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Utilization of *n*-alkyl-β-D-glucopyranosides in enantiomeric separation by micellar electrokinetic chromatography[1]

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Abstract

The *n*-octyl-β-D-glucopyranoside has been previously used in micellar liquid chromatography to separate racemic mixtures. We report here on the utilization of *n*-alkyl-β-D-glucopyranosides (from C_7 to C_{10}) as pseudo stationary phases in the separation by HPCE of racemic mixtures of amino acids, derivatized as carbamates. The result from this comparative study was that the best enantiomeric selectivity is obtained with the *n*-nonyl-β-D-glucopyranoside. Using this chiral surfactant we optimized different operating parameters such as: concentration of surfactant, ionic strength and pH of the mobile phase and temperature. Therefore it appeared that this new electrophoretic system is performing best at 18 °C, with an 80 m*M* concentration of surfactant in the electrolyte constituted by a $NaHCO_3$–NaOH buffer, the applied voltage being equal to 15 kV. The enantiomeric selectivity of this system, original because of the nature of the micellar additive (neutral micelle), seems to be a function of the analyte hydrophobicity.

Keywords: Enantiomer separation; Alkylglucopyranosides; Chiral selectors; Amino acids; Carbamates

1. Introduction

Separation of racemic mixtures into individual optical isomers to this day constitutes one of the major challenges for chemical analysis. Such separations appear to be fundamental for many industries and especially in the pharmaceutical industry. It is now well established that the pharmaceutical characteristics of two enantiomers corresponding to a chiral active principle can be very different [1]. These differences can induce physiological disorders which can be more or less important. Facing such a challenge, different chromatographic techniques have been considered over the last 20 years, to carry out such chiral separations, and even to purify such molecules.

Thus, gas phase chromatography (GC) and high-performance liquid chromatography (HPLC) have been widely used to carry out enantiomeric separations [2–4]. More recently, and especially these last 5 years, high-performance capillary electrophoresis (HPCE) has revealed by its principal variations [capillary zone electrophoresis (CZE), capillary gel electrophoresis (CGE), micellar electrokinetic chromatography (MEKC) and isotachaphoresis (ITP)] very interesting potentialities for the determination of the optical purity of drugs and other racemic mole-

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cules. The different works carried out in this field of analytical chemistry in full development have been the subject of many and excellent reviews [5–14].

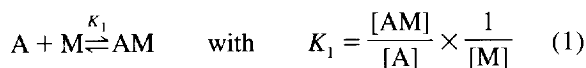
In HPCE the chiral separation is carried out using essentially a wide range of chemical selectors which are either trapped in a gel [15–17] or added to a buffer. In this last case, many different types of chiral selectors have been used: cyclodextrins [18–21], chiral metallic complexes [22,23], crown ethers [24], carbohydrates [25] or chiral surfactants as for example bile acids [26–31] or sodium *n*-decanoyl-L-valinate [32]. In fact, when the chiral selector is a surfactant, the electrophoretic technique used to carry out the separation is MEKC. The surfactant is then added to the electrophoretic buffer at such a concentration that it forms a chiral micelle which will constitute a micellar pseudo-stationary phase charged negatively and presenting consequently an electrophoretic mobility having an opposite direction to the electroosmotic flow. The enantiomeric recognition is carried out, in these conditions, by the chiral centers added in the micelle, as it is well established that in MEKC the analytes interact with the micelles [33,34].

The alkylglucopyranosides, non ionic surfactants constituted of a dextrorotatory glucose, optically pure and of a lipophilic alkyl chain, differentiate themselves from each other by the length of this alkyl chain and their anomeric form (α or β) [35] and also by the nature of the bond between glucose and the alkyl chain [36,37].

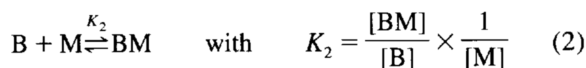
These non ionic surfactants which are characterized by high critical micellar concentrations (CMCs) therefore present a chiral character due to the presence of a glucose in the dextrorotatory form. These chiral surfactants used at first for the solubilization of membrane proteins in HPLC [38] have led, relatively recently with this technique and in the case of a *n*-octyl-β-D-glucopyranoside to satisfactory resolutions of racemic mixtures [39]. As the analytical chemists need new methods to resolve matrixes of increasing complexity, we have decided to study the potentiality of *n*-alkyl-β-D-glucopyranosides as chiral selectors in MEKC. The only application of glucopyranosides reported up to now in literature in HPCE is the utilization of octyl-β-D-glucopyranoside as an inhibitor of the formation of gel during use, by Ishihama and Terabe [40], of acid glycyrrhizic as chiral pseudo stationary phase in the presence of sodium dodecyl sulfate (SDS). Because of the nature of the surfactant used which is a chiral non ionic surfactant, the present work is, to our knowledge, the first attempt to use neutral chiral micelles for the resolution of racemic mixtures. To begin with, we have proceeded to a theoretical modelization of enantiomeric separation in these new electrophoretic conditions, then, in a second phase, we used *n*-alkyl-β-D-glucopyranosides for the resolution of racemic amino acids mixtures derivatized as carbamates.

2. Theoretical

The model which has been developed by Wren and Rowe to rationalize chiral separation of a racemic mixture in the case of capillary zone electrophoresis, and especially when the chiral selector is a cyclodextrin [41–43], can be transposed directly to a chiral selector in MEKC constituted by a non ionic chiral micelle, as it is the case for the *n*-alkyl-β-D-glucopyranosides. The enantiomeric resolution of a racemic charged mixture A/B by this non ionic chiral micelle (not having electrophoretic mobility of its own) can then be schematized by the two following equations:



$$A + M \overset{K_1}{\rightleftharpoons} AM \quad \text{with} \quad K_1 = \frac{[AM]}{[A]} \times \frac{1}{[M]} \qquad (1)$$



$$B + M \overset{K_2}{\rightleftharpoons} BM \quad \text{with} \quad K_2 = \frac{[BM]}{[B]} \times \frac{1}{[M]} \qquad (2)$$

where:

1. A and B are the enantiomers constituting the racemic mixture and presenting for each one of them an electrophoretic mobility in free solution, μ_f, of course identical but different from zero;
2. M is the non ionic chiral micelle and presents, consequently, an electrophoretic mobility corresponding to that of the electroosmotic flow, μ_{eo};
3. AM and BM are the diastereoisomeric complexes formed by each of the enantiomers and the micelle and possessing each, at first approximation, an electrophoretic mobility equal to that

of a micelle, i.e., the electroosmotic flow mobility.

The chiral selector being in the present case a non ionic chiral micelle, its concentration in the electrophoretic medium can easily be evaluated from:

1. the total concentration of the surfactant, c_T,
2. and the critical micellar concentration of this surfactant in operating conditions, c_o,

that is: $c_T - c_o$. (3)

In such conditions, the expression of the apparent electrophoretic mobility of an enantiomer, established formerly by Wren and Rowe [41,42] becomes:

$$\mu_a = \frac{\mu_f + \mu_{eo} K(c_T - c_o)}{1 + K(C_T - c_o)} \quad (4)$$

with $K = K_1$ in the case of an enantiomer A and $K = K_2$ in the case of an enantiomer B.

However, in order to avoid the difficulties resulting from fluctuations in the mobility of the electroosmotic flow, which are due to the problem of reproducibility at the level of the capillary surface, we will study the evolution of the effective electrophoretic mobility of enantiomers μ^{eff} as a function of experimental parameters.

The apparent (μ_a) and effective (μ^{eff}) electrophoretic mobilities of an enantiomer being linked together by the following expression:

$$\mu_a = \mu^{eff} + \mu_{eo} \quad (5)$$

it is possible to express the evolution of the effective electrophoretic mobility of an enantiomer, μ^{eff}, as a function of the total concentration of the surfactant by combining Eq. (4) and Eq. (5):

$$\mu^{eff} = \frac{\mu_f - \mu_{eo}}{1 + K(c_T - c_o)} = \frac{1}{1 + K(c_T - c_o)} \mu_f^{eff} \quad (6)$$

μ_f^{eff} being the effective electrophoretic mobility, in free solution, of the considered enantiomer.

In agreement with this last equation the variation of the effective electrophoretic mobility of an enantiomer as a function of the total concentration of the non ionic chiral surfactant is reported in Fig. 1.

As evidenced by this figure, for total concentrations of surfactants inferior or at the most equal to their CMC, the effective electrophoretic mobility of an enantiomer remains constant and equal to the one it presents when there is no surfactant for the pH and ionic strength considered, the expression $K(c_T - c_o)$ of Eq. (6) being:

1. either strictly zero, when $c_T = c_o$,
2. or negligible compared to 1, when $c_T < c_o$.

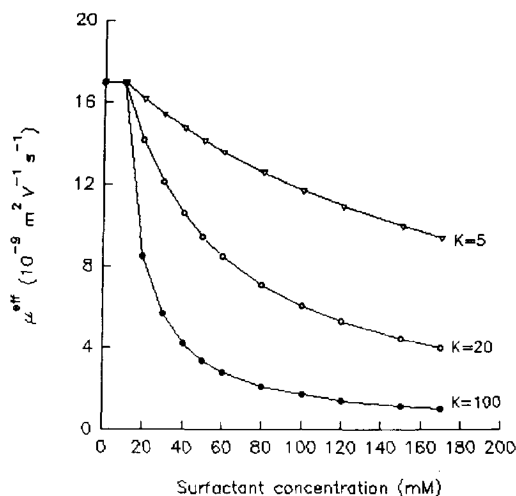


Fig. 1. Theoretical evolution of the effective electrophoretic mobility of some enantiomers as a function of the neutral chiral surfactant total concentration, with $c_o = 10$ mM and $\mu_f^{eff} = 17 \cdot 10^{-9}$ m^2 V^{-1} s^{-1} (determined in the case of tryptophan at pH 10.5 and without chiral surfactant).

For surfactant concentrations inferior to CMC only solvatophobic interactions exist between enantiomers and surfactant monomeric molecules. These forces of interaction are extremely weak and are therefore characterized by very small affinity constants K.

It is needless to say that for such concentrations in non ionic chiral surfactants no enantiomeric resolution is possible as the two enantiomers are not complexed and present the same electrophoretic mobility.

On the other hand, above the CMC of the non ionic chiral surfactant (c_o), the Fig. 1 shows an important decrease of the effective electrophoretic mobility of the enantiomer, μ^{eff}, as a function of the surfactant concentration used. This decrease is more marked if the complexation constant K of the

enantiomer by the non ionic chiral micelles is greater. This simply evidences the fact that the enantiomer being more and more efficiently complexed by the non ionic micelles migrates with an experimental electrophoretic mobility closer to the latter one, that is to say at the speed of the electroosmotic flow in the present case, resulting in an effective electrophorectic mobility straining towards zero.

What is more, and assuming that the affinity constants K_1 and K_2 of the two enantiomers A and B for the chiral micelle be effectively different, an enantiomeric resolution will be obtained for such concentrations of surfactant, the difference between the electrophoretic mobilities of two enantiomers being given for the following equation:

$$\Delta\mu = \frac{(K_2 - K_1)(c_T - c_o)(\mu_f - \mu_{eo})}{1 + (K_2 + K_1)(c_T - c_o) + K_1K_2(c_T - c_o)^2} = \frac{(K_2 - K_1)(c_T - c_0)}{1 + (K_2 + K_1)(c_T - c_o) + K_1K_2(c_T - c_o)^2}\,\mu_f^{eff} \quad (7)$$

Thus there will exist an optimal concentration of non ionic chiral surfactant for which the difference of electrophoretic behaviour between the two enantiomers A and B will be maximal. This concentration will depend of the values of constants K_1 and K_2 and of their difference.

It will obviously exist an optimal concentration of chiral surfactant for which the selectivity of the electrophoretic system will be maximum. In a classical way the selectivity of a chromatographic system is defined as the ratio of the partition coefficients of the two compounds separated, i.e., in the present case the partition coefficients of the enantiomers between the aqueous and micellar phases. Consequently, during the enantiomeric resolution in MEKC of a racemic by a chiral micelle the selectivity is equal to the ratio of the affinity constants of the enantiomers for the micelle with regards to the aqueous phase.

That is:

$$\alpha = \frac{K_2}{K_1} = \frac{\mu_f^{eff} - \mu_B^{eff}}{\mu_f^{eff} - \mu_A^{eff}} \times \frac{\mu_A^{eff}}{\mu_B^{eff}} \quad (8)$$

by expressing K_1 and K_2 in agreement with Eq. (6).

As it has been widely reported in literature [44], the efficiency of the electrophoretic system is given by the following equation:

$$N = \frac{V}{2D}\,\bar{\mu}_a \quad (9)$$

where $\bar{\mu}_a$ is the arithmetical average of the apparent electrophoretic mobilities of the two compounds separated and D their coefficient of molecular diffusion in the electrophoretic buffer.

In the case of an enantiomeric resolution of a racemic by a non ionic chiral micelle, the efficiency of the electrophoretic system can be written by combining the Eqs. (5,6,9):

$$N = \frac{V}{2D}\,\mu_{eo} + \frac{V}{2D} \times \frac{1}{1 + K(c_T - c_o)}\,\mu_f^{eff} \quad (10)$$

This equation shows, in the present case, the dependency of the efficiency of the electrophoretic system with the total concentration of the chiral surfactant. Moreover, this equation shows that the evolution of the electrophoretic system efficiency with the surfactant total concentration is not independent of enantiomer affinity for the chiral micelle and in particular this evolution is even more pronounced as the affinity constants of enantiomers for the chiral micelle are greater. However, if the efficiency of the electrophoretic system increases with the concentration of the electrophoretic buffer in neutral chiral micelles, it tends more or less rapidly (as a function of the affinity constant K of the enantiomers for this micelle) towards the same limit value which is equal to $(V/2D)\mu_{eo}$.

Thus this theoretical approach has allowed the placing in evidence, in the case of chiral resolution of racemics by means of non ionic chiral micelles, a precise dependence of fundamental parameters of micellar electrokinetic chromatography, i.e., the mobility (retention), the selectivity and the efficiency of the electrophoretic system with:

1. the total concentration of the surfactant,
2. the differences of the affinity constants of enantiomers to the chiral micelle.

Supported by these facts we have, in a second

period, applied this approach to the enantiomeric resolution of amino acids using a non ionic chiral micelle formed of *n*-alkyl-β-D-glucopyranosides by optimizing in turn each experimental parameter which is likely to modify the affinity constant of amino acids for such a micelle, namely:

1. the nature of *n*-alkyl-β-D-glucopyranoside,
2. the temperature,
3. the pH and the ionic strength of the electrophoretic buffer.

However we must point out that, during the optimization phase, it was not a question of neglecting the influence on the chiral resolution of amino acids, of other classical parameters of the MEKC including the applied tension and the total concentration of surfactant, this last parameter appearing to be crucial with regards to our theoretical approach.

3. Experimental

3.1. Reagents

The different buffers used during this study were prepared daily from 18 MΩ water generated by an Alpha Q purification system (Millipore, Bedford, MA, USA) and either of sodium bicarbonate of purity >99.7% (Prolabo, Paris, France), adjusted to the required pH with NaOH (98% purity, Prolabo), or of sodium salts of phosphoric acid of 99% purity (Aldrich, Saint Quentin Fallavier, France).

The diverse *n*-alkyl-β-D-glucopyranosides studied were all of 98% purity and were purchased from Sigma France (La Verpillière, France) like all the different racemics of amino acids of 98% grade. The latter not presenting any chromophore were derivatized as carbamates by ourselves in agreement with the protocol reported in the literature [45]. The structures of the derivatized products thus obtained were systematically checked by mass spectrometry. Due to their weak solubility in the electrophoretic buffers, these derivatives of racemic amino acids were dissolved in ethanol of RS HPLC grade (Carlo Erba, Rueil Malmaison, France) before injection.

3.2. Apparatus

All experiences were carried out on a P/ACE 2100 system (Beckman, Fullerton, CA, USA) fitted with an UV detector (λ_{detect}=214 nm) monitored by a computer PS/2 (IBM, Greenock, U.K.) using P/ACE software (Beckman). Data collection was performed using the same software.

Injections were systematically carried out by pressure [hydrodynamic injection during 1 s, injection pressure $3.47 \cdot 10^3$ Pa (0.5 p.s.i.)]. The used capillary columns were made of fused-silica untreated with regard to any covalently bonded stationary phase. Before use, many flushings were carried out through the capillary in the following order: 0.1 *M* HCl, acetonitrile, water and finally the buffer solution. These capillary columns all presented a total length of 57 cm and an internal diameter of 50 μm. The injection took place at anode whereas the detection was carried out near to the cathode, directly on the capillary, through a window made through the polyimide sleeve at 50 cm from the inlet.

Mass spectra were recorded on a Fisons ZAB HSQ mass spectrometer. The values of electrolytes pH were measured at the analysis temperature using a Model ϕ Beckman pH meter.

4. Results and discussion

The aim of this study being the enantiomeric resolution in MEKC of racemic mixtures for all amino acids presenting a large range of hydrophobicity (see Table 1) using as chiral selector a non ionic micelle, we started the optimization of this separation by testing the potentialities offered by different *n*-alkyl-β-D-glucopyranosides.

The various *n*-alkyl-β-D-glucopyranosides tested and reported in Table 2 are all commercialized. Their critical micellar concentrations, which are fundamental physicochemical parameters of surfactant molecules, are equally mentioned in this table.

4.1. Choice of the n-alkyl-β-D-glucopyranoside

This comparative study of diverse *n*-alkyl-β-D-glucopyranosides has been realized in a basic medium in order to confer a negative charge to

Table 1
Characteristics of the different amino acids studied [[46]]

Amino acids	Abbreviations	Degree of hydrophobicity	Isoelectric point
Tryptophan	Try	−34	5.89
Isoleucine	Ile	−18	5.98
Methionine	Met	−13	5.74
Valine	Val	–	5.96
Alanine	Ala	−5	6.00

amino acids. We used as racemic test the racemic mixture of tryptophan, i.e., the most hydrophobic amino acid among the range of samples we had (see Table 1). Among all those considered it is therefore the amino acid which must present the greatest interaction with the non ionic micelle. However, this interpretation does not take into account an eventual modification resulting from derivatization.

The study of the variation of the effective electrophoretic mobilities in basic medium of the different amino acids studied in the form of carbamates, as a function of the total concentration of surfactant, shows without ambiguity that this structural modification does not alter the relative hydrophobicity of the amino acids. As shown in Fig. 2, the tryptophan derivatized in the form of carbamate, in basic medium and for a given concentration of surfactant, has the smallest effective electrophoretic mobility of all the amino acids considered.

The theoretical approach developed previously has shown that effective electrophoretic mobilities of analyzed molecules are inversely proportional to affinity constants of these ones for the micelle, and therefore to their hydrophobicity. Such an observation reveals that the tryptophan derivatized as carbamate presents, amongst all the amino acids studied in the form of carbamates, the greatest affinity constant towards the micelle and therefore the greatest hydrophobicity. Moreover, applying the same approach a complete examination of effective electrophoretic mobilities found in basic medium, with the same concentration of surfactant in the electrophoretic buffer, for the whole of amino acids studied in the form of carbamates, shows clearly that the classification of these by increasing hydrophobicity is strictly identical to the classification that we can establish for the non derivatized amino acids from the values reported in Table 1.

Tryptophan, including its carbamate form, having amongst the whole of amino acids considered the strongest interactions with the micelle, and therefore a greater chiral selectivity, appeared as a good test sample in view of the choice of the chiral selector. In consequence we have evaluated with regards to this test sample the four n-alkyl-β-D-glucopyranosides reported in Table 2 as chiral selectors. To do this, by

Table 2
Critical micellar concentration (CMC) of n-alkyl-β-D-glucopyranosides evaluated as chiral selector in MEKC [[47]]

Surfactants	Abbreviations	CMC (mM)
n-Heptyl-β-D-glucopyranoside	βGC_7	–
n-Octyl-β-D-glucopyranoside	βGC_8	20–25
n-Nonyl-β-D-glucopyranoside	βGC_9	6.5
n-Decyl-β-D-glucopyranoside	βGC_{10}	2–3

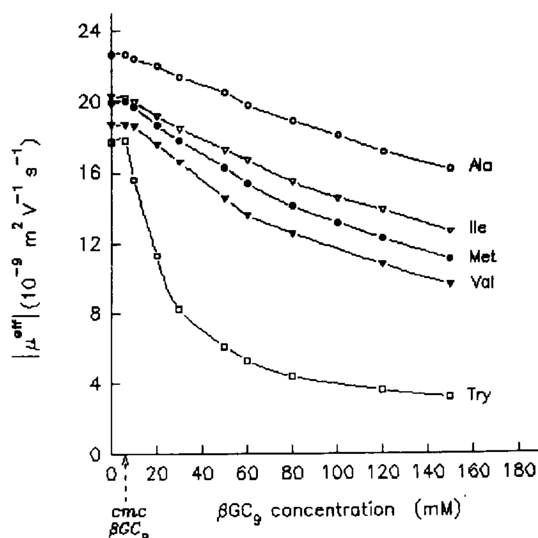


Fig. 2. Evolution of effective electrophoretic mobilities of different amino acids, derivatized as carbamates, by MEKC as a function of the surfactant concentration. (Operating conditions: temperature: 27 °C, applied voltage: 25 kV, buffer: $NaHCO_3$ (50 mM)–NaOH (100 mM) pH 10.5, surfactant βGC_9).

operating in basic medium, we have calculated the chiral selectivity of electrophoretic systems constituted successively by four n-alkyl-β-D-glucopyranosides for different concentrations of surfactant. We must point out that in the case of n-decyl-β-D-glucopyranoside it was necessary to add a weak amount of acetonitrile (7%) to obtain the total solubilization of this surfactant in the electrolyte.

The evolution of the chiral selectivity of the electrophoretic system ($\Delta\mu$) as a function of the concentration of surfactant for each of the considered n-alkyl-β-D-glucopyranosides is reported in Fig. 3.

Upon examination of this figure we need to make a few comments:

(i) whatever the nature of the chiral surfactant considered, there exists an optimal concentration of n-alkyl-β-D-glucopyranoside for which the enantiomeric selectivity of the electrophoretic system is maximal. We must point out that such a fact is in perfect agreement with our model.

(ii) whatever the nature of the n-alkyl-β-D-glucopyranoside considered, there is only chiral separation for surfactant concentrations higher than their CMC.

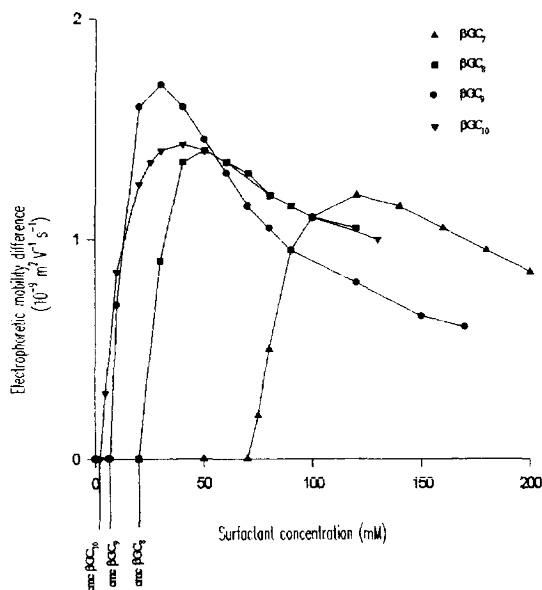


Fig. 3. Evolution of the system chiral selectivity as a function of the surfactant concentration and its nature. (Operating conditions: temperature: 27 °C, applied voltage: 25 kV, buffer: $NaHCO_3$ (50 mM)–NaOH (100 mM) pH 10.5, test sample: tryptophan derivatized as carbamate).

Such a situation reveals clearly that the solvatophobic interactions, which can exist between the amino acids and the monomers of the surfactant, are too weak to induce a chiral recognition. Such experimental evidence appears once again to be in perfect agreement with the theoretical approach that we have presented here.

(iii) finally, it is with n-nonyl-β-D-glucopyranoside that the highest chiral selectivity is obtained. Consequently, it is with this n-alkyl-β-D-glucopyranoside, used as a chiral selector, that we have pursued the optimization of the enantiomeric resolution of amino acids (derivatized as carbamates).

4.2. Concentration of surfactant

The chiral selector being the n-nonyl-β-D-glucopyranoside, we have studied the evolution of enantiomeric selectivity ($\Delta\mu$) for each of the amino acids, reported in Table 1, as a function of the concentration of this surfactant (see Fig. 4).

The selectivity of the electrophoretic system for an amino acid resulting from the difference between the experimental electrophoretic mobilities corresponding to its two enantiomers shows curves which present the same general appearance if we trace the evolution of the difference between effective electrophoretic mobilities corresponding to the two enantiomers.

Also conforming to our theoretical model, there exists, whatever the amino acid considered, a total concentration of non ionic chiral surfactant (in the present case n-nonyl-β-D-glucopyranoside) for which the selectivity of the electrophoretic system is maximal. These maxima, as shown in Fig. 4, are more pronounced if the amino acids considered show a more hydrophobic character (comparison of curves in Fig. 4).

However, in the case of alanine, which is the less hydrophobic of the amino acids studied, no enantiomeric resolution is obtained whatever the concentration of n-nonyl-β-D-glucopyranoside.

For the other four amino acids, there still exists a concentration of surfactant above which an enantiomeric resolution can be obtained. Moreover it is interesting to note that this concentration limit, above which the resolution of an amino acid racemic mixture becomes possible, is always at least equal or

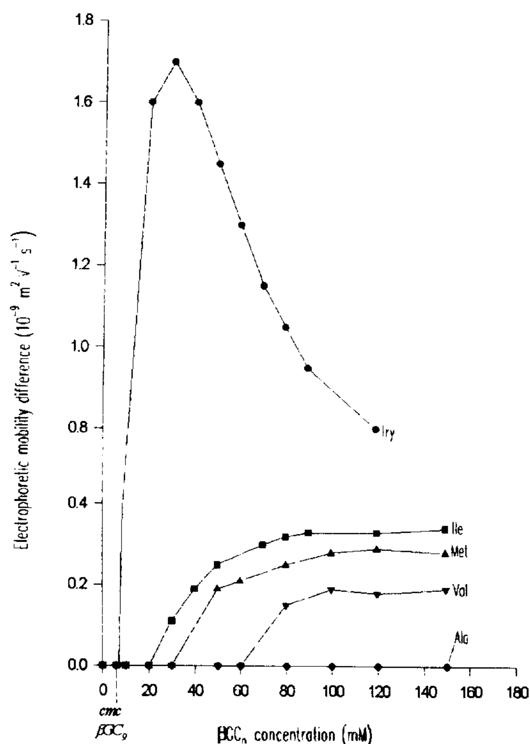


Fig. 4. Evolution of the electrophoretic system chiral selectivity as a function of the n-nonyl-β-D-glucopyranoside concentration in the case of different racemic amino acid (derivatized as carbamates) mixtures. (Operating conditions: temperature: 27 °C, applied voltage: 25 kV, buffer: $NaHCO_3$ (50 mM)–NaOH (100 mM) pH 10.5).

superior to the CMC of n-nonyl-β-D-glucopyranoside (6.5 mM for reminder). However the value of this concentration limit is not independent of the nature of the amino acid and appears to increase if the hydrophobicity of amino acid is weaker. This value varies from 6.5 mM in the case of tryptophan (the most hydrophobic amino acid among those studied) to a value above 20 mM in the case of methionine and greater than 70 mM in the case of valine.

In the same way, the maximal value attained for chiral selectivity appears to be dependent on the nature of the amino acid considered and appears to be greater if the amino acid presents a more pronounced hydrophobic character. So, this maximal resolution of value 1 in the case of alanine (the less hydrophobic amino acid amongst the whole of studied amino acids) is more than 1.5 in the case of tryptophan which corresponds, on the contrary, to the amino acid which is the most hydrophobic among all those studied. Consequently, it must be underlined that the product analyzed seems to play an important part in the mechanism of chiral recognition when using non ionic chiral micelles as selectors, such as those made of n-alkyl-β-D-glucopyranosides.

Consequently, a compromise must be found as for the concentration of surfactant. In fact it is necessary that the selectivity of the electrophoretic system for the tryptophan remains as close as possible to its maximal value while still obtaining for other amino acids analyzed a chiral selectivity already noticeable. A concentration of n-nonyl-β-D-glucopyranoside equal to 80 mM seems, on this basis, a good compromise.

In order to increase the chiral selectivity of the electrophoretic system and therefore trying to resolve the enantiomers of alanine we have in turn aimed to optimize the experimental parameters which could cause:

1. modification of the affinity constant for the micelle (temperature),
2. reinforcement of the hydrophilic character of the mobile phase (ionic strength) or of the hydrophobic character of amino acids (pH).

4.3. Temperature effect on chiral resolution

Evaluating the resolution:

1. either with the help of a classical formula [44], in the case of two well resolved enantiomers (case of tryptophan),
2. or by using Wren's approach [43] which is based on the peaks heights ratio, in the case of two partially resolved enantiomers (case of isoleucine and methionine),

we have followed the evolution of this one in a range of experimental temperatures between 16 °C and 30 °C. The evolution of chiral resolution as a function of temperature for tryptophan on the one hand and for isoleucine and methionine on the other hand is reported respectively in Fig. 5a and b. Unlike the approach elaborated by Wren leading to a maximal

value of the resolution equal to 1, we cannot, in such conditions, report on the same graph the evolution of the enantiomeric resolution of these three racemic mixtures as a function of temperature.

This point having been cleared, these figures show that, whatever the nature of the considered amino acid, the chiral resolution increases when the experimental temperatures decrease.

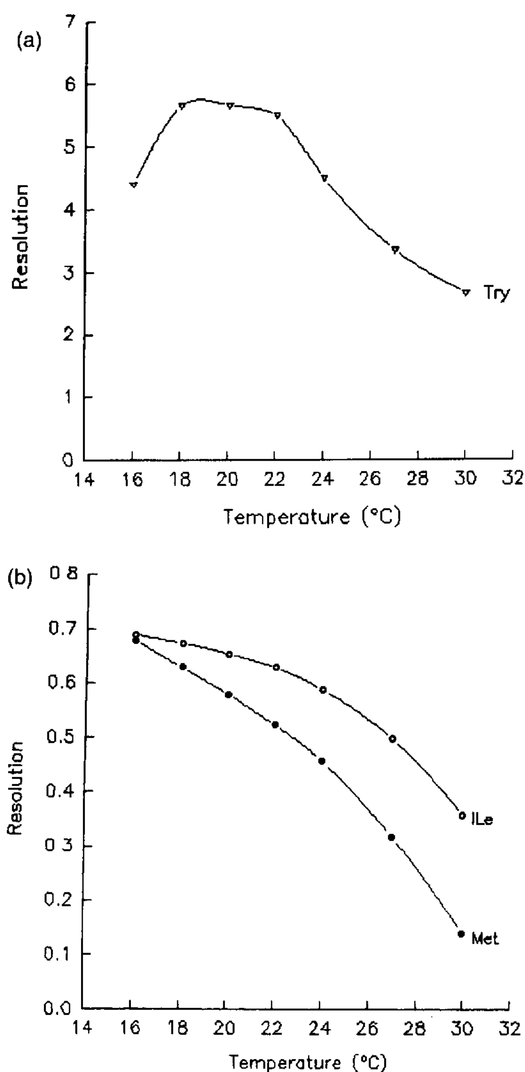


Fig. 5. Evolution of the enantiomeric resolution of amino acids (derivatized as carbamates) as a function of temperature: (a) tryptophan, (b) isoleucine and methionine. (Operating conditions: applied voltage: 15 kV, buffer: $NaHCO_3$ (50 m*M*)–NaOH (100 m*M*) pH 10.5, βGC_9 concentration = 80 m*M*).

Seeking for the origin of this improvement of enantiomeric resolution with the decrease of temperature, we examined the evolution of chiral selectivity and of the efficiency of the electrophoretic system as a function of the separation temperature.

The enantiomeric selectivity stays constant whatever the experimental temperature and the nature of the studied amino acids. In such conditions, it is quite logical that the considered electrophoretic system appears to be non resolutive for alanine, and at whatever temperature we try to carry out enantiomeric separation of this amino acid. The selectivity for this couple of enantiomers has been reported as equal to the unit at 27 °C.

In fact the improvement of the enantiomeric resolution by lowering the temperature finds its origin in a significant increase of the efficiency of the electrophoretic system as evidenced in Fig. 6 in the case of tryptophan.

The capillary electrophoresis system used can reach stable experimental temperatures if they are equal or superior to 5 °C below room temperature and an experimental temperature of 18 °C seems to be the optimal temperature. Consequently, we completed the optimization at this temperature.

Then we studied the influence of pH and ionic strength of the mobile phase on chiral resolution, these two experimental parameters being equally

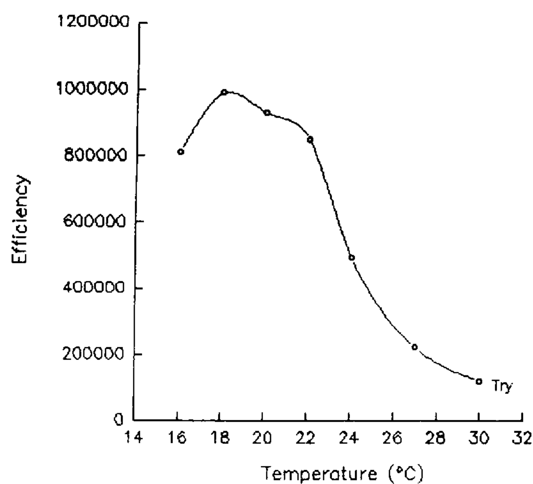


Fig. 6. Evolution of the electrophoretic system efficiency as a function of temperature. (Operating conditions: identical to those reported in Fig. 5, test sample: tryptophan derivatized as carbamate).

likely to modify the affinities of amino acids for the chiral micelle.

4.4. Influence of the pH on the chiral resolution

The chiral micelle, being non ionic, moves at the speed of the electroosmotic flow. So, the chiral resolution must be higher in the case of anionic racemics than for cationic racemics. So, we have explored the influence of pH on the enantiomeric resolution of amino acids only in the range of pH in which they possess a negative charge. These acids being derivatized as carbamates must present pK_a values close to that of the carboxylic function. The evolution of chiral resolution for four of five amino acids as a function of pH and for pH>4 are reported in Table 3, the ionic strength being maintained constant and equal to 90 mequiv. At this stage of the experiment we must point out that, whatever the pH chosen from the explored range, this new electrophoretic system, based on the use of n-alkyl-β-D-glucopyranosides, does not lead to a chiral separation in the case of valine.

When reading Table 3 it appears that enantiomeric resolution is merely altered by a modification of pH. It tends however to increase when we decrease the pH. Unfortunately this slight improvement is obtained at the cost of the time of analysis which increases considerably after the decrease of the electroosmotic flow, the surface of the fused-silica capillary becoming less and less negatively charged. So analysis time becomes quite prohibitive for pH inferior to 7. In reality for the zone of pH really accessible experimentally (in a reasonable analysis time) the state of ionisation of the considered amino acids is slightly or not modified and no noticeable change can be seen concerning the affinity of those for the micelle. Therefore a slight improvement of enantiomeric resolution results from the retention (reduction of the electroosmotic flow).

Facing such a situation, a pH of about 10 seems to lead to a better resolution per time unit.

4.5. Influence of ionic strength on chiral resolution

The pH being fixed at a value of 10.5 we have then studied the consequential effects of a modification of the electrolyte ionic strength on the enantiomeric resolution (see Table 4).

In this study it appears that the chiral resolution of racemic mixtures of the studied amino acids increases when the ionic strength of the electrolyte increases. As for the pH this improvement of resolution is mainly the result of an increase of retention, the selectivity of the electrophoretic system remaining constant. This increase of migration time is due essentially to a decrease of the electroosmotic flow. However, whatever the considered amino acid, above a certain ionic strength the chiral resolution ceases to increase and seems to tend towards a limit. Moreover, a very unstable baseline associated with the use of high concentrations of buffer in electrolyte also imposes a limitation on the use of an electrophoretic buffer presenting strong ionic strength.

Table 3
Evolution of the resolution and elution times of four amino acids (derivatized as carbamates) as a function of pH

Amino acids	pH											
	5.6		6.7		7.7		8.7		10.2		11.4	
	t_R min	R_s [a]	t_R min	R_s [a]	t_R min	R_s	t_R min	R_s	t_R min	R_s	t_R min	R_s
Tryptophan[c]	>40	n.d.	>40	n.d.	12.1	6.5	9.7	5.9	8.7	5.7	7.3	2.5
Isoleucine[d]	>40	n.d.	>40	n.d.	17.1	0.9	12.4	0.8	11.0	0.8	8.8	0.4
Methionine[d]	>40	n.d.	>40	n.d.	17.8	0.9	12.8	0.7	11.3	0.7	9.0	0.4
Valine	>40	n.d.	>40	n.d.	19.0	[b]	13.4	[b]	11.8	[b]	9.3	[b]

Operating conditions: temperature: 18 °C, buffer: Na_2HPO_4–Na_3PO_4 ionic strength=90 mequiv., concentration of βGC_9=80 mM).

[a] n.d.= not determined.

[b] Small resolution corresponding to a partial resolution.

[c] Resolution is evaluated using classical formula [44].

[d] Resolution is evaluated using Wren's approach [43].

Table 4
Evolution of the enantiomeric resolution of amino acids (derivated as carbamates) according to the ionic strength of the electrolyte

Amino acids	Ionic strength				
	54.0 mequiv.	67.5 mequiv.	90.0 mequiv.	135.0 mequiv.	270.0 mequiv.
Tryptophan[a]	4.5	4.6	5.4	6.8	6.8
Isoleucine[b]	0.6	0.65	0.7	0.8	0.8
Methionine[b]	0.5	0.6	0.6	0.7	0.8

Operating conditions: temperature: 18 °C, buffer: $NaHCO_3$–NaOH pH = 10.5, concentration of βGC_9 = 80 m*M*).
[a] Resolution is evaluated using classical formula [44].
[b] Resolution is evaluated using Wren's approach [43].

Consequently, a 90 mequiv. ionic strength appears optimal.

Having come to this stage of optimisation it remained to determine the tension that we had to apply in order to obtain the best separation possible in a minimum time of analysis.

4.6. Optimisation of the applied voltage

A study carried out with tryptophan and alanine as test samples, that is to say the amino acids constituting the two poles of the range of studied hydrophobicity, showed the clear dependence of the optimal applied voltage and of the affinity of amino acid for the chiral micelle. So, in the case of tryptophan, the most hydrophobic amino acid among those considered, the optimal applied voltage is situated in the range 5–10 kV. Then for such a voltage an extremely high efficiency is obtained (N/m = 1 900 000), efficiency which decreases, although remaining quite high, when the applied voltage increases. On the contrary in the case of alanine, the less hydrophobic amino acid among those studied, the efficiency of the electrophoretic system increases according to the applied voltage up to a value equal to 15 kV. As shown above, the less hydrophobic amino acids present chiral selectivities lower than in the case of tryptophan. Consequently, we considered 15 kV as the optimal value of the applied voltage.

The optimization of enantiomeric separation of the five amino acids being finished we proceeded to the analysis of a mixture containing the racemics of five acids derivatized as carbamates:

1. at a temperature of 18 °C,
2. with an applied voltage of 15 kV,
3. with the electrophoretic buffer made of $NaHCO_3$ (50 m*M*)–NaOH (100 m*M*) at pH = 10.5 to which was added as a chiral selector the *n*-nonyl-β-D-glucopyranoside at a concentration equal to 80 m*M*.

The electropherogram obtained in these optimal conditions is reported in Fig. 7.

This electropherogram shows that by using a new class of chiral selectors, *n*-alkyl-β-D-glucopyranosides, it becomes possible to perform in about 10 min the enantiomeric separation of four of the five considered amino acids. However, in these conditions, these various racemic mixtures are not always separated in a satisfactory way. If the racemic tryptophan is resolved remarkably by this new electrophoretic system, the separation of enantiomers of isoleucine on the one hand and methionine on the

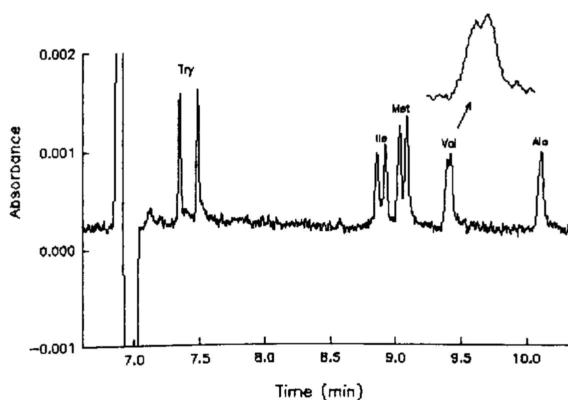


Fig. 7. Enantiomeric separation of five amino acids (derivatized as carbamates) by MEKC with *n*-nonyl-β-D-glucopyranoside as chiral selector. (Operating conditions: fused silica capillary 57 cm × 50 μm, hydrodynamic injection of 1 s, applied voltage: 15 kV, temperature: 18 °C, λ_{detect} = 214 nm, buffer: $NaHCO_3$ (50 m*M*)–NaOH (100 m*M*) pH 10.5, *n*-nonyl-β-D-glucopyranoside concentration = 80 m*M*).

other hand being practically acceptable, unfortunately no chiral resolution is obtained for alanine and only a partial separation is obtained in the case of the racemic valine. However, this is the very first use in capillary electrophoresis of these chiral surfactants and these first results are more than encouraging.

5. Conclusion

The subject of this present study was to apprehend the potentialities of *n*-alkyl-β-D-glucopyranosides, chiral surfactants having never been used up to now as chiral selectors in capillary electrophoresis, with regards to enantiomeric resolution in micellar electrokinetic chromatography. The results obtained during this first approach were more than encouraging. Effectively, the *n*-nonyl-β-D-glucopyranoside allowed after optimisation of operating conditions the resolution, in a reasonable time, of racemic mixtures of amino acids derivatized as carbamates and corresponding to a relatively wide range of hydrophobicity. Moreover, it is interesting to note that these new chiral electrophoretic systems enable the attainment, in certain conditions, of efficiencies superior to those reported up to now for MEKC in the literature. In this way they constitute presently a new class of chiral selectors, besides cyclodextrins and bile salts for example, which can be used to try to take up one of the most difficult challenges in analytical chemistry: the resolution and quantification of racemic mixtures. In order to widen their field of application in the direction of enantiomeric resolution of racemics corresponding to non ionic and non potentially ionisable molecules, we will study in the near future the possibility of conferring a charge either by using them as co-micelles in presence of sodium dodecyl sulfate for example or by complexing them.

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