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Non-covalent associations of cyclomaltooligosaccharides (cyclodextrins) with carotenoids in water. A study on the α- and β-cyclodextrin/ψ,ψ-carotene (lycopene) systems by light scattering, ionspray ionization and tandem mass spectrometry☆

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Abstract

Water-soluble complexes of the dietary carotenoid ψ,ψ-carotene (lycopene **1**) with cyclomaltohexaose (α-cyclodextrin, αCD) and cyclomaltoheptaose (β-cyclodextrin, βCD) have been prepared and characterized via multiangle light scattering (MALS), ionspray/electrospray ionization (IS/ESI) mass spectrometry (MS) and tandem MS. MALS experiments point out that large aggregates of particles, on the nanometer-size scale, are present in water, with meaningful differences in the shape of the αCD/**1** aggregates with respect to βCD/**1** analogues. The true 1:1 αCD/**1** inclusion complex has been observed by IS/ESIMS and confirmed by tandem MS. The structure of CD/**1** aggregations in water is proposed which are consistent with the combined MALS and MS experimental results.

Keywords: Cyclodextrins; Dietary carotenoids; Electrospray ionization/ionspray mass spectrometry; Tandem mass spectrometry; Inclusion complexes; Light scattering; Lycopene; Nanoparticles

1. Introduction

The natural cyclomaltooligosaccharides (cyclodextrins, CDs) are concave macrocycles capable of expressing molecular recognition by the formation of noncovalent inclusion (or host–guest) complexes with suitably sized lipophilic molecules hosted into their cavity. CD complexes are currently used in the pharmaceutical, cosmetic and food industries, where they can: (i) enhance the water solubility of lipophilic guests; (ii) control the release of volatile guests; and (iii) protect labile guests from degradation promoted by external agents (e.g., air and light).[1–3]

Carotenoids are a class of natural, highly lipophilic, air- and light-sensitive substances, among which ψ,ψ-carotene (lycopene, **1**), *trans*,*trans*-β-carotene (**2**), and those essential to vision, lutein (**3**) and zeaxanthin (**4**), are most important dietary compounds.[4,5]

Dietary carotenoids are relevant members of the nonenzymatic pool of low-molecular-mass free-radical scavengers that are relegated to cell defense against endogenous and exogenous reactive oxygen species (ROSs, e.g., superoxide ions, hydrogen peroxide, hydroxyl radicals, nitrous oxide, etc.).

Recent literature reports show increasing interest in the roles played by redox phenomena within cells in some fundamental aspects of life, such as aging and age-related diseases, including their pathogenesis.[6] The effects of endogenous and exogenous ROSs are indeed limited by either enzymatic scavengers (superoxide dismutase, catalase etc.) or low-molecular-mass molecules (flavonoids, carotenoids, glutathione, vitamin C, etc.). The complex equilibrium between ROS and anti-ROS defenses determines the level of oxidative stress. There

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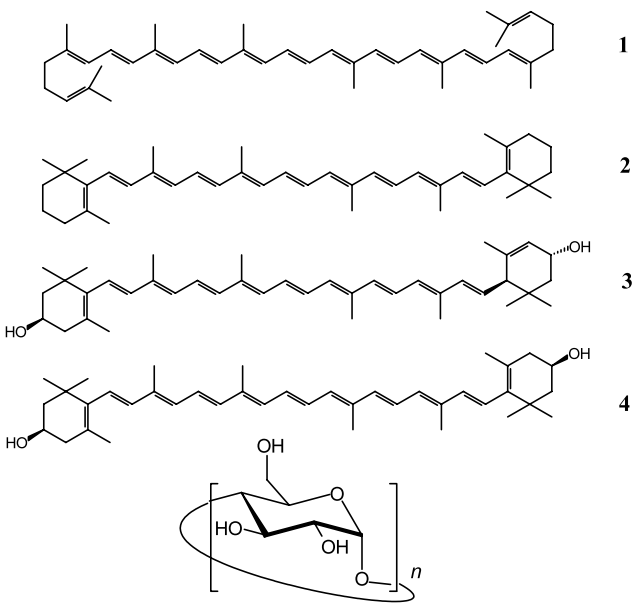

n=6 α-cyclodextrin (αCD)
n=7 β-cyclodextrin (βCD)
n=8 γ-cyclodextrin (γCD)

is experimental evidence that oxidative stress is involved in the mechanism of cell aging and the chemical modification of cellular proteins and DNA.[6]

The generally elevated hydrophobicity and sensitivity of carotenoids to external agents, such as air and light, may constitute a serious problem for bioavailability, formulation, and manipulation of carotenoid-based drugs and cosmetics. Hence, many efforts have been made in order to boost properties such as compatibility with polar solvents, typically water, and resistance to light or air-mediated oxidation. Two different approaches are reported in the literature: (i) molecular encapsulation of carotenoids into the cavity of CD in an aqueous medium,[7–9] or (ii) preparation of nanosized carotenoid hydrosols by polymer- or surfactant-controlled precipitation from water–alcohol mixtures.[10,11]

In Part 1 of this paper,[7] we described the preparation of quite stable, intensely pink-colored water solutions of **2** with βCD or γCD (Structures **1**–**4**). These solutions were able to retain in the aqueous phase most of **2** upon extraction with apolar organic solvents.[12] NMR and light-scattering experiments provided clues that such CD/**2** non-covalent associations in water form large aggregates, likely micelles.[7] We are presently extending the preparation and characterization of CD complexes with other dietary carotenoids, at first with **1**, which is the most attractive one, as it exhibits the highest relative ease of one electron transfer, i.e., the strongest reducing properties, among the current dietary carotenoids and vitamin E.[4]

The present study has two main and complementary purposes: (i) to get insights into the mechanism of formation of aggregates of CDs with highly hydrophobic carotenoids in water. Indeed, the therapeutic intake of antioxidants, and carotenoids especially, is still under investigation. Any improvement in the bioavailability of carotenoids will be beneficial for either clinical trials or knowledge of their in vivo antioxidant activity;[6,13,14] and (ii) to assess analytical and structural tools for a correct characterization of carotenoid/CD adducts.

2. Results and discussion

Light scattering.—Our previous study on complexes between CDs and **2**,[7] has demonstrated that multi-angle light scattering (MALS) allowed one to obtain an estimation of the dimension of the particles in solution. Furthermore, the dimension of the particles could also be determined even at unknown, but very low concentrations. This result is particularly remarkable when the sample is only partially soluble, and/or part of the sample remains on the filter. An identical method to the previous study has been used for the characterization of complexes between CDs and **1**.

As usual, following Zimm[15] the fundamental equation of a MALS experiment is the following:

$$\frac{K\cdot c}{R(\theta)} = \frac{1}{M_w\cdot P(\theta)} + 2A_2\cdot c \qquad (1)$$

where $R(\theta)$, Rayleigh factor, is related to the intensity of the scattering, θ is the angle between the detector and the primary incident light, $P(\theta)$, form factor, is related to the angular variation of the scattering and c denotes the concentration of the solution. From a MALS experiment, we could obtain three important molecular parameters: M, A_2 and $\langle s^2\rangle^{1/2}$. M denotes the molar mass, the weight-average M_w for a polydisperse sample; A_2 is the second virial coefficient; $\langle s^2\rangle^{1/2}$ is the dimension of the particles, i.e., the root-mean-square radius. A description of the other parameters of Eq. (1) can be found in Part 1 of this work.[7]

To demonstrate the presence of large particles of complexes in solution, we have used the dimension $\langle s^2\rangle^{1/2}$ which is related to the form factor $P(\theta)$. A large particle cannot be considered as a single point of scattering, and consequently, destructive interference of the scattered light can occur. The intensity of the scattering, $\theta > 0°$, in the presence of destructive interference is lower and depends on the angle θ, the shape, and the dimension of the particles. The form factor $P(\theta)$ was introduced to account for destructive interference effects. Debye showed[16] that the reciprocal of the form factor $P(\theta)^{-1}$ may be approximated by:

$$P(\theta)^{-1} = 1 + 1/3\mu^2\langle s^2\rangle + \ldots \qquad (2)$$

where $\mu = (4\pi/\lambda)\sin(\theta/2)$ is a function of the angle θ, wavelength λ and $\langle s^2\rangle^{1/2}$. Hence, we can estimate the

dimension $\langle s^2\rangle^{1/2}$ from the initial slope of the $P(\theta)$ versus $\sin^2(\theta/2)$ plot.

At least from a qualitative point of view, the CD/**1** complexes were more soluble with regard to the CD/**2**

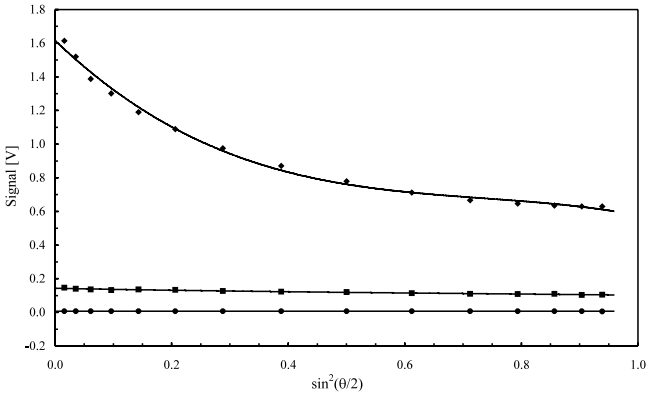

Fig. 1. Comparison of the intensity of scattering of three samples: (●) α-cyclodextrin; (◆) α-cyclodextrin/lycopene, 0.45 μm filter; (■) α-cyclodextrin/lycopene, 0.20 μm filter.

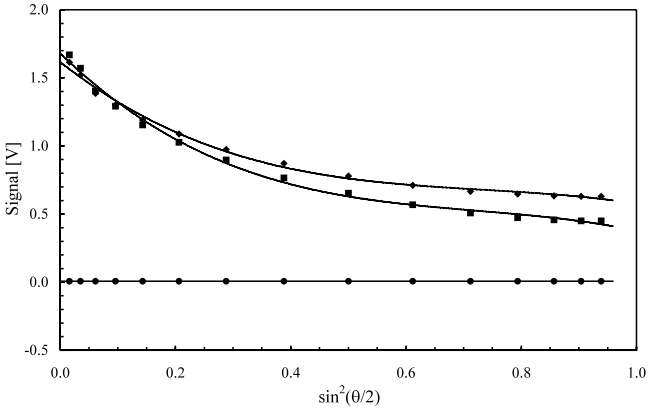

Fig. 2. Comparison of the intensity of scattering of three samples, 0.45 μm filter: (●) α-cyclodextrin; (◆) α-cyclodextrin/lycopene; (■) β-cyclodextrin/lycopene.

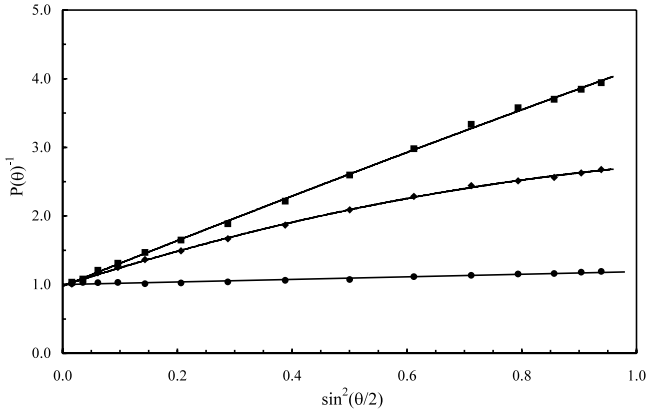

Fig. 3. $P(\theta)^{-1}$ vs. $\sin^2(\theta/2)$ plot of three samples: (●) α-cyclodextrin; (◆) α-cyclodextrin/lycopene; (■) β-cyclodextrin/lycopene.

Table 1
Root-mean-square radius $\langle s^2\rangle_z^{1/2}$, of αCD/**1** and βCD/**1** complexes filtered through a 0.45 μm filter

Complex	$\langle s^2\rangle_z^{1/2}$ (nm)
αCD/**1**	109.6 ± 1.5
βCD/**1**	116.1 ± 0.7

complexes previously investigated.[2] Apparently, the CD/**1** complexes were entirely soluble and did not obstruct the 0.45 μm nor 0.2 μm filter. This is an important difference with the CD/**2** complexes, which entirely remained on the 0.2 μm filter. Fig. 1 shows a comparison of the scattering of three solutions whose nominal concentration was approximately 0.1 mg/mL: (i) αCD used as reference (●); (ii) αCD/**1** complex filtered through a 0.45 μm filter (αCD/**1**, ◆); (iii) αCD/**1** complex filtered through a 0.2 μm filter (■). The scattering of the 0.45 μm solution with respect to the αCD solution is impressive. Furthermore, also the scattering of the 0.2 μm αCD/**1** solution is meaningful as it means that a considerable part of the particles can remain in solution even after such a filtration.

The behavior of both αCD and βCD complexes with **1** after 0.45 μm filtration is illustrated by the plots of Fig. 2 (see caption for details). It is pointed out in Fig. 1 that **1** is able to form soluble complexes with both αCD and βCD hosts. Besides the pattern of the angular variation of the scattering of the two complexes, αCD/**1** and βCD/**1** are quite different. This means that the particles differ for both dimension and shape.

Like in the previous study,[7] with regard to the dimensions of the complexes, we can also estimate some quantitative results. Fig. 3 shows the $P(\theta)^{-1}$ versus $\sin^2(\theta/2)$ plot for three solutions. The filled circles refer to the reference αCD solution, filled diamonds to the αCD/**1** complex and filled squares to the βCD/**1** complex. The value of $\langle s^2\rangle^{1/2}$ was estimated for the two different complexes by using Eq. (2). It is well known that for a polydisperse sample MALS estimates the z-average of the dimension of the particles $\langle s^2\rangle_z^{1/2}$. The $\langle s^2\rangle_z^{1/2}$ values of the two complexes are reported in Table 1.

The $\langle s^2\rangle_z^{1/2}$ value of the two complexes is very high and demonstrates the presence of large aggregates. Besides, the difference between the $\langle s^2\rangle_z^{1/2}$ values of the two complexes, ca. 6%, is not meaningful. This result is apparently strange because, as pointed out previously, the angular pattern of the scattering of the two complexes is different. This difference is particularly evident in the Fig. 3. The $\langle s^2\rangle_z^{1/2}$ value is estimated from the initial slope of the $P(\theta)^{-1}$ versus $\sin^2(\theta/2)$ plot. Obviously, in this case, the initial slope for the two complexes was substantially equal, and considering the

different angular pattern of the scattering, a trivial conclusion is that the shape of the aggregates is different. The analysis of the $P(\theta)^{-1}$ versus $\sin^2(\theta/2)$ plot confirms that the shape of the βCD/**1** complex is more compact with regard to the αCD/**1** complex. Moreover, an accurate analysis of the plot for the latter complex highlights a meaningful downward curvature. In general, in dilute polymeric solutions the origins of the curvature could be various: polydispersity, excluded volume, and stiffness of the macromolecules. In our specific case, the curvature of the αCD/**1** plot probably means the contemporary presence of large and small particles. In other words, we can conclude that the particles of the βCD/**1** complex are more homogeneous in dimension than those of the αCD/**1** complex.

Mass spectrometry.—The solutions containing αCD/**1** and βCD/**1** complexes were analyzed by electronebulization mass spectrometry (MS), namely ionspray (IS) or electrospray ionization (ESI), with a triple quadrupole or an ion-trap analyzer, respectively. The structural features of the ions related to supramolecular associations of **1** and CDs were investigated by tandem MS, which has already proved to be a useful approach for the study of gaseous charged non-covalent associations and host–guest systems.[17] Independently of the ionization method (IS or ESI) and of the analyzer type (triple quadrupole or ion trap) employed, significant and substantially identical MS and tandem MS results were obtained by operating only in the negative-ion mode, whilst the positive-ion mode failed to provide any useful response of **1**–CDs associations. This fact confirms what was reported in one of the first attempts to exploit electronebulization MS sources for the analysis of water dispersions of **2** and CDs.[18] Since the practical identity of IS and ESI spectral features, only the IS data are reported herein. Fig. 4 displays a detail of the negative-ion polarity MS spectrum obtained after electronebulization of a solution containing the αCD/**1** complex (see Section 3). The peak at m/z 1507.0 is consistent with the 1:1 deprotonated association of αCD with **1** $[\alpha CD/\mathbf{1} - H^+]^-$. The large shape ($\sim 2$ amu, full width at half height, FWHH) of this peak, however, suggests that it should be an envelope of overlapped signals having mass differences less than unit and thus remaining unresolved by the low-resolution settings of the analyzers, probably due to the isotopic peaks of multicharged associations, e.g., $[n\alpha CD/\mathbf{1} - nH^+]^{n-}$ species, in which the αCD/**1** molecular ratio remains 1:1 (see above). Stronger evidence for the presence of such $[n\alpha CD/\mathbf{1} - nH^+]^{n-}$ species could have been achieved by resolving the natural isotope peaks with a high-resolution instrument, which, unfortunately, was not available to us. Analogously, the quite broad signal (~ 1 amu, FWHH) centered at 534.2 Th (Fig. 4, bottom) is consistent with an unresolved cluster of peaks due to either $[n\mathbf{1} - nH^+]^{n-}$ and probably also to oxidized species such as the radical anion $[\mathbf{1} - H_2]^-$. As observed by a referee "oxidation/reduction is a more likely cause, but it is not easy to prove". The tandem MS collision induced decomposition (CID) of m/z 1507.0 parent ion (Fig. 5) sheds light on this point by showing the predominant fragment ion of m/z 971.0 (52% relative abundance with respect to the base peak at m/z 1507.0), assigned to deprotonated αCD, i.e., $[\alpha CD - H^+]^-$. The mass difference of 536 Da between the precursor and m/z 971.0 fragment corresponds to the loss of one neutral molecule of **1**, and, therefore, it strongly supports the presence of gaseous 1:1 deprotonated αCD/**1** association within the m/z 1507.0 parent ion. Moreover, the CID spectrum of this parent ion (Fig. 5) provides direct evidence for the above-suggested presence of $[n\alpha CD/\mathbf{1} - nH^+]^{n-}$ multicharged clusters and also of oxidized species of **1**, e.g., the radical anion $[\mathbf{1} - H_2 + e^-]^-$, as the fairly abundant daughter ion of m/z 1069.5 (30% relative abundance) is consistent with self-associations of **1** such as $[\mathbf{1} + (\mathbf{1} - H_2) + e^-]^-$ (nominal mass 1070 Da) and/or $[(\mathbf{1} - H^+) + (\mathbf{1} - 2H)]^-$ (nominal mass 1069 Da). This proves that the m/z 1507.0 parent ion encloses more than one molecule of **1**; therefore, $[n\alpha CD/\mathbf{1} - nH^+]^{n-}$ multicharged clusters of 1:1 αCD/**1** molecular ratio must be present within the m/z 1507.0 parent ion.

These findings, however, do not rule out different types of aggregates. The odd-electron charged species generated by the electronebulization process are expected to be highly reactive and may lead, in line of principle, to covalent adducts of αCD, such as $[\alpha CD + \mathbf{1} - 2H^+]^-$, $(C_{76}H_{114}O_{30})^-$. The corresponding peak in the mass spectrum is expected at a nominal m/z value of 1506 Th, which, at the actual resolution settings, would be *de facto* overlapped to the signal due to the genuine inclusion complex of deprotonated αCD with neutral **1** at m/z 1507. Thus, the peak at m/z 1507.0 could represent the superimposition of at least three different classes of aggregates: (i) true 1:1 host–guest αCD/**1** complexes; (ii) a collection of $[n\alpha CD/\mathbf{1} - nH^+]^{n-}$ multicharged clusters with 1:1 αCD/**1** molecular ratio; and (iii) possibly covalent adducts generated by the reaction of charged odd-electron derivatives of **1** and neutral αCD during the electronebulization process. These results indicate that the large aggregates in water described above in the subsection on light scattering are likely to be composed of an inner shell made of **1** only and an outer shell made of CD molecules, right at the solvent interface. The formation of the host–guest αCD/**1** complex, and of possible covalent reaction products of αCD with activated derivatives of **1**, is thus expected to occur at the inner/outer shell interface.

The βCD/**1** complex behaved differently with respect to the αCD/**1** analogue. The negative-ion polarity full scan IS spectrum showed an interesting peak at m/z 1701.5 that could be consistent with $[\mathbf{1} + \beta CD +$

$MeOH - H^+]^-$ multicomponent adduct, which is analogous to our previous assignment on the homologous system βCD/**2**.[18] However, tandem MS experiments on their parent ion (Fig. 6) gave only the fragment at m/z 1133, assigned to deprotonated βCD, i.e., $[\beta CD - H^+]^-$. This points out that the ionic species at m/z 1701.5 is indeed $[(\beta CD)_3 - 2H^+]^{2-}$, i.e., a doubly charged βCD cluster, which can be obtained as well from a sample of pure βCD (data not reported here). This finding also provides us with the opportunity to revise the wrong assignment suggested preliminarily[18] for the peak at m/z 1701 at an early stage of the work, without the support of tandem MS experiments.

The issue of the striking difference in the mass spectrometric response of the αCD/**1** complex with respect to the βCD/**1** analogue should be correctly addressed and deserves further investigation. Indeed, experimental evidence for the presence of 1:1 complexes could be collected only in the case of the αCD/**1** adduct, despite

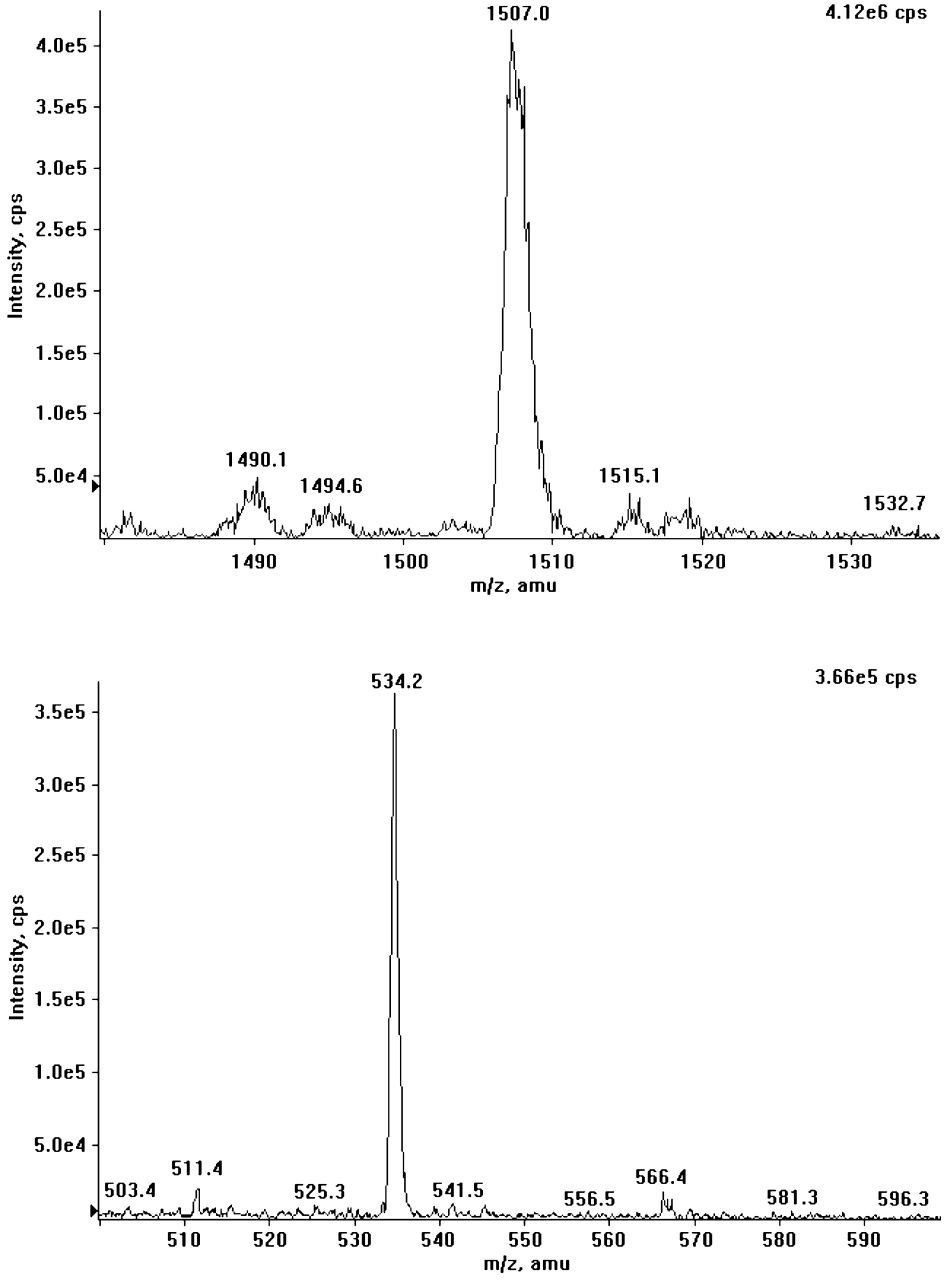

Fig. 4. Top: detail of negative-ion mode ionspray mass spectrum of the αCD/lycopene complex showing the peak at m/z 1507.0 (for assignments see *Mass spectrometry* in Section 2). Bottom: detail of negative-ion mode ionspray mass spectrum of the αCD–lycopene complex in 1:1 water–acetonitrile solution, showing the peak at m/z 534.2 (for assignments see *Mass spectrometry* in Section 2).

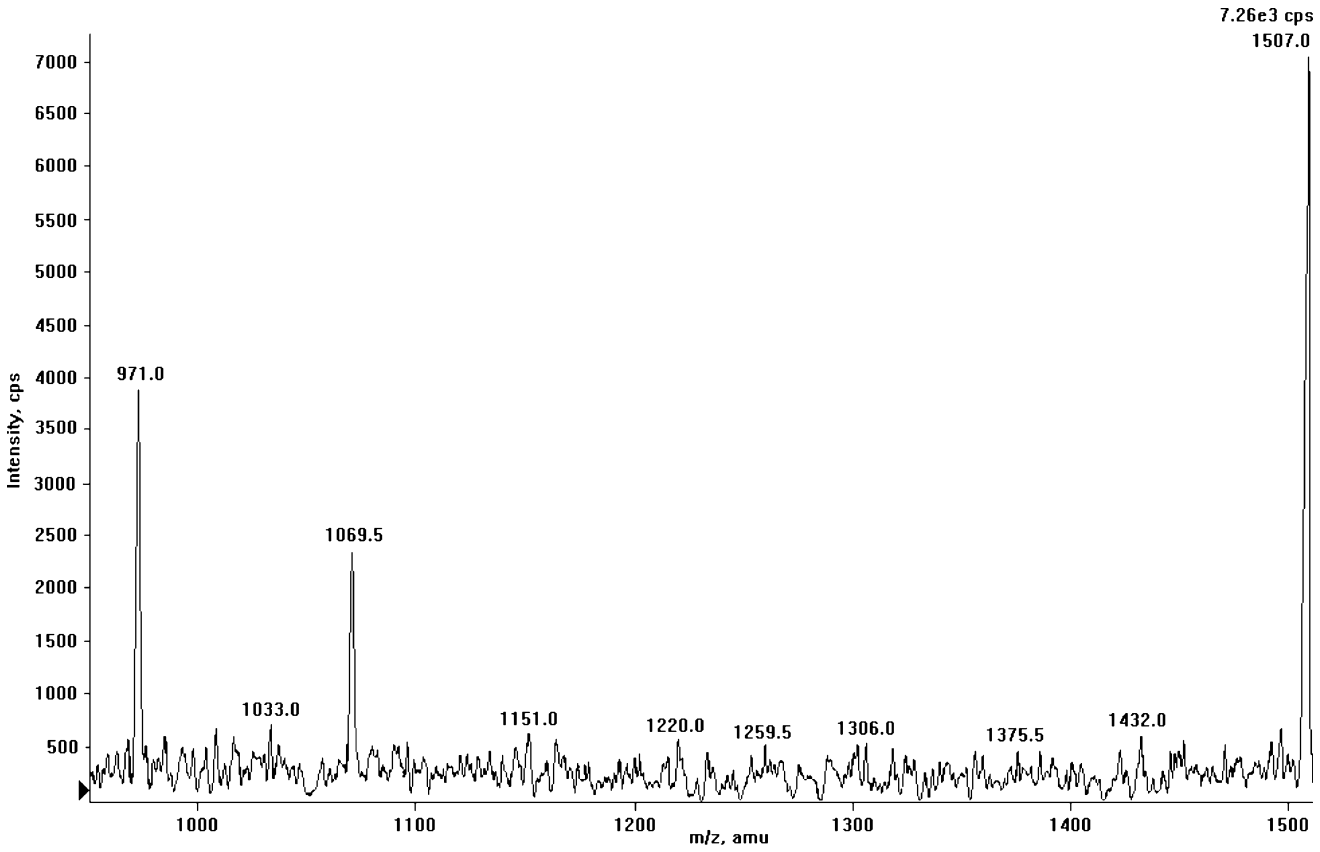

Fig. 5. Tandem MS collision-induced dissociation spectrum of m/z 1507 parent ion generated by the negative-ion mode ionspray ionization of the αCD/lycopene complex in 1:1 water–acetonitrile solution (see *Mass spectrometry* in Section 2 for details).

the smaller cavity diameter of αCD with respect to βCD. Nevertheless, the data do not rule out a participation of the 1:1 inclusion mechanism to the formation of the βCD/**1** complex, but rather suggest that only the 1:1 αCD/**1** complex is able to survive the charging and desolvation/declustering processes within the ion source.

In conclusion, the experimental results reported in the present work point out that both αCD and βCD are capable of giving raise to stable associations with highly apolar lycopene in water by a collection of different mechanisms, the formation of stoichiometric inclusion complexes providing only one of the possible contributions. Other possible types of associations could be generated by the assembly of nanoparticles of **1** surrounded by a shell of CD molecules acting as an amphiphilic solvating agent. This hypothesis is substantiated by the MALS data of the previous subsection, indicating that aggregates of the nm size scale of both αCD/**1** and βCD/**1** are present in solution. Experimental evidence for the presence of 1:1 complexes, however, could be collected only for the αCD/**1** adduct, suggesting that simple arguments based on the steric hindrance of host and guest were oversimplified. The detection of stoichiometric CD/**1** complexes in the case of αCD only could be related at least to two factors: (i) the different relative stability of the 1:1 complexes of αCD and βCD with compound **1**; and (ii) the relative importance of two deeply different mechanisms contributing to the stability of the aggregates in water: first, the formation of genuine inclusion complexes of defined stoichiometry and, second, the assembly of small particles of **1** coated by CD at the interface between the particles themselves and bulk water.

3. Experimental

Materials and preparation of the samples.—Lycopene **1** was extracted from ripe hips of *Rosa rubiginosa* by an isolation procedure previously described.[19] Both αCD and βCD were purchased from Aldrich Chemical Co. and were used without any further purification. The complexes were prepared by mixing **1** with αCD or βCD in 1:4 molar ratio and dissolving the mixtures in water, following the same procedure reported previously for the preparation of CD/**2** complexes.[7] Freeze-drying of the water solutions gave pale-pink solid samples of the αCD/**1** and βCD/**1** complexes.

Mass spectrometry.—Mass spectra were recorded on a Perkin–Elmer Sciex API 2000 triple quadrupole mass spectrometer with a TurboIonSpray™ source and on a Bruker Esquire 3000 ion-trap mass spectrometer (ITMS) with external ESI source. The solutions for mass spectrometric analysis were prepared by dissolving an aliquot of freeze-dried complex in mixtures of 1:1 water–MeOH or 1:1 water–MeCN. The samples were introduced into the ion source by an infusion pump operating with a flow rate of 5 μL/min. Typical instrument settings for the triple quadrupole were as follows: source potential at 5600 V, declustering potentials ranging from 20 to 50 V, resolution of 0.7 amu full width at half height (FWHH) on the first and third quadrupoles. Tandem MS experiments were performed with a LINAC (Concorde, Canada) collision cell with N_2 as collision gas at the nominal pressure of 8 mTorr (1 Torr = 133.3 Pa). For ESI-ITMS and related tandem MS experiments, nitrogen was used as the nebulizer gas at a pressure of 9 psi and as dry gas at a flow rate of 5 L/min at temperature of 200 °C. Capillary exit (CapEx) was −82 V, cut-off 50 m/z and accumulation time 2500 μs. Fragmentation was performed using cut-off 160 m/z and amplitude 0.6 V.

Light scattering.—Measurements were performed by a multi-angle laser light scattering (MALS) photometer Dawn DSP-F from Wyatt (S. Barbara, CA, USA) in water at rt. Details of the MALS hardware and software have been described in detail elsewhere.[20] The experimental methodology followed the scheme of a previous study on similar complexes between CDs and **2**.[7] The MALS photometer uses a vertically polarized laser of 632.8 nm of wavelength and simultaneously measures the intensity of the scattered light at 15 fixed angular locations in the range 14.5–171.1° in water. Measurements were performed using the K5 flow cell to reduce the scattering volume. As in the previous study, the instrument was calibrated using toluene, and the angular normalization of the 15 photodiodes was carried out by a concentrated solution of bovine serum albumin (BSA) globular protein. Each solution was prepared by mixing a weighed amount of the sample with the solvent. One half of each solution was filtered through a 0.2 μm cellulose acetate filter, and the residual solution was filtered through a 0.45 μm filter.

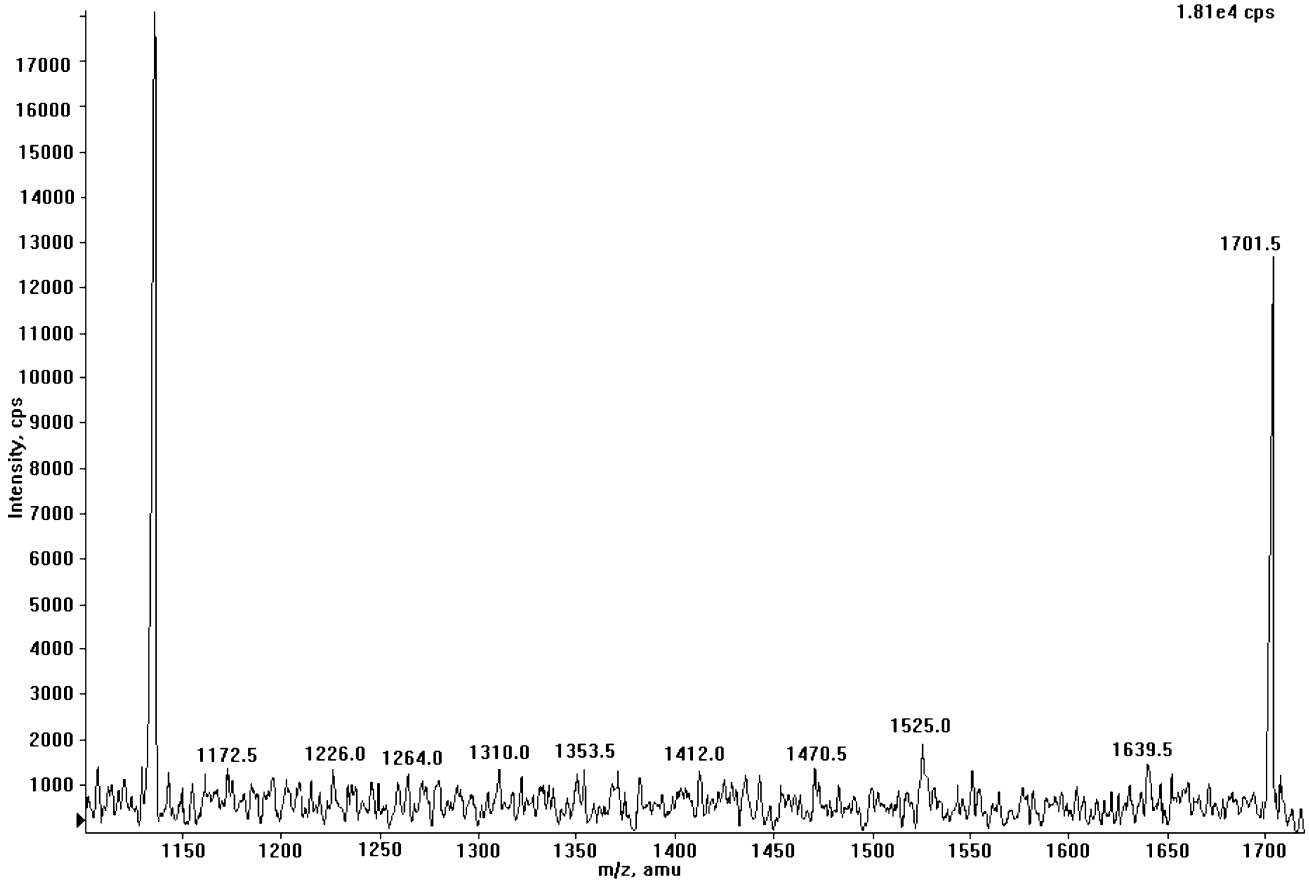

Fig. 6. Tandem MS collision-induced dissociation spectrum of m/z 1701.5 parent ion generated by the negative-ion mode ionspray ionization of the βCD/lycopene complex in 1:1 water–acetonitrile solution. The parent ion at m/z 1701.5, corresponding to $[(\beta CD)_3]^{2-}$ affords only the fragment of m/z 1133.0, assigned to $[\beta CD - H^+]^-$.

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