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Effect of drying method on mechanical, thermal and water absorption properties of enzymatically crosslinked gelatin hydrogels

RAYSSA C. SIMONI[1], GISLAINE F. LEMES[2], SANDRA FIALHO[1], ODINEI H. GONÇALVES[2], ANGELA M. GOZZO[1], VIVIANE CHIARADIA[3], CLAUDIA SAYER[3], MARIANNE A. SHIRAI[2] and FERNANDA V. LEIMANN[2]

[1]Departamento Acadêmico de Alimentos/DALIM, Universidade Tecnológica Federal do Paraná, Campus Campo Mourão/UTFPR-CM, Via Rosalina Maria dos Santos, 1233, Caixa Postal 271, 87301-899 Campo Mourão, PR, Brazil
[2]Programa de Pós-Graduação em Tecnologia de Alimentos/PPGTA, Universidade Tecnológica Federal do Paraná, Campus Campo Mourão/UTFPR-CM, Via Rosalina Maria dos Santos, 1233, Caixa Postal 271, 87301-899 Campo Mourão, PR, Brazil
[3]Departamento de Engenharia Química e Engenharia de Alimentos, Universidade Federal de Santa Catarina, Centro Tecnológico/CTC, Campus Reitor João David Ferreira Lima, Caixa Postal 476, 88040-970 Florianópolis, SC, Brazil

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ABSTRACT

Enzymatically crossliked gelatin hydrogel was submitted to two different drying methods: air drying and freeze drying. The resulting polymeric tridimensional arrangement (compact or porous, respectively) led to different thermal and swelling properties. Significant differences ($p < 0.05$) on thermal and mechanical characteristics as well as swelling in non-enzymatic gastric and intestinal simulated fluids (37 ºC) were detected. Water absorption data in such media was modelled according to Higuchi, Korsmeyer-Peppas, and Peppas-Sahlin equations. Freeze dried hydrogel showed Fickian diffusion behavior while air dried hydrogels presented poor adjustment to Higuchi model suggesting the importance of the relaxation mechanism at the beginning of swelling process. It was possible to conclude that the same gelatin hydrogel may be suitable to different applications depending on the drying process used.

Key words: air drying, freeze drying, hydrogel, transglutaminase.

INTRODUCTION

Hydrogels are a promising type of 3-dimensional crosslinked hydrophilic polymeric networks presenting interesting characteristics for biomedical applications since they do not dissolve in water at physiological temperature and pH conditions (Pal et al. 2007). Food packaging sector may benefit from hydrogels in applications as timetemperature indicators (Pereira Jr et al. 2015), nutraceuticals release in functional foods (McClements et al. 2009), fat replacers (Chung et al. 2014, Manzocco et al. 2013) and rheology control (Shewan and Stokes 2013).

Correspondence to: Fernanda Vitória Leimann
E-mail: fernandaleimann@utfpr.edu.br / fevitoria@gmail.com

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Gelatin hydrogels synthesis may be achieved by crosslinking with glutaraldehyde (Prata et al. 2008), genipin (Devi and Maji 2010) or transglutaminase (Carvalho and Grosso 2006). Transglutaminase (TGase) is a transferase that forms both inter and intramolecular isopeptide bonds by crosslinking glutamine and lysine residues from (Heidebach et al. 2009).

Gelatin is a protein obtained by the hydrolysis of collagen from animals bones and skin and has been commonly used for pharmaceutical and medical applications because of its biodegradability and biocompatibility in physiological environments (Khan and Schneider 2013). Furthermore, gelatin presents appealing physico-chemical properties such as (i) great capacity for modification at amino acids level, (ii) low immunogenicity and cytotoxicity, (iii) FDA approval as a clotting agent and exudate-absorbing construct, (iv) hydrogel formation by facile, low cost procedures (Einerson et al. 2002).

Although crosslinking extension plays an important role in hydrogels properties, attention must be paid to the drying method since it may lead to modifications on the tridimensional arrangement of the polymeric network during water removal (Wang et al. 2012). Freeze dried hydrogels presents more porous structure altering the release of loaded compounds (Risbud et al. 2000, Shalaby et al. 1991). Porosity must be controlled because it directly affects water uptake and thus the release of active molecules from hydrogels (Díaz-Bandera et al. 2013, Koo et al. 2014).

Production of hydrogels from transglutaminase crosslinked gelatin is well stablished in the literature (Dong et al. 2008, Lim et al. 1999, Prata et al. 2008). However, the effect of the drying method on such hydrogels still needs to be elucidated, mainly in respect of swelling, mechanical and thermal properties. In this work, enzymatically crosslinked gelatin hydrogels with the same composition were dried by freeze drying and conventional oven drying and their properties were investigated, namely the equilibrium of swelling, water absorption mechanism and thermal properties.

MATERIALS AND METHODS

MATERIAL

Gelatin and glycerol (Vetec, analytical grade) were used in the hydrogel synthesis. The enzyme transglutaminase (TGase, ACTIVA GM, 110 units/g) was kindly supplied by Ajinomoto Inc. Hydrochloric acid, sodium chloride, monobasic potassium phosphate and dibasic potassium phosphate (Vetec, analytical grade) were used in the preparation of non-enzymatic gastric and intestinal simulated fluids. Potassium bromide (KBr, spectroscopic grade, Sigma-Aldrich) was used in the Fourier Transform Infrared analyses.

HYDROGEL SYNTHESIS AND DRYING

Hydrogel synthesis was carried out according to Chambi and Grosso (2006) with some modifications. First, gelatin (4.2 g, 7 g/100 g_{water}) was dissolved in distilled water (60 mL) at 50 °C under magnetic stirring and then glycerol (1.05 g, 25 g/100 $g_{gelatin}$) was added to the solution. This solution was cooled at 25 ºC and transglutaminase (763 mg or 20 active units/$g_{gelatin}$) was added under magnetic stirring. Finally, the resulting solution was poured into a silicone vessel (4 x 9 cm) and the crosslinking reaction took place 50 °C for 15 minutes. After this time, the hydrogels were immediately transferred to an oven and kept at 85 °C for 10 minutes in order to inactivate the enzyme. The obtained hydrogel was dried under two different conditions: (i) air convection oven at 40 °C for 24 hours; or (ii) freeze-drier (Liotop 101L, Liobras) at -50

°C and 150 μmHg for 24 hours followed by freezing at 90 °C for 5 hours. All samples were kept in a glass desiccator until analyses. The procedures were repeated three times for each experimental condition.

SCANNING ELECTRON MICROSCOPY

Freeze dried hydrogels (FDH) and air dried hydrogels (ADH) were submitted to Scanning Electron Microscopy (JEOL JSM-6390LV) for morphological observations. Samples were frozen in liquid nitrogen, fractured and gold coated before analyses.

EQUILIBRIUM DEGREE OF SWELLING

Equilibrium degree of swelling (EDS) was determined according to Wang et al. (2012). The dried samples (3 x 3 cm) were kept in an oven at 40 ºC during 24 h and then weighted (W_0). After that, samples were immersed in distilled water (200 mL) at 25°C during 24 h and weighted (W_{24}). The equilibrium degree of swelling in water (EDS) was calculated using Equation (1).

$$EDS\ (\%) = \frac{W_{24} - W_0}{W_0} \times 100 \qquad \text{Equation (1)}$$

DYNAMIC SWELLING STUDIES

To evaluate the influence of the drying treatments on the swelling behavior, FDH and ADH were allowed to swell from the dried state in simulated gastric fluid (pH 1.2; 2 h, 37 ºC) and subsequently in simulated intestinal fluid (pH 7.4; 4 h, 37 ºC) (Argin et al. 2014). Non enzymatic simulated fluids were prepared according to The United State Pharmacopeia (1999). The hydrogels weight was recorded at timed intervals up to 360 min. Swelling kinetics were determined by the swelling degree at each time interval and calculated using Equation 2, where W_s is the weight of the swollen hydrogel after a specific swelling time.

$$Swelling\ \ degree\ \ (\%) = \frac{W_s - W_0}{W_0} \times 100 \qquad \text{Equation (2)}$$

For the water absorption analysis, the swelling ratio (Q) was calculated using Equation 3 and adjusted to the Higuchi, Korsmeyer-Peppas and Peppas-Sahlin equations (Equations 4, 5 and 6, respectively), where M_t is the mass of absorbed water at time t and M_f represents the absorbed water at an infinite time. Non-linear least squares fitting method was used to determine the parameters in each equation using Statistica 7.0 software (Statsoft).

$$Q = \frac{W_s}{W_0} \qquad \text{Equation (3)}$$

$$M_t / M_f = k' t^{1/2} \qquad \text{Equation (4)}$$

$$M_t / M_f = k\, t^n \qquad \text{Equation (5)}$$

$$M_t / M_f = k_d\, t^m + k_r\, t^{2m} \qquad \text{Equation (6)}$$

DIFFERENTIAL SCANNING CALORIMETRY

Hydrogels thermal properties were determined by Differential Scanning Calorimetry (DSC, Perkin Elmer, DSC 4000) calibrated with indium and zinc standards. Samples (approximately 10 mg) previously conditioned at 25 ºC for 2 weeks in desiccator) were placed in aluminum pans and then heated at 20 $^{o}C.min^{-1}$ from 0 to 250 ºC under gaseous nitrogen (20 $mL.min^{-1}$). Glass transition temperatures (Tg) were determined as the point of inflexion in the base line caused by the discontinuity of samples specific heat capacity. Melting temperature (Tm) was recorded as the onset temperature of the endothermic peak (Farris et al. 2011).

PUNCTURE TEST

Hydrogels puncture strength and puncture deformation were determined by the puncture test at a TA.XT Express Enhanced texturometer (Stable Micro Systems). Hydrogels were cut in circular samples (diameter l_0 = 30 mm) after swollen for 24 h (see EDS determination). After that, hydrogels were fixed in a 52.6 mm diameter cell and the perforation was conducted with a 3 mm diameter spherical probe and test velocity equal to 1 mm/s. Puncture strength (P) and the displacement of the probe (D) at break were determined directly from the force displacement curves. Puncture strength value was divided by the thickness of the sample (five random points with a digital caliper rule, Ford) and expressed in N/mm (Sabato et al. 2001). Equation 7 was used to calculate puncture deformation ($\Delta l/l_0$) of the swollen hydrogels, considering that the stress was perfectly distributed along the film at the breaking point (Alves et al. 2011).

$$\frac{\Delta l}{l_0} = \frac{[(D^2 + l_0{}^2)^{1/2} - l_0}{l_0} \qquad \text{Equation (7)}$$

STATISTICAL ANALYSIS

The results are expressed as mean ± standard error of the mean. Data were subjected to Students t-test using Statistica 7.0 software and the level of significance used was $p < 0.05$.

RESULTS AND DISCUSSION

Air dried hydrogel (ADH) has a vitreous aspect when compared to the freeze dried hydrogel (FDH) samples, which showed an opaque, whitish appearance. At Figure 1, SEM images from hydrogels surface (FDH-S and ADH-S) and fragile fractures (FDH-F and ADH-F) showed the microstructural modification caused by the drying treatments. ADH sample presented a smooth surface as well as a compact, continuous structure. Cao et al. (2007) noted that the cross-section of native gelatin films presented fibrillary orientation and when gelatin was crosslinked with ferulic acid and tannin acid, an apparent decrease in free volume could be observed. Jiang and Tang (2013) also observed that TGase crosslinking of gelatin resulted in films with a more compact microstructure. Gelatin films produced by Cao et al. (2007) and Jiang and Tang (2013) were dried at room temperature and even the crosslinked films presented fibrillary aspect. Betul et al. (2003) evaluated the effect of drying temperature on wheat gluten films microstructure and observed smoother surfaces when 50 ºC was applied. Denavi et al. (2009) concluded by Scanning and Transmission Electron Microscopy that the drying conditions of soy protein films (70 ºC and 30%RH) could promote

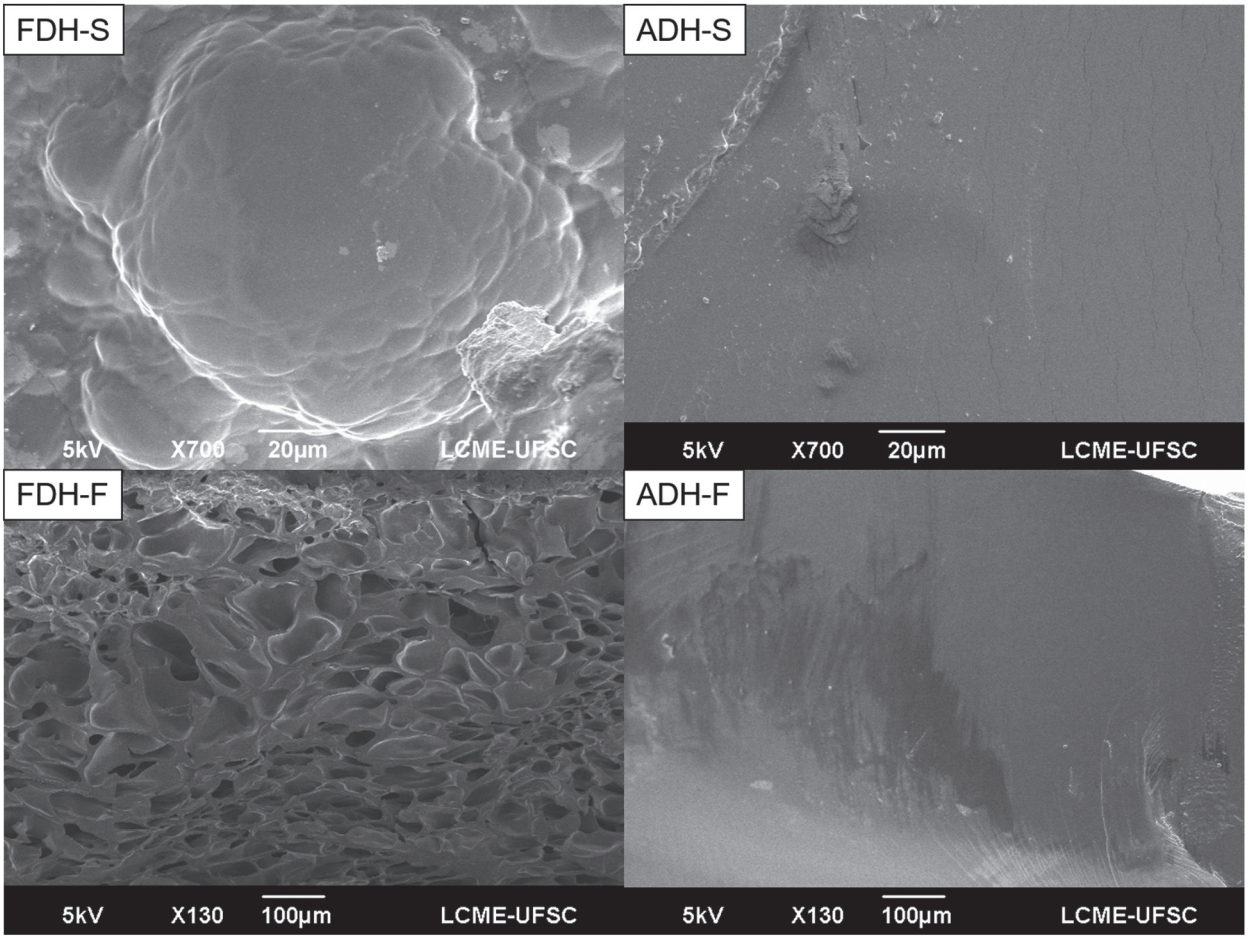

Figure 1 - Scanning Electron Microscopy images of: freeze dried gelatin hydrogel surface (FDH-S) and fragile fracture (FDH-F); air dried hydrogel surface (ADH-S) and fragile fracture (ADH-F). Magnifications: 700x surface and 130x fracture.

the formation of a higher number of hydrophobic interactions within the film structure, consequently a more compact structure. The ADH samples produced at the present work presented a smooth aspect at both surface and fracture suggesting that the drying method influenced the gelatin network conformation and interactions resulting in a smother microstructure.

FDH samples presented a rough surface and internal porous structure with sizes varying from 10 to 150 µm. During freeze drying, materials are dried by sublimation of ice crystals under vacuum at a temperature below the ice freezing point (Kang et al. 1999). In such cases, pore sizes may be controlled only the size of ice crystals formed during the freezing process. Peng and Chen (2011) commented that the free water in the polymeric solution is frozen causing polymer chains to gather and condense leading to a honeycomb structure after ice sublimation. On the other hand, for the air-drying method, there is more time to water evaporate leading to an equilibrium polymeric chain conformation (Ahmad et al. 2015, Zhang et al. 2013). Risbud et al. (2000) have found pore sizes equal to 39 µm in freeze-dried films of glutaraldehyde crosslinked chitosan/polyvinylpyrrolidone.

The equilibrium degree of swelling (EDS), puncture strength and puncture deformation found for the air dried and freeze dried hydrogels are presented in Table I. No significant difference ($p > 0.05$) was

detected in EDS due to the drying method indicating that final water uptake was the same for both cases disregarding internal materials morphology. Risbud et al. (2000) studied the effect of different pH on the swelling ratio (relation between swollen and dried hydrogel's weight) of air dried and freeze dried chitosan-polyvinylpyrrolidone hydrogels only during 180 min. Results obtained by these researchers indicate that after 180 min at pH 7 hydrogels reached values of 8 and 2.2 ($g_{wet\ hydrogel}/g_{dry\ hydrogel}$) for freeze dried and the air dried hydrogels respectively. Probably the hydrogels evaluated at the present work presented the same swelling degree due to the significant larger period of time evaluated.

Mechanical characterization provided that puncture strength for ADH and FDH were statistically equal. Alves et al. (2011) obtained puncture strength values up to 12 N for gelatin-poly(vinyl alcohol) hydrogels and Sabato et al. (2001) up to 52 N/mm for a blend of soy protein isolate and whey protein isolate. It is worth noting that analyses were carried out here with swollen hydrogels, which presented lower resistance due to the relaxation of the crosslinked chains. Puncture deformation (Table I) presented a significant difference ($p < 0.05$) between the treatments as FDH deformed approximately four times more than ADH. Since the crosslinking degree is the same for both samples the effect of the crosslinking extent evaluated by other authors (Johnson et al. 2004) is not worth in this case. Also, in some cases the mechanical performance of hydrogels is more related with chain distribution and entanglement than with the incorporated water content (Lopes and Felisberti 2003). The increase in mechanical strength due to the drying process is important since improving mechanical properties of hydrogels usually comes as at the cost of reducing swelling degree. In the present work it is evident that chain conformation of gelatin is different for FDH and ADH.

TABLE I
Equilibrium degree of swelling (EDS%), puncture strength (N), puncture deformation ($\Delta l/l_0$, %) and thermal properties obtained for gelatin hydrogels.

Treatment	EDS (%)	Puncture strength (N/mm)	$\Delta l/l_0$ (%)	Tg (ºC)	Tm (ºC)	ΔH (J/g)
Freeze dried (FDH)	377.41 ± 12.00	0.545 ± 0.378	4.85 ± 0.89	27.03	47.40	120.75
Air dried (ADH)	418.06 ± 26.36	0.749 ± 0.178	1.29 ± 0.95	49.15	31.56	177.78
p-value	0.917359	0.444234	0.009019	-	-	-

Kinetics of swelling degree in non enzymatic simulated gastric and intestinal fluids (Figure 2) showed significant differences ($p < 0.05$) after 180 min swelling. FDH kept swelling in simulated intestinal fluid while air-dried hydrogel presented the same swelling degree value until 360 min of analysis. Risbud et al. (2000) have found significant differences between air- and freeze-dried chitosan/PVP blend hydrogels crosslinked with glutaraldehyde. Freeze dried hydrogels exhibited superior pH-dependent swelling properties at lower pH values, which could be attributed to their porous nature. At the present study, the major dependence was observed for the simulated intestinal fluid which presented pH equal to 7.4. According to Zhu et al. (2012), gelatin acts as base in acidic medium forming NH_3^+ and -COOH while proteins become positively charged. In alkaline medium, protein acts as an acid donating H^+ thus forming $–COO^-$ while $–NH_2$ groups become negatively charged. Between pH 3 and 7 both mechanisms are present (NH_3^+ and COO^-). They observed that in basic medium gelatin hydrogels swelling was higher and concluded that this behavior was due to the presence of the hydrophilic functional groups (mainly COO) in the gelatin structure. Moreover, gelatin hydrogels can be hydrolyzed to form carboxyl groups, which also result in increasing of the swelling ratio.

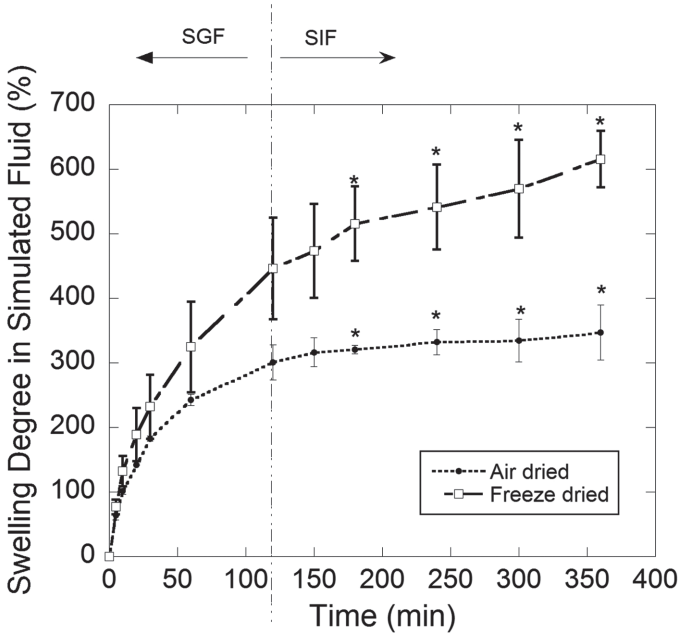

Figure 2 - Swelling degree of the gelatin hydrogels in free enzyme gastric (SGF) and intestinal (SIF) simulated fluids: (●) air-dried (ADH) and (□) freeze-dried (FDH); *significant difference ($p < 0.05$).

The higher swelling degree of freeze dried gelatin hydrogel (Figure 2) could be explained by the presence of hydrophilic functional groups (mainly COO-) and by the polymeric network hydrolysis that was favored in the more porous structure of the freeze-dried samples. Larger amounts of unbound water are present at highly swollen hydrogels allowing an improved solute release (Kim et al. 2003). This could be an important characteristic to release entrapped molecules at a higher rate on the intestinal tract.

Thermal characterization of FDH and ADH is presented in Figure 3 and Table I. Glass transition temperature (Tg) values obtained for both cases were lower than pure gelatin. However, it is worth noting that samples were equilibrated in a desiccator with silica during 2 weeks after analysis and the crosslinking degree from both samples did not presented significant difference ($p > 0.05$). Although gelatin presented a highly hydrophilic character, its glass transition temperature (Tg) in the dry state was 217 °C, but strongly decreases to approximately 0 °C according to the Fox-Flory equation for water contents up to 25 wt %. According to An increase in Tg is also expected for high crosslinking degree (Apostolov et al. 1999). According Farris et al. (2011), lower Tg values could be explained by an 'inhibition effect' exerted by the crosslinker, which prevented the recovery of the structurally ordered microcrystalline domains (micro crystallites) leading to a more amorphous final molecular structure. Furthermore, FDH presented lower Tg corroborating the amorphous structure hypothesis since the polymeric matrix remained at the same conformation during the drying step, unlike the ADH. FDH fusion enthalpy was also reduced (Table II) showing a that a more amorphous structure was created during the freeze drying when compared to the air dried sample.

Water absorption data and model parameters for ADH and FDH are presented in Table II and Figure 4. It is possible to observe a fast swelling in the early stages (before 60 min) in both cases suggesting that swelling took place following a chain relaxation mechanism (Argin et al. 2014). PeppasSahlin

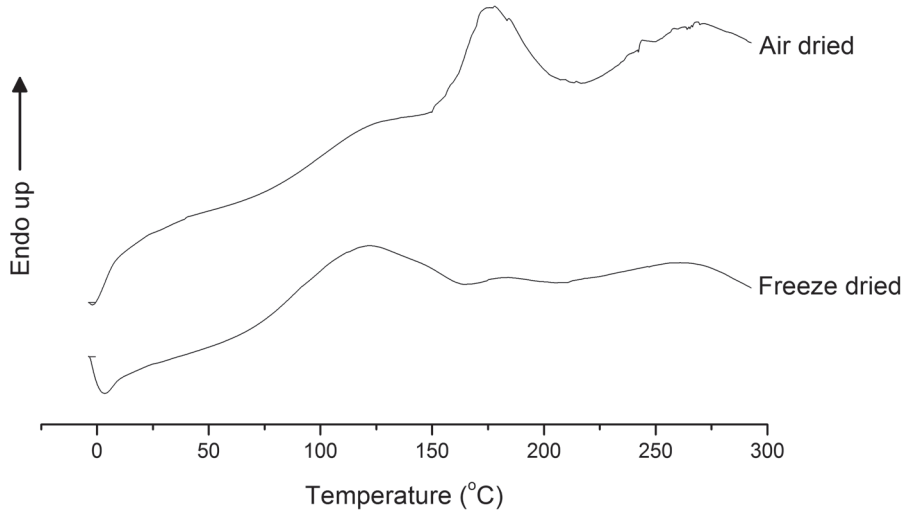

Figure 3 - DSC thermograms of freeze-dried (FDH) and air-dried (ADH) gelatin hydrogels.

TABLE II
Water absorption model parameters for air (ADH) and freeze dried (FDH) gelatin hydrogels using Higuchi, Korsmeyer-Peppas, and Peppas-Sahlin Equations.

Treatment	Higuchi model		Korsmeyer-Peppas			Peppas-Sahlin			
	k'	R^2	k	n	R^2	k_r	k_d	m	R^2
Freeze dried	0.0605	0.9380	0.1641	0.3109	0.9975	0.1428	-0.000030	0.3505	0.9994
Air dried	0.0670	0.7646	0.2945	0.2178	0.9904	0.2424	-0.000007	0.2765	0.9986

k' ($min^{-0.5}$), Higuchi kinetic constant; k (min^{-n}), Korsmeyer-Peppas kinetic constant; n is the release exponent describing the mode of the transport mechanism; k_d ($min^{-0.43}$), diffusional constant; k_r ($min^{-0.86}$), relaxational constant; m is the purely Fickian diffusion exponent for a system of any geometrical shape; r^2, correlation coefficient.

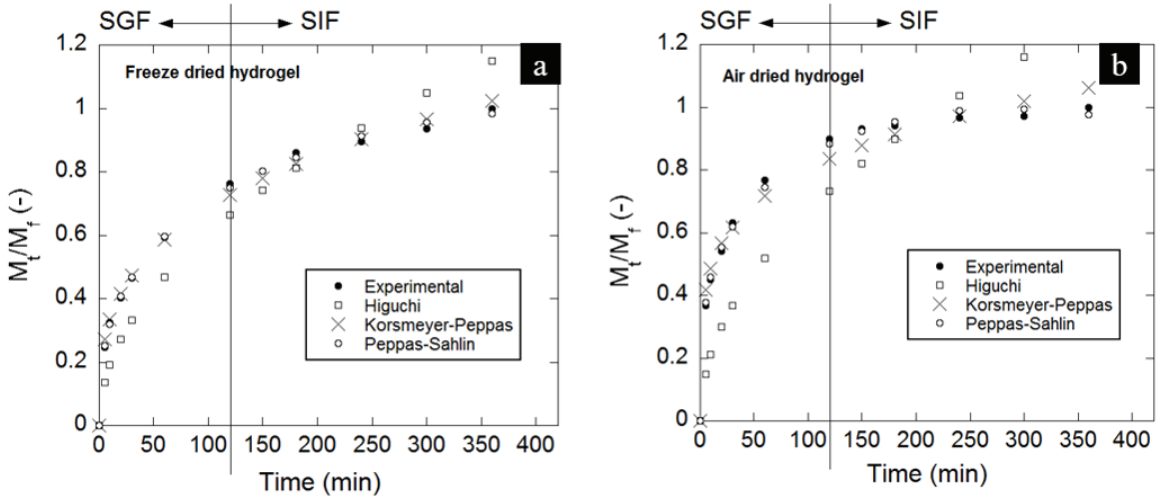

Figure 4 - Water absorption data of gelatin hydrogels (**a**) freeze-dried (FDH); (**b**) air-dried (ADH); in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF). Experimental data (●) and modeled data obtained by Higuchi (□), Korsmeyer-Peppas (×), and Peppas-Sahlin (○) equations.

model presented the best fit to the experimental results for both ADH and FDH (R^2 = 0.9986 and 0.9994, respectively). The negative values obtained for k_d should be interpreted in terms of a relaxation mechanism insignificant compared to the diffusion process. The low *n* value determined by the Korsmeyer-Peppas equation and the significantly higher values obtained for k_d compared to k_r by the Peppas-Sahlin equation indicates that the swelling of gelatin FDH in the simulated intestinal fluid followed a diffusioncontrolled mechanism (Argin et al. 2014). Also, for FDH a good fit to the Higuchi model was achieved. In the case of the ADH the lack of adjustment to Higuchi model suggests that the relaxation mechanism is important at the beginning of swelling process, where water is incorporated to the polymeric matrix leading to the expansion of its structure. These results are in agreement with the morphological characteristics presented at Figure 1. It is wotrh noted that, according to Bajpai et al. (2008), if the glass transition temperature of the polymer is below the experimental temperature, the polymer will be in the rubbery state and polymer chains may have high mobility allowing fast penetration of the solvent into the hydrogel matrix, resulting in a Fickian diffusion mechanism. This is the case for the freeze dried hydrogel since water absortion data was obtained at 37 ºC while its Tg was 27 ºC.

CONCLUSIONS

Enzymatically crosslinked gelatin hydrogels were dried by freeze drying and air drying. They presented signifficant differences on their thermal characteristics and swelling behavior in non enzymatic simulated gastric and intestinal fluids due to the changes which may be atributed to different polymeric chain conformation caused by the drying method. Freezedried hydrogel presented porous structure, amorphous chain conformation and a lower glass transition temperature leading to a Fickian difusion behaviour during swelling. This conclusion was confirmed by the Peppas-Sahlin model adjustment. On the other hand, air dried hydrogels presented a compact, non porous structure and higher glass transition temperature. These results suggests that the same gelatin hydrogel may be suitable to distinct applications depending on the drying process used.

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