Whey protein based colloidal gas aphrons combined with solid–liquid extraction as an integrated green separation of phenolics from fruit based by-products

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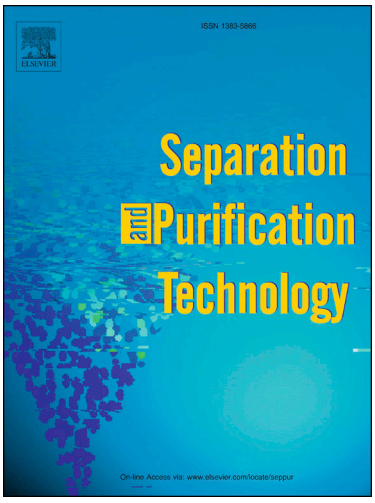

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Whey protein based colloidal gas aphrons combined with solid-liquid extraction as an integrated green separation of phenolics from fruit based by-products

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Abstract

Colloidal gas aphrons (CGA) are microbubbles created by the intense stirring of a surfactant solution that can be used as a separation method for biomolecules. The main objective of this work was to investigate for the first time the use of whey protein as a natural surfactant for CGA generation. Furthermore, their application for separating phenolic compounds from hydroalcoholic extracts obtained from fruit based by-products (grape marc and red goji berry). Additionally, to investigate if this surfactant-rich fraction could confer an advantage in stabilising anthocyanins during storage. First, a hydroalcoholic extract was obtained from each feedstock; then whey protein isolate (WPI) generated CGA were applied and compared with Tween 20 generated CGA. Recovery performance was assessed based on total phenolics, flavonoids, and antioxidant capacity. CGA generated with a 1.5% (WPI) displayed comparable characteristics (gas hold-up and stability) to those generated with Tween 20 (10 mM). The CGA separation process with WPI led to a recovery of up to 97% of phenolic compounds but a loss of antioxidant capacity under the tested conditions. Hydrophobic interactions as well as hydrogen bonding between phenolics and WPI could be responsible for the successful separation that could also hinder the radical scavenging activity. In contrast, these interactions could be responsible for the stabilising effect on anthocyanins observed during storage. Overall, CGA generated with WPI have resulted in an integrated separation method that by combining it with hydroalcoholic extraction leads to the effective separation of phenolics and their pre-formulation in a whey protein rich solution with stabilisation effect.

Keywords: Whey protein, colloidal gas aphrons, anthocyanins, phenolics, grape marc, red goji, degradation.

1. Introduction

Phenolic compounds, the biggest family of phytochemicals with more than 8000 structures, are of particular interest since they have demonstrated to aid in cognitive and metabolic disorders such as diabetes (Fallah et al., 2020; Russo et al., 2019; Yang et al., 2017), as well as to have anti-inflammatory and antimicrobial properties (Koh et al., 2023; Sonu et al., 2018). The number of research on the extraction of phenolic compounds from agri-food by-products has been increasing in the past decades since their valorisation is a way to reduce food waste with the added advantage of being a low-cost source (Moreno-González & Ottens, 2021; Muhlack et al., 2018; Sagar et al., 2018). In this context, by-products such as grape marc, obtained from the wine-making industry, have a high content of phenolics, mainly flavonoids such as anthocyanins (Cortés et al., 2020; Hegedüs et al., 2022; Pertuzatti et al., 2020; Sinrod et al., 2021; Spigno et al., 2007). Red goji berry pomace, on the other hand, is a less studied by-product composed mostly of skins and pulp and is rich in carotenoids and phenolics, like flavonoids and phenolic acids (Kosińska-Cagnazzo et al., 2017; Kulczyński & Gramza-Michałowska, 2016; Skenderidis et al., 2017).

Typically, these added value compounds are obtained by hydroalcoholic extraction. Further processing either involves a second extraction to maximise recovery and/or solvent removal. Although ethanol is considered a green solvent and classified as GRAS, i.e., suitable for food applications, its high flammability can limit its application at an industrial scale. Colloidal gas aphrons (CGA) are surfactant-stabilized microbubbles generated by the intense stirring of a surfactant solution at high speeds (5000-1000 rpm) (Jarudilokkul et al., 2004; Lye & Stuckey, 1998; Prasad et al., 2015). They present properties such as adherence of molecules to the surfactant bubble surface, which can be modulated according to the type of surfactant (ionic or non-ionic) used and higher stability than conventional foams (Jauregi et al., 2000). These characteristics and their ease of pumping from one point to another make them particularly interesting as a separation method which can be easily scalable using a flotation column in batch or counter-current mode (Dermiki et al., 2021). In our previous research, we demonstrated that phenolic compounds can be extracted from hydroalcoholic extracts of grape marc with CGA at high yields (Carullo et al., 2022; MohdMaidin et al., 2018; Spigno et al., 2015). In addition, using a food grade surfactant such as Tween 20 avoids the need for a subsequent step for its removal and, it could aid the formulation of the extracted compounds (MohMaidin 2019). As concluded by Jauregi & Dermiki, (2013), if CGA are applied following an integrated approach, i.e. if removal of surfactant is not required, this separation could be more advantageous than others such as, supercritical carbon dioxide extraction and solvent extraction.

Phenolic compounds, present a myriad of challenges when formulating them. Their physicochemical properties and their high susceptibility to structural changes due to solubility, temperature, pH, storage conditions, and oxidation can lead to a reduction in their bioactivity (Brglez Mojzer et al., 2016; Cao et al., 2021; Manach et al., 2004). Great

efforts have been made towards their stabilisation and protection, commonly through methods such as encapsulation and covalent and non-covalent complexation (Guo & Jauregi, 2018; Kaderides et al., 2020; McClements, 2018; Motilva et al., 2016; Wen et al., 2017). In recent work we have demonstrated that the non-ionic surfactant used for the generation of CGA (Tween 20) had a stabilisation effect on the anthocyanins as compared to the ethanolic raw extract, (MohdMaidin et al., 2019). Tween 20 is a synthetic but food grade surfactant. Yet, the use of these synthetic surfactants possesses limitations due to the quantities that can be used safely in foods and the lack of nutritional value.

Natural molecules with surfactant properties, such as saponins, have been explored for CGA separation. However, they possess limitations, especially in food applications, due to astringency and possible side effects depending on the concentration used (Sharma et al., 2023). On the other hand, whey protein, a by-product generated from cheese making and widely used as a food ingredient, presents surface activity properties such as those of low molecular weight surfactants, i.e. the ability to create stable foams (Cao et al., 2018; Luck et al., 2002; Nastaj & Sołowiej, 2020). Moreover, whey proteins have been extensively researched due to their capacity to interact with phenolic compounds and the stabilisation effect this interaction confers (Gong et al., 2021; Guo & Jauregi, 2018; Li et al., 2022; Li & Girard, 2023; Tazeddinova et al., 2022).

The main objective of this work was to investigate for the first time the use of whey protein as a natural surfactant for CGA generation and their further application for the separation of phenolic compounds from hydroalcoholic extracts obtained from different fruit based by-products with different phenolics composition, grape marc and red goji. Furthermore, to investigate if this surfactant-rich fraction could confer an additional advantage in stabilising anthocyanins during storage.

2. Materials and Methods

2.1 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. The grape marc was ground to a particle size of <2mm. 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).

2.2 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%), (+)-catechin hydrate (≥98%), gallic acid (≥98%), formic acid, 2,2′-azino-bis (3-ethylbenzothiazoline- 6-sulfonic acid) diammonium salt (ABTS) (≥98%), potassium persulfate (≥99%), (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) (≥97%), TPTZ (2, 4, 6-tris(2-pyridyl)-s-triazine) and ferric chloride hexahydrate were purchased from Sigma- Aldrich (Massachusetts, US). HPLC grade water, and acetonitrile were purchased from Sigma Aldrich (Massachusetts, US), and Thermo Fisher (Massachusetts, US). Delphinidin-3-o-glucoside (>99%); cyanidin-3-o-glucoside (>98%); petunidin-3-o-glucoside (> 98%) and malvidin-3-o-glucoside (>99%) were purchased from Extra synthese (Paris, France).

2.3 CGA production and characterisation

First, an aqueous solution of whey protein isolate with a final protein concentration of 15 mg/mL (1.5% w/v) was prepared in a 500 mL volumetric flask and left overnight at room temperature for complete hydration. This concentration was chosen based on previous experiments where the minimum protein concentration to obtain stable CGA was determined (data not published). 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 SLT 2 high-speed impeller (Silverson Machines, UK). The gas hold-up ε, the volume of air incorporated into the dispersion, was determined as follows:

Equation (1) $$\varepsilon = \frac{VCGA - V0}{VGA} x\,100$$

V_{CGA} is the volume of aphrons, and V_o is the volume of liquid used in the generation of CGA, expressed as percentage.

The stability of the foams was determined based on the time taken for half of the liquid volume ($V_o/2$) to drain (T v/2). For this, measurement of the volume drained from CGA was taken every minute for a 15 min period. The time was calculated from the equation derived from the graphical representation of the drained volume against time; in the equation, the drained volume was fixed to $V_o/2$=200mL to obtain the time it takes to drain half of the initial volume. Each CGA were done in triplicate.

2.4 Extraction of phenolic compounds from grape marc and goji berry skins

The grape marc solvent extraction was carried out following the methodology developed previously by MohdMaidin et al. (2018). The extraction was performed at 8:1 solvent to sample ratio (v/w) using a hydroalcoholic solution of 60% ethanol as solvent, under magnetic stirring for two hours at 60°C. After the extraction, the solids were separated through vacuum filtration using #1 Whatman paper. For the red goji berry skins, the extraction was carried out using a hydroalcoholic solution of 60% ethanol at 15:1 solvent to solute ratio (v/w), under magnetic stirring for 40 minutes at 60°C (conditions

established by the supplier). After this, the solids were separated through vacuum filtration using #1 Whatman paper. Grape marc extract (GME) and red goji skins extract (RGSE) were stored at -18 °C for further analysis and CGA processing. The extractions were carried out in duplicate and analysed in triplicate.

2.5 Separation of phenolic compounds with CGA

CGA made of WPI, labelled CGA(WPI) and CGA made of Tween 20, labelled CGA(Tween 20), were used to separate phenolic compounds from GME and RGSE. The separation was carried out based on the optimum conditions found in previous works, i.e., 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; Spigno et al., 2015). For the separation, 65 mL of extract 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, UK) 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 liquid phase (LP) at the bottom (drained liquid). 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:

Equation (2)
$$\text{Recovery (\%)} = \frac{M_{feed} - M_{liquid\ phase}}{M_{feed}} \times 100$$

Where M_{feed} is the initial mass of phenolic compounds in the GME or RSGE, and $M_{liquid\ phase}$ is the mass of phenolic compounds determined in the liquid phase. The determination of phenolics in the aphron phase led to an overestimation due to interference by the surfactant and this is why it was determined in the liquid phase.

2.6 Degradation of anthocyanins over time

To determine the stability of anthocyanins during storage, accelerated storage conditions were tested. The method was selected based on previous work done in the research group on the storage time effect on anthocyanins in Roselle beverages (Omoarukhe et al., 2023). Aliquots of GME, before and after CGA separation with both surfactants, were stored under accelerated conditions at 40°C in a controlled temperature cabinet (SANYO, GALLENKAMP) and 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 calculated based on the determination of protein content in the liquid fraction. The protein content of CGA(WPI) was 13.8 mg/mL, and for CGA(Tween 20), the surfactant concentration was 7.68 mM.

The changes were determined by calculating the degradation kinetics of the four main anthocyanins in GME. To corroborate the first order kinetic behaviour, the natural logarithms of each anthocyanin content were plotted against time and tested if it followed a linear relationship as described by the equation below:

Equation (3) $$-ln\left(\frac{A_t}{A_0}\right) = k * t$$

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 straight-line slope 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:

Equation (4) $$t\frac{1}{2} = \frac{Ln(2)}{k}$$

2.7 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-equilibration. 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 following external standards: delphinidin-3-o-glucoside; cyanidin-3-o-glucoside; petunidin-3-o-glucoside, and malvidin-3-o-glucoside (Appendix A.1).

2.8 Phenolic compounds analytical determinations

Total Phenol Content (TPC)

The total phenol content was determined by the Folin-Ciocalteu method (Singleton & Rossi, 1965). For the assay, 75 µL de Folin-Ciocalteu reagent [1:10] diluted in water v/v, 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. Results are expressed as milligrams of gallic acid per 100 grams of dry weight (mg GAE/ 100 g dw) or percentage of gallic acid equivalents (% GAE), adapted from equation 2.

Total Monomeric Anthocyanin Content (TMAC)

Total monomeric anthocyanin levels were measured by the pH differential method (Lee et al., 2005). For this, each sample was combined in a 1:20 ratio (v:v) with potassium chloride or sodium acetate buffers (pH 1.0 and 4.5, respectively) in separate wells of a 96-well microplate. 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 using the following formula:

Equation (5) $$\text{Monomeric Anthocyanins} = \frac{A \times MW \times DF \times 1000}{\varepsilon \times 1}$$

Where ***A*** is the difference in absorbance of (A520nm – A700nm)pH 1.0 – (A520nm – A700nm)pH 4.5; ***MW*** is the molecular weight of the main anthocyanin 493.43 g/mol for malvidin 3 glucoside (M3G); ***DF*** is the dilution factor; ***1000*** is the factor for conversion from g to mg; ***ε*** is the molar extinction coefficient, in L x mol^{-1} x cm^{-1} for M3G = 28 000; ***1*** is pathlength in cm. The results are expressed as milligrams of malvidin 3-glucoside equivalents or percentage of malvidin 3-glucoside equivalents (% M3GE), adapted from equation 2.

Total Flavonoid Content (TFC)

The analysis was carried out using the aluminium chloride methodology (Zhishen et al., 1999). For the analysis, 100 µL of each sample were mixed with 430 µL of the A solution (1.8 mL of 5% sodium nitrite with 24 mL of distilled water) in an Eppendorf and incubated for 5 min. Afterwards, 30 µL of 10% aluminium chloride were added and let it rest for 1 min. Finally, 440µL of solution B (12 mL of sodium hydroxide 1M with 14.4 mL of distilled water) were added. From this reaction, 150 µL were transferred 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. Results are expressed as milligrams of catechin equivalents per 100 grams of dry weight (mg CE/ 100 g dw) or the percentage of catechin equivalents (% CE) adapted from the equation 2.

2.9 Antioxidant Capacity (AOC)

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

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 5 mL of 7mM ABTS solution (50 mL volume, stored in an amber flask and kept under refrigeration at 0–4 °C up to one month) and 88 µL of 140mM potassium persulfate

277 solution (10 mL volume, stored in an amber flask and dark place at room temperature for
278 up to one month). Then, the mixture was kept in the dark at room temperature for at
279 least 16 hours before use.

280 For the assay, the working solution of the $ABTS^{\cdot+}$ was obtained by diluting the $ABTS^{\cdot+}$
281 stock solution with distilled water to an absorbance of 0.70 ± 0.02 measured at 734 nm.
282 After this, 5 µL of sample were added to 245 µL of $ABTS^{\cdot+}$ adjusted working solution. The
283 mixture was then incubated at room temperature in the dark for 5 min, and the
284 absorbance (ABS_{sample}) was recorded at 734 nm, using a microplate reader (FLUOstar
285 Omega, BMG Labtech). The results are expressed in micromoles of Trolox equivalents per
286 gram of theoretical quantity of dry weight of marc or skins found in the feedstock (µmol
287 TE/ g dw), calculated from a Trolox standard curve ranging from 0.02 to 0.4 mg/mL. The
288 analysis was done in triplicate for each sample.

289 *FRAP - Ferric Reducing Antioxidant Power*

290 To measure the total antioxidant activity by FRAP, the methodology established by Benzie
291 & Strain (1996) was used with minor modifications. First, FRAP stock reagents were
292 prepared as follows: 300 mM acetate buffer (pH=3.6, 2.699 g sodium acetate trihydrate
293 and 16 ml (16.8 g) of glacial acetic acid dissolved in 1 L of deionized water), 2,4,6-
294 tripyridyl-s-triazine (TPTZ) (10 mM) in 10 ml HCl (40 mM), and 20 mM ferric chloride
295 hexahydrate aqueous solution. The FRAP reagent was mixed with 25 mL of acetate buffer,
296 2.5 mL of TPTZ and 2.5 mL of ferric chloride solutions. For the assay, 10 µL of the
297 sample/standard were added to 300 µL of FRAP reagent in an Eppendorf and vortexed
298 for 10 s. Then 100 µL of this mixture were transferred into the microwell plate (96-well,
299 NUNC, FB), and absorbance was measured at 595 nm (FLUOstar Omega, BMG Labtech).
300 The results are expressed as micromoles of ascorbic acid equivalents per gram of
301 theoretical quantity of dry weight of marc or skins found in the feedstock (µmol AAE/ g
302 dw), using an ascorbic acid standard curve: 0.02 to 0.2 mg/mL. The analysis was done in
303 triplicate.

304 *2.10 Statistical Analysis*

305 The differences between the surfactants in gas hold-up, stability and CGA separation
306 performance, were analysed using a t-test for independent samples. For comparison of
307 antioxidant capacity of the extract and the separated CGA of the two surfactants, the
308 data was analysed using a one-way ANOVA, detecting differences with the Tukey test.
309 The significance level was defined at $p<0.05$ for all the analyses. All statistical analyses
310 were done using IBM® SPSS® Statistics 27. The results are reported as mean ± SD.

3. Results and Discussion

3.1 CGA characterisation

To demonstrate the capacity of WPI for CGA generation, the gas hold-up and drainage of CGA generated with this surfactant were compared against those of Tween 20. The latter is a food-grade synthetic surfactant that has been previously used for the separation of phenolic compounds from hydroalcoholic extracts. The results of this comparison are shown in Table 1. Overall, it was observed that CGA(WPI) have a lower gas hold-up and stability than CGA(Tween 20). However, CGA characterisation results are within the range of stability (450s – 700s) and gas hold-up (57-72%) reported for other surfactants, including Tweens and Spans (Dahmoune et al., 2013; Dermiki et al., 2009; Fuda et al., 2004; MohdMaidin et al., 2018; Spigno et al., 2015). These results demonstrate that whilst WPI displayed lower gas hold-up and stability than Tween 20, the tested concentration produced CGA within the range of stable CGA reported previously.

Table 1. Gas hold-up and half-life time of CGA generated with Tween 20 and WPI

Surfactant	Gas Hold Up (%)	T v/2 (s)
Tween 20 10 mM	67.22 ± 2.84 [a]	628.07 ± 2.65 [a]
WPI 15 mg/mL	56.50 ± 1.34 [b]	516.54 ± 18.22 [b]

Values are expressed as mean ± SD (*n=3)*. Different letters in the same column denote significant difference $p<0.05$.

3.2 Hydroalcoholic extracts characterisation

Prior to the CGA separation, a hydroalcoholic extract was obtained from each feedstock. The characterisation of the extracts (Table 2) showed that the phenolic content differed greatly in composition, which was expected due to the natural composition of each by-product. Goji berry extract had been previously characterized by the authors (data not published), showing that phenolic acids (caffeic acid) and flavonoids (quercetin-3-O-rutinoside, quercetin, kaempferol-3-O-rutinoside, and catechin) are the major phenolic compounds, explaining the content of TPC and TFC. Other studies have reported the presence of anthocyanins in goji berries (Kosińska-Cagnazzo et al., 2017; Kulczyński & Gramza-Michałowska, 2016; Liu et al., 2020); however, in this study, no anthocyanins were detected; this could be related to different factors like extraction and analytical methods used here. For grape marc, it has been widely reported that it contains a high

content of flavonoids, such as anthocyanins (delphinidin, malvidin, cyanidin, petunidin, and peonidin in their glycosylated forms) and phenolic acids (caffeic acid, ferulic acid, syringic acid) (MohdMaidin et al., 2018), which explain the high content of TPC, TFC, and the presence of TMAC.

Table 2. Phenolic compounds determination for grape marc (GME) extract and red goji skin extract (RSGE).

Feedstock	TPC	TMAC	TFC
GME	2034.98 ± 240.30	27.37 ± 2.82	2015.00 ± 108.99
RGSE	1029.61 ± 2.06	ND	254.95 ± 43.81

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 are expressed as mean ± SD (*n=6)*. ND: Not detected

3.3 Separation of phenolic compounds by CGA(WPI)

CGA separation performance for each surfactant and each feedstock is shown in Fig 1. The results show that the recovery of TPC and TFC from grape marc was above 90%, with similar values for both surfactants (Fig 1-A). Thus, whey protein behaves similarly to Tween 20 in terms of phenolic compounds separation. For TMAC in grape marc, a lower recovery of anthocyanins than total phenols and flavonoids was obtained with both surfactants (Fig 1-C). Whilst the recovery with WPI was the lowest of the two surfactants, recovery with WPI was still over 60%. A different trend was observed for red goji extract, where the highest recovery of total phenols and flavonoids was observed in CGA(WPI) (Fig 1-A; 1-B) and similar TPC to that obtained for grape marc (about 90%).

The similar performance of Tween 20 and WPI in terms of TPC and TFC yields might be explained by the type of interactions occurring between the surfactants and phenolics during their separation. Several studies have established that hydrophobic interactions drive the separation of phenolic compounds when using Tween 20 (Carullo et al., 2022; Noriega et al., 2018; Sazdanić et al., 2023). Here, we hypothesised that these same hydrophobic interactions would lead to a successful separation using WPI. In general, phenolic compounds interact with the hydrophobic pocket of the protein. However, other interactions such as hydrogen bonding and van der Waals forces have demonstrated to play an important role when it comes to binding of protein and

362 phenolics (Cao & Xiong, 2017; Gong et al., 2021; Meng & Li, 2021; Ozdal et al., 2013; Skrt
363 et al., 2012) which can explain the high recoveries obtained with WPI.

364

365 On the other hand, the significant difference in anthocyanin recovery between CGA(WPI)
366 and CGA(Tween) is likely to be related to the pH of the surfactant solutions and the
367 change of pH during the separation (Table 3). WPI stock solution displayed a pH above
368 the isoelectric point of whey proteins (4.5-5.5); therefore, the overall charge of the
369 protein was negative (Fuda et al., 2005; Fuda & Jauregi, 2006). In the case of GME and
370 RSGE, the addition of WPI(CGA) led to a rise in pH whilst a decrease for Tween 20 (Table
371 3).

372

Table 3. pH of GME, RSGE, surfactants and CGA phases after separation

		WPI (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 as mean ± sd (n=2)

373

374 The aphron phase in RSGE had a pH above the isoelectric point of whey protein (protein´s
375 net charge negative) , while GME showed a slightly lower pH in the AP, closer to the
376 isoelectric point of whey proteins (protein´s net charge close to zero). Therefore, despite
377 proteins in GME and RSGE CGA(WPI) being differently charged, similar recoveries of TPC
378 and TFC were attained for both. These findings support our hypothesis that CGA(WPI)
379 separation is driven mainly by hydrophobic interactions and hydrogen bonds.
380 Nonetheless, pH greatly affected the recovery of anthocyanins. The increased pH in the
381 CGA(WPI) aphron phase of GME could be the reason for anthocyanin's reduced recovery.
382 Anthocyanins are highly susceptible to structural changes above pH 2, where the
383 ionisation of the flavylium ion gives place to other structures, like chalcones and quinones
384 (Andersen & Jordheim, 2010; Cabrita et al., 2000; Enaru et al., 2021; Martín et al., 2017).
385 Therefore, this could be the reason for the better recovery of anthocyanins with
386 CGA(Tween 20) (pH 3.79) than CGA(WPI) (pH 5.81). In summary, phenolic compounds
387 can be recovered effectively with CGA generated from whey protein. However, the pH of
388 the resultant extraction mixture will need to be adjusted for pH-sensitive compounds.

Nevertheless, the isoelectric point of the whey proteins will need to be taken into account (for major whey proteins, it is about pH = 4-5) as at that pH, they will precipitate.

3.4 AOC after CGA separation

Following the successful separation and recovery of various phenolic compounds with WPI, it was crucial to determine the implications of this process on the AOC in the aphron phase. ABTS results (Fig. 2-A) show that a similar decrease of AOC occurred in GME for both surfactants, around 40%. For RSGE, the reduction was less than 10%. FRAP results (Fig. 2-B) showed a more noticeable decrease in GME than in RGSE with the two surfactants. However, this was more pronounced for CGA(WPI), where the initial activity was reduced by 50% approximately.

Previous studies have reported a reduction of AOC during CGA separation with Tween 20, attributing this to phenolic oxidation (Spigno et al., 2015). In the case of CGA(WPI), it has been reported previously that a major cause for the decrease in AOC is likely due to the complex protein-phenolic interactions, the type of phenolic compounds and the medium (Almajano et al., 2007; Cao & Xiong, 2017; De La Cruz-Molina et al., 2023; de Morais et al., 2020; von Staszewski et al., 2011). it is important to note that both extracts have a different phenolic profile. GME displayed a higher content of flavonoids (Table 2) that are known to interact more strongly with WPI than phenolic acids, which are abundant in RSGE (Cao & Xiong, 2017). Other studies report that a masking effect is likely to occur depending on the method due to the competition during the antioxidant activity analysis between the protein and hydroxyl groups of the phenolics (Ozdal et al., 2013; Stojadinovic et al., 2013).

In summary, despite the high recovery of phenolics attained with both surfactants, the antioxidant activity of the extracts was reduced after CGA separation but especially in the case of GME, and this effect was even more pronounced with WPI, possibly due to protein-phenolic interactions.

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

It was seen in the previous section that WPI possibly causes a decrease in AOC during CGA separation. Hence, it was important to understand the effect of CGA separation on the stability of anthocyanins since they are highly susceptible to degradation and they will have more or less stability depending on the structure, medium, temperature, and storage conditions (Andersen & Jordheim, 2010; Martín et al., 2017; Vidana Gamage et al., 2022). Thus, the impact of whey protein on the stability of GME's anthocyanins was studied. Figure 3 shows the degradation of anthocyanins in GME and GME-CGA over time which follow first-order kinetics. These results agree with previous studies for grape marc anthocyanins degradation, noting that while degradation is complex, it generally follows

first-order kinetics (Andersen & Jordheim, 2010; Hellström et al., 2013; Lavelli et al., 2016; MohdMaidin et al., 2019).

Both surfactants show a pronounced stabilising effect of M3G. Very steady degradation of this anthocyanin in the CGA of both surfactants as compared to the extract (control) is shown in Fig 3. Both WPI and Tween showed a stabilisation effect with high life increasing from 4 days in the extract to 8 and 10 in the whey and Tween 20 CGA respectively (Table 4). This stabilisation effect was also observed for C3G but there is almost no effect for P3G and D3G. Clearly, surfactants improve the stability of specific anthocyanins, possibly because of interactions between surfactant and anthocyanin and the degradation pattern could be influenced by the structure of anthocyanins (Appendix A.2).

Table 4. Half-lives (t ½, days) and degradation rate constant (k, d-1) of anthocyanins processed in control (GME), CGA(WPI) 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 (D3G)	3	0.2146	2	0.3419	3	0.2359
Cyanidin 3-O-glucoside (C3G)	3	0.2326	6	0.1187	6	0.1125
Petunidin 3-O-glucoside (P3G)	4	0.1595	4	0.1931	5	0.1456
Malvidin 3-O-glucoside (M3G)	4	0.1711	8	0.0859	10	0.0700

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 in positions 3' and 5' makes 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 note 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, 2010; Martín et al., 2017; Mattioli et al., 2020).

This set of results showed that for most of the primary anthocyanins in GME, the degradation was slower in both CGA than in the crude extract. Thus, extending the half-lives of the analysed anthocyanins, possibly due to the stabilisation effect of the interactions between surfactants and phenolics.

3.6. Integrated separation method

Figure 4 shows an example of how CGA could be integrated within the conventional process and how it would compare with other processes that are currently applied to grape marc such as, evaporation or ultrafiltration, in which the removal of the solvent or purification need to be carried out as a subsequent step. In this example, all the processes end with the drying or encapsulation of the extract however, the only one that integrates a pre-formulation step is CGA separation since there is no need to remove the protein that is present in the aphron phase. Moreover, the recycling of the protein in the liquid phase generated after the CGA 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. While something similar can occur in the case of evaporation, in the case of CGA, the collection of the liquid phase after the separation does not require the use of specialized equipment.

Other surfactant based separations have been developed that could represent a green solid-liquid extraction alternative to hydroalcoholic extraction. For example, Atanacković Krstonošić et al. (2023) describe solid-liquid extraction using a surfactant rich solvent as a green solvent that effectively extracts polyphenols with high antioxidant activity from grape marc. In this method, the solubilisation power of the surfactant in the form of micelles is the basis of the separation. Yet although it is an interesting method that leads to high yields of phenolics with high antioxidant activity, in comparison with the one proposed here, it requires: (i) higher amount of surfactant, 3% w/v vs 1.5% in the case of CGA (ii) higher solvent to solids ratio, 100:1 vs 64:1 with CGA (calculated based on solvent to solids ratio 8:1 and CGA to extract volume ratio 16) (iii) longer separation time, 45 minutes vs 10 minutes for CGA; for further comparisons between the different surfactant based methods including CGA see Atanacković Krstonošić et al. (2023). Overall, from the example and the results described above, it can be concluded that CGA can be advantageous as compared to other separations, especially because it allows the integration of the separation and formulation steps.

4. Conclusions

In this work, it was shown for the first time that CGA generated from whey proteins can successfully separate phenolics from natural extracts, and the recovery of phenolics was comparable to that of CGA generated from Tween 20. The results suggest that hydrophobic interactions between phenolics and whey protein are responsible for the successful separation, as well as hydrogen bonding. The strong interactions between phenolics and protein lead to high yields but can also hinder the radical scavenging activity, as observed in AOC reduction. On the other hand, these interactions can be responsible for the stabilising effect observed during storage. Overall, CGA generated with WPI have resulted in an integrated separation method that, by combining it with hydroalcoholic extraction, leads to the effective separation of phenolics and their pre-formulation in a whey protein rich solution with stabilisation effect. Thus, the combination of CGA generated with food grade surfactants (Tween 20 and whey protein) and the conventional solid-liquid extraction opens the perspective for a cost effective, integrated and sustainable process for the extraction and formulation of phenolics from by-products. Cost effectiveness will need to be further assessed by conducting a detailed techno-economic assessment. Nevertheless, CGA generated with whey protein, which is a food-grade surfactant derived from a by-product, can be an attractive and sustainable separation method for food applications.

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix

References

Almajano, M. P., Delgado, M. E., & Gordon, M. H. (2007). Changes in the antioxidant properties of protein solutions in the presence of epigallocatechin gallate. *Food Chemistry*, *101*(1), 126–130. https://doi.org/10.1016/j.foodchem.2006.01.009

Andersen, Ø. M., & Jordheim, M. (2010). Anthocyanins. In *eLS* (Issue October). Wiley. https://doi.org/10.1002/9780470015902.a0001909.pub2

Atanacković Krstonošić, M., Sazdanić, D., Ćirin, D., Maravić, N., Mikulić, M., Cvejić, J., & Krstonošić, V. (2023). Aqueous solutions of non-ionic surfactant mixtures as mediums for green extraction of polyphenols from red grape pomace. *Sustainable Chemistry and Pharmacy*, *33*, 101069. https://doi.org/10.1016/j.scp.2023.101069

Benzie, I. F. F., & Strain, J. J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Analytical Biochemistry*, *239*(1), 70–76. https://doi.org/10.1006/abio.1996.0292

Brglez Mojzer, E., Knez Hrnčič, M., Škerget, M., Knez, Ž., & Bren, U. (2016). Polyphenols: Extraction Methods, Antioxidative Action, Bioavailability and Anticarcinogenic Effects. *Molecules (Basel, Switzerland)*, *21*(7). https://doi.org/10.3390/molecules21070901

Cabrita, L., Fossen, T., & Andersen, Ø. M. (2000). Colour and stability of the six common anthocyanidin 3-glucosides in aqueous solutions. *Food Chemistry*, *68*(1), 101–107. https://doi.org/10.1016/S0308-8146(99)00170-3

Cao, H., Saroglu, O., Karadag, A., Diaconeasa, ta, Zoccatelli, G., Adam Conte-Junior, C., Gonzalez-Aguilar, G. A., Ou, J., Bai, W., Mara Zamarioli, C., Alexandre Pedro de Freitas, L., Shpigelman, A., Campelo, P. H., Capanoglu, E., Lik Hii, C., Mahdi Jafari, S., Qi, Y., Liao, P., Wang, M., Zou, L., Bourke, P., Simal-Gandara, J., Xiao, J., & Jesus Simal-Gandara, C. (2021). Available technologies on improving the stability of polyphenols in food processing. *Food Frontiers*, *2*, 109–139. https://doi.org/10.1002/fft2.65

Cao, Y., & Xiong, Y. L. (2017). Interaction of Whey Proteins with Phenolic Derivatives Under Neutral and Acidic pH Conditions. *Journal of Food Science*, *82*(2), 409–419. https://doi.org/10.1111/1750-3841.13607

Cao, Y., Xiong, Y. L., Cao, Y., & True, A. D. (2018). Interfacial properties of whey protein foams as influenced by preheating and phenolic binding at neutral pH. *Food Hydrocolloids*, *82*, 379–387. https://doi.org/10.1016/J.FOODHYD.2018.04.020

Carullo, D., Bosi, M., Dermiki, M., Bassani, A., Jauregi, P., & Spigno, G. (2022). Exploring different strategies of separation of antioxidant compounds from winery by-products via surfactant-assisted processes for process intensification and integration. *Food and Bioproducts Processing*, *131*, 191–201. https://doi.org/10.1016/J.FBP.2021.11.007

Cortés, A., Moreira, M. T., Domínguez, J., Lores, M., & Feijoo, G. (2020). Unraveling the environmental impacts of bioactive compounds and organic amendment from grape marc. *Journal of Environmental Management*, *272*, 111066. https://doi.org/10.1016/J.JENVMAN.2020.111066

Dahmoune, F., Madani, K., Jauregi, P., De Faveri, D. M., & Spigno, G. (2013). *Fractionation of a Red Grape Marc Extract by Colloidal Gas Aphrons*. *32*. www.aidic.it/cet

De La Cruz-Molina, A. V., Gonçalves, C., Neto, M. D., Pastrana, L., Jauregi, P., & Amado, I. R. (2023). Whey–pectin microcapsules improve the stability of grape marc phenolics during digestion. *Journal of Food Science*, *88*(12), 4892–4906. https://doi.org/10.1111/1750-3841.16806

de Morais, F. P. R., Pessato, T. B., Rodrigues, E., Peixoto Mallmann, L., Mariutti, L. R. B., & Netto, F. M. (2020). Whey protein and phenolic compound complexation: Effects on antioxidant capacity before and after in vitro digestion. *Food Research International*, *133*(February), 109104. https://doi.org/10.1016/j.foodres.2020.109104

Dermiki, M., Gordon, M. H., & Jauregi, P. (2009). Recovery of astaxanthin using colloidal gas aphrons (CGA): A mechanistic study. *Separation and Purification Technology*, *65*(1), 54–64. https://doi.org/10.1016/j.seppur.2007.12.023

Dermiki, M., J. Garrard, I., & Jauregi, P. (2021). Selective separation of dyes by colloidal gas aphrons: Conventional flotation vs countercurrent chromatography. *Separation and Purification Technology*, *279*. https://doi.org/10.1016/j.seppur.2021.119770

Enaru, B., Dret, G., Daria Pop, T., & StˇanilˇaStˇanilˇStˇanilˇa, A. (2021). *Anthocyanins: Factors Affecting Their Stability and Degradation*. https://doi.org/10.3390/antiox10121967

Fallah, A. A., Sarmast, E., Fatehi, P., & Jafari, T. (2020). Impact of dietary anthocyanins on systemic and vascular inflammation: Systematic review and meta-analysis on

randomised clinical trials. *Food and Chemical Toxicology*, *135*. https://doi.org/10.1016/J.FCT.2019.110922

Fuda, E., Bhatia, D., Pyle, D. L., & Jauregi, P. (2005). Selective separation of β-lactoglobulin from sweet whey using CGAs generated from the cationic surfactant CTAB. *Biotechnology and Bioengineering*, *90*(5), 532–542. https://doi.org/10.1002/BIT.20412

Fuda, E., & Jauregi, P. (2006). An insight into the mechanism of protein separation by colloidal gas aphrons (CGA) generated from ionic surfactants. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, *843*(2), 317–326. https://doi.org/10.1016/j.jchromb.2006.06.032

Fuda, E., Jauregi, P., & Pyle, D. L. (2004). *Recovery of Lactoferrin and Lactoperoxidase from Sweet Whey Using Colloidal Gas Aphrons (CGAs) Generated from an Anionic Surfactant, AOT*. https://doi.org/10.1021/bp034198d

Gong, S., Yang, C., Zhang, J., Yu, Y., Gu, X., Li, W., & Wang, Z. (2021). Study on the interaction mechanism of purple potato anthocyanins with casein and whey protein. *Food Hydrocolloids*, *111*, 106223. https://doi.org/https://doi.org/10.1016/j.foodhyd.2020.106223

Guo, Y., & Jauregi, P. (2018). Protective effect of β-lactoglobulin against heat induced loss of antioxidant activity of resveratrol. *Food Chemistry*, *266*(January), 101–109. https://doi.org/10.1016/j.foodchem.2018.05.108

Hegedüs, I., Andreidesz, K., Szentpéteri, J. L., Kaleta, Z., Szabó, L., Szigeti, K., Gulyás, B., Padmanabhan, P., Budan, F., & Máthé, D. (2022). *The Utilization of Physiologically Active Molecular Components of Grape Seeds and Grape Marc*. https://doi.org/10.3390/ijms231911165

Hellström, J., Mattila, P., & Karjalainen, R. (2013). Stability of anthocyanins in berry juices stored at different temperatures. *Journal of Food Composition and Analysis*, *31*(1), 12–19. https://doi.org/10.1016/J.JFCA.2013.02.010

Jarudilokkul, S., Rungphetcharat, K., & Boonamnuayvitaya, V. (2004). Protein separation by colloidal gas aphrons using surfactant. *Separation and Purification Technology*, *35*(1), 23–29. https://doi.org/10.1016/S1383-5866(03)00111-4

Jauregi, P., & Dermiki, M. (2013). Separation of value-added bioproducts by colloidal gas aphrons (CGA) flotation and applications in the recovery of value-added food products. *Separation, Extraction and Concentration Processes in the Food, Beverage and Nutraceutical Industries*, 284-e314. https://doi.org/10.1533/9780857090751.1.284

Jauregi, P., Mitchell, G. R., & Varley, J. (2000). Colloidal gas aphrons (CGA): Dispersion and structural features. *AIChE Journal*, *46*(1), 24–36. https://doi.org/10.1002/aic.690460105

Kaderides, K., Mourtzinos, I., & Goula, A. M. (2020). Stability of pomegranate peel polyphenols encapsulated in orange juice industry by-product and their incorporation in cookies. *Food Chemistry*, *310*(October 2019), 125849. https://doi.org/10.1016/j.foodchem.2019.125849

Koh, Q. Q., Kua, Y. L., Gan, S., Tan, K. W., Lee, T. Z. E., Cheng, W. K., & Lau, H. L. N. (2023). Sugar-based natural deep eutectic solvent (NADES): Physicochemical properties, antimicrobial activity, toxicity, biodegradability and potential use as green extraction media for phytonutrients. *Sustainable Chemistry and Pharmacy*, *35*, 101218. https://doi.org/10.1016/J.SCP.2023.101218

Kosińska-Cagnazzo, A., Weber, B., Chablais, R., Vouillamoz, J. F., Molnár, B., Crovadore, J., Lefort, F., & Andlauer, W. (2017). Bioactive compound profile and antioxidant activity of fruits from six goji cultivars cultivated in Switzerland. *Journal of Berry Research*, *7*(1), 43–59. https://doi.org/10.3233/JBR-160144

Kulczyński, B., & Gramza-Michałowska, A. (2016). Goji Berry (Lycium barbarum): Composition and Health Effects - A Review. *Polish Journal of Food and Nutrition Sciences*, *66*(2), 67–75. https://doi.org/10.1515/pjfns-2015-0040

Lavelli, V., Torri, L., Zeppa, G., Fiori, L., & Spigno, G. (2016). Recovery of Winemaking By-Products. *Italian Journal of Food Science*, *28*(4), 542–564. https://doi.org/10.14674/1120-1770/ijfs.v507

Lee, J., Durst, R. W., & Wrolstad, R. E. (2005). Determination of total monomeric anthocyanin pigment content of fruit juices, beverages, natural colorants, and wines by the pH differential method: Collaborative study. *Journal of AOAC International*, *88*(5), 1269–1278. https://doi.org/10.1093/jaoac/88.5.1269

Li, D., Yang, Y., Yang, X., Wang, Z., Yao, X., & Guo, Y. (2022). Enhanced bioavailability and anti-hyperglycemic activity of young apple polyphenols by complexation with whey protein isolates. *Journal of Food Science*, *87*(3), 1257–1267. https://doi.org/10.1111/1750-3841.16062

Li, N., & Girard, A. L. (2023). Impact of pH and temperature on whey protein-proanthocyanidin interactions and foaming properties. *Food Hydrocolloids*, *134*, 108100. https://doi.org/10.1016/j.foodhyd.2022.108100

Liu, B., Xu, Q., & Sun, Y. (2020). Black goji berry (Lycium ruthenicum) tea has higher phytochemical contents and in vitro antioxidant properties than red goji berry (Lycium barbarum) tea. *Food Quality and Safety*, *4*(4), 193–201. https://doi.org/10.1093/fqsafe/fyaa022

Luck, P. J., Bray, N., & Foegeding, E. A. (2002). Factors determining yield stress and overrun of whey protein foams. *Journal of Food Science*, *67*(5), 1677–1681. https://doi.org/10.1111/J.1365-2621.2002.TB08704.X

669 Lye, G. J., & Stuckey, D. C. (1998). Structure and stability of colloidal liquid aphrons.
670 *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, *131*(1–3), 119–
671 136. https://doi.org/10.1016/S0927-7757(96)03931-3

672 Manach, C., Scalbert, A., Morand, C., Rémésy, C., & Jiménez, L. (2004). Polyphenols:
673 food sources and bioavailability. *The American Journal of Clinical Nutrition*, *79*(5),
674 727–747. https://doi.org/10.1093/AJCN/79.5.727

675 Martín, J., Kuskoski, E. M., Navas, M. J., Asuero, A. G., Martín, J., Kuskoski, E. M., Navas,
676 M. J., & Asuero, A. G. (2017). Antioxidant Capacity of Anthocyanin Pigments.
677 *Flavonoids - From Biosynthesis to Human Health*. https://doi.org/10.5772/67718

678 Mattioli, R., Francioso, A., Mosca, L., & Silva, P. (2020). Anthocyanins: A Comprehensive
679 Review of Their Chemical Properties and Health Effects on Cardiovascular and
680 Neurodegenerative Diseases. *Molecules*, *25*(17), 3809.
681 https://doi.org/10.3390/molecules25173809

682 McClements, D. J. (2018). Encapsulation, protection, and delivery of bioactive proteins
683 and peptides using nanoparticle and microparticle systems: A review. In *Advances*
684 *in Colloid and Interface Science* (Vol. 253, pp. 1–22). Elsevier B.V.
685 https://doi.org/10.1016/j.cis.2018.02.002

686 Meng, Y., & Li, C. (2021). Conformational changes and functional properties of whey
687 protein isolate-polyphenol complexes formed by non-covalent interaction. *Food*
688 *Chemistry*, *364*(February), 129622.
689 https://doi.org/10.1016/j.foodchem.2021.129622

690 MohdMaidin, N., Michael, N., Oruna-Concha, M. J., & Jauregi, P. (2018). Polyphenols
691 extracted from red grape pomace by a surfactant based method show enhanced
692 collagenase and elastase inhibitory activity. *Journal of Chemical Technology and*
693 *Biotechnology*, *93*(7), 1916–1924. https://doi.org/10.1002/jctb.5459

694 MohdMaidin, N., Oruna-Concha, M. J., & Jauregi, P. (2019). Surfactant TWEEN20
695 provides stabilisation effect on anthocyanins extracted from red grape pomace.
696 *Food Chemistry*, *271*, 224–231. https://doi.org/10.1016/j.foodchem.2018.07.083

697 Moreno-González, M., & Ottens, M. (2021). A Structured Approach to Recover Valuable
698 Compounds from Agri-food Side Streams. *Food and Bioprocess Technology*, *14*(8),
699 1387–1406. https://doi.org/10.1007/s11947-021-02647-6

700 Motilva, M. J., Macià, A., Romero, M. P., Rubió, L., Mercader, M., & González-Ferrero, C.
701 (2016). Human bioavailability and metabolism of phenolic compounds from red
702 wine enriched with free or nano-encapsulated phenolic extract. *Journal of*
703 *Functional Foods*, *25*, 80–93. https://doi.org/10.1016/j.jff.2016.05.013

704 Muhlack, R. A., Potumarthi, R., & Jeffery, D. W. (2018). Sustainable wineries through
705 waste valorisation: A review of grape marc utilisation for value-added products.
706 *Waste Management*, *72*, 99–118. https://doi.org/10.1016/J.WASMAN.2017.11.011

Nastaj, M., & Sołowiej, B. G. (2020). The effect of various pH values on foaming properties of whey protein preparations. *International Journal of Dairy Technology*, *73*(4), 683–694. https://doi.org/10.1111/1471-0307.12705

Noriega, D., Zuñiga, M. E., Soto, C., MohdMaidin, N., Michael, N., & Jauregi, P. (2018). Colloidal Gas Aphrons separation to obtain polyphenol rich fractions from artichoke agro-industrial discards. *Food and Bioproducts Processing*, *110*, 50–59. https://doi.org/10.1016/j.fbp.2018.04.007

Omoarukhe, E. D., Harbourne, N., & Jauregi, P. (2023). The Effect of Cinnamon and Ginger Spices on Anthocyanins in Sweetened Roselle Beverages. *Beverages*, *9*(1), 24. https://doi.org/10.3390/beverages9010024

Ozdal, T., Capanoglu, E., & Altay, F. (2013). A review on protein-phenolic interactions and associated changes. In *Food Research International* (Vol. 51, Issue 2, pp. 954–970). https://doi.org/10.1016/j.foodres.2013.02.009

Pertuzatti, P. B., Mendonça, S. C., Alcoléa, M., Guedes, C. T., Amorim, F. da E., Beckmann, A. P. S., Gama, L. A., & Américo, M. F. (2020). Bordo grape marc (Vitis labrusca): Evaluation of bioactive compounds in vitro and in vivo. *Lwt*, *129*(January), 109625. https://doi.org/10.1016/j.lwt.2020.109625

Prasad, K. N., Spigno, G., Jauregi, P., Misra, N. N., & Cullen, P. J. (2015). Emerging macro- and micromolecules separation. *Food Waste Recovery: Processing Technologies and Industrial Techniques*, 227–248. https://doi.org/10.1016/B978-0-12-800351-0.00010-9

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., & Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology and Medicine*, *26*(9–10), 1231–1237. https://doi.org/10.1016/S0891-5849(98)00315-3

Russo, B., Picconi, F., Malandrucco, I., & Frontoni, S. (2019). Flavonoids and Insulin-Resistance: From Molecular Evidences to Clinical Trials. *International Journal of Molecular Sciences*, *20*(9), 2061. https://doi.org/10.3390/ijms20092061

Sagar, N. A., Pareek, S., Sharma, S., Yahia, E. M., & Lobo, M. G. (2018). Fruit and Vegetable Waste: Bioactive Compounds, Their Extraction, and Possible Utilization. *Comprehensive Reviews in Food Science and Food Safety*, *17*(3), 512–531. https://doi.org/10.1111/1541-4337.12330

Sazdanić, D., Krstonošić, M. A., Ćirin, D., Cvejić, J., Alamri, A., Galanakis, C., & Krstonošić, V. (2023). Non-ionic surfactants-mediated green extraction of polyphenols from red grape pomace. *Journal of Applied Research on Medicinal and Aromatic Plants*, *32*. https://doi.org/10.1016/j.jarmap.2022.100439

Sharma, K., Kaur, R., Kumar, S., Saini, R. K., Sharma, S., Pawde, S. V., & Kumar, V. (2023). Saponins: A concise review on food related aspects, applications and health

implications. In *Food Chemistry Advances* (Vol. 2). Elsevier Ltd. https://doi.org/10.1016/j.focha.2023.100191

Singleton, V. L., & Rossi, J. A. (1965). Colorimetry of Total Phenolics with Phosphomolybdic-Phosphotungstic Acid Reagents. *American Journal of Enology and Viticulture*, *16*, 144–158. http://www.ajevonline.org/content/16/3/144.full.pdf+html

Sinrod, A. J. G., Li, X., Bhattacharya, M., Paviani, B., Wang, S. C., & Barile, D. (2021). A second life for wine grapes: Discovering potentially bioactive oligosaccharides and phenolics in chardonnay marc and its processing fractions. *Lwt*, *144*(February), 111192. https://doi.org/10.1016/j.lwt.2021.111192

Skenderidis, P., Petrotos, K., Giavasis, I., Hadjichristodoulou, C., & Tsakalof, A. (2017). Optimization of ultrasound assisted extraction of of goji berry (Lycium barbarum) fruits and evaluation of extracts' bioactivity. *Journal of Food Process Engineering*, *40*(5), 1–12. https://doi.org/10.1111/jfpe.12522

Skrt, M., Benedik, E., Podlipnik, Č., & Ulrih, N. P. (2012). Interactions of different polyphenols with bovine serum albumin using fluorescence quenching and molecular docking. *Food Chemistry*, *135*(4), 2418–2424. https://doi.org/10.1016/j.foodchem.2012.06.114

Sonu, K. S., Mann, B., Sharma, R., Kumar, R., & Singh, R. (2018). Physico-chemical and antimicrobial properties of d-limonene oil nanoemulsion stabilized by whey protein–maltodextrin conjugates. *Journal of Food Science and Technology*, *55*(7), 2749–2757. https://doi.org/10.1007/s13197-018-3198-7

Spigno, G., Amendola, D., Dahmoune, F., & Jauregi, P. (2015). Colloidal gas aphrons based separation process for the purification and fractionation of natural phenolic extracts. *Food and Bioproducts Processing*, *94*, 434–442. https://doi.org/10.1016/j.fbp.2014.06.002

Spigno, G., Tramelli, L., & De Faveri, D. M. (2007). Effects of extraction time, temperature and solvent on concentration and antioxidant activity of grape marc phenolics. *Journal of Food Engineering*, *81*(1), 200–208. https://doi.org/10.1016/j.jfoodeng.2006.10.021

Stojadinovic, M., Radosavljevic, J., Ognjenovic, J., Vesic, J., Prodic, I., Stanic-Vucinic, D., & Cirkovic Velickovic, T. (2013). Binding affinity between dietary polyphenols and β-lactoglobulin negatively correlates with the protein susceptibility to digestion and total antioxidant activity of complexes formed. *Food Chemistry*, *136*(3–4), 1263–1271. https://doi.org/10.1016/j.foodchem.2012.09.040

Tazeddinova, D., Toshev, A. D., Abylgazinova, A., Rahman, Md. R., Matin, Md. M., Bin Bakri, M. K., & Ayan, O. (2022). A review of polyphenol and whey protein-based conjugates. *BioResources*, *17*(4). https://doi.org/10.15376/biores.17.4.Tazeddinova1

Vidana Gamage, G. C., Lim, Y. Y., & Choo, W. S. (2022). Sources and relative stabilities of acylated and nonacylated anthocyanins in beverage systems. *Journal of Food Science and Technology*, *59*(3), 831–845. https://doi.org/10.1007/s13197-021-05054-z

von Staszewski, M., Pilosof, A. M. R., & Jagus, R. J. (2011). Antioxidant and antimicrobial performance of different Argentinean green tea varieties as affected by whey proteins. *Food Chemistry*, *125*(1), 186–192. https://doi.org/10.1016/j.foodchem.2010.08.059

Wen, P., Zong, M. H., Linhardt, R. J., Feng, K., & Wu, H. (2017). Electrospinning: A novel nano-encapsulation approach for bioactive compounds. *Trends in Food Science and Technology*, *70*(May), 56–68. https://doi.org/10.1016/j.tifs.2017.10.009

Yang, L. P., Ling, W. H., Du, Z. C., Chen, Y. M., Li, D., Deng, S. Z., Liu, Z. M., & Yang, L. L. (2017). Effects of anthocyanins on cardiometabolic health: A systematic review and meta-analysis of randomized controlled trials. *Advances in Nutrition*, *8*(5), 684–693. https://doi.org/10.3945/AN.116.014852

Zhishen, J., Mengcheng, T., & Jianming, W. (1999). The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. *Food Chemistry*, *64*(4), 555–559. https://doi.org/10.1016/S0308-8146(98)00102-2

Figure Captions:

Figure 1. (A) Recovery of total phenol content, (B) total flavonoid content, and (C) total monomeric anthocyanins content from grape marc and red goji skins extract separated through CGA(WPI) and CGA(Tween 20). * Denotes a significant difference $p<0.05$.

Figure 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 phenolics source ($p<0.05$). Values are expressed as mean±sd (n=6).

Figure 3. Decrease of anthocyanins content over time represented as natural logarithm of the ratio of anthocyanins at a given time at time zero during storage at 40°C for 30 days. Values are expressed as mean ± sd (n=2).

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820 **Figure 4.** Diagram of incorporation of CGA in the process chain of grape marc
821 byproduct.

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823 **Figure A.1.** HPLC chromatogram of mixing standards of Delphinidin, Cyanidin, Petunidin,
824 and Malvidin (20 µmol) at 520 nm wavelength.

825 **Figure A.2.** Structure of anthocyanins used in this work. Figures taken from the Royal
826 Society of Chemistry-ChemSpider database (www.chemspider.com).

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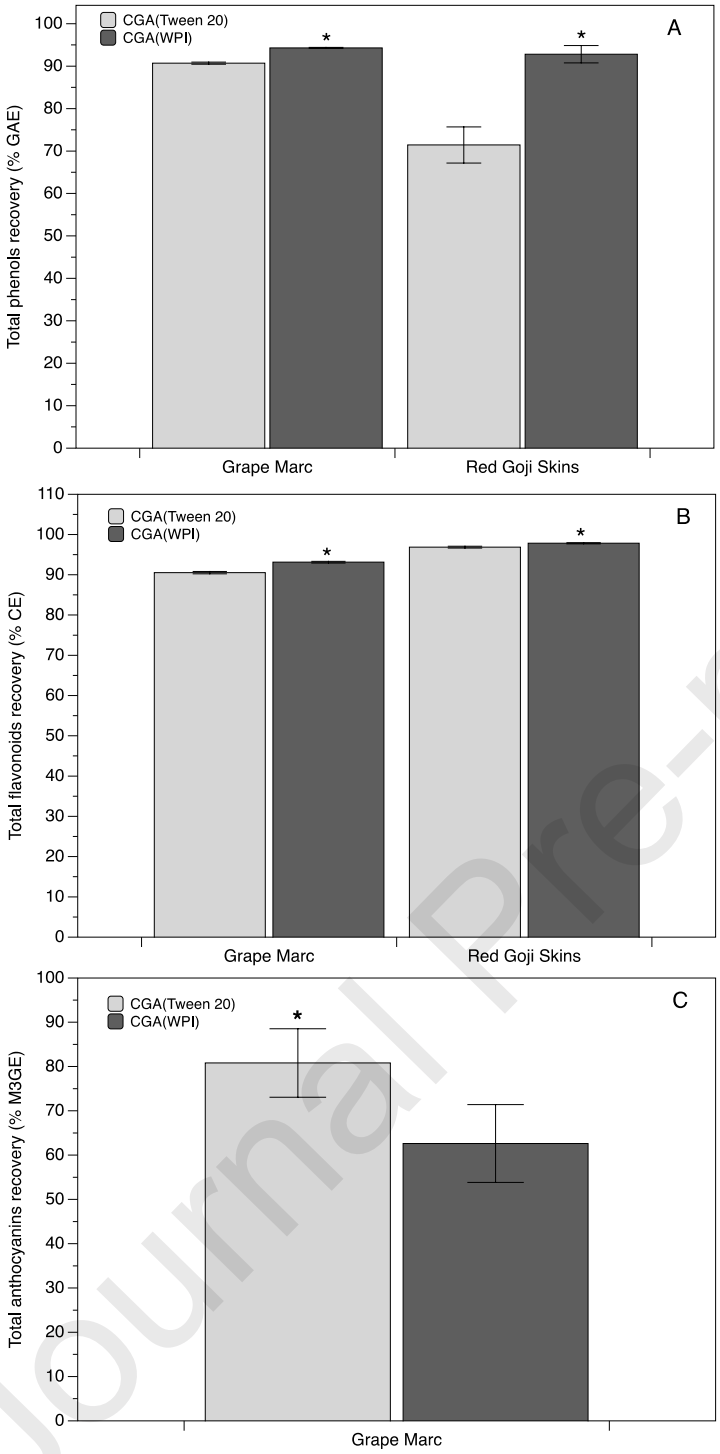

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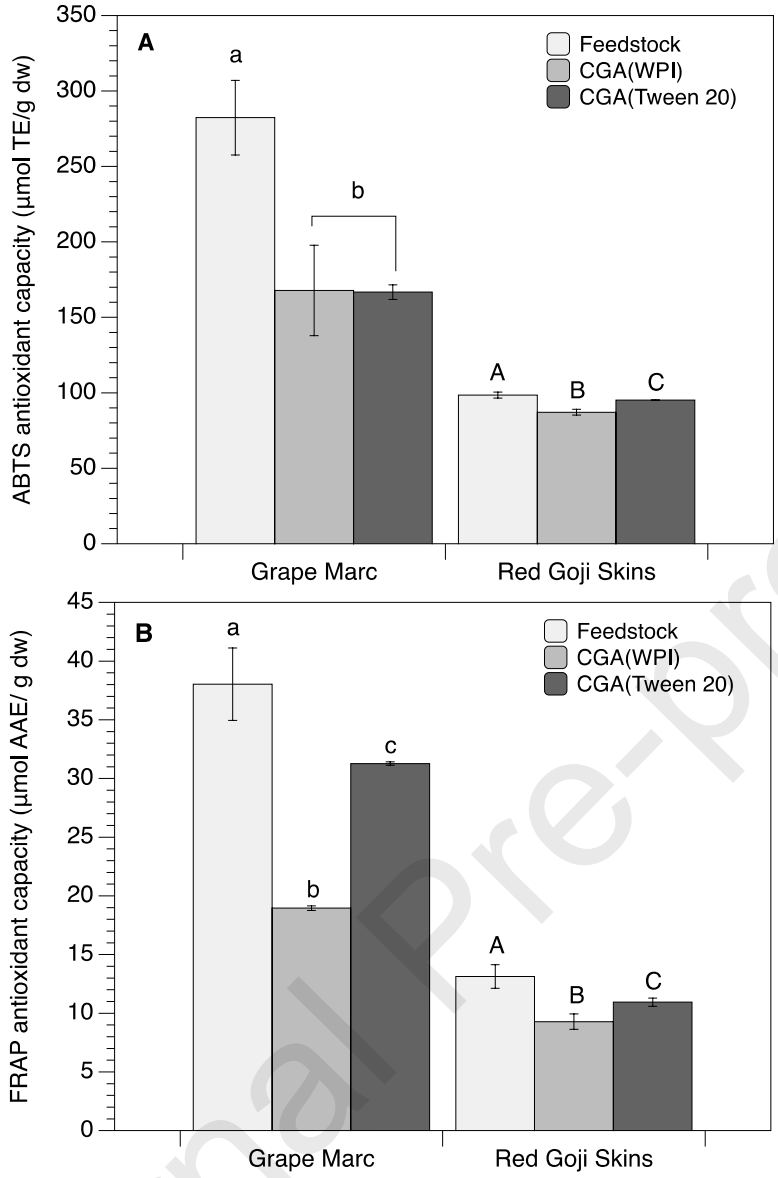

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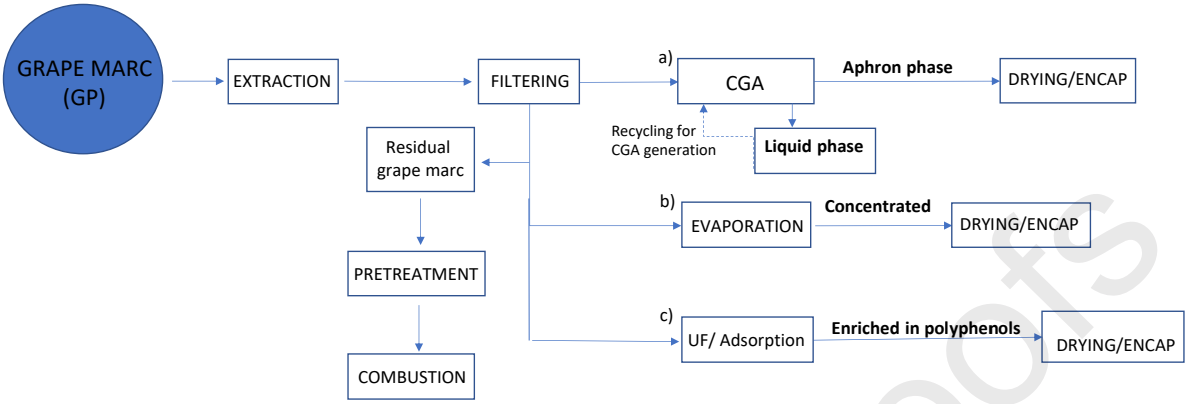

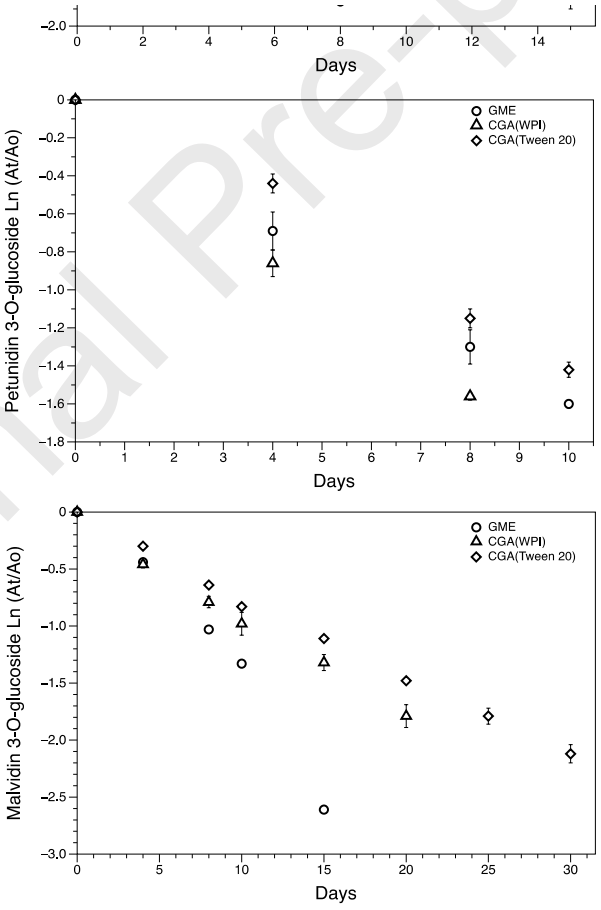

Highlights

- Successful recovery of phenolic by whey protein (WPI) generated CGA
- Separation of phenolics was similar with CGA(WPI) as with CGA(Tween)
- CGA(WPI) separation leads to reduction of antioxidant activity

- CGA made of whey protein can protect anthocyanins from degradation
- CGA separation integrates separation and pre-formulation of phenolics

Author statement

Aimara V. De La Cruz Molina: conceptualization, data curation, formal analysis, methodology, investigation, writing – original draft, review and editing

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