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Structural and physicochemical characterization of the inclusion complexes of cyclomaltooligosaccharides (cyclodextrins) with melatonin

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Abstract

The stoichiometry, geometry, stability, and solubility of the inclusion complexes of melatonin (MLT) with native cyclomaltooligosaccharides (α-, β- or γ-cyclodextrins, CDs) are determined experimentally by high-resolution NMR spectroscopy, calorimetric and solubility measurements, and mass spectrometry. The observed differences are discussed in terms of molecular recognition expression of the host–guest (h–g) interactions within the hydrophobic CDs cavities of different size. The 1:1 h–g stoichiometry in water solution prevails at low CD concentrations; the trend to form higher order associations is observed at increasing CD concentrations. The stability order β-CD > γ-CD > α-CD for the complexes in water solution and β-CD > α-CD > γ-CD for the protonated or alkali-cationated complexes in the gas phase are rationalized on the grounds of the structural data from NMR spectroscopy and of the thermodynamic parameters from calorimetric measurements. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: Melatonin; Cyclodextrins; Inclusion complexes; NMR; Calorimetry; Solubility; Mass spectrometry

1. Introduction

The non-covalent inclusion or host–guest (h–g) complexes of drugs with the natural cyclomaltooligosaccarides (cyclodextrins, CDs),[1] mainly those with β-CD, which is approved as a food additive and is available commercially at low cost, find current application in pharmaceutical preparations.[2] Indeed, substantial improvements of aqueous solubility, stability, bioavailability, release control, and palatability of an included drug are likely achieved by such complexes and some related multicomponent associations.[3–5]

Melatonin (MLT) is the main hormone secreted by the pineal gland of mammals; its structure (*N*-acetyl-5-methoxytryptamine) was determined by Lerner and coworkers in 1958.[6] The occurrence of MLT in bacteria,[7] protista,[8] plants,[9] and mammals[6,10] strongly suggests that this simple molecule explicates important biological activities linked to vital processes in virtually all these organisms. In mammals, MLT is mainly produced by the pineal gland during the hours of darkness, and it plays an important role as a regulator of sleep and seasonal reproductive cycles. MLT is produced also by other organs, e.g., retina,[11] lens,[12] Harderian gland,[13] ovary,[14] testes,[15] and bone marrow,[16,17] and it can mediate several cellular, neuroendocrine, and physiological processes.[10] Moreover, MLT raised current interest as a potent free-radical scavenger and as an established endogenous antioxidant.[18] For instance, MLT is shown to be five times more effective than glutathione as a scavenger of hydroxyl radicals,[19] twice more effective than vitamin E in inactivating water-sol-

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uble peroxy radicals,[20] and as much as 500 times more powerful than dimethyl sulfoxide in protecting from radiation-induced chromosome damage.[21] In addition, the reactivity as a scavenger of lipoperoxy radicals[22,23] and nitric oxide[24,25] has been reported for MLT.

For such relevant biological properties associated with an extremely low toxicity,[26] MLT can perform as a valuable drug for prevention or cure of several diseases; its present breadth of applications, ranging from sleep-induction and antiageing action to cancer therapy, could be further extended by several clinical or pre-clinical studies in progress.[27]

Chemically, MLT is scantly soluble in water and is quite sensitive to light; its indole ring is a suitable lipophilic structure for the formation of h–g complexes by insertion into the rigid hydrophobic cavity of the CDs,[1,2] as has already been reported for some indole derivatives, e.g., indomethacin,[28] tryptophane[29] and, most recently, 5-methoxytryptamine.[30,31] While some patents on MLT complexes with natural or modified CDs have appeared recently,[32–34] the scientific literature has reported only spectrofluorimetric analytical assays of MLT in the presence of CDs or modified CDs,[35,36] or their use as a vehicle[37,38] or as a stabilizing agent for MLT.[39]

The present study has been carried out as a coincident part of distinct research programs on: (i) the structural and physicochemical characterization of CDs h–g complexes with molecules of biological interest;[3–5,30,31,40–42] and (ii) the localization of MLT in micellar systems with both hydrophobic or hydrophilic domains,[43] respectively. The scope was to investigate on the molecular recognition differences expressed in terms of stoichiometry, geometry, stability, and solubility of the inclusion complexes of MLT with native α-, β- or γ-CDs, as a result of the h–g interactions within the hydrophobic CD cavities of different size and/or of possible non-covalent binding of MLT with the external hydrophilic surface of CDs. The experimental approaches have been performed by high-resolution NMR spectroscopy, mass spectrometry (MS), solubility and calorimetric measurements.

2. Materials and methods

Materials.—Melatonin (Aldrich, 99.5%), α-, β-, γ-cyclodextrins (Fluka), water (Fluka HPLC Grade) and acetonitrile (LabScan HPLC Grade) were used as received.

NMR spectroscopy.—NMR spectra were carried out on an Avance 500 spectrometer (Bruker, Karlsruhe, Germany) at 305.0 ± 0.1 K. All chemical shifts were referenced to internal methanol (3.310 ± 0.001 ppm) according to Funasaki et al.[44] Phase-sensitive two dimensional rotating frame nuclear Overhauser enhancement correlation experiments (2D-ROESY) were carried out using the pulse sequence proposed by Desvaux et al.[45] for enhancing sensitivity and reducing artifacts due to TOCSY type magnetization transfer. Data were collected over 2 K points in $F2$, 512 increments zero filled to 1 K in the processing stage. Spin-lock radiofrequency power and offset were calibrated for $\theta = 40°$, with $\theta = \arctan[(\gamma B_1/2\pi)/\Delta\nu]$ and $\Delta\nu$ = frequency difference (Hz) between hard pulse and spin lock. The samples of the complexes for ROESY experiments were prepared as follows: 15.0, 17.5 and 20.0 mg of α-, β-, and γ-CD, respectively, were mixed with 5.0 mg of MLT each in Eppendorf vials, suspended in 1 mL of D_2O, sealed and magnetically stirred for 24 h. The suspensions were then filtered, and the clear solutions (ca. 15 mM in CDs) were transferred into NMR tubes. The stoichiometry of the complexes was assessed via the continuous variation method (Job plot)[46] according to the following procedure: (i) a satd stock solution of MLT in D_2O was prepared, stirred for 24 h at room temperature, and then filtered (solution A); (ii) a 10-mM stock solution of cyclodextrin in D_2O was prepared (solution B); (iii) volumes V_A and V_B of solutions A and B, respectively, were pipetted into NMR tubes for chemical shift titration. The NMR solutions were prepared by systematically increasing V_B and keeping constant $V_A + V_B = 1$ mL for each sample. The molar ratio MLT/CD was obtained from the NMR spectra by integration of the anomeric proton (H-1′) of the CD and the aromatic protons of MLT. All the integrals were evaluated over a constant frequency interval and after accurate baseline correction by using a fourth order polynomial. All the experiments were repeated three times for the sake of reproducibility.

Mass spectrometry (MS).—Electrospray ionization MS (ESIMS) and ESIMS/MS experiments were performed with a benchtop LCQ Deca (ThermoFinnigan) ion trap using the following instrumental parameters: source voltage 5.02 kV, sheath gas flow rate 0.43 L min^{-1}, capillary voltage 36.8 V, capillary temp 70 °C, ion gauge pressure 1.3×10^{-5} torr [1 torr = 133.3 Pa], convetron gauge 1.35 torr operating in MS or in MS/MS mode. MS/MS experiments were performed in the resonance mode, by applying a supplementary RF voltage on the two end caps of 0.75 V at $Q = 0.3$, with activation time 30 ms and mass isolation window 2 a.m.u.

The mixture of α-CD, β-CD, and γ-CD and MLT (1:1:1:4 molar ratio) in water solution was prepared as follows: three 100-mL mother solutions of α-CD, β-CD and γ-CD were prepared by weighing 64.7, 69.3 and 74.9 mg of α-CD, β-CD, and γ-CD, respectively, in three different flasks containing a 1:1 water–acetonitrile solution. The final, already clear, solutions were deposed in an ultrasonic bath for 15 min; then, 4.4 mg (1.9×10^{-2} mmol) of MLT were added to a solution

obtained mixing 7.1, 7.7, and 8.2 mL of the α-CD, β-CD and γ-CD (4.73×10^{-3} mmol each) mother solutions, respectively. The final solution was deposed in an ultrasonic bath for 1 h and analyzed without any further purification by direct infusion (5 μL min^{-1}) in the ESI source. The ESIMSs spectra were recorded in the mass range 100–2000 Th. The ESIMS/MS spectra were recorded by the selection of the appropriate precursor corresponding to the protonated MLT/α-CD, β-CD or γ-CD complex of m/z 1205, 1367 and 1529, respectively.

All the experiments were repeated three times for the sake of reproducibility.

Solubility measurements.—All solutions were prepared by weight. At each CD concentration, the solubility (S) of MLT was determined by visual inspection of samples prepared at various CD/MLT molar ratio and kept in a thermostatic bath at 25 °C. In the case of γ-CD, the solubility was determined at [γ-CD] < 0.022 mol kg^{-1}, i.e., much below the γ-CD solubility in water. This was because at higher γ-CD concentration the solution becomes opalescent in the presence of MLT.

Solubility data (S, [CD]) of the investigated systems are summarized in Table 1.

Calorimetric measurements.—Calorimetric measurements were carried out at 25 °C with a thermal activity monitor (LKB) equipped with a flow-mix cylinder (LKB 2277-204). The solutions were driven by two peristaltic pumps (Gilson, minipulse 2) and the flow rates (0–0.003 g s^{-1}) were determined by mass.

As a standard procedure, aqueous solutions of the CDs were mixed in the calorimetric cell with pure water, and the signal was taken as baseline. After replacing water with an aqueous solution of MLT at fixed initial concentration (0.002 mol kg^{-1}), the measured heat flux was attributed to the CD/MLT complex formation process.

Preliminary experiments showed that the enthalpy of dilution of MLT solution is negligible. The experimental molar enthalpy (ΔH_{exp}) of the complex formation was calculated using the equation:

$$\Delta H_{exp} = \frac{q \times 10^3}{[MLT] \times \Phi} \quad (1)$$

where [MLT] is the stoichiometric concentration of the melatonin in the mixed solution, q is the calorimetric signal, and Φ is the total flow rate. It must be pointed out that ΔH_{exp} can be identified with the molar enthalpy ($\Delta H°$) of the complex formation when the MLT is totally complexed. The calorimetric data (ΔH_{exp}, [MLT], [CD]) of the investigated systems are summarized in Table 2.

3. Results and discussion

NMR spectroscopy.—Complexation of MLT with α-, β-, and γ-CD caused the expected chemical shift variations,[47] namely shielding of CD inner protons H-3′ and H-5′ and deshielding of the aromatic protons of MLT (H-2, H-4, H-6, and H-7, see Fig. 1 for atom numbering). The complexation induced chemical shift variation ($\Delta\delta$) of H-3′ as a function of the guest concentration was used to assess the stoichiometry of the complexes in solution via the Job method (see Section 2). The Job plots relating to the complexes of α-, β-, and γ-CD with MLT are reported in Fig. 2. The plot of α-CD/MLT complex showed a sharp maximum at [α-CD]/([α-CD] + [MLT]) = 0.50, corresponding to 1:1 stoichiometry. Less defined pictures came from the other two plots: both β-CD/MLT and γ-CD/MLT complexes displayed a broad maximum at CD molar fraction of 0.56, corresponding to neither 1:1 nor to 2:1 host-to-guest ratio (the 1:1 and 2:1 host–guest stoichiometries would provide maxima of the Job plots at molar fractions of 0.50 and 0.67, respectively). A possible rationale for this unexpected behavior could be the presence of multiple equilibria in solution, namely the formation of the 1:1 host–guest associations with a small contribution of

Table 1
Solubility data (S, [CD]) of melatonin in aqueous solutions of cyclodextrins at 25 °C

[α-CD] (mol kg^{-1})	S (mol kg^{-1})	[β-CD] (mol kg^{-1})	S (mol kg^{-1})	[γ-CD] (mol kg^{-1})	S (mol kg^{-1})
0	0.00187	0	0.00187	0	0.00187
0.00198	0.00230	0.00202	0.00301	0.00105	0.00203
0.00540	0.00262	0.00507	0.00360	0.00199	0.00255
0.0142	0.00330	0.0102	0.00401	0.00693	0.00240
0.0250	0.00371	0.0150	0.00430	0.00995	0.00296
0.0502	0.00390	0.0178	0.00453	0.0149	0.00282
0.0795	0.00465	0.0202	0.00550	0.0203	0.00331
0.0900	0.00522	0.0220	0.00549		
0.1166	0.00642	0.0231	0.00580		
		0.0248	0.00581		

Table 2
Calorimetric data (ΔH_{exp}, [MLT], [CD]) for the 1:1 complex formed by melatonin and cyclodextrins

α-CD			β-CD			γ-CD		
[CD] (mol kg^{-1})	[MLT] (mol kg^{-1})	$-\Delta H_{exp}$ (kJ mol^{-1})	[CD] (mol kg^{-1})	[MLT] (mol kg^{-1})	$-\Delta H_{exp}$ (kJ mol^{-1})	[CD] (mol kg^{-1})	[MLT] (mol kg^{-1})	$-\Delta H_{exp}$ (kJ mol^{-1})
0.0103	0.00124	1.10	0.00425	0.00109	2.95	0.00156	0.00137	0.22
0.0113	0.00117	1.22	0.00502	0.000937	3.36	0.00219	0.00112	0.34
0.0125	0.00108	1.36	0.00616	0.000705	3.90	0.00253	0.000983	0.40
0.0140	0.000968	1.47	0.00792	0.00120	4.37	0.00376	0.000494	0.53
0.0164	0.000961	1.69	0.00866	0.00112	4.77	0.00784	0.00120	0.77
0.0186	0.000814	1.96	0.00931	0.00101	4.97	0.01045	0.000940	1.00
0.0214	0.000632	2.22	0.0101	0.000929	5.35	0.01305	0.000679	1.05
0.0250	0.000380	2.46	0.0103	0.00102	5.37	0.01567	0.000416	1.37
0.0659	0.000681	4.71	0.0136	0.000703	6.60	0.02876	0.000452	2.10
0.0794	0.000412	5.38	0.0153	0.000544	6.87	0.06007	0.000452	2.45
			0.0177	0.000322	7.83			
			0.0192	0.000182	9.74			

the 2:1 stoichiometry. Similar cases have been recently discussed by Kokkinou et al.[48]

The ROESY experiments provided us with details on the solution geometry of the complexes. Figs. 3–5 report the expansion of the ROESY contour plots showing the intermolecular transverse NOEs (ROEs) between CDs inner protons (H-3′ and H-5′) and MLT. The terminal CH_3 of the acetyl moiety is not reported as it is not involved in any intermolecular ROE. The spectrum related to the α-CD/MLT complex pointed out the presence of NOEs of H-3′ with H-6, H-4, and H-7 of MLT. Interestingly, no contact between H-3′ and H-2 was detectable. The interpretation of intermolecular cross-peaks involving H-5′ was difficult, due to severe overlap of the signal of H-5′ with other glucose protons (e.g., the two diastereotopic H-6') and the singlet of the 5-OCH_3 group of MLT. However, a quite neat cross-peak due to H-6–H-5′ contact could be accounted for. These data are consistent with the guest molecule approaching the host's cavity from the secondary OH rim, namely the larger rim of the truncated cone. MLT is not deeply inserted into the cavity of α-CD, and the major site of interaction is the indole ring portion defined by C-4, C-5, and C-6. Conversely, the solution geometry of the β-CD/MLT complex inferred from the ROE data is consistent with a deep penetration of the indole ring into the molecular cavity of the host. Indeed, intense cross-peaks of the inner β-CD protons (H-3′ and H-5′) with both H-4 and H-7 were detected, along with a significant dipolar interaction of H-3′ with H-2. Noticeably, no cross-peak connecting H-3′ to H-6 could be found, in contrast to the findings for the α-CD/MLT complex (vide supra). ROE data indicate that MLT enters the cavity by the larger rim of β-CD truncated cone, and the indole ring system is allocated inside the cavity with H-6 protruding out of the primary OH rim, in close proximity to the H-5′ but far away from the H-3′. The opposite pattern is experienced by H-2, consistent with the observed dipolar interaction with H-3′ but not with H-5′. The ROE data for the γ-CD/MLT complex did not point out a specific mode of host–guest interaction, as previously discussed in the case of α- and β-CD. All the aromatic protons of MLT gave rise to detectable cross-peaks with both H-3′ and H-5′, showing that the inclusion of MLT into γ-CD actually takes place. Although the interpretation of MLT/H-5′ contacts requires caution, due to spectral overlap of H-5′ with H-6's, it should be pointed out that ROE data did not allow us to derive a characteristic geometry of interaction, as discussed above for α-CD/MLT (shallow insertion) and β-CD/MLT (deep insertion).

Volume integration of the significant cross-peaks of the ROESY spectra provided a less qualitative description of the inclusion process of MLT. The volume of intramolecular H-2/CH_2α cross-peak (see Fig. 1 for atom labeling) was used as an internal reference. All the

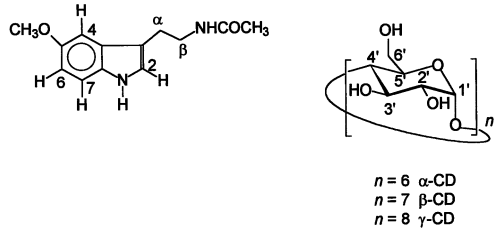


Fig. 1. Structure of melatonin (MLT) and cyclomaltooligosaccarides (α-,β- and γ-CDs).

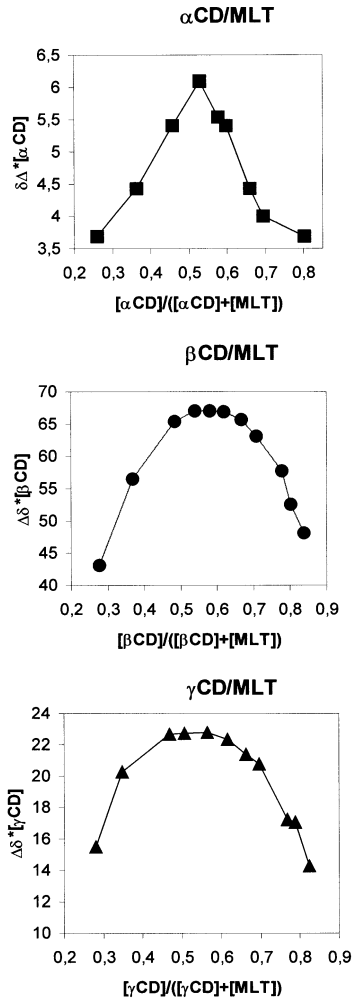


Fig. 2. Job plots for the CD/MLT complexes.

cross-peak volumes related to intermolecular ROEs were then normalized by using such reference. The results are summarized in Table 3. The hypothesis of 'deep insertion' mentioned above for β-CD/MLT is substantiated by the values related to H-3′/H-4 and H-3′/H-7 (high), H-3′/H-2 (medium) and H-3′/H-6 (null) cross-peaks. The figures obtained for the crosspeaks involving H-5′ are consistent with the proposed model: (a) H-4 and H-7 oscillating inside the cavity in close proximity to both H-3′s and H-5′s; (b) H-6 interacting appreciably with H-5′s; and (c) H-2 closer to H-3′ than to H-5′. In a similar way, the measured volumes for the α-CD/MLT complex confirm that the guest interacts with the wide-diameter side of the host mainly via the benzene ring; a deep insertion of the indole ring into the cavity is ruled out by the null value of the H-3′/H-2 volume. The values of H-3′/H-6 and H-3′/H-2 volumes reported in Table 3 show the order α-CD > γ-CD > β-CD and β-CD > γ-CD > α-CD, respectively, and suggest that these parameters can be exploited as descriptors of the degree of guest insertion into the CDs cavity. These orders are consistent with the following points: (i) the benzene ring of the indole system enters the cavity by the wide-rim side of the host; (ii) having defined an arbitrary molecular axis by connecting H-6 to H-2, the inclusion takes place with such axis parallel to the C_n symmetry axis of the CDs ($n = 6$, 7 and 8 for α-, β-, and γ-CD, respectively); (iii) according to the points (i) and (ii), the intensities of H-3′/H-6 and H-3′/H-2 cross-peaks must show a complementary pattern, as indeed observed, and can be used to account for a different degree of penetration: β-CD > γ-CD > α-CD.

In Scheme 1 are the just qualitatively sketched structures of the three 1:1 CD/MLT complexes in solution.

Solubility.—The solubility of MLT in aqueous solutions of CDs as a function of concentration [CD] is plotted in Fig. 6. The observed trends can be rationalized in terms of the formation of soluble complexes. In particular, at sufficiently low CD concentration ([CD] < 0.02 mol kg^{-1}), the diagrams indicate the complex formation with a 1:1 stoichiometry. Above this value, the rapid increase of S with [CD] suggests the formation of higher order complexes. In particular the opalescence of the solution observed at higher [γ-CD] in the presence of MLT could be most probably due to the formation of bulky CD/MLT aggregates.[49]

Calorimetry.—The experimental molar enthalpy (ΔH_{exp}) of complex formation as a function of the cyclodextrin to MLT molar ratio (R) is plotted in Fig. 7.

As it can be seen, the plots show a continuous variation of ΔH_{exp} with R. In particular ΔH_{exp} becomes more negative as R increases. To interpret this behavior we assume that a single 1:1 complex is formed by melatonin and cyclodextrin, according to the equilibrium:

$$\mathrm{MLT} + \mathrm{CD} \rightleftarrows \mathrm{MLT{\cdot}CD} \quad (2)$$

Assuming unitary activity coefficients, the corresponding equilibrium constant K_b can be written as:

$$K_b = \frac{[\mathrm{MLT{\cdot}CD}]}{([\mathrm{MLT}] - [\mathrm{MLT{\cdot}CD}])([\mathrm{CD}] - [\mathrm{MLT{\cdot}CD}])} \quad (3)$$

where [MLT] and [CD] are the stoichiometric concentrations of the melatonin and cyclodextrin, and [MLT·CD] is the equilibrium concentration of the complex.

Eq. (3) can be rearranged as follows

$$[\mathrm{MLT{\cdot}CD}] = \frac{[\mathrm{MLT}] + [\mathrm{CD}] + (1/K_b) - \sqrt{([\mathrm{MLT}] + [\mathrm{CD}] + 1/K_b)^2 - 4[\mathrm{MLT}][\mathrm{CD}]}}{2} \quad (4)$$

On the other hand, the molar enthalpy $\Delta H°$ of the complex formation is related to the equilibrium concentration of the complex by the equation:

$$\Delta H^\circ = \frac{q \times 10^3}{[\text{MLT·CD}] \times \Phi} \quad (5)$$

from which:

$$\frac{q \times 10^3}{\Phi} = \Delta H^\circ[\text{MLT·CD}] \quad (6)$$

According to Eq. (6), the experimental quantity q/Φ plotted against [MLT·CD] gives points lying along a straight line constrained to pass through the origin (Fig. 8).

Besides, the [MLT·CD] values can be obtained by means of Eq. (4) if the K_b values are known. For each system, the K_b value giving the best linear plot of q/Φ versus [MLT·CD] (i.e., the lowest standard deviation of the plotted points from the best straight line fitted using the least-squares method) was taken as the best estimate of this quantity. The corresponding ΔH° was obtained from the slope of the best straight line. The results of this analysis are reported in Table 4.

A perusal of Table 4 shows that the CD/MLT complex formation is driven by a favorable enthalpic effect. In addition to specific interactions of MLT with CD and water, a further contribution could arise from the release of 'low-entropy' water molecules from the hydrophobic region of the MLT solvation shell and from the CD cavity during the penetration process into the cavity.[49]

On the other hand, complex formation is hindered by the negative entropic term arising from the partial loss of molecular translational and rotational degrees of freedom due to CD/MLT aggregation.

The delicate balance of these enthalpic and entropic contributions could account for, at least qualitatively, the different stability of the CD/MLT complexes. In spite of the small negative enthalpic effect arising from a loose-fitting, the comparable stability of the γ-CD/MLT complex can be attributed consistently to the release of more 'low-entropy' water molecules and/or to a smaller loss of molecular translational and rotational degrees of freedom.

Mass spectrometry.—The so called 'soft' ionization MS methods, namely electrospray (ESI), ionspray (IS) and fast-atom bombardment (FAB), have been exploited in recent years for the study of CDs h–g complexes.[3,5,30,31,40,42,50–55] Gaseous entities with the mass and elemental composition corresponding to the

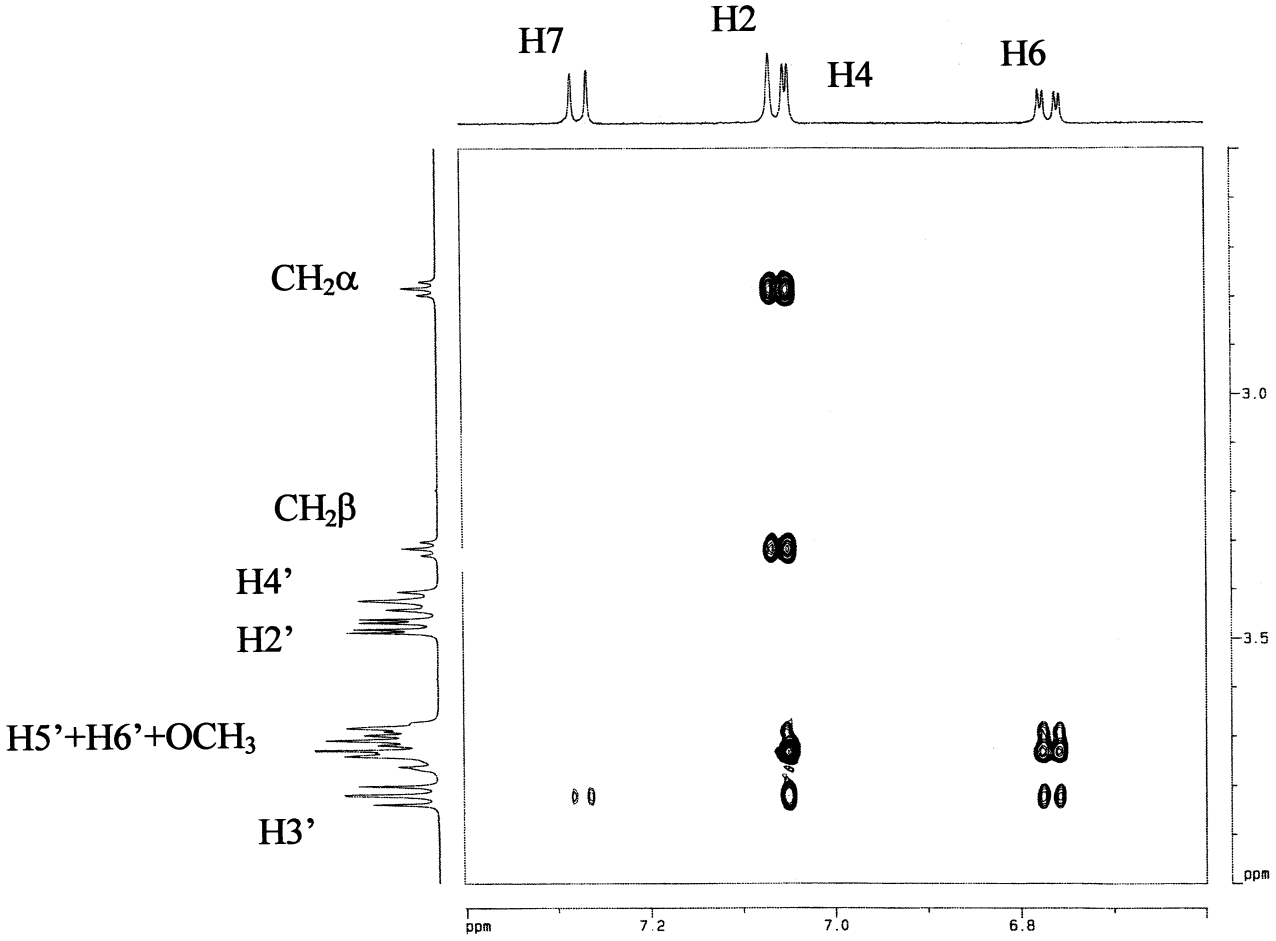


Fig. 3. Expansion of contour plot of ROESY experiment on the α-CD/MLT complex showing the intermolecular ROE region.

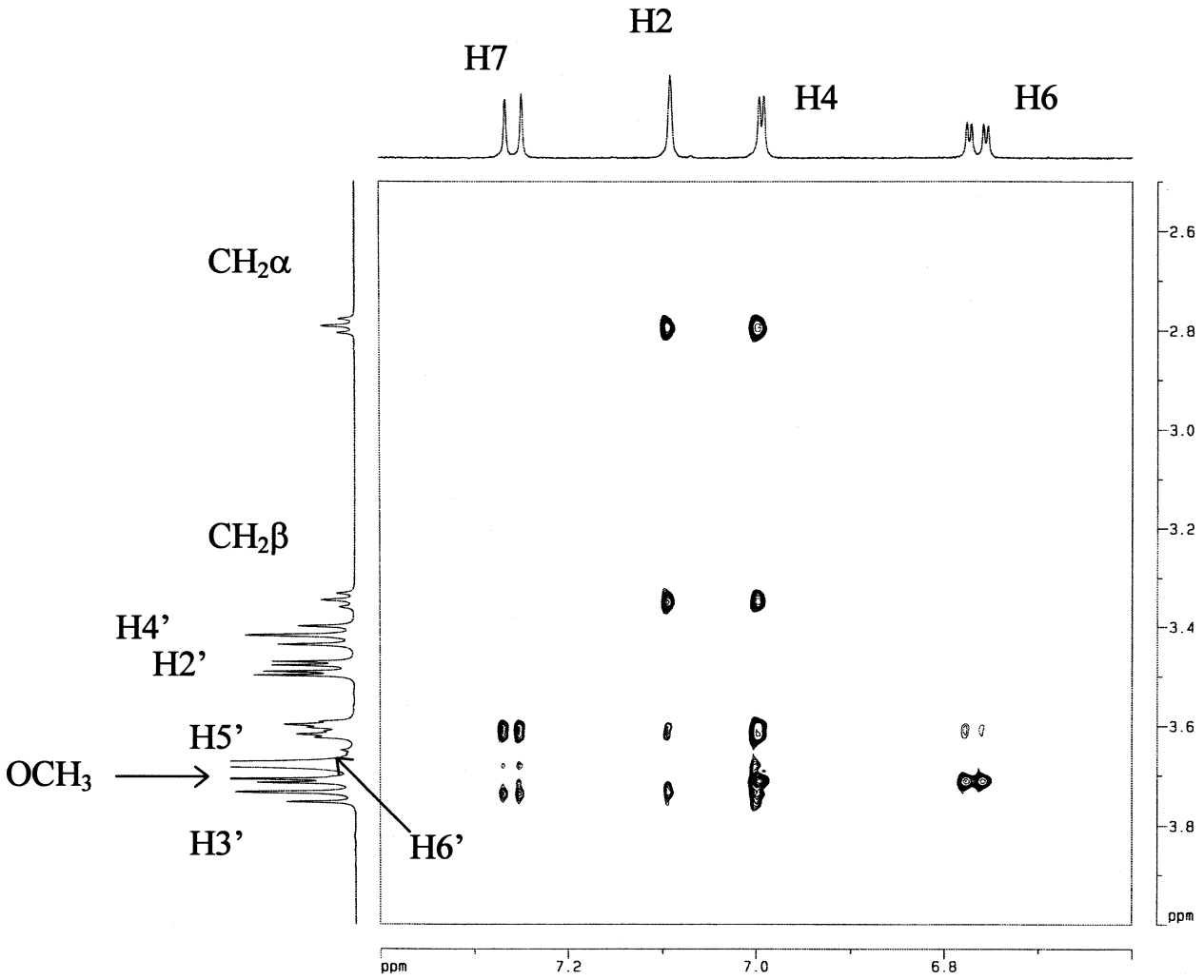


Fig. 4. Expansion of contour plot of ROESY experiment on the β-CD/MLT complex showing the intermolecular ROE region.

protonated, deprotonated, cationated or otherwise charged h–g complexes have actually been detected from the related species in solution, and these results indicate that MS methodology is a rapid and convenient analytical tool. Nevertheless, questions have been raised about the actual surviving capability of h–g intact gaseous charged associations. It has been argued that the 'hydrophobic effect', i.e., the main driving force for the inclusion of lipophilic guests into CDs cavity in water or hydrophilic media, could not operate in the gas-phase in the absence of solvents. The possibility that 'false positive' results could be obtained by the detection of just electrostatic adducts, as ion–molecule MS artifacts (e.g., proton-bound host and guest heterodimers), rather than of true intact gaseous charged h–g associations was advanced.[42b,42c,51] Still however, studies by collision-induced dissociation (CID) and tandem-MS performed on various CD h–g complexes with neutral guests[40,42,50,53,55] or with salts of acidic or basic guests (e.g., 5-methoxytryptamonium chloride[30] or some multicomponent systems of current pharmaceutical interest[3–5]), on cationated double macrocyclic inclusion associations[52] and on mechanical mixtures of the guest molecule with non-cyclic CD analogues[42b,42c] provided reliable evaluation of the strength of the noncovalent interactions, discrimination among CDs and related noncyclic oligosaccharides as ligands, and evidence of molecular and/or chiral recognition in the gas phase.

Most recently nondestructive MS experiments by exchange reactions of the included guests with neutrals or hydrogen/deuterium performed with D_2O within the cell of Fourier transform ion-cyclotron resonance mass spectrometers provided strong evidence for the formation of true gaseous inclusion complexes of native (or methylated CDs) with amino acids or amines.[54,55] These experimental results were also supported by molecular dynamics calculations that provided appropriate simulations of such gas-phase noncovalent inclusion processes.[54,55]

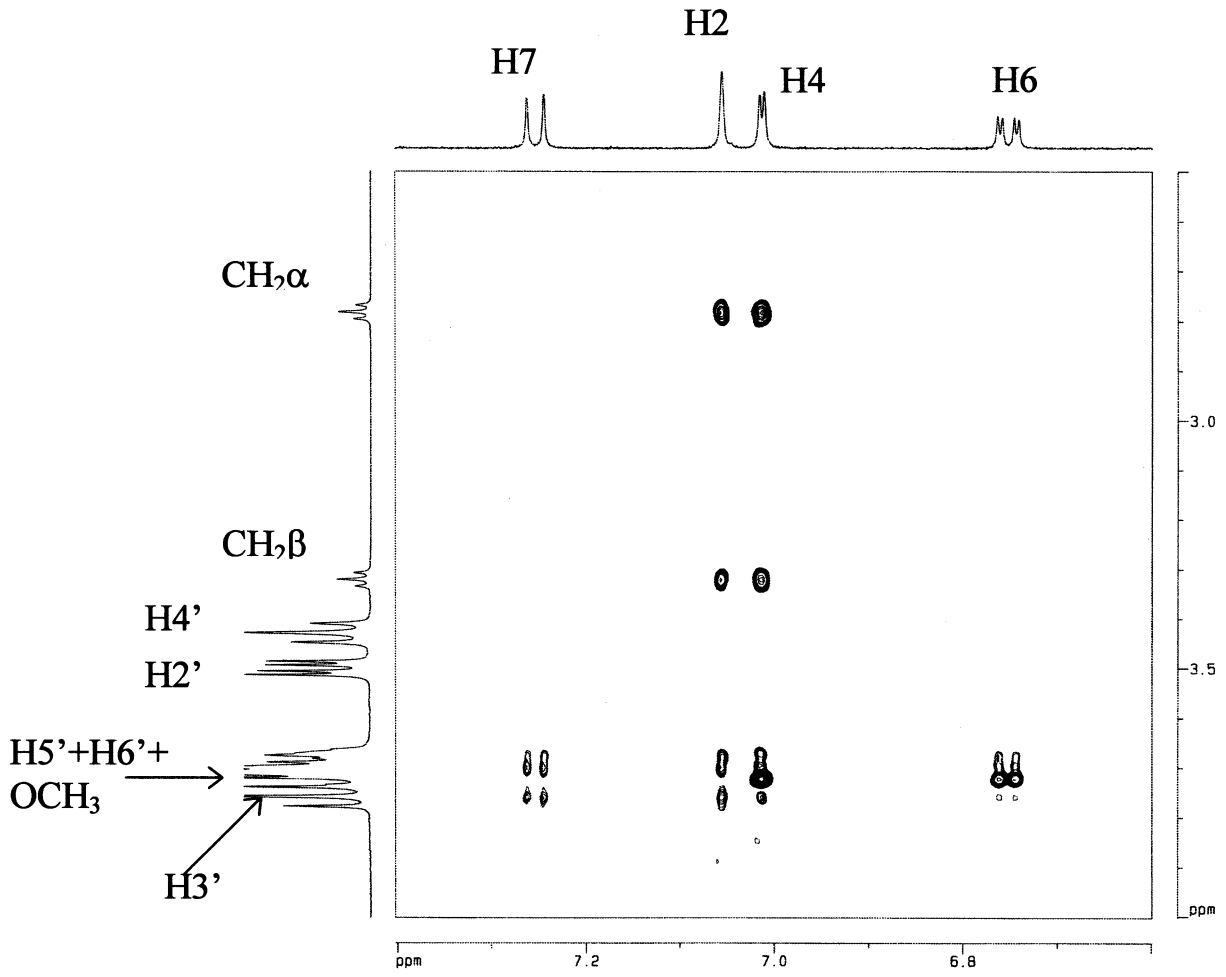


Fig. 5. Expansion of contour plot of ROESY experiment on the γ-CD/MLT complex showing the intermolecular ROE region.

The h–g complex of MLT with β-CD was previously studied by MS.[40] A gaseous protonated 1:1 h–g association (m/z 1367) was obtained by IS or FABMS. CID of such m/z 1367 species showed protonated β-CD and/or its cage fragmentation products to be formed in spite of the higher gas phase proton affinity of MLT with respect to that of β-CD. Such gas-phase endothermic guest-to-host proton transfer was attributed to the proton affinity decrease of the guest due to the dissociation reaction of protonated MLT by loss of neutral acetamide; the product of such a reaction, even at an incipient stage, appeared a relatively stronger acid, capable of performing an exothermic protonation of β-CD.[40]

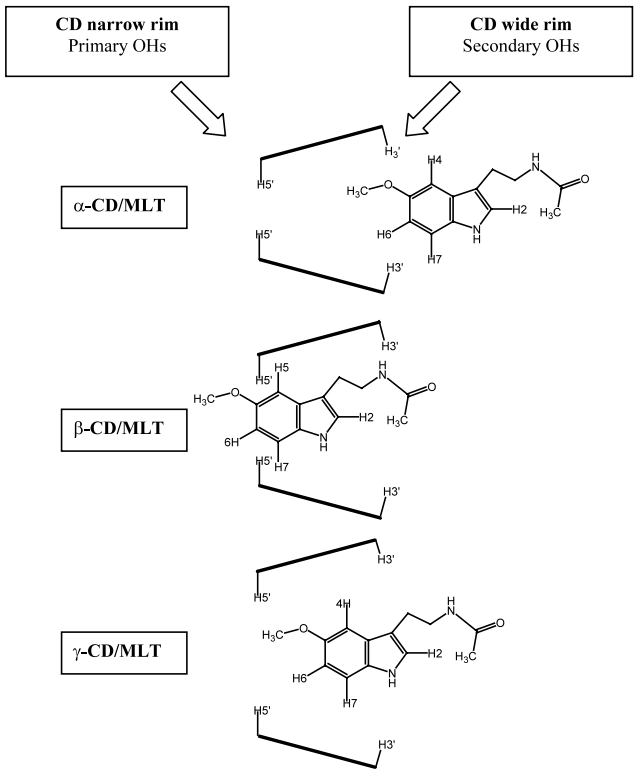
We have performed new experiments by ESI and tandem MS with a water solution of α-CD, β-CD, γ-CD, and MLT in 1:1:1:4 molar ratio. The scope was to measure under identical experimental conditions the relative abundance of the three gaseous charged 1:1 CD/MLT associations in order to provide a reasonable estimate of their relative gas-phase stability, to find out any possible correlations with the stability of the corresponding h–g complexes in solution and eventually to reveal any molecular recognition differences among these host ligands toward MLT in the gas phase. The ESIMS spectrum (Fig. 9) shows the peaks of the 1:1 associations of α-CD, β-CD, or γ-CD with MLT as protonated species at m/z 1205, 1367, 1529 or as sodi-

Table 3
Volumes of intermolecular ROESY cross-peaks [a]

CD protons	MLT protons			
	H-6	H-4	H-2	H-7
α-CD-H-3′	0.54	0.62	0.0	0.26
γ-CD-H-3′	0.24	0.19	0.33	0.37
β-CD-H-3′	0.0	2.10	0.49	0.69
α-CD-H-5′	[b]	[b]	[b]	[b]
γ-CD-H-5′	[b]	[b]	[b]	[b]
β-CD-H-5′	0.60	1.56	0.37	1.10

[a] Normalized to the volume of intramolecular H-2/$CH_2\alpha$ cross-peak for each spectrum.

[b] Volume not evaluated due to peak overlap.



Scheme 1. Qualitative sketch of the structures of the three 1:1 CD/MLT complexes in solution.

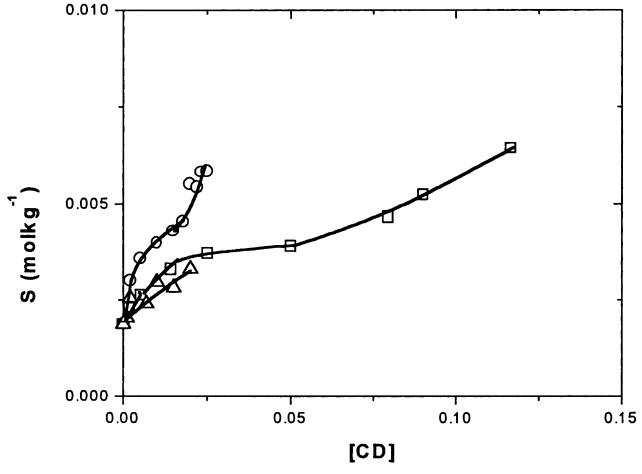


Fig. 6. MLT solubility as a function of CD concentration. (α-CD, □; β-CD, ○; γ-CD, △).

ated species at m/z 1226.5, 1389, 1550.5, respectively. Even minor amounts of related potassium adducts are detected. The spectral data of Fig. 9 show protonated species of the 1:1 CD/MLT complexes always more abundant than the corresponding alkali cationated ones. However, the relative abundance of alkali adducts was widely variable and just reflected the degree of alkali contamination of instrument and/or samples. Although the differences among these relative abundance data of either protonated and alkali cationated gaseous adducts is not striking, the order β-CD > α-CD > γ-CD does emerge. Hence, the higher stability of the β-CD complex, due to the deeper and more exothermic inclusion of MLT into the host cavity in solution, is observed also in the gas phase, i.e., even in absence of solvent and of the related 'hydrophobic effect'. Interestingly the stability order α-CD/MLT > γ-CD/MLT of the protonated or cationated complexes in gas-phase appears reversed with respect to that observed in water solution. This could be accounted for by the fact that the stability of the γ-CD/MLT complex in solution-phase, as evidenced by calorimetric results reported above, is consistently due to the positive entropic term attributable (at least in part) to the extrusion of water molecules from the γ-CD cavity, a process which does not apply in the gas phase. Moreover the shallow guest penetration into the α-CD cavity allows the binding of the protonated amidic group of MLT, leaning out from

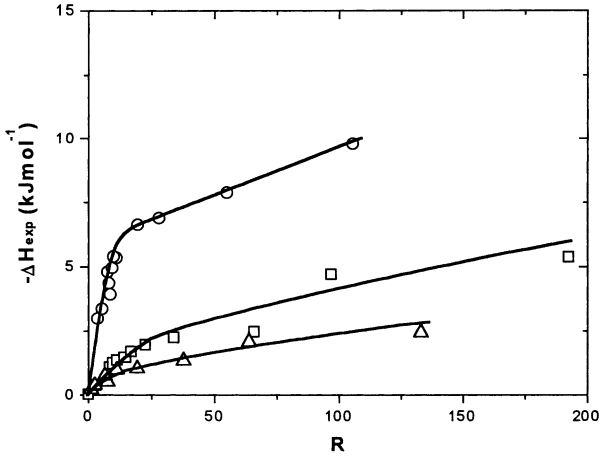


Fig. 7. ΔH_{exp} as a function of the CD/MLT molar ratio R. The continuous lines represent a guide for the eyes (α-CD, □; β-CD, ○; γ-CD, △).

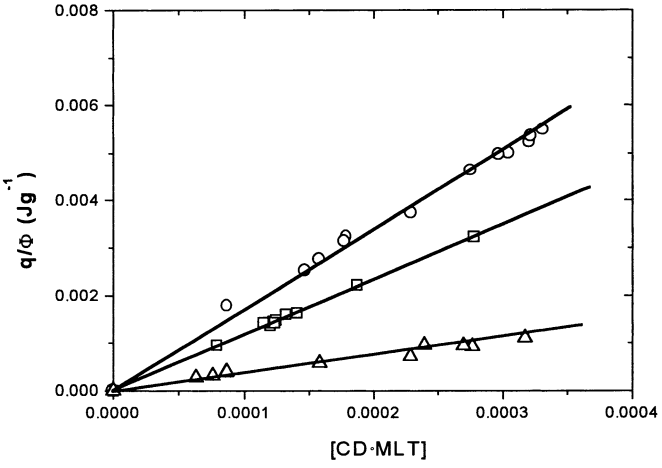


Fig. 8. Linear fit of the experimental data according to Eq. (6) (α-CD, □; β-CD, ○; γ-CD, △).

Table 4
Association constants and thermodynamic parameters for the formation of melatonin–cyclodextrin complexes at 25 °C

Complex	K_b (M^{-1})	$\Delta H°$ (kJ mol^{-1})	$\Delta G°$ (kJ mol^{-1})	$T\Delta S°$ (kJ mol^{-1})
α-CD/MLT	10.50 ± 0.04	-11.68 ± 0.07	-5.82 ± 0.07	-5.9 ± 0.1
β-CD/MLT	48.0 ± 0.6	-16.6 ± 0.1	-9.6 ± 0.3	-7.0 ± 0.4
γ-CD/MLT	39 ± 2	-3.5 ± 0.1	-9.1 ± 0.4	$+5.6 \pm 0.5$

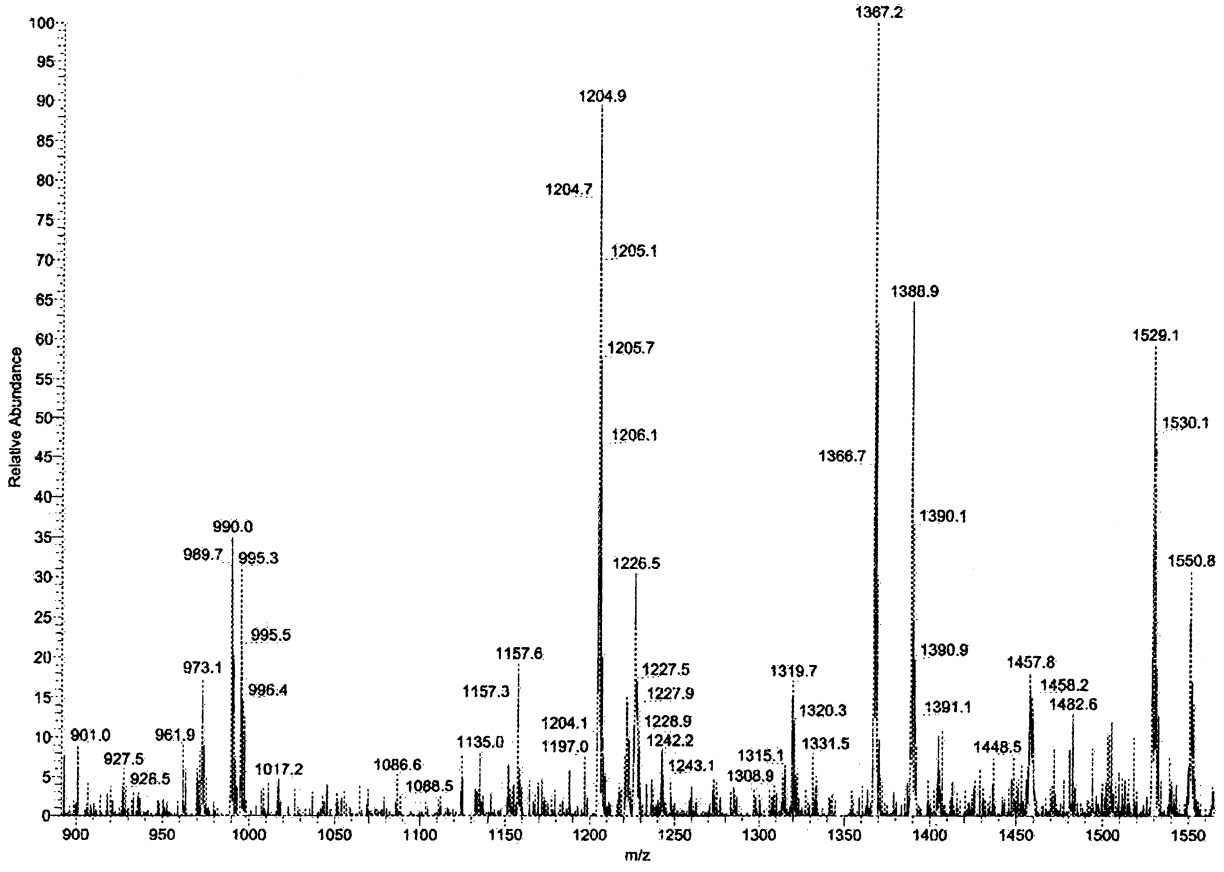


Fig. 9. Electrospray ionization mass spectrum of a water solution of α-CD, β-CD, γ-CD and MLT in 1:1:1:4 molar ratio.

the cavity, to the oxygen atoms of the rim or of the external surface of the CD through proton bridge and/or H-bonding, which can contribute cooperatively to the gaseous association stability, as previously proposed for other CD h–g complexes of guest molecules with hydrophilic partial structures.[5c,42c]

Eventually, the gas phase guest-to-host endothermic proton transfer with cage fragmentation of the protonated host by CID tandem MS experiments previously reported for the protonated adduct of β-CD[40] is reproduced as well for the corresponding α-CD or γ-CD complexes (CID spectra not shown).

In conclusion the present study on the h–g complexes of native α -, β-, and γ-CDs with MLT shows significant molecular recognition differences among these host ligands. In water solution, the observed differences in terms of stoichiometry, geometry, stability, and solubility resulting from high-resolution NMR spectroscopy, solubility, and calorimetric measurements are regarded as the expression of the h–g interactions of MLT within the hydrophobic CD cavities of different sizes and/or of possible noncovalent binding of MLT with the external hydrophilic host surface. Solubility trends indicate the 1:1 h–g stoichiometry to be favored at low CD concentrations with possible formation of higher order complexes and/or larger supramolecular aggregates, in particular for γ-CD, at increasing CD concentrations. The medium size β-CD cavity allows the best fitting for MLT by a deep guest penetration resulting in a relatively more stable and tighter h–g complex, according to the relatively higher values of association constant, exothermicity of com-

plexation, and decrease of entropy; on the other side, the smallest size α-CD cavity allows just a 'shallow' guest penetration by the insertion of the benzene ring of MLT from the host wide-side rim, leading to a an h–g complex with the lowest association constant; the widest size γ-CD cavity can well accept a deep inclusion of the MLT molecule. However, the degree of guest penetration into the CD cavity would not be the only molecular recognition expression determining the complex stability. The association constant of the γ-CD/MLT complex is indeed intermediate between those of α-and β-CD complexes; the lowest enthalpy and the positive entropy of the γ-CD/MLT complexation process strongly support a looser guest accommodation into the wider host cavity.

As a result of the MS experiments, the gas-phase order of stability of the protonated or alkali-cationated associations is β-CD/MLT > α-CD/MLT > γ-CD/MLT. The rank position of α-CD/MLT and γ-CD/MLT appears reversed with respect to that observed in solution and is accounted for either by the absence in the gas phase of the energetic contributions of desolvation in the inclusion process in solution, and/or by the possibility for charged α-CD/MLT gaseous association to gain additional stability by establishing proton bridge or H-bonding between the protonated or cationated amide group, not inserted into the cavity and oxygen atoms at the rim or the external surface of the CD.

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