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Mass Spectrometry as a Complementary Approach for Noncovalently Bound Complexes Based on Cyclodextrins

Mihaela Silion, Adrian Fifere, Ana Lacramioara Lungoci, Narcisa Laura Marangoci, Sorin Alexandru Ibanescu, Radu Zonda, Alexandru Rotaru, and Mariana Pinteală

Abstract

An important and well-designed solution to overcome some of the problems associated with new drugs is provided by the molecular encapsulation of the drugs in the cyclodextrins (CDs) cavity, yielding corresponding inclusion complexes (ICs). These types of non-covalent complexes are of current interest to the pharmaceutical industry, as they improve the solubility, stability and bioavailability of the guest molecules. This review highlights several methods for cyclodextrin ICs preparation and characterization, focusing mostly on the mass spectrometry (MS) studies that have been used for the detection of noncovalent interactions of CDs inclusion complexes and binding selectivity of guest molecules with CDs. Furthermore, the MS investigations of several ICs of the CD with antifungal, antioxidants or fluorescent dyes are presented in greater details, pointing out the difficulties overcome in the analysis of this type of compounds.

Keywords

Mass spectrometry · Electrospray ionization · Cyclodextrin · Inclusion complex · Noncovalent interactions

Abbreviations

CB7	Cucurbit[7]uril
CDs	Cyclodextrins
CID	Collision-induced dissociation
Cy-β-CD	Cysteinyl-β-CD
DM-β-CD	Heptakis(2,6-di-O-methyl)-b-cyclodextrin
ESI-MS	Electrospray ionization mass spectrometry
FT-IR	Fourier transform infrared spectrometry
HP-β-CD	Hydroxypropyl β-cyclodextrin
ICs	Inclusion complexes
LC-MS/MS	Liquid chromatography mass spectrometry
m/*z*	Mass/charge
MALDI-MS	Matrix-assisted laser desorption ionization mass spectrometry
MCT-β-CD	Mono-chlorotriazine-β-cyclodextrin
MH	Maltohexaose
MS	Mass spectrometry
M-β-CD	Methyl β-cyclodextrin
NMR	Nuclear magnetic resonance spectroscopy
NOE	Nuclear Overhauser enhancement spectroscopy
ROESY	Rotating-frame Overhauser spectroscopy
SBE-β-CD	Sulfobutyl ether β-cyclodextrin
SO3-β-CD	β-cyclodextrin sulfate
Su-β-CD	Succinyl-β-cyclodextrin
XRD	X-ray diffraction

41.1 Introduction

Cyclodextrins (CDs) are some of the best known molecules able act as host and form inclusion complexes (ICs). A wide range of molecules have been entrapped by CDs, forming ICs, regardless of their aggregation state, with one or two

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guest molecules being entrapped by one, two or three CDs [1]. The ICs can present different proprieties compared to the forming components, most of the time ICs leading to better solubility, bioavailability and dissolution rate of the guest compounds with poor or no water solubility. The pharmaceutical industry is one of the leading users of this type of complexes in order to overcome the problems associated with new drug molecules [2–5]. Moreover, the CDs have the ability to form ICs in situ, improving the dissolution of guest molecules even if no real complexation happens in the solid state [6].

In theory any characterization technique that can observe changes in physicochemical properties, (changes in solubility, pKa values, UV-Vis absorbance, fluorescence, chemical reactivity and stability, etc.) can be used to determine the stability constants and the stoichiometry of the formed complexes [7–9]. Furthermore, since the complexation process influences the properties of the complexation media, monitoring these media changes (i.e. osmotic pressure, freezing point, vapor pressure, viscosity) can be applied to study the complexation. However, only a few of these methods can be used to get structural information on the guest/host complexes. Usually, noncovalent complexes based on cyclodextrins can be detected by nuclear magnetic resonance (NMR) spectroscopy, Fourier transform infrared spectrometry (FT-IR), X-ray diffraction (XRD), conductometric titration, spectrophotometric and fluorometric techniques [10–15]. Nevertheless, these methods provide little information regarding the molecular weight and binding stoichiometry of the complex. Other techniques such as mass spectrometry (MS) especially electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI), have become a powerful means of studying host guest complexes. MS offers several important advantages over NMR spectroscopy and other techniques, namely a superior sensitivity, faster speed and the ability to monitor the exchange in molecular complexes, which allows to detect the existence of multimolecular complexes. Therefore, MS can be used to explore the formation of higher order molecular complexes with low water solubility [1]. Even more, during the last 25 years, ESI and MALDI MS techniques due to relatively soft ionization conditions, have become very effective methods for direct determination of molecular association of weak non-covalent bonding [16–24].

In this chapter we summarize the existent scientific literature concerning the study of noncovalently bound complexes based on cyclodextrins by MS technique. As the subject is a vast one and, subsequently, the number of publications greatly increase year by year, the examples presented in detail in this review are mostly focused on the CDs inclusion complexes studied in our laboratory.

41.2 Cyclodextrins and Inclusion Complex Formation

CDs are cyclic oligosaccharides where α-D-glucopyranose units are coupled without free-rotation through α-1,4 bonds, contributing to the formation of a toroidal or a truncated shape (Fig. 41.1a). Due to their intramolecular hydrogen bonds, the flexibility of CDs depends on the number of glucopyranose units in the following order: γ-CD > α-CD > β-CD [8, 25]. Due to the low reactivity, CDs are the most used molecules in the formation of ICs, protecting against bioconversion, photo, thermal or oxidative degradation processes their guest molecules [26].

The outsides of CDs are hydrophilic [5] but their insides are as a hydrophobic "pocket" with the dimensions dictated by the number of glucopyranose units. The existence of the hydrophobic "pocket" ensures the location of an appropriate hydrophobic drug, making possible the inclusion complex formation in mild conditions by non-covalent interactions and without changes in the drug structures (Fig. 41.1b) [4, 27]. The formation of ICs is due to the high enthalpy release of water molecules from the CDs cavities [6, 27], to which it is added hydrogen bonds formation, electrostatic and van der Waals forces, hydrophobic interactions, as well as changes of conformational tensions in the case of α-CD [28].

An innovative method for the synthesis of polymers is when instead of an insoluble monomer in a suitable solvent for the polymerization process its IC is used [29–32]. Multiple studies were performed with α-, β- and γ-CDs for the formation of ICs, but lately their derivatives with biological active groups or groups for increased water solubility were developed, such as: methyl β-cyclodextrin, hydroxypropyl β-cyclodextrin, β-cyclodextrin sulfate, sulfobutyl ether β-cyclodextrin, etc.

However, due to their cavity dimension, ability to form IC with high stability, lower cost compared with the other CDs, β-CDs remain the most used CDs in the formation of ICs. It should be mentioned that the β-CDs chemical derivatives can complex with the molecules of the same size as unmodified β-CDs but in a different stoichiometry, such as fullerene C60 is forming IC with β-CDs in a molar of 1/2 while with sulfobutyl ether β-cyclodextrin in a 1 to 1 ratio due to the intake of the carboxyl groups [33].

Different method are applied for the preparation of ICs, such as: co-precipitation [34–36], freeze drying [37–41], microwave irradiation [42–44], kneading method [45, 46] and the co-evaporation method [47], but the choice of one or the other method is imposed by the characteristics of both the host and guest properties, cost, yield, simplicity, swiftness and last but not least scale-up ease.

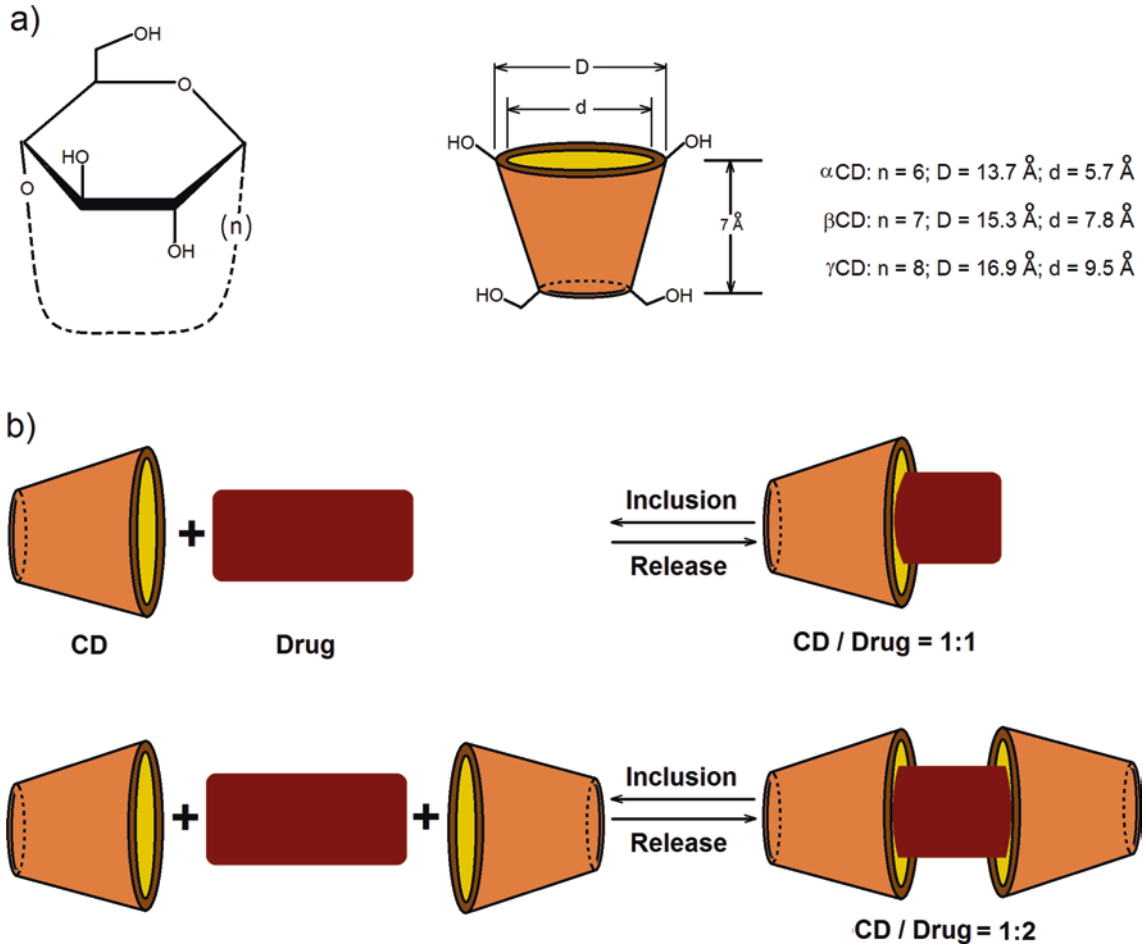


Fig. 41.1 Schematic representation of (**a**) molecular structure and conformation of CD and (**b**) the possibilities of drug-CD ICs formation

41.2.1 Investigation of Inclusion Complexes

Several paper and reviews describe the methods for developing new drug formulations based on ICs and their biomedical application [5, 6, 8, 16, 48, 49]. However, only few reviews contain the details of analytical methods that are typically employed to study CDs and their ICs [50, 51]. One of these reviews, published by Singh et al., describes the usual techniques used for characterization of ICs in solution and in the solid state, focusing on drug delivery applications [52]. Later, Mura et al. published two review articles describing the analytical techniques for characterization of drug–CDs ICs, in both solid and liquid phases [50, 51]. *The authors have done a* comprehensive overview of the main analytical tools which can be used for the characterization of ICs in solution and in solid state, with their advantages and disadvantages. Recently, Narayanan et al. published a new paper updating the reviews mentioned above [15].

Whereas, CD inclusion complexes occur in both solution and solid state, various techniques have been employed to characterize them. In the solid state, potential guest molecules can be inside the CD-IC cavities or simply present as a physical mixture. While, in the liquid, an equilibrium exists between the complexed and uncomplexed host CDs and [53]. Thus, the main analytical techniques used for characterization of CDs inclusion complexes in solution include: *Spectroscopic techniques*: nuclear magnetic resonance (NMR), ultraviolet/visible (UV-VIS) , circular dichroism, fluorescence, electron spin resonance; *Electroanalytical techniques*: polarography, voltammetry, potentiometry, conductimetry; *Separation techniques*: High performance liquid chromatography (HPLC), capillary electrophoresis; *Polarimetry* and *Isothermal titration calorimetry* [50]. In the solid systems, the CD inclusion complexes can be investigated using *Thermal analysis techniques*: differential scanning calorimetry (DSC), thermal gravimetric analysis, hot stage microscopy; X-ray diffraction: single crystal X-ray diffraction, powder X-ray diffraction; *Spectroscopic techniques*: FT-IR spectroscopy, attenuated total reflectance (ATR)-FTIR spectroscopy, Raman and *Scanning electron microscopy* [51]. Other techniques such as electrospray (ESI) and matrix-assisted laser desorption ionization

(MALDI) mass spectrometry are also widely used studying host-guest complexes, with high sensitivity and rapidity, at a very low level of sample consumption [1].

41.3 Mass Spectrometry as a Complementary Approach for Cyclodextrins Based Inclusion Complexes

In the last decades, soft ionization mass spectrometry such as ESI-MS and MALDI-MS gave access to a broad variety of analytes with complex structures, including biomolecules and biopolymers [54, 55]. In the last 25 years, the focus has change from simple analysis of organic and biological molecules to new structures that are stabilized by noncovalent interactions [56]. In this context, ESI-MS proved to be an efficient tool for the study of supramolecular assemblies and noncovalent complexes.

ESI-MS as the "soft" ionization method, can permit transfer of the complex ions, from the liquid or solid phase into the gas phase. This allows users to establish the stoichiometry of an inclusion complex, to estimate the energy of the host–guest interaction [16, 57] and to study the gas-phase reactions of the ICs with different ligands [58, 59]. Even though, the structures of these complexes in solution and in the gas phase can differ the direct correlation between gas-phase complex and the solution behavior is often good for ESI-MS [17, 60].

Initially it was considered that an inclusion complex was produced in the gas phase if the specific mass to charge ratios corresponded to the expected value for the ICs [61–63]. Several studies have reported a large number of guest molecules including amino acids with aromatic and nonaromatic side chains and peptides [61, 62, 64]. The guest molecules used in these studies presented at least one polar that allowed it to form hydrogen bonds or dipole–dipole interactions with hydroxyls of the CD. Due to their electrostatic nature these forces become stronger in the gas phase and most likely are the dominant forces in the stabilization of the complex in the solvent free environment. Such types of electrostatic interactions between binding partners in the gas phase leads to a large fraction of nonspecific adducts in the mass spectra of CDs bound to aliphatic ligands.

ESI-MS can contribute to the investigation of noncovalent complexes, due to the following data provided by mass spectrometric experiments:

- Offers access to stoichiometry data on weakly bound complexes by determination of the exact *m*/*z* ratio, the isotope pattern and elemental composition of an ion;
- Through collision induced fragmentation reactions, information may be gained on the arrangement of noncovalently bound subunits in the complex;
- Different methods can be developed to take a closer look at chiral recognition processes that involve chiral receptors and chiral guests;
- Can be used as a detector for solution—phase processes, the dynamic processes occurring in noncovalently bound complexes can thus be monitored;
- Gives information on impurities and defects by detection of ions with *m*/*z* ratios different from that expected for the complex.

However, it is extremely important to carefully interpret mass spectra of ICs as they will not always provide all the necessary information on each and every sample under study. Based on the ESI-MS of reference compounds that do not form ICs in solution, Cunniff et al. determined that the protonated complexes observed in the gas phase were products of electrostatic interactions, and not inclusion [64]. This was also evidenced by molecular modeling calculations suggested that ICs in the gas phase may come from nonspecific complexes in solution phase. Regardless of the way of starting the calculations, with the analyte on the rim or outside the CDs, the analyte ended up in the inner cavity. A similar event could also happen during electrospray ionization. Polar interactions, such as electrostatic attraction and hydrogen bonds are generally weaker in solution than in the gas phase, thus some cluster ions that cannot be detected in solution, can be observed in the gas phase. Conversely, as nonpolar interactions are weaker in gas phase, complexes already confirmed by other spectral methods, that exist in solution, cannot be found in the gas phase due to their decomposition [64, 65]. Taking all this into account, it can be concluded that in the gas phase, only the CDs complexes with relatively polar compounds can be observed. Moreover, another issue comes from the structure of ions that can be attributed to ICs. Polar compounds, such as CDs, are known to give loosely and nonspecifically bonded clusters in MS. Only a careful examination of each individual case and a vigilant selection of references can differentiate between the "real" inclusion complex and a nonspecific cluster ion.

One good way of differentiating between the two cases, is by comparing CDs with a linear oligosaccharide, such as maltohexose, as host for the same guest. If both the CD and the linear oligosaccharide give the same abundance of complex ions, then nonspecific complexation is the most likely to dominate the CD. If the most intense peak corresponds to the complex ion only in the case of the CD, then most likely, a specific inclusion complex is observed [17].

Another method to tell apart the nonspecific structure of CDs and its ICs is the use of breakdown curves. These plots represent the relative intensity of the complex ion peak as a function of the collision energy, allowing the comparison between noncovalent binding energies and, consequently, to differentiate between non-specific struc-

Table 41.1 Representative examples of CDs inclusion complexes studied by MS

CDs host	Drug guest	Stoichiometry CDs:guest	MS ionization	References
β-CD	Piroxicam, terfenadine	1:1	ESI +	[63, 66]
β-CD	Diclofenac sodium	1:1	MALDI	[67]
β-CD, γ-CD	Cinchonine	1:1, 2:1 1:1:1	ESI +	[68]
β-CD, γ-CD	Oleanolic acid	1:1, 2:1 1:1:1	ESI –	[19]
β-CD	1,6-Diphenyl-1,3,5-hexatriene	1:1, 2:1, 1:2	ESI +/–	[69]
β-CD, HP-β-CD	Flurbiprofen Naproxen Nimesulide Progesterone Cholesterol	1:1 1:1 1:1, 1:2 1:1, 1:2 1:1, 1:2	ESPI + ESI +	[24, 70]
γ-CD γ-CDSA	Fullerene C60	2:1	ESI – MALDI	[33, 71]
α-CD, β-CD, γ-CD	Camostat mesylate	1:1	ESI +	[72]
α-CD, β-CD, γ-CD	A007 prodrugs	1:1, 1:2	ESI –	[73]
α-CD, β-CD, γ-CD M-β-CD, HP-β-CD	Terbinafine; naftifine	1:1	ESI+	[74]
β-C-D	Propiconazole nitrate	1:1	ESI +	[75]
HP-β-CD	Losartan potassium	1:1	ESI +/–	[76]
β-CD	Adamantyl-containing Ru ligand	1:1	ESI +/–	[77]
β-CD	Alendronate sodium	1:1	ESI +	[23]
C-β-CD	Albendazole	1:1	ESI +	[78]
β-CD, γ-CD	Fisetin	1:1	ESI +	[79]
Cy-β-CD	Baicalein	1:1	MALDI +	[80]
β-*CD*, γ-*CD*	1,4-Naphthoquinolines	1:1 2:1	ESI +	[81]
β-CD, γ-CD	Trans-polydatin	1:1	ESI +	[82]
α-CD, β-CD	Probenecid	1:1	ESI +	[83]
β-CD, CB7, 18C6	Norepinephrine	1:1	ESI +	[84]
HP-β-CD	Kamebakaurin	1:1	ESI +	[85]
α-CD, MH	Moringin	1:1	ESI +	[86]
β-CD	Metyrapone	1:1	ESI +	[87]
β-CD	Indolizinyl-pyridinium salt	1:1 2:1	ESI +	[88]
β-CD	Cumaric acid	1;1	ESI -	[89]
DM-β-CD	Quercitrin, hyperoside, rutin	1:1	ESI +	[22]
β-CD	Tetracaine-hydrochloride	1:2	ESI +	[90]
β-CD	Chloralose	1:1	ESI +	[91]

tures and ICs [17]. Some representative MS studies that have been used for the detection of CDs ICs are summarized in Table 41.1.

41.4 Cyclodextrin Based Inclusion Complexes with Biomedical Applications Characterized by Mass Spectrometry

Due to their ability to mask the toxicity, increase the solubility and protect against degradation or bioconversion, CDs have been used more and more as hosts for different molecules good potential applications. The ability of antifungal agents, anti-oxidant molecules or fluorescent dyes to form ICs with CDs has been investigated by MS in our laboratory and several examples are presented in the next section.

Method: All compounds obtained in our laboratory have been analyzed using an Agilent 6520 Series Accurate-Mass Quadrupole Time-of-Flight (Q-TOF) LC/MS. The solutions were introduced into the ESI source via a syringe pump at a flow-rate of 0.02–1 mL/min. The Q/TOF MS parameters used were: positive ion mode, drying gas (N2) flow rate 7.0–9.0 L/min; drying gas temperature 325 °C; nebulizer pressure 15 psig, capillary voltage 4500 V; fragmentation voltage 100–300 V; the full-scan mass spectra of the investigated compounds were acquired in the *m*/*z* range of 100–3000. In the MS/MS experiment, nitrogen was used as collision gas, the mass selected monoisotopic parent ions were isolated in the quadrupole with an isolation width of 2 *m*/*z* and

fragmented by collision with N_2 gas molecules. The relative CID energies for the dissociation of samples in the positive ion mode were 10–80 eV. The mass scale was calibrated using the standard calibration procedure and compounds provided by the manufacturer. Data were collected and processed using MassHunter Workstation Software Data Acquisition for 6200/6500 Series, version B.01.03.

41.4.1 CDs-Antifungal Agents Inclusion Complex Confirmation via MS

There are a lot of antifungal agents available and their application, topical or systemic, is dictated mostly by their solubility and/or toxicity [92–94].

In this context a solution to circumvent these problems is the use of ICs. The complexation of antifungal agents started with the amphotericins class [95–98], *but later extended to azoles—itraconazole* [99], *econazole* [100–102] *and clotrimazole* [103, 104] with new antifungal agents ICs being patented and commercialized each year. The complexation of two antifungal drug naftifine (NF) and terbinafine with CDs has been investigated using ESI-MS in the positive ion mode. Uzqueda et al., assert that ESI-MS can provide a direct proof of the formation of 1:1 non covalent complexes in the gas phase. Thus, the 1:1 stoichiometry determined by ESI are in agreement with those obtained by ^{1}H-NMR and UV spectroscopy [74].

Garcia et al. [78] report the use of ESI-MS to investigate the ability of β-CD citrate derivative to form ICs with albendazole (ABZ). The analysis in positive ionization mode allowed us to observe a molecular ion with *m/z* 1574. This revealed that the inclusion complex was formatted in a ratio 1:1 ABZ: C-β-CD citrate derivative, which is in agreement with the results obtained from the solubility diagrams. In addition, by the fragmentation of molecular ion 1:1 ABZ:C-βCD is confirmed the stoichiometry of the complex and the ROESY assays showed that the tail and the aromatic ring of ABZ were inside the cavity of derivative.

As recently described by Marangoci et al., ESI-MS was used to investigate the stoichiometry and formation of the inclusion complex between NO_3PCZ and unsubstituted β-CD [75].

Propiconazole (PCZ) is a triazole developed and marketed by Janssen Pharmaceutics (Belgium) as an antifungal pesticide. Protonated propiconazole nitrate (NO_3PCZ), a derivative of propiconazole, was proved to have a better antifungal activity and lower acute toxicity, comparable to those of commercial azole drugs [75, 105] which makes it an interesting candidate for clinical use. As most clinical azoles (with fluconazole as the sole exception), NO_3PCZH has the major inconvenient of having a poor water solubility, which severely reduces its bioavailability. To address this issue, the formation of a host-guest ICs with native and modified β-CD as a host carrier molecule was investigated with good results [28, 75, 106]. ICs of NO_3PCZH with three substituted CDs derivatives (SBE7-β-CD, MCT-β-CD and SO_3-β-CD) were investigated as new antifungal systems and compared to a previously published complex of NO_3PCZ with unsubstituted β-CD [105].

The β-CD, PCZ and NO_3PCZ were initially analyzed in positive mode, to contrast the relative CID energies between β-CD and its complex. In the positive mode, ions of $[\beta\text{-CD} + H]^+$ and $[\beta\text{-CD} + Na]^+$ are in great abundance. Peaks at *m/z* 1135 and *m/z* 1157 corresponds to the single charge protonated and sodium adduct of β-CD, respectively. The doubly charged state of β-CD ions at *m/z* 568 can also be observed in the mass spectra.

The ESI-MS spectra for PCZ and NO_3PCZ are recorded in the positive mode and examples of them are displayed in Fig. 41.2. In the case of PCZ (mixture of four stereoisomers) the ESI–MS spectrum is shown in Fig. 41.2a, and the zoom isotope peaks of PCZ isomers are also shown as an inset in the figure at *m/z* 342 and 344. In addition, the PCZ is represented by the singly charged sodium adduct at *m/z* 364 and 707 associated with the monomer $[PCZ + Na]^+$ and the dimer $[2PCZ + Na]^+$, respectively. These results indicate the possibility of dimerization of PCZ in solution as well as in the gas phase. The nitration reaction of PCZ and proposed structure of NO_3PCZ is shown in Scheme 41.1.

The ESI–MS analysis of NO_3PCZ in water methanol mixture (1:1 v/v) (Fig. 41.2b) revealed the formation of proton charged propiconazole nitrate at *m/z* 405, which confirms that the nitration reaction of PCZ was successfully carried out.

The formation of β-CD-NO_3PCZ complexes is clearly evident from the singly charged species $[\beta\text{-CD-}NO_3PCZ + H]^+$ at *m/z* 1540 as shown in Fig. 41.3. In addition, the most abundant peak at *m/z* 1157 corresponds to β-CD sodium adduct, while a peak at *m/z* 1199 can be attributed to NO_3β-CD.

In order to obtain information on the binding strength of the non-covalent complex, MS/MS experiments on β-CD, PCZ, NO_3PCZ and their ICs were performed. A confirmatory experiment of the formation of $[\beta\text{-CD-}NO_3PCZ + H]^+$ complex, made by using different relative collision energies (10–50 eV), put out the its specific peak at *m/z* 1540 (Fig. 41.4).

The peak at *m/z* 1197 is the result of the PCZ molecule loss from inclusion complex, by dissociation process of NO_3PCZ in ESI conditions and recombination of NO_3 ion with β-CD. In addition, the linear fragments with 162 Da (the mass of one glycoside unit) sequence correspond to the typical fragmentation of β-CD, and is formed through scission of the 1,4-glycosydic bonds between glycoside units. Two distinct processes are involved in the fragmen-

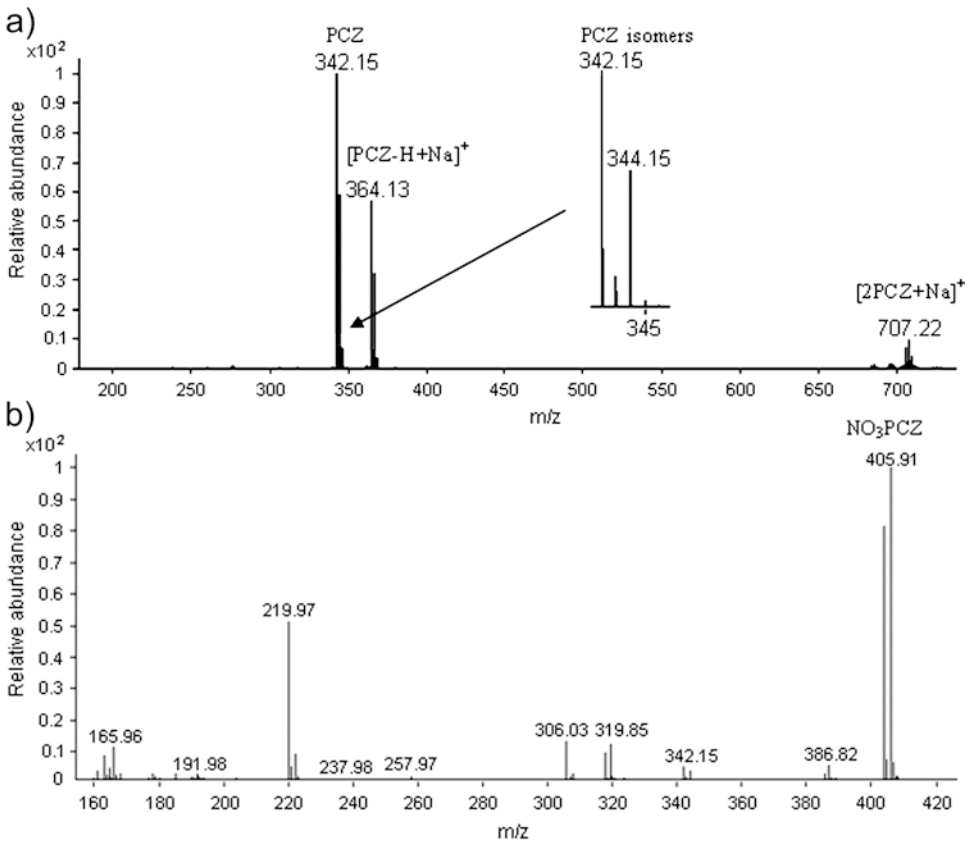
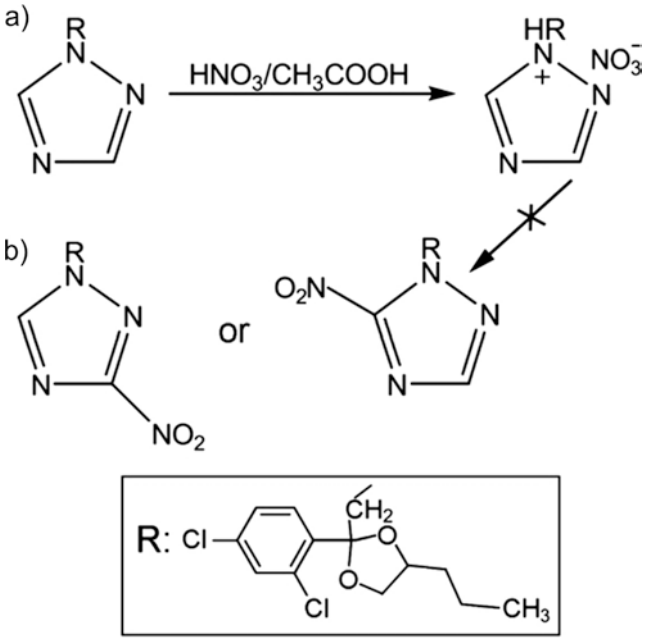


Fig. 41.2 Positive ESI mass spectrum of (**a**) PCZ in methanol solution and (**b**) NO_3PCZ in 1:1 v/v water/methanol solution. Reprinted from Results in Pharma Sciences, 1, Marangoci, N. et al., Inclusion complex of a new propiconazole derivative with beta-cyclodextrin: NMR, ESI-MS and preliminary pharmacological studies, 27–37., Copyright (2011), with permission from Elsevier [75]



Scheme 41.1 Reaction of 1,2,4-triazoles with acidic nitrating agents (**a**) and the proposed structure of NO_3PCZ (**b**). Reprinted from Results in Pharma Sciences, 1, Marangoci, N. et al., Inclusion complex of a new propiconazole derivative with beta-cyclodextrin: NMR, ESI-MS and preliminary pharmacological studies, 27–37., Copyright (2011), with permission from Elsevier [75]

tation of β-CD-NO_3PCZ: neutral loss of PCZ and the β-CD dissociation by consecutive losses of one glycosidic unit [75].

The β-CD, NO_3PCZ and the inclusion complex have been analyzed by UV-VIS spectroscopy in distilled water, in order to prove complex formation by measuring the differences in absorbance values due to the complexation of β-CD with NO_3PCZ. From the UV-Vis absorption spectra (Fig. 41.5) can be observed that the band of 200–220 nm is strongly decreasing in intensity for the inclusion complex, comparing to those of NO_3PCZ.

Through the docking process and molecular dynamics simulations for β-CD-NO_3PCZ inclusion complex, several possible orientations of the NO_3PCZ were investigated, in order to examine the possible inclusion pathways and to detect the possible global minimum (Fig. 41.6) [105].

In conclusion the formation of the inclusion complex was proven by MS and further confirmed by UV-Vis and molecular simulation. Also, considering antifungal potencies, low toxicities, synthetic accessibilities and good pharmaceutical properties, β-CD-NO_3PCZ inclusion complex may be an excellent promising antifungal agent and may lead to for further preclinical studies.

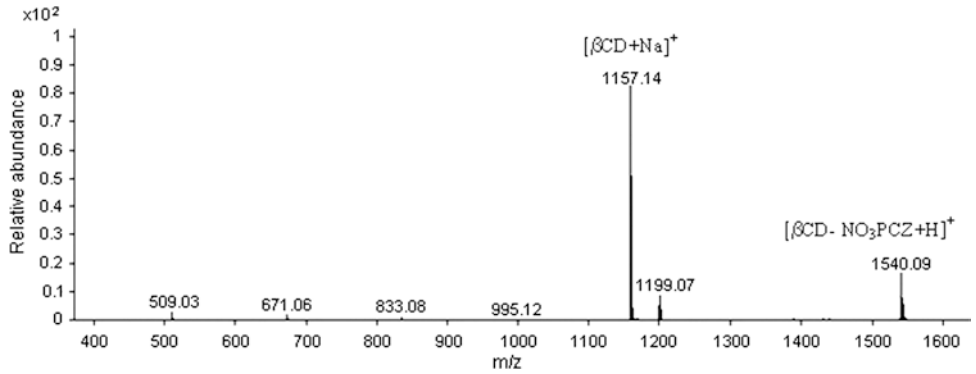


Fig. 41.3 Positive ESI mass spectrum of β-CD-NO_3PCZ inclusion complex in 1:1 v/v water- methanol solution. Reprinted from Results in Pharma Sciences, 1, Marangoci, N. et al., Inclusion complex of a new propiconazole derivative with beta-cyclodextrin: NMR, ESI-MS and preliminary pharmacological studies, 27–37, Copyright (2011), with permission from Elsevier [75]

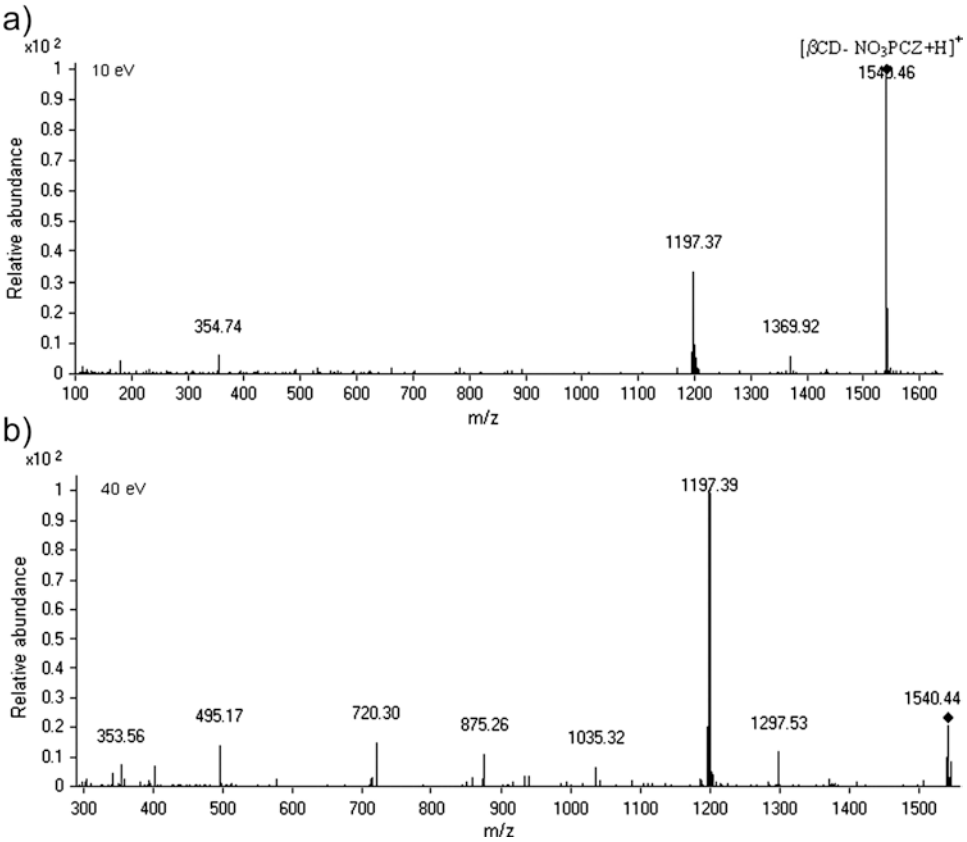


Fig. 41.4 Collision-induced dissociation (CID) spectrum of the βCD-NO_3PCZ at the collision energy 10 and 40 eV. Reprinted from Results in Pharma Sciences, 1, Marangoci, N. et al., Inclusion complex of a new propiconazole derivative with beta-cyclodextrin: NMR, ESI-MS and preliminary pharmacological studies, 27–37, Copyright (2011), with permission from Elsevier [75]

41.4.2 CDs Inclusion Complex with Natural Compounds Confirmation via MS

Natural phenolic compounds are derivatives of phenol, which have various biological activities, such as antioxidant, anti-inflammatory and anti-cancer protection. The most studied natural phenols are the flavonoids, which include several thousand compounds including flavonols, flavones, flavanol (catechins), flavanones, anthocyanidins and isoflavonoids. Unfortunately, most of these compounds suffer from poor solubility, stability and bioavailability, but their encapsulation into CDs provides an alternative approach to remove these problems.

In this context, several papers have been published on the study of CDs inclusion complexes with natural phenolic compounds, such as rutin [20, 107–109], quercetin [107, 110, 111], morin [112], rosmarinic acid [113, 114] caffeic acid [115] chlorogenic acid [116, 117], hydroxytyrosol [118], bacalein [80], resveratrol [119], isoquercitrin [120], kaempferol [121], protocatechuic acid [122] and many

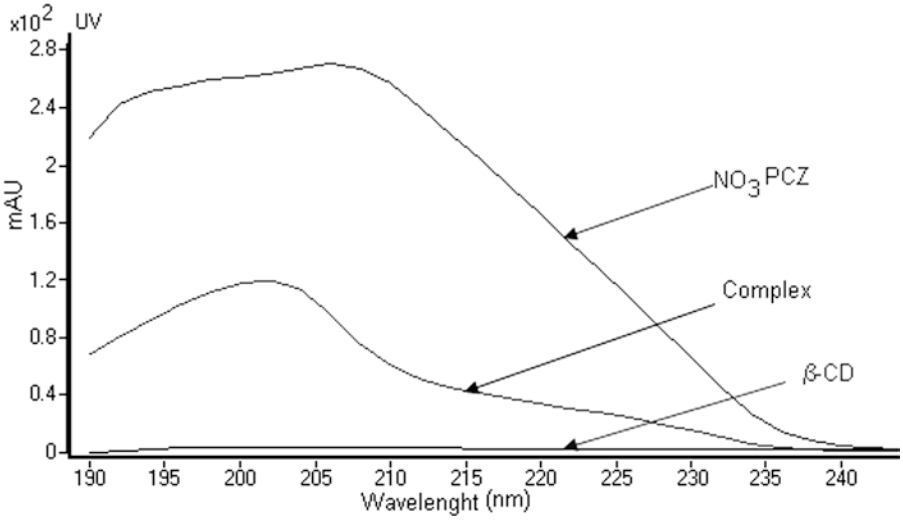


Fig. 41.5 UV-Vis spectra of NO_3PCZ, inclusion complex and βCD. Reprinted from Results in Pharma Sciences, 1, Marangoci, N. et al., Inclusion complex of a new propiconazole derivative with beta-cyclodextrin: NMR, ESI-MS and preliminary pharmacological studies, 27–37., Copyright (2011), with permission from Elsevier [75]

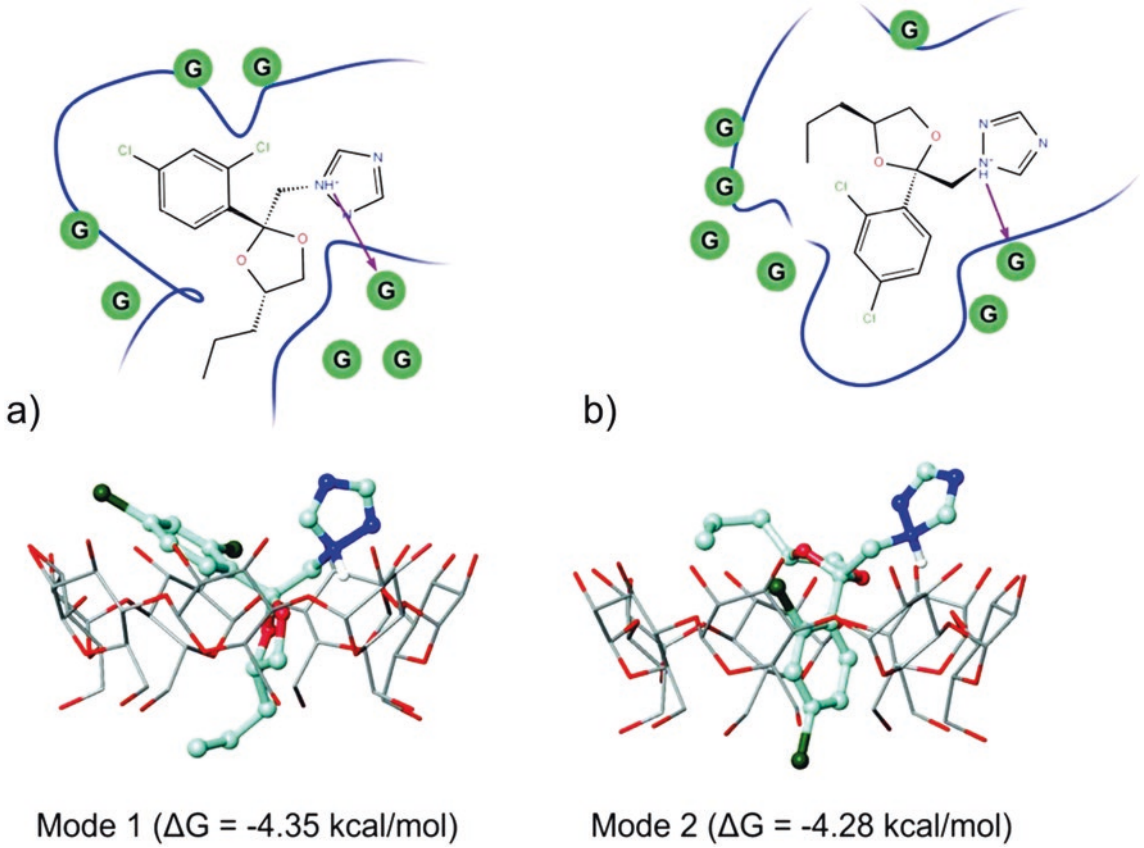


Fig. 41.6 Conformations of two types of β-CD-NO_3PCZ ICs. The two clusters represent different modes of complexation with close binding free energies (and, consequently, close Ka values) which may coexist in solution: (**a**) the dioxolanyl cycle of NO_3PCZ was located inside the β-CD ring, near its glycoside residues, and that the 1,2,4-triazolylmethyl moiety of NO_3PCZH participates, through its NH^+ group, in a hydrogen bond with a CD glycosidic oxygen; (**b**) the hydrogen bond occurs with the 1,2,4-triazolylmethyl moiety, but the dioxolanyl ring sit is more exposed to the solvent, in a parallel plane with the secondary rim of CD, which also favoured hydrogen bond formation. Reproduced with permission from *New journal of chemistry, Royal Society of Chemistry* [105]

others. In these papers, the formation of ICs was confirmed by DSC, XRD, ^{1}H NMR, UV-Vis, FT-IR, fluorescence spectroscopy. For some compound MS technique has been used to investigate inclusion complex formation of antioxidants and to determine the corresponding association constants [19, 20, 79, 115].

The formation and the stoichiometry of inclusion complex between α-,β- and γ-CD with rutin (R) were investigated by ESI-MS in positive and negative ion mode [20]. Authors suggests that the specific ICs between rutin and CDs with a 1:1 stoichiometry can be confirmed by ESI-MS/MS using the relative peak intensities and relative collision-induced dissociation (CID) energies. From competition experiments with the three CDs species in equimolar amounts and rutin in excess, under identical MS/MS experimental conditions, they have elucidated also the relative gas phase stabilities of the complexes. In this experiment, the abundances of the CDs–R complexes are in the different order to those of the free CDs, which confirm that the formation of specific ICs, rather than non-specific electrostatic adduct. The similar studies have used ESI-MS in order to verify the formation and the binding stoichiometry of the Fisetin/β- and γ-CDs ICs [79]. Experiments using equivalent amount CDs and excess amount of FIT were performed under identical experimental conditions. The results were in a good agreement with molecular modelling studies and suggested that the FIT molecule was almost coaxially included in the CDs cavity with a 1:1 ratio between FIT and CDs. Other phenolic compounds such as cumaric acid, caffeic acid, trans-ferulic acid and p-coumaric acid were investigated by ESI-MS if they form ICs with CDs [70, 89, 115]. The experimental and computational studies show that all phenolic compounds are forming 1:1 ICs with β-CD indicated that the phenolic compounds are entrapped in the CDs cavity.

A representative study has demonstrated that ESI-MS provides a suitable and rapid method for analysis of DM-β-CD inclusion complexes with three flavonoid glycosides such as quercitrin (Qr), hyperoside (Hr) and rutin (R) [22]. From the difference in binding constants, it was found that the interactions between the sugar moiety and the aglycone between flavonoid glycosides affect non-covalent binding of glycosides in the order: DM- β -CD-Q > DM- β -CD-R > DM- β -CDH. In addition, the CID experiments demonstrates that the aglycone moiety is accommodated into the DM-b-CD cavity and the glycoside moiety is located outside the cavity.

Recently, Lungoci et al. have proposed the development of new ICs based on protocatechuic acid (PCA) with native and modified β-CD (anionic SBE-β-CD). PCA is a natural phenolic compound originated in many medicinal plants. Due to the antioxidant and anti-inflammatory properties, PCA could be used as a protective agent against cardiovascular diseases and neoplasms. The mechanism is associated with inhibition of generation and scavenging of free radicals by donating a hydrogen atom or an electron [122]. Also, PCA has been used as a template for polymeric core-shell magnetic nanoparticles and to stabilize magnetic particles in water, leading to increasing stability and improving pharmacokinetic properties offering the possibility of guidance and therapeutic action [123].

The SBE-β-CD_PCA inclusion complexes were then used for obtaining the nanocarriers based on nanoparticles functionalized with branched polyethyleneimine of low molecular weight, for active drug delivery [122]. The formation of the inclusion complex was confirmed by NMR and DSC analyses. Furthermore, PCA, β-CDs and SBE-β-CD_PCA complexes were evaluated by ESI MS in the negative ionization mode. The mass spectrum of PCA showed two characteristic peaks at *m/z* 153 corresponding to PCA deprotonated form $[M - H]^-$ and at *m/z* 109 which may be generated by a neutral loss of a COO^- from PCA (*spectrum is not shown*). As a representative example, the corresponding ESI-MS mass spectrum for the βCD-PCA inclusion complex is shown in the Fig. 41.7 (the results are not published).

The negative ESI mass spectrum of βCD-PCA inclusion complex in water is shown on Fig. 41.7, where the most abundant peak at *m/z* 1287 corresponds to $[\beta\text{-CD-PCA-H}]^-$, while the peak at *m/z* 1210 can be attributed to $[\beta\text{-CD-PCA-COO}^-\text{-2HO}^-]^-$ the complex which losses of a carboxylic acid and two *hydroxyl* group. The results are consistent with the earlier solubility studies, and the 1:1 stoichiometry of the CDs-PCA inclusion complexes shows a correlation between the gas phase and the solution phase.

The results are consistent with the earlier solubility studies, and the 1:1 stoichiometry of the CDs-PCA inclusion complexes shows a correlation between the gas phase and the solution phase. Furthermore, the formation of the inclusion complex was also confirmed by NMR and DSC analyses.

41.4.3 CD Inclusion Complex with Fluorescent Dye Confirmation via MS

The fluorescent dyes have been most extensively employed as staining agents in biological tissues, and as guests for tissues macrocyclic hosts in aqueous media [81, 88, 124, 125]. However, fluorescent dyes suffer from several fundamental problems including toxicity, low water solubility and poor membrane permeability when used for bio-labelling and bio-imaging. The inclusion complexation behavior of some fluorescent dyes guest molecules with native α-, β-, and γ-CDs and modified CDs has been investigated by ESI-MS [81, 125]. Wanger et al. have studied the binding of the fluorescent molecule Nile Red (NR) with several native and modified CDs [125]. The CDs-NR complexes formation in

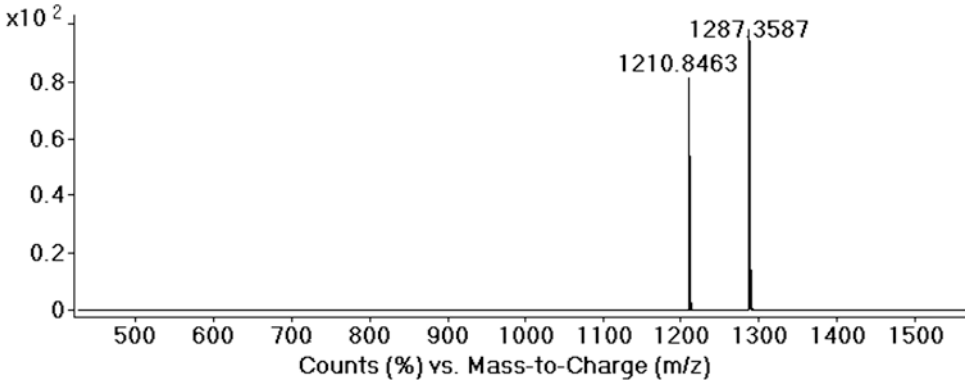


Fig. 41.7 Negative ion ESI mass spectrum for the βCD-PCA inclusion complex

solution, with 1:1 and/or 2:1 ratio have been detected by ESI MS. In these cases, the ESI-MS experiments have allowed direct and clear determination of the ICs formation and their stoichiometry, but cannot provide information about the nature of these complexes such as the magnitude of the binding constants or/and the specific orientation of the NR guest inside the CDs cavity.

Another detailed study regarding the factors can affect the formation of 2:1 host-guest ICs of 1,4-naphthoquinolines derivatives (PAN) in β and γ-CD under ESI-MS conditions, have been reported by the same group [81]. In order to explain their ESI-MS results, the authors calculated the energies of CDs-PAN complexes using molecular modeling, considering that the relative abundance of ICs with differing stoichiometries in the ESI-MS experiments, is directly related to the energy and thus stability of these complexes. The comparison of the data obtained from the ESI-MS of the 19 PAN fluorescent dyes and two CDs (β and γ-CD), and the corresponding data of calculated energies for 1:1 and 2:1 complexes obtained via molecular modelling, have been made [81]. For the majority of the CDs-PAN complexes under excess CDs conditions 1:1 stoichiometry was observed and only six lead to 2:1 host–guest ratios [81].

Therefore, the size and the specific nature of the substituents involved shows that both steric and electronic factors must be taken into account in predicting which CDs inclusion complexes stoichiometries will be stable enough to be observed in the ESI-MS experiments. Recently, Pricopie et al., reported a new study on supramolecular host–guest inclusion complex between a fluorescent indolizinylpyridinium salt (IPy) and β-CD. The authors have made a correlation between the results obtained from the solubilization tests with the results obtained from ESI MS. They noticed that at the 1:1 ratio the cold solution was still cloudy showing an incomplete solubilisation of the guest, leading to the conclusion that higher amounts of β-CD were needed for the stabilization of the inclusion complex [88]. On the other hand, a higher ratio between guest and β-CD may lead to the formation of more complex supramolecular assemblies containing the inclusion complex formed from one guest molecule and two or more β-CD units.

To check this hypothesis, a similar approach with Wanger and al [81]. was used, by the addition of β-CD in excess in order to assure the formation and observation of only the most stable 2:1 β-CD_IPy adduct ions. ESI-MS experiments were performed for the reaction complex containing different ratios between compound β-CD and IPy (1.5:1; 2:1 and 3:1). Unfortunately, for all situation didn't result in a single species of 2:1, but rather the reaction solution β-CD_ IPy presented a mixture of 1:1 and 2:1 species.

The positive ion ESI-MS spectra (Fig. 41.8a) for the β-CD_ IPy at a 1.5:1 ratio revealed several peaks, including the weak abundant peak at *m/z* 1654 corresponding to the 1:1 species of β-CD_ IPy complex noted as $[IPy\text{ -Br} + \beta\text{-CD}]^+$ and the peak at *m/z* 2788 corresponding to the 2:1 species of β-CD_ IPy complex noted as $[IPy\text{ -Br} + 2\beta\text{-CD}]^+$. In all the investigated samples the formation of a host–guest inclusion complex was observed. More interestingly, on further increasing the stoichiometry between the host and guest (3:1 ratio), molecular peaks (Fig. 41.8 b and c) for both 1:1 $[IPy\text{ -Br} + 2\beta\text{-CD}]^+$ and 1:2 complexes $[IPy\text{ -Br} + 2\beta\text{-CD}]^+$ were still visible with higher intensities.

In addition, in order to explain the obtained ESI-MS results, binding energy of β-CD_ IPy complexes was theoretically calculated using molecular modeling. The docking simulations indicated that the molecule of IPy is able to form both 1:1 and 1:2 complexes with β-CD, the selected and optimized molecular docking models are reported in Fig. 41.9.

According to these results, for the 1:1 inclusion complex, the bipyridyl moiety of IPy is embedded in the hydrophobic cavity of β-CD. Interestingly, in the case of the 1:2 inclusion complex the marginal phenyl and methoxy moieties of IPy were deeply embedded in the hydrophobic cavity of the β-CD molecules. The obtained theoretical data suggest that both the species could exist in the solution.

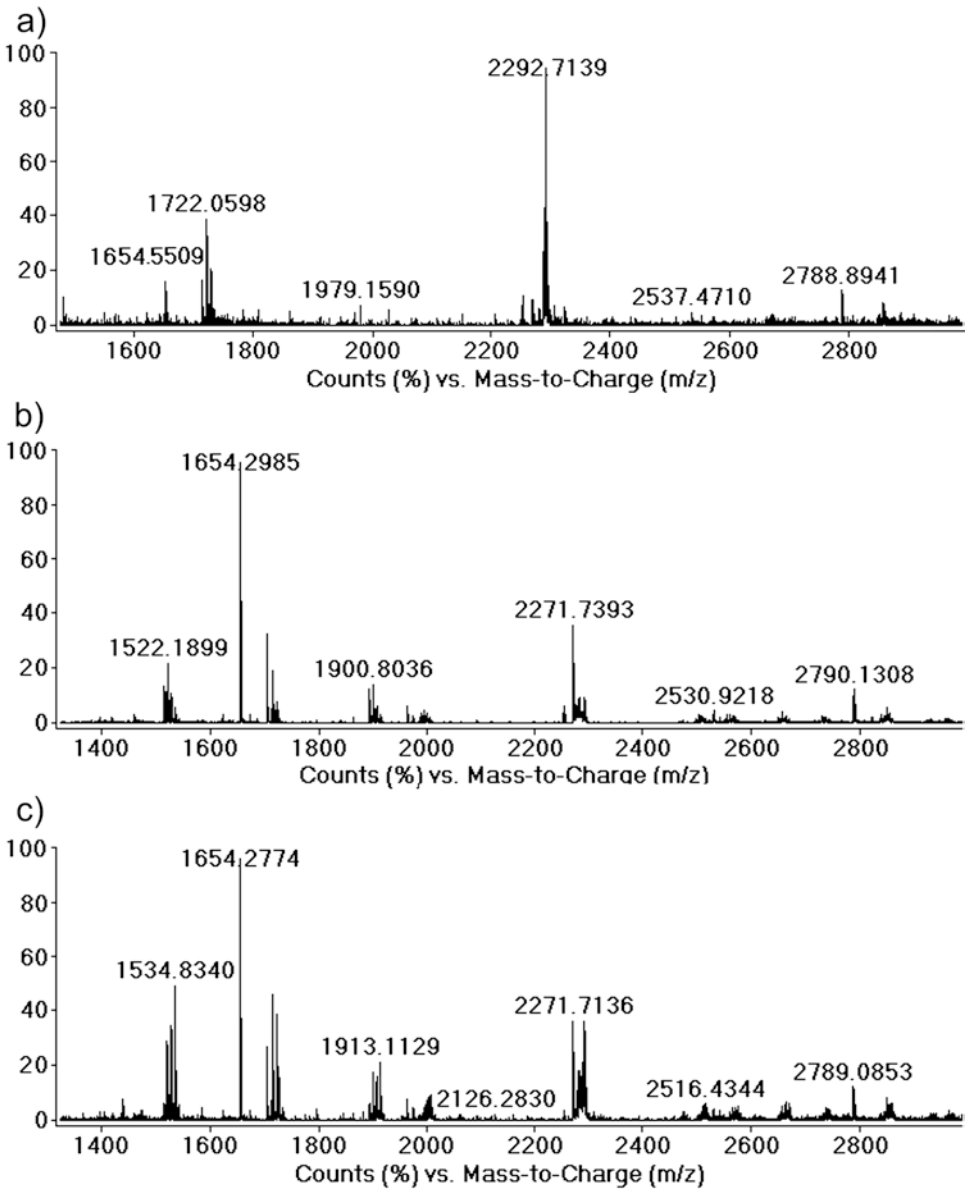


Fig. 41.8 Positive ion ESI-MS spectrum of β-CD_4 at ratio of (**a**) 1.5:1, (**b**) 2:1 and (**c**) 3:1 ratio. Reproduced with permission from Polymer Chemistry, 9, Pricope, G. et al., Novel cyclodextrin-based pH-sensitive supramolecular host-guest assembly for staining acidic cellular organelles, 968–975., Copyright (2018), with permission from The Royal Society of Chemistry [88]

41.5 Conclusions

Cyclodextrins have been used more and more as hosts for different guest molecules, with good potential medical applications, due to their ability to mask their toxicity, increase their solubility, and protect them from degradation or bioconversion.

Mass spectroscopy has had an increased use in the last period in the study of inclusion complexes. This review presented several methods to prepare inclusion complexes of CDs, the possibility to characterize them and some MS studies on CDs inclusion complexes with antifungal agents, antioxidant molecules or fluorescent dyes. The ESI-MS experiments have offered a direct and clear determination of the ICs formation and their stoichiometry but cannot always provide data about the nature of these complexes, such as the magnitude of the binding constants and/or the specific orientation of the guest inside the CDs cavity. The size and the specific nature of the substituents involved shows that both steric and electronic factors must be considered in predicting which CDs inclusion complexes stoichiometry's will be stable enough to be observed in the ESI-MS experiments.

A careful examination of the MS results, together with a good selection of references, can make the difference between distinguishing real inclusion complexes and non-specific complexes. Other complementary techniques such as NMR, UV-Vis and molecular modelling can help to further confirm the MS results.

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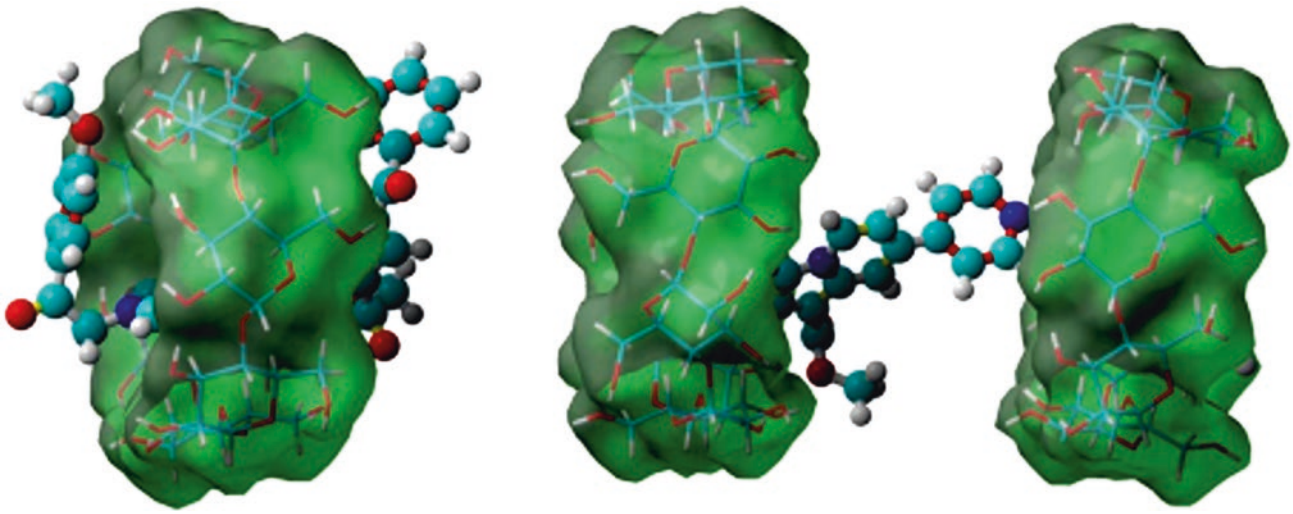

Fig. 41.9 Molecular docking models of IPy in complex with β-CD showing the possibility of the 1:1 (left) and 1:2 (right) inclusion complex formation. Reprinted from Polymer Chemistry, 9, Pricope, G. et al., Novel cyclodextrin-based pH-sensitive supramolecular host-guest assembly for staining acidic cellular organelles, 968–975., Copyright (2018), with permission from The Royal Society of Chemistry [88]

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