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Characterization of a new methylated β-cyclodextrin with a low degree of substitution by matrix-assisted laser desorption/ionization mass spectrometry and liquid chromatography using evaporative light scattering detection

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Abstract

A new methylated β-cyclodextrin with a low degree of substitution was characterized by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and high-performance liquid chromatography (HPLC) with evaporative light scattering detection. Using α-cyano-4-hydroxycinnamic acid as the matrix and the thin layer method as the deposition procedure, MALDI-TOF-MS revealed that the mixture was composed of CDs bearing from 2 to 8 methyl groups with an average degree of substitution (DS) of 0.7 (i.e. 0.7 methyl groups per glucopyranose unit). Using a Purospher Star RP-18 endcapped column with acetonitrile–water mobile phase in gradient elution mode, HPLC was employed at analytical scale to obtain a chromatographic fingerprint of the crude mixture and at semi-preparative scale to fractionate it. MALDI-TOF-MS of these fractions revealed that the overall retention of the different derivatives, which depicts their polarity, was mainly driven by the DS and increased with the number of methyl groups on the CD moiety.

Keywords: Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; High-performance liquid chromatography; Evaporative light scattering detection; Methylated β-cyclodextrins; Degree of substitution

1. Introduction

Cyclodextrins (CDs) are torus-shaped cyclic oligosaccharides composed of six, seven and eight α-1,4-linked D-glucopyranose units for the α-, β- and γ-CD, respectively [1]. These hydrophilic molecules present hydrophobic cavities allowing the formation of complexes with many guest molecules of appropriate size. Moreover, they have the ability to discriminate between structurally related compounds such as isomers or enantiomers. Thanks to these properties, CDs and their derivatives are used in a wide variety of industrial and pharmaceutical applications [2] as well as in analytical chemistry where they are employed as separating agents in several techniques [3,4].

Some of the most widely used derivatives are the methylated β-CDs (Me-β-CDs). Indeed their properties (low toxicity, high aqueous solubility and binding capacity, reasonable price) allow their use in the pharmaceutical industry as drug carriers (solubilization, stabilization) [5] and in analytical chemistry as chiral selectors in techniques such as capillary electrophoresis [6].

However, selective methylation of the native β-CD is not easy because the hydroxyl groups present at positions 2, 3 and 6 of each glucopyranose unit are in competition during the reaction [7]. Most of the commercially available Me-β-CDs are therefore mixtures of CDs bearing different numbers

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of methyl groups at different positions. In other words, these mixtures contain several degrees of substitution (DS, defined as the number of methyl groups per glucopyranose unit) that each have a large number of positional and regional isomers. The heterogeneity of such mixtures makes their characterization essential.

Several methods have already been reported to accurately analyze mixtures of intact Me-β-CDs. Mass spectrometry has been extensively used for the determination of their substitution pattern and average DS. Several ionization techniques have been employed such as fast atom bombardment [8,9], plasma desorption [10], matrix-assisted laser desorption/ionization [11–13] and electrospray [13–16]. In addition, separative methods such as liquid chromatography [8,9,17–19] and subcritical fluid chromatography [16,19] have often been used to obtain a fingerprint of mixtures and identify their major components. In the same goal, capillary electrophoresis has been employed with indirect detection [20]. Another way to gain deeper insight into the composition of Me-β-CDs is to analyze the glucose derivatives obtained after chemical hydrolysis [8,9,21]. This method allows the determination of the average DS and provides information on the position of methyl substituents on the glucopyranose units.

All these methods were mainly designed for the analysis of dimethyl-β-CDs (DM-β-CDs, DS = 2) that possess an average of 14 methyl groups. These derivatives are of special interest since, compared with that of native β-CD, the aqueous solubility of Me-β-CDs increases as the number of methyl groups reaches around 13–14 and then decreases with additional substituents [5].

However, to our knowledge the analysis of Me-β-CDs with a low degree of substitution (DS < 1) is poorly described. Yet their properties are different from those of DM-β-CDs especially polarity (or hydrophobicity) and solubility, as mentioned earlier, and they may therefore require specific analytical conditions.

The present study reports the characterization of a new Me-β-CD having an average DS in the range 0.4–0.7. This product was designed by the manufacturer as a pharmaceutical grade CD suitable for encapsulation of active products [22] and present a good water solubility (which increases with temperature unlike DM-β-CDs), an enhanced ability to form inclusion compounds and initial results indicate a good potential for biological tolerance.

The crude commercial mixture was analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) to determine its substitution pattern and its average DS and by high-performance liquid chromatography (HPLC) with evaporative light scattering detection (ELSD) to obtain a chromatographic fingerprint. Then, in order to better understand the chromatographic behaviour and the substitution of the different derivatives, fractions obtained by semi-preparative chromatography were analyzed by MALDI-TOF-MS.

2. Experimental

2.1. Reagents

HPLC-grade acetonitrile (MeCN) and methanol (MeOH) were purchased from SDS (Peypin, France) and deuterium oxide (D_2O) from Aldrich (Milwaukee, WI, USA). 18 MΩ deionized water was produced by an Elgastat UHQ II system (Elga, Antony, France).

α-Cyano-4-hydroxycinnamic acid (CHCA) and 2,5-dihydroxybenzoic acid (DHB) were purchased from Aldrich (Milwaukee, WI, USA), 1-hydroxy-isochinolin (HIC) and 3-hydroxypicolinic acid (HPA) from Fluka (Buchs, Switzerland) and sodium chloride (NaCl) from Prolabo (Paris, France).

β-CD and Me-β-CD (Kleptose® Crysmeb Exp, Lab 3487) were obtained from Roquette (Lestrem, France). The Kleptose® Crysmeb is described as a new generation of Me-β-CD produced through selective methylation of β-CD using a technology patented by Roquette and characterized by an average DS in the range 0.4–0.7 [22].

2.2. Nuclear magnetic resonance

The ^{1}H NMR experiments were carried out at 25 °C on a DPX 250 MHz Advance apparatus (Bruker Biospin, Rheinstetten, Germany). Calibration was made with the D_2O signal.

Samples of β-CD and Me-β-CD were prepared in D_2O at a concentration of 5 mg in 0.75 mL. The solutions were evaporated to dryness under nitrogen stream and reconstituted in 0.75 mL of D_2O. This operation was repeated two times in order to ensure a complete exchange of the hydroxyl protons.

The ^{1}H NMR spectrum of the Me-β-CD was identical to that obtained by the supplier [22] and the DS calculated from the integration was found to be 0.68 which is in the expected range of 0.4–0.7.

2.3. Mass spectrometry

The mass spectrometry experiments were performed on a linear MALDI-TOF model Omniflex system (Bruker Daltonics, Bremen, Germany) in positive ionization mode using a nitrogen laser (λ = 337 nm, 2 ns pulse width). The acceleration voltage was 17.70 kV and the detector signal was digitized at a sampling rate of 500 MHz. Spectra were recorded between 0 and 5000 Da. Calibration was made with the native β-CD. The lowest possible laser power was applied to induce the desorption and ion formation.

Matrices solutions were prepared in MeOH at a concentration of 50 mM. Me-β-CD solutions were prepared in water containing 0.5 mM NaCl. The crude mixture was dissolved to a concentration of 0.5 mM and the fractions collected by semi-preparative chromatography were evaporated to dryness under nitrogen stream and reconstituted in the appropriate volume to obtain a concentration factor of 100 compared to the initial volume.

The analyzed samples were prepared in two different ways. For the thin layer method, 0.5 μL of matrix solution were deposited on the target and evaporated prior to deposition of 0.5 μL of Me-β-CD solution. For the dried droplet method, 1 μL of a mixture of equal volumes of matrix and Me-β-CD solutions was deposited. The spectra presented were obtained with the thin layer method, unless otherwise stated.

2.4. High-performance liquid chromatography

The chromatographic system consisted of a Lachrom L-7100 quaternary pump (Merck-Hitachi, Darmstadt, Germany), a Rheodyne model 7725 injection valve (Rheodyne, Cotati, CA, USA) fitted with a 20 μL injection loop, a Sedex 55 evaporative light scattering detector (Sedere, Alfortville, France) (drift tube temperature: 50 °C, nebulizer gas pressure: 2.2 bar, photomultiplier: 9). Data were collected by an EZChrom Elite version 2.5 software (Merck, Darmstadt, Germany).

The chromatographic columns were Nucleosil 50-5-C8 endcapped (250 mm × 4 mm; 5 μm) and Nucleosil Phenyl (150 mm × 4.6 mm; 7 μm) from Macherey-Nagel (Düren, Germany), Zorbax Phenyl (250 mm × 4.6 mm; 7 μm) from DuPont (Wilmington, DE, USA), Hypercarb (100 mm × 4.6 mm; 5 μm) from ThermoQuest Hypersil (Runcorn, UK) and Purospher Star RP-18 endcapped (125 mm × 4 mm; 5 μm) from Merck (Darmstadt, Germany). Separations were carried out at room temperature in gradient elution mode (H_2O:MeCN 95:5 to 55:45 in 40 min) at 1 mL/min.

Solutions of the Me-β-CD crude mixture were prepared in water at a concentration of 2 g/L for analytical chromatography and 10 g/L for semi-preparative chromatography. Prior to their analysis, the fractions collected by semi-preparative chromatography were evaporated to dryness under nitrogen stream and reconstituted in the appropriate volume of water to obtain a concentration factor of 20 compared to the initial volume.

3. Results and discussion

3.1. Analysis of the crude mixture by MALDI-TOF-MS

In order to determine its substitution pattern and its average DS, the Me-β-CD was first analyzed by MALDI-TOF-MS. This technique is a tool of choice for the characterization of oligosaccharides and their derivatives [23].

Four different matrices, already employed for the analysis of CD derivatives, were compared: CHCA [11,12,24], DHB [12,13,25], HIC [25] and HPA [24]. First, laser shots were performed on the matrix crystals without analytes in order to check the absence of peaks between 1000 and 1500 Da which is the mass range of Me-β-CDs. None of these matrices exhibited any peaks in this mass range. Different ionization thresholds were found according to the matrices: only 40% of the laser power was required to ionize CHCA whereas 60% was found necessary for DHB and HIC and 80% for HPA.

Then the Me-β-CD was analyzed with each matrix in order to determine the most suitable one. When HIC and HPA were employed, signals were difficult to obtain and led moreover to low resolution mass spectra. These two matrices were therefore dropped. The use of CHCA and DHB gave comparable spectra with equivalent mass resolutions but signals were more easily obtained with CHCA. This matrix was thus selected for the continuation of the study.

Under these MS conditions, the different derivatives were detected as monocharged sodiated pseudo-molecular ions $[M + Na]^+$. The formation of potassium adducts was prevented by the addition of NaCl, as already reported [13], because the determination of the average DS requires the quantification of the different derivatives which is easier when only one type of adduct is obtained. The mass spectrum (Fig. 1) shows seven derivatives bearing from 2 to 8 methyl groups. Each derivative is represented by an isotope distribution identical with that obtained for the native β-CD in the same conditions. The mass difference of 14 Da between two consecutive derivatives corresponds to the methylation of a hydroxyl group. The resolution, defined as $R = M/\Delta M_{FWHM}$ where M is the m/z ratio of a peak and ΔM_{FWHM} its full width at half maximum, was measured on the highest monoisotopic peak of each distribution. It was found between 2000 and 2500.

The gaussian profile of the peaks clearly illustrates the type of mixture produced by the methylation of CDs and the difficulty of obtaining only one given derivative. This profile was similar to that reported by the supplier and obtained by electrospray-MS [22]. The DS value was calculated from the peak heights which has been proven to be an accurate method [11] and was found to be 0.71 which is in good agreement with the NMR result.

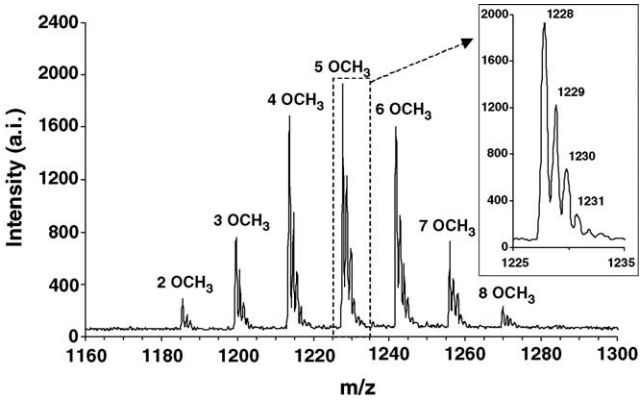

Fig. 1. Matrix-assisted laser desorption/ionization time-of-flight mass spectra of the methylated β-cyclodextrin. The number of methyl groups per cyclodextrin is indicated above the corresponding isotope distribution. The spectrum is the addition of 50 laser shots.

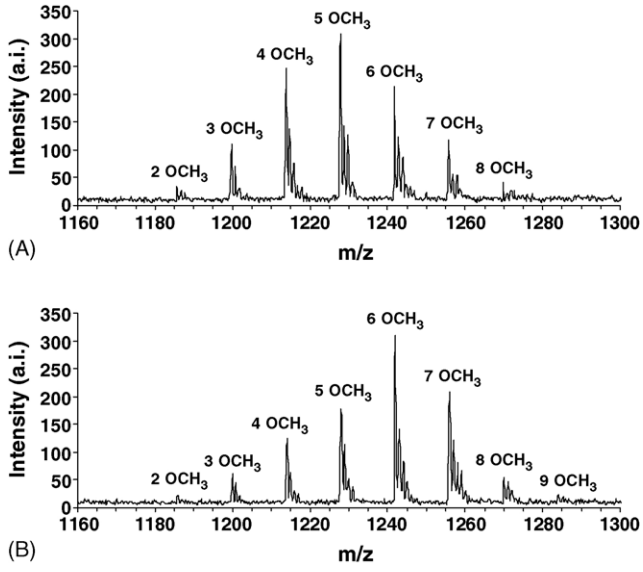

Fig. 2. Matrix-assisted laser desorption/ionization time-of-flight mass spectra of the methylated β-cyclodextrin. Samples were prepared according to the thin layer method (A) and the dried droplet method (B). The number of methyl groups per cyclodextrin is indicated above the corresponding isotope distribution. Each spectrum is the addition of 10 laser shots.

It should be noted that the previous spectrum was obtained by depositing the sample on the target with the thin layer method [26] (see Section 2). Preliminary studies indicated that the sample preparation had a great influence on the results. With the thin layer method, mass spectra were reproducible and the calculated average DS was constant. In contrast, mass spectra obtained with the commonly used dried droplet method were more variable and showed an increase in the relative intensities of the most methylated derivatives compared to the least ones (Fig. 2), which led to a considerable over-estimation of the DS. To our knowledge, such a variation in Me-β-CD MALDI-TOF mass spectra with the deposition method has never been reported. It is known that with several ionization techniques (MALDI, atmospheric pressure chemical ionization and electrospray), the ionization efficiency of DM-β-CDs increases with the number of methyl groups per CD [13]. The rate of increase is the lowest for MALDI and in our case, this increase was only observed with the dried droplet method. This could be explained by an influence of the number of methyl groups per CD on the properties of analyte–matrix crystals, a phenomenon that cannot be involved with the thin layer method.

MS allows a fast characterization of Me-β-CD mixtures through the determination of the substitution pattern and average DS. However, this technique cannot distinguish the different isomers of the same derivative. To obtain additional information on the Me-β-CD composition, HPLC, which can separate the substitution isomers, was used to obtain a chromatographic fingerprint of the mixture and evaluate its real complexity.

3.2. Analysis of the crude mixture by HPLC-ELSD

The detection of these non UV-absorbing molecules was carried out by an evaporative light scattering detector. This detection mode has already been reported to be a tool of choice for the analysis of CD derivatives [27].

It has been reported that the resolution of complex mixtures of DM-β-CDs, characterized by an average DS close to 2, is considerably influenced by the nature of the HPLC column packing materials [17,18]. Indeed, among various kinds of C8, C18 and phenyl bonded phases, silica and monomeric based packing materials which present both reinforced hydrophobic and polar interactions show the best performances. The Nucleosil 50-5-C8 [17] and Zorbax phenyl [18] columns were the most suitable alternatives of the different supports to obtain the best separations. Besides, it has been reported that MeCN:H_2O mobile phases are more efficient and selective than MeOH:H_2O mixtures [18].

The present study was therefore undertaken with these two columns using MeCN:H_2O mobile phases. The separation of the different mixture components was found to require a gradient elution mode. Fig. 3 shows that the fingerprints obtained with these two columns are similar and characterized by an equivalent selectivity. The same analysis was performed on a Nucleosil phenyl column in order to observe the influence of the packing material and of the bonded group. The chromatogram obtained with this column showed a poorer selectivity compared to the previous ones which underlines the importance of the choice of column for the analysis of Me-β-CDs.

Since the present Me-β-CD was less methylated than DM-β-CDs, it was also less hydrophobic or more polar. In order to get a richer fingerprint, a support with reinforced hydrophobic interactions ought therefore to be more appropriate to increase the separation of derivatives differing by the number of methyl groups. The Purospher Star RP-18

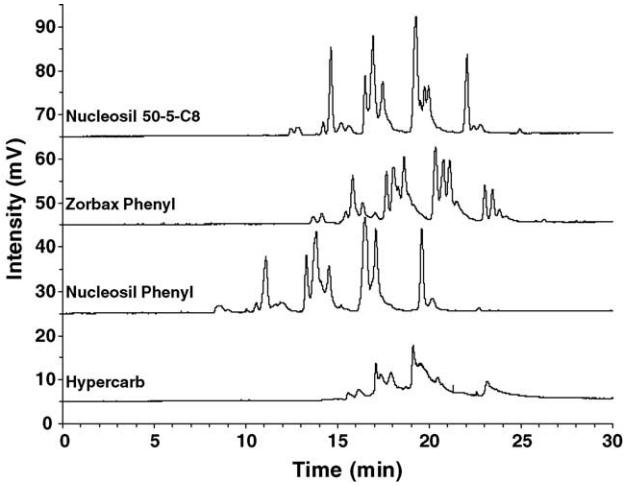

Fig. 3. High-performance liquid chromatography of the methylated β-cyclodextrin. Mobile phase: H_2O:MeCN 95:5 to 55:45 in 40 min; flow rate: 1 mL/min; evaporative light scattering detection.

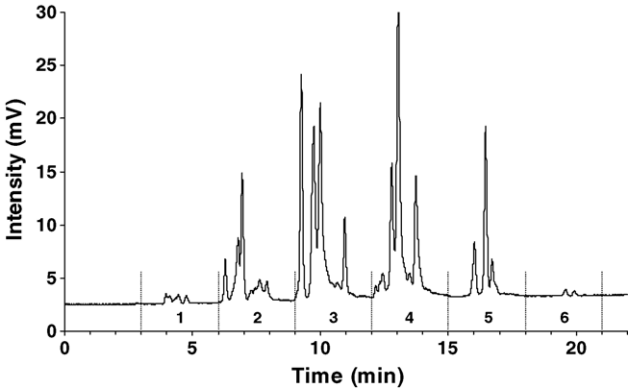

Fig. 4. High-performance liquid chromatography of the methylated β-cyclodextrin. Column: Purospher Star RP-18 endcapped (125 mm × 4 mm; 5 μm); mobile phase: H_2O:MeCN 95:5 to 55:45 in 40 min; flow rate: 1 mL/min; evaporative light scattering detection. Dotted lines and Arabic numerals indicate the borders and number of each fraction collected by semi-preparative chromatography, respectively.

endcapped column was therefore tested. Indeed, its characteristics (highly pure metal-free silica, complete cross-linked modification and deactivation of the surface resulting in a polymeric structure) make this support highly hydrophobic. This column was found to provide a slightly better selectivity than the others as well as a better efficiency (Fig. 4). Since the increase of the hydrophobic interactions was found advantageous, a Hypercarb column was employed. This chromatographic support presents stronger hydrophobic interactions than classical C18 columns and has moreover the ability to separate closely related compounds [28] such as the three isomers of the monosubstituted sulfobutyl ether-β-cyclodextrins [29]. However, as shown in Fig. 3, the chromatogram obtained with this column was characterized by a low resolution of the different groups of peaks as well as a low efficiency.

The study was therefore carried out with the Purospher Star RP-18 endcapped column which showed the best results. Fig. 4 shows that the chromatogram obtained under these conditions was composed of many peaks with different intensities, indicating the heterogeneity of the mixture and especially the wide range of polarity, which is depicted by the retention. In contrast, the main components of DM-β-CDs were separated in isocratic elution mode [8,9,17–19]. In brief, the polarity range of all the constituents present in Me-β-CD mixtures having low DS is wider than in DM-β-CD mixtures. The addition of a methyl group seems, therefore, to bring about a more marked alteration in the properties of CDs bearing only five methyl groups than for CDs already bearing 14.

In order to acquire more information about the different components which were detected, fractionation of the crude mixture was envisaged. As indicated in Fig. 4, the chromatographic peaks fall into six groups according to their retention times and these different groups were first thought to roughly correspond to CDs bearing different numbers of methyl groups, the most retained derivatives being the most methylated, as reported for DM-β-CDs [17,18]. The separation was scaled up by increasing the sample concentration from 2 to 10 g/L. The resolution of the different components was not altered by this overload, showing the high capacity of the column. Six fractions were therefore collected corresponding to the six different groups of peaks.

3.3. Analysis of the fractions collected by semi-preparative chromatography

In order to get the maximum amount of material during each injection, the ELSD, which is a destructive detector, was disconnected from the chromatographic system. The fractionation was therefore carried out without detection and was based on the retention time only. After collection, each fraction was analyzed by HPLC in order to check its purity. Fig. 5 shows that the fractions were free from any components belonging to a neighbor group.

Each fraction was then analyzed in MALDI-TOF-MS in order to determine its composition. As shown in Fig. 6, it was obvious that the different groups of peaks (Fig. 4) did not correspond to the different derivatives. In fraction 1, only one derivative was detected but the other fractions were found to contain two or three different derivatives. For instance, in fraction 4, CDs bearing five, six and seven methyl groups were detected. The first conclusion was therefore that CDs with different numbers of methyl groups could have close retention times. Besides, the same derivative could be found in more than a single fraction. For instance, CDs bearing six methyl groups were detected in fractions 3, 4

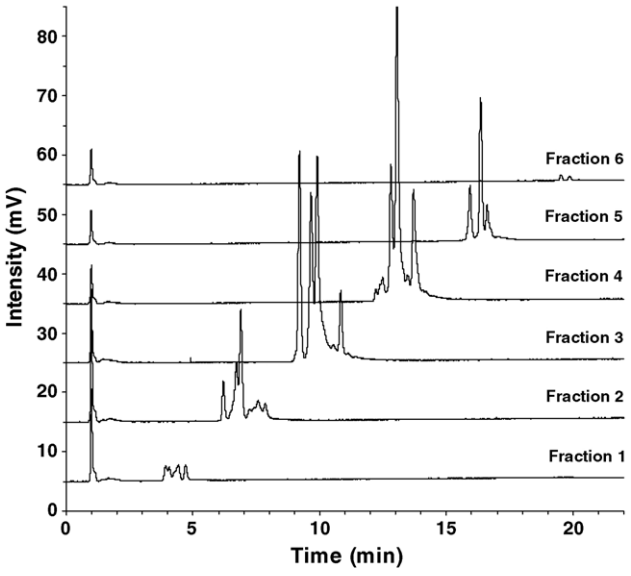

Fig. 5. High-performance liquid chromatography of the methylated β-cyclodextrin fractions. Column: Purospher Star RP-18 endcapped (125 mm × 4 mm; 5 μm); mobile phase: H_2O:MeCN 95:5 to 55:45 in 40 min; flow rate: 1 mL/min; evaporative light scattering detection.

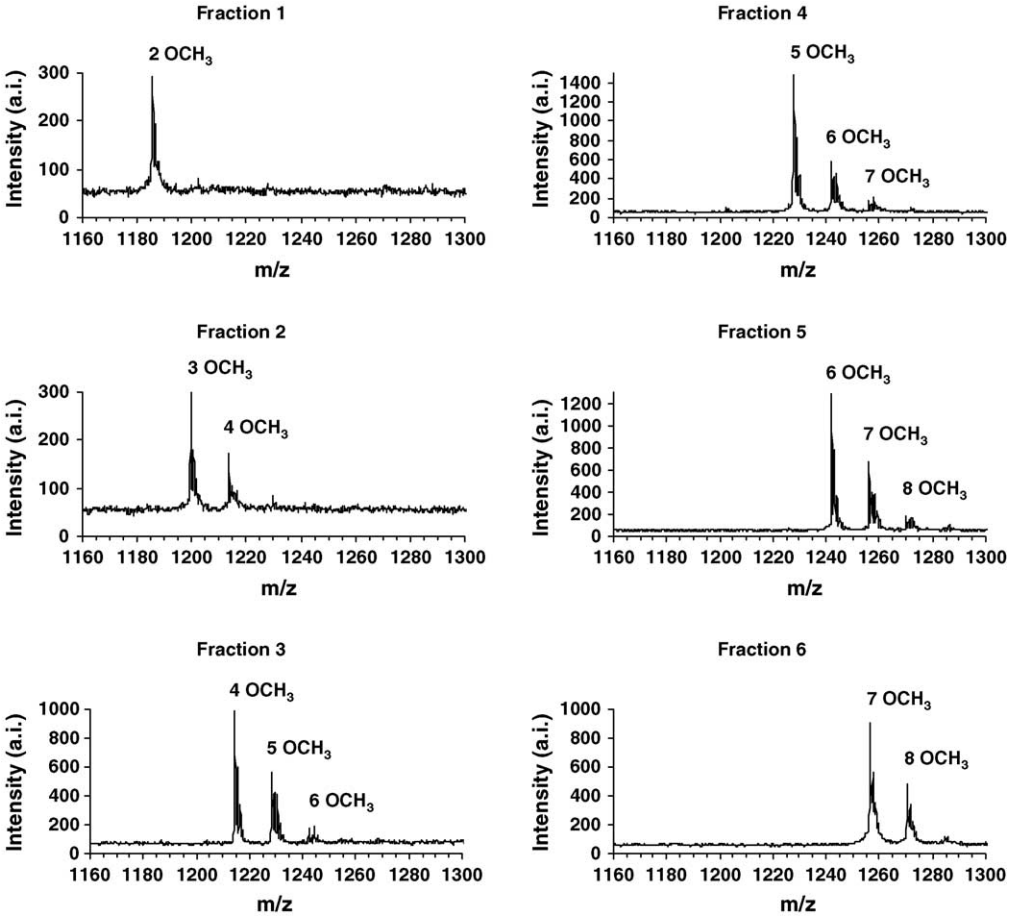

Fig. 6. Matrix-assisted laser desorption/ionization time-of-flight mass spectrum of the methylated β-cyclodextrin fractions. The number of methyl groups per cyclodextrin is indicated above the corresponding isotope distribution. Each spectrum is the addition of 50 laser shots.

and 5. The second conclusion was that different isomers of the same derivative could have a broad range of retention times.

In brief, the chromatographic retention of the different derivatives, which depicts their polarity, was not only influenced by the DS. Indeed, several derivatives were more retained than their over-methylated homologues, as shown in Fig. 6 where CDs bearing five methyl groups were detected in fraction 4 whereas CDs bearing an additional group were present in the previous fraction. Therefore, polarity seemed to be also influenced by the position of the methyl groups on the cyclodextrin moiety which is constituted by seven glucopyranose units each having three different possible positions of substitution (position 2, 3 or 6).

However, the DS was actually the key factor influencing retention. The average number of methyl groups per CD (i.e. DS × 7) was calculated for each fraction and represented as a function of the average retention time of the fraction. As shown in Fig. 7, a good correlation ($r^2 = 0.99$) was found, indicating that retention was mainly driven by the DS. In summary, despite a slight influence of the positions of substitution, the overall polarity of the Me-β-CD derivatives was a function of their DS.

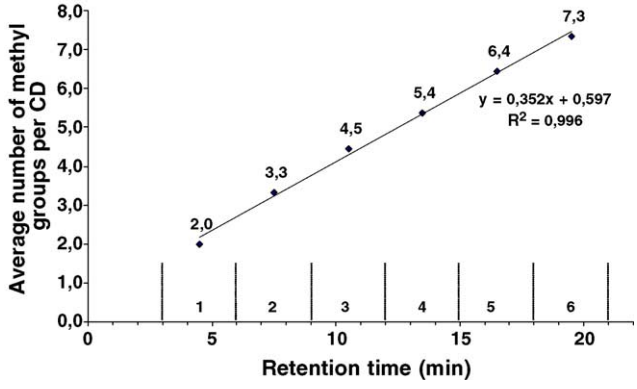

Fig. 7. Average number of methyl groups per cyclodextrin in the fractions as a function of their average retention times. Dotted lines and Arabic numerals indicate the borders and number of each fraction collected by semi-preparative chromatography, respectively.

4. Conclusion

The accurate characterization of this new Me-β-CD required a careful choice of the analytical tools and methods,

especially the column employed in HPLC and the sample deposition method used for MALDI-TOF-MS analysis.

These Me-β-CDs derivatives, characterized by a low DS, showed marked differences with DM-β-CD mixtures. This was illustrated by their wider range of polarity and their different ionization efficiencies according to the deposition method.

In order to obtain more information about this type of Me-β-CDs, the analysis of this mixture by on-line liquid chromatography–electrospray ionization mass spectrometry is currently in progress.

Acknowledgements

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