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Characterization of sulfobutyl ether-β-cyclodextrins mixtures by anion-exchange chromatography using evaporative light scattering detection

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Abstract

An analytical method based on anion-exchange chromatography (AEC) using volatile eluent ion and evaporative light scattering detection was developed for the analysis of mixtures of sulfobutyl-ether-β-cyclodextrins (SBE-β-CDs). A systematic investigation of the retention mechanism of pure SBE-β-CD standards has been studied on a silica quaternary ammonium exchanger (Vydac 302 IC column). The influence of the nature and concentration of volatile anions (acetate, formate, trifluoroacetate), the addition of the organic modifier in the mobile phase as well the nature of the stationary phase have been evaluated under isocratic elution conditions. Satisfactory analysis of two commercial and two home-made SBE-β-CD samples was achieved on the Vydac 302 IC column by using ammonium acetate as ion eluent in water–acetonitrile (70:30) under a salt concentration gradient mode. This method provides for SBE-β-CD samples, an efficient and characteristic liquid chromatography fingerprint which depicts the mixture complexity and determines an average degree of substitution (DS) for each sample.

Keywords: Evaporative light scattering detection; Retention mechanism; Mobile phase composition; β-Cyclodextrin; Sulfobutyl ether; Volatile anions

1. Introduction

Sulfobutyl ether derivatives of β-cyclodextrin have found industrial applications (drug formulation, cosmetics) [1–3] but are also widely used as chiral selectors for the separation of enantiomers by capillary electrophoresis [4–12]. The sulfobutyl-ether-β-cyclodextrins (SBE-β-CDs) are said to be randomly substituted because they are mixtures due to number, position and glucose unit, thus one has degree of substitution, positional isomerization (2, 3 or 6) and regioisomerization (glucose unit 1–7 for beta CD). The knowledge of the composition of these modified cyclodextrins is important because this composition has an influence on their enantiomeric selectivity [8–12] and may also be of interest in industrial applications.

The analysis as well as the isolation in high purity of these cyclodextrins is still a real problem. Capillary electrophoresis [13,14] as well as mass spectrometry [15] can be performed for the characterization of SBE-β-CDs but only a separative technique

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such as liquid chromatography allows the production of pure SBE-β-CD components on a micro or macroscale. Recently, we developed an analytical chromatographic method to obtain the chromatographic fingerprint of one SBE-β-CD sample using ion–pair reversed-phase chromatography and evaporative light scattering detection [16]. This separation was observed on a Spherisorb ODS 1 column by using heptylammonium formate as an ion-pairing agent under a concentration gradient. This technique allowed us to obtain a characteristic fingerprint depicting the mixture complexity and it is suitable for checking batch-to-batch variability. Nevertheless, the on-line identification with mass spectrometry was not possible due to the formation of clusters constituted of heptylammonium formate and SBE-β-CD, preventing easy interpretation of the fragments. Moreover, the ion-pairing agent used prevented a purification by preparative chromatography.

Taking into account the polarity and the multicharged nature of SBE-β-CDs, it seems advisable to investigate an analytical method based on the anion-exchange chromatography (AEC) to achieve separation of the cyclodextrins. Mixtures of SBE-β-CDs were first fractionated [17,18] by preparative anion-exchange chromatography using DEAE-Sephadex A-25 resin. The isolated fractions have been characterized and it was established that the components of the mixture are resolved based on the differences in charge. Until now, AEC has been never considered as an analytical tool able to provide a characteristic SBE-β-CD fingerprint in a simple run.

In AEC, the coulombic interaction competition between the eluite ion and eluent ion for the charged functional group in the stationary phase is the primary retention mechanism [19]. But many researchers have investigated some types of secondary non-ionic retention mechanisms on silica or polymeric anion-exchanger supports. The choice of eluent ion is depending on two conditions: the eluent ion must allow satisfactory retention of the anionic solute on the stationary phase and it must be compatible with the detection mode selected for the analysis. As a direct UV detection cannot be used for SBE-β-CD, an evaporative light scattering detector (ELSD) has been chosen. This detection mode is universal since the analyte has a lower volatility than the mobile phase, it is compatible with a gradient elution mode and requires the complete volatilization of the mobile phase. Moreover, this detection mode has proved to be good choice for cyclodextrin analysis [16,20,21]. Furthermore, the use of volatile mobile phases is highly suitable to isolate pure cyclodextrins. In this paper, a systematic investigation of the retention mechanism of SBE-β-CD anions on a silica quaternary ammonium exchanger is described. The nature and concentration of anion eluents (acetate, formate, trifluoroacetate) have been studied as well as the influence of the organic modifier in the mobile phase.

In this report, we describe the application of the AEC method to study the composition of two commercial SBE-β-CD mixtures and of two home-made SBE-β-CD samples and we established an average degree of substitution (DS) for each mixture with the ELSD.

2. Experimental

2.1. Instrumentation

The liquid chromatographic apparatus consisted of a Thermo Separation Products (Les Ullis, France) Model Spectra SERIES P4000 pump, a Rheodyne (Cotati, CA, USA) Model 7125 injection valve fitted with a 20-μl stainless loop, a Sedere (Vitry/Seine, France) Model Sedex 55 evaporative light scattering detector (ELSD) and a Shimadzu (Kyoto, Japan) CR-5a integrator. The usual ELSD settings were as follows: drift tube temperature 50°C, nebulizer gas pressure 2.2 bar; photomultiplier 8.

The anion-exchanger column was the Vydac 302 IC column (250×4.6-mm I.D., 100 μequiv/g) from Interchim (Montluçon, France). Two other columns were used: the Nucleosil® Anion II (250×4.6 mm, 50 μequiv/g) from Macherey-Nagel (Düren, Germany) and the IonPac® AS4A-SC (250×4 mm, 20 μequiv/g) from Dionex (Sunnyvale, USA). Flow-rate was 1 ml min^{-1} and under these conditions, the pressure was about 60 bar with the different mobile phases.

2.2. Chemicals

Eluent constituents were purchased as follows: HPLC-grade acetonitrile, methanol from J. T. Baker (Noisy Le Sec, France); water from an Elgastat UHQ II System from Elga (Villeurbanne, France). Acetic acid, formic acid, trifluoroacetic acid and ammonium hydroxide were obtained from Fluka (St. Quentin Fallavier, France) as well β-cyclodextrin. The 1,4-butane sultone was purchased from Aldrich (St. Quentin Fallavier, France). For LC analysis, the SBE-β-CD sample was prepared at 2500 mg l^{-1} in deionized water.

PHOEBUS, an application program help for buffer studies (Sedere Co, Franklin MA, USA) was used for the mobile phase preparation. The mobile phase was a mixture of ammonia and acetic, formic or trifluoroacetic acid. It was prepared by imposing the acetate, formate or trifluoroacetate concentration value and a pH value of 4. The ammonium concentration was calculated by the software package. For each mobile phase, the pH value was checked with a Beckman (Gagny, France) pH meter model Φ 10.

Captisol® (sample A) and Advasep® (sample B), commercialized by Cydex Inc. (Overland Park, KS, USA) are given with an average degree of substitution of 7 and 4, respectively.

2.3. Preparation and characterization of sulfobutyl ether derivatives of β-cyclodextrin

Sulfobutyl ether derivatives of β-cyclodextrin were synthesized according to the procedure of Rajewski and Stella [22] and as reported in our previous work [16]. The CE method [13,14] provides characterization of heterogeneity of sulfoalkyl ether β-cyclodextrin derivatives versus the degree of substitution.

The average degree of substitution of synthesized SBE-β-CD samples was determined to be 7 (sample C) and 4.5 (sample D) by the CE method.

3. Results and discussion

The analysis in AEC has been already achieved for anionic oligosaccharides. Carboxymethyl-cellulose have been separated on a CarboPac PA-1 pellicular anion-exchange column (polymeric support with a high exchanger capacity) with pulsed amperometric detection and under gradient elution conditions of sodium hydroxide and sodium acetate [23]. But, by this method only mono, di or trisubstituted carboxymethyl-cellulose were characterized. More recently, fragments of heparin, a sulfated oligosaccharide, were analysed on a strong anion-exchange silica-based column (S5-SAX, Phase separations Ltd.) with UV-detection and under gradient elution of sodium chloride [24]. However, this method allowed us to separate with a low resolution only two decasaccharides substituted by 14 and 15 sulfates, respectively. In the two cases, the non-volatile mobile phase used is not suitable to isolate pure cyclodextrins without a further step in purification. Moreover, the described methods were inadequate for separating SBE-β-CD mixtures which were constituted of both low- and high-charge compounds.

The highly charged character of the components contained in the commercial SBE-β-CD mixtures would entail strong ion-exchange interactions with the anion exchanger. Consequently, we have first investigated the chromatographic behaviour of SBE-β-CD on a low-capacity exchanger and we have selected a silica-based rather than a polymeric anion exchanger to reduce the possible secondary hydrophobic interactions between the solutes and the stationary phase. Our study was mainly carried out on a Vydac 302 IC column, a low capacity anion-exchanger (quaternary amine, 100 μequiv/g), based on a high-purity 10-micron large-pore silica [25].

Nine SBE-β-CD fractions, containing from mono- to nona-SBE substitutions, were isolated from a mixture of SBE-β-CDs using preparative anion-exchange chromatography as described in Ref. [22]. After characterization by mass spectrometry, they were used as standard solutes and listed as DS 1 to DS 9 for this preliminary study. With the view to develop simple and ordinary conditions for SBE-β-CD analysis, we first tested the elution strength of some commonly used eluent anions under isocratic conditions. Aqueous solutions of acetate (CH_3COO^-), formate ($HCOO^-$) and trifluoroacetate (CF_3COO^-), for which the compatibility with the ELSD has already been demonstrated [26,27] and currently used in anion-exchange chromatography [28], were evaluated as the mobile phase. Because

not only a 2–6 pH range was required when using the Vydac 302 IC column, but also a good buffer capacity of the eluent, the pH of each mobile phase was adjusted to a value of 4.

Even when the elution strength of the aqueous mobile phase was increased by changing the nature (acetate, formate, trifluoroacetate) and concentration of the eluent anion (until 300 m*M*), it was not possible to elute the SBE-β-CD fractions (DS 1 to DS 9) sufficiently fast under simple chromatographic conditions (aqueous solution of salt as mobile phase in an isocratic mode). In fact, in spite of very high solubility of SBE-β-CDs in water, it appeared that the strong ion-exchange interactions were reinforced by considerable adsorption of these anionic compounds onto the support. These results led us to add organic modifier such as acetonitrile or methanol to the mobile phase to regulate the SBE-β-CD retention. As expected in anion-exchange chromatography when organic analytes are retained by adsorption, the SBE-β-CDs decreased in retention as the concentration of organic modifier increased in the mobile phase without variation in the eluent ion concentration [29,30]. As previously noticed, SBE-β-CDs are soluble in water–acetonitrile or water–methanol mixtures under a percentage of organic modifier equal to or less than 50%. Therefore, the proportion of these organic solvents could not exceed this value in the eluent. As yet observed in ion–pair reversed-phase chromatography [16], the elution strength of acetonitrile was greater than that of methanol for SBE-β-CD analysis. Even at high concentrations of salt, 50% of methanol in the mobile phase did not allow the elution of the most charged fractions. Hence, the effects of the eluent anion concentration on SBE-β-CD retention were investigated in water–acetonitrile mixtures as mobile phase under isocratic elution conditions.

3.1. Influence of the nature and concentration of eluent anions

Three eluent anions have been selected for this study: acetate, formate and trifluoroacetate. Whatever the percentage of acetonitrile in mobile phase (from 20% to 50%), by increasing the CF_3COO^- concentration in the eluent, it was not possible to elute the most charged SBE-β-CDs. Moreover, a CF_3COO^- concentration above 100 m*M* in the mobile phase increases drastically the ELSD background noise, whereas this phenomena was not observed with CH_3COO^- or $HCOO^-$ even at a concentration of 300 m*M*. Therefore, CF_3COO^- was not retained as the eluent anion for further studies.

Table 1 summarizes the retention factors (*k*) and

Table 1
Retention factor (*k*) and separation factor (α) for SBE-β-CD fractions (from DS=3 to 9) on Vydac 302 IC column (250×4.6 mm I.D.) versus the concentration of formate and acetate used as eluent anions in mixture water–acetonitrile (70:30) as mobile phase

Ammonium salt	Eluent anion concentration (m*M*)	Retention factor							Separation factor					
		$k_{DS\ 3}$	$k_{DS\ 4}$	$k_{DS\ 5}$	$k_{DS\ 6}$	$k_{DS\ 7}$	$k_{DS\ 8}$	$k_{DS\ 9}$	$\alpha_{DS\ 3,\ DS\ 4}$	$\alpha_{DS\ 4,\ DS\ 5}$	$\alpha_{DS\ 5,\ DS\ 6}$	$\alpha_{DS\ 6,\ DS\ 7}$	$\alpha_{DS\ 7,\ DS\ 8}$	$\alpha_{DS\ 8,\ DS\ 9}$
Formate	50	5.50	a	a	a	a	a	a	–	–	–	–	–	–
	100	0.40	0.87	2.30	6.54	18.44	a	a	2.17	2.64	2.77	2.81	–	–
	150	b	0.33	0.70	1.80	3.10	7.10	a	–	2.12	2.57	1.72	2.30	–
	200	b	0.26	0.41	0.85	1.50	3.45	6.40	–	1.57	2.07	1.76	2.30	1.85
	250	b	b	0.25	0.40	0.70	1.90	2.20	–	–	1.28	1.75	2.43	1.15
Acetate	50	1.90	a	a	a	a	a	a	–	–	–	–	–	–
	100	0.27	0.63	1.6	5	17	a	a	2.33	2.54	3.15	3.40	–	–
	150	b	0.28	0.5	1.2	2.9	10	a	–	2	2.40	1.60	3.45	–
	200	b	0.14	0.27	0.6	1.20	2.8	4.30	–	1.93	2.22	2	2.35	1.53
	250	b	b	0.12	0.25	0.50	1.40	1.60	–	–	2.10	2	2.80	1.14

[a] Compounds not eluted ($k>20$).
[b] Compounds eluted in the void volume.

the separation factors (α) for seven SBE-β-CD fractions (from DS 3 to DS 9) under isocratic elution conditions using CH_3COO^- and $HCOO^-$ as the eluent ion in water–acetonitrile mixture (70:30) as mobile phase. Whatever the nature of the eluent ion, its addition, at a 50 m*M* concentration, resulted in the elution in void volume of the least charged SBE-β-CDs, DS 1 and DS 2. As expected in AEC [31], the SBE-β-CD fractions decreased in retention as the concentration of eluent ion increased. In the 50–250 m*M* range of the eluent ion concentration, the elution strength of CH_3COO^- was greater than that of $HCOO^-$. Whatever the eluent ion nature, considerable differences in retention were observed between the least charged fractions and the most charged ones. A 100 m*M* concentration of eluent ion was necessary to obtain satisfactory retention and separation for DS 3 to 7 but under these isocratic conditions, the interactions between the most charged fractions and the stationary phase were so strong that they prevented the elution of these fractions. At a similar elution strength, it appeared that the acetate in the water–acetonitrile system was more selective than the formate in water–acetonitrile system. For the DS 4–DS 5 pair, for example, a similar $k_{DS\ 4}$ value (close to 0.27) was observed with 150 m*M* acetate or 200 m*M* formate in mobile phase. However, a greater $\alpha_{DS\ 4,\ DS\ 5}$ value was obtained with acetate (2.54) than with formate (1.57). For the DS 5–DS 6 pair, the same observation was done by comparing the k and α values obtained for 250 m*M* formate (1.28) or 200 m*M* acetate (2.22) in mobile phase. Therefore, CH_3COO^- has proved to be the most suitable eluent ion to promote the separation of SBE-β-CD fractions: it is more eluting and more selective than $HCOO^-$. In order to optimize the conditions for the analysis of different SBE-β-CD mixtures, it was necessary to investigate the factors that contributed to SBE-β-CD retention in these chromatographic systems. Table 2 summarizes the results of linear regression analysis for solute retention (DS 1 to DS 9) versus acetate concentration (studied concentrations: 12.5, 25, 50, 60, 75, 100 m*M* for DS 1 to DS 3 and 100, 125, 150, 175, 200, 250 m*M* for DS 4 to DS 9). A good linearity, characteristic of ion-exchange [18,27,29], was obtained independently from the organic modifier concentration (correlation coefficient r^2 better than 0.98 and 0.999). The slope *S*, is usually found to be close to the ratio y/x, where y is the net charge of the eluite and x the charge of the eluent ion. Because acetate is a monovalent ion, the theoretical slope of

Table 2
Linear regression analysis of log $k = -S$ log[CH_3COO^-]+constant[a] for SBE-β-CD with acetate eluent ion in different mixtures water–acetonitrile[b]

SBE-β-CD fraction	x/y[c]	ACN percentage in mobile phase					
		20% ACN		30% ACN		50% ACN	
		S	Correlation coefficient (r^2)	*S*	Correlation coefficient (r^2)	*S*	Correlation coefficient (r^2)
DS 1	1	1.08	0.9995	1.01	0.9995	1.05	0.9985
DS 2	2	1.80	0.9997	1.71	0.9985	1.71	0.9990
DS 3	3	2.87	0.9985	2.75	0.9851	2.74	0.9999
DS 4	4	2.13	0.9925	2.20	0.9812	2.12	0.9879
DS 5	5	2.57	0.9683	2.76	0.9824	2.67	0.9979
DS 6	6	3.15	0.9979	3.15	0.9935	3.19	0.9921
DS 7	7	3.69	0.9944	3.78	0.9944	3.78	0.9983
DS 8	8	3.78	0.9839	3.81	0.9904	4.71	0.9951
DS 9	9	4.30	0.9995	3.85	0.9935	4.77	0.9934

[a] *S* is a constant (slope).
[b] Studied concentrations of the eluent ion: 12.5, 25, 50, 60, 75 and 100 m*M* for DS 1, DS 2 and DS 3 and 100, 125, 150, 175, 200 and 250 m*M* for DS 4, DS 5, DS 6, DS 7, DS 8 and DS 9.
[c] y is the net charge of SBE-β-CD fraction and x is the charge of the eluent ion.

the linear curve should be equal to the net charge of each SBE-β-CD fraction. The results were close to that for the least-substituted derivatives (DS 1, DS 2 and DS 3) and this supported the idea that normal ion-exchange was occurring predominantly between the eluent ion and these eluite ions. However, when the net charge of the SBE-β-CD fraction is higher than 3, the value of S departs significantly from the y/x ratio and is not affected by a variation of the organic modifier percentage in mobile phase. As a result, the apparent value of the ionic charge of the multicharged SBE-β-CD fractions seems to be lower than the real value. These results indicate that the y/x ratio depends on other specific properties of both the polycharged cyclodextrins and the stationary phase. The deviations from the ideal slope can be attributed to steric problems. Indeed, the conical form of the hydrophobic cage of the SBE-β-CD and the presence of the SBE groups onto the rings should induce steric hindrance and consequently the polyvalent ions cannot access ion-exchange sites. To conclude, on an anion-exchange column, the more charged the cyclodextrin is, the more retained it is. However, the SBE-β-CD retention cannot be explained by the net charge concept alone and it is in good agreement with the results obtained for other polycharged compounds [31].

Two other columns were tested for the analysis of the SBE-β-CDs. The Nucleosil® Anion II (250×4.6 mm) seemed interesting to elute the anionic cyclodextrins due to its features close to the Vydac column. Indeed, this column has a low anion-capacity (50 μeq/g) and contains spherical silica packing material. The IonPac® AS4A-SC (250×4 mm) is also a low anion-capacity exchanger (20 μeq/g) and composed of a polyethylvinylbenzene support. For the least charged fractions of SBE-β-CD (DS<4), with the Nucleosil® Anion II and the IonPac® AS4A-SC, the elution strength when using acetate was close to the one obtained on the Vydac column. However, the most charged fractions were not eluted on the two supports, even at very high CH_3COO^- concentration and with 50% of acetonitrile added in the mobile phase. This observation showed that the elution of the SBE-β-CD fractions depends strongly on the nature of the chosen support, especially for the most charged DS (>4) that are more difficult to elute due to higher electrostatic interactions with the anion-exchanger.

3.2. Characterization of several SBE-β-CD samples

In the light of these results, it appeared that the use of anion-exchange bonded-silica column (Vydac) with salt gradient elution should provide an efficient means of isolating the different SBE-β-CD fractions in complex mixture due to the great difference of retention of these compounds under these chromatographic conditions. The four SBE-β-CD samples (A–D) typically showed a complex profile of resolved peaks (Figs. 1 and 2). Samples A and C, characterized by the CE method with a similar average DS (close to 7), were analysed under the same gradient elution condition based on increasing acetate concentration from 50 m*M* to 300 m*M* in water–acetonitrile (70:30) in 35 min (Fig. 1). Samples A and C showed broadly similar chromatographic profiles but with differences in the relative proportions of each of the peaks within the profile. The presence of β-CD and side products not totally eliminated by dialysis (peaks eluted before 7 min) was noticed only for the home made SBE-β-CD sample C. By analysing the nine SBE-β-CD fractions (DS 1 to DS 9) under the same chromatographic conditions, the different peaks in Fig. 1 have been identified as corresponding to SBE derivatives respectively with a defined DS of 2–10 for sample A and a DS of 4–12 for sample C. The distribution of the peaks is centered around the largest peak with DS 7 for sample A and with DS 8 for sample C. A total separation between the different fractions were achieved with respect to the DS for the two samples. The elution conditions used were inadequate for the two other samples because they entailed a too fast elution of these mixtures. Samples B and D, less charged, required a weaker concentration of acetate added in the mobile phase. Fig. 2 shows their characteristic fingerprints obtained under the gradient elution composed of an initial concentration of acetate of 10 m*M* (for 5 min) and a final concentration of acetate of 250 m*M* reached in 20 min. Likewise, the elution of β-CD and side products (before 7 min) was observed only for the synthesized mixture. The comparison of the substitution profiles

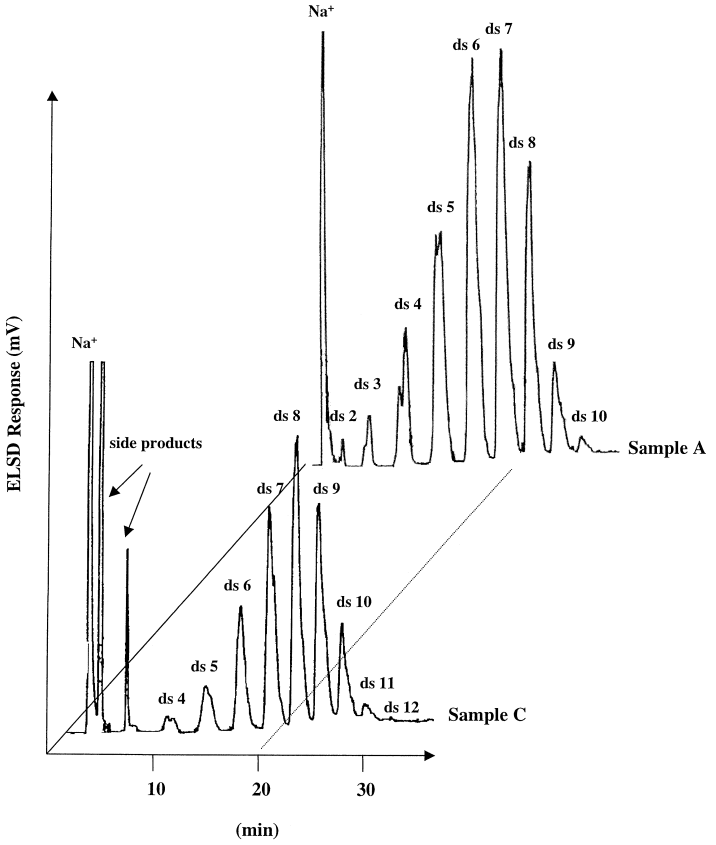

Fig. 1. Chromatographic fingerprints of sample A and sample C under gradient elution conditions. Column Vydac 302 IC (250×4.6 mm I. D). Eluent A: 50 m*M* of ammonium acetate in water–acetonitrile (70:30). Eluent B: 300 m*M* of ammonium acetate in water–acetonitrile (70:30). Gradient conditions: from 100% A to 100% B in 35 min. Flow-rate: 1 ml min^{-1}, evaporative light scattering detection, model Sedex 55, drift tube temperature 50°C, nebulizer gas pressure 2.2 bar; photomultiplier 8.

constituted of nine peaks (DS 1 to 9) in the two cases shows that the commercial SBE-β-CD is slightly more charged with a fingerprint centered on DS 5 for sample B and on DS 3 for sample D.

The calculation of an average degree of substitution has been made for the four SBE-β-CD samples by considering an equal response coefficient for each degree of substitution with the ELSD. Table 3 reports the average DS for each sample. The calculated DS were slightly different from the one obtained by CE with an indirect detection (with more or less a difference in 1 unit DS).

Thus, with fast equilibration time (less than 10 min even at high concentration of salt), no surface modification, reproducible analyses, the use of the AEC on the Vydac 302 IC column is an alternative method to the characterization of the SBE-β-CD. The combined AEC–ELSD process alone was suitable for the separation and purification of the SBE-β-CDs in one step.

4. Conclusion

This paper reports the first investigation in which the SBE-β-CD has been carried out using high-performance anion-exchange chromatography. This technique provides characteristic fingerprints with respect to the degree of substitution for complex mixtures of SBE-β-CDs. It offers the possibility to check batch-to-batch variability in addition to the current CE method. With the use of a volatile eluent,

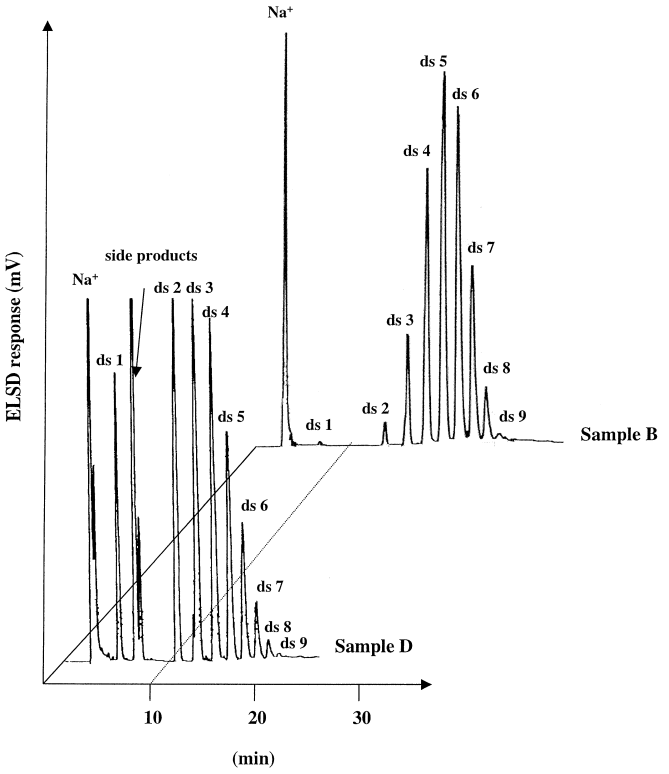

Fig. 2. Chromatographic fingerprints of sample B and sample D under gradient elution conditions. Column Vydac 302 IC (250×4.6 mm I. D). Eluent A: 10 m*M* of ammonium acetate in water–acetonitrile (70:30). Eluent B: 250 m*M* of ammonium acetate in water–acetonitrile (70:30). Gradient conditions: from 100% A for 5 min to 100% B in 20 min. Flow-rate: 1 ml min^{-1}, evaporative light scattering detection, model Sedex 55, drift tube temperature 50°C, nebulizer gas pressure 2.2 bar; photomultiplier 8.

the transposition for the preparative anion-exchange chromatography is conceivable for the purification of SBE-β-CD samples and the collection of fractions with pure degrees of substitution. Moreover, the LC–ELSD conditions are favorable to develop a LC–MS method in order to better characterize the SBE-β-CDs. Work is in progress to perform the analysis of the anionic cyclodextrins with LC–MS coupling.

Table 3
Average DS of different SBE-β-CD samples calculated from peak areas (A_i)[a]

SBE-β-CD sample	DS=Σ(ds×A_i(%)/100)
Sample A	6.4
Sample B	5.3
Sample C	7.7
Sample D	3.5

[a] Each DS was detected with an evaporative light scattering detector and eluted on Vydac 302 IC column (250×4.6 mm I.D.) under gradient elution conditions as depicted in Figs. 1 and 2 .

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