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Complexation of Apple Antioxidants: Chlorogenic Acid, Quercetin and Rutin by β-Cyclodextrin (β-CD)*

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Abstract

The complexation and antioxidant activity of the major apple polyphenols: Chlorogenic Acid (CA), Rutin (Rt) and Quercetin (Qc) with β-cyclodextrin (β-CD) were studied, by fluorescence spectroscopy and Ferric Reducing/Antioxidant Power Assay (FRAP) techniques. All polyphenols formed 1:1 stoichiometry complexes. Their stability constants decreased in the order Qc (1138 M^{-1}) > CA (465 M^{-1}) > Rt (224 M^{-1}). The complex formation was also confirmed in the solid state by DSC measurements. Qc showed the highest antioxidant activity, followed by Rt and CA. In all cases, the complexation process produced a slight increment in their antioxidant activity (between 7 and 14%), more evident for Rt and CA than for Qc.

Abbreviations: CA – chlorogenic acid; CD – cyclodextrin; β-CD – β-cyclodextrin; FRAP – Ferric reducing antioxidant power; HSA – Human Serum Albumin; λ_{em} – emission wavelength; λ_{exc} – excitation wavelength; Qc – quercetin, Rt – rutin; TPTZ – 2,4,6-tripyridyl-s-triazine.

Introduction

Dietary intake of natural antioxidants has recently received increased attention due to the epidemiological evidence that correlates a regular intake of these products with protection against several diseases [1]. Fruits and vegetables are the main antioxidant suppliers in the human diet. Among them, apple is important not only for its high antioxidant content, but also for its acceptance among the general consumer population. Vinson *et al.* [2] reported that 22% of the fruit phenolics consumed in the United States came from apple. Recently, Eberhardt *et al.* [3] found that 100 g of fresh apples have an antioxidant activity equivalent to 1500 mg of vitamin C, and more important, that apple phenolic extracts inhibited proliferation of a human cancer cell line. The major antioxidants present in apple are polyphenols, which include phenolic acids (chlorogenic, cinammic, gallic acid, etc.) and flavonoids (catechin, quercetin, quercetin glycosides, etc.). Generally, these polyphenols are distributed in the whole fruit, with higher concentrations present in the peel rather than in the flesh [4].

After peeling, apples develop undesirable brown color, mainly due to oxidation of endogenous polyphenols into quinones, and their subsequent polymerization into brown products. This process is an enzymatic oxidation that depends on both, polyphenol oxidase (PPO) activity and polyphenols type and concentration [5]. Polyphenol oxidation decreases both commercial and nutritional value of apple products. Therefore, several studies have been carried out aimed to control enzymatic browning in these fruits, using different antibrowning agents, such as ascorbic acid derivatives, 4-hexylresorcinol and cyclodextrins (CD) among others [6–9].

Natural CD are cyclic oligomers, with a truncated cone shape, built up from 6, 7 or 8 glucopyranose units linked by α-(1–4)glycosidic linkages, named α-, β- and γ-CD, respectively (Figure 1a). They posses the ability to form inclusion complexes with a variety of organic molecules, a property used to modify their physicochemical properties (increasing the bioavailability and

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stability of poorly soluble drugs, among others) [10]. It has been reported that they can act as apple PPO inhibitors due to their capacity to form inclusion complexes with PPO substrates [6, 8, 9, 11].

Several papers have been published on the complexation of natural apple polyphenols by CDs, dealing mostly with the complexation behavior of chlorogenic acid (CA) and rutin (Rt) [12–21]. In these studies, the complex formation has proven useful both in enhancing the solubility and bioavailability of polyphenols, and also in protecting phenolic substrates from PPO, therefore reducing enzymatic browning of apple juices.

Due to the lack of information on the antioxidant capacity behavior of CD-complexed polyphenols, the aims of this paper were: (a) to study the complexation, in solution and in the solid state, of the major apple phenolic acid, CA (Figure 1b) and flavonols, Qc (Figure 1d) and its glycoside derivative Rt (Figure 1c) by β-CD, and (b) to determine the effect of the complexation process on their antioxidant capacity. In order to measure this effect, the Ferric Reducing/Antioxidant Power Assay (FRAP) was used for the first time with CD complexes.

Experimental

Materials

Qc (3, 3′, 4′, 5, 7-pentahydroxyflavone), Rt (3-(6-O-(6-deoxy-α-L-mannopyranosyl)-β-glucopyranosyl)oxy)-2-(3′,4′,5,7-tetrahydroxyflavone) and CA (1,3,4,5-tetrahydroxycyclohexane carboxylic acid 3-[3,4-dihydroxycinnamate]) were purchased from Sigma (USA). 2,4,6-tripyridyl-s-triazine (TPTZ), was purchased from Acrōs Organics (USA). β-CD (Kindly supplied by Wacker Biohem, USA) was recrystallized twice from distilled water and dried in a vacuum oven. Phosphate buffers were prepared from K_2HPO_4 and KH_2PO_4, both from Jalmek A.C.S. grade (Mexico). All other reagents used were A.C.S grade from Acrōs Organics (USA).

Fluorescence measurements

Steady-state fluorescence measurements were performed with a Shimadzu RF 5301 PC spectrofluorimeter (Shimadzu Scientific Instruments, USA). All emission spectra were recorded with 5 nm excitation and emission slits. Stability constants of Qc and Rt were determined according to the methodology described by Haiyun *et al.* [21], with some modifications. Briefly, stock solutions of both flavonols in the range of 10^{-3}–10^{-4} M in methanol were daily prepared and kept protected form light. A 0.012 M β-CD stock solution in water was prepared and used in all experiments. Samples for fluorescence measurements were prepared from these stock solutions, having a final flavonol concentration of $2–5 \times 10^{-5}$ M, with a 2% (v/v) final methanol concentration, and different CD concentrations (2.5×10^{-4}–1×10^{-2} M). The pH of these solutions was adjusted to 7.0 by adding Phosphate buffer (Total phosphate concentration: 0.05 M). All samples were kept 30 min at 25 ± 1 °C and protected from light before measurement, in order to allow for the formation of the complex formation, as suggested by Haiyun *et al.* [21]. Emission fluorescence spectra were acquired in the 465–600 nm interval, at a fixed excitation wavelength of 440 nm. In the case of CA, a 10^{-4} M

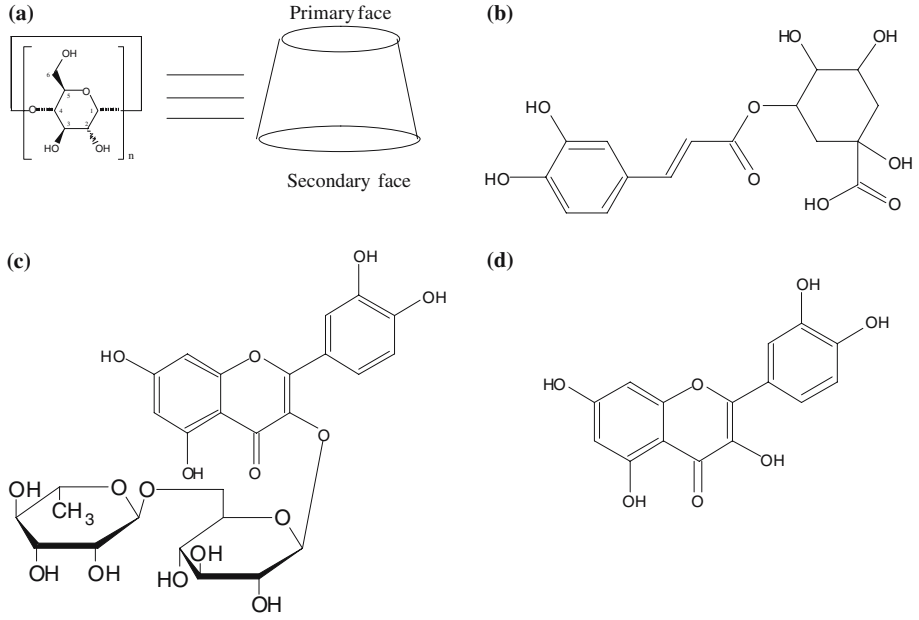


Figure 1. Chemical structure of molecules used in this study. (a) β-cyclodextrin, β-CD; (b) Chlorogenic acid, CA; (c) Rutin, Rt and (d) Quercetin, Qc.

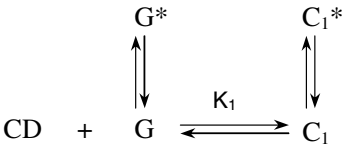
water stock solution was daily prepared and kept protected from light. From these stock solution, samples for fluorescence measurement were prepared just before the measurement, with a final CA concentration in the range of $4–5 \times 10^{-6}$ M. Buffer and CD concentrations were the same than that for Qc and Rt. Emission fluorescence spectra were acquired in the 365–600 nm interval, at a fixed excitation wavelength of 320 nm.

Differential scanning calorimetry

Thermograms of the pure polyphenols, CD, physical mixture and complexes were measured in a Netzsch DSC 200PC Phoxx (Netzsch Group, Germany) calorimeter. Experimental conditions were as follows: temperature range, 30–350 °C; scanning rate, 10 °C min^{-1}; sample weight 4–6 mg. Samples were analyzed by duplicate. Physical mixtures were prepared by mixing equimolar amounts of β-CD and polyphenols in a mortar. To obtain the solid β-CD–polyphenol complexes, the following methods were performed: β-CD–Qc complex was prepared according to Pralhad and Rajendrakumar [22]. Briefly, equimolar quantities of both components (10 mM) in water were prepared and 0.5 ml of 25% ammonia was added to dissolve the Qc, stirred for 2 h, frozen at −80 °C and freeze dried for 48 h in a Labconco Freezone 6. Similar procedures were used for Rt and CA, with the only difference that no ammonia was used for CA.

Calculation of the stability constants from fluorescence measurements

The stability constants of the complexes formed between β-CD and the polyphenols were determined from the fluorescence experimental data recorded, assuming the following equilibrium:



(1)

where G* and C_1* are the guest and the complex in the excited state, they are the only fluorescent species, and that there is no association or dissociation process in the excited state [10, 23]. K_1 is the stability equilibrium constant, for the system, defined as a function of the equilibrium concentrations of the three species present in the system:

$$K_1 = \frac{[C_1]}{[CD] \cdot [G]} \tag{2}$$

Under the experimental condition previously described, the measured fluorescence intensity at any wavelength (F^{λ}), can be related to the initial CD concentration by:

$$F^{\lambda} = \frac{F_H^{\lambda} + F_{C_1}^{\lambda} K_1 [CD]_0}{1 + K_1 [CD]_0}, \tag{3}$$

where F_H^{λ} and $F_{C_1}^{\lambda}$ are the fluorescence intensities at any wavelength for the free and complexed polyphenols, and $[CD]_0$ is the initial CD concentration. Experimental data of F^{λ} as a function of $[CD]_0$ was fitted to Equation (3) using a commercial nonlinear least-squares fit program (SIGMA PLOT 8.0), using as initial parameters (K_1 and $F_{C_1}^{\lambda}$) those obtained from the analysis of the experimental data (fluorescence intensities at a fixed wavelength) using the Benesi–Hildebrand equation (double reciprocal plot) [24]:

$$\frac{1}{\Delta F} = \frac{1}{\Delta F_c} + \frac{1}{K_1 \Delta F_c [CD]_0}, \tag{4}$$

where ΔF is the difference on fluorescence between free guest and the fluorescence at the different CD concentrations, and ΔF_c is the difference on fluorescence between free guest and 1:1 complex.

Antioxidant capacity (FRAP assay)

Antioxidant capacity was determined by the FRAP assay according to Benzie and Strain [25] and modified by Pulido *et al.* [26]. FRAP reagent was daily prepared, and kept at 37 °C, by mixing 35 ml of 0.3 M acetate buffer, pH 3.6 with 3.5 ml of a 10 mM TPTZ solution in 40 mM HCl plus 3.5 ml of 20 mM $FeCl_3 \cdot 6H_2O$. Measuring solutions were prepared by mixing 900 μl of FRAP reagent with 90 μl of distilled water and 30 μl of sample or methanol (blank). Methanolic solutions of Fe^{2+} in the range of 100–3000 μM were prepared from a 3000 μM $FeSO_4 \cdot 7H_2O$ stock solution, to obtain the calibration curves. Absorbance was measured at 595 nm, every 30 s, during 30 min, using an Agilent 8453 spectrophotometer (Agilent Technologies, USA). To determine the complexation effect over the antioxidant activity of phenolics, 250 μM methanolic polyphenol stock solutions were prepared, their ferric reducing ability determined by the FRAP assay, and compared with that obtained with the complexes. All complexes were obtained by mixing 60 μl of the methanolic polyphenols solution with 180 μl of a 14 mM β-CD solution, and kept 30 min at 37 ± 1 °C protected from light. For this assays 120 μl of the complex solutions were mixed with 900 μl of FRAP reagent and absorbance was recorded.

Statistical analysis

Stability constant values represent the mean ± SEM of three replicates. FRAP values represent the mean ±

SEM of four replicates. *t*-Student tests ($p < 0.05$) were performed with the FRAP data.

Results and discussion

Steady-state fluorescence measurements

Complexation of CA

The variation of the fluorescence emission spectra of CA in the presence of different β-CD concentrations (Figure 2a) shows that the addition of CD produces a slight increase in the fluorescence intensity of CA (2- to 3-fold at the maximum CD concentration), as well as a small blue shift of the emission spectrum (approximately 10–15 nm). This increase in the fluorescence intensity of CA is characteristic of the inclusion of several fluorescent probes into the CD cavity [10, 23, 27, 28], due to the modification of its surrounding microenvironment, when part or all of the fluorescent molecule enters into the hydrophobic cavity.

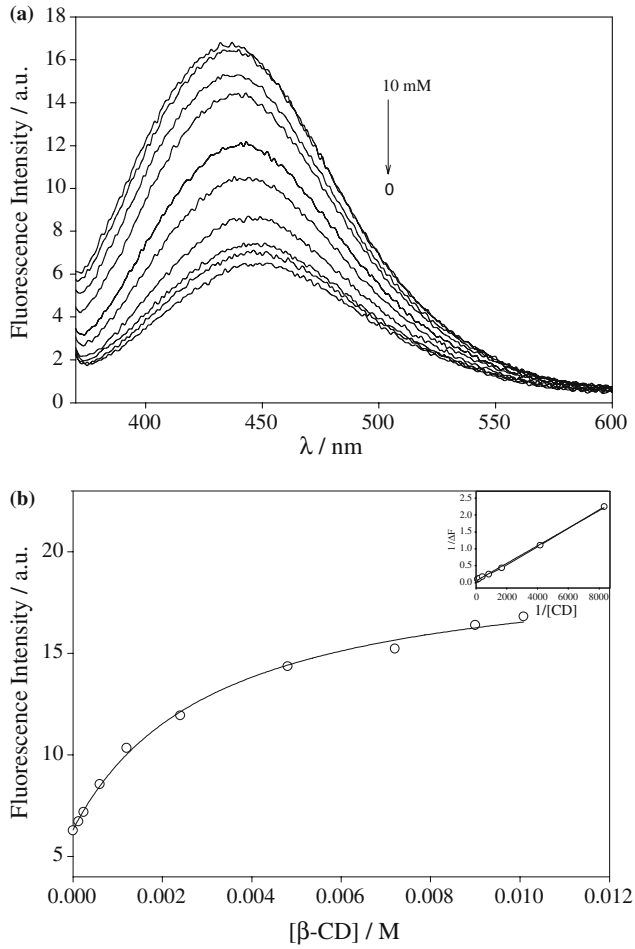


Figure 2. (a) Emission fluorescence spectra of CA in the presence of different β-CD concentrations varying from zero to 10.8 mM. $\lambda_{exc} = 320$ nm. $[CA]_0 = 1–5 \times 10^{-6}$ M. pH 7.0, 25 ± 1 °C. (b) Fluorescence intensities of CA at 437 nm *versus* different β-CD concentrations. Inset in (b): double reciprocal plot of the same data. Lines are the curves fitted to the experimental data.

In order to determine the stoichiometry and stability constant of the CA: β-CD complex, experimental data were first analyzed using the Benesi–Hildebrand method (double reciprocal plot). The inset in Figure 2b shows a straight line for the plot of ΔF^{-1} at 347 nm *versus* $[CD]^{-1}$ over all the β-CD concentrations studied, which means that the CA: β-CD complex has a 1:1 stoichiometry, in agreement with results published by other authors using different analytical techniques [12, 13, 15, 17, 20]. Using Equation (4), a first approximation to the stability constant (K_1) and the maximum fluorescence Intensity for the 1:1 complex ($F^{\lambda}_{C_1}$) were determined by dividing the independent variable by the slope, and from the reciprocal of the independent variable, respectively. These values were used as initial parameters in order to determine the stability constant by fitting the experimental data to Equation (3) by a non-linear method. Figure 2b shows the increase in the fluorescence intensities at 347 nm *versus* initial CD concentration. The stability constants determined by both linear and non-linear method are shown in Table 1. These values (420 and 465 M^{-1}) are in concordance with the values reported by Rodrigues *et al.* [20] for the linear analysis of ^{1}H-NMR shift displacement of both CD and CA protons (504 M^{-1}) and by Irwin *et al.* [12, 13, 15, 17] by ^{1}H-NMR shift displacement and UV–Vis spectroscopy at different temperature, pH and methanol:water molar fractions (412–597 M^{-1}).

Complexation of rutin

The complexation of Rt by β-CD has been carried out by different analytical techniques, including phase solubility diagram [19], microcrystalline cellulose TLC [16], capillary electrophoresis [14], UV–Vis spectroscopy [14] and fluorescence [14, 21]. All these studies determined a 1:1 stoichiometry for the Rt:β-CD complex, however, there are some discrepancies in the stability constant values reported in these papers (K_1 values ranged from 100 to 600 M^{-1}) Due to the low solubility of both Rt and Qc, the methodology proposed by Haiyun *et al.* [21], that uses 2% methanolic solution in phosphate buffer pH 7.0, was used in this work. A moderate increase on the fluorescence intensity (4- to 5-fold at the maximum CD concentration) of Rt was observed as β-CD concentration increased, with a maximum at 530 nm (Figure 3a).

Table 1. Stability constants (K_1) for the complexes formed between β-CD with the three polyphenols under study, obtained by linear (Benesi–Hildebrand) and non-linear analysis of fluorescence data presented on Figures 2, 3 and 4

Guest	K_1/M^{-1} (linear fit)	K_1/M^{-1} (non-linear fit)
CA	420 ± 50[a]	465 ± 26
Rt	250 ± 48	224 ± 14
Qc	1284 ± 106	1138 ± 52

[a] SEM value for $n = 3$.

Once the complexation of Rt with β-CD was confirmed by the increment on the fluorescence intensity of a constant Rt concentration with increasing amounts of β-CD (Figure 3b), the stoichiometry and stability constant of this system were determined in the same manner as for CA (Table 1). Insert in Figure 3b shows a linear behavior for the double reciprocal plot, confirming that the complex has a 1:1 stoichiometry. Both linear an non-linear stability constant values (250 and 224 M^{-1}) are in good agreement with the value obtained by Haiyun *et al.* [21] using the Benesi–Hildebrand method (265 M^{-1}).

Complexation of quercetin

In contrast with CA and Rt, for which several studies on their complexation with β-CD have been carried out, there is a lack of information on the complexation of Qc with any CD. There are only two papers where its complexation behavior has been analyzed. One only reports that during the formation of a ternary complex between β-CD derivatives, Qc and Al^{3+} or Zr^{4+}, an increment on the Qc fluorescence spectra is observed [29]. The other one reports the complexation of Qc with β-CD and hydroxypropyl-β-CD in solution trough the phase solubility method, and in the solid state (DSC, X-ray and scanning electron microscopy) [22]. Due to the low solubility of Qc in water, and in order to be able to compare the complexation behavior of Qc and Rt with β-CD, the complexation of Qc was studied using the same experimental conditions used for Rt. The variation of fluorescence emission spectra of Qc by addition of β-CD is presented in Figure 4a. When this figure is analyzed, several aspects should be taken in consideration: (i) under the same experimental conditions, the Qc system shows higher fluorescence intensities than those of Rt (Figure 3a). This may be

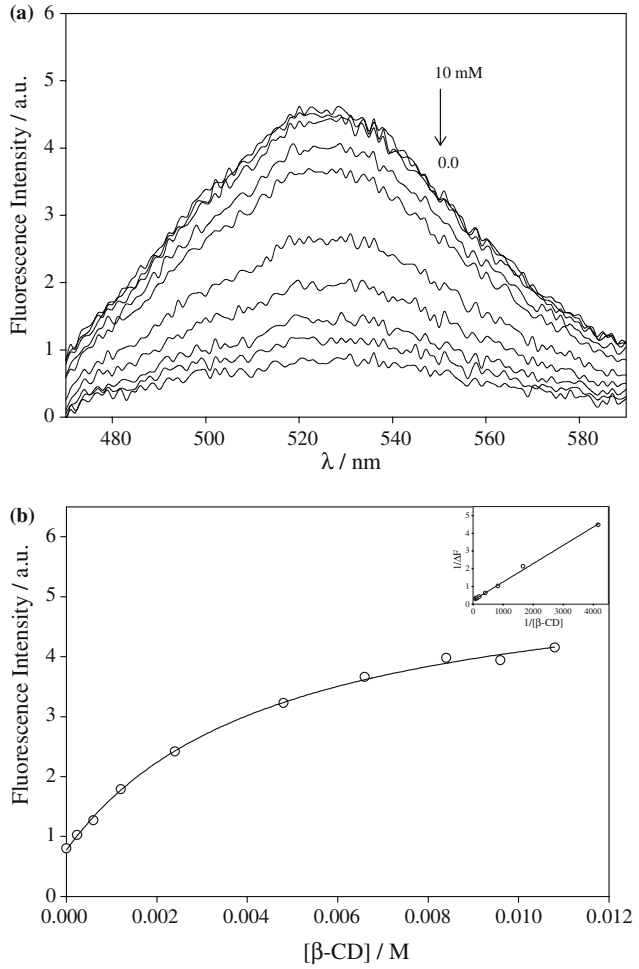


Figure 3. (a) Emission fluorescence spectra of Rt in the presence of different β-CD concentrations varying from zero to 10.8 mM. $\lambda_{exc} = 440$ nm. $[Rt]_0 = 2–9 \times 10^{-5}$ M. pH 7.0, 25 ± 1 °C. Total methanol concentration 2% (v/v). (b) Fluorescence intensities of Rt at 530 nm *versus* different β-CD concentrations. Inset in (b): double reciprocal plot of the same data. Lines are the curves fitted to the experimental data.

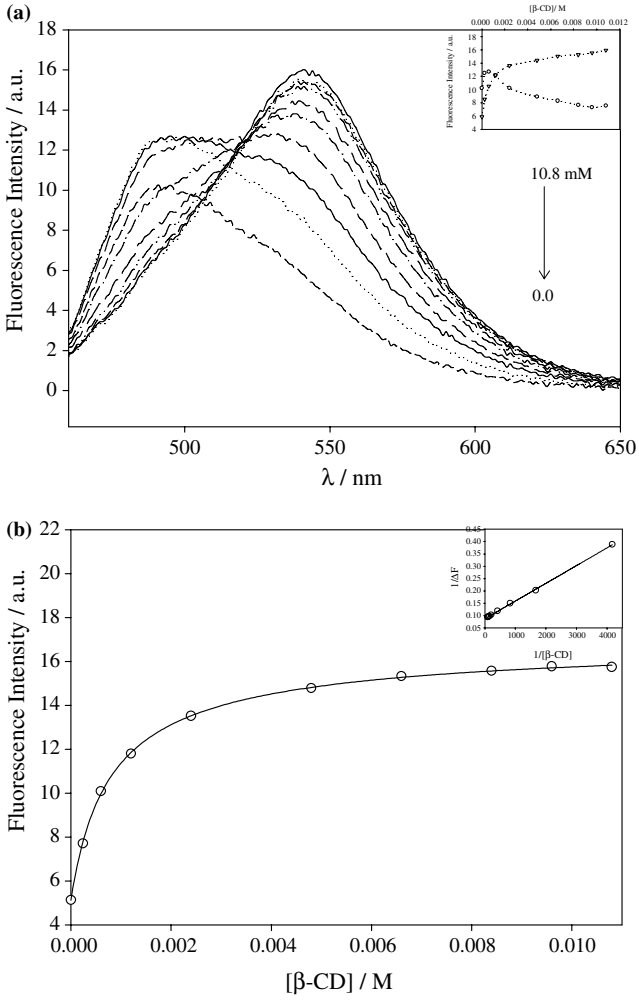


Figure 4. (a) Emission fluorescence spectra of Qc in the presence of different β-CD concentrations varying from zero to 10.8 mM. $\lambda_{exc} = 440$ nm. $[Qc]_0 = 2–9 \times 10^{-5}$ M. pH 7, 25 ± 1 °C. Total methanol concentration 2% (v/v). Inset in (a): Fluorescence intensities of Qc at 495 nm (circle) and 540 nm (triangle) *versus* different β-CD concentrations. (b) Fluorescence intensities of Qc at 540 nm *versus* different β-CD concentrations. Inset in (b): double reciprocal plot of the same data. Lines are the curves fitted to the experimental data.

rationalized in terms of the substitution group at C-3, that being a glycoside group in Rt, may partially inhibit the transformation of the 5-OH···O=C intramolecular hydrogen bond into an intermolecular hydrogen bond to the solvent, which occurs freely in Qc due to its hydroxyl group in C-3 [30]. ii) In the absence of CD in the system, Qc dissolved in 2% (v/v) methanol solution shows a maximum at 495 nm, similar to the normal fluorescence of Qc species dissolved in a $<1\%$ ethanol, reported by Sengupta and Sengupta [31]. At low CD concentrations there is an increase in the fluorescence intensity at this wavelength, however, for CD concentrations higher than 1×10^{-3} M there is a systematic decrease in the fluorescence intensity (See inset in Figure 4a). At the same time, there is a continuous increment in the fluorescence intensity at 540 nm, as the CD concentration is increased from 0 to 10.8 mM. The appearance of this fluorescence band has been assigned to proton transfer tautomer species formation due to the interference with the internal H-bond of Qc during its binding with HAS [30, 31]. Studies carried out in order to determine the complexation of metals with Qc, have also shown this increment in the fluorescence intensity at (or near) this wavelength [32, 33]. Gupta and Panda [34] determined that the binding of Qc with tubulin accounts for the increment on the fluorescence of Qc at 535 nm. All this information suggests that the formation of a complex between Qc and β-CD is taking place.

From the straight line obtained in the double reciprocal plot of the fluorescence intensity of Qc at 540 nm *versus* β-CD concentration, a 1:1 stoichiometry, with a stability constant of 1286 M^{-1} was calculated for this system (inset in Figure 4b). This stability constant was used as initial parameter in the non-linear fitting, obtaining a K_1 value of 1138 M^{-1}. Figure 4b shows the experimental data, as well as its non-linear fitting curve. The stability constant determined in this study is higher that that obtained by Pralhad and Rajendrakumar [22] from phase solubility analysis (402 M^{-1}), however, this discrepancy is not unusual in the complexation of poorly soluble drugs by CD [35], where higher values are obtained by spectroscopic methods when compared with the solubility phase method.

The stability constants for the three systems studied, are summarized in Table 1, which indicates that the stability of the complexes decreased in the order Qc > CA > Rt. From the analysis of the molecular structure of CA, Rt and QC (Figure 1), it is possible to see that the three of them posses an ortho-diphenol ring. From ^{1}H-NMR studies, Irwin *et al.* [12] proposed that during the complexation of CA by β-CD, this diphenol ring enters into the CD cavity. Similar results were observed by Haiyun *et al.* [21] for the complexation of Rt, by both one and two dimension ^{1}H-NMR analysis. Even though no structural (NMR nor X-ray) analyses were carried out for the Qc:β-CD system, from the complexation behavior described for CA and Rt by other authors, one may assume that the complexation of this flavonol will also take place in the diphenol ring. The higher stability constant of Qc respect Rt, could be explained in terms of steric hindrance, since the glycoside group of Rt may complicate the inclusion of the diphenol inside the CD cavity.

Complexation in the solid state

As described in the experimental section, solid products were obtained, by freeze-drying solutions containing equimolar mixtures of each polyphenol and β-CD. Considering that the three polyphenols formed 1:1 stoichiometry complexes with β-CD in solution, DSC analysis where carried out, in order to determine if the solid products obtained were physical mixtures or real complexes. For this, the thermograms of pure polyphenols, β-CD, physical polyphenol–β-CD mixture and isolated products where compared for each polyphenol. It is well known that if a complex is formed in the solid state, its thermogram should be different to that of the physical mixture, which will show the combination of the host (CD) and guest (polyphenol). In the complex thermogram, the melting peak of the guest is usually absent, as a result of the interaction between the guest and the CD cavity [21, 22, 33].

Pure compounds presented the following endothermic peaks: β-CD presented a broad endothermic peak around 100 °C and a decomposition endothermic peak

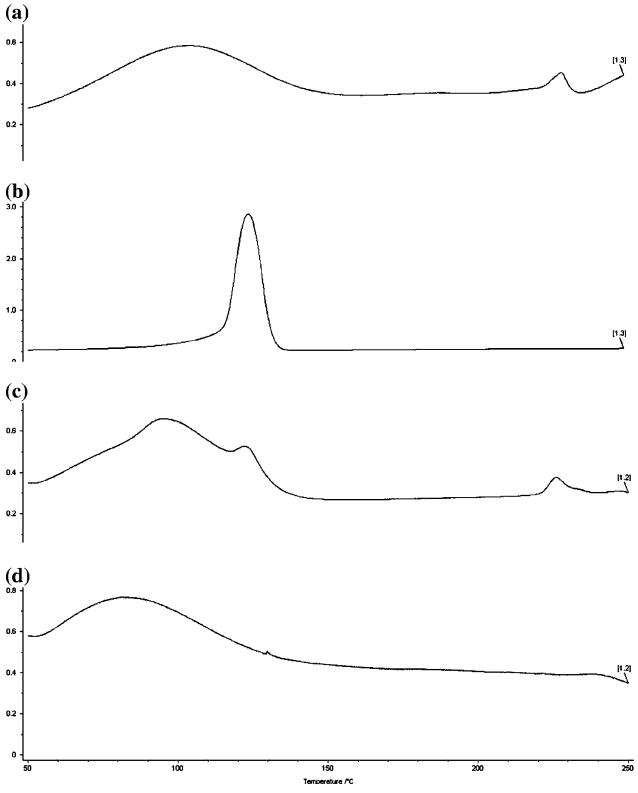


Figure 5. DSC curves: (a) β-CD, (b) Qc, (c) physical mixture of β-CD and Qc, (d) inclusion complex between β-CD and Qc. Range temperature: 30–350 °C, scanning rate: 10 °C min^{-1}; sample weight: 4–6 mg.

at 227 °C; CA presented two endothermic melting peaks, a small one at 199 °C (may be due to impurities) and a larger one at 212 °C; Rt showed a broad endothermic melting peak at 184 °C; and Qc presented a endothermic peak at 123 °C as it becomes anhydrous and an endothermic melting peak at 323 °C.

In all cases, the thermograms of the polyphenol–β-CD physical mixtures presented the endothermic peaks, from polyphenols, and β-CD. The solids isolated from the equimolar polyphenol:β-CD solutions, presented the following endothermic peaks: β-CD:CA solid showed two broad endothermic peaks around 100 and 242 °C; β-CD:Rt and β-CD:Qc solids only showed a broad peak around 100 °C due to the lost of hydration water.

Antioxidant capacity of free and complexed polyphenols

The study of the complexation by β-CD of fruit polyphenols may be interesting at least from two points of view: (i) the possibility of using these complexes as enhancers of either the solubility or the natural antioxidant activity of these compounds; and (ii) in order to gain insight on the ability of β-CD to prevent enzymatic browning in fruits [6, 8, 9, 11]. The first type of studies has been carried out by Nagase *et al.* [36] for the solubility enhancement of the seleno-organic antioxidant drug ebselen. In this paper, the first point of view is also addressed, since there is no evidence that the antioxidant capacity behavior of complexed antioxidants has been previously studied.

Several methods have been developed to quantify the total antioxidant activity of pure compounds or real samples. Two of the most popular methods are 'oxygen radical absorption capacity' (ORAC) [37, 38] and FRAP [25, 26]. The main difference between this two methods is that ORAC follows a hydrogen atom transfer pathway, where the antioxidant and a peroxyl radical form a stable antioxidant radical that stops the radical chain oxidation [38]. On the other hand, FRAP follows a single electron transfer pathway between the antioxidant and a Ferric complex:

$$\mathrm{Fe(TPTZ)_2(III) + AntiOH \rightarrow Fe(TPTZ)_2(II) + AntiOH^{+\cdot}} \quad (5)$$

In other words, FRAP measures the reducing capacity of the antioxidant compounds toward ferric ions [25, 26]. Since there are no studies on the antioxidant capacity of CD complexes, in this work, the FRAP assay was used in order to study if the complexation of CA, Rt and Qc modifies their antioxidant behavior.

Having in mind that FRAP is a method that measures the antioxidant capacity of a molecule through the reduction of Fe^{3+} to Fe^{2+} measuring the absorbance at 595 nm due to the Fe^{2+}TPTZ complex, it was first necessary to investigate if β-CD could interfere with the FRAP measurement due to complexation of TPTZ with β-CD. In order to eliminate this possibility FRAP values for different fixed Fe^{2+} concentrations, in the interval 0–3000 μM, were determined in the absence and presence of β-CD. No difference was detected between both kind of measurements, confirming that β-CD does not interfere with the FRAP assay (data not shown).

Due to the low solubility of Qc and Rt in water, and in order to be able to compare the antioxidant capacity results with those form the complexation behavior. FRAP analysis were carried out in methanolic solutions. The overall regression equation for the calibration curve was $y = (0.00055 \pm 2.9 \times 10^{-5})x + (0.1776 \pm 0.0105)$ and the limit of detection and quantification was 120 and 400 μM Fe^{2+}, respectively. The original FRAP methodology proposed by Benzie and Strain [25] established a 4 min interval before the determination of the FRAP value. However, as shown in Figure 6, and in agreement with Pulido *et al.* [26], when 250 μM polyphenol solutions (and solutions of 250 μM polyphenol in the presence of 14 mM β-CD solution) were incubated with FRAP reagent, even after 30 min the

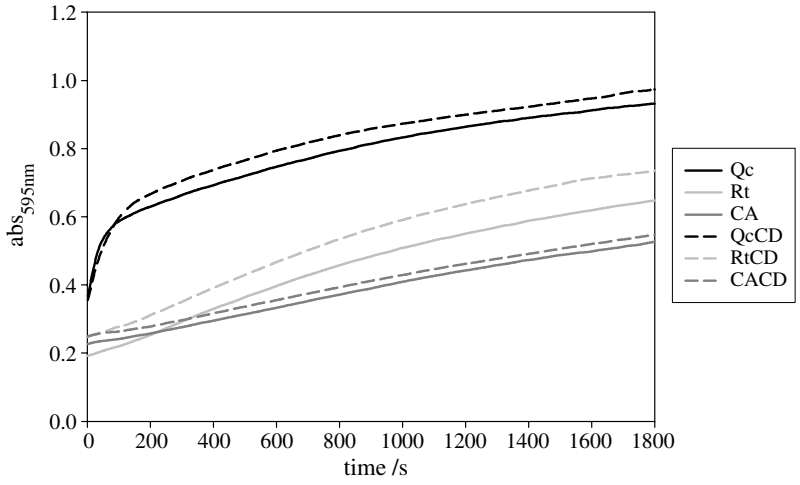


Figure 6. FRAP reaction kinetics for 250 μM methanolic solutions of free and complexed polyphenols measured at 595 nm.

reaction was not complete. For this reason, FRAP values were determined at 4 and 30 min, observing an increment in the FRAP value that ranked between 45 (for Qc) to 120% (for Rt). In this figure, it is also possible to observe that in all cases the complex showed a higher reducing ability, i.e. the complexes formed behave as better antioxidants than polyphenols alone. It is also possible to see that this increment in the antioxidant activity is more prominent in Rt.

Even though the original FRAP method reports the FRAP value as μM trolox equivalent/g of sample, since the aim of this study was to compare the reducing ability between the free and complexed polyphenols, the ferric reducing ability of 250 μM solutions of polyphenols (free and complexed), is reported as μM Fe^{2+} produced by the antioxidants, from a constant Fe^{3+} concentration. FRAP values where determined at 4 and 30 min, and are summarized in Table 2 and Figure 7. When these results are compared, several aspects need to be pointed out. (i) The FRAP value of polyphenols decreased in the order Qc > Rt > CA, in agreement with Pulido *et al.* [26] that observed that the reducing ability of polyphenols depended on several factors, such as the number of hydroxyl groups, the degree of conjugation between them, and specifically for flavonols (Qc and Rt), that they met three conditions: (a) the presence of 3′,4′-dihydroxyl groups (Ring B Qc Figure 1), (b) the presence of 2,3 double bond in conjunction with 4-oxo group in the heterocyclic (Ring C), that permits the conjugation between rings A and C, and (c) the

Table 2. Comparison of FRAP values at 4 and 30 min (μmol equivalent of Fe^{2+}/l) of 250 μM methanolic solutions of Qc, Rt and CA free and in the presence of β-CD 14 mM, calculated using Fe^{2+} calibration curves in methanol (Mean ± SEM; $n = 4$)

	Fe^{2+} 4 min	Fe^{2+} 30 min
Qc	1424 ± 73	2073 ± 130
Rt	660 ± 58	1454 ± 73
CA	569 ± 23	1063 ± 65
Qc:CD	1515 ± 107	2225 ± 227
Rt:CD	770 ± 23	1608 ± 70*
CA:CD	673 ± 11*	1215 ± 27*

* Statistical difference ($p < 0.05$) between complexed and free polyphenols.

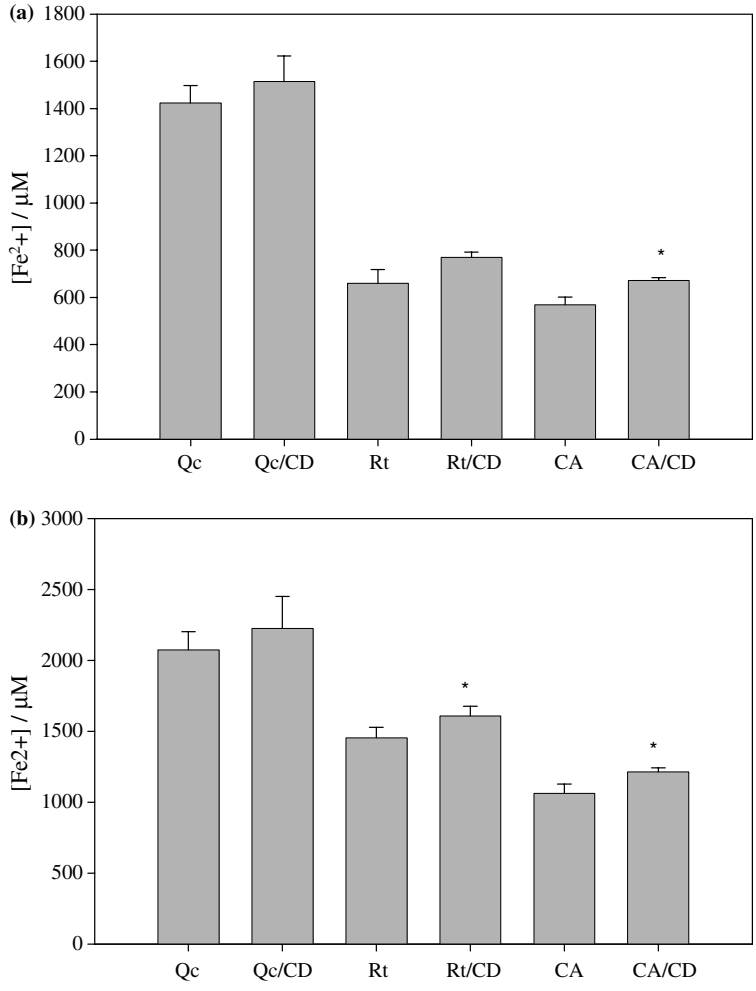


Figure 7. Ferric reducing ability (μmol equivalent of Fe^{2+}/l) of 250 μM methanolic solutions of Qc, Rt and CA free and in the presence of β-CD 14 mM determined at 4 min (a) and 30 min (b). * Statistical difference ($p < 0.05$) between complexed and free polyphenols.

presence of hydroxyl groups at positions 3 and 5. According to this criteria, Qc, that meets the three flavonoid conditions has a higher reducing ability than Rt, that has a glycoside group at position 3. Similar results were observed by Mayer *et al.* [39], when the antioxidant activity was measured *in vitro* as their capacity of protecting lipoproteins against oxidation. In the case of CA, its low reducing ability may be explained in terms that it does not have as many conjugated hydroxyl groups as Qc or Rt.

When the reducing ability of the complexes was determined, it showed, in all cases, a slight increment, as compared to the free polyphenol. This increment is more evident for Rt and CA (about 18%) than for Qc (less than 10%). Statistically, at 4 min, the increase in FRAP value was only significant for CA, being significant for Rt and CA at 30 min. It is important to point out that this is the first study in which the antioxidant activity of complexed polyphenols has been measured.

Even though no further studies were carried out, this increment in the reducing ability can be explained by considering that when the polyphenols are complexed by β-CD, the ortho-diphenol group penetrates into the less polar CD cavity, there may be a modification in the redox behavior of the polyphenols similar to the one described by Isao *et al.* [40] for nitrogen heterocyclic molecules.

From the results previously presented, it may be concluded that the complexation of the studied polyphenols by β-CD may be of great interest, since it not only stabilizes them, and increases their solubility, but also increases their antioxidant capacity, further work has to be carried out, in order to study their antioxidant capacity in biological systems, as well as their behavior as PPO substrates, in order to study the possibility of using them as a natural enrichment source for fruit products with longer shelf life.

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