The rutin/β-cyclodextrin interactions in fully aqueous solution: spectroscopic studies and biological assays

M.L. Calabrò[a], S. Tommasini[a,*], P. Donato[a], R. Stancanelli[a], D. Raneri[a], S. Catania[c], C. Costa[b], V. Villari[d], P. Ficarra[a], R. Ficarra[a]

[a] *Dipartimento Farmaco–Chimico, Facoltà di Farmacia, Università di Messina, Viale Annunziata, 98168 Messina, ME, Italy*
[b] *Medicina del Lavoro, Policlinico Universitario, Via Consolare Valeria 1, 98125 Messina, ME, Italy*
[c] *Dipartimento Farmaco–Biologico, Facoltà di Farmacia, Università di Messina, Viale Annunziata, 98168 Messina, ME, Italy*
[d] *CNR, Istituto per i Processi Chimico-Fisici, IPCF-CNR, Via La Farina 237, 98123 Messina, ME, Italy*

Abstract

In the present work the feasibility of β-cyclodextrin complexation was explored, as a tool for improving the aqueous solubility and antioxidant efficacy of rutin. By means of ^{1}H NMR, UV–vis and circular dichroism spectroscopy the single aromatic ring of rutin was found to be inserted into the β-cyclodextrin cavity to form a 1:1 inclusion complex. The effect of β-cyclodextrin on the spectral features of rutin was quantitatively investigated, in fully aqueous medium, by holding the concentration of the guest constant and varying the host concentration. The associated binding constants were estimated to be 142 ± 20 and $153 \pm 20 \, M^{-1}$, respectively, on the basis of the observed UV–vis absorption and circular dichroism intensities. The antioxidant activity of rutin was also investigated, as affected by molecular encapsulation within β-cyclodextrin (batophenanthroline test; comet assay; lipid peroxidation); the inclusion complex revealed improved antioxidant efficacy that may be in part explained by an increased solubility in the biological moiety.
© 2004 Published by Elsevier B.V.

Keywords: Rutin; β-Cyclodextrin; ^{1}H NMR; UV–vis; CD; Binding constant; Solubility measurements; Batophenanthroline test; Comet assay; Lipid peroxidation

1. Introduction

Rutin (quercetin-3-rhamnosylglucoside, Fig. 1), a natural flavone derivative, is well known for its significant scavenging properties on oxidizing species such as OH radical [1–3], superoxide radical [4–5] and peroxyl radical [6–7]. As an outcome of these biological effects, it has been widely used in treating disease, its several pharmacological activities including antiallergic [8], anti-inflammatory and vasoactive [9], antitumor [10], antibacterial, antiviral and antiprotozoal properties [11]. In addition, hypolipidaemic [12], cytoprotective [13], antispasmodic [14] and anticarcinogenic [15]

activities have also been reported. It is worth considering that, being a non-toxic, non-oxidizable molecule, rutin offers an advantage over myricetin, quercetagenin and other flavonoids, which on some occasions behave as pro-oxidant agents and catalyse oxygen radicals production [16]. Its poor solubility yet imposes some restraints to further pharmaceutical use. It is generally acknowledged that non-covalent complexes formed by cyclodextrins with several guest molecules offer a variety of physicochemical advantages over the free drugs, including the possibility for increased water solubility, bioavailability and solution stability [17–21].

The interaction of rutin with native and modified β-cyclodextrin has been studied by other authors and the host–guest binding constants (equilibrium constants) were determined by different analytical techniques: cellulose TLC [22], UV and fluorescence spectroscopy, RP-HPLC and free
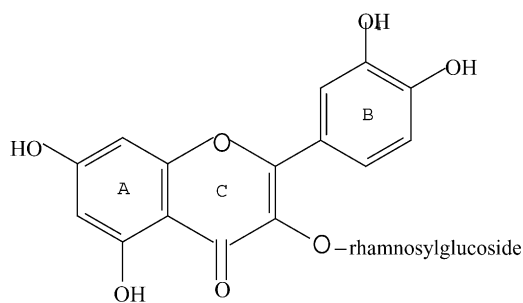
Fig. 1. The structure of rutin.

solution CZE [23]. Furthermore, the spatial configuration of the β-cyclodextrin complex was proposed on the basis of NMR and molecular modelling results [24]. On the other hand, the antioxidant activity of rutin has not yet been investigated, as affected by molecular encapsulation within cyclodextrins. Besides, all the studies cited above were carried out in binary aqueous–organic solvent mixtures. There is evidence that the nature of the solvent can influence or control the nature of the complex, being a critical factor in determining complex stability. Observed solvent effects may arise from solvent–solvent interactions (solvophobic effect); solute–solvent interactions (solvation effect); solute–solute interactions (inter- or intra-solute effect) [25]. The free energy change of complex formation resulting from these contributions will show significant differences, relative to fully aqueous systems. Moreover, the structures of cyclodextrin complexes may be quite different according to the type of solvent, since host and guest molecules will adopt a mutual orientation which allows polar portions of the guest to interact with the cyclodextrin and the solvent. The stoichiometry and stability constant of a complex are commonly estimated from the measurement and treatment of analytical data, using a wide selection of methods [26]. The choice of a model for experimental data processing, as well as the experimental conditions, can largely affect the numerical results achieved and this can account for the difference between the binding constants reported in the literature for the same substrate. A careful preliminary study of the interacting molecules is in all cases needed, in order to classify the complexation behaviour unambiguously; that is particularly true in the case of rutin, which is capable of generating colloidal dispersion coexisting with the solution phase at the equilibrium. To the best of our knowledge, there is no literature describing the interactions between rutin and cyclodextrins in aqueous media, despite the interest for its potential relevance to a variety of biological phenomena, as well as for pharmaceutical applications. The inclusion process alters the spectral features of the guest molecules, as reflected by changes in their UV spectra; UV–vis spectroscopy is one of the most widely used method for studying inclusion complex formation, due to its high sensitivity and feasibility, though lacking in selectivity [27,28]. By virtue of the chiral properties of cyclodextrins, circular dichroism spectroscopy is another technique employed for

the purpose, in a similar way as UV–vis spectroscopy, with the advantage of greater specificity than absorption, since the optical activity of a given molecule can only be interfered by another chiral compound [29]. One of the early methods for investigating binding interactions has been ^{1}H NMR, since the complexation phenomenon leads to shifts in the proton signals of both the host and guest molecules. Even being a powerful tool, NMR does suffer from some limitations, especially in the case of low soluble compounds, or low critical aggregation concentrations [30]. In this paper, the interactions between rutin and β-cyclodextrin were studied in aqueous solution by several spectroscopic techniques: UV–vis absorption, ^{1}H NMR and circular dichroism (CD) spectroscopy; the stoichiometry and estimated binding constants are also reported. Moreover, the protection provided by the solid rutin/β-cyclodextrin complex against oxidative damage was assayed by different in vitro tests: bathophenanthroline test, comet assay and lipid peroxidation; the differences between the free and complexed drug are also highlighted.

2. Experimental

2.1. Chemicals

The following reagents and solvents were used: rutin (3,3′,4′,5,7-pentahydroxyflavone-3-rutinoside, $C_{27}H_{30}O_{16}$, MW 610.5) from Extrasynthese (Genay, France); β-cyclodextrin (β-CyD, $C_{42}H_{70}O_{35}$, MW 1135.00), HPLC grade, from Fluka Chemie (Switzerland). They were employed without any further purification. Water used throughout the study was double-distilled and deionised, then filtered through 0.22 μm Millipore filters (Bedford, USA). Deuterium oxide (D_2O, deuterium content 99.9%) and all chemicals for biological activity tests were supplied by Sigma–Aldrich Chemie (Germany). All solvents employed in the spectrophotometric analyses were of spectroscopic reagent grade, from Merck (Darmstadt, Germany). All other materials were HPLC grade.

2.2. Apparatus and procedure

The UV–vis absorption spectra were obtained with a Perkin-Elmer UV–vis double beam spectrophotometer mod. Lambda 45, equipped with a PC for data processing (software: UV-Win Lab, from Perkin-Elmer, Norwalk, USA). 0.1 cm pathlength rectangular quartz cells (Hellma) were employed in the 200–500 nm spectral range (scanning speed 60 nm/min; slit = 2). Concentrations of the samples to be measured were adjusted so that the extinction values did not exceed $E = 1.0$ at a given wavelength. UV–vis spectroscopy was also employed to quantify the amount of rutin in the β-CyD complexes, investigated in the biological assays. The amount of drug was calculated by interpolation of a calibration curve previously obtained in water. Baseline was established for each measurement placing in the reference compartment an

aqueous solution of β-CyD at the same concentration of the sample. All the data shown represent the average of, at least, three determinations.

The circular dichroism spectra were collected using a JASCO J-500A spectropolarimeter, from Japan Spectroscopy Co. (Tokio, Japan), furnished with a 150 W xenon lamp. The instrument was interfaced with a PC for CD signals reading. The measurements were performed under nitrogen flux at $25 \pm 1\,°C$ and the samples were contained in rectangular quartz cuvettes of 0.1 cm pathlength. The acquisition parameters were: wavelength range 200–500 nm at steps of 1 nm, bandwidth 2 nm, time constant 0.5 s, sensitivity 2 mdeg./div. The instrument was calibrated by using a 0.06% aqueous solution of ammonium D-10-camphorsulphonate, from Jasco.

Spectra were recorded in triplicate; subtraction of the blank from the CD signals of the samples was performed using the cyclodextrin aqueous solutions at each concentration value. Data were averaged and subjected to a smoothing procedure with the Savitzky–Golay algorithm.

Proton NMR spectra were recorded at $25 \pm 0.1\,°C$ on a Varian Gemini 300 MHz spectrometer, from saturated solutions of the analytes (pure β-CyD and solid complexes with the flavonoid) dissolved in D_2O. The chemical shifts were referred to the residual proton signal of the solvent (HDO) at 4.8 ppm. No internal ^1H NMR reference was added, since the possibility of its binding to β-CyD could not be excluded.

2.3. Solubility measurements

Phase-solubility studies were performed with a Haake C25 thermostated bath, equipped with a Haake F6 controller which allowed an accuracy of $\pm 0.01\,°C$. Excess rutin was added to unbuffered aqueous solutions of β-CyD ($0.0–9.0 \times 10^{-3}$ M) in 10 ml capped tubes, then sonicated in a water bath (Bandelin RK 514, Berlin, Germany) for 15 min. Tubes were sealed to avoid changes due to evaporation and magnetically stirred for 116 h in a thermostated bath at $25 \pm 0.01\,°C$, shielded from light to prevent any degradation of the molecules. After the equilibrium was reached, suspensions were filtered through Whatman® PTFE 0.45 μm filters (Bedford, USA). An aliquot from each vial was withdrawn by 1 ml glass syringe (Poulten & Graf GmbH, Germany) and assayed spectrophotometrically to evaluate the amount of flavonoid dissolved. Dilution was intentionally avoided, to prevent any possible interference with the chemical equilibrium, particularly considering the presence of colloidal particles. Experiments were carried out in triplicate, solubility data were averaged and used to calculate the binding constants for rutin/β-CyD complex formation, by UV–vis as well as by CD spectroscopy.

2.4. Preparation of the solid complex

Rutin/β-CyD inclusion complex was obtained suspending adequate amounts of both molecules in 40 ml purified water.

Stirring was carried out for 116 h, under controlled temperature ($25 \pm 0.01\,°C$) and shielded from light to prevent any degradation of the molecules. After that time, solutions were filtered through Whatman® PTFE 0.45 μm filters to eliminate the free flavonoid which had not reacted. Water was at last evaporated under vacuum (30 °C), yielding the solid complex as a pale yellow powder which was washed with a little water, dried up to constant weight and kept into desiccator.

2.5. Biological activity tests

2.5.1. Bathophenanthroline test

The bathophenanthroline test was performed according to Yoshino and Murakami [31] with slight modifications. The sample contained 980 μl of 10 mM Tris–HCl (pH 7.1), 10 μl of 0.05 mM $FeSO_4$ and 10 μl of the solution containing rutin (2.3 μM final concentration). A fresh stock solution of $FeSO_4$ was prepared daily. All the mixtures were incubated at $37 \pm 0.1\,°C$ in spectroscopic quartz cells (1 cm optical pathlength, Hellma); 500 μl of 1 mM bathophenanthroline disulphonate were added at different intervals and the absorbance at 540 nm was measured against a proper blank. For all tests rutin, both free and complexed, was solubilized in DMSO and diluted to the desired concentration with EtOH/H_2O (1:1 (v/v)); the solvent mixture resulted ineffective in all the biological tests performed. Experiments were carried out in triplicate.

2.5.2. Comet assay

The single cell gel electrophoresis was performed as described by Singh et al. [32], on human leucocytes from peripheral venous blood. Freshly collected whole blood was incubated for 1 h at $37 \pm 0.1\,°C$ with 10 μM benzo[a]pyrene (BaP) to induce DNA damage. Rutin (free and complexed, 2.3 μM final concentration) was pipetted immediately before BaP. Frosted microscope slides (prepared in triplicate per sample per experiment) were covered with 140 μl of 0.75% regular melting point agarose; a second 110 μl layer of 0.5% low melting point agarose mixed with leucocytes incubation mixture was pipetted out. After solidification, a third layer of 110 μl low melting point agarose was pipetted out on the slides and allowed to gel at 4 °C. Slides were immersed in lysing solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris–HCl pH 10, 1% Sarkosyl with 1% (v/v) Triton X-100 and 10% (v/v) DMSO) and kept at 4 °C overnight. Slides were then placed in alkaline buffer (300 mM NaOH and 1 mM EDTA, pH 13) for 20 min to allow DNA unwinding; electrophoresis was conducted in the same solution for 25 min at 0.6 V/cm. Finally, slides were washed 3 min × 5 min in 0.4 M Tris–HCl buffer (pH 7.5), fixed in methanol for 10 min and stained with 50 μl propidium iodide (2.5 mg/ml). Comets were viewed under a fluorescence Nikon E800 microscope with a 200× objective. Fifty cells were randomly selected in each slide and scored from 0 to 4 on the basis of comet tail length; undamaged cells (score 0) looked like an intact nucleus without tail, while the damaged cells appeared as

comets. Final score was calculated by the formula: score = (n cells scored 1) + ($2n$ cells scored 2) + ($3n$ cells scored 3) + ($4n$ cells scored 4).

2.5.3. Lipid peroxidation

The antioxidant activity of the compounds against lipid peroxidation induced by $FeSO_4$ was estimated in rat liver microsomes by measurement of malondialdehyde (MDA) formation, using the thiobarbituric acid method as described by Husain and Somani [33]. Microsomal fraction was obtained as previously described [34] and total protein content was measured according to the method by Germanò et al. [35].

Heat-inactivated microsomes (0.5 mg/ml proteins) were incubated for 1 h at $37 \pm 0.1\,^\circ$C with 100 μM ascorbic acid, 10 μM $FeSO_4$ and rutin (2.3 μM) or vehicle. Peroxidative damage was stopped adding two volumes of a mixture composed of thiobarbituric acid (0.374% (w/v)) and trichloroacetic acid (15% (w/v)) in 0.25 N HCl. After 15 min at 85 °C and cooling, the precipitate was removed by centrifugation and the supernatant was measured at 532 nm against a blank containing all the reagents except the test sample.

2.6. Statistical analysis

Statistical analysis was carried out to study the significance of the results of the biological activity tests comparing free and complexed rutin with the controls. Dunn's test, a nonparametric multiple comparison test based on Kruskal–Wallis rank sums, was performed to analyse the Comet scores. ANOVA, followed by Newman–Keuls multiple comparison test, was used to compare lipid peroxidation in terms of MDA production.

3. Results and discussion

3.1. UV–vis spectroscopy studies

Table 1 shows the UV–vis absorption spectral data of rutin in aqueous solution, free and complexed with β-CyD. The UV absorption spectrum of rutin (5×10^{-6} M) in aqueous solution consists of two main bands, centred at 256 and 351 nm; these absorption bands have been predicted to arise from π, π^* electronic transitions and are highly absorptive. According to Mabry et al. [36], the first maximum derives from the π, π^* electron rearrangement in the phenyl group, whereas the maximum at longer wavelengths is attributed to the benzene ring of the cinnamoilic moiety that can be

considered as an acyl-disubstituted benzene chromophore. Contributions from the n, π^* transition of the C=O bond cannot be detected, as probably hindered by the former ones; the fine vibrational structure of the spectrum is obscured due to the polarity of the solvent and the strong solvent–solute interactions. The UV spectrum of rutin is quite different in the presence of β-CyD; Table 1 shows the UV spectral data of the rutin/β-CyD complex, dissolved in water (free and bound rutin about 22 and 78%, respectively). Both absorption bands shifted towards longer wavelengths (namely, 256–260 and 351–355 nm), while the intensity ratio of the two maxima decreased (from 1.44 in rutin to 1.24 in the complex). The red shifts observed in the presence of β-CyD are clearly indicative of a chromophores' displacement, upon host binding, to a more hydrophobic environment. Preliminary studies had showed that, depending on the concentration and due to its poor solubility, free rutin forms in water semi-macroscopic stable aggregates, which make the dispersion clear in the visible region of the spectrum but opalescent in the UV.

The presence of colloidal particles imposed particular attention in performing spectroscopic measurements, since they were found capable of light-scattering in the UV spectral region investigated. A calibration curve was made for free rutin, by dissolving increasing amounts of the drug, exactly weighed, in the desired volume of purified water. A plot of the measured absorbances at the appropriate wavelength (maximum at 256 nm) against the molarity of rutin gave a straight line ($R^2 = 0.997$) in the concentration range from 0.0 up to 5.5×10^{-5} M, above which a well-defined plateau was observed, due to the presence of a second, colloidal phase (even though no precipitate was formed). The numeric value of 5.5×10^{-5} M (0.034 mg/ml) represents rutin maximum water solubility (S_0) at $25 \pm 0.1\,^\circ$C. This finding is in perfect agreement with that firstly reported by Higuchi and Connors [37]. The effect of β-CyD on the UV absorption spectra of rutin was quantitatively investigated by holding the concentration of the guest constant ($2S_0$, equal to 1.1×10^{-4} M) and varying the host concentration between 0.0 and 9.0×10^{-3} M, as fully described in Section 2.3. The absorption maxima were progressively shifted to longer wavelengths in the presence of β-CyD, accompanied by isosbestic points at 275 and 400 nm [38]. These findings suggested the formation of a 1:1 rutin/β-CyD inclusion complex. The absorption intensities of the maxima increased as a function of β-CyD concentration, indicating an increased apparent solubility of the guest molecule, up to 6.0×10^{-3} M β-CyD, above which they were nearly constant. At this point, rutin must be fully complexed by β-CyD. The binding constant for the 1:1 rutin/β-CyD complex is

$$K = \frac{\left[R \cdot CyD\right]}{\left[CyD\right] S_0} \tag{1}$$

in which [$R \cdot CyD$], [CyD] and S_0 represent the equilibrium concentrations of the complexed substrate, free cyclodextrin and free substrate, respectively. Since free cyclodextrin

Table 1

UV–vis absorption spectral data of rutin (5×10^{-6} M) in water, alone and complexed with β-CyD (free and bound rutin in the inclusion complex about 22 and 78%, respectively)

	Absorbances (nm)	A_1/A_2
Rutin	256; 351	1.44
Rutin/β-CyD	260; 355	1.24

concentration is higher than the cyclodextrin amount in the complexed form, it holds that $C_{CyD} \cong [CyD]$. Eq. (1) can be written as

$$K \cong \frac{[R \cdot CyD]}{C_{CyD} S_0} \qquad (2)$$

The rutin concentration, $C_R - S_0$ (C_R being the analytical rutin concentration), which is not soluble in the aqueous phase, acts as a reservoir for the formation of the complex when the cyclodextrin is added. The measured molar extinction coefficient of the free rutin at 256 nm was ε_0 20 000 mol^{-1} cm^{-1}, whereas the molar extinction coefficient for bound rutin was measured as $\varepsilon_c = A_F - A_0/S_0$ 28 000 mol^{-1} cm^{-1}, A_F being the saturated absorbance value for which all the rutin concentration, $C_R - S_0$, is in the complexed form and A_0 the absorbance value of rutin when $C_{CyD} = 0$.

In the presence of a given amount of β-CyD, at the analytical concentration of C_{CyD}^i, the apparent absorption of the solution will be $A_i = \varepsilon_0 l S_0 + \varepsilon_c l [R \cdot CyD] = A_0 + \varepsilon_c l [R \cdot CyD]$ with l the length of the optical cell. Therefore, the concentration of the complexed rutin can be obtained from the absorbance values: $[R \cdot CyD] = (A_i - A_0)/(\varepsilon_c l)$. Plotting this quantity as a function of the total concentration of cyclodextrin (see Fig. 2), a linear behaviour was obtained, indicating a 1:1 stoichiometry of the complex. From the slope of the linear fit (correlation coefficient, $R^2 = 0.987$) the estimated binding constant for the 1:1 rutin/β-CyD inclusion complex was obtained equal to $K = 142 \pm 20$ M^{-1} (log $K = 2.15$). Experiments were carried out in triplicate, each data point represents the mean ± 0.0055 S.D. (calculated R.S.D. $= 2.9\%$). It has been reported that a high correlation coefficient does not necessarily mean a 1:1 stoichiometry of the complex, the selection of the method significantly affecting the success rate of the experiment. Particularly, the double reciprocal plot for a 2:1 complex can in one case satisfy the linear relationship [39]. However, the checked plot for a 1:2 complex did not show a straight line, confirming a 1:1 complex formation.
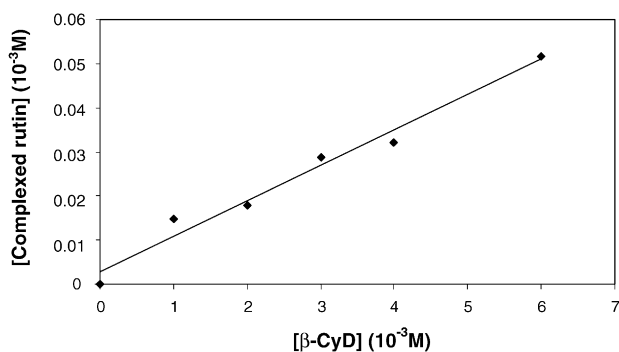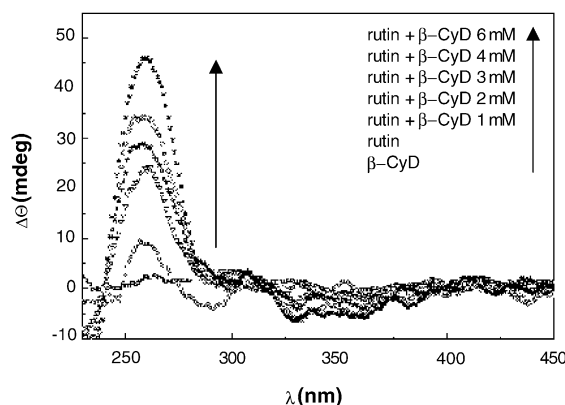

Fig. 3. The effect of various β-CyD concentrations on the CD spectra of rutin in water. Spectra were recorded in triplicate and smoothed by the Savitzky–Golay algorithm.

3.2. Circular dichroism studies

Fig. 3 shows the CD spectra in aqueous solution of β-CyD, rutin alone and in the presence of β-CyD at various concentrations ($0.0-9.0 \times 10^{-3}$ M). Rutin exhibits a positive band with a maximum at 260 nm. Induction of circular dichroism in an achiral chromophore by complexation to a chiral receptor is a well known phenomenon, which has been widely applied as a means for deducing complex structure; Zhdanov et al. [40] have reviewed this subject. As expected due to the chiral environment of β-CyD, induced CD can also be seen in this host–guest systems. In the presence of β-CyD in fact, a broadened ICD band is observed in the region 325–375 nm. This independent induced CD band has a negative sign, its intensity being much lower than the intrinsic CD peak of rutin. The presence of the chiral selector also induces a positive Cotton effect on this latter band, whose signal proportionally increased with increasing CyD concentration, up to 6.0×10^{-3} M, above which it was nearly constant. The calculated molar ellipticity of free rutin was $\theta_c \approx 220°$ cm^{-1}, raising up to $\theta_c \approx 715°$ cm^{-1} in the presence of β-CyD. For the negative induced band as well, the CD signal obtained was a function of β-CyD concentration; this effect was however limited to few mdeg., even though the corresponding UV band in the UV absorption spectrum was highly absorptive. The transition centred at 350 nm corresponds to the cinnamoilic chromophore, whereas the one at 260 nm may be attributed to the phenyl group of the molecule, on the basis of the UV spectrum. CD spectra of complexes are generally characterized by their sign, magnitude and wavelength of the location. According to the symmetry rule, the sign of the induced Cotton effect depends upon the spatial relationship between the asymmetrical centre and the perturbed chromophore, while the magnitude of the observed Cotton effect is related to the rigidity of the complex formed. The Kirkwood–Tinoco theory of polarizabilities [41], developed for the CyD case, gives this rule: if the transition dipole moment of the guest chromophore is aligned parallel to the axis

Fig. 2. UV–vis determination of the amount of complexed rutin as a function of β-CyD concentration at 25 ± 0.1 °C. The differences in the UV–vis absorbance intensities (at 260 nm) were normalized for the measured extinction molar coefficient of the complex ($R^2 = 0.987$; slope 0.008). Each data point represents the mean ± 0.0055 S.D. of three experiments (calculated R.S.D. $= 2.9\%$).

of symmetry of the CyD, then the sign of the induced Cotton effect for that transition will be positive, whereas if the dipole moment is aligned perpendicularly to the cavity axis, the ICD sign will be negative. This rule applies to a chromophore that resides inside the cavity; if it is located outside, then the signs of the ICD will be opposite to this [42]. Also, it can be definitely affirmed, on the basis of several experimental approaches, that the non-polar portions of guest molecules will be inserted into the CyD cavity, even though few exceptions to this behaviour have been reported, ascribed to steric factors [43].

Examining the case of the rutin/β-CyD complex, the higher positive effect on the first CD band, peaking at 260 nm, apparently suggests that the phenyl chromophore (single ring of rutin) could be located inside the cavity, accordingly to what claimed by Haiyun et al. on the basis of NMR and molecular modelling results [24]. The observed positive CD bands in the presence of β-CyD indicate that the electric dipole moment, lying in parallel with the long axis of the chromophore, is parallel to the symmetry axis of the CyD cavity. Furthermore, the higher intensity of these CD signals suggests a smaller tilt angle (θ) between the long axis of the phenyl group of rutin and the symmetry axis of β-CyD, most likely caused by the steric repulsion between the –*ortho* hydroxyl groups and the hydrophobic inner surface of β-CyD. Hence, the bicyclical portion of the guest would be located outside the cavity. The lower intensity of the induced CD band for this chromophore could be attributed to the formation of strong hydrogen bonding between the carboxyl group of the cinnamoilic moiety and the hydroxyl group on the edge of β-CyD, resulting in a larger tilt angle (θ) against the symmetry axis of the cavity. The Cotton effect of free rutin, which is as well observed in the absence of β-CyD, can be explained by the rotation barrier of the moiety, which apparently is high enough to maintain the non-planarity of the molecule. The non-planar structure of the lower energy conformations must be responsible for the observed positive CD signal. The effect of β-CyD on the CD spectra of rutin was quantitatively investigated by holding the concentration of the guest constant ($2S_0$, equal to 1.1×10^{-4} M) and varying the host concentration between 0.0 and 9.0×10^{-3} M, as fully described in Section 2.3. As can be seen in Fig. 3, both the positive and negative CD peaks increased with the increasing β-CyD concentrations, up to 6.0×10^{-3} M, above which their intensities were nearly constant. Attempted fit of the ellipticity data for the negative ICD signal failed however, due to the low signal-to-noise ratio. On the other hand, from the ellipticity changes for the positive band (centred at 260 nm), plotted in a similar way as the UV absorption data, the rutin/β-CyD 1:1 binding constant could be estimated. Let us assume that θ_F is the saturated CD signal for which all the rutin concentration is in the complexed form, θ_I is the observed CD signal in the presence of a given amount of /β-CyD, and l the optical cell pathlength. The plot of complexed rutin concentration, $[R \cdot CyD] = (\Delta\theta_i - \Delta\theta_0)/([\theta_C]l)$, as a function of the total cyclodextrin concentration (the measured molar ellipticity
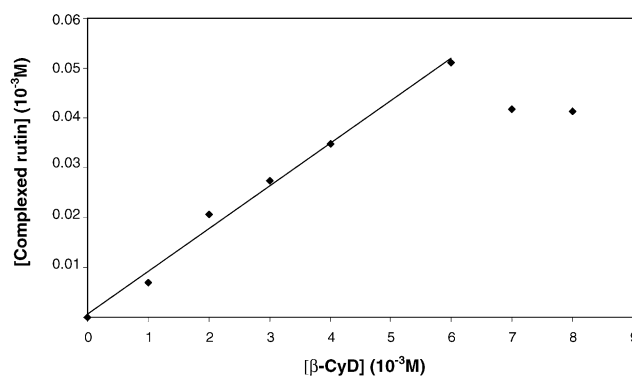

Fig. 4. CD determination of the amount of complexed rutin as a function of β-CyD concentration at $25 \pm 1\,^\circ$C. The ellipticity changes for the positive band (at 260 nm) were normalized for the measured molar ellipticity of the complex ($R^2 = 0.997$; slope 0.0086).

of complexed rutin is $[\theta_C] = (\Delta\theta_F - \Delta\theta_0)/S_0 \approx 715^\circ$ cm^{-1}) was a straight line (correlation coefficient, $R^2 = 0.997$, slope 0.0086, see Fig. 4). From the fit the binding constant for a 1:1 inclusion complex was obtained, $K = 153 \pm 20$ M^{-1} ($\log K = 2.18$), in good agreement with the results of the UV–vis spectroscopy studies.

3.3. ^{1}H NMR studies

The ^{1}H NMR signals of free β-CyD in D$_2$O are listed in Table 2. The free β-CyD resonance positions at $25 \pm 0.1\,^\circ$C, relative to the solvent line (residual HDO at 4.78 ppm) were: H$_1$ at 5.060 ppm (doublet), H$_2$ at 3.6386 ppm (doublet of doublets), H$_3$ at 3.95 ppm (triplet), H$_4$ at 3.575 ppm (triplet), H$_5$ at 3.838 ppm (doublet of triplets), with H$_6$ protons nearly overlapping (3.870 ppm). As expected, in D$_2$O solution the active H$_2$, H$_3$ and H$_6$ hydroxylic protons were not detected and only the resonances of the non-exchanging hydrogens attached to the carbons could be observed. The effects of rutin on the spectrum of β-CyD were investigated by dissolving the powdered complex in 0.5 ml deuterium water. As the host–guest system is in the NMR chemical shift fast-exchange limit, the free and complexed form of β-CyD gave only an NMR proton signal, due to the fast exchange between the free and bound β-CyD at equilibrium. That means, for the β-CyD protons, the measured resonance positions are the av-

Table 2
The ^{1}H NMR signals of free and complexed β-CyD in D$_2$O

Nucleus	δ (ppm)		$\Delta\delta$
	Free /β-CyD	Rutin/β-CyD	
H$_1$	5.060	5.044	0.016
H$_2$	3.638	3.623	0.015
H$_3$	3.956	3.926	0.030
H$_4$	3.575	3.559	0.019
H$_5$	3.838	3.807	0.031
H$_6$	3.870	3.846	0.024

Resonance positions at $25 \pm 0.1\,^\circ$C, relative to the solvent line (residual HDO at 4.78 ppm).

erage of the chemical shifts of the free β-CyD molecules and that of the complexed ones, weighted by the fractional population in each state. As it can be appreciated in Table 2, the lower field triplet assigned to the H_3 protons' resonance was shifted to higher field in the presence of the host molecules; an upfield shift was observed for the H_5 protons' resonance as well, which originally overlapped H_6 protons' signal.

On the contrary, no appreciable chemical shift was observed for the H_2, H_4 and H_6 protons. NMR studies of cyclodextrin complexes with aromatic guests, carried out by several researchers, have shown that H_3 and H_5 protons are located inside the β-CyD cavity, the former creating a ring near the large opening, the latter being near the smaller one. The H_6 protons are located on the upper surface and directed inwards into the cavity; all other protons are located on the exterior [44]. That gave further evidence for the inclusion complex formation between rutin and β-CyD, since only when the phenyl group of the molecule penetrates into the cavity, can the H_3 and H_5 protons be susceptible to the anisotropic shielding of the aromatic ring, while the exterior protons remain relatively unaffected. On the other hand, if the association would have taken place outside the cavity, the external H_1, H_2 and H_4 protons should have been the most affected. Also, the observed $\Delta\delta$ value for the H_3 protons is considered to be related to the stability of the complex formed, whereas that of the H_5 protons indicates the penetration depth of the aromatic moiety into the cavity. It can be therefore confirmed that the binding site for rutin is the single aromatic ring inserting from the primary edge of β-CyD.

3.4. Bathophenanthroline test

This test evaluates the presence of ferrous ion measuring the UV absorption of the Fe^{2+}–bathophenanthroline disulphonate complex. It allowed assessing the effects of rutin and its complex with β-CyD in removing iron from the reaction medium, either chelating the metal or enhancing the rate of Fe^{2+} auto-oxidation. Ferrous ion can participate in the generation of reactive oxygen species (ROS), its subtraction therefore inhibits the formation of these radical species thus protecting the biological substrate from oxidative damage. Fig. 5 shows that rutin increases the subtraction of Fe^{2+} at the concentration tested. It is also evident that rutin, when complexed with β-CyD, enhances Fe^{2+} auto-oxidation or chelation to a bigger extent with respect to free rutin. The iron chelation by rutin may be responsible of its antioxidant activity. The complex formed reduces the accessibility of iron to oxygen molecules and the consequent production of hydroxyl radicals.

3.5. Comet assay

The results obtained from the single cell gel electrophoresis of human leucocytes are shown in Fig. 6. Decreased damage is associated with lowered score and indicates a protective effect of the antioxidant treatment. Concentrations
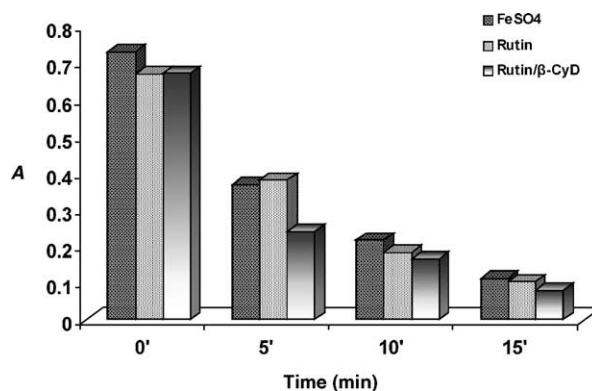

Fig. 5. Effect of 2.3 μM β-CyD and of 2.3 μM free and complexed rutin on Fe^{2+}–bathophenanthroline disulphonate complex. Values are the mean of three values in three different experiments.

were normalized for the rutin molarity (2.3 μM free rutin) in the BaP/cells reaction mixture. Treatment with 2.3 μM β-CyD did not induce damage per se. Significantly lower DNA damage in the stressed cells was observed with all the antioxidant compounds tested, however, complexation with the cyclodextrin improved the protective effect of rutin.

3.6. Protection from lipid peroxidation

To evaluate the antioxidant activity of the compounds against lipid peroxidation induced by $FeSO_4$ in rat liver microsomes, concentrations were normalized for rutin molarity (2.3 μM free rutin) in the peroxidant reaction mixture. Free rutin provided remarkable protection against the peroxidative damage ($P < 0.001$). Complexation with β-CyD improved the antioxidant activity of rutin, resulting more effective in reducing MDA production (data shown in Table 3). Moreover, cyclodextrins have been reported to complex ferrous ion [45], consequently we also searched for a direct antioxidant activity of non-complexed β-CyD; results indicate that 2.3 μM free cyclodextrin exerts a protective effect from lipid peroxidation. It seems reasonable to hypothesize that this effect can
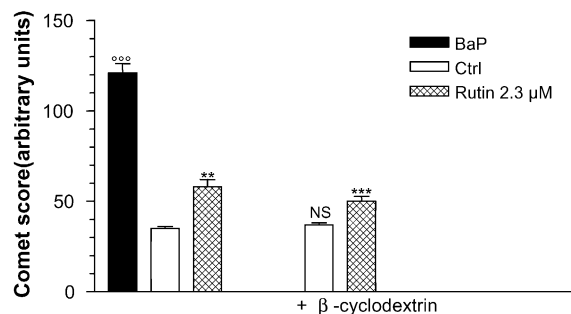

Fig. 6. Effect of 2.3 μM β-CyD on undamaged human leucocytes and of 2.3 μM free and complexed rutin on BaP-mediated DNA damage by comet assay. Each bar represents the mean of three experiments, each in triplicate, with 1 S.D. error bars shown. By means of Dunn's test, the comet score of the treated cells was compared to that of untreated but damaged cells ([**] $P < 0.01$; [***] $P < 0.001$); BaP damage and the effect of the uncomplexed β-CyD were compared to undamaged control cells ([°°°] $P < 0.001$; NS = not significant).

Table 3
Effect of 2.3 μM β-CyD and of 2.3 μM free and complexed rutin on FeSO$_4$/ascorbic acid-induced lipid peroxidation on rat hepatic microsomal fraction

Basal	BaP	β-CyD	Rutin 2.3 μM	Rutin/β-CyD
0.237 ± 0.027	27.120 ± 5.214	5.343 ± 0.890[**]	0.316 ± 0.086[***]	0.021 ± 0.001[°°]

Values represent the mean ± S.D. of MDA concentration in three experiments, each in triplicate. Basal: undamaged microsomes. The effect of the uncomplexed cyclodextrin and rutin was compared to a control ([**]$P < 0.01$; [***]$P < 0.001$); MDA levels in the samples treated with the rutin/β-cyclodextrin complex were compared to free rutin ([°°]$P < 0.01$).

be due to chelation of the inducer of peroxidative damage, Fe^{2+}, by the macrocycle.

4. Conclusions

Circular dichroism, ^{1}H NMR and UV–vis spectroscopy provided valuable information about the nature of rutin/β-cyclodextrin interactions in aqueous medium, which are of special interest for their potential relevance to a variety of biological phenomena, as well as for pharmaceutical applications. UV–vis and CD data derived from rutin solubility in the presence of various concentration of β-CyD showed that the aqueous solubility of the drug increased proportionally with an increase of interacting β-CyD; furthermore, the estimated binding constants for the rutin/β-cyclodextrin 1:1 inclusion complex revealed a good agreement between the two techniques. The inclusion process altered the spectral features of the molecules, as reflected by changes in NMR protons' resonances, UV–vis and CD spectra. UV–vis spectroscopy is one of the most widely used methods for studying inclusion complex formation, due to its high sensitivity and feasibility, though lacking in selectivity. CD spectroscopy is particularly suitable for probing conformational and configurational structures and dynamic changes, combining the sensibility of UV–vis spectroscopy with the advantage of greater specificity, since absorbances due to the presence of achiral molecules do not interfere, as long as they do not show a strong UV absorption in the wavelength range of the CD measurements. Solid rutin/β-CyD complexes were obtained successfully and assayed by different biological tests. Antioxidant protection against oxidative stress may decrease the rate of damage of biological macromolecules and hence help to prevent toxic injury, ageing and age-related diseases including cancer. Scavenging of free radicals and binding of iron are two important and well known mechanisms supporting the antioxidant properties shown by rutin. The inclusion complex formation with β-cyclodextrin further improved its antioxidant activity, most likely as a result of the increased solubility in the biological moiety.

References

[1] S. Gang, W. Wang, J. Wu, Radiat. Phys. Chem. 55 (1999) 285–291.

[2] S.R. Husain, J. Cillard, P. Cillard, Phytochemistry 8 (1987) 2489–2497.

[3] X.J. Zhang, J.L. Wu, Z.L. Cai, S.D. Yao, Z.H. Zuo, N.Y. Lin, Radiat. Res. 142 (1995) 227–235.

[4] J. Robak, R.J. Gryglewski, Biochem. Pharmacol. 37 (1988) 837–845.

[5] S.V. Jovanovic, S. Steeken, M. Tosic, B. Marjanovic, M.G. Simic, J. Am. Chem. Soc. 116 (1994) 4864–4876.

[6] W. Bors, W. Heller, C. Michel, M. Saran, Method in Enzyme, vol. 186, Academic Press, New York, 1990.

[7] J. Torel, J. Cillard, P. Cillard, Phytochemistry 45 (1986) 383–395.

[8] S. Chen, J. Gong, F. Liu, U. Mohammed, Immunology 100 (2000) 471–480.

[9] N. Ihme, H. Kiesewetter, K.H. Hoffman, A. Birk, A. Muller, K.I. Grutzner, Eur. J. Clin. Pharmacol. 50 (1996) 443–447.

[10] E.E. Deschner, J. Ruperto, J. Wong, H.L. Newmark, Carcinogenesis 12 (1991) 1193–1204.

[11] W. Panasiak, M. Wleklik, A. Oraczewska, M. Luczak, Acta Microbiol. Pol. 38 (1989) 185–199.

[12] S.Y. Park, S.H. Bok, S.M. Jeon, Y.B. Park, S.J. Lee, T.S. Jeong, M.S. Choi, Nutr. Res. 22 (2002) 283–295.

[13] K.H. Janbaz, S.A. Saeed, A.H. Gilani, Fitoterapia 73 (2002) 557–563.

[14] R. Mata, A. Rojas, L. Acevedo, S. Estrada, F. Calzada, I. Rojas, Planta Med. 63 (1997) 31–40.

[15] R.P. Webster, M.D. Gawde, R.K. Bhattacharya, Cancer Lett. 109 (1996) 185–191.

[16] W.F. Hodnick, F.S. Kung, W.J. Roettger, C.W. Bohmont, R.S. Pardini, Biochem. Pharmacol. 35 (1986) 2345–2357.

[17] T. Loftsson, M.E. Brewster, J. Pharm. Sci. 85 (1996) 1017–1025.

[18] M.L. Calabrò, S. Tommasini, P. Donato, D. Raneri, R. Stancanelli, P. Ficarra, R. Ficarra, C. Costa, S. Catania, C. Rustichelli, G. Gamberini, J. Pharm. Biomed. Anal. 35/2 (2004) 365–377.

[19] S. Scalia, A. Casolari, A. Iaconinoto, S. Simeoni, J. Pharm. Biomed. Anal. 30 (2002) 1181–1189.

[20] K.L. Park, K.H. Kim, S.H. Jung, H.M. Lim, C.H. Hong, J.S. Kang, J. Pharm. Biomed. Anal. 27 (2002) 569–576.

[21] J.M. Moreno-Cerezo, M. Cíaz, D. Cíaz, M. Córdoba-Borrego, J. Pharm. Biomed. Anal. 26 (2001) 417–426.

[22] M. Lederer, E. Leipzig-Pagani, Anal. Chim. Acta 329 (1996) 311–314.

[23] S. Letellier, B. Maupas, J.P. Gramond, F. Guyon, P. Gareil, Anal. Chim. Acta 315 (1995) 357–366.

[24] D. Haiyun, C. Jianbin, Z. Guomei, S. Shaomin, P. Jinhao, Spectrochim. Acta A 59 (2003) 3421–3429.

[25] K.A. Connors, Chem. Rev. 97 (1997) 1325–1357.

[26] J.M. Bosque-Sendra, E. Almansa-Lopez, A.M. Garcìa-Campana, L. Cuadros-Rodrìguez, Anal. Sci. 19 (2003) 1431–1439.

[27] H. Basan, N.G. Göger, N. Ertas, M.T. Orbey, J. Pharm. Biomed. Anal. 26 (2001) 171–178.

[28] G. Granero, C. Garnero, M. Longhi, J. Pharm. Biomed. Anal. 29 (2002) 51–59.

[29] M. Blanco, J. Coello, H. Iturriaga, S. Maspoch, C. Pèrez-Maseda, Anal. Chim. Acta 407 (2000) 233–245.

[30] J.E. Forman, R.E. Barrans, D.A. Dougherty, J. Am. Chem. Soc. 117 (1995) 9213–9228.

[31] M. Yoshino, K. Murakami, Anal. Biochem. 257 (1988) 40–44.

[32] N.P. Singh, M.T. Mc Coy, R.R. Tice, E.L. Schneider, Exp. Cell. Res. 175 (1988) 184–191.

[33] K. Husain, S.M. Somani, J. Appl. Toxicol. 17 (1997) 189–194.

[34] M.M. Bradford, Anal. Biochem. 72 (1976) 248–254.

[35] M.P. Germanò, R. De Pasquale, V. D'Angelo, C. Catania, V. Silvari, C. Costa, J. Agric. Food Chem. 50 (2002) 1168–1171.

[36] T.J. Mabry, K.R. Markham, M.B. Thomas (Eds.), The Systematic Identification of Flavonoids, Springer-Verlag, New York, 1970.

[37] T. Higuchi, K.A. Connors, Adv. Anal. Chem. Instr. 4 (1965) 117–212.

[38] H.A. Archontaki, M.V. Vertzoni, M.H. Athanassiou-Malaki, J. Pharm. Biomed. Anal. 28 (2002) 761–769.

[39] G. Pistolis, A. Malliaris, Chem. Phys. Lett. 303 (1999) 334–346.

[40] Y.A. Zhdanov, Y.E. Alekseev, E.V. Kompantseva, E.N. Vergeichik, Russ. Chem. Revs. 61 (1992) 563.

[41] K. Harata, H. Uedaria, Bull. Chem. Soc. Jpn. 48 (1975) 375–384.

[42] M. Kodaka, T. Fukaya, Bull. Chem. Soc. Jpn. 62 (1989) 1154–1159.

[43] G.M. Bonora, R. Fornasier, P. Scrimin, U. Tonellato, Carbohydr. Res. 223 (1992) 71–90.

[44] D.J. Wood, F.E. Hruska, W.J. Saenger, J. Am. Chem. Soc. 99 (1977) 1735–1740.

[45] M.E. Lindsey, G. Xu, J. Lu, M.A. Tarr, Sci. Total Environ. 307 (2003) 215–229.