Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Pharmaceutical and Biomedical Analysis
35 (2004) 365–377
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

# Effects of α- and β-cyclodextrin complexation on the physico-chemical properties and antioxidant activity of some 3-hydroxyflavones

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

[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] *Dipartimento di Scienze Farmaceutiche, Università di Modena e Reggio Emilia, Via Campi 183, 41100 Modena (MO), Italy*

Received 4 August 2003; received in revised form 3 December 2003; accepted 3 December 2003

**Abstract**

Inclusion complexes of some flavonols (3-hydroxyflavone, morin and quercetin) have been obtained with α- and β-cyclodextrins, by the co-evaporation method. Different analytical techniques (DSC, XRPD, FT-IR, $^1$H-NMR, UV-Vis) have been employed for a throughout investigation of the structural characteristics of such supramolecular aggregates, which exhibited distinct spectroscopic features and properties from both "guest" and "host" molecules. The stoichiometric ratios and stability constants describing the extent of formation of the complexes have been determined by phase-solubility studies; in all cases type-$A_L$ diagrams have been obtained (soluble 1:1 complexes). The effect of molecular encapsulation on the flavonols antioxidant activity has been afterwards evaluated, by means of different biological assays (Bathophenanthroline test; Comet assay; Lipid peroxidation). Complexation with cyclodextrins further improved the antioxidant activity, increasing drugs solubility in the biological moiety.


*Keywords:* Flavonols; α- and β-Cyclodextrins; DSC; XRPD; FT-IR; $^1$H-NMR; UV-Vis; Phase-solubility; Bathophenanthroline test; Comet assay; Lipid peroxidation

## 1. Introduction

Flavonoids have recently attracted a great interest as potential therapeutic agents against a large variety of diseases, most of which involve radical damage. These polyphenolic compounds, ubiquitous in higher plants, are also frequent components of major dietary constituents.

The biological and medicinal properties of flavonoids have been reviewed extensively, so there is a wealth of data on their activity as reducing agents, hydrogen-donating antioxidants and singlet oxygen quenchers; in some cases metal chelating properties have been proposed [1–7]. The chemical features of

* Corresponding author. Tel.: +39-090-6766407; fax: +39-090-355613.
*E-mail address:* stommasi@pharma.unime.it (S. Tommasini).

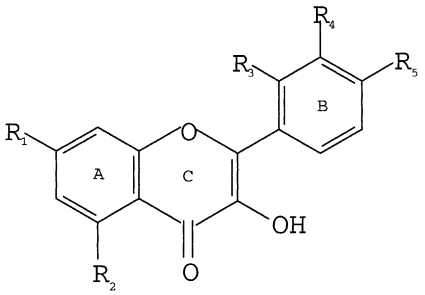
0731-7085/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2003.12.005

flavonoids are predictive of their antioxidant activity, in the case of flavonols, possessing a free 3-OH group and a 2,3-double bond in conjunction with a 4-oxo function in the C ring, phenoxy-radicals produced are stabilised by resonance effects. Quercetin, in particular, satisfies all the proposed criteria for effective radical scavenging, since the *o*-dihydroxy-structure in the B ring confers higher stability to the radical form and participates in electronic delocalisation [8–13]. Anti-tumour promoting activity has also been reported for morin and quercetin, thus, proving useful as chemopreventive agents in human carcinogenesis [14–18].

The therapeutic usefulness of these potential benefits is, however, limited by the unfavourable physicochemical properties of these compounds, especially their very poor water solubility. A higher hydrosolubility could lead to a better bioavailability and furthermore, biological assays could be more easily performed.

On the other hand, pharmaceutical applications of cyclodextrins (CyDs) as additive and drug-complexing agents have been growing rapidly, as reflected in the increasing number of medicinal products being placed on the market as CyD-based formulations. CyDs have a doughnut-shaped hydrophobic cavity in which various types of "guest" molecules may be clathrated, either in the solid phase or in aqueous solution [19–23]. Encapsulation of a drug molecule will advantageously affect many of its physico-chemical properties, thus leading to enhanced solubility, dissolution rate, membrane permeability and bioavailability of slightly soluble drugs, including some flavanones [24–29]. Furthermore, there is generally an improvement in their stability to air and light [30–32]. By taking advantage of CyD-complexation, many attempts have also been made to reduce the untoward side effects associated with drugs [33,34]. Finally, several analytical and spectroscopic techniques benefit from CyD-inclusion of the analytes and the associated enhanced selectivity and sensitivity [35–37]. Different methods are available for the synthesis of CyD-guest complexes, depending on the physico-chemical properties of the "guest" molecules: co-precipitation, lyofilisation or freeze-drying, kneading, neutralisation, grinding or co-pulverizing [38].

The present work has involved the synthesis, isolation and characterisation of crystalline inclusion complexes of some flavonols, i.e. 3-hydroxyflavone (3-OH–F), morin and quercetin (Fig. 1) with α- and β-CyDs. A careful evaluation of all the factors involved in the different complexation methods led to a set-up the optimal conditions, by taking into account some unfavourable characteristics of the "guest" molecules, mainly their low solubility in many solvents. The combined use of different characterization techniques, according to the physical state considered, gave by far the best results in terms of reliability of the models. The stoichiometric ratios and stability constants describing the extent of formation of the complexes were obtained by phase-solubility studies. The effect of CyD-complexation on the flavonols antioxidant activity was afterwards evaluated, by means of different in vitro tests.




| | |
|---|---|
| $R_1, R_2, R_3, R_4, R_5$= H | 3-Hydroxyflavone |
| $R_1, R_2, R_3, R_5$= OH; $R_4$= H | Morin |
| $R_1, R_2, R_4, R_5$= OH; $R_3$= H | Quercetin |

Fig. 1. Chemical structures of the flavonoids under study.

## 2. Experimental

### 2.1. Chemicals

The following reagents and solvents were used. 3-hydroxyflavone ($C_{15}H_{10}O_3$, MW 238.2), morin ($C_{15}H_{10}O_7 \cdot 2H_2O$, MW 338.27) and quercetin ($C_{15}H_{10}O_7 \cdot 2H_2O$, MW 338.27) from Extrasynthese (Genay, France). α-Cyclodextrin (α-CyD, $C_{36}H_{60}O_{30}$, MW 972.86) and β-cyclodextrin (β-CyD, $C_{42}H_{70}O_{35}$, MW 1135.00), both HPLC grade, from Fluka Chemie (Switzerland). All the above materials were employed without any further purification. Water was distilled prior to use, then filtered through 0.22 μm Millipore filters (Bedford, USA). Potassium bromide and

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 of analytical reagent grade.

### 2.2. Phase-solubility studies

Phase-solubility studies were performed with a Haake C25 thermostated bath, equipped with a Haake F6 controller which allowed an accuracy of 0.01 °C.

Excess amounts of the flavonols were added to 10 ml tubes containing unbuffered aqueous solutions of α- (0.01–0.14 M) and β-CyD (0.001–0.014 M). Flasks were sealed to avoid changes due to evaporation and the solutions were magnetically stirred for 72 h in a thermostated bath at 25 °C. After the equilibrium was reached, suspensions were filtered by Acrodisc® LC PVDF 0.45 μm filters (Gellman Science). An aliquot from each vial was adequately diluted and assayed spectrophotometrically to evaluate the amount of each flavonoid dissolved. Experiments were carried out in triplicate. Processing of the data was done by the Statistica® software (Statsoft).

### 2.3. Preparation of the solid complexes

The inclusion compounds of the three flavonols (3-hydroxyflavone, morin and quercetin) with α- and β-CyDs were prepared by three different methods: kneading, co-evaporation, thin layer.

Kneaded products were obtained by wetting in a mortar physical mixtures of the substances (in a molar ratio 1:1), with a minimum amount of a 1:1 (v/v) methanol–water mixture. Mixtures were grounded thoroughly with a pestle until a homogeneous paste was obtained, which was then dried under vacuum up to constant weight (room temperature).

Co-evaporated products were obtained from organo-aqueous solutions (1:2, v/v) containing equimolecular amounts of the two partners. Only water-miscible organic solvents were employed (acetone and methanol), able to dissolve the flavonoids and give a clear solution when added to the aqueous solution of cyclodextrin. Both concentrations of the two solutions were adjusted in order to avoid the extemporaneous precipitation of any of the components at the time of mixing. Stirring was carried out for at least 72 h, under controlled temperature (25 °C) and shielded from light to prevent any degradation of the flavonoid molecule. After this time, solutions were cooled to facilitate the precipitation of the inclusion compounds, as it is known from literature that complexation is an exothermic reaction. Since spontaneous precipitation of the complexes did not occur, the following procedure was established for their recovering. The organic solvent was evaporated in a rotary vacuum evaporator, hence solutions were filtered to eliminate the free flavonoid which had not reacted. Water was at last evaporated under vacuum (30 °C), providing the solid complexes still containing an excess amount of the free cyclodextrins.

The last procedure consisted in a variant of the co-evaporation method above described. According to this procedure, the flavonoids were crystallised from a suitable organic solvent (acetone or methanol) in such a way as to obtain a thin layer of the substance all around the bottom of a flask and an aqueous solution containing an equimolecular amount of CyD was then added. The following steps consisted in stir, filtration and finally evaporation of the water, under the same conditions as the previous method.

### 2.4. Differential scanning calorimetry (DSC)

The DSC curves were obtained using a Perkin-Elmer differential scanning calorimeter model DSC-4, calibrated with indium (99.99% purity, m.p. 156.6 °C), at heating rates of 10 °C/min. One to five milligram samples were heated in aluminium crimped pans under nitrogen gas flow, in the 40–350 °C range and measurements were made in duplicate. Software used: Thermal Analysis Data Station (TADS).

### 2.5. X-ray powder diffractometry (XRPD)

Powder X-ray diffraction patterns were taken with a Philips PW 1050/25 X-ray diffractometer, by using a Ni-filtered Cu Kα radiation ($\lambda = 1.5418$ Å), in the $2^\circ \leq 2\theta \geq 50^\circ$ range, at 25 °C (target, Cu; voltage, 40 kV; current, 20 mA; time constant, 4 s; angular speed, 1°/min; slit, −0.1° and −1°). Samples were finely ground in an agate mortar and measurements were made in duplicate.

### 2.6. Fourier transform-infrared spectroscopy (FT-IR)

The FT-IR spectra acquired by Diffuse Reflectance Infrared Fourier Transform (DRIFT) were taken from potassium bromide mixtures of the samples, obtained by gently mixing in an agate mortar. A FT-IR Perkin-Elmer 1600 spectrophotometer was used for the analysis, in the frequency range between 4000 and 450 $cm^{-1}$.

### 2.7. Proton magnetic resonance ($^1H$-NMR)

Proton NMR spectra were recorded at 25 °C on a Varian Gemini 300 MHz spectrophotometer, from saturated solutions of the analytes (pure CyDs and solid complexes with flavonols in a molar ratio 1:1) dissolved in $D_2O$. The chemical shifts are referred to $D_2O$ signal at 4.8 ppm at 25 °C.

### 2.8. UV-visible spectroscopy (UV-Vis)

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). Quartz cells with a 10 mm pathlenght (Hellma) were employed in the 220–400 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 each flavonoid included in the CyD-complexes, investigated in biological assays. The drug concentration was calculated by interpolation of calibration curves previously obtained in $H_2O$–EtOH (1:1, v/v) solutions of the free substances.

### 2.9. Biological activity tests

#### 2.9.1. Bathophenanthroline test

The Bathophenanthroline test was performed according to Yoshino and Murakami [39], 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 the flavonols (2.3 μM final concentration). Stock solution of $FeSO_4$ was prepared daily. All the mixtures were incubated at 37 °C in spectroscopic cells; 500 μl of 1 mM bathophenanthroline disulfonate were added at different intervals and the absorbance at 540 nm was measured.

#### 2.9.2. Comet assay

The single cell gel electrophoresis was performed as described by Singh et al. [40] on human leucocytes from peripheral venous blood. Freshly collected whole blood was incubated for 1 h at 37 °C with 10 μM benzo[a]pyrene (BaP) to induce DNA damage. Frosted microscope slides (prepared in triplicate per sample per experiment) were covered with 140 μl of 0.75% regular melting 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 × 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 damaged cells appeared as comets. Final score was calculated as follows: Score = ($n$ cells scored 1)+(2$n$ cells scored 2)+(3$n$ cells scored 3) + (4$n$ cells scored 4).

#### 2.9.3. 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 [41]. Microsomal fraction was obtained as previously described [42] and total protein content was measured according to the method by Bradford [43].

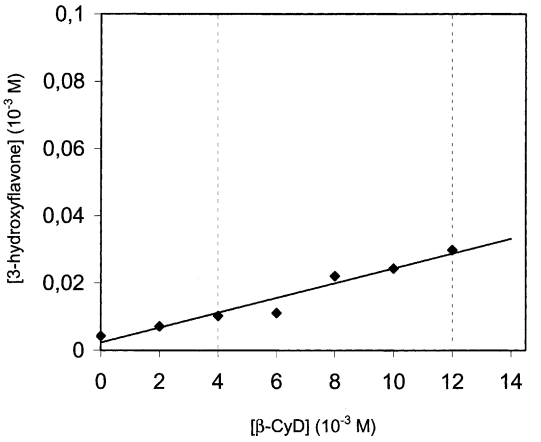



Fig. 2. Solubility of 3-OH–F as a function of β-CyD concentration in water at 25 °C. Each data point is the mean of three measurements.

Heat-inactivated microsomes (0.5 mg/ml proteins) were incubated for 1 h at 37 °C with 100 μM ascorbic acid, 10 μM $FeSO_4$ and flavonols (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 test sample.

### 2.10. Statistical analysis

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

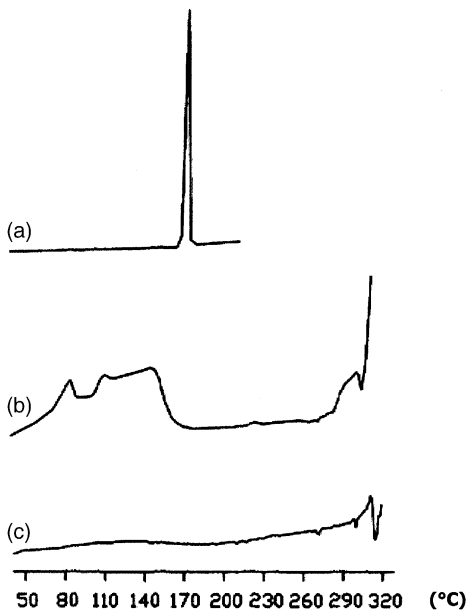



Fig. 3. DSC thermal curves: 3-OH–F (a); α-CyD (b); corresponding inclusion complex (c).

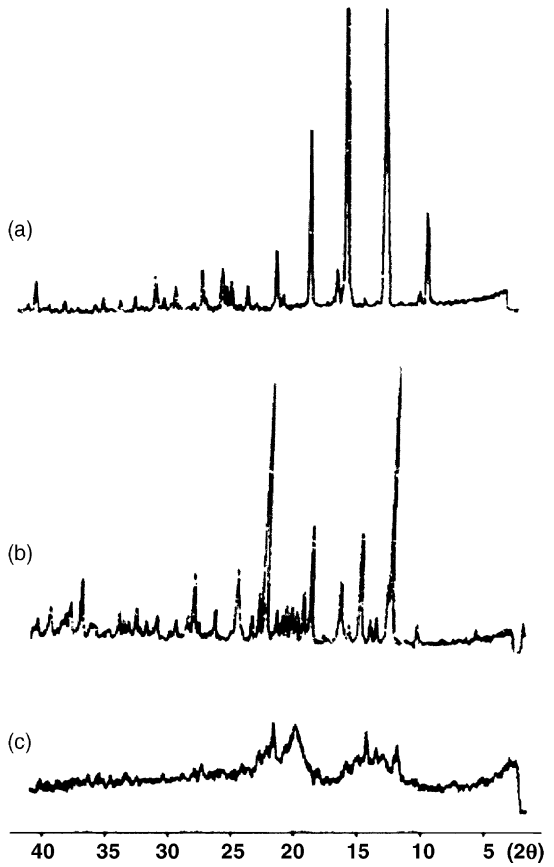



Fig. 4. X-ray diffraction profiles: 3-OH–F (a); α-CyD (b); corresponding inclusion complex (c).

## 3. Results and discussion

### 3.1. Phase-solubility studies

The stoichiometric ratios and stability constants were derived from the changes in the solubility of the substrates in the presence of increasing amounts of CyDs, measured by UV absorbance. For all the drug/CyD systems, type-$A_L$ diagrams were obtained, meaning that the solubility of the flavonoids is apparently increased by the presence of the macrocycle. Assuming a 1:1 stoichiometry of the complexes, stability constants, $K_c$, were calculated from the straight-line portion of the phase-solubility diagram [44], according to the following equation:

$$K_c = \frac{\text{slope}}{\text{intercept}\,(1 - \text{slope})} \quad (1)$$

Table 1
XRPD patterns for 3-OH–F/α-CyD system

| α-CyD | | | 3-OH–F | | | Inclusion complex 3-OH–F/α-CyD | | |
|---|---|---|---|---|---|---|---|---|
| °2Θ | *d* (Å) | $I/I_0$ | °2Θ | *d* (Å) | $I/I_0$ | °2Θ | *d* (Å) | $I/I_0$ |
| 5.2 | 16.9 | 3 | | | | | | |
| | | | 9.1 | 9.71 | 18 | | | |
| 9.9 | 8.93 | 7 | 9.7 | 9.11 | 2 | | | |
| 12.0 | 7.37 | 100 | | | | 11.9 | 7.43 | 77 |
| 12.3 | 7.19 | 21 | 12.3 | 7.19 | 90 | 12.1 | 7.31 | 49 |
| 13.0 | 6.80 | 9 | | | | | | |
| 13.6 | 6.50 | 9 | | | | 13.5 | 6.55 | 43 |
| 14.4 | 6.14 | 40 | | | | 14.3 | 6.19 | 86 |
| 15.2 | 5.82 | 6 | 15.4 | 5.75 | 100 | | | |
| 15.9 | 5.57 | 22 | | | | 15.8 | 5.60 | 40 |
| | | | 17.2 | 5.15 | | | | |
| 18.2 | 4.87 | 42 | 18.3 | 4.84 | 34 | | | |
| 18.7 | 4.74 | 18 | | | | | | |
| 19.2 | 4.64 | 10 | | | | | | |
| 20.0 | 4.43 | 12 | | | | 20.0 | 4.43 | 100 |
| 20.4 | 4.35 | 9 | 20.4 | 4.35 | 3 | | | |
| 20.8 | 4.27 | 11 | 20.9 | 4.24 | 12 | | | |
| 21.7 | 4.09 | 94 | | | | 21.7 | 4.09 | 92 |
| 22.2 | 4.00 | 17 | | | | 22.2 | 4.00 | 43 |
| 22.7 | 3.91 | 9 | 23.2 | 3.83 | 4 | 22.8 | 3.90 | 43 |
| 24.0 | 3.70 | 26 | 24.5 | 3.63 | 6 | | | |
| | | | 24.9 | 3.57 | 4 | | | |
| 25.7 | 3.45 | 11 | 25.2 | 3.53 | 8 | | | |
| 26.9 | 3.31 | 5 | 26.8 | 3.32 | 8 | | | |
| 27.4 | 3.25 | 22 | | | | 27.4 | 3.25 | 37 |
| 27.8 | 3.20 | 8 | 28.0 | 3.18 | 5 | | | |
| 28.7 | 3.10 | 7 | | | | | | |
| 29.3 | 3.04 | 3 | 29.8 | 2.99 | 2 | | | |
| 30.2 | 2.96 | 9 | 30.5 | 2.93 | 7 | | | |
| 31.9 | 2.80 | 15 | | | | | | |
| 32.6 | 2.74 | 10 | 32.2 | 2.78 | 3 | | | |
| 32.9 | 2.72 | 10 | | | | | | |
| 33.2 | 2.69 | 13 | 33.3 | 2.64 | 2 | | | |
| 35.5 | 2.53 | 5 | 34.8 | 2.57 | 2 | | | |
| 36.3 | 2.47 | 21 | | | | | | |
| 37.1 | 2.42 | 12 | | | | | | |
| 37.4 | 2.40 | 8 | 37.8 | 2.38 | 2 | | | |
| 38.6 | 2.33 | 12 | | | | | | |
| | | | 40.0 | 2.25 | 6 | | | |

Each data point is the average of three determinations. Fig. 2 shows the phase-solubility diagram obtained for 3-hydroxyflavone with b-CyD as an example ($K_c$ 958.63 $M^{-1}$). Stability constant calculated for β-CyD complex was greater than the α-CyD one ($K_c$ 16.12 $M^{-1}$) and a similar behaviour was observed for morin (α-CyD-system: $K_c$ 49.31 $M^{-1}$; β-CyD-system: $K_c$ 330.95 $M^{-1}$) and quercetin (α-CyD-system: $K_c$ 19.17 $M^{-1}$; β-CyD-system: $K_c$ 128.69 $M^{-1}$).

### 3.2. Solid complexes

The selection of the method of preparation of the inclusion compounds was highly dependent on the solubility of the flavonoids and CyDs in a common solvent, which implied the formation of a homogeneous system and therefore a more intimate contact between the interacting molecules. Since the flavonols are not water-soluble, an organic solvent had to be employed. In solution, the relative hydrophobicity of the CyD cavity implies that the substitution of internal water molecules with less polar molecules is thermodynamically favoured. Among the water-miscible solvents considered, methanol was preferred to acetone because, being more polar, it has a smaller affinity both for the drug and for the lipophilic microenvironment of the CyDs. For these reasons, it is less competitive for inclusion into the cavity, and a better complexation yield could be accomplished.

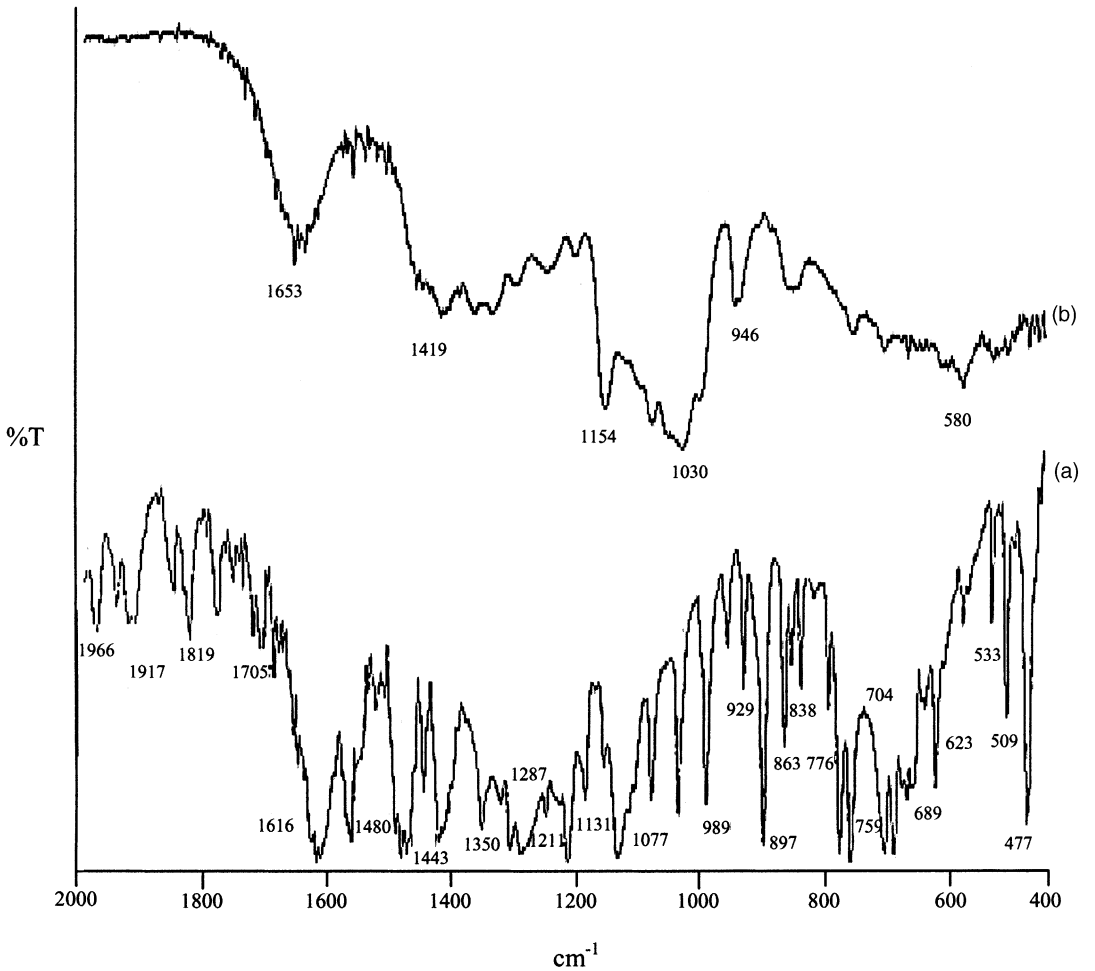



Fig. 5. FT-IR spectra: 3-OH–F (a); inclusion complex with β-CyD (b). Acquired by DRIFT.

### 3.3. DSC

DSC was employed to check any variation of crystalline properties of the flavonoids due to the interactions with the CyDs. In the DSC curves of the complexes, the absence of the melting endothermic peaks of the flavonoids (i.e. ∼170 °C for 3-hydroxyflavone; ∼130 and 290 °C for morin; ∼140 and 320 °C for quercetin) could be noticed and only a reduced peak around 100 °C was evident, as a result of displacement of bound water in the cavity by the guest (crystallization water). In the case of morin and quercetin, however, things were somewhat complicated since the peaks arising from fusion and loss of water were partially hindered by the CyDs typical transitions. This result definitely proves a change in crystallinity of the drug, presumably due to an inclusion process. An example is shown in Fig. 3.

### 3.4. XRPD

X-ray powder diffraction patterns confirmed the results of DSC analysis: the diffraction peaks relevant to crystalline flavonoids were no longer detectable in all the CyD-systems, and the reduction of the degree of crystallinity of the drugs could be taken as an indication of complexation. A reduced number of signals were noticeable in the complexes, of remarkably lowered intensity, thus meaning a greater amorphousness of the inclusion compounds, compared to the free molecules (see Fig. 4 for an example). In more detail, the differences in interplanar distances, diffraction angles and relative diffraction peak intensities borne out the formation of different crystalline structures (data reported in Table 1).

### 3.5. FT-IR

FT-IR spectroscopy allowed the analysis of changes in the spectral features of the "guest" molecules. Diffuse reflectance is a very useful technique in the analysis of powders, or whenever the roughness of the analyte surface is not negligible for the incident radiation wavelength. As for the FT-IR spectra of the inclusion compounds, the existence of new species with different spectroscopic features is confirmed by the disappearance of the characteristic bands of the free flavonoids, associated with aromatic bending and stretching (around 1100 and 1600 $cm^{-1}$), –OH phenolic bending (above 1200 $cm^{-1}$). All other flavonols characteristic vibrations showed at least a reduced intensity: 3-hydroxyflavone's (1211; 989; 897; 863; 776; 759 $cm^{-1}$); morin's (1246; 1173; 974; 828; 637 $cm^{-1}$); quercetin's (1170; 1132; 1092; 1015; 864 $cm^{-1}$). This proved not only an interaction but a real inclusion, since in the case of a physical mixture

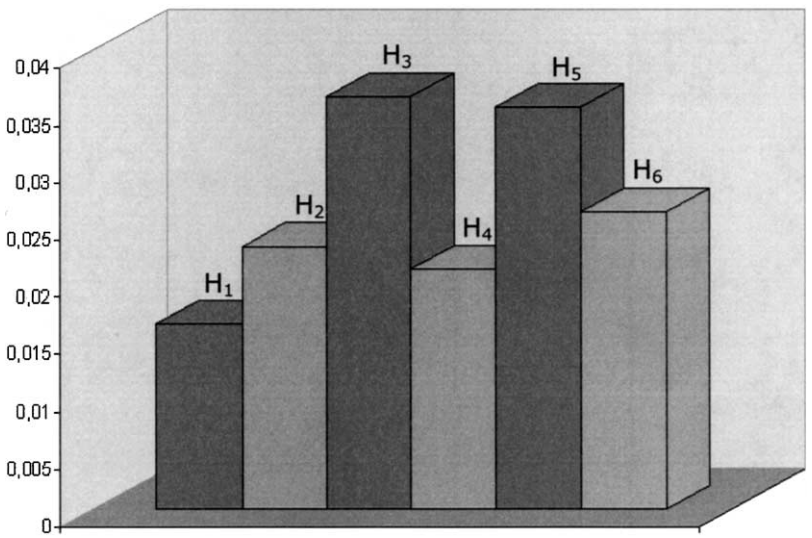



Fig. 6. Magnitude of the *up-field* shift observed for the β-CyD protons after interaction with 3-OH–F. $^1$H-NMR spectra recorded in $D_2O$; signals referred to residual HDO.

only a superimposition of the signals would have been observed. An example is reported in Fig. 5.

### 3.6. $^1$H-NMR

Proton magnetic resonance was very useful for clarifying at a molecular level the nature of drug/CyD interaction, though introducing two main difficulties: firstly, the use of any reference standard was not advisable, since the organic molecule proved to compete for inclusion in the CyD cavity, hence in some cases spoiling the complexes. The HDO residual signal itself was therefore used as an internal standard. Moreover, signals of the flavonoids were hardly detectable in the spectra of the complexes, due to their very poor water-solubility and to the presence of a great amount of CyD, either complexed and free (in the latter case as an impurity). The absence of new peaks arising from the pure complexes indicated that, on the NMR time scale, the inclusion process is a fast exchange regime. Observation of the $^1$H-NMR spectra of the inclusion compounds showed that a number of signals relevant to CyDs moiety experienced shift, $H_3$ and $H_5$ located inside the torus being by far the most affected, while

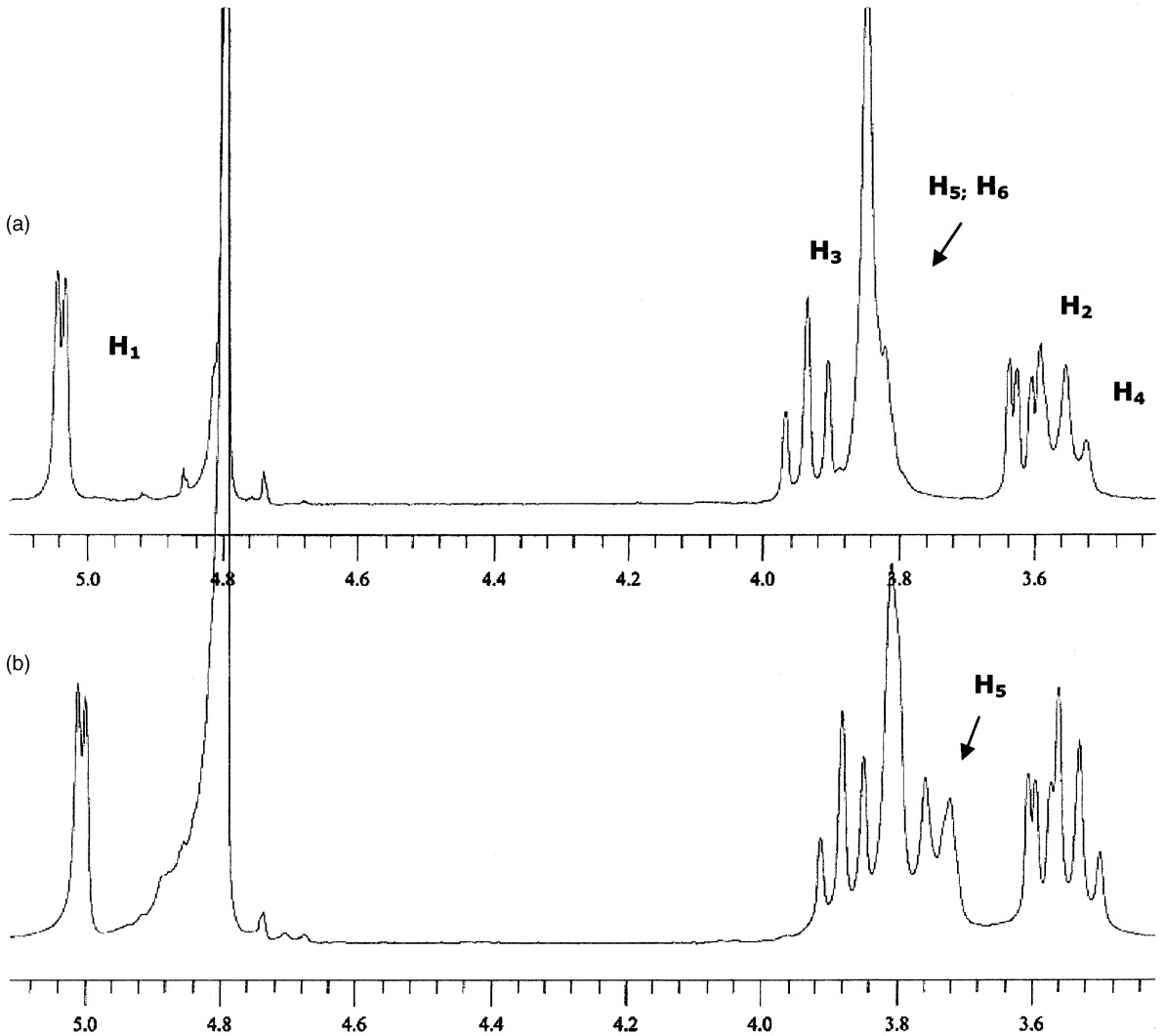



Fig. 7. $^1$H-NMR spectra: free β-CyD (a); inclusion complex with 3-OH–F (b). Spectra recorded in $D_2O$; signals referred to residual HDO.

the external protons $H_1$, $H_2$ and $H_4$ were shifted much less by the inclusion process. The larger *up-field* shift observed for the internal protons was undoubtedly due to the ring current effects generated by the circulating π electrons of the aromatic "guest" (that is magnetic anisotropy), clearly proving the reality of the inclusion. $H_6$ proton, located on the cavity rim, was also shifted, but to a smaller extent. Proton spectrum of β-CyD, performed both in the presence and in the absence of the flavonoid "guest", showed that $H_5$ signal is not detectable in the free CyD, since hindered by $H_6$, whereas it becomes evident in the complex, as a consequence of the up-field shift (Figs. 6 and 7 show data obtained for 3-hydroxyflavone/β-CyD system as an example).

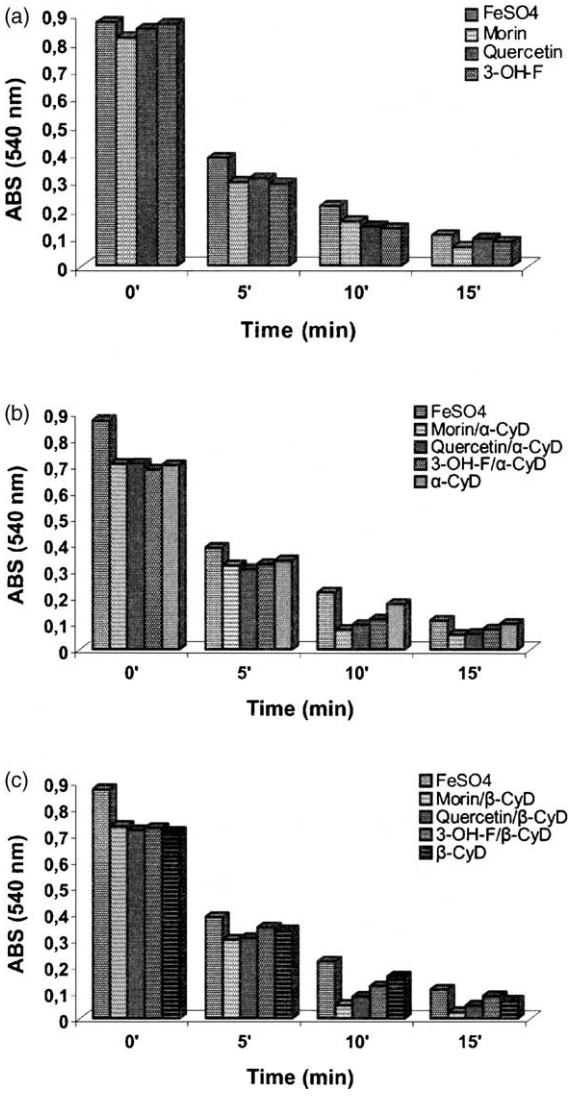



Fig. 8. (a–c) Effect of 2.3 μM flavonols, free (a), complexed with α-CyD (b) and β-CyD (c), on the autooxidation of ferrous ion with bathophenanthroline disulfonate.

### 3.7. Bathophenanthroline test

This test evaluates the presence of ferrous ion measuring the UV absorption of the $Fe^{2+}$-bathophenanthroline disulfonate complex. It allowed to assess the effects of flavonols and their complexes with α- and β-cyclodextrin in removing the iron from reaction medium, either chelating the metal or enhancing the rate of $Fe^{2+}$ autooxidation. Iron can participate in the generation of reactive oxygen species (ROS) and its substraction inhibits the formation of ROS, thus protecting the biological substrate from oxidative damage.

Fig. 8a shows that all the flavonols increase the substraction of $Fe^{2+}$ at the concentration tested. Fig. 8b and c show that the flavonols complexed with α- and β-CyD enhance $Fe^{2+}$ autoxidation or chelation to a bigger extent in respect to the flavonols not complexed. The effect is better showed with β-CyD; in particular morin enhances the $Fe^{2+}$ autooxidation markedly when complexed with β-CyD.

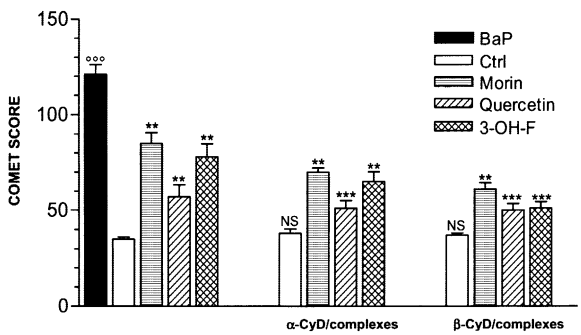



Fig. 9. Effect of 2.3 μM α- and β-CyD on undamaged human leucocytes and of 2.3 μM free and complexed flavonols on BaP-mediated DNA damage by comet assay. Each bar represents the mean of three experiments, each in triplicate, with 1 SD error bars shown. By means of Dunn's-test, comet score of treated cells was compared to that of untreated but damaged cells (** $P < 0.01$; *** $P < 0.001$); BaP damage and the effect of uncomplexed CyDs were compared to undamaged control cells (°°° $P < 0.001$; NS: not significant).

The iron chelation by flavonols may be responsible for their antioxidant activity. The complexes formed reduce the accessibility of iron to oxygen molecules and the consequent production of hydroxyl radicals.

### 3.8. Comet assay

The results obtained from the single cell gel electrophoresis of human leucocytes are shown in Fig. 9. Decreased damage is associated with decreased score and indicates a protective effect of antioxidant treatment. Concentrations were normalized for flavonoid molarity (2.3 μM free flavonoid) in the BaP/cells reaction mixture. Significantly less DNA damage in stressed cells was observed with all the antioxidant compounds tested, with quercetin giving the lowest comet scores. Treatment with 2.3 μM α- and β-CyD did not induce damage *per se*. Complexation with both cyclodextrins improved the protective effect of the three flavonols, but lower scores resulted from the treatment of cells with β-complexes.

### 3.9. 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 flavonoid molarity (2.3 μM free flavonoid) in the peroxidant reaction mixture. All the free flavonols provided remarkable protection against the peroxidative damage ($P < 0.001$), with quercetin giving the lowest MDA production. Complexation with both

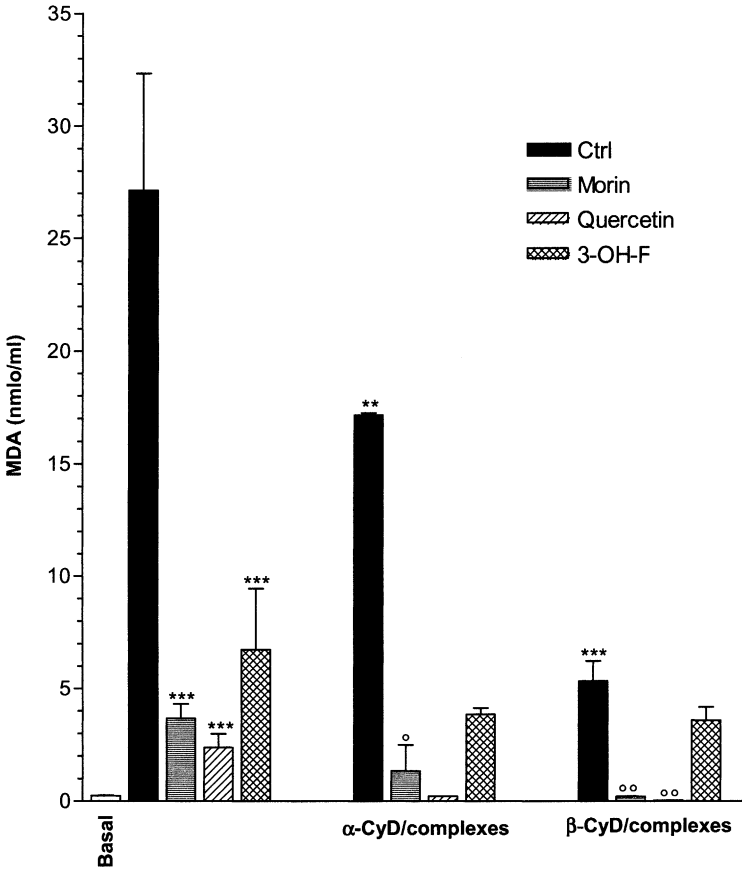



Fig. 10. Effect of 2.3 μM α- and β-CyD and of 2.3 μM free and complexed flavonols on $FeSO_4$/ascorbic acid-induced lipid peroxidation on rat microsomal fraction. Each bar represents the mean ± S.D. of MDA production in three experiments, each in triplicate. Basal = undamaged microsomes. The effect of uncomplexed CyDs and flavonols was compared to control (** $P < 0.01$; *** $P < 0.001$); MDA levels in samples treated with flavonol/CyD complexes were compared to free flavonols (° $P < 0.05$; °° $P < 0.01$).

cyclodextrins improved the antioxidant activity of the flavonols, but β-CyD resulted the most effective in reducing MDA production for all the three complexed flavonols. As cyclodextrins have been reported to complex ferrous ion [45], we also searched for a direct antioxidant activity of non-complexed α- and β-CyD; results indicate a protective effect from lipid peroxidation of 2.3 μM cyclodextrins, more evident for the β-compound, which is presumably due to chelation of the inducer of peroxidative damage, $Fe^{2+}$. Data illustrated in Fig. 10.

## 4. Conclusions

The preparation of the inclusion compounds was accomplished from liquid mixtures of the "host" and "guest" molecules, by the co-evaporation method. This had proved to be the most suitable procedure, regarding both the drug stability and the amount of complexes obtained. Among the water-miscible solvents considered, methanol was the chosen one since, due to its polarity, it showed smaller affinity for the CyD lipophilic microenvironment than the drug. For these reasons, it was less competitive for inclusion into the cavity, and a better complexation yield could be accomplished.

The combined use of different analytical techniques, according to the physical state considered, enabled to fully characterize the inclusion compounds obtained. These showed different structural and thermic properties and distinct spectroscopic features from the free molecules (flavonols and CyDs). Both α- and β-cyclodextrins were successful in complexing the flavonols, but it most be pointed out that inclusion in β-CyD was in all cases more effective. The higher degree of interaction could be attributed to the larger dimension of β-CyD cavity, compared to the α-CyD one; these results were supported by the phase-solubility studies. For all the drug/CyD systems, type $A_L$ diagrams were obtained (1:1 complexes), meaning that the solubility of the flavonols is increased by the presence of the macrocycle. However, stability constants calculated for the flavonol/β-CyD systems were higher than those of the α-CyD ones. The highest value obtained for 3-hydroxyflavone was presumably due to the higher hydrophobicity of the molecule, which therefore exhibits a greater affinity for CyD cavity. Biological assays borne out later evidence on these results. Antioxidant protection against oxidative stress may decrease the rate of DNA and membrane damage and hence help to prevent toxic injury, ageing and age-related diseases including cancer. Scavenging of damaging free radicals and binding of iron are two important antioxidant mechanisms shown by flavonols. Complexation with cyclodextrins further improved their antioxidant activity, increasing their solubility in the biological moiety.

## References

[1] J.A. Manthey, K. Grohmann, N. Guthrie, Curr. Med. Chem. 8 (2001) 135–153.
[2] H. Ohshima, Y. Yoshie, S. Auriol, I. Gilibert, Free Radic. Biol. Med. 25 (1998) 1057–1065.
[3] A. Bocco, M.E. Cuvelier, H. Richard, C. Berset, J. Agric. Food Chem. 46 (1998) 2123–2129.
[4] R. Bouhamidi, V. Prévost, A. Nouvelot, Life Sci. 321 (1998) 31–38.
[5] L.G. Korkina, I.B. Afanas'ev, Adv. Pharmacol. 38 (1997) 151–163.
[6] V. Cody, E. Middleton, J.B. Harborne, A. Beretz, Plant Flavonoids in Biology and Medicine. Part II. Biochemical, Cellular and Medicinal Properties, Alan R. Liss, Inc., New York, 1988.
[7] T. Miyake, T. Shibamoto, J. Agric. Food Chem. 45 (1997) 1819–1822.
[8] G. Cao, E. Sofic, R.L. Prior, Free Radic. Biol. Med. 22 (1997) 749–760.
[9] C.A. Rice-Evans, N.J. Miller, in: C.A. Rice-Evans, L. Packer (Eds.), Flavonoids in Health and Disease, Marcel Dekker, Inc., New York, 1998, pp. 199–218.
[10] S.V. Jovanovic, S. Steenken, Y. Hara, M.G. Simic, J. Chem. Soc., Perkin Trans. 2 (1996) 2497–2504.
[11] C.A. Rice-Evans, N.J. Miller, G. Paganga, Free Radic. Biol. Med. 20 (1996) 933–956.
[12] S.A.B.E. van Acker, D.J. van den Berg, M.N.J.L. Tromp, D.H. Griffioen, W.P. van Bennekom, W.J.F. van der Vijgh, A. Bast, Free Radic. Biol. Med. 20 (1996) 331–342.
[13] P. Cos, L. Ying, M. Calomme, J.P. Hu, K. Cimanga, B. van Poel, L. Pieters, A.J. Vlietinek, D. Vanden Berghe, J. Nat. Prod. 61 (1998) 71–76.
[14] H. Fujiki, T. Horinchi, K. Yamashita, H. Hakii, M. Suganuma, H. Nishino, A. Iwashima, Y. Hirata, T. Sugimura, in: V. Cody, E. Middleton, J.B. Harborne (Eds.), Plant Flavonoids in Biology and Medicine: Biochemical, Pharmacological and Structure Activity Relationships, Alan R. Liss, Inc., New York, 1986, pp. 429–440.
[15] E.E. Desckner, J. Ruperto, G. Wong, H.L. Newmark, Carcinogenesis 12 (1991) 1193–1196.
[16] V. Elangovan, N. Sekar, S. Govindasamy, Cancer Lett. 87 (1994) 107–113.

[17] G. Block, Nutr. Rev. 50 (1992) 207–213.
[18] W.S. Chang, Y.J. Lee, F.J. Lu, H.C. Chiang, Anticancer Res. 13 (1993) 2165–2170.
[19] W. Saenger, Angew. Chem. Int. Ed. Engl. 19 (1980) 344–362.
[20] J. Szejtli, Cyclodextrins and their Inclusion Complexes, Akademiai Kiado, Budapest, 1982.
[21] J. Szejtli, Cyclodextrin Technology, Kluwer Academic Publishers, Dordrecht, 1988.
[22] D. Duchene, New Trends in Cyclodextrins and Derivatives, Editions de Santé, Paris, 1997.
[23] D. Duchene, Cyclodextrins and their Industrial Uses, Editions de Santé, Paris, 1997.
[24] T. Loftsson, M.E. Brewster, J. Pharm. Sci. 85 (1996) 1017–1025.
[25] R.A. Rajewoki, V.J. Stella, J. Pharm. Sci. 85 (1996) 1142–1169.
[26] T. Irie, K. Uekama, J. Pharm. Sci. 86 (1997) 147–162.
[27] R. Ficarra, P. Ficarra, M.R. Di Bella, D. Raneri, S. Tommasini, M.L. Calabrò, M.C. Gamberini, C. Rustichelli, J. Pharm. Biomed. Anal. 23 (2000) 33–40.
[28] R. Ficarra, P. Ficarra, M.R. Di Bella, D. Raneri, S. Tommasini, M.C. Calabrò, A. Villari, S. Coppolino, J. Pharm. Biomed. Anal. 23 (2000) 231–236.
[29] R. Ficarra, S. Tommasini, D. Raneri, M.L. Calabrò, M.R. Di Bella, C. Rustichelli, M.C. Gamberini, P. Ficarra, J. Pharm. Biomed. Anal. 29 (2002) 1005–1014.
[30] D. Duchene, F. Glomot, C. Vantion, STP Pharma. 1 (1985b) 323–332.
[31] K. Uekama, S. Narisawa, F. Hirayama, M. Otagiri, K. Kawano, T. Ohtami, H. Ogino, Int. J. Pharma. 13 (1983) 235–261.
[32] J. Mielcorek, J. Pharm. Biomed. Anal. 15 (1997) 681–686.
[33] K.H. Fromening, J. Szejtli, Cyclodextrins in Pharmacy, Kluwer Academic, Dorohecht, The Netherlands, 1994.
[34] E. Albers, B.W. Muller, B.W. Muller, Cuz CrC, Rev. Ther. Drug Carrier Syst. 12 (1995) 311–337.
[35] S. Li, W.C. Purdy, Chem. Rev. 92 (1992) 1457–1470.
[36] R.J. Hurtubise (Ed.), Phosphorimetry: Theory, Instrumentation and Applications, VCH, New York, 1990, pp. 305–334.
[37] R. Ficarra, G. Mondio, F. Mondio Mondio, S. Tommasini, R. Stancanelli, A. Villari, P. Ficarra, in: Proceedings of the Communication Presented at the Drug Analysis 2002 Symposium, Bruges, Belgium, 21–25 April 2002.
[38] F. Hirayama, K. Uekama, in: D. Duchene (Ed.), Cyclodextrins and their Industrial Uses, Editions de Santé, Paris, 1997, pp. 131–172.
[39] M. Yoshino, K. Murakami, Anal. Biochem. 257 (1988) 40–44.
[40] N.P. Singh, M.T. McCoy, R.R. Tice, E.L. Schneider, Exp. Cell Res. 175 (1988) 184–191.
[41] K. Husain, S.M. Somani, J. Appl. Toxicol. 17 (1997) 189–194.
[42] M.P. Germanò, R. De Pasquale, V. D'Angelo, C. Catania, V. Silvari, C. Costa, J. Agric. Food Chem. 50 (2002) 1168–1171.
[43] M.M. Bradford, Anal. Biochem. 72 (1976) 248–254.
[44] T. Higuchi, K.A. Connors, Adv. Anal. Chem. Instrum. 4 (1965) 117–212.
[45] M.E. Lindsey, G. Xu, J. Lu, M.A. Tarr, Sci. Total Environ. 307 (2003) 215–229.