MICROENCAPSULATION OF NATURAL ANTIOXIDANTS FROM *PTEROSPARTUM TRIDENTATUM* IN DIFFERENT ALGINATE AND INULIN SYSTEMS

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ABSTRACT: The bioactivity of natural antioxidants from plant extracts is well known. Still, the effectiveness of these natural antioxidants, namely polyphenols, depends on preserving their stability, which can be increased by microencapsulation. The aim of this study was to protect natural antioxidants from the aqueous extract of Portuguese wild herb *Pterospartum tridentatum* by encapsulation in alginate hydrogel microbeads. Microbeads were prepared by electrostatic extrusion technique: plain Ca-alginate microbeads and Ca-alginate microbeads with 10 and 20 mass% of inulin as a filler substance. Total polyphenol content (TPC) and the radical scavenging activity using ABTS and DPPH cations were determined. The release studies of polyphenols from microbeads were performed. The microbeads were analysed by Fourier transform infrared spectroscopy (FTIR), scanning electron microscopy (SEM) and optical microscopy (OM). Encapsulation efficiency (EE) was in the range from 49 to 73%. Antioxidant assays and release studies showed that alginate-inulin microbeads appeared to be suitable dosage forms. The inclusion of inulin contributes to improved microbeads structure, as well as to nutritional values of food. Thereby, potential applications of these microbeads could be functional food products, an increasingly valued market.
Key words: *encapsulation, electrostatic extrusion, antioxidants, Pterospartum tridentatum, alginate*

INTRODUCTION

From the ancient times, herbs and spices were being added to different food and beverages to improve flavour. It has been also shown that bioactive compounds commonly found in herbs contain health benefits because of their significant antimicrobial and antioxidant capacities and anticarcinogeni activities (Cao et al., 1999). Various bioactive compounds, such as alkaloids and flavonoids, have been identified in aqueous extracts of *Pterospartum tridentatum* which is wild herb widely used in traditional medicine and cuisine (Vitor et al., 2004). *Pterospartum tridentatum* L. is a European endemic species belonging to the subfamily *Papilionoideae* (Talavera, 1999) and known as *carqueija* or *carqueja* in Portugal. Some authors refer the use of this herb in popular medicine for colds, stomach aches, intestinal problems, kidney disease, liver and bladder problem. However, most of bioactive compounds are very sensitive to many factors. The effectiveness of these natural antioxidants, namely polyphenols, depends on preserving their stability, which can be increased by microencapsulation.

Microencapsulation is an effective method to protect bioactive components, preserve their stability during processing and storage and prevent undesirable interactions with food matrix (Wandrey et al., 2009, Nedović et al., 2011). Electrostatic extrusion is technique used for encapsulation of the compounds by production of spherical, small and uniform microparticles which are desirable in food applications (Manojlović et al., 2008, Kostić et al., 2012).

The goal of the present study was to develop *P. tridentatum* extract formulations aimed at delivery of bioactive compounds in functional food products. The extract was encapsulated

in alginate and alginate-inulin microbeads by electrostatic extrusion and the obtained microbeads were characterized from the aspect of TPC and antioxidant activity. Besides hydrogel microbeads, freeze-dried forms of microbeads were also assessed, as they are convenient for long-term applications.

MATERIAL AND METHODS

Na-alginate (medium viscosity) was purchased from Sigma. Folin-Ciocalteu, Na-carbonate, Ca-chloride and Na-citrate were of analytical grade and supplied by Sigma-Aldrich (St. Louis, MO, USA). 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)diammonium salt (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), sodium chloride, potassium persulfate were obtained from Sigma -Aldrich (Germany). Inulin was generously gifted from a local milk factory.

Preparation of microbeads

The appropriate amount of *P. tridentatum* aqueous extract (2 mg mL^{-1}) was added to Na-alginate water solution (1,5% w/v) to prepare extract-alginate solution. In addition, extract-alginate solution was mixed with 10 and 20 mass% of inulin to prepare extract-alginate-inulin solutions. Both types of solutions were submerged to electrostatic droplet generation in order to produce alginate and alginate-inulin microbeads entrapping extract compounds as described in the next paragraph.

The obtained solutions were extruded through a blunt stainless steel needle (23 G) at a constant flow rate of 25,2 mL h^{-1}, by a syringe pump (Razel Scientific Instruments, Stamford, CT, USA). The extrusion was performed under an applied electric field between the positively charged needle and grounded collecting solution. The potential difference was controlled by a high voltage unit (Model 30R; Bertan Associates, Inc., New York, USA) and kept at a constant voltage of 7,0 kV. Collecting solution was mixture of *P.tridentatum* extract and Ca-chloride 1,5% (w/v). After ions exchange, alginate droplets formed insoluble hydrogel microbeads with the extract entrapped in. The microbeads were left in the cross-linking solution for 30 min and then used for further analysis (Bugarski et al., 2004). Hydrogel microbeads were observed under optical microscope (Olympus CX41RF, Tokyo, Japan) and average diameter was measured with the image analysis program Cell^{A} (Olympus, Tokyo, Japan).

In order to produce freeze-dried beads, hydrogel microbeads were frozen at −80°C for 1 h before freeze drying, which was carried out at −50°C at a pressure of 30 Pa for 10 min and 10 Pa for 24 h. The surface morphology of carefully freeze-dried microbeads was determined by scanning electron microscopy (SEM, model RESCAN MIRA3XMU, Czech Republic).

Fourier transform infrared spectroscopy (FTIR)

The interactions between the different components of the alginate-inulin systems were analysed by Fourier transform infrared (FTIR) using a FTIR spectrophotometer (BOMEM, Hartmann & Braun). Microbeads samples were dried in a vacuum desiccator, triturated with micronized KBr powder and compressed into discs by pressing the powders. Discs and scanned from 4000 to 450 cm^{-1} at a resolution of 4 cm^{-1} at room temperature.

Determination of total phenol content (TPC)

TPC was determined using the Folin-Ciocalteau reagent, according to a modified method of Lachman et al (1998). In brief, 70 µL of the sample was pipetted into a 15 mL volumetric flask containing 350 µL of Folin–Ciocalteu reagent, 4,2 mL of distilled water and 1,05 mL of 20% (w/v) Na-carbonate, and the volume was made up with distilled water. After 2h, the absorbance of was measured at 765 nm against a blank sample. Gallic acid was used as the standard and the results expressed as mg L^{-1} of gallic acid equivalents (GAE).

Encapsulation efficiency

Encapsulation efficiency (EE%) was calculated as the amount of TPC encapsulated in microbeads (m_b) divided by the TPC of the solution used for the preparation of microbeads (m_s), as shown in equation 1:

$$EE\% = m_b/m_s \times 100 \quad (1)$$

Quantification of TPC in microbeads (m_b) was performed after dissolving microbeads in 2% (w/v) Na-citrate solution (in a weight ratio of 1:5), using a Vortex mixer to chemically dissolve them at room temperature. TPC was determined using the Folin–Ciocalteu method according to the procedure described in the upper section.

Determination of free radical-scavenging ability

The radical scavenging activity of the extract was determined according to Dudonne et al. (2009). The DPPH• solution in ethanol ($6x10^{-5}$M) was mixed with 100 µL sample and after 30 min the decrease in absorbance at 515 nm was measured (A_E). A blank sample contained 100 µL of ethanol (A_B). Percentage of cation inhibition was calculated using equation 2:

$$\% \text{ inhibition} = [(A_B - A_E)/A_B] \times 100 \quad (2)$$

where (A_B) is absorbance of the blank sample and (A_E) is absorbance of the sample.

The free radical scavenging capacity of the extracts was also studied using the ABTS radical assay. ABTS• was produced according to Re et al. (1999). An appropriate solvent blank reading was taken (A_B). Extract solutions (30 µL) was mixed with 3 mL of ABTS• solution and absorbance reading was taken after 6 min (A_E). The percentage of inhibition of ABTS• was calculated using equation 2.

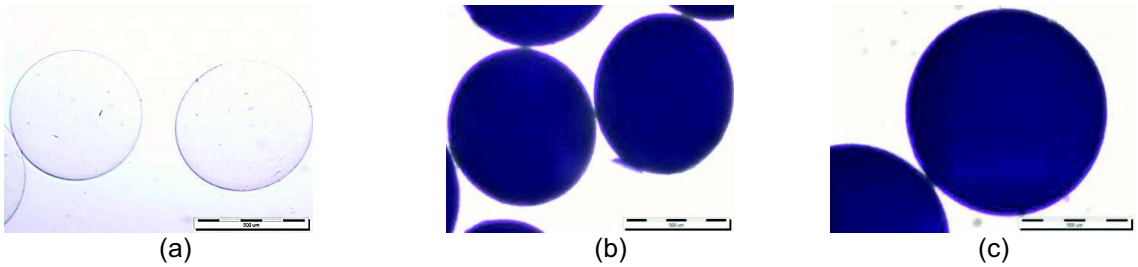
In order to test radical scavenging activity of the extract after microencapsulation, certain amount of hydrogel microbeads with encapsulated extract was suspended in 3 mL of distilled water. The samples were left on an orbital shaker operating at 100 rpm and when the extract was completely released the samples were analysed on TPC, DPPH and ABTS.

Release studies

The release studies of polyphenols from freshly prepared hydrogel microbeads were performed at laboratory conditions. About 5 g of microbeads was suspended in 12,5 mL of distilled water. The samples were submitted to continuous agitation on an orbital shaker operating at 100 rpm (New Brunswick Scientific Co., Inc., Edison, NJ). At defined time intervals (2, 5, 10, 20, 30, 45, 60 min), an aliquot (70 µL) was taken for analysis of TPC.

RESULTS AND DISCUSSION

The obtained hydrogel alginate and alginate-inulin microbeads were analysed by optical microscope. Alginate microbeads encapsulating *P. tridentatum* extract appeared spherical with a quite smooth surface and average diameter of ~500 µm (Fig. 1a). Alginate microbeads with 10 mass% of inulin were slightly distorted from a perfect sphere and they are larger compared to alginate microbeads, ~700 µm (Fig. 1b). Alginate microbeads with 20 mass% of inulin had spherical appearance with the average diameter of ~800 µm (Fig. 1c).



(a) (b) (c)

Figure 1. Photos of alginate microbeads encapsulating *P. tridentatum* extract: (a) alginate microbeads; (b) alginate microbeads with 10 mass% of inulin; (c) alginate microbeads with 20 mass% of inulin.

After the freeze-drying, removed water accounts for 93,9, 86,0 and 78,6% of the initial weight for alginate, alginate with 10 mass% and 20 mass% of inulin, respectively. Microbeads treated by freeze-drying process were analysed by SEM. SEM was used to give information about features of the surface and the influence of inulin on surface morphology. Freeze-drying damaged the walls of alginate microbeads so they got irregular shape, with the surface of a spongy texture (Fig. 2 Ia, Ib, Ic). The problem of the gel collapse during freeze-drying process was significantly reduced by addition of inulin. The microbeads with 10 mass% (Fig. 2 IIa, IIb, IIc) and 20 mass% (Fig. 2 IIIa, IIIb, IIIc) of inulin have a rather smooth surface with a preserved round structure. SEM micrographs were also analysed using ImageJ application. The outcomes showed that the average diameter of alginate freeze-dried microbeads was 212,5±32,6 μm. Microbeads with 10 mass% of inulin, had average diameter 553,9±72,3μm and the one with 20 mass% of inulin, 578,8±83,5μm. The average diameters were determinate on a sample of 100 microbeads.

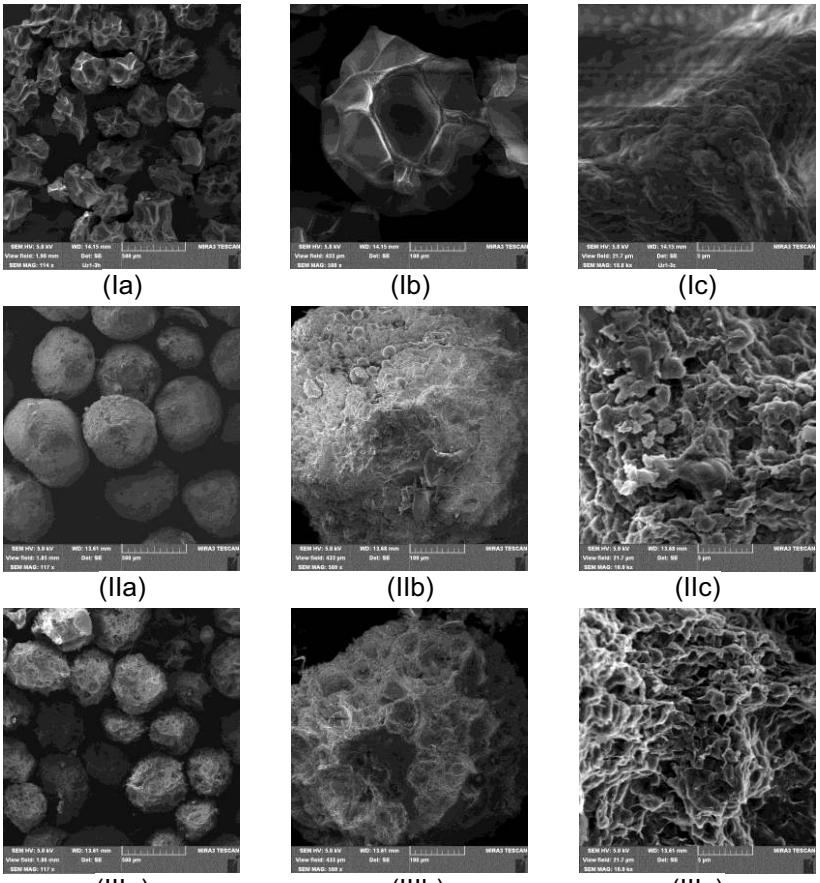


Figure 2. SEM micrographs of the freeze-dried microbeads: (I) alginate microbeads, (II) alginate microbeads with 10 mass% of inulin, (III) alginate microbeads with 20 mass% of inulin, (a) low magnitude, (b) medium magnitude, (c) high magnitude

The results of TPC and EE% for all microbeads are given in table 1. Depending on the addition on inulin, TPC in microbeads ranged from 0,24 to 0,33 mg GAE g_{beads}^{-1} and the highest amount of TPC was detected in alginate microbeads with 20 mass% of inulin. The results indicate that this type of microbeads has the highest encapsulation capacity. This can be explained by reduction of pore size of alginate in the presence of fillers (Rassis et al., 2002). In that way prevention of leakage of the encapsulated compounds was achieved.

Table 1. TPC, DPPH and ABTS of *P. tridentatum* extract and TPC, EE%, DPPH and ABTS of *P. tridentatum* extract encapsulated in hydrogel microbeads

SAMPLE	TPC (mg GAE g_{beads}^{-1})	EE(%)	DPPH(%)	ABTS(%)
Fresh extract	0,35[a]	/	57,5	47,7
Alginate microbeads	0,24	49,0	33,6	30,3
Alginate microbeads with 10 mass% of inulin	0,30	63,8	39,5	31,5
Alginate microbeads with 20 mass% of inulin	0,33	73,8	40,7	34,6

[a]*The unit is mg GAE mL^{-1}*

In this study, the antioxidant capacity of microbeads was determined by two analytical assays: (a) by ABTS radical cation ($ABTS^{*+}$) decolourization assay and (b) by DPPH radical photometric assay. The results are expressed as the percentage of radical inhibition and compared to antioxidant activity of the fresh extract (Tab. 1). The inhibition of the DPPH• radical with fresh extract (2 mg ml^{-1}) was around 57% and the inhibition of ABTS• was 47%. Upon encapsulation, antioxidative activity of extract compounds was preserved at a high level, as confirmed by both assays and alginate microbeads with 20 mass% of inulin showed the highest antioxidative potential.

FTIR was used to identify functional groups and characterise the relationship between the matrix and the extract components. In spectra of alginate freeze-dried microbeads (Fig.3) the strong and broad absorption band has been observed at 3430 cm^{-1} due to –OH stretching. This peak is slightly wider and moved to 3427 and 3417 cm^{-1} for samples with 10 and 20 mass% of inulin, respectively, due to overlap with the corresponding band in the case of inulin, located at 3380 cm^{-1} (pure inulin spectrum is not shown). The strong asymmetric stretching absorption band at 1625 cm^{-1} and weaker symmetric stretching band near 1440 cm^{-1} appeared due to the presence of carboxylate anions COO− (Singh et al., 2009; Vijaya et al., 2008). The weak peak at 2920 cm^{-1} becomes stronger with increase in the amount of inulin, because this peak is related to the $–CH_2$ groups present in both, alginate and inulin. Similarly, the band at 1030 cm^{-1} becomes stronger and sharp, due to the C-O-C stretching of inulin (Fares et al, 2011). There are some changes in the peak intensity in the range of 900-1500 cm^{-1} between FTIR spectra of the native (not shown) and extract-encapsulating microbeads. The results of FTIR analyses indicate the absence of chemical interactions between extract compounds and alginate, thus it can be deduced that alginate hydrogel is a compatible material for encapsulating biochemical active compounds extracted from plants.

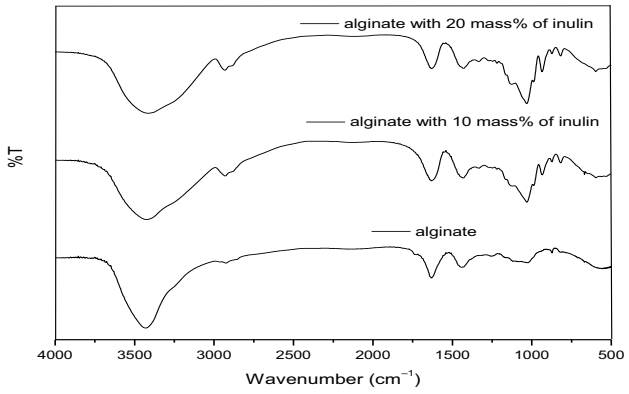


Figure 3. FTIR spectra of microbeads with encapsulated *P. tridentatum* extract

The release profiles for all three types of microbeads encapsulating *P. tridentatum* extract are shown on Fig. 4. The polyphenolic compounds were released after 30-40 min and the presence of inulin enabled better retention of polyphenols, especially in the first few minutes. For sake of comparison, two minutes after sinking microbeads in water, almost 90% of polyphenols was already released from alginate microbeads, while at the same time, about 30% was still captured within the alginate matrix containing 20 mass% of inulin.

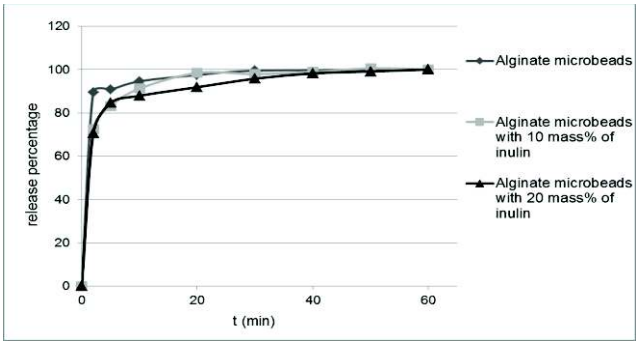


Figure 4. Release profiles of polyphenols from freshly prepared microbeads encapsulating *P. tridentatum* extract.

CONCLUSIONS

Encapsulation of aqueous *P. tridentatum* extract within alginate and alginate-inulin microbeads has been assessed. The obtained microbeads displayed significant polyphenol content. The best results were achieved with alginate-inulin microbeads, as they were treatable by freeze-drying. They were also richer in polyphenols and they protected the extract from release better than plain alginate forms. The antioxidant activity of the extract was preserved after microencapsulation at a high level, especially in case of alginate microbeads with 20 mass% of inulin.

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