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Accepted Version

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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 (2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) assay. Moreover, the extract effectively neutralised reactive oxygen species (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 a higher antioxidant activity by the ferric reducing antioxidant power (FRAP) assay than the free extract after gastrointestinal digestion (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.**

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., 2021; 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 gastrointestinal tract (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 are assessed using a Caco-2 cell line and compared against the commercial antioxidant compound Trolox.

2. Materials and methods

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), fetal bovine serum (FBS) Superior, Hanks' balanced salt solution (HBSS) Resazurin sodium salt, 2',7'-Dichlorofluorescin diacetate (DCFH-DA), 3-Morpholinosydnonimine (Sin-1), (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), and tert-butyl hydroperoxide (tBOOH) were purchased from Sigma-Aldrich. Minimum essential medium Eagle (MEM) (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.

2.1 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 (GME) was freeze-dried and stored at -18 °C for further analysis, described in sections 2.6 and 2.7.

2.2 *In vitro* cell culture studies

Cell culture

Caco-2 cell line (ATCC, HTB-37) from human colon epithelial carcinoma was routinely expanded in MEM, supplemented with 20% 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- ethylenediaminetetraacetic acid (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 phosphate buffered saline (PBS) solution, 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 total phenolic content (TPC) 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) dimethyl sulfoxide (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 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.

2.3 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.

2.4 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 (FTIR) Spectroscopy 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.

2.5 *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

249 access to the commercially available enzyme, and amylase was not used in the oral phase
250 since there was no starch in the sample.

251 W-P-GME particles (200 mg) or free GME (100 mg) were resuspended in 1 mL of
252 distilled water and digested. The sample was diluted 1:1 (v/v) in oral digestion with
253 simulated salivary fluid, $CaCl_2$ 0.3 M and water. The tubes were incubated in an orbital
254 incubator (Fisher Scientific) for 2 min at 37 °C and 150 rpm. For gastric digestion (GD),
255 a pepsin solution (2000 U/mL) in water was prepared based on the previously determined
256 activity. The 2 mL of oral phase were diluted 1:1 (v/v) with simulated gastric fluid, pepsin
257 solution, $CaCl_2$ 0.3 M, HCl 1 M (to pH 3.0) and water. The samples were incubated for 2
258 h at 37 °C and 150 rpm. A 1.8 mL sample was collected after the 2 h of GD. For intestinal
259 digestion (ID), bile solution and pancreatin were prepared in simulated intestinal fluid.
260 The 2.2 mL of gastric phase were diluted 1:1 (v/v) with simulated intestinal fluid,
261 pancreatin solution, bile, $CaCl_2$ 0.3 M, NaOH 1 M (to pH 7.0) and water. The samples
262 were incubated for 2 h at 37 °C and 150 rpm. Then the samples were put in an ice water
263 bath for 30 min to stop the enzyme´s activity.

264 After digestion, each digested sample was centrifugated in a Ministar blueline
265 microcentrifuge (fixed speed 2,000 $\times g$) at room temperature for 5 min. The supernatants
266 were collected and stored for analysis. Digestion of polyphenols was evaluated according
267 to the analytical determinations described in sections 2.6 and 2.7 after GD and after
268 gastrointestinal digestion (GID).

269 The residual values of polyphenols were calculated as a percentage of the total mass of
270 TPC (mg) remaining after GD and after the overall GID in relation to the initial mass. In
271 the case of the antioxidant capacity, the values correspond to the trolox equivalents (TE)

(mg) for ABTS and ascorbic acid equivalents (AAE) (mg) for FRAP remaining after each phase of the digestion in relation to the initial ones.

2.6 Analytical determinations

Total Phenolic Content

The total phenolic content (TPC) was determined by the Folin-Ciocalteu method (Singleton & Rossi, 1965). For the assay, 75 μL de Folin-Ciocalteu reagent (1:10) was added in a 96-well microplate, with 15 μL of the sample and 60 μL of 7.5% Na_2CO_3. The samples were incubated in the dark for 30 min. After this time, the microplate was read at 765 nm in a microplate reader (Synergy H, BioTek, Vermont, USA). The results were quantified from a Gallic acid calibration curve ranging from 0.1 to 1.0 mg/ml and expressed as milligrams of gallic acid equivalents (GAE) per gram of dried extract (mg GAE / g de).

Total Monomeric Anthocyanin Content

Total monomeric anthocyanins content (TMAC) levels were quantified by the AOAC Official Method 2005.02 pH differential method (Lee et al., 2005). A sample of GME was combined in a 1:20 ratio (v:v) with potassium chloride and sodium acetate buffers (pH 1.0 and 4.5, respectively) separately. After an equilibration period of 15 min, the absorbance of each solution was measured at 520 and 700 nm in a microplate reader (Synergy H, BioTek, Vermont, USA). The values were calculated with the following formula.

Equation 3

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

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 malvidin 3-O-glucoside (493.43 g/mol)
- DF= dilution factor
- ε= molar absorption: 28,000 L/mol · cm

The results were expressed as milligrams of malvidin 3-O-glucoside equivalents per litre (mg M3GE/L)

Total Flavonoid Content

The total flavonoid content (TFC) was measured using the aluminium method (Zhishen et al., 1999) with some modifications. Briefly, 100 μL of the sample were added to an Eppendorf tube, and 430 μL of solution A (1.8 mL of 5% $NaNO_2$ mixed with 24 mL of distilled water) was added to the sample and incubated for 5 min. Later 30 μL of 10% $AlCl_3$ were added and left to rest for 1 min. Finally, 440 μL of solution B (12 mL of NaOH 1M mixed with 14.4 mL of distilled water) was added without further incubation. 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 (Synergy H1, BioTek, Vermont, USA). The absorbance was compared with a Catechin standard curve ranging from 0.1 to 1 mg/ml. The results were expressed as milligrams of Catechin equivalents (CE) per gram of dried extract (mg CE/ g de).

2.7 Antioxidant Capacity assessment by ABTS and FRAP methods

The total antioxidant activity of all samples was measured by ABTS (2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) assay (Re et al., 1999) with some modifications. The ABTS•+ stock solution was prepared by mixing 5 ml of 7 mM ABTS solution and 88 µl of 140 mM potassium persulfate ($K_2S_2O_8$) solution. Then, the mixture was kept in the dark and at room temperature for at least 16 h before use. The working solution of ABTS•+ was obtained by diluting the ABTS•+ stock solution with distilled water to an absorbance of 0.70 ± 0.02 at 734 nm. Then, 5 µl of the sample was added to 245 µl of ABTS•+ working solution, and the mixture was homogenised and then incubated in the dark for 5 min. The absorbance of the control and the samples were recorded at 734 nm using a microplate reader (Synergy H1, BioTek, Vermont, USA). The scavenging activity of each sample on ABTS•+ was calculated from a Trolox standard curve at concentrations of 0.04 to 0.4 mg/mL. Results were expressed as micromole Trolox equivalents (TE) per gram of dry extract.

For the Ferric Reducing Antioxidant Power (FRAP) assay (Benzie & Strain, 1996), 10 µl of the sample was added to 300 µl of FRAP reagent in a microcentrifuge tube and vortexed for 10s. Then, in triplicate, 100 µl of this mixture was transferred into a 96-well microplate, and absorbance was measured at 595 nm in a microplate reader (Synergy H1, BioTek, Vermont, USA). An ascorbic acid standard curve from 0.01 to 0.2 mg/mL was used for the quantification. Results were expressed as micromole ascorbic acid equivalents (AAE) per gram of dry extract.

2.8 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.

3. Results and discussion

3.1 Characterization of grape marc extract

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 total phenolic content (TPC), total flavonoid content (TFC) content, and antioxidant capacity (Table 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 gallic acid equivalents (GAE)/ g de, respectively, when conventional extraction of polyphenols from grape marc. However, we obtained a lower content of total monomeric anthocyanin content (TMAC), which might be explained by a combination of factors such as extraction method, grape variety, growing region, and processing, these conditions play a significant role since not all grapes bear the same TMAC (Rinaldi et al., 2020; Spigno et al., 2015).

3.2 Biocompatibility of grape marc extract

Studying the potentially toxic effects of bioactive compounds is essential to determine whether they are safe to consume without harming the host. The grape marc extract (GME) showed a dose-responsive effect after 24 h of incubation with Caco-2 cells (Fig. 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. 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.

3.3 Cellular antioxidant activity (CAA) of grape marc extract

Reactive oxygen species (ROS) are natural by-products of cell activity and essential signaling molecules (Zhang et al., 2016). However, an imbalance between oxidant-producing systems and antioxidant defense 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 2'-7'-Dichlorodihydrofluorescein (DCFH) fluorescence, testing GME at non-toxic concentrations (33 and 67 GAE μg/mL based on TPC). As shown in Fig. 2A, both GME concentrations decreased the intracellular ROS basal levels, comparing with the control (cells treated with Hanks' balanced salt solution (HBSS)) to a similar level to the one observed for (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (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.

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 μg GAE/mL based on TPC for 4 h. Then, cells were stimulated with 5 μM of the oxidising agent 3-Morpholinosydnonimine (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. 2B, 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 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and ferric reducing antioxidant power (FRAP) methods. Wang et al. (2016)

induced ROS production using t-BOOH (tert-butyl hydroperoxide) 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 (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 (2,2'-azobis(2-amidopropane)) 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.

3.4 Encapsulated GME morphology, size, and Z-potential

The morphology and size of the encapsulated GME were studied through scanning electron microscopy (SEM) analysis. Fig. 3A shows the formation of large crystals with a wide distribution of submicron and micron particles during freeze-drying of GME (Table 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. 3B), while microparticles loaded with GME (W-P-GME) (Fig. 3C) kept their spherical shape but presented some wrinkles in their surface. 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 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 whey protein isolate (WPI) alone. (Carra et al., 2022; Moreno et al., 2018). The results obtained here (1μm) demonstrate that nano spray drying significantly affects the particles' reduction size. 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.

3.5 Fourier Transform Infrared (FTIR) analysis

FTIR analysis was used to examine interactions between the biopolymers and GME. The infrared spectra of the carriers, GME and microparticles are shown in Fig. 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,

460 1740, 1610 and 900-1250 cm^{-1} corresponding to the C-H stretching of the CH, CH2 and
461 CH3 groups, C=O stretching vibration of the ester carbonyl, C=O stretching of the
462 vibration the carbonyl group, C-O-C and O-H of pyranose rings respectively (Khodaiyan
463 & Parastouei, 2020).

464 Looking at the infrared spectra of W-P and W-P-GME, slight shifts in the amide I and
465 amide II regions were observed compared to WPI (1517 to 1535 cm^{-1}). These shifts can
466 be attributed to the interaction between carboxyl groups of pectin and the charged amino
467 groups of the main WPI proteins' composition (beta-lactoglobulin, alpha-lactalbumin,
468 and serum albumin) (Raei et al., 2018). An increase in the intensity was observed for the
469 W-P particles, which can be attributed to the rise in random coils and the previously
470 mentioned interaction between WPI and pectin (El-Messery et al., 2020; He et al., 2016).
471 However, when GME is added, a decrease in intensity is observed. This result is
472 consistent with those obtained by Meng and Li (2021), where Gallic acid, chlorogenic
473 acid, and epigallocatechin gallate-WPI complexes showed decreased intensity in the
474 amide I band. This change can be attributed to the reduction of α-helical structures as a
475 result of protein conformational modifications upon phenolics complexation by hydrogen
476 bonding and hydrophobic interactions between the phenolic compounds and hydrophobic
477 groups of the protein, so there are not only interactions but also changes in the secondary
478 structure of the proteins (Bourassa et al., 2013; He et al., 2016). According to previous
479 reports, W-P-GME did not show any characteristic band from GME, indicating that
480 phenolics distinct peaks can be hidden when in contact with other biopolymers like WPI.
481 This change could mean the formation of complexes that reduce the bending and
482 stretching of the bonds in GME polyphenols.

483 **3.6 *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. 5. These results represent the fraction of TPC (or activity which, is quantified as Trolox equivalents (TE) or ascorbic acid equivalents (AAE)) remaining after gastric digestion (GD) or gastrointestinal digestion (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. 5A). 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 GD; 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. 5A), 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). 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% 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. 5B & 5C). 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 FTIR 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.

4. Conclusions

A raw ethanolic extract of a winery by-product (grape marc) with antioxidant capacity was successfully encapsulated using whey protein isolate (WPI) and pectin and nano spray drying (73% yield), resulting in spherical smoothed-surface microparticles with an average size of 1 μm, polydispersity index (PDI) of 0.717, and a surface charge (Z-potential) close to -30 mV. The Fourier Fourier Transform Infrared (FTIR) analysis of the microparticles confirmed the complexations between WPI, pectin and the phenolics in grape marc extract (GME) through non-covalent interactions. The developed encapsulation system protected the GME phenolics and the antioxidant activity during gastrointestinal digestion (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.

Funding

The research leading to these results received funding from the European Union's H2020. Research and Innovation Programme under the Maria Sklodowska-Curie grant agreement no. 778388 (H2020 MSCA-RISE-2017 project Food for Diabetes and Cognition (FODIAC), and the SbDtoolBox - Nanotechnology-based tools and tests for Safer-by-Design nanomaterials, with the reference NORTE-01-0145-FEDER-000047, funded by Norte 2020 – North-Regional Operational Programme under the PORTUGAL 2020 Partnership Agreement, through the European Regional Development Fund (ERDF).

Acknowledgements

577 The authors would like to thank the Food Processing group at INL, especially Arlete
578 Marques and Isabel Bourbon.

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