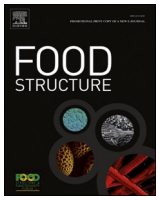
Food Structure 32 (2022) 100256

Contents lists available at ScienceDirect

Food Structure

journal homepage: www.elsevier.com/locate/foostr



Impact of gelatine coating on the performance of tannin-loaded pectin microbeads obtained through external gelation

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ARTICLE INFO

Keywords:
Amidated pectin
Gelatin
Microencapsulation
Polyphenol
Quebracho
Chestnut

ABSTRACT

One of the limitations of external gelation for microencapsulation is that small water-soluble compounds tend to diffuse out of the microbeads, resulting in low encapsulation efficiencies. In this work we propose a one-step approach for hydrogel microbead formation and simultaneous coating using external gelation. We explored amidated pectin as the encapsulation matrix for two different tannin-rich extracts (from chestnut and quebracho). The inclusion of tannin extracts contributed to the improvement of the structure of the microbeads through their interactions with pectin. By adding gelatine to the gelling bath, the microbeads were coated with the protein. This led to a significant increase in microencapsulation efficiency, which in some cases almost doubled compared to non-coated microbeads. Thanks to the binding of tannins with gelatine, coated microbeads loaded with the greatest amount of tannin extracts (20% w/w) presented the best retention of the bioactive compounds. A 14-days storage release study showed that these microencapsulation systems only experienced a slight loss of tannins during this period, with quebracho extract exhibiting greater retention than chestnut extract. Overall, by exploiting interactions in the pectin/tannins/gelatine ternary system, the proposed strategy for microbead production and coating in a single step allowed the development of a simple and more efficient microencapsulation approach for tannin extracts through external gelation.

1. Introduction

As in the last decades consumer demand for new healthy products has risen, there has been an increased commercial interest in researching and developing new functional foods (Ozen, Pons, & Tur, 2012). The big challenge is to incorporate natural bioactive ingredients to add potential to the food products over and above the mere calories from macronutrients. Among these new functional ingredients, tannins are bioactive compounds that have been shown to prevent or delay the evolution of different diseases (Molino, Casanova, Rufián Henares, & Fernandez Miyakawa, 2019). From the chemical point of view, tannins can be divided between hydrolysable tannins and condensed tannins. Hydrolysable tannins consist of esters of gallic acid with a core sugar, often glucose. The most representative molecule of the family is tannic acid. Condensed tannins are constituted by flavan-3-ol or flavan-3,4-diol units linked by carbon–carbon bonds, forming then oligomeric and polymeric proanthocyanidins(Khanbabaee, van Ree, & Ree, 2001). The susceptibility to hydrolytic cleavage is what differentiates the hydrolysable from the condensed tannins (Serrano, Puupponen-Pimiä, Dauer, Aura, & Saura-Calixto, 2009).

The easiest approach to create new functional food products is the addition of bioactive ingredients to already existing food matrices (Kaur & Das, 2011). Nevertheless, direct incorporation of tannins to foodstuffs could be a tricky issue, as these compounds are well known for their unpleasant flavour characterized by bitterness and astringency (Silva *et al.* 2017; Soares et al., 2018). In addition, the use of excessive amounts

Abbreviations: ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid, CHE, Chestnut; CSLM, Confocal laser scanning microscopy; FRAP, Ferric reducing antioxidant power; ME, Microencapsulation efficiency; QUE, Quebracho; SEM, Scanning electron microscopy.

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https://doi.org/10.1016/j.foostr.2022.100256
Received 18 October 2021; Received in revised form 13 January 2022; Accepted 19 January 2022
Available online 31 January 2022

of tannins may also alter the structure of some foodstuffs. These drawbacks are due to the chemical property of tannins to bind and precipitate proteins, among other molecules. In view of that, microencapsulation could be a strategic approach to coat or embed tannins and deliver them into the organism, avoiding off-flavours or direct contact with food matrices.

Of the various microencapsulation approaches available, hydrogel capsules have been widely exploited for food applications, thanks to easy, food-grade formulation conditions and the stiffness of the gelled capsules once they are formed (L. G. Gómez-Mascaraque, Martínez-Sanz, Fabra, & López-Rubio, 2019). One of the most used methods to produce these microbeads is extrusion, which consists of extruding the feed solution containing the bioactive ingredient through an orifice. The generated droplets drip into a gelling bath where the microbeads are formed (Whelehan & Marison, 2011). Alginate is the most widely used polysaccharide to produce hydrogel beads through this technique, but other biopolymers, such as gums, pectin or carrageenan, have also been explored for this purpose (L. G. Gómez-Mascaraque, Llavata-Cabrero, Martínez-Sanz, Fabra, & López-Rubio, 2018; Naqash, Masoodi, Rather, Wani, & Gani, 2017; W. Sun & Griffiths, 2000). However, these have been mainly applied as co-materials in association with alginate, in order to give a more stable structure with a greater encapsulation efficiency (Aguirre-Calvo, Molino, Perullini, Rufián-Henares, & Santagapita, 2020).

Pectin is a biological processing by-product obtained mainly from citrus or apple fruits and it has been used as thickening agent, gelling agent and stabilizer in several fields of the food industry thanks to its biodegrability and biocompatibility (Li et al., 2021). It can also be utilized as dietary fibre, prebiotic and fat replacer (Naqash et al., 2017). From a chemical point of view, these hydrocolloids are a group of anionic polysaccharides characterised by the presence of α-1,4-linked D-galacturonic acids in their backbone (Chan, Choo, Young, & Loh, 2017). They can be divided basically into two groups according to the degree of methoxylation (DM) of these galacturonic acid units: Low-Methoxyl pectin (LMP) with a DM $<$ 45% and High-Methoxyl pectin (HMP) with DM $>$ 45%. Moreover, the yield of this biopolymer could be improved through chemical modification (aminolysis), obtaining amidated pectin (AP) with better gelling ability than LMP. This derivatization consists of replacing part of the methyl-ester groups (de-esterification) in the galacturonic acid residues, generally with ammonia, obtaining primary amide groups. (S. Y. Chan et al., 2017; Yang, Xie, & He, 2011). Analogously to LMP, AP can gel with an "egg-box" structure, in the presence of divalent cations (e.g. calcium)" (Axelos & Thibault, 1991; Grant, Morris, Rees, Smith, & Thom, 1973). Moreover, pectin can also be modified by replacing methyl-ester groups on carboxyl groups, obtaining primary amide groups. This aminolysis leads to an alteration of the properties of pectin, improving its gelling properties. The degree of amidation is, thus, a characteristic parameter in amidated pectin (Chan et al., 2017), indicating the molar ratio of primary amide groups present to galacturonic acid units (comprising both free and replaced).

However, upon gelling, pectins form networks of highly porous nature with due to their weak binding to cross-linking agents, so that the use of pectin has been limited to targeted delivery of probiotics, rather than molecules characterized by smaller dimensions (Naqash et al., 2017). The addition of fillers, such as starch or rice bran, or chemical cross-linking agents (i.e. chlorhexidine) have been proposed to counteract the high porosity and increase the efficiency of pectin microbeads (E. S. Chan et al., 2011; Lascol *et al.* 2018). Among crosslinkers, natural ones are preferable synthetics for food application. In view of that, Mamet, Yao, Li, and Li (2017) showed the generation of intermolecular cross-linking between the carboxyl groups of pectin molecules and the phenolic hydroxyl groups of tannins. The hydrogen bonds and hydrophobic interactions led to a new aggregated network formed by the gels containing tannins, enhancing the pectin hydrogel properties (Mamet *et al.* 2017). In view of that, tannins could be used not only as carried molecules, but at the same time they could act as enhancer for the formation of the structure of the microbeads.

Gelatine is largely employed for pharmaceutical production and has recently attracted interest being used as microencapsulation matrix for food ingredients (Chambi & Grosso, 2006; L. Gómez-Mascaraque, Lagarón, & López-Rubio, 2015; Saravanan & Rao, 2010). This protein is obtained from partial hydrolysis of collagen that contains repeating tri-peptide sequences of glycine-aa_1-aa_2, where amino acids aa_1 and aa_2 are mainly proline and hydroxyproline (L. G. Gómez-Mascaraque, Soler, & Lopez-Rubio, 2016). Gomez-Mascaraque et al. (2018) recently developed ι-carrageenan/gelatine hydrogel capsules by extruding ι-carrageenan solutions onto a gelatine bath. The additional gelatine coating provided a better encapsulation yield for the polyphenols used as model bioactive compounds, by exploiting polyelectrolyte interactions between biopolymers (L. G. Gómez-Mascaraque et al., 2018). The authors could also observe that the presence of polyphenolic compounds had a great impact on the structure of the capsules. However, the system presented some limitations for their practical application, such as the big dimensions of the capsules (1–2 mm of diameter).

In this work, we adapted and improved the one-step coating approach previously developed to produce gelatine-coated pectin hydrogel microbeads. Their performance as microencapsulation vehicles for two different tannin extracts, with distinct composition and structure, was compared to that of uncoated pectin microbeads. The microstructure and encapsulation efficiency of the microbeads was studied and compared, as well as their release properties during storage.

2. Materials and methods

2.1. Materials

Pectin AGLUPECTIN LA-20 P (degree of amidation 22–25, degree of methoxylation 20–25, protein content 0.5% (w/w)) was supplied by JRS Silvateam Food Ingredients S.r.l. (Italy). Gelatine from porcine skin, with reported gel strength of 175 g Bloom, was supplied by Sigma-Aldrich (Ireland). Tannin extracts from quebracho (QUE) and chestnut (CHE) were provided by Silvateam Spa (Italy), as powder. Both QUE and CHE were obtained by hot water extraction from wood and bark. QUE was previously characterized by Pasch, Pizzi, and Rode (2001) and it is a phytocomplex constituted by profisetinidin condensed tannins with a polymerization degree up to 6.25 (Pasch *et al.* 2001). The detailed composition of CHE, consisting of a mix of hydrolysable ellagitannins, with 30% of isomers castalagin and vescalagin as representative substances, was previously described by Pasch & Pizzi (2002). Fast green FCF, calcium chloride dihydrate, sodium citrate and potassium bromide were obtained from Sigma-Aldrich (Ireland).

2.2. Preparation of the feed formulations

Porcine gelatine was dissolved in distilled water at 40 °C, under magnetic stirring. Then it was incorporated to $CaCl_2$ solution, to obtain a final $CaCl_2$ concentration of 0.1 M and gelatine concentration of 1% (w/v), avoiding gelation at room temperature. Pectin 2% (w/v) aqueous solution was prepared by magnetic stirring at room temperature. This concentration was selected according to our preliminary trials, achieving the maximum concentration which could be pumped through a 100 μm nozzle, as described in Section 2.3. Lower concentrations were not sufficient to form spheres. Increasing concentrations of QUE and CHE with respect to the mass of pectin (0%, 10% and 20% w/v) were subsequently added to the pectin solutions and mixed until a homogeneous dispersion was obtained.

2.3. Preparation of the hydrogel microbeads

Pectin solutions were filtered through 0.8 μm pore syringe filters for aqueous media (Sartorius, Germany). Microbeads were produced

Table 1
Coding used to indicate the different systems developed. Aqueous pectin solution was prepared at 2% (w/v). The concentrations of tannin extracts are referred with respect to the mass of the pectin (w/w). Porcine gelatine was incorporated to $CaCl_2$ solution, for a final concentration of 1% (w/v). QUE, quebracho tannin extract; CHE, chestnut tannin extract.

	Type of tannin	Tanninconcentration (w/w)	Gelatine
P	–	–	×
PC10	CHE	10	×
PQ10	QUE	10	×
PC20	CHE	20	×
PQ20	QUE	20	×
PG	–	–	✓
PC10G	CHE	10	✓
PQ10G	QUE	10	✓
PC20G	CHE	20	✓
PQ20G	QUE	20	✓

according to Gómez-Mascaraque et al. (2019) with slight modifications. We used an Inotech Encapsulator IER-50 (Inotech Biosystems Intl. Inc., Switzerland) to generate microbeads by extrusion of the pectin solutions through a 100 μm nozzle at a flow rate of 2.5 mL/min into the gelling bath (140 mm diameter) containing 250 mL of $CaCl_2$ or gelatine/$CaCl_2$ solutions. The gelling bath was located at a distance of 16 cm from the nozzle and maintained under constant agitation. Pectin droplet formation and break up was aided by a nozzle vibration frequency of 1240 Hz and an applied voltage of 1.3 kV, as optimized in preliminary trials. The collection time was set at 4 min for each batch, and the microbeads were cured within the gelling solution for 90 min before being filtered and thoroughly washed with deionized water. The produced microbeads were directly analysed. Table 1 summarises the coding used to indicate the different systems developed.

2.4. Water content

To estimate the water content, the microbeads (ca. 1 g) were filtered and accurately weighted before and after freeze-drying. The quantity of water was calculated in accordance with the Eq. (1), where m_h is the mass of the hydrated microbeads and m_d is the mass of the dried microbeads. Measurements were performed in three independent batches.

$$Water \quad (\%) = \frac{m_h \quad - \quad m_d}{m_h} \quad \times \quad 100 \tag{1}$$

2.5. Morphological characterization of the hydrogel microbeads

The morphology of fresh microbeads was studied by optical microscopy, taking images at 4x and 10x magnification using a digital microscopy system Olympus BX51 (Olympus Corporation, Japan). A digital camera head ProgRes CT3 (Jenoptik, Jena, Germany) and ProgRes CapturePro software (v 2.10.0.0) were used for image capturing. Size distributions were obtained from a minimum of 200 measurements, by using the ImageJ software (v. 1.52q).

The microstructure of the freeze-dried microbeads was also studied by scanning electron microscopy (SEM). Samples were stuck to the sample holders using carbon tape and sputter-coated with chromium under vacuum, using an Emitech K575X sputter coater (Quorum Technologies, UK). The samples were then imaged using a Gemini field emission scanning electron microscope (ZEISS, Germany) equipped with a secondary electron detector, at an accelerating voltage of 2 kV and a working distance of 4–6 mm.

Fresh microbeads were also observed using a Leica TCS SP5 confocal laser scanning microscope (CSLM) (Leica Microsystems CMS GmbH, Wetzlar, Germany) after staining the protein with Fast green FCF. 100 μL of 0.1% Fast green FCF (aq.) were added to 1.5 mL of microbead suspensions. Aliquots of the stained samples were then transferred to cavity slides and covered with 0.13 mm coverslips. Fast green FCF was excited at 633 nm using a He/Ne laser, and the corresponding emission filter was set at 660–710 nm. Leica LAS AV software (v 2.7.3.9723) was used to acquire digital images of 1024 × 1024 pixels in size.

2.6. Determination of gelatine content in the microbeads

The protein content of the gelatine-coated microbeads was estimated by measuring the nitrogen content of the samples through the Dumas method (L. G. Gómez-Mascaraque *et al.* 2019), using a LECO FP-528 N Analyzer (LECO Instruments UK Ltd., Cheshire, UK). As the pectin used for the present study was amidated and thus contained nitrogen, the nitrogen content of the microbeads prepared in the absence of gelatine was also measured and subtracted from the total nitrogen content obtained for the gelatine-coated microbeads. The obtained difference was then multiplied by a nitrogen conversion factor of 6.17, which was the experimental percentage of nitrogen in the commercial gelatine obtained through the same Dumas method (Eq. (2)). The determinations were conducted in triplicate.

$$\% \quad Gelatine \quad content = (\%N_{CPG} \quad - \quad \%N_{CP}) * 6.17 \tag{2}$$

Where $\%N_{CPG}$ is the nitrogen content in gelatine-coated pectin microbeads and $\%N_{CP}$ is the nitrogen content in their non-coated counterparts.

2.7. Tannin content and antioxidant capacity

Two different methods have been used to estimate the tannin content in the microbeads: UV 280 nm assay and Folin-Ciocalteu (quantification of phenolic content) assay. The antioxidant capacity was evaluated with the ABTS assay. The analyses were conducted on both the feed solutions and the microbeads. A weight of 1.5 g of fresh microbeads (for microencapsulation efficiency, Section 2.8) or 30 mg of dried microbeads (for retention analysis, Section 2.9) were dissolved in 5 mL of sodium citrate (2% w/v) solution, during 24 h, in order to dissolve pectin and release the contents of the microbeads.

UV280 nm assay. This is a direct method to determine the tannin content, which is measured through optical density at wavelength of 280 nm using a 10-mm quartz cuvette (Piccardo & González-Neves, 2013). The determination was carried out with a Cary 100 Bio UV-Vis spectrophotometer (Agilent, Ireland). Calibration was performed with aqueous solutions of the original tannin extracts, CHE or QUE, (5 – 100 ppm) as reference standard.

Folin-Ciocalteu assay. The protocol of Moreno-Montoro, Olalla-Herrera, Gimenez-Martinez, Navarro-Alarcon, and Rufián-Henares (2015) was applied, with slight modifications. In brief, 60 μL of sodium carbonate (10%) were mixed with 15 μL of Folin-Ciocalteu reagent, 30 μL of sample and 195 μL of distilled water. After an incubation at 37 °C for 60 min, the absorbance of the samples was measured at a wavelength of 760 nm. The amount of phenolic compounds was determined according to a calibration curve obtained with aqueous solutions of the original tannin extracts, CHE or QUE, (1 – 100 ppm) as reference standards.

ABTS assay. The antioxidant capacity, measured as radical scavenging activity, was evaluated following the procedure by Re *et al.* (1999). Calibration was performed with aqueous solutions of the original tannin extracts, CHE or QUE, used as reference standard (1 – 50 ppm).

Absorbances for ABTS and Folin-Ciocalteu assay were recorded with a Synergy HT microplate reader (Bio-Tek Intruments, USA) with a transparent 96-well polystyrene microplate (Biogen Científica, Spain). All the measurements were executed in triplicate on independent duplicates and results were expressed as weight percentage of tannins in the microbeads.

The three techniques above described were used as direct or indirect methods to estimate the presence of tannins in the measured samples.

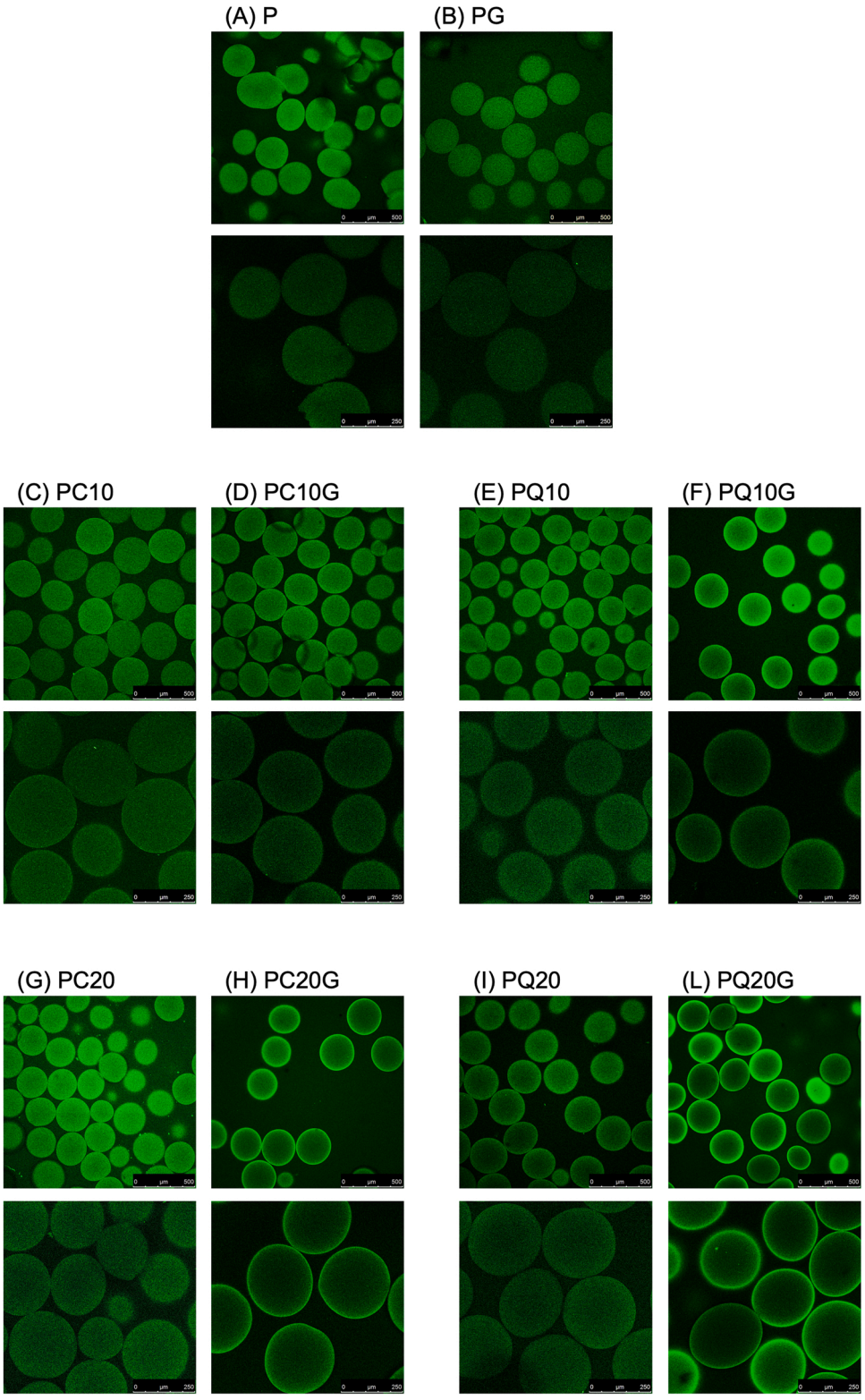

Fig. 1. Confocal laser scanning microscopy (CSLM) images of the different systems of microbeads, at two different magnifications (obtained with 10x and 20x objectives, respectively). (A) P, (B) PG, (C) PC10, (D) PC10G, (E) PQ10, (F) PQ10G, (G) PC20, (H) PC20G, (I) PQ20, (L) PQ20G.

All of them were used to calculate the microencapsulation efficiency (ME%) and to study the effect of storage on the retention of tannins.

2.8. Microencapsulation efficiency (ME%)

The ME% was calculated following the equation below:

$$ME(\%) = \frac{TTC_m}{TTC_i} \times 100 \quad (3)$$

where TTC_m was the total tannin content of the trapped extract in the fresh microbeads after 90 min of curation, and TTC_i was the total tannin content measured in the initial feed solution used for the

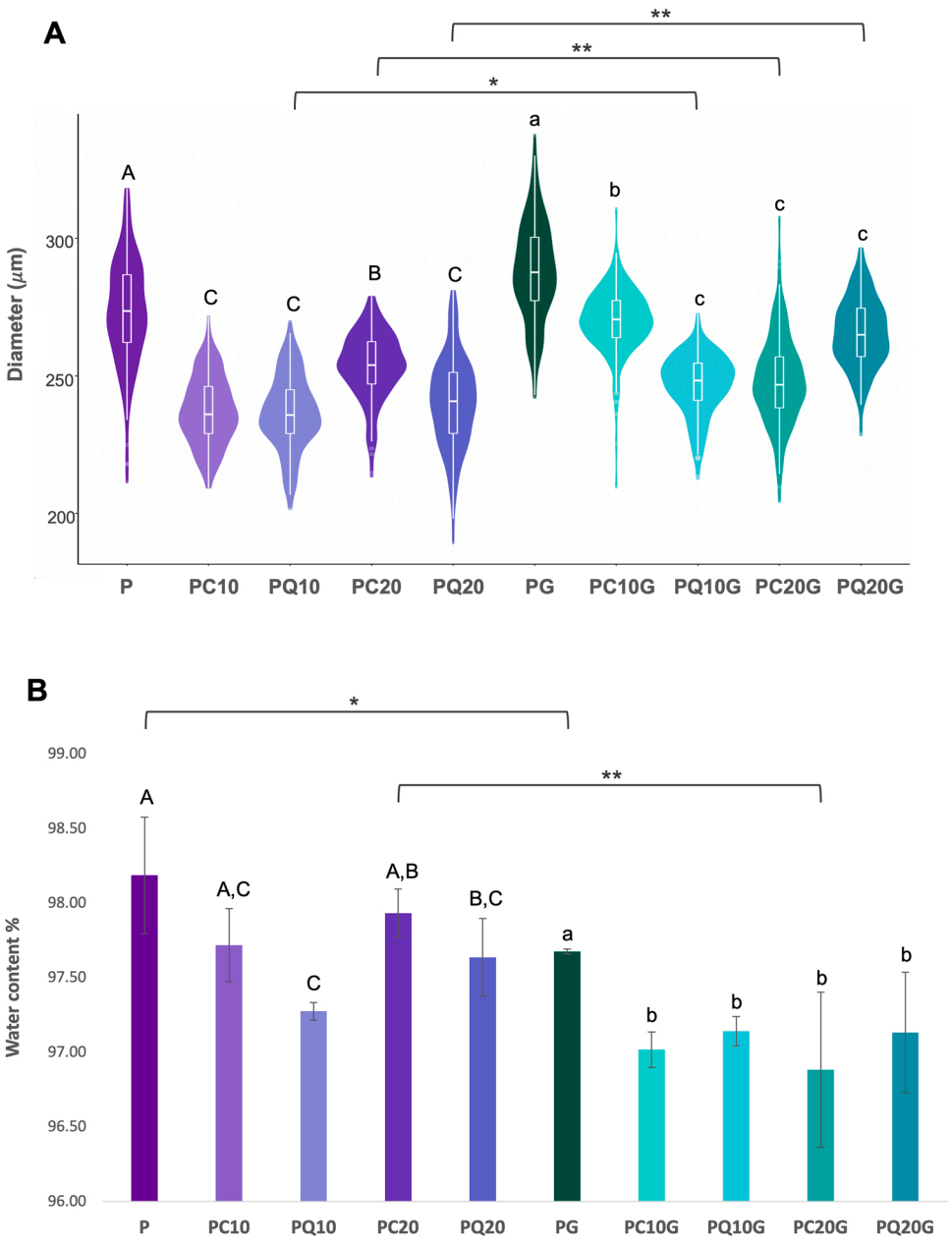

Fig. 2. (A) Size distribution reported as diameter (μm) and (B) water content of the microbeads. * Indicate statistically significant differences by Student's t-test between uncoated and coated microbeads: *p < 0.05, **p < 0.01. Capital and small letters indicate statistically significant differences by ANOVA and Bonferroni post-hoc test (p < 0.05) among uncoated and coated microbeads, respectively.

microencapsulation and estimated with the methods described in Section 2.7.

2.9. Retention of tannins during long-term storage

The microbeads suspensions were stored under refrigeration and their tannin content was analysed after selected time intervals for a 14 day period, to study the retention of these compounds in the microbeads during storage. The first sampling was carried out after 90 min of curation in the gelling bath (T0). After that, 7 batches of 1.5 g of fresh microbeads were washed with distilled water and stored in 10 mL of distilled water, under refrigeration (at 4 °C). After selected time intervals (1 h, 3 h, 6 h, 24 h, 48 h, 7d, 14d) two batches of samples was filtered, stored at − 80 °C and freeze-dried until further analysis. Tannin retention was expressed as the percentage of tannins remaining in the microbeads after each selected time period, in relation to the amount initially encapsulated.

$$Tannin \quad retention \quad \% = \frac{TTC_{Tx}}{TTC_{T0}} \times 100 \tag{4}$$

where TTC_{Tx} was the total tannin content in the microbeads measured at each of the selected time intervals and TTC_{T0} was total tannin content in the microbeads measured at T0.

2.10. Statistical analyses

t-Student's test was performed to compare two independent samples. The statistical significance of the differences among samples was calculated by one-way ANOVA with Bonferroni *post-hoc*. All the statistical analyses were realised using the SPSS software (version 23, SPSS, Chicago, IL, United States).

3. Results and discussion

3.1. Morphology

Fig. 1 shows CLSM micrographs of the various microbeads systems produced in the study, providing information on the morphology and size distribution. Supplementary Fig. S1 shows complementary images obtained by optical microscopy.

Microbeads generated with pectin without the addition of tannins nor gelatine (P) predominantly showed an irregular, non-spherical shape. The presence of small vermiform appendices in this sample is highlighted with arrows in Fig. S1. Pectins are generally not considered good matrices for microencapsulation through external gelation on their own because, due to their low viscosity and gelling mechanism, they do not gel fast enough, resulting in morphological distortions (Chan, Lee, Ravindra, & Poncelet, 2009; Davarcı, Turan, Ozcelik, & Poncelet, 2017). For this reason, and because pectins are considered to form relatively weak gels, they are frequently exploited as excipient to improve the microstructure of other hydrogel network systems, in terms of size, compactness as well as their interconnectivity (Aguirre Calvo, Santagapita, & Perullini, 2019; Díaz-Rojas et al., 2004; Mitrevej, Sinchaipanid, Rungvejhavuttivittaya, & Kositchaiyong, 2001), rather than on its own. The formation of microbeads observed for the pectin chosen for this study, despite their imperfections, could be due to the partial amidation of the hydrocolloid. Indeed, this chemical modification is reported to enhance pectin gelling properties, without requiring the addition of other ingredients, such as sugars, in certain cases (Chan et al., 2017).

The presence of tannins noticeably contributed to an improvement of the morphology of the microbeads (Figs. 1 and S1), indicating tannin-pectin interaction. The capacity of amide groups in pectin to establish multiple hydrogen bonds with negatively charged carboxylate groups and their free electron pair of tannins has been reported (Adamczyk, Adamczyk, Smolander, & Kitunen, 2011; Buchweitz, Speth, Kammerer, & Carle, 2013). Even though PC10 and PQ10 microbeads still presented some imperfections, a further increase in tannin concentration (PC20, PQ20) allowed obtaining more spherical microbeads almost free of defects. However, an excess of tannins could have a detrimental effect. In our preliminary studies, a tannin content higher than 20% (w/w) resulted in the impossibility of pumping the feed formulation through the 100 μm nozzle. Moreover, previous work reported that the incorporation of tannic acid 30% (w/w) within gelatine-coated ι-carrageenan microbeads resulted in negative structural changes, causing a rigid and fragile skin-like surface, which also limited gelatine adhesion on the surface of the microbeads. The authors suggested that the high affinity of tannic acid towards gelatine bath might have competed with the interactions between ι-carrageenan and gelatine, resulting in a lower protein binding in the gelling bath (Gómez-Mascaraque, Martínez-Sanz, Hogan, López-Rubio, & Brodkorb, 2019).

The presence of gelatine in the gelling bath also contributed to an improvement of the morphological characteristics of the microbeads, even in the absence of tannins. Indeed, PG formed more spherical microbeads and more free of defects compared to P (Figs. 1 A and B, and S1 A and F).

Table 2
Content of nitrogen (%N) and amount of gelatine attached to the microbeads. Different letters indicate statistically significant differences by ANOVA and Bonferroni post-hoc test ($p < 0.05$).

	%N		% N	% gelatine
P	1.698 ± 0.019	**PG**	2.835 ± 0.090 ^{A}	7.278 ± 0.557 ^{A}
PC10	1.649 ± 0.014	**PC10G**	3.728 ± 0.021^{B}	12.83 ± 0.311^{B}
PQ10	1.538 ± 0.067	**PQ10G**	3.716 ± 0.216^{B}	13.44 ± 1.335^{B}
PC20	1.785 ± 0.062	**PC20G**	3.747 ± 0.382^{B}	12.11 ± 2.355^{B}
PQ20	1.711 ± 0.077	**PQ20G**	3.755 ± 0.105^{B}	12.62 ± 0.648^{B}

By staining the microbeads with Fast Green FCF, a dye commonly used for protein staining (Jiménez-Munoz, Brodkorb, Gómez-Mascaraque, & Corredig, 2021), CSLM was used to investigate the presence of a gelatine layer on the surface of the microbeads. However, all the produced microbeads, even those prepared without gelatine, exhibited some fluorescence. This might be due to the presence of a small protein content in the amidated pectin (0.5%), as specified in the technical sheet. Moreover, the amide groups of pectin used might have interacted to some extent with the dye. Nevertheless, CSLM allowed a preliminary assessment of the impact of tannins in the formation of the gelatine coating. While in PG the fluorescence intensity was homogeneous within the microbeads, a greater fluorescence intensity was observed on the surface of the coated microbeads containing tannins (PC10G, PQ10G, PC20G, PQ20G), especially as the tannin content increased (Fig. 1). This suggested that the presence of tannins promoted the binding of gelatine on the surface of the microbeads, which was further confirmed in Section 3.3. As previously described, tannin-protein interactions depend, among other factors, on the concentration (Molino et al., 2019). Further topographical information can be found in the SEM images (Supplementary Fig. S2) in which the presence of fibrous material can be distinguished in the gelatine-coated microbeads, especially in the case of PC20G and PQ20G.

No notable differences were observed in the microstructure of the obtained microbeads when the condensed (quebracho) or the hydrolysable (chestnut) tannins were used, suggesting that the addition of tannins, in general, has a positive impact on the morphology of amidated pectin microbeads produced by the external gelation technique.

3.2. Size and water content of microbeads

Size distribution and water content of the microbeads produced through the extrusion-external gelation method are shown in Fig. 2. The non-coated microbeads (without gelatine), P, exhibited the largest size ($D = 274 \pm 19$ μm). The incorporation of tannins resulted in a statistically significant reduction ($p < 0.05$) in microbead size in comparison to those obtained in absence of tannins. Tannins have been reported to enhance the gelling properties of pectins, acting as crosslinkers and improving hydrogel network formation (Mamet et al., 2017). More specifically, condensed tannins with a relatively low degree of polymerisation (DP 5), and similar to that of the tannins used in this study, were shown to penetrate into the junction zones and stabilize them through hydrogen bonds pectin. In this sense, a lesser interaction between chestnut tannins and pectin could correspond to the minor efficiency in the formation of structured network, resulting in more swelling (bigger sizes). It is worth mentioning that, among tannin-loaded microbeads, PC20 presented the highest size ($p < 0.05$, P PC20 = 253 ± 12 μm) compared to the others. Within the gelatine-coated group, again the absence of tannins determined the highest size for PG (PG D = 288 ± 17 μm). Comparing the two different groups of microbeads, i.e. non-coated vs. gelatine-coated, the differences in their size distribution were not statistically different between the two groups. However, in general, the attachment of an external layer of gelatine resulted in an increase in the size compared to uncoated microbeads, and significant differences were observed for some individual samples, i.e. between PQ10 and PQ10G ($p = 0.011$), between PQ20 and PQ20G ($p = 0.001$), and between PC20 and PC20G ($p = 0.004$) (Fig. 2A).

These findings were also reflected in the water content results. In both groups, the presence of tannins resulted in a decrease in microbeads water content, with the production of smaller and denser microbeads. Even though no statistical differences were found between the two main groups (i.e., gelatine-coated vs. uncoated microbeads), in general gelatine-coated microbeads presented a lower water content than the non-coated ones, and significant differences were observed for some of the formulations, i.e. between P and PG ($p = 0.031$), and between PC20 and PC20G ($p = 0.001$). As for the size distribution, PC20G showed the lowest values, confirming that different chemical composition and

concentration play a significant role in the extent of interactions with the two biopolymers, as discussed above.

3.3. Protein content

In order to estimate the amount of gelatine adhered to the coated microbeads, we quantified the protein content (Table 2). As we mentioned earlier, the commercial pectin used for the present was amidated and the microbeads without gelatine contained a certain amount of nitrogen per se. Therefore %N due to the presence of pectin for each microbead system was subtracted (Table 2). To calculate the % of gelatine attached, % of N in the commercial gelatine used for this study was measured and used to estimate the nitrogen-to-protein conversion factor (6.17) for gelatine.

Even though the gelatine coating in PG could not be observed in the CLSM images, a certain amount of gelatine was detected (% 7.3 ± 0.6). This indicate that there is an interaction between pectin and gelatine. Some authors exploited the attraction between these materials, with their complexation, to realize microencapsulation by complex coacervation for drug delivery (Saravanan & Rao, 2010). However, the amount of gelatine present in the coated microbeads without tannins (PG) resulted significantly lower ($p < 0.05$) than in the tannin-loaded microbeads. Conversely, no statistically significant differences were detected among the different gelatine-coated microbeads loaded with tannins. This was indicative that the ability of tannins to bind to proteins facilitated the uptake of additional gelatine beyond the amount that could be bound due to pectin-gelatine polyelectrolyte interactions only. Supporting these data, Sun, Cameron, Manthey, Hunter, and Bai (2020) found that the addition of tannic acid to pectin/gelatine coacervate helped in improving its properties, leading to the production of better performing microbeads.

It is known that tannins have a peculiar affinity to bind to proteins, by establishing cross-links with the implication of bonds of a different nature. A different chemical composition, such as molecular weight and degree of galloylation, could determine distinctive interplay between tannins with gelatine (de Freitas & Nuno, 2012; Molino et al., 2019). However, gelatine−tannin interaction can also be limited by the interference of other molecules, pectin among them, which could compete with proteins for binding tannins. This issue is not yet totally understood because, some authors showed that the presence of polysaccharides, in particular mannoproteins, can inhibit the evolution of tannin aggregate particle size but not their generation (Riou, Vernhet, Doco, & Moutounet, 2002). The different affinity for pectin and/or gelatine could be the reason for a different intra-group microbead size distribution.

In a previous work, gelatine-coated ι-carrageenan microbeads incorporating 30% tannic acid, which, similarly to chestnut extract, belongs to hydrolysable tannins, were found to exhibit a much lower protein content than those measured in this study, despite the control microbeads in the absence of tannic acid, and those containing other type of phenolic compounds uptaking up to 40% gelatine (Gómez-Mascaraque et al., 2019). The authors attributed the lower gelatine adherence to the competing interaction of tannic acid with gelatine rather than ι-carrageenan. In our systems, instead of hindering the attachment of gelatine, the presence of tannins helped the formation of the gelatine coating, adhering more protein than the control (PG). This is evidence of the complexity involved in the interactions in these ternary systems, where the type and concentration of polyphenols and biopolymers will determine whether the tannins have a synergistic effect towards the gelatine uptake or not.

3.4. Microencapsulation efficiency

A phytocomplex such as those examined in this study could not be quantified by a single universal method. For this reason, three different spectrophotometric techniques (UV 280 nm, Folin-Ciocalteu and ABTS assays) were used to estimate the capacity of tannin retention (CHE or QUE) by the different microencapsulation systems. More specifically, the three selected techniques provided information on different aspects of the extracts: the presence of tannins in solution, thanks to the high absorption at 280 nm exhibited by tannins, the total polyphenol content, and the radical scavenging capacity. To calculate the tannin amount in the samples as accurately as possible, CHE or QUE were used to produce calibration curves as reference for samples containing the respective extracts. Thus, results could be reported as mg of tannins (CHE or QUE) per mg of encapsulation matrix (Table S1 of the Supplementary Material). Finally, the ME% was calculated as the percentage of the tannin content in the original feed solutions that was detected in the microbeads (Table 3). In general, the three techniques applied generated similar trends, with some slight variations.

Table 3
Microencapsulation efficiency (ME %) measured with three different methods: UV 280 nm, Folin-Ciocalteu and ABTS assays and expressed as a percentage. Data are reported as means ± SD. QUE, quebracho tannin extract; CHE, chestnut tannin extract; Different capital letters indicate statistically significant differences within the same extract and method of analysis ($p < 0.05$) by ANOVA and Bonferroni post-hoc test.

		UV 280 nm	Folin-Ciocalteu	ABTS
CHE	PC10	37.97 ± 6.518^{A}	21.02 ± 3.207^{A}	32.14 ± 2.089^{A}
	PC20	35.05 ± 0.217^{A}	19.96 ± 0.979^{A}	28.60 ± 7.554^{A}
	PC10G	56.65 ± 8.550^{B}	32.31 ± 3.277^{B}	43.10 ± 4.994^{B}
	PC20G	59.51 ± 4.253^{B}	46.36 ± 1.982^{C}	44.42 ± 6.596^{B}
QUE	PQ10	40.05 ± 2.702^{A}	20.73 ± 1.671^{A}	42.69 ± 12.69^{A}
	PQ20	37.99 ± 0.406^{A}	$27.39 \pm 1.965^{A,B}$	33.01 ± 7.525^{A}
	PQ10G	66.58 ± 1.311^{B}	32.51 ± 3.937^{B}	58.34 ± 11.06^{B}
	PQ20G	60.89 ± 1.432^{C}	45.50 ± 6.76^{C}	62.20 ± 9.250^{B}

As we discussed previously, the incorporation of tannins could improve the structure of pectin microbeads, which was attributed to the interactions between both components. The presence of amide groups and a small amount of protein impurities (0.5%) in the commercial pectin used for this study led to hydrogen bonding with tannins, which contributed to their retention within the microbeads to some extent (Table 3), considering the fact that they are relatively small water-soluble molecules.

The inclusion of the highest amount of tannins in the feed formulations (20% w/w) resulted in PC20 and PQ20 being the microbead systems with the highest tannin content. Even though PC10 and PQ10 showed slightly better values of ME%, these were not statistically significant. Gómez-Mascaraque & Lopez-Rubio (2019) reported that the reduction of the ratio matrix-to-encapsulated compound could result in a decrease of the ME%. In this study, no saturation of the pectin-tannin interaction sites was observed at the ratios assayed.

Coating with gelatine resulted in a significant improvement in the ME%, in some cases observing an almost 2-fold increase in ME% compared to the uncoated counterparts. For gelatine-coated microbeads, the highest %ME was achieved for the formulations containing the highest concentrations of tannins (PC20G and PQ20G). The well-known ability of tannins to bind proteins (Molino et al., 2019) might have played a fundamental role in the improvement of their encapsulation. In this sense, some of the excess tannins that could not interact with pectin and diffused out to the gelling bath were able to bind the gelatine being absorbed on the surface of the microbeads, being retained and avoiding their loss.

3.5. Retention of tannins during storage

For the development of products intended for functional food application, it is important to ensure a stable content of the bioactive compounds along the shelf-life of the products. Thus, the potential diffusion of water soluble compounds, when they are encapsulated within hydrogel microbeads, should be prevented as much as possible. To evaluate this, the retention of tannins from both microbead systems

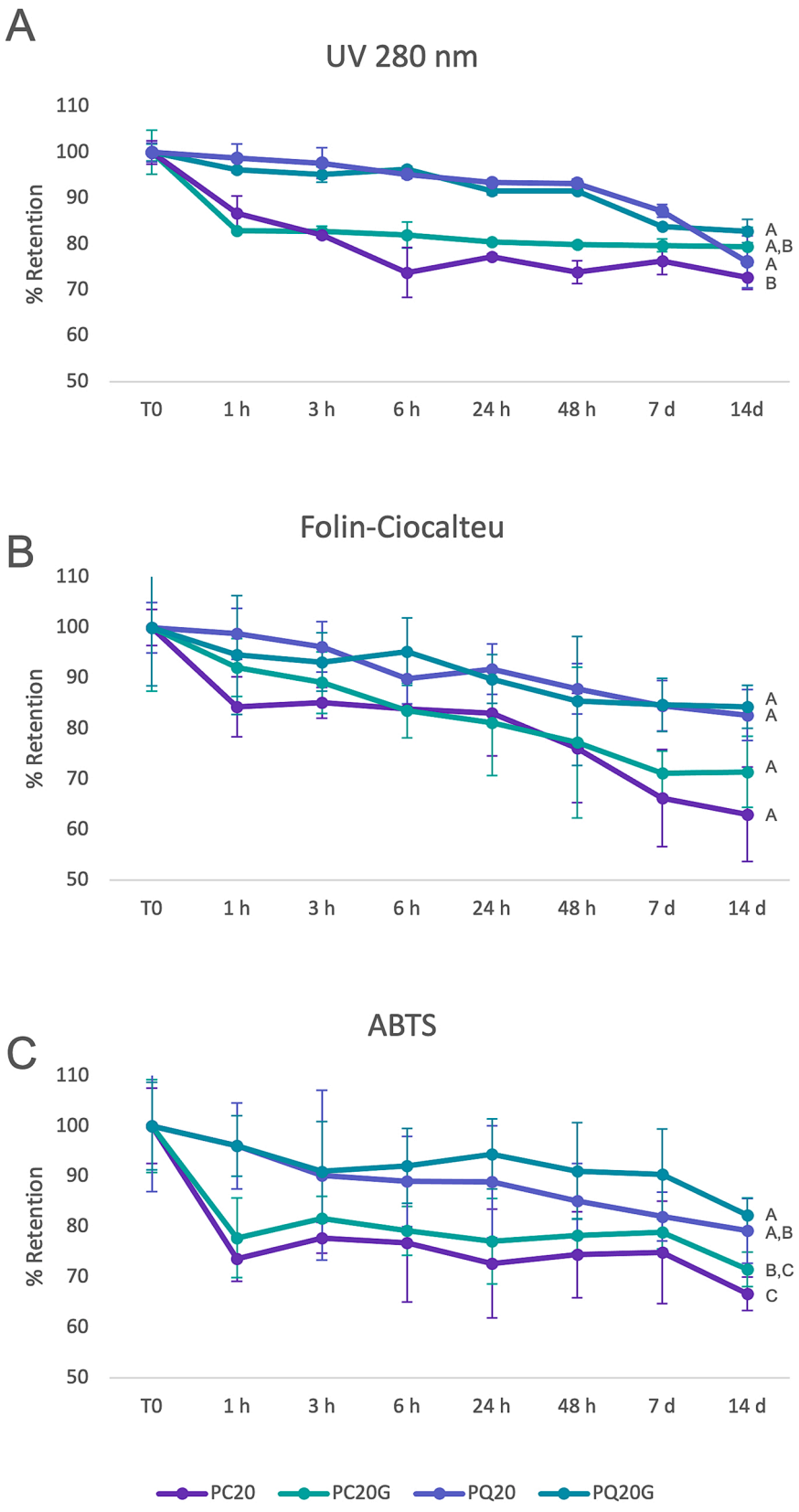

Fig. 3. Effect of storage on tannin retention of fresh microbeads, evaluated with three different methods: (A) UV 280 nm, (B) Folin-Ciocalteu and (C) ABTS assays. Tannin retention is expressed as percentage of the tannins initially encapsulated (ME%) remaining in the microbeads after each selected time period. Different letters indicate statistically significant differences by ANOVA and Bonferroni post-hoc test ($p < 0.05$) among the profiles of the different samples.

(with and without gelatine coating) was monitored over a storage period of 14 days. The microbeads with the greatest tannins content (PC20, PQ20, PC20G, PQ20G) were selected for this study. Fig. 3A, B and C show the results generated by UV 280 nm, Folin-Ciocalteu and ABTS assays, respectively.

Similar trends were observed through the three methods, so there was a correspondence between the measured tannin content and the antioxidant capacity exerted. In general, all systems presented over time a slow and gradual release of the tannin extracts, demonstrating a good retention ability over the 14-day study period. The release of tannins from the gelatine-coated microbeads exhibited the same trend as their uncoated-counterparts, indicating that the additional protein layer did not affect to a large extent the release kinetics of the systems.

The incorporation of quebracho extract in PQ20 and PQ20G resulted in a greater retention ability, resulting in a lower loss of bioactive compounds during storage, compared to PC20 and PC20G. The microbeads containing quebracho tannin extract had also exhibited a greater microencapsulation efficiency compared to their counterparts containing chestnut tannins. Thus, not only did microbeads containing quebracho tannins incorporate a greater amount of tannins, but a lower loss of these tannins was also observed during storage. This suggests that condensed tannins were able to establish stronger interactions with pectin than hydrolysable tannins. The differences in chemical structure between the two types of tannins might be responsible, at least partially, for the observed differences. Condensed tannins have a relatively flat structure, while the structure of chestnut ellagitannins is more complex, which may result in greater steric hindrance (Radebe, Rode, Pizzi, Giovando, & Pasch, 2013; Radebe, Rode, Pizzi, & Pasch, 2013).

The slight loss of tannins during the 14-days storage, once again, support that the interactions between tannins and pectin play an important role in the creation of a network, capable of efficiently maintaining tannins trapped. However, despite the gelatine-coating can improve the ME% in a statistical significantly manner compared to non-coated microbeads, it is unable to make a great improvement in tannin retention over time. In this sense, it has to be highlighted that uncoated microbeads already gave good results in this respect.

4. Conclusions

In the present study, amidated pectin was used as an alternative microencapsulation matrix through external gelation. The addition of tannin extracts as model bioactive compounds resulted also in a tool to improve structural features of the microbeads themselves, such as a better morphology and reduced swelling and microbead size. The microbeads with the highest tannin content (PC20 and PQ20) showed better morphology and encapsulation efficiency, presumably due to their interactions with the polysaccharide. These bindings allowed the microbeads to show good encapsulation efficiency, limiting the loss of tannins from the matrix.

Coating the microbeads by including gelatine in the gelling bath proved to be a good approach to improve the morphology of pectin microbeads. Moreover, this 1-step approach for microbead formation and coating considerably improved the %ME of the systems due to the interaction of the tannins with gelatine, which also resulted in greater amounts of the protein to attach to the microbeads.

The release of tannins from the fresh microbeads over a 14-days period was low, ensuring a minimum loss of bioactive compounds during storage in all the system evaluated. Although the gelatine coating did not significantly contribute to preventing the tannin release, it was a successful approach to achieve a higher tannin loading in the microbeads.

Incorporation of quebracho tannins resulted in greater microencapsulation efficiencies and better retention ability during storage than for the chestnut tannins, which indicated stronger interactions of the condensed tannins with pectin compared to the hydrolysable tannins.

These encouraging results suggest that the developed systems of microencapsulation, in particular those gelatine-coated, could be applied to embed phenolic compounds into fresh foods with a high water content. In order to reach a global overview for food applications, future work should investigate the structural changes the encapsulation systems undergo during gastrointestinal digestion, and the consequent release of the bioactive compounds.

Funding

This work was funded by Teagasc, the Irish Agricultural and Food Development Authority (Fermoy, Ireland), by the "Plan propio de Investigación y Transferencia" of the University of Granada under both programs "Intensificación de la Investigación, modalidad B" and by the European Research Commission (Research Executive Agency) under the research project Stance4Health (Grant Contract Nº 816303) granted to José A. Rufián-Henares.

Declaration of Competing Interest

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

Acknowledgments

This paper will be part of the doctoral thesis of S.M., conducted within the context of the "Nutrition and Food Sciences Program" at the University of Granada.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.foostr.2022.100256.

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