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## **Influence of hydroxypropyl- $\beta$ -cyclodextrin on the photostability and antiradical activity of Trolox**

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### **Abstract**

Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a derivative of vitamin E, can undergo photolysis upon UV irradiation. In the present study the photodegradation kinetics of Trolox were thus investigated in different systems in the absence and in the presence of titanium dioxide, a physical sunscreen present in several cosmetic products, that can act as photocatalyst. In all the considered media Trolox degraded under UVB light following pseudo-zero order kinetics, probably by a mechanism of photooxidation. The rate of Trolox photodegradation was lower in O/W emulsions than in aqueous solution and in gel; furthermore it was significantly influenced by the presence of TiO<sub>2</sub>. Aiming to increase Trolox stability, it was complexed with hydroxypropyl- $\beta$ -cyclodextrin: the inclusion complex was characterized by phase solubility studies, spectrophotometry and differential scanning calorimetry. The irradiation experiments indicated that complexation with hydroxypropyl- $\beta$ -cyclodextrin was effective in reducing the photodegradation rate of Trolox. Moreover the host molecule favored the uptake of Trolox into the porcine skin, as shown by in vitro permeation studies. Nevertheless the assay of peroxidation, based on the reaction of malondialdehyde with thiobarbituric acid, indicated that the antioxidant activity of Trolox was maintained even after inclusion in cyclodextrin.

### **Keywords**

Hydroxypropyl- $\beta$ -cyclodextrin, Lipoperoxidation, UVB-induced degradation, Skin uptake, TiO<sub>2</sub>, Trolox

## Introduction

Skin represents a major target of oxidative stress since it is continuously exposed to external oxidant aggressions, such as ultraviolet (UV) radiations, ozone or chemicals. Repeated exposure to such aggressions is responsible for skin damage and contributes to the development of cutaneous diseases. Oxidative stress is the consequence of an imbalance between pro- and antioxidant species. The former essentially concerns highly reactive oxygen or nitrogen species (ROS or RNS) e.g. superoxide anion, hydrogen peroxide, hydroxyl radical or nitric oxide. Naturally produced by the organism, these metabolites play a role in many physiological process such as cell respiration, neurotransmission or immune reactions. Nevertheless, excessive production leads to the oxidation of lipids, proteins and nucleic acids contributing to the cellular dysfunction and death. To avoid oxidative stress, cells are equipped with two major antioxidant defense systems. The first concerns enzymes which catalyse ROS or RNS degradation, such as superoxide dismutase or glutathione reductase. The second involves antioxidants like vitamins that reduce ROS and RNS by oxido-reduction reactions [1]. Vitamin E is one of the most potent endogenous antioxidants and has been extensively reported to exert protective effects in experimental models involving free radical formation [2, 3]. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) is chemically related to vitamin E: the chromanol structure provides the antioxidant activity while the carboxyl group imparts a moderate water-solubility [4]. Although Trolox has been researched extensively on its anti-radical activity, it has some drawbacks such as poor water solubility and photo-instability [5, 6]. The main purpose of the present study was to increase the photostability of Trolox by complexation with hydroxypropyl- $\beta$ -cyclodextrin (HP- $\beta$ -CD). Therefore the effect of complexation on the degradation rate of Trolox upon UVB irradiation was investigated, in the absence and in the presence of the well-studied and widely used TiO<sub>2</sub>, an additive in many cosmetic products [7] that can induce photocatalytic reactions [8]. The complexation of Trolox with HP- $\beta$ -CD was studied by DSC and by the phase-solubility method. The stability constant (K<sub>st</sub>) was calculated both according to the Higuchi and Connors method [9] and by spectrophotometric analysis [10]. The penetration of Trolox in porcine ear skin was investigated in vitro by comparing pure Trolox with Trolox included in cyclodextrin. Furthermore the UVB-induced lipid peroxidation of porcine skin was investigated in vitro to evaluate the influence of complexation in HP- $\beta$ -CD on the antioxidant activity of Trolox. The peroxidation assay was then carried out on linoleic acid solution employed as skin lipid model.

## Experimental

### Materials

Cetearyl alcohol, hydroxyethyl cellulose (Natrosol®MR, HEC), dimethicone (silicone oil 350 mPa s), polyethylene glycol sorbitan monolaurate (Tween®20), octyl octanoate (Tegosoft® EE) were purchased from A.C.E.F (Piacenza, Italy). Citric acid, ascorbic acid, hydrochloric acid, sodium azide were purchased from Fluka (Milan, Italy). Ethanol, methanol, 1-butanol, ferrous sulfate were purchased from Carlo Erba (Milan, Italy). Sodium monohydrogen phosphate, sodium dihydrogen phosphate, sodium chloride, sodium citrate dihydrate, dichloromethane, sodium dodecyl sulfate (SDS) were Merck (Milan, Italy) products. Ceterayl alcohol/cetearyl glucoside (Montanov®68), cetearyl alcohol/cocoglucoside (Montanov®82), arachidyl alcohol/behenyl alcohol/arachidyl glucoside (Montanov®202), alkyl polyglucose (Oramix®CG110) were kind gifts from Seppic (Paris, France). Titanium dioxide was a kind gift from Degussa Goldschmidt (Pandino, Cr, Italy), hydroxy propyl- $\beta$ -cyclodextrin (HP- $\beta$ -CD) was a kind gift from La Roquette (France). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and 2-thiobarbituric acid (TBA) were Sigma Aldrich (Milan, Italy) products.

#### Apparatus

A Modulyo freeze dryer system (Edwards, West Sussex, UK) was used to prepare the inclusion complex of Trolox and HP- $\beta$ -CD. The irradiation test was carried out in Pyrex® glass cells (5 mL volume) under a solar box equipped with a TL40/12RST40T12 UVB lamp (Philips, Milan, Italy). The dye MDA-TBA adduct was detected by a Lambda 2 UV-Vis spectrophotometer (Perkin Elmer, Waltham, MA, USA). HPLC analysis was performed by using an apparatus (Shimadzu, Tokyo, Japan) consisting of a SPD-2A UV-Vis detector, a LC-6A pump unit control, a C-R3A chromatopac integrator and a RP-C18 column (80  $\times$  4.6 mm; 5  $\mu$ m). Trolox was eluted with a mixture of methanol/water/HCl (55/45/0.4) at a flow rate of 0.8 mL/min. The detection wavelength was 290 nm and the retention time was around 4.0 min. The thermograms were obtained by a DSC-7 power compensation (Perkin Elmer, Waltham, MA, USA). A 660/H ultrasonic cleaner (Elma, Singen, Germany) was employed to disperse TiO<sub>2</sub>. O/W emulsions and HEC gel were prepared employing a DLS stirrer (Velp Scientifica, Milan, Italy), a SL-2 (Silverson, Bucks, England) and a T25 basic Ultra-Turrax® (IKA, Staufen, Germany) homogenizers. The measurements of pH were carried out with a microprocessor pHmeter (Hanna Instruments, Milan, Italy). A 90 Plus Particle Size Analyzer (Brookhaven, New York, USA) was used to determine the zeta potential of TiO<sub>2</sub>. A Servapor cellulose membrane (MWCO: 1000 Da; Serva, Milan, Italy), was used to study Trolox diffusion profiles.

#### Preparation of inclusion complex and physical mixture

Freeze drying technique was used to prepare the inclusion complex of Trolox and HP- $\beta$ -CD. A mixture of Trolox and cyclodextrin (1:2 molar ratio) was prepared in water, at pH 3.8, and shaken for 24 h in the dark.

After equilibration the suspension was filtered and freeze dried before re-dissolution (10 mg) in 10 mL of methanol to assess the active loading by HPLC. The average percentage of active loaded was around 2.1% w/w.

The physical mixture was prepared in the same molar ratio (1:2) by mixing appropriate amounts of solid components in a glass mortar.

#### Characterization of inclusion complex

##### *Solubility diagram and stability constant*

Phase solubility studies were performed according to the Higuchi-Connors method [9].

An excess of Trolox (20 mg in 20 mL of water) was added to a series of vials containing increasing amounts of HP- $\beta$ -CD, at pH 3.8. The closed vials were shaken in the dark for 24 h at room temperature. After equilibration, each sample was centrifuged and analyzed by HPLC to detect the concentration of Trolox. A phase diagram was constructed by plotting the total molar concentration of Trolox found in the solution against the molar concentration of cyclodextrin added to the system. The stability constant ( $K_{st}$ ) was calculated from the initial rise of the curve according the following equation:

$$K_{st} = \text{slope} / S_0(1 - \text{slope})$$

where the slope is obtained from the least squares linear regression of the molar concentration of Trolox in solution versus the molar concentration of cyclodextrin in water and  $S_0$  is the intrinsic solubility of Trolox (0.55 mM) in the absence of cyclodextrin. The previous equation was applied by assuming a 1:1 stoichiometry for the inclusion complex.

##### *Spectrophotometric determination of stability constant*

The UV-Vis spectra of aqueous solutions of Trolox ( $1.0 \times 10^{-4}$  M) in the absence and in the presence of HP- $\beta$ -CD at increasing concentrations were recorded at 25 °C from 200 to 400 nm. The study was performed at pH 3.8, 5.5 and 6.5. Assuming that in solution the following equilibrium exists:



the stability constant of the complex is given by

$$K_{st} = [\text{Trolox/CD}] / [\text{Trolox}][\text{CD}] \quad (2)$$

and from Eq. 3:

$$A = (A_0 + A_{CD}K_{st}[\text{CD}]) / (1 + K_{st}[\text{CD}]) \quad (3)$$

which can be linearized to

$$A - A_0 / A_{CD} - A = K_{st}[\text{CD}] \quad (4)$$

In this equation  $A$  is absorbance in the presence of HP- $\beta$ -CD,  $A_0$  is absorbance in the absence of HP- $\beta$ -CD,  $A_{CD}$  is absorbance at highest HP- $\beta$ -CD concentration. The stability constant ( $K_{st}$ ) was obtained from the slope of the plot of  $(A - A_0)/(A_{CD} - A)$  against molar concentration of HP- $\beta$ -CD [10].

#### *DSC studies*

The samples were placed in conventional aluminum pans and then heated under nitrogen flow at a scanning speed of  $10\text{ }^\circ\text{C min}^{-1}$  from 20 to 200  $^\circ\text{C}$ . The weight of each sample (pure Trolox, Trolox/HP- $\beta$ -CD complex and physical mixture) was such that it contained a constant amount of active.

#### *Zeta potential evaluation of TiO<sub>2</sub>*

The suspension of TiO<sub>2</sub> particles was properly diluted with KCl (10 mM) and placed in the cell where a conductance of about 300  $\mu\text{S}$  was established. The determinations were repeated three times at  $25\text{ }^\circ\text{C} \pm 0.1\text{ }^\circ\text{C}$ .

#### *Photodegradation study*

##### *Sample preparation*

In order to investigate the effect of HP- $\beta$ -CD on the photostability of Trolox ( $1.0 \times 10^{-3}\text{ M}$ ) upon UV irradiation, the test was performed separately on pure Trolox and on the complex in the absence and in the presence of TiO<sub>2</sub> (0.025% w/w). The runs were carried out in water, in a gel and in two different O/W emulsions. The gel was prepared by simply dispersing the appropriate amount of HEC (2% w/w) in water heated to about 80  $^\circ\text{C}$ , mechanically stirring until room temperature was reached. The aqueous and gel samples were prepared at pH 3.8. The first emulsion (emulsion A) was prepared by dispersing the melted lipid phase (3 g of Montanov<sup>®</sup> 68 and 20 g of Tegosoft<sup>®</sup> EE) in water (77 g) heated to 70  $^\circ\text{C}$ . After homogenization the emulsion was mechanically stirred until it reached room temperature; the pH was properly adjusted to 3.8 or 5.5. The second emulsion (emulsion B) was prepared by adding, under homogenization, the melted lipid phase (3 g of Montanov<sup>®</sup> 202, 2.2 g of cetearyl alcohol, 0.5 g of dimethicone) to the hot (80  $^\circ\text{C}$ ) aqueous phase containing 3 g of Montanov<sup>®</sup> 82. The emulsion was then mechanically stirred until room temperature and the pH was adjusted to 3.8.

##### *Irradiation runs*

All the samples (10 mL) were placed at 10 cm from the UVB lamp, in closed Pyrex<sup>®</sup> cells upon magnetic stirring and at scheduled times of 30 min, Trolox concentration was detected by HPLC. The radiation intensity of the lamp, measured by means of a CO.FO.ME.GRA multimeter, was  $2.6 \times 10^{-4}\text{ W/cm}^2$  for UVB radiations; this experiment can be considered an accelerated test of stability.

## Trolox diffusion through hydrophilic membrane

Diffusion runs were carried out using a multi-cell rotating device consisting of a series of donor and acceptor compartments each having a volume of 1.5 mL and area of 2.0 cm<sup>2</sup>. A dialysis membrane with a cut off of 1000 Da was employed; the donor phase consisted of an aqueous solution containing Trolox pure or complexed with HP- $\beta$ -CD at the concentration of  $5.0 \times 10^{-4}$  M while the receptor phase consisted of 0.1 N phosphate buffer at pH 7.4. At fixed times, up to 2 h, the receiving phases were analyzed by HPLC and replaced with 1.5 mL of fresh buffer solution.

## In vitro skin permeation and uptake studies

This experiment allowed determination of the amount of Trolox pure or complexed which penetrates through the porcine skin. The method used was in line with the guidelines published by Diembeck et al. [11]. Ear skin from pig was obtained freshly from a local slaughterhouse. Most of the underlying tissue was removed with a surgical scissor, making sure that the basal membrane was still present. The skin previously frozen at  $-18$  °C was pre-equilibrated in 0.9% saline solution at 25 °C for 2 h before the experiments. Each circular piece of this skin was sandwiched between the two halves of a vertical Franz cell with the SC side facing the donor compartment. The donor phases (1 mL) consisted of: aqueous solutions of Trolox or of Trolox/HP- $\beta$ -CD at concentration of both  $5.0 \times 10^{-4}$  M and  $5.0 \times 10^{-3}$  M; Trolox ( $5.0 \times 10^{-4}$  M), pure or complexed, in 20% w/w Tween®20. The pH of all the donor phases was 3.8. The receiving compartments were filled with 6.0 mL of 0.5% w/w SDS aqueous solution which was continuously stirred with a small magnetic bar. Each donor cell was sealed with parafilm and covered with aluminum foil to prevent light exposure. At hourly intervals for 6 h, the receiving phases were taken, replaced with fresh SDS solution and HPLC analyzed. At the end of the experiment (24 h) the receiving phases were analyzed while each skin piece was removed, washed with isotonic PBS, cut up and extracted with 5 mL of methanol. After 2 h of magnetic stirring, the suspensions were centrifuged and assayed by HPLC. The skin uptake was expressed as  $\mu\text{g}$  of Trolox per cm<sup>2</sup> of skin diffusion area ( $\mu\text{g}/\text{cm}^2$ ).

## Assay of peroxidation on different substrates

### *Porcine skin*

To assess the effect of Trolox on UVB induced skin lipid peroxidation we evaluated the formation of MDA [12] in porcine ear skin obtained from the animals as previously described. This assay is based upon the formation of a pink adduct (absorption maximum 532 nm) between TBA and MDA, a colorless end product of lipid peroxide decomposition. MDA was detected spectrophotometrically according to the TBA test



described by Kovachich and Mishra [13] with the following modification. In one series of experiments ear porcine skin slices were incubated for 24 h in Trolox-containing medium (1.25 mg/mL), shielded from the light. In another series of experiments ear porcine skin slices were incubated, under the same conditions, in Trolox/HP- $\beta$ -CD-containing medium. The incubation medium used for control slices was free of Trolox. After incubation the slices were transferred to ascorbic acid (100  $\mu$ M) and ferrous sulphate (25  $\mu$ M) aqueous solution in Pyrex<sup>®</sup> cells and then irradiated for 3 h under UVB lamp with the SC side facing the light source. After irradiation the slices were dried under vacuum and then incubated for 24 h in 10 mL of dichloromethane to extract MDA. The organic solvent was evaporated under vacuum by Rotavapor and the residue was reconstituted with 3 mL of 8.0% w/w SDS aqueous solution. A 0.2 mL aliquot of each sample was added to 0.3 mL of distilled water, 1.5 mL of 1.0% w/w phosphoric acid and 1 mL of 0.6% w/w TBA. The mixture was heated for 45 min at 90–100 °C in water bath. After cooling in ice-bath to terminate the reaction, 1-butanol (4 mL) was added, centrifuged and the clear supernatant was spectrophotometrically analyzed to detect the TBA-MDA adduct. The final concentration of MDA generated during the reaction was calculated using a molar extinction coefficient of 8167 M<sup>-1</sup> cm<sup>-1</sup>.

#### *Linoleic acid*

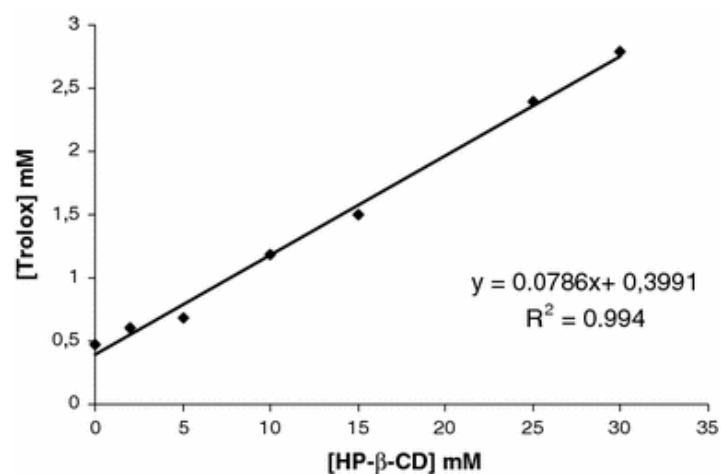
To further confirm the antioxidant activity of Trolox the UV-light induced peroxidation was also assessed on linoleic acid, as a model skin fatty acid. Briefly, 0.3 mL of distilled water, 1.5 mL of 1.0% w/w phosphoric acid and 1 mL of 0.6% w/w TBA were added to 0.2 mL of linoleic acid at 1.0% w/w concentration in 4.0% SDS aqueous solution, previously added with Trolox or with the complex. MDA formation was monitored in the same conditions above reported for the skin.

## **Results and discussion**

### Characterization of inclusion complex

#### *Solubility diagram and stability constant*

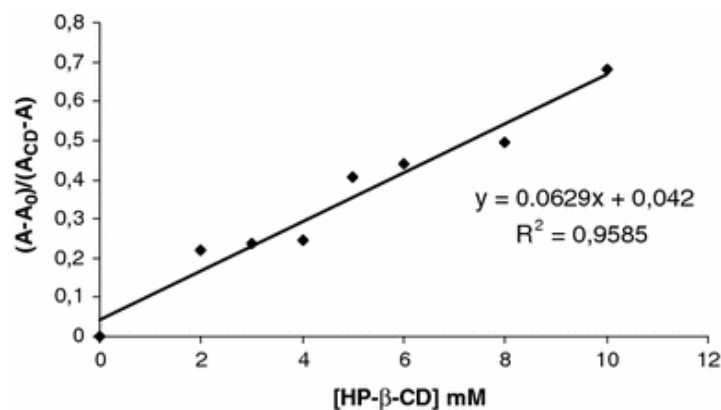
A quantitative investigation of the inclusion complexation of Trolox in HP- $\beta$ -CD was performed according to the Higuchi and Connors method [9]. The solubility diagram of Trolox and HP- $\beta$ -CD concentration (pH 3.8) is reported in Fig. 1. It was observed that the solubility of Trolox increased as the concentration of HP- $\beta$ -CD was increased, displaying an A-type phase solubility diagram. A value of 155 M<sup>-1</sup> was obtained for K<sub>st</sub>, calculated as described earlier.



**Fig. 1** Phase solubility diagram for Trolox at different HP-β-CD concentrations, at pH 3.8

*Spectrophotometric determination of stability constant*

The addition of HP-β-CD from 0.0 to 10.0 mM to Trolox aqueous solution resulted in an increased absorbance intensity of the peak but it did not cause a shift in  $\lambda_{max}$ . This result indicated the interaction between the active and the cyclodextrin. In Fig. 2 the plot of  $(A - A_0)/(A_{CD} - A)$  against HP-β-CD concentrations at pH 5.5 is reported as example. The value of  $K_{st}$  was calculated at different pH from the slope of the plot. It was found that the constant  $K_{st}$  increased as the pH of the medium decreased, as summarized in Table 1.



**Fig. 2** Plot of the ratio  $(A - A_0)/(A_{CD} - A)$  in the absence and in the presence of different concentrations of HP-β-CD, at pH 5.5

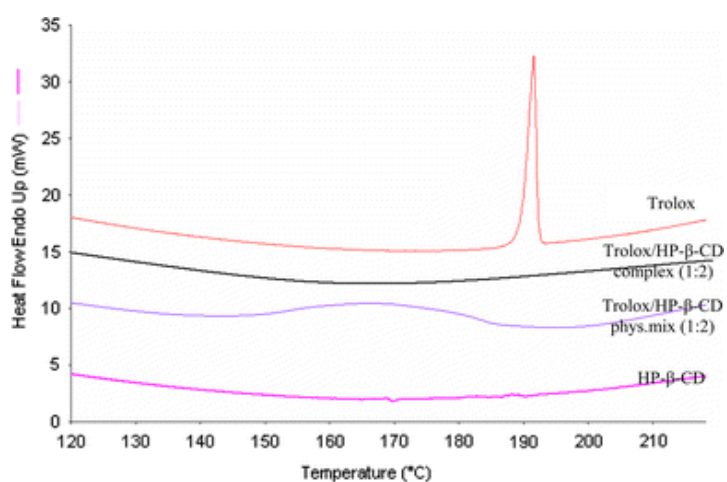
**Table 1** Stability constant ( $K_{st}$ ) values for Trolox/HP- $\beta$ -CD obtained spectrophotometrically at different pH of the medium

pH	$K_{st}$ ( $M^{-1}$ )
3.8	247
5.5	63
6.5	14

These data show that the inclusion complexation was really influenced by Trolox protonation–deprotonation equilibrium. In these terms, it would appear that the undissociated Trolox molecule has a better fit in the cyclodextrin cavity, while dissociated Trolox, due to its negative charge and hydrophilicity, might interact with the hydrophobic sites of the cyclodextrin preventing the complex formation.

#### *DSC studies*

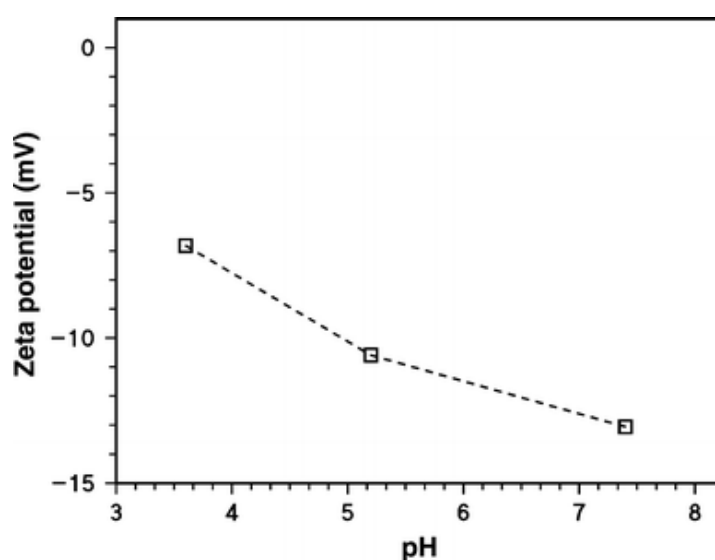
The DSC thermograms gave further information about the interaction between Trolox and HP- $\beta$ -CD. As shown in Fig. 3, the DSC curve of pure Trolox displayed one endothermic peak at 195 °C while the thermogram of HP- $\beta$ -CD did not show any peak. The disappearance of melting peak in the thermogram of the complex indicated the presence of an interaction between the two components: the peak of Trolox was not detected since the crystalline active molecule was contained within the cavity of the cyclodextrin. In the thermogram of the physical mixture the shape and the shift of the peak indicated the existence of a new solid phase in which the Trolox was dispersed with a lower crystallinity than the pure Trolox.



**Fig. 3** DSC thermograms of Trolox, Trolox/HP- $\beta$ -CD (1:2 mol) complex, Trolox/HP- $\beta$ -CD (1:2 mol) physical mixture and HP- $\beta$ -CD

Zeta potential evaluation of  $\text{TiO}_2$

The adsorption of Trolox at the  $\text{TiO}_2$  surface is the dominant factor influencing the photocatalytic process. The purpose of this analysis was to examine the effects of pH on the photocatalytic properties of  $\text{TiO}_2$ : different pH levels could influence the surface charge of the photocatalyst consequently affecting the absorption of Trolox and concomitantly the photo-chemical reactions. Fig. 4 shows the zeta potentials of  $\text{TiO}_2$  suspension as a function of pH level.



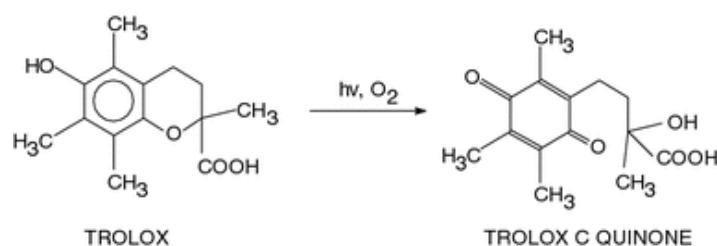
**Fig. 4** Zeta potential of  $\text{TiO}_2$  as a function of pH

It can be seen that the zeta potential of  $\text{TiO}_2$  was negative over the entire pH range but it shifted to more negative values in alkaline solutions where both Trolox and the surface of  $\text{TiO}_2$  possessed negative charges resulting in a repulsive interaction between the two species. On the contrary low pH level would enhance Trolox adsorption at  $\text{TiO}_2$  surface consequently favoring the photocatalytic interactions. Interestingly, the photocatalytic activity of  $\text{TiO}_2$  resides in its ability to transform other molecules upon radiation adsorption causing the formation of electron/hole ( $e^-/h^+$ ) couples. Such an activity is always in competition with the thermal recombination of electrons and holes [6].

Photodegradation study

Trolox absorbs radiation in the UV region, with a peak at 290 nm and, as previously reported [6], upon UVB irradiation it displays a peak at 273 nm that can be attributed (on the basis of literature data [14]) to a

photodegradation product, the Trolox C quinone (see Scheme 1). It is an oxidation product typical of lipoperoxidation reactions when Trolox is used as an antioxidant and it suggests that the photodegradation of Trolox involves oxygen as partner in the reaction. In our previous paper [15] it has been reported that less polar environments (hydro-alcoholic mixtures, micelle solutions) are able to inhibit the charge-transfer reactions that cause Trolox photooxidation. This phenomenon is reasonable under the hypothesis that the photolysis process involves charge-transfer reactions, such as photoionization or heterolytic bond cleavage, that are usually favored in more polar media. The opposite phenomenon occurs when homolytic bond cleavage and radical formation are involved.



**Scheme 1** Reaction of photooxidation of Trolox

The irradiation runs were carried out separately on pure Trolox and on Trolox/HP- $\beta$ -CD complex in order to investigate the protective effect of the cyclodextrin on the photooxidation of the active molecule; the influence of the media (water, gel, emulsion A and emulsion B) was also examined as reported above. Irradiation runs were performed in the absence and in the presence of  $\text{TiO}_2$  to assess the possible photocatalytic effect of this substance.

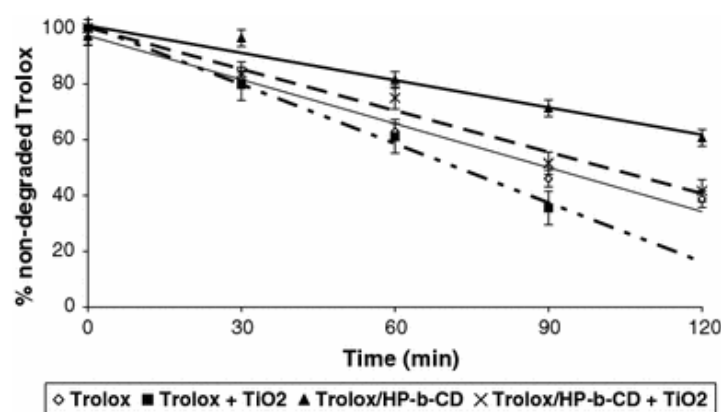
In all the media Trolox degraded under UVB light following pseudo-zero order kinetics probably by a mechanism of photooxidation. Table 2 reports the kinetic constants of Trolox photodegradation rates in water.

**Table 2** Kinetic constants of Trolox ( $1.0 \times 10^{-3}$  M) degradation rates in water upon UVB irradiation, in the absence and in the presence of  $\text{TiO}_2$ , at pH 3.8

Samples in water	k ( $\text{M s}^{-1}$ )
Trolox without $\text{TiO}_2$	$1.13 \times 10^{-7}$
Trolox with $\text{TiO}_2$	$1.21 \times 10^{-7}$
Trolox/HP- $\beta$ -CD without $\text{TiO}_2$	$0.46 \times 10^{-7}$
Trolox/HP- $\beta$ -CD with $\text{TiO}_2$	$0.97 \times 10^{-7}$

The results reported above indicate that in water the Trolox degradation rate decreased when it was included in HP- $\beta$ -CD cavity. It is interesting to observe that also the photocatalysis induced by TiO<sub>2</sub> was inhibited by the complexation with the cyclodextrin, probably because the oxide was excluded from the hydrophobic site of the guest molecule due to its negative charge (see Fig. 4). Accordingly, the cyclodextrin could separate Trolox from TiO<sub>2</sub> that, being more polar, stayed in the external aqueous phase.

The second medium considered in the present study was a HEC gel in which Trolox was dispersed at  $1.0 \times 10^{-3}$  M concentration, pure or complexed with HP- $\beta$ -CD, in the absence or in the presence of TiO<sub>2</sub> (Fig. 5). Similarly to water, also in this case the cyclodextrin exerted a protective effect both against Trolox direct photolysis and against the photocatalytic activity of TiO<sub>2</sub> (Table 3).



**Fig. 5** Time evolution of Trolox ( $1.0 \times 10^{-3}$  M) in HEC gel, pure and complexed with HP- $\beta$ -CD, in the absence and in the presence of TiO<sub>2</sub>, at pH 3.8

**Table 3** Equations and kinetic constants of Trolox ( $1.0 \times 10^{-3}$  M) degradation in HEC gel upon UVB irradiation, in the absence and in the presence of TiO<sub>2</sub>, at pH 3.8

Samples in HEC gel	Equations	k (M s <sup>-1</sup> )
Trolox without TiO <sub>2</sub>	$y = -0.3262x + 100.96$	$0.87 \times 10^{-7}$
Trolox with TiO <sub>2</sub>	$y = -0.4965x + 100.23$	$1.18 \times 10^{-7}$
Trolox/HP- $\beta$ -CD without TiO <sub>2</sub>	$y = -0.5258x + 97.334$	$0.54 \times 10^{-7}$
Trolox/HP- $\beta$ -CD with TiO <sub>2</sub>	$y = -0.7072x + 101.02$	$0.82 \times 10^{-7}$

To expand the study on the influence of the nature of the medium on the photodegradation of Trolox, irradiation under UVB was performed also on a fluid O/W emulsion (emulsion A). Table 4 reports the rate constants of photodegradation of Trolox, pure or complexed with HP- $\beta$ -CD, at pH 3.8 and 5.5.

**Table 4** Kinetic constants of Trolox ( $1.0 \times 10^{-3}$  M) degradation rates in emulsion A upon UVB irradiation, in the absence and in the presence of TiO<sub>2</sub>, at pH 3.8 and 5.5

Samples in emulsion A	k (M s <sup>-1</sup> ) pH 3.8	k (M s <sup>-1</sup> ) pH 5.5
Trolox without TiO <sub>2</sub>	$0.47 \times 10^{-7}$	$0.83 \times 10^{-7}$
Trolox with TiO <sub>2</sub>	$0.96 \times 10^{-7}$	$0.40 \times 10^{-7}$
Trolox/HP- $\beta$ -CD without TiO <sub>2</sub>	$0.17 \times 10^{-7}$	$0.10 \times 10^{-7}$
Trolox/HP- $\beta$ -CD with TiO <sub>2</sub>	$0.24 \times 10^{-7}$	$0.31 \times 10^{-7}$

Kinetic constants in emulsion A (Table 4) indicate that the inclusion of Trolox in the cavity of the cyclodextrin significantly decreased its photolytic and photocatalytic degradation rate, as already observed in water and in gel. However compared to the aqueous media, in emulsion A the rates of Trolox photodegradation were lower. Our hypothesis is that, when Trolox is in the internal hydrophobic phase of the emulsion, it experiences an apolar environment that should inhibit the photooxidative reactions. Furthermore in emulsion A, TiO<sub>2</sub> catalyzed the photodegradation of the active even though, by increasing the pH of the medium, its catalytic activity significantly decreased.

Finally, the irradiation experiment was carried out on a consistent O/W emulsion (emulsion B) containing Trolox at  $1.0 \times 10^{-3}$  M concentration, pure or complexed with HP- $\beta$ -CD, in the absence or in the presence of TiO<sub>2</sub> (Table 5).

**Table 5** Kinetic constants of Trolox ( $1.0 \times 10^{-3}$  M) degradation rates in emulsion B upon UVB irradiation, in the absence and in the presence of  $\text{TiO}_2$ , at pH 3.8

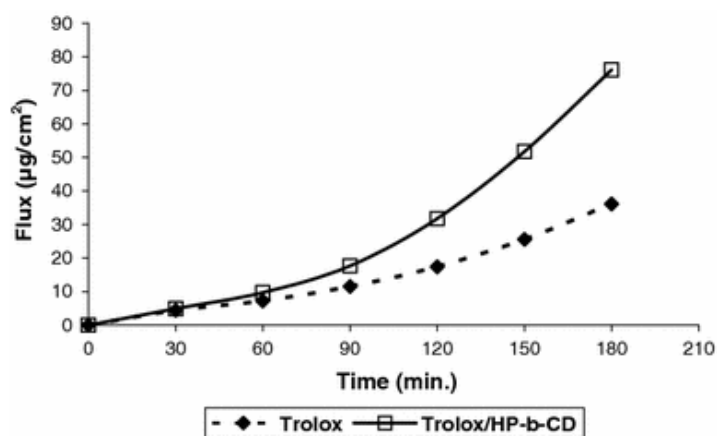
Samples in emulsion B	k ( $\text{M s}^{-1}$ )
Trolox without $\text{TiO}_2$	$0.17 \times 10^{-7}$
Trolox with $\text{TiO}_2$	$0.11 \times 10^{-7}$
Trolox/HP- $\beta$ -CD without $\text{TiO}_2$	$0.07 \times 10^{-7}$
Trolox/HP- $\beta$ -CD with $\text{TiO}_2$	$0.01 \times 10^{-7}$

Results of Table 5 evidence that HP- $\beta$ -CD exerts a certain protective effect against both the photolysis and the photocatalysis of Trolox. Moreover it is interesting to observe that the rates of Trolox photodegradation are slower in the presence than in the absence of  $\text{TiO}_2$ , indicating that the oxide could act as a sunscreen. By comparing Tables 4 with 5 it can be noted that emulsion B is more protective than emulsion A toward the photodegradation of Trolox probably due to its composition that confers more structure.

#### Trolox diffusion through hydrophilic membrane

Trolox and HP- $\beta$ -CD association was also studied by evaluating the diffusion through an artificial membrane with a cut off of 1000 Da. The choice of this barrier was justified by the structural characteristic of the drug: this membrane allows the permeation of Trolox (MW = 250.29) but it is impermeable to the host molecule. The purpose of the present study was to investigate the effect of cyclodextrin on the permeability of the active through the membrane in an effort to predict, to a limited extent, the permeability through the skin. A plot of Trolox permeation in the absence and in the presence of HP- $\beta$ -CD is shown in Fig. 6.





**Fig. 6** Flux ( $\mu\text{g}/\text{cm}^2$ ) of Trolox, pure or included in HP- $\beta$ -CD, through hydrophilic membrane

The diffusion of Trolox through the membrane was faster from the complex solution than from the pure active solution suggesting a possible interaction between the host molecule and the pores of the barrier. On the basis of this finding, it can be stated that cyclodextrin affected the trans-membrane transport enhancing the active diffusion. Nevertheless it is generally recognized that cyclodextrin increased drug availability at the surface of the biological barrier by keeping the hydrophobic drug molecules in solution unlike conventional penetration enhancers, such as alcohol and fatty acids, that disrupt the lipid layers of the biological barrier [16].

#### In vitro skin permeation and uptake studies

Data of Trolox transepidermal permeation and skin uptake, determined in vitro on porcine ear skin, are summarized in Table 6. All systems show a negligible percutaneous permeation within the first 6 h while a certain Trolox content ( $\mu\text{g}/\text{mL}$ ) was observed in all the receiving phases after 24 h. In aqueous solutions, pure Trolox at  $5.0 \times 10^{-4}$  M and at  $5.0 \times 10^{-3}$  M gave skin accumulation of respectively 2.45 and 27.01  $\mu\text{g}/\text{cm}^2$ , while the complex (Trolox/HP- $\beta$ -CD) at the same concentrations gave respectively 4.5 and 42.13  $\mu\text{g}/\text{cm}^2$ . This enhancement effect could be due to the fact that the cyclodextrin increases the availability of the active molecule at the surface, in agreement with the results of diffusion through the membrane previously discussed.

**Table 6** Permeation through porcine skin and uptake of Trolox, pure and complexed with HP- $\beta$ -CD

Samples	[Trolox] (M)	Permeation within 6 h ( $\mu\text{g/mL}$ )	Permeation after 24 h ( $\mu\text{g/mL}$ )	Uptake after 24 h ( $\mu\text{g/cm}^2$ )
Trolox in water	$5.0 \times 10^{-4}$	Negligible	0.70	$2.45 \pm 1.2$
Trolox/HP- $\beta$ -CD in water	$5.0 \times 10^{-4}$	Negligible	1.12	$4.50 \pm 2.3$
Trolox in 20%Tween <sup>®</sup> 20	$5.0 \times 10^{-4}$	Negligible	0.69	$2.77 \pm 1.3$
Trolox/HP- $\beta$ -CD in 20% Tween <sup>®</sup> 20	$5.0 \times 10^{-4}$	Negligible	0.72	$2.52 \pm 1.5$
Trolox in water	$5.0 \times 10^{-3}$	Negligible	4.90	$27.01 \pm 2.6$
Trolox/HP- $\beta$ -CD in water	$5.0 \times 10^{-3}$	Negligible	11.1	$42.13 \pm 2.8$

Moreover Table 6 shows that Trolox permeation and accumulation values from micellar solution of pure active and of the complex were similar indicating that the enhancement effect of the cyclodextrin is negligible in such media.

#### Assay of peroxidation on different substrates

##### *Porcine skin*

To verify the antiradical activity of Trolox once permeated into the porcine ear skin, in the absence and presence of HP- $\beta$ -CD, MDA levels were monitored by TBA assay as described above. Results are summarized in Table 7.

**Table 7** MDA levels derived from skin slices irradiated under UVB light for 3 h

Sample	MDA ( $\mu\text{mol/g}$ )
Skin un-treated (control)	2.96
Skin Trolox-treated	1.40
Skin (Trolox/HP- $\beta$ -CD)-treated	1.52

A comparison between Tables 7 and 6 suggests that, despite higher Trolox accumulation from the complex than from pure active, the antioxidant activity of the two species was about equal, as deduced from MDA levels registered in the absence and in the presence of HP- $\beta$ -CD. Our hypothesis is that, over a certain range of concentration, the antiradical activity of Trolox is unrelated to its concentration.

#### *Linoleic acid*

To further investigate the influence of HP- $\beta$ -CD on the antioxidant activity of Trolox, the peroxidation assay was carried out on micellar (SDS) solutions of linoleic acid added with pure Trolox, with the complex or untreated (control). The addition of Trolox or Trolox/HP- $\beta$ -CD, at the same concentration and for the same incubation time, led to MDA levels of respectively 0.091 and 0.14 nmol/mL, against a MDA level of 0.20 nmol/mL for the control. These results suggest that the complexation with HP- $\beta$ -CD partially increased the peroxidation level. Anyway, compared to the control, a certain antioxidant activity of the complexed Trolox was observed.

#### **Conclusions**

The present investigation underlines the possibility of increasing the photostability of Trolox, a widely used antioxidant, by including it in HP- $\beta$ -CD. The decrease in the degradation rate was more evident in O/W emulsion than in hydrophilic media where generally photooxidative reactions are favored. Nevertheless this study demonstrates that the complexation with HP- $\beta$ -CD enhances Trolox uptake in porcine ear skin without hindering its antiradical activity.

These data suggest that the complex Trolox/HP- $\beta$ -CD could play a primary role in protection against oxidative stress of skin and might have great application potential.

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