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ENCAPSULATION OF ANTIOXIDANT PHENOLIC COMPOUNDS IN ZEIN ULTRA-THIN FIBERS VIA ELECTROSPINNING

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ABSTRACT

Phenolic compounds are of great interest for the food industry, in particular due to their antioxidant capacity. Nevertheless, their relevance as bioactive substances is often hindered by poor stability and solubility. Phenolic compounds can be encapsulated for better maintaining their bioactivity. In this work, we explore an alternative for the encapsulation of phenolics using zein (Z), a food-grade biopolymer, as the carrier material. In particular, gallic acid (GA) and naringenin (NAR) were homogeneously incorporated in ultrathin zein fibers by means of a simple one-step electrospinning process. Morphology, cargo stability and cargo-carrier molecular interaction were studied. The phenolics release behavior was analyzed in aqueous media at different pH conditions. Pure Z fibers present a ribbon-like structure of variable dimensions, characteristically ranging between 230 – 396 nm in width up to 0.8 µm. Incorporation of the antioxidants did not visibly affect this morphology. Loading values were 4.93 ± 0.15% (GA) and 5.12 ± 0.60% (NAR). Phenolic loadings remained stable for the period observed (~3 months) at room storage conditions. Release studies revealed a burst release trend with a cumulative release threshold minimum for pH 2 and maximum for pH 7. Results show that this is a promising approach for phenolic compounds encapsulation.

KEYWORDS: nanoencapsulation, phenolic compounds, antioxidants, zein, electrospinning, nanofibers.

ENCAPSULACIÓN DE COMPUESTOS FENÓLICOS ANTIOXIDANTES EN FIBRAS ULTRAFINAS DE ZEÍNA A TRAVÉS DE ELECTROSPINNING

RESUMEN

Los compuestos fenólicos son de interés en la industria alimentaria debido a su capacidad como antioxidante. Sin embargo, su relevancia como bioactivos es frecuentemente limitada por sus bajas estabilidad y solubilidad. Estos pueden ser encapsulados para conservar mejor su bioactividad. En este trabajo exploramos una alternativa de encapsulación de fenólicos usando zeína (Z) como material vehículo. Ácido gálico (GA) y naringenina (NAR) fueron incorporados homogéneamente en fibras ultrafinas de zeína por un proceso simple de *electrospinning*. Se estudió la morfología, estabilidad e interacción cargo-vehículo y se analizó la liberación de los compuestos en medio acuoso a diferentes pH. Las fibras de Z

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presentaron forma de cinta de dimensiones variables, de ancho típicamente entre 230 y 396 nm, hasta 0,8 µm, que no se modificó por la incorporación de los antioxidantes. Las cargas fueron de 4,93 ± 0,15 % (GA) y 5,12 ± 0,60 % (NAR), que permanecieron estables por 3 meses en almacenamiento a temperatura ambiente. Los estudios de liberación revelaron una rápida liberación con un límite de compuesto liberado dependiente del pH (mínimo a pH 2 y máximo a pH 7). Los resultados demuestran que este es un enfoque promisorio para encapsulación de antioxidantes.

PALABRAS CLAVE: nanoencapsulación, compuestos fenólicos, antioxidantes, zeína, *electrospinning*, nanofibras.

ENCAPSULAÇÃO DE COMPOSTOS FENÓLICOS ANTIOXIDANTES EM FIBRAS ULTRAFINAS DE ZENIA A TRAVES DE ELECTROSPINNING

RESUMO

Os compostos fenólicos são de interesse na indústria da alimentação dividido a sua capacidade como um antioxidante. No entanto, a sua relevância como bioativos é frequentemente limitada pela sua estabilidadee solubilidade baixas.Os compostos fenólicos podem ser encapsulados para preservar melhor a sua bioatividade. Em este trabalho, vamos explorar uma alternativa de encapsulação de fenólicos sando zeína (Z) como material de suporte. Ácido gálico (GA) e naringenina (NAR) foram incorporados homogeneamente em fibras ultrafinas de zeina por um simples processo de electrospinning. Se estudou sua morfologia, a estabilidade e a interação cargo-veículo e se analisou a libertação dos compostos em meio aquoso a diferentes pH. As fibras de Z apresentaram forma de fitas de dimensões variáveis, de largura tipicamente entre 230 e 396 nm, até 0,8 µm, que não se modificou pela incorporação de antioxidantes. As tensões foram de 4,93 ± 0,15% (GA) e 5,12 ± 0,60% (NAR), mantendo-se estáveis durante 3 meses de armazenamento a temperatura ambiente. Os estudos de libertação revelaram uma libertação rápida com um limite de composto liberado dependente do pH (mínimo a pH 2 e máximo a pH 7). Os resultados mostram que esta é uma abordagem promissora para a encapsulação de antioxidantes.

PALAVRAS-CHAVE: nanoencapsulação, compostos fenólicos, antioxidantes, zeína, electrospinningnanofibras.

1. INTRODUCTION

Phenolic compounds are more than eight thousand naturally occurring substances with different bioactive functionalities, usually found in plant and plant products (Harborne, *et al.*, 2000). Flavonoids are the largest group of plant phenolics and the variations in their structure result in the major flavonoid classes: flavonols, flavones, flavanones, flavanols, isoflavones and anthocyanidins. Phenolic acids constitute another important class of phenolic compounds that can be classified as hydroxybenzoic and hydroxycinnamic acids, according to their structure.

Phenolics are considered among the largest contributors to the antioxidant potential of plant-origin food products (Larson, 1998). Probably the most largely described feature of almost all the groups of phenolic compounds is their antioxidant capacity. This is due to their redox properties, which play an important role in scavenging free radicals and oxygen species or decomposing peroxides (Nijveldt, *et al.*, 2001); such properties varies from one specific polyphenol to the other, and are related to their chemical structure (Foti, *et al.*, 1996; Natella, *et al.*, 1999; Silva, *et al.*, 2000; Heim, *et al.*, 2002).

Polyphenols are currently the major group of interest amongst plant-origin bioactive compounds (Cowan, 1999; Aridogan, *et al.*, 2002; Belščak-Cvitanović, *et al.*, 2011; Gou, *et al.*, 2011). In the food industry, phenolic compounds have gained great attention as possible functional ingredients in food formulations because they have been claimed to have abilities to promote human health, e.g., reduction in the incidence of some degenerative diseases including cancer and diabetes (Conforti, *et al.,* 2009; Kim, *et al.*, 2009), reduction in risk factors of cardiovascular diseases (Jiménez, *et al.,* 2008), anti-mutagenic and anti-inflammatory effects, etc. (Parvathy, *et al.*, 2009).

The presence and distribution of functional groups in their molecules (e.g., hydroxyl, carbonyl, amide, aromatic rings, carbon-hydrogen skeleton of sugars,

etc.) define the hydrophobicity of phenolic compounds, which is critical for many of the typical biological roles of polyphenols (including antimicrobial activity and oxidation-inhibitor capacity) (Haslam, 1996). There are a wide range of water-solubility values for each class of phenolic compounds and, although in the natural state polyphenol-polyphenol interactions usually ensure some minimal solubility in aqueous media, some plant polyphenols may be difficulty soluble in water (Haslam, 1996) and, regretfully, a low water and poor bioavailability are limiting factors for their use as bioactive agents (Shulman *et al.,* 2011). Moreover, most of the phenolic compounds with proven bioactivities are quite reactive, and therefore labile at some extent towards a number of environmental factors. The relevance of phenolic compounds as bioactive agents can therefore be limited by their low solubility and stability.

In order to preserve the structural integrity, phenolics need to be sheltered by a finishing formulation able to protect them and deliver them to the physiological targets without losing any bioactivity (Fang and Bhandari, 2010; Munin and Edwards-Lévy, 2011).

A valid way for stabilization and encapsulation of phenolic compounds is electrospinning (Fernández, *et al.*, 2009; Li, *et al.*, 2009; Neo, *et al.*, 2013b). Electrospinning is a straightforward technique that consists in the application of intense electrical fields to polymer solution for inducing the formation of a Taylor's cone; this causes a continuous drawing of the solution from a syringe needle towards a collecting surface that has been previously connected to ground. During the drawing process the solvent is evaporated and randomly oriented ultra-thin fibers, which often exhibit thicknesses within the nanoscale (1 - 100 nm), are collected in the form of macroscopically homogeneous membranes on the collector. The process is quick and takes place at room temperature, which is optimal for avoiding temperature-induced degradation. Moreover, the nonwoven electrospun membranes have high surface area-to-volume ratios and porosities, and the polymer features might rapidly exhibit changes in response to external stimulus; all these characteristics are desirable for delivery systems of therapeutic agents. Indeed, Shen, *et al.* (2011) suggested that incorporating bioactive compounds in ultrathin fibers by this technique allows the improvement and enhancement of their functionalities thanks to the nano-scale effects.

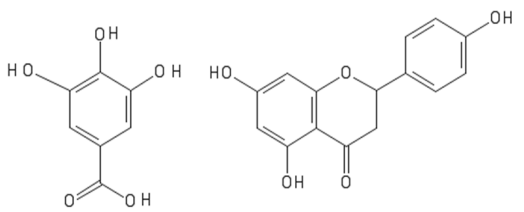
In this work, two types of highly antioxidant phenolic compounds of very different hydrophobicity, namely gallic acid (GA) (phenolic acid, water-solubility: ~1.4 x 10^4 mg/kg at 23°C) and naringenin (NAR) (flavanone, poorly water-solubility: ~1.6 x 10^1 mg/kg at 23°C), are encapsulated by blend electrospinning in ultrafine fibers made of zein (Z) a hydrophobic protein extracted from corn maize. The stability and morphology of the systems is studied along with the type of possible interactions cargo-carrier; the release of the antioxidants in aqueous media is studied at different pH conditions.

2. MATERIALS AND METHODS

2.1 Chemicals

Gallic acid, naringenin and zein (from maize, Z3625 CAS 9010-66-6) were purchased from Sigma Aldrich (Milan, Italy). Chemical structures of GA and NAR are shown in **Figure 1**. With regard to zein, there are currently four classes of zein recognized: α, β, γ, and δ. These classes are expressed sequentially in maize and are found to interact with each other for stability. Zein from corn was reported to be approximately 35% α-zein, which includes 2 prominent bands of 22 and 24 kDa. β-zein fails to enter an SDS-PAGE gel without reduction. Reducing SDS-PAGE analysis shows that β-zein has 3 major bands of 24, 22, and 14 kDa (Esen, 1986). The amino acid sequences of this zein have been published (Phillips and McClure, 1985). Ethanol (absolute), citric acid monohydrate, sodium citrate, sodium chloride, potassium biphosphate, potassium phosphate, were purchased from Sigma-Aldrich (Milan, Italy) or Fluka analytical (Spain). For the release experiments, naringenin solubility studies and analytical determinations of the antioxidants concentration, citrate buffer (0.1 M; pH 2.0, adjusted with HCl and 0.1 M pH 4.5) and phosphate buffer (0.1 M; pH 7.0 and pH 8.0) were used. In every case double distilled water was used; chemicals and solvents were used without any further purification protocol.

Figure 1. Chemical structures of (*left*) gallic acid (GA) and (*right*) naringenin (NAR).



2.2 Preparation of the phenolic-loaded zein nanofibers by electrospinning

Gallic acid-zein and naringenin-zein ethanolic solutions for production by electrospinning of Z-GA and Z-NAR encapsulation systems were prepared as described by Neo, *et al.* (2013b) with some modifications. The polymer solutions were prepared by dissolving zein (25% w/w) in a hydroalcoholic solution (ethanol:water 4:1) containing the previously dissolved desired amount of gallic or naringenin (in such a way for obtaining 5% w/w of the phenolic with respect to electrospun material, i.e. of dry matter), under constant stirring at room temperature. Solutions were placed in a 10 mL syringe with a metallic needle and put in a KDS 100 (KD-Scientific, USA) syringe pump. The needle was connected to a high voltage generator (Spellman SL150) by an alligator clip. Applied voltage was 16 kV and flow rate of electrospinning solution was 0.5 mL/h and the collection time was 1 h. A foil-covered copper tray located at 12 cm from the needle tip was used as a collector for the electrospun fibers. The electrospinning set up and process is schematized in **Figure 2**. After electrospinning, the fibers (still attached to the foil) were taken off and then left to dry at room conditions, before storing them in plastic envelopes.

2.3. Morphology of the fibers

The fiber morphology of the electrospun materials was studied by Field-Emission Scanning Electron Microscopy (FE-SEM). FE-SEM microphotographies were acquired by a FE-SEM ULTRA 55-44-22, evaluated by secondary (SE2) and backscattered electrons (AsB) detectors. Samples were coated with platinum and examined at 5 kV. SEM images were obtained at the facilities of Universidad Politécnico de Valencia (Valencia, Spain), thanks to the kind support of the Center for Molecular Recognition – IDM and Food Research and Innovation Group.

2.4. Fourier transform infrared spectroscopy (FT-IR)

FT-IR Spectrometer (Spectrum 100, PerkinElmer) with an universal attenuated total reflectance (ATR) accessory, featuring a single-reflection sampling plate with a 1.8 mm round germanium surface, was used to qualitatively study the interaction between the phenolics and the polymeric matrix (zein) in the encapsulation systems. The spectra of the pure phenolic compounds (powders), the neat zein fibers and the phenolic-zein composites was recorded between 600 and 3600 cm^{-1}, with a resolution of 1 cm^{-1} and averaged over 10 scans, without any sample pretreatment. Spectrum 6.0 software was used for data acquisition and analysis.

2.5. Loading value, loading efficiency and stability during storage

For evaluating the loading efficiency of the electrospinning processes, a portion of the nanofibrous membrane was weighed (typically from 10 to 30 mg), dissolved in 10 mL ethanol (for assuring complete dissolution of both cargo and carrier), stirred for 3 h and sonicated for 10 min. The concentration was then determined as follows:

For gallic acid: An amount of the dissolution was diluted properly in a pH 7 buffer and its concentration was determined by means of cyclic voltammetry. Current was measured for applied potentials from 0 to 1 V with screen-printed electrodes DS 410 (DropSens, Spain), consisting in a cobalt-phtalocyanine/carbon working electrode, carbon counter electrode and silver reference electrode. The resulting voltammograms of GA at the pH 7 buffer conditions present two oxidation peaks nearby 0.15 V and 0.4 V; assessment was done by measuring the

oxidation current at 0.4 V and correlating it with previously built calibration curves at the same conditions.

[Calibration curve (at pH 7 buffer): *current at 0.4 V* (A) = 3.52 x 10^{-7} * *GA concentration* (mg/L) + 2.40 x 10^{-7}; linearity range: 0.5 - 8 mg/L; R^2 = 0.999].

For naringenin: An amount of the dissolution was diluted properly in ethanol and its concentration was determined by means of UV-Vis spectrophotometry. The absorbance spectra of the solution was recorded for the wavelength range from 800 nm to 200 nm (UV-VIS Carry 100 BIO). In ethanol, naringenin presents an absorbance peak at 288.7 nm; assessment was done by measuring the absorbance of the peak and correlating it with previously built calibration curves.

[Calibration curve: *absorbance* = 0.131 * *NAR concentration* (mg/L) – 0.275; linearity range: 3 – 25 mg/L; R^2 = 0.991]

The corresponding value of phenolic amount assessed in the membrane was then reported to the corresponding nanofibrous membrane weight for determining the *loading value* (e.g., g of compound/g of nanofibrous material, or g of compound/g of zein). The percentage loading efficiency of the electrospinning process is defined as: *[experimentally determined loading value / theoretical loading value] x 100*; where the *theoretical loading value* is given by the proportional amount of compound and zein in the electrospinning solution prepared. The membranes were kept inside plastic Petri dishes at room temperature (~24°C) and the loading value was determined at the end of a 3 months storage time. All the results were obtained with at least 4 replicates.

2.6. Release studies of GA and NAR at different pH

Cumulative release [mg of phenolic / g of nanofibers] was calculated as follows:

$$cumulative\ release = C_w \times \frac{V}{m}$$

Where C_w is the concentration of the phenolic compound in the releasing aqueous medium at any given time [e.g, mg of phenolic / ml of release medium]; V is the total release medium volume [ml] and m is the weight of nanofibers [g]. For the release behavior description and discussion, the percent cumulative release was preferred. Since the maximum cumulative release possible is given by the amount of compound encapsulated in the nanofibers, percent cumulative release was calculated as follows:

$$cumulative\ release\ \% = \frac{cumulative\ release}{loading\ value} \times 100$$

In this equation, *loading value* refers to the experimentally determined loading value (see section 2.5).

Determination of C_w for gallic-acic: A proper amount of the Z-GA membranes (typically from 10 to 17 mg), was weighed and placed in a beaker containing 40 mL of the corresponding buffer (releasing medium), at pH 2.0, 4.5, 7.0 for Z-GA, gently stirring. A screen-printed sensor allowed to record cyclic voltammograms (0 – 1 V) (as explained above in section 2.4) directly in the releasing media, after 1, 3, 5, 7, 9, 11, 26, 39 and 53 min; oxidation peak currents were then correlated to the gallic acid concentrations in the releasing medium (C_w) by using pH-specific calibration curves.

Determination of C_w for naringenin: A proper amount of the Z-NAR membranes (ranging typically 1 to 3 mg) was weighed and placed in a glass containing 50 mL of the corresponding buffer (releasing medium), at pH 2.0, 4.5, 7.0, with gentle stirring. After times of 1, 3, 5, 7, 25, 45, 52 min, a 1 mL sample of the releasing medium was taken, its absorbance spectra was recorded from 800 to 200 nm and, immediately after, it was re-added to the releasing medium for minimizing losses or dilution/concentration-effects. Naringenin presents absorbance peaks at 287.7 nm at pH 2.0, 287.9 nm at pH 4.5 and 321,6 nm at pH 7.0; assessment was done by measuring the absorbance of the peak and correlating it with previously built pH-specific calibration curves. Note: since solubility in water for this compound is very low (which was determined to be 16 ± 1.3 mg/L in water at 23°C), it is worth to say that attention was paid in order to guarantee that maximum naringenin concentrations (C_w) were well below the naringen solubility, in order to ensure bulk release conditions.

All the release experiments were done at least by duplicate.

3. RESULTS AND DISCUSSION

3.1 Production and morphology of Z-GA and Z-NAR nanofibrous encapsulation systems

At the electrospinning working conditions it was possible to obtain pure zein electrospun mats that macroscopically looked smooth of a slight pale-yellow color, but appeared whiter compared to zein powder. A brief scheme of the electrospinning set up and process is presented in **Figure 2**. SEM pictures showed that these mats are formed by randomly-oriented bead-free ribbon-like fibers in the sub-micron scale (**Figure 3a**) with the typical morphology of zein electrospun fibers at the selected working conditions i.e., 25% (w/w) of zein in 80% hydroalcoholic mixtures (w/w) (Yao, *et al.*, 2007). The pure zein fibers displayed smooth surfaces and typical diameters ranging between 230 to 396 nm, although some thicker fibers were observed (even above 0.8 µm).

Under similar electrospinning conditions, it was possible to obtain fibers loaded at 5% (g per 100 g of electrospun mat) with GA and NAR (Z-GA and Z-NAR, correspondingly). The phenolic-loaded electrospun mats showed no difference with respect to the pure zein fibers neither macroscopically nor microscopically (**Figure 3b-c**). The fact that no physical separation or particles from these fibers was observed and that the fibers surface appeared smooth just as the pure polymeric electrospun material, suggests that the incorporation of the phenolic compound occurs homogeneously within the fibrous matrix.

Figure 2. Schematic representation of the electrospinning setup and process: The hydroalcoholic solution of zein and the phenolic compound, contained in a syringe with a metallic needle, is pumped through a syringe pump (a) and a drop is formed in the needle tip. A high voltage is applied to the needle (b). When the electrostatic charge of the solution reaches a critical value, there is the formation of a Taylor's cone in the needle tip, with a consequent ejection of a charged jet of the polymer solution towards a grounded collector (c). The solvent evaporation occurs quickly and solvent-free fibers are collected in the form of nanofibrous membranes or mats (d).

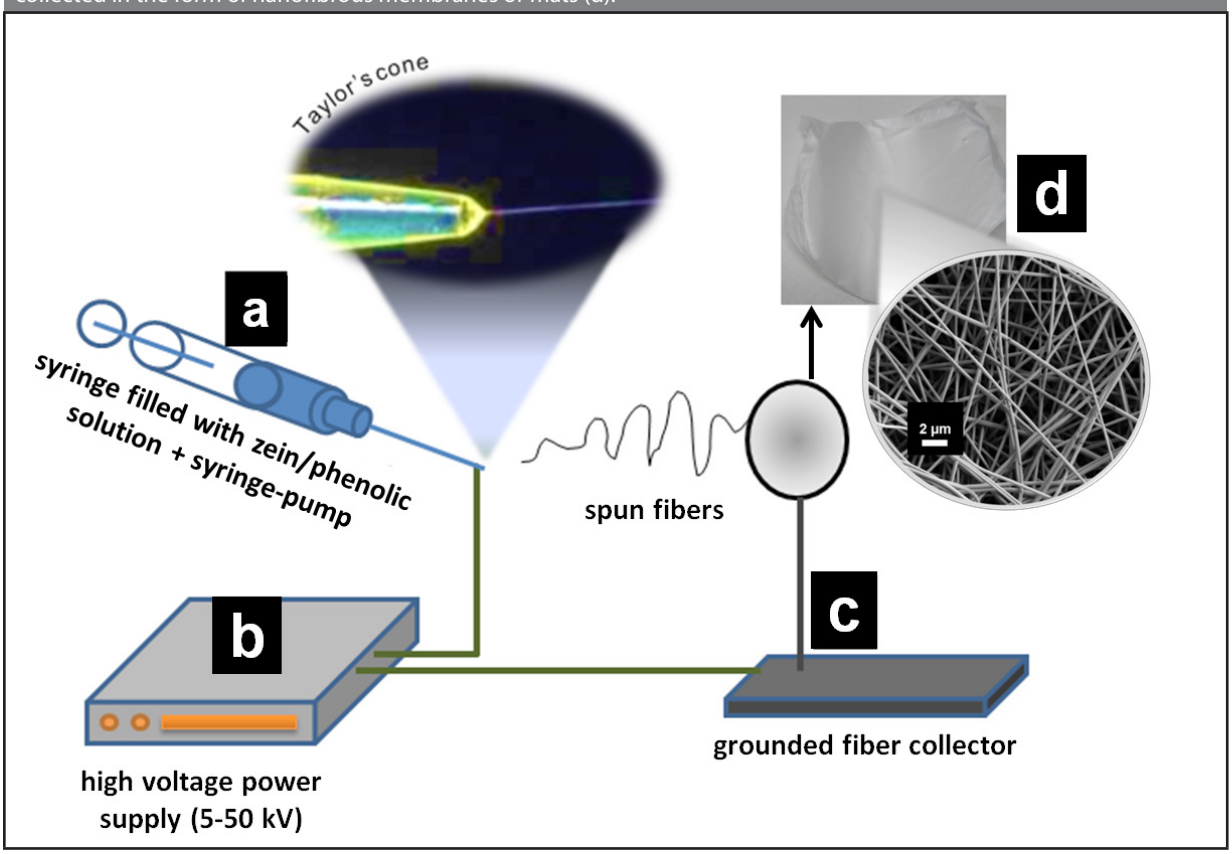
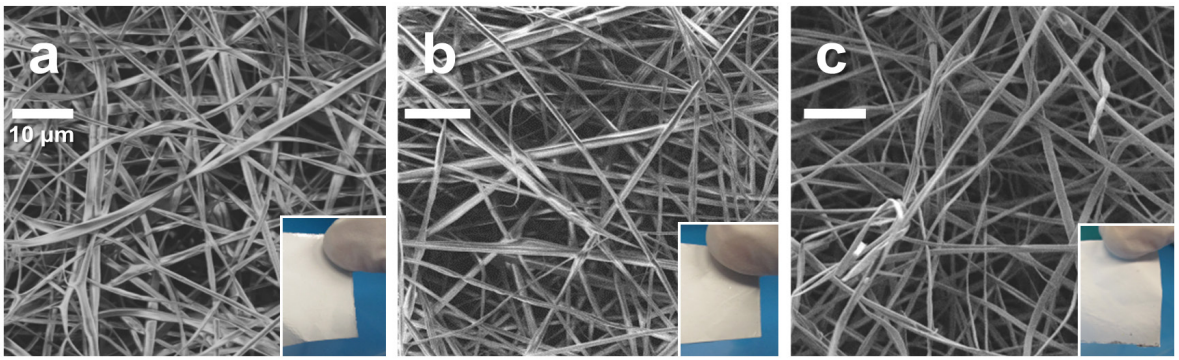


Figure 3. SEM micrographs of (a) pure zein, (b) Z-NAR and (c) Z-GA, electrospun fibers (all the white bars correspond to 10 µm); in the inserts, a photograph with a detail of their macroscopic appearance.



Neo, *et al.* (2012; 2013a) obtained similar results when electrospinning zein-gallic acid blends at these working conditions. Thanks to their observations, it is possible to know that the phenolic compound is distributed within the fibers likely in the form of well-separated aggregates rather similar to amorphous nanoparticles (not as crystals). Albeit for the scope of this work the zein fibers were loaded with 5% of the cargo, it was possible to obtain by this methodology bead-free zein fibers carrying up to 20% a phenolic compound, probably entailing a significant increase of the fiber diameters. Such high loadings are possible because of the high solubility of these type of phenolic compounds in the zein electrospinning solvent, namely ethanol-water mixtures.

3.2. Zein-phenolics interaction

The FT-IR spectra of the pure phenolics in the powder form and the ultra-thin fibers (pure zein and Z-NAR and Z-GA composites), are shown in **Figure 4**. Pure zein electrospun fibers (**Figure 4a**) presents the characteristic peaks of zein at 3298 cm^{-1} (N-H stretching vibrations), 2960 cm^{-1}, 2930 cm^{-1} and 2873 cm^{-1} (C-H stretching vibrations of aliphatic groups), 1650 cm^{-1} (corresponding to amide I) and 1531 cm^{-1} (corresponding to amide II). The amide I peak is due to the carbonyl stretch vibrations, while the amide II peak is derived from N–H bending and C–N stretching vibrations (Neo, *et al.*, 2013b).

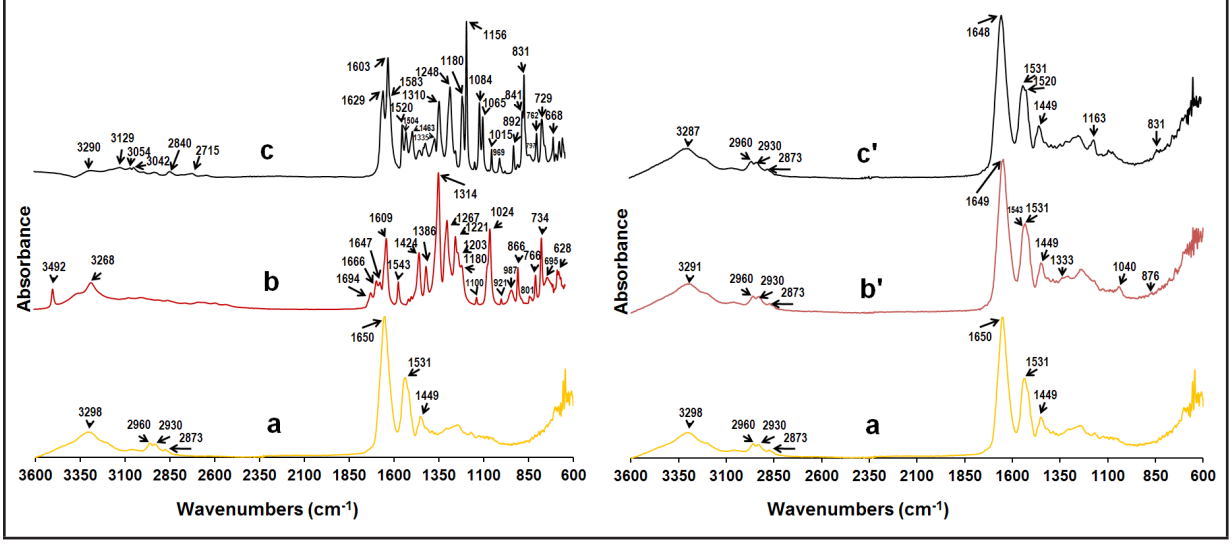
Gallic acid powder exhibits characteristic peaks at 3492 cm^{-1} and 3268 cm^{-1}, which are due to stretching of the O-H groups. The bands between 1609 cm^{-1} and 1386 cm^{-1} are characteristic of the C-C/C-H stretching and bending vibrations of the aromatic ring, whereas the O-H and C-H bending vibrations of the phenol alcohol cause the characteristic peaks within the region between 1314 cm^{-1} and 1180 cm^{-1}. The stretching and bending vibrations of C-O groups are observable between 101 cm^{-1} and 668 cm^{-1} (Mohammed-Ziegler and Billes, 2002).

With regard to naringenin (**Figure 4c**), it exhibits characteristic peaks at 3290 cm^{-1} due to phenolic O-H stretching vibrations, at 3129 cm^{-1} due to C-H aliphatic stretching and at 3054 cm^{-1} due to C-H stretching in the aromatic ring; a series of typical intense peaks are located nearby at 1629 cm^{-1}, 1603 cm^{-1} and 1583 cm^{-1}, the first due to the C=O stretching vibrations and the two latter due to C=C stretching vibrations in the left aromatic ring (see **Figure 1**). Other bands that are produced by the C=C stretching vibrations in the right aromatic ring are located at 1504 and 1248 cm^{-1} (Unsalan, Erdogdu and Gulluoglu, 2009; Tan, *et al.,* 2009). HC-C bending vibrations of the right aromatic ring are responsible for the 1335 cm^{-1} and 1211 cm^{-1} peaks (the latter visible but not pointed in **Figure 4c**), whereas HC-C bending and stretching vibrations of the carbons located between the central and left rings is responsible for the peak at 1180 cm^{-1}. The most intense peak, at 1156 cm^{-1}, is probably due to C-OH bending vibrations occurring at the left aromatic ring. The peaks at 1084 cm^{-1} and 969 cm^{-1} are due to the C-O stretching of the central ring, whereas that of 831 cm^{-1} corresponds to the same type of stretching of the left aromatic ring. C-C stretching

in the right aromatic ring causes the peak at 1015 cm^{-1}. Among the bands located at the lower wavenumbers of the naringenin spectrum, C-C-O bending of the central ring causes the peak at 892 cm^{-1} and C-C-C=O bending causes the peak at 729 cm^{-1}.

In the Z-NAR and Z-GA spectra (**Figures 4c** and **4b**, respectively), it is possible to appreciate that some characteristic peaks of the pure phenolic compounds completely disappear, for both gallic acid (3492, 1267, 1100 and 734 cm^{-1}) and naringenin (3129, 2715, 1629, 1603, 969 and 892 cm^{-1}). Indeed, as expected from a composite with a homogeneously distributed cargo, the FT-IR spectra of appear very similar to that of the pure zein nanofiber. However, a detailed analysis of composite fibers spectra reveal that there are important differences. The characteristic peak of the N-H stretching vibrations of pure zein nanofibers shifted from 3298 cm^{-1} to 3291 cm^{-1} (Z-GA) and 3287 cm^{-1} (Z-NAR), while no band shifting is observed in the C-H antisymmetric stretch mode of zein (2960 cm^{-1}) in any of the two composites. The intensity of the amide I peak, located at 1650 cm^{-1} in the pure zein nanofibers slightly shifts to 1649 cm^{-1} in Z-GA and to 1648 cm^{-1} in Z-NAR. Neo, *et al.* (2013b) obtained similar results for zein-gallic acid nanofibrous composites, noticing that two components are present in this amide I band; a component centred around 1645 cm^{-1}, mainly due to random coil structures, and a smaller component around 1607 cm^{-1}, mainly due to intermolecular β sheets. These authors observed that the shape and relative intensities of both the amide I and II bands change when increasing the gallic acid loaded amounts (5, 10 and 20%). They hypothesized that the observed increase in the component centered around 1607 cm^{-1} was caused by the increased presence of gallic acid, which has an intense peak around 1609 cm^{-1} (as mentioned above, due to stretching and bending vibrations of the aromatic ring). A similar situation could be expected for naringenin that presents a similar band around 1603 cm^{-1} (**Figure 4b** and **4c**), which would explain the wider shape of the 1648 cm^{-1} band. The spectra of Z-GA and Z-NAR nanofibers reveal the presence of additional bands, which are due to the interaction between the phenolic compounds and their protein matrix. New bands appear at 1543, 1333, 1040 and 876 cm^{-1} in Z-GA (**Figure 4b**) and at 1520, 1163 and 831 and cm^{-1} in Z-NAR (**Figure 4c**). For Z-GA, the peak appeared at 1543 cm^{-1} can be attached to the C-C/C-H stretching and bending vibrations of the aromatic ring of gallic acid. On the other hand, the other new peaks were different to those in the spectra observed for the pure gallic acid, and could be due to the formation of oligomeric structures. Oligomer forming structures are originated by C–C coupling between the aromatic rings and C-O-C bonding that involves phenolic

Figure 4. FT-IR spectra of different samples: (a) pure zein fibers; (b) gallic acid powder; (c) naringenin powder; (b') Z-GA; (c') Z-NAR.



side chains (Božič, Gorgieva and Kokol, 2012; Neo, *et al.*, 2013b). For Z-NAR, instead, all the new peaks appeared can be explained by the high-intensity characteristic bands of the pure naringenin.

In sum, the changes in the infrared spectra, in particular those observed in the N-H and amide bands, clearly indicate that phenolics interact at the molecular level with the zein in the phenolic-zein electrospun fibers, which is in agreement with previous findings in similar encapsulation systems (Neo, *et al.*, 2013b), probably forming protein-complexed forms of the phenolics.

3.3. Loading, loading efficiency and storage stability

The theoretical loading values of these encapsulation systems was 5% (w/w) of electrospun mat. Results showed that the loading of fresh membranes was 4.93 ± 0.15 % (w/w) for **Z-GA** and 5.12 ± 0.60 % (w/w) for **Z-NAR**, meaning that no bioactive is lost during electrospinning process (100% loading efficiency).

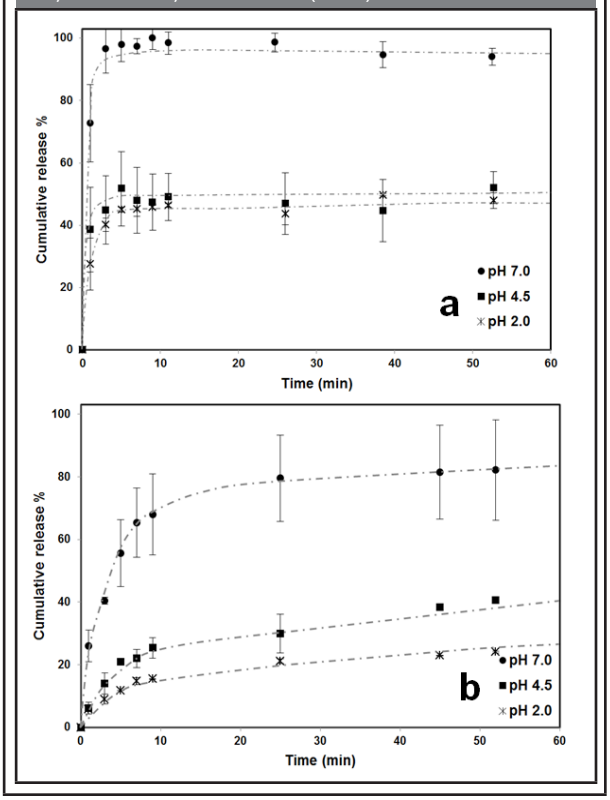
There was no significant variation after nearly three months of storage at room temperature (RH < 60%) (p < 0.05), showing that the cargo was stable in the encapsulation system. It is worth to notice that the hydroalcoholic gallic acid solutions used for Z-GA were be prepared freshly (ideally the same day of electrospinning) since the solutions of this phenolic acid can lead to losses, whereas naringenin solutions showed a greater stability at the same conditions.

3.4. Phenolics release in aqueous media

In vitro release studies of the phenolic compounds encapsulated in the zein fibers were carried out in aqueous media, to test the influence of environmental pH; release was studied at three different values of pH: 2.0, 4.5 and 7.0. The results of the percentage of cumulative release for the first hour of experiment are presented in **Figure 5** as percentage cumulative release against time.

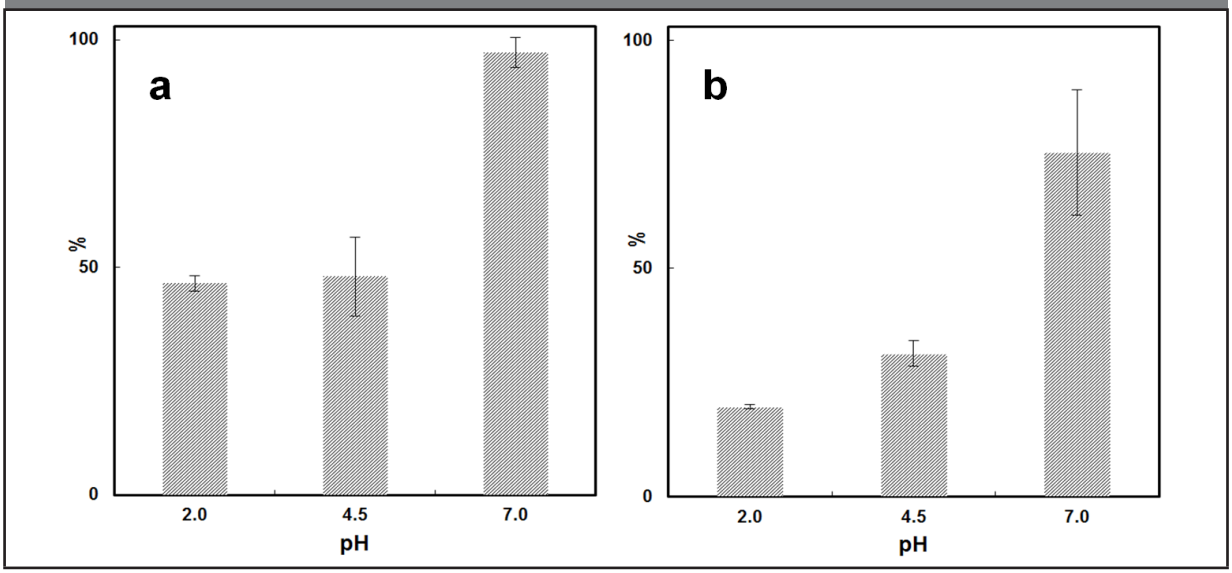
At a first instance it appears that all the curves behave diffusively, with a long time plateau value observed for t > 25 min in the case of naringenin and for t > 3min in the case of gallic acid. Let Cw/∞, be the concentration of the phenolic in the aqueous phase, in the equilibrium: results presented in **Figure 5** indicate that pH 7.0 favors a neat increase of Cw/∞, suggesting that specific interactions of the compound with the matrix (i.e. hydrogen bonds and electrostatic interactions between naringenin or gallic acid and zein) cannot be assumed negligible with respect to the osmotic pressure controlling the diffusion and release of compounds. The values of the percent cumulative release in the equilibrium are presented in **Figure 5**, as a function of pH.

Figure 5. Release profiles of (a) GA and (b) NAR from Z-GA and Z-NAR respectively, in aqueous media at pH 2.0, 4.5 and 7.0, until 60 min (n = 2).



For both the tested bioactives these interactions appear more important when the pH is low (i.e., a lower value of Cw/∞, is observed), indicating that the overall phenolic release can be modulated by the environmental acidity. The release of gallic acid is much more rapid and is nearly completed after 5 min at pH 7; at this time, zein membranes at pH 2 have released only half of the initially loaded bioactive. The same behavior is observed for the release of naringenin, albeit in this case a complete release of the phenolic is apparently never reached. If we consider that at 1 h the system is in equilibrium, the partition coefficient, $Kc = Cp/Cw/\infty$, where Cp and Cw are the concentration in the polymeric matrix and the aqueous

Figure 6. Percent cumulative release of phenolic compounds (a: GA, b: NAR) released to the medium at pH 2.0, 4.5 and 7.0 at the equilibrium (plateau value) (n = 2).



phase respectively, are pH 2 > pH 4.5 > pH 7.0, for naringenin and pH 2 = pH 4.5 > pH 7.0 for gallic acid ($p < 0.05$).

To better understand the diffusion velocity, *Cw*, has been normalized for its long time plateau value (Cw/∞) and plotted against the square root of time ($t^{0.5}$). This approach allows for direct comparison between the different release rates of the. same bioactive from membranes at different pH. In **Figure 7** the profiles of naringenin release are plotted against $t^{0.5}$. The linear behavior confirms a Fickian diffusion release (Kost and Langer, 2001; Luykx, *et al.*, 2008). Quite interestingly, we found no significant differences on the release rate (given by the slopes in **Figure 7**) for samples maintained at pH 2, 4.5 or 7. We conclude that pH affects the *amount* of naringenin released once the plateau is reached, whereas it does not have a visible effect on the *apparent velocity* in the diffusion-driven stage of release. A similar conclusion can be hypothesized for the release of gallic acid. Unfortunately, the few experimental data for GA release we collected before the plateau is reached do not support a direct evidence of this process. It seems clear though, that its release at all the conditions tested follows the trend of a burst release phenomenon (Huang and Brazel, 2001).

For both the phenolics the more drastic differences in the bioactive release occur in the passage from pH 4.5 to pH 7.0. These results suggest a strong effect of the cargo-carrier chemical affinity on the release behavior, which results greatly affected by the pH of the aqueous environment. Our proposal for elucidating this behavior is that the release phenomenon is influenced by the pH-dependent surface charging of zein, and by the pH-dependent ionization of the encapsulated compounds, which suffer important changes within this pH range, as explained below:

With respect to the pH-dependent surface charging of zein, according to de Folter, *et al.*, (2012) who investigated the stability and electrostatic repulsions of colloidal zein at different pH, the isoelectric point of corn zein (same commercial reference as used in this work) is ca. pI 6.5. The ζ- potential of acqueous zein particles is maximum at pH 4 (+60 mV), close to zero between 6.2 and 6.5, and -20 mV at pH 7. In sum, in aqueous media the neat charge of zein at pH 2.0 and 4.5 is *very* positive whereas it tends to be *slightly* negative at pH 7.0.

On the other hand either NAR or GA acid suffer ionization changes within this same range of pH that could help to further explain changes in the cargo-carrier affinities. In the first place, NAR undergoes a first dissociation around pH 6.8 (**Figure 8a**). With regard to GA, this molecule has 4 potential acidic protons, the first one corresponding to $pKa \approx 4.5$ (carboxylic acid) and the

rest to 8.7, 11.4 and 13.1 (Slabbert, 1977; Ji, *et al.*, 2006). Moreover, the dissociation of a GA-derived free radical (**Figure 8b**) has been determined (Eslami, *et al.,* 2010) with a *pKa* = 5. In sum, at pH 7.0 the major species of both phenolics will be anionic, whereas at pH 4.5 and 2.0 they will be mainly in the undissociated form (although for GA more precisely a mixture could be expected at pH 4.5).

Figure 7. Cumulative amount of naringenin released, normalized by the long time plateau value (Cw/ Cw|∞,) as a function of square root time, at pH 2.0, 4.5 and 7.0.

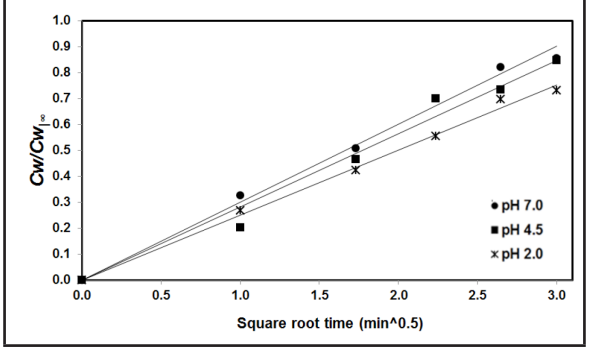


Figure 8. (a) Dissociation of the 7-hydroxy group of NAR to form its first oxoanion, *pK* = 6.8. **(b)** Top: dissociation of gallic (carboxylic) acid, *pK* ≈ 4.5; bottom: dissociation of phenolic hydrogen (3- or 4-hydroxy group) of the gallate radical of gallic acid, *pK* = 5.

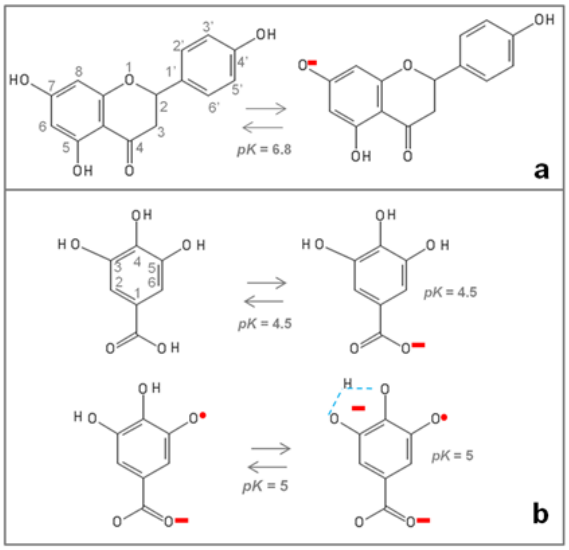
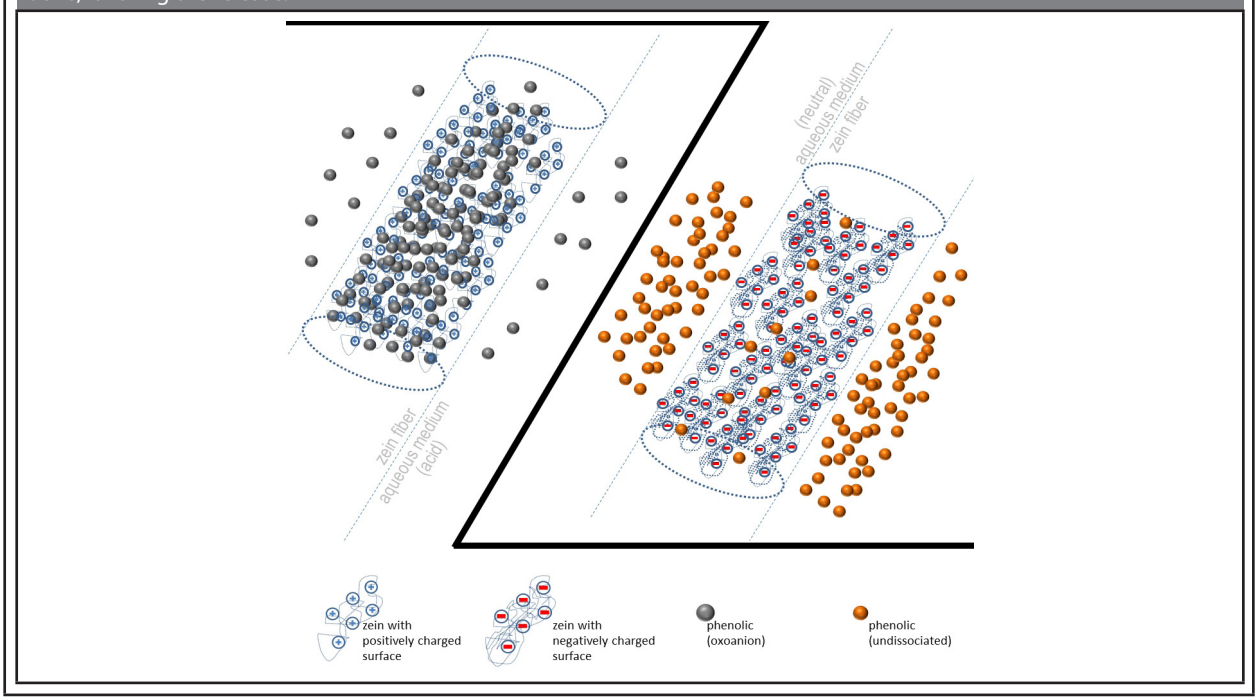


Figure 9. Proposed scheme of the pH-dependent total release of phenolics from the zein-phenolic fibers phenomenon. At lower pH (left) phenolics are in a more stable interaction state with zein and partially complexated. At neutral pH (right), an increased cargo affinity to the medium and electrostatic repulsion with the proteins weakens the cargo-carrier interactions, favoring the release.



At the acidic conditions of this study (pH 2.0 and 4.5), the undissociated forms would be more affine to the positively charged polymeric fibers, facilitating the complex formation with the protein, which would reflect in a higher tendency to remain encapsulated. At pH 7.0, in contrast, the oxoanions surely have an increased solubility in the aqueous phase and, moreover, the phenolic-protein interaction would be less stable due to electrostatic repulsion with negative charges of the protein, thus favoring a larger release rate to the aqueous phase. This hypothesis is schematized in **Figure 9**.

The encapsulation systems Z-GA and Z-NAR act as a pH-responsive, fully-edible carrier with great potential to be used as an active antioxidant ingredient or packaging/coating material. We foresee a particular potential for applications in which the pH increases "downstream" (e.g., encapsulation systems for release of phenolics targeted at the small intestine). In such cases these materials could be used for a fast release of antioxidants at early stages (pH < 2) while "keeping a reserve" of antioxidants to be delivered later (when pH increases up to 7).

4. CONCLUSIONS

A simple electrospinning process allows to obtain ultra-thin fibers of zein with the simultaneous encapsulation of significant loads of different types of highly-antioxidant phenolic compounds. The results indicated that release of the loaded phenolics into aqueous environments is pH-dependent, in the sense that higher ratios of the loaded compounds are released at pH 7.0 compared to those at pH 4.5 or lower, probably due to pH-dependent differences in the molecular cargo-carrier interactions. The process does not entail the use of toxic solvents and the resulting encapsulation system is fully edible, which would expedite its use in food formulation, food packaging and pharmacological applications.

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