Storage stability and simulated gastrointestinal release of spray dried grape marc phenolics

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Abstract

Maltodextrin (MD), whey protein isolate (WPI) and pea protein isolate (PPI) have been used in this work as carrier agents in the spray drying of grape marc extract, and the obtained microparticles have been characterized in terms of drying yield, morphology (SEM), size distribution, chemical structure (FTIR), chemical composition (total phenolic and anthocyanin content), and antioxidant activity (ORAC). WPI was found to be the most effective carrier for optimizing drying yield, requiring less than 0.5:1 carrier:extract solids ratio to achieve the usual 50% yield benchmark. *In vitro* gastrointestinal release of total phenolics and anthocyanins indicated Fickian diffusion, with the Higuchi and Korsmeyer-Peppas models providing the best fit for the data. An accelerated stability test carried out at 40 and 60°C revealed first-order kinetics for anthocyanin degradation. WPI formulated microparticles showed severe degradation at 40 and especially 60°C (complete degradation after 4 days), followed by MD and PPI microparticles. Similarly, a long term stability test carried out at room temperature (25°C) over six months showed that total phenolic content did not diminish for any of the carriers, but anthocyanin content suffered a 20% loss after 6 months for PPI and MD formulated samples, and a 50-65% loss for WPI formulated samples.

Keywords

Grape phenolics, spray drying, storage stability, degradation kinetics modelling, release profiles modelling, microstructure.

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1. Introduction

Grape phenolics, particularly anthocyanins, have attracted the interest of researchers for years due to their anti-oxidant, anti-cancer, anti-inflammation and anti-microbial properties, as well as reducing the incidence of cardiovascular diseases and improving the cognition and neuronal function with aging and neurodegenerative diseases [1-4]. They are abundantly present in grape marc, a residue of the wine making industry consisting of the grape seeds and skins that are left after pressing. Due to their properties, these compounds present potential applications in food [5, 6] and cosmetic [7, 8] products, but proper formulation is essential to ensure their bioavailability and resistance to degradation by preserving the core material from untoward environmental conditions. Spray drying is a simple yet efficient technique where an aqueous solution containing the active principles for formulation is uniformly mixed with the carrier materials, and this mixture is then fed into a spray dryer and atomized with a nozzle or spinning wheel. Water is evaporated by the hot air contacting the atomized material and the powder is then collected in a cyclone separator [9]. Spray drying has been widely used in the food industry since the late 1950s [9, 10], and carbohydrates (such as maltodextrin, gum Arabic or modified starch), lecithin, chitosan and certain proteins (such as gelatin derivatives and dairy proteins) are all commonly used wall materials [10-13]. Since phenolic compounds can be easily degraded by environmental factors such as light, oxygen and temperature, microencapsulation by spray drying can help stabilize these compounds and prolong their shelf life. The improved stability of spray dried phenolics has been previously studied for fruit and vegetable extracts such as *cagaita* [14], *corozo* [15], pomegranate peel [16], *açai* [17], black carrot [18], blueberry [19], blackcurrant [20], bayberry [21], Hibiscus Sabdariffa L. [22] and cranberry [23]. In the particular case of spray dried grape extract, de Souza *et al*. [24] studied the stability of anthocyanins stored at room temperature during 120 days and reported that the loss of anthocyanins was inversely proportional to the concentration of carrier material.

Additionally, the generally poor bioavailability of phenolic compounds is currently a challenge, as only a small proportion of the molecules remain available following oral administration, due to insufficient gastric residence time, low permeability and/or solubility within the gut [10]. Bioavailability issues, added to the natural instability of these compounds during food processing and in the gastrointestinal tract, limit their activity and potential health benefits, and make proper formulation a crucial factor. *In vitro* dissolution tests traditionally used in drug development have become an important tool in the field of novel food ingredients and nutraceuticals to assess the release profiles of bioactive compounds in simulated media such as salivary, gastric and intestinal fluids. The results can then be fitted using the different empirical models available for drug release, which will give an indication of the release mechanism, the mass transport mechanisms involved and the release kinetics [25]. Release profiles in simulated gastrointestinal media have been previously reported for microencapsulated strawberry [26], bilberry [27] and cinnamon [28] polyphenols, but to the best of our knowledge there are no in-depth studies available for release profiles of formulated grape phenolics.

In a previous work, we examined the behaviour of three different carrier agents (maltodextrin, whey protein isolate and pea protein isolate) in the spray drying formulation of polyphenol-rich grape marc extract [29]. While maltodextrin has been extensively researched as one of the most common choices when spray drying bioactives, whey and pea protein are relatively novel carriers for formulation of phenolic compounds (especially the latter) and our results indicated that they are both suitable candidates with satisfactory performance in terms of phenolic and

anthocyanin retention as well as cellular antioxidant activity. In the present work, the spray drying efficiency of these carriers and the short and long term storage stability of the formulated microparticles has been assessed, as well as the release mechanism of the phenolics in simulated gastric and intestinal fluids.

2. Materials and methods

2.1. Materials

The polyphenols-rich grape marc extract used in all experiments was supplied by the winery *Grupo Matarromera* (Valladolid, Spain). It consisted of an aqueous extract of red grape marc (harvest 2014), which had been obtained by their patented extraction technology [30]. Natural carriers used in this study were maltodextrin (DE18), whey protein isolate (90% protein) and pea protein isolate (75% protein); all were purchased from Myprotein (Northwich, UK).

Folin-Ciocalteou reagent was purchased from Panreac (Barcelona, Spain). Gallic acid was purchased from Sigma-Aldrich (St Louis, MO, USA). AAPH [2,2'-azobis(2-methylpropionamidine) dihydrochloride], disodium fluorescein and Trolox [(±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid] were purchased from Sigma-Aldrich (St Louis, MO, USA).

2.2. Spray drying of grape marc extract

The grape marc extract was manually mixed with the correspondent carrier by mechanical stirring. The carrier:extract solids ratios for the three different natural carriers studied (MD, WPI and PPI) varied between 0.3:1 and 2:1 (i.e. 0.3 and 2 g of carrier per gram of dry extract, respectively). The solids content of the aqueous extract was measured at 3.53 wt%, and the total soluble solids of the extract plus carrier mixture ranged between 4.6 and 10.6%.

Drying was performed in a GEA Mobile Minor™ spray dryer model MM Basic (Düsseldorf, Germany) equipped with a rotary atomizer. In all experiments the inlet temperature, feed rate, atomizer pressure and aspirator rate were kept at 140°C, 21.5 g/min, 0.6 MPa and 40 kg/h, respectively. The resulting outlet temperature was 81°C. These operating parameters were selected based on our previous work [29]. The equipment was run for 15 minutes with distilled water before each experiment in order to reach a stable outlet temperature. The feed mixture was constantly stirred and pumped into the equipment at using a peristaltic pump (Watson Marlow 520S, Wilmington, MA, USA). The spray dried powder was recovered from the cyclone only (discarding any microparticles deposited on the dryer chamber), transferred into sealed glass containers and stored in desiccators containing silica gel at room temperature in absence of light.

The drying yield, or product recovery, was calculated according to the following formula, based on dry matter measurements:

$$Drying\ yield\ (\%) = \frac{obtained\ spray\ dried\ powder\ (g)}{grape\ marc\ extract\ solids\ (g) + carrier\ (g)} \times 100 \quad (1)$$

2.3. Freeze drying of grape marc extract

A sample of grape marc extract was freeze dried in the absence of carrier to be used as control. The extract was iced overnight at -25°C and then freeze dried under vacuum (0.180 mbar) for 96 h using a Telstar Lyoquest -55 unit (Terrassa, Spain).

2.4. Release study

Dissolution assays were performed in duplicate on a Copley DIS6000 unit (Nottingham, UK) in simulated gastric and intestinal fluids prepared according to USP26 / NF21 [31]. Simulated gastric fluid consisted of a 0.034 M sodium chloride solution acidified with hydrochloric acid to a pH of 1.2. Simulated intestinal fluid consisted of a 0.05 M monobasic potassium phosphate solution basified with sodium hydroxide to a pH of 6.8.

Samples of powder (ca. 1000 mg) were placed in a 40 mesh basket attached to the stirring shaft and lowered into 475 mL of solution at 37°C ± 0.5°C. The mixture was stirred at 100 rpm for 3 h and 3 mL aliquots were taken at pre-defined intervals (5, 10, 15, 30, 60, 120 and 180 min). The sample volume was replaced with fresh buffer solution. The aliquot was filtered through a 0.45 μm regenerated cellulose syringe filter and the filtrate was analysed directly to quantify the total amount of polyphenols and anthocyanins. The presented values are the mean of two independent dissolution tests and are expressed in terms of % dissolved polyphenols and anthocyanins, i.e. the actual polyphenol or anthocyanin concentration in the solution divided by the polyphenol or anthocyanin loading of the microparticles (determined as in sections 2.6.3 and 2.6.4 respectively) and multiplied by 100.

2.5. Stability study

An accelerated stability study was carried out at 40 and 60°C during 14 days. Samples were placed in controlled temperature ovens and subsamples were taken on days 2, 4, 7, 9, 11 and 14 and characterized; the results are expressed as TPC, TAC and ORAC antioxidant activity retention, i.e. TPC, TAC or ORAC values at time *t* divided by the initial TPC, TAC or ORAC values and multiplied by 100. A freeze dried sample was used as control in this study.

Further, a long-term stability study was performed by placing samples in a controlled temperature room (constant temperature of 25°C) with daylight conditions (350 lux) during six months. A duplicate of each sample was covered in tin foil to avoid light exposure in order to independently assess the effect of storage time and light exposure. Samples were characterized once a month (TPC, TAC and ORAC antioxidant activity).

2.6. Characterisation methods

2.6.1. Moisture content and water activity

Approx. 1g microparticles were dried at 105°C until constant weight, and moisture content was calculated based on the weight difference before and after drying. Analysis were carried out in duplicate.

Water activity of the samples was measured with a Rotronic Hygropalm HP-23-AW-A (Bassersdorf, Switzerland). The temperature was maintained at 21.5°C ± 0.5°C. All determinations were done in duplicate.

2.6.2. Undissolved solid content of the spray drying solutions

Undissolved solid content of the spray drying solutions was performed by dissolving 0.366 g of carrier in 10 mL of milliQ water to produce a concentration equivalent to that used in the experiments with a 1:1 carrier:extract solids ratio. This ratio was chosen as a reference to be able to compare the three carriers. The mixture was stirred at 600rpm for 2 hours and filtered (0.45 μm) to remove any undissolved material, followed by oven drying at 105°C. Solubility

was calculated as the weight obtained after drying divided by the weight (dry basis) of carrier employed. Measurements were carried out in triplicate.

2.6.3. Total phenolic content

Analysis of total phenolic content (TPC) was carried out using the Folin-Ciocalteou method as described previously [29]. In short, 20 mg of powder were diluted in 10 mL of a mixture of 50% degassed distilled water acidified to pH 1 with sulphuric acid and 50% ethanol. The powder was sonicated for 15 minutes to ensure complete dissolution. An aliquot of 40 μL was diluted in 3 mL of milliQ water, and allowed to react during 5 minutes with 200 μL of Folin-Ciocalteu reagent, after which 600 μL of sodium carbonate (20%, saturated) was added. The mixture was then allowed to rest for 30 minutes at 40°C. Absorbance of each sample was measured at 765 nm against a blank sample in a UV 2550 Shimadzu spectrophotometer (Kyoto, Japan) and compared against a gallic acid calibration curve. Results are expressed as mg gallic acid equivalents per gram of dry product (mg_{GAE}/g_{DB}) [32]. Analysis were carried out in duplicate.

2.6.4. Total anthocyanin content

Analysis of total monomeric anthocyanin content (TAC) was carried out using a pH differential method described elsewhere [29]. An aliquot of 0.5mL of the sample prepared for TPC analysis (see section 2.6.3) was filtered using a regenerated cellulose syringe filter and added to 2mL of a 0.025 M potassium chloride buffer (pH 1.0). An equivalent sample was added to a 0.4 M sodium acetate buffer (pH 4.5). After 15 minutes, absorbance of both samples was measured at 520 nm in a UV 2550 Shimadzu spectrophotometer (Kyoto, Japan), and an additional measure at 700 nm was made to correct for haze. The difference between these measurements is proportional to the concentration of anthocyanin [33, 34]. Results are expressed as malvidin-3-glucoside equivalents per gram of dry product (mg_{MVD}/g_{DB}). The molar extinction coefficient and the molecular weight used for malvidin-3-glucoside were 28000 L/cm/mol and 493.2 g/mol, respectively [33]. Analysis were carried out in duplicate. TAC recovery was calculated as the percentage of TAC in the powder obtained compared to the theoretical value based on the extract.

2.6.5. Oxygen radical absorbance capacity

Antioxidant activity was measured by the Oxygen Radical Absorbance Capacity assay (ORAC) in a Fluostar Optima (BMG-Labtech) as described elsewhere [29]. The assay measures the ability of the antioxidant species in the sample to inhibit the oxidation of disodium fluorescein (FL) catalyzed by peroxyl radicals generated from the thermal decomposition of AAPH. The samples were prepared by diluting 100 μL aliquots of the TPC samples (see section 2.6.3) in 10 mL of 75 mM phosphate buffer solution (pH 7.4). An aliquot of these samples (25 μL) or a Trolox standard were placed in the wells of a 96 well-plate, and 150 μL of a 100 nM fluorescein solution were added. Each measurement is an average of 7 repetitions, as 7 wells were used per sample. This mixture was incubated at 37°C during 30 minutes. AAPH solution (25 μL 240 mM) was then added rapidly using a multichannel pipette. Fluorescence was recorded every minute during 90 minutes and the antioxidant capacity was estimated by integrating the area under the kinetic curves for each well (Optima-MARS Data Analysis). The results are presented as Trolox equivalent antioxidant capacity (TEAC) per gram of dry product ($\mu mol_{TEAC}/g_{DB}$). Analysis were carried out in duplicate.

2.6.6. Particle size distribution

A Malvern Mastersizer 2000 (Malvern, UK) was used to measure the particle size of the spray-dried microparticles within a stream of air at 0.2 MPa.

2.6.7. FTIR

The spray dried microparticles were characterized by Fourier Transform Infrared Spectroscopy FT-IR using a Bruker Alpha spectrometer with a Platinum-ATR single diffraction sampling module (Billerica, MA, USA). The spectra were measure as the average of 60 scans at a resolution of 4 cm^{-1}. The ATR signal was transformed to transmittance and the obtained spectra were normalized after the correction of the baseline.

2.6.8. Scanning electron microscopy

SEM images were obtained with a FEI® Quanta 200 ESEM microscope (Hillsboro, OR, USA) operating under High Vacuum using the Beam Deceleration Mode. Samples were measured at a landing energy ranging 1-2 kV and the signal was acquired by the Backscattered Electron Detector (BSED) working in Mode A (1 sector used only). The beam deceleration voltage applied was 4 kV.

2.7. Statistical methods

Spray drying variables were optimized in a previous work [29] and only the type of carrier and the carrier:extract solids ratio were studied here (see Table 2). Due to limited amount of grape marc extract available from the same batch, no repetitions were conducted on these experiments. Characterization of the obtained microparticles was carried out at least in duplicate and analysis of data was performed using Microsoft Excel.

3. Results and discussion

3.1. Grape marc extract spray drying yield

Fig. 1 shows the effect of carrier material type and concentration on the yield of spray-dried powders. A drying yield of 50% is taken to be the benchmark for successful spray drying [35-37]. An experiment to spray dry the grape marc extract in the absence of carriers was performed but the solids were stuck to the dryer chamber wall and no powder was recovered in the collection vessel, as is usually the case with sugar-rich foods [35]. When a carrier material was added to the extract in a 1:1 ratio, MD and WPI provided the highest yields (75.6 and 77.6% respectively), whereas the yield obtained with PPI was much lower (17.4%) and below the 50% threshold.

Further experiments were carried out with MD and WPI to determine the minimum amount of carrier required for efficient drying. Addition of 0.6:1 MD resulted in a drying yield of 49.3%, whereas a lower amount of WPI was necessary (between 0.3:1 and 0.5:1). These results are consistent to those reported by Fang and Bhandari, who observed that WPI was considerably more efficient than MD to overcome the stickiness problems during spray drying of sugar rich bayberry juice [37]. This was attributed to the preferential migration of proteins and surface coverage to the air/water interface of the microparticles during the drying process, which reduced the stickiness between particle/particle and particle/dryer wall, whereas the mechanism of maltodextrin was to increase the overall T_g of the bayberry powders.

Interestingly, the results obtained with PPI were vastly different from those with WPI: drying yields were noticeably lower, even with a 2:1 carrier:extract solids ratio. This could be caused by the low solubility of PPI in the aqueous extract, which led to the presence of suspended,

undissolved particles. Pea protein is known to have poor aqueous solubility, especially at low pH values [38]. The undissolved solid content of PPI in water for the concentration equivalent to the 1:1 ratio was measured at 93.2%, i.e. only 6.8% of the added PPI was dissolved, whereas MD and WPI achieved full dissolution for that same concentration. The suspended solids may induce the formation of bigger droplets during the atomisation step that adversely affect the drying rate, decreasing the drying yield [11, 39]. Solubility of the protein decreases with increasing molecular weight, and in our case the molecular weight of PPI was significantly larger than WPI: the main proteins present in PPI are legumin (320-380 KDa), vicilin (170 KDa) and convicilin (290 KDa) [38], whereas α-lactalbumin (14 KDa) and β-lactoglobulin (18 KDa) are the main proteins in WPI [40]. Wang *et al*. reported a positive relationship between the solubility of different proteins and their effectiveness as spray-drying additives [41]. Bhandari *et al.* reported that low molecular weight proteins such as WPI may improve the recovery and quality of spray dried powder [37], whereas Jayasundera *et al.* reported superior drying performance of sodium caseinate (as a model dairy protein) over PPI when spray drying model sugar-rich foods and attributed it to the low solubility of pea protein [42].

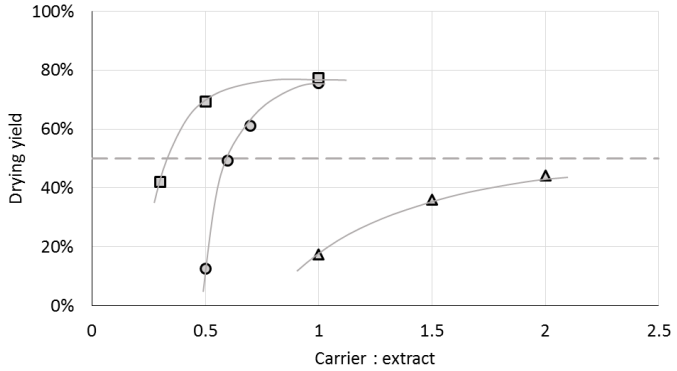

Fig. 1. Drying yield of grape marc extract containing different carrier:extract solids ratios for MD (○), WPI (□) and PPI (△). A drying yield of 50% (dashed line) shows the threshold for efficient drying.

The characterization of the grape marc extract is shown in Table 1, and the results obtained for the different formulations are shown in Table 2. Lower carrier:extract solids ratios lead to higher concentration of bioactive compounds (TPC, TAC) per gram of powder. This is an expected 'concentration' effect produced by the diminishing amount of carrier and agrees with previously reported results [43-45]. Water activity of the formulated microparticles was in all cases below the threshold for microbial growth inhibition, which has been reported to be $a_w <$ 0.61 [46].

Table 1. Characterization of the grape marc extract, as received and after freeze-drying.

	TPC (mg_{GAE}/g_{DB})	TAC (mg_{MVD}/g_{DB})	ORAC ($\mu g_{TEAC}/ g_{DB}$)	Total solids content (%)
Extract, as received	211 ± 3.8	3.87 ± 0.14	2043 ± 68	3.53 ± 0.02
Extract, freeze-dried	219 ± 2.6	3.72 ± 0.19	3178 ± 273	85.4 ± 1.7

Table 2. Drying yield, total phenolic content (TPC), total anthocyanin content (TAC), antioxidant activity (ORAC), moisture content (MC), water activity (a_w) and TAC recovery of grape marc extract spray-dried using different carriers and carrier:extract solids ratios.

Carrier	Carrier:extract solids ratio	Drying Yield (%)	TPC (mg_{GAE}/g_{DB})	TAC (mg_{MVD}/g_{DB})	ORAC ($\mu g_{TEAC}/ g_{DB}$)	MC (%)	a_w
MD	1:1	75.6	128 ± 8.2	2.39 ± 0.22	3684 ± 341	11.0 ± 0.1	0.292 ± 0.002
MD	0.7:1	61.0	135 ± 2.8	2.49 ± 0.05	2739 ± 23	11.1 ± 0.1	0.168 ± 0.006
MD	0.6:1	49.3	142 ± 1.5	2.60 ± 0.01	2995 ± 60	11.0 ± 0.1	0.141 ± 0.004
MD	0.5:1	12.6	162 ± 0.2	3.21 ± 0.05	4853 ± 36	15.4 ± 0.2	0.350 ± 0.003
WPI	1:1	77.3	116 ± 0.3	1.96 ± 0.09	2841 ± 107	11.1 ± 0.1	0.332 ± 0.014
WPI	0.5:1	69.3	159 ± 1.8	2.73 ± 0.16	4545 ± 23	14.2 ± 0.1	0.331 ± 0.001
WPI	0.3:1	42.1	167 ± 9.7	2.34 ± 0.18	2900 ± 146	14.8 ± 0.0	0.236 ± 0.004
PPI	2:1	44.2	52 ± 1.8	1.14 ± 0.05	849 ± 165	6.6 ± 0.1	0.269 ± 0.003
PPI	1.5:1	36.1	74 ± 2.4	1.12 ± 0.02	1204 ± 38	6.5 ± 0.2	0.191 ± 0.005
PPI	1:1	17.4	96 ± 0.1	1.73 ± 0.03	2787 ± 148	7.4 ± 0.0	0.183 ± 0.016

3.2. Particle morphology and size distribution

Scanning electron microscopy of the samples spray dried with 1:1 carrier revealed different morphologies: MD and WPI produced irregular spherical microparticles due to their skin forming capacity, whereas PPI microparticles had an amorphous appearance (Fig. 2). The poor skin forming ability of PPI has been linked with low powder recovery and particle agglomeration [36], and we observed a similar appearance for spray dried PPI in a previous work [29]. The dents and shrinkages observed on the surface on MD and WPI microparticles are usually attributed to the fast evaporation of water and resulting collapse of the microparticles during the drying process [47]. In the case of WPI particles, the development of resistive skin on the particle surface impedes the diffusion of the internal vapour, causing outward pressure on the skin that results in blow-holes on the surface [48]. Similar blow-holes have been observed for other WPI spray dried particles [49, 50].

The particle size distribution (Fig. 3) of MD and WPI microparticles is similar, with a mean particle diameter ($D_{4,3}$) of 16.2 and 15.0 μm respectively. The amorphous, irregular form of PPI formulated microparticles gives them a larger mean particle size (see Table 3) compared to MD and WPI. These numbers are consistent with the particle size observed by SEM (Fig. 2). The span is lowest for MD microparticles, indicating a narrower particle size distribution; this can be also seen in Fig. 3. As mentioned in section 3.1, the low solubility of PPI in the extract and the consequent presence of suspended solids in the feed mixture may interfere with the atomisation step, resulting in large particles such as the ones obtained in this work.

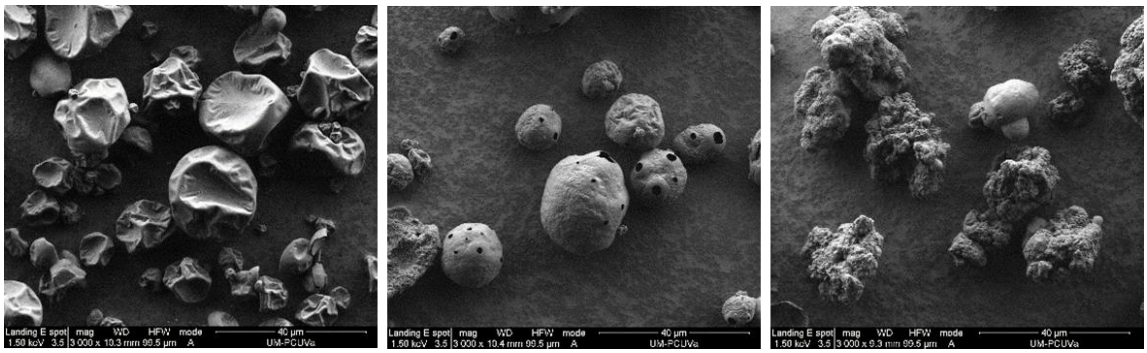

Fig. 2. Scanning electron micrographs for grape marc extract spray-dried with 1:1 MD (left), 1:1 WPI (center) and 1:1 PPI (right)

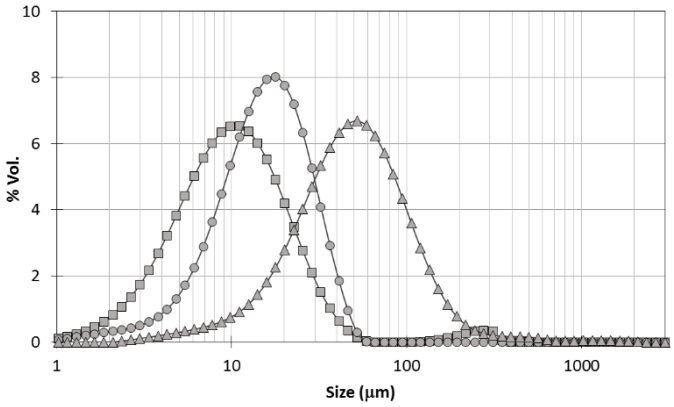

Fig. 3. Particle size distribution of grape marc extract spray-dried using 1:1 MD (○), 1:1 WPI (□) and 1:1 PPI (△).

Table 3. Particle size distribution, volume mean diameter ($D_{4,3}$) and particle span of grape marc extract spray-dried using different carriers ($D_{v,10}$ represents the value below 10%; $D_{v,50}$ represents the value below 50%; $D_{v,90}$ represents the value below 90%).

Carrier	Ratio	$D_{v,10}$ (µm)	$D_{v,50}$ (µm)	$D_{v,90}$ (µm)	$D_{4,3}$ (µm)	Span = $(D_{v,90}-D_{v,10})/D_{v,50}$
MD	1:1	6.0 ± 0.5	14.6 ± 0.4	28.8 ± 0.2	16.2 ± 0.3	1.6 ± 0.1
WPI	1:1	3.5 ± 0.3	9.4 ± 0.3	22.9 ± 0.7	15.0 ± 0.8	2.1 ± 0.2
PPI	1:1	16.4 ± 0.4	45.7 ± 0.8	111.3 ± 6.0	62.7 ± 9.5	2.1 ± 0.1

3.3. FTIR analysis

Infrared spectra of the freeze-dried extract, carriers and samples are shown in Fig. 4. The two protein carriers show characteristic peaks at 1630 and 1519 cm^{-1} corresponding to the amide I and II bands, respectively. The amide I band is due to C=O stretching vibrations of the peptide bonds, whereas the amide II band is caused by C–N stretching vibrations in combination with N-H bending [51, 52]. MD shows three characteristic bands at 1150 and 1080 cm^{-1} corresponding to the C–O bond, and at 1025 cm^{-1} corresponding to the angular deformation of the C=H and $C=H_2$ bonds, all from groups found in the carbohydrates [53]. These peaks, as well as the amide bands mentioned above, are also visible in the formulated microparticles but not in the F/D extract, indicating that the extract was covered by the proteins in the spray drying process.

The F/D extract spectrum shows several bands between 1680 and 900 cm^{-1} from phenolic compounds found in grapes. The strong peak at 1600 cm^{-1} found in the F/D extract sample may be assigned to the vibration of the C=C bond, typical of aromatic systems [53]. This peak is

absent from the pure carrier samples but visible in the formulated samples, especially in the MD 1:1 sample. The large band found from 3000 to 3600 cm^{-1} in all samples is related to the hydrogen bonded O–H stretching of carbohydrates, carboxylic acids, and residual water [54]. The peaks found near 2930 cm^{-1} are produced by the asymmetric and symmetric stretching of the C–H bond of methyl groups [26, 53].

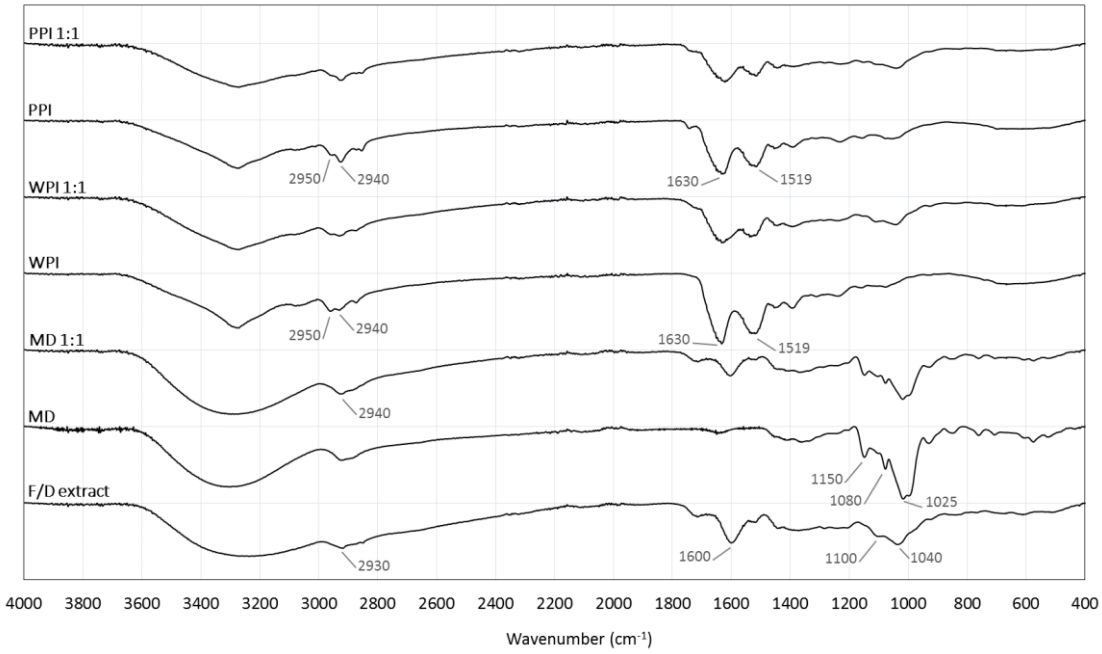

Fig. 4. Infrared spectra of freeze dried extract, carriers (MD, WPI and PPI) and formulated particles with a carrier:extract solids ratio 1:1 (MD 1:1, WPI 1:1 and PPI 1:1). Data has been offset for clarity.

3.4. *In vitro* release study

The microparticles formulated with 1:1 carrier:extract solids ratio were selected for the release study. The release profiles of TPC and TAC from microparticles over time in simulated gastric and intestinal fluid are shown in Fig. 5. All tests were carried out at 37°C over a period of 3 h, and a F/D sample of grape marc extract was used as control. It can be observed that MD formulated microparticles and the F/D extract have very similar TAC and TPC release profiles in both media, indicating that MD has no effect in the release. On the other hand, protein formulated microparticles show either a slower release rate or a lower final released fraction. In particular, WPI particles showed a clearly slower release rate in all cases. The smoother surface of these microparticles may lead to a slower release of the bioactive compounds due to a lower surface area in contact with the medium [55].

With protein carriers, the pH of the simulated solution has a strong effect on the release of the active compounds, since the solubility of proteins is known to increase at pH values above and below their isoelectric point (pI ~ 4.5) due to an increase in positive or negative net charges that produce repulsion forces among the molecules [56, 57]. At pH values around the isoelectric point, the protein is believed to precipitate and form agglomerates that inhibit the release of the core material. Assadpour *et al.* [58] studied the effect of pH on the release rate of folic acid from spray dried pectin-whey protein nano-capsules and reported increased release rates for alkaline conditions. In our case, release of total phenolics in F/D or MD microparticles (Fig. 5, left) was independent of the media and therefore the pH, whereas WPI microparticles showed

an increased release rate of phenolics as well as final release fraction in alkaline conditions (i.e. simulated intestinal fluid). PPI microparticles showed similarly fast release rates but the final release fraction was lower in alkaline conditions. The release of total anthocyanins (Fig. 5, right), however, is favoured at acidic conditions for all carriers due to their higher solubility at low pH [59].

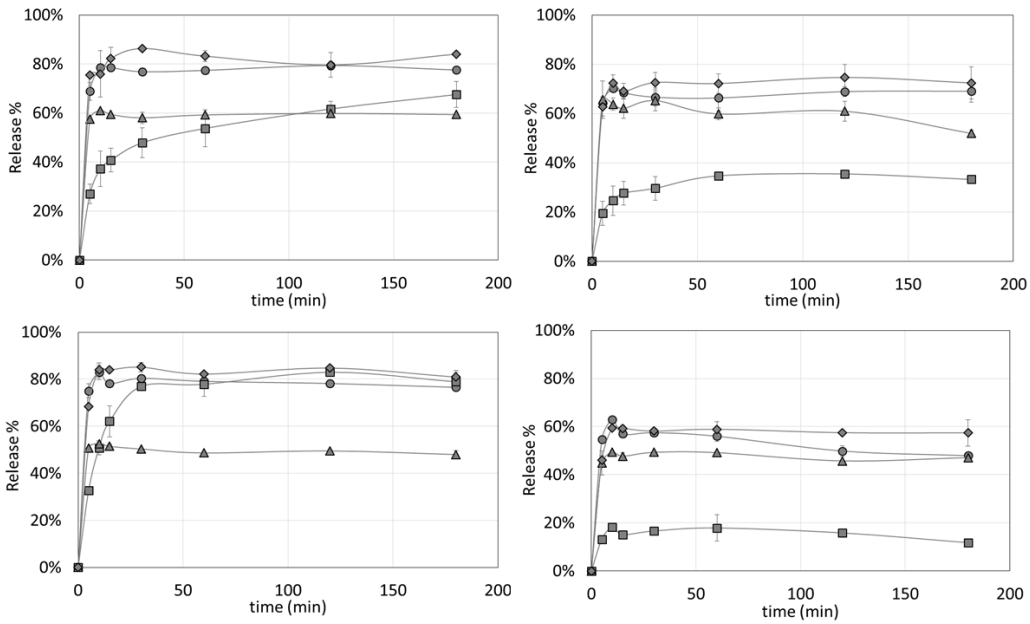

Fig. 5. Release profiles for TPC (left) and TAC (right) in simulated gastric (top) and intestinal (bottom) fluid using 1:1 MD (○), 1:1 WPI (□), 1:1 PPI (△) and F/D extract without carrier (◇). Lines added to guide the eye.

The experimental data was fitted using different empirical models for drug release. There are multiple kinetic models available for drug release, depending on the nature of the drug (or bioactive compound in this case), the release mechanism (dissolution, diffusion, partitioning, osmosis, swelling or erosion), the morphology or the solubility [25]. Of the main empirical models, three were selected to fit the data in this study: Korsmeyer-Peppas, Higuchi and Baker-Lonsdale.

The Korsmeyer-Peppas model [60] (Eq. 2) is a simple, semi-empirical model, exponentially relating the drug release to the elapsed time:

$$Q = kt^n \quad (2)$$

where Q is the fraction of drug released at time t, k is a constant and n is the diffusion exponent. This exponent characterizes the release mechanism and depends on the geometry of the system: for spherical microparticles, n=0.43 indicates diffusion-controlled drug release or Fickian diffusion, and n=0.85 indicates swelling controlled drug release or case II transport. When n is between 0.43 and 0.85, the drug release behaviour can be regarded as the superposition of both phenomena, which is called anomalous transport[25]. To determine the exponent n, it is recommended to use the portion of release curve until the point where $Q <$ 0.60.

The Higuchi model [61] was developed to study the release of soluble and poorly soluble drugs incorporated in semi-solid and/or solid matrixes, and describes drug release as a diffusion based on Fick's law where the fraction of drug released is proportional to the square root of time, according to Eq. (3).

$$Q = k\sqrt{t} \quad (3)$$

The Baker-Lonsdale model [62] was derived from the Higuchi model to describe the drug controlled release from a spherical matrix by diffusion, and has been utilized to analyze the results of drug release from microspheres and microcapsules according to Eq. (4):

$$\frac{3}{2}\left[1 - (1 - Q)^{2/3}\right] - Q = kt \quad (4)$$

Due to the very fast release of our microparticles in some cases (particularly for anthocyanin content), only certain data could be fitted, and in all cases using only the portion of the release curves before reaching the plateau. The results are shown in Table 4, with the Higuchi model providing the best fit for our data, followed closely by the Korsmeyer-Peppas model. The diffusion exponent *n* for the Korsmeyer-Peppas model was in all cases below 0.43, indicating Fickian diffusion, which agrees with Higuchi's interpretation.

Table 4. Kinetic release data of spray dried extract with different carriers (ratio 1:1) and freeze dried extract (control).

		Total Phenolic Content							Total Anthocyanin Content						
		Korsmeyer- Peppas			Higuchi		Baker-Lonsdale		Korsmeyer- Peppas			Higuchi		Baker-Lonsdale	
		R^2	*k*	*n*	R^2	*k*	R^2	*k*	R^2	*k*	*n*	R^2	*k*	R^2	*k*
Gastric fluid	MD	0.866	0.568	0.127	0.910	0.236	0.820	0.014	0.624	0.586	0.064	0.877	0.210	0.705	0.010
	WPI	0.967	0.205	0.236	0.943	0.100	0.975	0.002	0.958	0.144	0.220	0.982	0.076	0.971	0.001
	PPI	-	-	-	0.861	0.184	0.653	0.007	-	-	-	-	-	-	-
	F/D	0.868	0.653	0.080	0.886	0.243	0.684	0.016	0.6328	0.578	0.071	0.890	0.212	0.728	0.011
Intestinal fluid	MD	-	-	-	0.864	0.244	0.651	0.016	-	-	-	0.860	0.181	0.617	0.007
	WPI	0.874	0.213	0.350	0.985	0.149	0.992	0.006	-	-	-	0.873	0.048	0.394	0.000
	PPI	-	-	-	0.852	0.160	0.627	0.005	-	-	-	0.876	0.147	0.691	0.004
	F/D	0.868	0.508	0.197	0.936	0.248	0.879	0.017	0.864	0.319	0.244	0.948	0.173	0.879	0.007

3.5. Stability

3.5.1. Accelerated stability test at 40 and 60°C

A short-term stability study was carried out over a two week period at increased temperature (40 and 60°C) using a F/D grape marc extract sample as control and the 1:1 formulated samples. The results for TPC, TAC and ORAC retention are shown in Fig. 6. No loss of total phenolics was detected under either temperature for any of the samples. In the case of WPI formulated microparticles, TPC increased after the two week period. This effect was also reported by Flores *et al*. [19] when they studied the storage at 22, 37 and 45°C of blueberry pomace extract spray dried with WPI as wall material.

These authors also observed an increase in antioxidant capacity of the samples upon storage at different temperatures, with a final ferric reducing antioxidant power varying proportionally with increasing temperatures. In our work, we found that WPI formulated microparticles increased their antioxidant potential by 55% after two weeks storage at 60°C. These results also confirm that ORAC stability is high during the first month even under increased temperature, and this parameter is more affected by long-term storage (see Fig. 9).

Total anthocyanin content was the most affected parameter by storage under temperature, as seen in Fig. 6, and this could be related to the increase observed in antioxidant activity for WPI particles, particularly at 60°C. Thermally produced phenolic compounds from degradation or polymerization of anthocyanins may partially or fully compensate for loss in antioxidant activity arising from decreased monomeric anthocyanins [19]. Several authors have previously reported first-order kinetics for anthocyanin degradation [15, 18, 19, 22, 24, 27], as well as high instability of these compounds at elevated temperature [23]. WPI formulated microparticles showed severe degradation at 40 and especially 60°C (complete AC degradation after 4 days), followed by F/D, MD and PPI microparticles (see Fig. 7). The data was adjusted following first order kinetics according to Eq. (5) and the half-life period is calculated from Eq. (6) [15]. The degradation constant and half-life periods are shown in Table 5.

$$\ln\left({}^{C_0}/_{C_t}\right) = kt \tag{5}$$

$$t_{1/2} = {}^{\ln(2)}/_{k} \tag{6}$$

An increase in storage temperature led to an increase in degradation rate constants, particularly for WPI microparticles (219% increase) and the F/D grape marc extract control (111% increase). Mahdavi *et al*. [63] also observed that an increase in storage temperature led to an increase in degradation rate constants for MD encapsulated barberry anthocyanins. At 42°C, they reported a degradation rate of 0.013 days^{-1} and a half-life of 52.5 days for 4:1 MD microcapsules, which is in agreement with the results shown in Table 5. PPI formulated microparticles showed the lowest anthocyanin degradation rate (longest half-life periods) under both temperature conditions, with a degradation constant 97.5% (at 40°C) and 99% (at 60°C) lower than that for WPI microparticles, and a remarkable half-life period of over 11 months at 40°C. Flores *et al*. pointed out that the disadvantage of using WPI as a wall material in spray drying arises from significantly higher rates of anthocyanin degradation compared with polysaccharide-based wall materials such as MD [19]. Interestingly, this counteracts the advantages of using WPI described in section 3.1, i.e. its high spray drying efficiency. Similarly, PPI microparticles show remarkable stability, even at 60°C, with the lowest AC degradation rates, but the yields obtained during the spray drying step were poor, and larger amounts of carrier are required in order for the drying process to be considered efficient, thus reducing the final bioactive concentration in the product.

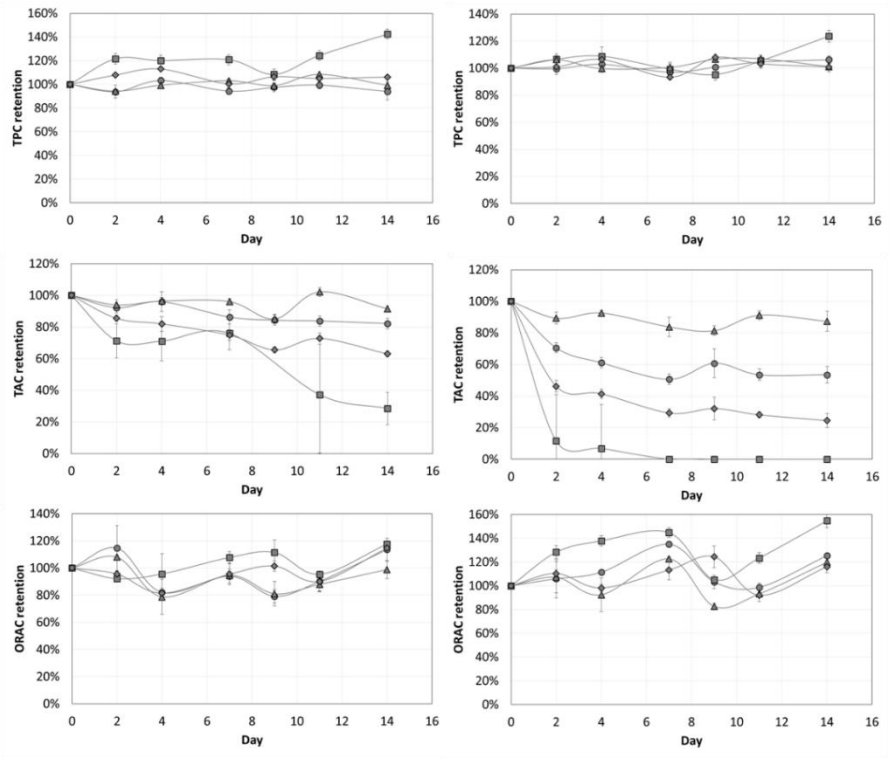

Fig. 6. TPC, TAC and ORAC retention of 1:1 MD (○), WPI (□), and PPI (△) formulated particles and F/D extract without carrier (◇) during accelerated stability test. Left: 40°C; right: 60°C. Lines added to guide the eye.

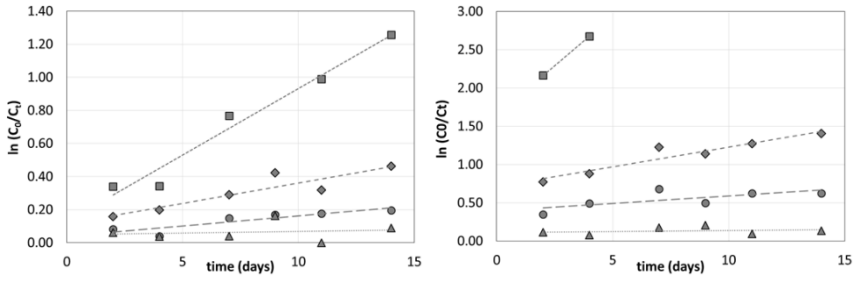

Fig. 7. First order kinetic plot for total anthocyanin degradation during accelerated stability test for 1:1 MD (○), WPI (□) and PPI (△) formulated particles and F/D extract without carrier (◇). Left: 40°C; right: 60°C.

Table 5. Anthocyanin first order degradation rate constants during accelerated stability test at different temperatures.

T (°C)	Carrier	k_{obs} (days^{-1})	$t_{1/2}$ (days)
40	MD	0.012	56.5
	WPI	0.080	8.6
	PPI	0.002	346.0
	None (F/D)	0.025	28.2
60	MD	0.020	35.5
	WPI	0.256	2.7
	PPI	0.003	258.7
	None (F/D)	0.052	13.4

Anthocyanin degradation is also directly related to colour stability. Polymerisation of AC with themselves or with non-anthocyanin phenolics can occur, forming red to brown products at moderate temperatures [64]. At 40°C, all samples maintained a similar macrostructure and colour stability after two weeks. However, at 60°C only PPI maintained retained its macrostructure and showed good colour retention, whereas WPI, F/D and MD showed agglomeration and/or discoloration. This can be observed in Fig. 8, where the colour of MD, WPI and F/D samples stored at 60°C shifted to brown/orange after 4 days.

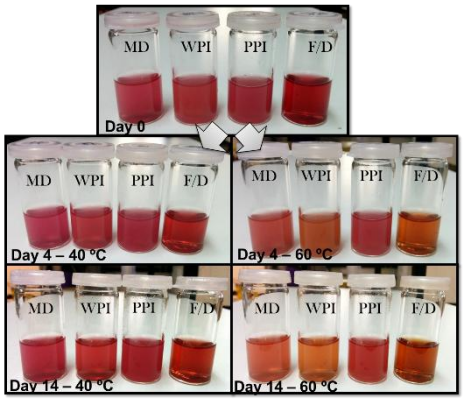

Fig. 8. Colour stability of spray dried grape marc extract samples over time at different temperature (samples dissolved in TPC solution, see section 2.6.3)

3.5.2. Long term stability test at room temperature

A 6 month stability study was carried out at 25°C with and without daylight exposure for the 1:1 formulated samples of all carriers. The changes in total phenolic and anthocyanin content as well as ORAC antioxidant activity with time are shown in Fig. 9. There is no clear effect of daylight exposure in either variable. Similarly, Ersus and Yurdagel studied the stability of black carrot anthocyanins spray dried using a range of maltodextrins when exposed to 3000 lx of light at 25°C over 64 days and reported that there was no significant effect of light in either the degradation constant or the half-life of the microparticles [18].

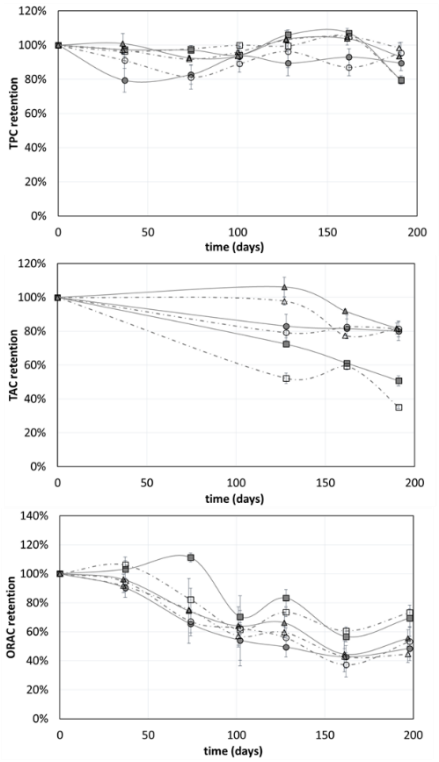

Fig. 9. TPC, TAC and ORAC retention of 1:1 MD (○), WPI (□) and PPI (△) formulated particles during 6 month storage at 25°C. Empty symbols with dashed lines represent samples under daylight conditions. Lines added to guide the eye.

Similarly to what was observed in the accelerated stability study, total phenolic content did not suffer any apparent loss after 6 month storage for any of the carriers, and therefore all three carriers can be considered efficient with regard to total phenolics stability. Total anthocyanin content suffered a 20% loss after 6 months for PPI and MD formulated samples, and a 50-65% loss for WPI formulated samples. Antioxidant activity also shows a decreasing trend with time (particularly after the first month), but in this case WPI microparticles show a higher retention of ORAC activity, which could be related to the higher AC degradation, as discussed above.

4. Conclusions

This study compares the efficiency of maltodextrin, pea and whey protein in terms of particle stability and release kinetics when used as carrier agents in the spray drying of grape marc phenolics. The results show that proteins are interesting alternatives to the frequently used maltodextrin, with each of the proteins studied offering specific advantages. Whey protein is a very effective drying aid in terms of drying yield, as the amount required to achieve efficient drying is much lower than for pea protein or maltodextrin. These WPI formulated microparticles showed a slower and more controlled release of total phenolics, both in gastric and intestinal simulated fluid. However, these microparticles offered poor stability, showing very low retention of anthocyanins in both the short and long term stability studies, and should only be considered for products intended for immediate use. On the other hand, pea protein offers a poor drying yield due to its lower solubility, requiring larger amounts of carrier in order for the drying process to be considered efficient, thus reducing the final bioactive concentration in the product. Additionally, these PPI microparticles showed almost instantaneous release of total phenolics and anthocyanins in both gastric and intestinal simulated fluids. However, PPI particles showed excellent stability, with very low anthocyanin degradation (and therefore good colour stability) at all temperatures, and could be considered

when a long shelf life of the product is required. Our results showed that the stability of the antioxidant capacity of the particles was dependent on storage time rather than temperature. Exposure of the particles to normal ambient light conditions during storage had no significant effect on stability of total phenolic, anthocyanin or antioxidant activity.

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