Complexation of olive oil antioxidant with cyclodextrins
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ABSTRACT
Oleuropein (OLE) is a major constituent of the olive leaf and green olives (\textit{Olea europaea}). It has antioxidant and therapeutic properties and is valued as a medicinal component. The molecule is a complex phenolic compound constituted by a heterosidic ester of the elenolic acid and dihydroxy ethanol. This work concerns the determination of the inclusion complex constants between OLE and cyclodextrins (CDs). The methodology was based on the competition of two guests for the CD cavity, one being a dye and the other OLE. The dye used was methyloorange (MO) and pH 3 was selected, since MO molar absorbivity at 500 nm is maximum. Solutions of MO $0.045 \times 10^{-3}$ mol L$^{-1}$, OLE $0.75 \times 10^{-3}$ mol L$^{-1}$ and $\alpha$-CD, 0 to $0.625 \times 10^{-3}$ mol L$^{-1}$, or $\beta$-CD, 0 to $5.0 \times 10^{-3}$ mol L$^{-1}$, with citrate buffer 0.05 mol L$^{-1}$ were used for determining the absorbance values. From these data and by appropriate mathematical modeling, thorough the supposition of equilibrium between the complexes, the constant of formation of OLE:CD complexes were obtained. The resulting complexation constants are: for OLE:$\alpha$-CD $K=1352.4$ M$^{-1}$ ($R=0.9975$) and for OLE:$\beta$-CD $K=1827.9$ M$^{-1}$ ($R=0.9991$). These results show that OLE has a greater affinity for $\beta$-CD than for $\alpha$-CD and given the relatively high constants, OLE:$\beta$-CD complexes have potential for giving longer shelf lives for OLE food and medicinal additive preparations. Solid forms of the inclusion complexes of oleuropein in cyclodextrins were prepared by the methods of kneading and physical mixture, and then characterized by Thermogravimetric Analysis (TGA) and Differential Scanning Calorimetry (DSC), showing higher degradation temperature for the complexes. The best preparation was obtained with complexation of oleuropein and $\beta$-CD at 1:2 molar ratio. Complexation is advantageous since it protects oleuropein from higher temperatures, which is useful in food and pharmaceutical applications.

\textit{Keywords:} oleuropein; cyclodextrin; complex; inclusion; DSC; TGA

INTRODUCTION
Oleuropein (Figure 1) is a bitter glycoside that can be isolated from the green leaves of the olive tree (WALTER et al, 1973). It is a heterosidic ester of the elenolic acid and dihydroxy ethanol (EFMORFOPOULOU & RODIS, 2004). It is the largest phenolic component of olives, but it is practically absent in olive oil due to its high solubility in water, its low solubility in the oil and the extensive enzymatic degradation that occurs during the olive oil production (MARTINS & PINHO, 2008). In the case of olive oil, the components that most contribute in the antioxidant activity are the 3,4 dihydroxyphenyl ethanol, in the simple and esterified forms (3,4-DHPEA and the 3,4-DHPEA-EDA), both obtained by oleuropein hydrolysis. However, as oleuropein is an antioxidant, it is easily decomposed when exposed to light, humidity and heat, and one of the possibilities of stabilization of this molecule is by...
molecular inclusion, for example, with cyclo-
dextrins (EFMORFOPOULOU & RODIS, 2004).

*In vivo* studies carried out on rabbits showed that oleuropein administration (about 10 to 20 mg kg\(^{-1}\) day\(^{-1}\)), can help in ischemia treatments (ANDREADOU et al., 2008). Another study revealed an anti HIV activity using oleuropein, where the synergism between the HAART substances (drugs used for the treatment against HIV virus) and oleuropein was demonstrated, being, therefore useful in the treatment of the disease (HUANG et al., 2003).

The main objective of our work was to prepare the complexes of oleuropein by molecular inclusion in \(\alpha\)-CD and \(\beta\)-CD and determine their physicochemical properties. Complexes were prepared by the kneading technique and compared with the simple physical mixture of the substances. Analyses by Differential Scanning Calorimetry (DSC), Thermogravimetric Analysis (TGA) and Derivative Thermogravimetry (DTG) were used to determine the thermal stability of the products of complexation. A model for the formation of the inclusion complexes at equilibrium, based on the competition with a dye, was developed. Absorbance tests with solutions containing oleuropein, cyclodextrins and a dye allowed calculating the stability constants for complex formation.

**MATERIALS & METHODS**

Materials: Oleuropein (molar mass 540.53 g mol\(^{-1}\)) was isolated from olive tree leaves and supplied by Professor Fátima Paiva-Martins (University of Porto). Other reagents such as methyl orange, alpha and beta-cyclodextrins were of analytical grade.

Methods: Determination of the complexation constants between oleuropein and cyclodextrins. The determination of the constant of formation of the oleuropein complex with the cyclodextrins (\(\alpha\)-CD and \(\beta\)-CD) demanded the development of a theoretical equation which allowed modeling the absorbance of a dye solution in presence of oleuropein. This model is based on the supposition of thermodynamic equilibrium between the complexes of oleuropein:CD and a dye:CD. Methyl orange (MO) was selected as the competing dye for complex formation, because when it is complexed pure with the CDs, the strength of its complexation constant has the appropriate intermediary value, to give measurable changes in the absorption when oleuropein is added to the solution. The model equation is:

\[
x = \left( \frac{DABS}{\Delta} \right) \left[ 1 + \frac{1}{K_1 \left( \frac{a - DABS}{\Delta} \right) + K_2 b} \right]
\]

where:

- \(a\) = total concentration of methyl orange in M;
- \(b\) = total concentration of oleuropein in M;
- \(x\) = total concentration of cyclodextrin in M;
- \(DABS\) = \(\text{ABS} - \text{ABS}_0\);
- \(\text{ABS}\) = absorption of each point of the curve in the wavelength of 500 nm;
- \(\text{ABS}_0\) = absorption of the point of concentration zero of CD;
- \(K_1\) = complexation equilibrium constant between Methyl orange and the CD;
- \(K_2\) = complexation equilibrium constant between oleuropein and the CD;
- \(\Delta = \sigma - \sigma_0 = \frac{\sigma - \text{ABS}_0}{a}\);
- \(\sigma = \text{absorção molar específica do corante alaranjado de metila}\);
- \(\sigma = \text{absorção molar específica do corante complexado com a \(\alpha\)-CD}\)

Absorbance tests.

a) Preparation of stock solutions in Citric acid/Sodium Citrate (CA/SC) buffer 0.05 mol L\(^{-1}\), pH 3.0: A stock solution of \(\alpha\)-cyclodextrin \(1.25 \times 10^{-2}\) mol L\(^{-1}\), \(\beta\)-cyclodextrin \(10.0 \times 10^{-3}\) mol L\(^{-1}\), oleuropein (OLE) \(3.0 \times 10^{-3}\) mol L\(^{-1}\) and methyl orange (MO) \(0.9 \times 10^{-3}\) mol L\(^{-1}\) was prepared in a volumetric balloon with CA/SC buffer.
b) Preparation of the work solution of methyl orange and oleuropein in buffer CA/SC 0.05 mol L\(^{-1}\), pH 3.0: A methyl orange stock solution 0.09 × 10\(^{-3}\) mol L\(^{-1}\), with oleuropein 1.5 × 10\(^{-3}\) mol L\(^{-1}\) was prepared in a volumetric flask (10 mL of the MO stock solution (prepared in the subsection a) in 20 mL of the buffer stock solution CA/SC, plus 50 mL of the oleuropein solution (prepared in the subsection a) and the remaining volume of the flask was completed with water.

c) Dilutions of cyclodextrin stock solution: 0 to 4 mL of cyclodextrin stock solution were put in 21 test tubes and the volume was completed with the buffer work solution CA/SC. Soon afterwards all the tubes were agitated in vortex.

d) Complexation curve between oleuropein and α-cyclodextrin with the presence of methyl orange: 1.5 mL of the MO:oleuropein (subsection b) work solution and 1.5 mL of the different dilutions of α-CD (subsection c) were put in 21 test tubes, so that the MO concentration in the cell was 0.045 × 10\(^{-3}\) mol L\(^{-1}\), the concentrations of α-CD varied from 0 to 0.625 × 10\(^{-3}\) mol L\(^{-1}\) and the oleuropein concentration was 0.75 × 10\(^{-3}\) mol L\(^{-1}\). The tubes were agitated in a vortex and the absorbance measurements were made at 500 nm. The zero was calibrated with distilled water.

e) Complexation curve between oleuropein and β-cyclodextrin with the presence of methyl orange: 1.5 mL of the MO:oleuropein (subsection b) work solution were put in 21 test tubes and 1.5 mL of the different β-CD dilutions (subsection b), so that the concentration of MO in the cell was 0.045 × 10\(^{-3}\) mol L\(^{-1}\), the β-CD concentrations varied from 0 to 5.0 × 10\(^{-3}\) mol L\(^{-1}\) and the oleuropein concentration was 0.75 × 10\(^{-3}\) mol L\(^{-1}\). The tubes were agitated in a vortex and the absorbance measurements were made at 500 nm. The zero was calibrated as above.

Complex preparation.

a) Kneading Technique. α-cyclodextrin (0.5442 g) or β-cyclodextrin (0.6780 g) was kneaded with 1 mL water, at ambient temperature, until homogenization (approximately 5 min). Then, oleuropein (0.2703 g) was added to the paste and kneading followed for a further period of 20 min. The resulting paste was dried at ambient temperature (25 °C) for 48 h.

b) Physical Mixture Technique. α-cyclodextrin (0.5442 g) or β-cyclodextrin (0.6780 g) and oleuropein (0.2703 g) were manually mixed, for a period of 10 min, and then dried at ambient temperature (25 °C) for 24 h. A simple physical mixture was prepared as above, but without any drying or heating of the product and served for comparison with the kneaded products.

The above-described protocols have used equimolar quantities of oleuropein and cyclodextrin. Another series of experiments was carried out with 1:2 (OLE:CD) molar ratio, with the quantities being 0.2703 g of oleuropein and α-cyclodextrin (1.088 g) or β-cyclodextrin (1.356 g).

Thermal analyses.

The thermal analyses of Differential Scanning Calorimetry (DSC) and Thermogravimetric Analysis (TGA) followed standard well known procedures. The equipment used was a Differential Scanning Calorimeter SHIMADZU, model DSC-50 and a SHIMADZU Thermobalance, model TGA-50 for Thermogravimetric Analysis.

RESULTS & DISCUSSION

Figures 2 and 3 show the absorbance data used to calculate the complex formation constants. The values of the constants for the formation of the complexes are:

For α-cyclodextrin: \(K_1 = 4557.2\, M^{-1}\), \(R = 0.9994\), for the methyl orange:α-CD complex, and
\(K_2 = 1352.4\, M^{-1}\), \(R = 0.9975\), for the oleuropein:α-CD complex.

For β-cyclodextrin: \(K_1 = 952.00\, M^{-1}\), \(R = 0.9996\), for the methyl orange:β-CD complex, and
\(K_2 = 1827.9\, M^{-1}\), \(R = 0.9991\), for the oleuropein:β-CD complex.

The comparison of the experimental data and the absorbance model for the complexation of oleuropein with cyclodextrins, in the presence of the competing dye, gave excellent results (Figures 2 and 3).

The complexation constant between β-CD and an olive tree leaves extract (rich in oleuropein, about 90.2%) was determined by Karathanos et al (2007) using the Higuchi & Connors (1965) method. This method uses phase solubility diagrams of oleuropein and cyclodextrin solutions of different concentrations. The authors obtained an AL type graph, with a complex with stoichiometric molar ratio 1:1, an equilibrium constant of
300 M\(^{-1}\) and an increase of 50% in oleuropein solubility. This was probably the first work involving the calculation of the equilibrium constant between the oleuropein and the β-CD.

The difference in the complexation constant values obtained in our experiments and those mentioned in the literature can be explained mainly by the differences in the nature of the method of analysis and some conditions inherent to the experiment, such as pH, temperature and other factors that influence the thermodynamic constants. For example, the HIGUCHI & CONNORS (1965) method can lead to the formation of aggregates which affect the value of the complexation constant (Messner et al., 2010).

**Figure 2.** Comparison between the complexation data of methyl orange (MO) dye and alpha-cyclodextrin (α-CD), in the absence (a) and in the presence (b) of oleuropein (OLE). Conditions: room temperature, citrate buffer 0.05 mol L\(^{-1}\), pH 3.0. MO concentration in the cuvette was 4.5 \(\times\) 10\(^{-5}\) mol L\(^{-1}\), OLE concentration was 7.5 \(\times\) 10\(^{-4}\) mol L\(^{-1}\).

**Figure 3.** Comparison between complexation data of methyl orange (MO) dye and beta-cyclodextrin (β-CD), in the absence (a) and in the presence (b) of oleuropein (OLE). Conditions: room temperature, citrate buffer 0.05 mol L\(^{-1}\), pH 3.0. MO concentration in the cell was 4.5 \(\times\) 10\(^{-5}\) mol L\(^{-1}\), OLE concentration was 7.5 \(\times\) 10\(^{-4}\) mol L\(^{-1}\).

Thermal analyses.
Thermal analysis data is shown at the Figures 4 to 6.
The DSC thermograms showed weak interactions between oleuropein and α-CD and their interpretation was hindered because the peaks associated with the fusion point of oleuropein and the loss of water were very close. However, in the thermogram product of OLE:α-CD complexation by kneading, the boiling point peak of OLE disappeared, indicating formation of the complex. In the thermograms of the physical mixtures a sharper fall in the peaks was observed, and the interpretation was hindered. For the oleuropein and β-CD complexation products, the complex formation could be confirmed in the preparation 1:2, as the fusion and boiling peaks disappeared completely. In the other cases, weaker interactions occurred and the corresponding peaks occurred late, compared with the peaks of the pure substance. In the analysis of the OLE:β-CD complexes (not only in the preparation 1:1, but also in 1:2), the disappearance of the fusion peak of pure BHA was observed, indicating complex formation, whereas in the thermograms of physical mixtures this peak appears well defined (more so in the preparation 1:1). Through the thermogravimetric analysis (TGA) tests involving α-CD, or β-CD and the oleuropein, the volatilization of the last compound occurred more slowly at higher temperatures, indicating complex formation.

![Figure 4](image1.png)

**Figure 4.** Left: DSC thermogram of pure substances: (a) Oleuropein, (b) β-cyclodextrin and (c) α-cyclodextrin. Right: Thermogravimetric curves of the pure substances: (a) oleuropein (b) α-cyclodextrin and (c) β-cyclodextrin.

![Figure 5](image2.png)

**Figure 5.** Left: DSC curves for the products obtained from the complex preparation techniques: (a) kneading 1:1, (b) kneading 1:2, (c) physical mixture (1:1), (d) physical mixture (1:2), with oleuropein: α-cyclodextrin given molar ratios. Right: TGA thermograms of products obtained from the complex preparation techniques: (a) kneading 1:1, (b) kneading 1:2, (c) physical mixture (1:1), (d) physical mixture (1:2), with oleuropein: α-cyclodextrin given molar ratios.

**CONCLUSION**

The constants of complexation of oleuropein with cyclodextrins were measured using the method of reading the absorbance of a solution of these substances in the presence of a dye. The resulting values for the
Oleuropein:α-CD and oleuropein:β-CD complexes were 1352.4 M⁻¹ and 1827.89 M⁻¹, respectively. These results indicated a greater affinity of oleuropein for β-CD, than for α-CD, caused probably by a better fitting of the oleuropein molecule inside the cavity of β-CD.

![DSC curves](image1)

![TGA thermograms](image2)

**Figure 6. Left:** DSC curves for the products obtained from the complex preparation techniques: (a) kneading 1:1, (b) kneading 1:2, (c) physical mixture (1:1), (d) physical mixture (1:2), with oleuropein: β-cyclodextrin given molar ratios. **Right:** TGA thermograms of products obtained from the complex preparation techniques: (a) kneading 1:1, (b) kneading 1:2, (c) physical mixture (1:1), (d) physical mixture (1:2), with oleuropein: β-cyclodextrin given molar ratios.

Solid forms of the inclusion complexes of oleuropein and cyclodextrins were efficiently prepared by the method of kneading and thermal analyses demonstrated higher degradation temperature for the complexes than the pure oleuropein. The oleuropein boiling point shifted from 230 °C to a reduced mass loss from 250 to 300 °C and the most suitable preparation was found for complexation of oleuropein and β-CD at 1:2 molar ratio.

It may be concluded that complexation is advantageous for protecting oleuropein from higher temperatures and since the best preparations involved the use of the β-CD, this is convenient from the point of view of process economics as β-CD is the cyclodextrin with the lowest price in the market.

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**REFERENCES**


