Carbohydrate Polymers 98 (2013) 726–735

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

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

Multiple complexation of cyclodextrin with soy isoflavones present in an enriched fraction☆

Francini K.J. Yatsu[a,*], Letícia S. Koester[a], Ivana Lula[b,c], Joel J. Passos[b], Rubén Sinisterra[b], Valquiria L. Bassani[a]

[a] Programa de Pós-Graduação em Ciências Farmacêuticas, Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul, Av. Ipiranga 2752, CEP 90610-000 Porto Alegre, RS, Brazil
[b] Departamento de Química, Instituto de Ciências Exatas, Universidade Federal de Minas Gerais (UFMG), CEP 31270-901 Belo Horizonte, MG, Brazil
[c] Instituto de Ciências Exatas e Tecnologia-IET, Centro Universitário de Belo Horizonte (UniBH), Av. Prof. Mário Werneck, 1685 – Estoril, CEP 30455-610 Belo Horizonte, MG, Brazil

ARTICLE INFO

Article history:
Received 18 May 2013
Received in revised form 26 June 2013
Accepted 27 June 2013
Available online 2 July 2013

Keywords:
Cyclodextrin
Daidzein
Genistein
Glycitein
Isoflavones

ABSTRACT

In the present study we evaluated the complexation of daidzein/genistein/glycitein, present in an isoflavone enriched fraction (IEF), with β-cyclodextrin and 2-hydroxypropyl-β-cyclodextrin (HPβCD). Based on the increased solubility and higher complexation efficiency, IEF and HPβCD solid complexes were prepared by kneading, freeze-drying, co-evaporation, spray-drying and microwave. The solid complexes were characterized using Fourier transformed-infrared spectroscopy, differential scanning calorimetry, X-ray diffraction, scanning electron microscopy, and nuclear magnetic resonance spectroscopy, and the isoflavone content and solubility were determined by liquid chromatography. The results suggest that the isoflavones daidzein, genistein and glycitein may be externally associated to HPβCD as well as that isoflavones/HPβCD inclusion complexes are formed through the insertion of B-ring into the cyclodextrin cavity. Except for the freeze-dried IEF/HPβCD solid complex, all complexes showed similar content and solubility. In conclusion, the three isoflavones showed to be able to simultaneously complex with HPβCD.

1. Introduction

Isoflavones are a subclass of flavonoids with a 15-carbon (C_6–C_3–C_6) backbone arranged as a 1,2-diphenylpropane skeleton. They are widely distributed in the plant kingdom, especially soybeans (*Glycine max*). Despite the main isoflavones present in soybean/soy based products are mostly in 6″-*O*-malonyl glycoside and 7-*O*-glucosides forms, only the aglycone forms (daidzein, glycitein and genistein), which are the biologically active forms, are absorbed (Setchell, 1998).

Due to its chemical structure similar to the estrogen (Kuiper et al., 1997), many biological activities have been ascribed for isoflavones, like reduced menopause related symptoms, prevention of osteoporosis and coronary heart disease, as well as prevention of prostate and breast cancer (Adlercreutz, 2002; Howes, Howes, & Knight, 2006; Yamaguchi, 2002). However, its use as a dietary supplement and pharmaceutical preparation is limited by its bitter taste, low water solubility, low stability and low bioavailability (Setchell et al., 2001; Ungar, Osundahunsi, & Shimoni, 2003).

Among conventional methods used to enhance the solubility and bioavailability of drugs with low water solubility, the preparation of inclusion complexes with cyclodextrins seems to be very promising. Cyclodextrins are macrocyclic, non-reducing maltooligosaccharides composed of glucose units linked by α-(1,4)glycosidic bonds (Szejtli, 1998). The most common cyclodextrins are the α-cyclodextrin, β-cyclodextrin (βCD), γ-cyclodextrin, which are composed of six, seven and eight glucopyranose units, respectively. Due to lack of free rotation of the bonds connecting the glucopyranose units, the cyclodextrins have a toroidal or cone shaped, where the primary hydroxyl groups are located on the narrow side of the torus while the secondary hydroxyl groups are located on the wider edge. This architecture provides the formation of an internal hydrophobic cavity, whereas the external surface is hydrophilic. The lipophilic cavity of cyclodextrin molecules provides a microenvironment into which appropriately sized non-polar moieties can enter to form

* Corresponding author. Tel.: +55 51 3308 3004; fax: +55 51 3308 5437.
E-mail addresses: franciniyatsu@gmail.com, franciniyatsu@yahoo.com (F.K.J. Yatsu), leticia.koester@ufrgs.br (L.S. Koester), ivanalula@ufmg.br (I. Lula), passosjj@gmail.com (J.J. Passos), sinisterra@ufmg.br (R. Sinisterra), valquiria@pq.cnpq.br (V.L. Bassani).

0144-8617/$ – see front matter © 2013 The Authors. Published by Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.carbpol.2013.06.062

a inclusion complex (Loftsson & Brewster, 1996). Moreover, toxicity studies have demonstrated that, when orally administered, cyclodextrins are practically non-toxic due to the lack of absorption from the gastrointestinal tract (Irie & Uekama, 1997).

Some studies have evaluated the formation of inclusion complexes of cyclodextrins with genistein and daidzein. Crupi et al. (2007) evaluated the mode of complexation between genistein and βCD, (2-hydroxypropyl)-β-cyclodextrin (HPβCD) and methyl-β-cyclodextrin by UV–vis absorption and FTIR–ATR spectroscopy. In the same year, the improvement of daidzein and genistein solubility by complexation with HPβCD at different host/guest molar ratios was also reported. (Stancanelli et al., 2007). Later, Daruhazi et al. (2008) reported the obtention of genistein/β- and γ-cyclodextrin complexes employing kneading method and Xavier et al. (2010) prepared genistein/βCD solid complexes with high drug loading by freeze-drying.

Although these studies have demonstrated the feasibility of obtaining inclusion complexes with isoflavones, none of them evaluated the simultaneous complexation of the three isoflavones with cyclodextrins, as well as the effect of different methods of complexation on solubility and complexation efficiency. Therefore, in the present study, we evaluated the complexation of an isoflavone enriched fraction (IEF) with βCD and HPβCD and prepared, by different methods, solid complexes of IEF/HPβCD, which were further characterized using Fourier transformed-infrared spectroscopy, differential scanning calorimetry, X-ray diffraction, scanning electron microscopy, and nuclear magnetic resonance spectroscopy. To the best of our knowledge, this is the first report of complexation study comprising multiple associations of molecules present in a fraction.

2. Materials and methods

2.1. Chemicals

Liquid chromatography (LC)-grade acetonitrile (Tedia, Fairfield, OH, USA), trifluoroacetic acid (Merck, Hohonbrunn, Germany), and purified water (Milli-Q^{TM} system, Millipore, Bedford, MA, USA) were used for mobile phase preparation. Daidzein (98%, HPLC purity), glycitein (97%, HPLC purity), and genistein (98%, HPLC purity) were obtained from Sigma–Aldrich (Steinheim, Germany). HPβCD and βCD were kindly supplied by Roquette Frères (Lestrem, France).

2.2. Isoflavone enriched fraction preparation

The IEF was produced from *Glycine max* dry extract. The extraction of the isoflavones was performed with ethanol 96 GL at the ratio of dry extract to ethanol of 1:20 (g/mL). The resulting mixture was heated at 60 °C for 2 h under constant agitation. The extractive solution was separated from the insoluble material by filtration and hydrolyzed with hydrochloric acid 0.5 M at 80 °C for 12 h under constant agitation. The resulting hydrolyzed solution was mixed with distilled water at the volume ratio of 1:5 (v/v) and maintained at rest for 12 h at 10 °C. The crystallized material was separated by decantation and filtration. The solid material was recrystallized in pure ethanol at 80 °C, filtrated and dried in oven at 40 °C for 24 h. The isoflavones content was determined by Liquid chromatography: 441.43 ± 3.89 mg/g of daidzein, 363.46 ± 1.04 mg/g of glycitein and 91.72 ± 0.95 mg/g of genistein.

2.3. Liquid chromatography analysis

The Liquid chromatography (LC) analysis was performed on a Shimadzu Prominence device coupled to diode array detection (DAD) instrument and an automatic injector (Kyoto, Japan). The stationary phase was a Phenomenex RP-18 column (Synergi Fusion 150 × 4.6 mm i.d.; particle size, 4 μm) guarded by a Waters precolumn (20 × 3.9 mm i.d.; particle size, 10 mm) (Milford, MA, USA). The mobile phase consisted of (A) trifluoroacetic acid 0.1% (v/v) and (B) acetonitrile:trifluoroacetic acid (100:0.01, v/v). The gradient elution was 20–25% B (0–10 min), 25–30% B (10–15 min), and 30–35% B (15–23 min). The column was washed with acetonitrile for 3 min and re-equilibrated with 20% B for 4 min before the next analysis. The flow rate was 1 mL.min^{-1} and the injection volume was 10 μL. The detection wavelength was 260 nm and the analysis was carried out at 40 °C. All the samples were solubilized in acetonitrile 50% (v/v) and sonicated for 30 min. The clear solution was filtered through a 0.45 μm PTFE membrane and injected. The method was validated over the concentration range of 0.1–10.0 μg.mL^{-1} according to ICH guidelines (ICH, 2005).

2.4. Phase-solubility study

Phase-solubility studies were performed with an IKA® stirred temperature controlled bath (IKA Werke GmbH & Co., Breisgau, Germany), which allowed an accuracy of 0.1 °C. A fixed amount of IEF exceeding its solubility was added to unbuffered aqueous solutions containing increasing concentrations of HPβCD or βCD in 2 mL glass vials. Vials were sealed to avoid changes due to evaporation and magnetically stirred for 2 days at 24.0 ± 0.01 °C or 37.0 ± 0.01 °C, protected from light to prevent any degradation of the molecules. After the equilibrium was reached, the suspensions were centrifuged for 30 min at 7000 rpm and filtered through a 0.45 μm PTFE membrane (Millipore HAWP) for direct analysis by LC. Experiments were carried out in triplicate.

The apparent stability constant (K_s), the complexation efficiency (*CE*) and the molar ratio of the isoflavones/cyclodextrins complexes were calculated based on the phase-solubility diagrams according to the following equations:

$$K_s(M^{-1}) = \text{slope}/(S_0 \times (1 - \text{slope}))$$

$$\text{CE} = \text{slope}/(1 - \text{slope})$$

$$\text{Molar ratio} = 1 + (1/\text{CE})$$

where S_0 is the intrinsic solubility of isoflavones in water (solubility of isoflavones in absence of cyclodextrin).

2.5. Preparation of isoflavone enriched fraction/(2-hydroxypropyl)-β-cyclodextrin complexes

2.5.1. Freeze-drying method

The required amount of IEF and aqueous solution of HPβCD were mixed at the molar ratio of 1:1 and stirred at 25 °C for 2 days protected from light. After this period, the dispersion filtered through a 0.45 μm PTFE membrane (Millipore HAWP), frozen at −18 °C for 24 h and then dried 48 h by lyophilization in an Edwards EF4 Modulyo freeze dryer (Edwards, UK).

2.5.2. Spray-drying method

The required amounts of IEF and HPβCD at the molar ratio of 1:1 were dissolved in ethanol 77% (v/v) at 60 °C. The resultant solution was cooled down to room temperature and dried in a Mini spray-dryer B-290 coupled to Inert loop B-295 (Büchi, Switzerland). The spray-dryer was operated under the following conditions: inlet temperature 130 °C, outlet temperature 50 °C, and flow rate of 3 mL min^{-1}.

2.5.3. Kneading/microwave method

Pre-weighed amounts of IEF and HPβCD at the molar ratio of 1:1 were grounded for 10 min in a ceramic mortar. The resultant mixture was further kneaded with ethanol 66% (v/v) for 45 min.

The pasty mass obtained was dried in a microwave at a power of 245 watts at 60 °C, crushed and sifted through sieve no. 80.

2.5.4. Microwave method

The required amounts of IEF and HPβCD at the molar ratio of 1:1 were dissolved in ethanol 66% (v/v) at 60 °C. The solution was further microwaved at a power of 245 watts at 60 °C for 90 s. The solvent was evaporated under vacuum and the collected residue was crushed and sifted through sieve no. 80.

2.5.5. Co-evaporation method

Accurately weighed amounts of IEF and HPβCD at the molar ratio of 1:1 were dissolved in ethanol 66% (v/v) and stirred on a magnetic stirrer at 60 °C. The pasty mass obtained was dried overnight in a vacuum desiccator, crushed and sifted through sieve no. 80.

2.5.6. Physical mixture

For comparison purposes, physical mixture (PM) was prepared at the molar ratio of 1:1 of IEF:HPβCD by simple blending in a vial for 30 min.

2.6. Scanning electron microscopy

The photomicrographs of the samples were taken using a Jeol JSM 6060 microscope (Tokyo, Japan) at a voltage of 10 kV. The samples were previously mounted on aluminum stubs using double-sided adhesive tape and vacuum-coated with a thin layer of gold.

2.7. Fourier transformed-infrared (FTIR) spectroscopy

Infrared spectra covering the range of 4000–400 cm^{-1} were obtained with a Spectrum BX FTIR spectrometer with *MIRacle ATR* accessory (Perkin Elmer, MA, USA). The spectra were an average of 18 scans at a resolution of 4 cm^{-1}.

2.8. Thermal analysis

Differential Scanning Calorimetry (DSC) curve was performed in a DSC-60 calorimeter (Shimadzu Co., Kyoto, Japan) using the following conditions: dynamic nitrogen atmosphere (50 mL min^{-1}) and heating rate of 10 °C min^{-1}. Samples were accurately weighted and submitted to further heat scanning from 25 to 500 °C in a sealed aluminum pan. An empty sealed aluminum pan was used as reference. Data analysis was carried out using TA Analysis Software.

2.9. X-ray diffraction

The X-ray diffraction (XRD) experiments were carried out on a Siemens D5000 diffractometer (Siemens, Berlin, Germany) equipped with a curved graphite crystal using Cu Kα radiation (λ = 1.5406 Å). The diffraction data were collected at room temperature in a Bragg–Brentano θ–2θ geometry. The equipment was operated at 40 kV and 20 mA with a scan range between 8 and 40. The diffractograms were obtained with a constant step, step size/time of 0.02 2θ/s.

2.10. Nuclear magnetic resonance spectroscopy

NMR spectra were recorded on Bruker DRX400-*AVANCE* spectrometer operating at 400 MHz, equipped with a 5 mm inverse probe with z-gradient coil and a 5 mm, direct detection, dual probe ($^1H/^{13}C$), respectively. DMSO-d_6 (isotopic purity at least 99.5%) was purchased from Aldrich and used as solvent, and tetramethylsilane (TMS) as internal standard (δ 0.0). One-dimensional ^1H and ^{13}C NMR spectra were acquired under standard conditions. Two-dimensional inverse hydrogen-detected heteronuclear shift correlation spectra were obtained by HSQC pulse sequence [^1J(C, H)] and HMBC pulse sequence [^nJ(C, H), n = 2, 3, and 4]. ^1H homonuclear correlation spectroscopy (COSY) and ^1H homonuclear 2D-ROESY (spinlock pulse = 600 ms) were used to confirm the assigments of the hydrogen signals. Nuclear Overhauser effect (NOE) correlations were used in order to confirm daidzein/glycitein/genistein/cyclodextrin interactions and to get information about the geometry of the supramolecular complexes. All experiments were recorded at 300 K.

2.11. Solubility of the isoflavones in IEF associated or non-associated to cyclodextrin

The solubility study of the isoflavones present in IEF and IEF associated to cyclodextrins were determined by adding an excess of sample into 2 mL of distilled water in a glass vial. The glass vial was capped and attached to a wrist shaker in a water bath at 25 ± 2 °C for 2 days with agitation. The samples were centrifuged 30 min at 7000 rpm, filtered through a 0.45 μm PTFE membrane and analyzed by LC. All the experiments were performed in triplicate.

2.12. Statistical analysis

The results were analyzed either by the student's test or by the analysis of variance (ANOVA) followed by Tukey's test for significance at $p < 0.05$.

3. Results and discussion

The phase-solubility diagrams of IEF/HPβCD and IEF/βCD at 24.0 ± 0.01 °C and 37 °C ± 0.01 °C are showed, respectively, in Fig. 1A and B. In both temperatures the aqueous solubility of IEF increased linearly with the concentration of HPβCD or βCD.

At 24.0 ± 0.01 °C, the effect of both cyclodextrins on the IEF water solubility and on the apparent stability constant and complexation efficiency of corresponding complexes is presented in Table 1.

Among the three isoflavones, genistein presented the highest S_0, which may be related to the presence of an additional hydroxyl group. In contrast, daidzein and glycitein presented the highest increase in their solubility in the presence of both cyclodextrins.

The phase-solubility diagrams presented A_L-type profile with slope less than unit in each case (Higuchi & Connors, 1965), which indicates that the increase in solubility was due to the formation of a 1:1 M complex in solution with all the three isoflavones. The K_s determined for all isoflavone/cyclodextrin complexes suggest a good interaction between the isoflavones and cyclodextrins, especially for daidzein and glycitein complexes. Isoflavones also demonstrated stronger interactions with HPβCD, which could explain the higher solubility of these complexes in water than that observed for isoflavone/βCD complexes.

Considering that isoflavones present low water solubility, it is more convenient to compare the CE than K_s values. For poorly soluble drugs (aqueous solubility <0.1 mM), the S_0 is in general much higher than the intercept of the phase-solubility diagram resulting in non-linearity of otherwise linear (A_L-type) phase-solubility diagram, which can lead to erroneous $K_{1:1}$ values (Carrier, Miller, & Ahmed, 2007). Based on CE values, HPβCD also presented the highest solubilizing power since a lower amount of this cyclodextrin was required to solubilize all isoflavones (1:7). In other words, to form a complex between one molecule of HPβCD and one molecule of isoflavone, approximately 7 molecules of HPβCD will remain free. This low efficiency results in a large excess of

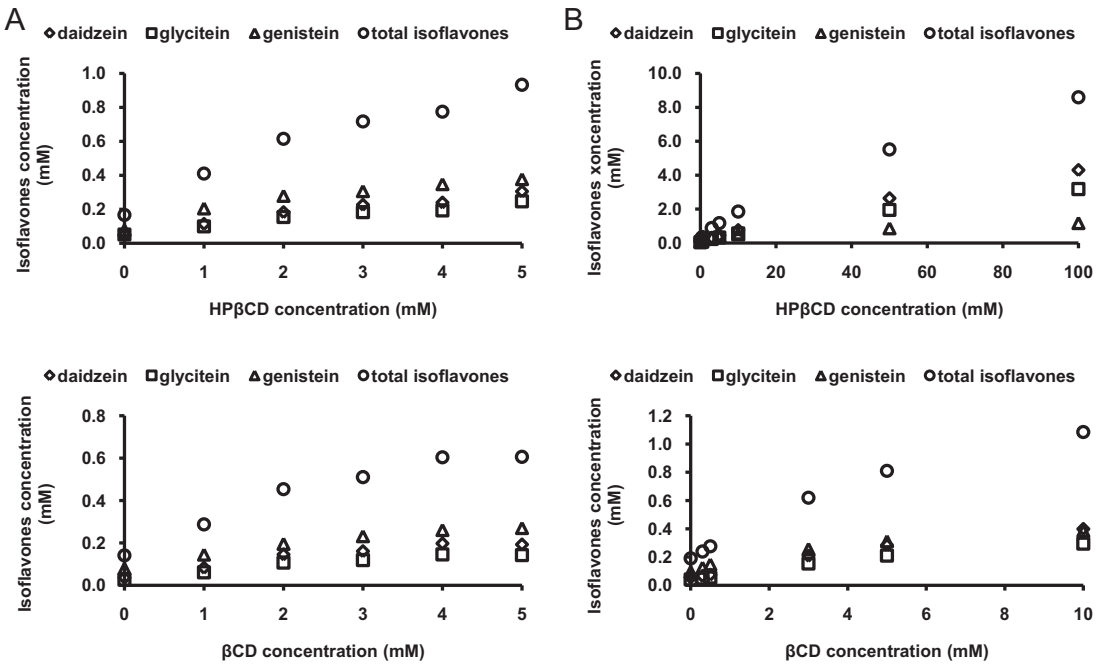

Fig. 1. Phase-solubility diagrams of isoflavone enriched fraction/(2-hydroxypropyl)-β-cyclodextrin and isoflavone enriched fraction/β-cyclodextrin at (A) 24.0 ± 0.01 °C and (B) 37.0 ± 0.01 °C.

uncomplexed HPβCD solubilized in water and represents an undesirable characteristic in the production of pharmaceutical forms since it represents a very large amount of excipient.

Recently, Borghetti, Lula, Sinisterra, and Bassani (2009) demonstrated that operating conditions (temperature, stirring time, and excess amount of quercetin) can influence the quercetin/β-cyclodextrin complexation efficiency in the phase-solubility studies. They showed that, with an excess amount of quercetin at higher temperature (37 °C), it was possible to enhance the complexation efficiency. For this reason, in an attempt to increase the complexation efficiency of both cyclodextrins, another phase-solubility study was performed at higher temperature (37 °C) with a higher cyclodextrin concentration range: 0–10 mM of βCD and 0–100 mM of HPβCD. The βCD concentration range was limited to 10 mM due its lower solubility in water.

At 37 °C (Fig. 1B), the phase-solubility revealed that, even in wider range of cyclodextrin concentrations, the profile of the phase-solubility diagrams was not altered (A_L type, slope < 1). However it resulted in a S_0 increase and, on the other hand, a significant reduction of the K_s and CE values for both cyclodextrins, especially for genistein (Table 1). These results suggest that the formation of isoflavones/cyclodextrins complexes is an exothermic process and that the higher cyclodextrin concentration was not able to overcome this effect.

The isoflavone aglycones contained in the IEF presented a water solubility higher than that reported for pure daidzein, which were 2.9 μg/mL (Wang, Komolpis, Kaufman, Malakul, & Shotipruk, 2001), 1.17 μg/mL (Zhao et al., 2011), 2.63 μg/mL (Borghetti et al., 2011), and 3.84 μg/mL (Ma, Zhao, Li, & Shen, 2012); and for pure genistein, which were 3.5 μg/mL (Wang et al., 2001), and 1.43 μg/mL (Wu, Ge, Zhang, Yu, & Zhang, 2010). No data about the glycitein solubility was found in the literature. A similar result was reported for an aglycone mixture of soy isoflavones (consisting of 55% genistein, 43% daidzein, and 1.8% glycitein). The authors verified that the solubility of genistein and daidzein in the mixture was 5.2- and 3.3-fold higher than that of the pure compounds in pH 6 buffer (Huang, Hung, & Fang, 2008). The explanation for this observation remains unclear and may involve several physical or physicochemical phenomena.

Thus, the complexation of isoflavone contained in IEF in the production of water-soluble complexes with cyclodextrins showed to be a promising strategy since the IEF/cyclodextrins complexes presented higher solubility and superior complexation efficiency. Based on the obtained results, IEF/HPβCD complexes were produced by different methods and characterized.

Table 1
Effect of cyclodextrins on the apparent stability constant and complexation efficiency of corresponding complexes at 24.0 ± 0.01 °C. and 37.0 ± 0.01 °C.

Temp. (°C)	Isoflavones	S_0 (mM)	K_s (M^{-1})		CE		Molar ratio (ISO:CD)		r^2	
			HPβCD	βCD	HPβCD	βCD	HPβCD	βCD	HPβCD	βCD
24	Daidzein	0.0493	1041	977	0.0514	0.0349	1:20	1:30	0.97	0.91
	Glycitein	0.0507	809	968	0.0411	0.0256	1:25	1:40	0.97	0.91
	Genistein	0.0765	775	503	0.0593	0.0398	1:18	1:26	0.91	0.94
	Total	0.1664	741	610	0.1678	0.1053	1:7	1:10	0.95	0.92
37	Daidzein	0.0557	738	616	0.0449	0.0369	1:23	1:28	0.99	0.96
	Glycitein	0.0450	682	622	0.0327	0.0270	1:32	1:38	0.99	0.96
	Genistein	0.0952	105	291	0.0102	0.0294	1:99	1:35	0.90	0.92
	Total	0.1944	397	465	0.0920	0.1003	1:12	1:11	0.98	0.95

temp = temperature, S_0 = apparent solubility, K_s = apparent stability constant, CE = complexation efficiency, CD = cyclodextrin, ISO = isoflavone, HPβCD = (2-hydroxypropryl)-β-cyclodextrin, βCD = β-cyclodextrin.

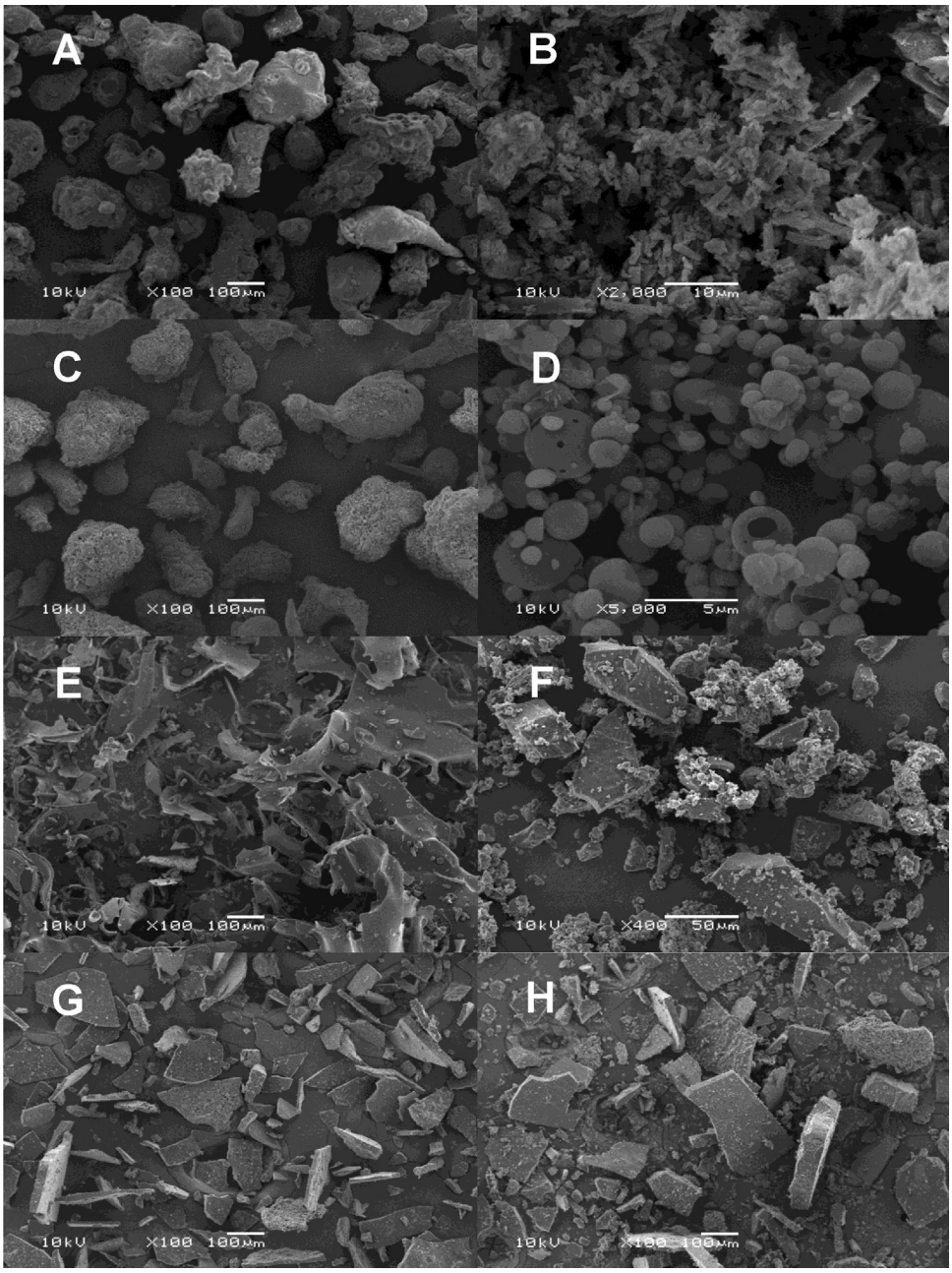

Fig. 2. Photomicrographs obtained by scanning electron microscopy of (A) (2-hydroxypropyl)-β-cyclodextrin (HPβCD), (B) isoflavone enriched fraction (IEF), (C) physical mixture of IEF and HPβCD (1:1), (D) spray-dried IEF/HPβCD complex, (E) freeze-dried IEF/HPβCD complex, (F) kneading/microwaved IEF/HPβCD complex, (G) microwaved IEF/HPβCD complex, and (H) co-evaporated IEF/HPβCD complex.

The photomicrographs obtained for HPβCD, IEF, IEF/HPβCD physical mixture and the corresponding solid complexes are presented in Fig. 2. IEF reveled particles with an acicular habit with fissures and irregular surface (Fig. 2B), whereas HPβCD particles were larger with rounded shape and sub-rough surface (Fig. 2A). In case of the IEF/HPβCD physical mixture, the particles of both IEF and HPβCD were clearly distinguishable from each other (Fig. 2C), which suggest that no interaction takes place between the two parent compounds in solid state. On the other hand, the solid complexes were completely different from those of IEF or HPβCD. The spray-dried IEF/HPβCD complex showed spherical particles with smooth surface (Fig. 2D) and the freeze-dried IEF/HPβCD complex showed to be an amorphous powder (Fig. 2E). Since the other complexes were crushed, they presented particles with shard shape and broken edges. Despite these results suggest the formation of a new single solid phase, they cannot confirm the formation of complexes between IEF and HPβCD.

Fig. 3 presents the thermograms of HPβCD, IEF, and the corresponding physical mixture and solid complexes. IEF presents a unique sharp endothermic peak at 300 °C and HPβCD a broad endothermic peak at 334 °C. In the IEF/HPβCD physical mixture, the intensity of the peak corresponding to the melting point of IEF is drastically reduced, which was expected since the amount of IEF in the physical mixture is very small. The IEF/HPβCD solid complexes showed a complete disappearance of IEF peak and the presence of slightly shifted peak of HPβCD. Therefore, although the products obtained are different from the physical mixture, with respect to the DSC profiles, they not provide enough evidence of true inclusion complexes.

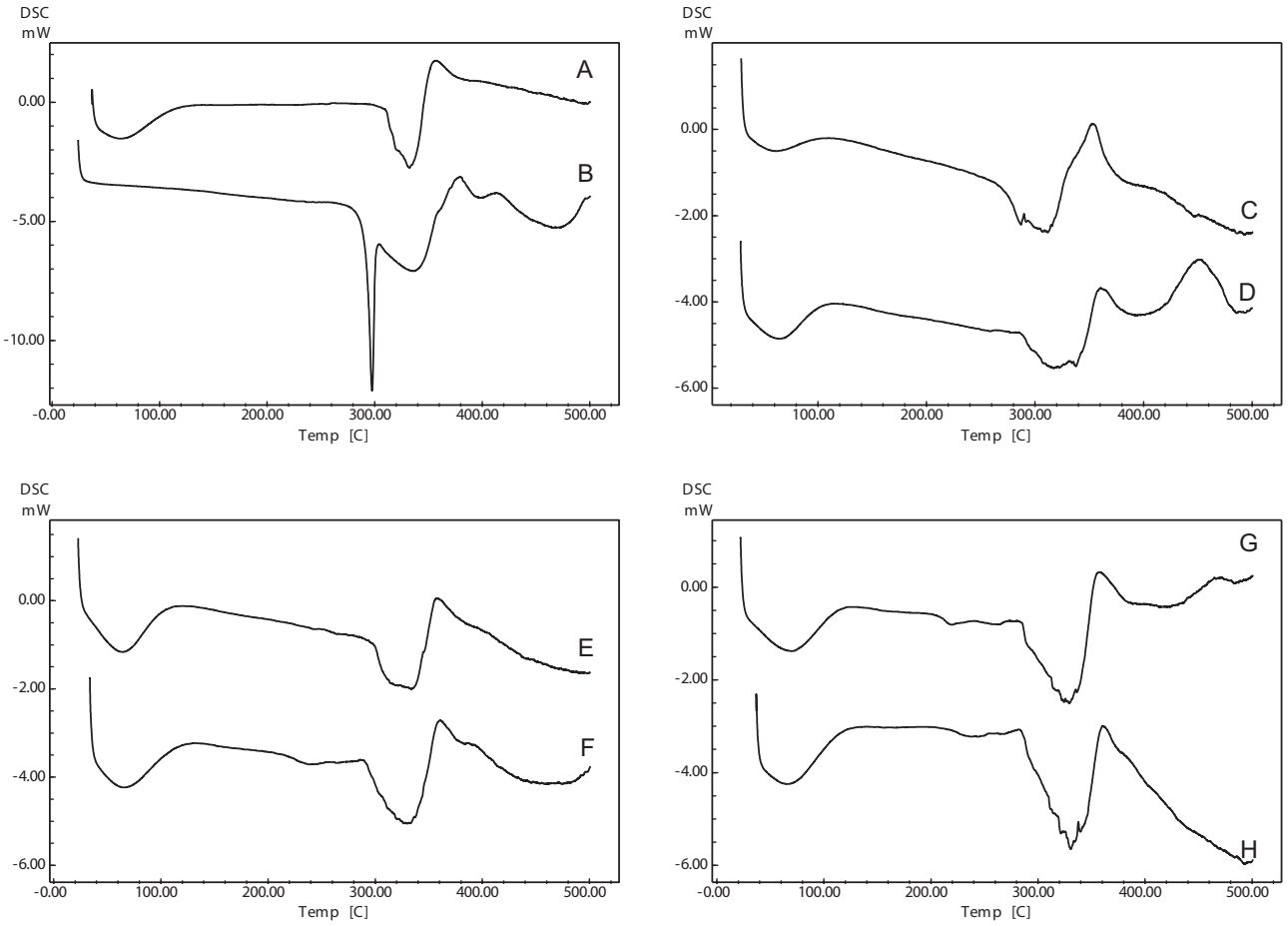

Fig. 3. The Differential scanning calorimetry curves of (A) (2-hydroxypropyl)-β-cyclodextrin (HPβCD), (B) isoflavone enriched fraction (IEF), (C) physical mixture of IEF and HPβCD (1:1), (D) freeze-dried IEF/HPβCD complex, (E) spray-dried IEF/HPβCD complex, (F) co-evaporated IEF/HPβCD complex, (G) microwaved IEF/HPβCD complex, and (H) kneading/microwaved IEF/HPβCD complex.

Additional evidence of IEF complexation was obtained by XRD diffractograms (Fig. 4). XRD pattern of IEF revealed a well-defined crystal, whereas HPβCD showed to be amorphous. The diffractogram of physical mixture showed peaks corresponding to the IEF, indicating the presence of IEF in the crystalline state. In contrast, microwaved, kneading/microwaved, and co-evaporated solid complexes exhibits considerable decrease of the diffraction peaks, suggesting that it is less crystalline than

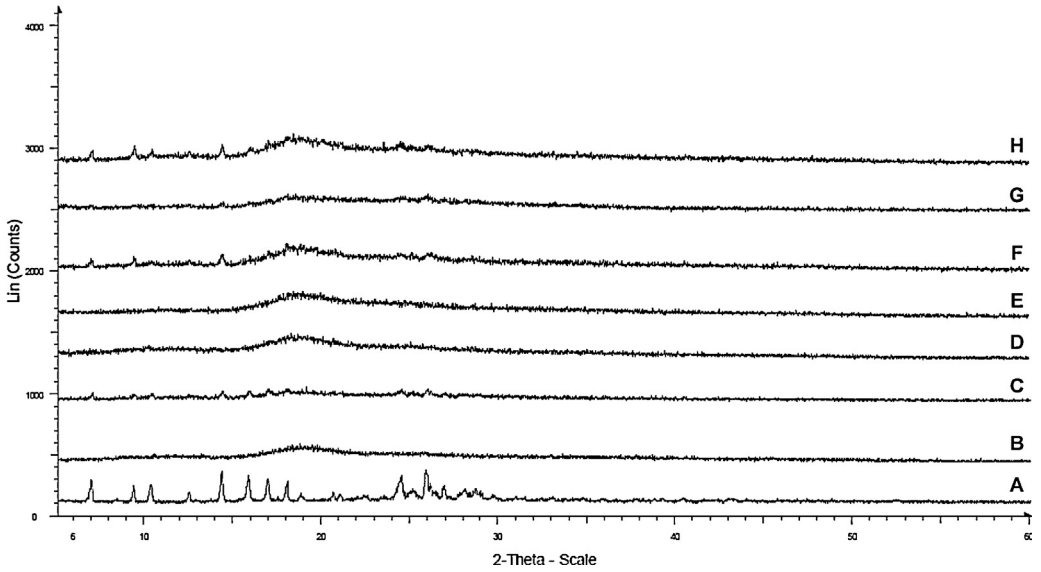

Fig. 4. X-ray diffractograms of (A) isoflavone enriched fraction (IEF), (B) (2-hydroxypropyl)-β-cyclodextrin (HPβCD), (C) physical mixture of IEF and HPβCD (1:1), (D) freeze-dried IEF/HPβCD complex, (E) spray-dried IEF/HPβCD complex, (F) microwaved IEF/HPβCD complex, (G) kneading/microwaved IEF/HPβCD complex, and (H) co-evaporated IEF/HPβCD complex.

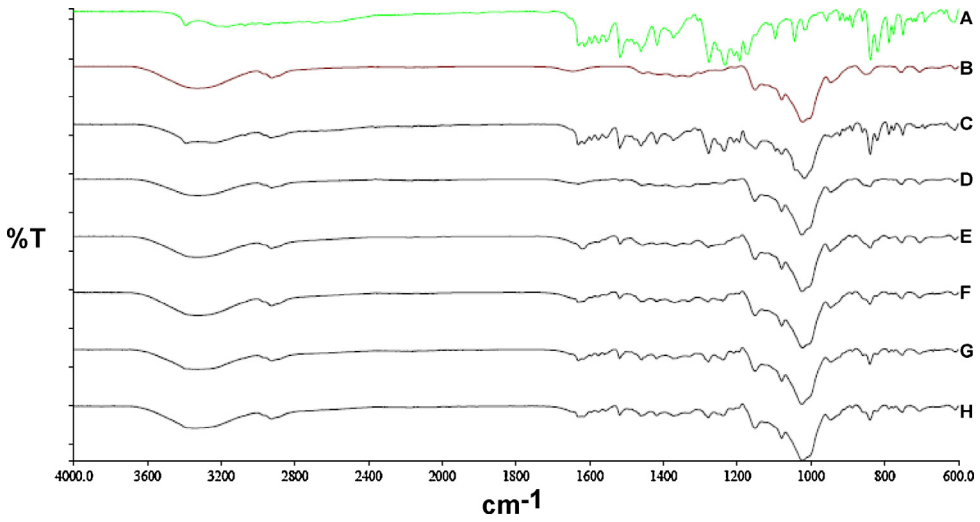

Fig. 5. FTIR spectrum (A) isoflavone enriched fraction (IEF), (B) (2-hydroxypropyl)-β-cyclodextrin (HPβCD), (C) physical mixture of IEF and HPβCD (1:1), (D) freeze-dried IEF/HPβCD complex, (E) spray-dried IEF/HPβCD complex, (F) microwaved IEF/HPβCD complex, (G) kneading/microwaved IEF/HPβCD complex, and (H) co-evaporated IEF/HPβCD complex.

the physical mixture. Moreover, the freeze-dried and spray-dried solid complexes diffractograms presented an absence of sharp peaks. These results strongly suggest that the complexes are in a totally different form and the isoflavones are interacting with HPβCD probably by the formation of inclusion complexes.

In attempt to elucidated the host:guest interactions, FTIR spectrum and ^{1}H NMR spectrum of IEF, HPβCD, IEF/HPβCD physical

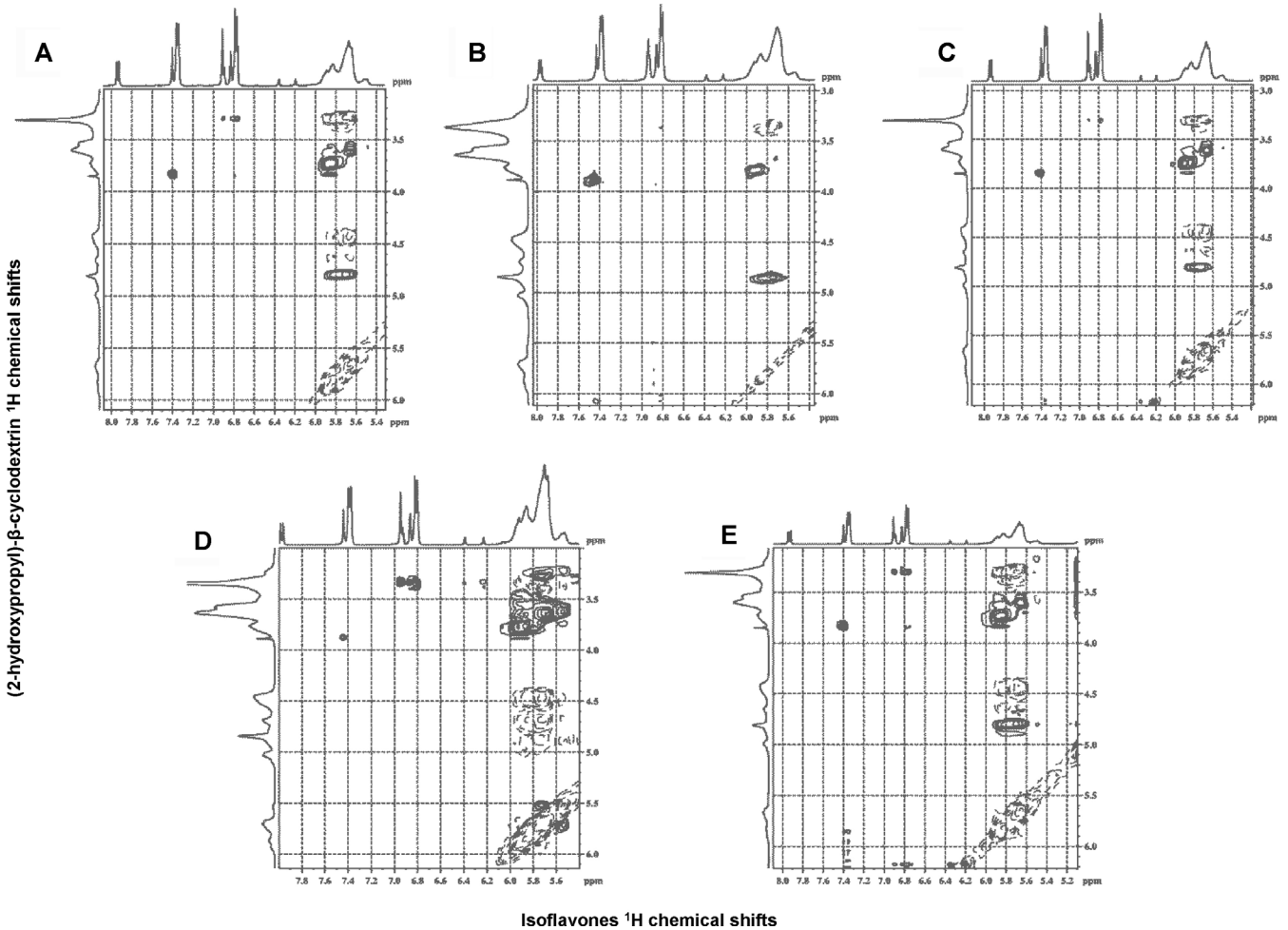

Fig. 6. Expanded region of 2D-ROESY counter maps of complexes (400 MHz, D_2O). (A) spray-dried IEF/HPβCD complex, (B) freeze-dried IEF/HPβCD complex, (C) microwaved IEF/HPβCD complex, (D) kneading/microwaved IEF/HPβCD complex, and (E) co-evaporated IEF/HPβCD complex.

mixture and IEF/HPβCD complexes were compared (Fig. 5, Table A.1 and Fig. A.1). FTIR spectrum of IEF (Fig. 5) showed characteristic absorption bands in 3394 cm^{-1} related to –OH free stretching frequency, 3224 cm^{-1} related to —OH bonded stretching frequency (characteristic of the hydrogen bond formed between —OH at the C_5 and the carbonyl group at C_4 in genistein), 3070 cm^{-1} related to C—H (sp^2) stretching frequency, 2939 cm^{-1} related to C—H (sp^3) stretching frequency (characteristic of the $—OCH_3$ from glycitein), 1630 cm^{-1} related to C═O stretching frequency, 1614 cm^{-1} related to C═C stretching frequency, 1194 cm^{-1} related to C—O stretching frequency, and 820 cm^{-1} related to stretching frequency of p-substituted phenyl. The FTIR spectrum of HPβCD showed prominent peaks at 3337.71 cm^{-1} (O—H stretching), 2925.65 cm^{-1} (C—H stretching), and 1651.49 cm^{-1} (H—O—H bending). The FTIR spectrum of physical mixture showed peaks corresponding to the original compounds.

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2013.06.062.

With respect to IEF/HPβCD solid complexes spectra, the characteristic absorption bands of IEF (3394 cm^{-1}, 3224 cm^{-1}, and 2939 cm^{-1}) could not be identified. The C═O stretching (1630 cm^{-1}), the C═C stretching (1614 cm^{-1}) and the C—O stretching (1194 cm^{-1}) peaks also appear bumped and diminished in intensity in the spectra of the complexes. These observations are consistent with those made by Crupi et al. (2007), which postulated that spectral changes, at high frequency, in the O—H stretching profile, and, at low frequency, in the C═O, C═C, C—O—C and C—O stretching vibrations could be attributed to the association, via H-bond, between the host and the guest during complexation process.

With respect to ^1H and ^{13}C NMR spectra, the chemical shifts of hydrogens and carbons of IEF and the chemical shifts of hydrogens IEF/HPβCD complexes are summarized in Table A.1. The comparison between the one-dimensional ^1H NMR spectra obtained for IEF and the five complexes are presented in Fig. A.1.

The hydrogen and carbons attributions of the EIF were compared with the ^1H NMR spectral data of the literature (Borghetti et al., 2011; Coward, Barnes, Setchell, & Barnes, 1993; Jha, Zilliken, & Breitmaier, 1980; Park et al., 1999; Sung, Choi, Lee, Park, & Moon, 2004; Xavier et al., 2010). The hydrogen and carbons chemical shifts observed in the EIF spectrum were consistent with a mixture of the three isoflavones.

The freeze-dried IEF/HPβCD complex only presented chemical displacements (downfield shift) in the hydrogen signals H_6 and H_8 (A ring) and OH-4′ (B ring) in the isoflavones. In the same way, the spray-dried IEF/HPβCD complex showed few chemical displacements (downfield shift) in the hydrogen signals H_5 and H_6 (A ring), and $H_{2'/6'}$ (B ring) in the isoflavones. On other hand, microwaved, kneading/microwaved, and co-evaporated IEF/HPβCD complexes demonstrated a significant number of chemical displacements (downfield shift) in the hydrogen signals H_2 (C ring); H_5, H_6, H_8, OH-5 and OCH_3 (A ring); and $H_{2'/6'}$ and $H_{3'/5'}$ (B ring) in the isoflavones. In all cases, this result suggests some perturbations in molecular electron density of isoflavones due to existence of the interactions between isoflavones and HPβCD.

Daruhazi et al. (2008) reported that genistein has formed an inclusion complex with βCD by the insertion of the B ring into the cyclodextrin cavity. In the same way, Borghetti et al. (2011) reported that daidzein would form an inclusion complex with βCD and HPβCD by the insertion of the B ring into the cyclodextrin cavity. On other hand, Xavier et al. (2010) reported that the complexation of genistein with β-CD occurs mainly through the insertion of the A-ring into the cyclodextrin cavity. Based in our results, the complexation can occur through the insertion of either A-ring or B-ring into the cyclodextrin cavity.

Table 2
Total content of isoflavone in the IEF/HPβCD solid complexes and its water solubility.

Isoflavones	Complexation methods									
	Spray-drying		Freeze-drying		Kneading/microwave		Microwave		Co-evaporation	
	Content (mg/g)	Solub. (μg/mL)	Content (mg/g)	Solub. (μg/mL)	Content (mg/g)	Solub. (μg/mL)	Content (mg/g)	Solub. (μg/mL)	Content (mg/g)	Solub. (μg/mL)
Daidzein (mean ± S.D.)	57.26 ± 0.02	8.69 ± 0.65	18.47 ± 0.04	4.31 ± 0.90	61.59 ± 0.02	6.40 ± 0.57	58.07 ± 0.07	9.33 ± 1.23	63.40 ± 0.05	9.85 ± 2.08
Glycitein (mean ± S.D.)	51.17 ± 0.02	0.77 ± 0.09	16.28 ± 0.03	0.56 ± 0.12	55.90 ± 0.03	0.59 ± 0.14	55.46 ± 0.01	1.12 ± 0.24	54.75 ± 0.03	0.91 ± 0.28
Genistein (mean ± S.D.)	11.76 ± 0.01	2.86 ± 0.25	5.05 ± 0.03	1.87 ± 0.32	12.65 ± 0.04	2.39 ± 0.27	12.58 ± 0.04	3.90 ± 0.66	13.47 ± 0.07	4.15 ± 0.96
Total (mean ± S.D.)	120.19 ± 0.05	12.32 ± 0.97	39.80 ± 0.10	6.74 ± 1.33	130.14 ± 0.05	9.38 ± 0.80	126.11 ± 0.03	14.35 ± 2.12	131.61 ± 0.01	14.90 ± 3.16

S.D. = standard deviation.

Fig. 6 presents expanded regions of ^{1}H homonuclear 2D-ROESY contour maps of IEF/HPβCD complexes. The expansion of the spray-dried, freeze-dried, microwaved, kneading/microwaved and co-evaporated IEF/HPβCD complexes spectra presented intermolecular cross-peaks between the aromatic doublets (7.44–7.24 ppm) of the $H_{2'/6'}$ of the isoflavones and the hydrogen H_5 (3.88–3.85 ppm) of HPβCD. They also presented interaction between the hydrogen H_8 and H_6 (6.94–6.86 ppm) and $H_{3'/5'}$ (6.81 ppm) of the isoflavones and the hydrogen H_4 (3.32–3.38 ppm) of HPβCD. Therefore, the ^{1}H NMR analysis suggests that the isoflavones/HPβCD inclusion complexes have been formed through the insertion of B-ring into the cyclodextrin cavity, since the hydrogens H_3 and H_5 of HPβCD are located inside the cyclodextrin cavity. Moreover, the results also suggest that isoflavones could be externally associated to the HPβCD since the hydrogen H_4 of HPβCD is located outside the cyclodextrin cavity. This type of association is known as association complexes and also contributes to the increase on the isoflavones solubility in water.

Once the IEF/HPβCD solid complexes presented strong evidences about the formation of inclusion complexes, the content of isoflavone in the IEF/HPβCD solid complexes and its solubility in water were evaluated (Table 2). Except for the freeze-dried IEF/HPβCD solid complex, all complexes showed similar content of isoflavone and solubility ($p < 0.05$).

In the freeze-drying method, the dispersion of IEF and HPβCD was filtered before the lyophilization step to remove the unsolubilized material. Therefore, the smaller content of isoflavones in the IEF/HPβCD solid complex was probably due to the elimination of the unsolubilized IEF. Moreover, the freeze-drying method was less successful in promoting the formation of complexes between IEF and HPβCD, since the freeze-dried IEF/HPβCD solid complex presented the lowest solubility. This result could be related to the use of ethanol as co-solvent in the spray-drying, kneading/microwave, microwave and co-evaporation methods. The solubilization of the isoflavones and HPβCD, promoted by the ethanol, could facilitate the hydrophobic interaction between the isoflavones and HPβCD, consequently promoting the formation of complexes.

4. Conclusions

This is the first report that evaluates the influence of different methods of complexation on the simultaneous host:guest interactions among three compounds present in a plant fraction (IEF) and cyclodextrins. For both cyclodextrins tested, the phase-solubility diagrams may be classified as AL-type, indicating the formation of a 1:1 IEF/cyclodextrins complex, although HPβCD prove to be more effective in increasing the solubility of isoflavones.

The characterization of solid complexes and corresponding physical mixture by DCS, FTIR, and XRD analysis suggest that isoflavones interact with HPβCD probably by the formation of inclusion complexes. The ^{1}H NMR analysis suggests that the isoflavones/HP-β-CD inclusion complexes have been formed through the insertion of B-ring into the cyclodextrin cavity. Moreover, the results also suggest that isoflavones could be externally associated to the HPβCD.

All results suggest that both inclusion complexes and association complexes between cyclodextrins and isoflavones could be effectively formed and, except for the freeze-dried IEF/HPβCD solid complex, all other solid complexes obtained showed similar isoflavone content and solubility ($p < 0.05$). The formation of such complexes could be regarded as a promising strategy for improving the bioavailability of the isoflavones aglycones in a plant fraction.

Acknowledgements

The authors are grateful to the Coordenacão de Aperfeiçoamento de Pessoal de Nível Superior (Rede de Nanobiotecnologia) and to the Conselho Nacional de Desenvolvimento Científico e Tecnológico for the financial support and the scholarships.

References

Adlercreutz, H. (2002). Phyto-oestrogens and cancer. *Lancet Oncology*, *3*(6), 364–373.
Borghetti, G. S., Lula, I. S., Sinisterra, R. D., & Bassani, V. L. (2009). Quercetin/beta-cyclodextrin solid complexes prepared in aqueous solution followed by spray-drying or by physical mixture. *AAPS PharmSciTech*, *10*(1), 235–242.
Borghetti, G. S., Pinto, A. P., Lula, I. S., Sinisterra, R. D., Teixeira, H. F., & Bassani, V. L. (2011). Daidzein/cyclodextrin/hydrophilic polymer ternary systems. *Drug Development and Industrial Pharmacy*, *37*(8), 886–893.
Carrier, R. L., Miller, L. A., & Ahmed, M. (2007). The utility of cyclodextrins for enhancing oral bioavailability. *Journal of Controlled Release*, *123*(2), 78–99.
Coward, L., Barnes, N. C., Setchell, K. D. R., & Barnes, S. (1993). Genistein, daidzein, and their beta-glycoside conjugates – antitumor isoflavones in soybean foods from american and asian diets. *Journal of Agricultural and Food Chemistry*, *41*(11), 1961–1967.
Crupi, V., Ficarra, R., Guardo, M., Majolino, D., Stancanelli, R., & Venuti, V. (2007). UV–vis and FTIR–ATR spectroscopic techniques to study the inclusion complexes of genistein with beta-cyclodextrins. *Journal of Pharmaceutical and Biomedical Analysis*, *44*(1), 110–117.
Daruhazi, A. E., Szente, L., Balogh, B., Matyus, P., Beni, S., Takacs, M., et al. (2008). Utility of cyclodextrins in the formulation of genistein: Part 1. Preparation and physicochemical properties of genistein complexes with native cyclodextrins. *Journal of Pharmaceutical and Biomedical Analysis*, *48*(3), 636–640.
Higuchi, T., & Connors, K. A. (1965). Phase-solubility techniques. *Advances in Analytical Chemistry and Instrumentation*, *4*(1), 117–212.
Howes, L. G., Howes, J. B., & Knight, D. C. (2006). Isoflavone therapy for menopausal flushes: A systematic review and meta-analysis. *Maturitas*, *55*(3), 203–211.
Huang, Z. R., Hung, C. F. K. L. Y., & Fang, J. Y. (2008). In vitro and in vivo evaluation of topical delivery and potential dermal use of soy isoflavones genistein and daidzein. *International Journal of Pharmaceutics*, *364*(1), 36–44.
ICH. (2005). Stability testing testing of new drug substances and products. In *International conference on harmonisation QIA (R2)*
Irie, T., & Uekama, K. (1997). Pharmaceutical applications of cyclodextrins. III. Toxicological issues and safety evaluation. *Journal of Pharmaceutical Sciences*, *86*(2), 147–162.
Jha, H. C., Zilliken, F., & Breitmaier, E. (1980). Carbon-13 chemical shift assignments of chromones and isoflavones. *Canadian Journal of Chemistry*, *58*(12), 1211–1219.
Kuiper, G. G., Carlsson, B., Grandien, K., Enmark, E., Haggblad, J., Nilsson, S., et al. (1997). Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. *Endocrinology*, *138*(3), 863–870.
Loftsson, T., & Brewster, M. E. (1996). Pharmaceutical applications of cyclodextrins. 1. Drug solubilization and stabilization. *Journal of Pharmaceutical Sciences*, *85*(10), 1017–1025.
Ma, Y., Zhao, X., Li, J., & Shen, Q. (2012). The comparison of different daidzein-PLGA nanoparticles in increasing its oral bioavailability. *International Journal of Nanomedicine*, *7*, 559–570.
Park, H. J., Park, J. H., Moon, J. O., Lee, K. T., Jung, W. T., Oh, S. R., et al. (1999). Isoflavone glycosides from the flowers of *Pueraria thunbergiana*. *Phytochemistry*, *51*(1), 147–151.
Setchell, K. D. (1998). Phytoestrogens: The biochemistry, physiology, and implications for human health of soy isoflavones. *The American Journal of Clinical Nutrition*, *68*(6 Suppl.), 1333S–1346S.
Setchell, K. D., Brown, N. M., Desai, P., Zimmer-Nechemias, L., Wolfe, B. E., Brashear, W. T., et al. (2001). Bioavailability of pure isoflavones in healthy humans and analysis of commercial soy isoflavone supplements. *Journal of Nutrition*, *131*(4 Suppl.), 1362S–1375S.
Stancanelli, R., Mazzaglia, A., Tommasini, S., Calabro, M. L., Villari, V., Guardo, M., et al. (2007). The enhancement of isoflavones water solubility by complexation with modified cyclodextrins: A spectroscopic investigation with implications in the pharmaceutical analysis. *Journal of Pharmaceutical and Biomedical Analysis*, *44*(4), 980–984.
Sung, J. H., Choi, S. J., Lee, S. W., Park, K. H., & Moon, T. W. (2004). Isoflavones found in Korean soybean paste as 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors. *Bioscience Biotechnology and Biochemistry*, *68*(5), 1051–1058.
Szejtli, J. (1998). Introduction and general overview of cyclodextrin chemistry. *Chemical Reviews*, *98*(5), 1743–1753.
Ungar, Y., Osundahunsi, O. F., & Shimoni, E. (2003). Thermal stability of genistein and daidzein and its effect on their antioxidant activity. *Journal of Agricultural and Food Chemistry*, *51*(15), 4394–4399.
Wang, H. Y., Komolpis, K., Kaufman, P. B., Malakul, P., & Shotipruk, A. (2001). Permeabilization of metabolites from biologically viable soybeans (*Glycine max*). *Biotechnology Progress*, *17*(3), 424–430.

Wu, J. G., Ge, J. A., Zhang, Y. P., Yu, Y., & Zhang, X. Y. (2010). Solubility of genistein in water, methanol, ethanol, propan-2-ol, 1-butanol, and ethyl acetate from (280 to 333) K. *Journal of Chemical and Engineering Data*, *55*(11), 5286–5288.

Xavier, C. R., Silva, A. P. C., Schwingel, L. C., Borghetti, G. S., Koester, L. S., Mayorga, P., et al. (2010). Improvement of genistein content in solid genistein/β-cyclodextrin complexes. *Química Nova*, *33*(3), 587–590.

Yamaguchi, M. (2002). Isoflavone and bone metabolism: Its cellular mechanism and preventive role in bone loss. *Journal of Health Science*, *48*(3), 209–222.

Zhao, C., Wang, Y. T., Su, Y. Z., Zhang, H. F., Ding, L. X., Yan, X. F., et al. (2011). Inclusion complexes of isoflavones with two commercially available dendrimers: Solubility, stability, structures, release behaviors, cytotoxicity, and anti-oxidant activities. *International Journal of Pharmaceutics*, *421*(2), 301–309.