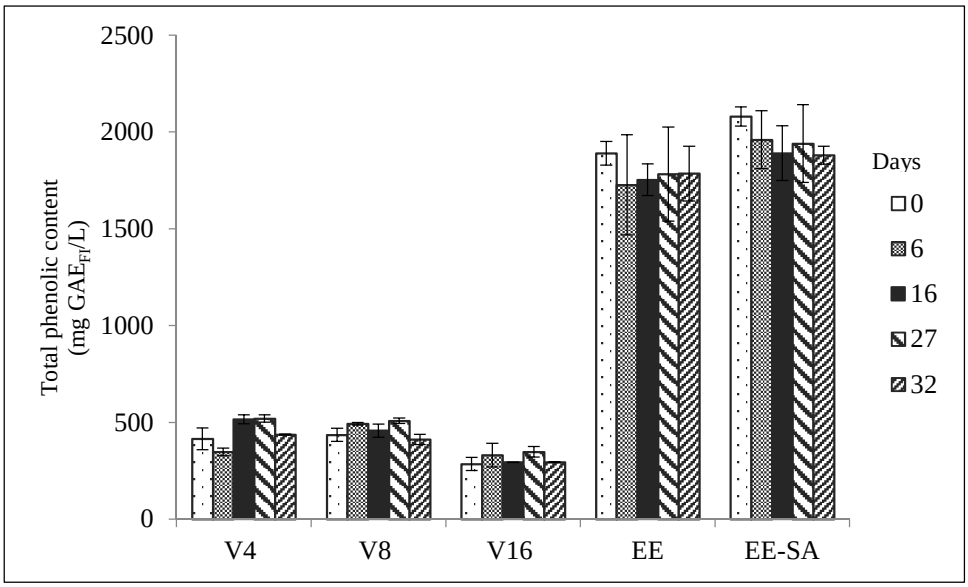


Figure 5.1 Total phenolic content of CGA processed and controlled samples over time. Error bars represent means ± SD, n = 2.Different letter indicates a significant difference using Tukey's test ($P<0.05$).

5.3.2 In vitro antioxidant activity

The antioxidant activity of the control samples and CGA processed samples stored over time was evaluated using ABTS assay. Figure 5.2(A) shows a decrease in antioxidant activity in the controlled samples and the CGA processed samples overtime. With the exception of EE, the reduction in antioxidant activity was significant for all samples during the first 5 days. Further decrease was observed in V4 after 12 days, however in V8, V16 and EE-SA samples no further reductions or even slight increases in antioxidant activity were observed.

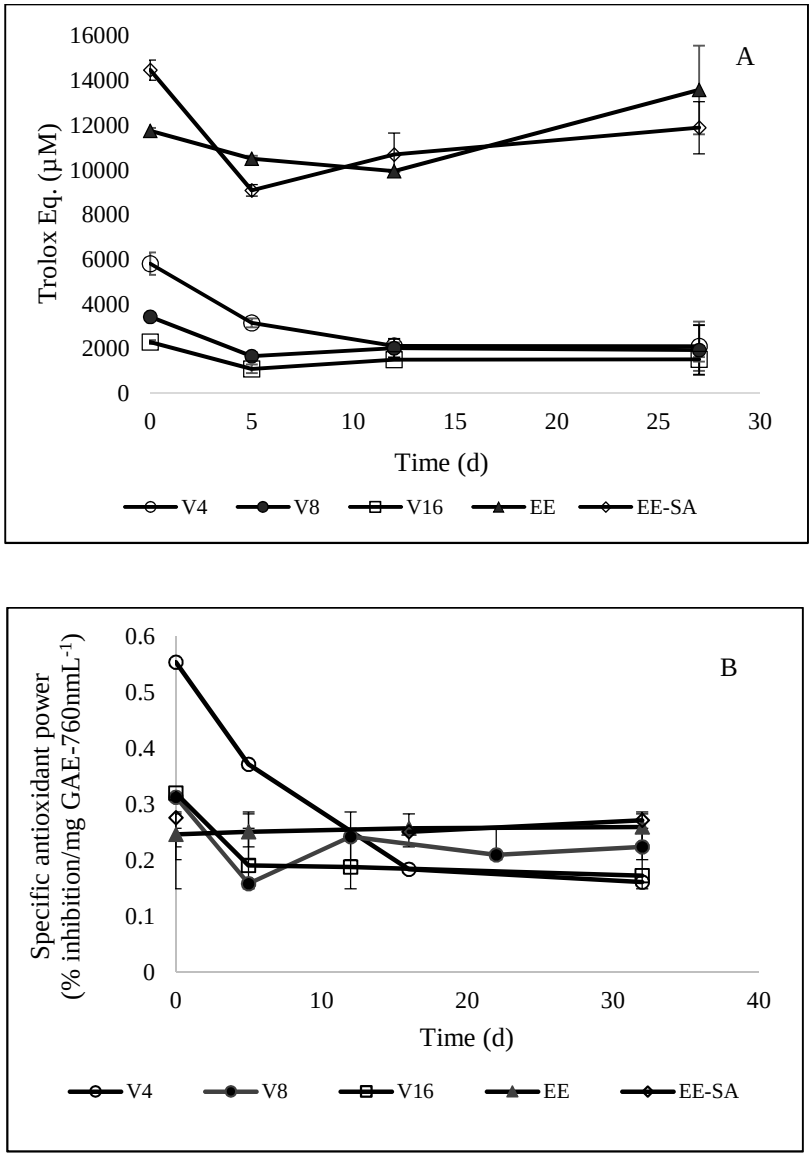


Figure 5.2 Antioxidant activity profile expressed as Trolox Equivalent (µM) (A) and specific antioxidant power (% inhibition/mg GAE-760nmL-1) (B) of different samples during storage. Error bars represent means ± SD, n = 2.

However, when the specific antioxidant power (calculated as percentage of inhibition by the total phenol content measured with Folin-Ciocalteu assay) was calculated, a more distinct pattern was observed (Fig 5.2B). In general, the specific antioxidant power decreased over time although there were slight losses in the total phenol content (Fig. 5.1).Rapid loss of antioxidant power was observed in V4 from day 0 to day 16, although losses for V8, V16 and EE-SA were only observed until day 5. It

was interesting to note that there was no specific antioxidant loss in EE. This could be related to the total phenolic content as depicted in Figure 5.1 where the losses in EE was not significantly different ($p \leq 0.05$) between the time points. This implied that the losses in TP and antioxidant activity in EE were in the same proportion hence the antioxidant efficiency was almost constant over time (Fig 5.2B).

However, this was not the case for the CGA samples where minimal losses of total phenolic content were noted but important changes in antioxidant activity. Therefore, this suggests that in these samples the antioxidant activity may not solely derive from the total phenolic content. Over estimation of total phenolic content could possibly happen by the action of proteins presence in the crude extracts and the CGA processed samples.

5.3.3 Total anthocyanins content

Degradation of anthocyanins has been previously studied in wine and its residues (Bimpilas, Panagopoulou, Tsimogiannis, & Oreopoulou, 2016; Clemente & Galli, 2011, 2013; Lavelli et al., 2017). The patterns of degradation involving anthocyanins are complex, but the degradation rate generally follows first-order kinetics (Amendola et al., 2010; Buckow, Kastell, Terefe, & Versteeg, 2010). However, the information on anthocyanins degradation in the presence of surfactant is lacking. In this study, the degradation of anthocyanins in the controlled samples (EE and EE-SA) were compared with the CGA processed samples stored at 20°C, following the first-order kinetics (Fig. 5.3).

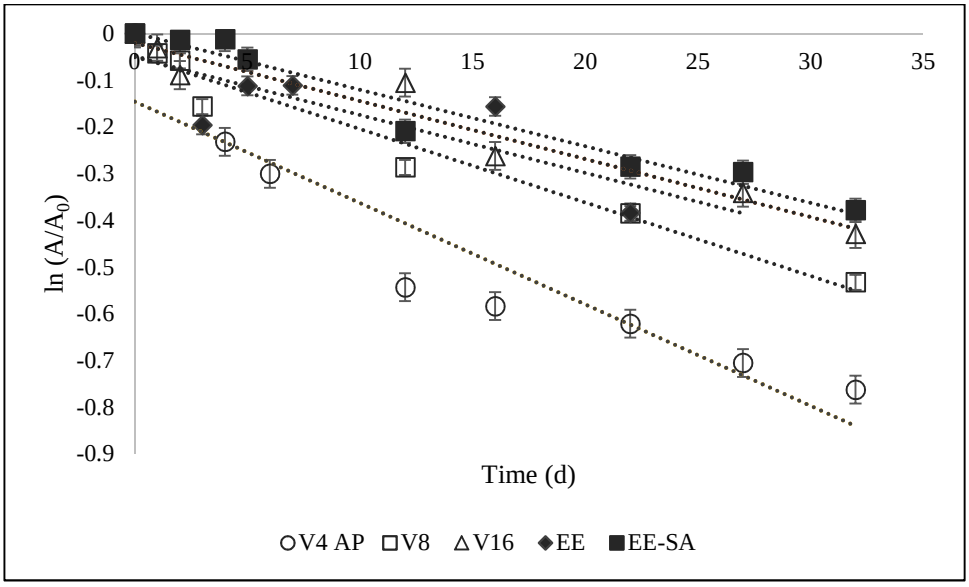


Figure 5.3 Time course for the decrease of anthocyanins (A/A_0) concentration in samples measured by pH differential method, during storage at 20°C. Error bars represent means ± SD, n = 2. Dotted lines represent the fitting of data with first-order kinetics. Rate constants are reported in Table 5.1.

In Table 5.1 the first-order rate constant (*k*) of all samples is shown. The first-order rate constant for anthocyanins degradation ranged between 0.0124 and 0.0217 d^{-1}.Although the R^2 values for V4 and EE were lower than the others, the first-order rate constant was assumed. The justification for this was that the pH of these samples ranging from 3.5-3.8, therefore the degradation of the anthocyanins and thus the ionisation of flavilium ion follow this degradation order(West & Mauer, 2013). In the controlled samples, the first-order rate constant of EE was higher (k = 0.0159 d^{-1}) than EE-SA; the first-order rate constant of EE-SA was the lowest among all samples (k = 0.0121 d^{-1}). This suggests that the addition of sorbic acid increased the stability of anthocyanins during storage although sorbic acid has only been reported to prevent microbial growth during storage (Troller & Olsen, 1967).

Table 5.1 First-order empirical rate constants (k) and half-life for anthocyanins.

Sample	R^2	K (d^{-1})	$t_{1/2}$ (d)	Losses (%)*
V4	0.8861	0.0217 ± 0.0019	31	53.35
V8	0.9585	0.0157 ± 0.0024	44	41.30
V16	0.9385	0.0124 ± 0.0015	55	34.90
EE	0.8131	0.0159 ± 0.0012	43	41.04
EE-SA	0.9583	0.0121 ± 0.0011	57	31.53

Rate constants are expressed as means ± SD, n = 2.
*losses calculated after day 32

Generally, the first-order rate constant decreased with the increased in volume ratio and thus with an increased in the surfactant content in the CGA processed samples (ranging from 6.07-8.56mM TWEEN20). The first-order rate constant of V16 was comparable to the one of EE-SA, whilst V8's was close to those in EE. Meanwhile, V4 was found to degrade the fastest over time. Thus, it can be said that V16 had a comparable stability to EE-SA, followed by the V8 sample and finally the V4 sample.

The extraction of grape pomace with water containing 3% of citric acid has also been proposed to recover phenolic-rich coloured extracts with 36-62% of total anthocyanins obtained, intended to be used as food ingredients (Cardona, Lee And, & Talcott, 2009). However, the colour degradation of these water-based extracts at 30°C is fast, with first-order rate constant of 0.0364 and 0.038 for cold and hot pressed extractions, respectively. In the present study, the first-order rate constants were lower indicating more stable extracts. The most stable sample was the EE-SA (k = 0.0121d^{-1}) suggesting a stabilisation effect of sorbic acid. Comparable results were obtained for V16 CGA with k= 0.0124d^{-1}. However, it must be pointed out that the stabilisation effect in the CGA processed samples was only achieved at certain concentration of surfactant, in the case of V16 it was estimated as8.56mM of TWEEN20. In the case of V4 and V8 CGA

samples which contain 6.07mM and 7.56mM TWEEN20 respectively, the stabilisation effect was minimal and even below the stability of EE control.

However, the ethanolic extract of grape pomace obtained from micronized grape skin powder and maltodextrin-encapsulated grape skin extracts had low first-order rate constant of 0.0033-0.0014 d^{-1}(Lavelli et al., 2017). The possible reason for this could be due to the drying and encapsulation ofthe grape skin extracts, lowering their water activity which was largely responsible for the degradation of polyphenols. Therefore these results are not comparable with the present results, as this study assessed the stability of anthocyanins in a liquid form. Moreover, the drying process will require higher overhead costs and needs high energy and pressure input which would add up greatly to the overall costs.

The half-lives of anthocyanins of EE and EE-SA stored at 20°C were 43 and 57 d, respectively (Table 5.1). In comparison to the CGA samples, V4 had the shortest half-life of 31 d, followed by V8 (44 d) and V16 (55 d). Comparing the CGA processed samples to the controlled samples; the half-life of V16 was almost similar to EE-SA, but longer than that of EE. Meanwhile, V8's half-life was close to that of EE. These half-life values were higher than the one reported for the blueberry juice stored at 25°C ($t_{1/2}$ = 4.4 d), possibly due to the different types of anthocyanins present (Buckow et al., 2010). Similarly, when the percentage of anthocyanin losses after 32 days of storage were determined, EE-SA had the least losses of 31.53%, closely followed by V16 of 34.90%. Quite similar anthocyanins losses were noted in EE and V8. In a study of blue grape anthocyanins stored at 15-35°C, the loss of anthocyanins in grape juice sample with sorbic acid was 77% when stored for 60 days (Thakur & Arya, 1989). In comparison to the EE-SA, the losses of anthocyanins in EE-SA were small; although it must be highlighted that Thakur & Arya (1989) had their sample stored for 60 days at a wider

range of temperature. In the present study, the loss of anthocyanins was the highest in V4 which coincided with the lowest TWEEN20 concentration in this sample. Therefore, it can be conclude that the surfactant had a comparable stabilisation effect to the sorbic acids. This further confirmed that the surfactant might play an important role in protecting the anthocyanins from oxidation, thus extending the half-life.

5.3.4 HPLC-DAD analysis of anthocyanins

The HPLC-DAD analysis showed that all samples had 13 anthocyanin peaks which were identified in the beginning and at the end of storage study. The identified anthocyanins were: delphinidin, cyanidin, petunidin, peonidin and malvidin with different glycosyl acylation attached (Fig. 5.4).In red wines and their pomace made from *V. vinifera* grapes, the main anthocyanins detected were of 3-o-monoglucosides of the free anthocyanidins including pelargonidin-3-o-glucoside, cyanidin-3-o-glucoside, delphinidin-3-o-glucoside, peonidin-3-o-glucoside, petunidin-3-o-glucoside and malvidin-3-o-glucoside (Drosou et al., 2015; He et al., 2012; Kammerer et al., 2004). However, in this study, pelargonidin-3-o-glucoside was not detected. The chemical structure of the anthocyanins identified was illustrated in Figure 5.5.

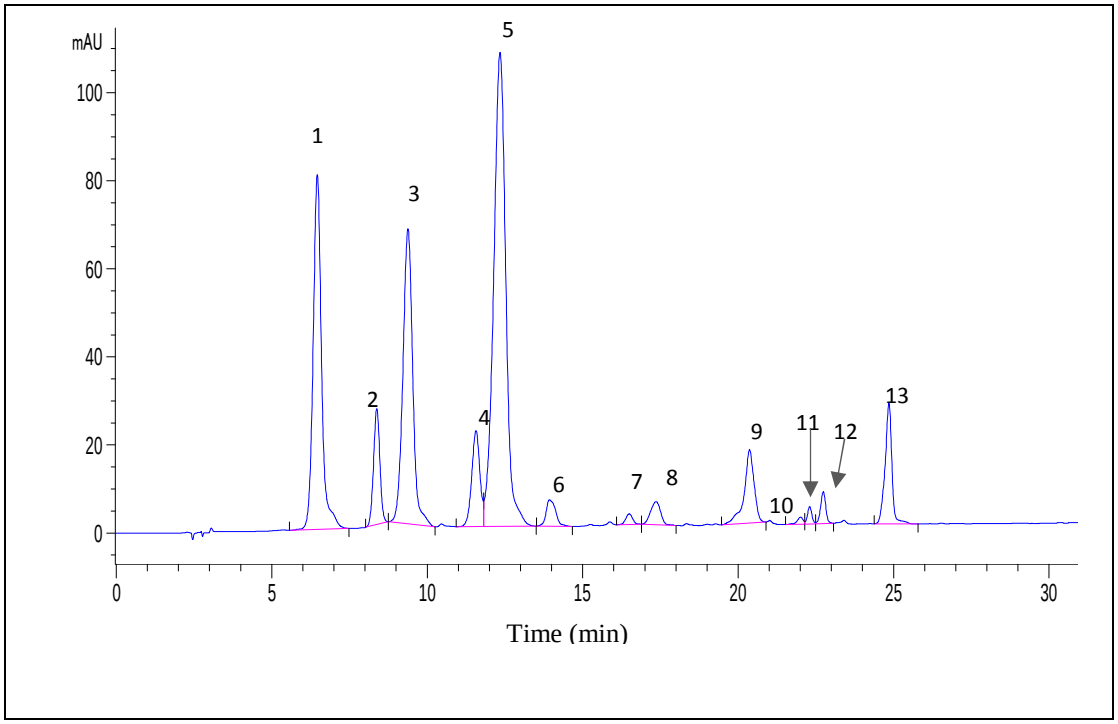
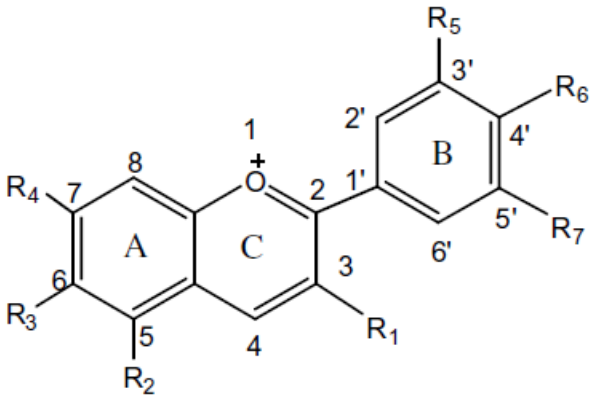


Figure 5.4 Typical HPLC profile of anthocyanin in ethanol extract of grape pomace at 520nm. Peak 1: delphinidin 3-glc, 2:cyanidin 3-glc, 3:petunidin 3-glc, 4:peonidin 3-glc, 5: malvidin 3-glc, 6:delphinidin 3-acetylglc, 7:cyanidin 3-acetylglc, 8:petunidin 3-acetylglc, 9:malvidin 3-acetylglc, 10:cyanidin 3-o-p-coumaroylglc, 11:petunidin 3-o-p-coumaroylglc, 12:peonidin 3-o-p-coumaroylglc, 13:malvidin 3-o-p-coumaroylglc.

In all the samples, the most abundant anthocyanin was malvidin-3-o-glucoside (0.68mg/g) which was in agreement with other studies (Bimpilas, Panagopoulou, Tsimogiannis, & Oreopoulou, 2016; Morais, Ramos, Forgács, Cserháti, & Oliviera, 2002) followed by delphinidin3-o-glucoside (0.58mg/g). This composition was the same as those found in blueberry (Buckow et al., 2010). Both pigments were typically responsible for the purple and purple-blue which could be seen in the colour of the ethanolic extract.



Name	Substitution pattern							
	R1	R2	R3	R4	R5	R6	R7	Colour
Delphinidin	OH	OH	H	OH	OH	OH	OH	Blue-red
Cyanidin	OH	OH	H	OH	OH	OH	H	Orange-red
Malvidin	OH	OH	H	OH	OMe	OH	OMe	Blue-red
Petunidin	OH	OH	H	OH	OMe	OH	OH	Blue-red
Peonidin	OH	OH	H	OH	OMe	OH	H	Orange-red

Figure 5.5 Structural identification of anthocyanidins (aglycons) (Castañeda-Ovando, Pacheco-Hernández, Páez-Hernández, Rodríguez, & Galán-Vidal, 2009).

Different anthocyanins had different degradation kinetics in the samples (Table 5.2). Among the four anthocyanins, delphinidin was the least stable anthocyanins, with the exception of EE. This can be seen in their short half-lives (29-41d) and their faster degradation kinetics (k = 0.0126-0.0195d^{-1}). Fleschhut, Kratzer, Rechkemmer, & Kulling (2006) reported that an increase in hydroxyl groups in the B ring of the anthocyanin nucleus could result in a decrease in the stability which could possibly account for the anthocyanins loss. However, this was not observed in EE where cyanidin degraded faster than delphinidin, but both of them seemed to be less stable than petunidin and malvidin indicating that methylation of hydroxyl-groups in B ring increased the stability of anthocyanins. Our results were comparable to those reported by Hellstrom et al., (2013) for delphinidin and cyanidin in the blackcurrant and chokeberry juices stored at 21°C with half-lives between 16-44 days.

Table 5.2 Half-lives ($t_{1/2}$, day) of different anthocyanins in controls and CGA samples, stored at 20°C

Compound/Sample	**V4** ***K*** **(d^{-1})**	**V8** ***K*** **(d^{-1})**	**V16** ***K*** **(d^{-1})**	**EE** ***K*** **(d^{-1})**	**EE-SA** ***K*** **(d^{-1})**
Delphinidin 3-glc	35 0.0195	36 0.0190	29 0.0233	41 0.0168	41 0.0168
Cyanidin 3-glc	42 0.0163	55 0.0126	52 0.0132	33 0.0204	42 0.0162
Petunidin 3-glc	50 0.0136	44 0.0155	52 0.0132	50 0.0136	49 0.0139
Malvidin 3-glc	55 0.0126	57 0.0121	71 0.0097	59 0.0116	65 0.0116

*3-glc, 3-o-glucoside

Malvidins are known to be the most stable as compared to other anthocyanins due to the absence of two hydroxyl groups in the B ring structure. This was clearly evident as they had the longest half-life as compared to other anthocyanins across all samples. Interestingly, malvidin in V16 sample had longer half-life (71d), with slowest degradation rate (k = 0.0097 d^{-1}) than in EE-SA (k = 0.0116d^{-1}, $t_{1/2}$ = 65d) which agrees with the observed protecting effect of the surfactant. Results from that study showed that the stability of polyphenols was influenced by the hydrophobic/hydrophilic balance in the solution. Moreover, the colloidal aggregates would influence the oxidation rate if the polyphenol was incorporated in or closely interacting with the aggregates(Lin et al., 2007).

According to Hellström et al., (2013), the effect of sugar moiety was minor as compared to the effect induced by the type of the core anthocyanidin. In these extracts, malvidin- and delphinidin 3-glucosides were the two major groups. Delphinidin glucosides exhibited greater temperature sensitivity due to their three hydroxyl group in the B ring in comparison to malvidin derivative which had only one –OH group attached to it (Buckow et al., 2010). This can be clearly seen in the half-life of malvidins ($t_{1/2}$ = 55-71 days) across all samples. Moreover, the stability of anthocyanins was also reduced

by the number of hydroxyl groups in the A ring with the absence of dihydroxyl group in the B ring (Buckow et al., 2010). The matrix of samples also had a major impact on the stability of anthocyanins where anthocyanins in juices were more prone to degradation as compared to those in smoothie (Hellström et al., 2013). A study in anthocyanins stability from encapsulated grape skin showed significant increase in the half-life of anthocyanins up to 452 days. The study also proved that by lowering the water activity of the encapsulated grape skin powder can double the half-life up to 998 days (Lavelli et al., 2017). In fruit juices, several factors can influence the stability of anthocyanins, such as pH, presence of ascorbic acid and anthocyanin degrading enzymes (Buckow et al., 2010). Finally, the degree of glycosylation also might possibly affect anthocyanins stability; the higher the degree of glycosylation, the more stable it became.

Co-pigmentation of anthocyanins with other compounds is considered as an important mechanism of colour stabilisation in plants. Anthocyanins can form co-pigments with metal ions, other phenolic compounds or through self- association (Castañeda-Ovando et al., 2009). Co-pigmentation through self-association is not very likely because in order for it to take part, the concentration of the anthocyanin should be greater than 1mmol//L. Hydroxycinnamic acids and flavonols were reported as the best cofactors in wine (Bimpilas et al., 2016). Co-pigmentation can be influenced by the anthocyanins and co-pigment structure, and also by the concentration of anthocyanins to the co-pigment (Eiro & Heinonen, 2002). In the present study, the ratio of anthocyanins and co-pigments might not be sufficient for the co-pigmentation to occur since no additional phenolic acids were added to the samples. Thus, the stability effect on the anthocyanins observed in this study could be postulated due to the TWEEN20 and the addition of sorbic acid.

5.3.5 Colour stability and pH

Grape pomace extract has high levels of anthocyanins. However, anthocyanins undergo degradation during processing and storage, thus affecting colour characteristics. In the present study, the colour stability of the controlled samples and the CGA processed samples were investigated. Colorimetric parameters chroma (*C*) and hue (*h*) were determined and the ΔE was applied to see colour changes over time. The effects are comparable to those observed in anthocyanins, yet with some exceptions.

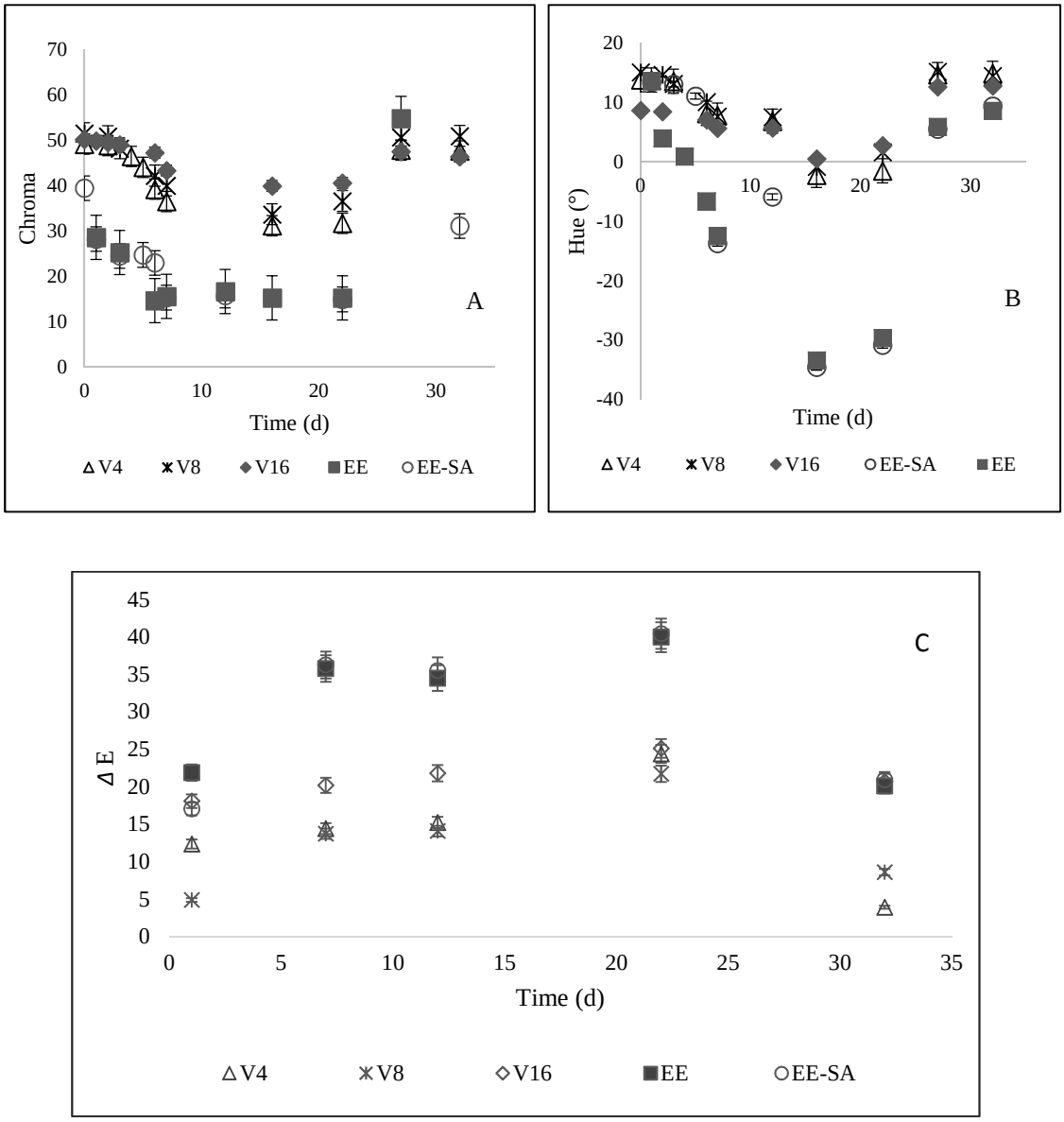


Figure 5.6 Chromaand hue values of samples during storage (A and B), totalcolour difference (ΔE) between samples (C). Error bars represent means ±SD, n = 2.

Figure 5.6(A and B) showed the changes of colour in chroma and hue angle for the controlled samples and the CGA processed samples over time. In this study, the CGA processed samples were compared against EE and EE-SA. The results obtained showed that EE and EE-SA had a similar trend; ΔE values overlapped with each other. At day 0, both samples had dark red colour (c = 39.4, hue = 6.9). Over time, both chroma and hue values decreased rapidly by day 22, from dark red tending towards blue-black shade (c = 14.9, hue = -29.6). The same trend was observed in all of the CGA processed samples, although the chroma and hue angles decreased steadily as compared to EE and EE-SA. At day 0, all of the CGA processed samples had almost similar colour of dark red shade (c = 49.2-51.5; hue = 8.7-15.1). However, the chroma (c = 31.7-40.6) and hue angle (hue = -1.4-2.8) values decreased in all of the CGA processed samples over time. In short, V4 turned from dark red to light red, tending towards blackish; V8 turned from dark red to light red, tending towards browning and finally, V16 turned from dark red to light red, tending towards pinkish. Therefore, these results showed that minimum colour changes were observed in V16, which correlated with the lower degradation rate determined above and confirms the stabilisation effect by the surfactant. Furthermore, the colour changes of samples can be further explained by ΔE (Fig. 5.6C). ΔE indicates the magnitude of the color difference between fresh and stored grape extracts. In the controlled samples (EE and EE-SA), huge changes could be seen as compared to other CGA samples in every time points. In all the CGA processed samples, the changes were not significantly different ($p>0.05$) in the first 12 days of storage. However, higher magnitude of changes was observed in day 22, and minimal changes were observed in day 32.

Anthocyanins differ from each other by the number and position of the hydroxyl, and methoxyl substituent groups in the B ring of the molecule. The hydroxylation pattern

of the anthocyanins in the B ring can directly affect the hue and colour stability due to the effect on the delocalized electron path length in the molecule (He et al., 2012). Anthocyanins with more hydroxyl groups in the B ring can contribute more to blueness meanwhile the degree of methylation in the B ring can increase redness. The rapid decrease in red colour of EE and EE-SA might be explained by the half-life of a particular anthocyanin (Table 5.2). In both controlled samples, cyanidin-3-glucoside and petunidin-3-glucoside had the shortest half-lives between 16-21 days. Both anthocyanins were responsible for the red and dark red colour respectively, which could explain the losses of dark red colour in both controlled samples after 21 days. Both anthocyanins had two hydroxyl group attached to the B ring, which increased the blueness of the colour, as found in these samples. In the case of the CGA processed samples, V4 appeared to have the same result as EE and EE-SA, which possibly explained the short half-life of cyanidin-3-glucoside. However, in the case of V8 and V16, delpinidin-3-glucoside had the shortest half-life, 31 and 36 days respectively. This could have contributed to the colour changes observed, from dark red to light red, tending towards brownish and pinkish. Delphinidin-3-glucoside was responsible for the blueness as it had three hydroxyl groups attached to the B ring. Although most studies showed that delphinidin-3-glucoside exhibited a greater thermal sensitivity due to their three hydroxyl substitution group, this was not clearly observed in this study; thus the correlation between anthocyanin stability and chemical structure is still unclear (Rice-Evans, Miller, & Paganga, 1996).However, the huge colour changes in EE-SA could not be explained by the slowest anthocyanins loss in this sample. This suggests that the reaction of colour stability in this sample is yet to be determined as does not simply correlate with anthocyanins degradation. The mechanisms of stabilisation of anthocyanins by

TWEEN20 are yet to be determined, but we propose that the micelles might play a role in encapsulating the anthocyanins, thus protects them against oxidation during storage.

Overall, the pH of all samples dropped and then increased slightly throughout the storage period (Fig. 5.7). Although the reason is unclear, it may be due to the formation of phenolic acids like gallic acid which has brought the samples to be more acidic than before. The increase in pH later at the end of storage possibly indicated the oxidation of these intermediate compounds.

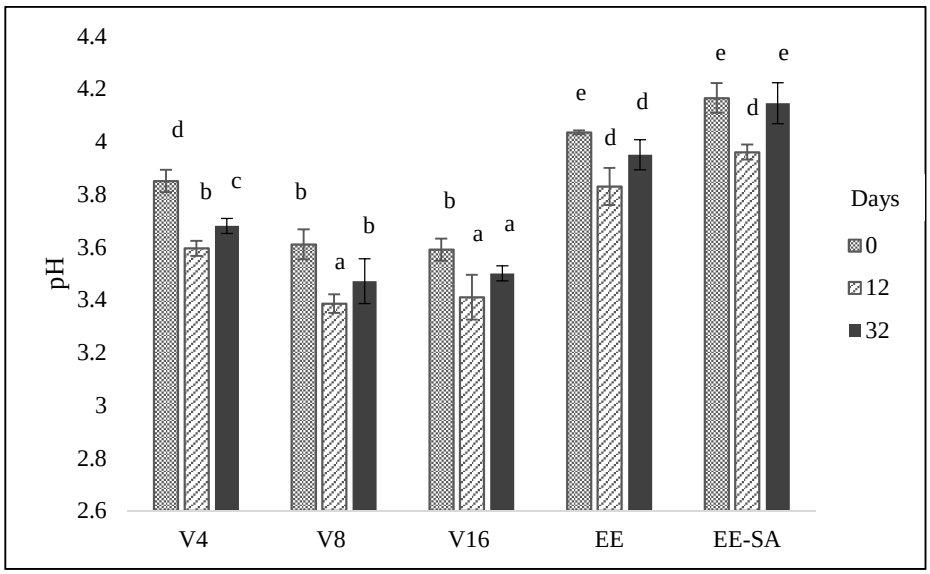


Figure 5. 7pH values of all samples during storage. Error bars represent means ±SD, n = 3. Different letter indicates a significant difference using Tukey's test ($P<0.05$).

5.4 Conclusions

Red grape pomace is a well-known source of anthocyanins. The results obtained in this study suggest that the presence of TWEEN20 at a certain concentration greatly increased the stability and thus enhanced the shelf life of grapes anthocyanins in CGA processed samples when stored at 20°C. The stability of anthocyanins CGA processed samples increased with concentration of TWEEN20. Among the CGA processed samples, V16 showed minimal degradation of anthocyanins over time with a half-life of 55 days and overall anthocyanins losses of 34.90%. This result was comparable to the controlled samples of EE-SA with 31.53% loss of anthocyanins ($t_{1/2}$= 57d), confirming the stabilisation effect of the anthocyanins by the addition of sorbic acid. Malvidin-3-o-glucoside was found to be the most stable anthocyanin ($t_{1/2}$ 55-71 d), which was in agreement with most other reported studies. A good correlation between the colour changes and the degradation rate of individual anthocyanins was also observed in this study; i.e degradation of cyanidins and petunidins resulted in loss of dark red colour in the control samples.

Overall, this study shows that the surfactant could offer additional stabilisation to the anthocyanins and the results obtained here were superior to others reported for wet formulations of anthocyanins. The mechanisms of stabilisation of anthocyanins by TWEEN20 may be related to solubilisation effect of the anthocyanins by the micelles. Finally, the main findings in this study show the advantages of CGA, not only as an integrative separation technique but also in food formulation.

5.5 References

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CHAPTER 6: General conclusions and suggestions for future work

6.1 Conclusions

Polyphenols from grape pomace, efficiently extracted using organic solvents may need the solvent removed before formulation for food and/or cosmetic applications. In the present work the aim was to investigate the application of colloidal gas aphrons (CGA) to the crude grape pomace extracts for the recovery and formulation of polyphenols. In particular, the focus of this study was to investigate if the second extrction into the CGA phase would lead to an enrichment of the most active polyphenols and if the surfactant in the CGA would result in any advantage for their formulation. Based on the previous knowledge obtained in related research, this study focused on using TWEEN20for the generation of CGA, a non-ionic surfactant that is considered safe for consumption and has the least irritating effect on the skin,.

First polyphenols were extracted from the pomace by applying two different extractions: (i) ethanolic extraction (EE) (ii) hot water extraction (HWE); both are considered green solvents. Different polyphenols composition was noted in both extracts. The EE was particularly rich in anthocyanins and flavonols whilst the HWE contained high amounts of phenolic acids and flavonols. Although the total phenolic content in HWE was lower than in EE, its antioxidant activity showed that HWE had a more potent effect in quenching the radicals, as measured by ABTS assay.

Similar recovery of polyphenols was obtained from both EE and HWE with CGA generated with CTAB and TWEEN20, with higher antioxidant capacity found in CGA fractions separated with TWEEN20 than with CTAB; hence TWEEN20 was selected to be used forthis study. Alsousing the non-ionic surfactant had the added advantage that the final product was considered non-toxic and safe and no additional step for surfactant

removal was necessary. The selectivity in relation to sugar was particularly good with EE at low CGA to feed volumetric ratio; but this was not in the case of HWE where similar high recoveries were obtained for polyphenols and sugar at all volumetric ratios. In the case of proteins, similar recovery trend was observed in both extracts; similar recoveries for proteins and polyphenols were obtained, thus low selectivity in proteins was obtained. Overall, it should be highlighted that for both grape pomace extracts in the further CGA separation process, the most important operating parameters were volumetric ratio and drainage time. The recovery of polyphenols increased with the volumetric ratio but decreased after the ratio 16 while in the case of drainage time, the opposite trend was observed, increase in the drainage time resulted in decreased polyphenols recovery. Consequently, since both the EE and HWE showed similar recovery trend, the selection of the solvent forthe solid-liquid extraction process, in this case was tailored made to the final application/product intended. For example, the ethanolic extraction led to an extract particularly rich in anthocyanins that could be of particular interest for application as a natural colouring either to be applied in food or cosmetics and could also be interesting for applications as functional food product. HWE on the other hand extracted most of the phenolic acids which can be useful in formulating products rich with natural antioxidants.

The selected CGA fractions were tested for their activity against skin related enzymes. The main findings here (chapter 3) showed that both ethanolic and water extracts of grape pomace had high inhibitory activity and that further separation with CGA led to high inhibitory potency. The key polyphenols identified in HWE were gallic acid, chlorogenic acid, quercetin and kaempferol, whilst quercetin, resveratrol and gallic acid were the major polyphenols in EE. The derived CGA fraction from HWE had gallic acid, syringic acid and kaempferol and the derived CGA fraction from EE had quercetin and the anthocyanins as the key polyphenols. Moreover, the increased in the potency of

the CGA fractions suggested that the TWEEN20 might play a role in solubilising the polyphenols; facilitating the delivery of these key polyphenols to the enzymes. However, these polyphenols need to pass the great barrier of stratum corneum layer in order to reach the targeted enzymes inside the dermis layer. Therefore, a step further was taken in assessing the permeability of these polyphenols in surfactant rich solutions onto porcine skins (chapter 4). The main results showed that the presence of TWEEN20 increased the penetration and thus permeability of individual polyphenols (gallic acid and resveratrol) across the skins and artificial membrane. However, a smaller amount of polyphenols from the crude grape pomace extract and the CGA fraction penetrated across the membrane. This could possibly be due to the complexity of the samples which hindered the diffusion of particular polyphenols across the membrane. Overall, CGA fractions from grape pomace extracts showed high potency in inhibiting the enzymes related to skin aging and their application in cosmetic formulations seems promising given the surfactant's role in facilitating the diffusion of the polyphenols across the skin.

In order to consider the application of CGA in the liquid formulation of the anthocyanins as a natural food colouring, the stability of the anthocyanins from the EE and its derived CGA fractions were evaluated during storage at 20°C for 32 days (chapter 5). The main findings here showed that stability of anthocyanins in CGA samples increased with the concentration of TWEEN20. It is important to highlight that the highest stability of anthocyanins in the CGA fraction was achieved with the concentration of 8.58mM of TWEEN20 and the result was comparable to the EE-SA (ethanol extract with addition of sorbic acid) in terms of half-life and losses of total anthocyanins. A good correlation was also obtained between the degradation of individual anthocyanins and the colour changes of all samples during storage. Therefore these findings here suggest that the stability of anthocyanins in the CGA processed fraction was aided by the surfactant

that may protect them from oxidation/degradation. This again shows that CGA can be used not only as a separation method but can also facilitate the formulation step.

Overall in this study it has been shown that the application of CGA can lead to the recovery and at the same time formulation of polyphenols with potential application in cosmetics and food products. The inhibitory activity potency of polyphenols extracted in CGA fractions which were rich in surfactant was higher than that of the crude extracts, and also the anthocyanins stability was higher in the CGA fractions (with high surfactant concentration) than in the crude extracts, as shown by the results in chapter 3, 4 and 5. As a final conclusion, CGA is an interesting and promising recovery-formulation technique especially with TWEEN20 as the surfactant since its removal is no longer needed; in fact as shown in this study the surfactant has a positive effect by increasing the solubility of polyphenols, protecting and possibly selectively "encapsulating" polyphenols in the micelles. Finally CGA is applicable to the recovery of different types of by-products and can contribute to development of "greener" processes by reducing the use of solvents and hence addressing the environmental concerns.

6.2 Suggestions for future work

The present study proposed a separation process for the recovery of polyphenols from grape pomace ethanolic and water extracts using colloidal gas aphrons. The separation was conducted conventionally, in a flotation column operated in batch mode where the CGA were generated prior the mixing (or contact) with the extracts. However, alternative ways to apply CGA generation could still be explored andalso CGA could be applied to different by-products. Here, we had conducted a preliminary experiment on carrot pomace using an *in situ* generation of CGA. In this trial, the carrot pomace was

mixed with a 1mM SDS surfactant solution. The mixture was subjected to intense stirring for 5 mins and heated up using a hot plate to 60°C. This led to the generation of foams. After the stirring had stopped, the mixture was allowed to separate for another 5 minutes. The liquid phase was pipetted out while the aphron/foam phase was allowed to collapse completely and residues were filtered. Total phenolic content, total carotenoids and antioxidant activity of both phases were analysed (Table 6.1).

Table 6. 1 Total phenolic content and total carotenoids for the carrot pomace extracted using CGA

Analysis	Aphron/Foam	Liquid	Separation Factor (SF)
Total Phenol – Folin Index (mg GAE/g fresh weight)	40.36 ± 3.55	4.43 ± 2.10	9.11 ± 2.51
Total Carotenoid (µmoles/ g fresh weight)	19.58 ± 2.45	14.24± 3.11	1.21 ± 0.63
Antioxidant activity (% inhibition)	6.37	4.44	-

Values represent mean ± standard deviation (n = 3).

The results showed higher content of phenolic content in the aphron/foam phase as compared to the liquid phase. This was evident by the high separation factor of 9. The high separation factor indicated the affinity of compounds to be in the aphron/foam phase. As for the total carotenoids, the recovery was higher in the aphron/foam phase but not significantly different from the liquid phase. This result was further supported by the separation factor which was around 1, indicating a uniform partition of the carotenoids between the two phases. Furthermore, the recovery of carotenoids could be influenced by the pH of the mixture. Dermiki et al., (2009) found that maximum recovery of astaxanthin (which belongs to xanthophyll group) was achieved under strong alkaline solution even

though they are highly hydrophobic. The antioxidant activity, calculated as percentage of inhibition showed a slightly higher inhibition in the aphron phase. Interestingly, this result coincided with the total carotenoids results, which possibly suggested that the main contributors to this activity could possibly be the carotenoids whilst the polyphenols extracted may not reacted towards this assay. In the extraction of polyphenols from plant matrix, these polyphenols must be released from the food matrix first by disrupting the plant tissue, followed by the removal of the debris and liquid-liquid or liquid-solid extraction (Amorim-Carrilho et al., 2014). These steps were mainly represented in this experiment, where disruption of the matrix could be done by the intense stirring, followed by the solid-liquid extraction by the surfactant. From the observations above, it is possible to conclude that the recovery of polyphenols could be done by means of an *in situ* generation of CGA, avoiding the solid-liquid extraction by the organic solvents. However, several important parameters like pH, volume of surfactant to the pomace and moisture content should be further investigated.

In addition to the work conducted in Chapter 2 where flotation column was used, it will be more interesting to investigate the optimisation process by continuous operation, where feed and CGA are introduced simultaneously by using two different pumps. This will be interesting to investigate the scale-up of the CGA separation. Moreover, the size and dimension of the column could be increased to improve recovery and perhaps its selectivity. Alternatively in batch mode additional steps could be taken by subjecting the drained liquid phase to a second or more flotation steps to improve the recovery of polyphenols. With regard to integration of the steps taken before CGA separation, the suspension of grape pomace-extract could directly be used for CGA generation without having to filter the pomace residues. This technique will allow the combination of extraction and purification steps in recovering and formulating the active CGA fractions

to be applied in food and non-food industries. In this study, although the grape pomace residues were not utilised, this residues should be further used, probably as a source of dietary fibre by turning them into grape pomace flour. This step can be further investigated in future to fully utilise the grape pomace, leaving no by-product behind, in line with the zero waste concept in the food industries.

Concerning the inhibitory activities of grape pomace extracts and CGA fractions to collagenase and elastase enzymes, more pure extracts is probably desired in order to establish the connection between the activities and the type of polyphenols. This can be achieved by subjecting the extracts to HPLC coupled with fractions collector. Regarding the permeability of the polyphenols on the skins, the results showed very minimal penetration of CGA fraction into the skin. Still, this experiment has provided an interesting result to be used in future. The formulations (in this case, the ethanolic extract and CGA fraction) could be more concentrated to achieve certain thermodynamic activity in order for flux to occur. Moreover, the extract could be semi-purified using solid-phase-extraction or fractionated to group the polyphenols according to molecular weight or hydrophilicity depending on the type of stationary phase and the eluents used. This is seen as an important step as purification of extract is limited in CGA separation with the studied parameters. Further work in evaluating the polyphenols in the different layers of the skins could be done to improve this experiment.

Chapter 5 revealed that the CGA processed fractions improved the stability of anthocyanins and the colour quality. However, this study can be further carried out in a real food matrix. For instance, the CGA fractions can be incorporated into yoghurts, ice creams or biscuits fillings and assess the stability of anthocyanins and the colour properties. This will be very interesting to investigate because it represents the concept of “from waste to shelves”. Also, the application of this CGA fraction could be widen

and be very useful if it offers additional stability against pH variations. It is known that anthocyanins are unstable at higher pH and that the colour changes from dark red/purple to blue with the increased of pH. Therefore, the food and non-food industries suffer from providing the blue natural-based colour. It would be a great advantage if the stability of these anthocyanins in the CGA fractions show some stabilisation at higher pH.

With a growing interest towards environmentally friendly extraction processes, the use of CGA generated from TWEEN20 on the ethanolic and hot water grape pomace extracts as conducted in this study contributes toward the development of “green” extraction of polyphenols, minimising the use of solvents and also offering the additional advantage of facilitating their formulation for food and non-food applications.

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Appendix A: Calibration curves

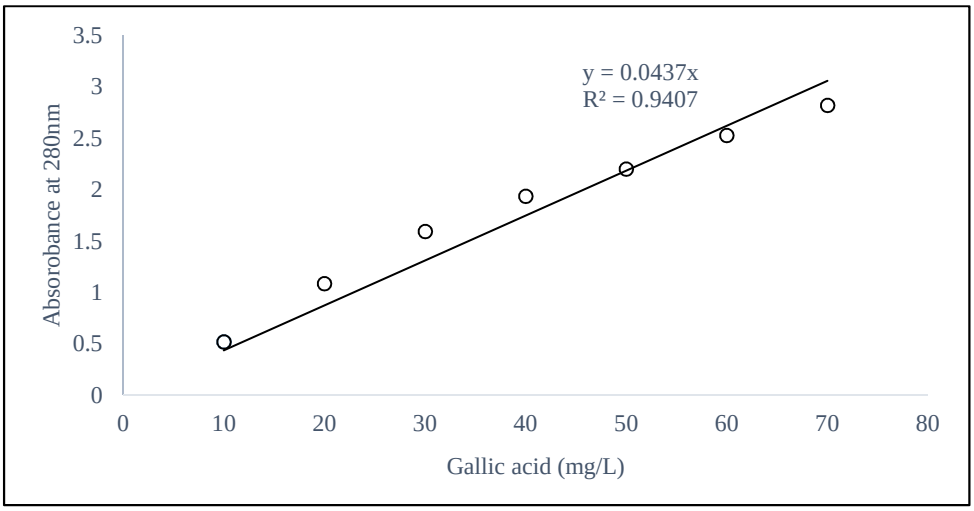


Figure A1: Calibration curve for the estimation of gallic acid using direct measurement method

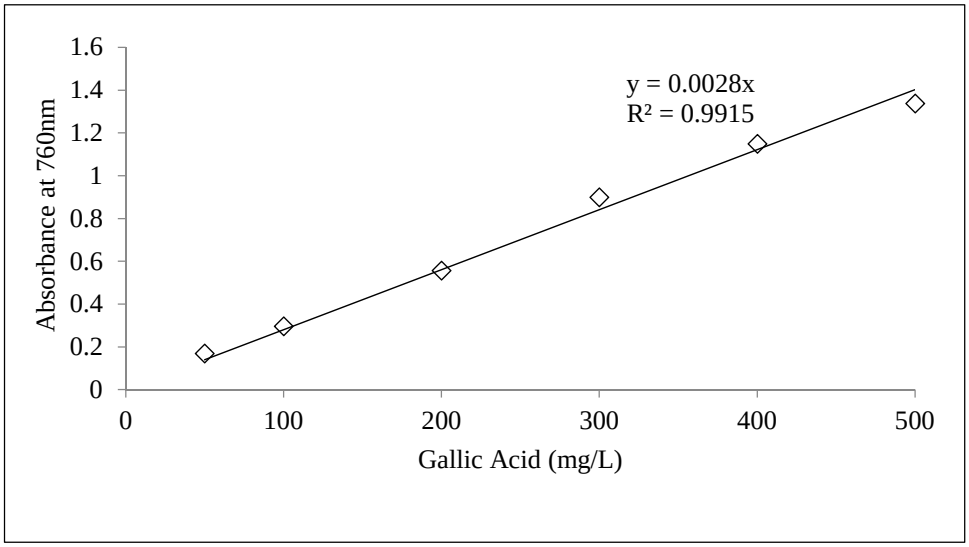


Figure A2: Calibration curve for the estimation of gallic acid using Folin-Ciolcateau method

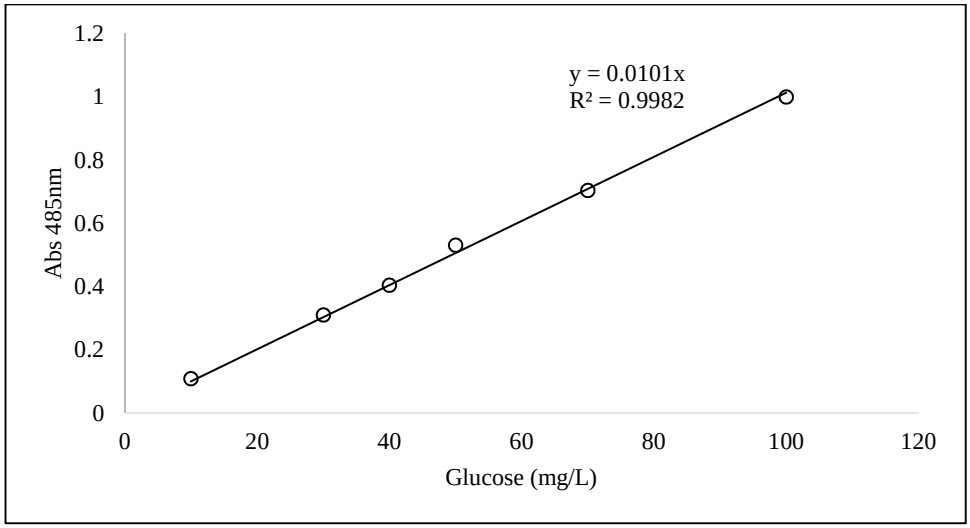


Figure A3: Calibration curve for the estimation of glucose

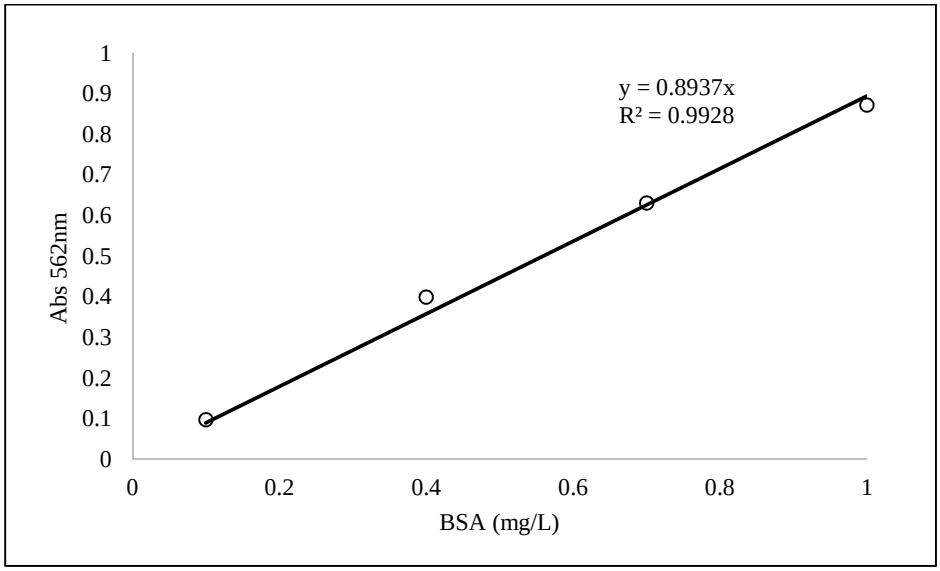


Figure A4: Calibration curve for the estimation of BSA using the BCA method

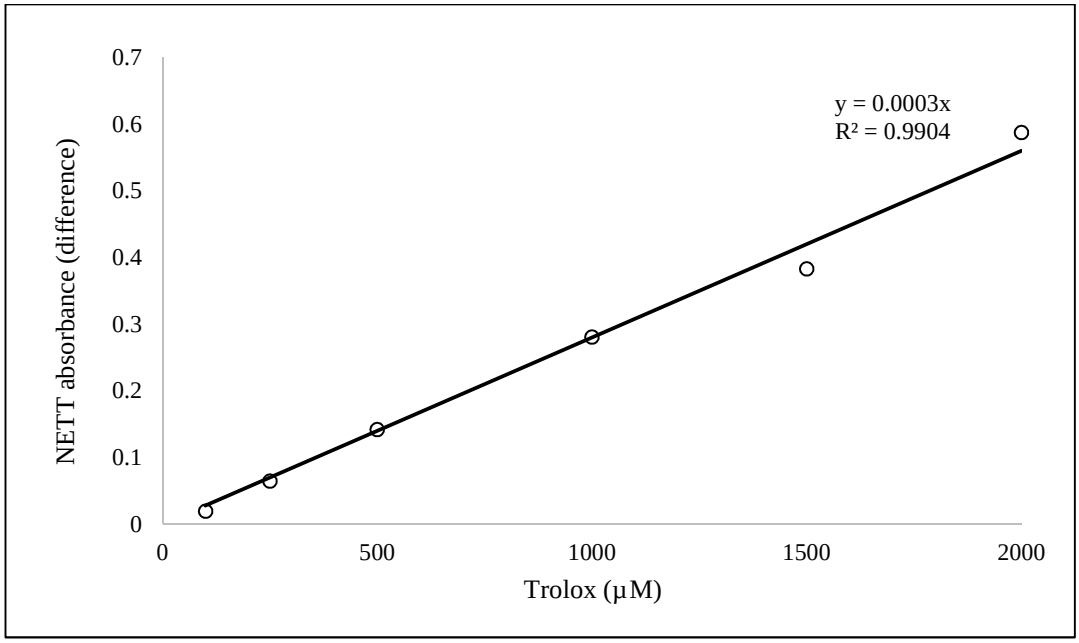
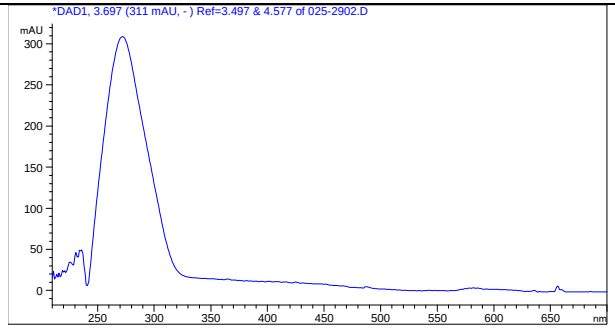
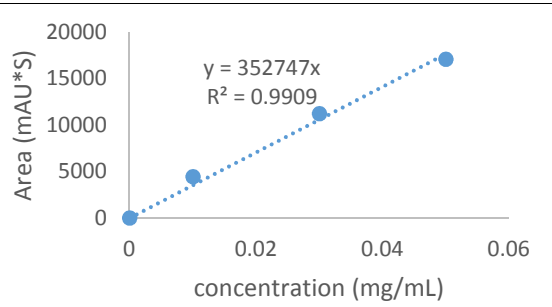
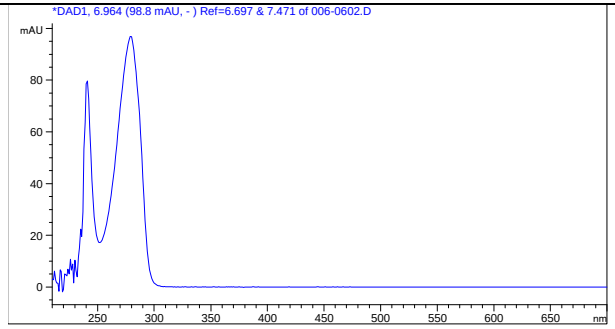
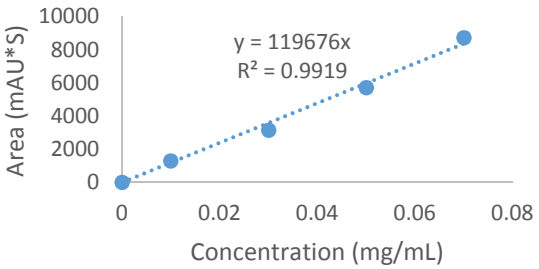
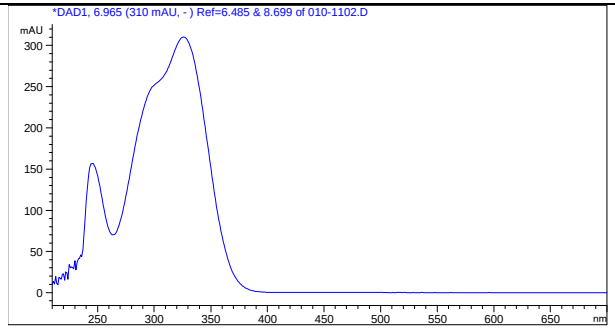
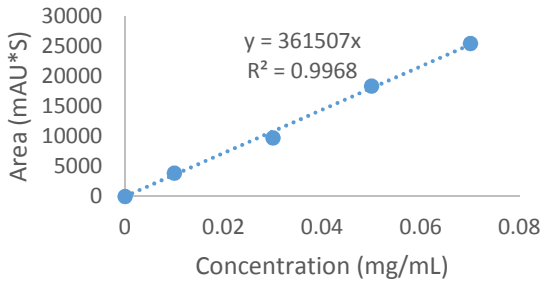
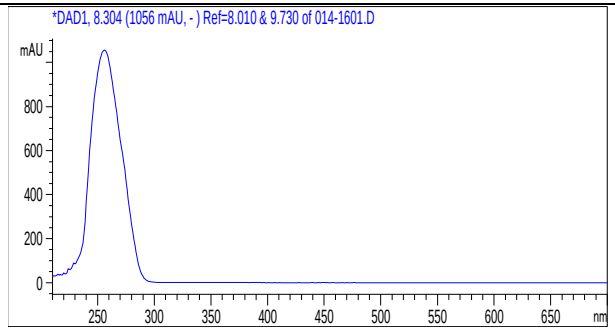
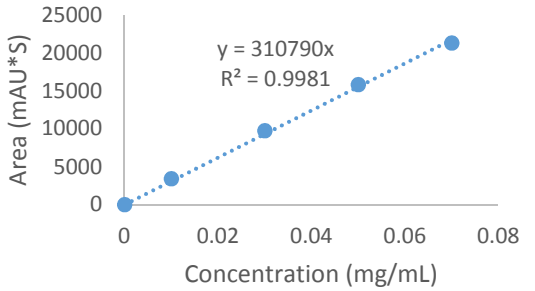
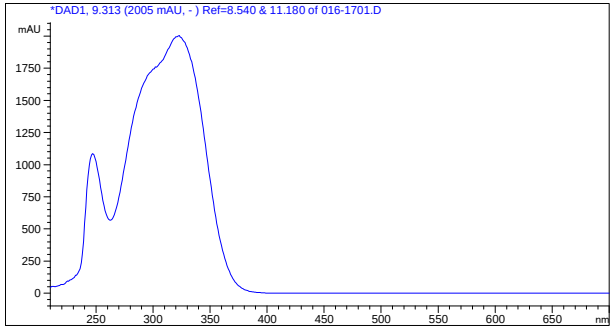
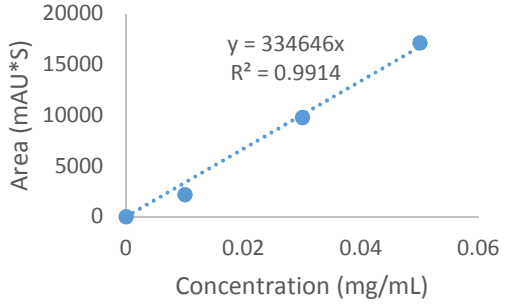


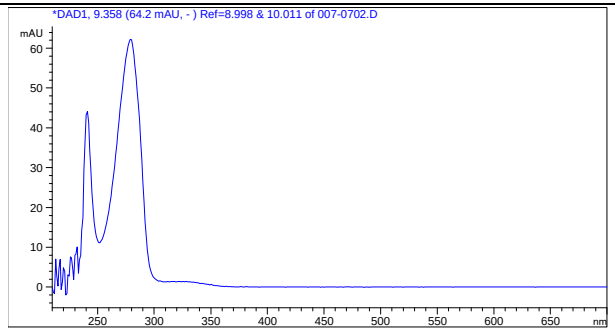
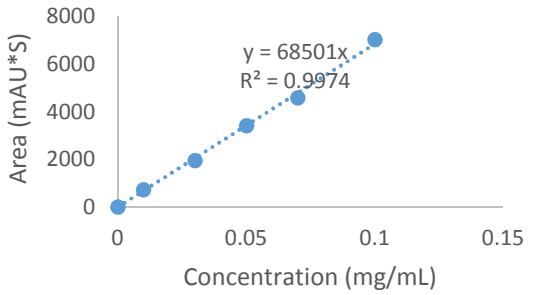
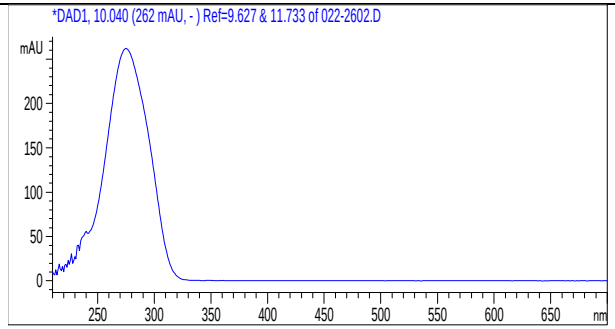
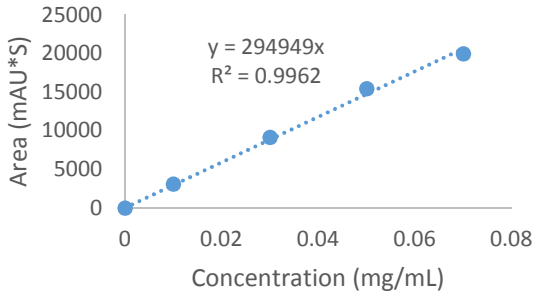
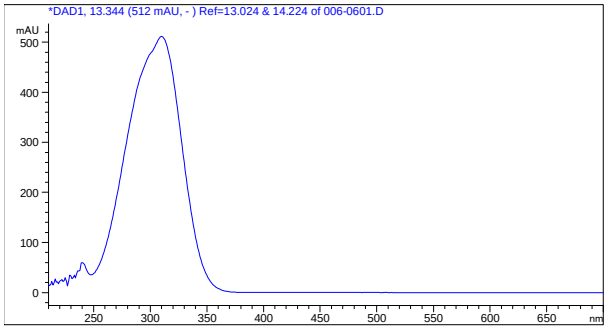
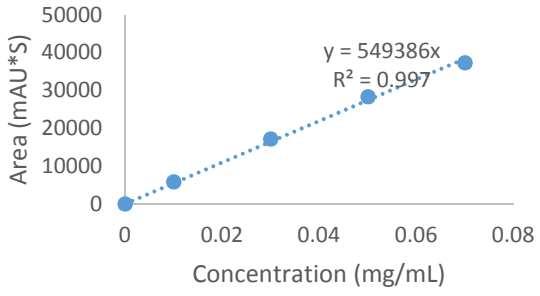
Figure A5: Calibration curve of antioxidant activity to the function of Trolox using ABTS method

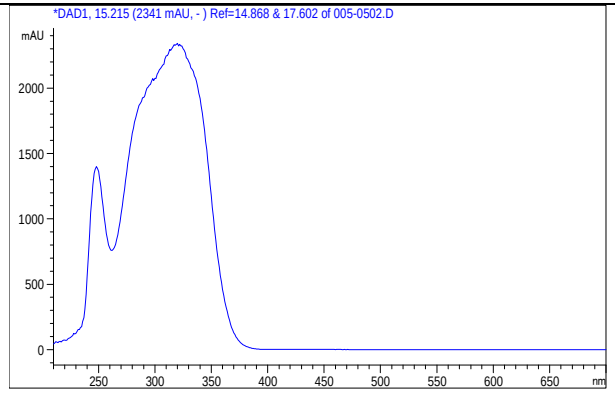
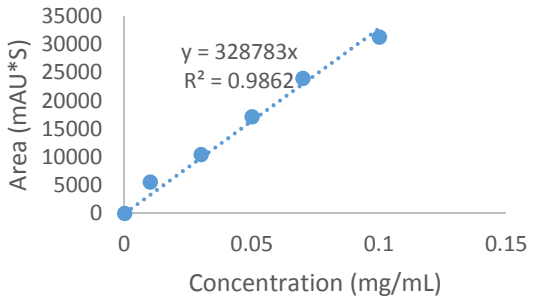
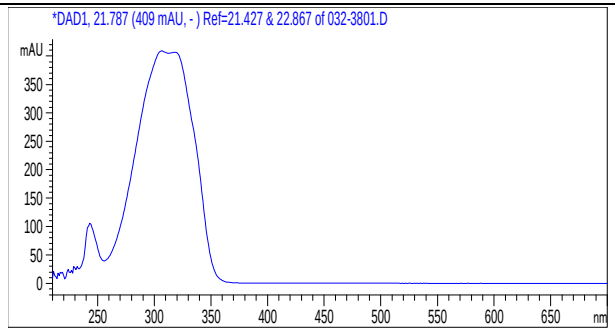
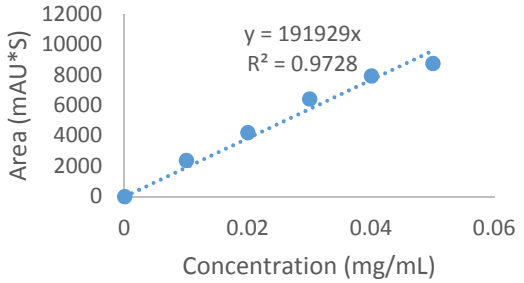
Appendix B: HPLC calibration curves and chromatograms

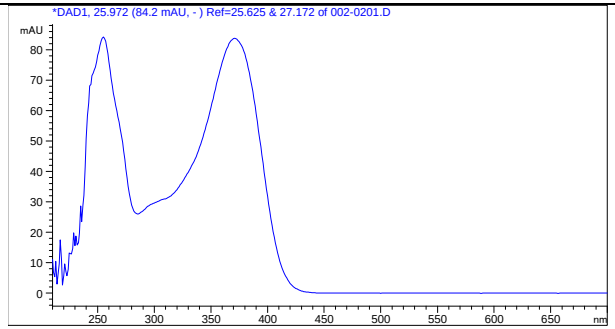
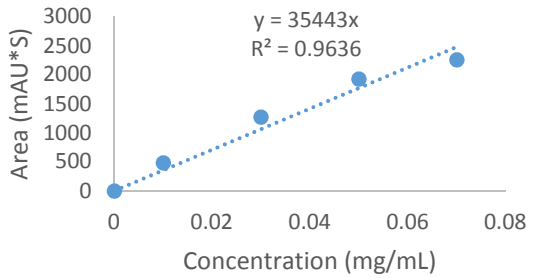
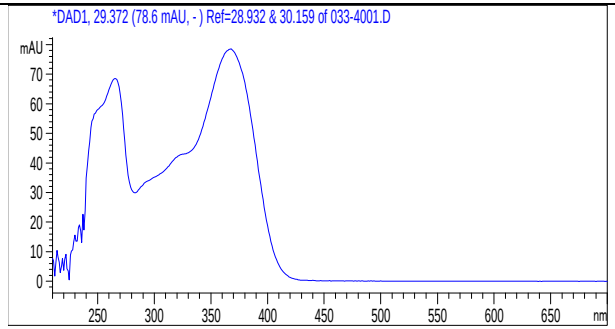
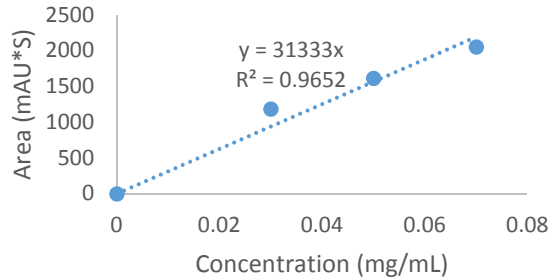
Table A1: Calibration curves and spectra of individual polyphenols analysed by HPLC

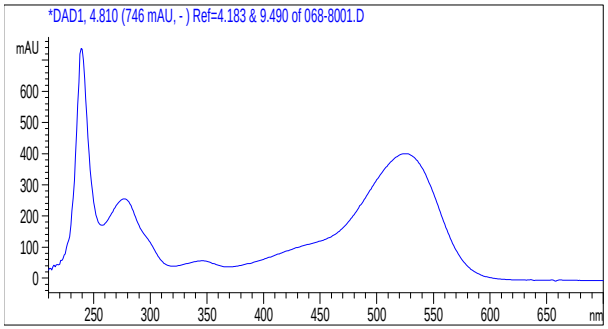
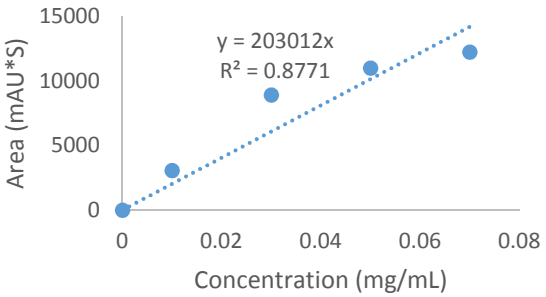
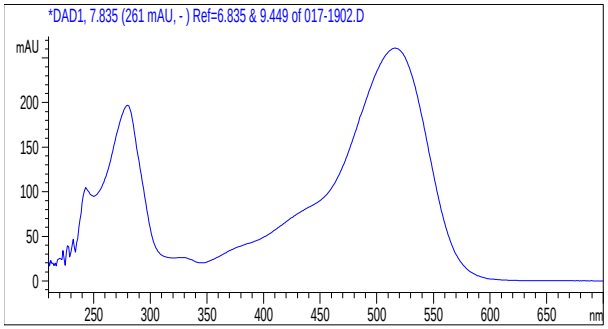
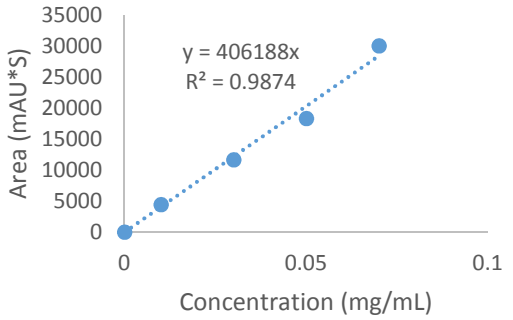
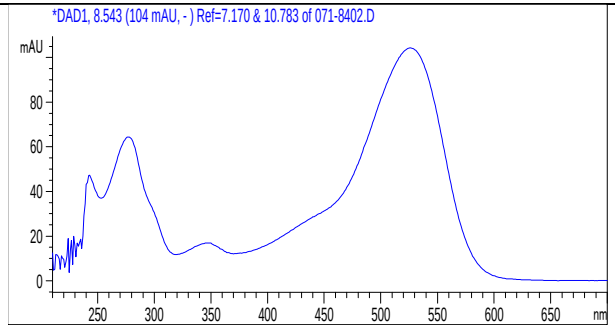
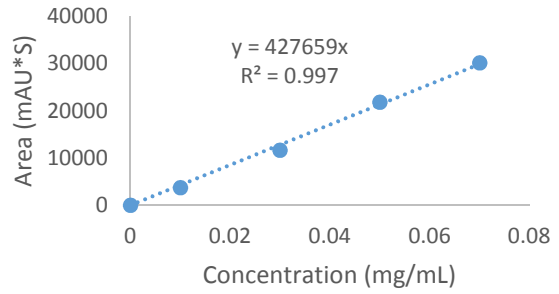
Hydroxybenzoic acids (280nm) and Hydroxycinnamic acids (320nm), Flavanols (280nm), Flavonols (320nm)			
Compound	Retention time	Spectra	Calibration curve
Gallic acid	3.6		
(+)-Catechin hydrate	6.7		

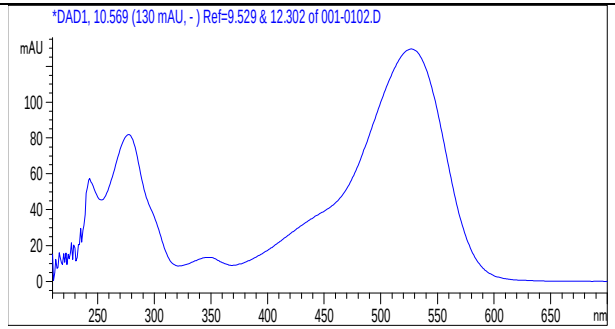
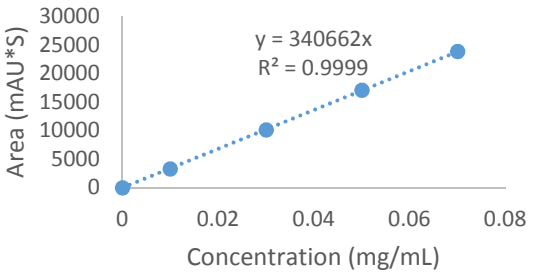
Chlorogenic acid	6.9		
p-hydroxybenzoic acid	8.3		
Caffeic acid	9.2		

(-)-Epicatechin	9.4		
Syringic acid	10.0		
p-coumaric acid	13.3		

Ferulic acid	15.3		
Resveratrol	21.7		

Quercetin	25.6		
Kaempferol	29.4		
Anthocyanins (520nm)			
Compound	Retention time	Spectra	Calibration curve

Delphinidin-3-o-glucoside	4.8		
Cyanidin-3-o-glucoside	7.8		
Petunidin-3-o-glucoside	8.5		

Malvidin-3-o-glucoside	10.8		

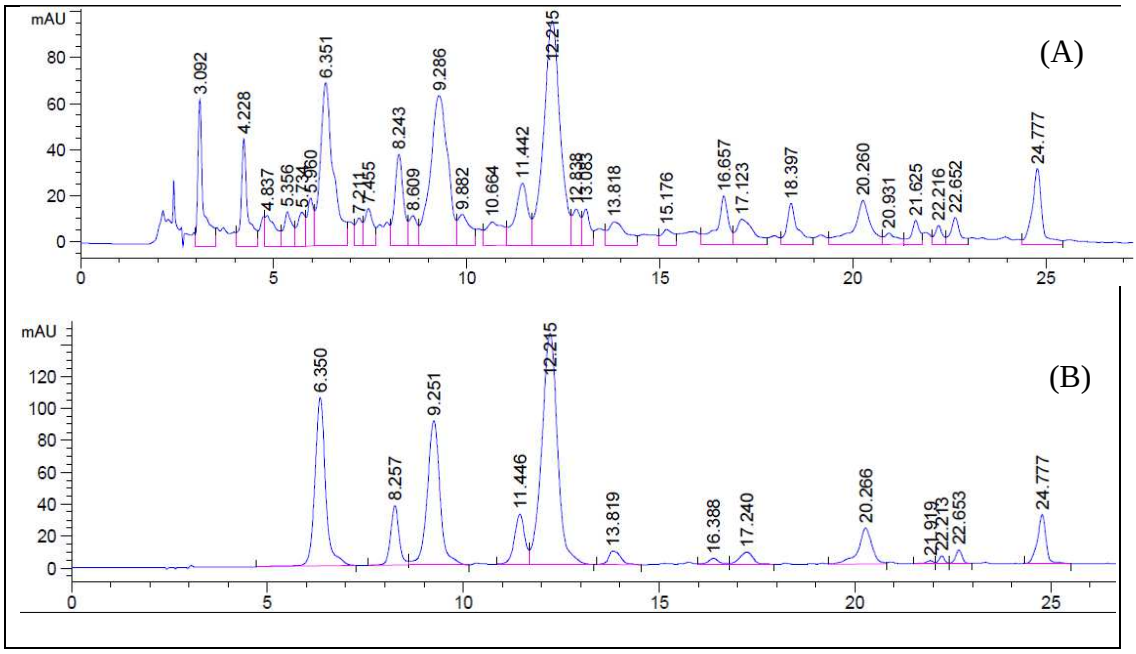
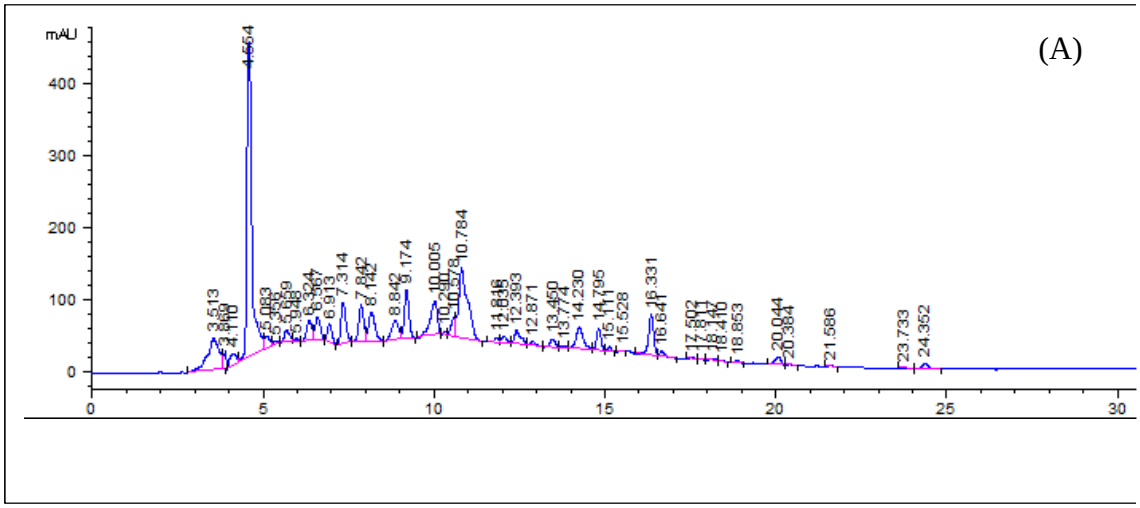


Figure B1: HPLC analysis of ethanolic extract of grape pomace at i) 280nm and ii) 520nm. Dilution factor = 10



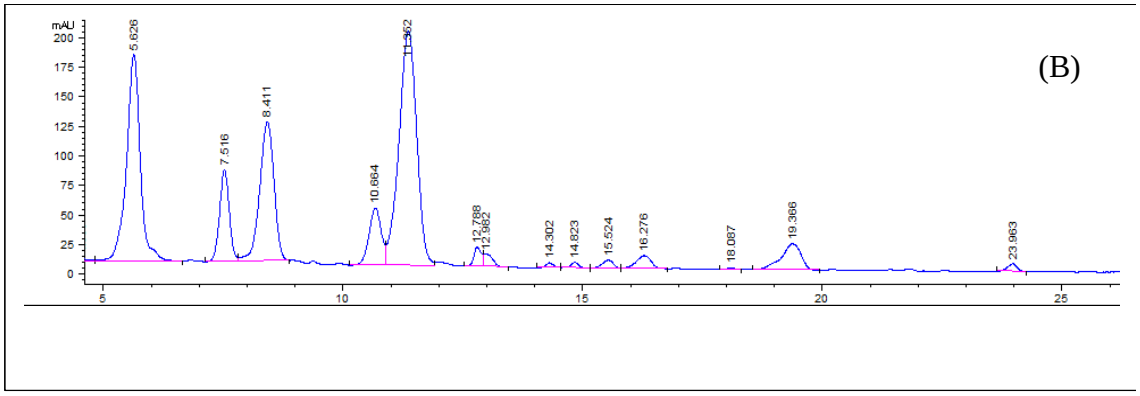


Figure B2: HPLC analysis of hot water extract of grape pomace at A) 280nm and B)520nm. Dilution factor = 5 for (A) and o for (B).

Appendix C: Stability of CGA

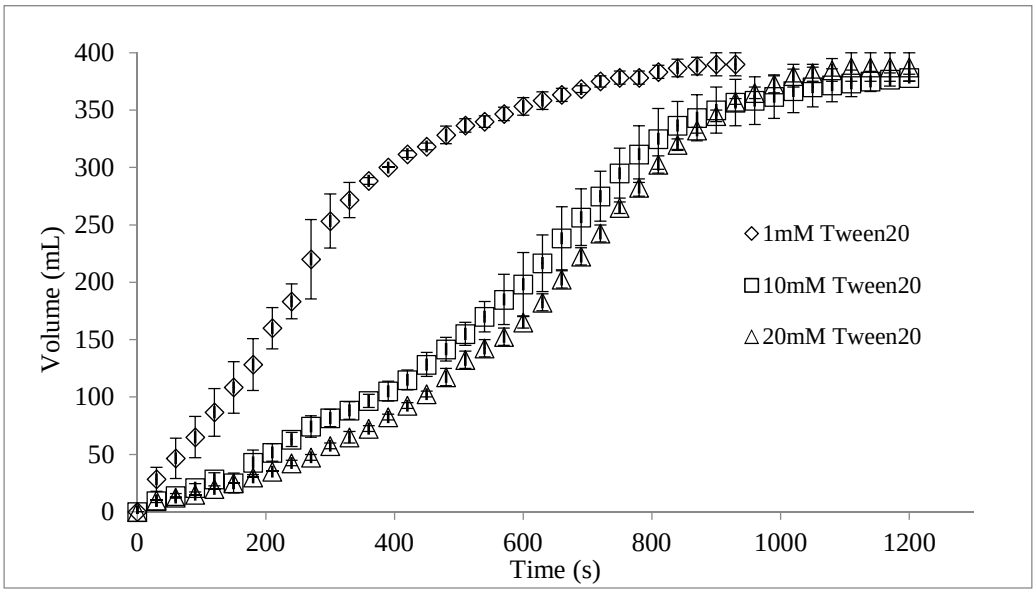


Figure C1: Drainage of liquid with time for different concentration of TWEEN20

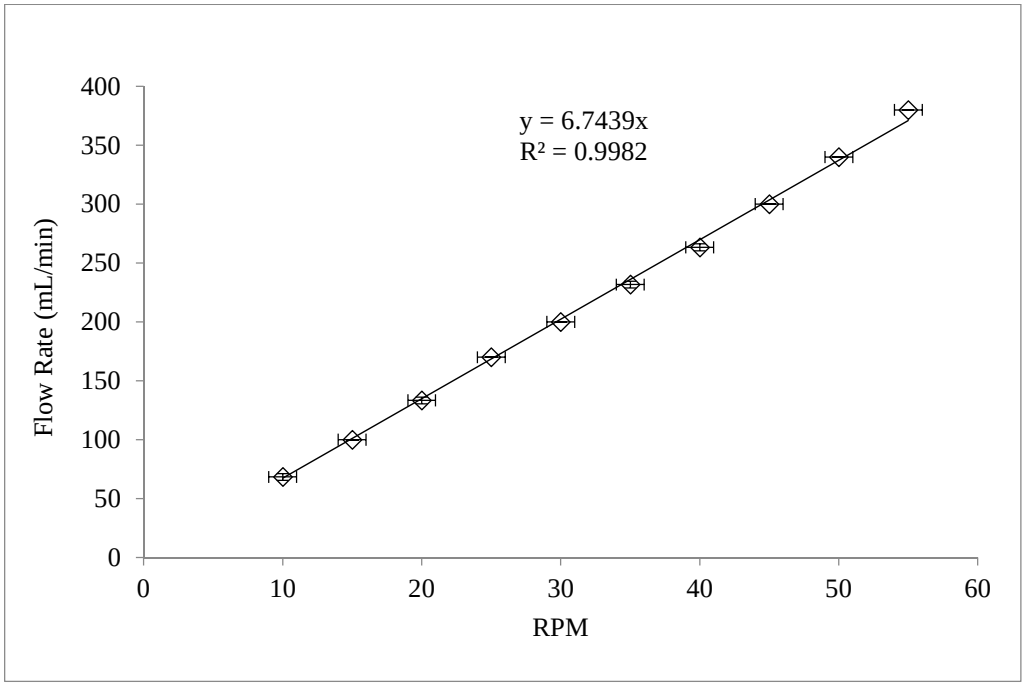


Figure C2: Calibration curve between flowrate (mL/min) to the function of RPM

Appendix D: Separation of grape pomace with CGA and storage study samples

Plate 1 A): The view of recovery of polyphenol from grape pomace extract using CGA by flotation column. B): The recovered phases; left: aphron and right: liquid phase.

Plate 2: The CGA and the control samples at day 0 (A) and day 32 (B)