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**Settore scientifico disciplinare di afferenza:** CHIM/08

STUDIES ON THE INTERACTION OF  
SURFACTANTS AND NEUTRAL  
CYCLODEXTRINS BY CAPILLARY  
ELECTROPHORESIS. APPLICATION TO  
CHIRAL ANALYSIS.

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“Quando il saggio indica la luna,  
lo stolto guarda il dito.”  
(proverbio cinese)

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To my Mum

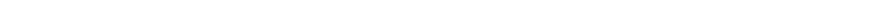
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## Preface

In their pioneering papers of 1984 e 1985 Terabe et al. discovered that chromatographic process can take place in a CE environment. In details the separation is achieved using additive (named *separation carrier* or *pseudostationary phase*) having a velocity different from that of the analytes to be separated. In the last two decades electrokinetic chromatography (EKC) has been mainly regarded as a special mode of capillary electrophoresis (CE), becoming a powerful technique that brings speed, reproducibility and automation to the intensive methods of classical electrophoresis.

Usually the additive present in the electrophoretic background, pseudostationary phase, is a micelle-forming ionic surfactant. This approach is named Micellar Electrokinetic Chromatography (MEKC). It was also realized very early by Terabe that the presence of micelles is not a prerequisite of EKC: over time many variants of EKC have been developed by employing as a separation carrier 'polymeric micelles', microdroplets, other types of colloidal phases, dissolved linear polymers and dendrimers or oligomeric units. Despite all these variants however, MEKC is the most known and wieldiest applied technique. It is important to note that among the many ionic surfactants available, sodium dodecyl sulfate (SDS) is the most used. It possesses a long alkyl chain as the hydrophobic group and an ionic group as the polar group. The micelle formed by SDS is believed to be spherical in shape, with the polar groups being located in the outer zone of the micelle and the alkyl groups constituting the hydrophobic core.



A further additive able to establish interactions with the analytes that have to be separated can be supplemented into the background electrolyte (BGE) together with the primary surfactant. The solutes can interact differently with both the separation media and an improved selectivity is often achieved. One of the most used dual-pseudostationary phase systems is represented by cyclodextrin-modified micellar electrokinetic chromatography (CD-MEKC), mainly applied in chiral analysis and for the resolution of complex mixtures of hydrophobic compounds. The presence of CDs introduces new equilibria in the separation medium (e.g. inclusion of SDS monomer into the CD cavity). The aim of this PhD thesis is a study to provide a contribution towards the adequate understanding of the interactions between the components of the most commonly used BGE in CD-MEKC: SDS and neutral CDs. This information can be useful in order to properly apply these coupled systems in selective (and enantioselective) analysis of complex samples. In presence of micelles and cyclodextrins in the electrophoretic background, the analytes can establish different kind of interactions: (i) analytes-micelles, (ii) analyte- cyclodextrin, (iii) analyte-(cyclodextrin-surfactant's monomer). Furthermore, the interaction between cyclodextrin and micelles should be taken into account as well. The research was carried out with the aim to investigate the interaction between neutral cyclodextrins and sodium dodecyl sulfate (SDS).

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**Chapter 1:**  
**Sodium Dodecyl Sulfate**  
**Critical Micelle Concentration**

## Sodium Dodecyl Sulfate and Critical Micelle Concentration

### Introduction

It has been widely recognized that the physical properties of surfactant solutions, such as surface tension, osmotic pressure, electrical conductivity, and solubility (as a function of temperature), show an abrupt change in the neighbourhood of the critical concentration occurs. This unusual behaviour was first investigated by McBain on fatty acid salts in dilute aqueous solution (1, 2) and later by Hartley (3). Other evidence for molecular aggregation was obtained from vapour pressure measurements and the solubility of organic material. The formation of colloidal-sized clusters of individual surfactant molecules in solution is now better known as micellization. Although first suggested by McBain, the earliest concrete model for spherical micelles is attributed to Hartley *et al.* (4). The process of surfactant clustering or micellization is primarily an entropy-driven process (5, 6). When surfactants are dissolved in water, the hydrophobic group disrupts the hydrogenbonded structure of water and therefore increases the free energy of the system. Surfactant molecules therefore concentrate at interfaces, so that their hydrophobic groups are removed or directed away from the water and the free energy of the solution is minimized. The distortion of the water structure can also be decreased (and the free energy of the solution reduced) by the aggregation of surface-active molecules into clusters (micelles) with their

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hydrophobic groups directed toward the interior of the cluster and their hydrophilic groups directed toward the water.

However, the surfactant molecules transferred from the bulk solution to the micelle may experience some loss of freedom from being confined to the micelle. In addition, they may experience an electrostatic repulsion from other similarly charged surfactant molecules in the case of surfactants with ionic head groups. These forces increase the free energy of the system and oppose micellization. Hence, micelle formation depends on the force balance between the factors favouring micellization (van der Waals and hydrophobic forces) and those opposing it (kinetic energy of the molecules and electrostatic repulsion). The explanation for the entropy-dominated association of surfactant molecules is called the “hydrophobic effect” or “hydrophobic bonding” (7).

The concentration at which micelles first appear in solution is called the critical micelle concentration (CMC) and can be determined from the discontinuity or inflection point in the plot of a physical property of the solution as a function of the surfactant concentration.

Representing the surfactant by  $S$ , the micellization process can be described by the reaction:



where  $S_n$  is a micellar aggregate composed of  $n$  surfactant molecules. The aggregation number  $n$  (which represents the number of surfactant molecules in a micelle) has been found to increase with increasing length of the hydrophobic group and decrease with increasing size of the hydrophilic group (10). In general, the greater the hydrocarbon chain length of the surfactant molecules, the greater the aggregation number of

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micelles. Also, those factors that increase the aggregation number tend to decrease the CMC. For example, increasing the alkyl chain length of a surfactant decreases the CMC. The presence of electrolyte also decreases the CMC, due to the “salting out” effect. When surfactant monomers are salted out by the presence of an electrolyte, micellization is favoured and the CMC is decreased.

It is important to emphasize that CMC represents the concentration of free surfactant monomers in a micellar solution under given conditions of temperature, pressure, and composition.

### *Structure of a Micelle*

The recognition that surfactant association structures can mimic biological structures has sparked considerable interest in self-assembled surfactant aggregates such as cylindrical, lamellar, and reverse micelles.

Lipid aggregates known as liposome are common in physiological systems, and tailored liposomes are used, for example, as drug-delivery vehicles or in cosmetics (8). Self-assembled structures such as micelles or reversed micelles (surfactant aggregates with hydrophilic head groups shielded from, and lipophilic tails sticking out to an organic solvent) also play an increasingly important role in catalysis and separation processes in engineering and environmental science and technology (9–10).

In aqueous media, for example, surfactants with bulky or loosely packed hydrophilic groups and long, thin hydrophobic chains tend to form spherical micelles, while those with short, bulky hydrophobic chains and small, close-packed hydrophilic groups tend to form lamellar or cylindrical micelles.

## Chapter 1: SDS and CMC

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At concentrations slightly above the CMC, micelles are considered to be of spherical shape. Changes in temperature, surfactant concentration, or additives in the solution may change the size, shape, aggregation number, and stability of the micelles.

### *Dynamic properties of surfactants solution*

Micelles are often drawn as static structures of spherical aggregates of oriented surfactant molecules. However, micelles are in dynamic equilibrium with individual surfactant molecules that are constantly being exchanged between the bulk and the micelles.

Additionally, the micelles themselves are continuously disintegrating and reassembling. There are two relaxation processes involved in micellar solutions. The first is a fast relaxation process referred to as  $\tau_1$  (generally on the order of microseconds), which is associated with the quick exchange of monomers between micelles and the surrounding bulk phase. This process can be considered to be the collision between surfactant monomers and micelles. The second relaxation time,  $\tau_2$  (on the order of milliseconds), is attributed to the micelle formation and dissolution process (i.e., the lifetime of the micelle).

It has been shown that in certain surfactants such as non-ionic surfactants and mixed surfactant systems,  $\tau_2$  can be as long as minutes!

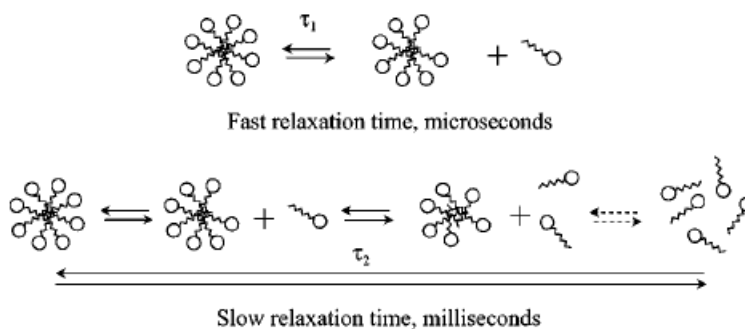


Fig. 1: Mechanisms for the two relaxation times,  $\tau_1$  and  $\tau_2$ , for a surfactant solution above CMC (11).

Figure 1 shows the two characteristic relaxation times,  $\tau_1$  and  $\tau_2$ , associated with micellar solutions. Micelle formation and disintegration is analogous to the equilibrium between water and water vapour at a given temperature and pressure. For a closed system containing liquid water and water vapour in equilibrium, the number of water molecules per unit area per second evaporating from the surface is equal to the number of water molecules condensing at the surface. Thus, the total number of molecules in the vapour phase or in the liquid does not change with time, so the rate of condensation is equal to the rate of evaporation. The same principle holds for a micellar solution. Under equilibrium conditions, the rate of micelle formation is equal to the rate of disintegration into surfactant monomers.

Micellar relaxation kinetics show dependence on temperature, pressure, and concentration, as well as on the addition of other species such as short-chain alcohols. It was shown that the  $\tau_2$  of an SDS micelle decreases with increased concentration of C1– C5 alcohols (11). These kinetics have been studied by various techniques such as stopped-flow,

temperature-jump, pressure-jump, and ultrasonic absorption (12). The two relaxation times can be used to calculate two important parameters of a micellar solution: (a) the residence time of a surfactant molecule in a micelle and (b) the average lifetime or stability of a micelle.

The described methods provide a direct description and characterization of the dynamic properties of micelles. However other physical-chemical methods can be applied to gain information on the micelle architecture (13).

**Sodium Dodecyl Sulfate: determination of critical micelle concentration by capillary electrophoresis.**

The optimization of the analytical conditions and the separation of analytes in capillary electrophoresis (CE) have been the subjects of an important field of research (12-15). The study of the micellization process being a key parameter in the optimization of analytical conditions in CE, particularly in Micellar Electrokinetic Chromatography (MEKC), a good understanding of the micellization of a surfactant is of paramount importance; thus, the determination of the CMC of surfactants under the operating conditions of a system is certainly desirable. A number of methods, including electrical conductivity (16–19), surface tension (20), light scattering (21,22), spectrophotometry (18,19,23), cyclic voltammetry (24), NMR (25), speed of sound (26), CE (27–28), etc., have been used to determine the CMC of surfactants. The CMC value of a surfactant is affected by the operating conditions of an electrophoretic system, such as the nature of the buffer electrolyte, the type and composition of the electrolyte solution (28, 34), buffer pH (29,30), the ionic strength of the electrolyte solution (28,29,31), the type



## Chapter 1: SDS and CMC

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of counter-ion of the electrolyte solution (32), the type of counter-ion of the surfactant (32), the presence of various organic modifiers (33), temperature (35), and the nature of solubilised solutes (36). CE is conveniently applied to the determination of the CMC in an electrophoretic system under any of the operating conditions, which are desirable to be applied. Several approaches based on CE technique have been proposed to determine the CMC values of surfactants (38-40, 27–28). Among them, three major methods are emphasized: the first method, proposed by Terabe et al. (41), is based on the linear relationship of the retention factor of a solute with micelle concentration using MEKC technique. It is the *zonal method*, which is based on the migration speed monitoring of a micelle-interacting marker injected as an analyte in a capillary filled with a surfactant solution.

The second method is based on the variation of the effective electrophoretic mobility of a marker compound as a function of surfactant concentration in the premicellar and micellar regions. By plotting the effective electrophoretic mobility of a marker compound against surfactant concentration, a sharp change in slope can be observed at the CMC (27–32). The third method is based on the measurements of the electric current of micellar electrolyte solutions as a function of surfactant concentration using CE instrumentation at a given applied voltage (42). The practical requirements for making CMC measurements and the CMC values of surfactants determined by CE methods are presented. In addition, difficulties, uncertainty, and misconceptions that may arise in the CMC determination are discussed.

### Methodological approaches

*Method based on the retention model—micellar electrokinetic chromatography method*

It has been known that the effective electrophoretic mobility of a neutral solute ( $\mu_{\text{eff}}$ ) in MEKC is proportional to the mobility of the micellar phase ( $\mu_{\text{mc}}$ ) and is given by (43):

$$\mu_{\text{eff}} = (k/1 + k) \mu_{\text{mc}} \quad (1)$$

where  $k$  is the retention factor of the solute and the term  $k/(1 + k)$  represents the mole fraction of the solute in the micellar phase. Eq. (1) can be rearranged and expressed as:

$$k = \mu_{\text{eff}}/\mu_{\text{mc}} - \mu_{\text{eff}} \quad (2)$$

In CE, the electrophoretic mobility of a solute is related to the migration times by:

$$\mu_{\text{eff}} = (1/t_r - 1/t_{\text{eo}})(L_t L_d/V) \quad (3)$$

where  $t_r$  and  $t_{\text{eo}}$  are the migration time of the solute and that of the neutral marker, respectively,  $L_t$  and  $L_d$  the total length of the capillary and the distance from the upstream end to the detector, respectively, and  $V$  is the applied voltage. By substituting migration times for the mobilities in Eq. (2), the retention factor can be expressed in terms of migration times as:

$$k = \frac{t_r - t_{\text{eo}}}{t_{\text{eo}} (1 - t_r/t_{\text{mc}})} \quad (4)$$

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where  $t_{mc}$  is the migration time of a micelle marker. Accordingly, the retention factor of a neutral solute can be calculated from the migration times. For an anionic solute, the effective electrophoretic mobility can be described as the weighted average of the mobility of the solute in the micellar phase and its own mobility in the aqueous phase and is given by:

$$\mu_{eff} = \left(\frac{k}{1+k}\right)\mu_{mc} + \left(\frac{1}{1+k}\right)\mu_0 \quad (5)$$

where  $\mu_0$  is the mobility in the absence of micelles in the aqueous phase. Similarly, Eq. (5) can be rearranged and expressed as:

$$k = \frac{\mu_{eff} - \mu_0}{\mu_{mc} - \mu_{eff}} \quad (6)$$

and

$$k = \frac{t_r - t_0}{t_0(1 - t_r/t_{mc})} \quad (7)$$

where  $t_0$  is the migration time of the anionic solute in the absence of micelles.

For an acidic solute, the situation becomes more complicated. The effective electrophoretic mobility is expressed as the weighted average of a solute with the mobility of the micellar phase and its own mobility in the aqueous phase as described in Eq. (5). However, depending on the pH of the buffer electrolyte, the electrophoretic mobility of an acidic solute in the absence of micelles is expressed as

$$\mu_0 = \frac{K_a}{K_a + [H^+]} \mu_{A^-} \quad (8)$$

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SDS and CMC

where  $\mu_{A^-}$  is the mobility of the fully dissociated species and  $K_a$  is the acid dissociation constant. Therefore, the retention factor of an acidic solute can be expressed as the weighted average of the retention factor of its undissociated form ( $k_{HA}$ ) and that of the fully dissociated form ( $k_{A^-}$ ) as:

$$k = \left( \frac{[H^+]}{[H^+] + K_a} \right) k_{HA} + \left( \frac{K_a}{[H^+] + K_a} \right) k_{A^-} \quad (9)$$

$$k = \frac{k_{HA} + (K_a/[H^+])k_{A^-}}{1 + K_a/[H^+]} \quad (10)$$

It should be noted that, in this case, the interactions of a selected acidic solute with surfactant monomers are usually assumed to be negligibly small. For a basic solute with an anionic surfactant as a micelle forming agent, the equilibrium involves ion-pairing interaction between the cationic solute and the anionic micelles. Again, the effective electrophoretic mobility of a basic solute can be described as the weighted average of a solute with the mobility of the micellar phase and its characteristic mobility in the aqueous phase by the equation:

$$\mu_{\text{eff}} = \frac{K_b / (1 + K_b + K_b K_1)}{1 + k} \mu_c + \frac{k}{1 + k} \mu_{\text{mc}} \quad (11)$$

where  $\mu_c$  is the electrophoretic mobility of the non ion-paired species,  $K_b$  is defined as the base constant of the solute divided by the concentration of the hydroxide ion, and  $K_1$  is the product of the CMC and the ion-pairing equilibrium constant. Thus,  $k$  can be expressed as:

$$k = \frac{\mu_{\text{eff}} - [K_b / (1 + K_b + K_b K_1)] \mu_c}{\mu_{\text{mc}} - \mu_{\text{eff}}} \quad (12)$$

where

$$\mu_c = \mu_0(1 + K_b)/K_b.$$

On the other hand,  $k$  is related to the partition coefficient of a solute between the micellar and aqueous phases ( $P_{mw}$ ) and the phase ratio ( $V_{mc}/V_{aq}$ ) by the equation:

$$k = (P_{mw}V_{mc})/V_{aq} \quad (13)$$

The phase ratio is governed by three parameters as shown in the following equation :

$$\frac{V_{mc}}{V_{aq}} = \frac{\tilde{V}(C_T - CMC)}{1 - \tilde{V}(C_T - CMC)} \quad (14)$$

where  $\tilde{V}$  and  $C_T$  are the molar volume and total surfactant concentration, respectively. At low micellar concentrations, the phase ratio is approximately equal to  $\tilde{V}(C_T - CMC)$ . In this case,  $k$  is linearly related to  $C_T$  by the following equation:

$$k = P_{mw}\tilde{V}(C_T - CMC) \quad (15)$$

By plotting  $k$  against  $C_T$ , the CMC of a surfactant can be easily determined from Eq. (15).

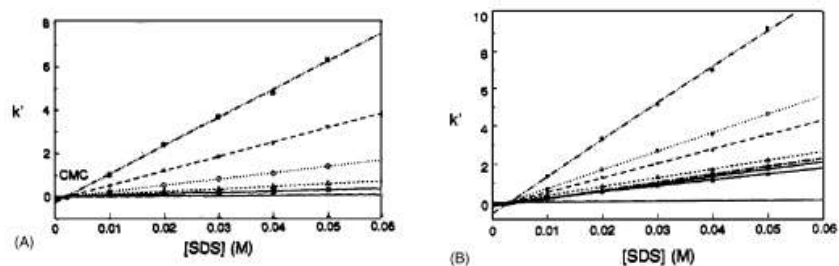


Fig. 2 (26): Relationship between  $k$  and [SDS] for A) some neutral solutes: 2-naphthol (■), toluene (\*), nitrobenzene (○), phenol (Δ) and resorcinol (□) and B) some anionic solutes (chlorophenols, CPs): 2CP (□), 3CP (Δ), 23CP (○), 25CP (\*), 245CP (■), 246CP (▲) and pentaCP(●). Electropherograms measured in 50mM phosphate buffer at pH 7.0 (40°)

Fig. 2A shows the plots of  $k$  versus sodium dodecylsulfate (SDS) concentration for some neutral solutes (benzene derivatives) applying the Eq. (4) to evaluate  $k$  values and in fig. 2B anionic solutes (chlorophenols) using Eq. (7) also to evaluate  $k$  values. As shown, all lines almost pass through the same intercept, and the slope of the line ( $P_{mw}$ ) increases with the hydrophobicity of the compound. The results indicate that a stronger interaction between the selected test solute and the surfactant may yield a smaller error in the determination of the CMC value (26).

*Method based on the mobility model—capillary electrophoresis (mobility) method*

In the evolution of the effective electrophoretic mobility of a marker compound as a function of surfactant concentration in the pre-micellar and micellar regions, a dramatic change in slope at a particular surfactant

concentration is observed. This particular concentration is a good indication of the CMC of the surfactant.

The method based on this concept was first introduced by Jacquier and Desbene (27) using naphthalene as a marker compound for determining the CMC of SDS. A sharp change in slope was observed at around 5mM when mobility curves were plotted as a function of SDS concentration in the premicellar and micellar regions. The mobility equations for describing the migration behavior of naphthalene in the premicellar and micellar concentration regions are given by:

$$\mu_{\text{eff}} = \frac{K_{\text{solv}}[C_T]}{1 + K_{\text{solv}}[C_T]} \mu_{\text{solv}} \quad (C_T < \text{CMC}) \quad (16)$$

and

$$\mu_{\text{eff}} = \frac{K_{\text{solv}}[\text{CMC}]}{1 + K_{\text{solv}}[\text{CMC}]} \mu_{\text{solv}} + \frac{K_{\text{mc}}[M]}{1 + K_{\text{mc}}[M]} \mu_{\text{mc}} \quad (C_T > \text{CMC}) \quad (17)$$

where  $K_{\text{solv}}$  and  $\mu_{\text{solv}}$  are the binding constant and the limiting mobility of solvophobic complexes formed between the test solute and surfactant monomers through solvophobic interactions,  $K_{\text{mc}}$  is the binding constant of the solute to the micelles, and  $[M]$  is the micelle concentration which is equal to  $(C_T - \text{CMC})/n$ , where  $n$  is the aggregation number. When the interaction between the selected neutral solute and surfactant monomers becomes significantly strong, the mobility equation for describing the migration behaviour of solutes in the micellar concentration region needs to be modified as follows (44):

$$\mu_{\text{eff}} = \frac{K_{\text{AS}}(\text{CMC})\mu_{\text{AS}} + K_{\text{AM}}[M]\mu_{\text{mc}}}{1 + K_{\text{AS}}(\text{CMC}) + K_{\text{AM}}[M]} \quad C_T > \text{CMC} \quad (18)$$

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where  $K_{AS}$  and  $\mu_{AS}$  (corresponding to  $K_{solv}$  and  $\mu_{solv}$ , respectively, in Eq. (17)) are the binding constant and the limiting mobility of the complexes formed between the neutral solute (A) and surfactant monomers(s). Different mobility equations should be derived for describing the migration behaviour of various types of test solutes in different electrophoretic systems. For example, the effective electrophoretic mobility of a negatively charged solute ( $A^-$ ) in the premicellar and micellar concentration regions, respectively, can be described by the following equations (36,37):

$$\mu_{\text{eff}} = \frac{\mu_{A^-} + K_{A-S}[S]\mu_{A-S}}{1 + K_{A-S}[S]} \quad (\text{below the CMC}) \quad (19)$$

and

$$\mu_{\text{eff}} = \frac{\mu_{A^-} + K_{A-S}(\text{CMC})\mu_{A-S} + K_{A-M}[M]\mu_{\text{mc}}}{1 + K_{A-S}(\text{CMC}) + K_{A-M}[M]} \quad (\text{above the CMC}) \quad (20)$$

where  $\mu_{A^-}$  is the electrophoretic mobility of the negatively charged solute,  $K_{A-S}$  and  $\mu_{A-S}$  are the binding constant and electrophoretic mobility, respectively, of the negatively charged solute associated with the anionic surfactant monomers, and  $K_{A-M}$  is the binding constant of the charged solutes to the micelles.

*Practical requirements for making critical micelle concentration measurements: micellar electrokinetic chromatography method and capillary electrophoresis (mobility) method*

A suitable marker compound for making CMC measurement using CE technique the following features should be met. First, the test solutes should have a high UV molar absorptivity for easy detection when using



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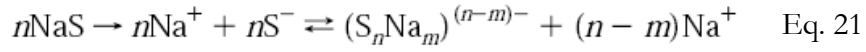
CE instruments with spectrometric detection (27). Second, depending on the nature of surfactants, the test solute should be able to incorporate into the micelles to a certain extent. For example, according to linear solvation energy relationship (LSER) studies (45–47), SDS micelles possess a hydrophobic interior structure with strongly hydrogen bond donating character. Thus, solutes with a greater hydrophobicity and/or with strongly hydrogen bond accepting character intend to incorporate into SDS micelles to a greater extent. This is because the greater the difference in the binding constants of the selected solute with surfactant monomers and with the micelle is, the more dramatic the change in the slope of the curves of the electrophoretic mobility as a function of the total surfactant concentration at the CMC is. Consequently, the more precise will be the CMC value determined. Third, a test solute should have a proper solubility in a micellar electrolyte solution, because lack of solubility may result in a low separation efficiency of the electrophoretic system and uncertainty in the measurement of migration times, thus leading to systematic errors in the determination of the CMC value (27). In order to solve solubility problems and/or to improve separation, selectivity and/or resolution in CE, an organic modifier is frequently added to the electrolyte solution. However, addition of organic modifiers to the micellar electrolyte solutions may induce the modification of the micelle structure and/or micelle properties. In order to obtain consistent and reproducible results, the temperature in the capillary should be well controlled and the electric field should be kept constant for an experiment set.

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**Method based on the measurements of electric current using capillary electrophoresis instrumentation- capillary electrophoresis (current) method**

Cifuentes et al. (42) reported a method of measuring electric current by a CE instrument, based on the concept that the conductivity of ionic surfactants in an electrolyte solution depends on the aggregation state of the surfactant (48). This approach essentially consists of a CE version of the traditional method of measuring the CMC value by conductivity.

Assuming that the micelle is composed of  $n$  ionic monomers or amphiphiles (S-) and it carries  $m$  co-ions (e.g., Na+) induced within the micelle, at concentration not greatly above the cmc, it can be written:



This equilibrium shows that, at concentration of surfactant  $c > \text{cmc}$ , amphiphiles are mainly in a micellar form, i.e.,  $n$  molecules of surfactant plus  $m$  co-ions will aggregate to form a micelle, while at  $c < \text{cmc}$ , surfactant will be in a monomeric form, moving freely in solution in a way similar to that of co-ions at these low concentrations.

On the other hand, in a CE instrument, applying a voltage  $V$  on a capillary of radius  $r$  and total length  $l$ , filled with a surfactant solution, gives an electric current  $I$  according to Ohm's law (50):

$$V = \frac{l}{\pi r^2 (\sigma_{\text{Na}^+} + \sigma_{\text{S}^-} + \sigma_{\text{mic}})} I$$

where  $\delta_{\text{Na}^+}$ ,  $\delta_{\text{S}^-}$ , and  $\delta_{\text{mic}}$  are the specific conductivities of the co-ion, amphiphile, and micelle, respectively. It can be easily deduced from the

## Chapter 1: SDS and CMC

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equilibrium (eq.21), at  $c < \text{cmc}$ , the main contribution to the overall conductivity of the solution comes from  $\delta_{\text{Na}^+}$  and  $\delta_{\text{S}}$ , while at  $c > \text{cmc}$ , the main contribution comes from  $\delta_{\text{mic}}$  with a low number of co-ions, e.g., sodium, moving freely in solution at these high concentrations. The observed decrease in conductivity of solutions of ionic surfactants above the cmc is explained through both inclusion within the micelle of co-ions to that of amphiphiles and the increase in resistance to migration of the micelle caused by the ionic atmosphere of co-ions surrounding this ordered structure.

Thus, in CE, by plotting electric current against different surfactant concentrations at a given voltage, experimental points must fit into two lines whose slopes should be different depending on the range of  $c$  considered. It can be easily observed that the increase of the circulating current is linear under and above the CMC value; moreover, because of the lower conductivity of the micelles compared to monomer of SDS, the slope after the micellization is less abrupt. That is, the two slopes correspond to the monomeric and micellar states of the surfactant. In Fig. 3, the plot of the electric current values obtained for different concentrations of SDS in water at 25 °C is shown. As expected, experimental points fit into two straight lines of different slope. As can be seen in Figure 3, this variation of slope takes place at a SDS concentration of  $\sim 8$  mM.

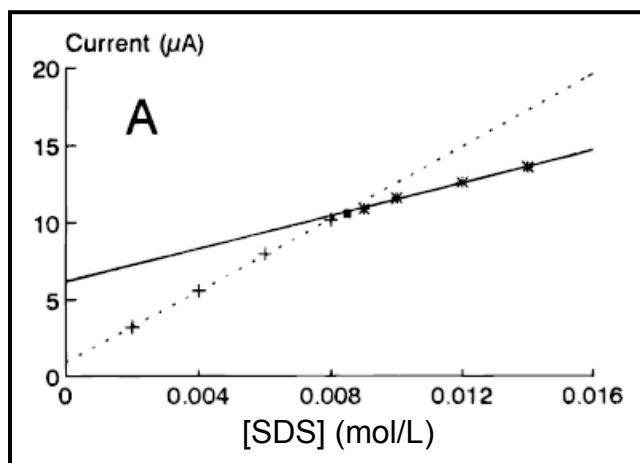


Fig.3: plot of electrical current vs concentration of SDS Fused silica capillary: 75  $\mu\text{m}$  i.d. and 47 cm length. Temperature was kept at 25  $^{\circ}\text{C}$ . SDS in water; current measured at 20 kV.

*Practical requirements for making critical micelle concentration measurements:  
Capillary electrophoresis (current) method.*

As the electric current of an electrolyte system measured by a CE instrument is usually very small, care should be taken to manipulate the sensitivity of this method by choosing the adequate capillary size. Capillary diameter and length, and voltage to be allied should be chosen in order to obtain the adequate current values, preferably, in the 1-100  $\mu\text{A}$ . Especially for low-conductivity surfactant solutions, capillary diameter and voltage should be increased and capillary length decreased. Proper control of Joule heating also requires power to be kept dissipated and the temperature should be strictly controlled.

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**Chapter 2:**  
Micellar Electrokinetic Chromatography

## Micellar Electrokinetic Chromatography

### Introduction

Capillary Electrophoresis (CE) is one of the most important separation techniques in analytical chemistry, comparable to Gas Chromatography (GC) and High Performance Liquid Chromatography (HPLC). CE is suitable in the separation of both small solutes and biomolecules (proteins, peptides, DNA) due to its unique selectivity, high resolution, high efficiency and small sample requirements as well as for the automation of the analytical procedure.

Electrokinetic chromatography (EKC), introduced by Terabe and co-workers in 1985 (1,2), belongs to the family of electromigration separation techniques.

According to the IUPAC recommendation (3), EKC is a separation techniques:

*'based on a combination of electrophoresis and interactions of the analytes with additives (e.g. surfactants), which form a dispersed phase moving at a different velocity [...than the analytes (editorial note)]. In order to achieve separation either the analytes or this secondary phase should be charged'.*

It employs electrokinetic phenomena (electrophoresis and electroosmosis) for the separation of constituents in a sample. EKC invariably also involves chemical equilibria, for example distribution, ion exchange and/or complex formation. In particular, EKC is defined as a capillary electromigration separation technique employing a separation carrier. The separation carrier, also called pseudostationary phase (microdroplet, a micelle, a dendrimer, or a dissolved polymer), interacts

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with the solutes to be separated while its migration velocity is, in general, virtually unaffected by the interaction. If the solutes to be separated do not possess any charge, the separation carrier must have an intrinsic electrophoretic mobility.

Micellar EKC (MEKC) is a 'special case of EKC, in which the separation carrier is a micellar dispersed phase in the capillary' and microemulsion EKC (MEEKC) 'is a special mode of EKC, where a microemulsion is employed as the dispersed phase'.

Micellar electokinetic chromatography (MECK or MECC) can be considered as an hybrid between electrophoresis and chromatography

The separation of neutral species by MEKC is accomplished by the use of charged surfactants in the running buffer. At a concentration above the critical micellar concentration (CMC), for example 8 or 9 mM for sodium dodecyl sulfate (SDS), aggregates of individual surfactant molecules (micelles) are formed. Micelles are essentially spherical with the hydrophobic tails of the surfactant molecules oriented towards the centre to avoid interaction with the hydrophobic buffer, and the charged heads oriented toward the buffer. A representation of micelles is depicted in (fig.1).

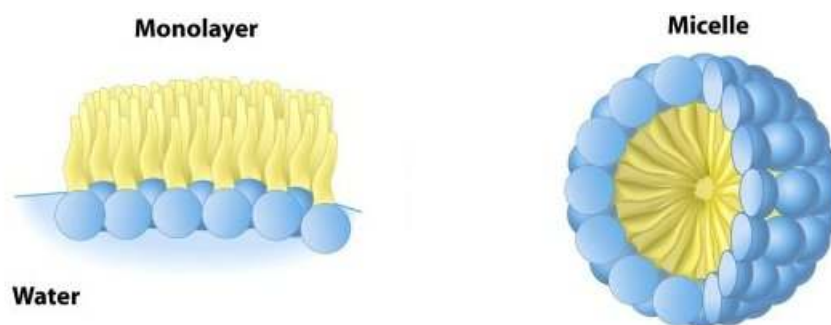


Fig.1: Ionic micelles

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Depending on the charge, the micelles migrate either with or against the electrosmotic flow (EOF), depending on the charge. Anionic surfactants, such as sodium dodecyl sulfate (SDS) migrate toward the anode, that is, in the opposite direction to the EOF. Since the EOF is generally faster than the migration velocity of the micelles at neutral or basic pH, the net movement is in the direction of the EOF. During migration, the micelles can interact with solutes in a chromatographic manner through both hydrophobic and electrostatic interactions.

The more the solute interacts with the micelle the longer is its migration time since the micelle carries against the EOF. When the solute is not in contact with the micelle it is simply carried with the EOF. The more hydrophobic compounds interact more strongly with the micelle and are retained longer.

### **Resolution**

The separation mechanism of neutral solutes in MEKC is essentially chromatographic and can be described using modified chromatographic relationship. The ratio of the total moles of solute in the micelle (pseudo stationary phase) to those in the mobile phase, the capacity factor,  $k'$ , is given by:

$$k = \frac{t_s - t_0}{t_0 (1 - t_s/t_{sc})} \quad \text{Eq.1}$$

where  $t_0$  = migration time of the surrounding (mobile) phase, the electrosmotic flow (EOF),  $t_s$  = migration time of the solute zone,  $t_{sc}$  = migration time of the front of the separation carrier, in specific the migration time of the micelles, also called  $t_m$ .

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This Equation is modified from the normal chromatographic description of  $k$  to account for movement of the pseudostationary phase. Note that if  $t_{sc}$  ( $t_m$ ) becomes infinite (that is when the micelle becomes truly stationary) the equation reduces to its conventional form.

Resolution of two species in MEKC can be described by

$$R = \underbrace{\left(\frac{N^{1/2}}{4}\right)}_{\text{Efficiency}} \underbrace{\left(\frac{\alpha-1}{\alpha}\right)}_{\text{Selectivity}} \underbrace{\left(\frac{k'_2}{k'_2+1}\right)}_{\text{Retention}} \underbrace{\left(\frac{1-(t_0/t_m)}{1-(t_0/t_m)k}\right)}_{\text{Retention}} \quad \text{eq. 2}$$

Where  $N$  is the number of theoretical plate,  $\alpha$  is the selectivity factor given by  $k_1/k_2$ , and  $k_1$  and  $k_2$  are the retention factors of the solutes. It is possible to see from eq. 2 that resolution can be improved by optimizing efficiency, selectivity and/or capacity factor. With regards to the capacity factor, this can be adjusted by varying the surfactant concentration. A potential problem with the use of ionic surfactants, especially at high concentration, is the increase in generated current.

In the separation of neutral solutes all the solutes elute between  $t_0$  and  $t_m$ . Neutral hydrophilic compounds that do not interact with the micelle elute with the EOF and those are totally retained by the micelles elute with the micelles. While the time window is often fairly small, the peak capacity can be very high due to the high efficiency. It is desirable to employ conditions that open the time window, that is moderate EOF and micelles exhibiting high mobility.

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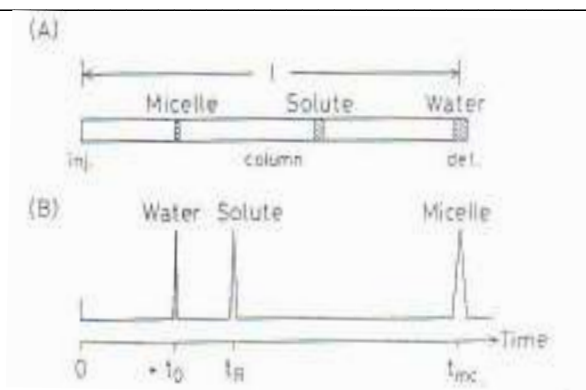


Fig.2: Time window in MEKC: A) separation inside the capillary; B) explanation of time window through an electropherogram.

In MEKC, as in capillary zone electrophoresis, the higher the applied voltage, the higher the  $N$  value and the higher the resolution, unless the temperature increase too high. Usually a high voltage of 10-30 kV is applied to perform the MEKC separation and 100000-300000 theoretical plates are obtained with a 50cm length and 50 $\mu$ m i.d. capillary tube within a relatively short time.

The retention factor  $k$  is important in increasing the resolution in MEKC. The optimum  $k$  value to give the highest resolution is determined by the following equation:

$$k_{opt} = (t_{mc}/t_0)^{1/2} \quad \text{Eq. 3}$$

The result given by eq.3 suggests that the retention factor should not be close to either extreme for separation by MEKC.

The selectivity factor  $\alpha$  is the most important and the most effective term to increase resolution and can be manipulated through selection of surfactants and modification of the buffer solution.

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MEKC

**Selectivity**

The selectivity can be manipulated in MEKC; varying the physical nature (size, charge, geometry) of the micelles by using different surfactants, can yield dramatic changes in selectivity. Surfactants can be anionic, cationic, zwitterionic or mixture of each (table 1).

	Biological detergents	CMC (mM)	Aggregation number
Anionic	SDS	8.0	62
Cationic	DTAB	14	50
	CTAB	1.3	78
Non Ionic	Octylglucoside	-	-
	n-Dodecyl- $\beta$ -D-matoside	0.16	-
	Triton X100	0.24	140
Zwitterionic	CHAPS	8.0	10
	CHAPSO	8.0	11
Bile Salt	Cholic Acid	14	2-4
	Deoxycholic acid	5	4-10
	Taurocholic acid	10-15	4

Table 1: Most used surfactants

In all cases, separation parameters such as temperature, buffer concentration, pH, or additives in background electrolyte (urea, metal ions, or cyclodextrins), can be changed for improving both the selectivity and the resolution of the system.

## **Resolution Optimization**

The choice of micelle and of modifier added to the aqueous phase is the most effective and important means of enhancing resolution. Another key parameter that effects the resolution is the temperature.

### **Choice of micelle**

#### *Ionic Surfactant*

For the separation of neutral analytes, the micelle used in MEKC must be ionic, thus obtained from ionic surfactant or mixtures of ionic and non-ionic surfactants. Some typical surfactants with their CMC and aggregation number are listed in table 1.

Surfactants have a hydrophobic chain and an ionic hydrophobic group within each molecule and both groups affect selectivity in MEKC. SDS is the most widely used surfactant; it possesses a long alkyl chain as the hydrophobic group and an sulfate group as the polar group. SDS is devoid of UV-vis absorption and it can be supplemented to the BGE without any cut-off in the spectrophotometric detection. When the analyte is incorporated into the micelle three types of interactions are possible:

- The analyte is adsorbed onto the surface of the micelle by electrostatic or dipole interaction;
- The analyte behaves as a surfactant by participating in the formation of the micelles;
- The analyte is incorporated into the core of the micelle.

The effect of the surfactant's molecular structure on the separation selectivity will differ according to the type of interaction involved. The hydrophilic, or ionic group, is generally more important in determining



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selectivity than is hydrophobic group since most analytes interact with the micelle surface.

The influence of surfactant type on the separation selectivity can be investigated through linear solvation energy relationships (LSER) (4). Using LSER methodology useful information about the nature of solute interactions with different types of surfactant aggregates can be obtained. One result of these studies is the selectivity differences in MEKC between several anionic surfactants are primarily due to hydrogen-bonding interactions rather than the dipolar interactions.

Cationic micelles show substantially different selectivity for neutral and for ionic solutes, as compared with anionic micelles because of the different polar group on this surfactant (5). Most cationic surfactants have an alkyltrimethylammonium group and their counter ions are halides. Cetyltrimethylammonium bromide (CTAB) is the most popular cationic surfactant used in CE, mainly to reverse the direction of the EOF due to the adsorption of the cationic surfactant on the capillary wall (6). Nevertheless it is not widely used as a micelle forming surfactant in MEKC, probably due to its UV absorption in the short wavelength region and the generation of bromine in anodic vial during electrophoresis. However, CTAB or the corresponding chloride (CTAC) shows significantly different selectivity compared to anionic surfactants (7), while the migration order of analytes still follows the order of increasing distribution constant, as in the case of anionic micelles. According to studies using the solvation parameter model, CTAB differ significantly from SDS concerning its hydrogen bond acidity and

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basicity. Therefore the use of a cationic surfactant instead of SDS is a promising alternative to change the selectivity.

### *Nonionic surfactant*

Nonionic surfactants themselves do not possess electrophoretic mobility and cannot be used as a pseudostationary phase in a conventional MEKC of neutral solutes. Still, nonionic surfactant micelles are useful for the separation of charged compounds, especially for peptides with closely related structures (8). Since the separation principle is the same as with ionic surfactants, we can classify the technique with non-ionic micelles as being an extension of MEKC. Non-ionic surfactants can be employed as pseudostationary phases in MEKC in a combination with ionic surfactants.

### *Biological Surfactants-Bile salts*

Bile salts which are a group of steroidal surfactants are common biological surfactants used in MEKC as an alternative. Some of the bile salts, which have been employed in MEKC separations include sodium cholate (SC), sodium taurocholate, (STC), sodium deoxycholate (SDC) and sodium taurodeoxycholate (STDTC). These surfactants are considered to form a helical micelle with a reversed micelle conformation (9). Also they have a relatively low solubilising power and they are capable of chiral discrimination (10).

### *Micelle polymers*

These additives form monomolecular micelles (aggregation number of 1), so that enhanced stability and rigidity of the micelle can be obtained as well as easier control of the micelle size as compared with micelles formed by a conventional low molecular mass surfactant. Since the CMC

of these polymer is essentially zero, the net micellar concentration is constant or independent of the concentration and composition of a buffer, pH, additives and temperature, and hence better reproducibility in the migration time can be expected in micelle polymer-MEKC than in low molecular mass surfactant-MEKC (11). In addition micelle polymer-MEKC is suitable to be adapted for a on-line coupling of MEKC with mass spectrometry (MS).

#### *Mixed Micelles*

It is known that mixed micelles are formed more than two different surfactants are dissolved in a solution. Mixed micelles consisting of ionic and a non-ionic surfactant are particularly useful in MEKC. Because they have larger surface and lower surface-charge density than ionic micelles, they provide higher retention factor and a narrower migration time and also different from that of an ionic micelle (12).

#### **Choice of buffer solution**

In general, the constituents of the buffer, in which the pseudostationary phase is dissolved do not significantly affect the resolution, but the pH of the buffer is an important factor in manipulating resolution of ionisable analytes. A ionized form of the analyte having the same charge as the micelle will be incorporated into the micelle to a lesser extent than its uncharged form.

#### **Choice of Temperature**

The distribution coefficient is dependent on the temperature and an increase in temperature causes reduction of the migration time because of the decrease in the distribution coefficient. The temperature rise also results in increases of the velocity of EOF and micelle by the same

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extent owing to the lowered viscosity of the separation electrolyte. The dependence of the distribution coefficient on temperature is different among analytes. Although the temperature does not have a significant impact on selectivity and resolution it will seriously affect the migration time. It is essential for the reproducibility of the results to keep temperature precisely constant.

### **Choice of Additives**

The aqueous phase in MEKC corresponds to the mobile phase in RPLC and therefore the various mobile phase modifiers developed in RPLC are also applicable in MEKC.

#### *Organic Solvents*

Water-miscible organic solvents, such as methanol and acetonitrile, reduce the retention factor and alter separation selectivity. A high concentration of organic solvent may break down the micellar structure and therefore it is recommended that the volume fraction of the solvent should not exceed 20%. The addition of the organic often reduces the electrosmotic velocity, thereby extending the migration time window they increase resolution, probably resulting from the reduced charge on the micelle due to swelling caused by the organic solvent (13, 14).

#### *Cyclodextrins*

Cyclodextrins (CDs) are popular in the field of chromatography. Most of the techniques using CDs are based on their capability to recognize specific molecules that fit the hydrophobic cavity. Native CDs are devoid of electrophoretic mobility; however CD derivatives with ionic group substituents can be used as a pseudostationary phase instead of a micellar separation carrier in EKC. This is especially effective for the

## Chapter 2: MEKC

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separation of the number of neutral compounds, including aromatic isomers and racemic mixture (2, CD-MEKC chapter).

CDs have also been used as additives to micellar solutions for CD-modified MEKC (CD/MEKC). When CD is added to a micellar solution, the analyte is distributed among three phases: micelle, CD, and water (aqueous phase). From the viewpoint of the electrophoretic separation, CD exerts a remarkable effect on the apparent retention factor between micellar and nonmicellar phases. Highly hydrophobic compounds tend to be almost totally incorporated into the micelle because of their low solubility in water. CD is water soluble and capable of including hydrophobic compounds into hydrophobic cavity. The inclusion complex formation equilibrium constant for the analyte depends on steric parameters. Thus a fraction of the hydrophobic analyte will be included in the cavity, even in the presence of micelles. Therefore the migration time or the apparent retention factor of hydrophobic analyte that forms an inclusion complex with a CD will decrease with an increase in CD concentration. Separation selectivity among highly hydrophobic analytes depends solely on the difference in their distribution ratio between CD and the micellar phase, because such hydrophobic analytes can be assumed to be insoluble in water. CD-MEKC is mainly applied because of the chirality of CD in enantioselective analysis of neutral chiral compounds.

### *Ion-pair reagents*

The partition process between analytes and ionic micelles depend on their charge. If they have the same charge the analyte's inclusion is not possible. On the other hand if they show opposite charge the interaction

## Chapter 2: MEKC

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will be too strong. An ion-pair reagent will strongly modify the interaction between ionic analytes and ionic micelle. For example, a cationic ion-pair reagent will enhance the interaction between an anionic analyte and an anionic micelles by forming the ion-pair with the analyte and the micelle. Differently, a cationic ion-pair reagent will reduce the interaction between a cationic analyte and an anionic micelle by competing with the analyte for combining with the micelle. The effect of the ion-pair reagent on the migration time or separation selectivity depends on the molecular structure of the ion-pair reagent itself.

### *Urea*

A high concentration of urea is known to increase the solubility of hydrophobic compounds in water as well as to hinder hydrogen bond formation in the aqueous phase. The addition of high concentration of urea to the SDS solution enabled the MEKC separation of highly hydrophobic compounds.

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**Chapter 3:**  
Cyclodextrins



## Cyclodextrins

### Introduction

Supramolecular chemistry is a discipline of chemistry which involves all intermolecular interactions where covalent bonds are not established between the interacting species: i.e., molecules, ions, or radicals. The majority of these interactions are of the host-guest type. Among all potential hosts, the cyclodextrins seem to be the most important ones, for the following reasons:

- (1) They are seminatural products, obtained from a renewable natural material, starch, by a relatively simple enzymatic conversion.
- (2) They are produced in thousands of tons per year amounts by environmentally friendly technologies.
- (3) As a result of point 2, their initially high prices have dropped to levels where they become acceptable for most industrial purposes.
- (4) Through their inclusion complex forming ability, important properties of the complexed substances can be modified significantly. This unprecedented “molecular encapsulation” is already widely utilized in many industrial products, technologies, and analytical methods.
- (5) Any of their toxic effect is of secondary character and can be eliminated by selecting the appropriate CD type or derivative or mode of application.
- (6) As a result of point 5, CDs can be consumed by humans as ingredients of drugs, foods, or cosmetics.

## Chapter 3: CDs

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*Discovery Period: From 1891 to the 1930s.*

The first reference to a substance which later proved to be a cyclodextrin, was published by Villiers (1), in 1891. Digesting starch with *Bacillus amylobacter* (which probably was not a pure culture, but also contained heat-resistant spores of *Bacillus macerans*), he isolated about 3 g of a crystalline substance from 1000 g starch, and determined its composition to be  $(C_6H_{10}O_5)_2 \cdot 3H_2O$ . Villiers named this product “cellulosine”, because it resembled cellulose with regard to its resistance against acidic hydrolysis and because it did not show reducing properties. Even at that time, he observed that two distinct crystalline “cellulosines” were formed, probably  $\alpha$ - and  $\beta$ -CDs. Twelve years later, Schardinger (2), who studied various isolated strains of bacteria that survived the cooking process and which were thought to be responsible for certain cases of food poisoning, published a report that digesting starch with such a microorganism resulted in the formation of small amounts of two different crystalline products. These substances seemed to be identical with the “cellulosines” of Villiers. Schardinger continued to study these crystallized dextrans, with the expectation that they would shed some light on the synthesis and degradation of starch. He named the isolated microbe *Bacillus macerans* (3,4). He observed that the crystalline dextrans formed characteristic iodine adducts upon the addition of iodine-iodide solution. He reported that about 25-30% of the starch could be converted to crystalline dextrans (with an additional larger amount of amorphous dextrans). In all of his experiments, the major crystalline product was the so-called  $\beta$ -dextrin. The simplest means to distinguish between the  $\alpha$ - and  $\beta$ -dextrans was the iodine reaction. The crystalline  $\alpha$ -

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dextrin/iodine complex in thin layers is blue when damp and gray-green when dry while the crystalline  $\beta$ -dextrin/iodine complex is brownish (red-brown) damp or dry(5). It can be said that the fundamentals of cyclodextrin chemistry were laid down by Schardinger.

In the 24 years following Schardinger's last CD publication (in 1911), it was Pringsheim (6,7), who played the leading role in cyclodextrin research. He published extensively, with a number of co-authors, but their papers are of limited value. The greatest weakness in these studies lies in the fact that they worked with incompletely separated fractions, and used inadequate methods, e.g., cryoscopic molecular weight determinations. Pringsheim's numerous papers contain many unfounded speculations, and the majority of the published experimental data are unreliable. This group's merit is, however, the discovery that the crystalline dextrans and their acetates have a high tendency to form complexes with various organic compounds.

During the following 35 years, until encouraging results of adequate toxicological studies became available and these deterred many scientists from developing CD-containing products for human use. By the end of the 1960s, the methods for the laboratory-scale preparation of cyclodextrins, their structure, physical and chemical properties, as well as their inclusion complex forming properties had been discovered. Summarizing the literature available at that time, the conclusions could be condensed into three points:

- (a) Cyclodextrins are very interesting, promising molecules, worth further study, particularly because of their industrial possibilities.

### Chapter 3: CDs

- (b) Cyclodextrins are very expensive substances, available only in small amounts as fine chemicals.
- (c) Cyclodextrins are apparently highly toxic; therefore, their utilizations for human consumption seems to be questionable.

#### *Structural Features*

Cyclodextrins comprise a family of three well known industrially produced major, and several rare, minor cyclic oligosaccharides. The three major cyclodextrins are crystalline, homogeneous, nonhygroscopic substances, which are torus-like macro-rings built up from glucopyranose units. The  $\alpha$ -cyclodextrin comprises six glucopyranose units,  $\beta$ -CD comprises seven such units, and  $\gamma$ -CD comprises eight such units. The most important characteristics of the CDs are summarized in Table 1. The nomenclature of CDs is not exact. Maltose is a disaccharide, i.e., a cyclomaltopentaose could be interpreted as a 10 glucopyranose containing cyclic oligosaccharide. Otherwise, this is the five-membered pre- $\alpha$ -CD.

	$\alpha$	$\beta$	$\gamma$
no. of glucose units	6	7	8
mol wt	972	1135	1297
solubility in water, g 100 mL <sup>-1</sup> at room temp	14.5	1.85	23.2
$[\alpha]_D^{25}$ °C	150 ± 0.5	162.5 ± 0.5	177.4 ± .5
cavity diameter, Å	4.7–5.3	6.0–6.5	7.5–8.3
height of torus, Å	7.9 ± 0.1	7.9 ± 0.1	7.9 ± 0.1
diameter of outer periphery, Å	14.6 ± 0.4	15.4 ± 0.4	17.5 ± 0.4
approx volume of cavity, Å <sup>3</sup>	174	262	427
approx cavity volume in 1 mol CD (ml)	104	157	256
in 1 g CD (ml)	0.10	0.14	0.20
crystal forms (from water)	hexagonal plates	monoclinic parallelograms	quadratic prisms
crystal water, wt %	10.2	13.2–14.5	8.13–17.7
diffusion constant at 40 °C	3.443	3.224	3.000
hydrolysis by <i>A. oryzae</i> $\alpha$ -amylase	negligible	slow	rapid
$V_{max}$ value, min <sup>-1</sup>	5.8	166	2300
relative permittivity (on incorporating the toluidinyl group of 6- <i>p</i> -toluidinyl naphthalene 2-sulfonate) at pH = 5.3, 25 °C	47.5	52.0	70.0
(on incorporating the naphthalene group)	a	29.5	39.5
pK (by potentiometry) at 25 °C	12.332	12.202	12.081
partial molar volumes in solution mL mol <sup>-1</sup>	611.4	703.8	801.2
adiabatic compressibility in aqueous solutions mL (mol <sup>-1</sup> bar <sup>-1</sup> ) × 10 <sup>4</sup>	7.2	0.4	-5.0

<sup>a</sup> Naphthalene group is too bulky for the  $\alpha$ -CD cavity.

Table 1: Structural characteristic of  $\alpha$ -,  $\beta$ -,  $\gamma$ -CD (28).

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As a consequence of the  ${}^4C_1$  conformation of the glucopyranose units, all secondary hydroxyl groups are situated on one of the two edges of the ring, whereas all the primary ones are placed on the other edge. The ring, in reality, is a cylinder, or better said a conical cylinder, which is frequently characterized as a doughnut or wreath-shaped truncated cone. The cavity is lined by the hydrogen atoms and the glycosidic oxygen bridges, respectively. The nonbonding electron pairs of the glycosidic oxygen bridges are directed toward the inside of the cavity producing a high electron density there and lending to it some Lewis base characteristics. The C-2-OH group of one glucopyranoside unit can form a hydrogen bond with the C-3-OH group of the adjacent glucopyranose unit. In the CD molecule, a complete secondary belt is formed by these H bonds, therefore the  $\beta$ -CD is a rather rigid structure. This intramolecular hydrogen bond formation is probably the explanation for the observation that  $\beta$ -CD has the lowest water solubility of all CDs. The hydrogen-bond belt is incomplete in the R-CD molecule, because one glucopyranose unit is in a distorted position. Consequently, instead of the six possible H-bonds, only four can be established fully. The  $\gamma$ -CD is a noncoplanar, more flexible structure; therefore, it is the more soluble of the three CDs.

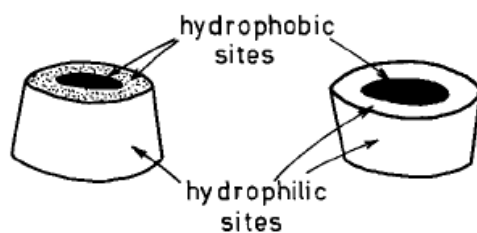


Fig. 3. Schematic representation of the hydrophobic and hydrophilic regions of an  $\alpha$ -CD cylinder (28).

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Figure 3 shows a sketch of the characteristic structural features of CDs. On the side where the secondary hydroxyl groups are situated, the diameter of the cavity is larger than on the side with the primary hydroxyls, since free rotation of the latter reduces the effective diameter of the cavity. For a long time, only the three parent (or major) CDs ( $\alpha$ -,  $\beta$ -, and  $\gamma$ -CD) were known and well characterized. During the past decade, a series of the larger CDs has been isolated and studied (30). For example, the nine-membered  $\delta$ -CD was isolated from the commercially available CD conversion mixture by chromatography. The  $\delta$ -CD had greater aqueous solubility than the  $\beta$ -CD, but less than that of  $\alpha$ - and  $\gamma$ -CD. It was the least stable among the CDs known at that time; their hydrolysis rate increases in the order of  $\alpha$ -CD <  $\beta$ -CD <  $\gamma$ -CD <  $\delta$ -CD. The  $\delta$ -CD did not show any significant solubilization effect on slightly soluble drugs in water, except in the cases of some large guest molecules such as spironolactone and digitoxin, e.g. the solubility of spironolactone increased about 30-fold in the presence of  $\delta$ -CD (30). Table 2 illustrates the complex-forming ability of the larger CDs. These results are in accord with the results of computer graphic studies. The larger CDs are not regular cylinder shaped structures.

compound	inclusion complex formation constant (M <sup>-1</sup> )							
	$\alpha$ -CD	$\beta$ -CD	$\gamma$ -CD	$\delta$ -CD	$\epsilon$ -CD	$\xi$ -CD	$\eta$ -CD	$\theta$ -CD
benzoic acid	16	23	3	3	3	5	4	5
2-methylbenzoic acid	13	13	7	6	6	5	6	7
3-methylbenzoic acid	26	40	6	3	5	6	7	8
4-methylbenzoic acid	36	66	8	2	4	6	6	7
2,4-dimethylbenzoic acid	45	42	8	3	4	5	7	6
3,5-dimethoxybenzoic acid	47	63	10	8	9	10	9	12
salicylic acid	11	65	13	9	8	8	9	10
3-phenylpropionic acid	35	79	7	2	3	5	4	6
4-tert-butylbenzoic acid	51	457	59	60	4	9	19	31
ibuprofen	55	2600	59	1013	—	10	44	225
1-adamantanecarboxylic acid	114	501	42	8	—	4	4	8

Table 2: inclusion complexing capacity of different CDs, studied by Capillary Electrophoresis (28)

They are collapsed, and their real cavity is even smaller than in the  $\gamma$ -CD (Figure 4).



Fig. 4. “Collapsed cylinder” structure of the  $\delta$ -CD (28).

The driving force of the complex formation, the substitution of the high enthalpy water molecules in the CD cavity, is weaker in the case of larger CDs; therefore, their utilization as inclusion complexing agents will probably remain rather restricted.

### **CD Inclusion Complexes**

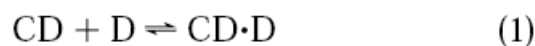
In an aqueous solution, the slightly apolar cyclodextrin cavity is occupied by water molecules which are energetically unfavoured (polar-apolar interaction), and therefore can be readily substituted by appropriate “guest molecules” which are less polar than water. (Figure 5.) The dissolved cyclodextrin is the “host” molecule, and the “driving force” of the complex formation is the substitution of the high enthalpy water molecules by an appropriate “guest” molecule. One, two, or three cyclodextrin molecules contain one or more entrapped “guest” molecules. Most frequently the host : guest ratio is 1:1. However 2:1, 1:2, 2:2, or even more complicated associations, and higher order equilibria exist, almost always simultaneously.

The formed inclusion complexes can be isolated as stable crystalline substances. Upon dissolving these complexes, an equilibrium is established between dissociated and associated species, and this is

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expressed by the complex stability constant  $K_{1:1}$ . The association of the CD and guest (D) molecules, and the dissociation of the formed CD/guest complex is governed by a thermodynamic equilibrium.



$$K_{1:1} = \frac{[\text{CD}\cdot\text{D}]}{[\text{CD}][\text{D}]} \quad (2)$$

The most important primary consequences of the interaction between a poorly soluble guest and a CD in aqueous solution are as follows:

- ❖ The concentration of the guest in the dissolved phase increases significantly, while the concentration of the dissolved CD-decreases. This latter point is not always true, however, because ionized guests, or hydrogen-bond establishing (e.g. phenolic) compounds may enhance the solubility of the CD.
- ❖ The spectral properties of the guest are modified. For example, the chemical shifts of the anisotropically shielded atoms are modified in the NMR spectra. Also when achiral guests are inserted into the chiral CD cavity, they become optically active, and show strong induced Cotton effects on the circular dichroism spectra. Sometimes the maximum of the UV spectra are shifted by several nm and fluorescence is very strongly improved, because the fluorescing molecule is transferred from the aqueous milieu into an apolar surrounding.
- ❖ The reactivity of the included molecule is modified. In most cases the reactivity decreases, i.e., the guest is stabilized, but in many cases the CD behaves as an artificial enzyme, accelerating various reactions and modifying the reaction pathway.



### Chapter 3: CDs

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- ❖ The diffusion and volatility (in case of volatile substances) of the included guest decrease strongly.

And in the solid state:

- ❖ The formerly hydrophobic guest, upon complexation, becomes hydrophilic; therefore its chromatographic mobility is also modified.
- ❖ The complexed substance is molecularly dispersed in a carbohydrate matrix, forming a microcrystalline or amorphous powder, even with gaseous guest molecules.
- ❖ The complexed substance is effectively protected against any type of reaction, except that with the CD hydroxyls, or reactions catalyzed by them.
- ❖ Sublimation and volatility are reduced to a very low level.
- ❖ The complex is hydrophilic, easily wettable, and rapidly soluble.

When, in an aqueous system, the formation of the CD inclusion complex can be detected, e.g. by NMR or circular dichroism, or through a catalytic effect; this does not mean that a well-defined crystalline inclusion complex can be isolated. The two main components of the driving force of the inclusion process are the repulsive forces between the included water molecules and the apolar CD cavity on one hand, and between the bulk water and the apolar guest, on the other hand. This second factor does not exist in the crystalline (dry) state. Therefore it is not uncommon that the complex formation is convincingly proved in solution, but nevertheless the isolated product is nothing other than a very fine dispersion of the CD and the guest.

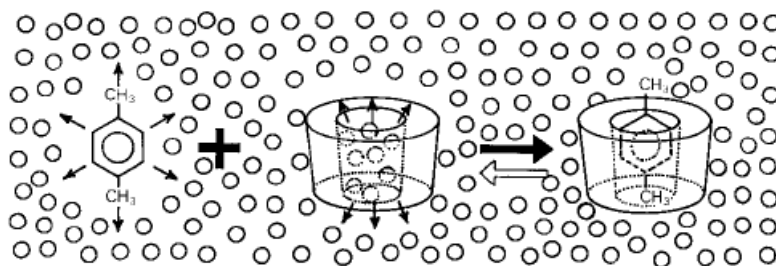


Fig. 5. Schematic representation of CD inclusion complex formation. *p*-Xylene is the guest molecule; the small circles represent the water molecules (28).

### Applications

Several researches are addressed to the study of the CD inclusion phenomena. These works are generally not directly practice-oriented, dealing with energetic and kinetics of inclusion, X-ray, FTIR, liquid and solid-phase NMR, EPR, circular dichroism, Raman spectroscopy, enhancement of luminescence and phosphorescence, thermal analysis, interaction of CDs with specific guest types, enzyme modeling with CDs and CD derivatives, preparation, analysis of cyclodextrin complexes, etc. These methods, as well as the correlation between the complexation and various structural and external parameters form the basis for all practical applications of CDs.

Most of the investigations are dedicated to the pharmaceutical application of CDs. The majority of drug molecules are poorly soluble in water, consequently their biological absorption is slow and frequently far from being complete. Moreover many drug molecules are rather sensitive to oxidation, thermo-decomposition, light, ions, other ingredients of the pharmaceutical formulation, etc.

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Most drug molecules are ideal complex-forming partners for cyclodextrins, because their polarity, molecular mass, and structure enable them to get included into the CD cavity. This is a very productive field, and considering the lengthy development and strict requirements for approval of a new chemical entity (a cyclodextrin complex of a well-known drug molecule is always considered to be a new chemical entity) it must be considered as a significant achievement that more than a dozen drugs are already approved and marketed in cyclodextrin-complexed form. In the coming years this field will display the most intensive development.

Not every cyclodextrin or cyclodextrin derivative can be administered to humans, partly because some of those cyclodextrins have such a high affinity toward the cell-membrane lipid components of the organism that depending on their concentration, may result in haemolysis, or else their synthesis is too expensive.

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**Chapter 4:**  
Cyclodextrins Modified Micellar  
Electrokinetic Chromatography

## **Cyclodextrin-modified micellar electrokinetic chromatography (CD-MEKC)**

### **Introduction**

In conventional MEKC, highly hydrophobic compounds tend to be totally incorporated into micelle and hence migrate at the same velocity as that of the micelle and they cannot be separated.

To solve this problem several approaches have been applied: addition of organic solvents (1,2), use of bile salts instead of a long alkyl chain surfactant (3,4) and addition of CDs and addition of urea (5) to the micellar solution.

Among these methods, CD-modified MEKC (CD-MEKC) (6), which employs CD together with an ionic micellar solution has been demonstrated to be useful in the separation of hydrophobic complex mixtures, but also in the separation of enantiomeric compounds (7).

In CD-MEKC, usually, negatively charged micelles are employed and they migrate in the direction opposite to the EOF, while uncharged CDs migrate with the same velocity of the EOF.

As CD is electrically neutral, it migrates at an identical velocity with the bulk solution. Therefore, the distribution of the analyte between the micelle and the non micellar aqueous phase including CD directly affect the resolution. Addition of CD to the micellar solution reduces the partitioning of the analyte to the micelle by increasing the fraction of the analyte in the non micellar aqueous phase. If major fraction of the

analytes is incorporated into the micelle or is completely included into CD, the separation will be unsuccessful.

The capacity factor,  $k$ , of a highly hydrophobic solute in CD-MEKC is given by:

$$k = \frac{n_{mc}}{n_{CD}} = K \frac{V_{mc}}{V_{CD}} \quad \text{Eq.1}$$

where  $n_{CD}$  and  $n_{mc}$  are the total amounts of the solute included by CD and those of the solutes incorporated by the micelle,  $V_{CD}$  and  $V_{mc}$  are the volumes of CD and the micelles and  $K$  is the distribution coefficient.

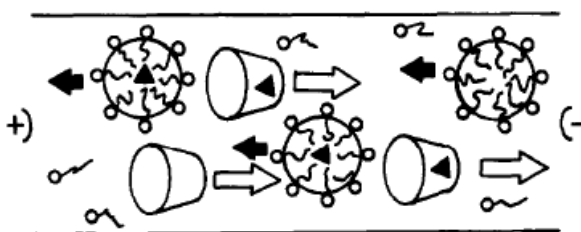


Fig.1: Schematic illustration of the separation principle of CD-MEKC. Filled arrows indicate the electrophoretic migration of the micelle and open arrows the electroosmotic migration of CD (6).

The capacity factor is proportional to the phase (volume) ratio of the micelle to CD. The distribution coefficient means the relative affinity of the solute between CD and the micelle. The ratio of the solute incorporated in the micelle depends on its hydrophobicity but the inclusion-complex formation of the solute with CD depends on the concordance of the solute molecular size with the cavity diameter of CD in addition to the hydrophobicity. Consequently, the selectivity in CD-



MEKC will be mostly determined by the tendency of the solute to form an inclusion complex with CD.

Fig. 2 shows the separation of a mixture of naphthalene and four tricyclic and three tetracyclic aromatic hydrocarbons by  $\gamma$ -CD-MEKC. The elution order means that smaller PAHs are more easily included in the CD cavity or the larger PAHs are incorporated by the SDS micelle to a greater extent.

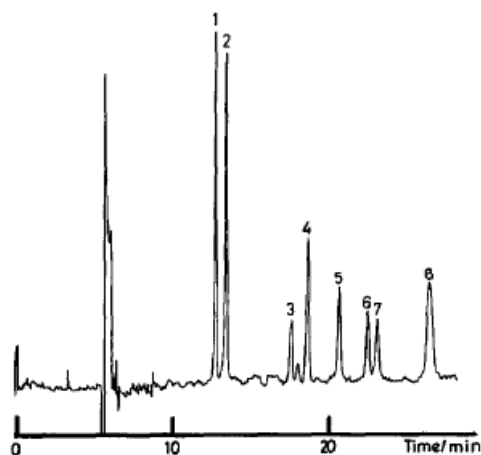


Fig. 2:  $\gamma$ -CD-MEKC separation of a mixture of naphthalene and four tricyclic and three tetracyclic aromatic hydrocarbons: 1 = naphthalene; 2 = acenaphthene; 3 = anthracene; 4 = fluorene; 5 = phenanthrene; 6 = chrysene; 7 = pyrene; 8 = fluoranthene. Separation solution, 30 mM  $\gamma$ -CD, 100 mM SDS and 5 mM urea in 100 mM borate buffer (pH 9.0); applied voltage, 20 kV (6)

Three CDs,  $\alpha$ -,  $\beta$ - and  $\gamma$ -CD are the most popular, but various derivatives have also been developed for modifying the selectivity or increasing the solubility in water.

$\beta$ -CD and its derivatives, such as the methylated ones, have cavity size suitable for inclusion of a wide range of analytes.

CDs are also useful for enantiomeric separation: one important conceptual point in enantioselective capillary electrophoresis is that enantioseparation is commonly not based on the principle of zonal electrophoretic separation. In fact, it is based on the result of different migration velocities caused by different charge densities of analytes. The enantiomers of a chiral compound possess the same charge densities. Therefore none of the potential migration forces in CE, such as the electrophoretic mobility of the analyte, the EOF, their combination or a transport by nonenantioselective carrier (as a SDS's micelle) is, in principle, able to differentiate between the enantiomers. The prerequisite for separation of enantiomers is, thus, the enantioselective interaction with chiral selectors included in the electrophoretic background.

*CDs and SDS: inclusion complex*

A significant property of CDs is the ability in including organic or inorganic compounds into their cavity, both in the solid state and in solutions, to form inclusion complex (8,9). They are also able to include surfactant molecules. Inclusion complexes, between CDs and surfactant monomers, as SDS, with a 1:1 stoichiometry are usually assumed, although inclusion complexes with a 2:1 stoichiometry may occasionally be reported (10,11). The addition of  $\beta$ -CD to the pre-micellar solution system appears to encapsulate surfactant molecules and to shift the equilibrium in favor of the formation of inclusion complexes. Thus, influencing considerably the micellization of surfactant. (12). As a

consequence, the CMC value of a surfactant increases with increasing concentration of, for example,  $\beta$ -CD.

The study of the association of CDs/surfactants (SDS) has been carried out e.g. by a conductivity technique. (13). This field of investigation involved not only the analytical chemistry aspect, but also those of biological chemistry; in fact e.g. interesting studies are inherent to the effect of CDs on phospholipids, as major constituent of cell membranes (14).

#### **Conductometric study of the association of CDs with ionic surfactants**

The conductance method is based on the electrolytes properties of conducting electricity. The conductivity is due to ions migration and it is regulated by Ohm's law:

$$I=V/R \text{ (Eq.1)}$$

where V is the potential difference and R is the resistance. The resistance reciprocal is the conductance, measured in  $\Omega^{-1}$  (Siemens). It depends on the concentration and on the mobility of the ions present in the solution and on the temperature.

When the conductance of an electrolyte is considered, it is useful to measure the equivalent conductivity,  $\Lambda$ , that means a certain volume  $V_e$  in which a equivalent gram of electrolyte is dissolved and it can be measured by two electrodes (conductometric cell) at a distance of 1 cm and having an area of  $V_e \text{cm}^2$ .

It can be defined also the molar conductivity, that is strictly connected to the molar concentration of the electrolyte present in the solution.

By means this technique the stoichiometry and the inclusion complex of CD in presence of different surfactants were assumed.

In 1985, Satake et al, by developing a conductometric and potentiometric method, found the association constant of  $\alpha$ -CD with ionic surfactant (15).

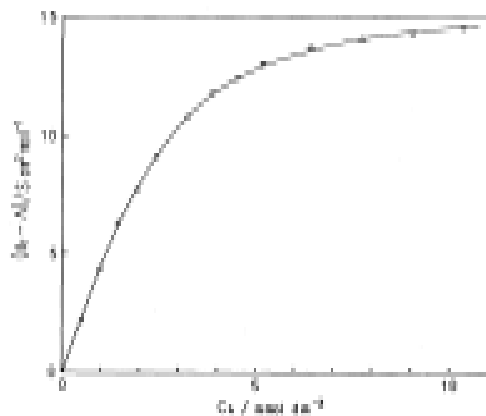
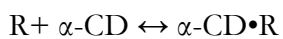


Fig. 3: Plots of  $\Lambda_f - \Lambda$  vs CD concentration ( $C_h$ ) for sodium 1-nonasulfonate solution at 25°C. The solid line is calculated from eq. 5 (15)

In this study the difference of equivalent conductance of the surfactant monomer in presence and in absence of  $\alpha$ -CD has been plotted against the increasing concentration of the cyclodextrin.

By assuming a 1:1 reaction scheme:



where R refers to the amphiphilic ion, the association constant K can be written as:

$$K = \frac{[\text{R}\alpha\text{CD}]}{[\text{R}][\alpha\text{CD}]}$$

It can be described:

$$[R] = C - [R\alpha CD]$$

$$[\alpha CD] = C_h - [R\alpha CD]$$

Where C is the total concentration of the ionic surfactant, and  $C_h$  is the concentration of the CD.

The fraction of R associated with  $\alpha CD$  can be expressed as:

$$f = [R\alpha CD] / [R]$$

so:

$$[R] = C - fC = C(1-f)$$

$$[\alpha CD] = C_h - fC;$$

The association constant, K, can be written as:

$$K = fC / [C - (1-f)(C_h - fC)]$$

That can be formulated as:

$$K = f / (1-f)(C_h - fC) \text{ Eq.3}$$

If we denote the ionic equivalent conductivities of R and  $\alpha\text{-CD}\cdot\text{R}$

by  $\lambda_f$  and  $\lambda_a$ , we may write:

$$\Lambda_f - \Lambda = f(\lambda_f - \lambda_a) \text{ Eq.4}$$

The use of eq.2 and 3 for the evaluation of K requires a knowledge of  $\lambda_a$ .

Unfortunately, however, the value of  $\lambda_a$  is somewhat difficult to estimate, since  $\Lambda$  decreases slightly but continuously even at higher  $\alpha\text{-CD}$  concentrations. It follows immediately from Eq.2 and 3 that:

$$\Lambda_f - \Lambda = \frac{\lambda_f - \lambda_a}{2KC} [K(C + C_h) + 1 - \sqrt{\{K(C + C_h) + 1\}^2 - 4K^2 C C_h}] \text{ Eq.5}$$

On the basis of Eq.5, the values of K and  $\lambda_f - \lambda_a$  can be assumed from the plot of  $\Lambda_f - \Lambda$  vs.  $C_h$  by using a non-linear least squares method (Gauss-Newton algorithm). The fig.3 shows the calculated conductivity curve

## Chapter 4: CD-MEKC

from eq.5. The agreement between experimental point and calculated curves (line) is quite satisfactory over a whole range of  $C_h$  indicating the validity of a 1:1 reaction scheme.

The association constants and the conductometric parameters thus determined are summarized in table 1. In this table is also given the standard free energy change of complex formation,  $\Delta G^\circ$ .

Amphiphile	C mmol dm <sup>-3</sup>	Temp °C	$\lambda_c$ Scm <sup>2</sup> mol <sup>-1</sup>	$\lambda_f - \lambda_c$ Scm <sup>2</sup> mol <sup>-1</sup>	K dm <sup>3</sup> mol <sup>-1</sup>	$-\Delta G^\circ$ kJ mol <sup>-1</sup>	
C <sub>12</sub> E <sub>8</sub> /SO <sub>3</sub> Na	n=5	2.947	38	32.5	12.2	66	19.3
		2.365	25	77.0	18.9	68	20.1
		1.539	40	106.7	26.9	52	20.7
	n=6	2.885	38	50.8	11.8	150	21.8
		2.584	25	74.0	18.1	177	22.8
		1.995	40	100.4	24.7	158	25.5
	n=7	2.665	25	75.6	16.7	456	25.8
		2.838	38	49.8	9.8	1090	25.9
		2.489	25	75.1	15.0	1058	27.2
	n=8	1.891	40	99.2	20.2	914	28.2
		2.512	38	49.0	8.9	2218	27.6
		2.981	25	71.2	11.5	2178	29.0
n=10	1.761	40	96.5	17.0	2158	30.8	
	2.982	25	70.2	12.2	4090	30.6	
	2.547	25	89.4	11.8	4138	30.6	
n=12	2.434	25	80.6	11.0	4548	30.7	
	2.597	25	88.0	12.0	3658	30.3	
SDS	8.740	25	89.1	8.7	7048	31.9	
DAC	2.825	25	96.7	13.2	4760	30.9	
DTAB	2.824	25	97.4	12.5	4880	31.0	
DPC	1.391	25	89.7	18.8	4980	31.0	

Table 1: The Conductometric and Thermodynamic Parameters of  $\beta$ CD-Amphiphilic systems (15).

Other studies were carried out to determine the binding ratio of  $\beta$ -cyclodextrin to various surfactants and to ascertain the effect this inclusion process had upon micellization.

Conductance measurements were undertaken to determine the stoichiometry and association constants of CD-surfactant inclusion complexes in the pre-CMC and post-CMC regions (16).

Figure 4 shows the effect upon the molar conductances of pre-micellar SDS solutions after adding of  $\beta$ CD.

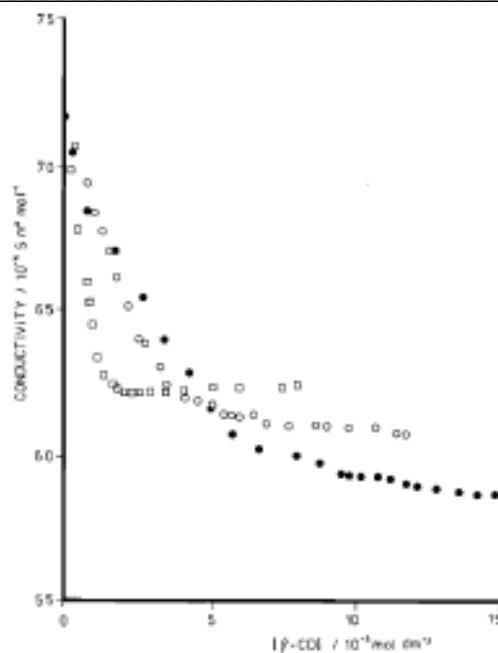


Fig.4: Molar conductivity of SDS in  $\beta$ CD solutions below the CMC: open squares,  $1.00 \times \text{mol dm}^{-3}$  SDS; closed circles,  $5.00 \times \text{mol dm}^{-3}$  SDS (16).

The molar conductance decreased sharply as  $\beta$ CD was added presumably because the dodecyl sulfate ion having been complexed by  $\beta$ CD was less effective as a charge carrier. At a certain concentration in  $\beta$ CD, this linear decrease of molar conductance with  $\beta$ CD concentration halted rather abruptly to show no or little further decrease with further  $\beta$ CD additions.

The stoichiometry at which this halt occurred was 1.2: 1  $\beta$ CD:SDS for  $1 \times 10^3 \text{mol dm}^{-3}$  SDS; 1.1: 1 for  $2.00 \times 10^3 \text{mol dm}^{-3}$  SDS; 1.1:1 for  $3.00 \times 10^3 \text{mol dm}^{-3}$  SDS; and 1.1: 1 for  $5.00 \times 10^3 \text{mol dm}^{-3}$  SDS. This

indicates that the chief inclusion complex of  $\beta$ CD with SDS in this range is 1:1 with possibly a little 2: 1 complex also present. The plateau in the molar conductance beyond the 1 : 1 stoichiometry indicates that the SDS has been almost totally complexed. The molar conductance at this stage is due only to the charged inclusion complex and  $\text{Na}^+$  ions originating from the surfactant.

Above the CMC, the molar conductance pattern for SDS solutions containing  $\beta$ CD is less simple, but consistent as a function of SDS concentration. Figure 5 demonstrates this for three post-CMC SDS systems. The consistent trend in all four systems was a sharply rising conductance with  $\beta$ CD addition to a maximum, which was more swiftly reached in more dilute micellar systems, followed afterwards by a drop in conductance in which two linear portions could be discerned as with the pre-CMC SDS systems.



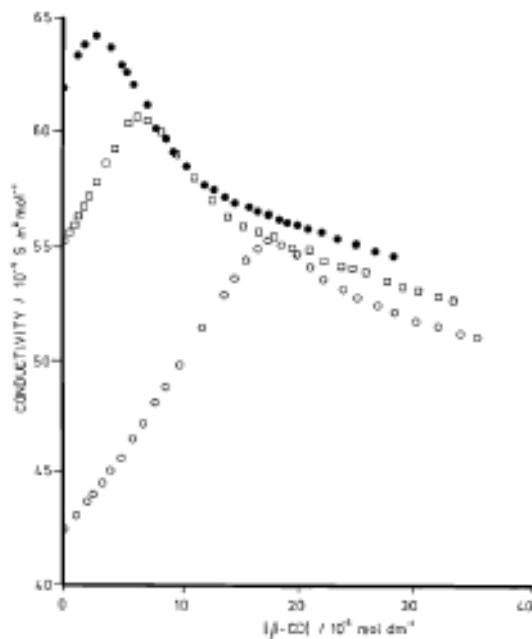


Fig. 5: Molar conductivity of SDS in  $\beta$ CD solutions above the CMC: closed circles,  $0.95 \times 10^{-2} \text{ mol dm}^{-3}$  SDS; open squares,  $1.20 \times 10^{-2} \text{ mol dm}^{-3}$  SDS; open circles,  $2.00 \times 10^{-2} \text{ mol dm}^{-3}$  SDS (16).

The addition of  $\beta$ CD to the micellar SDS thus appears to break up the micelles releasing monomeric SDS and given  $\text{Na}^+$  ions. The former subsequently complex with  $\beta$ CD in the 1:1 stoichiometry observed in the pre-CMC range and the latter largely give rise to a marked increase in conductance. At the maximum of the curve, the micelles have disappeared and subsequent addition of  $\beta$ CD simply removes free SDS anions largely in a 1: 1 complex.

The maximum of the curve may thus be interpreted as the CMC of the mixed surfactant system.

## Chapter 4: CD-MEKC

These values are plotted in Fig. 6 and it can be said that addition of cyclodextrins to SDS systems generally leads to an increase in the CMC.

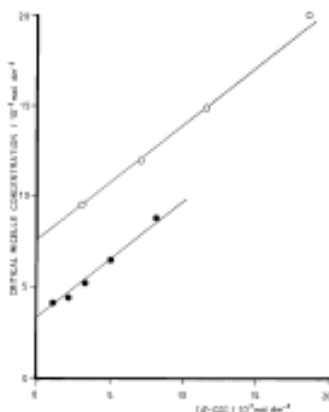


Fig.6: Effect of  $\beta$ CD on the CMC values of SDS and TTAB (16).

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## **Experimental Part**

**Studies on the interaction of surfactants and cyclodextrins by capillary electrophoresis. Application to chiral analysis in real sample.**

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## Abstract

In the present study, mixed systems composed of SDS in the presence of neutral cyclodextrins were considered. Firstly, the effect of the CDs on the CMC of the surfactant was evaluated by CE experiments. Furthermore, a new CE approach based on electric current measurement was developed for the estimation of the stoichiometry as well as of the binding constants of SDS-CDs complexes. The results of these investigations were compared to those obtained with a different technique, electronic paramagnetic resonance (EPR). The obtained results suggested that methylated CDs, in particular (2,6-di-*O*-methyl)- $\beta$ -cyclodextrin (DM- $\beta$ CD), strongly affect the micellization of SDS in comparison to the other studied CDs. This effect also paralleled the chiral CD-MEKC performance, as indicated by the enantioresolution of ( $\pm$ )-Catechin, which was firstly selected as a model compound representative of important chiral phytomarkers. Then a CD-MEKC system, composed of sodium dodecyl sulfate as surfactant (90 mM) and hydroxypropyl- $\beta$ -cyclodextrin (25 mM) as chiral selector, under acidic conditions (25 mM borate – phosphate buffer, pH 2.5) was applied to study the thermal epimerisation of epi-structured catechins, (-)-Epicatechin and (-)-Epigallocatechin, to non epi-structured (-)-Catechin and (-)-Gallocatechin. The latter compounds, being non-native molecules, were for the first time regarded as useful phytomarkers of tea sample degradation. The proposed method was applied to the analysis of more than twenty tea samples of different geographical origins (China, Japan, Ceylon), having undergone different storage conditions and manufacturing processes.

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## **Chapter 5:**

Capillary electrophoretic study on the interaction between sodium dodecyl sulfate and neutral cyclodextrins. Application to chiral separations.

## Experimental

### *Materials*

Sodium dodecyl sulfate (SDS),  $\alpha$ -cyclodextrin ( $\alpha$ CD),  $\beta$ -cyclodextrin ( $\beta$ CD),  $\gamma$ -cyclodextrin ( $\gamma$ CD), (2-hydroxypropyl)- $\beta$ -cyclodextrin (HP- $\beta$ CD) were from Fluka (Milan, Italy). Heptakis (2,6-di-*O*-methyl)- $\beta$ -cyclodextrin (DM- $\beta$ CD), pentyphenylketone (PFK) and 4'-*tert*-butylacetophenone (TBA) were from Aldrich (Milan, Italy). *Tert*-butylbenzylketone (BBK) was synthesized as previously described (19). ( $\pm$ )-Catechin, ( $\pm$ )-Ibuprofen and ( $\pm$ )-Ketorolac were from Sigma (Milan, Italy). Methanol, phosphoric acid, sodium hydroxide, sodium hydrogen phosphate and all the other chemicals were from Carlo Erba Reagenti (Milan, Italy). Water used for the preparation of solutions and running buffers, was purified by a Milli-RX apparatus (Millipore, Milford, MA, USA).

### *Apparatus*

Electrophoretic experiments were performed on a HP<sup>3D</sup>CE capillary electrophoresis instrument from Agilent Technologies (Waldbronn, Germany). Fused-silica capillaries (50  $\mu$ m id, 38.5 cm total length, 30 cm length to the detector) were from Composite Metal Service (Ilkley, UK); the data were collected on personal computer equipped with the integration software Agilent Rev. A. 09. 01.

### *Methodological approaches*

Prior to the first use, fused-silica capillaries were washed sequentially with NaOH 1 M, NaOH 0.1 M and water. Each of the washing cycles was carried out for ten minutes at 60 °C. The experiments for the determination of: (i) CMC of SDS in the presence of cyclodextrins, (ii)



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association constants and stoichiometry of the complexes SDS:CD and (iii) mobility of selected neutral solutes in SDS/CD running buffers, were carried out by the experimental procedures described below.

#### *CMC determination by the electric current method.*

Firstly, a capillary conditioning cycle was performed flushing with both NaOH 0.1 M and water for five minutes. The solutions to be tested were flushed into the capillary for 5 min and a voltage of 20 kV was applied at constant temperature (25 °C). The electric current circulating in the capillary was measured by the CE instrumentation after an equilibration time of 2 min and the obtained values were plotted against the concentration of SDS (mM). The regression lines in submicellar and micellar regions were determined by the least-square method using the software package OriginPro 7.5. The intersection point of the two regression lines was assumed as the CMC.

#### *Estimation of the association constant and complex stoichiometry (SDS:CD).*

The determination of association constants and stoichiometry of complexes SDS:CD, were carried out using the CE apparatus, by measuring the decrease in electric current of a submicellar SDS solution when it is supplemented by increasing amount of neutral CD. Precisely, the experiments were performed at 25 °C after a capillary conditioning cycle (5 min at the pressure of 50 mbar) with the solution to be tested. The electric current was measured after a 2 min equilibration time at a constant voltage of 30 kV. Firstly, the experiment was carried out on an aqueous solution of SDS 2.5 mM (Cs) (the control solution); successively the experiments were conducted on solutions obtained by supplementing the control solution with the selected CD at increasing concentrations in

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the range 0.5 – 10 mM. The electric current decrease respect to the control solution ( $\Delta I$ ), occasioned by the addition of the CD, was plotted as function of CD concentration ( $C_c$ ), and the data were elaborated by MatLab 7.1 (Austin, TX, USA).

#### *Electrophoretic mobility of neutral probes in SDS/CDs solutions.*

Effective electrophoretic mobility ( $\mu_e$ ) was determined for the neutral analytes BBK, PFK and TBA, each dissolved at 0.01 mg/mL concentration in a water/methanol solution (90/10, v/v). The level of methanol in the solution was chosen to be the lowest allowing for the complete dissolution of the analytes; the presence of methanol in the samples solutions was exploited, as the neutral marker, in the determination of the electroosmotic flow (EOF). The effective electrophoretic mobility was calculated using the conventional equations. Prior the analysis, the capillary was conditioned with NaOH 0.1 M, water and the running buffer, for 5 min. The sample solutions were loaded into the capillary by hydrodynamic injection at 50 mbar for 2 s. The UV detection wavelength was 210 nm and the applied voltage was 30 kV. The running buffer was sodium phosphate 10 mM (pH 8.0) supplemented with the studied cyclodextrins ( $\beta$ CD, HP- $\beta$ CD and DM- $\beta$ CD) at 20 mM concentration. The concentration of SDS was varied within a wide range (17 – 164 mM) and the calculated effective mobility for each of the studied analytes was plotted against the SDS concentration.

#### *EPR experiments*

*Tert*-butylbenzyl nitroxide (TBBN) radical is generated by mixing a solution of the corresponding amine (*tert*-butyl benzyl amine; 0.8 mM)

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with a solution of the magnesium salt of monoperoxyphthalic acid (Aldrich, technical grade) 0.8 mM. In order to achieve a sufficiently large radical concentration, the mixed solution is generally heated at 60 °C for 1-2 min. Aliquots from a concentrated surfactant and CD solution are added to the solution of nitroxide to yield the required concentrations. Samples are then transferred in capillary tubes (1 mm i.d.) and the EPR spectra are recorded. Digitized EPR spectra are transferred to a personal computer for analysis using digital simulations carried out with a program developed in our laboratory and based on a Monte Carlo procedure (20).

#### *Chiral separations*

Chiral CE analysis of (±)-Catechin, (±)-Ibuprofen and (±)-Ketorolac was performed using the “short-end” injection mode (effective capillary length was 8.5 cm; total length was 38.5 cm) at the constant voltage of 15 kV. The temperature was maintained at 15 °C and the UV detection was fixed at 200 nm. Hydrodynamic injection of aqueous sample solutions of the analytes (0.01 mg/mL) was carried out at 25 mbar for 2 s. The running buffer was an aqueous 50 mM phosphoric acid solution at pH 2.5 (adjusted with NaOH 0.1 N). This was supplemented with 20 mM of the studied CDs; the BGE contained also SDS at different concentrations in the range 30 – 70 mM.

## Results

### *Effect of CDs on the critical micelle concentration (CMC) of SDS.*

The CMC of ionic surfactants can be conveniently determined by using CE instrumentation (chapter 1).

In the present study, in order to test the reliability of the method, the CMC of SDS

was preliminary evaluated in deionized water; the CMC was found to be 7.9 mM (RSD % 2.1;  $n = 3$ ) in good agreement with the values reported in the literature (14, 23). Successively, the CMC of SDS was determined in the presence of  $\alpha$ CD,  $\beta$ CD,  $\gamma$ CD, HP- $\beta$ CD, DM- $\beta$ CD. In Fig. 1 a representative plot current - SDS concentration in the absence and in the presence of either  $\beta$ CD and DM- $\beta$ CD (10 mM) is depicted. As expected and according to previous reports (10 - 16), the presence of cyclodextrin significantly increased the CMC of SDS.

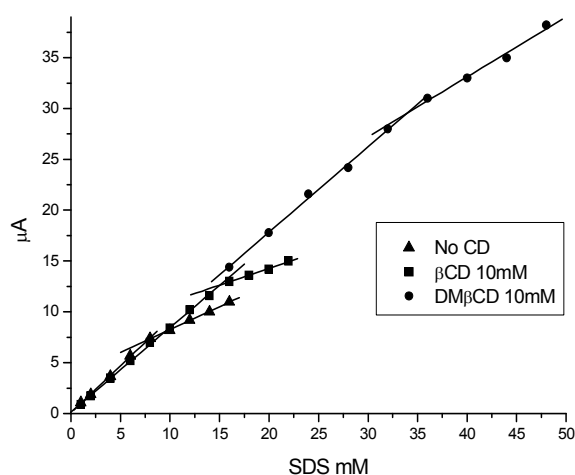


Fig. 1: Plot of the CE current ( $\mu$ A) versus the SDS concentration (mM).  
Experimental conditions: Total capillary (id  $50\mu$ m) lengths: 38.5 cm;

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applied voltage 20 kV; temperature 25 °C. ▲ in water; ● in presence of DM-βCD (10mM), ■ in presence of βCD (10mM)

Table 1 shows the obtained CMC values of SDS, determined in the presence of different CDs each at the concentration of 10 mM. It can be observed that the inhibition of micellization can be related to the nature of the CD. In particular, βCD and HP-βCD showed similar micellization inhibition whereas the CMC values obtained with methylated CD was hardly determined because the slopes of the straight lines did not vary abruptly (Fig. 1); this effect was particularly pronounced at CD concentrations higher than 15 mM.

CD type	CMC* (mM)
no CD	7.9
αCD	13.3
βCD	15.1
γCD	21.4
HP-βCD	14.6
DM-βCD	34.0

Table 1. CMC values of SDS in the presence of CDs (10 mM), determined by the electric current method.

\*The obtained values are the mean of three determinations (n = 3; RSD % < 1.8).

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A further investigation was carried out to evaluate the effect of the CD concentration on the CMC values. In these experiments  $\beta$ CD, HP- $\beta$ CD and DM- $\beta$ CD were used in the concentration range of 2 – 15 mM and the CMC of SDS was estimated by the electric current method. By plotting the obtained CMC versus the CDs concentration, linear trends (Fig. 2) were obtained ( $r > 0.999$ ) for  $\beta$ CD and HP- $\beta$ CD, whereas using DM- $\beta$ CD, the variation of CMC did not follow a linear profile, suggesting an unconventional ability in micellization inhibition.

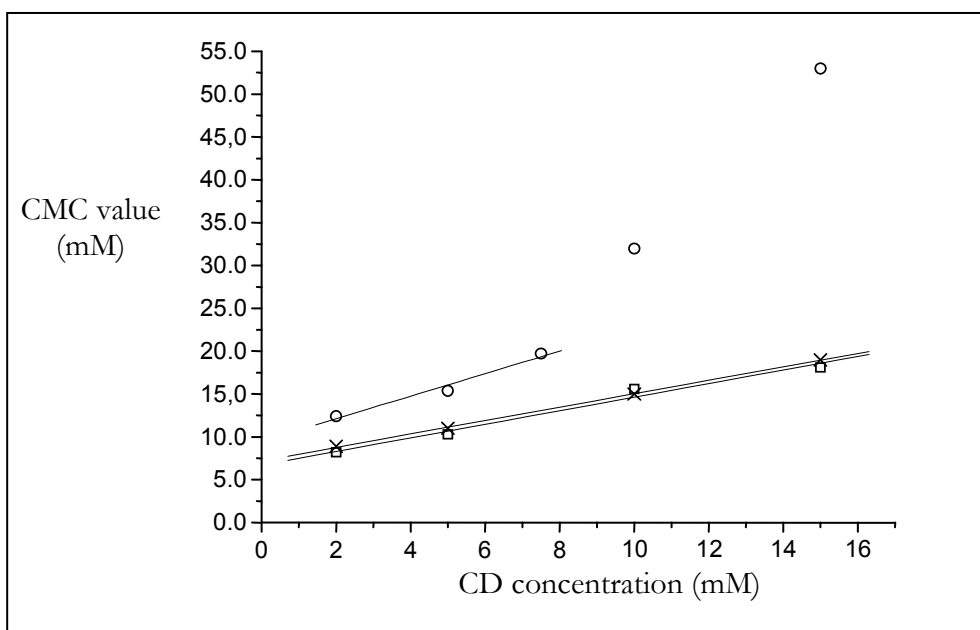


Fig. 2: Variation of the CMC of SDS in the presence of different concentration of CDs. Experimental conditions: Total capillary (id 50 $\mu$ m) lengths: 38.5 cm; applied voltage 20 kV; temperature 25 °C. Symbols:  $\circ$  DM-  $\beta$ CD;  $\square$   $\beta$ CD;  $\times$  HP-  $\beta$ CD.

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*Study of the association of  $\beta$ CDs and SDS by CE*

In order to achieve information on the micellization of SDS in the presence of CDs, the stoichiometry of the complexes SDS:CDs and the related association constants needed to be estimated. To this purpose an original approach based on CE electric current measurements has been developed. The method is based on the variation observed in the electric current circulating in the CE capillary filled with a submicellar SDS solution, when increasing CD amounts were added. Precisely, the surfactant concentration was kept constant at a low level (2.5 mM) and the CD concentration was progressively increased in the range 0.5 – 10 mM. Because of the association of ionic surfactant monomers to the cyclodextrin, a decrease of electric current is observed. In fact, the electric current parallels the behaviour of conductance, which has been widely reported as a parameter used in several studies on surfactant – cyclodextrin interaction (9, 11, 24 – 26). According to Ohm's laws, the current intensity  $I$  circulating in a capillary filled with ionic surfactant solution at submicellar level, is proportional to the ionic conductivity  $\kappa$ . In these experiments low current intensity values are observed, however the CE instrumentation allows the electric current to be measured with adequate sensitivity and precision ( $\pm 0.1 \mu\text{A}$ ) under controlled temperature ( $\pm 1 \text{ }^\circ\text{C}$ ); these conditions allowed for reliable determinations. Further, the small sample volume required and the opportunity of automation, represent valuable advantages over the conventional conductance experiments.

Once the electric current of the SDS solution was recorded as a base value (absence of CD), the decrease ( $\Delta I$ ) values, occasioned by

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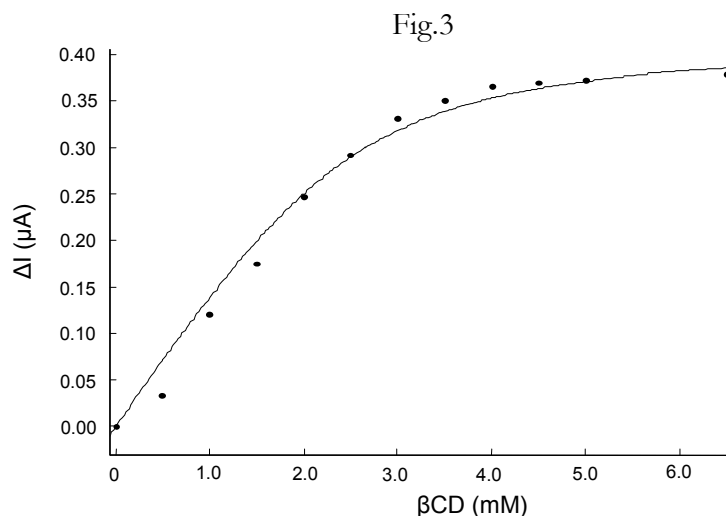
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supplementing the plain solution (control solution) with the studied CD, were determined. The investigation was carried out using  $\beta$ CD, HP- $\beta$ CD and DM- $\beta$ CD. The  $\Delta I$  values obtained respect to the base current, were plotted against the correspondent CD concentrations; the stoichiometries of the inclusion complexes were deduced form the breaks in the current profiles. Moreover, the association constants  $K$  for 1:1 complexation can be obtained by applying a nonlinear least-square regression to an equation (Eq. 1), derived in analogy with that proposed by Satake *et al.* for conductance experiments (22, 23).

$$\Delta I = \frac{\Delta i}{2KC_s} \left\{ K(C_s + C_c) + 1 - \left( \left[ K(C_s - C_c) + 1 \right]^2 - 4K^2 C_s C_c \right)^{\frac{1}{2}} \right\} \quad (\text{Eq. 1})$$

where  $\Delta i$  (difference in the ionic equivalent electric current) is proportional to the ionic equivalent conductivity as  $I$  is proportional to the equivalent conductivity ( $\Lambda$ );  $C_s$  is the surfactant concentration (in the control solution) and  $C_c$  the CD concentration. In Fig. 3a the graph related to the experiments using  $\beta$ CD is illustrated.





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Fig 3. Plots of the decrease of CE electric current ( $\Delta I$ ;  $\mu\text{A}$ ) versus the  $\beta$ -CD concentration (mM). The measurements were carried out at a constant concentration of SDS (2.5 mM) in water. Conditions: total capillary (id 50  $\mu\text{m}$ ) length: 38.5 cm; applied voltage of 30 kV; temperature 25  $^{\circ}\text{C}$ . The data points represent the experimental values and the line represents the calculated curve from Eq. 1.

The solid line shows the calculated electric current curve from the reported equation, whereas the points show the experimental data. The obtained regression coefficient ( $r^2 = 0.9843$ ) suggests a good fit with the proposed 1:1 stoichiometry. Thus, the estimated association constant  $K$  ( $3021 \text{ M}^{-1} \pm 98$ ;  $n = 3$ ) can be reasonably assumed as a reliable data which, in fact, was found to be in agreement with those reported in the literature (25, 26). Concerning HP- $\beta$ CD (Fig. 4), the 1:1 stoichiometry was also confirmed by the good correlation coefficient ( $r^2 = 0.9876$ ) and the  $K$  value was found to be  $2871 \text{ M}^{-1} \pm 107$  ( $n = 3$ ).

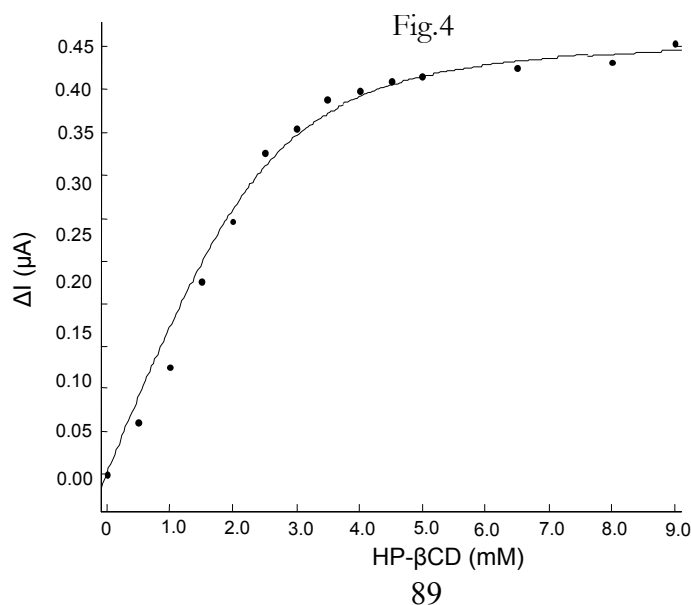


Fig. 4. Plots of the decrease of CE electric current ( $\Delta I$ ;  $\mu\text{A}$ ) versus the  $\beta$ -CD concentration (mM). The measurements were carried out at a constant concentration of SDS (2.5 mM) in water. Conditions: total capillary (id 50  $\mu\text{m}$ ) length: 38.5 cm; applied voltage of 30 kV; temperature 25  $^{\circ}\text{C}$ . The data points represent the experimental values and the line represents the calculated curve from Eq. 1.

It is noteworthy that the similarity of the data obtained for the  $K$  values of  $\beta\text{CD}$  and HP- $\beta\text{CD}$  are paralleled by the CMC values (Table 1). This observation can prove both the reliability of the proposed methods and the analogy of the interaction of  $\beta\text{CD}$  and HP- $\beta\text{CD}$  with SDS. Differently, the experiments carried out with DM- $\beta\text{CD}$  showed a characteristic profile (Fig. 5) that did not fit with the proposed model (Eq. 1), according to a 1:1 stoichiometry. Thus, in order to better characterize the interaction between SDS and DM- $\beta\text{CD}$  our attention was focused on the estimation of the partitioning rate of a radical probe in the different pseudo phases by using EPR methods.

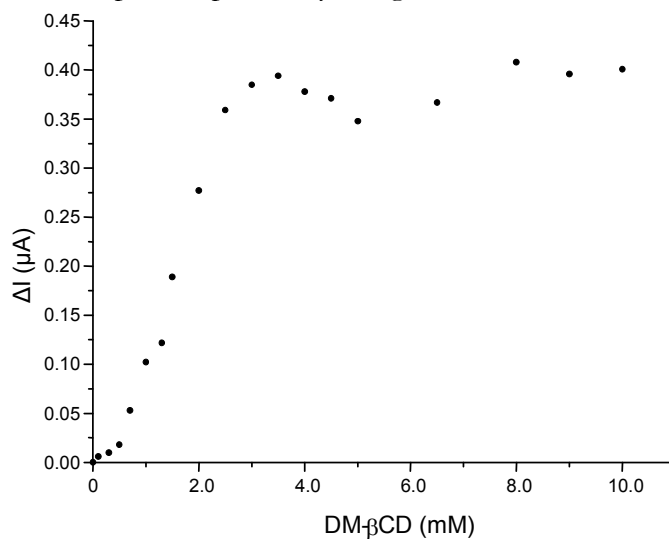


Fig.5

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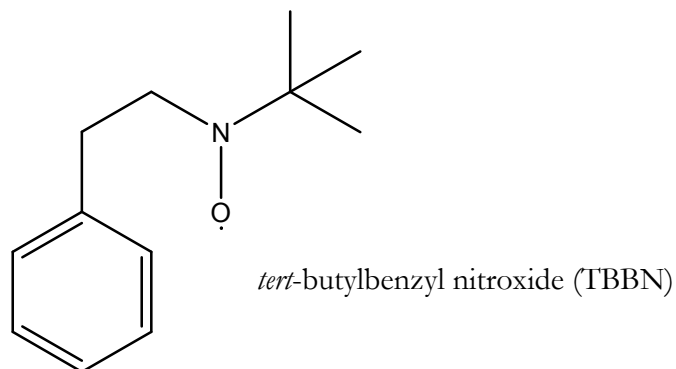
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Fig.5: Plot of the decrease of CE electrical current ( $\Delta I$ ;  $\mu A$ ) versus the DM- $\beta$ CD concentration (mM). The measurements were carried out at the constant concentration of SDS (2.5 mM) in water. Conditions: total capillary (id 50 $\mu$ m) lengths: 38.5 cm; applied voltage 30 kV; temperature 25 °C. The data points represent the experimental values.

#### *EPR studies on the interaction of SDS and neutral cyclodextrins.*

We have recently shown that EPR spectroscopy is suitable for studying the partitioning rate of a dialkyl nitroxides, namely , in CD/micelle systems (27). The method is based on the significant differences in the EPR parameters shown by TBBN when it experiences water, cyclodextrin cavity or micellar environment. Under these conditions, the EPR spectra also show a strong line width dependence on temperature both in the presence of SDS micelle and CD, indicating that the lifetime of the radical in the associated and free form is comparable to the EPR timescale. Because of this favourable feature, the analysis of the line shape makes possible to measure the rate constants for the partition of the probe in the pseudo-phases.



The EPR spectra at 25°C of TBBN, produced by reaction of the magnesium salt of monoperoxyphthalic acid (0.8 mM) with *tert*-butyl benzyl amine (0.8 mM) in the presence of SDS/ $\beta$ CD, SDS/HP- $\beta$ CD and SDS/DM- $\beta$ CD in water, are shown in Figure 6.

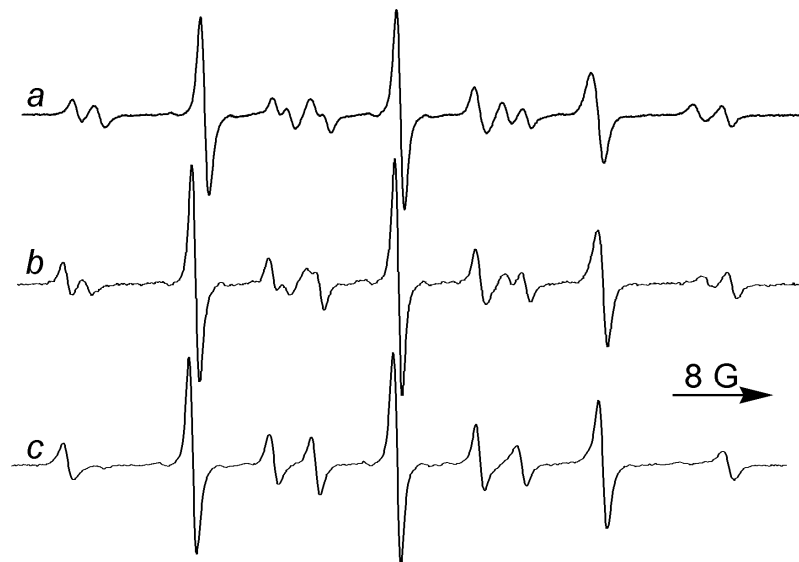


Fig. 6. EPR spectra of TBBN recorded in water at 298 K in the presence of SDS 49 mM. (a)  $\beta$ CD 16 mM; (b) HP- $\beta$ CD 16 mM; (c) DM- $\beta$ CD 16 mM.

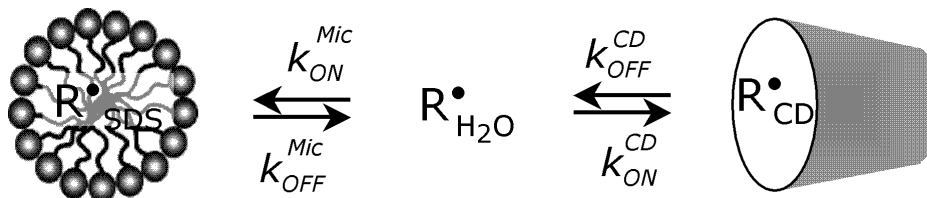
All the spectra can be correctly reproduced only by assuming the kinetic scheme reported in Scheme 1 in which the radical probe is exchanging, with a rate comparable to the EPR time scale, between the water phase and both the CD cavity and the micellar pseudo-phase. Simulation of the exchange-broadened EPR spectra, assuming a three-jump model as illustrated in Scheme 1, led to the determination of the rate constants (see Table 2) of the exchange processes.

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[SDS] / mM	CD	$k_{ON}^{MIC} / s^{-1}$	$k_{OFF}^{MIC} / s^{-1}$
33	no CD	$6.2 \times 10^6$	$3.9 \times 10^6$
26	$\beta$ CD	$8.0 \times 10^5$	$3.9 \times 10^6$
33	“	$3.0 \times 10^6$	$3.9 \times 10^6$
49	“	$6.2 \times 10^6$	$3.9 \times 10^6$
26	HP- $\beta$ CD	$7.8 \times 10^5$	$5.0 \times 10^6$
33	“	$2.9 \times 10^5$	$5.0 \times 10^6$
49	“	$6.0 \times 10^5$	$5.0 \times 10^6$
33	DM- $\beta$ CD	$1.6 \times 10^7$	$4.4 \times 10^7$
49	“	$1.5 \times 10^7$	$2.8 \times 10^7$
74	“	$1.5 \times 10^7$	$1.5 \times 10^7$

Table 2. EPR rate constants at 298 K for the partition of TBBN in the micellar location ([CD]=16 mM).



Scheme 1.

*CE mobility of neutral analytes in mixed systems (SDS/CDs).*

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The three different systems investigated by EPR (SDS/ $\beta$ CD, SDS/HP- $\beta$ CD and SDS/DM- $\beta$ CD) were used as BGEs in the separation of three different neutral analytes, namely: *tert*-butylbenzylketone (BBK), pentyl-phenylketon (PFK) and 4'-*tert*-butylacetophenone (TBA).

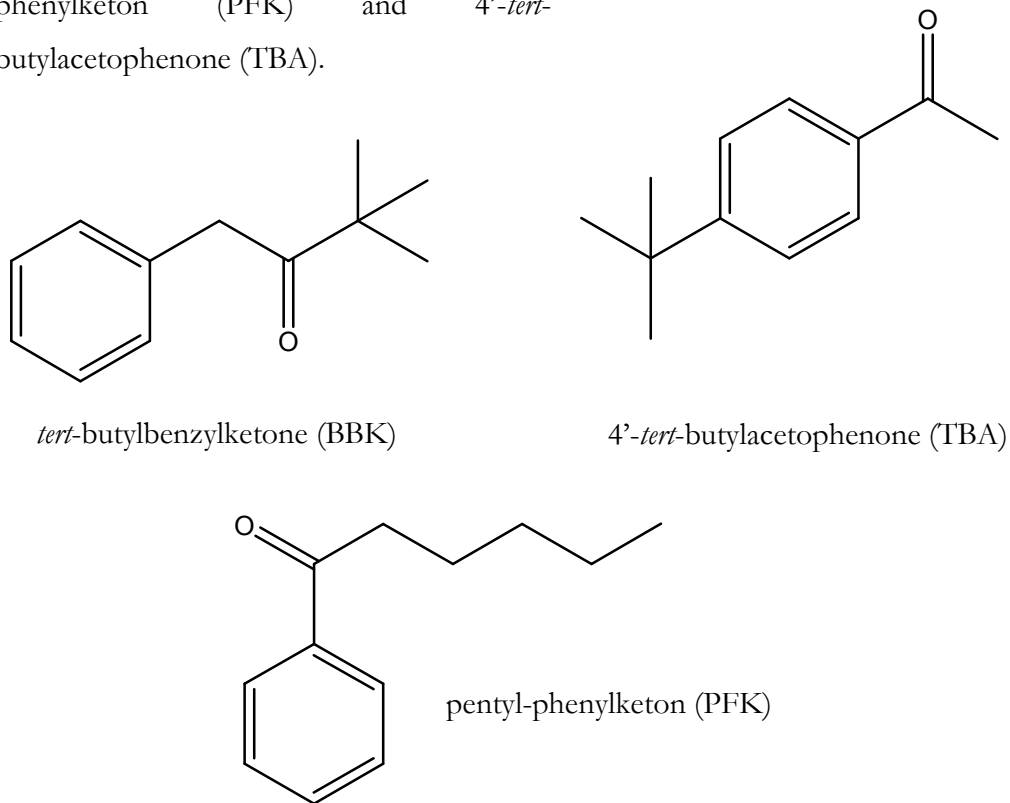


Fig. 7. Chemical structure of the three probe used in mobility studies.

Compound BBK represented the diamagnetic analogue of TBBN used in EPR experiments, whereas PFK and TBA were related isomers. The calculated (28) Log P values resulted to be 3.12, 3.76 and 3.68, respectively; they were similar to that of naphthalene (3.40), reported to

be one of the most suitable marker for studies of CMC by CE mobility methods (29).

The CE mobility of the neutral probes was determined at a fixed CD level (20 mM) in the presence of SDS, which was varied in a wide range of concentration. The variation of the effective electrophoretic mobility of neutral analytes in CD-MEKC systems, was plotted versus the surfactant concentration. The calculation of the effective mobility ( $\mu_e$ ) only required the electroosmotic mobility to be measured, whereas the mobility of the micelle may be neglected (3, 14, 29). This aspect simplified the study being the micelle mobility measurements very questionable in complex systems such as a mixture surfactant – cyclodextrin.

As shown in Fig. 8a, in the presence of  $\beta$ CD the mobility profiles of the selected probes show a marked break point, whereas in the presence of HP- $\beta$ CD (Fig. 8b) and most of all, using DM- $\beta$ CD (Fig. 8c), the change of mobility is, in general more gradual.

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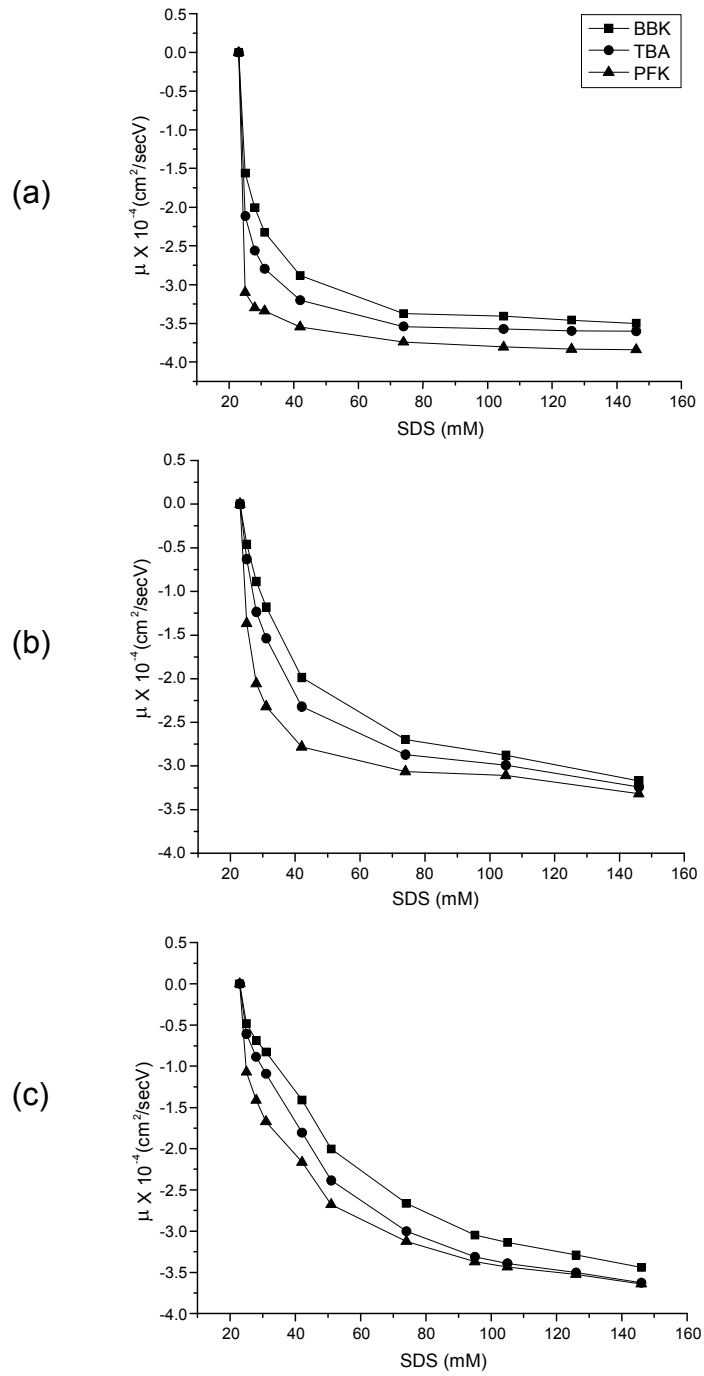


Fig.8



Fig.8. Effect of SDS concentration on the effective mobility of neutral analytes (BBK, TBA and PFK, 0,01mg/mL) at a constant concentration of: (a)  $\beta$ CD (20mM); (b) HP- $\beta$ CD (20mM) and (c) DM- $\beta$ CD (20 mM). Conditions: Total capillary (id 50 $\mu$ m) lengths: 38.5 cm (30 cm, effective length); applied voltage 30 kV; hydrodynamic injection, 50 mbar x 2 s; UV detection at 210 nm; temperature 25 °C. BGE: sodium phosphate 10 mM, pH 8.0.

*Application to chiral separations.*

The three studied systems, namely SDS/ $\beta$ CD, SDS/HP- $\beta$ CD and SDS/DM- $\beta$ CD were used in enantioselective CD-MEKC analysis of ( $\pm$ )-Catechin, which was chosen as a model compound. There are important evidences that catechins (natural polyphenols from green tea, wine, fruits etc.) have a role against cancer, cardiovascular diseases and other degenerative diseases (30); the chiral analysis of these compounds is recently become of interest because it was shown that (–)-Catechin can be considered as a non-native enantiomer and its presence in food and nutraceuticals should be ascribed to epimerisation reactions occurring in manufacture processes involved in food technologies (18, 31, 32). Furthermore, it was found that the native (+)-enantiomer is more bioavailable than the (–)-diastomer (32, 33). Thus the enantioresolution of ( $\pm$ )-Catechin was tried in order to evaluate the effectiveness of the studied SDS/CD systems as potential chiral BGEs. Under acidic conditions (pH 2.5 in a 10 mM phosphate buffer), Catechin is undissociated and the mobility of its enantiomers has to be ascribed only to their different partition/inclusion in the complex, chiral pseudostationary phase. In addition, the EOF was strongly suppressed

because of the acidic pH and, in the presence of anionic SDS micelles, higher was the SDS concentration and faster was the anodic migration of the neutral solute. The chiral separation of ( $\pm$ )-Catechin was carried out at constant concentration of CDs (20 mM) whereas the SDS level was varied (50, 60 and 70 mM); the obtained electropherograms are shown in Fig. 9. Using HP- $\beta$ CD in the presence of the relatively lowest amount of SDS, the migration of Catechin enantiomers was not achieved within 30 min (Fig. 9b); this result suggests a strong competition of the CD over the anionic carrier, in the sequestering ability exerted on the analyte. Differently, in the presence of  $\beta$ CD and DM- $\beta$ CD the Catechin enantiomers migrated in short time with good resolution (Fig. 9a and 9c). As expected, the increase of SDS concentration allowed for a faster migration of the enantiomers in all the systems (Fig. 9a - c). Interestingly, as shown in the figures 9a and 9b, baseline enantioresolution was maintained also at high SDS concentration using either the  $\beta$ CD and HP- $\beta$ CD. Differently, in the presence of DM- $\beta$ CD high enantioresolution was achieved only at low SDS concentration; the increase of SDS diminished the migration time but a considerable deterioration of separation was observed.

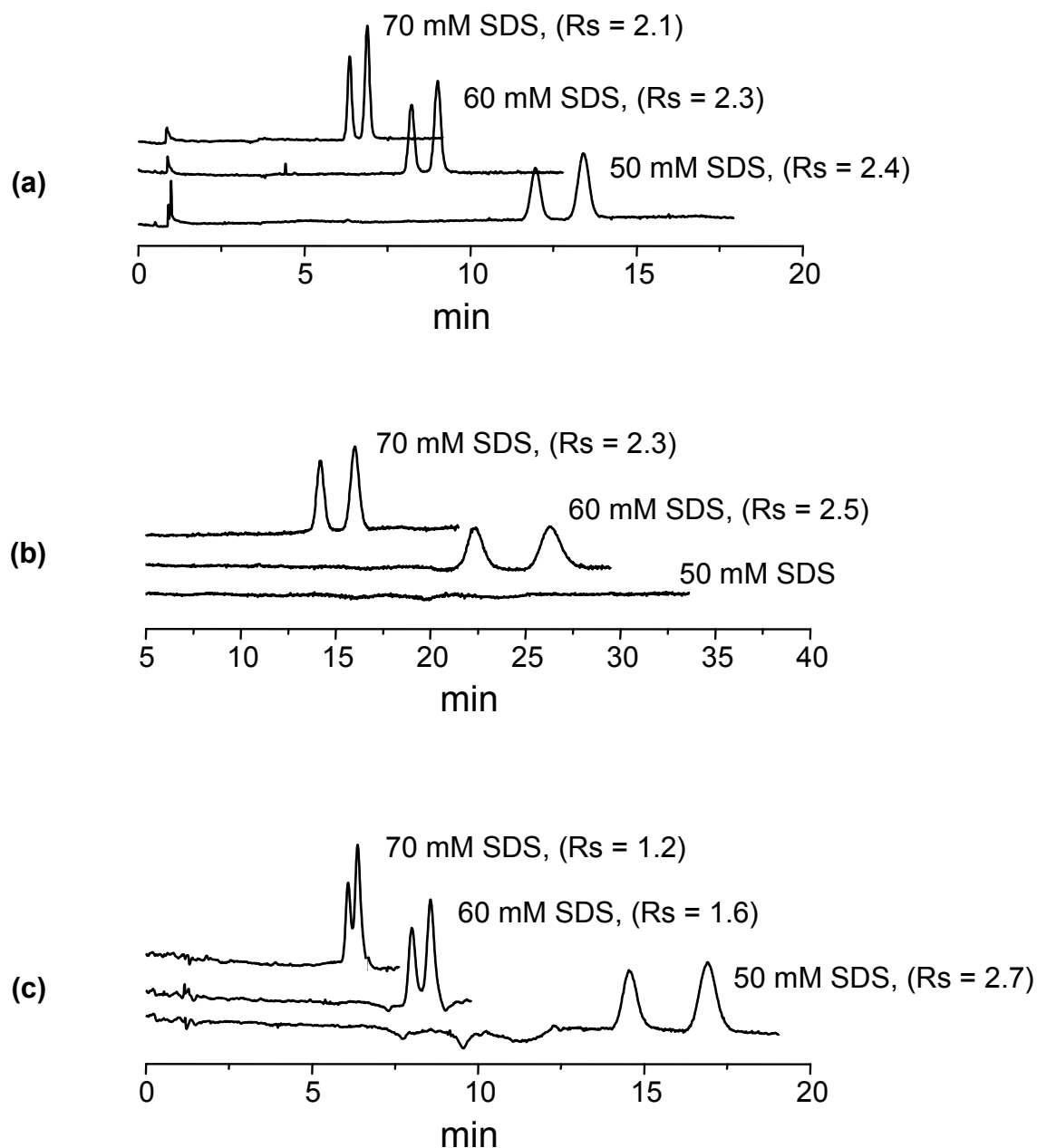


Fig 9. Electropherograms of ( $\pm$ )-Catechin (0.01 mg/mL) under CD-MEKC conditions using different CDs (at 20 mM): (a)  $\beta$ CD, (b) HP-

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$\beta$ CD and (c) DM- $\beta$ CD, in the presence of different SDS concentrations.

Conditions. Total capillary (id 50  $\mu$ m) length: 38.5 cm (8.5 cm, effective length); applied voltage, 15 kV; hydrodynamic injection, 25 mbar x 2 s; UV detection at 200 nm; temperature, 15 °C. BGE: sodium phosphate 50 mM, pH 2.5.

## Discussion

*Association of SDS to CD by electric current CE experiments.*

In mixed systems such as surfactant - CD, it is assumed that the surfactant aggregation occurs only when all the available CD cavities are occupied by the surfactant monomers. Thus, the CMC ( $CMC_{app}$ ) will be equivalent to the combined concentrations of the surfactant monomers associated to the CD and of free dissolved monomers in equilibrium with the micellized surfactant ( $CMC_{real}$ ) (12). However, as shown by the CMC values determined by the CE current method in the presence of a constant amount of different CDs (Table 1), the nature of the CD plays an important role in micellization inhibition. In particular, using DM- $\beta$ CD, the CMC was strongly increased compared to the other CDs. Furthermore the slopes of the straight lines describing the variation of CE electric current under increasing SDS concentration, showed a slight slope variation between the submicellar and micellar regions, in the presence of the methylated CD. In Fig. 1 the described behaviour is shown for DM- $\beta$ CD in comparison to  $\beta$ CD. The limited difference in the slopes between the two parts of the graph for DM- $\beta$ CD, could be explained by invoking a small difference between the micelle size (above the CMC) and the size of SDS aggregates in the submicellar region. This behaviour was in fact also reported in micellization of SDS in the

presence of organic solvents such as acetonitrile. In the latter case, the generation of small surfactant – solvent mixed aggregates was suggested to be responsible for the gradual change in electric current agreeing with the multiple equilibria model (stepwise aggregation model) (15, 34). Accordingly, it should be hypothesized that the strong inhibition effect of the methylated CD on the micellization of SDS, might lead to the formation of intermediates aggregation states of the surfactant monomers.

A further evidence of the unusual interaction of DM- $\beta$ CD with SDS is provided by the results shown in the graphs of Fig. 2. The CMC of SDS increases linearly with the increasing concentration of  $\beta$ CD and HP- $\beta$ CD; in these instances it could be proposed that the ability of the CD in preventing the micellization (micellization inhibition) can be expressed by the slope of the straight line. From this point of view  $\beta$ CD and HP- $\beta$ CD behave similarly; differently, the experimental data distribution shown in Fig. 2 for DM- $\beta$ CD, can suggest that the micellization inhibition is strongly related to the CD concentration. On this basis, the unusually high CMC of SDS observed in the presence of relatively high DM- $\beta$ CD concentrations, could be the result of high association constants, complexation stoichiometries different from the simple 1:1 model and/or modification of the micelle aggregation number.

The complexation stoichiometry of SDS to CD, appeared to be as a fundamental parameter in this study and it was approached by means of an original method based on CE electric current measurements. The obtained results confirmed that the interaction of DM- $\beta$ CD with SDS

significantly deviated from the behaviour of  $\beta$ CD and HP- $\beta$ CD (Fig. 3 and Fig. 4). In fact, while the mathematical analysis of the experimental data obtained for  $\beta$ CD and HP- $\beta$ CD was simple and suggested a complexation pattern 1:1, that for DM- $\beta$ CD led to a non unique numerical solution. Similar situations are often encountered in solving thermodynamic equations dealing with inclusion complexation involving cyclodextrins. In such cases, more sophisticated models with variable stoichiometry could be invoked, and the possibility of dimerization of 1:1 complexes should not be excluded (35). By these considerations and due to the lack of further information about more sophisticated data treatments as well as other physicochemical data, we eventually did not apply models deviating from the simple 1:1 stoichiometry. This study was thus approached by using EPR, a spectroscopic technique which could offer a different perspective on the aggregation organization of SDS/CD systems.

*EPR studies.*

EPR data can be employed in the determination of thermodynamic and kinetic parameters of an organic spin probe in the three different “pseudo phases” (SDS, CD and water). The rate constant for the partitioning of TBBN radical in water, SDS and cyclodextrin cavities (see Table 2) indicates that the change in the nature of the macrocyclic host has a dramatic effect on the partitioning behaviour of the radical probe. Actually a dramatic reduction of the residence time of the radical guest in the micelle is observed along the series  $\beta$ CD, HP- $\beta$ CD, DM- $\beta$ CD. With  $\beta$ CD and HP- $\beta$ CD the rate of escape ( $k_{\text{OFF}}^{\text{MIC}}$ ) from the micelle is not affected by the CD concentration and the increase in the radical probe

fraction partitioned in the micellar phase observed by increasing the concentration of SDS is due to an increase of the pseudo-first order rate of solubilization ( $k_{\text{ON}}^{\text{MIC}}$ ). On the contrary, in the presence of DM- $\beta$ CD the increase of the amount of TBBN partitioned in the micellar phase observed when increasing the concentration of SDS is related to the decrease of the rate of escape ( $k_{\text{OFF}}^{\text{MIC}}$ ) from the micelle. This last result is a clear indication that methylated CD is strongly interacting with SDS micelle, very presumably, by altering the micellar structure in agreement with the results found by CE experiments. On the other hand, these hypothesis is paralleled by the EPR evidences showing that DM- $\beta$ CD significantly alters the structure of the micelles by lowering their aggregation number as well as the polydispersivity and by increasing the solvent penetration (27).

*CE mobility studies.*

Studies on CE mobility of selected neutral probes as well as on enantioseparation of bioactive compounds, were performed to investigate on the applicative repercussions of the different SDS/CD systems.

In CD-MEKC experiments involving both neutral solutes and CDs, the effective electrophoretic mobility of the analytes could approach to zero (equal to the EOF) if: (i) the CMC is not achieved, or (ii) the solutes are strongly retained into the CD cavities also in the presence of charged micelles. The inclusion of a solute into the CD cavity is a process in competition with its partitioning into the micelle; these two coupled equilibria are affected by the nature of the solute, thus in our investigation different probes were considered.

Under variation of SDS level, the mobility of the probes varied by following the hydrophobicity order (PFK the most retained into the micelle and BBK the less retained) independently from the used CD type (Fig. 8). Furthermore, all the neutral markers migrated concomitantly to the EOF ( $\mu_e = 0$ ) approximately at the same SDS concentration independently from the CD nature. Precisely, below 25 mM (SDS) the mobility of the probes resulted to be nearly overlapped to the system peak assigned to the EOF. This finding confirmed that the  $CMC_{real}$  is lower than  $CMC_{app}$ , the latter estimated as the sum of CD concentration plus the CMC of SDS in water ( $\approx 8$  mM). A further observation was related to the very different trend of the mobility profiles that were found to be strongly dependent on the CD type. Using DM- $\beta$ CD (Fig. 8c) the mobility of the probes decreased more gradually compared to the decrease observed with HP- $\beta$ CD and  $\beta$ CD (Fig. 8a and Fig. 8b). This behaviour suggests that the methylated cyclodextrin could affect the micellization of the surfactant by promoting the formation of aggregates characterized by a lower partitioning ability toward the solutes. On the other hand, these hypothesis is paralleled by the EPR evidences showing that DM- $\beta$ CD significantly alters the structure of the micelles by lowering their aggregation number as well as the polydispersivity and by increasing the solvent penetration.

*Application to chiral separations.*

The chiral separation of ( $\pm$ )-Catechin was performed under acidic pH in order to suppress the dissociation of phenolic moieties; the compound resulted in its neutral form and, under these conditions, the enantioseparation can be assumed merely as the result of the partitioning



behaviour of the enantiomers between SDS and CD. Simple analytical parameters, namely the migration time and chiral resolution of the racemic compound, could be considered useful in describing the organization of the complex mixed medium.

As shown in the electropherograms of Fig. 9, ( $\pm$ )-Catechin resulted to be enantioresolved using each of the studied neutral CD; this could suggest a similarity in the interaction of the solute with the three different CDs. By supplementing these systems with increasing SDS amounts, a competition between the solute and the SDS monomers in gaining the CD cavity, takes place. The lost in enantioresolution observed at increasing SDS concentrations can be assumed as a parameter which is related to the binding SDS - CD. The electropherograms (Fig. 9a and 9b) suggest that the enantioselectivity of  $\beta$ CD and HP- $\beta$ CD was poorly affected by the variation in SDS concentration. In these examples, the interaction between SDS and  $\beta$ CD or HP- $\beta$ CD, seems to be slightly affected by the variation of their mutual concentrations and it could be hypothesized that once the saturation of CD by SDS (1:1 stoichiometry) has occurred, the two “pseudostationary phases”, CD and micelle, are not involved in further coupled equilibria (secondary complex equilibria). Differently, in the systems using DM- $\beta$ CD, the mutual variation of the concentration of both the “pseudostationary phases”, could lead towards a continuous alteration of the combination pattern SDS:CD that affects the enantioselectivity.

Although these findings are far from a description of a general behaviour, from our experiments using ( $\pm$ )-Catechin,  $\beta$ CD and HP- $\beta$ CD proved to be suitable in developing CD-MEKC systems with relatively

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ease enantioselectivity tuning by means of changing the concentration of both SDS and CD in a wide range (18). On the contrary the system involving DM- $\beta$ CD showed to be less robust and versatile; in particular, the enantioseparation was obtained in a restricted range of SDS concentration.

On the other hand, it is worth considering the potential of DM- $\beta$ CD in appropriate selected conditions. In fact, in a exploratory experiment this cyclodextrin was found to be enantioselective for ( $\pm$ )-Ibuprofen and ( $\pm$ )-Ketorolac (Fig. 10) whereas HP- $\beta$ CD and  $\beta$ CD failed in this attempt.

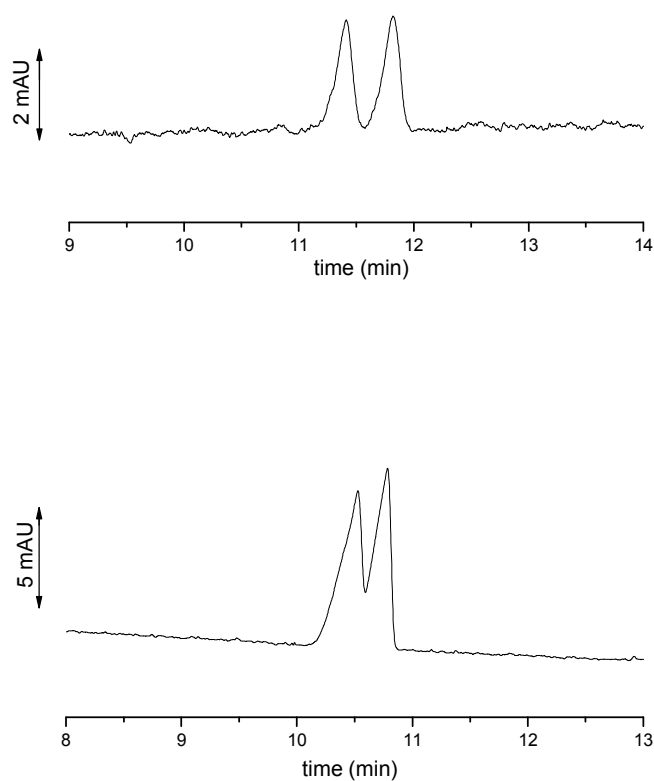


Fig. 10. Electropherograms of (a) rac-Ketorolac (0.01 mg/mL) and (b) rac-Ibuprofen (0.01 mg/mL) under CD-MEKC conditions using DM-

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$\beta$ CD (20 mM) as chiral selector, in the presence of SDS 50 mM.

Conditions: Total capillary (id 50  $\mu$ m) length: 38.5 cm (8.5 cm, effective length); applied voltage, 15 kV; hydrodynamic injection, 25 mbar x 2 s; UV detection at 200 nm; temperature, 15 °C. BGE: sodium phosphate 50 mM, pH 2.5.

### **Conclusion**

Capillary electrophoresis has been demonstrated to be a useful tool to study the interaction between the ionic surfactant SDS and neutral cyclodextrins. Electric current measurements performed by CE instrumentation were applied in the determination of CMC of SDS in mixed systems involving the presence of CDs. Further, for the first time, a similar approach was proposed for the estimation of inclusion complexation constants of neutral CDs with SDS as the guest. From the data obtained, DM- $\beta$ CD was shown to deviate from the simple 1:1 complex model that was observed to be valid for  $\beta$ CD and HP- $\beta$ CD; the complex interaction of DM- $\beta$ CD and SDS was confirmed by EPR studies.

The results suggest that the macrocycle DM- $\beta$ CD can be considered less suitable in the development of robust chiral CD-MEKC systems. On the other hand, it should be pointed out that DM- $\beta$ CD at relatively low SDS concentration levels, maintains a chiral discrimination ability which can be usefully exploited. These results underline the importance of an adequate understanding of the micelle-CDs interactions in order to properly apply this complex systems able to enhance the general

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selectivity and in particular the enantioselectivity toward biologically important compounds.

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## **Chapter 6:**

Differentiation of green tea samples by chiral CD-MEKC  
analysis of catechins content.

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**Introduction**

Important evidence from *in vivo* studies shows that green tea catechins play a role against biomarkers for cancer, cardiovascular diseases and other degenerative diseases (1, 2). Recently, structure-activity relationships of tea compounds have been investigated and the catechol functionality in the catechins has been considered to be responsible for the protective effects exerted by green tea against a wide range of human diseases (3, 4). Analysis of the content of catechins in green tea, matcha, tea beverages, green tea extract dietary supplements as well as in biological fluids, is thus very important in a variety of studies including epidemiological and nutritional studies designed to examine the possible relationships between tea consumption and the incidence of cancer and cardiovascular diseases (1 – 4). In these fields, the most of the works focus on the determination of (-)-Epicatechin, (+)-Catechin, (-)-Epigallocatechin, (-)-Epicatechin gallate, (-)-Gallocatechin gallate and (-)-Epigallocatechin gallate; also methylxanthines such as Caffeine and Theobromine are often considered because of their relevant presence in tea extracts. Reversed-phase HPLC has been conveniently applied in these determinations using, gradient elution and UV detection (5 – 9), except in a few cases (10, 11). In these reports, the analysis was performed to evaluate the quality of the extracts, as well as to provide the differentiation of tea samples based on geographical origin (7), seasonal variations (8) and processing (9). Electrochemical detection (12 – 17) and hyphenation LC-MS (18 - 20) were applied in analysis of biological samples (plasma, saliva etc.).



## Differentiation of green tea samples by chiral CD-MEKC analysis of catechins content.

The necessity to simultaneously analyse the phenolic compounds (catechins-related components) and methylxanthines has made micellar electrokinetic chromatography (MEKC) a suitable approach for this task. Neutral conditions are usually selected in order to minimize the degradation of catechins occurring under basic pH (21 – 24); however, as reported by Wörth *et al.*, acidic conditions should be preferred to further improve their stability during separation (25). On this basis it has been previously reported effective microemulsion electrokinetic chromatographic (MEEKC) methods for catechins analysis at pH 2.5 in *Cistus* extracts and green tea (26, 27). The acidic conditions were also exploited using different MEKC systems involving cyclodextrins (CDs) in the separation buffer to improve the selectivity of separation. This approach was applied to the analysis of catechins in chocolate and *Theobroma cacao* (28, 29). By means of this chiral method, and for the first time, the presence of (-)-Catechin, a non-native enantiomer, was demonstrated only in the processed food (chocolate), suggesting that it was yielded by epimerisation of (-)-Epicatechin during the manufacturing processes starting from *Theobroma cacao*.

In the present study, a fast MEKC approach mediated by hydroxypropyl- $\beta$ -cyclodextrin (HP-  $\beta$  CD) as chiral selector was optimised for the enantioseparation of ( $\pm$ )-Catechin and ( $\pm$ )-Gallocatechin. The method allowed for the simultaneous separation of the methylxanthines and the most represented green tea catechins.

First, the proposed CD-MEKC was applied to study the thermal epimerisation of (-)-Epigallocatechin to (-)-Gallocatechin. It was demonstrated that the occurrence of (-)-Gallocatechin in tea extracts

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should only be ascribed once epimerisation has taken place as a consequence of manufacturing processes involving heating. (-)-Galocatechin, being a non-native enantiomer, was thus considered, for the first time, as a useful chiral marker of tea degradation.

Subsequently, the validated method was applied to the quantitation of catechins and methylxanthines in more than twenty tea samples of different geographical regions (China, Japan, Ceylon), having undergone different storage conditions and/or manufacturing processes.

**Materials and methods***Materials*

Catechin standard references ( $\pm$ )-Catechin hydrate ( $(\pm)$ -C), (-)-Catechin ((-)-C), (+)-Catechin hydrate ((+)-C), (-)-Epicatechin ((-)-EC), (-)-Epigallocatechin ((-)-EGC), (-)-Epicatechin gallate ((-)-ECG), (-)-Epigallocatechin ((-)-EGC), (-)-Epigallocatechin gallate ((-)-EGCG), (-)-Galocatechin ((-)-GC), (-)-Galocatechin gallate ((-)-GCG), Caffeine (CF), Theophylline (TF) and Theobromine (TB) were from Sigma-Aldrich (Milan, Italy). Syringic acid, sodium dodecyl sulfate (SDS) and hydroxypropyl- $\beta$ -cyclodextrin (HP- $\beta$ CD) were from Fluka (Buchs, Switzerland). (+)-Galocatechin ((+)-GC) was purchased from Molekula (Germany). Boric acid, phosphoric acid, sodium hydroxide, and all the other chemicals of analytical grade, were purchased from Carlo Erba Reagenti (Milan, Italy). Water used for the preparation of solutions and running buffers, was purified by a Milli-Q apparatus (Millipore, Milford, MA, USA). The analysed tea samples were obtained from Grosserbe s.r.l. (Bologna, Italy).

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Fused-silica capillaries (50  $\mu\text{m}$  id, 30 cm total length, 8.5 cm length to the detector) were from Composite Metal Service (Ilkley, UK).

#### *Apparatus*

Electrophoretic experiments were performed by a HP<sup>3D</sup>CE instrument from Agilent Technologies (Waldbronn, Germany). The data were collected on a personal computer, equipped with the software HPCE version A 09 (Agilent Technologies).

The separations were obtained at a constant voltage of 15 kV and the cartridge temperature was 25°C. The detection was carried out by using the on-line DAD detector and the quantitation was performed at the wavelength of 200 nm. Hydrodynamic injections were performed at 25 mbar for 2 s. New capillaries were conditioned by flushing sequentially 1M sodium hydroxide, 0.1M sodium hydroxide and water in the order, for 10 min each. Between the injections the capillary was rinsed with 0.1M sodium hydroxide, water and running buffer for 3 min each.

#### *Solutions*

Borate-phosphate buffer used as the background electrolyte (BGE) was prepared at a concentration of 25 mM and pH 2.5 by following a standard procedure. The running buffer solution was obtained by dissolving SDS (90 mM) and HP-  $\beta$ CD (25 mM) in the BGE.

#### *Calibration graphs*

The calibration was carried out for the catechins: (-)-EC, (+)-C, (-)-C, (-)-EGC, (-)-EGCG, (-)-ECG, (-)-GCG, (-)-CG, (-)-GC and (+)-GC, and for the methylxanthines CF and TB in the ranges reported in Table 1. The linearity of the response was evaluated by analyzing mixed standard solutions of the analytes in the presence of Syringic acid as internal

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standard (100 µg/mL). Triplicate injections were made for each calibration point and the ratios of the corrected peak area (area/migration time) of the analyte to internal standard were plotted against the concentration of each of the analysed compounds.

#### *Epimerisation studies*

Experiments on thermal epimerisation of catechins were carried out on individual aqueous standard solutions of (-)-EC and (-)-EGC at a concentration of 100 µg/mL each and in the presence of Syringic acid as internal standard. The solutions were heated in capped Scott glass tubes, at the temperatures of 70, 80 and 90 °C using a Thermoblock Falc system mod. D200 (Falc Instruments, Bergamo, Italy). The epimerisation of (-)-EC to (-)-C and that of (-)-EGC to (-)-GC was followed by analyzing (during 15 – 120 min) aliquots of the solutions using the chiral CD-MEKC method. In detail, aliquots of the stock solutions subjected to heating were withdrawn after 15, 25, 60 and 120 min and cooled in an ice-bath for 5 min. The solutions were then stored in a refrigerator at 4 °C and analysed (within 3 h) by means of the validated CD-MEKC. Three sets of experiments were performed on independent stock solutions of (-)-EC and (-)-EGC; each of the time points was analysed in triplicate. The catechin amount was calculated by external standard method and the data were plotted to obtain the kinetic profiles.

#### *Preparation of tea samples*

Tea leaves were pulverized and infusions were then prepared by extraction of 1 g of the obtained powder with 60 mL of 85°C water for 5 min. The infusion was filtered through a filter paper and diluted 1:2 with the internal standard (Syringic acid, IS) aqueous solution (100 µg/mL).

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After an additional filtration through membrane filter (0.2  $\mu\text{m}$ ), the samples were directly injected into the CE apparatus.

**Results and discussion***Optimisation of the CD-MEKC*

An aqueous test mixture of three methylxanthines (CF, TB and TF) and the studied green tea catechins (EC, ECG, EGC, EGCG, GCG), including the racemic mixtures ( $\pm$ )-C and ( $\pm$ )-GC in the presence of the internal standard, was employed to find the optimum CD-MEKC separation conditions. Some aspects were considered essential for optimisation of separation conditions: (i) the acidic pH was selected to preserve the catechins from degradation during separation (25 – 27); (ii) the method was aimed at obtaining a fast and simultaneous separation of catechins and xanthines; (iii) the method had to be enantioselective for ( $\pm$ )-C and ( $\pm$ )-GC.

Chiral CE analysis of some catechins in green tea was reported to be successfully performed using neutral CDs (e.g. 6-*O*- $\alpha$ -D-glucosyl- $\beta$ -cyclodextrin (32)) as chiral selectors. Based on our previous studies HP- $\beta$ CD, was selected using SDS as surfactant at acidic pH values (28, 29). The strong suppression of the electroosmotic flow (EOF) at low pH values, allowed for the anodic migration of the analytes (including the cationic methylxanthines) partitioned into the SDS micelle. Under these conditions, a very short capillary (effective length of 8.5 cm) and a relatively high SDS concentration was necessary to achieve fast analysis. Once the neutral chiral selector HP- $\beta$ CD was added to the SDS solution, a slower anodic migration of the solutes was observed, mainly because of their inclusion complexation in the neutral cyclodextrin. This process

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occurs in competition with the partitioning of the solutes in the anionic carrier (SDS micelles) (33). In the present study it was found that the use of a borate/phosphate buffer (25 mM, pH 2.5) improved the peak shapes and reduced the migration time, compared to the results obtained with a Britton-Robinson (pH 2.5) running buffer used in previous applications (28, 29). This effect was particularly beneficial because high levels of the neutral cyclodextrin could be added to the BGE to increase the enantioresolution without significant prolongation of the migration times. Using HP- $\beta$ CD (25 mM) in the presence of SDS (90 mM) in a pH 2.5 borate/phosphate (25 mM) running buffer, the enantioresolution of ( $\pm$ )-C, ( $\pm$ )-GC, and the complete separation of methylxanthines and the other most represented green tea catechins, were obtained as shown in Fig. 1.

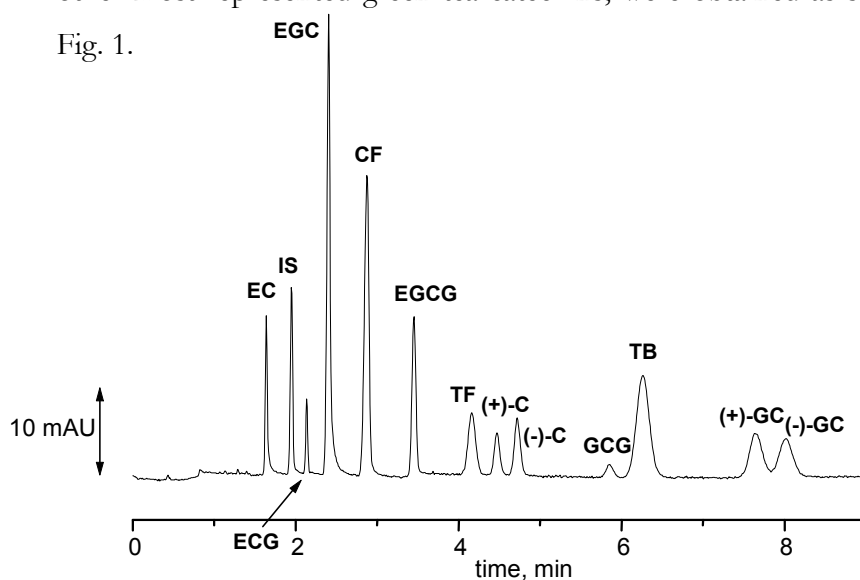


Fig.1: CD-MEKC electropherogram of a standard mixture of catechins and methylxanthines. Separation conditions: 25mM borate-phosphate buffer (pH 2.5) supplemented with SDS 90mM and HP- $\beta$ CD 25mM.

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Fused capillary (50 $\mu$ m id, 30cm total length, 8.5 cm effective length).

Voltage 15kV, temperature 25°C, hydrodynamic injection at 25mbar for 2sec, detection at 200nm.

#### Validation

*Linearity, Sensitivity, Reproducibility and Selectivity*

Analyte	Conc. range $\mu$ g/mL	a	b	$r^2$	LOD $\mu$ g/mL	LOQ $\mu$ g/mL
(-)-EC	3.0-300.0	0.0381( $\pm$ 0.00067)	0.06036( $\pm$ 0.11851)	0.9995	0.1	0.3
(-)-ECG	5.0-150.0	0.0349( $\pm$ 0.00097)	-0.0049( $\pm$ 0.01455)	0.9990	0.1	0.3
(-)-EGC	10.0-300.0	0.0608( $\pm$ 0.0008)	0.08455( $\pm$ 0.13646)	0.9997	0.05	0.2
CF	10.0-500.0	0.0382( $\pm$ 0.00047)	0.2530( $\pm$ 0.2776)	0.9990	0.1	0.3
(-)-EGCG	20.0-600.0	0.0303( $\pm$ 0.0004)	0.0246( $\pm$ 0.0603)	0.9998	0.1	0.3
(+)-C	0.5-50.0	0.0306( $\pm$ 0.00036)	-0.00525( $\pm$ 0.0020)	0.9998	0.1	0.3
(-)-C	0.3-30.0	0.0310( $\pm$ 0.00088)	-0.00865( $\pm$ 0.0130)	0.9999	0.1	0.3
(-)-GCG	5.0-50.0	0.0303( $\pm$ 0.00107)	0.01661( $\pm$ 0.0191)	0.9995	0.15	0.4
TB	1.0-100.0	0.0255( $\pm$ 0.0001)	-0.00405( $\pm$ 0.0063)	0.9999	0.2	0.6
(-)-GC	1.0-50.0	0.0205( $\pm$ 0.00017)	0.02259( $\pm$ 0.00549)	0.9996	0.4	1.0
(+)-GC	1.0-50.0	0.0187( $\pm$ 0.00021)	0.03122( $\pm$ 0.00098)	0.9998	0.7	2.0

Table 1: Regression curves data of catechins for five calibrations points:

$y = ax + b$ , where y is the relative corrected peak area (area/migration time) of analyte to internal standard, x is the analyte concentration ( $\mu$ g/mL), a is the slope, b is the intercept and  $r^2$  is the correlation coefficient.

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Table 1 reports the parameters of the calibration graph obtained by linear regression analysis of the corrected peak area ratio (corrected peak area of the analytes versus the corrected peak area of the internal standard) against the concentration ( $\mu\text{g/mL}$ ) of the analytes. The sensitivity data LOQ and LOD were obtained by diluting standard solutions till a signal-to-noise ratio of 10:1 and 3:1 respectively, was achieved. A wavelength of 200 nm was selected for all the measurements.

In Table 2 the inter-day ( $n = 3$ ) and intra-day ( $n = 9$ ) repeatability of migration time and corrected peak area, obtained after repeated injections of a standard solution at the concentration of about  $50 \mu\text{g/mL}$  of each analyte, is reported.

	RSD% (tm)		RSD% (Area/tm)	
	Intra-day (n=3)	Inter-day (n=9)	Intra-day (n=3)	Inter-day (n=9)
EC	0.261	0.450	0.972	0.935
IS	0.146	0.568	0.957	0.953
ECG	0.268	1.542	0.065	4.786
EGC	0.060	0.361	1.344	4.070
CF	0.324	0.902	0.810	0.494
EGCG	0.126	1.462	1.928	2.977
TF	0.299	0.980	0.327	1.241
(+)-C	0.182	0.959	0.468	3.067
(-)-C	0.173	1.035	0.180	1.284
GCG	0.180	1.217	7.135	8.016
TB	0.298	1.165	6.358	6.680
(+)-GC	0.623	1.100	5.732	3.455
(-)-GC	0.549	1.113	2.573	3.368



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Table 2: repeatability data in CD-MEKC analysis of catechins and methylxanthines at a concentration level of 50 g/mL (IS is the internal standard).

The obtained values prove the high reproducibility of the method and the opportunity for a selective identification of the considered phytomarkers by comparison of the migration time. Identification was also carried out by spiking experiments with solutions of certified analytes and by comparing the related UV spectra recorded using the DAD detector within the wavelength range 200 – 400 nm.

#### *Accuracy*

Accuracy was evaluated on (–)-EGCG, the most abundant catechin in green tea and on (–)-EC and (+)-C, which are among the most representative polyphenols in the plant kingdom. A tea product labeled Kokeicha Green Tea (from Japan) was selected for recovery studies. In detail, standard solutions of the three considered catechins at three different concentration levels were added to the infusion (60 mL of water containing 1.0 g of the pulverized tea) and subjected to heating (85 °C for 5 min) in capped glass tubes. After CD-MEKC analysis, the catechin amount was calculated by the external standard method; the obtained data are reported in Table 3.

In particular, total error (TE) was evaluated as the parameter that takes into account both accuracy and precision. In fact, TE describes the quality of a test result, providing a worst case estimate of how large the errors might be in a single measurement. As reported in Table 3, the values were calculated to be less than 10% for each of the analytes within

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the examined concentration range. Considering the application of the CD-MEKC method to complex biological samples, the accuracy was adequate.

Table 3: accuracy for the assay of catechins.

Analyte	Spiked (mg/g)	Total Error (TE)
(-)-EGCG	30.0	2.5
	15.0	2.1
	7.50	4.3
(-)-EC	6.0	9.1
	3.0	8.9
	1.5	5.3
(+) -C	0.60	9.2
	0.30	9.1
	0.15	8.1

#### Epimerisation studies

As reported, the epi-structured catechins (2,3-*cis* form) undergo epimerisation to the nonepi-structured catechins (2,3-*trans* form) during heat-processing (34, 35). Detailed studies on the kinetics of thermal epimerisation, carried out on green tea catechins such as (-)-EC, (-)-EGCG and (-)-ECG, to (-)-C, (-)-GCG and (-)-CG, respectively, followed a first order reaction and the rate constants of reaction kinetics followed the Arrhenius equation (34 – 39).

It has been previously showed the importance of chiral separation of the native (+)-C from the non-native (-)-C in actual samples of chocolate (29). The non-native (-)-C was suggested to be a useful marker of epimerisation occurring in *Theobroma cacao* during manufacturing processes (29, 40). On the same basis, (-)-GC can be regarded herein, as

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an additional marker of degradation in tea extracts. Actually, analogously with Catechin, in the plant kingdom the native Gallocatechin corresponds to the (+)-enantiomer. Thus, the presence of the (-)-GC in processed tea should only be related to epimerisation from (-)-EGC, which is quite present in fresh, unfermented green tea (39).

In order to verify the reliability of (-)-GC as a potential chiral marker of thermal degradation, the kinetics of epimerisation of (-)-EGC to (-)-GC, was studied for the first time using the CD-MEKC method, and then compared under the same conditions to that of the well known conversion of (-)-EC to (-)-C. Aqueous solutions (100 µg/mL) of the epi-structured compounds were heated and after 15, 25, 60 and 120 min, aliquots of the solutions were analysed by CD-MEKC. The content of the remaining catechins in their epi-forms and that of the yielded nonepi-forms were plotted against the time (concentration profile). The corrected peak area ratios (analyte/internal standard) were expressed as the percentage of the yielded (-)-C and (-)-GC (Fig. 2a-b) and were calculated by assuming as the reference the corrected peak area ratio of the analytes at the beginning of the experiments ( $t_0$ ) at room temperature. Under the applied experimental conditions the non-epi-structured catechins appeared to increase. However as it can be seen (Fig. 2b), at the temperature of 80°C and 90°C the concentration of (-)-GC reached a maximum (after 60 min), then decreased. This behaviour, observed at a lesser extent, also for the time course of formation of (-)-C (Fig. 2a, at 90°C), was ascribed to thermal degradation of catechins which is reported to occur concurrently to their epimerisation (35).

Fig.2a

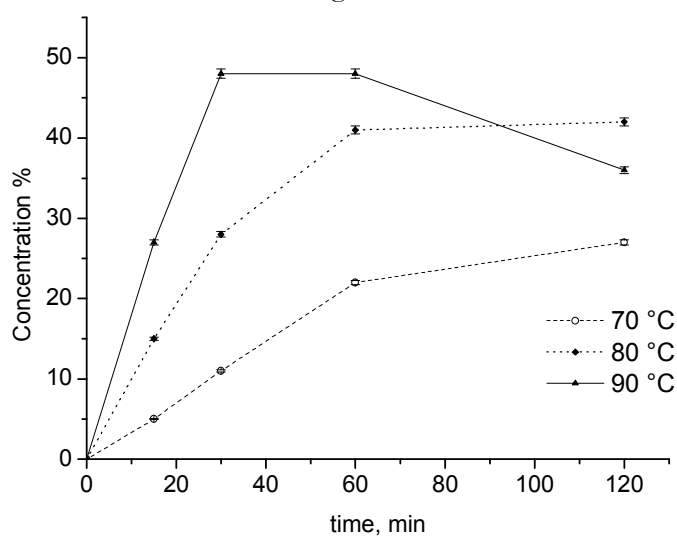


Fig.2b

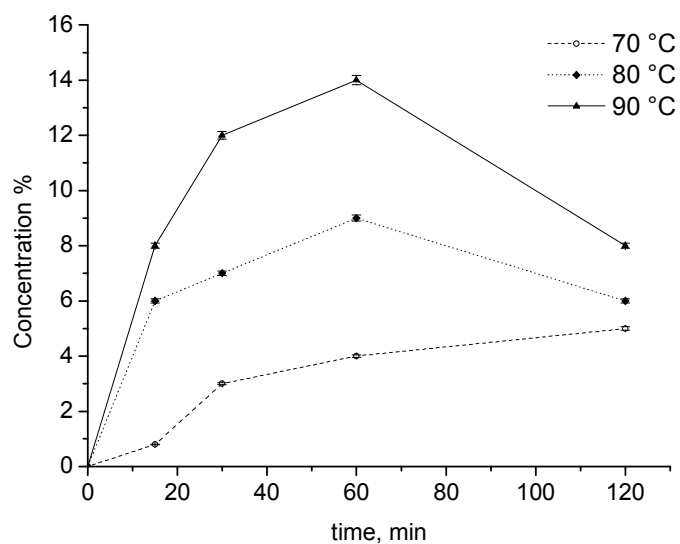


Fig.2a: Time course of formation of (-)-C when aqueous solution of (-)-EC (100 $\mu$ g/mL) is heated at different temperatures. Data are expressed as mean  $\pm$ SD of n=3 samples.

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Fig.2b: Time course of formation of (-)-GC when aqueous solution of (-)-EGC (100 $\mu$ g/mL) is heated at different temperatures. Data are expressed as mean  $\pm$ SD of n=3 samples

In Fig. 3a-b, the time trend related to the remaining percentage of (-)-EC and (-)-EGC, is reported. By plotting the  $\ln(x/x_0)$ , where  $x$  is the remaining concentration of the epi-structured catechins and  $x_0$  is the initial concentration, versus the time (min) at different temperatures, linear relationships were obtained and the corresponding rate constant  $k_y$  were derived as the slopes of the obtained straight lines. These results suggested apparent first order degradation kinetics according to previous studies (34-38).

According to the Arrhenius equation:

$$k_y = Ae^{-Ea/RT} \quad (1)$$

where,  $A$  is the frequency factor;  $Ea$  is the activation energy;  $R$  is the ideal gas constant and  $T$  is the temperature in Kelvin, the  $\ln(k_y)$  values were plotted against the inversed temperature ( $1/T$ ) to generate Arrhenius plots. The slopes of the obtained plots were found to be -5412.5 ( $r^2 = 0.947$ ) and -82025 ( $r^2 = 0.942$ ) for (-)-EC and (-)-EGC, respectively and the derived activation energy ( $Ea$ ) was 66.7 KJ/mol and 45.0 KJ/mol for (-)-EC to (-)-C and (-)-EGC to (-)-GC, respectively. It was thus concluded that the thermal conversion of the native (-)-EGC to the non-native (-)-GC occurs at a rate similar to that of the formation of (-)-C from (-)-EC. As a consequence, (-)-GC can be regarded as an additional chiral marker of green tea degradation.

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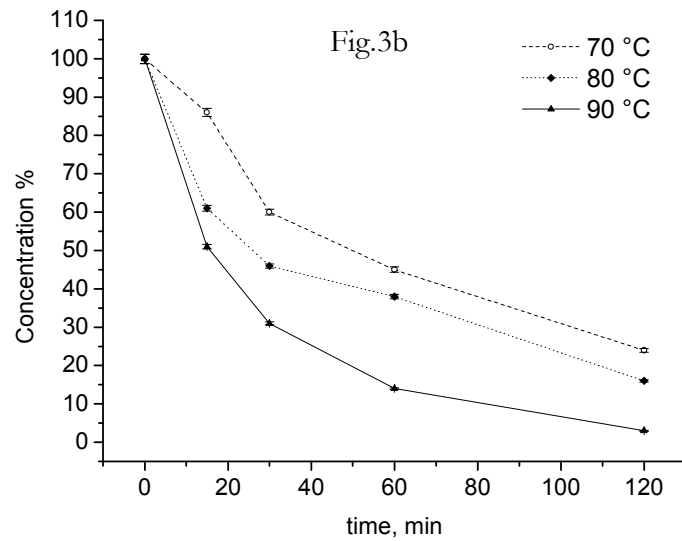
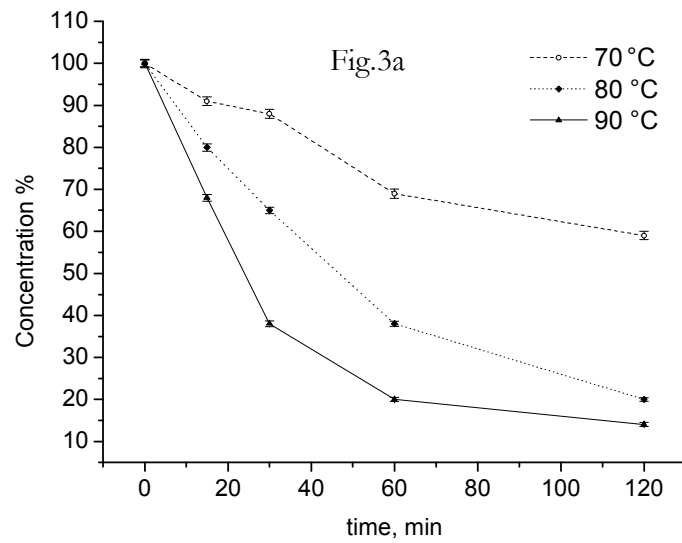


Fig3a: Time course of degradation of (-)-EC (100 $\mu$ g/mL) subjected to heating at different temperatures. Data are expressed as mean  $\pm$ SD of n=3 samples.

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Fig. 3b: Time course of degradation of (-)-EGC (100 $\mu$ g/mL) subjected to heating at different temperatures. Data are expressed as mean  $\pm$ SD of n=3 samples.

**Application to quantitative analysis**

The validated CD-MEKC method was applied to the identification and quantitation of the catechins in infusion of green tea from different regions of China, Japan and Ceylon. The extraction procedure simulated the brewing conditions and consisted in the infusion of pulverized tea leaves in hot water (85°C) for 5 min (17, 20, 27); after filtration and dilution with water, the samples were directly injected into the CE system for the analysis, in the presence of the internal standard. The amount of the considered analytes in the different tea samples was calculated by external standard method and is reported in Table 4. As can be seen, in some of the samples, the presence of the non-native catechins (-)-C and (-)-GC is observed and should be ascribed merely to manufacturing processes involving heating. As an example, the electropherogram of Kokeicha Green tea sample (from Japan) is reported in Fig. 4: both (-)-C and (-)-GC were detected, thus indicating that epimerization occurred. This result was consistent with the information provided by the supplier, who claims that Kokeicha Green tea is subjected to steam treatment before packaging.

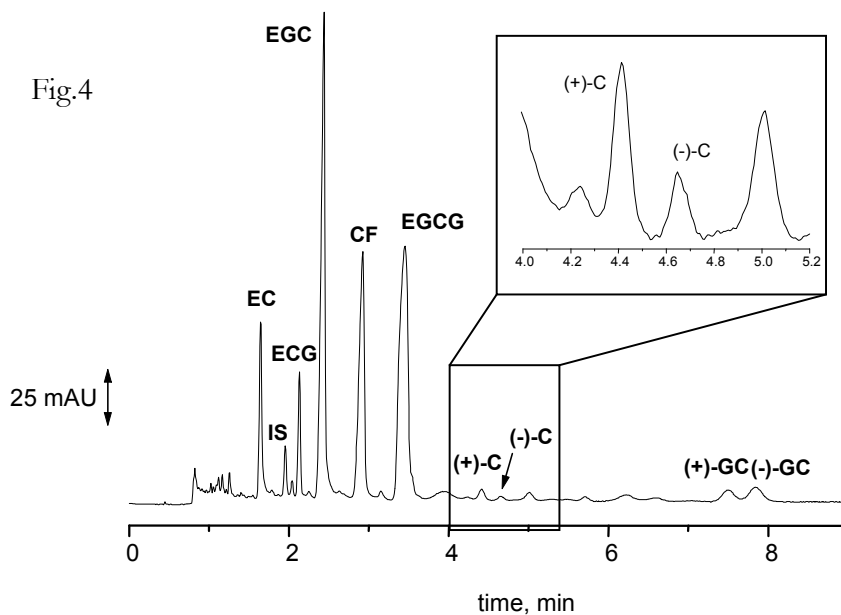


Fig.4: Electropherogram of extract from Kokeicha Green tea under optimised CD-MEKC conditions. Conditions and symbols as in Fig.1.

#### Concluding remarks

The proposed CD-MEKC, able to separate in about 8 min the most represented green tea catechins and methylxanthines, is the first method that allows simultaneous enantioseparation of Catechin and Galocatechin. The method was applied in the study of thermal epimerisation of the considered catechins to demonstrate the relevance of non-epistructured (-)-Catechin and (-)-Galocatechin as markers of degradation of tea samples. Different kinds of tea samples were analysed and the quantitative results were treated by factor analysis to study the data set in a multivariate way. It has been confirmed that the enantioselective quantitation of  $\tilde{(-)}$ -Catechin and  $\tilde{(-)}$ -Galocatechin can be



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useful in assessing thermal treatment, in fact, the obtained results were found to be consistent with the information, when provided, on the manufacturing processes (e.g. steam treatment, fermentation).

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“Gli Assiri e i Babilonesi sono i popoli più noti che hanno abitato la Mesopotamia, vasta pianura percorsa dal Tigri e dall’Eufrate.”  
(Rif. “Cosa curiosa” , volume per la prima classe delle scuole medie).

Questa frase è riportata per dimostrare che, anche se quello che avete trovato nella presente tesi vi sembra sbagliato, almeno qualcosa di vero lo avete letto (citazione da R.Gotti).

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