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Title: IMPROVED ANTIOXIDANT CAPACITY OF QUERCETIN AND FERULIC ACID DURING IN-VITRO DIGESTION THROUGH ENCAPSULATION WITHIN FOOD-GRADE ELECTROSPUN FIBRES

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Abstract: Two bioactive compounds, quercetin and ferulic acid, were encapsulated using the electrospinning technique within hybrid amaranth protein isolate (API):pullulan ultrathin fibres. Initially, the composition of the encapsulation structures was optimized, both in terms of matrix components ratio and to maximize the bioactive loading. The morphology and thermal stability of the developed encapsulation structures were evaluated, as well as the encapsulation efficiency and distribution within the fibres of both antioxidant compounds. Moreover, the release characteristics and protection ability of the encapsulation structures during an in-vitro digestion study were investigated. Smooth ultrathin electrospun fibres were obtained in which the antioxidants were homogeneously distributed. Through this methodology, it was possible to incorporate within the API:pullulan fibres up to 10 and 20 % (by weight) of quercetin and ferulic acid, respectively, which were released in a sustained manner during in-vitro digestion, keeping to a greater extent their antioxidant capacity in comparison with the non-encapsulated compounds.

HIGHLIGHTS

- Quercetin and ferulic acid were encapsulated using electrospinning
- Blends of amaranth protein isolate and pullulan were used as encapsulating matrices
- Sustained release of the antioxidants from the electrospun fibers was observed
- Encapsulation improved antioxidant capacity of bioactives during *in-vitro* digestion

IMPROVED ANTIOXIDANT CAPACITY OF QUERCETIN AND FERULIC ACID DURING *IN-VITRO* DIGESTION THROUGH ENCAPSULATION WITHIN FOOD-GRADE ELECTROSPUN FIBRES

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Abstract

Two bioactive compounds, quercetin and ferulic acid, were encapsulated using the electrospinning technique within hybrid amaranth protein isolate (API):pullulan ultrathin fibres. Initially, the composition of the encapsulation structures was optimized, both in terms of matrix components ratio and to maximize the bioactive loading. The morphology and thermal stability of the developed encapsulation structures were evaluated, as well as the encapsulation efficiency and distribution within the fibres of both antioxidant compounds. Moreover, the release characteristics and protection ability of the encapsulation structures during an *in-vitro* digestion study were investigated. Smooth ultrathin electrospun fibres were obtained in which the antioxidants were homogeneously distributed. Through this methodology, it was possible to incorporate within the API:pullulan fibres up to 10 and 20 % (by weight) of quercetin and ferulic acid, respectively, which were released in a sustained manner during *in-vitro* digestion, keeping to a greater extent their antioxidant capacity in comparison with the non-encapsulated compounds.

Keywords

Amaranth protein isolate, pullulan, encapsulation, electrospinning, quercetin, ferulic acid, *in-vitro* digestion, controlled release

Chemical compounds studied in this article

Quercetin (PubChem CID: 5280343); Ferulic acid (PubChem CID: 445858)

1. Introduction

Synthetic antioxidants are often used to preserve food quality, although natural antioxidants like carotenoids, vitamins, flavonoids and phenolic acids act as a natural defense in the organism to fight illnesses. In particular, food antioxidants have focused a great deal of attention due to their potential to prevent certain diseases like cancer and neurodegenerative or cardiovascular diseases (Giampieri, Alvarez-Suarez, & Battino, 2014; Mates, Segura, Alonso, & Marquez, 2011; Yang et al., 2013).

Quercetin is a flavonoid naturally present in vegetables, fruits, non-alcoholic drinks and medicinal plants. It is abundant in apples, onions, teas and red wines, where it is present as a glucoside. Many health-related beneficial effects have been ascribed to quercetin, including anti-inflammatory and antiallergic effects as well as having the potential to diminish or prevent cardiovascular disease and certain types of cancer (Campbell et al., 2006; Fawzy & Asker, 2009; Gupta et al., 2010; Makris & Rossiter, 2001). The limitations for incorporating quercetin into functional foods are related to its low water solubility, as well as its degradation when exposed to high temperature, oxygen and/or light (Buchner et al., 2006; Scalia & Mezzena, 2009; Zenkevich et al., 2007). Furthermore, its low bioavailability as well as the chemical instability when exposed to gastrointestinal track conditions, limits the biological activity and therefore the potential health benefits.

Ferulic acid is a hydroxycinnamic acid, with strong antioxidant properties. It is an abundant phenolic compound found in plant cell walls, but it is barely present as a free compound, but usually it is covalently bound to polysaccharides or proteins from the cell walls. In its free form, ferulic acid is a potent antimicrobial compound (Jeong et al., 2000) and has demonstrated to be a chemopreventive agent towards colon cancer (Kawabata et al., 2000). Ferulic acid is FDA approved and, among other uses, it is

added as a preservation agent to inhibit autooxidation of oils (Graf, 1992). Its drawbacks for direct addition to functional foods are the photodegradation and sensitivity to oxygen exposure.

Micro- and nanoencapsulation have been considered as plausible options for the protection of these antioxidant compounds (Braithwaite et al., 2014). For instance, Scalia et al. (2009) demonstrated that incorporating quercetin within lipidic microparticles effectively resulted in lower photodegradation of the bioactive. Similarly, the development of nanoencapsulation systems based on ciclodextrins, liposomes and quitosan have resulted efficient in improving the solubility, stability and bioavailability of quercetin in pharmaceutical applications (Barras et al., 2009; Wu et al., 2008; Zhang et al., 2007). Pool et al. (2012a & 2012b) developed PLGA and Eudragit nanoparticles loaded with quercetin, proving that the antioxidant activity and the biaccessibility within simulated small intestinal conditions were enhanced when quercetin was encapsulated. Quercetin has also been incorporated in nanoemulsion-based delivery systems improving its bioaccessibility compared with crystalline quercetine (Pool et al., 2013). Similarly, encapsulation systems for ferulic acid based on cyclodextrins, lipidic nanoparticles, micelles, and microemulsions have been developed (Anselmi et al., 2008; Carlotti et al., 2008).

Regarding the encapsulation techniques, in the last years, electrospinning has been broadly explored as a straightforward and versatile method, with a number of advantages when compared to traditional encapsulation techniques such as spray drying, coacervation or ionic gelation (Bhushani & Anandharamakrishnan, 2014; Zussman, 2011). In fact, this high-voltage-based technique has been recently used to develop electrospun fibres containing quercetin (Li et al., 2014; Peng et al., 2011) and ferulic

acid (Yan et al., 2014; Yu et al., 2010). However, even though during the process of fibre formation is normally considered that the solvent is mostly evaporated, for food-related applications it is highly desired that food-derived matrices and food-approved solvents are used for encapsulation. Recently, we reported about the development of novel amaranth protein-based electrospun fibres using a food contact solvent (Aceituno-Medina et al., 2013). Amaranth is a traditional under-utilized Mexican crop with highly nutritious grains and leafs. The aim of this work was to investigate the potential of these novel amaranth-based structures for the encapsulation and protection of two potent antioxidant compounds (quercetin and ferulic acid) to be incorporated into functional foods. To the best of our knowledge, this is the first time that an amaranth protein-based matrix is used for the encapsulation and protection of bioactives. The morphology of the developed electrospun fibres together with the encapsulation efficiency and bioactive stability and distribution within the fibres were studied. Moreover, the release profile and antioxidant capacity of the encapsulated functional substances during an *in-vitro* digestion study were also investigated and compared to that of the free compounds.

2. Materials and methods

2.1 Materials

Formic acid of 95% purity, the non-ionic surfactant polyoxyethylene sorbitan monooleate (Tween 80), quercetin, ferulic acid (99.98% of purity), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), potassium persulphate and pullulan (M_w~100,000 Da) were supplied by Sigma-Aldrich (St. Louis, MO, USA). All products were used as received, without further purification. The commercial amaranth protein concentrate (*Amaranthus hypochondriacus* L. Revancha variety) was supplied by Nutrisol (Hidalgo, Mexico). The Amaranth Protein Isolate (API) was prepared based on

the methodology previously reported by Martínez and Añón (1996) with some modifications. The protein isolate prepared under these conditions consisted in a mixture of different proteins with molecular weights ranging from 10-83 kDa (Aceituno-Medina et al., 2013). Briefly, the commercial amaranth protein concentrate (APC) was defatted with hexane for 12 h (10% (w/v) suspension). Then, the amaranth protein concentrate was suspended in water and its pH was adjusted to 9 with a 2 M NaOH solution. The suspension was stirred for 30 min at room temperature and, then, centrifuged 20 min at 9000 *g*. Then, the supernatant was adjusted to pH 5 with 2 M HCl and centrifuged at 9000 g for 20 min at 4°C. The pellet was resuspended in water, neutralized with 0.1 N NaOH and freeze-dried. The protein content was determined by the Kjeldahl technique (AOAC, 1996) using a conversion factor of 5.85.

2.2 Preparation of bioactive-containing solutions for electrospinning

In order to develop the electrospun fibres for encapsulation of the bioactives, blends of 50:50 (w/w) and 80:20 (w/w) API:pullulan blends with the surfactant Tween 80 (~20 % by weight with respect to the API content) were prepared using 95% formic acid as the solvent. The polymer content in solution was kept constant at 20% (w/v). The bioactives were incorporated in the solutions at 10 % (w/w) in the case of quercetin and 20% (w/w) in the case of ferulic acid with respect to the biopolymer blend weight. The solutions were gently stirred until homogeneous dispersions were obtained.

2.3 Characterization of the polymer solutions

The apparent viscosity (η_a) of the polymeric solutions at 100 s^{-1} was determined using a rotational viscosity meter Visco Basic Plus L from Fungilab S.A. (San Feliu de

Llobregat, Spain) using a Low Viscosity Adapter (LCP). The measurements were made in triplicate at 25ºC.

2.4 Development of encapsulation structures through electrospinning

The methodology to obtain API/pullulan fibres through electrospinning has been described elsewhere (Aceituno-Medina et al., 2013). All of the electrospinning experiments were carried out at room temperature in air. The electrospinning environmental conditions were maintained stable at 24°C and 60% RH by having the equipment enclosed in a specific chamber with temperature and humidity control. In this work, the specific conditions of the electrospinning process for obtaining the fibres loaded with the bioactives were: a tip-to-collector distance of 10 cm, a flow rate of the solution of 0.4 ml/h and the voltage was kept at 22 kV.

2.5 Optical and Scanning Electron Microscopy (SEM)

The presence and distribution of the bioactives within the electrospun API/pulllulan fibres were observed using a digital microscopy system (Nikon Eclipse 90i, Barcelona, Spain) fitted with a 12 V, 100 W halogen lamp and equipped with a digital imaging head which integrates an epifluorescence illuminator. A digital camera head (Nikon DS-5Mc) with a 5 megapixel CCD cooled with a Peltier mechanism was attached to the microscope. Nis Elements software (Nikon Instruments Inc., Melville, NY, USA) was used for image capturing and the Adobe Photoshop CS3 extended software was used for image processing and analysis.

The morphology of the bioactive-containing electrospun fibres was examined using SEM (Hitachi S-4100) after sputtering the samples with a gold–palladium mixture under vacuum. All SEM experiments were carried out at an accelerating voltage of 10

kV. Fibre diameters of the electrospun fibres were measured by means of the Adobe Photoshop 7.0 software from the SEM micrographs in their original magnification.

2.6 Raman microspectroscopy

The dispersion of the bioactives within the electrospun fibres were analyzed using Raman microspectroscopy. Raman images were taken with a Jasco NRS-3100 Confocal Micro- Raman spectrophotometer (Jasco Inc., Easton, MD, USA) using a short working distance 100x objective. The laser source used was a NIR excitation tuned at 785 nm to avoid excessive fluorescence in the Raman signal. Raman chemical images were carried out in the point by point mode by plotting the areas of the quercetin and ferulic acid bands at ~1322 and ~1606 cm^{-1}, respectively, and were constructed by taking 25 x 25 spectra equally spaced along the selected fibre sample area. Both bioactives are good Raman scatterers and, thus, very short acquisition times (i.e. 2-3 s) were needed to record intense spectra, which only showed the signal of the antioxidants within the electrospun fibres.

2.7 Encapsulation efficiency

To assess the bioactive encapsulation yield, approximately 3 mg of loaded fibres were placed in a Falcon tube with 5 ml of ethanol and stirred during 10 min at 300 rpm to remove the bioactive from the surface of the encapsulation structures. Then, the tubes were centrifuged at ~10000 g during 10 min at 20ºC. The supernatant was taken and stored for subsequent analysis and the precipitate was repeatedly resuspended in 5 ml of ethanol and centrifuged as explained above until no signal from the bioactive was obtained. The amount of quercetin and ferulic acid was monitored through UV-Vis spectrophotometry using a SP-2000UV spectrophotometer, through interpolation of the

absorbance maximum at 256 nm and 324 nm for quercetin and ferulic acid, respectively, within the calibration curves previously obtained.

2.8 Thermogravimetric Analysis (TGA)

Thermogravimetric analysis (TG) curves were recorded with a TGA-DTA Setaram Setsys equipment. The samples (ca. 10 mg) were heated from 50 to 800°C with a heating rate of 5°C/min under argon atmosphere.

2.9 Release kinetics during *in-vitro* digestion

The *in-vitro* digestion studies were carried out following the methodology proposed by Dinnella et al. (2007). For the gastric digestion, 4mg of fibres (10 tubes with 4 mg of sample each) were diluted with 5 ml of distilled water and acidified to pH 2 using 6M HCl under constant stirring. Later on, 0.6 ml of a porcine pepsin solution (160 mg/ml) in 0.1 M HCl were added to the previous solution; distilled water was added to reach a final volume of 10 ml. The mixture was protected from light and stirred for 2h at 37ºC. At different time intervals (10, 30, 60, 90 and 120 min) the amount of released bioactive was analyzed through UV-Vis spectrophotometry. The amount of released bioactive was calculated from the previously developed calibration curve. After gastric digestion, the pH of the digesta was increased to pH 5 with 0.045 M $NaHCO_3$ and 2.4 ml of pancreatin–bile solution (pancreatin 4 mg/ml, bile 25 mg/ml in 0.1 M $NaHCO_3$) were added. Then, the pH was increased to 7.0 with 0.005 M $NaHCO_3$, and the mixture, protected from light, was stirred at 37 °C for 2 h. Similarly as in the gastric stage, the released bioactive was analyzed at different time intervals (10, 30, 60, 90 and 120 min) through UV-Vis spectrophotometry following the same methodology as described above.

2.10 Antioxidant activity during *in-vitro* digestion

The antioxidant activity was quantified along the digestion process to assess the protective effect of the encapsulation structures developed. The antioxidant capacity was determined using the $ABTS^{\bullet+}$ radical cation method (Re et al., 1999) using Trolox in methanol as a standard. The measurements were carried out at 734 nm using a 96 well microplate reader (Molecular Devices Inc, Sunnyvale, CA, U.S.A.). Results are expressed as mmol Trolox equivalent antioxidant activity/g of bioactive.

The *in-vitro* digestion process was carried out following the same methodology as above, but in this case, 20 mg of fibres and the equivalent amount of free bioactive solubilized in ethanol were used. The methanolic extracts obtained after the gastric and intestinal stages were homogeneized and 20 µl of these solutions were added to 230 µl of the $ABTS^{\bullet+}$ solution (absorbance of 0.7± 0.02).

The inhibition percentages were calculated using equation 1. A decrease in the characteristic blue colour of the radical $ABTS^{\bullet+}$ upon bioactive reaction provides a measurement of the capacity that this compound has to stabilize the radical. This change in colour is indicated by a decrease in the absorbance maximum at 734 nm:

% inhibition = [(Abs control – Abs sample)/Abs control]* 100 (Eq. 1)

The equivalent Trolox antioxidant capacity (TEAC) of the different compounds after the *in-vitro* gastric and intestinal digestion processes was calculated taking into account, both the bioactive content of the fibres and the release percentage at the end of the gastric and intestinal digestion stages.

3. RESULTS AND DISCUSSION

3.1 Morphology of the encapsulation structures and distribution of the bioactives within the fibres

In a previous work, electrospun fibres made of an amaranth protein isolate (API) and pullulan in different concentrations were developed and it was found that up to 80% (w/w) API content, when the surfactant Tween 80 (~20 % by weight with respect to the API content) was added, continuous and relatively homogeneous fibres were obtained (Aceituno-Medina et al., 2013). Initially, the effect of bioactive incorporation within the electrospun fibres was studied and related to the viscosity of the initial solutions. The amount of bioactive that could be added to the electrospinning solutions giving raise to stable suspensions and, thus, to a continuous fibre production process was first optimized. Regarding quercetin incorporation, only the solutions with the composition 50:50 API:pullulan (w/w) were suitable for stable electrospinning with a maximum bioactive content of 10 % by weight with respect to the biopolymer blend weight. In the case of ferulic acid, it was possible to incorporate this antioxidant within the fibres with more API content, i.e. in the 80:20 (w/w) API:pullulan structures, and the maximum loading attained was 20 % (w/w). Greater loadings resulted in precipitation of ferulic acid. Therefore, these two compositions were selected for subsequent analysis.

As observed in Figure 1, addition of quercetin to the API:pullulan fibres did not significantly modify the viscosity of the solution, but resulted in average thinner diameters of the electrospun fibres. This result indicates that, probably, addition of quercetin resulted in increased conductivity of the solution, which is known to affect the whipping motion of the electrospinning jet, thus, leading to thinner fibres (Bhardwaj & Kundu, 2010). In the case of ferulic acid addition, a slight increase in solution viscosity

was observed, which is usually related to thicker fibre morphologies (Suwatong et al., 2007). In general, for both encapsulation structures, smooth and defect-free fibres were obtained, confirming that no phase separation occurred during the electrospinning process and suggesting a good dispersion of the bioactives.

INSERT FIGURE 1 ABOUT HERE

In order to confirm the effective encapsulation of the antioxidants, fluorescence optical microscopy was carried out. Quercetin is known to have an emission fluorescence wavelength between 500 and 600 nm (Ram et al., 2004; Sengupta y Sengupta, 2003), while ferulic acid exhibits an emission at approximately 425 nm (Ram et al., 2004) and, thus, this property was used to visually check the inclusion of the antioxidants within the electrospun structures. Figure 1 also shows the fluorescence images of the API:pullulan fibres containing both bioactives which, as observed, seemed to be well distributed along the fibres.

Raman spectroscopy was also used to evaluate, not only the distribution, but also the stability of the encapsulated compounds. First, the Raman spectra of both compounds was obtained, so as to identify the characteristic vibrational bands with the aim to construct the Raman images from the confocal microscopy images taken from the samples. Figure 2 shows both the individual Raman spectra from the bioactives and the corresponding confocal Raman images from the API:pullulan electrospun fibres containing the antioxidants, which were obtained considering the area of the characteristic vibrational bands from quercetin and ferulic acid at ~1322 cm^{-1} and ~1606 cm^{-1}, respectively.

INSERT FIGURE 2 ABOUT HERE

The Raman images confirmed that as inferred from the fluorescence microscopy images, the bioactives were evenly distributed along the fibres. From Figure 2 it seems that some agglomeration areas were present in the quercetin-containing structures, fact that could be also observed in the fluorescence image (cf. Figure 1E). However, no displacements were observed in the vibrational bands from the individual spectra taken from the Raman images, indicating that the encapsulation process itself did not affect the stability of the bioactive compounds. It is interesting to note that even in the image points of lower intensity (indicated in the Raman images as F and G for the quercetin and ferulic acid structures, respectively), some characteristic bands from the antioxidants could be discerned, again highlighting the good dispersion of the bioactives within the encapsulates.

3.2 Encapsulation efficiency

To evaluate the encapsulation efficiency, calibration curves from both antioxidants in ethanol were prepared. The amount of quercetin and ferulic acid within the developed API:pullulan electrospun fibres was then monitored through UV-Vis spectrophotometry through interpolation of the absorbance maximum at 256 and 324 nm for quercetin and ferulic acid, respectively, within the calibration curves previously obtained. Both the equations for the calibration curves and the encapsulation efficiency are compiled in Table 1.

INSERT TABLE 1 ABOUT HERE

From this table it can be observed that very good encapsulation yields were obtained for both bioactives in comparison with other encapsulation technologies (Barras et al., 2009; Zhang et al., 2008). The efficiency obtained for quercetin was higher, but it should be taken into account that the concentration of bioactive in this case was half the concentration of ferulic acid used.

3.3 Thermal stability of the encapsulation structures

Thermogravimetric analysis of the loaded encapsulation structures was carried out to investigate if the encapsulation process affected the thermal stability of both the antioxidants and the matrices. Table 2 summarizes the main results and includes the data for the bioactives alone and for the encapsulation structures without the bioactives for comparison purposes. Pure quercetin displayed two main degradation events with maximums at ca. 91ºC and 358ºC. The first one is related to water loss of the compound, which disappeared upon encapsulation. The temperature of the second degradation peak, which corresponds to the degradation temperature of this flavonoid, significantly decreased upon encapsulation down to 220ºC, probably due to the dissolution/dispersion of the bioactive before encapsulation. Similar results of decreased thermal stability were observed upon complexation of quercetin with gelatinized starch (Zhang et al., 2011). However, it should be noted that the degradation temperature within the capsules is still far from the usual temperatures used for food processing.

INSERT TABLE 2 ABOUT HERE

Regarding ferulic acid, a single degradation event was seen for the pure compound at ~210ºC. In a similar way as with quercetin, upon encapsulation within the electrospun

fibres, the degradation peak maximum temperature decreased to 177ºC. The other two degradation peaks observed when plotting the weight loss first derivate from the TGA curves of the encapsulation structures at ~300 and ~400ºC corresponded to the degradation of the API:pullulan blend and to the surfactant Tween 80, respectively (Aceituno-Medina et al., 2013). When comparing these maximums with those from the structures without bioactive, a slight decrease in the degradation temperature from the biopolymer blend was observed, while the degradation temperature of the surfactant remained almost unchanged (cf. Table 2).

3.4 Release kinetics of the bioactives during *in-vitro* digestion

It is important to understand how the bioactives are released from the encapsulation structures during the process of digestion along the gastrointestinal (GI) tract when exposed to different pH conditions and to the presence of enzymes. It has been reported that micro and nanostructured systems as well as hydrogels shrink in gastric conditions while they swell under intestinal conditions, fact that conditions the release of the encapsulated bioactive substances (Chen et al., 2004; Hoad et al., 2009; Rayment et al., 2009).

In this work, following the *in-vitro* digestion methodology proposed by Dinnella et al. (2007), the amount of bioactives released from the API:pullulan structures during this simulated process was quantified.

Figure 3 shows the release profiles for quercetin and ferulic acid. It can be observed that in the first 10 min of gastric digestion, ~22 and ~15% of quercetin and ferulic acid, respectively, were released from the electrospun fibres, which probably corresponded to the bioactive molecules closer to the fibre surface. These release mechanisms have been already described for sorgo and maize proteins (Taylor et al., 2009; Wang et al., 2005)

and are also related to the high specific surface of the ultrathin encapsulation structures. A sustained release of quercetin from the electrospun encapsulation structures was observed during the gastric stage, releasing ~52% of the total compound. Comparatively, the release of ferulic acid during the gastric stage was quicker and almost 60% of the antioxidant was released in only 30 min. Yu et al. (2010) incorporated ferulic acid in polyvinyl pyrrolidone nanofibres adding SDS as anionic surfactant and quantified the release in PBS medium. They observed a complete release of the bioactive in the first 10 min and, thus, when comparing with the present results, a considerable improvement in the release characteristics was observed using the API:pullulan structures.

INSERT FIGURE 3 ABOUT HERE

At the beginning of the intestinal *in-vitro* digestion process, another quick increase in the release profiles was observed for both bioactives (~19 and ~21% in the quercetin and ferulic acid release, respectively), which could be ascribed to the change in pH which probably affected the conformation of the amaranth protein encapsulating matrix, facilitating the release of the antioxidant s. Similar results have been reported for catechins encapsulated in protein-based matrices (Taylor et al., 2009). At the end of the *in-vitro* intestinal digestion stage, the amount of quercetin and ferulic acid released corresponded to 82 and 99% of the total content within the fibres, respectively. In general, a more sustained release of quercetin was observed, which was probably related not only to the different composition of the fibres (i.e. ratio between API and pullulan and bioactive concentration), but also to the interactions of the bioactives themselves with the encapsulating matrices.

3.5 Antioxidant capacity of encapsulated vs. free bioactives during *in-vitro* digestion

It is well-known that pH and exposure to light, oxygen and temperature, may diminish the antioxidant activity of nutraceutical compounds (Liang y Subirade, 2010; Sansone et al., 2011; Anselmi et al., 2008; Carlotti et al., 2008; Fernández et al., 2009). However, little is known about how this antioxidant activity is kept during the digestion process. Therefore, a further aim of the present study was to evaluate the antioxidant activity of the encapsulated bioactives after the *in-vitro* digestion study and to compare it with that from the non-encapsulated compounds. The $ABTS^{\bullet+}$ method has been widely employed for evaluation of the antioxidant activity of biological materials, pure compounds and plant extracts both from hydrophilic and lipophilic nature. The inhibition percentage represents the loss of blue-green colour of the radical $ABTS^{\bullet+}$, upon addition of the antioxidant, thus decreasing the absorbance values measured at 734 nm. The control samples used to evaluate the antioxidant activity loss were freshly prepared methanolic extracts of the bioactives at the same concentrations than those found in the fibres. Moreover, the antioxidant activity of the pure fibres (without bioactive) and of the enzymes employed in the *in-vitro* digestion process were also analysed, so as to check if they also contributed to the antioxidant activity recorded.

For the calibration curve, different concentrations of Trolox were prepared (from 50 to 800 mM) and the inhibition percentage for each of the concentrations was calculated, obtaining the following calibration curve equation:

$y = 25.176x + 3.9357 \qquad R^2 = 0.9925$ (Eq. 2)

The equivalent Trolox antioxidant capacity (TEAC) of the different compounds after the *in-vitro* gastric and intestinal digestion processes was calculated and the results are compiled in Table 3.

INSERT TABLE 3 ABOUT HERE

It is important to highlight that the TEAC for the encapsulated bioactives was calculated taking into account, both the bioactive content of the fibres and the release percentage at the end of the gastric and intestinal digestion stages. As commented on above, initially, the antioxidant capacity of the pure bioactives was evaluated, providing inhibition percentages of ~48.12 and ~52.17% for the quercetin and ferulic acid, respectively. From Table 3, it can be observed that the inhibition percentage obtained from the neat fibres and the enzymes used in the *in-vitro* digestion process was low in comparison with that from the bioactives, indicating that they did not have a relevant antioxidant capacity. Nevertheless, the obtained values were substracted from their respective systems, so as not to overestimate the antioxidant capacity of both the free and encapsulated bioactives. From these corrected values in Table 3, it is clearly observed that the conditions used during the *in-vitro* digestion process, effectively affected the antioxidant capacity of the non-encapsulated bioactives and more than 60 and 70% of the initial inhibition percentage obtained for the quercetin and ferulic acid, respectively, was loss at the end of the digestion process. In contrast, encapsulation of the bioactives within the API:pullulan electrospun encapsulation structures resulted in an effective protection for the antioxidant capacity, showing inhibition percentajes of the radical $ABTS^{\bullet+}$ which doubled those from the non-encapsulated compounds. These results are promising since they show the ability of these API:pullulan fibres to protect quercetin

and ferulic acid during the *in-vitro* digestion, keeping to a greater extent their antioxidant capacity.

4. CONCLUSIONS

Quercetin and ferulic acid were properly encapsulated within amaranth protein isolate (API):pullulan ultrathin fibres using the electrospinning technique. The composition of the fibres was optimized and smooth, defect free encapsulation structures were obtained which contained the antioxidants evenly distributed along the fibres. The thermal stability of the bioactives decreased upon encapsulation, probably due to the dissolution/dispersion of the antioxidants required for subsequent electrospinning. However, a sustained release of quercetin and ferulic acid from the API:pullulan electrospun fibres was observed during *in-vitro* digestion, fact which contributed to improved antioxidant capacity of the bioactives in comparison with the free compounds. Therefore, the developed electrospun structures show promisory results in terms of bioactive protection for application in functional foods.

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Table 1. UV-Vis calibration curve equations and encapsulation efficiencies for quercetin and ferulic acid incorporated within 50:50 and 80:20 Amaranth Protein Isolate:pullulan electrospun fibers, respectively.

Bioactive	**Wt.%**	**Calibration curve equation**	**Theoretical concentration (µg bioactive/mg fiber)**	**Real concentration (µg bioactive/mg fiber)**	**Encapsulation efficiency (%)**
Quercetin	10	$y = 0.0072x + 0.0211$ $R^2 = 0.988$	83.3	78.1 ± 0.1	93.6 ± 0.1
Ferulic acid	20	$y = 0.0090x + 0.0014$ $R^2 = 0.998$	147.1	123.1 ± 0.2	83.7± 0.1

Table 2. TGA maximums of the weight loss first derivate (TD) for the different encapsulation structures

Bioactive	**API:pullulan (bioactive)**	**T_{max1} (ºC)**	**T_{max2} (ºC)**	**T_{max3} (ºC)**
-	50:50 –*		306.9	401.1
	80:20 –*		310.9	407.3
Quercetin	- (100%)	90.9	357.8	
	50:50 (10%)	220.6	298.6	403.4
Ferulic acid	- (100%)	210.7		
	80:20 (20%)	177.26	306.8	406.3

* Reference: Aceituno-Medina et al. (2013)

Table 3. Inhibition % of the ABTS radical and Trolox equivalent antioxidant capacity (TEAC) for the electrospun fibers, pure bioactives and enzymes studied after *in-vitro* gastric and intestinal digestion.

Sample	Gastric Conditions		Intestinal Conditions	
	Inhibition %	TEAC (mmol Trolox/g bioactive)	Inhibition %	TEAC (mmol Trolox/g bioactive)
API:pullulan 50:50	3.52 ± 0.03	-	4.85 ± 0.03	-
API:pullulan 80:20	5.09 ± 0.01	-	8.98 ± 0.01	-
Quercetin	13.39 ± 0.01	4.65 ± 0.01	16.62 ± 0.04	7.90 ± 0.02
API:pullulan (Quercetin)	30.12 ± 0.26	24.56 ± 0.24	31.06 ± 0.12	19.63 ± 0.09
Ferulic acid	17.56 ± 0.15	3.87 ± 0.04	14.96 ± 0.09	3.96 ±0.03
API:pullulan (Ferulic acid)	37.69 ± 0.67	15.16 ± 0.30	33.89 ± 0.20	10.43 ± 0.71
Enzymes	2.56 ± 0.01	-	8.41 ± 0.01	-

Figure Captions

Figure 1. SEM (A-D) and fluorescence microscopy (E,F) images of: 50:50 (A, C, E) and 80:20 (B,D,F) API:pullulan fibers without bioactive (A, B) and containing 10wt.% quercetin (C, E) and 20wt.% ferulic acid (D,F). Scale markers correspond to 1 µm (C), 2µm (A, B, D) and 10 µm (E, F). SEM micrographs include the apparent viscosity (η_{app}) of the solutions and average diameter of the fibers obtained.

Figure 2. Individual Raman spectra of pure quercetin (A) and ferulic acid (B) and confocal Raman images of API:pullulan electrospun fibers containing quercetin (C) and ferulic acid (D) with the corresponding individual Raman spectra defined in the images as E, F, G and H.

Figure 3. Release kinetics of (A) quercetin and (B) ferulic acid from the API:pullulan fibers during an *in-vitro* digestion study.

Figure 1

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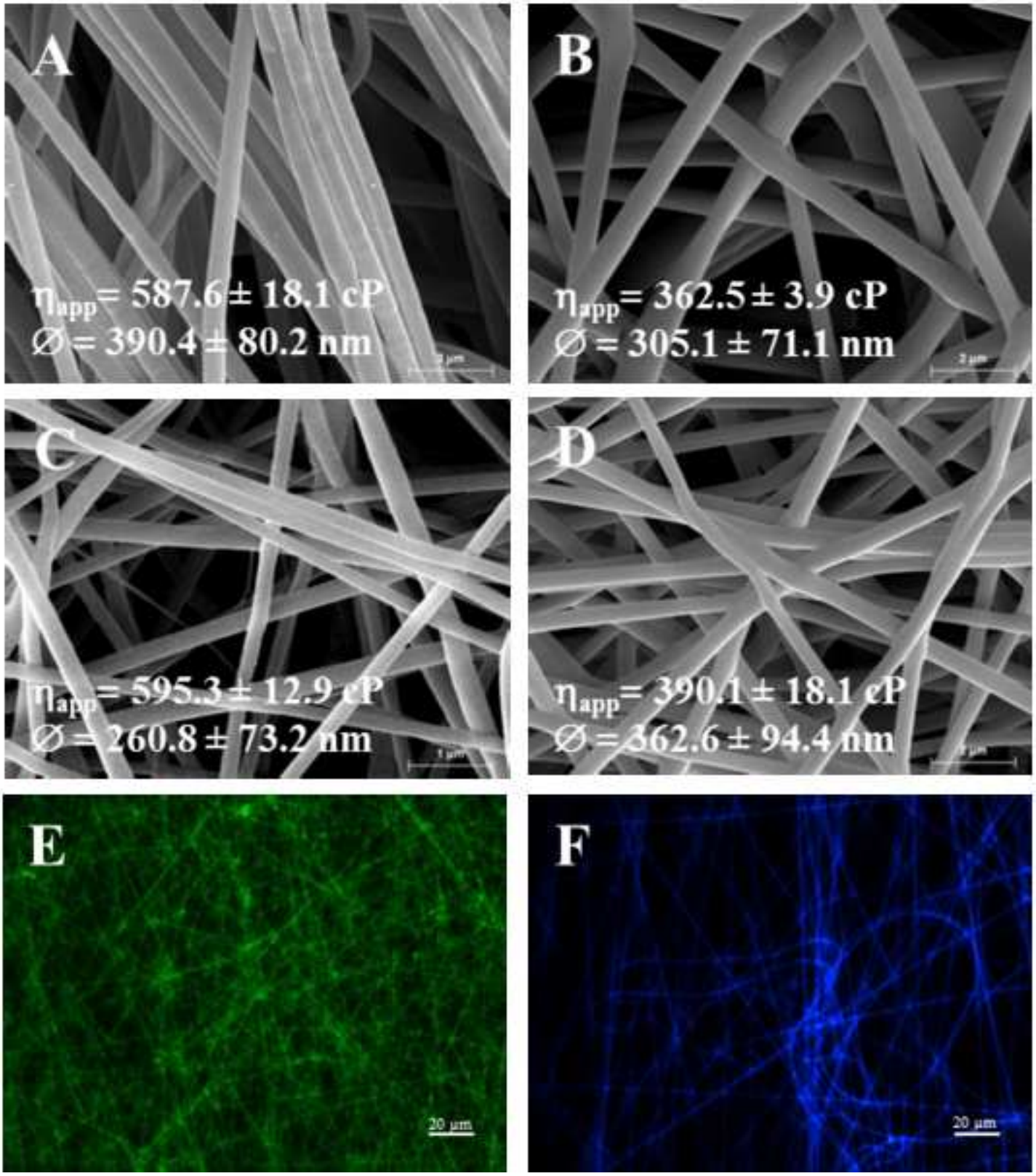

Figure 2

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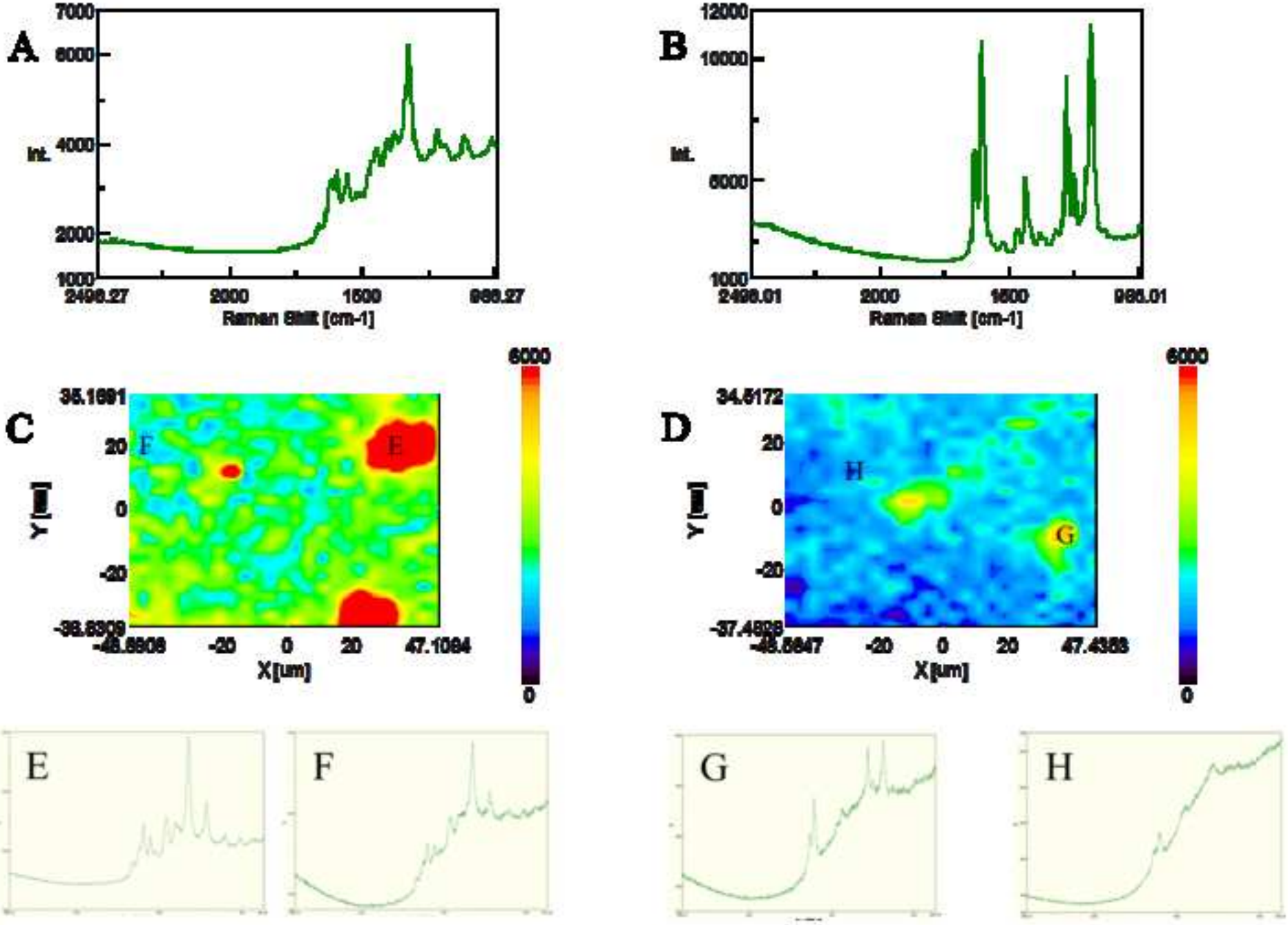

Figure 3A

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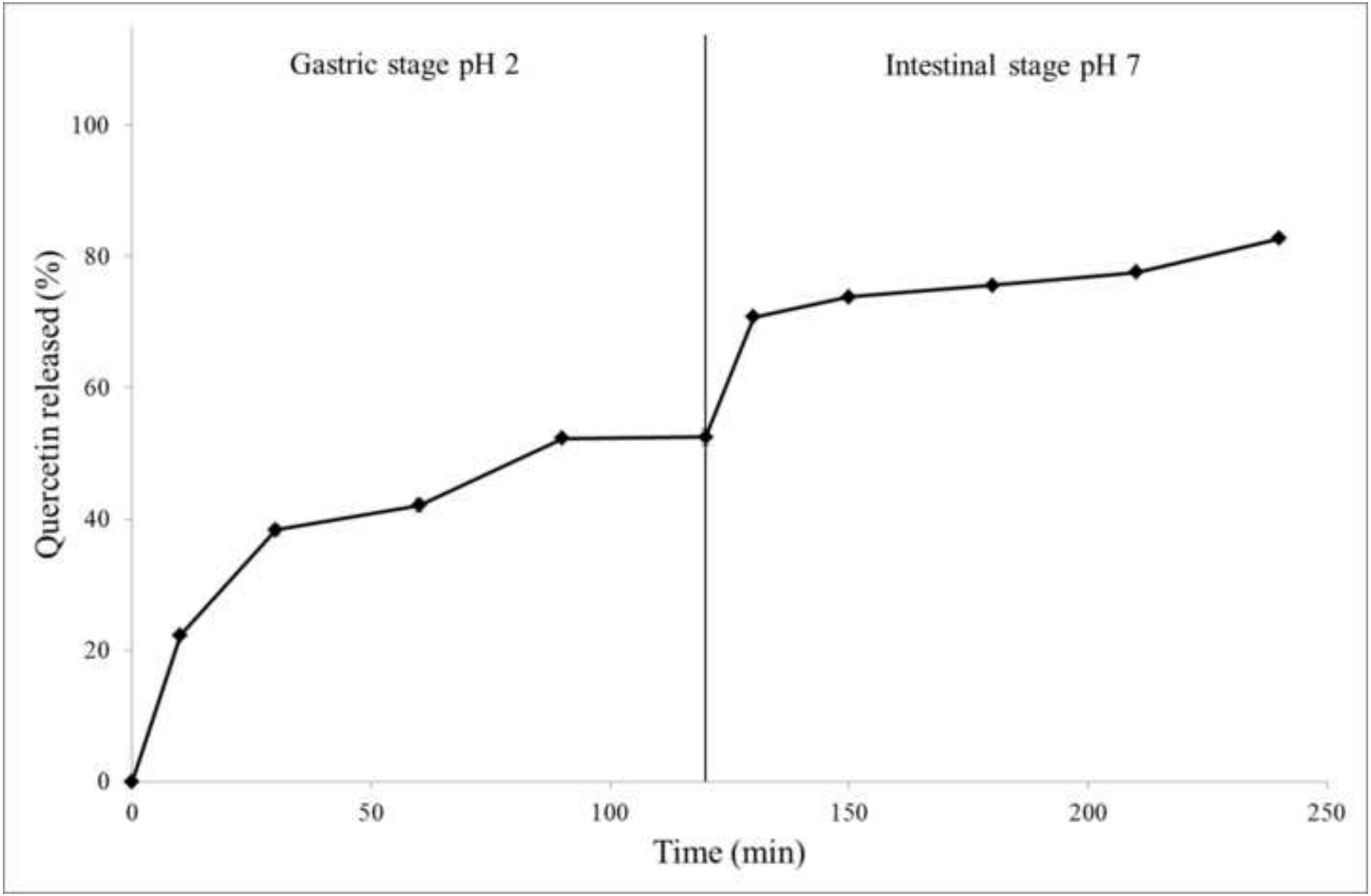

Figure 3B

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