Journal of Inclusion Phenomena and Macrocyclic Chemistry **34:** 85–96, 1999.

Interaction of β-Cyclodextrin with Unsaturated and Saturated Straight Chain Fatty Acid Anions Studied by Phenolphthalein Displacement

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(Received: 1 March 1998; in final form: 23 June 1998)

Abstract. The interaction of β-cyclodextrin (β-CD) with palmitoleate, linolenate, caprinate and caprylate was studied by the displacement of phenolphthalein (PHP) from the β-CD cavity. Absorbance values of β-CD–PHP solutions at 550 nm in 0.020 mol L^{-1} Na_2CO_3 buffer, pH 10.5, at 21.0 $\pm$ 0.5 °C, increased as the fatty acid anion was added. The concentration range of fatty acid anion used was 0.390–32.1 $\times$ 10^{-4} mol L^{-1} in the study of palmitoleate and linolenate, 1.92–80.0 $\times$ 10^{-4} mol L^{-1} in the study of caprinate and 0.770–32.0 $\times$ 10^{-3} mol L^{-1} in the study of caprylate. Concentrations of β-CD and PHP were 1.00 $\times$ 10^{-3} mol L^{-1} and 1.00–3.00 $\times$ 10^{-4} mol L^{-1} respectively. Data were fitted by nonlinear regression to a two step complexation model. Complex formation constants thus determined for the 1 : 1 and 1 : 2, fatty acid anion : β-CD complex were: $(1.2 \pm 0.2) \times 10^4$ and $(2.9 \pm 0.2) \times 10^2$ mol^{-1} L for palmitoleate, $(9.3 \pm 0.9) \times 10^3$ and $(1.1 \pm 0.1) \times 10^2$ mol^{-1} L for linolenate, $(4.1 \pm 0.2) \times 10^3$ and 81 ± 8 mol^{-1} L for caprinate, and $(5.2 \pm 0.7) \times 10^2$ and 27 ± 5 mol^{-1} L for caprylate respectively. The PHP–β-CD complex was also evaluated as a spectrophotometric sensor for the determination of olive oil acidity.

Key words: palmitoleic acid, linolenic acid, capric acid, caprylic acid, linoleic acid, oleic acid, β-cyclodextrin, phenolphthalein, complex formation constants, olive oil, free fatty acids, spectrophotometric probe.

1. Introduction

Cyclodextrins (CDs) are cyclic oligosaccharides composed of glucopyranose units bonded together via 1,4-ether linkages. The most extensively used CDs are α-, β-, and γ-CD which contain six, seven and eight glucopyranose units respectively, and their hydroxyethyl, hydroxypropyl and methyl derivatives. CDs form inclusion complexes with a variety of guest molecules including lipids [1]. These interactions result in interesting applications in analytical chemistry and technology. The complexation of α-CD with lipids has been used for detection in TLC plates [2], clarification of lipemic sera by either flocculation of serum lipoproteins and subsequent centrifugation [3, 4] or complexation-scavenging of nonesterified fatty acids

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resulting from lipolysis by lipase [5, 6]. Negative interference in the colorimetric determination of calcium in serum by nonesterified fatty acids was successfully removed by α-CD scavenging [7, 8]. The cholesterol complex of hydroxypropyl-β-CD has been used as a water soluble primary standard in enzymic cholesterol analysis [9]. The interaction of free fatty acids with β-CD has also been utilised for their removal from vegetable oils [10] and the protection of unsaturated fatty acids against oxidation [11].

Among the various techniques for the determination of CD (host)-ligand (guest) complex formation constants are competitive indicator techniques. These techniques are based on the displacement of an indicator molecule (probe) from the CD cavity and the change in UV-VIS spectra of the probe molecule upon complexation. Each probe can be used in a limited pH range depending on its pK_a. Commonly used probe molecules are phenolphthalein for β-CD complexation in alkaline pH [12, 13] and methyl-orange for both α- and β-CD complexation in acidic pH [14, 15].

Displacement of probe molecules from the CD cavity has also been utilised for the development of spectrophotometric analytical methods for the determination of host and guest molecules. Thus far methods have been developed for the determination of β-CD [16, 17], γ-CD [18], sodium dodecyl sulphate and N, N-dimethyl-N-dodecylamine oxide [19].

In this paper (i) the study of the interaction of β-CD with palmitoleate, linolenate, caprinate and caprylate by the phenolphthalein probe displacement technique is reported and (ii) the use of the phenolphthalein–β-CD complex as a spectrophotometric sensing element for the determination of olive oil acidity is evaluated.

2. Experimental

2.1. REAGENTS AND SOLUTIONS

Palmitoleic acid 99%, linolenic acid 99%, capric acid 99%, caprylic acid 99%, linoleic acid 99%, β-cyclodextrin and Na_2CO_3 were purchased from Sigma. Oleic acid 98% was purchased from Reidel–de Haen and phenolphthalein was purchased from Serva. The purity of fatty acids was determined by titration against KOH standard solutions in n-propanol.

A stock β-CD solution of 1.00×10^{-2} mol L^{-1} and a stock PHP solution of 1.00×10^{-3} mol L^{-1} were prepared by weighing and dissolving the appropriate amounts of β-CD and PHP in the 0.020 mol L^{-1} Na_2CO_3 buffer, pH 10.5. As it is known that β-CD complexes with methanol [15], no methanol was used in the solubilization of PHP to avoid interference in the determination of complexation constants.

A 10 a.d. (acidity degrees, w/w% free fatty acid content expressed as oleic acid) stock oleic acid standard solution was prepared by weighing the appropriate amount of oleic acid and dissolving in a low acidity olive oil ($\sim$0.2 a.d.) that had

been titrated according to the official method [20]. Standards of 0.3–3.6 a.d. were prepared by weighing the appropriate amounts of the 10 a.d. stock oleic acid as it is difficult to pipette olive oil due its viscous nature.

Solutions for the determination of palmitoleate and linolenate complex formation constants: Stock solutions of 1.20×10^{-2} mol L^{-1} palmitoleate and linolenate were prepared by weighing the appropriate amount of acid and dissolving in the Na_2CO_3 buffer. Solubilization was achieved by stirring, solutions were left for the foam to settle and pH was readjusted. As unsaturated fatty acids are prone to atmospheric oxygen oxidation, only freshly prepared solutions were used and care was taken to avoid direct sunlight exposure.

Solutions for the determination of caprinate and caprylate complex formation constants: Mixed working solutions containing β-CD and PHP were prepared using the β-CD and PHP stock solutions. Working solutions of 1.00×10^{-2} mol L^{-1} caprinate and 4.00×10^{-2} mol L^{-1} caprylate were prepared by weighing the appropriate amount of acid and dissolving in the respective β-CD and PHP mixed working solution. After solubilization pH was adjusted to 10.5.

2.2. INSTRUMENTATION AND MEASUREMENT

For the complexation study a Jasco V-550 UV-Vis spectrophotometer was used. The complexation study was conducted using a cell of 1 cm path length that was thermostated at 21.0 ± 0.5 °C by a water bath circulator.

Determination of palmitoleate and linolenate complex formation constants: Solutions of varying fatty acid anion concentrations were prepared by dispensing the appropriate volume of the stock fatty acid solution into 2.00 mL of the β-CD–PHP working solution and filling up to a final volume of 3.00 mL with the Na_2CO_3 buffer. Fatty acid anion concentration ranges used were 0.390–32.1 $\times$ 10^{-4} mol L^{-1}. The absorbance readings of these solutions at 550 nm, the λ_{max} of phenolphthalein, were used for the determination of stability constants.

Determination of caprinate and caprylate complex formation constants: A manual stepwise spectrophotometric titration mode was used. Working β-CD and PHP mixed solutions were titrated by the fatty acid anion working solutions. Fatty acid anion concentration ranges used were 1.92–80.0 $\times$ 10^{-4} mol L^{-1} in the study of caprinate and 0.770–32.0 $\times$ 10^{-3} mol L^{-1} in the study of caprylate.

3. Results and Discussion

3.1. DETERMINATION OF β-CD–FATTY ACID ANION COMPLEX FORMATION CONSTANTS

The reaction of β-CD and PHP is described by the equation

$$\mathrm{CD} + \mathrm{PHP} \overset{K_{\mathrm{PHP}}}{\rightleftharpoons} \mathrm{CDPHP} \qquad (1)$$

and that of β-CD and the fatty acid anions by

$$\mathrm{CD} + \mathrm{FA} \overset{K_1}{\rightleftharpoons} \mathrm{CDFA} \tag{2}$$

$$\mathrm{CDFA} + \mathrm{FA} \overset{K_2}{\rightleftharpoons} \mathrm{CDFA_2}. \tag{3}$$

As the only absorbing species is free PHP:

$$A = \epsilon_{\mathrm{PHP}} b[\mathrm{PHP}], \tag{4}$$

where ϵ_{PHP} is the PHP molecular extinction coefficient and b = 1 cm is the path length. The three mass balance equations, the expressions for the three formation constants (Equations (1)–(3)) and Equation (4) are used without solving by the Scientist software of Micromath Scientific for the determination of K_1 and K_2. The fitting method was nonlinear regression based on a modified Powell algorithm, where the dependent variable was the absorbance and the independent variable the total fatty acid anion concentration. Parameters for calculation are K_1 and K_2 along with K_{PHP}, while ϵ_{PHP} is fixed to the value experimentally determined. As in this spectrophotometric method absorbance readings are related to PHP concentration, a check on the linearity of the PHP calibration curve in 0.020 m $\mathrm{L^{-1}}$ $\mathrm{Na_2CO_3}$ buffer, pH 10.5 was conducted to reject absorbance readings lying out of the linear range. The linear range was found to be 0.068–1.6 $\times$ 10^{-4} mol $\mathrm{L^{-1}}$ where absorbance values measured were 0.16–3.8 A. Beers law was valid in this range (r = 0.999) and ϵ_{PHP} was found to be (2.38 $\pm$ 0.02) $\times$ 10^4 $\mathrm{mol^{-1}}$ L $\mathrm{cm^{-1}}$.

The use of the competitive technique for the determination of β-CD-fatty acid anion complexation constants presupposes that no interaction between ligand (fatty acid anion) and probe (PHP) molecules occurs. To check this assumption, the absorbances of solutions containing a fixed concentration of PHP (5.00 $\times$ 10^{-5} mol $\mathrm{L^{-1}}$) and increasing concentrations of fatty acid anions in the ranges studied were measured. This study proved that for palmitoleate, linolenate, caprinate and caprylate no interaction occurs. For linoleate and oleate a gradual decrease of absorbance as the fatty acid anion concentration increased was observed. This decrease was observed for concentrations higher than 1.3 $\times$ 10^{-3} mol $\mathrm{L^{-1}}$ and 0.9 $\times$ 10^{-3} mol $\mathrm{L^{-1}}$ that are close to the critical micelle concentrations for linoleate and oleate respectively [21]. This decrease was also found to be linearly related to fatty acid anion concentration. Furthermore the PHP calibration curve was also measured in the presence of the highest used oleic anion concentration (2.00 $\times$ 10^{-3} mol $\mathrm{L^{-1}}$). Beers law was found to be valid (r = 0.999) in the same range but ϵ_{PHP} was lower by $\sim$5%. These findings could be accounted for by the assumption that PHP partitions in the micellar phase where it decolorizes. According to these results no attempt was made to calculate complexation constants for linoleate and oleate.

Absorbance versus fatty acid anion concentration data for the unsaturated acids (palmitoleic and linolenic) and for the saturated acids (capric and caprylic) are

shown in Figures 1 and 2, respectively. Data for linoleate and oleate are similar to those presented in Figure 1B but saturation of absorbance vs concentration curves is observed at fatty acid anion concentrations higher than 1.3×10^{-3} mol L^{-1} and 0.9×10^{-3} mol L^{-1} for linoleate and oleate, respectively. This is due to the formation of fatty acid anion micelles. Up to those concentrations a gradual increase of absorbance upon anion addition was observed. The olive oil acidity determination described in this paper was based on this increase as olive oil consists mainly of oleic acid (>55%).

To determine the complexation stoichiometry a scan through the literature revealed that for oleate and elaidate complexation with hydroxypropyl-β-CD, the ratio of the 1 : 1 to 1 : 2 complexation constants is 750 and 29 respectively [27]. Analysis of dehydrated complexes of β-CD with fatty acids revealed 2 : 1 to 1 : 4.5, fatty acid : β-CD stoichiometries for saturated straight chain C_6—C_{12} fatty acids according to the length of their alkyl moiety [28], 1 : 2.3 and 1 : 3.6 for C_{18} derivatives [29] and 1 : 3, 1 : 4.7 and 1 : 3.4 for methyl derivatives of oleic, linoleic and methyl linolenic acid respectively [30]. However in dilute solutions such as those used in the experiments described here, no complexes higher than 1 : 2 should be expected, although in the crystalline state more than two β-CD molecules can be accommodated along the alkyl moiety of the fatty acids. For linoleate in solution there is a discrepancy between the reported stoichiometry: 1 : 1 [31] or 1 : 2 [32, 33].

Data were fitted using both the 1 : 1 and 1 : 2 complexation models. Fitting data to a 1 : 1, fatty acid anion : β-CD complexation model resulted in (a) relative standard deviations of determined K_1 and K_{PHP} values in the range of 60–90%, (b) K_{PHP} values in the range of 6–12 $\times$ 10^4 mol^{-1} L and (c) correlation coefficients of fits in the range of 0.8–0.95. In contrast, data fitting to a 1 : 2, fatty acid anion : β-CD model resulted in (a) relative standard deviations of K_1, K_2 and $K_{PHP} < 30\%$, (b) K_{PHP} values in the range of 2.6–3.3 $\times$ 10^4 mol^{-1} L, that are in reasonable agreement with literature values [12, 13, 22–26], taking into account differences in temperature and ionic strength and (c) correlation coefficients of fits >0.999. This comparison indicates that the complexation stoichiometry is 1 : 2, fatty acid anion : β-CD.

Results for the determination of complex formation constants are shown in Tables I and II for the unsaturated and saturated acids, respectively. Complex formation constants determined for the 1 : 1 and 1 : 2 fatty acid anion : β-CD complex were $(1.2 \pm 0.2) \times 10^4$ and $(2.9 \pm 0.2) \times 10^2$ mol^{-1} L for palmitoleate, $(9.3 \pm 0.9) \times 10^3$ and $(1.1 \pm 0.1) \times 10^2$ mol^{-1} L for linolenate, $(4.1 \pm 0.2) \times 10^3$ and 81 ± 8 mol^{-1} L for caprinate, and $(5.2 \pm 0.7) \times 10^2$ and 27 ± 5 mol^{-1} L for caprylate respectively. Unfortunately no literature values were found for comparison. Complex formation constants determined for the 1 : 1 (K_1) and 1 : 2 (K_2) complex are lower for saturated than unsaturated acids. This is in accordance with the finding that extraction of a mixture of saturated and unsaturated fatty acids by β-CD enhances the unsaturated fraction [11].

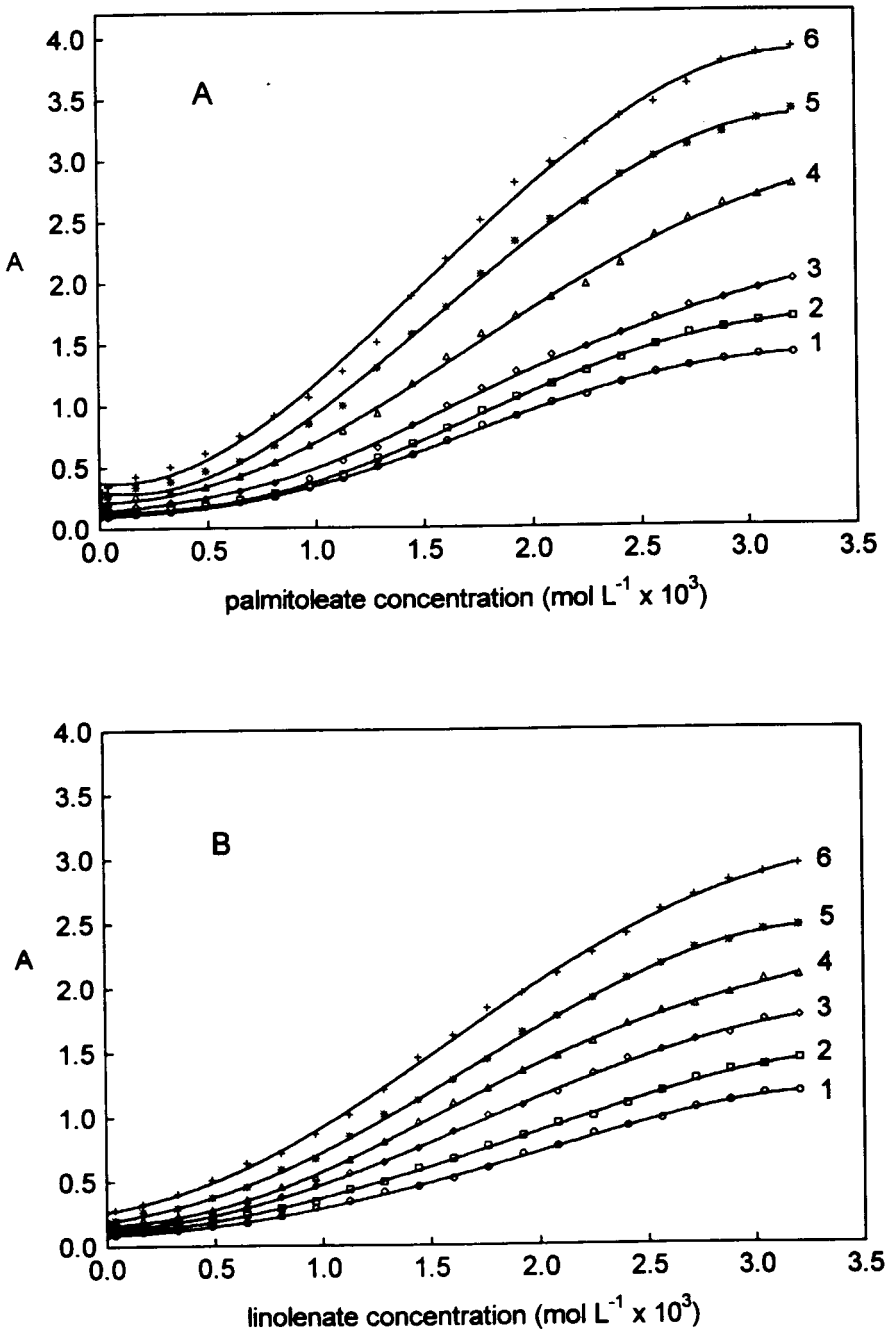

Figure 1. Absorbance data for the PHP displacement by the unsaturated fatty acid anion moiety in the β-CD cavity at 21.0 $\pm$ 0.5 °C and 1.00 $\times$ 10^{-3} mol L^{-1} β-CD. Data for (A) palmitoleate; and (B) linolenate; (1) 1.00, (2) 1.20, (3) 1.50, (4) 2.00, (5) 2.50, and (6) 3.00 $\times$ 10^{-4} mol L^{-1} PHP. The concentration range of fatty acid anion used was 0.390–32.1 $\times$ 10^{-4} mol L^{-1} for both acids.

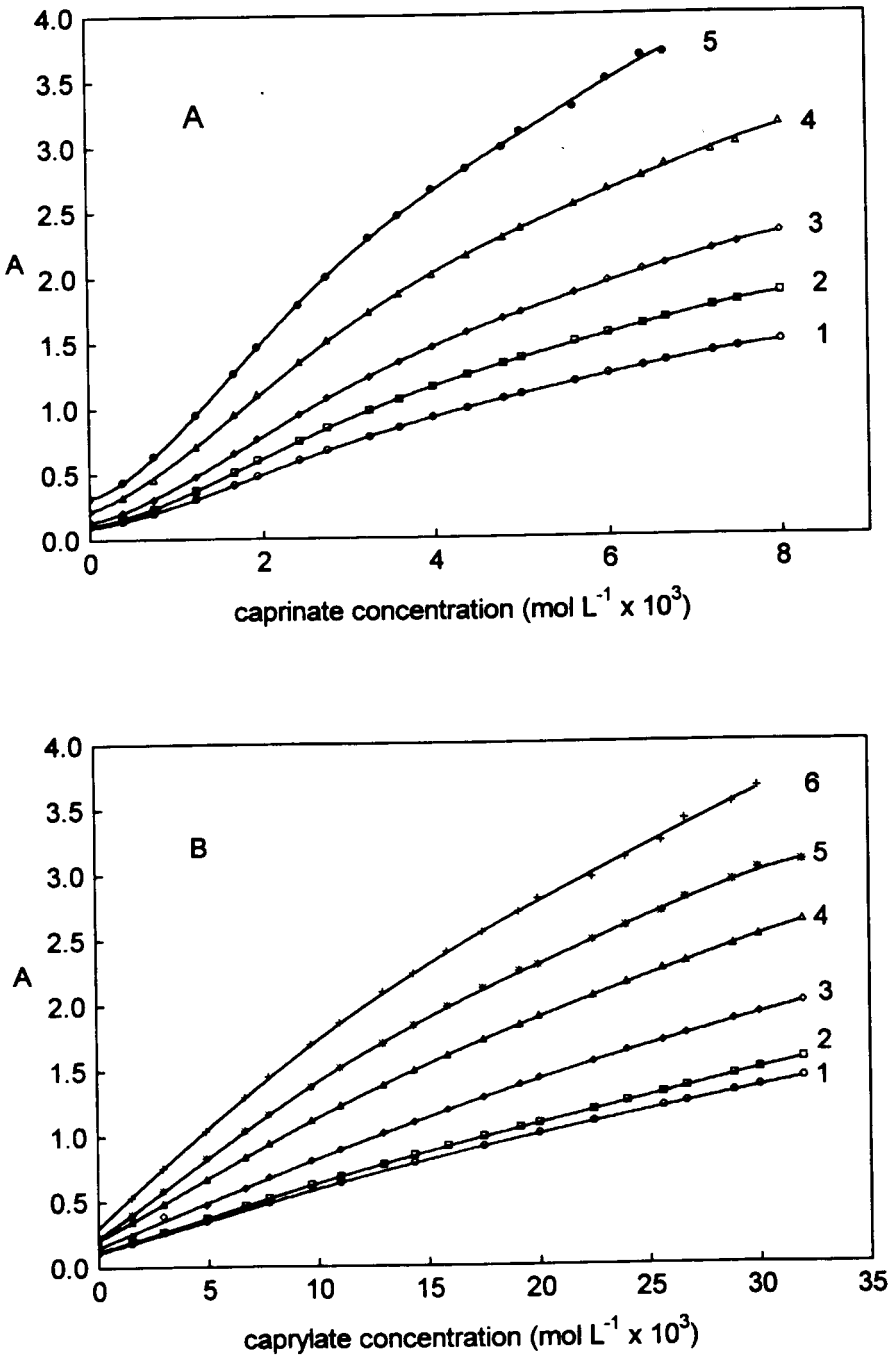

Figure 2. Absorbance data for the PHP displacement by the saturated fatty acid anion moiety in the β-CD cavity at 21.0 ± 0.5 °C and 1.00×10^{-3} mol L^{-1} β-CD. Data for (A) caprinate; and (B) caprylate; (1) 1.00, (2) 1.20, (3) 1.50, (4) 2.00, (5) 2.50, and (6) 3.00×10^{-4} mol L^{-1} PHP. The concentration range of fatty acid anion used was 1.92–80.0 $\times$ 10^{-4} and 0.770–32.0 $\times$ 10^{-3} mol L^{-1} for caprinate and caprylate respectively.

Table I. Complex formation constants for the unsaturated fatty acid anions palmitoleate and linolenate at 21.0 ± 0.5 °C

PHP concentration (mol $L^{-1} \times 10^4$)	Palmitoleate			Linolenate		
	$K_1 \pm$ SD (mol^{-1} L $\times 10^{-4}$)	$K_2 \pm$ SD (mol^{-1} L $\times 10^{-2}$)	$K_{PHP} \pm$ SD (mol^{-1} L $\times 10^{-4}$)	$K_1 \pm$ SD (mol^{-1} L $\times 10^{-3}$)	$K_2 \pm$ SD (mol^{-1} L $\times 10^{-2}$)	$K_{PHP} \pm$ SD (mol^{-1} L $\times 10^{-4}$)
1.0	1.5 ± 0.1	2.7 ± 0.5	3.0 ± 0.2	8.9 ± 0.6	1.0 ± 0.2	2.9 ± 0.1
1.2	1.07 ± 0.05	2.9 ± 0.3	2.9 ± 0.1	8.7 ± 0.5	1.2 ± 0.3	2.9 ± 0.1
1.5	1.01 ± 0.06	2.8 ± 0.3	3.2 ± 0.1	10.1 ± 0.6	1.2 ± 0.3	3.2 ± 0.1
2.0	1.1 ± 0.1	3.0 ± 0.4	3.1 ± 0.2	10.7 ± 0.6	1.1 ± 0.2	3.0 ± 0.2
2.5	1.4 ± 0.2	3.1 ± 0.5	3.3 ± 0.4	9.4 ± 0.8	1.0 ± 0.3	3.1 ± 0.3
3.0	1.2 ± 0.2	2.7 ± 0.6	3.1 ± 0.4	8.3 ± 0.7	1.0 ± 0.3	3.3 ± 0.2
Mean:	1.2 ± 0.2	2.9 ± 0.2	3.1 ± 0.1	9.3 ± 0.9	1.1 ± 0.1	3.1 ± 0.2

Table II. Complex formation constants for the saturated fatty acid anions caprinate and caprylate at 21.0 ± 0.5 °C

PHP concentration (mol $L^{-1} \times 10^4$)	Caprinate			Caprylate		
	$K_1 \pm$ SD (mol^{-1} L $\times 10^{-3}$)	$K_2 \pm$ SD (mol^{-1} L)	$K_{PHP} \pm$ SD (mol^{-1} L $\times 10^{-4}$)	$K_1 \pm$ SD (mol^{-1} L $\times 10^{-2}$)	$K_2 \pm$ SD (mol^{-1} L)	$K_{PHP} \pm$ SD (mol^{-1} L $\times 10^{-4}$)
1.0	3.8 ± 0.1	69 ± 3	3.0 ± 0.1	4.8 ± 0.5	36 ± 4	2.9 ± 0.2
1.2	4.3 ± 0.2	83 ± 4	3.2 ± 0.1	4.7 ± 0.3	29 ± 2	3.2 ± 0.1
1.5	4.4 ± 0.1	80 ± 3	3.2 ± 0.1	4.4 ± 0.3	30 ± 2	2.9 ± 0.1
2.0	4.1 ± 0.1	80 ± 4	3.0 ± 0.1	5.2 ± 0.2	22 ± 1	2.6 ± 0.1
2.5	4.0 ± 0.3	91 ± 8	2.9 ± 0.1	5.9 ± 0.3	22 ± 1	3.3 ± 0.1
3.0	ND	ND	ND	6.0 ± 0.5	24 ± 1	3.0 ± 0.2
Mean:	4.1 ± 0.2	81 ± 8	3.1 ± 0.1	5.2 ± 0.7	27 ± 5	3.0 ± 0.2

ND: not determined.

3.2. STUDY ON THE USE OF THE β-CD–PHP SYSTEM AS A SENSING ELEMENT FOR THE DETERMINATION OF OLIVE OIL ACIDITY

Displacement of PHP from the β-CD cavity by fatty acid anions might provide a suitable sensing system for the determination of olive oil free fatty acid content as for a limited concentration range the absorbance is almost linearly related to concentration. To exploit this, methanol was used for extracting free fatty acids from olive oil as β-CD also complexes triglycerides. As it is known that methanol perturbs the β-CD equilibrium by complexing with β-CD [15], experiments to measure the influence of methanol were conducted for 3, 5 and 33% (v/v) methanol concentration. In this study, solutions of 1.25×10^{-4} mol L^{-1} PHP and 0.500×10^{-3} mol L^{-1} β-CD containing varying oleate concentrations in the range 0–2.00×10^{-3} mol L^{-1} were used. On increasing the methanol concentration the absorbance values increase due to PHP displacement. At the 33% methanol concentration, addition of increasing oleate amounts produces smaller absorbance changes while the initial absorbance value before oleate addition is tripled (1.2 A). At 5% and 3% concentration absorbance data are shifted to higher values by 0.12 and 0.10 A units respectively. As there is only a slight difference between absorbance data at 3 and 5% (v/v) methanol, solutions containing 5% (v/v) methanol were used. Extraction of free fatty acids from olive oil was conducted by using a 1 : 2, 1 : 3 and 1 : 4 v/v ratio of olive oil : methanol. Then after centrifugation at 3600 rpm for 10 min, 1.00 mL of the supernatant was mixed with 5.67 mL of the Na_2CO_3 buffer and 1.00 mL of the resulting solution was mixed with 2.00 mL of the working solution containing 1.87×10^{-4} mol L^{-1} PHP and 0.750×10^{-3} mol L^{-1} β-CD. Calibration curves obtained were: $A = (0.53 \pm 0.02) + (0.27 \pm 0.01) \times C$ ($r = 0.991$), $A = (0.47 \pm 0.02) + (0.24 \pm 0.01) \times C$ ($r = 0.995$) and $A = (0.40 \pm 0.02) + (0.21 \pm 0.01) \times C$ ($r = 0.995$) for 1 : 2, 1 : 3 and 1 : 4 extraction ratio respectively, the concentration expressed in a.d. and the linear range extending from 0.4 to 3.6 a.d. This range is suitable for olive oil analysis as extra virgin olive oil has an acidity less than 1 a.d., fine virgin olive oil has an acidity less than 2 a.d. and courante virgin olive oil has an acidity less than 3.3 a.d. [34]. Results from recovery experiments are shown in Table III where recoveries are shown to range from 84.9 to 120%. Results from analysis of twenty samples differed from those obtained through the official EEC method [20] from −16 to +21%. The differences from the official method and the big range of % recoveries is due to the assumption that only oleic acid contributes to olive oil acidity or that the fatty acid composition of standards and samples is the same. Only under this assumption are calibration curves valid as each fatty acid anion has its own response in the β-CD–PHP system used. Analytical methods with similar limitations concerning the composition of the standards are the turbidimetric [35] and the phosphoric-vanillin [36] methods used in clinical laboratories for the determination of total lipids in serum where deviation in the fatty acid composition of samples from standards results in gross errors.

Table III. Recovery of oleic acid from commercial olive oils

Free fatty acid content (w/w% as oleic acid)			
Found	Added*	Recovered	Recovery,%
0.533	0.400	0.419	104.7
	0.800	0.705	88.1
	1.20	1.08	90.0
1.24	0.400	0.44	110
	0.800	0.679	84.9
	1.20	1.08	90.0
2.38	0.400	0.340	85.0
	0.800	0.96	120
	1.20	1.32	110
		Mean	98.1

*Added by weighing the appropriate amounts of oleic acid and olive oil.

4. Conclusions

The competitive indicator technique using PHP as a probe has proved to be useful for the determination of fatty acid anion–β-CD complex formation constants for saturated and unsaturated straight chain fatty acid anions. To our best knowledge the values determined in this work are the first published. PHP displacement from the β-CD cavity was also used to develop a method for the determination of olive oil acidity.

Acknowledgement

We gratefully acknowledge support, in the form of a scholarship to S.G.S., from the Greek State Scholarships Foundation.

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