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Effect of HP-β-cyclodextrins complexation on the antioxidant activity of flavonols

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ABSTRACT

The beneficial effects from phenolic compounds have been attributed to their antioxidant activity. Differences in the chemical structure of flavonols and their degree of substitution will influence phenoxyl radical stability and, thereby, their antioxidant properties. Cyclodextrins (CDs) can be used as a flavonol complexation agent, since they act as a substrate reservoir in a dose-controlled manner. In the present paper, the effect of complexing flavonols, kaempferol, quercetin and myricetin with HP-β-CDs on their antioxidant capacity is studied by means of the oxygen radical absorbance capacity-fluorescein (ORAC-FL) assay. This complexation phenomenon increased the antioxidant activity of the three flavonols, which reached a maximum level when each flavonol had been complexed in the hydrophobic cavity of CDs. The antioxidant activity increased because of the flavonol was protected against rapid oxidation by free radicals.

1. Introduction

The capacity of some plant-derived foods to reduce the risk of chronic diseases has been associated, at least in part, with the presence of non-nutrient secondary metabolites (phytochemicals) that have been shown to exert a wide range of biological activities. These metabolites are quite weak as bioactive compounds compared to pharmaceutical drugs, but, since they are ingested regularly and in significant amounts as part of the diet, they may have a noticeable long-term physiological effect (Espín, García-Conesa, & Tomás-Barberán, 2007). Polyphenols are a group of phytochemicals that exhibit a wide range of physiological properties, including anti-allergenic, anti-artherogenic, anti-inflammatory, anti-microbial, antioxidant, anti-thrombotic, cardioprotective and vasodilatory effects (Benavente-García, Castillo, Marín, Ortuno, & Del Río, 1997; Manach, Mazur, & Scalbert, 2005; Middleton, Kandaswami, & Theoharides, 2000; Puupponen-Pimiä et al., 2001; Samman, Lyons Wall, & Cook, 1998). The beneficial effects derived from phenolic compounds have been attributed to their antioxidant activity (Heim, Tagliaferro, & Bobilya, 2002), and are increasingly attracting the attention of food scientists, not only because of these beneficial effects on human health, but also in food preservation. In this sense, phenolic compounds could be a major determinant of the antioxidant potentials of foods which would therefore act as a natural source of antioxidants (Parr & Bolwell, 2000).

Flavonoids are low molecular weight polyphenolic compounds, which are widely distributed in vegetables and fruits. The basic flavonoid structure is the flavan nucleus, which consists of 15 carbon atoms arranged in three rings (C6–C3–C6), labelled as A, B and C (Fig. 1). Various classes of flavonoids (flavonols, flavones, flavanones, catechins or flavanols, anthocyanidins, isoflavones, dihydroflavonols and chalcones) differ in the level of oxidation and saturation of ring C, while individual compounds within a class differ in the substitution pattern of rings A and B. These differences in the structure and substitution will influence the phenoxyl radical stability and thereby the antioxidant properties of the flavonoids (Huber, Rupasinghe, & Shahidi, 2009).

The above-mentioned antioxidant properties reside mainly in their radical-scavenging activity. Repeated studies have shown that flavonoids with many hydroxyl groups are extremely effective antioxidants. In fact, hydroxylated flavonols, such as myricetin, quercetin and kaempferol, have been demonstrated to be particularly effective antioxidants in many studies (Cao, Sofic, & Prior, 1997; Soobrattee, Neergheen, Luximon-Ramma, Aruoma, & Bahorum, 2005; Wojdylo, Oszmianski, & Czemerys, 2007).

In recent years, complexation with cyclodextrins (CDs) has successfully been used to improve the solubility, chemical stability and bioavailability of a number of poorly soluble compounds, such as resveratrol (Martín Del Valle, 2004). CDs are a group of naturally occurring cyclic oligosaccharides derived from starch with six (α-), seven (β-) or eight (γ-cyclodextrins) glucose residues, linked by α(1 → 4) glycosidic bonds (Szejtli, 2004). In the pharmaceutical, cosmetics and food industries, CDs have been used as complexing agents to increase the water solubility of various compounds, such

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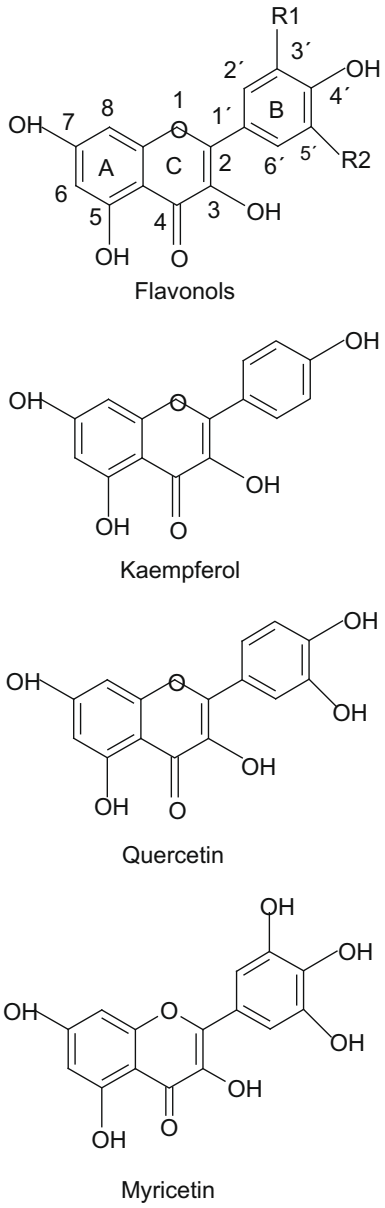

Fig. 1. Flavonols structures.

as drugs, vitamins and food colourants, increasing the solubility, stability and bioavailability of the guest molecule (Buschmann & Schollmayer, 2002; Loftsson & Brewster, 1996).

The ability of CDs to form inclusion complexes with guest molecules depends on the relative size of the internal cavity of CD to the size of the guest molecule or certain key functional groups within the guest. While the height of the CD cavity is the same for all types (7.9 Å), the number of glucose units determines the internal diameter of the cavity and its volume. Based on these dimensions β-CDs (cavity diameter 6–6.5 Å) will complex mainly aromatics and heterocycles (Martín Del Valle, 2004).

As recently described by our group, CDs can be used as flavonoids complexation agents, not only to increase the total flavonoid concentration in aqueous solutions, while the free flavonoids concentration remains constant, but also to decrease the free flavonoid concentration in aqueous solution, while the total concentration remains constant (Lucas-Abellán, Fortea, López-Nicolás, & Núñez-Delicado, 2007). In all cases, CDs acts as substrate reservoir in a dosage-controlled manner. In the case of resveratrol, its complexation with hydroxypropyl-β-cyclodextrins (HP-β-CDs) led to an increase not only in its aqueous solubility but also in its antioxidant activity due to its protection towards free radical attack (Lucas-Abellán et al., 2008).

The complexation of myricetin and quercetin in CDs has been described by our group and their complexation constants (K_c) were calculated by using both solubility and enzymatic methods (Lucas-Abellán, Fortea, Gabaldón, & Núñez-Delicado, 2008). However, it is still unknown whether entrapment in the internal cavity of CDs affects the antioxidant capacity of those flavonols.

Based on the statement described above and tacking into account radial distances, flavonols could be entrapped by β-CDs via phenoxyl group of ring A (C6–C8 = 2.68 Å) or ring B having one (C3′–C5′ = 2.68 Å), two (–OHC4′–OH–C5′ = 3.09 Å) or three OH groups (OH–C3′–OH–C5′ = 5.27 Å) (Fig. 1).

As continuing research examines the effect of antioxidants on health, the testing for antioxidant protection has become focus of attention in the dietary and natural products industry. Researchers associated with the natural product industry have pushed for a standardised method for measuring antioxidant capacity in natural products (Honzel et al., 2008). A large number of methods have been developed to evaluate the antioxidant capacity in foods, one of the most popular and best standardised chemical antioxidant methods being the oxygen radical absorbance capacity (ORAC) test (Ou, Huang, Hampsch-Woodill, & Prior, 2001). The ORAC method is based on the inhibition of the peroxyl-radical-induced oxidation initiated by thermal decomposition of azo-compounds, like 2,2′-azobis(2-amidino-propane) dihydrochloride (AAPH), and it is the only method that combines inhibition time and inhibition degree into a single quantity. This test is widely used for the evaluation and comparison of the antioxidant capacity of natural food products and plasma (Prior et al., 2007).

In the present paper, the effect of the complexation of three flavonols kaempferol, quercetin and myricetin, with HP-β-CDs on their antioxidant capacity against reactive oxygen species (ROS) has been studied by means of ORAC-fluorescein (ORAC-FL) assay (Ou et al., 2001). The antioxidant capacity of myricetin, quercetin and kaempferol in aqueous solution in the absence and presence of CDs is studied for the first time, using the ORAC-FL assay adapted to manual handling, and a conventional fluorescence plate reader.

2. Material and methods

2.1. Chemicals

FL, AAPH, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox C), myricetin, quercetin and kaempferol were purchased from Sigma (Madrid, Spain). HP-β-CDs were from TCI (Europe). All other chemicals used were of analytical grade.

A FL stock solution (4 μM) was made in 75 mM sodium phosphate buffer (pH 7.4) and was stored at −20 °C for 4 weeks. The FL solution was prepared daily in 75 mM sodium phosphate buffer (pH 7.4) by diluting the FL stock to a final concentration of 6 nM. Solutions of 0.25 mM Trolox C and 15 μM myricetin, quercetin and kaempferol in 75 mM sodium phosphate buffer (pH 7.4) were prepared and aliquoted into small vials for storage at −80 °C until use. A 127 mM AAPH solution in 75 mM sodium phosphate buffer (pH 7.4) was prepared daily.

2.2. ORAC-FL assay

The ORAC analyses were carried out on a Synergy HT multi-detection microplate reader, from Bio-Tek Instruments, Inc. (Winooski, USA), using 96-well polystyrene microplates with black

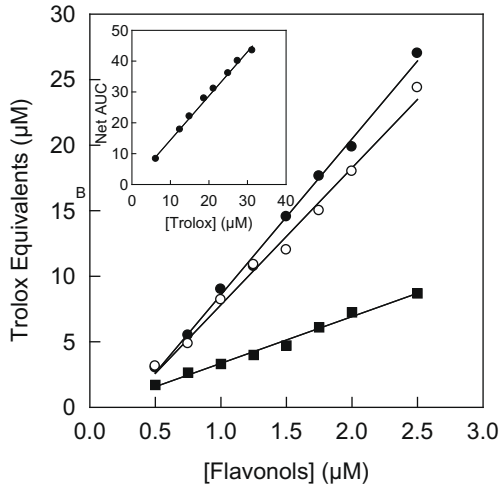

Fig. 2. Regression of μM Trolox equivalents of kaempferol (●), quercetin (○) and myricetin (■) at different concentrations. *Inset:* regression of net AUC of Trolox C on different concentrations of Trolox C. The net AUC = $AUC_{sample} - AUC_{blank}$.

sides and clear bottom, purchased from Nalge Nunc International. Fluorescence was read through the clear bottom, with an excitation wavelength of 485/20 nm and an emission filter of 528/20 nm. The plate reader was controlled by KC4, version 3.4, software. The oxygen radical absorbance capacity was determined as described by Dávalos, Gómez-Cordovés, and Bartolomé (2004), with slight modifications. The reaction was carried out in 75 mM sodium phosphate buffer (pH 7.4), and the final reaction mixture was 200 μL. FL (100 μL; 3 nM, final concentration) and myricetin, quercetin, or kaempferol in the absence or presence of β-CDs or HP-β-CDs (70 μL) solutions, were placed in the wells of the microplate. The mixture was preincubated for 30 min at 37 °C, before rapidly adding the AAPH solution (30 μL; 19 mM, final concentration) using a multichannel pipette. The microplate was immediately placed in the reader and the fluorescence recorded every 1.14 min for 120 min. The microplate was automatically shaken prior to each reading. A blank with FL and AAPH using sodium phosphate buffer instead of the antioxidant solution and eight calibration solutions using Trolox C (6.25, 12.5, 15.0, 18.75, 21.25, 25, 27.5 and 31.25 μM) as antioxidant were also used in each assay. The inhibition capacity was expressed as Trolox equivalents (mM), and is quantified by integrating of the area under the curve (AUC). All reaction mixtures were prepared in triplicate and at least three independent assays were performed for each sample. In order to avoid a temperature effect, only the inner 60 wells were used for experimental purposes, while the outer wells were filled with 200 μL of distilled water.

The results were expressed as μM Trolox equivalents by using the calibration curve of Trolox C ($y = 0.12 + 1.43x$) (Fig. 2, insert). The area under the fluorescence decay curve (AUC) was calculated by the following equation:

$$AUC = 1 + \sum_{i=1.14}^{i=120} f_i/f_0 \quad (1)$$

where f_0 is the initial fluorescence read at 0 min and f_i is the fluorescence read at time i. The net AUC corresponding to the sample was calculated by subtracting the AUC corresponding to the blank. Data processing was performed using Sigmaplot software package 9.0 (Jandel Scientific, Germany).

3. Results and discussion

Synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tertbutylhydroquinone (TBHQ) have been widely used as antioxidants in foods, but concerns over their safety have led to increased interest in natural antioxidants (Wanasundara & Shahidi, 1998). Synthetic antioxidants have in many cases been substituted by phenolic compounds and much research effect has been directed at these natural antioxidants, in particular flavonoids (Martínez-Valverde, Periago, Provan, & Chesson, 2002).

In the present paper, the effect of including of myricetin, quercetin and kaempferol in CDs on their antioxidant activity is studied by using the ORAC-FL method in the presence of increasing concentrations of CDs. These three flavonols were selected for study become of their on chemical structure characteristics, availability and prevalence in plants foods.

Fig. 2 presents the concentration-dependent data of antioxidant activity obtained for each flavonol tested: myricetin (filled square), quercetin (open circle) and kaempferol (filled circle). Least squares regression lines were computed between flavonols concentration and antioxidant activity (expressed as μM Trolox equivalents). As can be seen in Fig. 2, the regression analysis showed linear relation between each flavonol concentration and μM Trolox equivalents, yielding the equations: $y = -3.3 + 11.9x$; $y = -2.6 + 10.45x$ and $y = -0.2 + 3.56x$ for kaempferol, quercetin and myricetin, respectively. Considering that a slope of 1.0 in the lines of Fig. 2 would mean that the ORAC activity of 1 μM of the tested flavonol is equivalent to 1 μM Trolox, kaempferol (slope 11.9), quercetin (slope 10.45) and myricetin (slope 3.56) had ORAC activities from 3–12-fold greater than Trolox. These results agree with those presented by Tabart, Kevers, Pincemail, Defraigne, and Dommes (2009), in which the antioxidant activity of the same three flavonols was determined by several methods, including ORAC assay.

In general, the antioxidant activity of flavonoids depends on the structure and substitution pattern of their hydroxyl groups. As stated in other papers the presence of 3-OH group, which provides a catechol-like structure in ring C, is beneficial for the antioxidant activity of flavonoids. Additional hydroxyl groups at positions C5 and C7 of the A ring appear to be less important. The presence of the C2–C3 double bond with a 4-keto arrangement is known to be responsible for electron delocalisation from ring B, which it increases the radical-scavenging activity. The flavonols tested in this work have a different number of OH in ring B. We observed (Fig. 2) the antioxidant activity to be as follows: kaempferol > quercetin > myricetin, which is inversely proportional to the number of hydroxyl groups in the B ring. Kaempferol, which had only one OH group in position C4′ of ring B, showed a slightly higher antioxidant activity than quercetin (with a catechol moiety). Both flavonols appeared to be much better antioxidants than myricetin, which has a galloyl structure in ring B.

Once the antioxidant activity of each flavonol had been established, our purpose was to demonstrate the effect of the inclusion of kaempferol, quercetin and myricetin in HP-β-CDs on the antioxidant activity of the flavonols using the ORAC-FL assay.

The respective areas, under the curve, when the FL decay curves obtained in the presence of kaempferol (Fig. 3, insert), quercetin or myricetin (0.5 or 0.75 μM) (*data not shown*) in the absence of HP-β-CDs were smaller than those obtained in the presence of 1.75 mM HP-β-CDs. Because ORAC assay data combines inhibition time and inhibition degree in a single datum, it is important to note that any increase in the inhibition time and decrease in the inhibition degree of the FL decay curve was greater in the presence of flavonols–CD complexes in all cases than in the presence of flavonols alone (Fig. 3, insert).

When increasing concentrations of HP-β-CDs were added to the reaction medium at too flavonoid concentrations (0.5 or 0.75 μM), a clear increase in the antioxidant activity was observed in all cases (Figs. 3–5). It was not possible to measure the net AUC of kaempferol, quercetin and myricetin at concentrations higher than

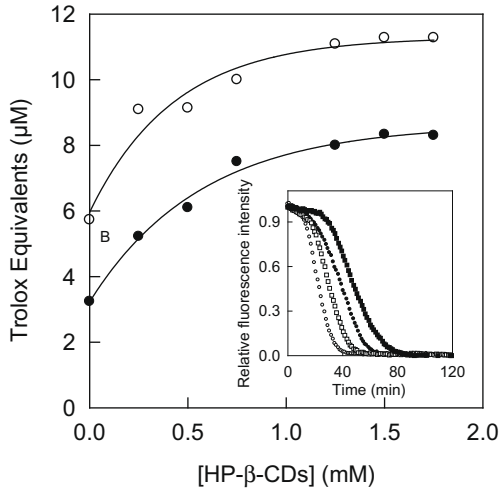

Fig. 3. Effect of HP-β-CDs concentration on the μM Trolox equivalents of kaempferol 0.5 μM (●) and 0.75 μM (○). *Inset:* FL fluorescence decay curves induced by AAPH in the presence of kaempferol alone (0.5 μM (○) and 0.75 μM (□)) and with HP-β-CDs 1.75 mM (kaempferol 0.5 μM (●) and 0.75 μM (■)).

0.75 μM in the presence of CDs, because the measuring times exceeded 2 h, which was established as optimum for the ORAC assay (Lucas-Abellán et al., 2008).

When the concentration of HP-β-CDs increased, the μM Trolox equivalents of flavonols also increased reaching saturation level at approximately 1 mM of HP-β-CDs in all cases. At the saturation level, flavonols showed approximately double the antioxidant activity in the presence than in the absence of CDs in all the cases studied (Figs. 3–5). This effect on antioxidant activity may have been due to the formation of inclusion complexes between these flavonoids and HP-β-CDs.

In order to corroborate that those three flavonols were complexed by HP-β-CDs in 75 mM sodium phosphate buffer (pH 7.4), their complexation constants, K_c were calculated by constructing phase solubility diagrams (*data not shown*). The K_c values obtained for kaempferol, quercetin and myricetin were 1411, 900 and 850 M^{-1}, respectively (Table 1). Kaempferol showed the highest K_c, whereas quercetin and myricetin had lower (and very similar) values.

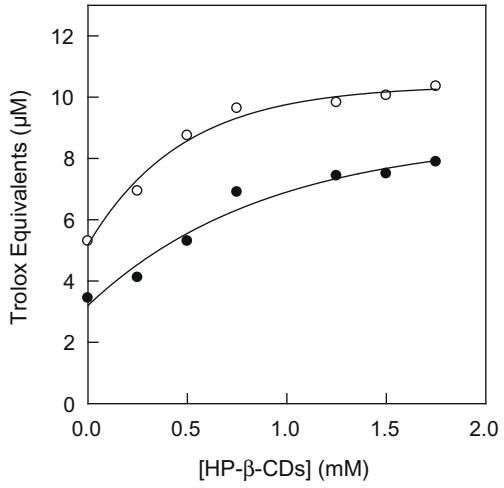

Fig. 4. Effect of HP-β-CDs concentration on the μM Trolox equivalents of quercetin 0.5 μM (●) and 0.75 μM (○).

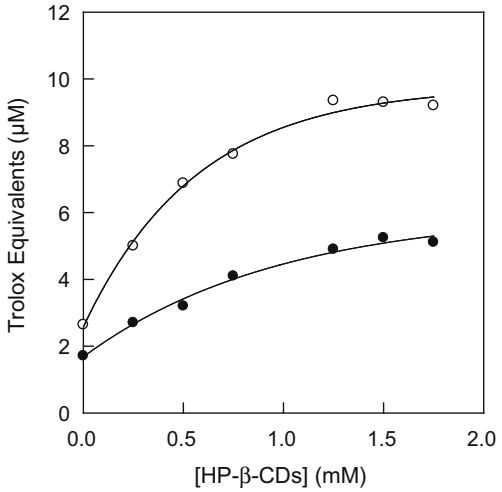

Fig. 5. Effect of HP-β-CDs concentration on the μM Trolox equivalents of myricetin 0.5 μM (●) and 0.75 μM (○).

As can be seen in Fig. 6, at an HP-β-CDs concentration of 1.75 mM, the concentration at which almost all flavonols were complexed, kaempferol presented the highest antioxidant activity (Fig. 6, black bars) and myricetin the lowest, as in the absence of HP-β-CDs. It is important to note that there was little difference between the antioxidant activities observed for the tree flavonols when all of them were totally complexed in HP-β-CDs. However,

Table 1
Complexation constant (K_c), aqueous solubility (S_0), free flavonol ([flavonol]$_f$) and antioxidant activity (AA) normalised with respect to the Trolox measurement.

Flavonol	K_c (M^{-1})	S_0 (μM)	[Flavonol]$_f$ (μM)			AA normalised respect to Trolox (μM TE/μM flavonol)	
			0 mM HP-β-CDs	0.25 mM HP-β-CDs	1.75 mM HP-β-CDs	0 mM HP-β-CDs	1.75 mM HP-β-CDs
Kaempferol	1411	4	0.75	0.556	0.217	11.9	15.1
Quecetin	900	26	0.75	0.612	0.291	10.4	13.5
Myricetin	850	62	0.75	0.619	0.3	3.6	11.2

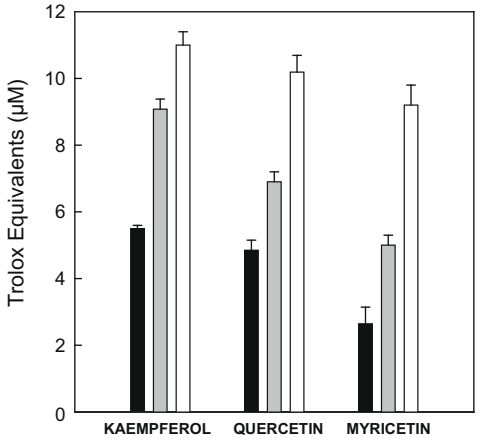

Fig. 6. Effect of HP-β-CDs concentration in the μM Trolox equivalents of kaempferol, quercetin and myricetin alone, 0.75 μM (black bars) and in the presence of HP-β-CDs 0.25 mM (grey bar) or HP-β-CDs 1.75 mM (white bars).

at 0.25 mM HP-β-CDs, a concentration at which not all the flavonol is complexed, the differences between the antioxidant activities of three flavonols were greater (Fig. 6, grey bars). As can be seen in Fig. 6, the increase in antioxidant activity at 0.25 mM HP-β-CDs was close to the increase observed at 1.75 mM in the case of kaempferol, while in the case of quercetin and myricetin, the antioxidant activity at 0.25 mM HP-β-CDs was much lower than at 1.75 mM. This can be explained taking by the higher K_c of kaempferol is compared with quercetin or myricetin.

At flavonol concentrations studied in this paper (up to 0.75 μM), kampferol, quercetin and myricetin were soluble in the reaction medium (Table 1). Therefore, the increase in μM Trolox equivalent observed in Figs. 3–5 when increasing CDs concentration were added to the reaction medium, cannot be attributed to the solubilisation of flavonols, as has been previously described for lycopen (Bangalore, McGlynn, & Scott, 2005) and α-tocopherol (Huang, Ou, Hampsch-Woodill, Flanagan, & Deemer, 2002). In all cases, when the flavonols were complexed, HP-β-CDs acted as a reservoir of controlled substrate dosage protecting flavonols against their rapid oxidation by AAPH radical. So, their antioxidant activity was prolonged and only reached a maximum when all the flavonols had been complexed (a minimum quantity of free flavonols was always present because the complexation phenomenon is a dynamic equilibrium).

To complete the study, an experiment was carried out in which increasing concentrations of each flavonol were added to the reaction medium at a fixed HP-β-CDs concentration (1.75 mM). In this case, the values of antioxidant activity normalised with respect to Trolox increased in all cases (Table 1).

In order to clarify whether the increase in antioxidant activity of flavonols was due to the protection afforded by HP-β-CDs, the free flavonol concentrations ($[\text{flavonols}]_f$) at each bar of Fig. 6, were calculated using the K_c value previously obtained for each flavonol (Table 1) and the following Eq. (2) (Lucas-Abellán et al., 2008):

$$[\text{flavonols}]_f = (-([\text{CD}]_t K_c - [\text{flavonols}]_t K_c + 1) + \sqrt{([\text{CD}]_t K_c - [\text{flavonols}]_t K_c + 1)^2 + 4K_c[\text{flavonols}]_t})/2K_c \quad (2)$$

where $[\text{flavonols}]_t$ is total flavonol and $[\text{CD}]_t$ is total CD.

As can be seen in Table 1, in the absence of HP-β-CDs, the concentration of free flavonol was the same in all cases. However, as shown in Fig. 6, kaempferol presented the highest antioxidant activity, due to its chemical structure. Moreover, as HP-β-CDs concentration increased, free flavonol concentration decreased in all cases, kaempferol being the most complexed flavonol, due to its higher K_c (Table 1). Due to the similar K_c value for quercetin and myricetin, both flavonols presented similar free concentrations at a fixed HP-β-CDs concentration, (Table 1). However, as can be seen in Fig. 6, quercetin presented greater antioxidant activity, as in the absence of HP-β-CDs, due to its chemical structure.

In summary, the antioxidant activity of kaempferol, quercetin and myricetin increased when as they are complexed in HP-β-CDs. Kaempferol presented the highest antioxidant activity both in the absence and presence of HP-β-CDs, while myricetin showed the lowest antioxidant activity.

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