LWT - Food Science and Technology 95 (2018) 129–134

Contents lists available at ScienceDirect

LWT - Food Science and Technology

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

Functional evaluation of microencapsulated anthocyanins from sour cherries skins extract in whey proteins isolate

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ARTICLE INFO

Keywords:
Microencapsulation
Anthocyanins
Whey proteins
Probiotics
Antioxidant activity

ABSTRACT

Sour cherries skins anthocyanins extract was encapsulated in whey proteins isolate, with an encapsulation efficiency of 70.30 ± 2.20%. The powder showed a satisfactory content in polyphenols, of which anthocyanins of 31.95 ± 0.65 mg CGE/100 g DW and flavonoids of 3.58 ± 0.73 mg CE/100 g DW. Confocal laser scanning microscopy showed 1–3 μm microparticles clustering into large coacervates ranging in size between 30 and 50 μm. The *in vitro* digestibility results suggested that whey proteins protected the anthocyanins from the gastric digestion, their release being facilitated into the intestine. The powder also stimulated the growth of *Lactobacillus casei* 431®. After 21 days of storage, the number of viable cells in the control decreased with almost 78%, in comparison with the enriched fermented milk, where a reduction with only 26% of viable cells was observed. Both the extract and the powder displayed a significant antioxidant activity, supporting the potential of using them as functional ingredients in food or nutraceuticals.

1. Introduction

The interest for the use of natural colorants as alternatives to synthetic dyes in food products and derivatives has gained a great importance in our days, both because of legislative requirements and consumer preferences. There is a wide growth in scientific data regarding the potential role of anthocyanins from different sources in nutraceutical and health benefits, which is given namely by the antioxidant activity. Anthocyanins are water-soluble pigments with antioxidant and biological properties that combat neurodegenerative diseases, diabetes, angiocardiopathy, cancer, inflammation and senium (He et al., 2017; Li et al., 2012). Sour cherry (*Prunus cerasus* L) skins are significant sources of anthocyanins, which potentially display a broad spectrum of health-promoting benefits. It has been reported that consumption of sour cherry anthocyanins increased the level of superoxide dismutase in serum, reduced the level of tumor necrosis factor-α, interleukin-6 and malondialdehyde in serum and PGE-2 in the paw of Freund's adjuvant-induced arthritis in rats (He et al., 2006, 2005). In the food industry, the sour cherries are processed in different products, such as juice, jelly, jam, marmalade, etc. However, the stability of anthocyanins depends on a combination of the environment and chemical factors such as pH, metal ions, exposure to light, temperature, oxygen, and enzymatic activities (Akhavan Mahdavi, Mahdi Jafari, Assadpoor, & Dehnad, 2016; Jafari, Mahdavi Khazaei, & Hemmati Kakhki, 2016; Khazaei, Jafari, Ghorbani, Hemmati, & Kakhki, 2014). Therefore, the food industry faces great challenges in using these fruits as a source of natural colorants in processed foods. Liu et al. (2014) suggested that anthocyanins can be directly absorbed by small intestinal epithelial cells, although it is difficult to transfer anthocyanins to the intestinal tract due to their instability in the adverse gastrointestinal environment (Gonzalez-Barrio, Borges, Mullen, & Crozier, 2010). Therefore, the challenge now is to protect these molecules from deterioration and to increase their bioavailability (He et al., 2017).

Encapsulation technology has been used in the food industry to protect the core material from adverse environmental conditions, such as undesirable effects of light, moisture, and oxygen, thereby contributing to an increase in the shelf life of the product, and promoting a controlled delivery of the encapsulate (Murali, Kar, Mohapatra, & Kalia, 2015; Shahidi & Han, 1993). Freeze drying is a suitable method for microencapsulation of heat-sensitive compounds, which are unstable in aqueous solutions and volatile flavors (Murali et al., 2015). The method was applied for microencapsulation of sour cherries extract rich in anthocyanins using a mixture of whey proteins isolate (WPI) and acacia gum as the shell material. The probiotic strain, *L. casei* 431®, was used

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https://doi.org/10.1016/j.lwt.2018.04.083
Received 13 January 2018; Received in revised form 30 March 2018; Accepted 27 April 2018
Available online 30 April 2018
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as a starter in the formulation of a fermented milk product enriched with the microencapsulated anthocyanins. Probiotics are defined as live microorganisms that have the property to confer beneficial effects on the host when consumed in adequate amounts (Sanders, 2003). According to Samona and Robinson (1994), the probiotic effect manifests only if the carrier food contains at least 10^6 CFU g^{-1}. Weekly, it is recommended the consumption of at least 300–400 g of probiotic food per consumer (Samona & Robinson, 1994).

In this study, the anthocyanins from sour cherries skins extract were microencapsulated in whey proteins isolates and acacia gum by coacervation and freeze-drying technique. The obtained powder was characterized in terms of encapsulation efficiency, phytochemicals, color, antioxidant activity and *in vitro* digestibility. The microstructure of the particles was analyzed by confocal scanning laser microscopy. Further, the powders were incorporated into fermented milk and the effect of the microencapsulated anthocyanins to act as a prebiotic for the *L. casei* 431® probiotic strain was tested.

2. Materials and methods

2.1. Chemicals and bacterial strain

Whey proteins isolate (protein content 95%) was purchased from Fonterra (New Zeeland). 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), ethanol, ethyl acetate, hexane and methanol (HPLC grade), sodium hydroxide, Ciocalteu phenol reagent and gallic acid were obtained from Sigma Aldrich Steinheim, Germany.

The probiotic strain *Lactobacillus casei* (*L. casei* 431®) was provided by Chr. Hansen, Denmark, as a freeze-dried commercial starter.

2.2. Sour cherries fruits and anthocyanins extraction

Sour cherries (*Prunus cerasus* L) were purchased from the local market (Galați) in June–July 2016. Fruits samples were washed and the skins and seeds were manually separated from the pulp, washed with distilled water and then blotted on paper towels to remove any residual pulp. Skins were freeze-dried and stored at a temperature of–20 °C until analyses. The extraction of anthocyanins from freeze-dried sour cherry skins was performed according to a previously described procedure, where the anthocyanins were extracted using 70% ethanol. Briefly, 1 g of freeze-dried sour cherry powder was extracted in 8 mL of 70% ethanol for 4 h on an orbital shaker. The mixture was further centrifuged at 9000 rpm for 10 min at 4 °C and the supernatant was collected and concentrated until dryness (AVC 2–18, Christ, UK). The extract was obtained by adding 10 mL of MilliQ water to 1 g of extract (Oancea, Turturică, Bahrim, Râpeanu, & Stănciuc, 2017a, 2017b).

2.3. Microencapsulation of anthocyanins

A stock protein solution (5% protein, w/w) was prepared by dissolving of WPI powder in deionized water. The WPI solution was allowed to stand overnight under stirring at room temperature to allow complete hydration. The WPI solution was mixed with 2% acacia gum solution and the pH was adjusted at 7.0 with 1 M NaOH. Further, 8 mL of sour cherries skin extract was added to the WPI-acacia gum mixture and the resulting solution was stirred for 2 h at 500 rpm and room temperature. Finally, the pH of the solution was adjusted at 3.5 using 1 M HCl. The sample was further frozen and stored at −70 °C. After that, the ice crystals were removed by freeze-drying (CHRIST Alpha 1–4 LD plus, Germany) at −42 °C under a pressure at 0.10 mBar for 48 h. Afterwards, the powder was collected and packed in metalized bags and kept in a freezer at −20 °C for later analyses.

2.4. Powder characterization

The methods previously described by Oancea et al. (2017a, 2017b) were used in this study for the encapsulation efficiency, extract and powder phytochemicals and color analysis, antioxidant activity and *in vitro* digestibility.

For the total anthocyanin content (TAC) the AOAC official method was used and the TAC content was expressed as mg of cyanidin 3–glucosides equivalents (CGE) per 100 g dry weigh (DW). The total phenolic content (TPC) was determined using a slightly modified Folin – Ciocalteu colorimetric method and the content was expressed as mg gallic acid equivalents (GAE) per 100 g DW. The total flavonoid content was determined using a modified version of the colorimetric method previously described by Dewanto, Wu, Adom, and Liu (2002), where 0.250 mL of extract was mixed with 1.25 mL of distilled water and 0.075 mL of 5% $NaNO_2$ and kept at room temperature to react for 5 min. Afterwards, 150 mL of 10% $AlCl_3 \cdot 3H_2O$ solution was added and the mixture was let to react for another 6 min. Finally, 0.5 mL of 1 M NaOH and 0.775 mL of deionized water were added and the absorbance was immediately measured at 510 nm. The results were expressed as mg catechin equivalents (CE) per 100 g DW.

The DPPH free radical scavenging activity (DPPH RSA) of the powder was determined by mixing 100 µL of extract with 3.9 mL of 0.06 mM DPPH methanolic solution. The mixture was kept in the dark for 60 min and the decrease in the absorbance was measured at 517 nm. The antioxidant activity was expressed as percentage of DPPH RSA and also as µmol Trolox equivalents (TE) per mg DW.

The total anthocyanin content (TAC) and the surface anthocyanin content (SAC) were carried out in order to further evaluate the encapsulation efficiency. To determine the TAC, 200 mg of microcapsules were mixed with 2 mL of methanol: acetic acid: water (50: 8: 42 v/v/v) and ultrasonicated for 20 min. For the SAC, 200 mg of microcapsules were simply washed with ethanol: methanol (1: 1 v/v) in a vortex for 1 min. Both, TAC and SAC mixtures were centrifuged at 9000 rpm for 5 min at 4 °C. The clear supernatant was collected and the quantification of anthocyanins was carried out according to the pH-differential method described by AOAC (2005). Encapsulation efficiency (% EE) was calculated according to Eq. (1):

$$\%EE = \frac{(TAC - SAC)}{TAC} \times 100 \quad (1)$$

The color measurements were performed using a Miniscan XE Plus 4500 L colorimeter (Hunter-Lab, Germany) where the sample was placed above the light source and the *L* (whiteness or brightness/darkness), *a* (redness/greenness), and *b* (yellowness/blueness) values were recorded.

For the *in vitro* digestion, the microcapsules were mixed with Tris-HCl buffer (10 mM, pH 7.5) at a ratio 10: 1 mg per mL buffer. The simulated gastric juice (SGJ), consisting of porcine pepsin (40 mg/mL in 0.1 M HCl) was added to the microcaspsules-Tris-HCl mixture in a ratio of 2: 1 and the pH was adjusted at 2.0 with 6 M HCl. Regarding the enteric digestion step, the simulated duodenal juice (2 mg pancreatin per mL 0.9 M sodium bicarbonate) and the mixture from the SGJ step were mixed in a ratio of 1:1 and the pH was adjusted to 7.0 with 0.1 M NaOH. During each step of the *in vitro* digestion, the samples were incubated for 2 h in an SI e 300R orbital shaking incubator (Medline Scientific, UK), at 100 rpm and 37 °C, and the TAC of the samples was measured every 30 min.

2.5. Confocal laser scanning microscopy

The anthocyanins from sour cherries, encapsulated in whey protein isolate - acacia gum (WPI-AG) mixture by freeze-drying, were studied by confocal laser scanning microscopy. To obtain a clear view of the particles microstructure details, a Zeiss confocal laser scanning system (LSM 710) equipped with a diode laser (405 nm), Ar-laser (458, 488,

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