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Journal of Chromatography A, 855 (1999) 645–656

JOURNAL OF CHROMATOGRAPHY A

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Electrospray mass spectrometry and supercritical fluid chromatography of methylated β-cyclodextrins

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Received 13 January 1999; received in revised form 10 March 1999; accepted 9 June 1999

Abstract

Five commercial dimethylated β-cyclodextrin (DM-β-CD) samples were analysed by electrospray (or ionspray) mass spectrometry (ESI-MS) and supercritical fluid chromatography (SFC) with evaporative light scattering detection. A silica and a nitro-bonded silica were selected using CO_2–methanol–acetonitrile–water and CO_2–methanol as mobile phase, respectively. An extensive optimisation scheme was performed for mobile phase selection. Both SFC systems were used for analyses of complex DM-β-CD samples. Peak identifications were made using off-line ESI-MS. Commercial DM-β-CDs are impure mixtures of homologues and isomers and analysis reveals that every manufacturer produces a different mixture.

Keywords: Mass spectrometry; Supercritical fluid chromatography; β-Cyclodextrins; Methylated cyclodextrin

1. Introduction

Cyclodextrins (CDs) are cyclic oligosaccharides consisting of 6 to 8 (1→4)-linked α-D-glucosyl residues denoted α, β, γ, respectively [1]. The formation of inclusion complexes with various substances [2] changes a variety of physical, chemical or biological properties of the guest molecule. The CD molecules have been modified chemically in an attempt to improve their complexation ability. From the thousands of CD derivatives described, the most popular commercially available ones are dimethylated β-CDs (DM-β-CDs). They have been used as chiral discriminating agents in liquid chromatography (LC) [3–5], thin-layer chromatography (TLC) [6], gas chromatography (GC) [7] and capillary electrophoresis (CE) [8–10]. However, the commercially available samples of so-called DM-β-CDs are a mixture of randomly methylated β-CDs. Indeed, this is true for almost all CD derivatives [11,12]. Some authors have focused on the analysis of such DM-β-CD mixtures using several separation methods such as: TLC [13,14], GC [13,15,16], LC [13–20] and CE [21]. Recently we described the first investigation of supercritical fluid chromatography (SFC) for the analysis of DM-β-CDs [18].

The aim of this paper is to describe a more detailed study of methylated β-CDs enabling a better

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0021-9673/99/$ – see front matter
PII: S0021-9673(99)00718-9

characterisation of commercial DM-β-CD samples. For this purpose electrospray mass spectrometry (ESI-MS) and SFC analyses were attempted.

2. Experimental

SFC analyses were conducted with a Model SF_3 Gilson apparatus (Villiers Le Bel, France), a Rheodyne (Berkeley, CA, USA) Model 7125 injector with a 20-μl sample loop, and a column oven CROCO-CIL (CIL-Cluzeau, St.-Foy-la-Grande, France). It was found that column temperature (from 20 to 100°C) and outlet pressure (from 100 to 210 bar) have little influence on retention using CO_2–MeOH but resolutions were often slightly higher at 41°C and 150 bar. Concerning ternary eluent optimisation, CO_2–methanol–X eluents were used (X= acetonitrile, tetrahydrofuran, dichloromethane, chloroform or methyl *tert*.-butyl ether) and experiments using CO_2–polar modifier in ratios from 85:15 to 60:40 by five steps. The methanol–X ratio in the modifier was from 100:0 to 20:80 by 10 steps.

SFC detection was performed with an evaporative light scattering detection (ELSD) system Model Sedex 55 (Sedere, Alfortville, France). The SFC interface of the ELSD system, equipped with a 5 cm×50 μm I.D. silica restrictor, was directly connected to the Gilson regulation pressure valve. The ELSD detector setting was as follows: photomultiplier, 7; evaporative temperature, 50°C; air pressure, 0.5 bar; nebulizer temperature, 50°C. Data were processed using a Shimadzu (Kyoto, Japan) Model CR 5A integrator.

ESI-MS (no distinction is made here between electrospray and pneumatically-assisted electrospray, called ionspray) of crude DM-β-CDs was carried out on a VG quatro II triple quadrupole (VG BioTech, Altringham, UK). Electrospray spectra were recorded in the positive mode and scanning was performed in the multi-channel analyser mode from m/z 1100 to m/z 1700 in 10 s. The solution was introduced into the ESI source at a constant flow-rate of 5 μl min^{-1}, using an infusion pump Model 22 (Harvard Apparatus, South Natick, USA). The ESI voltage was 3.08 kV and the orifice voltage was 50 V. For MS–MS, the pressure of gas cell was $2.7.10^{-3}$ mbar (Argon) and collision energy 18.0 eV.

Off-line ESI-MS of SFC collected fractions was carried out on an API 300 (Perkin-Elmer, Thornhill, Canada). Conditions were similar for both ESI-MS set-ups.

The following columns were used: LiChrospher 100 Diol 5 μm (150×4.6 mm I.D.), LiChrospher 100 CN 5 μm (150×4.6 mm I.D.), LiChrospher 100 RP 18 5 μm (150×4.6 mm I.D.), LiChrospher 100 RP 18 endcapped 5 μm (150×4.6 mm I.D.) from (Merck, Darmstadt, Germany), Nucleosil NO_2 10 μm (150×4.6 mm I.D.), Nucleosil C_8 5 μm (150×4.6 mm I.D.), Nucleosil phenyl 7 μm (150×4.6 mm I.D.), Nucleosil Diol 7 μm (150×4.6 mm I.D.), Hypersil silica 5 μm (250×4.6 mm I.D.) from Shandon (Cheshire, UK), Zorbax NH_2 7 μm (250×4.6 mm I.D.), Zorbax C_{18} 7 μm (250×4.6 mm I.D.), Zorbax phenyl 7 μm (250×4.6 mm I.D.), Zorbax TMS 7 μm (250×4.6 mm I.D.), Zorbax Sil 7 μm (250×4.6 mm I.D.) from Dupont (Wilmington, DE, USA), Pecosphere Silica (HS 3 silica) 3 μm (3.3×4.6 mm I.D.) from Perkin-Elmer (Überlingen, Germany).

Carbon dioxide was industrial grade (purity 99.7%, Air Liquide, Paris, France), methanol (MeOH), ethanol (EtOH), propanol, butanol, acetonitrile (ACN), dichloromethane (CH_2Cl_2), chloroform ($CHCl_3$), tetrahydrofuran (THF), methyl *tert*.-butyl ether (MTBE) (Mallinckrodt Baker, Noisy-le-Sec, France) were of analytical grade. Distilled water was from Stalabo (Cooperation Pharmaceutique Française, Melun, France).

β-Cyclodextrin W 7 (β-CD) was donated by Wacker (Lyon, France) and heptakis (2,3,6-tri-*O*-methyl)-β-cyclodextrin (TM-β-CD) was from Sigma (St. Louis, MO, USA). Five different commercial dimethyl-β-CD samples (A, B, C, D, E) were studied. The determination of methylation position has been previously described after hydrolysis by GC, LC and/or SFC [22] and it was shown that samples A and B essentially contained 2,3,6-tri-*O*-methyl units and 2,6-di-*O*-methyl glucopyranosyl units, whereas samples C and E contained 2,3,6-tri-*O*-methyl units, 2,6- and 3,6-di-*O*-methyl units and 3-*O*-methyl units. For sample D, all the possible substitution patterns were found. For ESI-MS, dimethyl-β-CDs were dissolved in water with 6 nmol l^{-1} NH_4HCO_3–100 pmol l^{-1} DM-β-CD as ionisation agent. Water proved to be the best solvent in

comparison with methanol or a methanol–water mixture. For SFC, solutes were dissolved in MeOH and injections were made in triplicate.

No differentiation will be made between what is sometimes called subcritical fluid chromatography (SubFC or sSFC) and SFC because ''transitions'' between these ''defined'' states are often undetectable chromatographically and the instrumentation used is identical.

3. Results and discussion

3.1. Electrospray mass spectrometry

With the development of soft ionisation methods, MS has been reported to be suitable for the study of native or modified cyclodextrins [23–26]. The composition of DM-β-CD mixtures was studied using ESI-MS. In order to quantify the different degrees of methylation, one adduct per degree of substitution (DS) is needed. We used ammonium salt as ionisation agent in order to obtain a single ammonium adduct [27]. Moreover, response factors estimated from 13 Me-β-CD to 18 Me-β-CD were very close [27]. However, we admit that the validity of this assumption can be dependant on the ESI-MS instrumentation and setting. This will be discussed later by comparison with SFC results.

The relative intensity of the ammoniated molecules was measured for each commercial DM-β-CD sample and results were calculated in relative percentages (Table 1). From 9 to 21 methyl (Me) groups per molecule were found. Methylation of the commercial DM-β-CD samples was heterogeneous and different from one sample to another. To characterise sample mixtures, the DS, which represents the number of methyl groups per anhydro glucose unit, is currently used (Table 1). There is no correlation between the DS value and the major product found in the sample because of permethylation (samples A, B, C, E) or under-methylation (sample D). Better characterisation of samples is achieved with ESI-MS than with DS. However, MS only provides information about the number of methyl groups per molecule, but no information about their disposition around the β-CD. In fact, DM-β-CD ''I'' and DM-β-CD ''II'', shown in Fig. 1 for example, both have 14 methyl groups in their ring and a DS of 2 but these compounds are positional isomers: indeed compound I is symmetrical (presence of a symmetric axis) whereas compound II is asymmetrical. The use of electrospray MS–MS was explored to obtain structural information. Is the 14 Me-β-CD found in commercial samples symmetrical or asymmetrical? For this purpose daughter ions from 14 Me-β-CD were recorded (Fig. 2). For sample A, the regularly

Table 1
Composition of five commercial DM-β-CDs (A–E) obtained by ESI-MS: molar relative percentages of methylated constituents and calculated degree of substitution (DS)

No. methyl groups per molecule	A	B	C	D	E
9 Me	–	–	–	2.5	–
10 Me	–	–	–	8	–
11 Me	–	–	–	20	–
12 Me	–	–	–	31	0.5
13 Me	–	–	–	25	8.5
14 Me	42	11	15	11	52
15 Me	46	26	36	2.5	25
16 Me	10	35	26	–	8
17 Me	2	19	13	–	3
18 Me	–	5.8	5.3	–	2
19 Me	–	1.8	2.5	–	1
20 Me	–	0.8	1.4	–	–
21 Me	–	0.6	0.8	–	–
DS	2.1	2.3	2.2	1.7	2.1

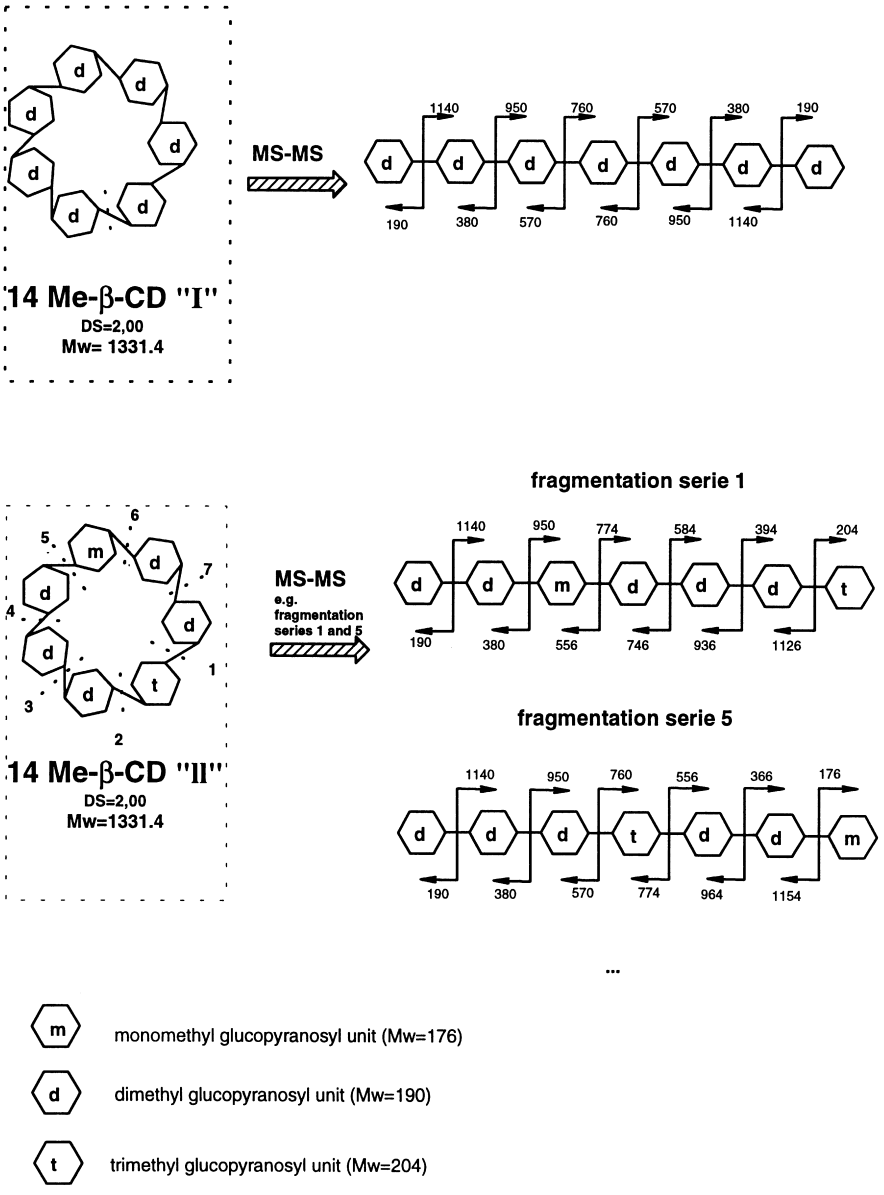

Fig. 1. Scheme of a symmetrical (14 Me-β-CD ''I'') and an asymmetrical (14 Me-β-CD ''II'') molecule as example and possible MS–MS fragmentations. For 14 Me-β-CD (II), seven fragmentation series are possible but only two are drawn. DS=Degree of substitution of the cyclodextrin.

distributed fragments are due to a sequential loss of dimethyl units (M_r=190) (Fig. 2a), consequently, the 14 Me-β-CD corresponded to a symmetrical DM-β-CD (Fig. 1, DM-β-CD; I). The same result was found for DM-β-CD (B).

In contrast, MS–MS spectra of 14 Me-β-CD of samples C, D and E (Fig. 2b) showed a loss of various fragments. These results indicate that the spectra of 14 Me-β-CD daughter of samples C, D and E corresponded to one (or more) asymmetrical DM-β-CD (see DM-β-CD; II, in Fig. 1 for example). Complete interpretation of such spectra (Fig. 2a) is

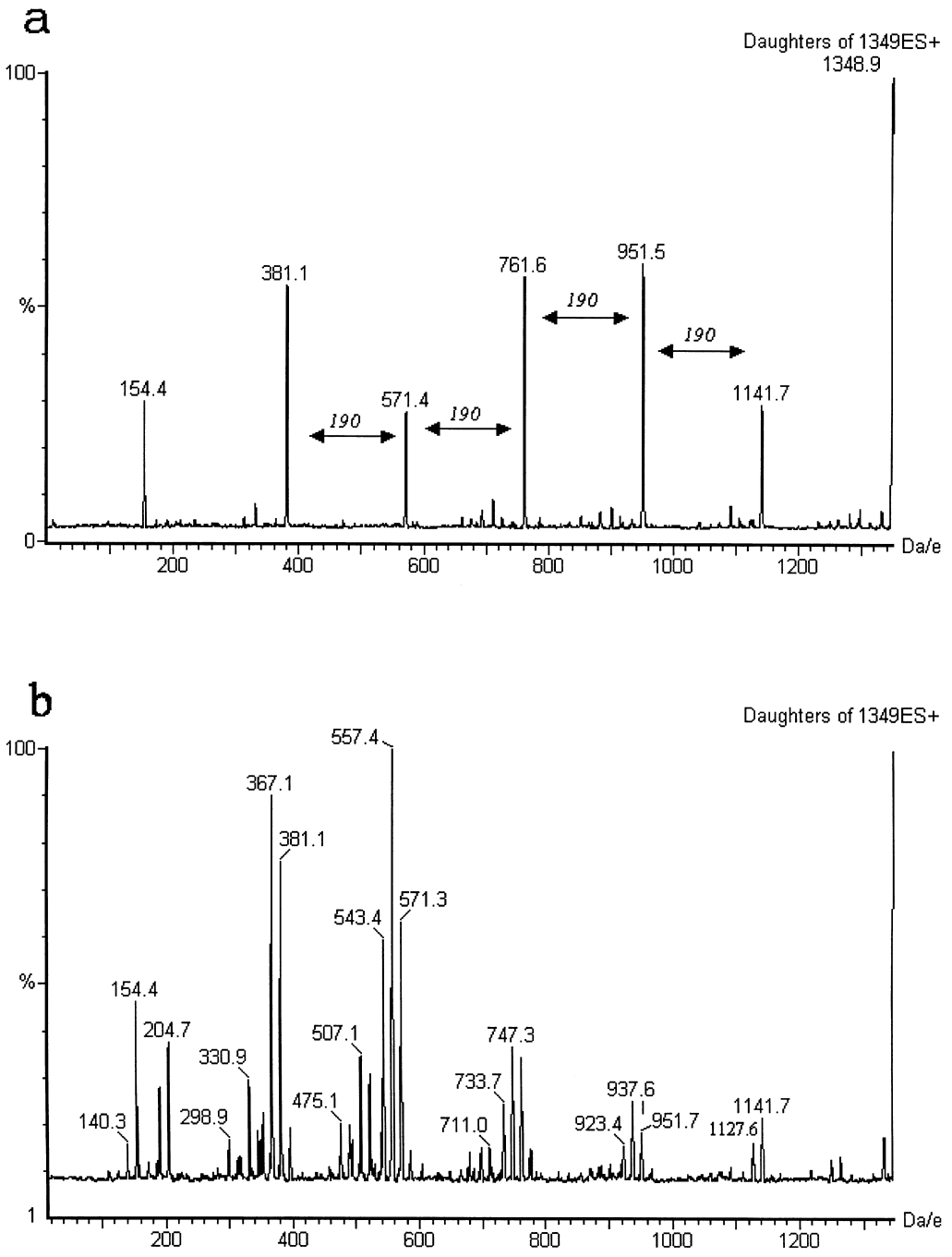

Fig. 2. Positive ion spray daughters MS–MS spectra of m/z 1349 ([14Me-β-CD+NH_4]$^+$ from two commercial samples. (a) DM-β-CD (A), (b) DM-β-CD (E).

not easy because, firstly, various fractionation series can be found for one molecule and, secondly, positional isomers corresponding to a given m/z are numerous. The most important fact is that MS–MS can distinguish symmetrical and asymmetrical 14 Me-β-CD. Consequently, chromatographic separations were attempted before ESI-MS analysis of fractions, in order to obtain more information about commercial samples.

3.2. Supercritical fluid chromatography

Initial SFC results of DM-β-CD analysis were very promising [18]. In the present work, various stationary and mobile phases were tested in order to obtain the best separation of the components of DM-β-CD (A). Using the best chromatographic conditions obtained, it will be shown that other commercial DM-β-CD samples (B, C, D and E) can be analysed and results compared.

3.2.1. Choice of the stationary phase

Various stationary phases (see Experimental: columns) were tested using CO_2–methanol eluent. Whatever the polarity of the stationary phase (e.g., silica or ODS silica), the retention order is always from the more methylated to the less methylated β-CD: permethylated CD (TM-β-CD) elutes before DM-β-CDs and β-CD is the last compound eluted, although the latter is seldom eluted with CO_2–MeOH (60:40). Moreover, retention times decrease when the methanol content increases, consequently a normal-phase partition process seems to be principally involved. Silanol interactions on apolar stationary phase are of great importance. Indeed, the capacity factor (k') is much higher for a non end-capped octadecyl stationary phase than for the corresponding end-capped phase (LiChrospher RP18 and RP18e).

Among the stationary phases tested, alkyl, phenyl and NO_2-bonded silica phases provide the highest retention values. Since the retention mechanism is not clearly explained, the choice of the stationary phase for further investigations is not easy. However, in terms of separation behaviour, the Nucleosil NO_2 stationary phase provided the best result with the CO_2–methanol eluent since six peaks were obtained [DM-β-CD (A), Fig. 3]. Other stationary phases tested provided only one or two peaks. Selectivities obtained on NO_2 stationary phases are probably due to specific interactions, alternatively, the formation of an inclusion complex may take place on the $-(CH_2)_3$-C_6H_4-NO_2 moiety. The presence of the NO_2 group on the aromatic ring seems to be essentially for selectivity because on phenyl stationary phase no separation occurred.

For the following studies, NO_2 medium polar stationary phase and the most polar stationary phase, bare silica (Hypersil silica column), which provides two peaks for DM-β-CD (A) (Fig. 4a) were selected. It will be shown that selectivities obtained using the latter stationary phase are quite different from the former.

3.2.2. Mobile phase optimisation using silica stationary phase

The influence of the mobile phase composition will firstly be described using Hypersil silica phase for DM-β-CD (A) analysis.

3.2.2.1. CO_2–alcohol mobile phase

Methanol is the most common polar modifier used in SFC or SubFC. Organic solvents such as acetonitrile, tetrahydrofuran, dichloromethane, chloroform and methyl *tert*.-butyl ether were tested and was found to be unsuitable for DM-β-CD analysis since, using contents up to 40% in CO_2, compounds were not eluted. So, the behaviour of various alcohols (methanol, ethanol, *n*-propanol, isopropanol) was investigated. Only methanol and ethanol have an adequate elution strength and can be used as polar modifiers. By comparing the results using methanol (Fig. 4a) and ethanol (Fig. 4b), it is obvious that the selectivities are quite different. Methanol and ethanol have a specific effect on selectivity. By using methanol–ethanol mixtures in various ratios as polar modifier, it was not possible to achieve separation of three peaks (one minor peak and two major peaks) as expected. Consequently, other ternary eluents were studied.

3.2.2.2. Ternary eluents

CO_2–methanol–X eluents were studied, where X was acetonitrile, tetrahydrofuran, dichloromethane, chloroform or methyl *tert*.-butyl ether. It was necessary to add methanol to obtain a sufficient elution strength. Tridimensional graphics were generated for

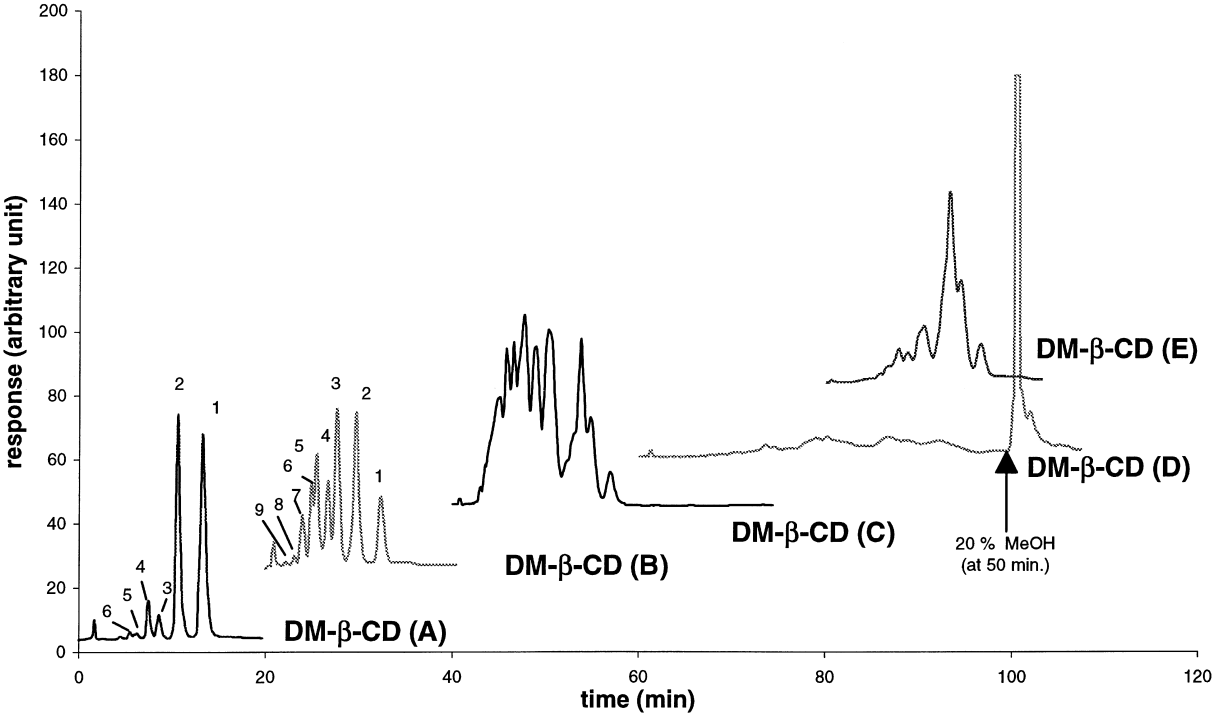

Fig. 3. SFC separations of the five commercial DM-β-CDs (A–E) on the NO_2 system. Column: Nucleosil NO_2 at 40°C, mobile phase: CO_2–MeOH (88:12) (for sample D, a CO_2–MeOH, 80:20, eluent was flushed at 40 min to elute under-methylated cyclodextrins), flow-rate: 3 ml min^{-1}, outlet pressure 150 bar, detection: ELSD.

each X solvent which can depict variation in k' or in selectivity (α). Only selectivities concerning CO_2–methanol–acetonitrile, which gave the best results, are shown in Fig. 5. Other solvents gave the same variations but with lower α values. For some CO_2–methanol–acetonitrile mixtures, three peaks (a, b and c) can be observed (Fig. 4d). By comparing Fig. 5a and b, one can conclude that when the acetonitrile content increases, the selectivity between peaks b and c (α_{bc}) increases but selectivity between peaks a and b (α_{ab}) decreases dramatically. The α_{bc} optimum is obtained with a CO_2–methanol–acetonitrile (70:6:24) eluent. The elution profile was similar to that obtained using CO_2–ethanol eluent (Fig. 4c). The α_{ab} optimum is obtained with a CO_2–methanol–acetonitrile (85:15:0) eluent (Fig. 5a). The best compromise for the separation of peaks a,b and c is to use a CO_2–methanol–acetonitrile (80:12:8) eluent (Fig. 4d). However, resolutions are quite poor.

3.2.2.3. Addition of water in the mobile phase

Water has been successfully used in packed column SFC in order to modify selectivities or to improve resolutions of carbohydrates [28]. Water from 0 to 2% in the modifier was added in a CO_2–MeOH–ACN mixture. When water content increases, the α_{bc} increases and the α_{ab} decreases. By using 0.9% water in the eluent (CO_2–MeOH–ACN–water, 70:5.8:23.3:0.9), the best selectivities and resolutions between peaks a, b and c of DM-β-CD (A) were obtained and, moreover, a fourth peak is resolved [Fig. 6, DM-β-CD (A)]. Compound(s) of this last peak were probably eluted in the tail of peak c (little shoulder) under the previous conditions.

3.2.3. Mobile phase optimisation using NO_2 stationary phase

A similar scheme of mobile phase optimisation was made using NO_2 stationary phase. Various

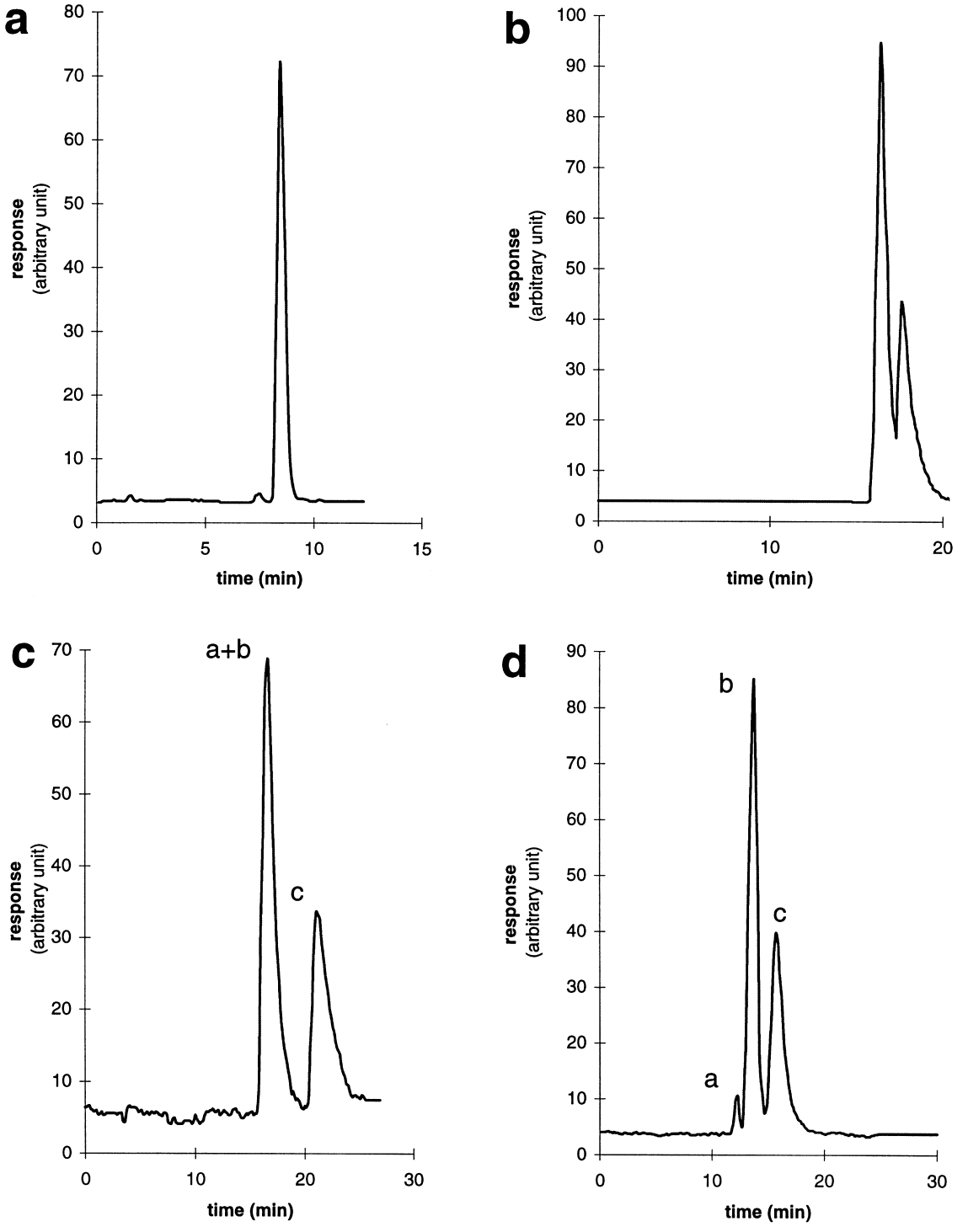

Fig. 4. SFC separation of DM-β-CD (A) on silica stationary phase. Column: Hypersil silica at 40°C, mobile phase: (a) CO_2–MeOH (85:15), (b) CO_2–EtOH (85:15), (c) CO_2–MeOH–ACN (70:6:24), (d) CO_2–MeOH–ACN (80:12:8), flow-rate: 3 ml min^{-1}, outlet pressure 150 bar, detection: ELSD.

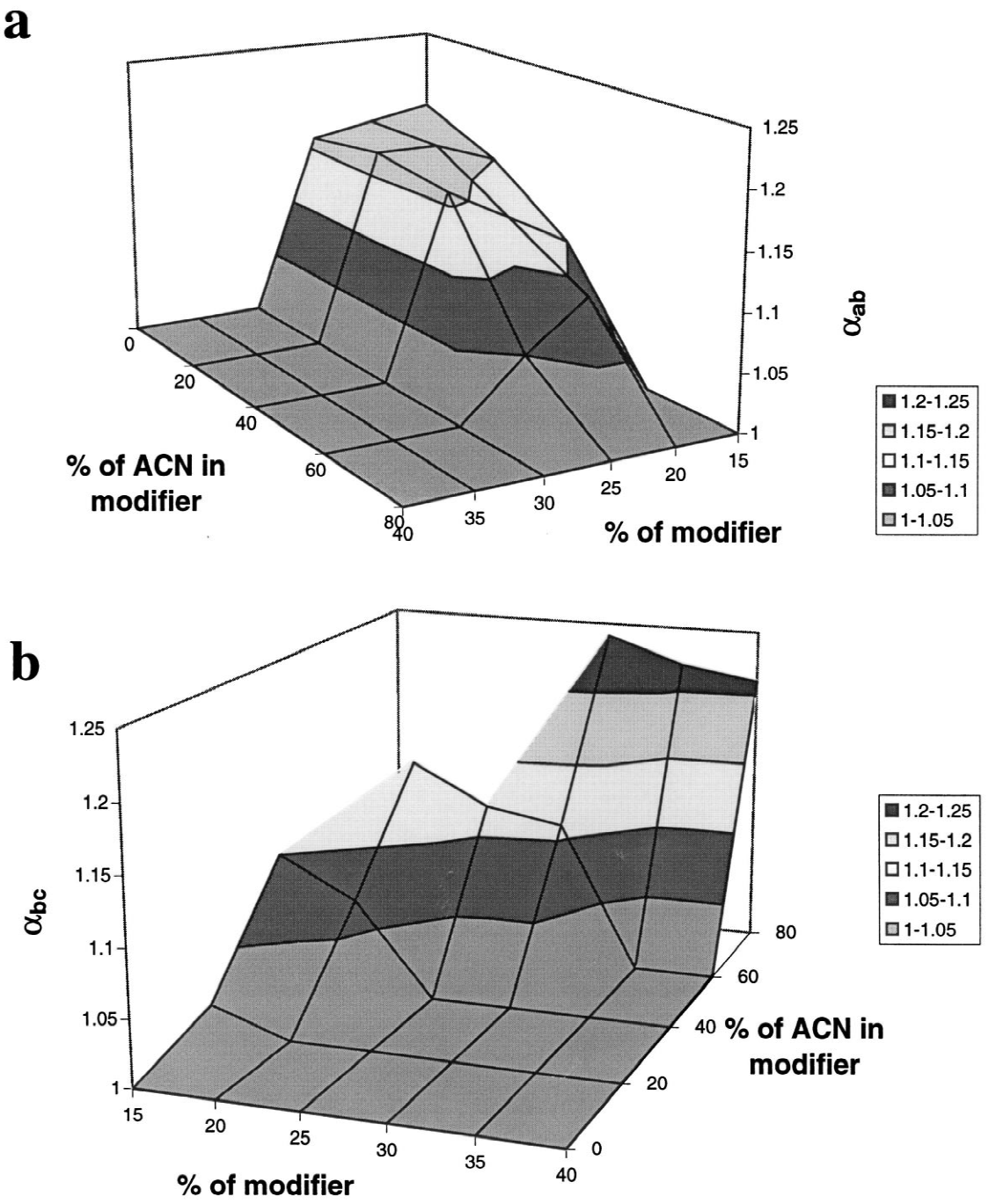

Fig. 5. Variations of selectivities on silica stationary phase between (a) peaks a and b of Fig. 4 (α_{ab}) and (b) peaks b and c of Fig. 4 (α_{bc}) as a function of the CO_2–modifier eluent. The modifier consisted of a MeOH–ACN mixture. Other conditions as in Fig. 4.

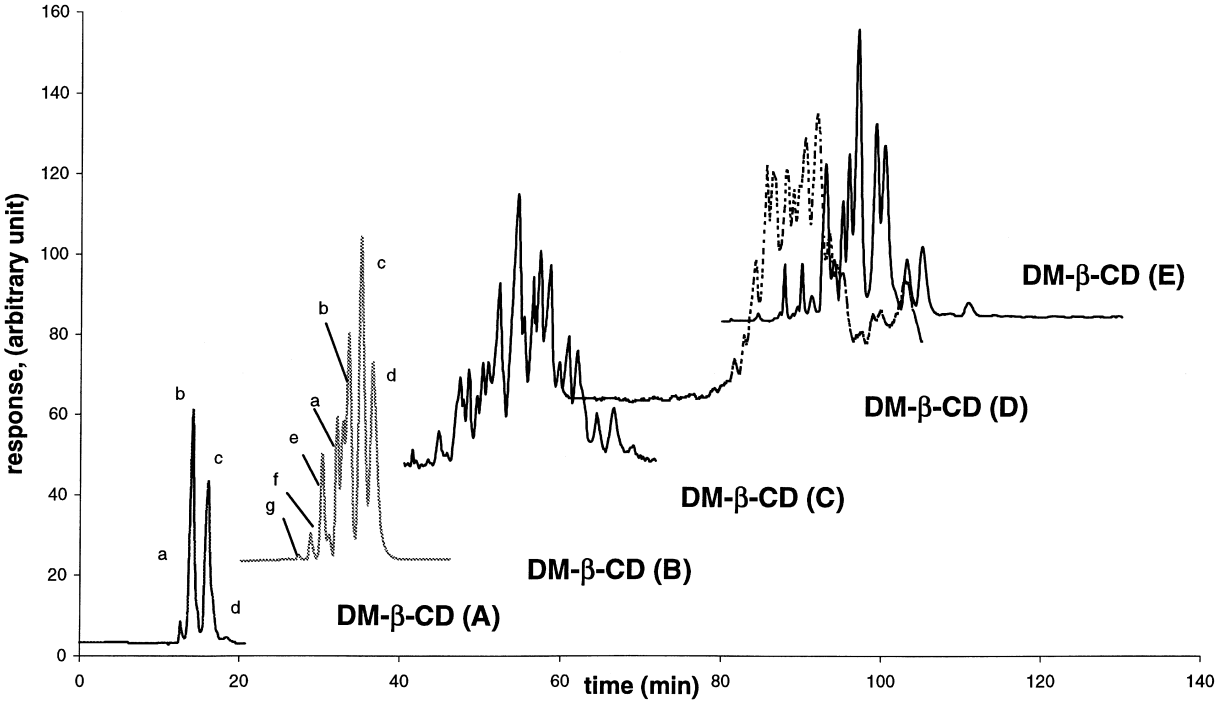

Fig. 6. SFC separations of the five commercial DM-β-CDs (A–E) on the silica system. Column: Hypersil silica at 40°C, mobile phase: CO_2–MeOH–ACN–water (70:5.8:23.3:0.9) (for sample D, a gradient elution was performed to elute under-methylated cyclodextrins: from time 20 to 30 min modifier was increased from 30 to 40%), flow-rate: 3 ml min^{-1}, outlet pressure 150 bar, detection: ELSD.

solvents were used in a binary CO_2–modifier eluent as in Section 3.2.2 and the same conclusions were drawn except for the fact that ethanol has no particular effect on the selectivities. Using CO_2–MeOH–X ternary eluents or addition of water, the selectivities and resolution were not improved. This result constitutes another characteristic of the NO_2 stationary phase in comparison to silica phase. One can conclude that the retention mechanisms are quite different and that CO_2–MeOH eluent is the best choice (Fig. 3a). In conclusion, two SFC systems can be used for DM-β-CD analysis: the silica stationary phase with CO_2–MeOH–ACN–water (70:5.8:23.3:0.9) eluent and the NO_2 stationary phase with CO_2–MeOH (87.5:12.5) eluent.

3.2.4. Comparison of various commercial DM-β-CD profiles using silica and NO_2 systems

Figs. 3 and 6 depict chromatographic profiles of five different commercial DM-β-CDs on both SFC systems. The under-methylated DM-β-CD (D) sample requires a gradient elution to be eluted. The five commercial DM-β-CDs did not exhibit the same elution profile from one system to another. Indeed, DM-β-CD (A) and (B) gave, respectively, six and nine chromatographic peaks on the NO_2 stationary phase whereas only four and seven peaks were obtained on the silica stationary phase. Identification of peaks was made by ESI-MS after fraction collection for DM-β-CDs A and B. Table 2 summarises the results obtained for both these DM-β-CDs. The different compounds of sample (A) were also found in sample (B) but in different amounts. As seen in Table 2, the elution order on the NO_2 stationary phase seems to be a function of the decreasing number of methyl groups per molecule: the more methylated the CDs, the shorter the elution time. On the contrary, this is not true using a silica phase system (Table 2). Another important fact is that SFC is able to separate positional isomers: e.g., peaks 3 and 4 or peaks a and d corresponded to the separation of one positional isomer of 16-Me-β-CD from

Table 2
Peak identification by ESI-MS as number of methyl groups per molecule[a]

NO_2 system		Silica system	
Collected peak	No. methyl groups per molecule	Collected peak	No. methyl groups per molecule
1	14	a	16
2	15	b	14+17
3	16	c	15
4	16	d	16
5	17	e	17+18
6	17	f	18
7	18	g	19
8	18		
9	19		

[a] Peak fractions were collected from separations described in Fig. 3a and b (NO_2 system) and Fig. 6a and b (silica system).

the other two. It was found that selectivities between the different positional isomers is better on the silica stationary phase than on the NO_2 system. This was a main advantage of the silica system.

Concerning the commercial DM-β-CDs (C, D, E), which were randomly methylated in positions 2, 3 or 6 (see Experimental), separation is better on silica system: e.g., sample E gave six peaks on the NO_2 system whereas 13 peaks were resolved on the silica system. The complementation of the two SFC analytical systems is important for such complex mixtures.

As ELSD gives a response relative to mass injected [29], it allows quantification of the relative

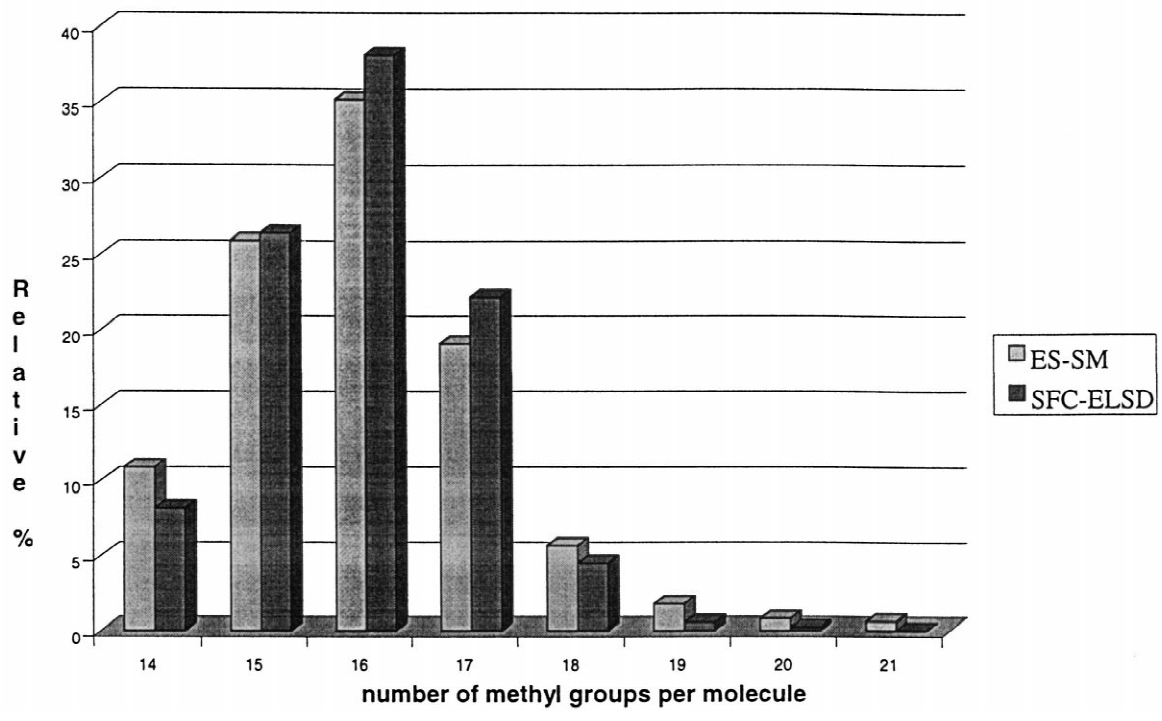

Fig. 7. Relative molar response of a commercial DM-β-CD (B) sample obtained by ESI-MS and SFC–ELSD as a function of methylation degree. Conditions as in Table 1 (ESI-MS) and Fig. 3 (SFC–ELSD). For SFC–ELSD quantitation, peak areas corresponding to the same number of methyl groups per molecule (Table 2, NO_2 system) were summarised.

percentages of methylated β-CDs. The area normalisation method was made only for samples A and B which provided good resolutions on the NO_2 system. As seen in Fig. 7, the results are very close to those obtained, in the first part, using ESI-MS analysis. Moreover, using the DS obtained from the suppliers, ESI-MS (Table 1) and SFC–ELSD are close. Using the area normalisation method, quantification is in accordance with the GC method after hydrolysis [22] and is possible without any calibration. This is important because pure standards are not commercially available.

4. Conclusions

A set of techniques have to be used for analytical studies of DM-β-CDs since such samples are very complex. ESI-MS, ESI-MS–MS and SFC can be easily used. SFC and off-line MS identification is a powerful technique which can be used as well as the classical hydrolysis–GC methods. On-line SFC–MS and SFC–MS–MS analyses are under progress. Silica and NO_2 stationary phases provide characteristic fingerprints and retention mechanisms are probably quite different. The composition of the simplest samples of DM-β-CDs (A) and (B) can be well characterised using the NO_2 system. The heptakis (2,6-di-*O*-methyl)-β-CD and hexakis (2,6-di-*O*-methyl)- (2,3,6-tri-*O*-methyl)-β-CD were clearly identified. For very complex samples, a more SFC characteristic fingerprint is obtained using the silica phase system.

Acknowledgements

This work was supported by Conseil Régional de la Région Centre (France).

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