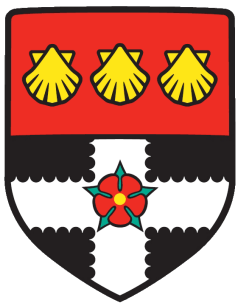

University of Reading

Recovery of polyphenols from agri-food by-products with whey protein stabilised colloidal gas aphrons

A thesis submitted as a partial fulfilment for the degree of Doctor of Philosophy

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June 2023

Declaration of authorship

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

Sincerely,

Aimara Victoria De La Cruz Molina

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June 2023

Acknowledgments

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Un día a la vez,

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Aimara V. De La Cruz-Molina, Catarina Gonçalves, Mafalda D. Neto, Lorenzo Pastrana, Paula Jauregi, Isabel R Amado. Whey-pectin microcapsules improve the stability of polyphenols bioactivity from grape marc extract during digestion. Journal of Food Science.

Poster presentation

Encapsulation of polyphenols by protein based encapsulants, presented at the 8th International Symposium on "Delivery of Functionality in Complex Food Systems". Porto, Portugal, July 2019.

Oral presentation

Whey-pectin nano-spray dried particles protect natural antioxidants from gastrointestinal digestion, presented at the 3° BioIberoamerica: Ibero-American Congress on Biotechnology, Braga, Portugal, April 2022.

General Abstract

Valorisation of food waste and losses is of great importance to achieve sustainability and a way to allocate these materials at a higher level in the waste management hierarchy. Moreover, food by-products are sources of important molecules such as proteins and polyphenols. In the present study, colloidal gas aphrons (CGA), surfactant stabilized micro bubbles with separation properties, are generated for the first time from whey protein (CGA(WPI) and investigated for separation and stabilisation of polyphenols extracted from fruit processing by-products.

The capacity of whey protein to generate CGA was measured and compared against CGA made of Tween 20. Phenolic extracts were obtained using ethanol and methanol for comparison; CGA were then applied to hydroalcoholic extracts. The recovery of polyphenols, antioxidant activity and anthocyanins stability under storage were then investigated. Additionally, the encapsulation through nano spray drying and *in vitro* digestion of a raw grape marc extract using whey protein and pectin was also assessed.

The main outcome was the production of stable CGA(WPI), with comparable characteristics to those of CGA(Tween 20). The application of CGA to various hydroalcoholic extracts showed that the type of polyphenols and surfactant influenced the recovery yields, especially anthocyanins, possibly due to near-neutral pH. CGA(WPI) and CGA(Tween 20) displayed a stabilisation effect for most of the evaluated anthocyanins. Both, separation and stabilisation, could be linked to the hydrophobic interactions occurring between surfactants and polyphenols, as well as hydrogen bonding in the case of CGA(WPI). Finally, the encapsulation through nano-spray drying led to microparticles with reduced size compared to spray drying, and improved bioaccessibility of polyphenols after *in vitro* digestion.

In conclusion, CGA(WPI) demonstrated to be effective in the separation of polyphenols, and a novel way to use whey protein and fruit by-products. Moreover, it opens the perspective for CGA to be used as a pre-formulation step for the development of functional foods and nutraceuticals.

Chapter 1: Introduction

Abbreviations

ACE: Angiotensin Converting Enzyme

AOT: Sodium bis(2-ethylhexyl)sulfosuccinate

BSA: Bovine Serum Albumin

CGA: Colloidal Gas Aphrons

CGA(WPI): Colloidal Gas Aphrons made of Whey Protein Isolate

CTAB: Cetyltrimethylammonium bromide

EFSA: European Food Safety Authority

FAO: Food and Agriculture Organization

FODIAC: Food for Diabetes and Cognition

GAE: Gallic Acid Equivalents

GAE_{TPI}: Gallic Acid Equivalents measured by Total Phenol Index

GRAS: Generally Recognized as Safe

IBA: International Blackcurrant Association

ME: Malvidin Equivalents

SDG: Sustainable Development Goals

SDS: Sodium Dodecyl Sulfate

TPC_{FI}: Total Phenol Content by Folin Index

UN: United Nations

WHO: World Health Organization

1.1 Introduction to this thesis

Along the food supply chain considerable amounts of food waste and losses are reported, with agri-food industry considered as one of the industries that generates significant amounts of waste, since it involves the production, post-harvest, manufacturing, transport, storage, and commercialization of food (Moreno-González & Ottens, 2021). However, big losses are also reported post-commercialisation. Because of the problematic this conveys, target 12.3 from the 2030 Agenda for Sustainability and Development aims to halve per capita global food waste at the retail and consumer levels, as well as reduce food losses along production and supply chain, including post-harvest (United Nations, 2023).

To achieve this goal, great efforts have been made for the valorisation of food waste and losses as part of the solution. The Waste Management Directive stablishes that waste can cease to be waste and give place to new materials what is known as by-products (Directive 2008/98/EC). In this sense, food by-products are of special interest since they are composed of parts with dense nutrient composition. For example, fibre, protein, and bioactive molecules (Schieber, 2017).

For example, food waste with the potential to turn into new materials are used as sources of polyphenols i.e., fruits that have gone through minimal processing, such as washing a packaging, and that during retail they have been discarded for several reasons. For example, fruits that have past their expiration date or show physical damage, but they could still be used and be safe for human consumption. Other examples include

unavoidable by-products generated during food manufacturing, such as fruit pomaces, that derive from the wine and juice manufacturing, constituted by skins, seeds, and stems, which are great sources of polyphenols are also constantly researched for the high amounts they constitute (Gómez-Mejía et al., 2019; Kapasakalidis et al., 2006; Li et al., 2020; Peixoto et al., 2018).

The first step to obtain the desired molecules is to extract them from the matrix and this is usually done with the help of organic solvents. The most common ones are methanol, acetone, ethanol, and their mixtures with water (Amendola et al., 2010; Brglez Mojzer et al., 2016; Spigno et al., 2007). However, when the application is directed for human consumption, safe solvents need to be used as not to represent a harm for the consumer, examples of this solvents are water and ethanol.

After the extraction, separation methods can be used to obtain richer fractions of bioactive molecules, for example colloidal gas aphrons (CGA). CGA are surfactant-stabilised microbubbles created from the intense stirring of the surfactant (>8,000 rpm) (Jauregi et al., 2000). These microfoams have been used for the separation and recovery of several products. For example, fine particles, proteins, colorants, and bioactive molecules such as, polyphenols (Alves et al., 2006; Fuda et al., 2005; Santos-Ebinuma et al., 2016; Spigno et al., 2015). So far, the use of CGA for the separation of polyphenols has been carried out only with synthetic surfactants. However, natural molecules with foamability properties offer the potential to be used for CGA generation. In this sense, proteins show similar properties to low molecular weight surfactants, such as foamability and low interfacial tension.

Proteins are abundant in many foods, and in some food by-products, like whey. This by-product has been widely used for its protein quality. CGA separation has been carried out with ionic (Dermiki et al., 2009; Spigno et al., 2010), non-ionic (MohdMaidin et al., 2018, 2019), and natural compounds with surfactant like properties (Kazemi et al., 2022; Saleem et al., 2019). The use of food grade surfactants, like polysorbates and sorbitans, and potentially, proteins, broadens the application of CGA into other areas such as food i.e., functional foods and nutraceuticals. In this way CGA can be investigated as a method that not only can extract polyphenols but can also facilitate their formulation and stabilisation.

As mentioned before whey proteins possess the capacity to form stable foams (Davis & Foegeding, 2007). Additionally, they have been widely studied for their nutritional value as well as their capacity to interact with other molecules such as polyphenols (Jauregi & Welderufael, 2010; Minj & Anand, 2020). The interactions between whey proteins and polyphenols have been exploited to create stable conjugates and complexes using different methods such as encapsulation (El-Messery et al., 2020; Oancea et al., 2018). The encapsulation has proven to be effective in the stabilisation and protection of polyphenols against pH, heat, oxidation, and *in vitro* digestion, for example (Guo et al., 2017; Guo & Jauregi, 2018).

1.2 Aim and Specific Objectives

This study aims to investigate the use of whey protein for CGA generation and its further application for separation, recovery, and encapsulation of polyphenols extracted from fruit processing by-products. The application of whey protein for CGA generation and polyphenols separation is investigated here for the first time. In addition, the encapsulation of polyphenols from grape marc with whey protein through nano spray drying was also investigated. The structure of this thesis and specific objectives are described below:

Chapter 1: Introduction and literature review: A brief introduction related to the current research on the use of fruit by-products as a source of polyphenols is presented. The use of whey proteins for stabilisation/protection of polyphenols through methodologies such as colloidal gas aphrons and encapsulation were reviewed, and research gaps were identified.

Chapter 2: Methods: A description of the shared methods used in Chapters 3, 4, 5, and 6 are presented.

Chapter 3: Extraction of polyphenols from different fruit by-products, and their relevance to FODIAC project: Introduction to the FODIAC project as well as the relevance of the selected fruit by-products as sources of polyphenols. Followed by phenolic extraction using conventional solid-liquid extraction with ethanol and methanol. The extracts were characterised in terms of polyphenols content and antioxidant activity by different

methods. The differences or similarities between them were discussed. The extracts obtained from fruit processing by-products (grape marc, goji berry skins, and blackcurrant pomace) were used in Chapters 4 and 5.

Chapter 4: Whey proteins as a surfactant for CGA generation and separation of polyphenols from blackcurrant pomace: In this chapter, the use of whey protein for CGA generation was evaluated since no previous studies have assessed this. Different concentrations and whey protein purities were taken into consideration for this. Once stable CGA made of whey protein (CGA(WPI) were obtained they were compared to those made of Tween 20 (CGA(Tween 20) and applied for the separation of polyphenols from blackcurrant pomace extract. This chapter served as a basis to determine the protein content needed to obtain stable CGA, and as proof of principle that CGA (WPI) could be used to recover polyphenols successfully from a raw fruit processing by-product.

Chapter 5: Separation of polyphenols from different phenolic composition feedstocks with CGA(WPI) and their stabilisation effect on anthocyanins: In this chapter, CGA(WPI) and CGA(Tween 20) were applied for the separation of polyphenols from extracts with different polyphenols composition (grape marc and red goji skin extract). The aim of this chapter was to determine if the separation with each surfactant could be influenced by the type of polyphenols in the extract. Additionally, the stabilisation effect of CGA(WPI) and CGA(Tween 20) on anthocyanins from grape marc extract during accelerated storage conditions was investigated.

Chapter 6: Whey protein and pectin encapsulation of polyphenols from natural extracts: In this chapter the use of whey protein and pectin for the encapsulation of raw grape marc extract was carried out with the aim to investigate if the encapsulation improved the polyphenols bioaccessibility. Moreover, the cytotoxicity and cellular antioxidant capacity of raw grape marc extract were also evaluated.

Chapter 7: General conclusions and future work: In this chapter the main outcomes from the study are described. Encountered limitations and recommendations for future studies are also presented.

1.3 Literature Review

1.3.1 Polyphenols classification and health benefits

According to the WHO, a healthy diet for adults should include five portions (400 g) of fruit per day to prevent malnutrition and a range of non-communicable diseases (World Health Organization, 2020). The benefits are attributed mainly to their type of macromolecules as well as the bioactive molecules found in them, such as polyphenols. Polyphenols are secondary metabolites in plants that comprise a vast family of molecules, with more than 8,000 reported to date. (Manach et al., 2004; Tsao, 2010). Its structure is composed by aromatic rings with several hydroxyl groups, and this structure is responsible for their bioactivity such as antioxidant, anti-inflammatory, and antimicrobials (Almajano et al., 2008; Nikbakht et al., 2021; Van de Velde et al., 2018). Moreover, polyphenols have shown the capacity to modulate the activity of some enzymes and cell receptors (Fraga et al., 2019).

Polyphenols are divided in different groups depending on their structure, such as phenolic acids, that can be classified as: hydroxycinnamic or benzoic acids, tannins (hydrolysable or condensed), stilbenes, and flavonoids; examples of these structures are shown in Figure 1.1. Among the vast number of polyphenols, flavonoids are the most extensive group, with around 4000 molecules identified (Tsao, 2010). Flavonoids are divided in subgroups, the most common types found in fruits are flavanones, flavones, flavan-3-ols, and anthocyanins; the latter being one of the most studied type of flavonoids (Panche et al., 2016). Flavonoids are of particular interest because they are associated

with health benefits, especially those related to the management of metabolic syndrome (Dias et al., 2022). For example, they have been attributed the capacity to increase glucose uptake, and insulin secretion, as well as to inhibit glucose production in *in vivo* models (Caro-Ordieres et al., 2020).

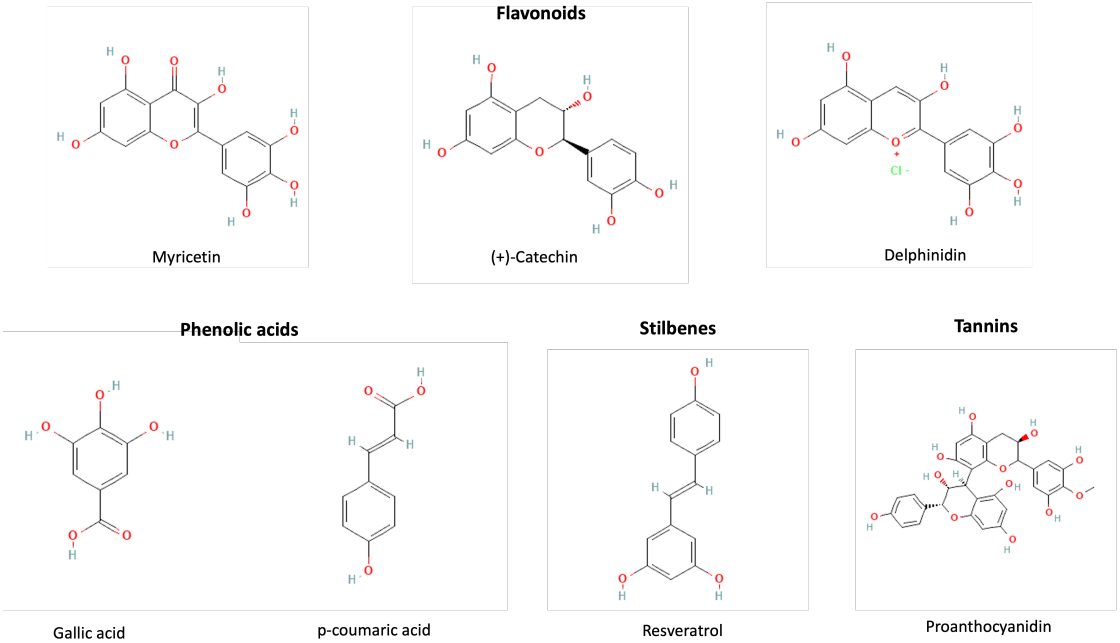

Figure 1.1. Main classification of polyphenols with examples and structures. Own creation figure with structures taken from Pubchem database (https://pubchem.ncbi.nlm.nih.gov/).

Some of the most researched flavonoids are anthocyanins, which are composed of a main anthocyanidin structure (aglycone) with carbohydrate residues and in some cases acyl group(s) in the third position of the C ring. In most instances anthocyanins are derived from six main anthocyanidin structures, with differences being the group found in positions 3' and 5' (Figure 1.2) (Andersen & Jordheim, 2010). Anthocyanins are responsible for the deep red and blue colour in most fruits; for example, berries, currants, and grapes have the highest anthocyanin content. Pigments from anthocyanins have been isolated from these fruits, especially grapes and currants, for use as colourants in

the food industry. They are approved by the European Commission and European Parliament under the E-163 name and recognized by the European Food Safety Authority (EFSA).

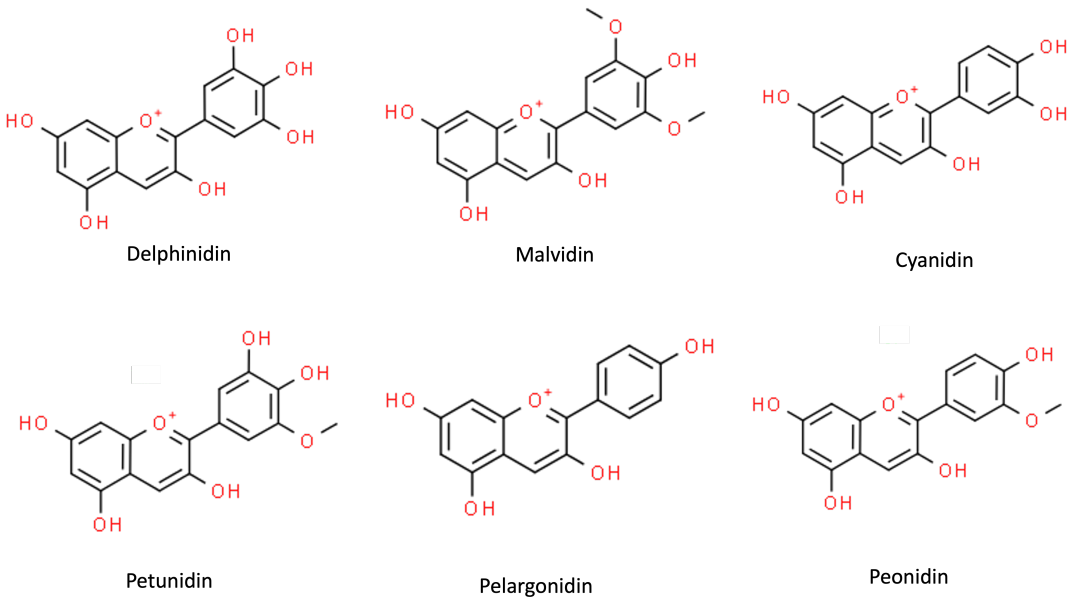

Figure 1.2. Primary anthocyanidins structures. Own creation figure with structures taken from ChemSpider database (www.chemspider.com).

Moreover, anthocyanins have been used in clinical trials to determine their effect on different diseases. Meta-analysis on clinical trials shows that the consumption of anthocyanins aid in the reduction of vascular inflammation markers related to cardiometabolic diseases, and the effect was more prominent when doses of >300 mg were administered (Fallah et al., 2020; Yang et al., 2017). These were administrated via capsules containing extracts rich in anthocyanins, pulverized extracts or fruits reconstituted in water, as well as purees, juices, and smoothies. Other randomised clinical trials have shown that the consumption of anthocyanins is linked to improvements in cognitive function in older adults (Devore et al., 2012; Kent et al., 2022).

1.3.2 Valorisation of fruit-based side-streams and by-products as source of polyphenols

In 2021, FAO reported a fruit production of 883 million tons, which accounts for an increase of 54% compared with the production 20 years ago (FAO, 2021). However, one of the main problems that this carry is the food loss and waste generated from their surplus and manufacturing. FAO's definition on food waste refers to the decrease in the quantity or quality of food resulting from decisions and actions by retailer, food services and consumers. On the other, had food loss is referred to the decrease in quantity or quality of food resulting from decisions and actions by food suppliers in the chain, excluding retail and food service providers and consumers (FAO, 2019). According to the FAOSTAT website a loss of more than 50% of fruits and vegetables is reported along the whole supply chain (FAOSTAT - www.fao.org/platform-food-loss-waste/flw-data/en/). In this context, agri-food industry is considered one of the industries that generates significant amounts of food is loss in here, since it involves the production, post-harvest, manufacturing, and commercialization (Moreno-González & Ottens, 2021).

Furthermore, United Nations stablished the 2030 Agenda for Sustainable Development composed of 17 goals, known as SDGs, as plan of action for the people, planet, and prosperity. It was stablished as the 12 SDG to ensure sustainable production and consumption, in specific SDG 12.3 aims to reduce by half the per capita global food waste at the retail and consumer levels, as well as losses along the production and supply chain, including those generated after post-harvest (United Nations, 2023). Hence, finding ways to tackle food loss and food waste is essential to achieve this purpose.

According to the waste management hierarchy (Fig 1.3) established by the Waste Framework Directive, waste can cease to be waste and become a secondary raw material, such as by-products (Directive 2008/98/EC). In this sense the avoidable and unavoidable materials generated along the supply chain have the potential to become by-products giving place to new products and materials (Leppänen et al., 2021; Schieber, 2017). Whilst waste prevention is the best option, when this is not achieved the goal should be always to leave disposal as last resort since this means send the waste to landfill.

Figure 1.3. Waste management hierarchy pyramid; taken from European Commission website (https://environment.ec.europa.eu/topics/waste-and-recycling/waste-framework-directive_en).

Several efforts have been made to valorise agri-food by-products, as they have proven to be sources of proteins, fibre, and bioactive molecules such as polyphenols (García-Becerra et al., 2016; Oancea et al., 2018; Sridhar & Charles, 2019). The valorisation of these by-products has been extensively researched in recent years, finding applications in several industries helping to move towards circular economies; since the aim of this production and consumption model is to extend the life cycle of products, implying waste

reduction and recycling whenever is possible, creating further value (European Commission COM(2020)98). Valorisation of these by-products could also lead to cost-effective and sustainable processes.

Across the agri-food industry different by-products are generated depending on the chain stage and type of processing. For example, berries, such as strawberries, blueberries, and raspberries are used in different food manufacturing processes. These berries are turned into jams, juices, purees, dried fruit, and added to foods such as yoghurt and bakery products. In this sense, their manufacturing does not generate large amounts of by-products because in most cases the whole fruit is in the final product. However, during their commercialisation, fruit waste generated from retail is often overlooked, or not handled in ways that allow for their further use.

On the other hand, there are other types of berries such as currants, whose production will be mostly for selected manufacturing processes, such as drinks. In the UK alone, approximately 8,000-11,000 tons of blackcurrant are produced annually (International Blackcurrant Association (IBA), 2021), mainly to be processed as juice. The manufacturing of blackcurrants into juice generates a by-product known as black currant pomace, the main component of which is currants skin. Industry has adopted some techniques to valorise this pomace since it amounts to several tons per year. Moreover, blackcurrant pomace has shown to be rich in fibre and polyphenols; therefore, it has been studied as an additive in foods such as meat, pasta, and bakery products (Diez-Sánchez et al., 2019; Quiles et al., 2018; Schmidt et al., 2018).

Compared to berries and currants, grapes generate larger amounts of side-streams and by-products. This fruit is one of the most produced fruits worldwide (73.5 million tonnes per year), and while grapes are a popular consumed as fresh or dry fruits, more than half of the world's production is destined for wine (OIV, 2020). In 2020, it was estimated that 165 million hectolitres of wine were produced in Europe, the largest wine producer in the world (FAO and OIV, 2016). Therefore, the production of wine generates millions of tons of a by-product known as grape pomace or grape marc.

During wine production approximately 20 to 25% of the grape utilised for wine making will end as grape marc. This by-product consists mainly of skin, stalks, seeds, and remaining pulp remaining after the crushing and pressing of the grapes to obtain their juice (Chowdhary et al., 2021; Gómez-Brandón et al., 2019; Sinrod et al., 2021; Spigno et al., 2017). Grape marc valorisation has been of great importance to avoid the underutilised material ending in landfills.

Moreover, its valorisation aids in overcoming environmental concerns, such as leaching and air pollution due to the incineration process (Chowdhary et al., 2021; Devesa-Rey et al., 2011; Galanakis, 2012; Spigno et al., 2017). Therefore, extensive research has been conducted to take advantage of this by-product. Some studies have focused on its use as bioethanol, vermicompost, extraction of grape seed oil, and colourants (Cortés et al., 2020; Farru et al., 2022; Mileva et al., 2023), with special interest in the extraction of polyphenols (Amendola et al., 2010; MohdMaidin et al., 2018; Pintać et al., 2018; Spigno et al., 2013).

Overall, the valorisation of the afore mentioned fruit by-products could lead to a substantial reduce of waste by the recycling and reuse (SDG 12.5). Additionally, their valorisation avoids for them to be disposed, and allowing them to fit in above levels of the waste management hierarchy pyramid (Fig 1.3).

1.3.3 Solvent extraction of polyphenols

Prior to their application and formulation, polyphenols must be extracted from the matrix in which they are embedded. This is an essential process that has been widely researched with the aim of obtaining the highest yield in an efficient and cost-effective manner. A key factor in the extraction of polyphenols is solvent selection, the principle of which is that polyphenols will be transferred from the matrix to the solvent according to their affinity based on their polarity (Gil-Martín et al., 2022). Most polyphenols have moderate polarity and can therefore be extracted with the help of solvents such as ethanol, methanol, acetone, and their combinations with water (Herrero et al., 2012). For less polar polyphenols, solvents such as ethyl acetate, chloroform, hexane, among others have been used. In both instances, aqueous mixture solutions of the solvents are recommended as they allow water to hydrate the food matrix (Belščak-Cvitanović et al., 2018).

However, in food, it is important to consider the type of solvent in terms of its toxicity since they cannot represent any potential harm to the consumer. Solvents like ethanol, acid buffers, water, and their combinations have been used in food because they are

generally recognised as safe (GRAS). Moreover, some of these solvents have the particularity of being consider “green solvents” for their low impact on the environment, health, and safety (Byrne et al., 2016; Capello et al., 2007). For polyphenol-based foods, ethanol is a solvent that meets these two criteria. Hence, its extensive use for the extraction of polyphenols.

Other parameters are of high importance in the extraction of polyphenols are the solvent-to-solid ratio, pH, temperature, and time as they will have a direct effect on the solubility and mass transfer (Herrero et al., 2012; Zwingelstein et al., 2020). Moreover, important attention must be paid to these parameters since changes in pH and temperature can have a detrimental effect on polyphenols. For example, the structure of anthocyanins is susceptible to changes near neutral pH. While at acidic pH they present their most stable structure. Figure 1.4 shows the changes in the structure of anthocyanins.

When the pH is <3, the flavylium cation of anthocyanins is in its stable form, whereas a pH above this can lead to two main scenarios. First, the opening of the pyrilium ring of the flavylium cation leads to hydration in the 2-position forming hemiketal and chalcones (highlighted in yellow). On the other hand, the proton transfer reactions related to the hydroxyl groups ionise the quinoidal bases (highlighted in purple and blue) (Andersen & Jordheim, 2010a; Cabrita et al., 2000; Vidana Gamage et al., 2022).

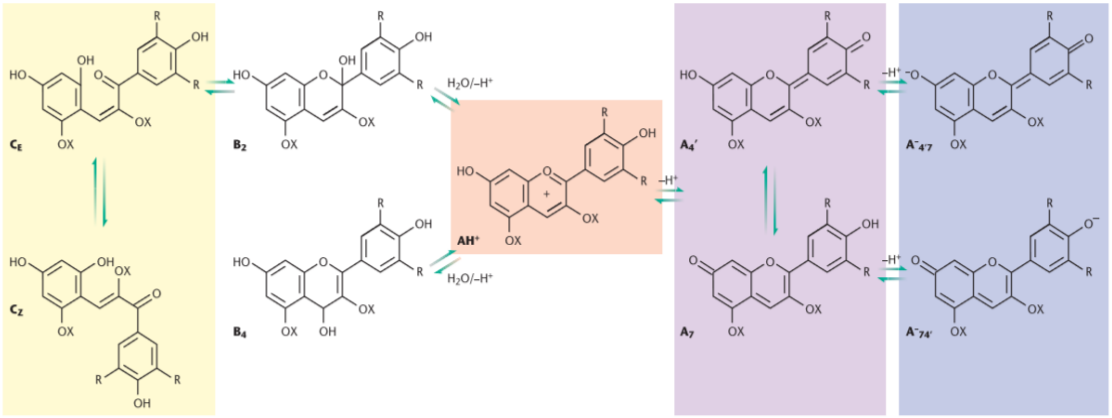

Figure 1.4. Change in structure of anthocyanins as a result of pH changes; taken from Andersen & Jordheim (2010)

Conventional solid/liquid extraction methods have been used for many years now, with the aim to release different polyphenols based on their solubility. These methods are simple, efficient, and affordable, hence their popularity (Gil-Martín et al., 2022). However, the main drawback is that a large amount of solvent is required, some of which are harmful to the environment. Therefore, the development of non-conventional methods has emerged.

Examples of non-conventional methods include microwave assisted and ultrasound extraction (Álvarez et al., 2016; Bonfigli et al., 2017; Garrido et al., 2019; González-Centeno et al., 2015; Hoskin et al., 2022; Natolino & Da Porto, 2020). These methods focus on the use of specific types of energy to aid the extraction of polyphenols from the cell wall in a more efficient manner, using less time and solvent. However, these methods have been noticed for some drawbacks such as cost, safety, and difficulty in scaling up.

Other methods such as supercritical fluid extraction and ionic liquid extraction focus more on the solvent rather than the applied energy (Aresta et al., 2020; Lima et al., 2017;

Ventura et al., 2017). These methods are of interest since green solvents can be used; for example, the non-toxic supercritical carbon dioxide and water are the most common for the supercritical fluid extraction. Supercritical carbon dioxide is a good solvent for polyphenols since it can extract non-polar and some polar low molecular weight compounds (da Silva et al., 2016; Gallego et al., 2019). In the case of ionic liquids there is vast number of molten salts that can be used. They have low combustibility and good solvation properties for polar and non-polar compounds (Lima et al., 2017; Martins et al., 2017; Ventura et al., 2017). Yet, some limitations apply to these methods, such as the specific (and expensive) equipment for supercritical fluid extraction and the low range of safe molten salts in the case of ionic liquids.

More recently, the extraction of polyphenols has moved in the direction of more sustainable methods such as natural deep eutectic solvents (NADES) extraction (Zannou & Koca, 2022), and surfactant-based methods (Dabetić et al., 2020; Sazdanić et al., 2023). These methods share similarities to the conventional ones as they are simple, efficient, and low-cost. Moreover, these two methods have the particularity that green solvents safe for human consumption can be used. NADES, such as choline chloride, sugars and polyols eutectic mixtures have been investigated for phenolic extraction giving good extraction yields (García-Roldán et al., 2023). The success of the extraction will be based in parameters such as density, viscosity, pH, temperature, and polarity of the eutectic mixture (Koh et al., 2023).

On the other hand, surfactant extraction has proven to be efficient in the extraction of polyphenols with surfactants such as Tweens, Brijs, poloxamers, and Triton X-100

(Sazdanić et al., 2023), due to the capacity of these surfactants to solubilize water soluble and insoluble molecules; thus, extracting them from the matrix in which they are imbedded. While these two methods show promise, the main challenge is the high number of combinations lead to a difficult tuning and optimisation of the process. Although, there is a vast number of methods, conventional ones such as hydroalcoholic solid-liquid extraction continue to be the most used in the extraction of polyphenols for food applications.

1.3.4 Colloidal gas aphrons (CGA) for separation of polyphenols

Following the extraction of polyphenols, enrichment, fractioning, and purification can been carried out using a range of methods. Among them are membrane processing, like microfiltration and ultrafiltration, adsorption technology, and colloidal gas aphrons (Abbott et al., 2020; Kelly et al., 2019). Colloidal gas aphrons (CGA) are surfactant-stabilized microbubbles (10-100 μm) generated by the intense stirring of a surfactant solution at high speeds (>8000 rpm) (Jauregi et al., 2000). CGA are composed of multilayers of surfactant molecules; these surfactant molecules adsorb at the interface with hydrophilic heads towards the aqueous phase and the hydrophobic tails towards the gas phase (Fig 1.5).

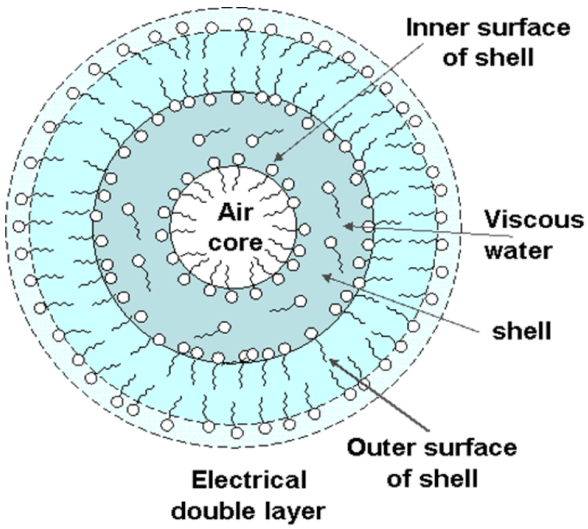

Figure 1.5. Proposed structure of CGA by Sebba, (1987).

Because of their unique structure CGA present important properties, such as:

- Adherence of molecules to the surfactant bubble surface. In the separation process, this property of CGA plays a key role as adherence is what gives CGA their selectivity in the separation and relies on the differences in interactions between the surfactant in CGA and the mixture components. The separation process can be driven by electrostatic and/or hydrophobic interactions between the surfactant bubbles and the molecules. The extent of such interactions depends on some aspects such as charge and hydrophobicity of the surfactant. The surface of the aphrons can be modify by using different surfactants. The outer surface can be negatively (ionic surfactant), positively (cationic surfactant) or non-charged (non-ionic surfactant) (Dermiki et al., 2009; Fuda & Jauregi, 2006; Jarudilokkul et al., 2004; Spigno et al., 2015).

- Higher stability than conventional foams. For their multilayer structure, when two aphrons collide, the force might not be enough to break the barrier of the layers, as opposed when two bubbles of conventional foam collide (Jauregi et al., 2000).
- The buoyancy of the encapsulated gas leads to an easy separation of the aphron phase from the bulk liquid.
- Small size of bubbles, this results in a larger interfacial area per unit volume and thus a larger capacity of adsorption of molecules. The size depends on the concentration and type of surfactant, ionic strength, and the presence of other molecules (Jauregi & Varley, 1998).

Their characteristics and their ease to pump from one point to another makes them particularly interesting as a separation method (Figure 1.6). Moreover, they offer the advantage to be an easily scalable and a cost-effective process depending on the surfactant used (Jauregi & Dermiki, 2010; Prasad et al., 2015).

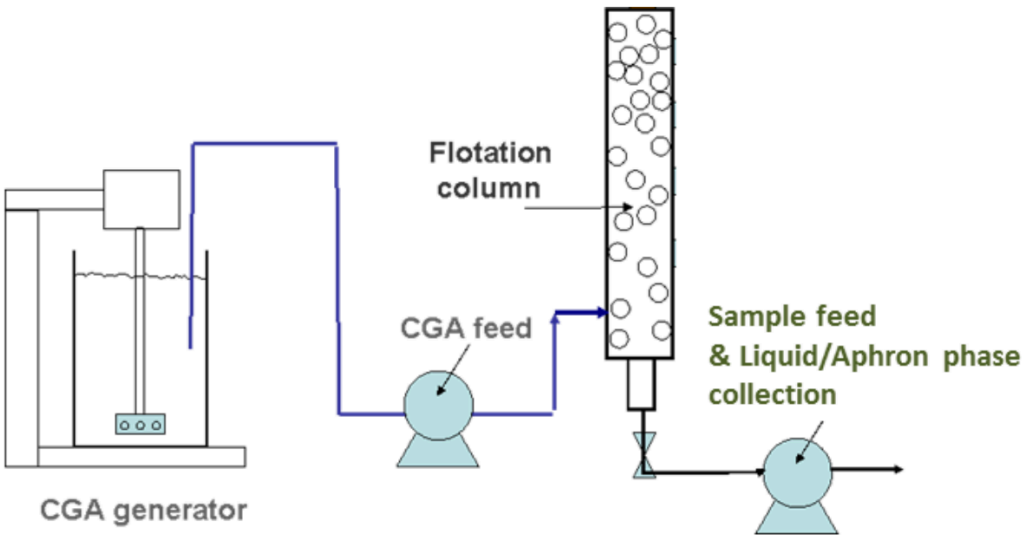

Figure 1.6. CGA separation process, taken from Dermiki et al. (2010)

So far most of the research in CGA has been on synthetic surfactants such as the anionic surfactant sodium bis(2-ethyl hexyl) sulfosuccinate (AOT), the cationic surfactant cetyltrimethylammonium bromide (CTAB) and the non-ionic Tween 20, and Tween 60 (Dermiki et al., 2009; Jauregi et al., 2000; Spigno & Jauregi, 2005). CGA separation using theses these surfactants have been used from different purposes such as fractionation, recovery, and purification (Dermiki et al., 2008; Fuda et al., 2005; Spigno et al., 2015). Fuda et al. (2004) used CGA made of AOT for the selective separation and purification of lactoferrin and lactoperoxidase from sweet whey by modifying the pH and ionic strength of AOT. The results show that CGA can be a promising technique, with the potential to obtain good recoveries in a cost-effective manner compared to the current techniques. However, before they can be used as such, many parameters need to be optimised as to obtain the highest recovery possible.

CGA have been also used for the fractioning and purification of natural phenolics derived from grape marc. Spigno et al. (2015), investigated the separation of gallic acid, caffeine, malvidin and quercetin from Pinot grape marc extract using CGA made of Tween 20 [10mM]. The results showed that the recovery of gallic acid was between 41 and 76%, for caffeine 55-79%, for malvidin 25-76%, and for quercetin 54-79%. The results showed that it is possible to apply CGA for the fractionation of natural extracts. Other studies carried out using CGA, show their versatility to be applied for the recovery of bioactive molecules, a summary of these studies is shown in Table 1.1.

Table 1.1. Studies on the separation of bioactive molecules using CGA, types of interaction and recoveries.

Bioactive molecules	Surfactant	Type of interactions	Recovery	Reference
β-lactoglobulin	CTAB [2mM]	Electrostatic	80-90%	Fuda et al. (2005)
Lactoferrin and lactoperoxidase	AOT [100mM]	Electrostatic	90%	Fuda et al. (2004)
Carotenoids from *Phaffia rhodozyma*	CTAB [2mM]	Electrostatic	97% from cell clarified solution	Dermiki et al. (2008)
Astaxanthin (hydroalcoholic solution)	CTAB [2mM], SDS [2mM], Tween 60 [20mM]	Electrostatic and Hydrophobic	65-70% with Tween; 40-55% with CTAB; 40-45% SDS	Dermiki et al. (2009)
Gallic acid from grape marc extract	CTAB [1-10mM]	Electrostatic	GAE_{TPI}: 20-70%	Spigno et al. (2010)
Grape marc polyphenols from Barbera grape pomace	CTAB [1mM] and Tween 20 [10mM]	Electrostatic and hydrophobic	GAE_{TPI}: 57-63%; ME_{538}: 57-80% - with CTAB GAE_{TPI}: 55-70%; ME_{538}: 40-65% - with Tween 20	Spigno et al. (2015)
	Tween 20 [10mM]	Hydrophobic	TPC_{FI}: 76-78%	Dahmoune et al. (2013)
	Tween 20 [10mM]	Hydrophobic	TPC_{FI}: 74-79%; ME: 77-85%	MohdMaidin et al. (2018)
	Tween 20 [20mM]	Hydrophobic	30-50%	Carullo et al. (2022)
Artichoke polyphenols	CTAB [1mM] and Tween 20 [10mM]	Electrostatic and hydrophobic	TPC_{FI}: Up to 64% with Tween 20, and up to 37% with CTAB	Noriega et al. (2018)

TPI: Total Phenol Index measured at 280 nm.
TFI: Total Folin Index: using the Folin-Ciocalteu method.
ME_{538}: Absorbance measured at 538 nm.

CGA application can be wide, not only for separation but could be used as formulation step. A recent study demonstrated that polyphenols from grape marc separated through CGA made with Tween 20, enhance the inhibitory activity of polyphenols against collagenase and elastase. These enzymes are involved in ageing processes (MohdMaidin et al., 2018). Furthermore, surfactants have shown the capability to improve polyphenols solubility, thus possibly their bioavailability and absorption. In the same context of formulation, CGA have been applied for the stabilisation of anthocyanins during storage conditions. The results showed that CGA made of Tween 20 delayed the degradation of anthocyanins from degradation and stabilise their pigments (MohdMaidin et al., 2019).

1.3.5 Whey protein

Whey is obtained during cheese production as a by-product and contains approximately 20% of the proteins in milk. Whey comprises 70% lactose, 10-14% protein, 9% minerals, 4% fat and 3% lactic acid (Jauregi & Welderufael, 2010; Yalçin, 2006; Zandona et al., 2021). Further processing will involve protein concentration by membrane filtration and spray drying with products containing up to 90% protein. Whey protein consists mainly of five globular proteins: β-lactoglobulin (50-60%), α-lactalbumin (15-25%), bovine serum albumin (6%), immunoglobulins (<10%) and in less percentage lactoferrin, lactoperoxidase, and proteose-peptones (de Wit, 1998; Fuda & Jauregi, 2006; Jauregi & Welderufael, 2010). These proteins present interesting properties, such as, solubility over

a wide range of pH and surfactant properties, such as the ability to form stable foams and emulsions (Pernell et al., 2002).

Whey utilisation has been of great interest since legislation on whey disposal has been implemented in some countries. Whey is considered a highly polluting by-product; therefore, notable efforts have gone into developing sustainable ways to reuse and add value to it. This by-product has now been used for different purposes ranging from functional foods and beverages to plastics, biofilms, and bioethanol are some examples (Zandona et al., 2021). However, most of whey is destined for human consumption since depending on the purity it can be considered a high-quality protein source.

In the food sector, whey proteins have gained considerable attention since they have shown the potential to act as dietary antioxidants (Adjonu et al., 2013; Athira et al., 2014; Le et al., 2015). Their antioxidant capacity, though, will vary depending on the whey treatment. Preheat treatment and hydrolysis of whey proteins have been reported to increase their antioxidant capacity compared to native whey (Corrochano et al., 2018). Moreover, peptides obtained by enzymatic hydrolysis of whey proteins have a range of bioactivities such as ACE inhibitory, antimicrobial, and opioid activity (Chamata et al., 2020, 2021; Jauregi & Welderufael, 2010; C. Zhao & Ashaolu, 2020).

Beyond the health properties of whey, this by-product is widely used for its techno-functional properties, such as foamability and emulsification (Gupta & Prakash, 2017; Ramos et al., 2017). Foamability properties of whey protein differ to those from other low molecular surfactants since their formation does not rely solely on charge or rapid

diffusion of the emulsifier at the interface. In the case of whey protein, the globular proteins absorb at the interface creating a viscoelastic film through intramolecular (Bos & Van Vliet, 2001; Foegeding & Davis, 2011; Wilde et al., 2004). The mechanical properties of the film will depend on the type of protein, strength of the interactions and concentration (Alves et al., 2022; Bos & Van Vliet, 2001; Hu et al., 2019). Studies show that the use of whey proteins in combination with bioactive compounds can bring stability in emulsions (Esfanjani et al., 2015), as well the protective effect of whey proteins against loss of antioxidant activity of resveratrol (Guo & Jauregi, 2018). Moreover, studies have assessed the use of polyphenols to improve the foamability properties of whey (Davis & Foegeding, 2007; Diaz et al., 2022). Additionally, whey proteins have been used to reduce astringency in wines (Jauregi et al., 2016).

1.3.6 Whey protein and polyphenols interactions

Whey proteins and polyphenols systems have been widely studied and great efforts have gone into the formulation of these systems. In this sense, findings can divide into two categories, i) the formation of conjugates via covalent interactions and ii) the formation of complexes via non-covalent interactions (Figure 1.7). The studies on whey protein-polyphenol complexes show that the interactions will vary depending on the type of proteins, protein concentration, type, structure and concentration of polyphenols, pH, and temperature (Ozdal et al., 2013).

Particular interest has been put into understanding how certain polyphenols influence the binding with whey proteins. Studies have shown that β-lactoglobulin complexed with

malvidin 3-O-glucoside, the main anthocyanin in grape marc, mainly through hydrophobic interactions and that the secondary structure of the protein was changed by this binding, resulting in a decrease of alpha-helix turns and an increase in beta-sheets (Z. He et al., 2016). In the case of alpha-lactalbumin, some studies have been carried out binding this protein to EGCG, and the findings show that the driving interactions are hydrophobic (Al-Hanish et al., 2016). Similar results have been seen when looking at the complexation of kaempferol and alpha-lactalbumin, where molecular docking results showed that out of five binding sites, three were found in the hydrophobic cavity of the protein (Diao et al., 2021). In the case of BSA, most of the studies agree that hydrogen bonding is driving these interactions (Frazier et al., 2006, 2010; Skrt et al., 2012).

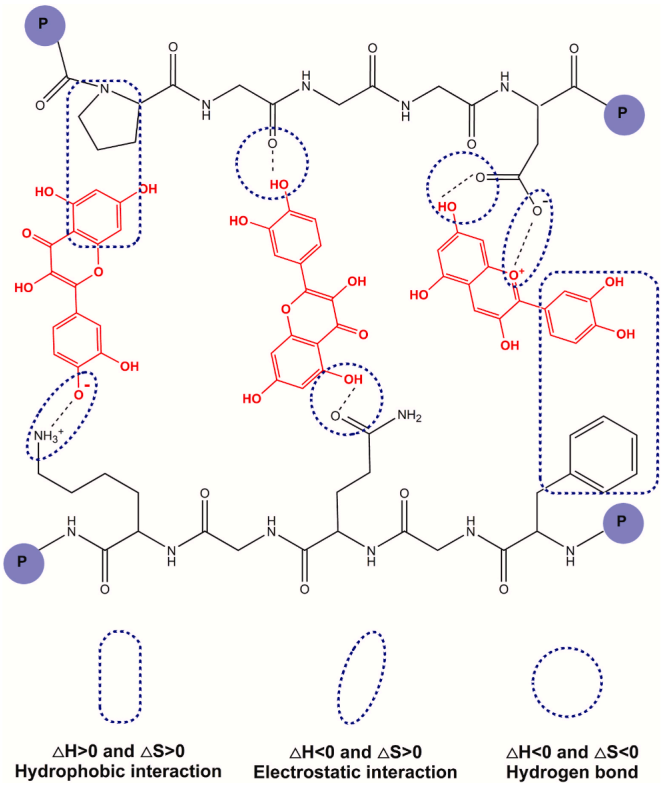

Figure 1.7. Non-covalent interactions between polyphenols and proteins. Taken from Li et al. (2021).

Most studies agree that the binding will be affected by three main aspects: size, glycosylation and number of hydroxyl groups in the C ring. Studies have shown that low molecular weight polyphenols have less binding affinity to whey proteins than bigger ones. In terms of glycosylation, it has been seen that polyphenols with more glycosides have less affinity than those that do not possess a sugar moiety. Finally, a decrease in hydroxyl group in the C ring of polyphenols will lead to a lower binding affinity (Cao & Xiong, 2017; Frazier et al., 2003; Skrt et al., 2012). When looking at the interactions occurring with specific compounds and whey protein isolates, it has been seen that in the case of petunidin 3-O-glucoside and whey protein isolate, they bind mainly by hydrogen bonding and van der Waals forces, which was corroborated by molecular docking (Gong et al., 2021).

1.3.7 Protection and formulation of polyphenols with whey proteins

The complexation of whey protein and polyphenols have been exploited for different purposes, one of them being encapsulation. These proteins have been extensively used for encapsulation, by for example, spray drying, and other emerging encapsulation methods such as nano spray drying, electrospinning, and electrospraying (Arpagaus, 2019; Hoskin et al., 2019; Pérez-Masiá et al., 2015; Wen et al., 2017; Yadav et al., 2020). Moreover, whey protein has been used in conjunction with surfactants and polysaccharides as nanocarriers for encapsulation of water soluble as well as insoluble bioactives (Maqsoudlou et al., 2020). For example, the delivery of curcumin loaded in nanoemulsions of whey protein and Tween 80 (Sari et al., 2015).

Encapsulation has been widely used for the preservation of polyphenols for applications in various industries. In food, encapsulation has been used with the aim of protecting polyphenols from processing and storage conditions as well as the targeted delivery of polyphenols in the colon (Silva et al., 2020). Research has shown that polyphenols have a beneficial effect on the gut microbiota, improving barrier function and inhibiting the pathogenesis of various gastrointestinal diseases (Zhang et al., 2021; Zhao & Jiang, 2021). The encapsulation of polyphenols with whey protein in combination with other materials such as pectin has been studied to this end (Assadpour et al., 2017; Esfanjani et al., 2015; Thongkaew et al., 2014).

Moreover, encapsulation with whey proteins has been studied to preserve and enhance other properties, such as alpha-amylase inhibitory activity (Wu et al., 2021), and cell antioxidant capacity (Kreatsouli et al., 2019). Although the encapsulation of polyphenols has been extensively studied, there is still room for improvement. The development of new technologies and analyses is a step closer to the fabrication of particles with a higher chance of achieving their particular goal, such as the effective release of polyphenols in the colon (Assadpour & Mahdi Jafari, 2019; S. Jafari et al., 2023; Maqsoudlou et al., 2020; Zhang et al., 2021).

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Chapter 2: Methods

Abbreviations

AAE: Ascorbic Acid Equivalents

AAPH: 2,2'-Azobis(2-amidinopropane) dihydrochloride

ABS control: Absorbance of the control

ABS sample: Absorbance of the sample

ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)

$ABTS^{\cdot+}$: monocation of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid

$AlCl_3$: Aluminium Chloride

AUC: Area Under the Curve

DPPH: 2,2-diphenylpicrylhydrazyl

Fe^{2+}: Iron (II)

Fe^{3+}: Iron (III)

FRAP: Ferric Reducing Antioxidant Power

g: gram

g/mol: grams per mol

HAT: Hydrogen Atom Transer

L/mol*cm: Litre per mol per centimiter

mg/mL: milligrams per millilitre

Na_2CO_3: Sodium Carbonate

$NaNO_2$: Sodium Nitrite

NaOH: Sodium Hydroxide

ORAC: Oxygen Radical Absorbance Capacity

pH: Potential of Hydrogen

SET: Single Electron Transfer

TE: Trolox Equivalents

TFC: Total Flavonoid Content

TMAC: Total Monomeric Anthocyanin Content

TPC: Total Phenol Content

TPTZ: 2,3,5-Triphenyltetrazolium chloride

µL: micro litre

µmol: micro mol

2.1. Introduction

In this chapter the common methods for the chapters are presented with a brief description of their principle and limitations.

2.2 Polyphenols analytical determinations

2.2.1 Total Phenol Content (TPC)

The total phenol content was determined by the Folin-Ciocalteu method (Singleton & Rossi, 1965). This method is based on the redox reaction between reducing molecules such as polyphenols, proteins, and sugars with the Folin-Ciocalteu reagent. In the reaction the blue chromogen is formed, and the coloration is proportional to the concentration of reducing substances. It is important to mention that this assay has been used as well to determine specific amino acids like tyrosine and tryptophan (present in whey protein) since these two have aromatic rings, like polyphenols, and have reducing properties (Folin & Ciocalteu, 1927). For this reason, other methods are needed to have reliable results regarding the content of polyphenols, especially in the presence of proteins and sugars. Folin-Ciocalteu has also been categorised as an antioxidant activity method, for its good correlation with other methods such as ABTS and DPPH (Lamuela-Raventós, 2018).

For the assay, 75 µL de Folin-Ciocalteu reagent [1:10] were added in a 96 well microplate, with 15 µL of the sample and 60 µL of 7.5% Na_2CO_3. For samples containing whey protein or Tween 20, 1mL of a 0.01% SDS solution was added to the stock of Na_2CO_3 to avoid

precipitation. The samples were incubated in the dark for 30 minutes after this time the microplate was read at 765 nm (FLUOstar Omega, BMG LABTECH, Offenburg, Germany), each sample was done in triplicate. The results were quantified from a Gallic Acid calibration curve ranging from 0.05 to 0.5 mg/ml, a new calibration curve was made for every experiment.

2.2.2 Total Monomeric Anthocyanin Content (TMAC)

Total monomeric anthocyanins levels were measured by the pH differential method (Lee et al., 2005). This assay is based on the reversible structural transformation monomeric anthocyanins can go under pH changes. Knowing that at pH 1 coloured oxonium is formed and at pH 4.5 colourless hemiketal is formed (Lee et al., 2005). Hence, the difference in absorbance is proportional to the concentration of pigment. Important to mention that degraded anthocyanins in their polymeric for are resistant to colour based on pH differences, so they are not measured with this assay (Lee et al., 2005; Mónica Giusti & Wrolstad, 2005). This method has shown good correlation with chromatography results such as HPLC, making it a good and efficient method for the quantification of monomeric anthocyanins (Lee et al., 2008). However, in the presence of protein or other substance that precipitates in the pH range of the study, special consideration needs to be taken, as the substances need to be diluted to not interfere, but anthocyanins still need to be in enough quantity to be measured.

For this, each sample was combined in a 1:20 ratio (v:v) with potassium chloride and with sodium acetate buffers (pH 1.0 and 4.5, respectively) in separate vessels. After an equilibration period (15 min), the raw absorbance of each solution was measured at 520 and 700 nm in a microplate reader (FLUOstar Omega, BMG LABTECH). The values were calculated with the following formula.

$$\text{Monomeric Anthocyanins} = \frac{A \times MW \times DF \times 1000}{\varepsilon \times 1}$$

Where:

- A= corrected absorbance value calculated as [($A_{520} - A_{700}$) pH 1.0 – ($A_{520} - A_{700}$) pH 4.5]
- MW= molecular weight of cyanidin 3-glucoside: 484.83 g/mol ; and malvidin 3-glucoside: 493.43 g/mol
- DF= dilution factor
- ε= molar absorptivity: cyanidin 3-glucoside: 26,900 L/ mol*cm; malvidin 3-glucoside: 28,000 L/ mol*cm
- 1= pathlength in cm

Results are expressed as milligrams of cyanidin 3-glucoside or malvidin 3-glucoside equivalents.

2.2.3 Total Flavonoid Content (TFC)

The total flavonoid content was measured based on the method by Zhishen et al. (1999) with some modifications. In this assay, the capacity of flavonoid to form chelates of Al (III)-flavonoids since flavonoids have high affinity to bind to metal ions like Al (III), this based on concentration and pH (Kasprzak et al., 2015). Additionally, the use of sodium

nitrite as nitrating agent with selectivity for phenolic hydroxyl groups (Shraim et al., 2021). However, the addition of sodium nitrite can bind with other compounds with hydroxyl groups, such as proteins. Moreover, this assay has been criticised for its variability depending on the reference flavonoid, and the assumption that all flavonoids in the sample have the same extension coefficient and absorption maxima (Shraim et al., 2021). It is still widely used, however, other analysis like chromatography should be considered to quantify these specific compounds.

For the analysis, 100 mL of the sample were added with 430 mL of the A solution (1.8 mL of 5% $NaNO_2$ with 24 mL of distilled water) and incubated for 5 minutes. Later 30 mL of 10% $AlCl_3$ were added and let it rest for 1 min. Finally, 440mL of solution B (12 mL of NaOH 1M with 14.4 mL of distilled water) were added. From this reaction 150 mL were translated to a 96 well microplate in triplicate. The samples were read at 496 nm in a microplate reader (FLUOSTAR OMEGA, BMG LABTECH). The absorbance was compared with a catechin standard curve ranging from 0.1 to 1 mg/ml, a new calibration curve was prepared for every experiment.

2.3 Antioxidant Capacity

Although there is a vast selection of antioxidant capacity methods, the ones used in this study have been selected for their mechanism, target radical(s), and applicability of the method to the selected samples. As well as for their wide use in food, i.e., phenolic extracts derived from fruits (Cano & Arnao, 2017). Generally, several methods are tested to evaluate the capacity of molecules by different radical scavenging mechanisms to

compare against each other and have a wider idea on how the samples reacts to different radicals (Lü et al., 2010; Nimse & Pal, 2015). It is important to mention that none of the applied methods can be extrapolated to *in vivo* biological systems. In fact, there is no method that allows for this, only cellular antioxidant activity and biomarker can give and indirect idea of how antioxidants scavenge radicals in *in vivo* systems (Sun et al., 2018; Yang et al., 2018). A description of each antioxidant method, mechanism, and relevance to this study is given below. For all the antioxidant activity assays a calibration curve was made every time the assay was performed.

2.3.1 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) - ABTS

ABTS, which stands for 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), is a widely used antioxidant method based on the scavenging activity of a compound to decrease the cation radical $ABTS^{\cdot+}$ (Cano and Arnao, 2017). The reaction mechanisms involved in this method are hydrogen atom transfer (HAT) and single electron transfer (SET). During HAT, the capability of a compound to quench free radicals by donating a hydrogen atom is measured. While, during SET, what is being measured is the capability of the compound to transfer and electron (Sun, Yang and Tsao, 2018).

ABTS method was selected since compared to others, it has the advantage to determine both hydrophilic and lipophilic compounds antioxidant capacity, and it can be performed in a wide pH range (Capanoglu et al., 2017). However, one of the main disadvantages is that this method cannot be correlated to *in vivo* activity since it not a radical found in nature and doesn't represent a physiological radical source (Cano & Arnao, 2017).

Moreover, especial consideration needs to be taken when analysing sample which contain protein as ABTS can give interference due to the capacity of proteins to donate hydrogen atoms. In fact, ABTS has been used to determine antioxidant activity in dairy products (Capanoglu et al., 2018). However, the activity is more prominent in hydrolysates of protein than its native form, since the hydrophobicity increases (Arranz et al., 2019; Corrochano et al., 2018).

The total antioxidant activity of all samples was measured by the ABTS assay at 734 nm, which was modified from Re et al. (1999). Briefly, the $ABTS^{\cdot+}$ stock solution was prepared by mixing a 5 mL of ABTS solution (7 mM, 50 mL volume, stored in an amber flask and kept under refrigeration at 0–4 °C up to one month) and 88 µL of potassium persulfate solution (140 mM, 10 mL volume, stored in an amber flask and dark place at room temperature for up to one month). Then the mixture was kept in the dark at room temperature for at least 16 h prior to use.

For the study of phenolic compounds, the working solution of the $ABTS^{\cdot+}$ was obtained by diluting the $ABTS^{\cdot+}$ stock solution with distilled water to an absorbance of 0.70 ± 0.02 measured at 734 nm. 5 µL of samples were added to 245 µL of $ABTS^{\cdot+}$ working solution, and the mixture was homogenized for 1 min in a vortex. The mixture was then incubated at room temperature in the dark for 5 min, and the absorbance (ABS_{sample}) was recorded at 734 nm, using a microplate reader (FLUOstar Omega, BMG LABTECH). The absorbance of $ABTS^{\cdot+}$ working solution was measured at the same wavelength and used as control ($ABS_{control}$). The scavenging activity of each sample on $ABTS^{\cdot+}$ was calculated

from a Trolox standard curve ranging from 0.02 to 0.4 mg/mLThe analysis was done in triplicate for each sample.

2.3.2 Ferric Reducing Antioxidant Power - FRAP

The FRAP method is based on the SET mechanism, were the capacity of a compound to reduce ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}) is measured by the change in colour during the reaction (Benzie & Devaki, 2017). In addition, FRAP is consider a relatively simple, quick, and inexpensive direct method. Moreover, it has the advantage to represent a common reaction that occurs *in vivo*, and the reaction in not affected by sugars and proteins; hence the selection of this method in the study. However, FRAP results cannot be extrapolated to *in vivo* biological systems and possible interference can occur if iron chelating substances are present in the sample, although they would have to be in high concentrations. Additionally, Fe^{+2} contamination that could lead to false higher values (Benzie & Devaki, 2017).

To measure the total antioxidant activity by FRAP, the methodology stablished by Benzie & Strain (1996) was used with minor modifications. In principle, the stock solution of the FRAP method goes as follows: 300 mM acetate buffer (pH=3.6, 2.699 g sodium acetate trihydrate and 16 ml (16.8 g) of glacial acetic acid dissolved in 1 l of deionized water), 2,4,6-tripyridyl-s-triazine (TPTZ) (10 mM) in 10 ml HCl (40 mM), and 20 mM ferric chloride hexahydrate aqueous solution.

The FRAP reagent was mixed then with 25 mL of acetate buffer, 2.5 mL of TPTZ and 2.5 mL of ferric chloride solutions. Then, 10 µL of the sample/standard were added to 300 µL of FRAP reagent in a microcentrifuge tube and vortexed for 10 s. Then 100 µL of this mixture, in triplicates, was transferred into the microwell plate (96well, NUNC, FB) and absorbance was measured at 595 nm (FLUOstar Omega, BMG LABTECH). Results were expressed as ascorbic acid equivalents (AAE), using an ascorbic acid standard curve: 0.02 to 0.2 mg/mL. Results were expressed as µmol of ascorbic acid equivalents per 100 grams of fresh weight (µmol AAE/ 100g fw) or 100 g dry weight (µmol AAE/ 100g dw). The analysis was done in triplicate.

2.3.3 2,2-diphenylpicrylhydrazyl - DPPH

In principle, DPPH, shares the same two mechanisms as the ABTS method, HAT and SET (Nenadis & Tsimidou, 2018). During the reaction the purple DPPH radical reacts with the antioxidant and its unpaired electron is stabilised, this is seen by the change in colour from purple to yellowish colour (Kedare & Singh, 2011). DPPH has the advantage to be a low cost, reproducible, applicable at ambient temperature, and has been accepted as an official method by the AOAC for food and beverage samples (AOAC SMPR 2011.011) (Sun et al., 2018). However, this radical is soluble in various organic solvent, but not water, meaning that certain compound like protein will precipitate thus is not a viable method for this kind of substances (Arnao, 2000; Nenadis & Tsimidou, 2018).

$DPPH^{\bullet+}$ assay was executed according to the method of Brand-Williams et al. (1995) with some modifications. The stock solution (600 µM) was prepared by dissolving 24 mg of DPPH in 100 mL of pure methanol and then stored at -20°C in the darkness. After, a

working solution (60 µM) was prepared by mixing 10 mL of the stock solution with methanol (90 mL), later the working solution was adjusted until final absorbance of 0.600 ± 0.100 at 515 nm. Briefly, 1.75 mL of the DPPH (working solution) was added to 0.250 mL of each corresponding extract. The reaction mixture was vortexed and kept at room temperature for 30 min in darkness. The absorbance was then measured at 515 nm using a UV spectrophotometer. Trolox was used as standard to prepare a calibration curve ranging from 0.003 to 0.04 mg/mL. Results were expressed as µmol of trolox equivalents per 100 g of fresh weight (µmol TE/ 100g fw). The analysis was done in triplicate.

2.3.4 Oxygen radical absorbance capacity assay (ORAC)

Whilst the previously mentioned methods measure the capacity of a compound or compounds to scavenge radicals by reducing them; ORAC measures the scavenging activity using a competitive reaction scheme (Huang & Tocmo, 2018). The advantage of ORAC is that during the reaction the antioxidant molecules in the sample compete with other known antioxidant to react with peroxyl radicals. The most common peroxyl radical sources are azo compounds like AAPH (2,2'-Azobis(2-amidinopropane) dihydrochloride), since they generate peroxyl radicals at a known rate (Dorta et al., 2018). Possibly the main drawback of this method is that it during the reaction other radicals are being formed and different mechanisms can be involved reflecting different results depending on the approach (Apak et al., 2016; Dorta et al., 2018). Moreover, it cannot be correlated to *in vivo* models.

ORAC assay was carried out according to the method designated by Dávalos et al. (2004). The reaction was carried out in phosphate buffer (75 mM, pH=7.4), using 20 µL of sample (corresponding dilutions) plus 120 µL of fluorescein (116.66 nM) in a black microplate (Nunc, Denmark) then the combination was preincubated (15 min, 37 °C). Later, 60 µL of 2,2'-azobis-(2-methylpropionamidine)-dihydrochloride (AAPH) (46.6 mM) were added and then incubated for 137 min at 40 °C. A totally of 104 measurements were read on a FlouSTAR OPTIMA fluorimeter (BMG Labtech, Offenburg, Germany) at 458 nm and 520 nm. A blank was made using phosphate buffer instead of the sample. The software used was a Fluostar Control version 1.32 R2. A calibration curve was made using Trolox (0.0002 to 0.0016 µmol TE/mL) as antioxidant standard and the results were expressed in µmol of trolox equivalents per 100 grams of fresh weight (µmol TE/ 100g fw). The analysis was done in duplicate for each extract.

Antioxidant curves were normalized to the curve of the blank corresponding to the same assay by multiplying original data by the factor $\text{fluorescence}_{\text{blank,t=0}}/\text{fluorescence}_{\text{sample,t=0}}$. By using the normalized curves, the area under the fluorescence decay curve (AUC) was calculated as:

$$\text{AUC} = 1 + \sum_{i=1}^{i=104} fi/f0$$

Where *f0* is the initial fluorescence average at 0 min and *fi* is the fluorescence media at time *i.* The AUC of each sample read was calculated by the following formula:

$$\text{AUC} = \text{AUC antioxidant} - \text{AUC control}$$

2.4. References

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Chapter 3: Extraction of polyphenols from fresh fruit by-products (FODIAC PROJECT), and fruit processing by-products

Abbreviations

AAE: Ascorbic Acid Equivalents

AAPH: 2,2'-Azobis(2-amidinopropane) dihydrochloride

ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)

ANOVA: Analysis of Varience

BCPE: Blackcurrant Pomace Extract

C_2C_3: Naphtalene

C3GE: Cyanidin 3 Glucoside Equivalents

CGA: Colloidal Gas Aphrons

CO_2: Carbon dioxide

DPPH: 2,2-diphenylpicrylhydrazyl

FDA: Food and Drug Administration

FODIAC: Food for Diabetes and Cognition

FRAP: Ferric Reducing Antioxidant Power

GAE: Gallic Acid Equivalents

GAE/100g fw: Gallic Acid Equivalents/ 100 grams of fresh weight

GME: Grape Marc Extract

GRAS: Generally Recognised as Safe

HCl: Hydrochloric acid

M3GE: Malvidin 3 Glucoside Equivalent

M3GE/100 g fw: Malvidin 3 Glucoside Equivalent per 100 grams of fresh weight

mM: micro molar

OH: Hydroxide

ORAC: Oxygen Radical Absorbance Capacity

QE: Quercetin Equivalents

QE/100g dw: Quercetin Equivalents/ 100 grams of dry weight

RGSE: Red Goji Skins Extract

SD: Standard Deviation

TFC: Total Flavonoid Content

TMAC: Total Monomeric Anthocyanin Content

TPC: Total Phenol Content

TPTZ: 2,3,5-Triphenyltetrazolium chloride

UCP: Universidade Católica de Portugal

µL: micro litre

µmol AAE/100g dw: micro mol of Ascorbic Acid Equivalents per 100 grams of dry weight

µmol AAE/100g fw: micro mol of Ascorbic Acid Equivalents per 100 grams of fresh weight

µmol TE/100g dw: micro mol of Trolox Equivalents per 100 grams of dry weight

µmol TE/100g fw: micro mol Trolox Equivalents per 100 grams of fresh weight

µmol TE/g dw: micro mol Trolox Equivalents per gram of dry weight

µmol TE/mL: micro mol Trolox Equivalents per mililitre

3.1 Introduction

It is known that a diet rich in fruits and vegetables has beneficial effects on health, this has led to an increase in their production and processing. However, globally, around half of these fruits and vegetables are lost or wasted along the supply chain, retail, and household level (FAO, 2019). Hence the importance to find ways to reuse and recycle the inputs generated. The European waste management directive (Directive 2008/98/EC) stablishes that waste can cease to be waste when this can be transformed into new materials, such as by-products. In this sense, food waste generated at retail level is of special interest. For example, fresh fruits that get to be discarded because they have passed their best before date, broken packaging, mild damage, but without signs of spoilage could be transformed into new materials instead of being wasted.

At food manufacturing level, the UK alone generates approximately 1.7 million tons of waste during the manufacturing of alcohol, soft drinks, and fruit juice (The Waste and Resources Action Program (WRAP, 2017). The principal by-product obtained by the previously mentioned industries is a pomace constituted by fruit peels, stems and seeds, and this pomace can represent up to 20% of the drink manufacturing. Research shows that fruit by-products are rich in fibre, protein, and bioactive compounds such as polyphenols, which make them an excellent source for the development of value-added products (García-Becerra et al., 2016; MohdMaidin et al., 2018; Oancea et al., 2018; Sessa et al., 2013).

For years polyphenols have drawn attention since their consumption is associated with beneficial effects on health (Naseri et al., 2018; Spínola et al., 2019). However, an essential step for the formulation is their extraction, a process that allows their separation and recovery from the matrix in which they are embedded. Polyphenols are classified based on their molecular structure which, will determine their polarity and solubility (Belščak-Cvitanović et al., 2018; Piccolella et al., 2019; Tsao, 2010).

For this reason, extraction is commonly carried out with the help of solvents like ethanol, methanol, acetone, and their combination with water (Herrero et al., 2012). The use of these solvents relies on their polarity and how this will enhance the mass transfer of polyphenols from the matrix based on their affinity (Gil-Martín et al., 2022). Methanol for example has a higher polarity than ethanol, and ethanol polarity is higher than the one of acetone. On the other hand, polyphenols polarity will be based on the number of benzene ring and hydroxyl group in their structures. The success of the extraction will depend on the balance of this two have in the solution (Galanakis et al., 2013; Gil-Martín et al., 2022; Paun et al., 2022)

In food however, it is important to consider the type of solvent and its concentration in terms of its toxicity since they cannot represent a potential harm for the consumer. The term used for solvents safe for human consumption is GRAS, which stands for Generally Recognized As Safe, this term was coined by the U.S. Food and Drug Administration (FDA), and has been used in an international context. In Europe those solvents that are safe for human consumption are regulated under the Directive 2009/32/EC. Among the safe for human consumption solvents that have been used for extraction of polyphenols are:

ethanol, CO_2, acetic acid, propylene glycol, ethyl lactate, triethylene glycol, and their mixture with water. These previously mentioned solvents have been used for the extraction of polyphenols (Castillo et al., 2022; García-Roldán et al., 2023; MohdMaidin et al., 2018); and while good yields have been reported, ethanol continues to be one of the most used for their capacity to extract a wide range of polyphenols.

The main extraction process parameters are solvent to solid ratio, contact time, temperature, and pH; these have a direct effect on the solubility and mass transfer of the extracted compound (Herrero et al., 2012; Zwingelstein et al., 2020). Many methods have been developed and optimized for the extraction of polyphenols by changing these parameters and applying different types of energy (Ameer et al., 2017; Ding et al., 2020; Gil-Martín et al., 2022). Although there is a vast knowledge on the extraction of polyphenols and techniques that continue developing (Chaves et al., 2020; Dabetić et al., 2020; García-Roldán et al., 2023), conventional solid-liquid extraction continues to be one of the most used methods due to the simple process and equipment it requires (Antony & Farid, 2022; Sridhar et al., 2021).

This work focuses on the extraction of polyphenols from fresh fruit discards and fruit processing by-products for two different purposes. One, is the valorisation of fresh fruit discards from retail as source of polyphenols to obtain extracts suitable for human consumption. This within the framework of the European project FODIAC (Food for Diabetes and Cognition (H2020-MSCA-RISE-778388)). This project aims to develop and integrate dietary solutions to tackle type 2 diabetes and mitigate cognitive function impairment among the elderly. On a different approach, hydroalcoholic extracts from

fruit processing by-products (supplied by FODIAC partners) were obtained to be used in further experiments involving the use of colloidal gas aphrons for polyphenols separation. It is important to highlight that while this work investigates the extraction of polyphenols, optimisation was not the aim.

3.2 Materials

Materials:

As part of FODIAC project academic and non-academic partners are involved, for this work all materials were supplied by non-academic partners. Berries and grapes discards were obtained from the FODIAC non-academic partner SONAE, one of the biggest retailers in Portugal, from its supermarket Continente. In the case of the fruit processing by-products, they were supplied by different FODIAC non-academic partners. 1) Winery Casa Emma (Italy) that after wine production, ends with different types of grape pomaces; some are processed and sold commercially as grape marc flour. 2) A&R House (UK), a company that processes different fruit pomaces, such as apple and blackcurrant pomaces, taking particular care in the preservation of their bioactive molecules. 3) EVRA (Italy), dedicates to the generation of extracts from different by-products from the Mediterranean area. The specifications of the fruit discards and fruit processing by-products are given below.

- Fresh blueberries, blackberries, raspberries, strawberries, and red grapes were supplied by SONAE's chain supermarket Continente (Porto, Portugal). The collected fruits were those past their best before date or with mild physical damage, but no evident spoilage signs.

- Dried blackcurrant pomace supplied by A&R House with the following characteristics: 2017 Blackcurrant pomace batch dried in a rotary dryer with a material minimum

passage through the system of 11 second at 97 °C; and the temperature of entering to the cooling of 85 °C.

- Casa Emma (Firenze, Italy) winery kindly supplied a sample of fresh frozen and vacuum packaged Sangiovese variety grape marc (skins, seeds, and stems) from the September 2019 harvest.
- Dried red goji berry skins (dried at 40-45°C) until moisture content below 5% was achieved) supplied from EVRA (Potenza, Italy).

Reagents:

Folin-Ciocalteu, ABTS (2,2′-azino-bis(3-ethylbenzothiazoline- 6-sulfonic acid) diammonium salt) ≥98.0%, DPPH (2,2-Diphenyl-1-picrylhydrazyl), TPTZ (2,4,6-tris(2-pyridyl)-s-triazine), AAPH (2,2’-azobis-(2-methylpropionamidine)-dihydrochloride), trolox (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid, 97% (238813), sodium carbonate, sodium nitrite, aluminium chloride, sodium hydroxide, hydrochloric acid, ferric, potassium chloride, sodium acetate, potassium persulfate, methanol and ethanol, ≥99.8% were purchased from Sigma-Aldrich.

3.3. Methods

3.3.1. Extraction of polyphenols from berries and grapes

The extraction of polyphenols from berries and grapes was carried out at the Catholic University of Porto (UCP) (FODIAC academic partner), Portugal with the purpose to obtain extracts rich in polyphenols, particularly anthocyanins to be used in further stages of the FODIAC project.

For this, two different batches of each fruit (grapes, strawberry, raspberry, blackberry, and blueberry) were homogenized and frozen for at least 24 h. After this, the fruits were allowed to thaw to facilitate the breakage of the cell wall. The extraction was done following the methodology by Ribeiro et al. (2020), and Oliveira et al. (2016), with some modifications. Briefly, each fruit was extracted in a 1:6 ratio (fruit:solvent) with 50% acidified ethanol (0.01% HCL), and 80% acidified methanol (HCl 0.01%); and homogenized for 1 min at 19,000 rpm in an Ultra-Turrax (IKA, Germany). The extracts were shaken at 1800 rpm for 1 hour at 25°C in an orbital shaker. Then, the extracts were centrifuged at 5000 rpm for 15 min at 4°C. The pellet and supernatant were stored at -20°C for further analysis. Additionally, and ethanolic extract composed of a mix of berries and grapes was carried out with the previous methodology using acidified ethanol. Each extract was made in duplicate.

3.3.2. Extraction of polyphenols from blackcurrant pomace, grape marc, and goji berry skins

The extraction of polyphenols from fruit processing by-products was carried out at the University of Reading, (FODIAC academic partner) UK, with the purpose to obtain extracts rich in polyphenols for further studies on CGA application, covered in the next chapters of this thesis.

For blackcurrant pomace and grape marc, a solvent extraction was applied to solubilise the polyphenols using a food grade solvent following the methodology developed previously by (MohdMaidin et al., 2018). The blackcurrant pomace and grape marc extraction was carried out in an 8:1 ratio (solvent:solute) using a hydroalcoholic solution of 60% ethanol under magnetic stirring for two hours at 60°C. After the extraction the solids were separated through vacuum filtration using #1 Whatman paper. The extract was stored at -18 °C for further analysis. The extraction was carried out in duplicate and analysed in triplicate. For the red goji berry skins, the extraction was carried out using a hydroalcoholic solution of 60% ethanol in a 15:1 ratio solvent:solute under magnetic stirring for 40 minutes at 60°C (conditions stablished by the supplier). After this, the solids were separated through vacuum filtration using #1 Whatman paper. The extract was stored at -18 °C for further analysis and CGA processing. The extraction was carried out in duplicate and analysed in triplicate.

3.3.3. Extracts characterisation

Since the extractions were carried out at different times and locations, polyphenols analytical determinations were carried out in different ways. The procedure of each method is described in Chapter 2, in section 2.2.

For extracts obtained from berries and grapes:

- Total Phenol Content (TPC): expressed in milligrams of gallic acid equivalents per 100g of fresh weight (mg GAE/100g fw).
- Total Monomeric Anthocyanins Content (TMAC): milligrams of cyanidin 3-glucoside or malvidin 3-glucoside equivalents per 100g of fresh weight (mg C3GE or M3GE/100g fw).

For extracts obtained from fruit processing by-products:

- Total Phenol Content (TPC): expressed in milligrams of gallic acid equivalents per 100g of dry weight (mg GAE/100g dw).
- Total Monomeric Anthocyanins Content (TMAC): milligrams malvidin 3-glucoside equivalents per L (mg M3GE/L).
- Total Flavonoid Content (TFC): milligrams of catechin equivalents per 100g of dry weight (mg CE/100g dw).

3.3.4. Antioxidant Capacity

Similarly, different antioxidant capacity methods were carried out depending on the time and location the extractions took place. The procedure of each method is described in Chapter 2, in section 2.3.

- For extracts obtained from berries and grapes: ABTS: values expressed as µmol of trolox equivalents per 100 grams of fresh weight (µmol TE/ 100g fw).
- DPPH: values expressed as µmol of trolox equivalents per 100 g of fresh weight (µmol TE/ 100g fw).
- ORAC: values expressed in µmol of trolox equivalents per 100 grams of fresh weight (µmol TE/ 100g fw). The analysis was done in duplicate.

Note: It is important to mention that due to time limitations it was not possible to assess the antioxidant activity of the mixed fruits hydroalcoholic extract.

For extracts obtained from fruit processing by-products:

- ABTS: values expressed in micro moles of trolox equivalents per 100 g dry weight (µmol TE/ 100g dw)
- FRAP: values expressed in micro moles of ascorbic acid equivalents per 100 g dry weight (µmol AAE/ 100g dw).

A diagram of the extraction process for fresh fruits (berries and grapes), and fruit processing by-products with the solvents and characterisation is shown in Figure 3.1.

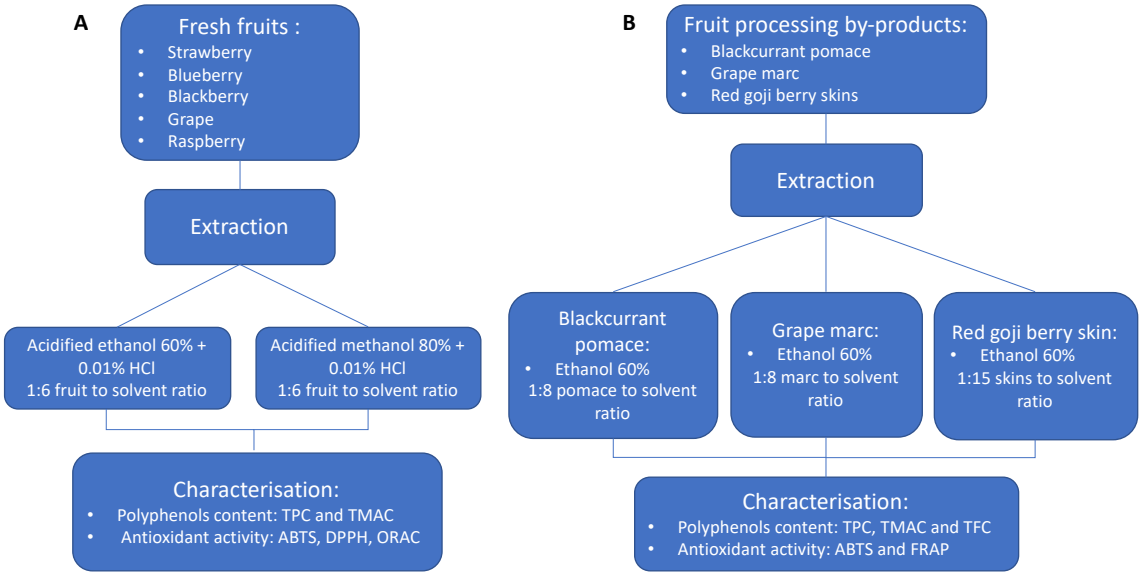

Figure 3.1. Diagram of the methodology used in this chapter for (A) fresh fruits, and (B) fruit processing by-products.

3.3.5. Statistical Analysis

Extractions were performed in duplicate and analysed in triplicate, except ORAC, which was analysed in duplicate. Differences in the extraction solvent used in berries and grapes was done applying the independent t-test analysis. For differences between berries and grapes, and fruit processing by-products the data was subjected to the One-Way ANOVA analysis where statistical differences were noted by the Tukey test. A Pearson's correlation test between polyphenols and antioxidant activity by ABTS and DPPH of the extracts from berries and grapes was carried out. The significance level was defined at $p<0.05$. The results were reported as means ± SD. All statistical analysis was done using IBM® SPSS® Statistics 27 software.

3.4 Results and Discussion

3.4.1. Berries and grapes polyphenols extraction

The total phenol content (TPC) of the extracts made of fruits from retail using methanol and ethanol is displayed in Figure 3.2. The results show that from all the analysed fruits, blackberry displayed the highest TPC with 317.66 mg GAE/ 100 g fw when using ethanol as solvent ($p<0.05$). Interestingly, blackberry methanolic extract was not significantly different than any of the berries ($p>0.05$), apart from strawberry that showed the lowest TPC among all fruits (131.09 mg GAE/100g fw) ($p<0.05$). Followed by blackberry, raspberry ethanolic extract displayed a TPC of 287 mg GAE/100g fw, while the methanolic extract had a content of 244.49 mg GAE/100g fw ($p<0.05$). For blueberry and grape similar values were found with ethanol, for both fruits no significant differences were detected between the solvents ($p>0.05$). Lastly, strawberry extracts showed the lower TPC among all the analysed fruits. However, a higher TPC was obtained with ethanol than methanol, 153.23 mg GAE/ 100 g fw versus 131.09 mg GAE/ 100 g fw, respectively ($p<0.05$).

The overall results show that for most of the fruits analysed, ethanol was a better solvent than methanol to extract TPC. Moreover, for the rest of fruits in which ethanol was not significantly better, it proved to be equal to methanol ($p<0.05$). The differences between solvents could be attributed to the polarity of compounds found in the selected fruits and their affinity to the solvents. These particular fruits (berries and grapes) are known to be rich in flavonoids such as anthocyanins and flavonols, as well as phenolic acids which are

moderately polar (Bucolo et al., 2018; Higbee et al., 2022; Pap et al., 2021). In this sense the extraction with ethanol probably benefited the most as its polarity is lower than methanol. The higher TPC values for some fruits, indicate that ethanol polarity is enough to allow the solubilisation of more polyphenols than methanol. Moreover, it is possible the acidification of the solvents was beneficial in the extraction since it allows polyphenols to be in a more acidic medium in which their structures are more stable (Gil-Martín et al., 2022; Sagar et al., 2018; Sridhar et al., 2021).

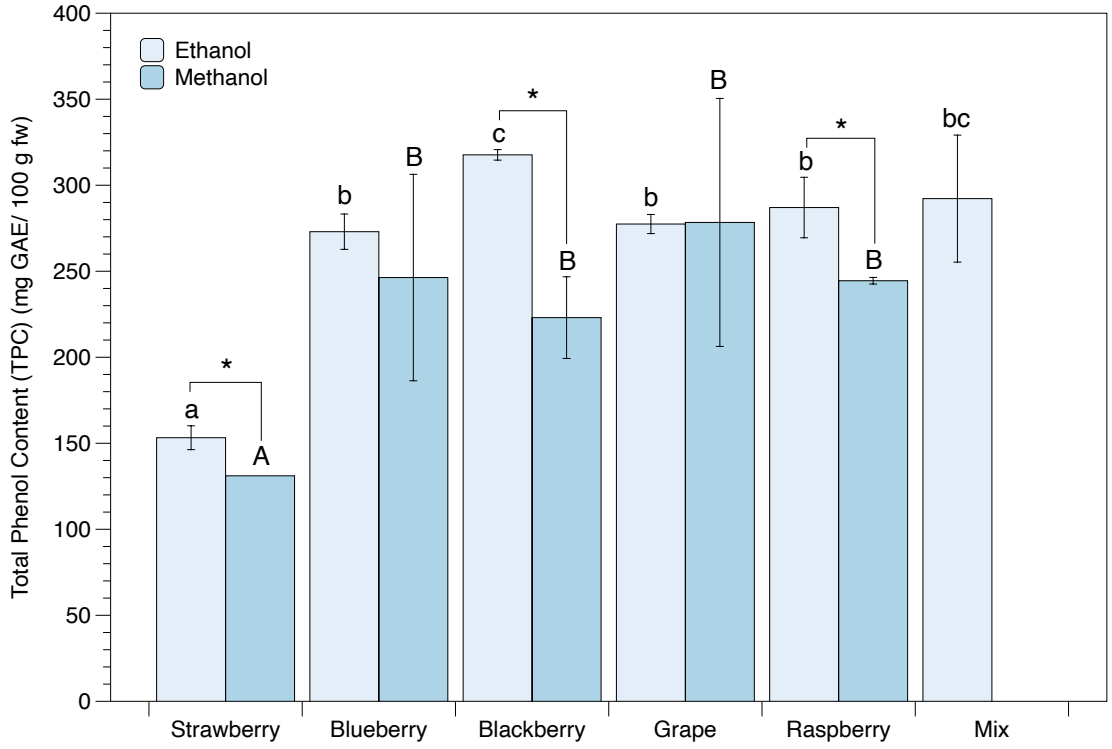

Figure 3.2. Total phenol content of fresh berries and grapes extraction with ethanol and methanol. Values are expressed in mean ± sd. *Denotes significant differences between solvents for the same fruit ($p<0.05$). Lower case letters denote significant differences between fruits using ethanol ($p<0.05$), uppercase letters denote significant differences between fruits using methanol ($p<0.05$).

After analysing the TPC content of the single fruits, it was decided that an extract containing a mix of the fruits could give relevant results. For the extraction the

methodology described in section 3.3.1, the extraction was carried out using equal parts of the single fruits. The results show that the extract had not only high content of TPC (292 mg GAE/ 100 g fw), but it was higher than all the single fruits ($p<0.05$), except for blackberry ($p>0.05$).

Regarding the total monomeric anthocyanin content (TMAC) (Fig. 3.3), the fruit with the highest TMAC was blueberry with both solvents ($p>0.05$); however, for ethanolic extractions blackberry showed to be equal as blueberry ($p>0.05$). Grape ethanolic extracts followed the highest TMAC values with 26.54 mg M3GE/100g fw, while its methanolic counterpart showed no significant differences between raspberry and blackberry. Moreover, for raspberry the use of ethanol or methanol did not have a significant effect ($p<0.05$) with values of 19.41 and 20.39 mg C3GE/100g fw for ethanol and methanol respectively. Strawberry was the fruit with less TMAC ($p<0.05$); with ethanol extracting more anthocyanins than methanol, 4.46 and 4.09 mg C3GE/100g fw ($p<0.05$), respectively.

Lastly, it was observed that the mixed fruit ethanolic extract TMAC was low when compared with the other fruits, 4.22 C3GE/100g fw ($p<0.05$), contrary to what was observed in the TPC. Overall, similar to what it was seen in TPC, ethanolic extracts displayed higher TMAC than methanol for most of the fruits, and for the rest no significate differences were between ethanol and methanol ($p>0.05$).

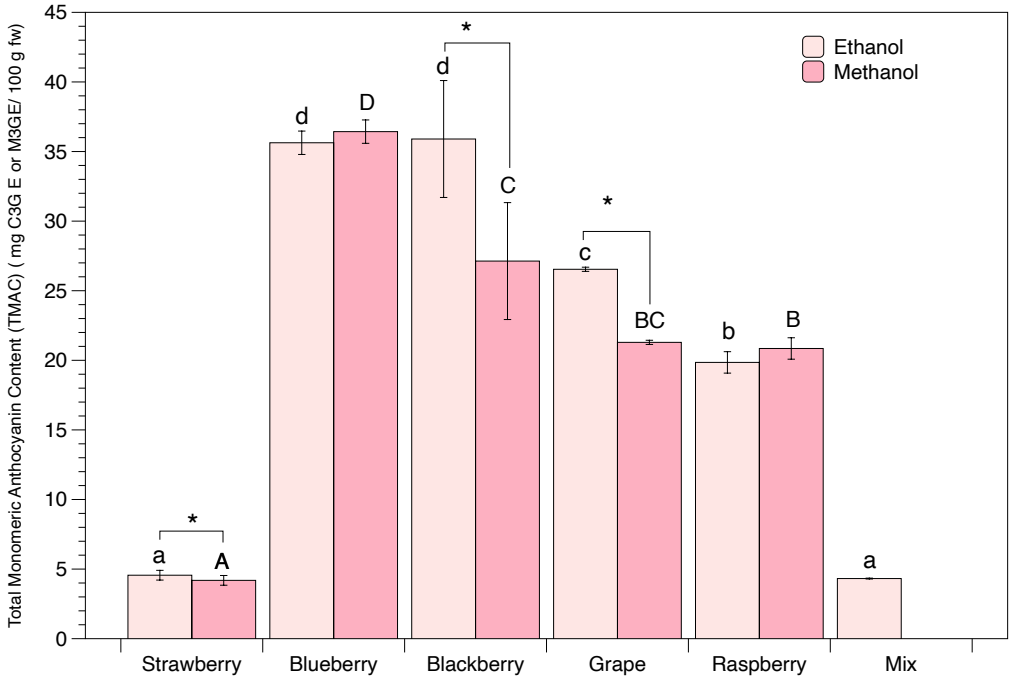

Figure 3.3. Total monomeric anthocyanin content of fresh berries and grapes extraction with ethanol and methanol. Values are expressed in mean ± sd. Strawberry, blackberry, raspberry values are expressed in cyanidin 3 glucoside equivalents, and blueberry and grape are expressed in malvidin 3 glucoside equivalents. *Denotes significant differences between solvents for the same fruit ($p<0.05$). Lower case letters denote significant differences between fruits using ethanol ($p<0.05$), and upper case letters denote significant differences between fruits using methanol ($p<0.05$).

Berries and grapes have been widely researched and their phenolic profile has been previously reported. A summary of the identified polyphenols in these fruits is shown in Table 3.1. It can be seen that in general, berries and grapes will have mainly flavonoids, especially glycosylated anthocyanins, hydroxybenzoic and hydroxycinnamic acids, flavonols, and flavan-3-ols. The phenolic profile reported is helpful to understand why ethanolic extractions were similar or better than methanol. For example, anthocyanins have more phenol groups and conjugations compared to phenolic acids. Flavonols as well will benefit from the extraction with ethanol, as they can have more than one aromatic

ring in their structure (Andersen & Jordheim, 2010a; Brglez Mojzer et al., 2016; Fu et al., 2016).

Table 3.1. Major phenolics detected by HPLC in the different fruit side-streams analysed in this chapter reported in literature.

Fruit	Major phenolics (detected by HPLC)	Reference
Strawberry	**Anthocyanins:** Cyanidin 3-O-glucoside, pelargonidin 3-O-glucoside	Mu¨ et al. (2004)
	Flavonoids: Quercetin, Kaempferol	Milosavljević (2020)
	Phenolic acids: Caffeic acid, ferulic acid, p-coumaric acid, ellagic acid	Kajd et al. (2011)
		Cervantes et al. (2020)
Blueberry	**Anthocyanins:** Delphinidin, malvidin, petunidin, cyanidin	Shibata et al. (2021)
	Flavonoids: Catechin, epicatechin, myricetin	Pico et al. (2022)
	Phenolic acids: Gallic acid, protocatechuic acid, chlorogenic acid, ellagic acid	Cervantes et al. (2020)
Blackberry	**Anthocyanins:** Cyanidin 3-O-glucoside, 3-rutinoside, delphinidin 3-xyloside, pelargonidin	Paun et al., (2022)
	Flavonoids: Catechin, epicatechin, myricetin, rutin	Zhu et al. (2023)
	Phenolic acids: Gallic acid, vanillic acid, protocatechuic acid, caffeic acid, syringic acid, ellagic acid	Gong et al. (2022)
		Oszmiański et al. (2015)
		Ivanovic et al. (2014)
		Jazić et al. (2019)
Grape	**Anthocyanins:** Malvidin, delphinidin, cyanidin, petunidin, peonidin, pelargonidin (glycosides)	Xu et al. (2011)
	Flavonoids: Catechin, epicatechin, myricetin, quercetin	Li et al. (2013)
	Phenolic acids: Gallic acid, vanillic acid, caffeic acid	MohdMaidin et al. (2018)
Raspberry	**Anthocyanins:** Cyanidin-o-sophoroside, 3-o-glucoside, 3-o-rutinoside; delphinidin and pelargonidin (glycosides)	Ponder & Hallmann (2019)
	Phenolic acids: chlorogenic acid, gallic acid, caffeic acid, ferulic acid, ellagic acid, *p*-coumaric acid	Teng et al. (2013)

	Flavonoids: quercetin, myricetin, kaempferol, rutin, catechin, epicatechin	Toshima et al. (2021) Teng et al. (2017) Chen et al. (2013)

3.4.2 Berries and grape extract antioxidant capacity

After the polyphenols determination the antioxidant capacity of the berries and grape extracts was assessed, the results are shown in Figure 3.4. For strawberry, the methanolic extract showed the highest antioxidant activity by ABTS 865.11 versus 706.87 µmol TE/100g fw ($p<0.05$) compared to the ethanolic extract, even though the ethanolic extract displayed a higher content of TPC and TMAC. For ORAC and DPPH, no significant differences were observed between solvents ($p>0.05$). Interestingly, the DPPH antioxidant capacity values were comparable to the ones of blueberry and grape methanolic extract by the same method ($p<0.05$), even though strawberry extracts displayed the lowest TPC and TMAC among all the fruits ($p>0.05$).

For blackberry, the ethanolic extract had the higher antioxidant capacity by ABTS and DPPH compared to the rest of the fruits 1777.27 and 1198.50 µmol TE/100g fw. As for ORAC, blackberry ethanolic extract did not show the highest capacity; however, the ethanolic extract displayed a significant higher activity than the methanolic extract, 5831.58 versus 4912.82 µmol TE/100g fw($p<0.05$).

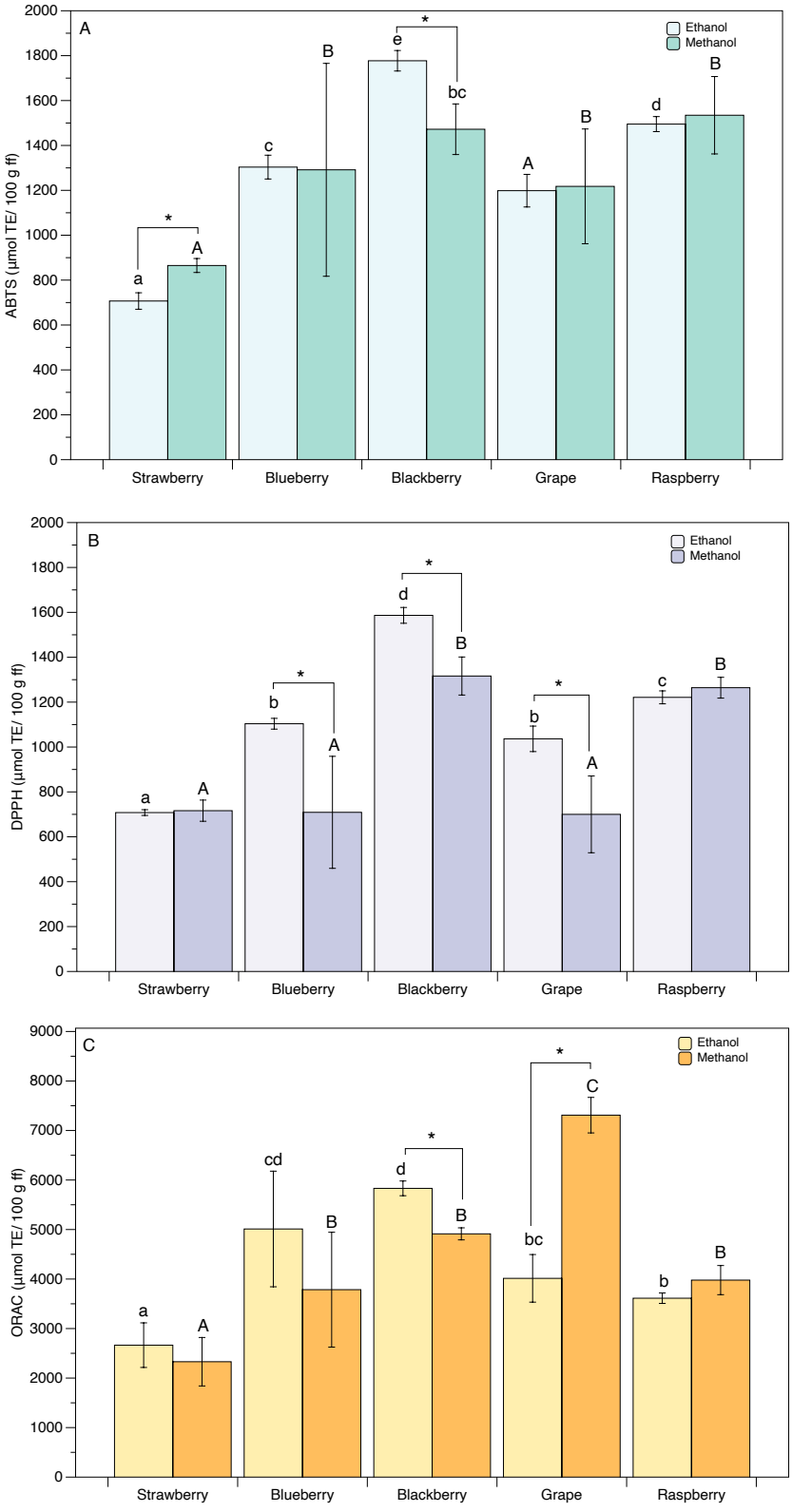

Figure 3.4. Antioxidant capacity by ABTS (A) DPPH (B) and ORAC (C) of berries and grape ethanolic and methanolic extracts. Values are expressed in mean ± sd. *Denotes significant differences between solvents ($p<0.05$). Lower case letters denote significant differences between fruits using ethanol ($p<0.05$), upper case letters denote significant differences between fruits using methanol ($p<0.05$).

Regarding blueberry extractions, it was seen that the only method in which the solvent played a significant part in the antioxidant capacity content was by DPPH, with 1103.72 and 709.05 µmol TE/100g fw, for ethanol and methanol respectively ($p<0.05$). For ABTS and ORAC no significant differences were seen between solvents ($p>0.05$). However, the antioxidant capacity in the blueberry ethanolic extract was 24% higher than the one in the methanolic counterpart (5011.25 and 3785.58 µmol TE/100g fw, for ethanol and methanol respectively).

For grape similar values were obtained between solvents for ABTS ($p>0.05$), while for DPPH it was seen that the ethanol had a higher antioxidant capacity than methanol, 1036.43 and 700.00 µmol TE/100g fw, respectively ($p<0.05$). On the other hand, for ORAC, the methanolic extract displayed almost double the capacity than ethanol even though the TMAC in the grape ethanolic extract is almost 20% higher than methanol. Lastly, it was seen that for raspberry similar values were obtained between ethanol and methanol ($p>0.05$), and the antioxidant activity was the second highest after blackberry by the ABTS and DPPH method for both ethanolic and methanolic extracts ($p<0.05$).

The overall results from the antioxidant capacity content in the extracts showed that for most of the fruits a higher antioxidant or equal content was found when using ethanol as a solvent than methanol. The only two instances in which a methanolic extract displayed a higher antioxidant activity was for strawberry by the ABTS method and grape by ORAC method. It is important to remember that no single antioxidant activity is the best, hence the use of multiples. Additionally, since their mechanisms are complex and not always, they can be compared against each other. For example, while ABST and DPPH are

combined mechanism methods, it has been seen that usually ABTS values tend to be higher than DPPH, especially for fruits and vegetables. This behaviour has been attributed to the capacity of ABTS to measure the activity of lipophilic and hydrophilic compounds, while DPPH benefits more hydrophobic compounds (Capanoglu et al., 2018).

When further analysing this, it was seen that for the ethanolic extracts there was a significant correlation between the TPC and TMAC with the antioxidant capacity by ABTS and DPPH ($p<0.05$) (Table 3.2). In the case of methanol, the only moderate but significant correlation was found between TPC and ABTS ($p<0.05$). These differences reinforce that the extraction with ethanol could be solubilising more polyphenols and probably with higher antioxidant capacity. Polyphenols with OH groups in the 3', 4' position in the B ring, as well as C_2-C_3 double bond. Additionally, the presence of OH in position 3 and 5 of the A ring, will have higher antioxidant capacity than those who don't meet these three requirements. By this criterion the polyphenols with the highest antioxidant capacity in the extracts will be anthocyanins followed by flavan-3-ols and flavanols, which at the same time will benefit the most by their affinity to ethanol because of their abundance of rings.

Table 3.2. Pearson's correlation analysis for total phenol content (TPC), total monomeric anthocyanins (TMAC), and antioxidant activity

	Ethanol		Methanol	
Correlation	R^2	Sig	R^2	Sig
TPC/ABTS	0.820	<0.001	0.671	<0.001
TPC/DPPH	0.878	<0.001	0.262	0.161
TMAC/ABTS	0.724	<0.001	0.504	0.005
TMAC/DPPH	0.700	<0.001	0.162	0.395

ABTS: (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)); DPPH: 2,2-diphenylpicrylhydrazyl
Note: Due to uneven set of data, ORAC correlation was not carried out

In summary, ethanol was better than methanol in the extraction of polyphenols from berries and grape extracts, which has the added advantage of being a solvent suitable for food formulations.

3.4.3. Extraction of polyphenols from fruit processing by-products and their antioxidant activity

The fruit processing by-products used in this section were selected for their high content of polyphenols as well as for their variety. The extracts obtained here were further processed by CGA separation which will be presented in the next chapters.

The results of the polyphenols characterisation are shown in Table 3.3. For blackcurrant pomace extract it was seen that this was the by-product with the highest content of polyphenols among the ones analysed. The results obtained here are higher than the ones obtained with hot water in the extraction of blackcurrant powder 1577.3 mg GAE/ 100g dw (Sadowska et al., 2019); and higher than the ones obtained with methanol by Michalska et al. (2017) in the extraction of polyphenol from blackcurrant pomace (707 mg GAE/100g dw).

Table 3.3. Polyphenols determination, and antioxidant capacity from fruit processing by-products.

Feedstock	TPC	TMAC	TFC
Blackcurrant pomace	3264.37 ± 32.31 ^{a}	300.87 ± 1.83 ^{a}	2273.28 ± 61.86 ^{a}
Grape marc	2034.98 ± 240.30 ^{b}	27.37 ± 2.82 ^{b}	2015.00 ± 108.99 ^{b}
Red goji skins	1029.61 ± 2.06 ^{c}	ND	254.95 ± 43.81 ^{b}

Total phenol content (TPC) values are expressed in mg gallic acid equivalents/ 100 g dry weight; Total Monomeric Anthocyanins Content (TMAC) values are expressed in mg of malvidin 3 glucoside/ 100 g dry weight; Total Flavonoid Content (TFC) values are expressed in mg catechin equivalent/ 100 g dry weight. Values expressed in mean ± SD *n=2*. Different letters in the same column denote significant differences ($p<0.05$). ND: NON-DETECTED

Grape marc is a by-product known for its high content of anthocyanins, and flavonols, as well as phenolic acids (Amendola et al., 2010; MohdMaidin et al., 2019; Pertuzatti et al., 2020; Spigno et al., 2007). Here, grape marc TPC results are similar to the ones reported by Pintać et al. (2018) for Italian grape marc who obtained values ranging from 1600 to 2400 mg GAE/100g dw, with 80% ethanol. Other studies, show slightly higher contents of TPC than here, for example Tsali & Goula, (2018), obtained a values of 2644 mg GAE/100g dw when using ethanol as solvent in combination with microwave assisted extraction.

However, a low content of anthocyanins was found here compared to the previous works in the group MohdMaidin et al. (2019). Lower values were also obtained compared to studies on grape marc flour were the TMAC is between 120 and 790 mg C3GE/100g dw (Monteiro et al., 2021). On the other hand, TFC values obtained here are higher than those obtained by Pintać et al. (2018), 500 to 625 mg CE/100g dry extract. It is possible the low content is mostly related to the variety of grape rather than the extraction process. It has been reported that great variability in the content of polyphenols can be obtained, mostly due to the variety of grape, country of origin, harvesting, among others (He et al., 2012; Pintać et al., 2018; Rinaldi et al., 2020).

Finally, the hydroalcoholic extraction of polyphenols from red goji skins was assessed. For this extract a TPC content of 1029.61 mg GAE/100g dw was achieved. This value is higher than the one reported previously by (Ağagündüz et al., 2021) who reported a TPC of 207.2 mg GAE/100g dry fruit. Moreover, high TFC content was found in the ethanolic extract compared to the values reported by Bolesławska et al. (2021) of red goji berry extracted with methanol; their values ranged from 36.8 to 561.8 mg QE/100g dw. In this study, no

anthocyanins were detected, however, this was expected since red goji berry has been reported to have low content of anthocyanins and flavonoids (Kosińska-Cagnazzo et al., 2017).

The antioxidant capacity of the by-product's extracts (Figure 3.5) follows the same pattern as the content of polyphenols: blackcurrant pomace > grape marc > red goji skins. For blackcurrant higher values were obtained as those reported by Michalska et al. (2017) when using 80% methanol to assess the antioxidant activity of blackcurrant pomace dried at different conditions, with values ranging from 182.1 to 111.3 µmol TE/ g dw. As for grape marc, a higher antioxidant capacity was achieved here in the ethanolic extract compared to the previously mentioned studies in grape marc flours (Monteiro et al., 2021). For red goji berry skin extract the antioxidant capacity content values obtained here are lower than the ones reported by the previously mentioned authors.

In summary, the extraction of polyphenols from food processing by-products with ethanol demonstrated that each by-product has a different content and variety of polyphenols based on the determinations of specific compounds such as anthocyanin and flavonoids. These results were expected not only because the by-products are inherently different, but they have also gone through different processes. For example, blackcurrant pomace has the advantage that it comes mainly from juice making meaning to all the polyphenols will be preserved in a better way compared to those obtained from grape marc, where apart from the juicing a fermentation process has been applied in which part of the polyphenols will be transferred to the wine. Hence, while both are rich in polyphenols, one might have the advantage over the other. From a practical point of view this offers the advantage that these particular fruit by-products can find applications in a

wide range of products. However, other aspects must be taken into consideration like the toxicity they represent, contaminants that could need purification steps depending on the application, and others (Socas-Rodríguez et al., 2021). Nonetheless, these results are important as they will be the base for further steps applied in this work.

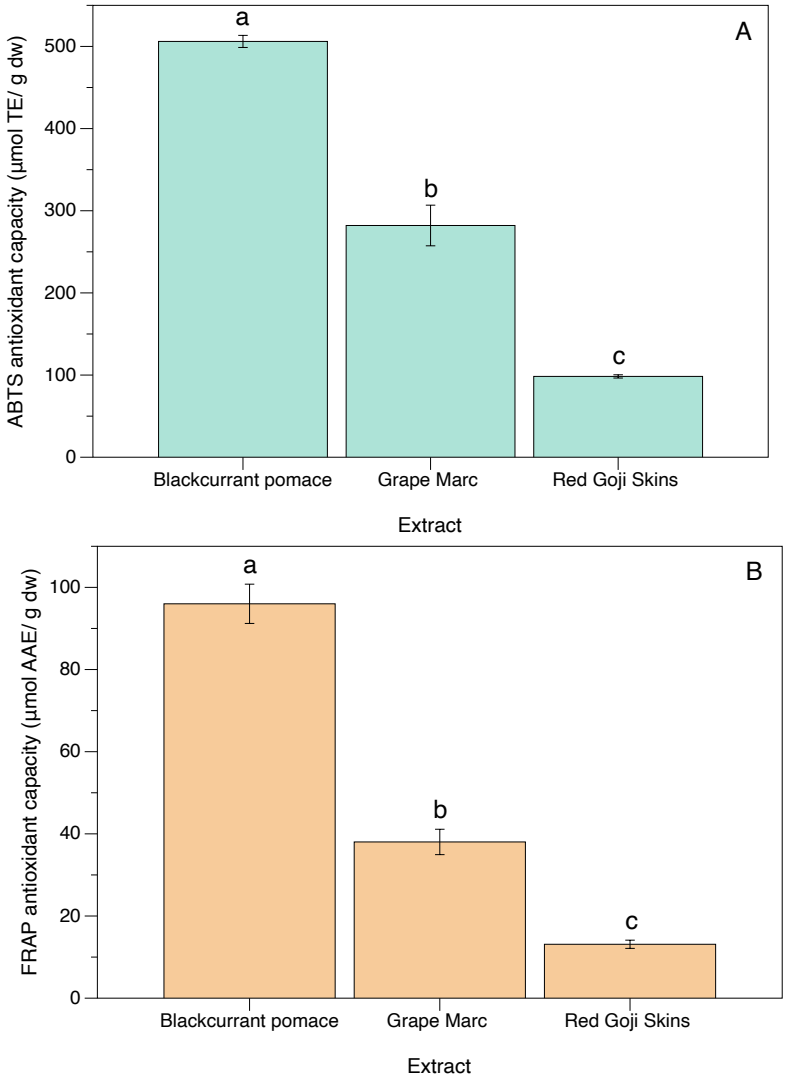

Figure 3.5. Antioxidant capacity by ABTS (A) and FRAP (B) from fruit processing by-products hydroalcoholic extracts. Different letters denote significant difference between extracts $p<0.05$.

3.5 Conclusions

In this chapter the extraction of polyphenols from berries and grapes considered fruit discards from retail, and fruit processing by-products was assessed. The results showed that in general, for berries and grapes the extraction of TPC and TMAC was equal or better compared to ethanol. This could be attributed to the better polarity match between ethanol and the different types of polyphenols resulting in their improved solubilisation and hence higher extraction yields. Regarding the antioxidant capacity a similar behaviour was observed, where ethanolic extracts had a higher content of antioxidant capacity, or at least it was equal to the capacity found in methanolic extracts. In the case of ethanol, the antioxidant capacity was correlated positively with TPC and TMAC ($p<0.05$).

These results are of great interest because the selected fresh fruits show the potential to be a source of polyphenols and antioxidant compounds. This translates to their valorisation and potential to become by-products instead of waste. Furthermore, proving that ethanol can have similar polyphenols yields as methanol is very important outcome for FODIAC project but also for further applications in functional food products. A more thorough characterisation of the ethanolic extracts would be of interest to determine the phenolic profiles. In addition, the cytotoxicity of these extracts should be evaluated.

Additionally, it was observed that the overall phenolic content of the selected fruit processing by-products varies significantly as well as their antioxidant capacity. This was expected due to the nature of each by-product. However, the characterisation of these fruit processing by-products was essential, as it is necessary for further processing with

CGA since different content and types of polyphenols may have an impact on their separation.

3.6. References

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Chapter 4: Whey Protein CGA Characterisation and its Application on Blackcurrant Pomace Extract

Abbreviations

AAE: Ascorbic Acid Equivalents

ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)

AP: Aphron Phase

BCA: Bicinchoninic acid

BCPE: Blackcurrant Pomace Extract

CGA: Colloidal Gas Aphrons

CGA(Tween 20): Colloidal Gas Aphrons made of Tween 20

CGA(WPI): Colloidal Gas Aphrons made of Whey Protein Isolate

FRAP: Ferric Reducing Antioxidant Power

LP: Liquid Phase

M3G: Malvidin 3 Glucoside

pH: Potential of Hydrogen

SD: Standard Deviation

TE: Trolox Equivalents

TFC: Total Flavonoid Content

TMAC: Total Monomeric Anthocyanins

TPC: Total Phenol Content

TPTZ: 2,3,5-Triphenyltetrazolium chloride

UW 80: Ultra Whey 80

UW XP: Ultra Whey XP

UW90: Ultra Whey 90

V_{AP}: Volume of Aphron Phase

V_{CGA}: Volume of CGA

V_{IL}: Volume of Initial Liquid

V_{LP}: Volume of Liquid Phase

WP: Whey Protein

WPI: Whey Protein Isolate

4.1 Introduction

For years polyphenols have drawn attention since their consumption is associated with beneficial effects on health. Polyphenols have the advantage to be found in most vegetables and fruits, especially in their skins, seeds, stems, and leaves (Manach et al., 2004; Tsao, 2010). In this sense, fruit by-products such as pomaces are excellent sources of polyphenols since usually, they are constituted by the aforementioned plant materials (García-Becerra et al., 2016; Sridhar et al., 2021; Sridhar & Charles, 2019). For example, blackcurrant pomace, a by-product obtained after the pressing of the fruit in the juice manufacturing, is composed mainly of the skins of the fruit, and research has shown that this pomace has a high content of dietary fibre, and bioactive compounds such as polyphenols, mostly anthocyanins, that have been additionally used as colorants (Alba et al., 2018; Azman et al., 2022; Michalska et al., 2017; Sójka et al., 2009).

Extraction of polyphenols from their matrix e.g., fresh fruit and their by-products, is the first step for their formulation. Many methods and solvents have been explored for the extraction of polyphenols, however, organic solvents such as ethanol, methanol, and acetone in combination with a source of energy such as direct heat, microwave extraction, and ultrasound are the most common ones. After the extraction further steps like separation and purification can be applied to obtain richer fractions of polyphenols. Surfactant-based separation with colloidal gas aphrons (CGA) is one of the methods to achieve this. CGA are surfactant-stabilized microbubbles (10-100 μm) generated by the intense stirring of a surfactant solution at high speeds (>8000 rpm). CGA are composed of multilayers of surfactant molecules that adsorb at the interface with hydrophilic heads

towards the aqueous phase and the hydrophobic tails towards the gas phase (Jauregi et al., 2000; Jauregi & Dermiki, 2010).

CGA are particularly interesting as a separation method because of their unique structure and characteristics such as the larger interfacial area, which favours interactions with other molecules. Moreover, they offer the advantage to be scalable and a cost-effective process depending on the surfactant that it is being used (Jauregi & Dermiki, 2010). Prior to the separation process, CGA characterisation is essential. The capability of a surfactant to create stable CGA will depend on how much air can be introduced in the solution as well as how fast the liquid will drain from the microbubbles. These variables are known as gas hold-up and half-life (Jauregi et al., 2000; Prasad et al., 2015).

Among the food safe surfactants used for CGA are Tween 20, Tween 60, and Span 80 (Carullo et al., 2022a; Dermiki et al., 2009; MohdMaidin et al., 2019; Spigno et al., 2015). Spans are sorbitan esters with a fatty acid tail, in the case of Span 80 the fatty acid is oleic acid (Fiume et al., 2019; Rose & Palkovits, 2012). On the other hand, Tweens are a family of surfactants composed by polysorbate molecules containing a hydrophilic head of ethylene glycol and a hydrophobic of fatty acid ester moiety. The use of these non-ionic surfactants has demonstrated to be efficient in the separation of bioactive molecules. Some of them, such as Tween 20, have demonstrated a particular affinity for anthocyanins (MohdMaidin et al., 2018; Spigno et al., 2015). Furthermore, surfactants have shown the capability to improve polyphenols solubility, thus possibly their bioavailability and absorption (MohdMaidin et al., 2018).

Moreover, CGA have been generated with other compounds with foaming properties, for example, rhamnolipids and saponins (Feng et al., 2009; Kazemi et al., 2022; Mukherjee et al., 2015; Saleem et al., 2019). In this context, other molecules with foaming capacity are proteins, such as those found in whey. *Whey* is a food by-product obtained during cheese production and consists mainly of the following proteins: β-lactoglobulin, α-lactalbumin, bovine serum albumin, and immunoglobulins (de Wit, 1998; Jauregi & Welderufael, 2010; Kilara & Vaghela, 2018). These globular proteins are amphiphilic molecules that can form stable foams (Foegeding et al., 2006; Luck et al., 2002; Pernell et al., 2002) ; therefore, they could offer the potential for CGA generation.

Whey proteins foaming properties come from their capacity to adsorb at the interface and interact with adjacently adsorbed proteins forming viscoelastic films (Bos & Van Vliet, 2001; Foegeding & Davis, 2011). Apart from foaming properties, whey proteins present interesting characteristics, like nutritional value, ability to form stable emulsions, and good solubility. Their solubility, though, is conditioned by pH and temperature (Alves et al., 2022; Hu et al., 2019; Moro et al., 2013). Near their isoelectric point (pH= 4.8-5.5), proteins will be less soluble due to aggregation. The same at temperatures over 40 °C, or hydrolysis, where the denaturation of these proteins will give place to aggregates (Alves et al., 2022; Hu et al., 2019; Nastaj & Sołowiej, 2020). However, controlled denaturation, by different methods like heat, solvents, hydrolysis, and pH is linked to foamability and stabilisation improvement (Althouse et al., 1995; Hu et al., 2019; Nastaj & Sołowiej, 2020; Van der Ven et al., 2002). The denaturation increases the number of hydrophobic patches, forming a cohesive interfacial film due to the intramolecular interactions

between aggregates and soluble protein (Alves et al., 2022; Foegeding et al., 2006; Foegeding & Davis, 2011).

Moreover, studies show that using whey proteins in combination with bioactive compounds can bring stability to foams made of whey protein (Cao et al., 2018; Rodríguez et al., 2015). Other studies have demonstrated the protective effect of whey protein against the loss of antioxidant activity of resveratrol (Guo & Jauregi, 2018). Along with stability and protection properties, whey protein concentrates and isolates are a relatively low-cost additive and have been widely used in the formulation of several food products for their functionality and versatility.

This work aims to study for the first time the use of whey protein as a surfactant to generate CGA and to characterise them in terms of gas hold-up and half-life. Furthermore, CGA generated from whey protein were applied to separate polyphenols from a hydroalcoholic extract of blackcurrant pomace. The separation of CGA using whey protein was compared to the one previously studied using the non-ionic surfactant Tween 20.

4.2 Materials

Materials

Surfactants: Whey protein concentrate, and isolate were supplied by Volac Ltd (see table 4.1 for specifications), and TWEEN® 20 was purchased from Sigma-Aldrich (UK).

Table 4.1. Protein, fat, lactose, and solubility index in whey protein reported by the manufacturer

Whey protein code name	Protein	Fat	Lactose	Solubility Index
UW 80	82	6	4	≤ 0.20
UW 90	93	0.2	2.5	≤ 0.50
UW XP	94	0.3	0.5	≤0.70

Protein, fat, and lactose values are expressed in percentage. Solubility index is expressed in mL. Note: The manufacturer reports solubility values measured by centrifugation of a 10% solution

Dried blackcurrant pomace was supplied by A&R House with the following characteristics: 2017 harvest blackcurrant pomace batch dried in a rotary dryer with a material minimum passage through the system of 11 secs at 97°C; and the temperature of entering to the cooling of 85°C.

Chemicals

ABTS: 2,2′-azino-bis(3-ethylbenzothiazoline- 6-sulfonic acid) diammonium salt (ABTS) ≥98.0% (HPLC) (A1888), potassium persulfate, trolox: (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (97%) , TPTZ: (2, 4, 6-tris(2-pyridyl)-s-triazine) (T1253), ferric chloride hexahydrate, Folin-Ciocalteu, sodium carbonate BioXtra, ≥99.0%, gallic acid, sodium hydroxide, (+)- catechin, aluminum chloride, sodium acetate, sodium

nitrate, potassium chloride, bicinchoninic acid solution, copper(II) sulfate pentahydrate 4% solution, and ethanol, ≥99.8% were bought from Sigma-Aldrich (UK).

4.3 Methods

4.3.1 CGA production and characterisation

The first step to demonstrate the capacity of whey protein to generate CGA was testing different concentrations to determine the minimum protein content for the generation of CGA. Protein concentration starting at 2mg/mL and in increments of 2 mg/mL, till reaching 12 mg/mL were tested. Additionally, to see the effect of whey protein composition on CGA generation and characteristics, one whey protein concentrate (UW 80), and two whey protein isolates (UW 90 and UW XP) were tested. Before the CGA generation the protein content was determined by the BCA method (see section 4.3.3)

An aqueous solution of 500 mL was prepared for each whey protein at a final protein concentration of 10, 15, and 20 mg/mL. The solutions were left overnight at room temperature for complete hydration. These concentrations were chosen based on previous experiments where the minimum concentration of protein to obtain stable CGA was determined (see Appendix 3.1). Due to low availability of some whey samples experiments with UW 90 and UW 80 were not performed in duplicate; however, for UW XP and Tween 20 two technical replicates were carried out.

In addition, an aqueous Tween 20 [10mM] solution was prepared to compare both CGA characteristics. All CGA were generated by stirring 400 mL of each solution at 8000 rpm for 5 min at room temperature using a high-speed impeller (Silversone SL2T). The gas hold-up ε; the amount of air incorporated into the dispersion, was determined as:

$$\varepsilon = {V_{CGA} - V_{IL}}/{V_{CGA}}$$

Where V_{CGA} is the volume of aphrons and V_{IL} is the volume of liquid used to form the CGA.

Stability of the foams in terms of drainage, typically known as half-life, was determined by calculating the time at which half of the initial liquid of surfactant used for CGA generation is drained. For this, measurement of the volume drained from CGA was taken every minute for a 15 min period. The half-life was calculated from the equation derived from the graphical representation of the drained volume against time (Appendix 3.1, and 3.2), in the equation the drained volume was fixed Vo/2=200mL in order to obtain the time that it takes to drain half-of the initial volume.

4.3.2 Separation of polyphenols with CGA

Prior the separation, a blackcurrant pomace hydroalcoholic (60% ethanol) extract (BCPE) was obtained using the methodology previously mentioned in Chapter 3. After this, CGA generated with whey protein, labelled CGA(WPI) and CGA made of Tween 20, labelled CGA(Tween 20), where used for the separation of polyphenols from BCPE. The separation was carried out based on the optimum conditions found in previous works, ie: extract to CGA volumetric ratio ($V_{extract}$:V_{CGA}) of 1:16, contact time of 5 min, and drainage time of 5 min (MohdMaidin et al., 2018, 2019). For the separation, 65 mL of BCPE were added to a flotation gas column (i.d 5cm, height: 50 cm) and 1040 mL of CGA(WPI) or CGA(Tween 20) were pumped with the help of a peristaltic pump (Watson Marlow) from the CGA generating container into the flotation glass column with the extract. An adjusted flow of

142 mL CGA/ min was set to allow a contact time of 5 min. After this, a period of 5 min drainage time was allowed for the separation of phases, aphron phase, AP(at the top) and (drained) liquid phase, LP (at the bottom) (see Figure 4.1 for full separation process). The volumes of aphron phase and liquid phase were measured. CGA were made in duplicate, and each duplicate was analysed in triplicate. The percentage recovery of total phenols, total flavonoids, and total anthocyanins in the aphron phase (AP) was calculated with the formula:

$$\text{Recovery (\%)} = \frac{\mathrm{M}_{feed} - \mathrm{M}_{liquid\ phase}}{\mathrm{M}_{feed}} \times 100$$

Where M_{feed} is the initial mass of polyphenols in the blackcurrant pomace and M_{liquid} phase is the mass of polyphenols determined in the liquid phase. For the antioxidant capacity the quantification was made directly on the aphron phase, this was estimated from the theoretical content of blackcurrant pomace processed for the CGA separation.

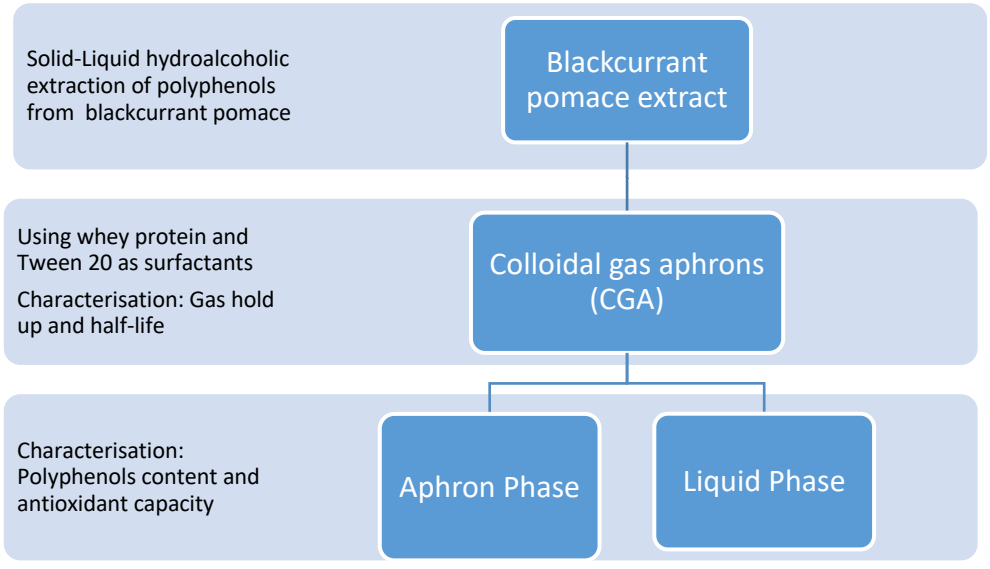


Figure 4.1. Schematic representation of the separation with CGA used in this chapter.

4.3.3 Analytical determinations

Protein determination by Bicinchoninic Acid (BCA) method

To protein determination was carried out by the BCA method following the protocol stablished by the supplier (Sigma-Aldrich). This method is based in the reduction properties of proteins to reduce Cu^{+2} to Cu^{+1} in an alkaline solution, and chelation of BCA that gives place to the colour purple formation. A limitation to this assay is that reductant molecules such as flavonoids have shown to interfere since being highly hydroxylated molecules, especially those with more than 3 hydroxyl groups can lead to considerable overestimation (Singh et al., 2020). Moreover, the presence of certain aminoacids such as cysteine, tyrosine, and tryptophan (present in whey protein) can interfere in the assay since they are copper chelating agents that could interfere by reducing the amount of colour produced (Cortés-Ríos et al., 2020).

Briefly in a 96 well microplate, 25 µl of sample were mixed with 200 µL of working reagent solution, made of 50 parts of Bicinchoninic Acid Solution per part of Copper (II) Sulfate Pentahydrate. The mixture was incubated for 30 min at 37ºC, after this time the absorbance was measured at 562 nm in a microplate reader (FLUOstar Omega, BMG LABTECH, Offenburg, Germany), each sample was done in triplicate. The results were quantified from a bovine serum albumin (BSA) calibration curve ranging from 0.4 to 1 mg/mL ($y=0.7956x + 0.0149$; R^2: 0.9964). Results are expressed as mg of BSA/ mL.

Phenolics determination

Total Phenol Content: results are expressed as percentage of gallic acid equivalents (% GAE) based on the equation in section 4.3.2.

Total Monomeric Anthocyanins Content: results are expressed as percentage of malvidin 3-glucoside equivalents (% M3GE) based on the equation in section 4.3.2.

Total Flavonoid Content: results are expressed in percentage of catechin equivalents (% CE) based on the equation in section 4.3.2.

4.3.4 Antioxidant Capacity Assays

ABTS: The results are expressed in micro moles of trolox equivalents per gram of theoretical quantity of blackcurrant pomace found in the feedstock (µmol TE/ g dw).

FRAP: The results are expressed as micro moles of ascorbic acid equivalents per gram of theoretical quantity of blackcurrant pomace found in the feedstock (µmol AAE/ g dw).

4.3.5 Precipitation of polyphenols from BCPE with whey protein

The precipitation of polyphenols was carried out by BCPE and a WP solution at the ratio corresponding to CGA separation, 1:16. First, 500 mL of a solution of WP [10 mg/mL] was prepared and let overnight at room temperature for total hydration of the proteins. After this 65 mL of BCPE was added to the 458 mL of the WP [10 mg/mL] solution, the volume of WP was calculated from the CGA gas hold-up experiments (section 4.3.1). BCPE and WP solution was mixed for 5 min, and let it rest until precipitation occurred. The final solution labelled WP+BCPE was centrifugated at 4,000 RPM for 15 minutes. The

supernatant and pellet were separated, and analytical determinations in section 4.3.3 and 4.3.4 were carried out in the supernatant.

4.3.6 Statistical Analysis

CGA separation was performed in duplicate, and each duplicate was analysed in triplicate. The data was subjected to the analysis of variance using IBM® SPSS® Statistics 27 software where statistical differences were noted using t-test analysis for the comparison of Tween 20 and UW XP characterisation, and recovery of polyphenols. For the antioxidant capacity after CGA separation One-Way ANOVA (Tukey test post-hoc) was applied. The significance level was defined at $p<0.05$. The results were reported as mean ± SD

4.4 Results and Discussion

4.4.1 CGA (WPI) characterisation

Characterisation of CGA is a critical factor for their application in the separation and recovery of molecules. This characterisation is based on the gas hold-up and stability of the generated foams, often referred to as half-life. The gas hold-up parameter lets us know how much air can be introduced in the initial solution at a particular concentration. Previous works on surfactants such as CTAB and Tween 20 show that gas hold-up ranges typically between 57% and 72% (when concentrations above critical micelle concentration) (Dahmoune et al., 2013; Fuda et al., 2004; MohdMaidin et al., 2018; Spigno et al., 2015). Secondly, CGA's stability (half-life) elucidates how fast the liquid is drained from CGA based on the time it takes to drain half of the volume of surfactant used for CGA generation. For this parameter, values in the range of 450 s to 700 s have been reported for CTAB, Tween 20, and Tween 60 (Dermiki et al., 2009; Fuda et al., 2004; Spigno et al., 2015).

Here, preliminary experiments were performed to find the minimum concentration to obtain stable CGA made of whey protein. The characterisation was done in a 2 mg/mL to 12 mg/mL protein concentration range. The results showed that CGA could be generated with protein concentrations as low as 2 mg/mL, based on the gas hold-up (Figure 4.2-A). However, at a protein concentration below 6 mg/mL, it was not possible to take the half-life (Figure 4.2-B) as the liquid from the CGA drained considerably fast not being able to determine a value. Stable CGA were achieved only with concentrations above 10 mg/mL, where gas hold-up and half-life values are within previously reported observations.

Therefore, 10 mg/mL was established as the minimum whey protein concentration for further studies with CGA.

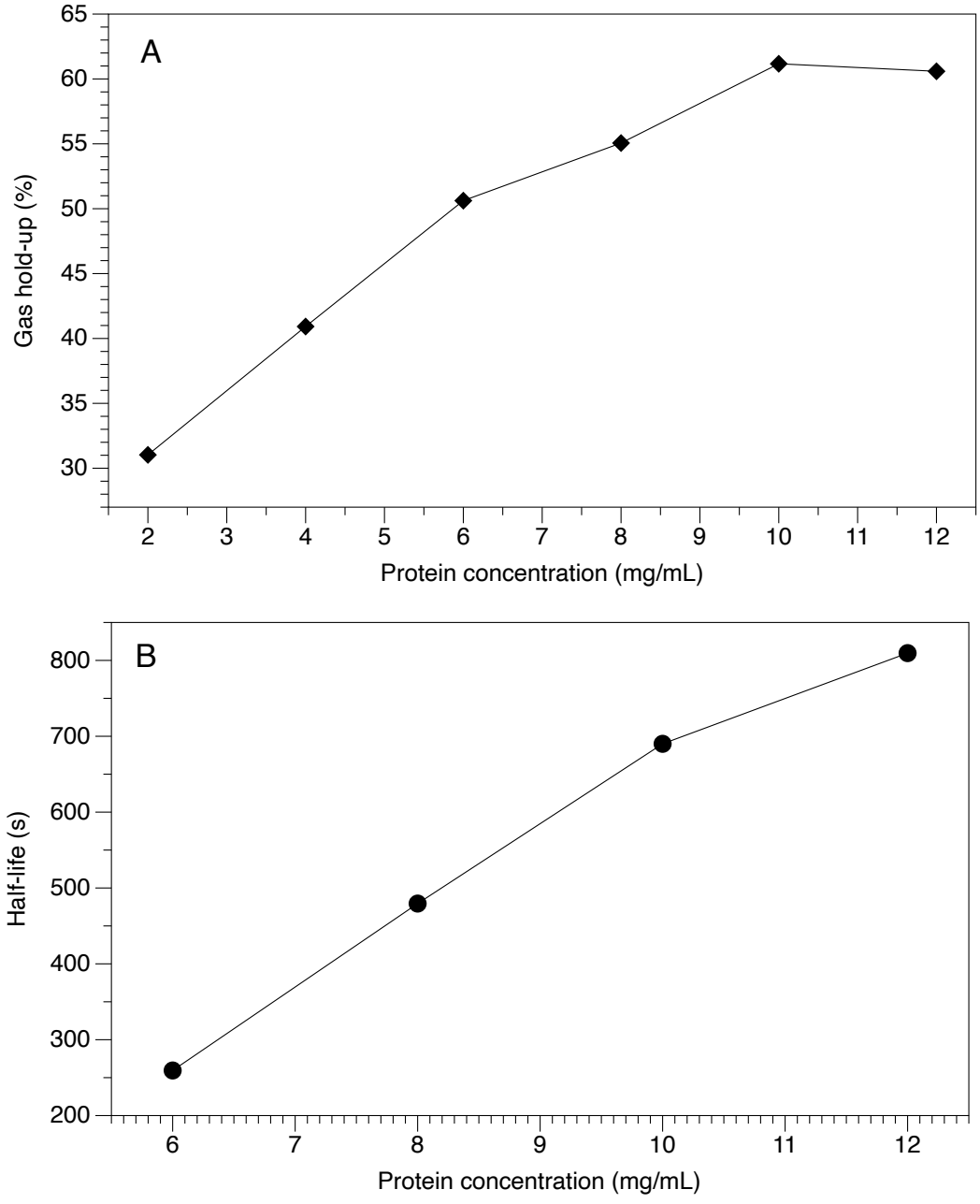


Figure 4.2. Gas hold-up (A) and half-life (B) of preliminary characterisation of CGA(WPI) using protein concentrations between 2 and 12 mg/mL.

Furthermore, the impact of different purity whey was assessed on CGA characteristics, i.e.: gas hold-up and stability. Whey samples supplied differed on protein, fat, and lactose content (Table 4.1).The CGA characterisation results are shown in Figure 4.3, from these results it is seen that both, gas hold-up and stability increased with increase in the concentration of whey. However, this behaviour is different depending on the purity of the whey protein.

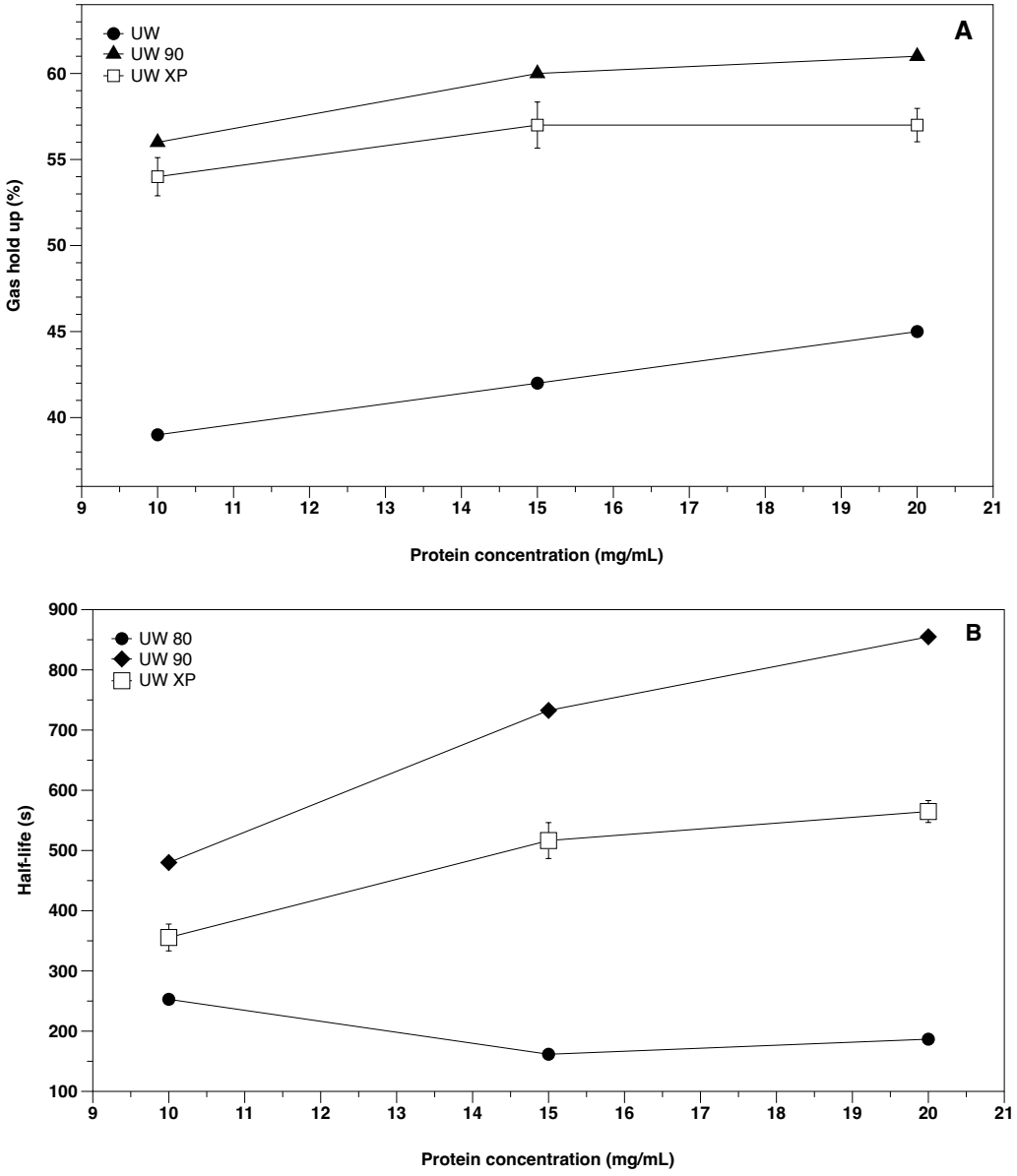


Figure 4.3 Whey protein CGA characterisation: (A) gas hold up and (B) half-life of different whey protein purity at different concentrations.

Differences in UW 80, UW 90 and UW XP could be explained based on differences in macromolecules composition. These results show that UW 80 has a lesser capability to form stable CGA since the gas hold-up and half-life were far from the typical range values, even at the highest protein concentration. While no differences between UW 90 and WP XP can be established, their gas hold-up and half-life were in the range of previously reported values using non-ionic surfactants for CGA generation (Carullo et al., 2022a; Dermiki et al., 2009; MohdMaidin et al., 2019; Spigno et al., 2015). Differences between UW 80 and the other two WP could be explained based on differences in the composition of fat and lactose.

It was seen that a higher fat content led to a decrease in the amount of air that could be introduced in the solution as well as the stability. Fat spreads on the active surface, and with this, a thinning of the liquid film occurs, leading to coalesce of bubbles and collapse (Huppertz, 2010; Kamath et al., 2008; Pilhofer et al., 1994), which could explain the reduced stability, especially in UW 80 (Fig 4.3-B). On the other hand, the presence of sugar on foams has been reported to have a stabilising effect, mainly attributed to the increase in viscosity, which slows down the liquid drainage in foams (Bos & Van Vliet, 2001; Kelly & Burgess, 1978; Yang & Foegeding, 2010). However, the increase in viscosity affects the capacity of air that can be introduced in the solution (Bong, 2010). So, an increase in viscosity of the active surface will results in a lower gas hold-up but higher stability. Based on the above results and availability UW XP at 15 mg/mL was selected to continue with CGA separation.

In order to understand how CGA(WPI) compared to CGA made of non-ionic surfactants, CGA(WPI) were compared to CGA(Tween 20) at 10mM. This Tween 20 concentration was

selected based on previous works that report this concentration as the optimum in terms of gas hold-up and half-life. The results here showed that CGA(Tween 20) had a higher gas hold-up and half-life than CGA(WPI) (Table 4.2). However, the progression of the volume drained from CGA(Tween 20) and CGA(WPI) follows a similar behaviour (Figure 4.4).

Table 4.2. Gas hold-up and half-life time of Tween 20 and UW XP

Surfactant	Gas Hold Up (%)	Half-life (s)
Tween 20 10 mM	67.22 ± 2.84 [a]	628.07 ± 2.65 [a]
UW XP 15 mg/mL	56.50 ± 1.34 [b]	516.54 ± 18.22 [b]

The values obtained here for CGA(Tween 20) were higher those obtained in previous studies. (Spigno et al., 2015) reported a 60% gas hold-up and 407 s half-life with 10 mM Tween 20. Similarly, (Noriega et al., 2018) obtained a CGA gas hold-up of 57% and 510 s half-life using Tween 20 [10mM]. In a recent study, (Carullo et al., 2022a) evaluated different concentrations of Tween 20 for CGA separation reporting that at 10mM, a 65% gas hold-up and 571 s half-life was observed, which are similar to the ones obtained here. While gas hold up and half-life values of CGA(WPI) generated with UW XP are significantly lower than those of CGA(Tween 20), they still fall under the previously reported values using non-ionic surfactants.

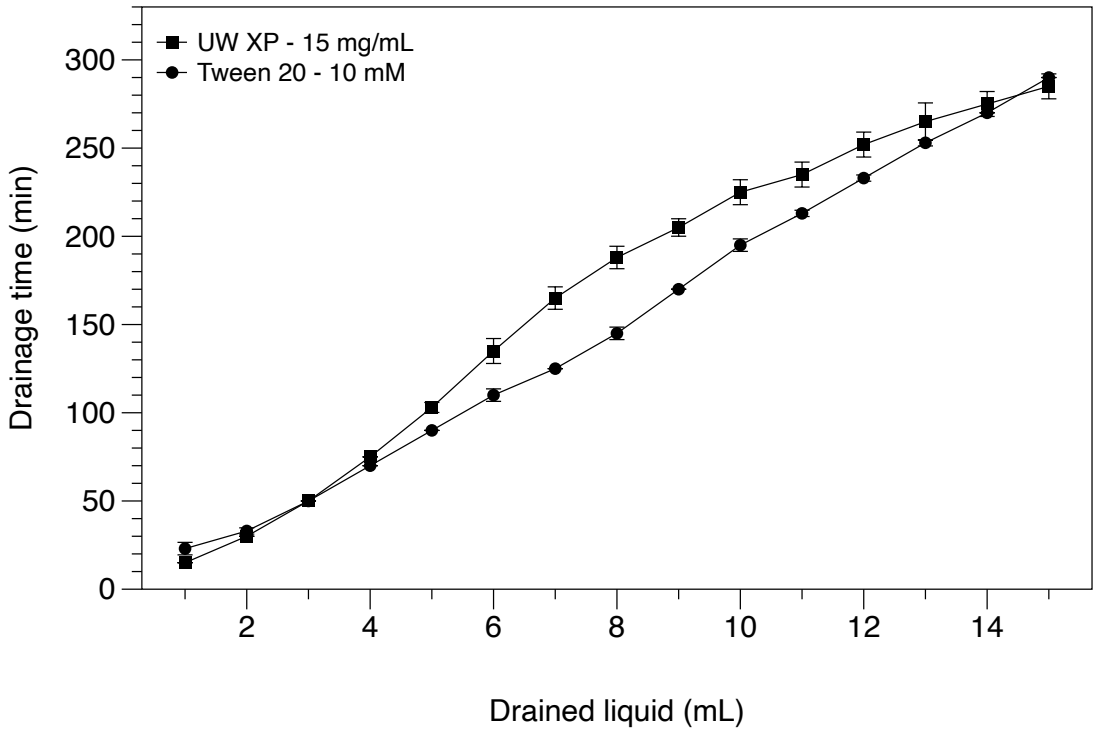


Figure 4.4. Progression of drainage time (min) against drained liquid (mL) for CGA generated with WPI and Tween 20.

In summary, the results demonstrated that whey protein can be used for CGA generation, and to obtain stable CGA a minimum protein concentration of 10 mg/mL is needed. Moreover, it was seen that whey protein purity heavily influences the stability of CGA, and therefore it needs to be considered. These preliminary results were valuable as they allowed to establish the protein concentration and purity of whey required to obtain stable CGA. Furthermore, at a protein concentration of 15 mg/mL CGA characterisation demonstrated that it is possible to generate CGA(WPI) with similar characteristics as those of CGA(Tween 20) reported previously in the literature.

4.4.2 Separation of polyphenols from BCPE by CGA(WPI) and CGA(Tween 20)

Previous work demonstrated that CGA generated from Tween 20 could be successfully applied to recover different phenolic compounds from natural extracts (Dahmoune et al., 2013; Dermiki et al., 2009; MohdMaidin et al., 2019; Spigno et al., 2015). These works noted that optimum recovery of polyphenols was obtained using Tween 20 [10mM] and a volumetric ratio of 1:16 ($V_{extract}$:V_{CGA}). Here, we applied these conditions for the separation with CGA(Tween 20). Since no studies have been carried out using whey protein for CGA generation or separation, the concentration (15 mg/mL) was selected based on the characterisation in the previous section. The volumetric ratio $V_{extract}$:V_{CGA} was kept at 1:16 to compare the outcomes of the recoveries.

Figure 4.5 shows that for CGA(Tween 20) and CGA(WPI) the recovery of TPC was 94% and 90% respectively. As for TFC, similar results were observed, for CGA(WPI) the recovery was of 90% whereas for CGA(Tween 20) was 93%. These results show that while the recovery of TPC and TFC were significantly different ($p<0.05$), a good recovery with CGA(WPI) was achieved. On the other hand, differences in recovery were more noticeable for TMAC, where the recovery was of 94% and 81% for CGA(Tween 20) and CGA(WPI) respectively; meaning a 14% higher recovery was achieved with CGA(Tween 20) than with CGA(WPI). In here is possible the pH of the resultant aphron phase (pH=4.48) had an incidence on the recovery due to protein solubility (less soluble near isoelectric point) and changes in the anthocyanins structure. Overall, for the analysed polyphenols, higher recoveries were achieved with CGA(Tween 20) than with CGA(WPI) ($p<0.05$). However, the recovery with CGA(WPI) was up to 90% for all the polyphenols, with TMAC being the lowest recovery yet above 80%.

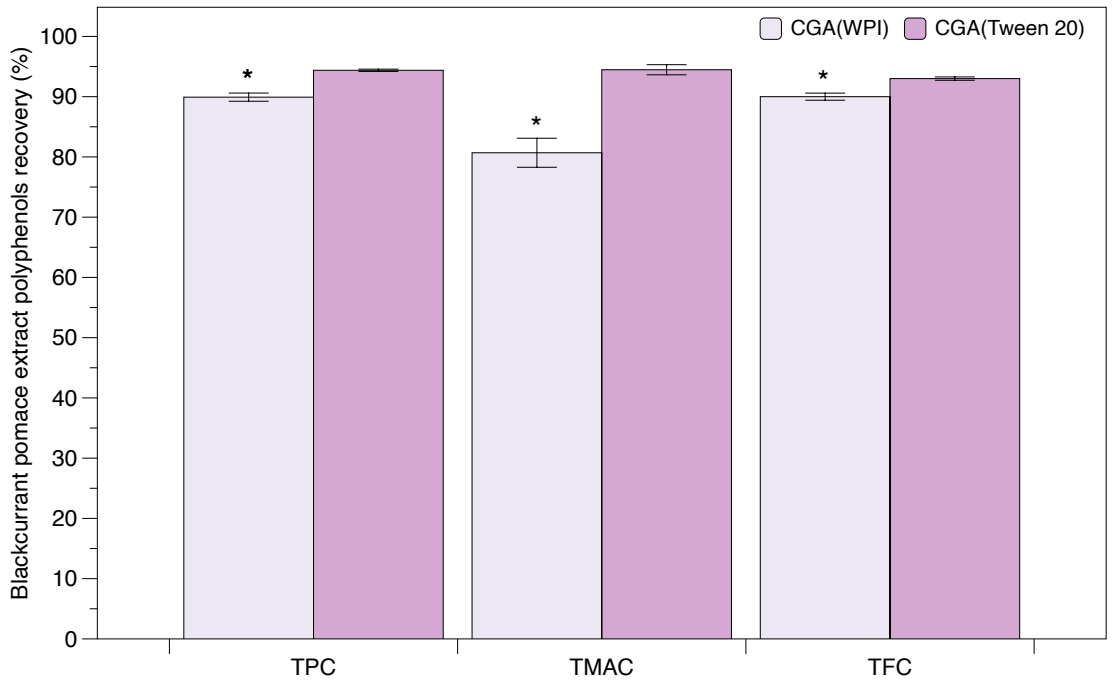


Figure 4.5. Recovery of polyphenols from blackcurrant pomace extract (BCPE) after CGA separation with WPI and Tween 20. *Denotes a significant difference at $p<0.05$.

No separation of polyphenols from blackcurrant pomace has been reported so far using surfactants. However, previous CGA works have been carried out on similar by-products, such as grape pomace. For example, (MohdMaidin et al., 2019) reported 83% recovery of TPC using Tween 20 CGA at 10mM. (Spigno et al., 2015) studied an anionic and no-ionic surfactant to separate polyphenols from grape marc. Their results showed a 78% and 76% recovery of TPC and TMAC, respectively, using Tween 20 CGA. These authors attributed polyphenols' high recovery with CGA(Tween 20) primarily to hydrophobic interactions between Tween 20 and polyphenols.

For CGA(WPI), interactions between WP and polyphenols are responsible for the successful separation. Interactions between whey proteins and polyphenols have been widely studied; finding that these interactions are mainly non-covalent such as hydrogen

bonds, and hydrophobic interactions (Frazier et al., 2010; Jauregi et al., 2016; Papadopoulou & Frazier, 2004). In this study, CGA(WPI) had a pH of 6.2 (above the IP of WP); therefore, proteins were negatively charged. In addition, polyphenols generally hold a negative charge due to their aromatic rings. Thus, there will be repulsive electrostatic interactions between proteins and polyphenols during the CGA separation. Therefore, the separation of polyphenols by whey proteins is likely driven by hydrophobic interactions and hydrogen bonds. The results obtained in this section demonstrate the successful use of CGA(WP) to separate polyphenols from BCPE.

4.4.3 Antioxidant activity of BCPE after CGA separation

Figure 4.6 shows the antioxidant activity after the CGA separation and the feedstock's initial content. It is shown CGA(WPI) has a higher ABTS antioxidant capacity ($p<0.05$) than CGA(Tween 20). On the other hand, the antioxidant capacity by FRAP displayed higher values ($p<0.05$) for CGA(Tween 20) than CGA(WPI). It is possible in here that the methods could be playing a part since it is known WP can interefere with ABTS assay (Cano & Arnao, 2017). However, this interference would not be enough to explain the considerable difference between CGA(Tween 20) and CGA(WPI). It is most probably that the difference is by the scavenging mechanism, ABTS measures hydrogen atom and electron transfer and, whilst FRAP only measures electron transfer. In this sense it is possible that after the the separation less sites for electron transfer are available in the phenolic structures due to interactions with the surfactants. These results are of importance since the recovery of polyphenols was higher for CGA(Tween 20), but it did not display a higher antioxidant activity overall.

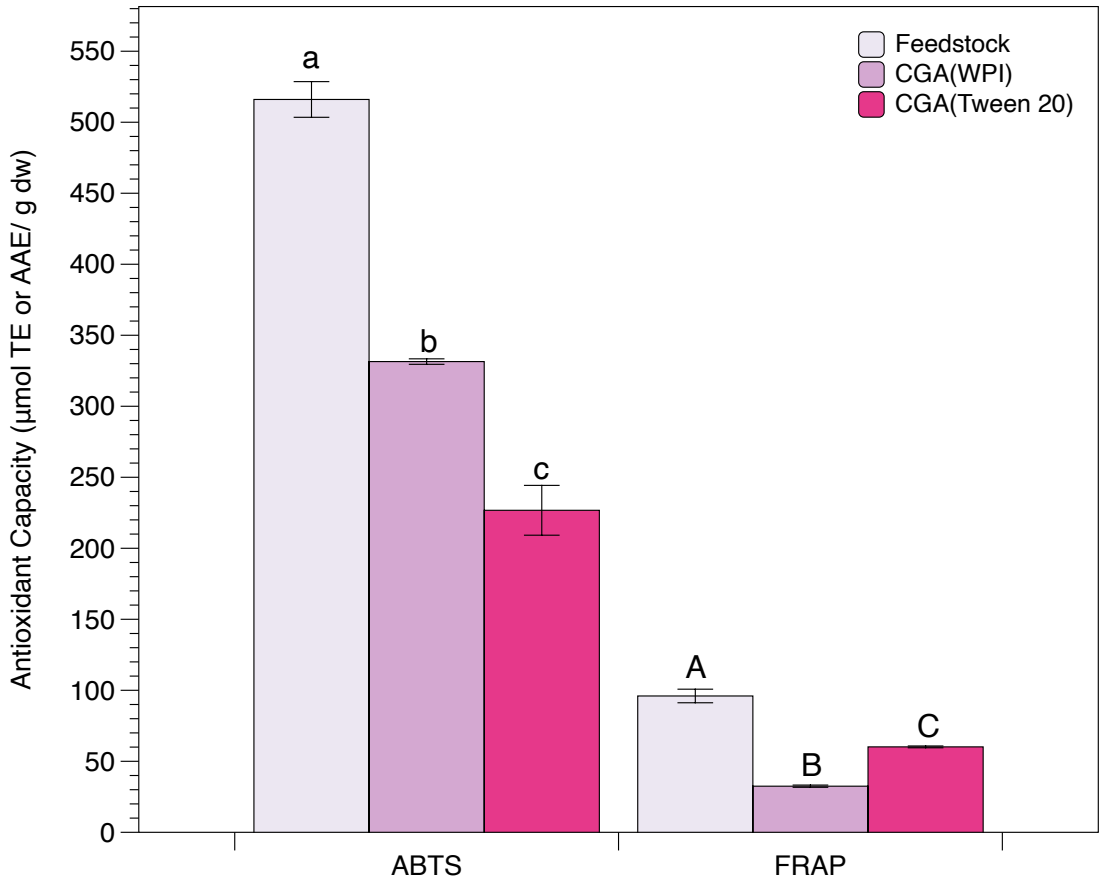


Figure 4.6. Antioxidant capacity of aphron phase of CGA(WPI), CGA(Tween 20), and blackcurrant pomace extract (BCPE) feedstock. Values of ABTS are expressed in µmol TE, and FRAP values are expressed in µmol AAE. Lower case letters denote significance differences $p<0.05$ for ABTS, and Upper case letters denote significance differences $p<0.05$ for FRAP.

More importantly, for both surfactants, the two antioxidant capacity methods showed less content in the AP than the initial antioxidant capacity found in the feedstock. This could be related to different factors such as interactions with the surfactants (Arranz et al., 2019) , susceptibility to pH changes, like is the case for anthocyanin (Andersen & Jordheim, 2010), and oxidation from the medium (Kishore et al., 2011). It has been reported that antioxidant activity of polyphenols can be affected by CGA separation with Tween 20. Spigno et al. (2015) found that some of the antioxidant capacity was lost during the separation. Polysorbates are prone to autoxidation and hydrolysis due to their fatty acid moiety, therefore is possible that polyphenols are acting as antioxidants to stabilise

the Tween 20 molecules, thus a reduction in the antioxidant capacity (Kishore et al., 2011; Pegues et al., 2021; Schmidt et al., 2020).

For CGA(WPI), part of the antioxidant capacity will be given by WP, since it has been demonstrated that WP possess bioactivity, in this study the antioxidant capacity of the WP was not assessed. Moreover, it is possible that interactions between WP and polyphenols influenced the antioxidant capacity, this behaviour has been previously reported (Almajano et al., 2007a; Loizzo et al., 2022; Oliveira & Pintado, 2015; von Staszewski et al., 2011). de Morais et al. (2020) reported that ECGC had a lower antioxidant capacity when comparing the original activity to the one of the WP-ECGC complex. The authors attributed this loss to a decrease in available hydroxyl groups due to hydrogen bonding formed in the WP-EGCG complex. The decrease in available hydroxyl groups could explain the decrease in the antioxidant capacity during the separation.

During the CGA separation, one interesting behaviour was observed regarding the drainage between CGA(WPI) and CGA(Tween 20). For CGA(WPI) the relation between AP to LP (V_{AP}/V_{LP}) was 0.798 ± 0.090, while for CGA(Tween 20) was 2.064 ± 0.048. These results are of importance since in the case of CGA(WPI) during the separation the drainage is 2.6 times higher than CGA(Tween 20), when letting a 5 min drainage time. V_{AP}/V_{LP} relationship could explain in some way the differences in the recovery of polyphenols and antioxidant activity between surfactants since the values show that CGA(WPI) drain at a significantly faster rate than CGA(Tween 20). Previous studies carried out on the separation of proteins from sweet whey with CGA made out of CTAB have shown that proteins like β-lactoglobulin were recovered in the CGA(CTAB) fraction. In the

form of insoluble aggregates. They observed that this aggregation during the separation improved drainage of the liquid within CGA phase. With this a higher selectivity was achieved while in the drained liquid other "contaminant" molecules were found (Fuda & Jauregi, 2006). Their observations help to support the statement that in this case aggregation of polyphenols and protein are occurring, which could explain the increased drainage from CGA(WPI). At the same time, this aggregation could lead to an improved separation, as polyphenols and protein will be complexed in the aphron phase.

Other factors such as ratio between polyphenols and whey protein as well as the ethanol content since ethanol can denature whey protein and cause precipitation, could have influenced the drainage and as well it is worth to investigate in depth. Overall, these results show that WP could be used as a surfactant to separate and preserve polyphenols antioxidant activity. However, optimum conditions are yet to be found to obtain a higher content antioxidant capacity in the AP.

4.4.4 Precipitation of polyphenols from BCPE with WP

Precipitation of polyphenols with WP is a common phenomenon that arises from their interactions. Interestingly, it was seen that during CGA separation, a precipitate was formed in the aphron phase of CGA(WPI). The degree to which precipitation occurs depends on factors such as the concentration, ratio and type of polyphenols and WP.

In order to understand if natural precipitation differs from the one in CGA(WPI), direct precipitation of BCPE with WP was carried out at the same ratio as in CGA separation (1:16). The results in Table 4.3 show that CGA(WPI) have a slightly higher, but significant

(p<0.05) TMAC and antioxidant capacity compared to the direct precipitation. No differences were observed between the two precipitations in the TPC. However, a significant decrease (p<0.05) in protein content was observed in CGA(WPI).

Table 4.3. Polyphenols, antioxidant capacity, and protein composition of supernatant from direct precipitation and CGA methods

Supernatant	TPC	TMAC	ABTS	BCA
Direct precipitation Aphron phase	0.382 ± 0.015	7.459 ± 0.448[a]	0.737 ± 0.021[a]	1.362 ± 0.013[a]
CGA(WPI)	0.392 ± 0.019	8.733 ± 0.511[b]	0.814 ± 0.026[b]	1.243 ± 0.010[b]

Values are expressed in mean ± sd. TPC (Total Phenol Content): mg GAE/mL; TMAC (Total Monomeric Anthocyanin Content): mg M3GE/L; ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)): mg TE/mL; BCA (Bicinchoninic acid assay): mg BSA/ mL. Different letters in the column denote significant differences p<0.05

The higher values for TMAC and ABTS could be related to CGA structure helping the solubilisation of polyphenols. These multi-layered microbubbles with high stability have a larger interfacial area (Jauregi et al., 2000; Jauregi & Dermiki, 2010; Jauregi & Varley, 1998; Spigno et al., 2015). With increased contact area, interactions between WP and polyphenols can be favoured. Moreover, the decrease in protein content in CGA(WPI) aids in supporting this statement since a lesser protein content could be associated with a decrease in free protein due to WP-polyphenols interactions. From this experiment, it is still unclear how the precipitation in CGA(WPI) differs from the spontaneous one since no drastic differences were seen.

4.5 Conclusions

In this chapter, the capacity of WP as a surfactant for CGA generation and separation of polyphenols was investigated. The characterisation results showed that it is possible to generate CGA with protein concentration as little as 2 mg/mL. However, to obtain stable CGA a minimum protein concentration of 10 mg/mL is needed. Moreover, it was seen that the purity of WP is critical; low-fat content and high lactose content was needed to produce stable CGA. Furthermore, stable CGA(WPI) generated with a protein concentration of 15 mg/mL showed similar characteristics (gas hold-up and half-life) to those of CGA(Tween 20). Therefore, 15 mg/mL was selected to generate CGA for the separation of polyphenols from blackcurrant pomace.

After obtaining stable CGA(WPI), their capacity to separate polyphenols from BCPE was assessed and compared to CGA(Tween 20). Although, significant differences were found in the recovery of polyphenols between CGA(WPI) and CGA(Tween 20). The recovery difference was less than 5% for TPC and TFC. However, a clear difference was seen for TMAC, where CGA(WPI) exhibited a lower recovery than CGA(Tween 20). This possibly due to the pH of CGA(WPI) since at near neutral pH anthocyanins are highly unstable to structural changes. Additionally, identification of polyphenols in each phase could give important information to elucidate which polyphenols are found in each phase and if there is a trend.

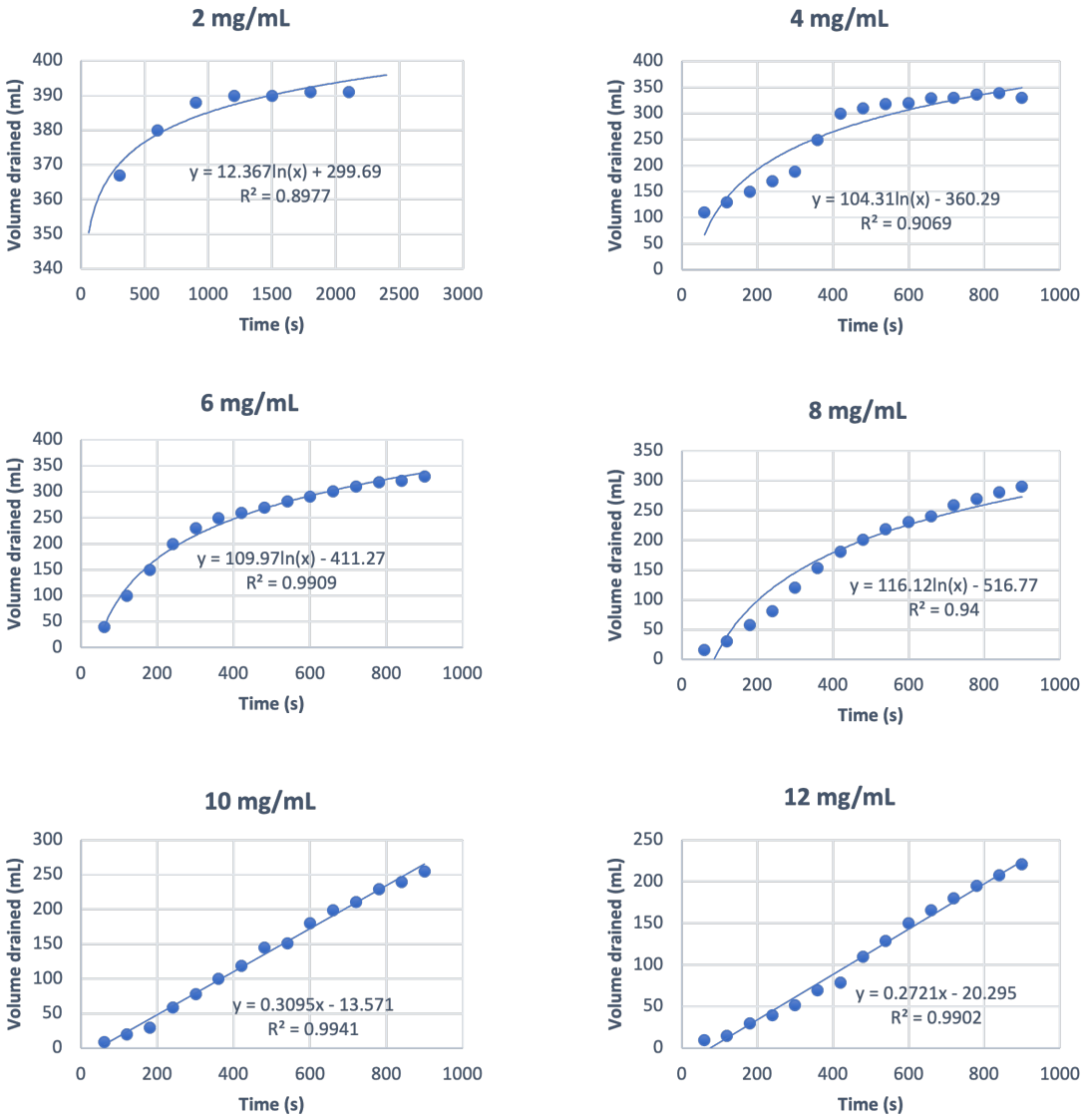
Regarding the antioxidant capacity, it was seen that CGA(WPI) and CGA(Tween 20) had a lower content than the one in the feedstock. Moreover, when comparing the antioxidant capacity of both CGA, the antioxidant capacity was higher for CGA(Tween 20).

This was likely due to the non-covalent interactions between whey proteins and polyphenols, causing a decrease or masking of the antioxidant capacity. Therefore, while a high recovery of polyphenols was obtained it did not correlate to a higher antioxidant capacity in the aphron phase of CGA(WPI) and CGA(Tween 20).

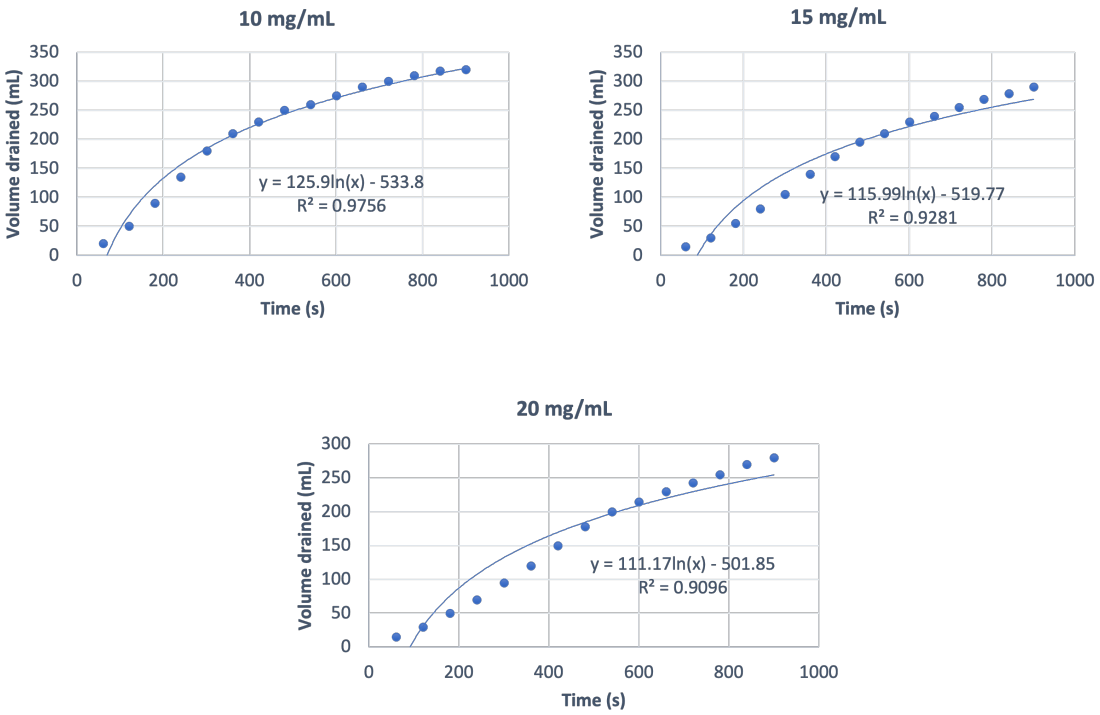
After CGA(WP) separation, precipitation of polyphenols from BCPE was observed, so direct precipitation was carried out to compare them. The results showed that a higher content of TMAC and antioxidant activity is found in the supernatant of CGA(WPI) than in the direct precipitation. These differences could indicate that CGA(WPI) aid in the solubilisation of polyphenols. Moreover, a lower protein content was observed in CGA(WPI) than in the direct precipitation; this could be related to a lower free protein content due to interactions with polyphenols. Additionally, it is possible the ethanol content might be playing a role since it is know that ethanol destabilises proteins, leading to precipitation.

However, the results obtained from the precipitation are not enough to make assumptions on the differences between CGA(WPI) and direct precipitation. It is still unclear to what extent this precipitation can be beneficial or detrimental; thus, more studies should be carried out. Notwithstanding, the results presented in this chapter show that WP can be used as a food-grade surfactant to generate CGA and separate polyphenols. Further work will focus on the use of CGA(WPI) for the separation of polyphenols from sources that differ from each other in the content and type of polyphenols.

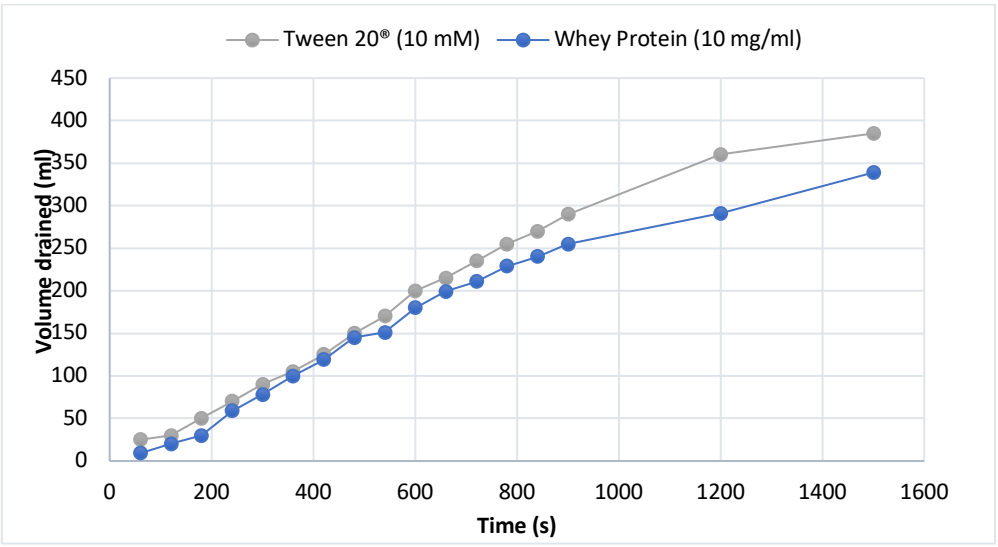
4.6 Appendix



Appendix 4.1. V/t of CGA(WPI) using UW 90 for concentrations of 2, 4, 6, 10, and 12 mg/mL.



Appendix 4.2. V/t of CGA(WPI) made of UW XP at 10, 15 and 20 mg/mL.



Appendix 4.3. Drainage rate for CGA Tween 20 [10mM] and whey protein 10 mg/mL.

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Chapter 5: Whey protein colloidal gas aphrons for separation of polyphenols from fruit processing by-products

Abbreviations

AAE: Ascorbic Acid Equivalents

ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)

BSA: Bovine Serum Albumin

C3G: Cyanidin 3 Glucoside

CGA: Colloidal Gas Aphrons

CGA(Tween 20): Colloidal Gas Aphrons made of Tween 20

CGA(WPI): Colloidal Gas Aphrons made of Whey Protein Isolate

D3G: Delphinidin 3 Glucoside

FRAP: Ferric Reducing Antioxidant Power

GME: Grape Marc Extract

HPLC-DAD: High Performance Liquid Chromatography – Diiodide Array Detector

HPLC: High Performance Liquid Chromatography

M3G: Malvidin 3 Glucoside

P3G: Petunidin 3 Glucoside

RGSE: Red Goji Skins Extract

SD: Standard Deviation

TE: Trolox Equivalents

TFC: Total Flavonoid Content

TMAC: Total Monomeric Anthocyanins

TPC: Total Phenol Content

TPTZ: 2,3,5-Triphenyltetrazolium chloride

WPI: Whey Protein Isolate

5.1 Introduction

The extraction of bioactive molecules such as polyphenols from agri-food by-products have been increasing in the past years, since they are not only cost effective and sustainable sources of polyphenols (Muhlack et al., 2018; Sagar et al., 2018), but also their valorisation is a way to move into the direction of circular economies (Moreno-González & Ottens, 2021). One of the pomaces that has been widely researched is grape marc (Cortés et al., 2020; Hegedüs et al., 2022; Pertuzatti et al., 2020; Sinrod et al., 2021; Spigno et al., 2007), this is a by-product obtained from the wine making industry, and has shown a high content of polyphenols, mainly flavonoids such as anthocyanins, as well as quercetin, myricetin, and rutin. Other compounds found in grape marc are stilbenes, tannins, and a variety of phenolic acids (Amendola et al., 2010; Li et al., 2013; MohdMaidin et al., 2019; Negro et al., 2003; Spigno et al., 2007).

Besides winemaking, one of the industries that generate several tonnes of pomaces per year is the juice manufacturing. Several pomaces such as orange, apple, berries, and currants have been widely studied since they are good sources not only of polyphenols but dietary fibre like pectin, for example (Azman et al., 2022; Benelli et al., 2010; Li et al., 2020; Rodríguez-Gutiérrez et al., 2019; Yates et al., 2017). In contrast, one of the less studied fruit by-products is goji berry pomace (Bora et al., 2019), the reason being that this is fruit that is consumed primary as dried fruit. However, some of the production has been destined for juice production, generating a pomace composed mostly of skins and pulp. Depending on the type of goji its phenolic profile will vary greatly, red goji for example, is rich in compounds such as carotenoids and polyphenols, like flavonoids

(catechin, myricetin, rutin) and phenolic acids (Kosińska-Cagnazzo et al., 2017; Kulczyński & Gramza-Michałowska, 2016; Skenderidis et al., 2017).

Fruit based by-products have shown to be excellent sources of polyphenols. However, these molecules present a myriad of challenges when formulating them. They are highly susceptible to structural changes that lead to a reduction in their bioactivity (Brglez Mojzer et al., 2016; Cao et al., 2021; Manach et al., 2004). Extensive research has gone into ways to protect polyphenols against degradation, showing that creating systems that favour interactions of polyphenols with other molecules, such as proteins, is an effective way to protect them (Gong et al., 2021; Li & Girard, 2023; Tazeddinova et al., 2022).

One of the most researched proteins for the stabilisation of polyphenols, is whey protein. The globular proteins that conform it have proven to exert a protective effect on polyphenols as well as to aid in their formulation. For example, whey proteins have shown a protective effect on resveratrol and its antioxidant capacity against heat-induced loss (Guo & Jauregi, 2018). These proteins have also been widely applied for the encapsulation of polyphenol from different sources, including by-products such as grape marc (Farrag et al., 2018). In addition, whey proteins have shown to improve sensory properties, such as reduce astringency in wine (Jauregi et al., 2016, 2021).

The stabilisation and protection of polyphenols has been carried out commonly through methods such as encapsulation and complexation. However, methods such as colloidal gas aphrons (CGA) surfactant microbubbles have shown the ability to interact with polyphenols, thus facilitating their separation (MohdMaidin et al., 2019; Spigno et al.,

2015b). In a recent study, CGA made of the non-ionic surfactant Tween 20 showed that these structures not only separate polyphenols in a surfactant rich fraction, but they also were able to stabilise anthocyanins (one of the most susceptible group of flavonoids to degradation) from grape marc during a period of 30 days at 20°C (MohdMaidin et al., 2018).

In the previous chapter of this work, it was established that whey protein can be used for CGA generation. Moreover, CGA(WPI) showed the ability to be applied for separation of polyphenols from raw blackcurrant pomace extract. For the separation it was seen that the previously found optimum conditions with non-ionic surfactants can be applied for CGA(WPI) separation. The purpose of this chapter is to gain an insight on the use of CGA(WPI) for the separation of polyphenols from fruit processing by-products extracts, whose content and type of polyphenols differ. Furthermore, the stabilisation effect of CGA(WPI) on anthocyanins from the grape marc extract during storage at accelerated conditions was investigated.

5.2 Materials

Materials:

Casa Emma (Firenze, Italy) winery kindly supplied a sample of fresh frozen and vacuum packaged Sangiovese variety grape marc (skins, seeds, and stems) from the September 2019 harvest. Dried red goji berry skins (dried at 40-45°C, until moisture content below 5% was achieved) were supplied from EVRA (Potenza, Italy). Whey protein isolate under the UW XP label was supplied by Volac Ltd (Hertfordshire, UK) with the following specifications: 94% protein, 0.3% fat and 0.5% lactose. TWEEN® 20 was purchased from Sigma-Aldrich (UK).

Reagents:

Folin-Ciocalteu (F9252), and sodium carbonate BioXtra, ≥99.0% (S7795), aluminum chloride anhydrous powder, 99.999%, sodium hydroxide, sodium nitrate, sodium acetate, potassium chloride, ethanol ≥99.8%, and formic acid were purchased from Sigma-Aldrich. HPLC grade water, and acetonitrile were purchased from Sigma Aldrich, and Thermo Fisher.

(+)-Catechin and gallic acid were purchased from Sigma Aldrich; delphinidin-3-o-glucoside (> 99%); cyanidin-3-o-glucoside (> 98%); petunidin-3-o-glucoside (> 98%) and malvidin-3-o-glucoside (> 99%); all purchased from Extra synthese (Paris, France).

Reagents for the ABTS method: 2,2′-azino-bis (3-ethylbenzothiazoline- 6-sulfonic acid) diammonium salt (ABTS) ≥98.0%, potassium persulfate ≥99.0%, (±)-6-hydroxy-2,5,7,8-

tetramethylchromane-2-carboxylic acid (trolox), 97%; for the ferric reducing antioxidant power (FRAP) method reagents: TPTZ (2, 4, 6-tris(2-pyridyl)-s-triazine) and ferric chloride hexahydrate were purchased from Sigma- Aldrich.

5.3 Methods

5.3.1 Separation with colloidal gas aphrons (CGA)

CGA(Tween 20) and CGA(WPI) were applied to grape marc extract (GME) and red goji skin extract (RGSE) obtained in previous experiments described in Chapter 3. The separation was carried out following the same methodology described in Chapter 4, section 4.2.1, 4.2.2. A summary of the conditions and evaluated parameters are described in Table 5.1.

Table 5.1. Experimental conditions for CGA separation used in this Chapter

Investigated variable	Fixed parameters	Evaluated outputs
Feedstock: grape marc, and goji berry skins extract	Hydroalcoholic extract volume: 65 mL	Recovery of various polyphenols
	WPI concentration: 15 mg/mL Tween 20 concentration: 10 mM	Antioxidant capacity in aphron phase
	Contact and drainage time (5 min)	Anthocyanins stability through storage time
	Volumetric ratio extract/CGA: 1:16	

5.3.2 Degradation of anthocyanins over time

To determine the stability of anthocyanins during storage time, accelerated storage conditions were selected based on previous work done in the research group on the storage time effect on anthocyanins in Roselle beverages where after a real time storage period of 180 days at 21°C it was determined that 1 day at 40°C was the equivalent to 6 days of the real time storage based on the first order kinetic model (Omoarukhe et al., 2023). Aliquots of grape marc extract labelled GME and CGA aphron phase labelled CGA(WPI) and CGA(Tween 20) were stored under accelerated conditions at 40°C in a cabinet (SANYO, GALLENKAMP), regularly monitored using a thermometer for 30 days; the changes were screened at day 0, 4, 8, 10, 15, 20, 25, and 30. The concentration of Tween 20 in CGA fraction was estimated from the volume of CGA and their corresponding liquid fractions. For WPI the protein concentration in CGA was estimated from the protein content determination in the liquid fraction.

The changes were determined by calculating the degradation kinetics of the four anthocyanins. For this, the natural logarithms of the anthocyanins content were plotted against time in order to test for first-order kinetics as described by the equation below:

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

Where A_0 is the initial anthocyanin content, A_t 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 obtained when plotting Ln (A_t/A_0) vs t.

From the equation above, the time taken for the anthocyanin content to halve, the half-life ($t_{1/2}$), can be derived as:

$$t\frac{1}{2} = \frac{Ln(2)}{k}$$

5.3.3 Identification of anthocyanins by HPLC

The anthocyanin content through the stability analysis was determined 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 (150mm×4.6mm i.d; 5µm particle size and 100Å pore size; part no.EXL-121-1546U) operated at 25 °C. The method was as follows: 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 min), 15–30% B (15 min), 30–50% B (10 min), 50–95% B (5 min) and 95–5% B (5 min), at a flow rate of 1 mL min^{-1}. The total run was 50 min and a pre-time of 10 min was allowed for re-equilibrating. The injection volume was 20 µL for pure standards and samples. The anthocyanins were detected at 520 nm and identified based on the retention times and by comparing the spectra with that of the external standards which were: delphinidin-3-o-glucoside (> 99%); cyanidin-3-o-glucoside (> 98%); petunidin-3-o-glucoside (> 98%) and malvidin-3-o-glucoside (> 99%).

5.3.4 Analytical determinations

To estimate the recovery of polyphenols analytical determinations were carried out according to the methodology described in Chapter 2 section 2.2. The results are expressed as:

- Total Phenol Content (TPC): milligrams of gallic acid per 100 grams of dry extract (mg GAE/ 100 g de) or percentage of gallic acid equivalents (% GAE), based on the equation in Chapter 4, section 4.3.2.
- Total Monomeric Anthocyanin Content (TMAC): percentage of malvidin 3-glucoside equivalents per 100 gram of dry weight (mg M3GE/ 100 g de), or percentage of malvidin 3-glucoside equivalents (% M3GE), based on the equation in Chapter 4, section 4.3.2.
- Total Flavonoid Content (TFC): mg of catechin equivalents per 100 gram of dry weight (mg CE/ 100 g de), or percentage of catechin equivalents (% CE), based on equation in Chapter 4, section 4.3.2.

5.3.5 Antioxidant Capacity

The antioxidant capacity was measured according to the methods described in Chapter 2, section 2.3. The results of ABTS are expressed in micro moles of trolox equivalents per gram of dry weight (µmol TE/ g dw). For FRAP, they are expressed in micro moles of ascorbic acid equivalents per gram of dry weight (µmol AAE/ g dw).

5.3.6 Statistical Analysis

All the experiments were performed in duplicate, and each duplicate was analysed in triplicate. The differences between surfactants in the CGA separation process were analysed using t-test for independent samples. For CGA antioxidant activity and stability of anthocyanins the data was analysed using a one-way ANOVA, detecting difference with the Tukey test. To determine if a correlation between polyphenols recovery and antioxidant capacity existed a Pearson's correlation analysis was carried out. The significance level was defined at $p<0.05$ for all analysis. All statistical analysis were done using IBM® SPSS® Statistics 27. The results were reported as means ± SD.

5.4 Results and Discussion

5.4.1 Separation of polyphenols from fruit processing by-products by CGA(WPI)

Prior to the CGA separation, a hydroalcoholic extraction was obtained from each feedstock (Chapter 3). The extracts were labelled as GME, for the grape marc extract, and RGSE, for red goji berry skin extract. It is important to mention that these two extracts differ in the type of polyphenols found naturally in them. Goji berry is known to have a higher content of phenolic acids and catechins. In comparison, grape marc's primary polyphenols are flavonoids, mainly anthocyanins, and phenolic acids. Moreover, they differ not only in the type but also in the content of polyphenols as it can be seen in Table 5.2 (taken from Chapter 3, table 3.3).

Table 5.2. Polyphenols determination, and antioxidant capacity from fruit processing by-products.

Feedstock	TPC	TMAC	TFC
Blackcurrant pomace	3264.37 ± 32.31 ^{a}	300.87 ± 1.83 ^{a}	2273.28 ± 61.86 ^{a}
Grape marc	2034.98 ± 240.30 ^{b}	27.37 ± 2.82 ^{b}	2015.00 ± 108.99 ^{b}
Red goji skins	1029.61 ± 2.06 ^{c}	ND	254.95 ± 43.81 ^{b}

Total phenol content (TPC) values are expressed in mg GAE/ 100 g dw; total monomeric anthocyanin content (TMAC) values are expressed in mg of M3GE/ 100 g dw; total flavonoid content (TFC) values are expressed in mg CE/ 100 g dw. Values expressed in mean ± SD *n=2*. Different letters in the same column denote significant differences ($p<0.05$).
ND: NON-DETECTED

CGA separation performance for each surfactant and each feedstock is shown in Fig 5.1. Recovery of polyphenols from GME was above 90%, with similar values for both surfactants, 94% and 91% for CGA(WPI) and CGA(Tween 20) respectively (Fig 5.1-A).

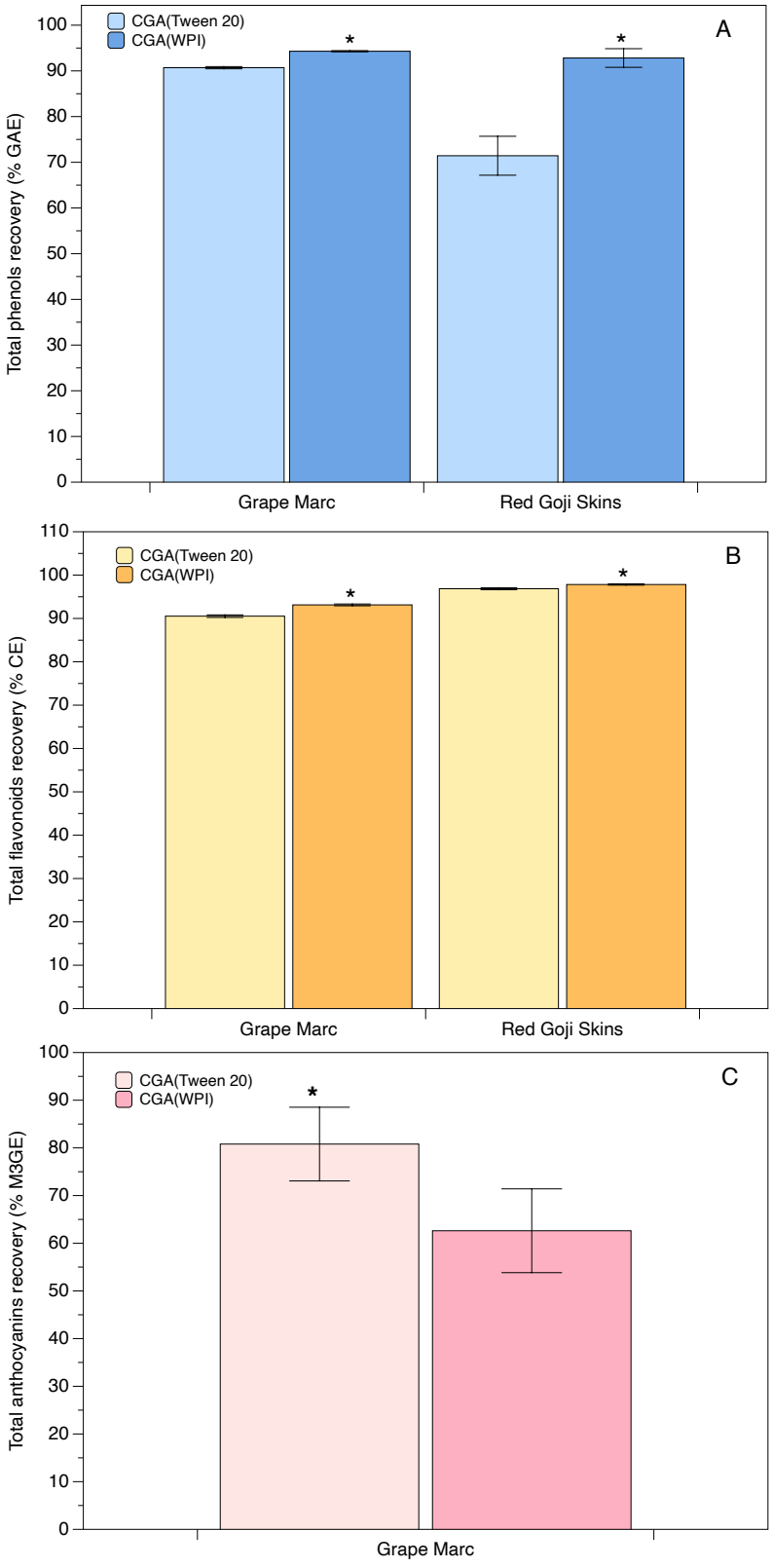


Figure 5.1. (A) Recovery of total phenol content (TPC), (B) total flavonoid content (TFC), and (C) total monomeric anthocyanins content (TMAC) from grape marc (GME) and red goji skins extract (RGSE) separated through CGA(WPI) and CGA(Tween 20). * Denotes a significant difference $p<0.05$.

As for TFC, for both feedstocks and CGA fractions the recovery was over 90% (Fig 5.1-B). However, for RGSE, the recovery was significantly different between surfactants ($p<0.05$), higher recovery was observed in CGA(WPI) (93%) than CGA(Tween 20) (71%). Regarding TMAC, a higher recovery was obtained with CGA(Tween 20), approximately 22% higher than with CGA(WPI) (Fig 5.1-C). Our results for anthocyanins recovery agree with other studies in which CGA made of Tween 20 have demonstrated higher recoveries for anthocyanins than other polyphenols (Carullo et al., 2022b; MohdMaidin et al., 2019; Spigno et al., 2015b). Interestingly, the results here showed that Tween 20, seems to have an affinity for other type of flavonoids.

Previous research has shown that polyphenols in hydroalcoholic solutions interact with non-ionic surfactants in CGA mainly by hydrophobic interactions (Carullo et al., 2022b; Dermiki et al., 2008b; Jarudilokkul et al., 2004; Jauregi & Dermiki, 2010; Noriega et al., 2018; Sazdanić et al., 2023). From these results on the recovery of polyphenols it seems that whey proteins are behaving in a similar way. Therefore, is possible that similar to Tween 20, hydrophobic interactions are playing a part in the successful separation of polyphenols.

Interactions between whey proteins and different types of polyphenols have been widely studied, and the findings reveal that the primary interactions are of the non-covalent type, such as hydrophobic interactions, hydrogen bonds, and van der Waals forces (Cao & Xiong, 2017; Frazier et al., 2010; Gong et al., 2021; Guo & Jauregi, 2018; Meng & Li, 2021). Moreover, the strength and magnitude of these interactions is influenced by many

factors such as pH, type of protein and concentration, structure and type of polyphenols, and the protein/polyphenols ratio (Guo & Jauregi, 2018; Ozdal et al., 2013).

Diverse studies have been carried out using different single whey proteins such as β-lactoglobulin, α-lactalbumin, bovine serum albumin (BSA), and lactoferrin in combination with different polyphenols (Al-Hanish et al., 2016; X. Chen et al., 2022; Diao et al., 2021; Frazier et al., 2006, 2010; Guo & Jauregi, 2018). Research shows that in general, polyphenols interact with the hydrophobic pockets of the main whey proteins (Skrt et al., 2012). These hydrophobic interactions have been associated with hydrogen bonding, but the binding seem to be a balance between the two (Ozdal et al., 2013). Also, the magnitude of these interactions will depend on the type of polyphenols, highly hydroxylated molecules promote more hydrogen bonding with the protein than smaller polyphenols (Capanoglu et al., 2017; Ozdal et al., 2013).

In summary, from these results, we can infer that CGA structure and characteristics, combined with whey protein-polyphenols interactions, enabled a successful separation. Moreover, the results obtained in this chapter and Chapter 4 agree that CGA(WPI) are suitable for the separation of polyphenols from extracts that differ in content and type of polyphenols. However, the results also showed that extracts with a high anthocyanin content do not benefit as much from the separation with WPI as they do with Tween 20. Something that will be discussed further in section 5.4.3.

5.4.2 Total antioxidant activity by ABTS and FRAP in CGA separation

After the CGA separation, it was essential to understand how the interactions between surfactants and polyphenols could influence their antioxidant activity. Figure 5.2 displays the antioxidant capacity in the feedstock's and aphron phases. For RGSE, an antioxidant capacity of 95 µmol TE and 11 µmol AAE by ABTS and FRAP, respectively were founded in CGA(Tween 20). For CGA(WPI), a content of 87 µmol TE and 9 µmol AAE by ABTS and FRAP respectively were observed. Overall, for RSGE a significantly higher antioxidant capacity was found in CGA(Tween 20) compared to CGA(WPI) ($p<0.05$). Furthermore, at least approximately 70% of the antioxidant capacity in the feedstock was present in both CGA.

For GME, the antioxidant capacity values were different depending on the method and significant differences were observed between CGA(WPI) and CGA(Tween 20) by FRAP ($p<0.05$). The results show that for both CGA(Tween 20) and CGA(WPI) approximately 60% of the activity from the feedstock was preserved after the separation. On the other hand, the FRAP method showed that 82% of the activity in the feedstock was present in CGA(Tween 20), compared to 50% in CGA(WPI). When analysing how the recovered polyphenols could correlate to the antioxidant capacity, the results showed that the only significant correlation was found between the TPC found in CGA(WPI) and FRAP ($r= 0.811$; $p=0.05$). No significant correlation ($p> 0.05$) was found between the rest of polyphenols and antioxidant capacity for both, GME and RSGE with both surfactants.

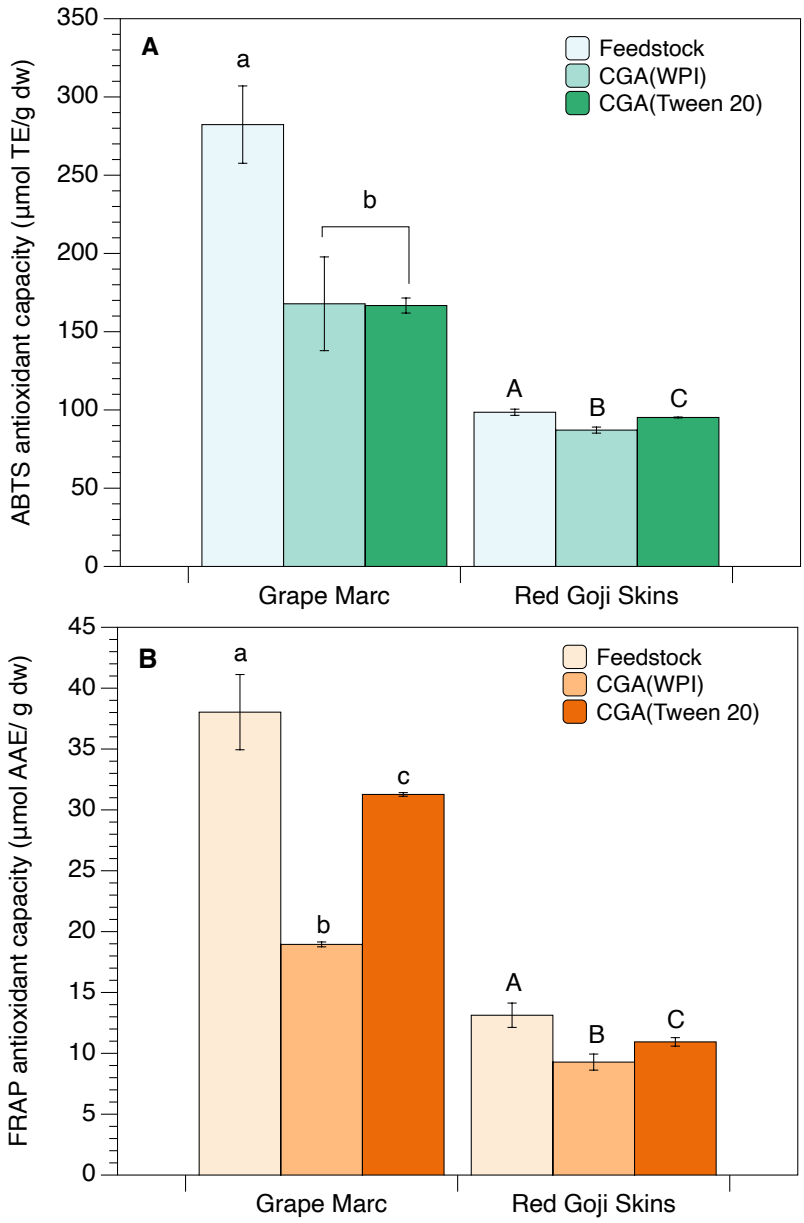


Figure 5.2. Antioxidant capacity by (A) ABTS and (B) FRAP in feedstock, CGA(WPI) and CGA(Tween 20) fractions. Different letters mean significant differences within the polyphenols source ($p<0.05$). Values are expressed as mean±sd (n=2).

However, it is important to recall what was observed in Chapter 4, that the antioxidant activity values of ABTS were higher than the ones by FRAP. Moreover, that FRAP values of CGA(WPI) were significantly lower than those of CGA(Tween 20). Supporting the inference that besides degradation and oxidation of polyphenols during the separation,

the measure mechanism of the methods will give place for differences depending on the type of polyphenols and interactions between surfactants.

Results obtained here with CGA(Tween 20) differ from those obtained by Carullo et al. (2022), where no loss of antioxidant capacity between the feedstock and the aphron phase was observed in the separation of grape marc polyphenols using Tween 20. However, loss of antioxidant capacity during CGA separation with Tween 20 has been previously reported. Spigno et al. (2015) found a slight loss of antioxidant activity in the aphron phase after Tween 20 CGA separation of polyphenols from grape marc. These authors argue that the lower content of antioxidant capacity with Tween 20 could have been due to possible oxidation of the polyphenols during the separation (Carullo et al., 2022b; Spigno et al., 2015b; Spigno & Jauregi, 2005).

In the case of CGA(WPI), it is likely that protein-polyphenols interactions as well as the medium, played an important role in the decrease of antioxidant activity. It has been discussed that interactions between whey protein and polyphenols might decrease the activity due to the hydrogen bonding between protein and polyphenols (Almajano et al., 2007b; de Morais et al., 2020; von Staszewski et al., 2011). However, some studies argue that the interactions could be creating a masking effect on the antioxidant capacity. This masking is likely due to the competition during the antioxidant activity analysis between the protein and hydroxyl groups of the polyphenols (Ozdal et al., 2013; Stojadinovic et al., 2013).

In CGA separation, parameters such as volumetric ratio, surfactant concentration, and polyphenols concentration will influence the separation as mentioned before (Dahmoune et al., 2013; MohdMaidin et al., 2018, 2019). However, in the antioxidant capacity other parameters, such as the medium, and interactions between surfactants and polyphenols could be playing an important role. These results obtained here are consistent with the ones obtained in Chapter 4; where regardless of the recovery of polyphenols during the CGA separation, the antioxidant capacity was affected. Therefore, it can be stated that while a good polyphenol separation is achieved, it does not necessarily correlate with high antioxidant capacity in CGA. However, in here, it seems that for extracts rich in phenolic acids and flavonoids such as RGSE, the antioxidant activity is affected to a less extent regardless of the surfactant.

5.4.3 pH influence in CGA separation

The pH of the surfactant aqueous solutions, extracts, liquid phase, and aphron phase was measured to see its effect on the separation. Table 5.2 shows that the WPI solution (pre-CGA) and aphron phase of RGSE had a pH of 6.20 and 6.16, respectively. For both the pH was above the isoelectric point of whey proteins. Therefore, whey proteins will be negatively charged at this pH (Fuda et al., 2005; Fuda & Jauregi, 2006). On the other hand, the aphron phase of GME showed a slightly lower pH, closer to the isoelectric point of whey proteins. So repulsive forces were minimised between protein molecules, thus

promoting hydrophobic interactions (Fuda et al., 2005; Fuda & Jauregi, 2006; Tian et al., 2017).

When evaluating these pH values and the results obtained from the recovery section, it is noticed that despite the pH difference in CGA(WPI) aphron phase for GME and RGSE, similar recoveries of TPC and TFC were attained for both. These findings support the argument that CGA(WPI) separation is driven mainly by hydrophobic interactions and hydrogen bonds. In addition, the increased pH in CGA(WPI) aphron phase of GME could be the reason for reduced recovery of anthocyanins. Anthocyanins are highly susceptible to structural changes above pH 2, where the ionisation of the flavylium ion gives place to other structures (Andersen & Jordheim, 2010b; Cabrita et al., 2000; Enaru et al., 2021; Martín et al., 2017). Therefore, this could be the reason for the better recovery of anthocyanins with CGA(Tween 20) (pH =3.79) than with CGA(WPI) AP (pH= 5.81). As well as the higher antioxidant capacity in CGA(Tween 20).

Table 5.2. pH of GME, RSGE, surfactants and CGA phases after separation

		UW XP (pH 6.20)		Tween 20 (pH 4.98)	
Feedstock	Extract	Liquid phase	Aphron phase	Liquid Phase	Aphron Phase
GME	4.56 ± 0.00	6.33 ± 0.00	5.81 ± 0.04	3.84 ± 0.06	3.79 ± 0.07
RGSE	5.68 ± 0.00	6.41 ±0.05	6.16 ± 0.03	4.79 ± 0.03	4.82 ± 0.01

Values are expressed in mean ± sd (n=2)

5.3.4 Stability effect of CGA on GME - kinetics of anthocyanins over storage time

It is known that anthocyanins are highly susceptible to degradation and will have more or less stability depending on the medium (Andersen & Jordheim, 2010b; Martín et al., 2017), structure (Vidana Gamage et al., 2022), and storage conditions, among others. Hence the interest to investigate how CGA can be used as polyphenols fractions enriched with whey proteins can aid stabilising anthocyanins. The protein content of CGA(WPI) was of 13.8 mg/mL, and for CGA(Tween 20) the surfactant concentration was of 7.68 mM.

Studies on anthocyanin stability in the presence of CGA made of Tween 20 have been carried out in the group (MohdMaidin et al., 2019). In this work, a similar methodology was used to investigate the effect of CGA(WPI) on stability of anthocyanins upon storage. For the stability analysis, four anthocyanins were selected based on their occurrence in grape marc established in previous studies (MohdMaidin et al., 2018, 2019). Delphinidin, petunidin, cyanidin and malvidin anthocyanidins in their 3-O-glucoside form were analysed through HPLC-DAD. The analysis showed that the four anthocyanins were present in the samples at the beginning of the storage study. Malvidin 3-O-glucoside (M3G) was the most abundant anthocyanin, followed by petunidin 3-O-glucoside (P3G), cyanidin 3-O-glucoside (C3G), and delphinidin 3-O-glucoside (D3G).

Degradation of anthocyanins followed first-order kinetics (Fig 5.3), in agreement with previously reported (Andersen & Jordheim, 2010b; Hellström et al., 2013; Lavelli et al., 2016; MohdMaidin et al., 2019). The anthocyanin M3G was the most stable of all. It was

present in CGA(Tween 20) during the entire duration of the study (up to 30 days), and up to day 20 in CGA(WPI). For GME the last day M3G was detected was day 15. C3G was the second more stable anthocyanin, mainly in CGA(WPI) and CGA(Tween 20). D3G and P3G were the less stable anthocyanins. Interestingly, D3G was detected on day 10 in GME but not in CGA(WPI) or CGA(Tween 20) after day 4. Overall, WPI and Tween 20 had a stabilising effect on M3G and C3G. While for D3G no stabilising effect was seen. In general, CGA(Tween 20) showed a slower loss over time than GME and CGA(WPI).

The degradation rate constant (k) and half-life of anthocyanins are shown in Table 5.3. From here it can be seen that the lowest half-life in the study was for D3G, particularly for CGA(WPI) (2 days) compared to GME and CGA(Tween 20) (3 days). For P3G, GME and CGA(WPI) showed the same half-life, and CGA(Tween 20) was slightly higher. On the other hand, C3G and M3G showed at least double the half-life of GME in the presence of CGA(WP) (8 days) and CGA(Tween 20) (10 days).

The GME and CGA(Tween 20) results agree with those obtained in the group by MohdMaidin et al. (2019). Their results show that for D3G, grape marc extract had a higher half-life and lower k than Tween 20 CGA (volumetric ratio of 16, same as here).

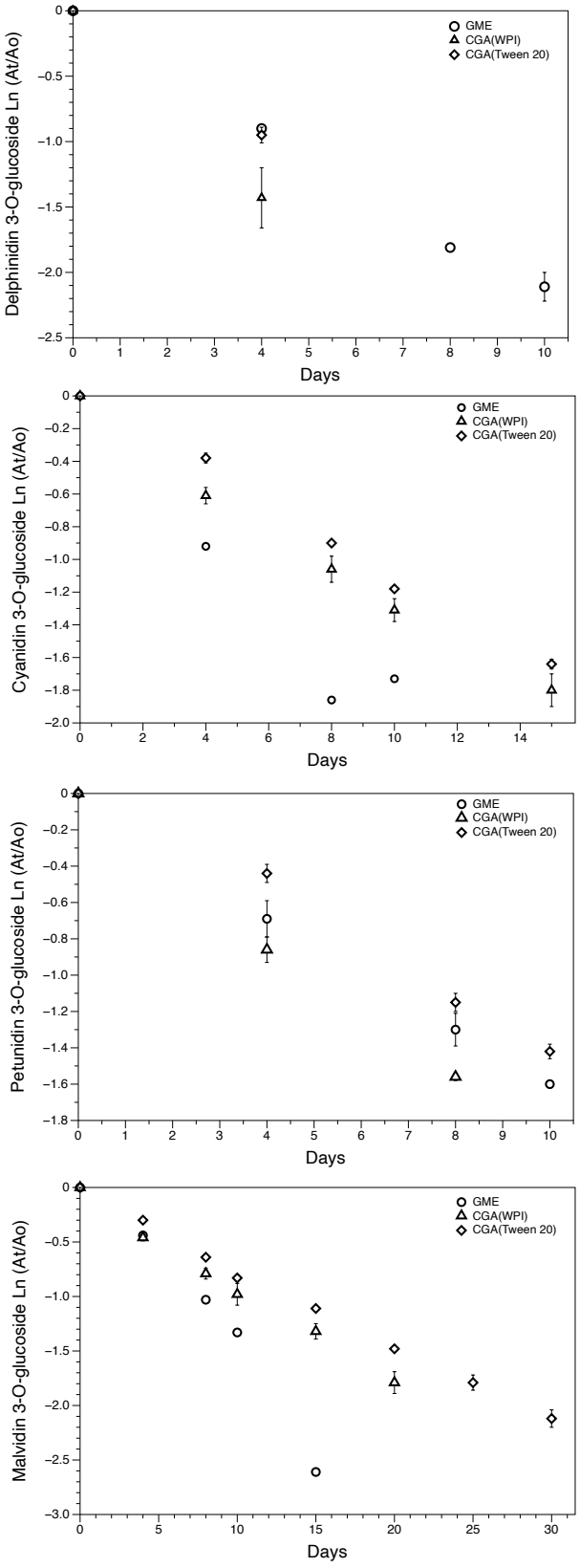


Figure 5.3. Decrease of anthocyanins over time represented as natural logarithm of the ratio of anthocyanins at a given time at time zero during storage at 40ºC for 30 days. Values expressed as mean ± sd (n=2).

Furthermore, C3G and P3G showed similar stability at CGA volumetric ratios of 16 and 4. As for M3G, this anthocyanin was the most stable in the grape marc extract and the different CGA volumetric ratios tested. A critical difference between our study and the previously mentioned one is the temperature at which anthocyanins were stored. For them the storage conditions were 20°C for 32 days, their results showed that for CGA Tween 20 at a volumetric ratio of 1:16, the half-life of anthocyanins ranged from 29 to 71 days. In contrast, the half-life range of the grape marc ethanolic extract was between 41 and 51 days.

Here, accelerated conditions were tested; for this, a temperature of 40°C was selected to observe the behaviour of anthocyanins at higher temperatures than in previous studies. The results showed that at 40°C, the half-life for CGA(Tween 20) ranged from 3 to 10 days, while for GME was 3 to 4 days. Interestingly, a similar anthocyanin degradation pattern was observed in this study and MohdMaidin et al. (2019). Here, the anthocyanin degradation goes as follows: D3G>P3G>C3G>M3G, while for them, it was D3G>P3G=C3G>M3G.

Table 5.3. Half-lives (t ½, days) and degradation rate constant (k, d-1) of anthocyanins processed in control (GME), CGA(WP) and CGA(Tween 20).

Anthocyanin/Sample	GME		CGA(WPI)		CGA(Tween 20)	
	$t_{1/2}$	K (d^{-1})	$t_{1/2}$	K (d^{-1})	$t_{1/2}$	K (d^{-1})
Delphinidin 3-O-glucoside	3	0.2146	2	0.3419	3	0.2359
Cyanidin 3-O-glucoside	3	0.2326	6	0.1187	6	0.1125
Petunidin 3-O-glucoside	4	0.1595	4	0.1931	5	0.1456
Malvidin 3-O-glucoside	4	0.1711	8	0.0859	10	0.0700

This pattern could be related to the structure of anthocyanins (Fig 5.4). For example, D3G is a highly hydroxylated molecule, making it more susceptible to degradation in aqueous systems and high temperatures (Cabrita et al., 2000; Vidana Gamage et al., 2022). On the other hand, the absence of hydroxyl groups on positions 3' and 5' make malvidins more stable compared to other anthocyanidins (Martín et al., 2017). Interestingly, here, C3G was more stable than P3G. This result was unexpected since the methoxy group in position 5' should give P3G more stability compared to C3G, which has no functional group in the 5' position. It is important to recall that the pH in CGA(WPI) and CGA(Tween 20) was above the pH in which anthocyanins are stable. Near neutral pH the flavylium cation is ionised, making the molecule more susceptible to structural changes (Andersen & Jordheim, 2010b; Martín et al., 2017; Mattioli et al., 2020).

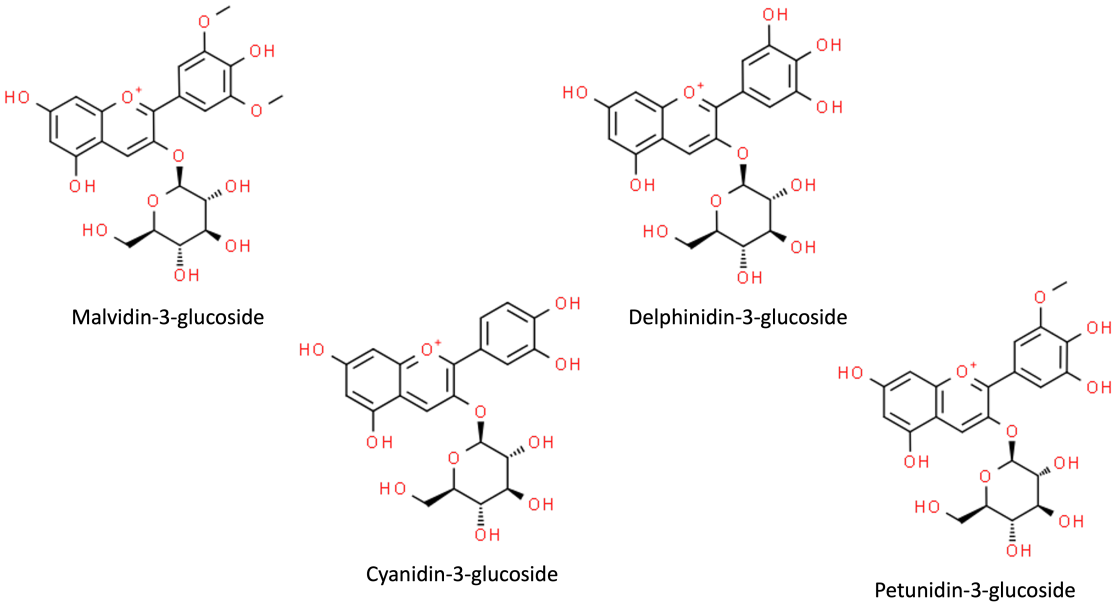


Figure 5.4. Structure of anthocyanins used in this work. Own creation figure with structures taken from Royal Society of Chemistry-ChemSpider database (www.chemspider.com).

Overall, these results, showed that the degradation in CGA(Tween 20) and CGA(WPI) was slower compared to the one in the crude extract. Moreover, they seem to have a similar behaviour as well. The stabilisation effect is due to the interactions between surfactant and polyphenols. However, anthocyanins such as delphinidin did not benefit from the CGA separation with neither of the surfactants. Nonetheless, for the rest of anthocyanins it was seen that the half-life was at least double than the one in the extract alone.

5.5 Conclusions

This chapter explored the influence of polyphenols from GME and RGSE in CGA separation with WPI and Tween 20. The results show that applying CGA(Tween 20) and CGA(WPI) to GME and RGSE led to a recovery of more than 70% TPC and 90% TFC. However, for anthocyanins in GME, the recovery was 22% higher in CGA(Tween 20) than in CGA(WPI). The results showed that overall, CGA(WPI) recovered more polyphenols than CGA(Tween 20) with the exemption of TMAC. However, this behaviour in CGA(WPI) could be associated to the pH in the CGA fraction.

For the antioxidant capacity it was seen that the separation significantly impacted the content in the CGA fractions compared to the content found in the feedstocks. More importantly, it was determined that the polyphenol recovery was not correlated with the antioxidant capacity. However, some interesting behaviours were seen in the antioxidant capacity of the feedstocks. For example, RGSE antioxidant capacity was not affected as much as it was for GME, and this was for both CGA fractions (possibly due to anthocyanins degradation at higher pH´s). In contrast, GME results showed that CGA(WPI) and CGA(Tween 20) have a prominent effect on the antioxidant capacity since the results differed significantly depending on the method. For CGA(Tween 20) antioxidant capacity, the oxidation of polyphenols during the separation is possibly responsible for the observed decrease. In the case of CGA(WPI), the interactions between whey proteins and polyphenols could be decreasing or masking the antioxidant capacity.

When analysing the pH values of the feedstocks and different fractions, the observations support that electrostatic interactions did not play a role in the separation. Moreover, pH in CGA(WPI) might have been responsible for the low content of anthocyanins since at near-neutral pH anthocyanins are highly susceptible to structural changes.

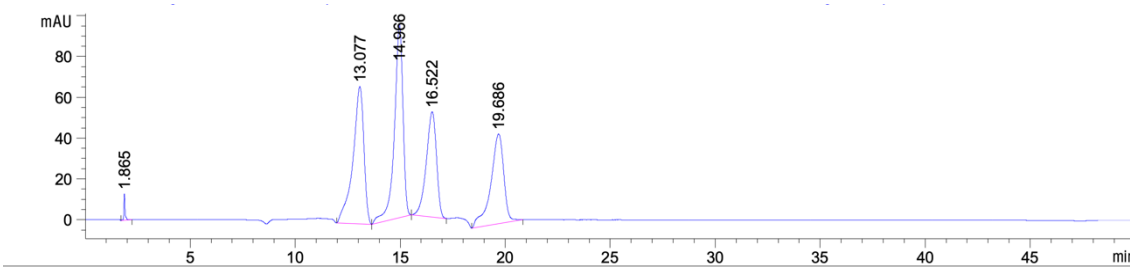
For the stability experiment, it was seen that under accelerated conditions, the half-life values ranged from 2 to 10 days in CGA fractions and 3 to 4 days in GME. Additionally, a degradation pattern was observed: D3G>P3G>C3G>M3G. Furthermore, the results showed that for D3G, the surfactants do not exert a stabilisation effect. Something worth exploring if was due to the medium or solely attributed to the surfactant. However, for M3G and C3G, there was a stabilisation effect with both surfactants, as the half-life was at least double than the one of GME. For the first time here, it was demonstrated that CGA(WPI) have a clear stabilising effect, especially for M3G and C3G.

The overall results in this chapter demonstrate that CGA(WPI) displays the versatility to be applied to different polyphenol extract feedstocks. Moreover, CGA(WPI) it shows the potential to aid in the stabilisation of anthocyanins such as M3G and C3G. However, modifications need to be done as to have a pH that allows for a higher recovery of anthocyanins. Nonetheless, CGA(WPI) shows the potential to be used in the formulation of polyphenol-based foods. Yet, further studies are needed to find conditions that allow a higher antioxidant capacity content. These results show that CGA could be a plausible option for formulating nutraceuticals with extracts that have as main anthocyanins M3G and C3G.

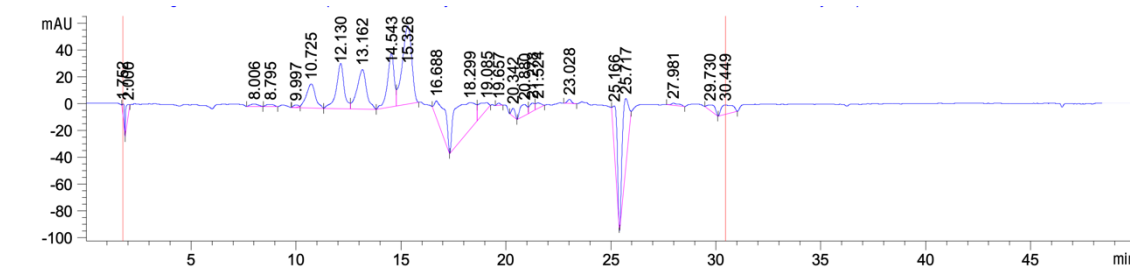
5.6. Appendix

Appendix Table 5.1. Regression coefficient of GME, CGA(WPI), and CGA(Tween 20)

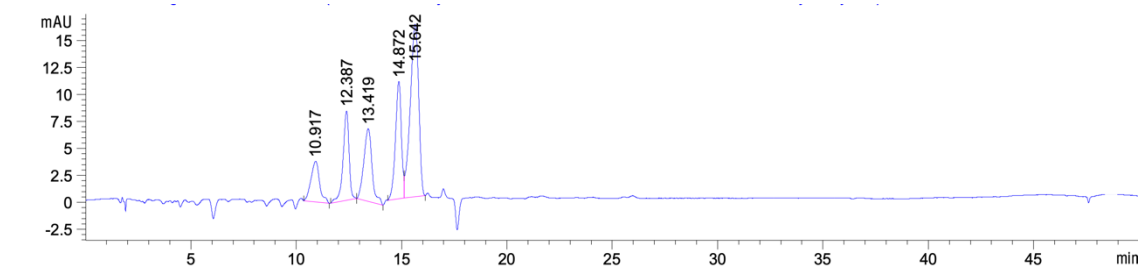
Anthocyanin/Sample	GME	CGA(WPI)	CGA(Tween 20)
	R^2	R^2	R^2
Delphinidin 3-O-glucoside	0.9967	1	1
Cyanidin 3-O-glucoside	0.999	0.9912	0.994
Petunidin 3-O-glucoside	0.999	0.9965	0.9891
Malvidin 3-O-glucoside	0.9619	0.9939	0.9964



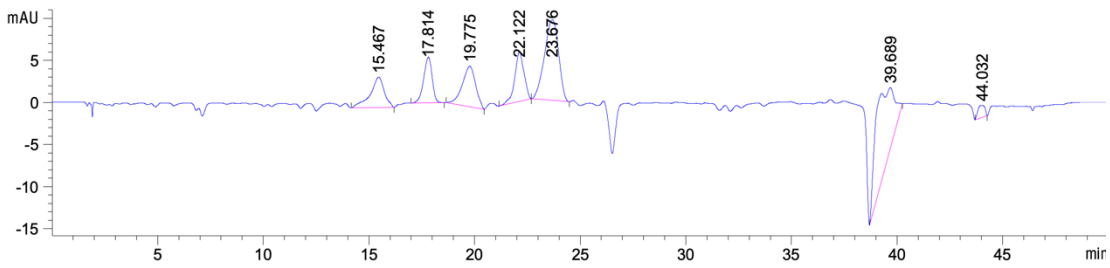
Appendix Figure 5.1. Standards 20 micro mol, Delphinidin, Cyanidin, Petunidin, and Malvidin



Appendix Figure 5.2. HPLC chromatogram extract day 0



Appendix Figure 5.3. HPLC chromatogram CGA(WPI) day 0



Appendix Figure 5.4. HPLC chromatogram CGA(Tween 20) day 0

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Chapter 6: Whey-pectin microcapsules improve the stability of grape marc phenolics during digestion

As resubmitted after corrections to the Journal of Food Sciene and Technology; with modifications on the methods section and FTIR results for this thesis.

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Abbreviations

AAE: Ascorbic Acid Equivalents

ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)

ATCC: American Type Culture Collection

CAA: Cellular Antioxidant Activity

DCF: Dichlorodihydrofluorescein

DCFH-DA: Dichlorodihydrofluorescein diacetate

DMSO: Dimethyl Sulfoxide

EDTA: Ethylenediaminetetraacetic acid

EY%: Encapsulation Yield percentage

FBS: Fetal Bovine Serum

FODIAC: Food for Diabetes And Cognition

FRAP: Ferric Reducing Antioxidant Power

FT-IR: Fourier Transform Infrared

GAE: Gallic Acid Equivalents

GD: Gastric Digestion

GID: Gastro Intestinal Digestion

GIT: Gastro Intestinal Tract

GME: Grape Marc Extract

HBSS: Hanks' Balanced Salt Solution

HTB-37: Caco-2 cell ATCC code

ID: Intestinal Digestion

MEM: Minimum Essential Medium

mg CE/g de: milligrams of catechin equivalents per gram of dry extract

MV3GE/L: Malvidin 3 Glucoside Equivalents per Litre

NEAA: Non-Essential Amino Acids

NSD: Nano Spray Drying

PBS: Phosphate Buffered Saline

PDI: Polydispersity Index

ROS: Reactive Oxygen Species

SD: Standard Deviation

SEM: Scanning Electron Microscope

Sin-1: 3-morpholinosydnonimine

t-BOOH: Tert-butyl Hydroperoxide

TE: Trolox Equivalents

TFC: Total Flavonoid Content

TMAC: Total Monomeric Anthocyanins

TPC: Total Phenol Content

TPTZ: 2,3,5-Triphenyltetrazolium chloride

W-P-GME: Whey – Pectin – Grape Marc Extract

W-P: Whey - Pectin

WPI: Whey Protein Isolate

µm: micro metre

Abstract

Grape marc is an agri-food residue from the wine industry valuable for its high content of phenolic compounds. This study aimed to develop an encapsulation system for grape marc extract (GME) using food-grade biopolymers resistant to gastric conditions for its potential use as a nutraceutical. For this purpose, a hydroalcoholic GME was prepared with total phenolics content of 219.62 ± 11.50 mg Gallic acid equivalents (GAE)/ g dry extract and 1389.71 ± 97.33 μmol Trolox equivalents (TE)/ g dry extract antioxidant capacity, assessed through ABTS assay. Moreover, the extract effectively neutralised ROS in Caco-2 cells, demonstrating an intracellular antioxidant capacity comparable to Trolox. The GME was encapsulated using whey protein isolate and pectin through nano-spray drying (73% yield), resulting in spherical microparticles with an average size of 1 ± 0.5 μm and a polydispersity of 0.717. The encapsulation system protected the microcapsules from simulated gastrointestinal digestion, where at the end of the intestinal phase, 82% of the initial phenolics were bioaccesible compared to 54% in the free GME. Besides, the encapsulated GME displayed similar (ABTS) or a higher antioxidant activity (FRAP) than the free extract after GID. These results show the potential of this encapsulation system for applying GME as a nutraceutical with a high antioxidant capacity and protective effect against cellular oxidation.

Keywords: grape marc phenolics, biopolymer, nano-spray drying, *in vitro* digestion, encapsulation.

6. 1 Introduction

Grape marc is a food by-product composed of the skins, seeds and stems recovered at the end of the winemaking process. This by-product has attracted significant attention due to its high phenolic content (Lavelli et al., 2016; Peixoto et al., 2018). Phenolics are a family of molecules with antioxidant properties, including phenolic acids and polyphenols such as flavonols and flavan-3-ols (Cao et al., 2021b; Tsao, 2010). It has been found that they can play a significant role in the management and prevention of several diseases, especially cardiovascular and type 2 diabetes (Dias et al., 2022; Fraga et al., 2019).

Phenolics are extensively researched for their properties but are challenging molecules. They are susceptible to temperature changes, moisture, oxygen, and high/low pH values. In addition, once ingested, they present low stability and bioavailability in the human body due to their low solubility and low membrane permeability (Ludwig et al., 2015; Scalbert & Williamson, 2000; Stalmach et al., 2009; Teng & Chen, 2019). For these reasons, phenolics are unlikely to be used in their pure form and encapsulation is foreseen as an alternative to improve their stability and preserve their properties within food products and bioavailability after consumption (Brezoiu et al., 2019; Sessa et al., 2013; Spigno et al., 2013). The encapsulation process involves using materials to embed, complex, or create a protective wall around bioactives, and by carefully selecting these materials, a targeted release of the bioactives can be achieved.

Polysaccharides and proteins are vastly used biopolymers for encapsulation, and interestingly, many of these materials can be obtained from by-products, like whey

protein isolate (WPI). WPI is a by-product of the cheese-making process, which contains proteins with high nutritional quality (de Wit, 1998; Jauregi & Welderufael, 2010; Yalçin, 2006). Furthermore, WPI forms complexes with polyphenols, stabilising them by improving their solubilisation and protecting their antioxidant activity from heat-induced loss (Guo & Jauregi, 2018). On the other hand, polysaccharides like pectin are found in the peel of citrus, apple, and other fruits. Pectin, as insoluble fibre, is poorly absorbed in the upper GIT, but pectinolytic enzymes produced by colonic microflora degrade the polysaccharide (Dongowski & Anger, 1996; Rehman et al., 2019). Pectin biodegradability is an interesting property to take advantage of as an effective carrier for the targeted release of bioactive compounds absorbed in the colon. Polyphenols can be absorbed in different parts of the GIT, and those reaching the colon are known to be metabolised by the microbiota into additional low molecular weight phenolic acids (Scalbert et al., 2002).

Besides, pectin has other interesting technological properties like emulsifying, gelling and complexation properties (Rehman et al., 2019). In particular, pectin is known for its interaction with WPI through covalent/non-covalent interactions, and their complexes have been studied for their application in food colloidal systems (Du et al., 2022). All these properties of pectin and WPI, together with their known interaction with polyphenols, are expected to protect these labile compounds from processing and digestive conditions, providing their selective release in the lower intestine where they can be absorbed.

Among the most used encapsulation methods is spray drying, an efficient, fast, cost-effective, and protective method to obtain dry particles (Annunziata et al., 2020; De La

Cruz-Molina et al., 2021; Fang & Bhandari, 2012). This encapsulation technique involves the formation of microcapsules by producing a mixture of bioactive compounds with carriers in solution or suspension and then atomising this mixture in a hot air stream to obtain a dry powder (Dias et al., 2022). Nano spray drying (NSD) has emerged as a technology to reduce particle size. With smaller particles, physiological fate is significantly enhanced due to the higher surface: volume ratio offering a higher penetration rate into the cells, stability, target release and bioavailability (Chopde et al., 2020; Jafari et al., 2021).

Several studies have been carried out to study the use of these protein-polysaccharide interactions for spray drying of grape by-products, and further *in vitro* digestion due to the excellent source of phenolics they represent (Brown Da Rocha & Zapata Noreña, 2020; Constantin et al., 2021; Du et al., 2022). However, few studies have investigated nano spray drying for raw extracts and their behaviour during gastrointestinal digestion. Desai et al. (2020) used nano spray drying to encapsulate a raw green coffee extract with maltodextrin; their findings showed that maltodextrin protected the chlorogenic acid and its antioxidant activity from digestion conditions and storage. Other works have used the nano spray dryer for the encapsulation of saffron and soy extracts; however, in these works, a purification of specific compounds was carried out before the encapsulation (Del Gaudio et al., 2016; Kyriakoudi & Tsimidou, 2018). Moreover, these mentioned studies investigate only the use of maltodextrin even though nano spray drying has been used for encapsulation of specific whey proteins such as bovine serum albumin and lactoferrin (Bourbon et al., 2020; Lee et al., 2011).

This work aims to produce nano spray dried microcapsules with whey protein-pectin as encapsulants for the encapsulation of a raw grape marc extract and to study the effect on the stability and bioaccessibility of the polyphenols. Moreover, the biocompatibility and antioxidant capacity of the extract is assessed using a Caco-2 cell line and compared against the commercial antioxidant compound Trolox.

6.2 Materials

Casa Emma Winery (Firenze, Italy) kindly supplied commercial grape marc flour from Sangiovese grapes. The grape marc flour is obtained by drying the grape marc at 42 °C for three days to preserve the phenolics. The grape marc is constantly mixed to avoid mould growth, and after the drying process, it is pulverised to a 250-micron particle size. The final product has the following specifications (supplied by the manufacturer): 8.53% moisture, 8% carbohydrates (from which sugars are 0.56%), 58.6% fibre, and 11.8% protein. Whey protein isolate was purchased from Volac International Ltd (Hertfordshire, UK) with the following specifications (supplied by the manufacturer): protein: 92% min, lactose: 0.9% max, fat: 0.8% max, pH: 5.8 min (10% sol). Pectin from citrus peel with ≥74.0% of galacturonic acid and ≥ 6.7% of methoxy groups; pepsin from porcine gastric mucosa ≥ 250 units/mg solid, pancreatin from porcine pancreas 8 x USP, bile, 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ), 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), FBS Superior, Resazurin sodium salt, 2',7'-Dichlorofluorescin diacetate (DCFH-DA), Sin-1, and (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) were purchased from Sigma-Aldrich. MEM Eagle (with 2 mM L-

Glutamine, 1 mM Sodium pyruvate, non-essential amino acids (NEAA)) and Penicillin-Streptomycin (10,000 U/mL-10 mg/mL, respectively) were from PAN-Biotech GmbH.

6.3 Methods

6.3.1 Extraction of phenolics from grape marc

A hydroalcoholic extraction was applied following the methodology previously developed in our group (MohdMaidin et al., 2018) to extract phenolics from grape marc. The extraction was carried out in an 8:1 ratio (solvent: solid) using a solution of 60% ethanol under magnetic stirring for 2 h at 60°C. After the extraction, the solids were separated through vacuum filtration using No. #1 Whatman paper. Later, the ethanol was removed from the extract using a rotavapor (RV 10 auto pro-V-C Complete, IKA, Staufen, Germany). Then, the grape marc extract was freeze-dried (GME) and stored at -18 °C for further analysis, described in sections 2.6 and 2.7.

6.3.2 In vitro cell culture studies

<u>Cell culture</u>

Caco-2 cell line (ATCC, HTB-37) from human colon epithelial carcinoma was routinely expanded in Minimum Essential Medium (MEM Eagle), supplemented with 20% fetal bovine serum (FBS), and 1% penicillin/streptomycin (final concentration of 100 U/mL and 100 µg/mL, respectively). The cells were kept in a humidified atmosphere of 5% CO_2, at

37 °C, in 75 cm^2 flasks. Cells were used in passages 33–52, being the cell culture media replaced every other day. Upon reaching confluency, cells were detached using 0.25% trypsin-EDTA solution, then pelleted by centrifugation at 300 ×*g* for 5 min and resuspended in fresh MEM at a concentration of 1×10^5 cells·mL−1. Cells were seeded onto 96-well plates at a density of 1×10^4 cells (100 µL of cellular suspension) per well and left to adhere for over 24 h.

Cell viability assay

The cytotoxicity of GME was determined indirectly by the resazurin conversion assay. After adhesion, the culture medium was removed, cells were washed twice with pre-warmed PBS, and 200 µL of samples or controls were applied and incubated for 24 h. GME was prepared as described in section 2.1, then further diluted with culture medium (10%, v/v) and tested at 33, 67 and 100 GAE µg/mL final concentrations based on TPC content in GME. These concentrations were chosen based on preliminary studies using concentrations reported by Freitas et al. (2020). Negative control was performed using cells growing in MEM (considered 100% cell viability), and 40% (v/v) DMSO was used as a positive control. After incubation, samples or controls were removed and washed twice with pre-warmed PBS. After this, 100 µL of 10% (v/v) resazurin in the culture medium (0.01 mg/mL final concentration) was added. The fluorescence intensity, proportional to the number of viable cells, was measured after 5 h of incubation using a microplate fluorescence reader (Synergy H1, BioTek, Vermont, USA) at an excitation wavelength of 560 nm and an emission wavelength of 590 nm. The % cell viability was expressed as the fluorescence of treated cells compared to that of cells growing in the culture medium.

Intracellular reactive oxygen species (ROS) quantification

The antioxidant activity of GME was determined in an *in vitro* cell assay using dichlorodihydrofluorescein diacetate (DCFH-DA) as a cell-permeable probe to detect intracellular ROS. After cell adhesion, the culture medium was removed, and 100 μL of 10 μM DCFH-DA solution was added to each well and incubated for 1 h. Afterwards, the solution was removed, and 100 μL of GME solubilised in HBSS was added to each well at a final concentration of 33 and 67 GAE μg/mL based on TPC content in GME, and incubated for 4 h. The fluorescence intensity was measured using a microplate fluorescence reader (Synergy H1, BioTek, Vermont, USA) at an excitation wavelength of 495 nm and an emission wavelength of 525 nm. Cells exposed to HBSS, Sin-1 (5 μM) and Trolox (50 μg/mL) were used as basal, positive, and negative controls, respectively.

Then, the protective effect of GME against oxidative stress was investigated using Sin-1 as an oxidative stress inducer. First, Caco-2 cells were exposed to GME at a 33 and 67 GAE μg/mL concentration based on TPC content in GME for 4 h. Then, Sin-1 was added to the cells at a final concentration of 5 μM and incubated for 1 h. The fluorescence intensity was measured every 15 min using a microplate fluorescence reader (Synergy H1, BioteK) at an excitation wavelength of 495 nm and an emission wavelength of 525 nm. Cells exposed to HBSS, Sin-1 (5 μM) and Trolox (50 μg/mL) were used as basal, positive, and negative controls, respectively.

6.3.3 Nano-spray drying (NSD)

First, 50 mL of 4% WPI and 0.4% pectin solutions were prepared separately and solubilised overnight at room temperature to ensure complete hydration. Then, 550 mg of GME was resuspended in the pectin solution (50 mL) and mixed with a magnetic stirrer for 5 min. This solution (pectin-GME) was mixed with the WPI solution (50 ml) and stirred for 10 min (magnetic stirring). Then the WPI-pectin-GME solution was centrifuged to remove any large undissolved particles and filtrated through a 0.45 µm PVDF filter before passing it through the NSD. The final solution had a final concentration of 2% WPI, 0.2% pectin and 0.55% GME. A solution containing the same proportion of WPI and pectin but no GME was prepared to compare physical characteristics. The encapsulation was performed using a Nano-spray Dryer B-90 (BÜCHI Labortechnik AG, Flawil, Switzerland). Compressed air was used as the drying gas, and the flow rate was set to about 100 or 110 L/min. The inlet temperature was set to 90°C, the spray rate to 65%, and the pump to 30%. WPI-pectin-GME (W-P-GME) and WPI-pectin (W-P) particles were stored at 4 °C.

6.3.4 Characterisation of the microparticles

Scanning electron microscopy (SEM)

The samples' surface morphology was evaluated through SEM using a Quanta FEG 650 (FEI, Oregon, USA). Dried samples were affixed on aluminium stubs covered by carbon ribbon and coated with gold, and samples were observed using an accelerating voltage of 5 kV under vacuum conditions.

Size and polydispersity index

The size of the particles was determined by analysing SEM images with the program ImageJ (National Institutes of Health, Maryland, USA). The scale was adjusted according to the parameters from SEM images, and the size of 175 particles was determined. After this, the mean and standard deviation was calculated, and from those values, the polydispersity index (PDI) was calculated with the following formula:

Equation 1

$$PDI = \sqrt{\frac{size\ \sigma}{size\ \bar{x}}}$$

Where σ is the standard deviation of the particle size and x is the mean size of the particles.

Yield

The drying yield was calculated from the ratio of total solids out (microcapsules) to total solids in (solids in extracts + encapsulants).

Equation 2

$$EY\% = \frac{Total\ solids\ out}{Total\ solids\ in} \times 100$$

Z-potential

The particles' surface charge (Z- potential) was measured by dynamic light scattering using an SZ-100 particle analyser (Horiba Scientific, Kyoto, Japan). Microparticles (1 mg/mL) were measured at 25 °C using a He-Ne laser (633 nm) in folded capillary cells.

Five independent measurements of each sample were done, and data were expressed as mean ±SD.

Fourier Transform Infrared Spectroscopy

Fourier Transform Infrared Spectroscopy (FTIR) determined functional groups and the bonding arrangement of sample constituents. FTIR analyses were carried out with an ALPHA II (Bruker, Ettlingen, Germany) spectrometer with a diamond composite in the 400–4000 cm^{-1} wavenumber region.

6.3.5 In vitro digestion

Particles were tested under simulated digestive conditions to evaluate the protective effect of polymeric particles on GME's activity and polyphenol content. First, the activity of the digestive enzymes (pepsin and trypsin in pancreatin) was quantified. Then, the experimental conditions were applied according to the *in vitro* static INFOGEST method (Brodkorb et al., 2019). The addition of gastric lipase was omitted due to the limited access to the commercially available enzyme, and amylase was not used in the oral phase since there was no starch in the sample.

W-P-GME particles (200 mg) or free GME (100 mg) were resuspended in 1 mL of distilled water and digested. The sample was diluted 1:1 (v/v) in oral digestion with simulated salivary fluid, $CaCl_2$ 0.3 M and water. The tubes were incubated in an orbital incubator (Fisher Scientific) for 2 min at 37 °C and 150 rpm. For gastric digestion (GD), a pepsin solution (2000 U/mL) in water was prepared based on the previously determined activity. The 2 mL of oral phase were diluted 1:1 (v/v) with simulated gastric fluid, pepsin solution,

$CaCl_2$ 0.3 M, HCl 1 M (to pH 3.0) and water. The samples were incubated for 2 h at 37 °C and 150 rpm. A 1.8 mL sample was collected after the 2 h of GD. For intestinal digestion (ID), bile solution and pancreatin were prepared in simulated intestinal fluid. The 2.2 mL of gastric phase were diluted 1:1 (v/v) with simulated intestinal fluid, pancreatin solution, bile, $CaCl_2$ 0.3 M, NaOH 1 M (to pH 7.0) and water. The samples were incubated for 2 h at 37 °C and 150 rpm. Then the samples were put in an ice water bath for 30 min to stop the enzyme´s activity.

After digestion, each digested sample was centrifugated in a Ministar blueline microcentrifuge (fixed speed 2,000 ×*g*) at room temperature for 5 min. The supernatants were collected and stored for analysis. Digestion of polyphenols was evaluated according to the analytical determinations described in sections 2.6 and 2.7 after GD and after GID. The residual values of polyphenols were calculated as a percentage of the total mass of TPC (mg) remaining after the gastric digestion (GD) and after the overall gastrointestinal digestion (GID) in relation to the initial mass. In the case of the antioxidant capacity, the values correspond to the TE (mg) for ABTS and AAE (mg) for FRAP remaining after each phase of the digestion in relation to the initial ones.

6.3.6 Analytical determinations

The methodology of this section is is described in Chapter 2 section 2.2

Total Phenolic Content: expressed as milligrams of Gallic acid equivalents per gram of dried extract (mg GAE/ g de).

Total Anthocyanin Content: expressed as milligrams of malvidin 3-O-glucoside equivalents per litre (mg M3GE/L)

Total Flavonoid Content: expressed as milligrams of Catechin equivalents per gram of dried extract (mg CE/ g de).

6.3.7 Antioxidant Capacity assessment by ABTS and FRAP methods

The methodology of this section is is described in Chapter 2 section 2.3

ABTS: expressed as micromoles of trolox equivalents per gram of dry extract (µmol TE/ g de).

FPRAP: expressed as micromoles of ascorbic acid equivalents per gram of dry extract (µmol AAE/ g de).

6.3.8 Statistical Analysis

The data were subjected to a One-Way ANOVA using IBM® SPSS® Statistics 27 software, where statistical differences were noted. Differences among different treatments were determined using independent samples t-test for particle size and gastrointestinal results. For the metabolic activity, differences were determined by Dunnett's multiple comparison test, as this is more suitable for the mean comparison of different experimental groups against a control group. The significance level was defined at $p<0.05$, and the results are reported as means ± SD.

6.4. Results and discussion

6.4.1 Characterization of GME

Hydroalcoholic extractions have proven to be efficient for extracting phenolics from grape by-products (MohdMaidin et al., 2018, 2019; Spigno et al., 2007, 2017). Indeed, we obtained a phenolics-rich extract with high TPC and TFC content and antioxidant capacity (Table 6.1). The phenolics content was higher than those reported by Pintać et al. (2018) and Aresta et al. (2020). They obtained 69 and 70 mg GAE/ g de, respectively, when extracting polyphenols from grape marc by conventional extraction. However, we obtained a lower content of TMAC, which might be explained since the content will vary depending on the extraction method, but also factors such as grape variety, region, and processing conditions play a significant role since not all grapes bear the same TMAC (Rinaldi et al., 2020; Spigno et al., 2015).

Table 6.1. Content of polyphenols and antioxidant activity in GME.

Total phenol content	219.62 ± 11.50
Total flavonoid content	151.69 ± 5.29
Total monomeric anthocyanins	12.80 ± 0.63
Antioxidant capacity by ABTS	1389.71 ± 97.33
Antioxidant capacity by FRAP	848.95 ± 43.99

TPC: mg GAE/g dry extract; TAC: mg MV3GE/ L; TFC: mg CE/ g dry extract
ABTS: µmol TE/ g dry extract; FRAP: µmol AAE/ g dry extract.
Values are represented as mean ± sd (n=6 from 3 replicates and 2 determinations)

6.4.2 Biocompatibility of GME

Studying the potential toxic effect of bioactive compounds is essential to determine whether they are safe to consume without harming the host. The GME showed a dose-responsive effect after 24 h of incubation with Caco-2 cells (Fig. 6.1). We observed cellular compatibility, *i.e.*, more than 70% of cell viability, for 33 and 67 µg/mL TPC based on GAE. However, cell viability below 70% was observed at the highest concentration tested (100 GAE µg/mL), which is considered toxic.

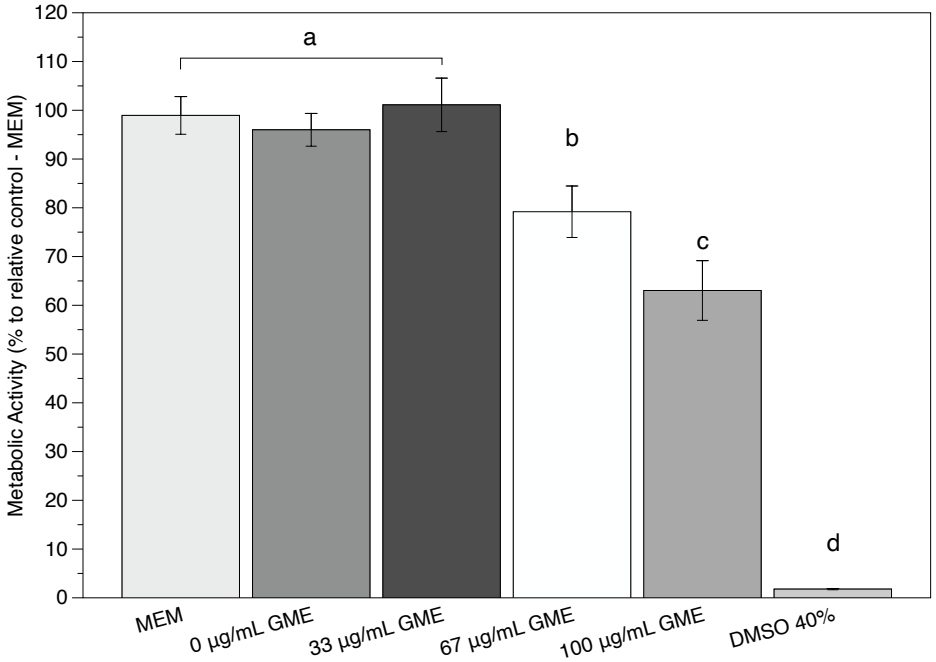

Figure 6.1. Viability of Caco-2 cells after 24 h-incubation with different concentrations of GME (33, 67 or 100 GAE µg/mL), measured through the resazurin assay. Culture medium (MEM) was used as a positive control (100% cell viability), and 40% DMSO (v/v) as a negative control. Values are the mean ± sd from 2 independent assays analyzed in quadruplicate. Different letters denote statistical significance ($p<0.05$) determined using the Dunnett method.

Studies in the grape phenolic extract have shown that concentrations between 0.1 to 10 µg/mL present no toxicity in Caco-2 cells with up to 93% viability (Wang et al., 2016). Another study by Costa et al. (2019) showed that concentrations of up to 2% of GME

were non-toxic for Caco-2 cells before and after simulated *in vitro* digestion. Also, Wolfe et al. (2008) observed that concentrations below 60 mg/mL of different extracts, e.g., wild blueberry, red grape, and strawberry, showed no cytotoxicity in HepG2 cells. However, in a preliminary assay, we observed that concentrations of 5 mg/mL GME, in the concentration range of some reports, were highly toxic (0% viability) for Caco-2 cells (data not shown), highlighting the importance of assessing each extract for its safe application.

6.4.3 Cellular antioxidant activity of GME

Reactive oxygen species (ROS) are natural by-products of cell activity and essential signalling molecules (Zhang et al., 2016). However, an imbalance between oxidant-producing systems and antioxidant defence mechanisms can trigger cell damage and cause cell death (Alfadda & Sallam, 2012). Cell-based assays have been used to assess the effectiveness of dietary antioxidant compounds (Kellett et al., 2018). Studies of intracellular oxidant production in Caco-2 cells were evaluated using DCFH fluorescence, testing GME at non-toxic concentrations (33 and 67 GAE µg/mL based on TPC). As shown in Fig. 6.2-A, both GME concentrations decreased the intracellular ROS basal levels, comparing with the control (cells treated with HBSS) to a similar level to the one observed for Trolox (50 µg/mL). This result suggests that GME can reduce ROS naturally produced by the Caco-2 cells, demonstrating a possible antioxidant effect (intracellular) against ROS.

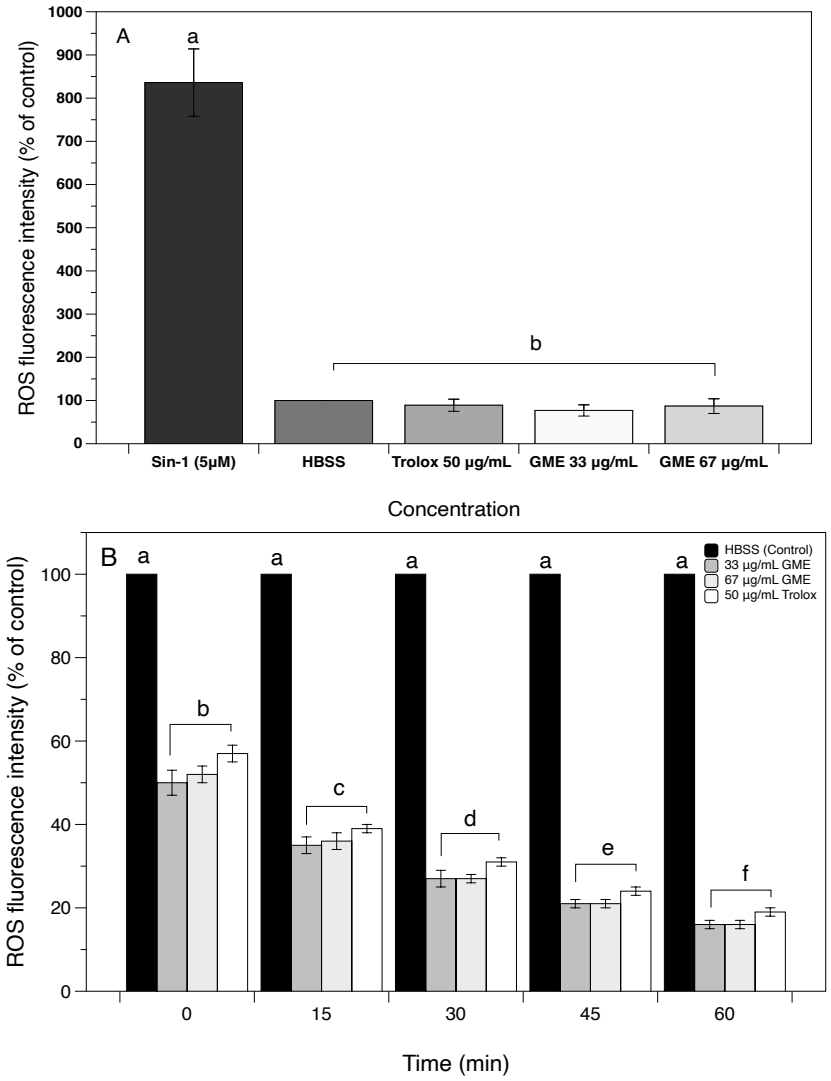

Figure 6.2. (A) Effect of different concentrations of GME (33 or 67 GAE µg/mL) on the ROS basal levels of Caco-2 cells after incubation for 4h, measured through the DCFH-DA assay. HBSS was used as a negative control, and Sin-1 (5 uM), as a positive control. (B) Protective effect of different concentrations of GME (33 or 67 GAE µg/mL) on the ROS levels of Caco-2 cells after incubation for 4h, followed by stimulation with 5 uM Sin-1 (oxidant) for 1 h. ROS was measured through the DCFH-DA assay. Cells treated with HBSS and stressed with Sin-1 were used as a positive control. Values are the mean ± sd of two independent assays analysed in quadruplicate. Different letters show statistical significance ($p<0.05$) determined using the Dunnett method.

To evaluate the potential protective effect of GME against intracellular oxidation, Caco-2 cells were pre-treated with GME at the non-toxic concentrations of 33 and 67 GAE µg/mL based on TPC for 4 h. Then, cells were stimulated with 5 µM of the oxidising agent Sin-1,

selected according to the literature (PD ISO/TS 19006:2016). Cells treated with HBSS and stressed with Sin-1 were used as a positive control. As shown in Fig. 6.2-B, cells pre-treated with non-toxic concentrations of GME significantly reduced intracellular ROS level produced after stimulation with Sin-1 compared to cells pre-treated with HBSS (control). This reduction was similar to that observed for treated cells with 50 µg/mL Trolox which was used as a potent antioxidant model compound.

GME showed a similar antioxidant effect to a well-known compound at similar concentrations, suggesting that GME polyphenols can effectively neutralise ROS-induced production (protective effect) in Caco-2 cells, demonstrating intracellular antioxidant capacity. The results of the CAA also corroborate the high antioxidant capacity of the GME observed by ABTS and FRAP methods. Wang et al. (2016) induced ROS production using t-BOOH in Caco-2 cells treated with grape phenolic extract for 1 h, and their results showed that concentrations of 0.1 to 10 µg/mL exert an antioxidant effect over ROS. Other studies have reported that concentrations of 100µ/mL, 200µ/mL and 500µ/mL reduced ROS production in Caco-2 cells treated with grape pomace extract for 5 h (I. M. Martins et al., 2017, 2020). However, at 500 µg/mL, the production of ROS was significantly reduced due to the pro-oxidant effect of polyphenols (Martins et al., 2020). Milinčić et al. (2021) observed an EC50 of ABAP radical at a 54 mg TPC/mL concentration of grape pomace skin extract on the same cell line. The concentrations used in the previously mentioned studies are considerably higher than the ones we reported, indicating that while grape pomace is an excellent source of antioxidants, the analyses of cell biocompatibility and antioxidant capacity need to be carried out before their formulation as nutraceuticals or functional food ingredients.

6.4.4 Encapsulated GME morphology, size, and Z-potential

The morphology and size of the encapsulated GME were studied through SEM analysis. Fig. 6.3-A shows the formation of large crystals with a wide distribution of submicron and micron particles during freeze-drying of GME (Table 6.2). For the nano spray dried particles, different morphologies were observed for the W-P particles with and without GME. Blank microparticles (W-P) had a spherical shape and smooth surface (Fig. 6.3-B), while microparticles loaded with GME (W-P-GME) (Fig. 6.3-C) kept their spherical shape but presented some wrinkles in their surface.

Table 6.2. Z-potential, size and PDI of particles

Particles	Z-potential	Size (µm)	PDI	Yield (%)
W-P-GME	-28.3 ± 6.1	1.00 ± 0.52 [a]	0.717	73
W-P	ND	1.16 ± 0.64 [b]	0.742	ND

Z-potential: based on 1mg/mL particle suspension in water; average ± sd from 5 measurements
ND: non determined
Different letters denote significate difference ($p<0.05$) using the independent samples t-test.

Moreover, no breakage was seen in W-P and W-P-GME. Regarding the size, W-P-GME particles showed a smaller and narrower size distribution than W-P particles (Table 6.2). Studies on the encapsulation of raw grape marc extract by conventional spray drying have reported sizes of 9.8 µm when using pectin and casein and 15 µm when using WPI alone. (Carra et al., 2022; Moreno et al., 2018). The results obtained (1µm) demonstrate that nano spray drying significantly affects the particles' reduction size.

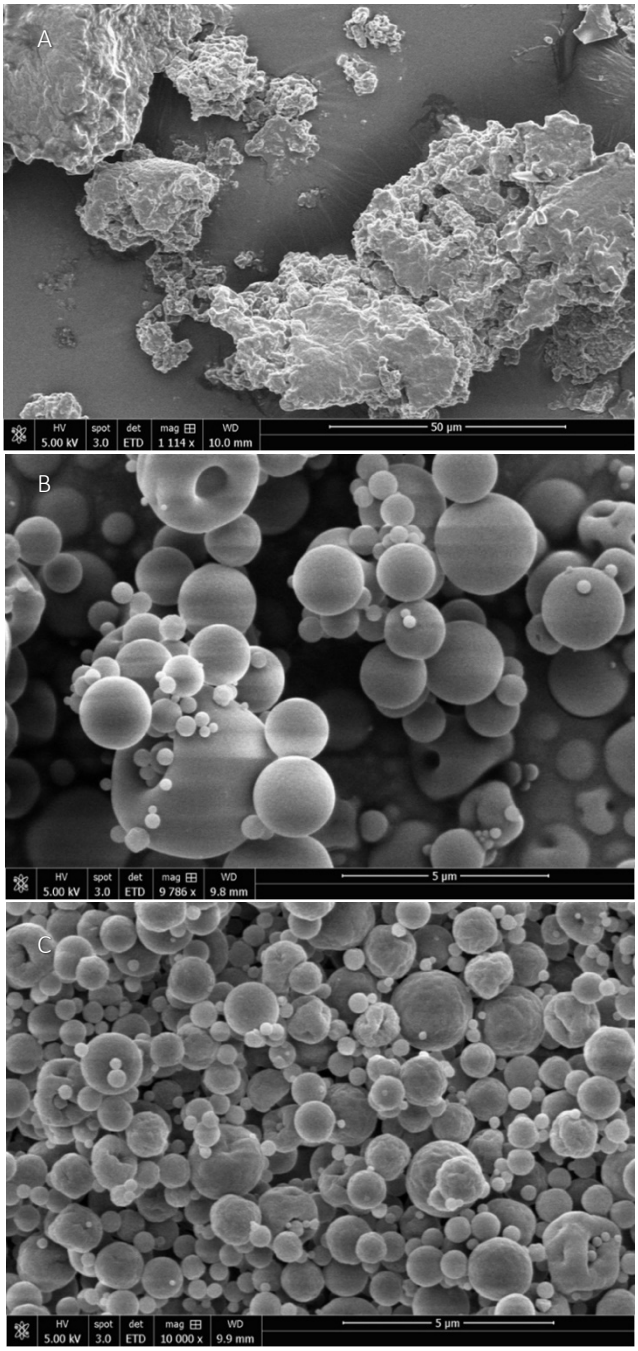

Figure 6.3. SEM images of GME (A), whey-pectin blank microparticles (B), and GME encapsulated in whey-pectin microparticles (C).

Moreover, the particles we obtained displayed a more homogeneous and well-defined particle shape than those in previously mentioned studies, where irregular and dented surfaces were obtained, and in the case of WPI, holes were seen in the microparticles (Moreno et al., 2018). The zeta potential of W-P-GME (Table 2) showed a medium to high particle surface charge, which confers the particles' colloidal stability.

6.4.5 FT-IR analysis

FT-IR analysis was used to examine interactions between the biopolymers and GME. The infrared spectra of the carriers, GME and microparticles are shown in Fig. 6.4. For WPI, characteristic amide I and II bands can provide information about protein secondary structures, and their change in vibration frequencies is related to the interaction between their functional groups. Amide I represents the C=O carbonyl stretching vibration of the peptide backbone (1600-1700 cm^{-1}), and the amide II band (<1550 cm^{-1}) represents the C-N stretching and N-H bending (López-Rubio & Lagaron, 2012; Meng et al., 2021). As for the GME, the characteristic bands of grape phenolic compounds were observed between 1700 and 900 cm^{-1}. The band at 1710 cm^{-1} was attributed to the stretching in the carbonyl group (C=O) band, 1600 and 1510 cm^{-1} bands correspond to the C=C stretching, characteristic of aromatic systems. The peak around 1440 cm^{-1} corresponds to the antisymmetric in-plane bending of -CH_3 related to aromatic rings and flavonoids (Moreno et al., 2018; Zhao et al., 2015). Characteristic peaks of pectin can be observed at 2920, 1740, 1610 and 900-1250 cm^{-1} corresponding to the C-H stretching of the CH, CH2 and CH3 groups, C=O stretching vibration of the ester carbonyl, C=O stretching of the vibration the carbonyl group, C-O-C and O-H of pyranose rings respectively (Khodaiyan & Parastouei, 2020).

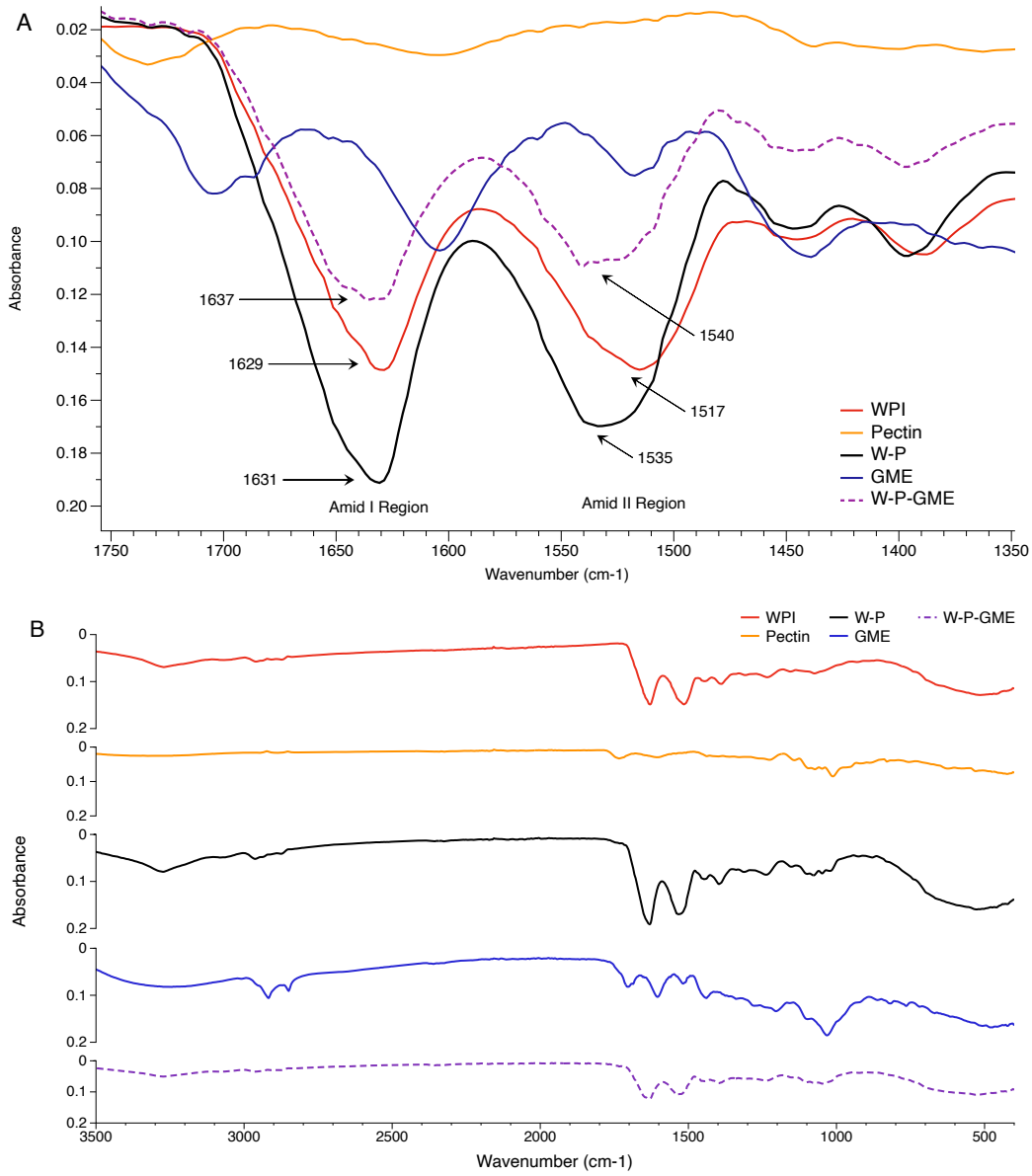

Figure 6.4. FT-IR of WPI, pectin, GME, whey-pectin blank microparticles, and GME encapsulated in whey-pectin microparticles. Amplified FTIR spectra of 1750 to 1350 cm^{-1} region displaying changes in amide I: Δ1629→1631→1637 and amide II: Δ1517→1535→1540 bands (A) and 3500 to 400 cm^{-1} region (B). The results of each material are the average of three independent spectra.
*Note: This figure has been modified for the purpose of the present thesis.

Looking at the infrared spectra of W-P and W-P-GME, slight shifts in the amide I and amide II regions were observed compared to WPI (1517 to 1535 cm^{-1}) (Fig. 6.4-A). These shifts can be attributed to the interaction between carboxyl groups of pectin and the charged amino groups of the main WPI proteins' composition (beta-lactoglobulin, alpha-

lactalbumin, and serum albumin) (Raei et al., 2018). An increase in the intensity was observed for the W-P particles, which can be attributed to the rise in random coils and the previously mentioned interaction between WPI and pectin (El-Messery et al., 2020; Z. He et al., 2016). However, when GME is added, a decrease in intensity is observed. This result is consistent with those obtained by Meng and Li (2021), where Gallic acid, chlorogenic acid, and epigallocatechin gallate-WPI complexes showed decreased intensity in the amide I band.

This change can be attributed to the reduction of α-helical structures as a result of protein conformational modifications upon phenolics complexation by hydrogen bonding and hydrophobic interactions between the phenolic compounds and hydrophobic groups of the protein, so there are not only interactions but also changes in the secondary structure of the proteins (Bourassa et al., 2013; Z. He et al., 2016). According to previous reports, W-P-GME did not show any characteristic band from GME, indicating that phenolics' distinct peaks can be hidden when in contact with other biopolymers like WPI. This change could mean the formation of complexes that reduce the bending and stretching of the bonds in GME polyphenols.

6.4.6 In vitro digestion of free and encapsulated GME

The results of the residual TPC and antioxidant activity for both free and encapsulated GME are shown in Fig. 6.5. These results represent the fraction of TPC (or activity which, is quantified as Trolox equivalents (TE) or ascorbic acid (AAE)) remaining after GD or GID, the latter indicating the bioaccessible fraction. Therefore, these values show the fraction

of TPC (or activity) that resisted the simulated gastrointestinal conditions in free GME. In contrast, for W-P-GME, these values account for the fraction of TPC that resisted the conditions and/or was encapsulated and effectively released from the microcapsules during digestion.

A different behaviour was observed for free and encapsulated GME, suggesting the microcapsules play an essential role in the phenolic content and their activity during digestion. For free GME, we observed that the TPC underwent some degradation due to the gastric conditions (acidic pH), as shown by a 76% residual TPC content (24% unaccounted for; Fig. 6.5-A). The moderate stability of GME polyphenols to gastric digestion agrees with previous studies (Li et al., 2023). The free GME suffered further degradation after intestinal conditions, resulting in a further 30% TPC loss in relation to that remaining after gastric digestion; low stability of polyphenols has been reported at neutral pH conditions (Li et al., 2023). So, after GID, the overall bioaccessible TPC was 54%. In the case of encapsulated GME, about 30% of TPC was unaccounted for after GD (Fig. 6.5-A), which may represent the fraction not released from the microparticles.

Indeed, high preservation of the TPC was expected during GD since strong electrostatic interactions stabilise the WPI- pectin complex at acidic pHs (3.6-4.5) (Raei et al., 2017), which should protect phenolics from degradation. However, some release of phenolics will still occur as WPI is susceptible to enzymatic hydrolysis, but pectin should have a stabilising effect in the system (Reichembach & Lúcia de Oliveira Petkowicz, 2021; Wusigale et al., 2020).

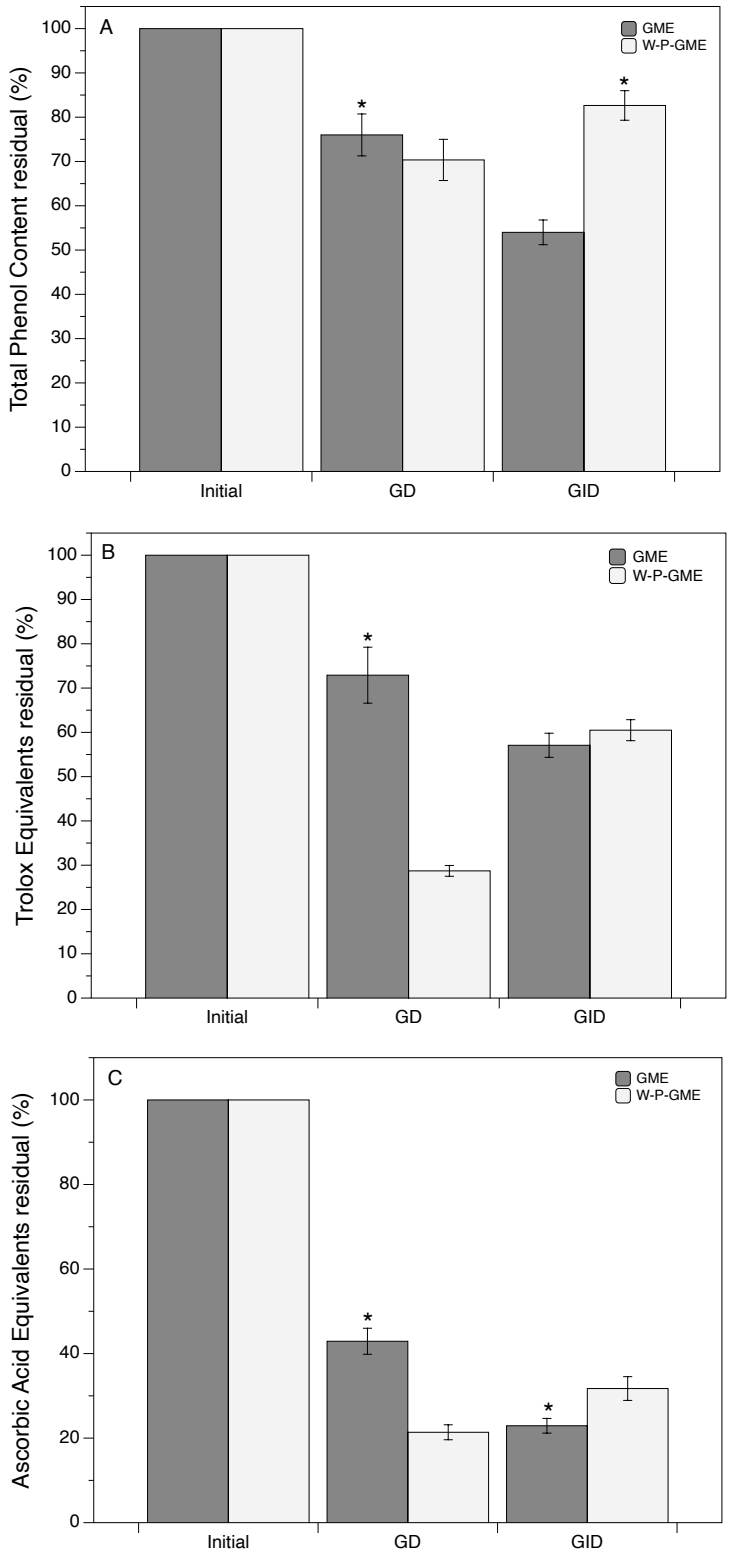

Figure 6.5. Results of the residual Total Phenol Content (A), Trolox Equivalents (B), and Ascorbic Acid Equivalents (C) of GME and GME encapsulated in whey-pectin microparticles after *in vitro* gastric digestion (GD) and gastrointestinal digestion (GID). Values are the mean ± sd from three independent assays, each analyzed in triplicate.* Denotes statistical significance between GME and W-P-GME ($p<0.05$) determined using the Tukey test. Note: This figure has been changed for the purpose of this thesis

Yet, the released fraction can also undergo similar degradation as that observed for the free extract (GME). Therefore, assuming the residual 70% TPC content in W-P-GME will undergo similar degradation as that of the free extract during GID, values close to 54 % of residual TPC (as in GME) would be expected however, it was found that 83% of the TPC remained after GID. This indicates a protective effect of the microcapsules, which resulted in about 30% encapsulation of the TPC in the gastric phase and their release at intestinal conditions, with an overall increase in the remaining TPC compared to free GME.

The behaviour of antioxidant activity during GID for both free and encapsulated GME showed a similar trend to TPC. Thus, the free GME showed a slight loss of activity after GD followed by a more pronounced decrease after GID, while for W-P-GME, the activity was slightly increased after GID compared to GD (Fig. 6.5-B & 6.5-C). Besides, free GME's bioactivity directly correlates with residual TPC values after GD and GID, achieving values of 73% and 57% of the initial activity, as assessed by the ABTS method. Although a similar trend was observed in both phases, lower values were recorded using the FRAP method.

For encapsulated GME, although a positive correlation was observed between residual TPC and antioxidant activity, the latter showed lower values than the residual TPC. For instance, 29 and 61% of the activity was observed using the ABTS method after GD and GID in W-P-GME. The reduced activity compared to the residual TPC might be due to released polyphenols from the capsules bearing lower antioxidant activity than those that were still encapsulated or that they might be complexed with the capsule components

since they are known to interact with whey proteins and their peptides (Guo & Jauregi, 2018), which has been confirmed by the FT-IR spectra.

Overall, the results of GID showed that the encapsulation succeeded in preserving the TPC and increasing their bioaccessibility. For the antioxidant activity, similar results to free GME were observed according to the ABTS method, and slightly higher activity according to the FRAP method.

6.5 Conclusions

A raw ethanolic extract of a winery by-product (grape marc) with antioxidant capacity was successfully encapsulated using WPI and pectin and nano spray drying (73% yield), resulting in spherical smoothed-surface microparticles with an average size of 1 μm, PDI of 0.717, and a Z-potential close to -30 mV. The FT-IR analysis of the microparticles confirmed the complexations between WPI, pectin and the phenolics in GME through non-covalent interactions. The developed encapsulation system protected the GME phenolics and the antioxidant activity during GID, improving bioaccessibility. The potent antioxidant intracellular protective effect of GME observed, and its improved resistance to GID when encapsulated compared to the free form suggest this encapsulation system could be a promising strategy towards preserving the antioxidant activity of this high-value-added by-product of the wine industry. The selected wall materials proved that the microcapsules resisted gastric conditions and could provide a targeted release in the lower intestine, where phenolic compounds are absorbed and can be metabolised by the microbiota. Although further studies are needed to test the stability, biocompatibility,

and *in vivo* bioactivity of the WPI-pectin-GME microcapsules, the presented results are promising towards using encapsulated GME as a nutraceutical.

Conflict of Interest

The authors declare that the research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

Data curation; Formal analysis; Methodology; Writing-original draft: Aimara V. De La Cruz Molina. *Conceptualisation:* Isabel Rodriguez, & Lorenzo Pastrana*. Funding acquisition:* Lorenzo Pastrana*. Resources:* Lorenzo Pastrana*. Writing-review & editing:* Isabel Rodriguez, Aimara V. De La Cruz Molina, Catarina Gonçalves, Mafalda D. Neto & Paula Jauregi. *Cell assays:* Catarina Gonçalves, Mafalda D. Neto. *Supervision:* Isabel Rodriguez, Lorenzo Pastrana, &Paula Jauregi.

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Chapter 7: General discussion and future work

Abbreviations

ADI: Acceptable Daily Intake

ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)

AOT: Sodium bis(2-ethylhexyl)sulfosuccinate

BCPE: Blackcurrant Pomace Extract

CGA: Colloidal Gas Aphrons

CGA(Tween 20): Colloidal Gas Aphrons made of Tween 20

CGA(WPI): Colloidal Gas Aphrons made of Whey Protein Isolate

CTAB: Cetyltrimethylammonium bromide

FAO: Food and Agriculture Organization

FODIAC: Food for Diabetes And Cognition

FRAP: Ferric Reducing Antioxidant Power

HPLC: High Performance Liquid Chromatography

RGSE: Red Goji Skins Extract

SDG: Sustainable Development Goals

TFC: Total Flavonoid Content

TMAC: Total Monomeric Anthocyanin Content

TPC: Total Phenol Content

UF: Ultra Filtration

UN: United Nations

WHO: World Health Organization

7.1 Conclusions

As a result of the fast-paced world we are living in now chronic conditions are on the rise (WHO, 2022). Research has shown that a healthy diet is a valuable tool in the management of these diseases. Fruits, like berries and grapes, haven been attributed health benefits due to their composition and bioactive molecules. Polyphenols found in them have proven aid with metabolic disorders such as diabetes and obesity (Ma & Chen, 2020; Yang et al., 2017). Unfortunately, it is estimated that at least half of the yearly fruit production ends up as waste (FAO, 2019), which is an alarming issue.

This problematic has been addressed by the United Nation as part of the 2030 Agenda, in the 12 SDG particularly 12.3, which aims to reduce food losses and waste along the production and supply chain (United Nations, 2023). In this context, great efforts have been made to find ways to reduce, reuse, and add value to unavoidable inputs such as by-products generated from different industries. By-products from agri-food industry are rich in valuable compounds such as fibre, protein, and bioactive molecules (Moreno-González & Ottens, 2021). Moreover, the valorisation of these by-products could not only lead to more sustainable processes but as well to promote good health and well-being which is part of the UN 2030 Agenda (SDG 3.b) (United Nations, 2023).

The aim of this study was to investigate the use of whey protein for the first time for CGA generation and its further application for separation, recovery, and encapsulation of polyphenols extracted from fruit by-products.

First, polyphenols from berries and grapes discards, and a mixture of them were extracted using ethanol and methanol. The extraction with an aqueous solution of ethanol demonstrated to be better or equal to methanol for the extraction of polyphenols, as well as overall antioxidant capacity. Moreover, ethanolic extractions of fruit processing by-products were obtained for further processing with CGA. The findings in this chapter contribute to the valorisation of fruits that otherwise would have been wasted, they showed the potential to be sources of polyphenols that could be further implemented in foods.

Next, the use of whey protein as a natural food grade surfactant was investigated for its foamability properties to generate CGA. Currently, the only food grade surfactants used for CGA generation are polysorbates and sorbitans. It was seen here for the first time that it is possible to generate stable CGA made from whey protein isolate (CGA(WPI)). However, to do so a minimum protein concentration of 10 mg/mL is needed. At this concentration, CGA(WPI) showed a gas hold up and half-life similar to those of CTAB, AOT, Tween 20, and Tween 60 (Dermiki et al., 2009; Fuda & Jauregi, 2006; MohdMaidin et al., 2019; Spigno et al., 2015). Additionally, a low-fat content WPI was necessary, since in here it was seen that from all the whey purities analysed the one with the highest fat content was not able to form stable CGA(WPI).

After CGA(WPI) characterisation, the separation of polyphenols from the fruit processing by-products was carried out using CGA(WPI) and compared to CGA(Tween 20). The separation of polyphenols from blackcurrant pomace extract (BCPE) with CGA(WPI)

resulted in a high recovery of polyphenols, comparable to CGA(Tween 20). However, the recovery of anthocyanins was lower with CGA(WPI). Similar recovery values can be attributed to hydrophobic interactions being the main interactions driving the separation with both surfactants. Regarding the antioxidant capacity, CGA(WPI) showed the highest antioxidant capacity by ABTS, while CGA(Tween 20) showed the highest antioxidant capacity by FRAP.

Moreover, it was seen that the drainage of CGA(WPI) was 2.6 times higher than that of CGA(Tween 20). This could be related to the formation of aggregates between WPI and polyphenols as observed in previous works, the formation of aggregates between surfactant and protein led to improved drainage. This difference in drainage could also explain the differences between the two surfactants in the antioxidant activity found the corresponding aphron phases. After CGA(WPI) collapse, precipitation was observed, and when compared to spontaneous precipitation, more anthocyanins and antioxidant capacity were found in the supernatant of CGA(WPI) than in that of the direct precipitation.

CGA separation of polyphenols from red goji skins (RGSE) showed a higher recovery of polyphenols with CGA(WPI) than with CGA(Tween 20). Moreover, more than 71% of the initial antioxidant capacity was observed in the CGA fractions of both surfactants. On the other hand, the CGA separation of polyphenols from grape marc (GME) showed that for CGA(WPI) and CGA(Tween 20) phenols and flavonoids recovery was over 90%. However, for monomeric anthocyanins, CGA(Tween 20) had a substantially higher recovery (23%) than CGA(WPI). Interestingly, similar behaviours were seen in the recovery of monomeric

anthocyanins from BCPE and GME with CGA(WPI). It is likely that the pH of the WPI solution had a major influence on the recovery since the pH was near neutral (pH=6.2). Around this pH anthocyanin's structure is highly unstable due to ionisation of the flavylium cation.

These results show that the composition of the extract is of significant importance for the separation with CGA. Previous reports show that CGA(Tween 20) have special affinity for anthocyanins, in here it was seen that it is possible that they have affinity to other types of flavonoids based on the recoveries shown in GME and RGSE. Interestingly, it was seen that CGA(Tween 20) does not benefit the separation of polyphenols to the same extent when the main polyphenols are not flavonoids. For CGA(Tween 20) the separation is driven by hydrophobic interactions. In this sense, smaller molecules, such as phenolic acids, are less hydrophobic than flavonoids because of the reduced number of rings; thus, fewer interactions occur. In the case of CGA(WPI), the recovery of TPC and TFC was similar for both the extracts. This indicates that, while hydrophobic interactions occur, other non-covalent interactions, such as hydrogen bonding, are influencing the separation, allowing for a higher recovery of polyphenols.

Moreover, it was seen that these interactions occurring between CGA(WPI) and anthocyanins from GME had a stabilisation effect on anthocyanins during storage. This was seen by the increase in half-life and lower degradation rate constant than the one from raw GME. Out of four anthocyanin, three of them were present at least double the time that in the raw GME. The high recovery of polyphenols with CGA (WPI) and the

stabilisation of anthocyanins due to the protein-polyphenols interactions act as formulation aid.

Encapsulation of polyphenols in the raw extract was investigated for their formulation and improved bioaccessibility. The encapsulation of raw GME with WPI and pectin by nano spray drying, showed that microparticles with a significant reduction in size and an enhanced morphology than those using conventional spray dryer. After gastrointestinal digestion the particles showed that at the end of the simulation most of the polyphenols were bioaccessible.

Overall, in this study, it has been shown that the extraction of polyphenols from berries and grapes discards, and fruit processing by-products with ethanol gave a high yield of polyphenols and antioxidant activity. Moreover, antioxidant activity of grape marc raw extract was tested in cells and showed the same activity as 50 µmol of Trolox. Polyphenol concentrations up to 67µg GAE/ g dw was considered safe according to cytotoxicity tests. Following the extraction of polyphenols, it was stablished that it is possible to obtain CGA with whey protein. CGA(WPI) showed the capability to separate polyphenols from crude extracts in a comparable way as CGA(Tween 20). Furthermore, the recovery with CGA(WPI) showed to be high regardless of the type of polyphenols in the extract. It is possible this is due to the different non-covalent interactions occurring in the system. Moreover, the use of CGA(WPI) can exert a protective effect on anthocyanins during storage, allowing a longer storage time, which can be beneficial from a production and consumption point of view. Finally, the encapsulation of GME through nano spray drying using WPI and pectin showed that this method and these materials enabled the

successful encapsulation of the polyphenols and their release at intestinal conditions resulting in improved bioaccessibility.

These main findings open the perspective for the formulation of functional products with CGA(WPI). The rich fraction of protein in combination with polyphenols could be of special interest to deliver a high protein portion, which can be especially beneficial for the elderly population or people with a protein deficit. Moreover, the bioactive molecules in this fraction have the potential to aid in chronic disorders, such as diabetes and cognitive functions. The use of whey protein and polyphenols complexes in conjunction with gastro-resistant polymers, such as pectin, offers the potential to be used in the supplementation of foods delivering safe and effective doses of polyphenols to the consumer.

It is important to mention that while protein polyphenols interactions have demonstrated to be beneficial from the stabilisation perspective as shown here. However, some important aspects need to be taken in consideration. During digestion the interactions between polyphenols and proteins can lead to a reduced digestibility of the protein due complexation making the protein unavailable for digestion (Stojadinovic et al., 2013). For the elderly special care should be taken here as they have a reduced number of enzymes that can break the protein (Melchior et al., 2023). However, an advantage of whey protein is that it is more digestible compared to other types of protein, especially plant based (Almeida et al., 2015; Melchior et al., 2023). Moreover, research, shows that the impact on bioaccessibility and digestibility is dependent on protein concentration and type of

polyphenols involved in the complexation (Accardo et al., 2023; de Morais et al., 2020; Li et al., 2022; Zang et al., 2021).

Another important point is the use and application of polyphenols for human consumption. While they have been extensively researched for their health benefits, in Europe the only regulated health claims that can be attributed to polyphenols are for olive oil (EC No 432/2012) and cocoa flavonols (EC No 1924/2006). In some cases, polyphenols can be considered toxic and harmful, this though, is much dependent on the dose they are administered and source (Hooper & Frazier, 2012; Scalbert et al., 2005; Younes et al., 2018). Hence the importance to carry out *in vitro* experiments that can be extrapolated to *in vivo* systems before formulation and clinical trials.

7. 2 Future work

This study proposes the use of fruit discards and fruit processing by-products for the extraction, enrichment, and formulation of polyphenols-based foods. Here it was observed that the hydroalcoholic extraction of polyphenols from berries and grapes was comparable to the yields obtained with methanol. Further studies should focus on the extraction of polyphenols combining these fruits to evaluate if it would be a more efficient way than the extraction of each individual fruit. Additionally, the obtained extracts here would benefit from phenolic characterisation using HPLC. Investigation of the solids obtained during the extraction could provide valuable information to determine if they can be used as dietary fibre. Additionally, the antidiabetic properties of the obtained extracts should be investigated to fulfil the main goal of FODIAC.

In Chapters 4 and 5, CGA(WPI) were generated based on the optimum conditions found for non-ionic surfactants to investigate if CGA could be generated from whey protein. Further work should focus on several aspects that could lead to more stable CGA. For example, the addition of sugars, as well as the partial denaturation of whey protein. Regarding the parameters that should be further investigated during the separation, are the stability of CGA during pumping, pH adjustment to improve the separation of anthocyanins, the use of aqueous extracts versus ethanolic, add WPI in the feed and generate CGA from there, and reducing contact time.

A higher protein concentration would be beneficial from a formulation perspective. However, it will have to be seen how the separation of polyphenols, and particularly the antioxidant activity could be influenced by this. Also, it worth to explore the solubility of whey protein at cold temperatures and microbial growth when the solution is left at room temperature. Moreover, a further step of encapsulation of CGA should be worth to explore as to CGA structure could be benefitting the interactions between protein and polyphenols. Additionally, the use of CGA(WPI) can be investigated for the colour preservation of anthocyanins. From an analytical perspective it would be good to use methods that allow to explore in depth the interactions between protein and polyphenols as well as CGA(WPI) structure. For this analysis such as FTIR, isothermal titration calorimetry, circular dichroisms, surface hydrophobicity, and X-ray diffraction could be used.

Regarding the encapsulation of raw GME, further studies should focus on the optimisation of the nano spray drying process. In this sense, it would be worth to explore if CGA(WPI) enriched with raw extracts from different by-products. Since in this work it has been proved that WPI can be used in nano spray drying, CGA(WPI) could be used as a pre formulation step. After the separation a third polymer can be added, such as inulin, which is a form of soluble fibre, and the encapsulation can follow. In here it would be important as well to assess the digestibility of protein and the polyphenols-protein complex after the *in vitro* digestion.

Additionally, it would be useful to investigate if the increment of pectin in the formulation could further enhance the protection under gastric conditions. Moreover, HPLC quantification and profiling of the compounds found in each digestion would be beneficial for observing and screening structural changes. Additionally, the cytotoxicity and cellular antioxidant activity of the microparticles should be assessed based on the final concentration found at the end of the digestion. Anti-inflammatory activity assays would be beneficial, since both WPI and polyphenols are known to possess this activity. Antimicrobial activity would also be good, as it could be used in packaging. In this context, films and edible packaging are of interest; however, permeability, solubility, and tensile strength would be important parameters to assess for example, among others.

As a whole process, the integration of the extraction of polyphenols from fruit by-products and its separation with CGA with whey protein, opens the perspective for a sustainable and cost-effective process of separation and formulation. From a techno-economic point of view, a comparison between whey protein isolate and Tween 20 cost

would need to be carried out, in here it would be good to consider the cost benefit they both represent. Whey protein has a higher acceptable daily intake (ADI) than polysorbates. For polysorbates is 10-25 mg/ kg of body weight per day (EFSA ANS Panel (EFSA Panel on Food Additives and Nutrient Sources Added to Food), 2015), while for protein is around 0.83 g/kg of body weight per day (EFSA NDA Panel (EFSA Panel on Dietetic Products, 2012). Additionally, flotation systems can represent a lower capital and operating cost than other separation methods such as microfiltration. CGA offers the possibility to be scaled as an industrial process. The separation could be carried out in batch and continuous mode using a flotation column set up.

Figure 7.1 shows an example of how CGA could compare to other processes that are applied currently applied to grape marc. One of the main advantages that CGA can offer is the potential it has to serve a pre-formulation step. Compared to evaporation or ultrafiltration, in which the removal of the solvent or purification are carried out as a first step, the use of whey protein as a surfactant does not have to involve any of those steps. In Figure 7.1, all the process end with the drying or encapsulation; however, the only one that offers the possibility of a pre-formulation step is CGA separation. Moreover, the recycling of the liquid phase recovered from the separation could be of great help from a techno economic point of view, as this could be used for following separations making a cost-effective process.

To do the recycling process of the liquid phase, the liquid phase would need to be characterised as to determine protein, polyphenols content, and antioxidant activity in the fraction. After this, a new characterisation would need to be carried out, and evaluate

if the recycling of the fraction is feasible. While the recovery of the solvent is something similar that occurs is some processes like in the case of the evaporation, the collection of the liquid phase after the separation does not require the use of specialized equipment. As seen in this example, and the results obtained in this work, CGA can be advantageous from different points of views, especially formulation.

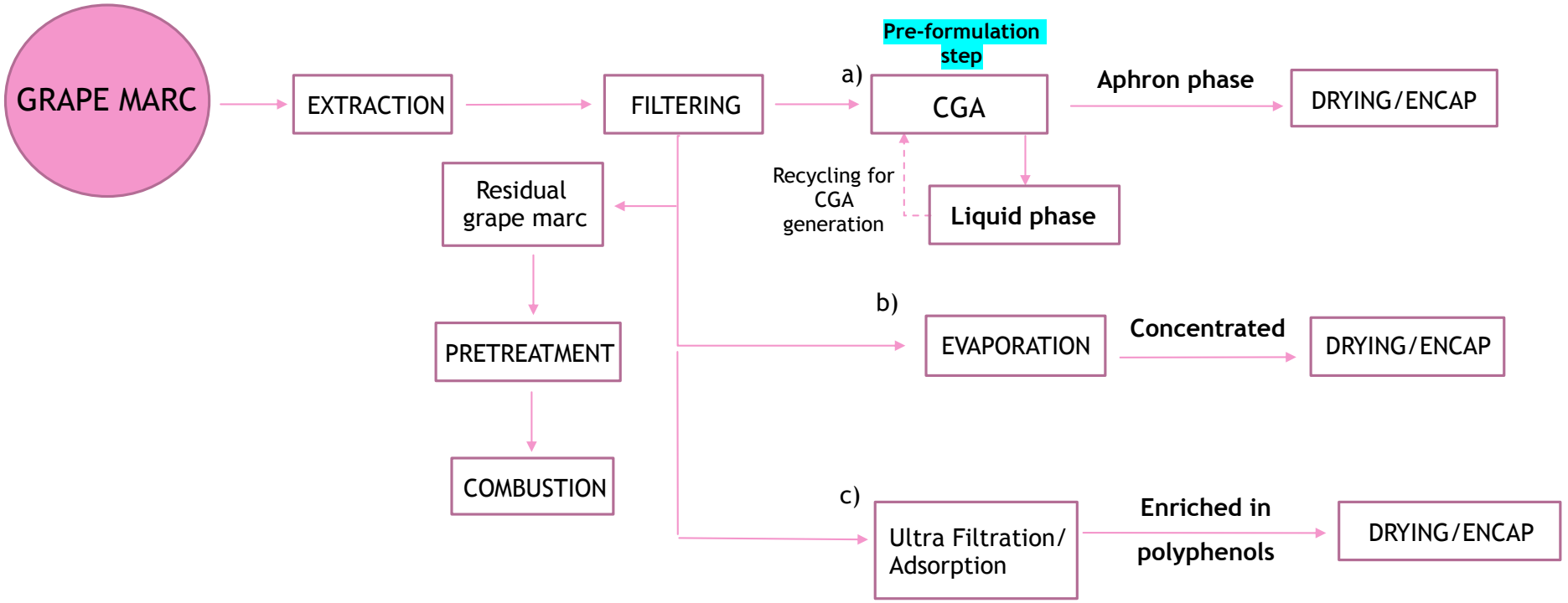

Figure 7.1. Diagram of incorporation of CGA in the process chain of grape marc by-product.

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