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Strategies to improve the solubility and stability of stilbene antioxidants: a comparative study between cyclodextrins and bile acids

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Running title: Solubility and stability of stilbene antioxidants

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Abstract

Aiming at the development of an active food packaging, the goal of this study was to increase stilbenes (resveratrol (RV), pterostilbene (PT) and pinosylvin (PS)) aqueous solubility and stability using hydropropyl-cyclodextrins (HP-CDs) and bile salts. To evaluate stilbene concentration, an HPLC-DAD method was validated. Stilbene solubility was improved by the formation of inclusion complexes and micellar systems with higher solubility values obtained for the inclusion complexes with cyclodextrins. Inclusion complexes revealed a 1:1 stoichiometry for RV and PT and a 1:2 for PS. Solid state characterization was carried out using X-ray diffraction, Fourier transform infrared spectroscopy and differential scanning calorimetry. ^{1}H NMR studies were also performed to characterize the prepared complexes. Photostability studies revealed that CDs were able to increase stilbene photostability at 4 ºC. This work showed that stable stilbene solutions can be achieved using hydroxypropyl-CDs, contributing for their incorporation in several materials for the food and pharmaceutical industries.

Keywords: stilbenes; cyclodextrins; bile salts; HPLC-DAD; photostability; inclusion complexes; micelles

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Introduction

Stilbenes comprise a class of plant polyphenols that have gained intense interest for their intricate structures and diverse biological activities (Shen, Wang & Lou, 2009). These compounds and their derivatives are of noticeable interest for areas as health and food biotechnology, drug research and development and medicine due to their potential in therapeutic or preventive applications (Shen et al., 2009). Stilbenes exist as both monomers and increasingly complex oligomers. The monomeric stilbene structure is relatively simple and characterized by two benzene rings joined by an ethylene bridge (Kasiotis, Pratsinis, Kletsas & Haroutounian, 2013). As a result of this ethylene bridge, stilbenes can occur as *cis*- and *trans*-isomers, of which the *trans*-isomer (E) is the most common configuration (Riviere, Pawlus & Merillon, 2012). So far, different pathways leading to *trans*-*cis* isomerization have been described such as double bond breakage by radicals, direct photoisomerization under solar or UV irradiation (Riviere et al., 2012) and thermal isomerization (Dugave & Demange, 2003). It is thought that *trans*-isomers are biologically more active than *cis*-isomers. However, the later are not so widely studied and only a few reports comparing the biological activity between the two isomers are available (Anisimova, Kiselevsky, Sosnov, Sadovnikov, Stankov & Gakh, 2011; Campos-Toimil, Elies, Alvarez, Verde & Orallo, 2007; Rius et al., 2010).

Stilbenes are widely considered phytoalexins in several plant families for their role in plant resistance to fungal pathogens. For instance, in some plants, stilbenes are constitutively expressed and, furthermore, their synthesis can also be induced in response to a large range of biotic and abiotic stress factors (Riviere et al., 2012). Resveratrol (3,5,4'-trihydroxystilbene, $C_{14}H_{12}O_3$) pterostilbene (3,5-dimethoxy-4'-hydroxystilbene, $C_{16}H_{16}O_3$) and pinosylvin (3,5-dihydroxystilbene, $C_{14}H_{12}O_2$) are known naturally occurring stilbene phytoallexins present in the

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wood pulp and bark of several trees, in *Vitis vinifera* leaves and berries, in peanuts, mulberries and several plant extracts, such as tea oils and herbal remedies (Bertacche, Lorenzi, Nava, Pini & Sinco, 2006; Lopez-Nicolas, Rodriguez-Bonilla & Garcia-Carmona, 2009a; Lopez-Nicolas, Rodriguez-Bonilla, Mendez-Cazorla & Garcia-Carmona, 2009b).

Over the past years, there has been an increased awareness and consumption of foods and supplements containing these stilbenes due to their perceived health benefits. For instance, resveratrol, a vastly studied stilbene, has been associated with several biological activities such as anti-oxidant, anti-inflammatory, analgesic, cardio-protective, neuro-protective, chemo-preventive, anti-aging and antimicrobial activities (Das, Lin, Ho & Ng, 2008; Paulo, Ferreira, Gallardo, Queiroz & Domingues, 2010). Pterostilbene and pinosylvin share many of resveratrol biological activities, including anti-cancer, anti-aging, and antimicrobial activities (Lee et al., 2005; Lin, Yue & Ho, 2009; Macickova et al., 2010; Park et al., 2012). Despite all these established biological activities, the poor solubility of these compounds and their sensitivity to external agents such as air, light, and oxidative enzymes can constitute a serious problem for their bioavailability and, therefore prevent stilbenes from achieving their desirable activity.

Several research studies have described that the complexation of polyphenols with cyclodextrins (CDs) and micellar systems led to a marked increase in their aqueous solubility and, even improved their stability and bioactivity (Dai, Chen & Zhou, 2008; Lu, Cheng, Hu, Zhang & Zou, 2009).

Bile acids are bi-planar natural compounds with two functionally different molecular surfaces: a hydrophobic convex surface of steroid core and a hydrophilic concave surface (Simonovic & Momirovic, 1997). This coexistence of polar and non-polar surfaces in molecules (amphyphilic molecules) induces their self-association in micelles, above a certain bile acid concentration (critical micellar concentration (CMC)), and influences their physical–chemical properties

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(Atanackovic, Posa, Heinle, Gojkovic-Bukarica & Cvejic, 2009). Micellar solutions of bile acids can solubilise poorly soluble organic substances, improving their resorption and enhancing cell permeability to substances (Atanackovic et al., 2009). Till now, various polymers and oligomers based on bile acids have been prepared for their potential biological and pharmaceutical applications as drug carrier systems, due to its ability to solubilise hydrophobic drugs, as molecular containers, non-polymeric hydrogelators and chemosensors (Atanackovic et al., 2009). CDs are cyclic oligosaccharides derived from starch containing six (αCD), seven (βCD), eight (γCD) or more (α-1,4)-linked α-D-glucopyranose units (Tong, 2001). Due to this specific structure of hydrophobic inner cavity and hydrophilic outer surface, CDs have the almost unique ability to form inclusion complexes with various organic, inorganic, biological and pharmaceutical molecules through non-covalent interactions (Dahan, Miller, Hoffman, Amidon & Amidon, 2010). Since natural CDs, in particular βCD, show limited aqueous solubility, new modified CDs with substitution of the hydrogen bond-forming hydroxyl groups have been developed. Examples of such modified CDs include the hydroxypropyl derivatives of βCD and γCD (i.e. HPβCD and HPγCD), the randomly methylated βCD (RMβCD) and others (Brewster & Loftsson, 2007). Up to now, all the properties of cyclodextrins or derivatives made them suitable for applications in analytical chemistry, agriculture, the pharmaceutical field, in food and toilet articles. In foods, CDs are used as molecular encapsulants to protect flavouring agents or other additives during the rigorous processing methods, extending the shelf life of the food item and preserving the product organoleptic properties. CDs are also used in the preparation of controlled release powdered flavours and confectionery items and are being used for the development of controlled-release active packaging systems (Del Valle, 2004; Koontz, Moffitt, Marcy, O'Keefe, Duncan & Long, 2010).

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Aiming at the development of an active food packaging for fresh products, the goal of this study was to increase RV, PT and PS aqueous solubility using hydropropyl-beta-cyclodextrin (HP-β-CD), hydropropyl-gamma-cyclodextrin (HP-γ-CD) and bile salts (sodium cholate and sodium deoxycholate). The system that enabled the highest fold-increase in the aqueous solubility of test compounds was further characterized by Fourier transform infrared spectroscopy (FTIR), differential scanning calorimetry (DSC), powder X-ray diffraction (DRX) and ^{1}H-NMR spectroscopy and its ability to improve test compounds photostability was also investigated.

Materials and Methods

Materials

Pinosylvin and pterostilbene were purchased from Sequoia Research Products Limited (Pangbourne, U.K.). *trans*-Resveratrol, sodium cholate and sodium deoxycholate were purchased from TCI Europe N.V. (Zwijndrecht, Belgium). Since stilbenes are sensitive to light, reagents and samples were stored in the dark. Regarding the CDs tested, hydroxypropyl-β-CD (HP-β-CD; KLEPTOSE® HP, M_w=1399 g/mol) was kindly provided by Roquette Freres S.A. (Lestrem, France) and hydroxypropyl-γ-CD (HP-γ-CD; M_w=1580 g/mol) was purchased from Sigma-Aldrich.

HPLC analysis of stilbenes

Stilbene quantification was carried out using an Agilent 1290 Infinity LC HPLC system (Waldbronn, Germany) equipped with an Agilent 1290 Infinity Diode Array Detector (G4212A DAD). Compound separation was performed using a Zorbax 300 SB-C_{18} reversed-phase

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analytical column (5 μm, 4.6 mm × 150 mm) acquired from Soquímica (Lisbon, Portugal). The composition of mobile phase was a mixture of 0.1% (v/v) formic acid solution in purified water (18.2 M Ω cm at 25°C) and acetonitrile (52:48, v/v). The mobile phase was filtered under vacuum (0.2 μm hydrophilic polypropylene filter) and degassed for 30 min in an ultrasonic bath before use. An isocratic flow of 1.0 mL/min was applied and the column oven was maintained at 25 ºC. The wavelength defined for monitoring stilbenes was 306 nm for *trans*-stilbenes isomers. The retention times obtained for *trans*-resveratrol, *trans*-pinosylvin and *trans*-pterostilbene were 1.8, 2.7 and 4.7 minutes, respectively. This method was validated according to the guidelines provided by the Food and Drug Administration (FDA) (Food and Drug Administration, 2001) and International Conference on Harmonization (ICH) (Harmonization, 2005) and the parameters studied were linearity, intermediate, intra- and interday precision and accuracy.

Critical micellar concentration (CMC) determination

In order to determine sodium cholate and sodium deoxycholate CMC, two methods were used. The first method consisted of a non-invasive method based on conductivity measurements (Posa, Guzsvany & Csanadi, 2009) and the second one was an invasive method based on Coomassie Brilliant Blue R-250 spectral shift (Courtney, Simpson & Beachey, 1986).

Phase solubility studies of stilbene-CD complexes

CD solutions were prepared at different concentrations (0, 0.025, 0.05, 0.10, 0.20 and 0.30 M^{-1}) in PBS buffer (pH 7.0). A volume of 500 μL of each CD solution was added to an excess amount of stilbenes (20 mg). The resulting suspensions were vortexed and sonicated for 1h in an ultrasonic bath with ice. The suspensions were kept protected from light on an orbital shaker at 25 ºC, 250 rpm for 24h. This equilibrium time was previous determined by preliminary studies.

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The resulting suspensions were filtered through 0.2 μm PVDF syringe filters (Millipore, MA, USA) to obtain clear solutions that were properly diluted with methanol:PBS (pH 7.4) (1:1) to the calibration range (1-100 μg/mL) and measured by HPLC after method validation.

The apparent stability constants ($K_{1:1}$ and $K_{1:2}$) were calculated according to the equations established by Higuchi and Connors (Higuchi & Connors, 1965).

Resveratrol solubility studies with bile salts

Solutions of bile salts were prepared in PBS buffer (pH 7.4) at the following concentrations: 0, 0.25, 0.5, 1.0, 1.5, and 2.0 CMC value (mM) for each bile salt respectively. An excess of stilbenes (0.5, 1 and 5 mg of resveratrol, pterostilbene and pinosylvin, respectively) was added to 500 μL of bile salts solutions and the suspensions were incubated protected from light in an orbital shake at 25 ºC, 250 rpm for 24h, since resveratrol solubility did not increase with increasing time periods. Solutions were sealed with parafilm and protected from light. Control solution was 1 mg/mL of stilbene in PBS buffer prepared in the same way. Before UPLC analysis, the samples were filtered through 0.45 μm PVDF syringe filters (Millipore, MA, USA), diluted with methanol:PBS (pH 7.4) (1:1) to the calibration range (1-100 μg/mL) and injected into the UPLC system.

Characterization of stilbene-CD complexes

Preparation of physical mixtures

Physical mixtures of stilbene and CD were prepared by simply blending uniformly in a mortar resveratrol, pterostilbene and pinosylvin with HP-β-CD or HP-γ-CD, previously sieved (200 μm mesh), with 1:1 or 1:2 molar ratios, depending on the stoichiometry obtained in the phase-solubility studies.

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184

185 Preparation of inclusion complexes for solid characterization

186 Inclusion complex samples were prepared as described previously in a 1:1 or 1:2 molar rates,
187 according to the apparent stability constants obtained, frozen at -80 ºC for at least 24 h,
188 lyophilized and stored in a dessicator until used.

189 Fourier Transform Infrared–Attenuated Transmitted Reflectance Spectroscopy (FTIR-ATR)
190 studies

191 Spectra were recorded using a Thermo Scientific Nicolet iS10 FT-IR spectrometer associated
192 with a Smart iTR* ATR horizontal reflexion accessory. FT-IR spectra acquisitions of stilbenes,
193 cyclodextrins, inclusion complexes and physical mixtures were directly performed in powder
194 samples with the application of 256 scans at a resolution of 4 cm^{-1} in the a spectral region
195 between 4000 and 450 cm^{-1}.

196 Thermal analysis

197 Differential scanning calorimetry (DSC) measurements of the pure compounds, physical
198 mixtures and inclusion complexes were carried out using a Shimadzu DSC-50 System
199 (Shimadzu, Kyoto, Japan) with a DSC equipped with a computerized data station TA-50WS/PC.
200 The thermal behaviour was studied by heating the samples (~2 mg) in a sealed aluminium pan
201 from 50–300 ºC, at a rate of 10 ºC/min and under a nitrogen flow of 10 mL/min, using an empty
202 pan sealed as reference. Indium (99.98%, mp 156.65 ºC, Aldrich, Milwaukee, USA) was used as
203 standard for calibrating the temperature.

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X-ray diffraction (XRD)

X-ray powder diffraction patterns were obtained at room temperature with a Rigaku, model DMAX III/C diffractometer system equipped with copper (Cu) as anode material and a graphite monochromator using a voltage of 30 kV and a current of 35 mA. The diffractograms were recorded in the 2θ angle range between 3–60º and the process parameters were set at: scan step size of 2θ/min with a total acquisition time of 1 h. Crystallinity was determined by comparing some representative peak heights in the diffraction patterns of the inclusion complexes and

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physical mixtures with a reference. The relation used for the calculation of the crystallinity was the relative degree of crystallinity (RDC) = I_{Sa}/I_{Ref}, where I_{Sa} is the peak height of the sample (inclusion complexes and physical mixtures) under investigation and I_{Ref} is the peak height of the same angle for the reference (Figueiras, Carvalho, Ribeiro, Torres-Labandeira & Veiga, 2007). For this analysis, the peak with the higher intensity was selected. Resveratrol, pterostilbene and pinosylvin were used as reference samples for calculating RDC values.

^{1}H Nuclear Magnetic Ressonance (NMR) studies

^{1}H NMR spectroscopy was performed at a temperature of 298 K on a Bruker Avance III 600 MHz spectrometer operating at 14.09 Tesla observing ^{1}H at 600.13 MHz. The spectrometer was equipped with a three-channel (^{1}H, ^{13}C and ^{15}N) cryoprobe and all spectra were processed with the software Topspin 2.0 (Bruker). Timethylphosphite (TMP) was used as internal standard at a concentration of 2% (v/v). Stilbene and cyclodextrin solutions were prepared at 2 mM in D_2O/DMSO-d_6 (1:10, v/v). Inclusion complexes solutions were prepared at 2 mM of each compound in D_2O/DMSO-d_6 (1:10, v/v).

Photostability studies

Stilbene inclusion complexes were prepared in the same way as for the FTIR studies, frozen at -80 ºC, lyophilized, reconstituted in water and diluted with methanol to a final concentration of 1 mg/mL. Stilbene solutions were also prepared at 1 mg/mL in methanol.

Photostability studies of pure and complexed stilbenes were performed under a standard fluorescent supermarket light (OSRAM L36 W/76). The samples were placed in tightly sealed glass vials kept at distance of 27 cm from the lamp for 76 days at 4 ºC and room temperature (20-22 ºC). Control samples for each temperature were kept in the dark for the same time period. In order to assess compound photostability, samples were taken at regular time intervals and the

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stilbenes were quantified by UPLC. Photodegradation was expressed by the reduction in the *trans* isomer of each stilbene throughout the assay by comparison with the initial values.

Results and Discussion

Resveratrol and its analogues pterostilbene and pinosylvin are known to possess a low aqueous solubility which has limited their use in numerous fields. Since cyclodextrins and micellar systems such as bile acids are known to increase the solubility of a vast range of compounds and are also able to protect them from external agents, in this work these two systems were chosen to incorporate resveratrol, pterostilbene and pinosylvin in order to evaluate their ability to increase compound solubility and stability.

Method validation

Usually, the methods preferred to quantify stilbenes in aqueous solutions are based on UV or fluorescence detection and can be performed with or without chromatographic separation. However, as stilbenes can occur as *trans*- or *cis*-isomers, a method capable of detecting and resolving these two isomers is more suitable for quantification. Considering that, in this work, we aimed at quantifying only the *trans*-isomers of stilbenes and, for this purpose, a methodology using high performance liquid chromatography (HPLC) coupled to UV detection method was developed and validated in terms of linearity, intermediate, intra- and interday precision and accuracy, following a 5-day validation protocol. The chromatographic conditions described allowed the successful elution of RV, PS and PT at 1.841, 2.743 and 4.748 min, respectively (Figure 1a). To evaluate the method's linearity, several solutions of stilbenes with concentrations ranging from 1 to 100 µg/mL were prepared (eight calibrators evenly distributed and five replicates) and analyzed as described above. Together with each calibration curve, three quality

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control samples (QC) at low (LQC: 1 μg/mL) and medium (MQC: 50 μg/mL) and high (HQC: 100 μg/mL) concentrations (n=3) were also analysed. Calibration curves were obtained by plotting the peak–area of each analyte against analyte concentration. The acceptance criteria included a determination coefficient of at least 0.99 and the calibrators' accuracy within a ±15 %, with the exception of the lower limit of quantitation (LLOQ), 1 μg/mL, where ±20 % was accepted. Due to the wide calibration range, weighted least squares regressions were adopted. Six weighting factors were evaluated for each analyte ($1/\sqrt{x}$, $1/x$, $1/x^2$, $1/\sqrt{y}$, $1/y$, $1/y^2$), and the one originating the best results was selected. Using each of those factors, the mean relative errors of each calibrator were calculated and their absolute value was summed. The weighting factor $1/x^2$ was chosen for all analytes since the sum of errors obtained was smaller while presenting simultaneously a mean R^2 value of at least 0.99 (Figure 1d). By means of these weighted least squares regressions, linear relationships were obtained ($R^2 \geq 0.99$) (Figure 1b), and the relative error [mean relative error (bias) between measured and spiked concentrations] was in accordance with the above-mentioned criteria (±15% for all concentrations, except at the lower limit of quantitation where ±20% was acceptable). The lowest concentration used for quantification (1 μg/mL) for each analyte is considerably higher than the LLOQ already described by other authors for resveratrol, pterostilbene and pinosylvin quantification, reaching limits within a 10-20 ng/mL range (Lin et al., 2009; Paulo, Domingues, Queiroz & Gallardo, 2011; Roupe, Halls & Davies, 2005). However, these limits are adequate for monitoring stilbene concentration in this work, as their concentrations in inclusion complexes and micelles are usually very high (Atanackovic et al., 2009; Das et al., 2008), even requiring serial dilutions to fall within the dynamic range of the assay.

Interday precision and accuracy (Figure 1c) were evaluated at eight concentrations (1, 2.5, 5, 10, 25, 50, 70 and 100 μg/mL). The calculated CVs were lower than 3.5% for all compounds at all

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283 concentration levels, while accuracy (in terms of mean relative error) was within a ±9% interval.
284 The CVs presented are lower than those described in literature for stilbene quantification by
285 HPLC-DAD (Careri, Corradini, Elviri, Nicoletti & Zagnoni, 2003; Paulo et al., 2011) and could
286 be related to the fact that an extraction procedure, usually a source of variability, is not
287 performed in this work (Juan, Maijo & Planas, 2010). Intra-day precision and accuracy (Figure
288 1d) were determined using 4 standard concentrations (1, 10, 50 and 100 5 μg/mL) prepared and
289 analyzed as mentioned above (six replicates for each concentration). The obtained coefficients of
290 variation (CVs) were lower than 3% for all the compounds at all tested concentrations,
291 presenting a mean relative error within a ±9% interval. Additionally, intermediate precision and
292 accuracy (Figure 1e) were assessed at 3 concentrations (15, 40 and 80 μg/mL) performed in
293 triplicate over the 5-day validation period (n=15). The results showed that the obtained CVs were
294 always lower than 3.7% and the relative error was within a ±6% interval from the target
295 concentration.
296 Overall, the HPLC-DAD method described allowed the quantification of RV, PT and PS *trans*-
297 isomers within a 6 min chromatographic run and was successful validated regarding all the
298 parameters evaluated, since CV and relative error values were always lower than 15% for all
299 criteria.

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300

301 **Solubilization of resveratrol with bile salts and CDs**

302 Bile salts

303 The physiological and therapeutic properties of some bile salts are, essentially, due to their
304 ability to form micelles, which facilitate the dissolution and release of molecules with poor
305 aqueous solubility (Reis, Moutinho, Matos, de Castro, Gameiro & Lima, 2004). Critical micellar
306 concentration (CMC) is a fundamental parameter in the evaluation of the biological activity of
307 bile salts. The CMC of a surfactant is defined as the solutes concentration at which micelles first
308 appear in solution and, in practical terms, is related to appreciable changes in surface tension,
309 solubilisation of other organic molecules, among other parameters (Reis et al., 2004). Many
310 different techniques can be used for determination of CMCs and are generally classified in
311 invasive (dye solubilization and spectral shift, etc.) and non-invasive methods (surface tension,
312 conductometry, etc.) (Roda, Hofmann & Mysels, 1983). In this context, two methods for
313 determining the CMC values of sodium cholate and sodium deoxycholate in buffered solutions
314 were evaluated. The chosen methods were conductometry (non-invasive) and Coomassie
315 Brilliant Blue R-250 dye solubilisation (invasive method). The CMC values obtained for sodium
316 cholate and sodium deoxycholate were, approximately, 10 and 3 mM, respectively
317 (Supplementary file S1) being similar to the ones described in the literature (Atanackovic et al.,
318 2009; Matsuoka & Moroi, 2002). As expected, each method yielded slightly different CMC
319 values, with the higher values being obtained for conductometry, which is in agreement with
320 previous results obtained by other authors (Poša, Guzsvány & Csanádi, 2010). Since, generally,
321 the values obtained using conductometry are over-estimated, the CMC values determined by this
322 method were used to assess the improvement of stilbene solubility by bile salts. As a
323 consequence of the membrane-toxic properties of micelles due to the disruption of membrane

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integrity, the highest concentration of bile salt used corresponded to 2xCMC (Atanackovic et al., 2009). Sodium cholate was able to increase RV, PT and PS solubility up to 0.2, 1.2 and 8.0 mg/mL, respectively (Figure 2a,b,c); whereas, sodium deoxycholate increased compounds' aqueous solubility to a smaller extent, yielding a maximum of 1 mg/mL of solubilised compound, in the case of PS. In the solubility diagrams obtained for RV and PT, there is a clear increase in solubility after reaching the CMC value, as expected.

Modified CDs

As an alternative agent for increasing stilbene solubility and stability, two modified CDs (hydropropyl-β-CD and hydroxypropyl-γ-CD) were evaluated in this work. These modified CDs were preferred over natural CDs, since their solubility in aqueous solutions is higher, thus resulting in higher solubility of the inclusion complexes formed (Challa, Ahuja, Ali & Khar, 2005). Hydrophilic CDs, namely HP-β-CD are considered non-toxic at low to moderate oral and intravenous doses and are more toxicologically benign than the natural β-CD (Irie & Uekama, 1997). Although the information regarding HP-γ-CD is limited, it is known that it possesses one of lowest haemolytic effect on human erythrocytes when compared with other natural or modified CDs (Brewster et al., 2007). The phase-solubility studies revealed that HP-γ-CD and HP-β-CD form inclusion complexes with RV and PT in a 1:1 stoichiometry (Figure 2d), due to the correlation coefficients obtained from phase-solubility diagrams, which exhibited a A_L type curve (linear increases of drug solubility as a function of CD concentration) whilst inclusion complexes of both CDs and PS showed a 1:2 stoichiometry and a A_N type curve (negatively deviating isotherms) (data not shown), meaning that the formation of higher order complexes is occurring (Brewster et al., 2007). This 1:2 stoichiometry is described for the first time for these complexes, considering that the only data available for these complexes referred a 1:1 stoichiometry (Lopez-Nicolas et al., 2009a). The differences obtained might be related with the

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348 design of the study and methodologies used, since different techniques (fluorescence
349 spectroscopy) and lower CD concentrations (≥0.1 M) were used in comparison to this work.
350 These A_N profiles have several explanations, including bulk changes imparted to the solvent by
351 the CD at various concentrations and also due to self-association of the CD at high
352 concentrations (Brewster et al., 2007). The apparent stability constants (K_S) obtained for RV are
353 lower than the ones previously described (Das et al., 2008; Lopez-Nicolas et al., 2009b), though
354 the maximum amount of resveratrol solubilised at the higher CD concentration (0.3 M) is within
355 the range of values reported (Das et al., 2008). In the case of PT, despite the K_S values for HP-β-
356 CD are within the same order of magnitude as the ones obtained by Lopez-Nicolas and co-
357 authors, they are slightly lower (Lopez-Nicolas et al., 2009b). For PS, the apparent stability
358 constants for both HP-γ-CD and HP-β-CD were significantly lower than the ones previously
359 obtained (Lopez-Nicolas et al., 2009a), which could be related with the phase-solubility profiles
360 obtained.

361 Bile salts *versus* CDs

362 When comparing stilbene solubility improvement, modified CDs allowed a greater increment in
363 compound solubility (4-6 log-fold increase) when compared to bile salts (≤4.5 log-fold increase)
364 (Figure 2e). In fact, CDs provided the obtention of inclusion complexes with solubilities more
365 than 700,000-fold higher than the aqueous solubility of test compound (S_0) while bile salts
366 enhanced stilbene solubility up to 25,000 times. The values obtained for resveratrol aqueous
367 solubility with HP-β-CD (38.74 mg/mL) (Figure 2d) was slightly higher than the one described
368 by Das and co-authors (30 mg/mL), probably due to the higher orbital rotation (250 rpm) used in
369 our work. The highest increase in solubility was verified for PT, the stilbene with the lowest
370 aqueous solubility (S_0=1.96x10^{-5} M). When comparing the solubility enhancement between RV
371 (S_0=1.43x10^{-4} M) and PS (S_0=1.91x10^{-3} M), modified CDs were able to solubilise RV to a

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372 greater extent than PS and bile salts enable the solubilisation of PS in higher amount, instead of
373 RV. Overall, CDs produced the highest improvements in stilbene aqueous solubility, which may
374 be a result of the low bile salt concentrations used, considering that, for instance, Gokturk and
375 co-authors obtained similar solubility for test compounds when using CDs and micelles, but the
376 surfactant concentration was much higher than the CMC (approximately 6xCMC) (Gokturk,
377 Caliskan, Talman & Var, 2012). Overall, these results clearly state the enormous impact of
378 cyclodextrins and bile salts in improving the aqueous solubility of several compounds, with 10^3
379 to 10^6-fold increase in the amount of solubilised compounds. Though the complexation of
380 stilbenes in modified CDs have already been described, the characterization of the inclusion
381 complexes formed has not yet been reported. Hence, the inclusion complexes between HP-γ-CD
382 and HP-β-CD and RV, PT and PS were further characterized by FTIR, XRD, DSC and ^{1}H NMR.
383

384 **Inclusion complex characterization**

385 Figures 3a–3f show the multiple FTIR spectra obtained for each one of the six inclusion
386 complexes formed. Each graph is composed by the FTIR spectra of inclusion complexes,
387 physical mixtures and respective stilbene and cyclodextrin. RV and PS spectra, in the range of
388 1700-700 cm^{-1}, exhibited four characteristic intense bands at 1600-1605, 1583-1585, 1381-1385,
389 960-964 cm^{-1} corresponding to C-C aromatic double bond stretching, C-C olefinic stretching, C-
390 O stretching and *trans* C-H olefinic stretching (Bertacche et al., 2006). For PT, only three intense
391 and visible bands in the selected range (1602, 1584 and 961 cm^{-1}) were identified but a fourth
392 intense band corresponding to the aromatic methoxy groups was visible at 2832 cm^{-1} (Panickera
393 et al., 2010) (data not shown). Regarding the inclusion complexes obtained for all stilbenes (RV,
394 PT and PS) (Figure 3a,d), the spectra showed changes in the spectral features of guest molecule,
395 since only the peak corresponding to C-C aromatic double bond stretching was identified with a

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band wavenumber slightly deviated from the ones obtained for guest molecules, which undoubtedly confirmed the presence of RV, PT and PS within the complex in the lyophilized systems. Taken together with the fact that physical mixtures exhibited the majority of guest molecule peaks with almost equal band wavenumbers, these evidences indicate the formation of weak interactions between CDs and stilbenes in these systems (Figueiras et al., 2007).

Power X-ray diffractometry was used to further confirm the formation of inclusion complex between stilbenes and HP-CDs. The X-ray diffraction (XRD) patterns for each compound, CD, physical mixtures and inclusion complexes are displayed in Figure 4. In addition to the XRD patterns, crystallinity was also determined by comparing some representative peak heights in the diffraction patterns of the inclusion complexes and physical mixtures with those of a reference (Supplementary file S2). In the X-ray diffractograms of RV, PT and PS was possible to observe several sharp peaks suggesting that these compounds are present in a crystalline form. Nonetheless, the XRD patterns for HP-CDs confirm its amorphous form, since no sharp peaks could be discriminated (Williams, Mahaguna & Sriwongjanya, 1998). In the case of physical mixtures, the XRD diffractograms are a superposition of stilbene and CD single component. However, there was a decrease in peak intensity meaning a possible loss in crystallinity that can be due to the amorphous character of the CD (Ribeiro, Figueiras, Santos & Veiga, 2008) used and also due to the mixing time of the physical mixture, possibly causing a starting complexation (Bertacche et al., 2006). Inclusion complexes revealed a stilbene amorphization with no detectable peak in the XRD patterns. Furthermore, these results are corroborated by the RDC values obtained for both physical mixtures and inclusion complexes, revealing a more pronounced decrease in the relative crystallinity of the inclusion complexes when compared to physical mixtures.

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Thermal analysis has routinely been used as a method to characterize cyclodextrins complexes. Supplementary file S3 shows the DSC thermograms of stilbenes, cyclodextrins, physical mixtures and inclusion complexes. The thermograms obtained for each stilbene were typical of crystalline anhydrous compounds with a sharp melting endotermic peak obtained at 268.45 ºC for RV, 94.87 ºC for PT and 157.18 ºC for PS which is in accordance with the melting points provided by the manufacturers. HP-β-CD and HP-γ-CD yielded a very broad endothermic effect, reaching a maximum value around 70-80 ºC, due to the release of water molecules from the CD cavity (Fernandes, Teresa Vieira & Veiga, 2002). In the DSC thermograms of all complexes, the absence of the melting endotermic peak of each stilbene can be noticed. This provided some evidence of inclusion complexation, since when guest molecules are embedded in the CD cavity, their melting points usually shift to a different temperature or disappear within the temperature range of CD decomposition (Karoyo, Sidhu, Wilson & Hazendonk, 2013). The endothermic peak of each stilbene was not visible in the DSC curves of the physical mixtures, as opposed to what should be expected. This fact could be due to the overlap of the CD dehydration peak with PT melting peak, to an excess amount of CD in PS mixtures due to the 1:2 stoichiometry or even to the fact that some complexation could have started during the mixing of the physical mixtures (Bertacche et al., 2006). Taken together, FTIR, XRD and DSC results undoubtedly confirmed the formation of inclusion complexes between stilbenes and CDs. However, a more thorough molecular characterization of the interactions involved in each complex formation was needed.

Over the past decades, NMR techniques have been used to study cyclodextrins inclusion complexes. The ^{1}H NMR spectra for each inclusion complex and stilbene are shown in Supplementary file S4, with the full spectra (stilbene and CD) (Supplementary file S4a, c and e) and only compound spectra (Supplementary file S4b, d and f) being represented for a better visualization of 1H NMR chemical shift displacements. As can be seen in full spectra, it was not

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possible to assign the peaks corresponding to HP-β-CD and HP-γ-CD due to the very broad peaks obtained, a result of the fact that these modified CDs consists of a mixture of a number of related derivatives with different degrees of substitution [$(C_3H_6O)_nH$] and isomeric forms (Fernandes, Carvalho, Pereira da Costa & Veiga, 2003). Therefore, it was only possible to observe the shifts of stilbene protons in the presence of HP-CDs, which provided information about the existence of an interaction in solution. The ^{1}H-chemical shifts of RV, PT and PS are reported in Table 1. As shown, an upfield shift was observed for almost all the protons of RV, PT and PS in the presence of both HP-β-CD and HP-γ-CD. The upfields of RV, PT and PS protons were larger for HP-γ-CD in comparison to HP-β-CD, implying a stronger interaction with HP-γ-CD, despite the similarity in the K_S values obtained for each CD. The hydroxypropyl substitution of CDs could result in steric hindrance around the guest molecules, making the entrance of the guest molecule inside CD hydrophobic cavity more difficult. Since HP-γ-CD has a larger cavity than HP-β-CD, this could result in the alleviation of the steric hindrance effect, thus promoting the entrance of the guest molecule inside the CD cavity (Fernandes et al., 2002). The largest upfield shifts were obtained for H_2, H_3, H_4 and H_5 stilbene protons, indicating that the double bond protons and closer aromatic protons are mainly involved in the formation of the complex (Bertacche et al., 2006; Boudad, Legrand, Lebas, Cheron, Duchene & Ponchel, 2001) as they experience significant perturbation upon complexation. Since this is the most hydrophobic moiety of the guest molecules, it would be expected to be included within the cyclodextrins cavity, leaving the hydroxyl groups in the extremities in contact with the hydrophilic medium. However, this perturbation in the double bond caused by a possible interaction with the CD did not result in any *cis-trans* isomerisation of stilbene, since no *trans*-isomer reduction was visible by HPLC-DAD upon complexation.

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Photostability studies

One of the main issues one working with stilbenes is their *cis-trans* isomerisation upon exposure to UV light (Paulo et al., 2011). Although only a few data are available, it is widely accepted that the *trans*-isomers have higher bioactivity than the *cis*-isomers (Waffo-Teguo et al., 2001). Therefore, the ability of the CDs in improving *trans*-stilbene stability was verified. Since the final application of these complexes is in the development of an active packaging for meat products, photostability studies were conducted by exposure to a standard supermarket fluorescent light to simulated meat packaging storage conditions. The photostability of RV, PT and PS and corresponding inclusion complexes was investigated under different storage temperatures (4 and 20 ºC), in the dark and under light exposure. Since the HPLC-DAD method was validated only for *trans*-isomers, it was only possible to quantify *trans*-stilbenes and, so, the results are expressed as the percentage of *trans*-isomer reduction over time. In general, both stilbenes and inclusion complexes are more stable at 4 ºC than at 20 ºC (Figure 5). Overall, the complexation of RV, PT and PS with HP- β-CD and HP-γ-CD, allowed an increase in stilbene photostability at 4 ºC, that was more significant in the case of HP- β-CD at least until 14 days of exposure. PS and PS complexes exhibited the highest photostability, since after 21 days of exposure, only a 30% reduction of *trans*-PS was observed. This increase in stilbene photostability could be related with the interaction of the CD with the protons involved in the *cis-trans* isomerisation, thus blocking, to some extent, the conversion of *trans*- to *cis*-stilbenes.

Conclusions

Nowadays, the use of antimicrobial and antioxidants as ingredients in active packaging is one of the main goals of the food industry. Due to the consumers' demand for natural alternatives and to stilbenes proved biological activities, they can be considered ideal candidates to be incorporated

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491 in these packaging systems. However, some problems related to these compounds' stability and
492 solubility need to be overcame to improve their efficacy in the package material. Toward this
493 aim, it was evaluated the increase in stilbene solubility by CDs and bile salts and concluded that
494 CDs proved to be more beneficial for increasing stilbene solubility with the advantage of being
495 less toxic to humans. Afterwards, a thorough characterization of the inclusion complexes formed
496 between RV, PT or PS and HP-β-CD or HP-γ-CD was performed, which confirmed that
497 formation of stable inclusion complexes and even shed some light about the molecular
498 interactions between guest molecules and cyclodextrins. Besides improving stilbene solubility,
499 CDs also increased their photostability, which can circumvent stilbene reactivity and encourage
500 their application not only in the food industry but also in the pharmaceutical industry.
501

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511

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650 ## Figure Captions

651

652 **Figure 1:** HPLC-DAD stilbene quantification linearity and validation data: a) typical HPLC-
653 DAD chromatogram (λ=306 nm), b) linearity data (n=5), c) inter-day precision and accuracy
654 (n=5), d) intra-day precision and accuracy (n=6) and e) intermediate precision and accuracy
655 (n=15). All concentrations are in μg/mL; CV, coefficient of variation; RE, relative error
656 [(measured concentration−spiked concentration/spiked concentration)×100]. When applicable,
657 values are presented as mean values ± standard deviation.

658

659 **Figure 2:** Comparison of stilbene solubilization by bile salts and cyclodextrins. Solubilization of
660 a) resveratrol, b) Pterostilbene, c) pinosylvin by buffered solutions of sodium cholate and
661 deoxycholate at 37 ºC, d) K_S values, stoichiometry and correlation coefficients for stilbene-HP-CD
662 complexes at 25 ºC and pH=7.0 and e) solubility enhancement using bile salts or
663 hydroxypropylated-CDs.

664

665 **Figure 3:** FTIR-ATR spectra of pure compounds (i), cyclodextrins (ii), physical mixtures (iii)
666 and inclusion complexes (iv) of a) HP-β-CD with resveratrol, b) HP-β-CD with pterostilbene, c)
667 HP-β-CD with pinosylvin, d) HP-γ-CD with resveratrol, e) HP-γ-CD with pterostilbene and f)
668 HP-γ-CD with pinosylvin.

669

670 **Figure 4:** X-ray diffractograms of pure compounds (i), cyclodextrins (ii), physical mixtures (iii)
671 and inclusion complexes (iv) of a) HP-β-CD with resveratrol, b) HP-β-CD with pterostilbene, c)

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672 HP-β-CD with pinosylvin, d) HP-γ-CD with resveratrol, e) HP-γ-CD with pterostilbene and f)
673 HP-γ-CD with pinosylvin.

674 **Figure 5:** Stilbenes (RV, PT and PS) and inclusion complexes with HP-β-CD and HP-γ-CD
675 photostability under exposure to a standard supermarket fluorescent light for 76 days at 4 ºC and
676 20 ºC.

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Highlights

- Novel HPLC-DAD method for the simultaneous determination of 3 *trans*-stilbenes
- Comparison of stilbene solubility in inclusion complexes and micellar systems
- Cyclodextrins increased stilbene solubility up to 6 log-fold increase
- Cyclodextrin-stilbene interaction occurs manily through the double bond protons
- Stilbene photostability was increased due to the inclusion in CDs

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Tables

Table 1: 600 MHz ^{1}H chemical shift (δ, ppm) of stilbene protons in free state and chemical shift displacements (Δδ, ppm) of stilbenes complexed with HP-β-CD and HP-γ-CD. Δδ is expressed as $\Delta\delta=\delta_{complex}-\delta_{free\ stilbene}$.

			with HP-β-CD		with HP-γ-CD	
Compound	**Assignment**	δ_{free}	$\delta_{complex}$	Δδ	$\delta_{complex}$	Δδ
Resveratrol	**H_1**	6.3251	6.2980	-0.0271	6.2254	-0.0997
	H_2	6.6649	6.5603	-0.1046	6.3942	-0.2707
	H_3	7.1659	7.0763	-0.0896	6.8123	-0.3536
	H_4	6.9841	6.8980	-0.0861	6.6216	-0.3625
	H_5	7.5278	7.4799	-0.0479	7.2598	-0.2680
	H_6	6.9288	6.9041	-0.0247	6.8123	-0.1165
Pterostilbene	**H_1**	6.5413	6.5678	0.0265	6.2960	-0.2453
	H_2	6.8561	6.6741	-0.1820	6.3386	-0.5175
	H_3	7.2431	7.0927	-0.1504	6.6350	-0.6081
	H_4	7.0682	6.8958	-0.1724	6.4440	-0.6242
	H_5	7.5470	7.4755	-0.0715	7.1109	-0.4361
	H_6	6.9414	6.9154	-0.0260	6.7820	-0.1594
	Methyl	3.8719	3.8619	-0.0100	3.7797	-0.0922
Pinosylvin	**H_1**	6.3600	6.3067	-0.0533	6.2324	-0.1276
	H_2	6.7137	6.5516	-0.1621	6.4340	-0.2797
	H_3	7.2399	7.1018	-0.1381	6.8775	-0.3624
	H_4	7.1508	7.0076	-0.1432	6.7827	-0.3681
	H_5	7.6350	7.5635	-0.0715	7.3806	-0.2544
	H_6	7.4562	7.4448	-0.0114	7.3570	-0.0992
	H_7	7.3647	7.3656	0.0009	7.2786	-0.0861

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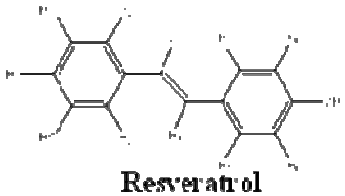
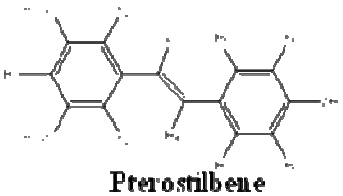
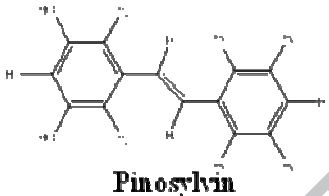


Resveratrol



Pterostilbene



Pinosylvin

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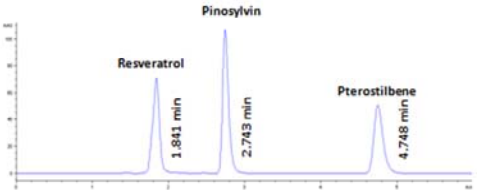
a)

Compound	Weight	Linearity	Slope	Intercept	R^2
Resveratrol			47.38 ± 1.81	-6.40 ± 1.49	0.9972 ± 0.002
Pterostilbene	$1/x^2$	1-100	37.89 ± 1.36	-5.27 ± 1.92	0.9978 ± 0.001
Pinosylvin			60.86 ± 0.55	-8.14 ± 1.49	0.9969 ± 0.001

b)



c)

Compound	Spiked	Measured	CV (%)	RE (%)
Resveratrol	1	1.032 ± 0.01	0.84	3.05
	2.5	2.39 ± 0.03	1.17	-4.61
	5	4.75 ± 0.10	2.16	-5.33
	10	9.39 ± 0.27	2.83	-6.58
	25	25.48 ± 0.60	2.36	1.82
	50	51.25 ± 0.55	1.08	2.42
	70	72.58 ± 0.74	1.02	3.55
	100	104.29 ± 1.22	1.17	4.11
Pterostilbene	1	1.03 ± 0.01	1.18	2.56
	2.5	2.41 ± 0.04	1.75	-3.95
	5	4.80 ± 0.11	2.33	-4.28
	10	9.51 ± 0.33	3.47	-5.22
	25	25.29 ± 0.60	2.36	1.11
	50	51.05 ± 0.84	1.66	2.04
	70	72.14 ± 0.99	1.37	2.95
	100	103.74 ± 1.00	0.97	3.60
Pinosylvin	1	1.03 ± 0.01	0.60	2.82
	2.5	2.43 ± 0.02	0.99	-3.03
	5	4.69 ± 0.06	1.19	-6.63
	10	9.25 ± 0.06	0.64	-8.17
	25	25.67 ± 0.31	1.21	2.59
	50	51.55 ± 0.43	0.84	3.00
	70	72.87 ± 1.31	1.80	3.91
	100	103.92 ± 0.79	0.76	3.77

d)

Compound	Spiked	Measured	CV (%)	RE (%)
Resveratrol	1	1.04 ± 0.02	1.80	4.03
	10	9.54 ± 0.01	0.14	-4.85
	50	51.61 ± 0.26	0.51	3.12
	100	103.85 ± 0.08	0.07	3.70
Pterostilbene	1	1.05 ± 0.02	1.66	5.13
	10	9.29 ± 0.01	0.15	-7.61
	50	52.13 ± 0.25	0.48	4.09
	100	105.02 ± 0.08	0.07	4.78
Pinosylvin	1	1.06 ± 0.02	2.27	5.38
	10	9.25 ± 0.26	2.84	-8.23
	50	50.88 ± 0.22	0.42	1.72
	100	102.84 ± 0.53	0.52	2.76

e)

Compound	Spiked	Mean Measured	CV (%)	RE (%)
Resveratrol	15	15.28 ± 0.45	2.94	1.74
	40	42.27 ± 0.59	1.40	5.34
	80	82.29 ± 3.04	3.69	2.65
Pterostilbene	15	15.07 ± 0.44	2.93	0.36
	40	41.66 ± 0.48	1.15	3.97
	80	81.84 ± 2.37	2.89	2.17
Pinosylvin	15	15.62 ± 0.26	1.68	3.92
	40	42.02 ± 0.34	0.80	4.80
	80	82.58 ± 0.59	0.71	3.12

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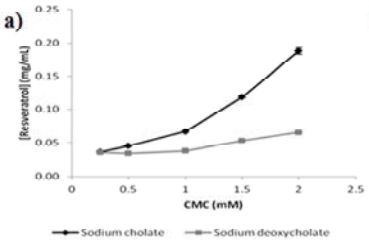
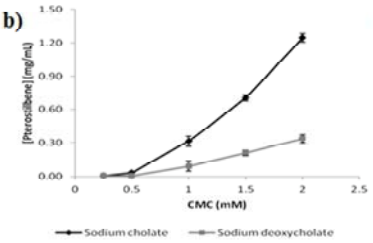
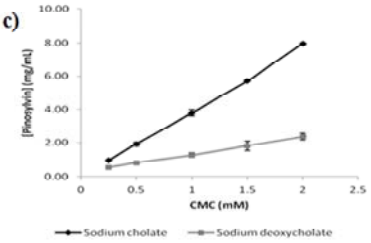
a)



b)



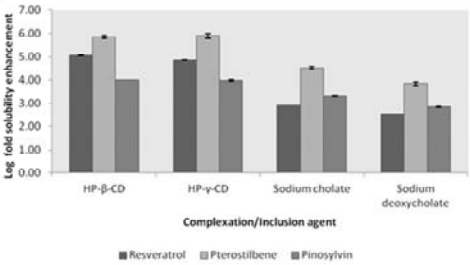
c)



d)

Inclusion complex	Maximum solubility (mg/mL)	K_S (M^{-1})	Stoichiometry	Correlation coefficient
Resveratrol-HP-β-CD	38.74±0.85	1682 ± 49	1:1	0.9991
Resveratrol-HP-γ-CD	22.76±0.64	1634 ± 68	1:1	0.9700
Pterostilbene-HP-β-CD	35.79±0.47	11730 ± 13	1:1	0.9998
Pterostilbene-HP-γ-CD	35.18±0.55	11705 ± 38	1:1	0.9982
Pinosylvin-HP-β-CD	40.14±0.21	14.0 ± 2.3	1:2	0.9988
Pinosylvin-HP-γ-CD	38.44±2.68	4.4 ± 0.3	1:2	0.9988

e)



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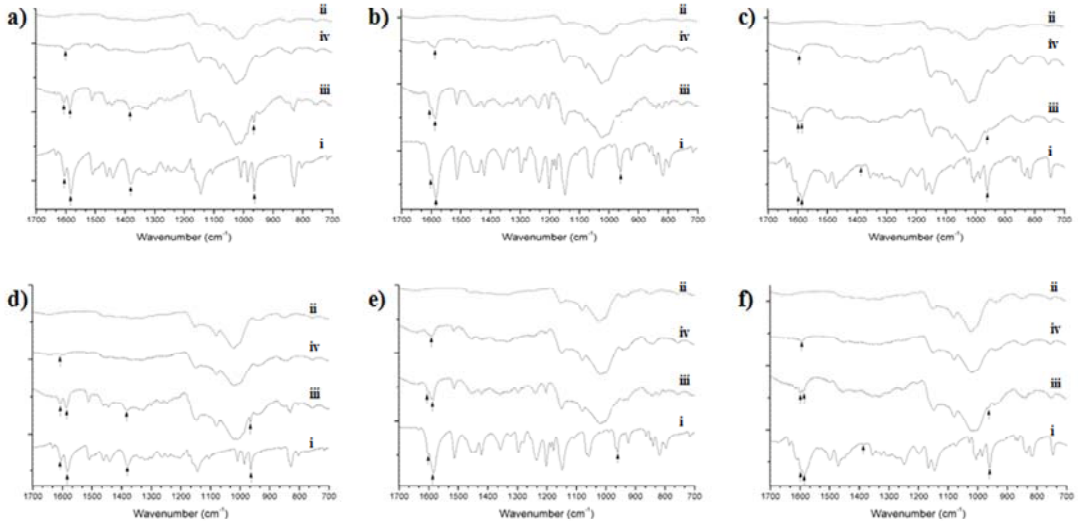

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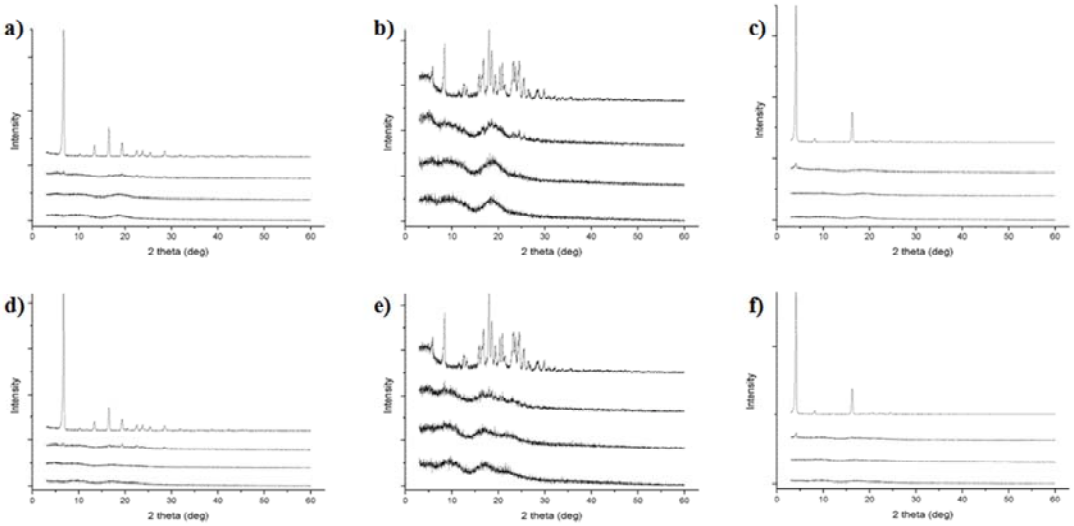

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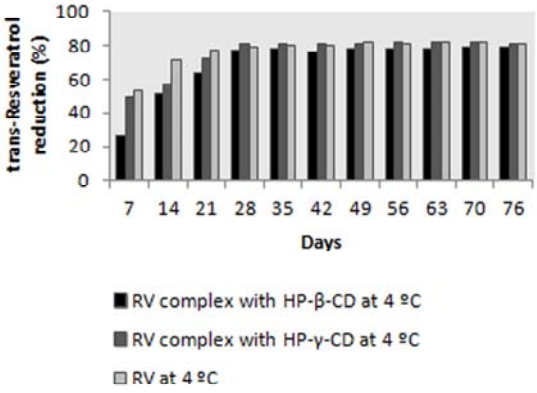
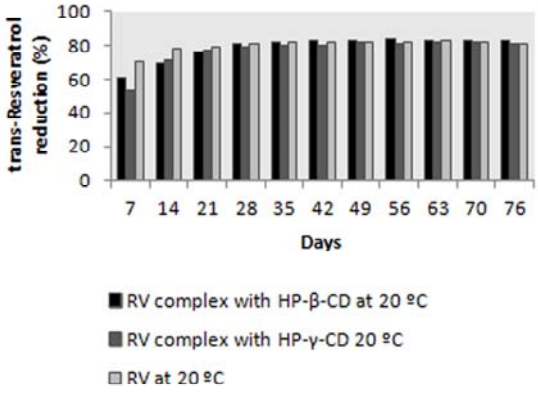

trans-Resveratrol reduction (%)
100
80
60
40
20
0
7 14 21 28 35 42 49 56 63 70 76
Days
RV complex with HP-β-CD at 4 ºC
RV complex with HP-γ-CD at 4 ºC
RV at 4 ºC


trans-Resveratrol reduction (%)
100
80
60
40
20
0
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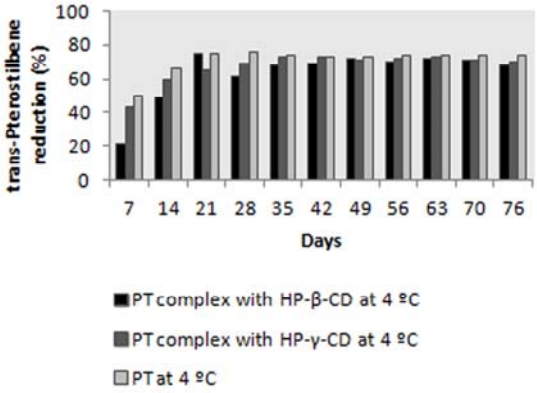
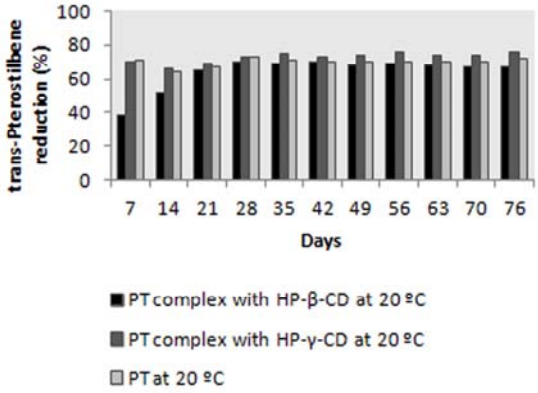
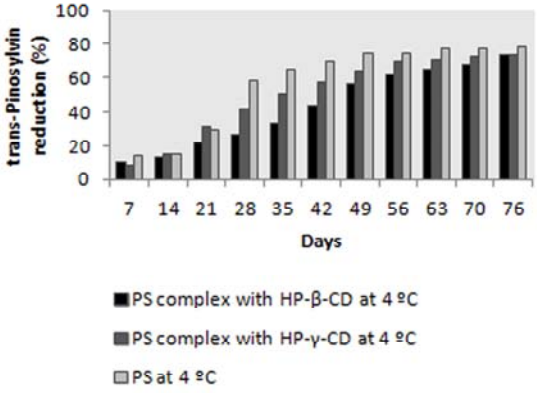
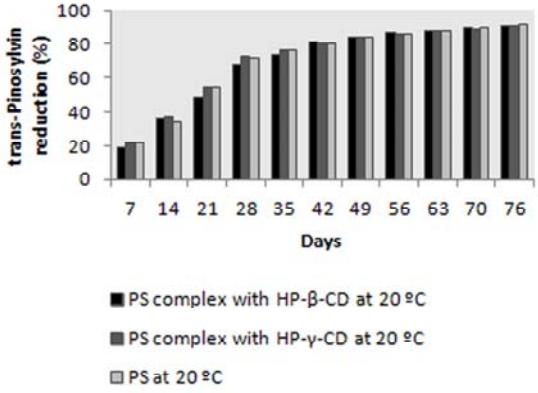
Days
RV complex with HP-β-CD at 20 ºC
RV complex with HP-γ-CD 20 ºC
RV at 20 ºC


trans-Pterostilbene reduction (%)
100
80
60
40
20
0
7 14 21 28 35 42 49 56 63 70 76
Days
PT complex with HP-β-CD at 4 ºC
PT complex with HP-γ-CD at 4 ºC
PT at 4 ºC


trans-Pterostilbene reduction (%)
100
80
60
40
20
0
7 14 21 28 35 42 49 56 63 70 76
Days
PT complex with HP-β-CD at 20 ºC
PT complex with HP-γ-CD at 20 ºC
PT at 20 ºC


trans-Pinosylvin reduction (%)
100
80
60
40
20
0
7 14 21 28 35 42 49 56 63 70 76
Days
PS complex with HP-β-CD at 4 ºC
PS complex with HP-γ-CD at 4 ºC
PS at 4 ºC


trans-Pinosylvin reduction (%)
100
80
60
40
20
0
7 14 21 28 35 42 49 56 63 70 76
Days
PS complex with HP-β-CD at 20 ºC
PS complex with HP-γ-CD at 20 ºC
PS at 20 ºC

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