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Whey–pectin microcapsules improve the stability of grape marc phenolics during digestion

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Abstract: Grape marc (GM) 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 GM 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 a total phenolics content of 219.62 ± 11.50 mg gallic acid equivalents (GAE)/g dry extract and 1389.71 ± 97.33 μmol Trolox equivalents/g dry extract antioxidant capacity, assessed through ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) assay. Moreover, the extract effectively neutralized reactive oxygen species 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 phenolics from simulated gastrointestinal digestion (GID), where at the end of the intestinal phase, 82% of the initial phenolics were bioaccessible compared to 54% in the free GME. [Correction added 27 December 2023; microcapsules has been corrected to phenolics.] Besides, the encapsulated GME displayed a higher antioxidant activity by the ferric reducing antioxidant power assay than the free extract after GID. These results show the potential of this encapsulation system for applying GME as a nutraceutical with a high antioxidant capacity and protective effect against cellular oxidation.

KEYWORDS
biopolymer, encapsulation, grape marc phenolics, in vitro digestion, nano spray drying

1 | INTRODUCTION

Grape marc (GM) 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 cheesemaking process, which contains proteins with high nutritional quality (de Wit, 1998; Jauregi & Welderufael, 2010; Yalçin, 2006). Furthermore, WPI forms complexes with polyphenols, stabilizing them by improving their solubilization 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 fiber, 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 metabolized 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 atomizing 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 the 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 NSD for raw extracts and their behavior during gastrointestinal digestion (GID). Desai et al. (2020) used NSD 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 NSD has been used for the 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 GM extract (GME) 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 kindly supplied commercial GM flour from Sangiovese grapes. The GM flour is obtained by drying the GM at 42°C for 3 days to preserve the phenolics. The GM is constantly mixed to avoid mold growth, and after the drying process, it is pulverized to a 250-μm 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% fiber, and 11.8% protein. WPI was purchased from Volac International Ltd 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 × 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 (*t*-BOOH) were purchased from Sigma-Aldrich. Minimum Essential Medium (MEM) Eagle (with 2 mM L-glutamine, 1 mM sodium pyruvate, nonessential amino acids) and penicillin–streptomycin (10,000 U/mL to 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 (Mohd-Maidin et al., 2018) to extract phenolics from GM. 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). Then, the GME was freeze-dried and stored at −18°C for further analysis, as described in Sections 2.6 and 2.7.

2.2 | In vitro cell culture studies

2.2.1 | 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 cm2 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 and 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.

2.2.2 | 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 and 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) 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.

2.2.3 | 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 reactive oxygen species (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. Afterward, the solution was removed, and 100 μL of GME solubilized 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) 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 solubilized 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). 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 | Characterization of the microparticles

2.4.1 | Scanning electron microscopy (SEM)

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

2.4.2 | Size and polydispersity index

The size of the particles was determined by analyzing SEM images with the program ImageJ (National Institutes of Health). 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 were calculated, and from those values, the polydispersity index (PDI) was calculated with the following formula:

$$PDI = \sqrt{\frac{\text{size}\sigma}{\text{size}\bar{x}}} \quad (1)$$

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

2.4.3 | Yield

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

$$EY\% = \frac{\text{Total solids out}}{\text{Total solids in}} \times 100 \quad (2)$$

2.4.4 | *Z*-Potential

The particles' surface charge (*Z*-potential) was measured by dynamic light scattering using an SZ-100 particle analyzer (Horiba Scientific). Microparticles (1 mg/mL) were measured at 25°C using an He–Ne laser (633 nm) in folded capillary cells. Five independent measurements of each sample were done, and data were expressed as mean ± SD.

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

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

After digestion, each digested sample was centrifugated in a MiniStar blueline microcentrifuge (fixed speed 2000 × g) at room temperature for 5 min. The supernatants were collected and stored for analysis. Digestion of polyphenols was evaluated according to the analytical determinations described in Sections 2.6 and 2.7 after GD and after GID.

The residual values of polyphenols were calculated as a percentage of the total mass of TPC (mg) remaining after GD and after the overall GID in relation to the initial mass. In the case of the antioxidant capacity, the values correspond to the Trolox equivalents (TE) (mg) for ABTS and ascorbic acid equivalents (AAE) (mg) for ferric reducing antioxidant power (FRAP) remaining after each phase of the digestion in relation to the initial ones.

2.6 | Analytical determinations

2.6.1 | Total phenolic content

The TPC was determined by the Folin–Ciocalteu method (Singleton & Rossi, 1965). For the assay, 75 μL de Folin–Ciocalteu reagent (1:10) was added to 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). The results were quantified from a gallic acid (GA) calibration curve ranging from 0.1 to 1.0 mg/mL and expressed as milligrams of GA equivalents (GAE) per gram of dried extract (mg GAE/g de).

2.6.2 | Total monomeric anthocyanin content

Total monomeric anthocyanin 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). The values were calculated with the following formula:

$$\text{Monomeric anthocyanins} = \frac{A \times MW \times DF \times 1000}{\varepsilon \times 1} \quad (3)$$

where A is the corrected absorbance value calculated as $[(A_{520} - A_{700})_{\text{pH 1.0}} - (A_{520} - A_{700})_{\text{pH 4.5}}]$; MW is the molecular weight of malvidin-3-*O*-glucoside (493.43 g/mol); DF is the dilution factor; ε is the molar absorption: 28,000 L/mol cm. The results were expressed as milligrams of malvidin 3-*O*-glucoside equivalents per litter (mg M3GE/L)

2.6.3 | Total flavonoid content

The total flavonoid content (TFC) was measured using the aluminum method (Zhishen et al., 1999) with some modifications. Briefly, the samples of 100 μL 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% $AlCl_3$ was added and left to rest for 1 min. Finally, 440 μL of solution B (12 mL of NaOH 1 M 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). 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 per gram of de (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 homogenized and then incubated in the dark for 5 min. The absorbance of the control and the samples was recorded at 734 nm using a microplate reader (Synergy H1, BioTek). The scavenging activity of each sample on ABTS•+ was calculated from a Trolox standard curve at concentrations of 0.04–0.4 mg/mL. Results were expressed as micromole TE per gram of dry extract.

For the 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 10 s. 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). An ascorbic acid standard curve from 0.01 to 0.2 mg/mL was used for the quantification. Results were expressed as micromole 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 an independent sample *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 TPC, 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 GAE/g de, respectively, when conventional extraction of polyphenols from GM. However, we obtained a lower content of 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 as not all grapes bear the same TMAC (Rinaldi et al., 2020; Spigno et al., 2015).

TABLE 1 Content of polyphenols and antioxidant activity in grape marc extract (GME).

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

Note: Values are represented as mean ± SD ($n = 6$ from three replicates).
Abbreviations: ABTS: TE μmol/g dry extract; FRAP: AAE μmol/g dry extract; TAC: MV3GE mg/L; TFC: CE mg/g dry extract; TPC: GAE mg/g dry extract.

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 GME showed a dose-responsive effect after 24 h of incubation with Caco-2 cells (Figure 1). We observed cellular compatibility, that is, 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 and 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 nontoxic for Caco-2 cells before and after simulated in vitro digestion. Moreover, Wolfe et al. (2008) observed that concentrations below 60 mg/mL of different extracts, for example, 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

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).

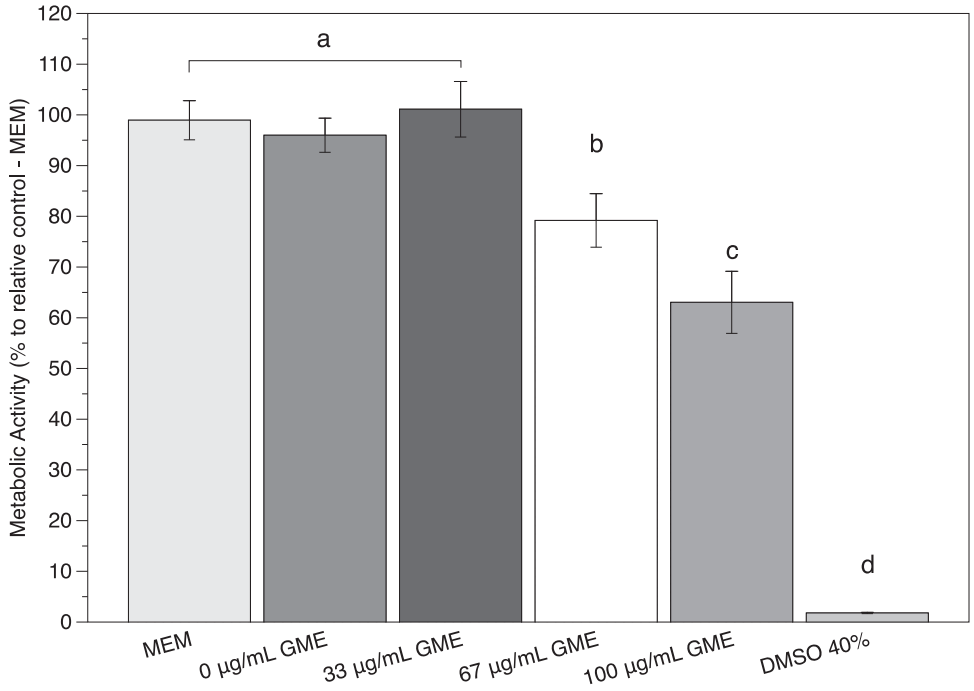

FIGURE 1 Viability of Caco-2 cells after 24 h-incubation with different concentrations of GME (33, 67, or 100 GAE µg/mL), measured through the resazurin assay. Culture medium (MEM) was used as a positive control (100% cell viability), and 40% DMSO (v/v) as a negative control. Values are the mean ± SD from 2 independent assays analyzed in quadruplicate. Different letters denote statistical significance ($p < 0.05$) determined using the Dunnett method.

Studies of intracellular oxidant production in Caco-2 cells were evaluated using 2′−7′-dichlorodih ydrofluorescein (DCFH) fluorescence, testing GME at nontoxic concentrations (33 and 67 GAE µg/mL based on TPC). As shown in Figure 2a, both GME concentrations decreased the intracellular ROS basal levels, comparing with the control (cells treated with HBSS) to a similar level to the one observed for (±)-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 pretreated with GME at the nontoxic concentrations of 33 and 67 µg GAE/mL based on TPC for 4 h. Then, cells were stimulated with 5 µM of the oxidizing agent 3-morpholinosydnonimine (Sin-1), selected according to the literature (PD ISO/TS 19,006:2016). Cells treated with HBSS and stressed with Sin-1 were used as a positive control. As shown in Figure 2b, cells pretreated with nontoxic concentrations of GME significantly reduced intracellular ROS level produced after stimulation with Sin-1 compared to cells pretreated with HBSS (control). This reduction was similar to that observed for treated cells with 50 µg/mL Trolox that 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 neutralize 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 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 0.1–10 µg/mL exert an antioxidant effect over ROS. Other studies have reported that concentrations of 100, 200, and 500 µg/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 prooxidant 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 although 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.

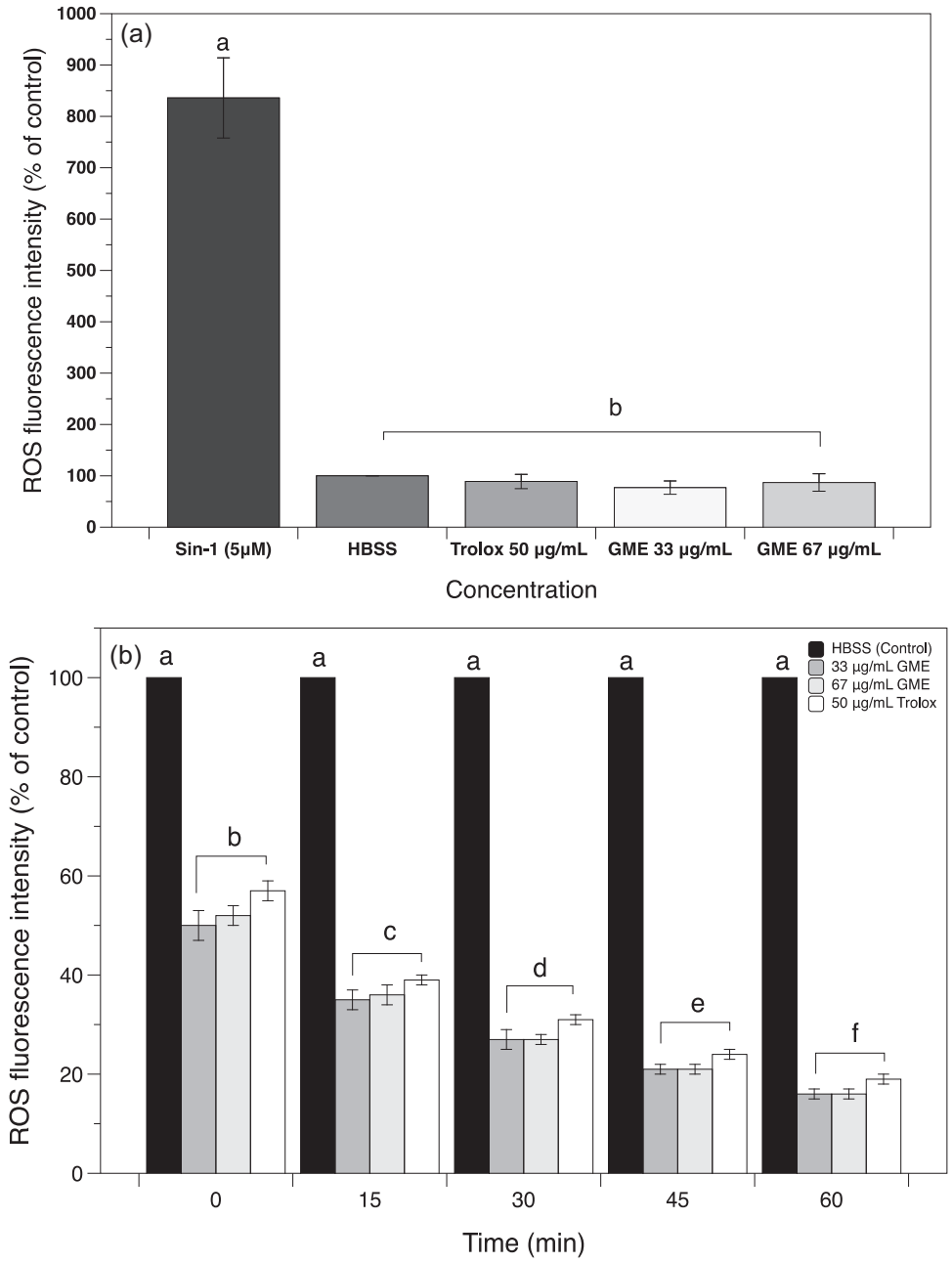

FIGURE 2 (a) Effect of different concentrations of GME (33 or 67 GAE μg/mL) on the ROS basal levels of Caco-2 cells after incubation for 4h, measured through the DCFH-DA assay. HBSS was used as a negative control, and Sin-1 (5 μM), as a positive control. (b) Protective effect of different concentrations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

3.4 | Encapsulated GME morphology, size, and *Z*-potential

The morphology and size of the encapsulated GME were studied through SEM analysis. Figure 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 (Figure 3b), whereas microparticles loaded with GME (W–P–GME) (Figure 3c) kept their spherical shape but presented some wrinkles in their surface. Moreover, no breakage was seen in W–P

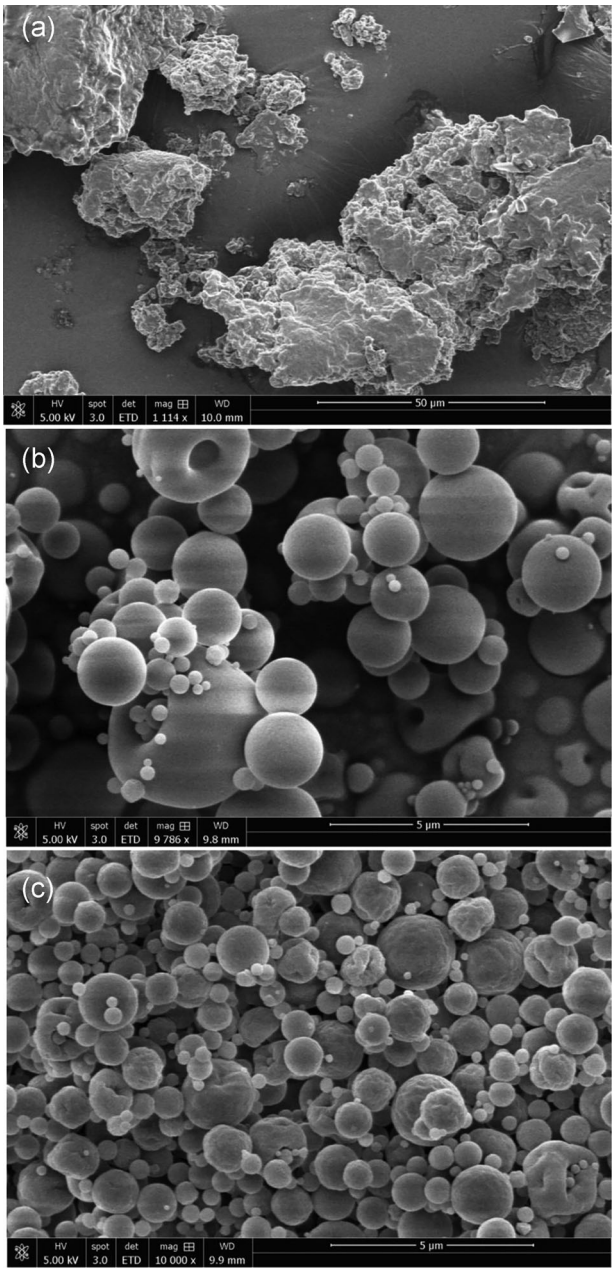

FIGURE 3 SEM images of GME (a), whey–pectin blank microparticles (b), and GME encapsulated in whey–pectin microparticles (c).

TABLE 2 *Z*-Potential, size, and polydispersity index (PDI) of particles.

Particles	*Z*-Potential	Size (µm)	PDI	Yield (%)
W–P–GME	−28.3 ± 6.1	1.0 ± 0.5[a]	0.7	73
W–P	ND	1.3 ± 0.7[b]	0.7	ND

Note: *Z*-Potential: based on 1 mg/mL particle suspension in water; average ± SD from five replicates. Size: average diameter ± SD of 175 particles in micrographs. PDI: SQRT, size std/size average. Different letters denote significant difference ($p < 0.05$) using the independent samples *t*-test. Abbreviations: ND, non-determined.

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 GME by conventional spray drying have reported sizes of 9.8 µm when using pectin and casein, and 15 µm when using WPI alone (Carra et al., 2022; Moreno et al., 2018). The results obtained here (1 µm) demonstrate that NSD significantly affects the particles' reduction in 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 Figure 4. For WPI, characteristic amide I and II bands can provide information about protein secondary structures, and their change in vibration frequencies is related to the interaction between their functional groups. Amide I represents the C=O carbonyl stretching vibration of the peptide backbone (1600–1700 cm^{-1}), and the amide II band (<1550 cm^{-1}) represents the C–N stretching and N–H bending (López-Rubio & Lagaron, 2012; Meng et al., 2021). As for the GME, the characteristic bands of grape phenolic compounds were observed between 1700 and 900 cm^{-1}. The band at 1710 cm^{-1} was attributed to the stretching in the carbonyl group (C=O) band, 1600 and 1510 cm^{-1} bands correspond to the C=C stretching, characteristic of aromatic systems. The peak around 1440 cm^{-1} corresponds to the antisymmetric in-plane bending of $-CH_3$ related to aromatic rings and flavonoids (Moreno et al., 2018; Zhao et al., 2015). Characteristic peaks of pectin can be observed at 2920, 1740, 1610, and 900–1250 cm^{-1} corresponding to the C–H stretching of the CH, CH_2, and CH_3 groups, C=O stretching vibration of the ester carbonyl, C=O stretching of the vibration of the carbonyl group, C–O–C and O–H of pyranose rings, respectively (Khodaiyan & Parastouei, 2020).

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

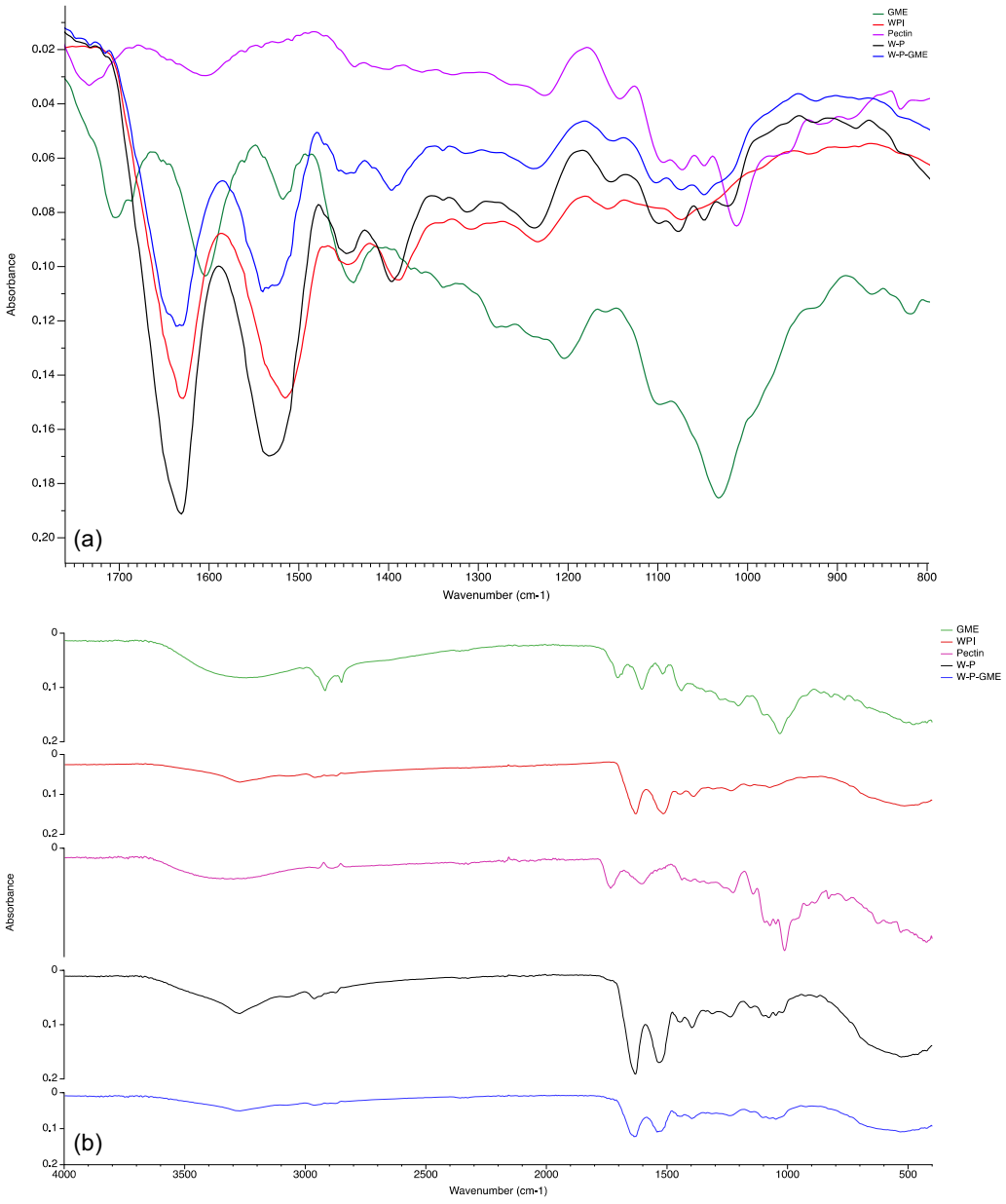

FIGURE 4 FT-IR of GME, whey–pectin blank microparticles, GME encapsulated in whey–pectin microparticles, pectin and whey. Amplified FTIR spectra of wavelengths 1750 to 800 cm^{-1} (a) and full spectra (b). The results of each material are the average of three independent spectra.

proteins' composition (β-lactoglobulin, α-lactalbumin, and serum albumin) (Raei et al., 2018). An increase in the intensity was observed for the W–P particles, which can be attributed to the rise in random coils and the previously mentioned interaction between WPI and pectin (El-Messery et al., 2020; He et al., 2016). However, when GME is added, a decrease in intensity is observed. This result is consistent with those obtained by Meng and Li (2021), where GA, chlorogenic acid, and epigallocatechin gallate–WPI complexes showed decreased intensity in the amide I band. This change can be attributed to the reduction of α-helical structures as a result of protein conformational modifications upon phenolics complexation by hydrogen bonding and hydrophobic interactions between the phenolic compounds and hydrophobic groups of the protein, so there are not only interactions but also changes in the secondary structure of the proteins (Bourassa et al., 2013; He et al., 2016). According to previous reports, W–P–GME did not show any characteristic band from GME, indicating that phenolics distinct peaks can be hidden when in contact with other biopolymers like WPI. This change could mean the formation of complexes that reduce the bending and stretching of the bonds in GME polyphenols.

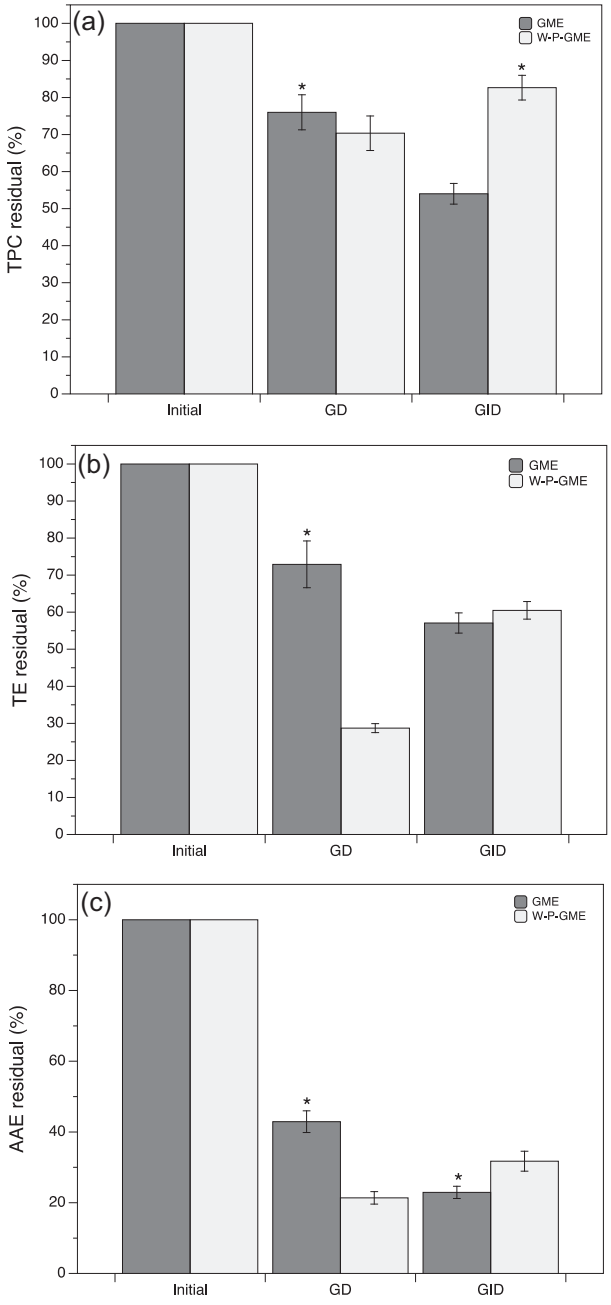

FIGURE 5 Results of the residual TPC (a), TE (b), and AAE (c) of GME and GME encapsulated in whey–pectin microparticles after in vitro gastric digestion (GD) and gastrointestinal digestion (GID). Values are the mean ± SD from three independent assays, each analysed in triplicate. *Denotes statistical significance between GME and W-P-GME ($p < 0.05$) determined using the Tukey test.

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 Figure 5. These results represent the fraction of TPC (or activity which is quantified as TE or AAE) remaining after GD or GID, the latter indicating the bioaccessible fraction. Therefore, these values show the fraction of TPC (or activity) that resisted the simulated gastrointestinal conditions in free GME. In contrast, for W–P–GME, these values account for the fraction of TPC that resisted the conditions and/or was encapsulated and effectively released from the microcapsules during digestion.

A different behavior was observed for free and encapsulated GME, suggesting that 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; Figure 5a). The moderate stability of GME polyphenols to GD 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; the 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 (Figure 5a), which may represent the fraction not released from the microparticles. Indeed, the high preservation of the TPC was expected during GD since strong electrostatic interactions stabilize 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 stabilizing 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 behavior 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, whereas for W–P–GME, the activity was slightly increased after GID compared to GD (Figure 5b,c). Besides, free GME's bioactivity directly correlates with residual TPC values after GD and GID, achieving values of 73% and 57% of the initial activity, as assessed by the ABTS method. Although a similar trend was observed in both phases, lower values were recorded using the FRAP method.

For encapsulated GME, although a positive correlation was observed between residual TPC and antioxidant activity, the latter showed lower values than the residual TPC. For instance, 29% and 61% of the activity was observed using the ABTS method after GD and GID in W–P–GME. The reduced activity compared to the residual TPC might be due to released polyphenols from the capsules bearing lower antioxidant activity than those that were still encapsulated or that they might be complexed with the capsule components as 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 (GM) with antioxidant capacity was successfully encapsulated using WPI and pectin and NSD (73% yield), resulting in spherical smoothed-surface microparticles with an average size of 1 μm, PDI of 0.717, and a surface charge (*Z*-potential) close to −30 mV. The FTIR analysis of the microparticles confirmed the complexations among WPI, pectin, and the phenolics in GME through non-covalent interactions. The developed encapsulation system protected the GME phenolics and the antioxidant activity during GID, improving bioaccessibility. The potent antioxidant intracellular protective effect of GME observed, and its improved resistance to GID when encapsulated compared to the free form suggest this encapsulation system could be a promising strategy toward 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 metabolized 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 toward using encapsulated GME as a nutraceutical.

AUTHOR CONTRIBUTIONS

Aimara V. De La Cruz-Molina: Writing—original draft; methodology; writing—review and editing; formal analysis; data curation. **Catarina Gonçalves**: Writing—review and editing; methodology; data curation. **Mafalda D. Neto**: Writing—review and editing; data curation; methodology. **Lorenzo Pastrana**: Conceptualization; funding acquisition; validation; visualization; project administration; resources; supervision; formal analysis; investigation. **Paula Jauregi**: Supervision; formal analysis; validation; visualization; writing—review and editing; conceptualization; investigation. **Isabel R. Amado**: Conceptualization; investigation; writing—original draft; writing—review and editing; validation; methodology; supervision; data curation; formal analysis.

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CONFLICTS 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.

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