ESI-Mass Spectrometry Analysis of Unsubstituted and Disubstituted β-Cyclodextrins: Fragmentation Mode and Identification of the AB, AC, AD Regioisomers

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The study of unsubstituted and disubstituted β-cyclodextrins (β-CDs) by ESI-mass spectrometry is reported, applying a cone-induced fragmentation in the presence of a twofold excess of sodium chloride, in order to gain information about the fragmentation of the different regioisomers. On the basis of the fragmentation pattern observed for the unsusbstituted β-CD, a statistical model shows that the fragments generated by every regioisomer of a disubstituted CD (AB, AC, and AD) are expected to differ in their relative intensity and, therefore, they can be used for correctly identifying the three different regioisomers. The model was tested on the three regioisomeric (AB, AC, and AD) diamino-β-CDs and ditosyl-β-CD and on the AC and AD regioisomers of dimesitylenesulphonyl-β-CD, allowing in every case through statistical analysis of the fragmentation pattern the correct assignment of every regioisomer on the basis of an ESI mass spectrum (single quadrupole analyzer, high cone voltage) of the pure compounds. The absolute intensities of the fragmentation peaks were voltage-dependent but their ratios was voltage-independent, indicating that no mass bias in peak ratios is introduced by the analyzer. Given the fast time of analysis and its general applicability, independently from the substituents, we propose our method as an easy way to identify the regioisomers of disubstituted β-CDs.   (J Am Soc Mass Spectrom 2003, 14, 124–135) © 2003 American Society for Mass Spectrometry

Cyclodextrins (CDs) are cyclic oligosaccharides formed by α-(1–4)-linked glucose units. The most common are cyclo-maltohexaose (α-CD), cyclo-maltoheptaose (β-CD), and cyclo-maltooctaose (γ-CD), which are composed of six, seven, and eight glucose units, respectively. They have the shape of a hollow truncated cone with a hydrophylic external surface, which makes them soluble in water, and a hydrophobic internal cavity, which allows the formation of inclusion complexes with a wide variety of compounds. This property was extensively studied by various experimental techniques and it was the subject of a great number of investigations (molecular and chiral recognition, chromatographic separations, biomimetic enzymes, etc.) as well as of important industrial applications (drug stabilization and delivery, aroma and color stabilization in foods and cosmetics, etc.) [1–5]. The performance is also due to the CD versatility,

attributable to the presence of a large number of hydroxy groups on the upper and lower rim, which provide unique opportunities to obtain a virtually unlimited number of derivatives by suitable modification with different neutral or charged groups [6, 7], thus allowing a fine tuning of their complexation and recognition properties as well as of their solubility in various solvents. However, the three types of hydroxy groups present at the 2-, 3-, and 6-positions compete for the derivatizing agent making the challenge of selective conversion a daunting task. Many efforts and strategies have been devised in order to obtain selectively functionalized CD derivatives: a great number of mono- and per-functionalized CDs have been reported, whereas only few di- and tri-modified derivatives are known, on account of the difficulties encountered in the preparation and structure determination of the regioisomers [1, 6, 7]. Regiospecific functionalization is generally achieved by using an excess of a reagent (p-toluenesulphonyl chloride, mesityl chloride, or 3-nitrobenzenesulphonyl chloride) [8, 9] or appropriate capping reagents (4,6-dimethoxybenzene-1,3-disulphonyl chloride, benzophenone-3,3'-disulphonyl chloride, biphenyl-4,4'-dis-

ulphonyl chloride) [10–12], which are rigid systems bearing the reactive sulfonyl chloride groups at the right distance for obtaining sulphonated AB, AC, and AD regioisomers with high selectivity. Generally, a chromatographic purification step is necessary to separate the different regioisomeric products from the unreacted β-CD. The availability of the pure regioisomers opens the possibility to obtain several derivatives with interesting properties via nucleophilic substitution of the sulphonate groups. As an example, diamino-β-CDs have been used as chiral selectors for enantiomeric separation of several chiral hydroxy acids and carboxylic acids in CE. Quite interestingly, the separation abilities were completely different for the three regioisomers, being the enantiomers of the hydroxy acids best separated in the presence of the AC isomer and the enantiomers of the carboxylic acids exclusively separated by the AB isomer [13]. The main problem with these compounds concerns the difficulty in assigning the regioisomeric substitution unambiguously, even with the modern high field and 2-D NMR techniques [14–19]. Frequently, enzymatic or chemical degradation methods or synthetic conversion to known compounds are used to identify the regioisomers. In particular, the additional reaction of a disulphonyl dichloride with a transannularly disulphonated β-CD was employed for assigning the 6A,6C- and the 6A,6D-disubstitution by Tabushi et al. [20], whereas the Korner's absolute method, based on the additional monosulphonation of polysulphonated CDs was applied by Fujita et al. [21]. Treatment with phenylmercaptan was used for assigning the 6A,6C,6E-trisubstitution by Fujita [22], whereas the conversion to the 3,6-anhydro derivatives was utilized by Yamamura et al. to identify isomeric pentakis(6-O-mesitylsulphonyl)-β-CDs [23]. Enzyme-based isomer determination was also developed by Fujita and coworkers, who hydrolized 6A,6B-disubstituted CDs with Taka-amylase to give specifically 6',6''-disubstituted maltotrioses and 6A,6C- and 6A,6D-isomers to give two molecules of 6'-monosubstituted maltose [8, 9]. A chemical degradation method, the "hex-5-enose method", was recently used by Pearce and Sinay to identify the diol AD regioisomer of perbenzylated Cds [24].

In order to develop a direct mass spectrometric method for identifying the CD regioisomers, we report in this paper the study of the ESI mass spectra of unsubstituted and 6,6'-disubstituted β-CDs, applying a cone-induced fragmentation in the presence of a twofold excess of sodium chloride. On the basis of the fragmentation observed for the unsubstituted β-CD, we have shown by a statistical analysis that different fragmentation patterns are expected for the three different regioisomers (AB, AC, and AD) of a generically 6,6'-disubstituted β-CD. According to this model, the three regioisomeric diamino-β-CDs and ditosyl-β-CDs and the AC and AD regioisomers of dimesitylenesulphonyl-β-CD showed different fragmentation pattern, allowing the correct assignment of the regioisomers by fast and easy mass spectral analysis.

Experimental

Chemical Syntheses

The AB, AC, and AD regioisomers of 6,6'-dideoxy-6,6'-diamino-β-cyclodextrin (diamino-β-CD) were first synthesized and purified by HPLC separately according to the literature procedures [11].

The synthesis of a mixture of AB, AC, and AD regioisomers of 6,6'-dideoxy-6,6'-ditosyl-β-cyclodextrin (ditosyl-β-CD) was also performed by a modified literature procedure [8]: 5.0 g (4.4 mmol) of β-CD and 1.68 g (8.8 mmol) of tosyl chloride were dissolved in 30 ml of dry pyridine at room temperature and the reaction was stirred for 8 h. The solution was then poured dropwise in 500 ml of acetone (slightly acidified with 3% of 0.01 N aqueous HCl). The precipitate was filtered, washed with acetone, and redissolved in DMF (minimum amount). The DMF solution was again poured dropwise in 500 ml of acetone (slightly acidified with 3% of 0.01 N aqueous HCl). The precipitate was filtered, washed with acetone and redissolved in water:methanol 1:1. A preparative HPLC (Waters Spherisorb ODS2 semiPrep Column, Waters, Milford, MA), 10 × 250 mm, 10 μm, linear gradient elution from water:methanol 72%:28% to water:methanol 32%:68% in 22 min, flow rate 4 ml/min) allowed the purification of four compounds, which were identified by ESI-MS as the mono-tosyl-β-CD (calculated for MNa$^+$: 1311 m/z, found 1311; yield: 29%) and the three regioisomeric ditosyl-β-CD (calculated for MNa$^+$: 1465 m/z, found for all three compounds: 1465 m/z; yields: 3.5–6.5%). From every pure regioisomer the corresponding diamino compound was also synthesized and purified according to the literature procedure [25].

The regioselective synthesis of a mixture of AC and AD regioisomers of 6,6'-dideoxy-6,6'-dimesitylenesulphonyl-β-cyclodextrin (dimesitylenesulphonyl-β-CD) was performed by the same procedure reported above for the ditosyl-β-CDs. 5.0 g (4.4 mmol) of β-CD and 1.92 g (8.8 mmol) of mesitylenesulphonyl chloride were dissolved in 30 ml of dry pyridine at room temperature and the reaction was stirred for 24 h. The solution was then poured dropwise in 500 ml of acetone (slightly acidified with 3% of 0.01 N aqueous HCl). The precipitate was filtered, washed with acetone, and redissolved in DMF (minimum amount). The DMF solution was again poured dropwise in 500 ml of acetone (slightly acidified with 3% of 0.01 N aqueous HCl). The precipitate was filtered, washed with acetone, and redissolved in water:methanol 1:1. A preparative HPLC (Waters Spherisorb ODS2 semiPrep column, 10 × 250 mm, 10 μm, linear gradient elution from water:methanol 72%:28% to water:methanol 32%:68% in 22 min, flow rate 4 ml/min) allowed the purification of three compounds, which were identified by ESI-MS as the monomesitylenesulphonyl-β-CD (calculated for MNa$^+$: 1339 m/z, found 1339 m/z; yield: 18.3%) and two regioisomeric dimesitylenesulphonyl-β-CD (calculated for MNa$^+$:

1521 m/z, found for both compounds 1521 m/z; yields: 2.5–2.6%). The two compounds were identified as the AC and the AD regioisomers. No peak corresponding to the AB regioisomer was detected in the HPLC analysis of the crude products. From every pure regioisomer the corresponding diamino compound was also synthesized and purified according to the literature procedure [25].

Mass Spectrometry Analyses

All the β-CD solutions for the mass spectrometry analyses reported here were prepared at a 50 μM concentration in the presence of sodium chloride (100 μM). Mass spectra were obtained on a Micromass ZMD (Manchester, UK) instrument by perfusing the solutions into the mass spectrometer at a 10 μl/min rate. MS conditions: ESI interface, positive ions, single quadrupole analyzer. Capillary voltage 3500 V, cone voltage 190 V, source temperature 80 °C, desolvation temperature 150 °C, cone gas (N$_2$) 60 l/h, desolvation gas (N$_2$) 450 l/h. Spectra were acquired in total ion mode (300–1700 Da), scan time 4.0 s, inter-scan delay 0.1 s.

Results and Discussion

β-CD Fragmentation

In order to understand the fragmentation mode of cyclic oligosaccharides, the fragmentation of the unsubstituted β-CD was investigated in more detail. Therefore, an ESI analysis (positive ions mode, single quadrupole analyzer) was performed by using a high cone voltage (190 V, details reported in the Experimental section), which ensures a small but significant fragmentation of the analyte and moreover, it favors the formation of singly charged rather than multiply charged ions, thus simplifying the resulting spectrum.

The mass spectrum, collected by the direct infusion of a 50 μM β-CD solution in bidistilled water was clearly dominated by the sodiated molecular ion (1157 Da), due to sodium traces always present in the glassware and in the bidistilled water and to the high affinity of the carbohydrates for this ion. The fragmentation pattern (data not shown) was characterized by low signal-to-noise ratios, hampering a clear interpretation of the spectrum. Given the high affinity of the β-CD molecule for the sodium ions, it was decided to favor the formation of the sodiated ions and therefore, the same experiment was repeated by adding sodium chloride to the β-CD solution (100 μM, NaCl:CD 2:1). The signal-to-noise ratio showed an increase of about 65-fold (data not shown). Therefore, in our hands, the addition of small amounts of salt gives rise to a stronger signal of the sodiated ions because of the high affinity of the β-CD for the sodium cation. Moreover, a simple fragmentation pattern was generated, with a high signal-to-noise ratio, generating a series of peaks always with a difference of 162 Da (1157/995/833/671/509/

347 m/z). This molecular mass corresponds to the monomeric residue of the β-CD (a dehydrated glucose molecule) and this may be interpreted by assuming that every fragmentation event takes place at the acetal junction and may occur via the complexation of sodium by the two acetal oxigens and subsequent sodium-assisted fragmentation (Figure 1a).

Thus, the first fragmentation event has the effect to open the CD ring (and therefore it is not visible in the mass spectrum since the opened sodiated CD still has a molecular mass of 1157 m/z), whereas the subsequent fragmentations generate sodiated fragments characterized by the following molecular masses: 995 (6 monomeric units), 833 (5 monomeric units), 671 (4 monomeric units), 509 (3 monomeric units), 347 (2 monomeric units), as schematically shown in Figure 1b.

It is important to notice that the fragmentation events can occur almost randomly at every acetal junction of the opened CD since all the possible fragmentation peaks (2,3,4,5, and 6 monomeric units) are present in the spectrum.

Identification of Isomeric Disubstituted β-CDs by Fragmentation: The Theoretical Approach

Assuming that a disubstituted β-CD will follow the same mechanism of fragmentation reported above, it is immediately evident that the fragmentation pattern will be more complicated. In fact, every fragment characterized by a given number of monomeric units will contain two, one, or no substituted glucose units, thus generating three different mass peaks.

The key feature for the correct identification of the regioisomers relies on the fact that the relative intensity of these three peaks is expected to be different for the AB, AC, and AD disubstituted CDs. The reason for this behavior is exemplified in Figure 2, in the case of the generation of the four-unit fragments.

By considering all the possible points of fragmentation, there are seven different possibilities for generating four-unit fragments. In the case of the AB isomer, three fragments out of seven will have two substituted glucose moieties, two out of seven will have one substituted glucose moiety and two out of seven will have unsubstituted glucose moieties. In the case of the AC isomer, on the other side, two fragments out of seven will have two substituted glucose moieties, four out of seven will have one substituted glucose moiety and one out of seven will have unsubstituted glucoses. Finally, in the case of the AD isomer, one fragment out of seven will have two substituted glucose moieties, six out of seven will have one substituted glucose moiety and no fragment with unsubstituted glucose moieties will be generated. The case of the four unit fragments can be extended to every fragment characterized by a defined number of monomeric units, as shown in Table 1.

Thus, if the fragmentation mechanism of disubstituted CDs is that which was previously proposed, it
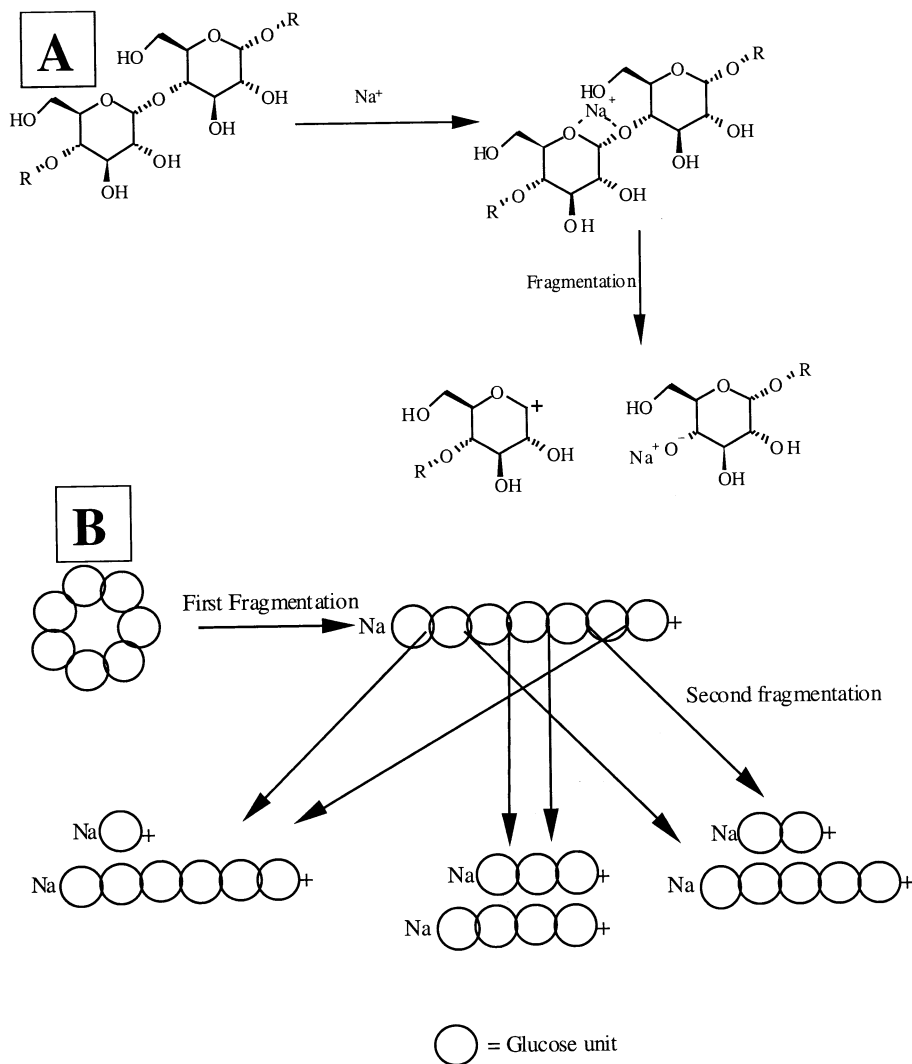
Figure 1. (a) Hypothetical mechanism of β-cyclodextrin fragmentation at the acetalic junction and (b) generation of the β-cyclodextrin fragments through a two-fragmentation random process. Every circle represent a glucose moiety.

would be possible to distinguish between the three regioisomers simply by evaluating the relative intensities of the di-, mono-, and unsubstituted fragments in every group characterized by the same number of monomeric units. In particular, the relative abundance of the monosubstituted fragments is expected to be quite different in the three cases (see Table 1).

Isomeric Disubstituted β-CD Identification by Fragmentation: The Case of Diamino-β-CDs

In order to verify if the theoretical analysis above reported could be applied to the identification of a real sample, we took into consideration the regioisomers of diamino-β-CD (Figure 3a).

Therefore, the AB, AC, and AD isomers were synthesized and purified by HPLC according to the literature procedure [11]. The ESI spectra of the pure compounds were obtained in the same conditions reported above (50 μM CD in the presence of 100 μM NaCl, cone voltage 190 V). The spectra are reported in Figure 4a.

All the spectra are dominated by the sodiated molecular ion (1155 Da), although many fragmentation peaks are evident. In order to verify if the peaks are consistent with the model, we first calculated the theoretical molecular mass of every sodiated fragment containing 2,3,4,5, or 6 glucose units and two, one, or no amino substitutents. Actually, almost all the fragmentation peaks observed in the spectra above reported have m/z ratios which exactly correspond to those calculated. In order to verify the general match between the fragmentation observed and the model, the percentage of abundance of every peak was calculated for every group of peaks corresponding to a fragment with the same number of monomeric units, according to the following equation:
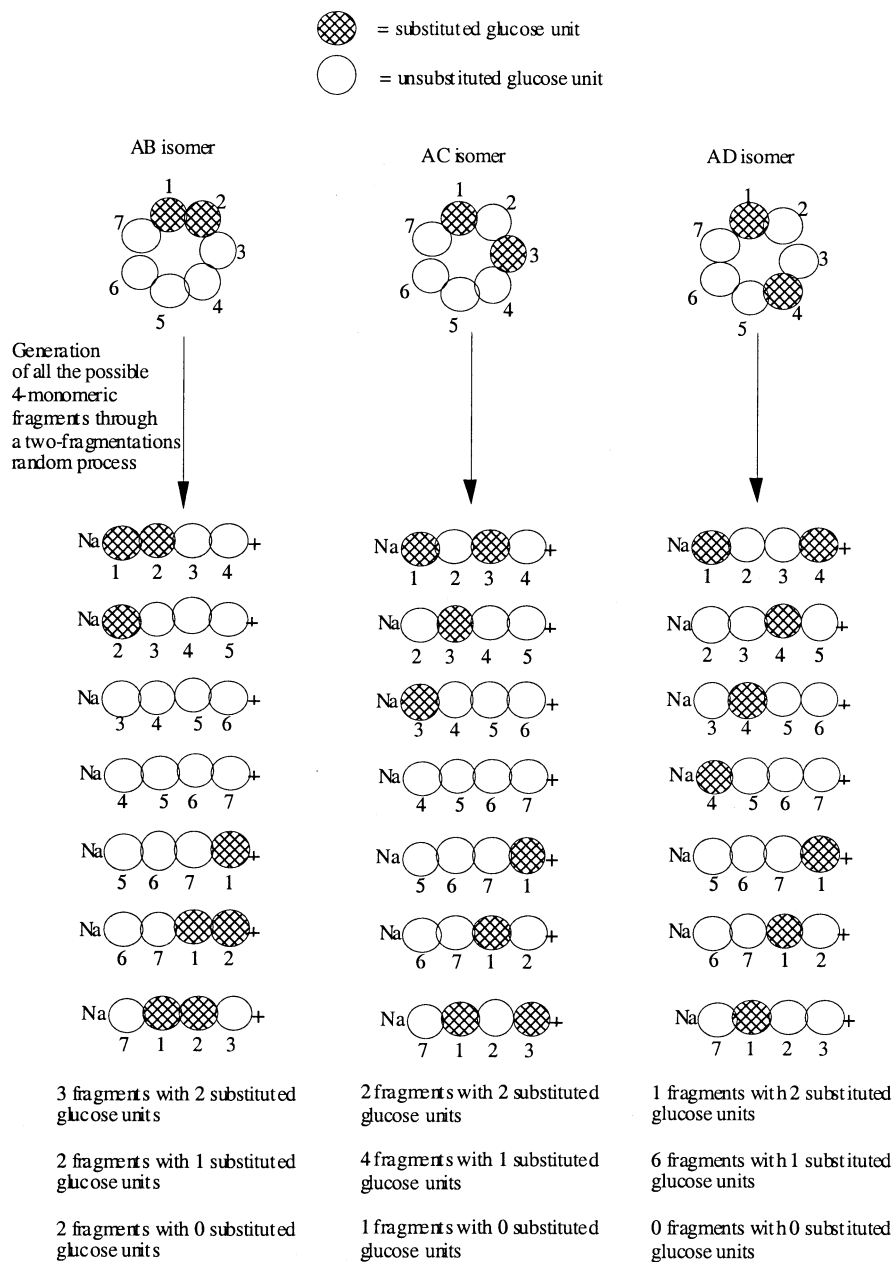
⊗ = substituted glucose unit

○ = unsubstituted glucose unit

AB isomer

AC isomer

AD isomer

Generation of all the possible 4-monomeric fragments through a two-fragmentations random process

AB isomer:

3 fragments with 2 substituted glucose units

2 fragments with 1 substituted glucose units

2 fragments with 0 substituted glucose units

AC isomer:

2 fragments with 2 substituted glucose units

4 fragments with 1 substituted glucose units

1 fragments with 0 substituted glucose units

AD isomer:

1 fragments with 2 substituted glucose units

6 fragments with 1 substituted glucose units

0 fragments with 0 substituted glucose units

Figure 2. Four-monomeric unit fragments generated by the two-fragmentation random process in the case of a disubsituted β-cyclodextrin. Every circle represents a glucose moiety. Substituted glucose moieties are represented as criss-crossed circles.

Table 1. Expected distribution of the unsubstituted, monosubstituted, and disubstituted peaks for every fragment characterised by a defined number of monomeric units in the case of AB, AC, and AD regioisomers, given a random fragmentation at the acetal junctions

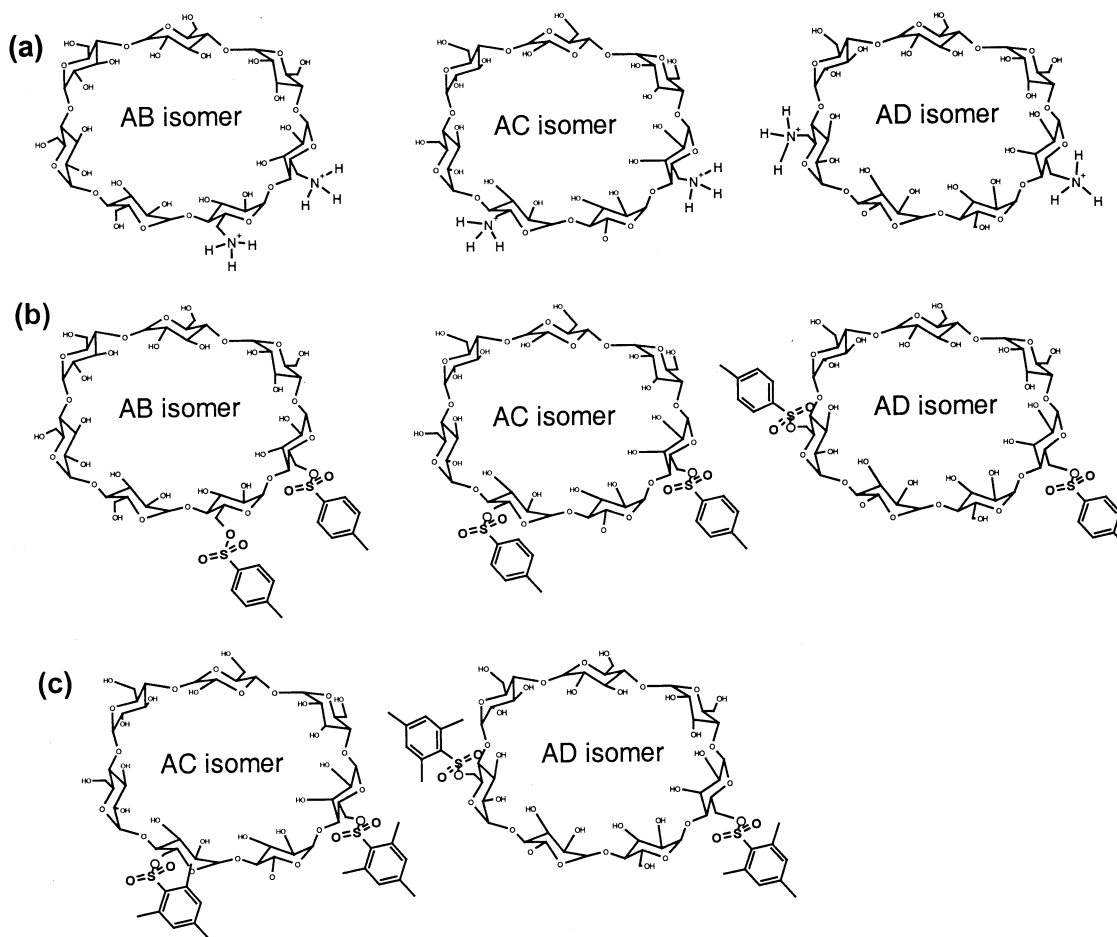|	AB isomer					AC isomer					AD isomer				
Fragments glucose units	2	3	4	5	6	2	3	4	5	6	2	3	4	5	6
Unsubstituted fragments	4/7	3/7	2/7	1/7	0/7	3/7	2/7	1/7	0/7	0/7	3/7	1/7	0/7	0/7	0/7
Monosubstituted fragments	2/7	2/7	2/7	2/7	2/7	4/7	4/7	4/7	4/7	2/7	4/7	6/7	6/7	4/7	2/7
Disubstituted fragments	1/7	2/7	3/7	4/7	5/7	0/7	1/7	2/7	3/7	5/7	0/7	0/7	1/7	3/7	5/7

Figure 3. The structure of the three 6,6'-dideoxy-6,6'-diamino-β-CDs (**a**), 6,6'-dideoxy-6,6'-ditosyl-β-CDs (**b**) and of two 6,6'-dideoxy-6,6'-dimesitylenesulphonyl-β-CDs (**c**).

$$Int.\ \%(nx) = \frac{Int.\ (nx)}{Int.\ (n0) + Int.(n1) + Int.(n2)} \times 100$$

(1)

where n is the number of glucose moieties in the fragment, x the number of the amino groups in the fragment and Int. indicates the absolute value of the intensity of the corresponding mass peak. The percentages of the peaks corresponding to the di-, mono-, and unsubstituted fragments with 2,3,4,5, and 6 glucose units were calculated and are reported in Table 2.

Iterated analyses of the same samples in the same experimental conditions showed that these percentages are very reproducible since oscillations in percentage values never exceeded the unit. Despite the presence of small deviations which make the percentages slightly different from the theory, it is quite easy to notice that the trends are strikingly similar to those expected (compare Tables 1 and 2). A statistical analysis was performed in order to outline those similarities: the relative percentages of the unsubstituted, the monosubstituted, and the disubstituted peaks were correlated to the expected number of fragments (out of seven). The similarity matrix showing the resulting Pearson coefficients is shown in Table 3.

AB, AC, and AD isomers appear to be most related to their theoretical values, having the highest Pearson coefficients always in agreement with the expected regioisomerism. Therefore, from the table shown, it seems possible to clearly distinguish between the three regioisomers simply by measuring the relative abundances of the fragments having different substituents. In order to achieve fast identification of the correct regioisomerism, beside this accurate statistical analysis, we identified as diagnostic peaks the two fragments having three glucose units and one or no amino group (*m/z* 508 and 509, respectively). In fact, the ratios between these peaks were found to be characteristic for every regioisomer, the 509 peak being higher in the AB isomer, the 508 peak being higher in the AD isomer, and having the two peaks more or less the same intensity in the AC isomer (Figure 4b). It should be emphasized that this trend is fully consistent with that predicted by the model.

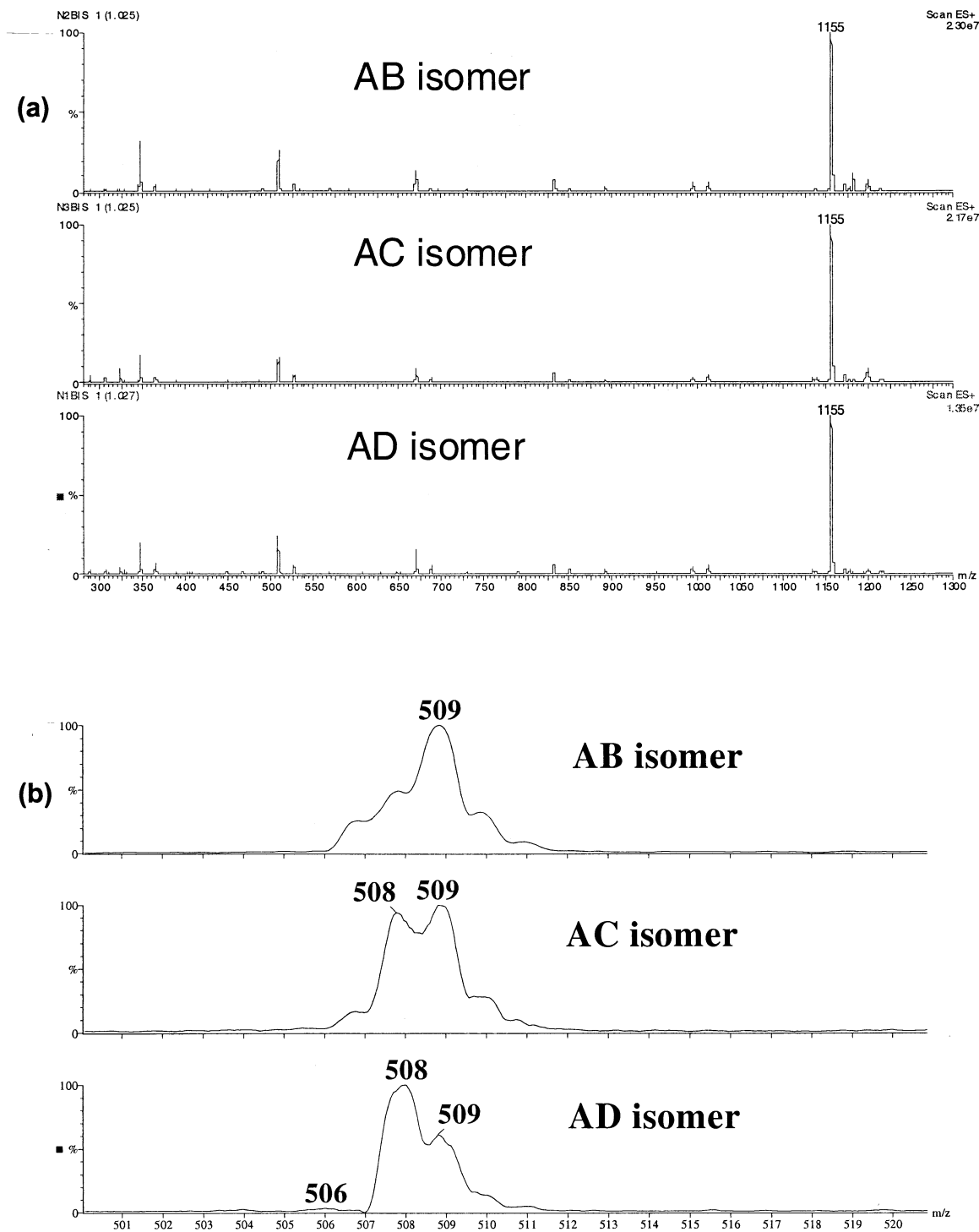It is important to notice that other peaks, beside those predicted by the model, are also present in the ESI

Figure 4. The spectra of the diamino-β-cyclodextrins: AB isomer (top), AC isomer (middle), AD isomer (bottom). (a) full spectra; (b) enhancement of the parts containing the diagnostic peaks 509 and 508.

spectra. Many of them differ from the calculated peaks reported in Table 2 for a value of 18 *m/z* and therefore are likely to be generated by the addition of a molecule of water. It should be considered that this side reaction has the obvious effect of modifying the relative intensities of the peaks, thus generating deviations from the predicted trend.

Isomeric Disubstituted β-CD Identification by Fragmentation: The Case of Ditosyl-β-CDs

In order to test the applicability of the method to a differently disubstituted β-CD and also in a case when the regioisomerism is not previously known, the three ditosyl-β-CD regioisomers (Figure 3b) were synthe-

Table 2. Relative abundances of the peaks found in the ESI spectra of the three regioisomers of the diamino-β-CDs, calculated according to formula 1 as percentage distribution of the unsubstituted, mono- and disubstituted fragments within the same number of glucose moieties

Fragments glucose units	AB isomer					AC isomer					AD isomer				
	2	3	4	5	6	2	3	4	5	6	2	3	4	5	6
Unsubstituted fragments	64	57	47	36	26	51	47	39	26	19	54	34	22	22	24
Monosubstituted fragments	25	29	34	43	50	46	45	46	53	34	44	64	62	48	34
Disubstituted fragments	11	15	19	21	23	3	9	14	21	46	2	2	15	31	43

sized as a mixture, simply by reacting the β-CD with two equivalents of tosyl chloride (details in the Experimental section).

The reaction mixture contained, as expected, three different disubstituted compounds (detected by HPLC analysis), which were separated by preparative HPLC (details in the Experimental section). Actually, the mixture could be analyzed directly in a HPLC-MS hyphenated system, provided a suitable NaCl solution is added as an additive in the HPLC eluents or as a post-column make-up liquid. The ESI spectra of the three pure compounds (labeled peak 1, 2, and 3) were obtained in the same conditions reported above (50 μM CD in the presence of 100 μM NaCl). The spectra are reported in Figure 5.

In all cases the sodiated molecular ion is the dominating peak but the interpretation of the spectra is not as straightforward as in the case of the diamino-β-CDs. First of all, it is easy to notice that many signals are present in the spectra. The two most intense, with m/z of 1293 and 1121, derive from two consecutive losses of 172 Da, which corresponds to the molecular mass of p-toluenesulphonic acid. This is consistent with the character of leaving group of the tosyl substitutent and it means that the first two events of fragmentation are the losses of the substitutents leading to dehydrated glucose units (possibly a 3,6-dehydro compounds).

Therefore, in order to correctly calculate the expected molecular masses of the fragments in every group defined by the number of the monomeric units, the disubstituted, the monosubstituted, and the unsubstituted peaks should be expected to differ by 18 Da, since the disubstituted will actually have two dehydrated units, the monosubstituted will have one dehydrated unit, and the unsubstituted will have no dehydrated

Table 3. Similarity matrix showing the Pearson coefficients of the correlations between the expected number of fragments generated for the three regioisomers of a generically disubstituted β-CD (Table 1) and the real percentages found in the ESI spectra of the diamino β-CDs (Table 2). In every column the highest correlations are marketed in bold

	Percentages found		
	AB	AC	AD
AB expected fragments	**0.172**	0.367	0.311
AC expected fragments	0.111	**0.720**	0.798
AD expected fragments	0.059	0.674	**0.885**

units. Although many peaks are present in the spectra, the most abundant fragments observed have m/z ratios exactly corresponding to those calculated. The other fragmentation peaks observed may probably be due to the different fragmentation reactions in the gas-phase, which may be triggered by the formation of dehydrated glucose moieties.

In every group of peaks corresponding to a fragment with the same number of monomeric units, the percentage of abundance of every peak was calculated according to eq 1. The percentages of the peaks corresponding to the di-, mono-, and unsubstituted (i.e., the di- and monodehydrated and the hydrated) fragments with 2,3,4,5, and 6 glucose moieties were calculated and are reported in Table 4.

As for the diamino-β-CDs, these percentages appeared to be very reproducible under the same experimental conditions. Moreover, in order to test the stability of the percentage values at different cone voltages (the value of 190 V used in all the experiments is about the maximum for our instrument), the mass spectra for all three samples were recorded at 150, 100, and 50 V. The analyses performed at the lowest cone voltage (50 V) resulted in spectra with no apparent fragmentation of the cyclodextrin derivatives, with the presence of only two peaks attributable to the mono- and doubly charged sodiated molecular ions (data not shown). In the cases of 100 and 150 V cone voltages, although the fragment peak absolute intensities decreased regularly by decreasing the voltage itself, the peak ratios remained remarkably similar to those measured at 190 V: oscillations in peak percentages never exceeded 2% (average 0.9%). As a typical example, the percentages calculated according to eq 1 for the monosubstituted (monodehydrated) fragment having three glucose moieties measured at different cone voltages are reported in Figure 6.

A statistical analysis was performed as above in order to correctly define every regioisomer: for every compound the relative percentages of the unsubstituted, of the monosubstituted and of the disubstituted peaks were correlated to the expected number of fragments (out of seven). The similarity matrix showing the resulting Pearson coefficients is shown in Table 5.

Also in this case the identification is unambiguously clear: Peak 1 has the highest coefficient in agreement with the AD isomer, peak 2 in agreement with the AC isomer, and peak 3 in agreement with the AB isomer. By comparing the relative elution order in reverse phase
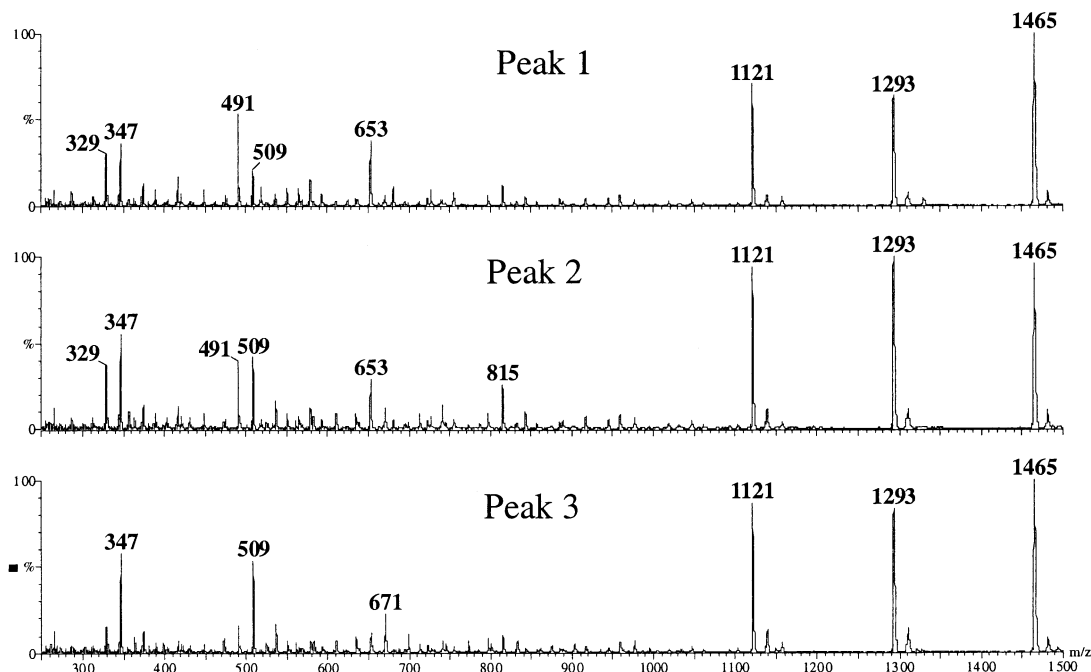
Figure 5. The spectra of the ditosyl-β-cyclodextrins synthesized as a mixture after peak purification.

HPLC, the proposed identifications were in agreement with those reported in the literature [8].

Likewise, as in the case of diamino-β-CDs, a quick identification of the regioisomerism may be achieved simply by checking the diagnostic peaks having three glucose units and one or no dehydrated group (m/z 491 and 509, respectively). In fact, the ratios betweeen these peaks were found again to be characteristic for every regioisomer, consistent with the proposed mode of fragmentation, the 509 peak being higher in the AB isomer, the 491 peak being higher in the AD isomer, and having the two peaks almost the same intensity in the AC isomer (see Figure 4).

In order to confirm the attributes, the corresponding diamino-β-CDs were also synthesized and purified starting from every purified regioisomer of ditosyl-β-CD, according to the literature procedure [11]. ESI-MS analysis of the synthesized compounds, performed as reported above, confirmed the expected regioisomerism for every pure diamino-β-CD, since the fragment percentages calculated from the spectra corresponded with those previously obtained.

It should be noted that the striking similarity between the fragmentation trends of the two differently substituted β-CDs is particularly interesting, since it demonstrates that the hypothesis on the fragmentation mode of disubstituted β-CDs has a general validity independently from the type of substituents. Moreover, the stability of the calculated percentages also at different cone voltages is a very important feature, since it means that the fragment peak ratios only depend from the intrinsic fragmentation of the cyclodextrin derivatives and therefore no instrumental bias is introduced, implying that the method may have a broad applicability independently from the fragmentation system (orifice, triple quadrupole, ion trap).

Isomeric Disubstituted β-CD Identification by Fragmentation: The Case of Dimesitylenesulphonyl-β-CDs

The method was finally applied, again with the regioisomerism not previously known, to another kind of

Table 4. Relative abundances of the peaks found in the ESI spectra of the three regioisomers of the ditosyl β-CDs, calculated according to formula 1 as percentage distribution of the unsubstituted (fully hydrated), mono- and disubstituted (mono and didehydrated) fragments within the same number of glucose moieties

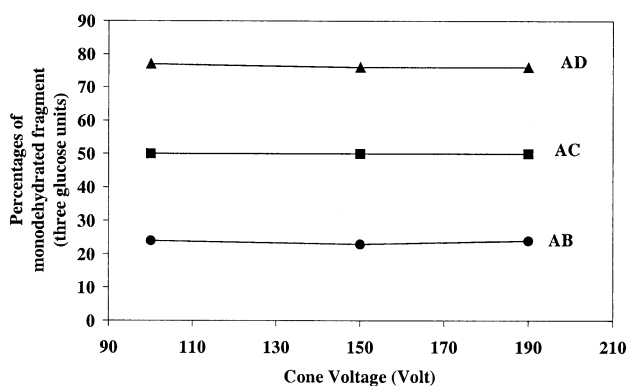| Fragments glucose units	Peak 1					Peak 2					Peak 3				
	2	3	4	5	6	2	3	4	5	6	2	3	4	6	
Unsubstituted fragments	52	27	13	14	12	57	49	24	10	10	73	68	50	27	17
Monosubstituted fragments	44	70	77	57	32	40	46	57	67	39	22	21	27	40	43
Disubstituted fragments	4	4	9	30	56	4	5	19	23	51	5	11	24	33	40

Figure 6. Percentages calculated according to eq 1 for the mono-substituted (monodehydrated) fragments having three glucose moieties measured at 100, 150, and 190 V cone voltage: AB regioisomer (circle), AC regioisomer (square), AD regioisomer (triangle).

Table 7. Similarity matrix showing the Pearson coefficients of the correlations between the expected number of fragments generated for the two regioisomers of a generically disubstituted β-CD (Table 1) and the real percentages found in the ESI spectra of the dimesitylenesulphony-β-CDs (Table 6). In every column the highest correlations are marketed in bold

	Percentages found	
	Peak 1	Peak 2
AB expected fragments	0.381	0.620
AC expected fragments	0.897	**0.788**
AD expected fragments	**0.973**	0.675

disulphonyl derivatives, i.e., the dimesitylenesulpho-nyl-β-CD regioisomers (Figure 3c), which were synthesized as a mixture with the same procedure reported for the ditosyl-β-CDs (details in the Experimental section). These derivatives were chosen in order to perform a regioselective synthesis since the more bulky mesitylene groups were thought to inhibit the formation of the vicinal AB regioisomer. Actually the reaction mixture contained only two different disubstituted compounds (detected by HPLC analysis), which were separated by preparative HPLC (details in the Experimental section). The ESI spectra of the two pure compounds (labeled peaks 1 and 2) were obtained in the same conditions reported above (50 μM CD in presence of 100 μM NaCl). The spectra are reported in Figure 7.

The interpretation is consistent with that above reported for ditosyl-β-CDs: The sodiated molecular ion (1521 m/z) is the dominating peak, the two most intense fragments (1321 and 1121 m/z) derive from two consecutive losses of 200 Da, which correspond to the molecular mass of mesitylenesulphonic acid, leading to dehydrated glucose units. Therefore, as for the ditosyl-β-CDs, in place of the disubstituted, monosubstituted, and unsubstituted fragments, there are fragments with two dehydrated units, one dehydrated unit, and no dehydrated units.

In every group of peaks corresponding to a fragment with the same number of monomeric units, the percentage of abundance of every peak was calculated according to eq 1. The percentages of the peaks corresponding

to the di-, mono-, and unsubstituted (i.e., the di- and monodehydrated and the hydrated) fragments with 2,3,4,5, and 6 glucose moieties were calculated and are reported in Table 6.

As before, these percentages are very reproducible even by changing the cone voltage from 190 to 100 V. A statistical analysis was performed as above in order to correctly assign the regioisomerism: for every compound the relative percentages of the unsubstituted, monosubstituted, and disubstituted peaks were correlated to the expected number of fragments (out of seven). The similarity matrix showing the resulting Pearson coefficients is shown in Table 7.

The identification confirmed the expected regioselectivity: Peak 1 has the highest coefficient in agreement with the AD isomer and peak 2 in agreement with the AC isomer.

As before, the analysis of the ratios of the diagnostic fragments having three glucose units and one or no dehydrated group (m/z 491 and 509, respectively) allowed the quick identification the correct regioisomerism, since consistent with the proposed mode of fragmentation and accordingly with the above reported disubstituted CDs, the peak at 491 m/z is higher in the AD isomer and has almost the same intensity of the peak at m/z 509 in the AC isomer (see Figure 7).

Again, in order to confirm the attributes, the corresponding diamino-β-CDs were also synthesized and purified, starting from both purified regioisomers of dimesitylenesulphonyl-β-CD, according to the literature procedure [11]. ESI-MS analysis of the synthesized compounds confirmed for both diamino-β-CDs the expected regioisomerism, since the fragment percentages calculated from the spectra corresponded with the AD and AC isomers previously obtained.

Table 6. Relative abundances of the peaks found in the ESI spectra of the two regioisomers of the dimesitylenesulphonyl-β-CDs, calculated according to formula 1 as percentage distribution of the unsubstituted (fully hydrated), mono- and disubstituted (mono and di-dehydrated) fragments within the same number of glucose moieties

Fragments glucose units	Peak 1					Peak 2				
	2	3	4	5	6	2	3	4	5	6
Unsubstituted fragments	52	23	9	8	6	61	51	32	12	6
Monosubstituted fragments	46	73	81	61	36	36	39	46	59	42
Disubstituted fragments	2	5	10	32	58	3	10	22	29	52

Table 5. Similarity matrix showing the Pearson coefficients of the correlations between the expected number of fragments generated for the three regioisomers of a generically disubstituted β-CD (Table 1) and the real percentages found in the ESI spectra of the ditosyl β-CDs (Table 4). In every column the highest correlations are marketed in bold

| | Percentages found | | |
	Peak 1	Peak 2	Peak 3
AB expected fragments	0.359	0.473	**0.546**
AC expected fragments	0.874	**0.828**	0.264
AD expected fragments	**0.960**	0.779	0.103

The last experiment also confirms the broad applicability of the method here proposed for a rapid identification of the regioisomerism of disubstituted β-CDs.

Conclusions

The data collected by studying the in-source fragmentation in ESI mass spectra in the presence of a twofold excess of sodium chloride support the hypothesis that unsubstituted and disubstituted β-CDs mainly randomly break at the acetal junctions, giving rise to different polyglucose sodiated fragments. This hypothesis was exploited in order to calculate the relative abundances of the di-, mono- and unsubstituted fragments generated by in-source fragmentation from the three different regioisomers (AB, AC, and AD) of differently disubstituted β-CDs. The model was tested on the three regioisomeric diamino-β-CDs, independently synthesized, obtaining results statistically consistent with the hypotheses. The three regioisomeric ditosyl-β-CDs and two regioisomeric dimesitylenesulphonyl-β-CDs, synthesized as a mixture and subsequently purified by HPLC as unknown samples, were also correctly and unambiguously identified by applying the method developed here.

The relative abundances of the fragmentation peaks were shown to be totally voltage-independent, indicating that this method may be extended to different mass spectrometers with different fragmentation systems without any instrumental bias. It should be pointed out that this measure can also be obtained from a simple single-quadrupole instrument by using the in-source fragmentation, thus allowing the application of this method without expensive MS/MS instruments.

These results are of importance from a theoretical point of view since they explain the main fragmentation mode of substituted β-CDs in ESI mass spectrometry, and perhaps even more important, from a practical one, by making available a very rapid procedure for the correct identification of disubstituted β-CD regioisomers. The latter result is of particular importance since the methods presently found in the literature are very cumbersome and/or time-consuming. Obviously the method can be applied only to the identification of pure regioisomers, although it could easily be extended to a HPLC-MS hyphenated technique in order to achieve an "on-line" identification.
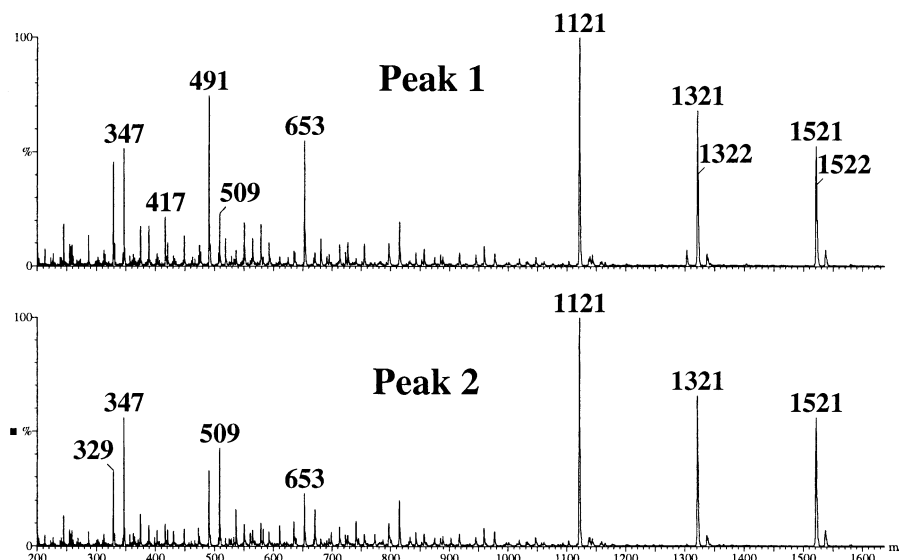
Acknowledgements

Figure 7.   The spectra of the two dimesitylenesulphonyl-β-cyclodextrins synthesized as a mixture after peak purification.

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