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Study of the inclusion complexes of aromatic molecules with cyclodextrins using ionspray mass spectrometry

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Abstract

The formation of inclusion complexes between cyclodextrins (cyclohexa-, cyclohepta-, and cyclooctamylose) and either 1-anilinonaphthalene-8-sulfonate or 2-*p*-toluidinylnaphthalene-6-sulfonate was investigated by ionspray mass spectrometry operated both in the positive and in the negative ion mode. This soft ionisation technique allowed the detection of the inclusion complexes; the presence of false positives was excluded by increasing the voltage at the orifice which caused breakage of the electrostatic adducts and some fragmentation of the free cyclodextrin molecules, but left the inclusion complexes intact. The spectra recorded in the negative mode showed the presence of complexes formed by two cyclodextrin molecules and one aromatic molecule; such stoichiometry was not detected in the positive mode. © 1996 Elsevier Science Ltd.

Keywords: Cyclodextrins; Ionspray mass spectrometry; Inclusion complexes

1. Introduction

Cyclodextrins are cyclic oligosaccharides constituted of glucose residues linked α(1-4) exhibiting a degree of oligomerisation which ranges from 6 to 12. Attention has

Abbreviations: α-Cdx, cyclohexa-amylose; amu, atomic mass units; ANS, 1-anilinonaphthalene-8-sulfonate; β-Cdx, cyclohepta-amylose; γ-Cdx, cycloocta-amylose; ISMS, ionspray ionisation mass spectrometry; ISV; ionspray voltage; OR, orifice voltage; PPG, polypropylene glycol; TNS, 2-*p*-toluidinylnaphthalene-6-sulfonate

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been focused especially on the commercially available cyclohexa- (α-Cdx), cyclohepta- (β-Cdx), and cycloocta-amylose (γ-Cdx) because they can host either hydrophobic or aromatic molecules in their cavity [1,2], when the size and the shape of the two interacting species are compatible. In fact, the hydroxyl groups belonging to the carbohydrate moieties arranged on the outer surface of the cyclic molecules form a hydrophilic surface and a hydrophobic cavity. The formation of inclusion complexes between cyclodextrins and molecules containing phenyl, naphthyl or other aromatic groups as well as long alkyl chains was studied in the past by means of different physico–chemical techniques (NMR spectroscopy [3,4], spectrofluorimetry [5,6], circular dichroism [7], and calorimetry [8,9]). In addition to this, a general review on host–guest chemistry studied by means of mass spectrometry has been published [10].

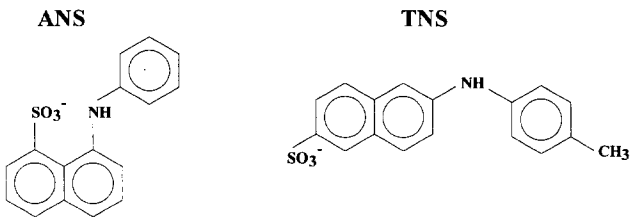
More recently, the complexation of amino acids by cyclodextrins was investigated using electrospray ionisation mass spectrometry [11–13]. The results obtained suggested the presence of false positives [13], most probably electrostatic adducts that are not distinguishable from the real inclusion complexes and that may lead the scientist to the wrong conclusions.

We now report on the ionspray mass spectrometry study of the inclusion complexes formed by α-Cdx, β-Cdx, and γ-Cdx with either 1-anilinonaphthalene-8-sulfonate (ANS) or 2-*p*-toluidinylnaphthalene-6-sulfonate (TNS). The existence of such inclusion complexes has been already demonstrated in aqueous solution by use of different techniques such as circular dichroism, fluorescence, and isothermal microcalorimetry [8]. The presence of false positives was excluded by applying a high voltage at the orifice.

2. Experimental

Ionspray mass spectrometry.—The mass spectra were recorded on a API-I PE SCIEX quadrupole mass spectrometer equipped with an articulated ion spray and connected to a syringe pump for the injection of the samples. The instrument was calibrated using a polypropylene glycol mixture (3.3×10^{-5} M PPG 425, 1×10^{-4} M PPG 1000, and 2×10^{-4} M PPG 2000), 0.1% acetonitrile, and 2 mM ammonium formate in 50% aq MeOH. The samples were injected at a flow rate of 7–10 μL/min. When the analyses were conducted in the positive mode, the ionspray voltage (ISV) was 5000 V and the orifice voltage (OR) was 50 V. In the negative mode, the ISV was set at -5000 V and the OR at -70 V. The spectra were recorded using a step size of 0.1 amu. Some experiments were performed varying the OR from 50 to 130 V, and from -70 to -180 V in the positive and in the negative mode, respectively.

Preparation of the samples.—The samples of α-Cdx, β-Cdx, and γ-Cdx (purchased from Sigma and used without further purification), as well as ANS and TNS (see Formula 1) were dissolved in 50% aq acetonitrile. The final concentrations used in the positive ion mode experiments were 0.2×10^{-4} M in cyclodextrins and 0.6×10^{-4} M in ANS (or TNS). For the negative ion mode experiments, the cyclodextrins were used at a final concentration of 3×10^{-4} or 1×10^{-3} M and ANS (or TNS) was 1×10^{-3} M. Ammonium acetate (0.6×10^{-4} M) and ammonia (5%) were used as ionising agents in the positive and negative ion mode, respectively.



3. Results and discussion

The ionspray mass spectrum of an equimolar mixture of the three cyclodextrins is reported in Fig. 1a. The three ions observed at m/z 990.6, 1152.6, and 1314.6 correspond to $[M + NH_4]^+$ species for each of the cyclodextrins examined. The signal intensity of these three ions decreased with increasing molecular weight; this observation has been previously reported for a group of oligosaccharides of structure $(Man)_n(GlcNAc)_2$ ($n = 5–9$) [14], thus indicating that the quantitative response of the mass spectrometer, at least for these compounds, depends on the molecular mass of the investigated species.

In Fig. 1b the spectrum of the mixture α-Cdx, β-Cdx, γ-Cdx and ANS is shown. Besides the ions corresponding to the ammonium adducts of the three cyclodextrins, ions not present in the previous spectrum were detected. They correspond to the complexes α-Cdx–ANS, β-Cdx–ANS, and γ-Cdx–ANS and their assignment is reported in Table 1. The same experiment was carried out with the three cyclodextrins and TNS; the spectrum obtained is reported in Fig. 1c and the assignment of the ions is described in Table 1. The ions detected in these two experiments clearly indicate the presence of a complex formed by the cyclodextrins and either ANS or TNS molecule, and we supposed that such complexes are inclusion complexes, since previous records in the literature demonstrated their formation using physico–chemical techniques [8].

In order to exclude the presence of electrostatic adducts formed during the ionspray process, spectra of the mixture of β-Cdx and TNS were recorded while varying the potential applied to the orifice from 50 to 130 V with steps of 10 V. We decided to use this experimental approach because it is known [15] that by increasing the voltage at the orifice, the breakage of the clusters and the fragmentation of the cyclodextrins take place. This experiment aimed to establish the higher stability of inclusion complexes under increasing orifice potential with respect to other non-specific adducts. In addition to this, we repeated the above experiment with a mixture of β-Cdx and GlcA, where the uronic acid was used as a blank, since it has never been reported in the literature that it is included by the cyclodextrin molecules. The spectra obtained, reported in Fig. 2a–d, clearly showed that when the orifice was set at 50 V, the ions corresponding to the complex β-Cdx–TNS and β-Cdx–GlcA were both present; when the potential applied to the orifice was 110 V, the ions corresponding to the complex β-Cdx–TNS were still present, while those corresponding to the complex β-Cdx–GlcA were not detected anymore. In Fig. 2b and d the peaks at 1135.4, 1157.3, and 1173.3 amu correspond to

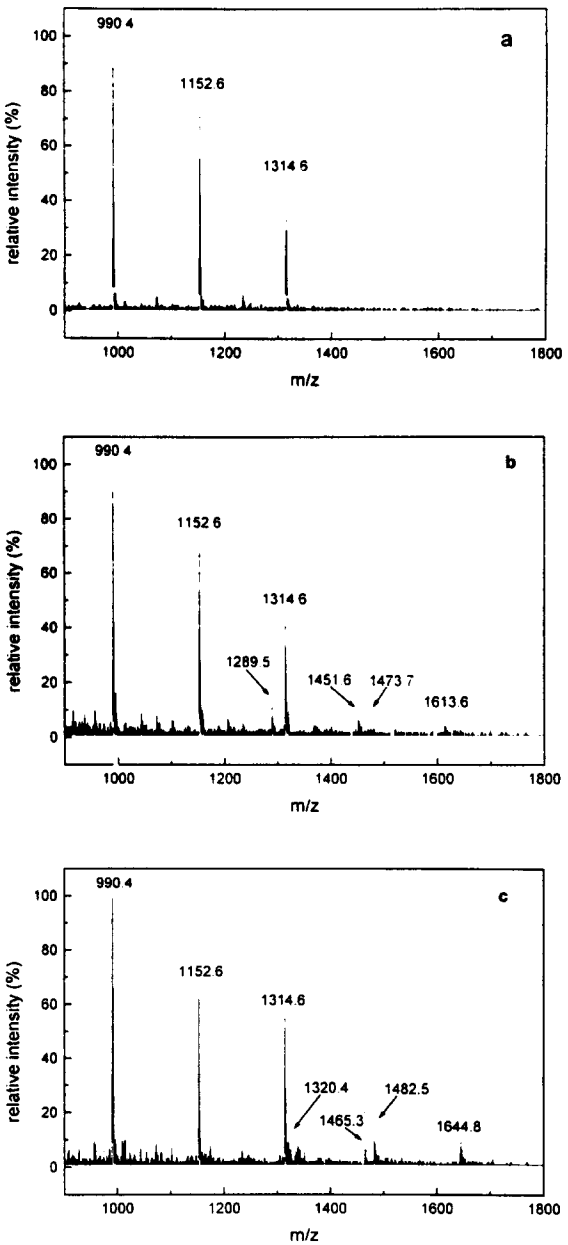


Fig. 1. Positive detection mode ISMS of α-Cdx, β-Cdx, and γ-Cdx (a), of α-Cdx, β-Cdx, and γ-Cdx with ANS (b) and of α-Cdx, β-Cdx, and γ-Cdx with TNS (c). The concentrations were 0.02 mM for each cyclodextrin and 0.06 mM for either ANS or TNS.

Table 1
Assignment of the observed ions for the experiments carried out on the mixtures of α-Cdx, β-Cdx, and γ-Cdx either with ANS or with TNS, performed in the positive ion mode

m/z	α-, β-, γ-Cdx + ANS	α-, β-, γ-Cdx + TNS
990.4	$[\alpha + NH_4]^+$	$[\alpha + NH_4]^+$
995.3	$[\alpha + Na]^+$	
1152.6	$[\beta + NH_4]^+$	$[\beta + NH_4]^+$
1289.5	$[\alpha + ANS(H) + NH_4]^+$	
1314.6	$[\gamma + NH_4]^+$	$[\gamma + NH_4]^+$
1319.4	$[\gamma + Na]^+$	
1320.4		$[\alpha + TNS(NH_4) + NH_4]^+$
1451.6	$[\beta + ANS(H) + NH_4]^+$	
1465.3		$[\alpha + TNS(H) + NH_4]^+$
1473.7	$[\beta + ANS(Na) + NH_4]^+$	
1482.5		$[\beta + TNS(NH_4) + NH_4]^+$
1613.6	$[\gamma + ANS(H) + NH_4]^+$	
1618.7	$[\gamma + ANS(Na) + NH_4]^+$	
1644.8		$[\gamma + TNS(NH_4) + NH_4]^+$

α refers to the α-Cdx molecular weight; β refers to the β-Cdx molecular weight, and γ refers to the γ-Cdx molecular weight. The detected counter ion for the ANS and TNS molecules are reported in brackets.

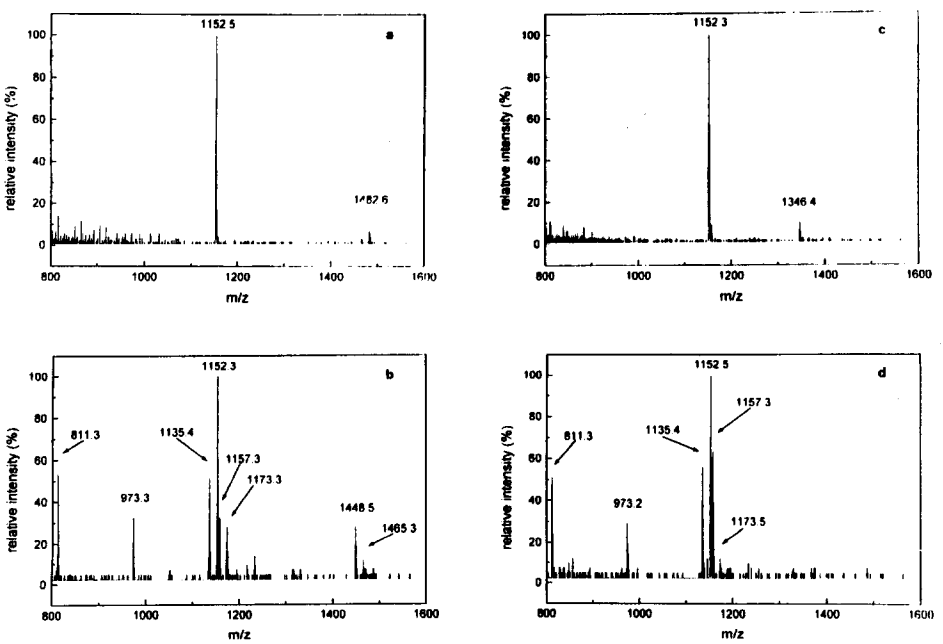


Fig. 2. Positive detection mode ISMS of the mixture β-Cdx and TNS at OR = 50 V (a) and OR = 110 V (b), and of the mixture β-Cdx and GlcA at OR = 50 V (c) and OR = 110 V (d). [β-Cdx] = [TNS] = [ANS] = 0.02 mM.

the protonated β-Cdx, and to its sodium and potassium adducts, respectively. The fragmentation of the β-Cdx was indicated by the presence of the peaks at 811.3 and 973.3 amu. The complex β-Cdx–TNS (Fig. 2b) was present in the protonated form (1448.5 amu) and as the ammonium adduct (1465.3 amu). This result indicated that the complex β-Cdx–GlcA is really an electrostatic adduct and not an inclusion complex, since the increase of the orifice potential is sufficient to break the complex. On the contrary, the presence of the complex β-Cdx–TNS at OR = 110 V or higher (data not shown) was persistent, thus suggesting more specific inclusion complexation involving higher association constants.

The exclusive use of ionspray mass spectrometry to determine the capacity of a molecule to form an inclusion complex with cyclodextrins is appealing, especially for the little amount of sample needed and the relatively short time required to perform these experiments. However, in the absence of other experimental data that indicate the existence of a particular inclusion complex, the conclusions inferred from the observation of the ionspray mass spectrum may not be correct, because of the presence of electrostatic adducts. The experimental approach of increasing the voltage at the orifice, described in this paper, could be successfully applied in order to unmask these false positives.

Interesting information about the interaction pattern of cyclodextrins with either ANS or TNS could also be obtained by operating the mass spectrometer in the negative ion mode. First of all, we expected that both the ANS and TNS complexes with cyclodextrins would be characterised by more intense ion peaks. In fact, since both ANS and TNS are negatively charged ions, the sensitivity of the complexes is very high. On the contrary, in the positive mode the occurrence of positively charged molecular species implies the presence of two positive charges, one to neutralise the fixed negative charge of either ANS or TNS, and the second to render the complex positively-ionised in order to be detected. The above consideration may explain the low intensities of the mass peaks relative to the complexes of either ANS or TNS with cyclodextrins when detected in the positive ion mode.

The mass spectra of the mixture of α-Cdx, β-Cdx, and γ-Cdx with ANS or TNS obtained in the negative mode are shown in Fig. 3a and b, respectively. The complete assignment of the ion peaks is reported in Table 2 for both the ANS and TNS experiments. The intensities of the peaks corresponding to the complexes are higher than those obtained in the positive mode (see Fig. 1). In addition to this, the spectrum of the mixture containing ANS shows a pattern different from that obtained in the presence of TNS. In the presence of TNS, the peak relative to the complex β-Cdx–TNS is by far the more intense among the peaks relative to the other complexes, i.e. α-Cdx–TNS and γ-Cdx–TNS. This observation is in agreement with the experimental findings obtained by means of independent techniques like fluorescence, circular dichroism, and isothermal microcalorimetry [8], which clearly showed that TNS forms more stable inclusion complexes with β-Cdx than with other cyclodextrins. For the sake of clarity, it has to be specified that the preferred interaction of ANS with γ-Cdx was not detected by means of mass spectrometry. This fact could be explained by the different experimental conditions (50% aq acetonitrile) needed for mass spectrometry experiments; in fact, the less polar acetonitrile co-solvent might have some influence on the cavity of the γ-Cdx, which is

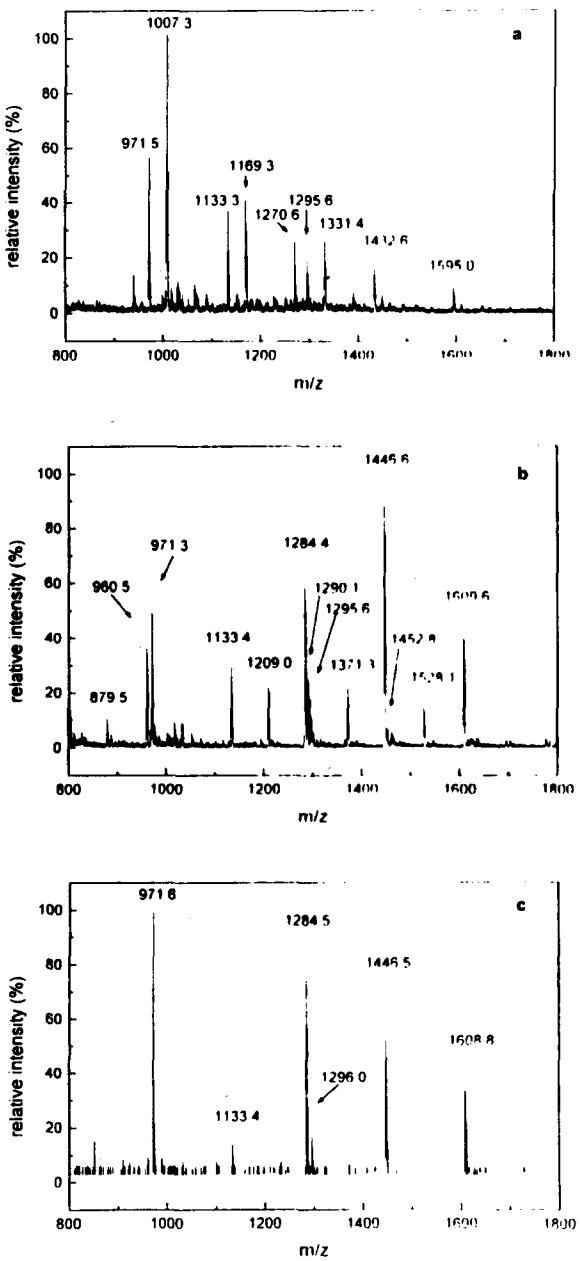


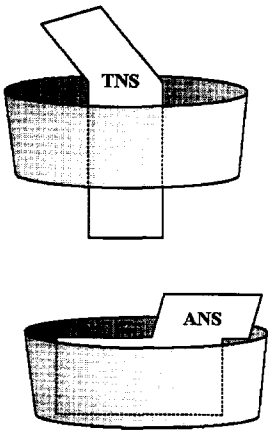
Fig. 3. Negative detection mode ISMS of α-Cdx, β-Cdx, and γ-Cdx with ANS at OR = −70 V (a), α-Cdx, β-Cdx, and γ-Cdx with TNS at OR = −70 V (b), and of α-Cdx, β-Cdx, and γ-Cdx with TNS at OR = −130 V (c). The concentrations were 0.33 mM for each cyclodextrin and 1 mM for either ANS or TNS.

Table 2
Assignment of the observed ions for the experiments carried out on the mixtures of α-Cdx, β-Cdx, and γ-Cdx with either ANS or with TNS, performed in the negative ion mode

m/z	α-, β-, γ-Cdx + ANS	α-, β-, γ-Cdx + TNS
879.5		$(\beta + 2TNS)^{2-}$
960.5		$(\gamma + 2TNS)^{2-}$
971.5	$(\alpha\text{-H})^-$	$(\alpha\text{-H})^-$
1007.3	$(\alpha + 2H_2O\text{-H})^-$	
1133.3	$(\beta\text{-H})^-$	$(\beta\text{-H})^-$
1169.3	$(\beta + 2H_2O\text{-H})^-$	
1209.0		$(\alpha + \beta + TNS\text{-H})^{2-}$
1270.6	$(\alpha + ANS)^-$	
1284.4		$(\alpha + TNS)^-$
1290.1		$(\beta + \beta + TNS\text{-H})^{2-}$
1295.6	$(\gamma\text{-H})^-$	$(\gamma\text{-H})^-$
1331.4	$(\gamma + 2H_2O\text{-H})^-$	
1371.3		$(\beta + \gamma + TNS\text{-}1)^{2-}$
1432.6	$(\beta\text{-ANS})^-$	
1446.6		$(\beta + TNS)^-$
1452.8		$(\gamma + \gamma + TNS\text{-H})^{2-}$
1528.1		$(\beta + \gamma + 2TNS)^{2-}$
1595.0	$(\gamma + ANS)^-$	
1609.6		$(\gamma + TNS)^-$

α refers to the α-Cdx molecular weight; β-Cdx molecular weight, and γ refers to the γ-Cdx molecular weight. ANS and TNS molecules are present with no counter ions.

larger than that of β-Cdx. In addition to the above observation, the spectrum obtained in the presence of TNS shows a number of peaks which were assigned to ternary complexes formed by two cyclodextrin molecules with the aromatic compound. Furthermore, the two cyclodextrin molecules involved in the complexation may be either of the same or of different type (e.g. β-Cdx/β-Cdx–TNS, β-Cdx/γ-Cdx–TNS, etc.); however, two α-Cdx molecules never belonged to the same complex, probably due to its small cavity. Similar evidence is not present in the spectrum relative to the mixture of α-Cdx, β-Cdx, and γ-Cdx with ANS, although the experimental conditions were exactly the same as those used in the TNS experiment. Molecular modelling calculations carried out for the TNS- and ANS–Cdx inclusion complexes [8] clearly showed that TNS enters into the Cdx cavity placing the long axis of the molecule parallel to the cylindrical axis of the cyclodextrin molecule (Scheme 1). This configuration leaves the toluidinyl moiety protruding out of the Cdx cavity being therefore available to complex a second cyclodextrin molecule. This possibility was already proposed by Kondo and co-workers [16]. The stacking phenomenon is not possible in the case of ANS–Cdx complexes, as far as can be inferred from molecular modelling calculations. In fact, the ANS molecule is less elongated than TNS and results well packed into the Cdx's cavity exhibiting the anilino-moiety interacting with one of the edges of the Cdx ring, and therefore not available for further complexation (see Scheme 1, ref. [8]). It is also well known that cyclodextrins molecules can self-associate to form channel-type structures (see for



Scheme 1.

example Saenger [1]). As a matter of fact, electrospray mass spectra of free cyclodextrins exhibiting ions corresponding to 2 or 3 cyclodextrins complexes have been reported in the literature [11]. Furthermore, the observation that the spectra recorded in the positive mode did not show multiple complexation of TNS could be related to the difficulty of obtaining triply charged complexes. In fact, in order to be included in the m/z range of instrument utilised in this study, multiple complexes could be only detected as doubly charged ions. However, since TNS bears a net negative charge, the actual number of positive charges must be three.

In order to exclude the presence of electrostatic adducts in the experiments carried out in the negative mode, spectra of the mixtures α-Cdx, β-Cdx, γ-Cdx and TNS, β-Cdx and TNS, and β-Cdx and GlcA were recorded while varying the OR from -70 V to either -130 or -180 V with -20 V steps. In the spectrum of α-Cdx, β-Cdx, γ-Cdx and TNS recorded at OR = -130 V (Fig. 3c) only the peaks corresponding to the free Cdx's and to the complexes of each Cdx with TNS in the molar ration of 1:1 were present. The higher voltage applied to the orifice resulted in the disappearance of the peaks corresponding to 2:1 and 1:2 complexes of Cdx and TNS, previously observed in the spectrum recorded at OR = -70 V (Fig. 3b). This finding can be easily explained by the lower stability of the complexes exhibiting a stoichiometry different from 1:1. The peak corresponding to the complex β-Cdx–TNS is no longer the most intense, but since the quantitative response of the mass spectrometer to the three cyclodextrins is not constant, it is better to look at the ratio Cdx–TNS/free Cdx. As it can be easily seen, this value is still higher for the β-Cdx–TNS complex than for the α-Cdx–TNS one.

The spectrum of the β-Cdx and TNS mixture recorded at OR = -180 V (Fig. 4b) showed that the complex β-Cdx–TNS is rather stable since its relative intensity did not decrease, while the sensitivity remained high, with respect to the experiment carried out at OR = -70 V (Fig. 4a).

The above experiment was also carried out on the β-Cdx and GlcA mixture (Fig. 4c–d). At OR = -70 V the intensity of the peak corresponding to the free β-Cdx was

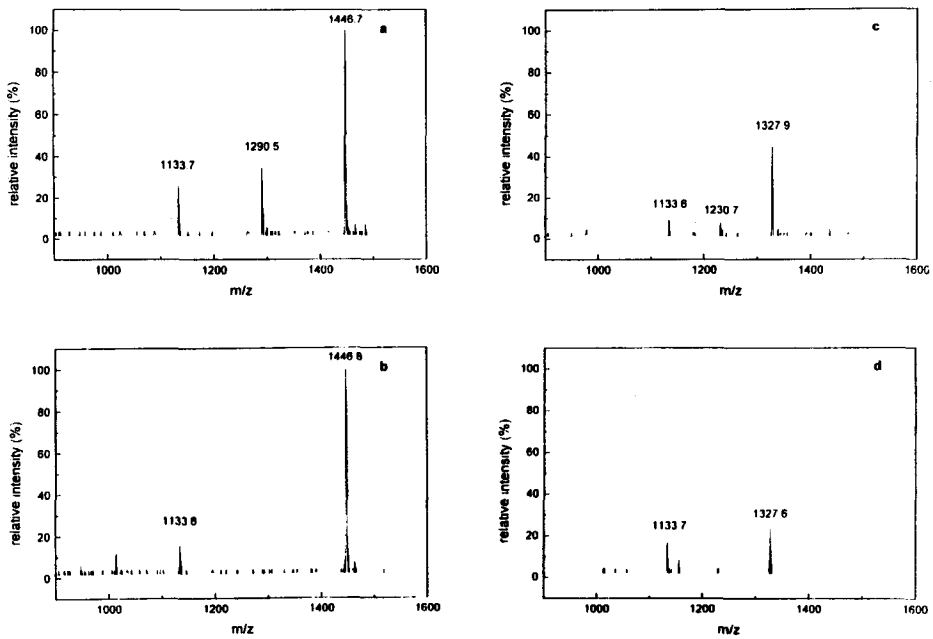


Fig. 4. Negative detection mode ISMS of the mixture β-Cdx and TNS at OR = −70 (a) and OR = −180 (b) and of the mixture β-Cdx and GlcA at OR = −70 (c) and OR = −180 (d). [β-Cdx] = [TNS] = [GlcA] = 1 mM.

almost one quarter of that relative to the complex β-Cdx–GlcA (peak at 1327.9 amu). The peak at 1230.7 amu refers to the complex β-Cdx/β-Cdx–GlcA which was not present in the spectrum recorded at OR = −180 V (Fig. 4d). Moreover, at OR = −180 V the intensity of the peaks corresponding to β-Cdx and β-Cdx–GlcA was almost the same. These results suggested that for higher values of OR, the complex β-Cdx–GlcA was disrupted with the consequent increase in the intensity of the free β-Cdx peak. The above data further indicated that the β-Cdx–TNS complex is far more stable than β-Cdx–GlcA which, from the experiments carried out in the positive mode, resulted from an electrostatic adduct. The lack of the complete disappearance of such a complex, as observed in the positive ion mode experiment, may be due to the presence of the fixed negative charge on the GlcA molecule. A similar persistent electrostatic adduct was observed in the positive mode in the case of β-Cdx–$NH4^+$, even for high values of voltage applied to the orifice.

In our opinion, in the present study two observations strongly point to the power of ionspray mass spectrometry. Firstly, the possibility of revealing the presence of specific complexes using a different orifice potential is very appealing, since it is a way to distinguish less stable adducts. Secondly, this soft ionisation technique led to the detection of the stoichiometric pattern of the Cdx's complexes with aromatic molecule revealing the presence of stoichiometries different from 1:1, in good agreement with molecular modelling calculations [8] and spectrofluorimetric data [16].

Acknowledgements

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References

[1] W. Saenger, *Angew. Chem. Int. Ed. Engl.*, 19 (1980) 344–362.
[2] J. Szejtli, *Carb. Polym.*, 12 (1990) 375–392.
[3] S.E. Brown, J.H. Coates, S.F. Lincoln, D.R. Coghlan, and C.J. Easton, *J. Chem. Soc. Faraday Trans.*, 87 (1991) 2699–2703.
[4] M. Suzuki, H. Takai, J. Szejtli, and E. Fenyvesi, *Carbohydr. Res.*, 201 (1990) 1–14.
[5] A. Munoz de la Pena, T.T. Ndou, J.B. Zung, K.L. Greene, D.H. Live, and I.M. Warner, *J. Am. Chem. Soc.*, 113 (1991) 1572–1577.
[6] S. Hashimoto and J.K. Thomas, *J. Am. Chem. Soc.*, 107 (1985) 4655–4662.
[7] K. Kano, *Inclusion Phenomena Molecular Recognition*, J.L. Atwood (ed.), Plenum NY, 1991, pp 243–249.
[8] V. Crescenzi, A. Gamini, A. Palleschi, and R. Rizzo, *Gazz. Chim. Ital.*, 116 (1986) 435–440.
[9] M. Kowblansky, *Macromolecules*, 18 (1985) 1776–1779.
[10] M. Vincenti, *J. Mass Spectrom.*, 30 (1995) 925–939.
[11] P. Camilleri, N.J. Haskins, A.P. New, and M.R. Saunders, *Rapid Commun. Mass Spectrom.*, 7 (1993) 949–952.
[12] R. Ramanathan and L. Prokai, *J. Am. Soc. Mass Spectrom.*, 6 (1995) 866–871.
[13] J.B. Cunniff and P. Vouros, *J. Am. Soc. Mass Spectrom.*, 6 (1995) 437–447.
[14] D.J. Harvey, *Rapid Commun. Mass Spectrom.*, 7 (1993) 614–619.
[15] R. Bakhtiar and S. Bulusu, *Rapid Commun. Mass Spectrom.*, 9 (1995) 1391–1394.
[16] H. Kondo, H. Nakatani, and K. Hiromi, *J. Biochem.*, 79 (1976) 393–405.