Chemistry of Natural Compounds, Vol. 40, No. 4, 2004

COMPLEXATION OF OLEUROPEIN AND *TRANS*-CINNAMIC ACID WITH CYCLODEXTRINS

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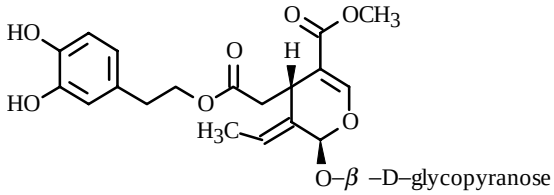
UDC 547.513.71

The complexation of oleuropein and trans-cinnamic acid with α-, β-, and γ -cyclodextrin has been studied in aqueous model systems by light scaterring. The influence of various parameters (pH, concentration, reaction time, nature of cyclodextrin) has been thoroughly examined. The formation of binary (1:1) inclusion complexes and the higher inclusion ability of β-CD for both compounds has been indicated. Trans-cinnamic acid was extracted from olive olive oil following its complexation with β-CD at satisfactory recovery levels.

Key words: *α*-, *β*-, *γ*-cyclodextrin, oleuropein, *trans*-cinnamic acid, antioxidants, inclusion complex.

Olive fruit and olive oil contain significant amounts of natural antioxidants, which contribute to the bitter taste of oil, are involved in the synthesis of thromboxane in human cell, have been associated with a low incidence of cardiovascular disease and cancer, and inhibit phospholipid oxidation [1–3]. It has been shown that the phenolic compounds which contribute most to the antioxidant activity of olive oil are 4-dihydroxyphenylethanol, the simple and ester form (3,4-DHPEA and 3,4-DHPEA -EDA), and an isomer of oleuropein aglycon (3,4- DHPEA-EA) [4, 5]. 3,4-DHPEA and 3,4-DHPEA-EA are hydrolysis products of oleuropein. Oleuropein, an intensely bitter glycoside, is present mainly in green olives and its structure, which is presented below, is specified as being that of a heterosidic ester of elenolic acid and dihydroxyethanol.



It has been shown to be a potent antioxidant endowed with anti-inflammatory properties, and it possesses antimicrobial activity against viruses, bacteria, yeasts, fungi, molds, and other parasites [6].

As is well known, antioxidant compounds are decomposed when exposed to oxygen, light, and heat. One of the new possibilities to stabilize them is the formation of inclusion complexes with cyclodextrins (CDs), a process which can actually be considered as molecular encapsulation. CDs are crystalline, homogeneous, non-hygroscopic cyclic oligomers of torus shape, built up from glucapyranose units. While the exterior of the molecule of CDs is hydrophilic, their nonpolar central cavity can selectively include various species. Although antioxidant compounds are a group of compounds for which CD complexation can potentially find a lot of applications, very few studies of the inclusion complexes have been reported.

The aim of this work is to elaborate a method for the optimization of production of complexes between CDs (host molecules) and the antioxidant compounds of olive oil (guest molecules). Unfortunately, the latter can be extracted in small amounts from olive oil and, due to their sensitivity, cannot easily be applied in model systems. For these reasons, we have used two compounds as indicators in order to evaluate the parameters which affect the phenomenon of complexation. The first one is oleuropein, a commercially available, antioxidant compound and the other is *trans*-cinnamic acid. The latter exists in small amounts in olive oil and because of its chemical and physical properties has proved to be a highly useful substrate in many studies of molecular complex formation [7].

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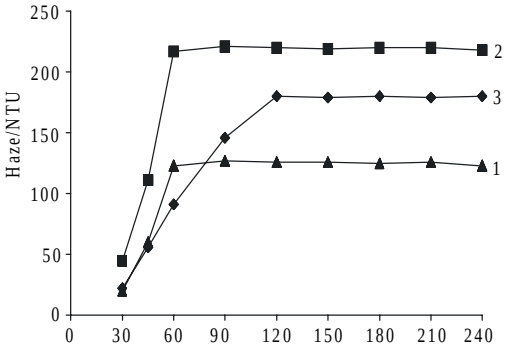

Fig. 1

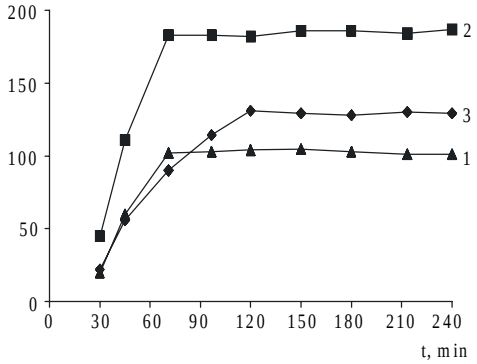

Fig. 2

Fig. 1. Haze produced in aqueous solutions of oleuropein with α- (1) , β (2), and γ-CD (3) (stoichiometric ratios1:1, pH 7) versus inclusion time.

Fig. 2. Haze produced in aqueous solutions of *trans*-cinnamic acid with α- (1), β -(2), and γ-CD (3) (stoichiometric ratios1:1, pH 4) versus inclusion time.

The turbidity of aqueous model systems of the aforementioned compounds with α-, β-, and γ-CD versus time were recorded. Since the CDs and the guest molecules were taken at concentrations such that they do not form haze by their own, at any temperature, the appearance of turbidity was considered as evidence of inclusion complex formation. The influence of various parameters, (pH, molar ratio, reaction time, nature of CD) has been thoroughly studied.

In the second part of the study, in order to confirm that the determined optimum conditions for complex formation can be applied to the antioxidant compounds contained in olive oil, a quantity of oil was spiked with *trans*-cinnamic acid and mixed with an aqueous solution of β-CD. The resulting complex was treated with organic solvents and the recovered amount of *trans*-cinnamic was estimated.

As shown in Figs 1 and 2, both oleuropein and *trans*-cinnamic acid produced more haze when complexed with β-CD, significantly less when reacted with α-CD, while complexation with γ-CD gave intermediate values. In all cases it appears that the amount of the guest molecules complexed with the three CDs has a linear relationship with the time of reaction, reaching a plateau in 1 hour in the case of α- and β-CD and in 2 hours in the case of γ-CD.

The results obtained can be explained if one takes into account the mechanism by which the phenomenon of complexation takes place, as well as the size of the cavity of each CD. The interior of the cavity of the CD molecule dissolved in water is less hydrophilic than the outer surface of the ring; therefore the presence of water molecules in the swallow is energetically unfavored. The apolar parts of the guest molecules dissolved in water are poorly hydrated, thus energetically unfavored too. The poorly hydrated guest molecule penetrates into the CD cavity, exposing from there the water molecules of high enthalpy, resulting in a lower free energy of the system. This seems to be the driving force of complexation. The greater the interaction between the complexed molecule and the cavity walls of the CD, the stronger the binding force between the components of the complex. The double in size cavity of β-CD, compared to that of α-CD, seems to include more effectively the molecules of *trans*-cinnamic acid and oleuropein. On the other hand, the guest molecules have much room to move inside the wider cavity of γ-CD and consequently they interact less with the walls of the cavity. It should also be pointed out that the cavity of γ-CD is so wide that it can accommodate many water molecules, and that their properties resemble water molecules in the bulk of the solvent [8]. As a result, the driving force of complex formation in the case of γ-CD is reduced and consequently the phenomenon of complexation is slower compared to that of the two other CDs. The picture that results from this interpretation of complex formation is of internal coordination.

In the case of *trans*-cinnamic acid, the haze formed in acidic solutions (pH 4) is higher than that in neutral solutions, whereas in alkaline media no observable haze occurs. Therefore the cinnamate ion seems to be a poor complex forming agent and we conclude that ionized compounds suitable for salt formation are considerably poorer complex forming agents, if they complex at all. This evidence may be helpful in determining the complex structure. Considering that the guest molecule will have a smaller effective dielectric constant if included in the apolar cavity of CDs than in water, the formation of an inclusion complex should be expected. This assumption is consistent with the literature, where it is reported that the un-ionized carboxylic acids complex more strongly than the carboxylate ions [7].

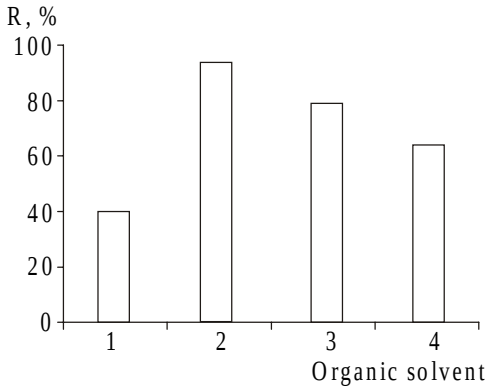

Fig. 3. % Release of *trans*-cinnamic acid from the inclusion complex with β-CD. Reaction time for complex formation: 1 h. 1– MeOH; 2 – EtOH; 3 – Ethyl ether; 4 – Chloroform.

In the case of oleuropein, haze is promoted in neutral solutions whereas a basic or acidic medium seems to be disadvantageous for complexation with all the CDs. This behavior can be explained based on the fact that oleuropein hydrolyzes into hydroxytyrosol and elenolic acid glucoside in alkaline solutions [9] while acid hydrolysis of oleuropein results in hydroxytyrosol, elenolic acid, and oleuropein aglycon [10].

The optimum stoichiometric ratio for complex formation between the three CDs and both guest compounds was found to be is 1:1. This picture of a binary (1:1) inclusion complex can be explained if we consider the cases in which different ratios can be obtained.

A complex containing two molecules of the guest compound and one molecule of CD can be formed in the following cases: 1) if the guest molecule is so small that two molecules can penetrate into the CD cavity. This is obviously not the case in our study; 2) if the second molecule, capable of hydrogen bonding, attaches to the CD surface, forming a complex with the composition 1:2 [11]. It seems that in our case the hydrogen bonds that may be formed are not strong enough to stabilize an "outer sphere" complex and consequently a 1:2 ratio cannot be achieved.

In the cases where stoichiometric ratios 2:1 or 3:1 have been reported, it is obvious that the guest molecule is included in a cavity formed by two or three host molecules. The length and chemical structure of the guest molecule are the major factors determining whether two or three host molecules can be associated in order to include the guest molecule [7]. It appears therefore that neither oleuropein nor *trans*-cinnamic acid can promote the association of the CDs in the model systems under study.

At this point we might need to discuss how internal complexation can affect the solubility of the resulting complex. The formation of a precipitate indicates that when the hydrophobic guest compound is included within the host molecule cavity, which has been evacuated from the included water molecules, the resulting complex has a lower solubility compared to that of the two individual species. This can be explained based on the possible modification(s) of the stereochemical structure of the complex, which can be attributed to the orientation of the guest molecule within the CD cavity and to the hydrophobic parts of the molecule that are excluded from it.

A quantity of olive oil was spiked with *trans*-cinnamic acid and reacted with an aqueous solution of β-CD under the determined optimum conditions, and the resulting complex was treated with organic solvents in order to extract the included guest molecule, the amount of which was determined with HPLC. The percent release of *trans*-cinnamic acid from the complex (%R) was defined as the amount of cinnamic acid measured by HPLC, after treatment of the complex with the solvents, over the amount complexed with the CD, times 100.

It is expected that organic solvents, such as methanol, ethanol, chloroform, and ethyl ether, which fit within the cavities of CDs more strongly than the guest molecule, should be capable of releasing the latter into the organic solution to various degrees, depending on the affinity of each solvent with the CD cavity. It becomes apparent from Fig. 3 that ethanol is the best solvent for the release of *trans*-cinnamic acid from its inclusion complex with β-CD. Ethyl ether and chloroform have weaker but comparable ability while methanol is less effective. Ethanol, being less polar than methanol, can penetrate more easily into the cavity of CDs. At the same time, ethanol molecules are more properly shaped than chloroform and ethyl ether to penetrate easily into the cavity of the CDs [12].

Summarizing the results, it has been proved that oleuropein and *trans*-cinnamic acid form binary complexes in aqueous solutions with α-, β-, and γ-CD and that β-CD is more effective for complexation with both guest molecules. In the case of oleuropein the maximum amount of complexation was observed in neutral medium while in the case of *trans*-cinnamic acidic medium is advantageous. It has also been proved that *trans*-cinnamic acid can be extracted from olive oil up to 98% after treatment of its complex with ethanol. Therefore, the interaction of antioxidant compounds with CDs may be considered a new proposal for their extraction from olive oil.

EXPERIMENTAL

Materials and Methods. Olive oil was purchased from the local market and was though purified with a 80/20 v/v mixture MeOH/H_2O to strip off the antioxidants. α-, β-CD (purity 99% and 97%, respectively) and *trans*-cinnamic acid (97%) were purchased from Sigma, and γ-CD (98.5%) from Merck.

Oleuropein was purchased from Extrasynthese. Methanol, ethanol, ethyl ether, and chloroform were of HPLC grade.

Buffer solutions CH_3COONa/CH_3COOH (0.002M) for pH range 3.2–4.4, citric acid – sodium citrate (0.001M) for the range 5.0–6.8, and sodium monohydrogen phosphate–sodium dihydrogen phosphate (0.002M) for the range 7.0–10.0 were prepared fresh daily in HPLC grade water.

Procedure for Complex Formation in Aqueous Model Systems. Two series of model systems were investigated:

1. In 15 ml of 2% aqueous solutions of β-CD adequate amounts of *trans*- cinnamic acid or oleuropein were added so that solutions at a molecular ratio of β-CD:guest molecule 1:1 were produced. The pH of the above solutions varied from 4–10. The temperature remained constant at 22°C;

2. At pH 4, in the case of *trans*-cinnamic acid and at pH 7, in the case of oleuropein, solutions of α, β-, or γ-CD:guest molecule at stoichiometric ratios 1:1, 1:2, 2:1 and 3:1 were prepared. The temperature was again kept constant at 22°C.

All the above solutions were shaken on a SBS orbital agitator at 100 rpm under N_2 atmosphere and their turbidity versus time was monitored.

Procedure for Spiking of Olive Oil with *trans*- Cinnamic Acid. 7.4 mg of *trans*-cinnamic acid was dissolved in 10 ml of antioxidant-free olive oil, contained in a 50 ml centrifuge tube. An aliquot of 10 ml of a 2% aqueous solution of β-CD (pH 4) was added to the centrifuge tube and the liquid phases were shaken on a SBS orbital agitator at 100 rpm, under nitrogen atmosphere, at 22°C for 1 h. The water and oil phases were separated from the precipitate by centrifugation at 4000g for 5 min. For the dissociation of the resulting complex, the precipitate was mixed with 5 ml ethanol for 10 min. Methanol, chloroform, and ethyl ether were substituted for ethanol to assess their potential to dissociate the guest – CD complex. Water was also substituted for CD in the blank. After centrifugation at 3000g for 4 min, the liquid phase was analyzed by HPLC.

Light scattering measurements were carried out with a VELP turbiditimeter. The instrument has a measuring range of 0-200 Nephelos Turbidity Units (NTU) and is equipped with two 1" diameter glass cuvettes with caps. Calibration of the instrument with known standards (10, 100, and 200 NTU) is necessary before its use.

The qualitative and quantative determination of cinnnamic acid was performed on a Waters HPLC system, consisting of a 600 pump and a 996 PDA detector. The chromatographic separation was achieved on a Nova Pak C18 column (4.6×250 mm) obtained from Waters, at room temperature. The mobile phase consisted of 2% acetic acid (A) and methanol (B) at the following gradient elution: 95% A – 5% B in 2 min, 75% A – 25% B in 5 min, 60%A – 40% B in 15 min, 50% A – 50% B in 25 min, 40% A – 60% B in 40 min.

External standard calibration of the HPLC system was performed. Calibration plots of *trans*-cinnamic acid were established over the range of 5–100 mg/ml, which was found to be linear.

REFERENCES

1. C. S. Rivas, J. C. Espin, and H. J. Wichers, *J. Sci. Food Agric.*, **80**, 1013, (2000).
2. A. Petroni, M. lasevieh, M. Salami, H. Papini, G. Montedoro, and C. Galli, *Thrombosis Res.*, **78,** 151, (1995).
3. F. Visioli, S. Bellosta, and C. Galli, *Biochem. Biophys. Res. Commun.*, **247,** 60, (1998).

4. M. Servilli, M. Baldioli, R. Selvagini, E. Miniati, A. Macchioni, and G. Montedoro, *JAOCS*, **76**, 7, (1999).
5. G. Montedoro, *J. Agric. Food Chem*.,**40**,1571, (1992).
6. O. B. Garcia, J. Castillo, J. Lorente, A. Orturo, and J.A. Del Rio, *Food Chem.*, **68**, 457, (2000).
7. K. Connors and T. Rosanske, *J. Pharm. Sci*., **69**, 173 (1980).
8. I. Tabushi, *J. Am. Chem. Soc*., **100**, 916 (1978).
9. M. Brenes, L, Rejano, P. Garcia, A. H. Sanchez, and A. Garrido, *J. Agric. Food Chem.*, **43**, 2702 (1995).
10. W. Walter, J. Fleming, and J. Etchelss, *Appl. Microbiol.*, 773 (1973).
11. A. Buvari, J. Szejtli, and L. Barcza, *Acta Chim. Acad. Sci.Hung*., **110 (1)**, 51, (1982).
12. M. Kazuyoshi, *Anal. Chem.,* **56,** (1980–1982).