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TANDEM MASS SPECTROMETRY CHARACTERIZATION OF ESTERIFIED CYCLODEXTRINS

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The tandem mass spectrometry (MS/MS) characterization of cyclodextrin derivatives, namely randomly esterified 6-O-(3-hydroxybutyril)-β-cyclodextrin (HBCD) and triacetyl-β-cyclodextrin (TABCD) is described. The chosen compounds share certain structural similarities which are exploited in order to establish a general approach in their tandem MS characterization. The TABCD commercial product is fully esterified and presents in single stage MS a single peak while HBCD presents a molecular weight distribution due to the variation of the substitution degree. HBCD product was obtained via ring opening of β-butyrolactone in the presence of β-cyclodextrin (CD). First, the specific fragmentation pathways are established for protonated and sodiated TABCD parent ions and, based on the established fragmentation behaviour, HBCD compounds are analyzed. Our findings indicate that in MS/MS analysis of esterified cyclodextrins the cleavage of the substituents can be selectively induced thus offering information on the substitution patterns. Moreover, we demonstrate, using tandem MS technique, that β-butyrolactone monomer units are attached to the CD molecule not as oligomer chains but as singly esterified molecules.

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1. Introduction

The last decade a growing need for novel inexpensive and green routes for synthesis of polymer architectures suitable for biorelated applications was observed. In the same time the characterization tools become more sophisticated in order to meet requirements for fast and accurate analysis. Thus, mass spectrometry arises as a technique of choice compared to other alternative techniques like NMR and IR spectroscopy [1]. MS can offer rapid answers to issues like molecular weight distribution, endgroup identification, comonomer composition, etc. Monodimensional MS provides information concerning the m/z (mass to charge ratio) of each polymer component allowing to determine the mass of the polymer chain to some extent, according to the mass accuracy of the mass spectrometer in use. This approach is commonly employed for already known polymer systems which have already established synthetic procedures. However, novel synthetic procedures require more than a single stage MS measurement for performing structural assignments. In such situations the structural characterization is performed by fragmentation experiments called multidimensional MS. Detection and interpretation of the fragmentation spectra ions allows reconstruction of the primary structure (connectivity) of the selected polymer architecture in the case of polyesters [2-8].

Complex structures like cyclodextrin (CD) derivatives received attention due to their potential biological applications [9]. The synthesis strategies consist in single or multiple step attachment of organic moieties or in using native CDs to initiate the polymerization, yielding CD

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end caped polymers. The preparation of CD-polyesters conjugates appeals to CDs in two ways, first as initiator and then as catalyst of the ring opening polymerization (ROP) [10-15].

The characterization of CD-oligomer derivatives is quite difficult to characterize because they present structural heterogeneity. Single stage MS can differentiate among different polymerization degrees (substitution degrees). However, isobaric peak series due to positional isomers can occur and the structural assignment should appeal to chromatographic separations or to tandem MS to perform analysis at molecular level as we previously described [12].

Several studies were performed on CD derivatives using as characterization tool ESI or MALDI MS [16-19]. Because of the complexity of the analyzed samples, chromatographic separation with offline [20] or online MS detection of the compounds [21, 17] is required. The MS characterization of polyester functionalized CDs used MALDI [10, 13, 14] or ESI MS [11, 12] to provide mass related data able to support, together with NMR spectroscopy, the structural assignment of the products at molecular level. However, only single stage MS without prior chromatographic separation was used in most cases [10, 12-14], despite the complexity of the analyzed mixtures.

The MS/MS studies for structural identification at molecular level of polyesters represent a subject of interest in the last period [3, 22-25]. The fragmentation occurs through the cleavage of the ester bonds by 1-4 H rearrangements. Tandem MS allows structural identification of polyester tethered CD as showed in one of our previous studies[12]. In the current paper we propose a thorough characterization of these compounds by using tandem MS aiming to establish the peculiarities of fragmentation processes and the usefulness of the resulted information in structural assessment of esterified CDs . The samples taken into consideration are random 3-OH butyrated β-CD obtained through solution ring opening polymerization of β-butyrolactone (BL) [26] and a commercial sample of TABCD.

2. Experimental

HBCD (6-O-3-OH butyril-cyclomaltoheptaose) samples were obtained as previously described [26], through solution ring opening polymerization of β-butyrolactone (BL) initiated by β-cyclodextrin in presence of sparteine. TABCD (triacetyl-β-cyclodextrin) was purchased from Aldrich.

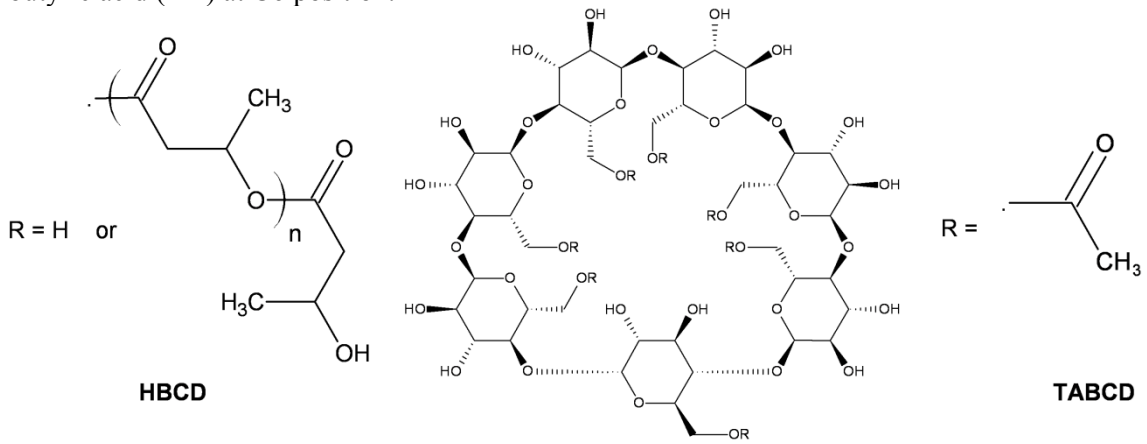
MS/MS experiments were conducted using the AGILENT 6520 LC ESI QTOF mass spectrometer equipped with a dual ESI source. The data were analyzed using the Mass Hunter software. The concentration of each solution was 0.1 g/L (acetonitrile/water 1:1 v/v mixture) for mass spectrometric analysis performed via direct infusion of the sample. The ESI MS parameters were set as follows: Vcap = 4000 V, fragmentor voltage = 200 V, drying gas temperature = 325 ^{o}C, drying gas flow = 10 L/min and nebulizer pressure = 35 psig. Nitrogen was used as spraying gas. The fragmentation was performed using nitrogen as collision gas at a pressure of 18 psig inside the collision cell. The TABCD and HBCD samples yielded fragment ions at variable Elab according to the type of ion. Samples were infused via an external syringe pump (KDS Scientific) with a flow of 0.05 mL/min. For protonated samples the injected solutions were spiked with 0.1 M formic acid solution (1/10 vol/vol of sample solution). For the sodiated samples NaI was used in the same proportion as formic acid.

3. Results and discussion

Cyclodextrins (CDs) are natural, cyclic oligosaccharides produced from starch. CDs with different degrees of polymerisation have been discovered but the most important are α-, β- and γ-CD composed of six, seven and eight α-D-(1-4) glucopyranoside moieties, respectively. Their structures are viewed as hollow, truncated cones where the C-6 primary alcohols crown the narrow rim while the wider rim is crowned by the secondary alcohols at positions C-2 and C-3 [27].

The fragmentation studies of CD and CD derivatives are trying to answer several questions related to specific structural details of these compounds. The collision induced dissociation (CID) fragmentation of cyclodextrins undergoes through the cleavage of the semiacetalic bonds resulting in daughter ions with a specific mass related to the number of glycoside structural units (m = n*162 Da, 162 Da represents the mass of one structural unit and n is the number of structural units) [28].

The compounds discussed in this paper are originating from β-CD and basically they can be described as esterified CD with acetic acid (TABCD) or 3-OH butyric acid [12, 26] (Scheme 1). TABCD is a commercial product with all OH groups modified with acetyl moieties while HBCD was obtained through ring opening of β-butyrolactone. The previous studies for structural elucidation were performed via LC ESI MS, COSY and HSQC NMR spectroscopy [26]. The obtained results showed that CD molecule is esterified with an average of 4 molecules of 3-OH butyric acid (BA) at C6 position.



Scheme 1. Structural description of the TABCD and HBCD compounds

The aim of the tandem MS experiments is to establish a fragmentation pattern related to these specific structures, namely esterified cyclodextrins. This pattern would be further useful for structural identification at molecular level of structurally similar compounds.

TABCD product, a fully esterified CD was first analyzed. A fragmentation behaviour similar to the one described generally for cyclic oligosacharides [29-34] with the cleavage of semiacetalic bonds, as depicted Scheme 2 - pathway C, was expected. However, the presence of the ester side groups may modify this behaviour.

The fragmentation in collision induced dissociation processes depends on various factors, among them being the nature of cations contributing to the formation of the parent ionic species. Therefore, both protonated and sodiated TABCD ionic species, were submitted to CID. The MS/MS spectrum of $[TABCD]^+$ is showed in Figure 1.

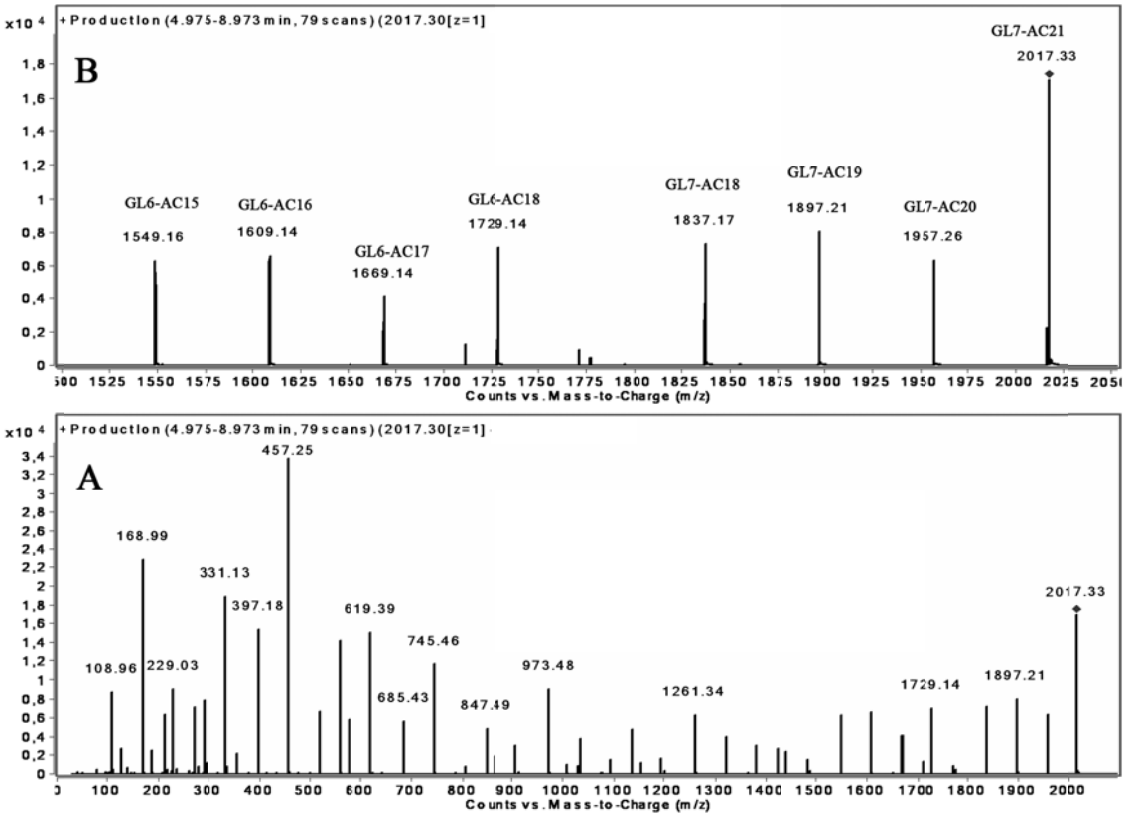
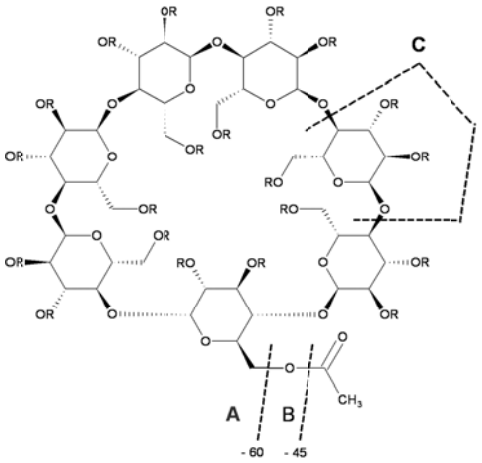


Fig. 1. A.MS/MS spectrum of $[TABCD]^+$ fragmentation energy E_{LAB}=20 eV; B. detailed view m/z span [1500-2050]

Several daughter ions which can be rationalized according to the fragmentation mechanism were identified. In order to simplify the interpretation of the MS/MS spectrum we may consider the TABCD sample as a copolymer having one co-monomer(GL) the remaining part from the glycoside ring after substracting 3 water molecules ($C_6H_4O_2$) and the other co-monomer the acetic acid (AC). Thus, our copolymer can be described as (GL_7-co-AC_{21}) as showed in Scheme 1. The GL units have a mass of 108 Da and the AC 60 Da. The m/z value obtained for the parent ion would be rationalized as 2017 = 108x7+60x21+1 (H). The observed fragments are resulted from two different CID processes, one at the level of semiacetalic bonds when the entire GL rings are lost as neutrals (C pathway), or only at the level of ester bonds when AC are lost as neutrals (Scheme 2). The ester bond can be cleaved on the alchil side (pathway A) through 1-4 H rearrangements [3] and the neutral loss has the value of 60 Da or on the acyl side (pathway B) and the neutral loss has the value of 42.



Scheme 2. Representation of the ESI QTOF fragmentation processes of $[TABCD]^+$ ionic species

The A and C types of processes are occurring in similar energetic conditions. We may remark that these fragmentation processes are consecutive, being given the nature of fragmentation on the QTOF mass spectrometer.

All the daughter ions are identified by their mass in the Table 1. In principle, the table cells contain all possible masses obtained from all the possible co-monomers combinations of A and C pathways and the values which were actually found in the MS spectrum are highlighted.

Table 1. Masses of the fragments[a] observed in the fragmentation (A and C pathways) of the $[TABCD]^+$ (GL units have the mass of 108; AC units have the mass of 60)

	GL1	*GL2*	*GL3*	*GL4*	*GL5*	*GL6*	*GL7*
AC0	109	217	325	433	541	649	757
AC1	169	277	385	493	601	709	817
AC2	229	337	445	553	661	769	877
AC3	289	397	505	613	721	829	937
AC4	349	457	565	673	781	889	997
AC5	409	517	625	733	841	949	1057
AC6	469	577	685	793	901	1009	1117
AC7	529	637	745	853	961	1069	1177
AC8	589	697	805	913	1021	1129	1237
AC9	649	757	865	973	1081	1189	1297
AC10	709	817	925	1033	1141	1249	1357
AC11	769	877	985	1093	1201	1309	1417
AC12	829	937	1045	1153	1261	1369	1477
AC13	889	997	1105	1213	1321	1429	1537
AC14	949	1057	1165	1273	1381	1489	1597
AC15	1009	1117	1225	1333	1441	1549	1657
AC16	1069	1177	1285	1393	1501	1609	1717
AC17	1129	1237	1345	1453	1561	1669	1777
AC18	1189	1297	1405	1513	1621	1729	1837
AC19	1249	1357	1465	1573	1681	1789	1897
AC20	1309	1417	1525	1633	1741	1849	1957
AC21	1369	1477	1585	1693	1801	1909	2017

[a]The values from the highlighted cells correspond to the peaks found in the MS spectrum from Figure 1.

In Fig. 1 and Table 1, a pattern related to the neutral loss of AC units can be identified. Unexpectedly, the AC units are lost according to their positioning on the GL units as no more than 3 AC units are lost (the load of one GL unit) and than a semiacetal bond can be cleaved. Probably the collision energetic conditions are leading to repeated consecutive losses of AC until one GL ring is cleaved and the process repeats until all the co-monomer units are depleted.

Besides the main peaks, there may be observed a less representative series resulted from the ester bond cleavage on the acyl side (pathway B). The lost fragments can be inferred using a similar algorithm but the neutral losses can have 42 Da value (Scheme 2).

However, when TABCD ionic species are obtained using Na+ cations the fragmentation spectrum is significantly changed (Fig. 2).

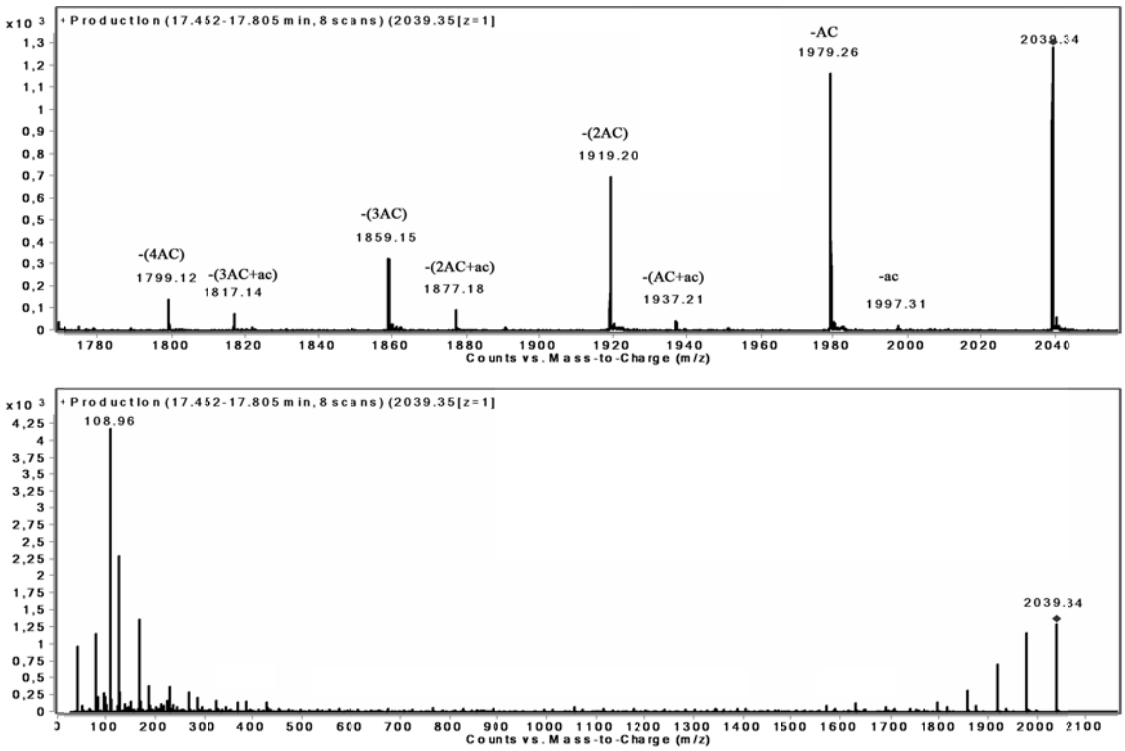


Fig. 2. MS/MS spectrum of $[TABCD+Na]^+$ E_{LAB}=120 eV: A. full spectrum; B. detailed spectrum in the region m/z = [1780 - 2060]

As expected, the collision energy for this type of adducts is increased as compared with protonated species and the fragmentation patterns are different. The fragmentation processes occur only through pathways A and B. Up to 4 AC consecutive neutral losses according to the main peak series (nominated AC) in the fragmentation spectrum from Figure 2B were observed. Also, a second peak series (nominated ac) with lower peak intensity generated by the consecutive fragmentation of the daughter ions from AC series was evidenced.

The charge induced fragmentations occur also consecutively and, due probably to charge positioning in the $[TABCD+Na]^+$ adduct, only the ester bonds are affected. This fragmentation pattern is obviously connected to the particular structure of esterified cyclodextrin and might be useful for structural elucidation of similar compounds.

This statement is supported by our next fragmentation experiment when we used own synthesized randomly substituted CD derivative, namely a 3-OH butyrared CD (structure given in Scheme 1). The main structural issue which should be established for this sample is related to the fact that the esterified carboxylic acid contains an OH group which may undergo further esterification to result in a polymer chain.

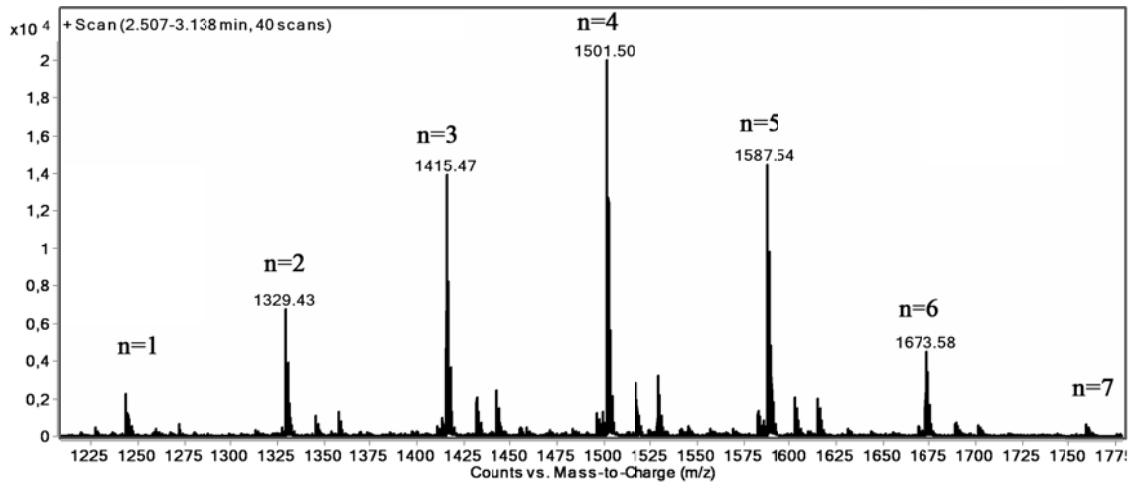


Fig. 3. ESI MS spectrum of [HBCD+Na]$^+$

HBCD sample consists of β-cyclodextrin derivatives with variable number of open β-butyrolactone units (the average substitution degree is 4). However, single stage MS experiment (Figure 3) can't confirm if the BL units are esterified on the CD as 3-OH butyric acid or as PHB (polyhydroxybutyrate) oligomer chains. In fact, the papers concerning the synthetic procedures of oligoester tethered CDs affirm that CD can initiate only one polyester chain [10, 11, 13]. However, we previously demonstrated [12] using MS and NMR that more than one chain can be initiated by a single molecule of CD in bulk conditions. The product analyzed in this paper was obtained through a synthetic procedure which insures that most of the BL units are singly linked to the CD, as proved by NMR [26]. Now, the task is to reach the same conclusion using only the MS/MS technique.

The fragmentation spectrum of the HBCD derivatives containing 5 BL units is presented in Figure 4. There may be observed that [HBCD]$^+$ species are fragmenting similarly to TABCD, being remarked only one type of cleavage, involving the semiacetalic bond (pathway C) as shown in Scheme 3.

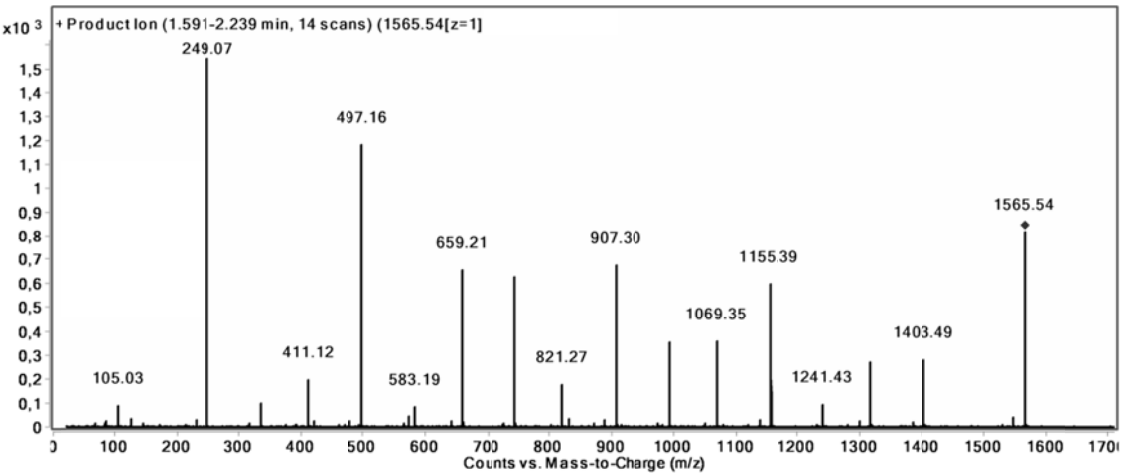
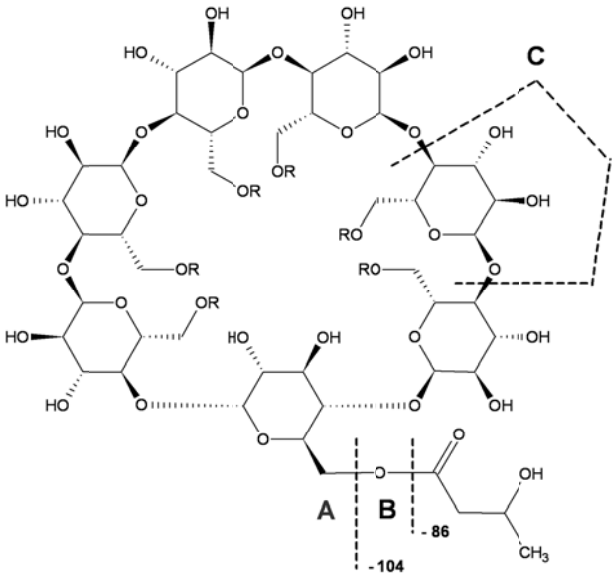


Fig. 4. MS/MS spectrum of [HB$_5$-CD$_7$]$^+$ E_{LAB}=15 eV



Scheme 3. Fragmentation pathways of HBCD

Analysing the HBCD fragmentation we may notice that the cleavage of the ester bonds is less obvious probably because the number of esterified OH groups is significantly smaller. However, knowing from the TABCD fragmentation experiment that CID processes on bothe ester and semiacetalic bonds may occur at similar collision energies we may suspect that 1,4-H arrangements take place also in the case of HBCD fragmentation but is not giving birth to significant fragments. The cleavage of ester bond (pathway A) is however confirmed by the presence of the 105 Da fragment which may be assigned as protonated 3-OH butyric acid.

The daughter ions observed in the fragmentation spectrum are rationalized in the Table 2, similarly to TABCD. HBCD parent ion took into consideration (structure given in scheme 1), with characteristic m/z = 1565, can be described as a copolymer, (G_7-co-HB_5), where G is the glycoside residue and HB is the 3-OH butyrate unit. Thus, the G units have a mass of 162 Da and the HB 86 Da. The m/z value obtained for the parent ion would be rationalized as the mass of the parent ion $[G_7\text{-}HB_5]^+$ 1565 = 162x7+86x5+1 (H).

Table 2. Masses[a] of the fragments observed in the fragmentation (C pathway) of the $[HBCD]^+$ (C units have the mass of 162; B units have the mass of 86)

	C1	*C2*	*C3*	*C4*	*C5*	*C6*	*C7*
B0	163	325	487	649	811	973	1135
B1	249	411	573	735	897	1059	1221
B2	335	497	659	821	983	1145	1307
B3	421	583	745	907	1069	1231	1393
B4	507	669	831	993	1155	1317	1479
B5	593	755	917	1079	1241	1403	1565

[a]The values from the highlighted cells correspond to the peaks found in the MS spectrum from Figure 4

The observed fragmentation pattern suggests that 3-OH butyrate is the substituent of CD but it doesn't clearly confirm if we have multiple esterification sites because the governing CID fragmentation mechanism is related to the cleavage of glycosidic interconnections and not directly targeted to the ester bonds. A fragmentation which would address directly the ester bond would be more conclusive. Therefore, knowing from the experiments effectuated on TABCD sample that Na cations induce mostly the 1,4 H rearrangements in esterified CD (pathway A) we chose to perform the fragmentation of $[G_7\text{-}HB_5\text{+Na}]^+$ parent ions (Figure 5).

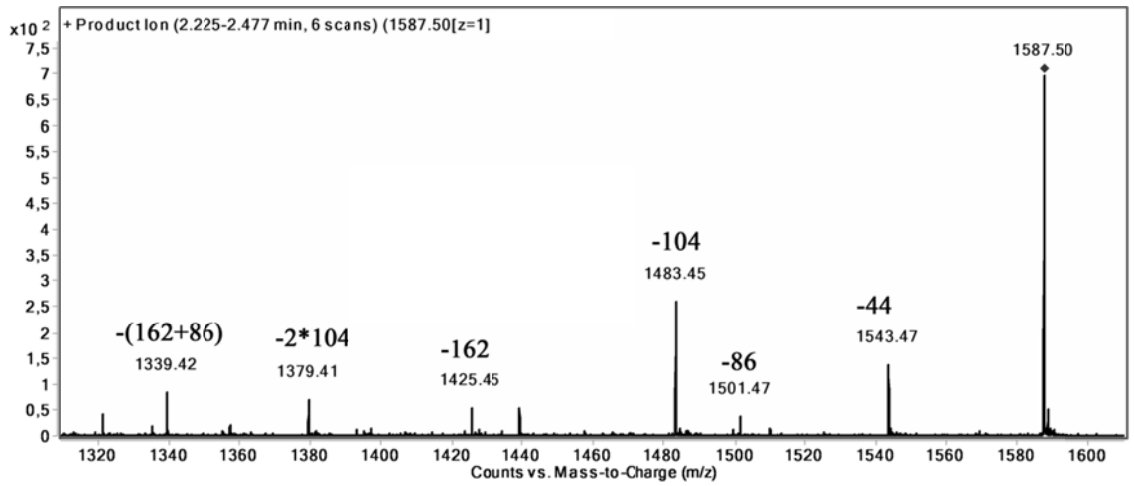


Fig. 5. MS/MS spectrum of $[G_7\text{-}HB_5+Na]^+$ E_{LAB}=110 eV (detailed view between m/z 1300-1600)

The MS/MS spectrum, presented in detailed view from Fig 5, contains several peaks corresponding to different fragmentation pathways. The most important pathway is the A fragmentation pathway which produce the daughter ions found at m/z = 1483 and 1379. These ionic species are formed through the loss of one (1483 fragment) and two (1379) molecules of 3-OH butyric acid (104 Da). The process involved is 1-4 H rearrangements and the fragmentations may be consecutive. A peak at 1501 Da resulted from pathway B, the cleavage of the ester bond on the acyl side was also observed. The third observed fragmentation pathway (C) concerns the cleavage of the semiacetalic bonds giving birth to the 1425 Da (loss of 162 Th) and 1339 Da (loss of 162+86 Th) daughter ions. The C pathway was not evidenced during the fragmentation of the [TABCD+Na]+ adducts and this difference may the consequence of the much lower substitution degree of the HB CD compared with TABCD. Probably the presence of the substitutions is shielding the 1,4 glycosidic bonds from interacting with the Na cation.

Another change in the fragmentation behaviour of HBCD is related to the appearance of the 1543 daughter ion (loss of 44 Th). The origin of this fragment remains unknown but we may suspect that charge induced dissociation processes may lead to cross-ring cleavages or even to the cleavage of the C-C bond at the level of methine C from 3-OH butyrate (loss of C_2H_4O).

Thus, fragmentation of sodiated HBCD derivatives is clearly showing that consecutive losses of 3-OH butyric acid (-104 Da) may occur in a similar manner as acetic acid moieties are stripped from the CD scaffold. The significance of the simultaneous loss of 3-OH butyric acid is related to the structural assignment of HBCD in the way that it proves beyond doubt that BL monomer units are grafted on different OH units and not as a single polymer chain.

4. Conclusions

This study presented for the first time the analysis at molecular level of esterified cyclodextrins like triacetyl-β-CD or 6-O-(3-hydroxybutyril)-β-cyclodextrin. The proposed fragmentation pathways allowed the rationalization of presumptively complicated MS/MS spectra. Once decrypted the fragmentation behaviour, pathways A, B and C the spectra interpretation reveal valuable structural details concerning the substitution patterns. It has been showed that moieties connected through ester bonds to CD can be selectively cleaved (pathways A and B) in collision induced dissociation processes of sodiated adducts. This behaviour is more pronounced as the substitution degree of CD increases. For a full substitution degree (case of TABCD) the cleavage of semiacetal bonds (pathway C) is suppressed in the favour of ester bond cleavages (pathways A and B).

We have demonstrated in this study that tandem MS is a useful tool in analysis of the esterified cyclodextrins and allows to determine the substitution patterns of esterified cyclodextrin derivatives .

Acknowledgements

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