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ORIGINAL ARTICLE

Complexation of zearalenone and zearalenols with native and modified β-cyclodextrins

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Abstract The complexation of the oestrogenic mycotoxin zearalenone (ZEN) and its metabolites α- and β-zearalenol (ZOLs) with native β-cyclodextrins (β-CD) and modified hydroxypropyl-β-CD and dimethyl-β-CD was studied by fluorescence spectroscopy, nuclear magnetic resonance spectroscopy and electrospray-mass spectrometry. The formation of the inclusion complex was confirmed by NMR studies of zearalenone:β-CD solution. NMR, ESI-MS and fluorescence data are in agreement with the formation of a 1:1 complex between zearalenone and β-CD, characterized by the deep insertion of the phenolic moiety inside the cavity of the CD from its secondary side. The complexes formed between the guests and native β-CD are characterized by high stability constants ($>10^4$), as measured by fluorescence titrations.

Keywords Zearalenone · Zearalenols · Cyclodextrins · Inclusion complexes · Binding constants

Introduction

Zearalenone (ZEN) is a toxic secondary metabolite produced by several species of *Fusarium* fungi, mainly *F. graminearum* and *F. culmorum*, which grow on several food commodities, especially cereals such as maize, barley, oats, wheat, and sorghum [1]. The structure of ZEN consists of a resorcinol moiety fused to a 14-membered macrocyclic lactone ring, which includes a *trans* double bond, a ketone, and a methyl side group. The structure is flexible enough to adopt a conformation able to bind to the mammalian oestrogen receptor, although with lower affinity than the natural oestrogen 17-β-estradiol: this results in severe effects on the reproductive system in several animal species, particularly pigs. The reduction of the C-6 ketone in the liver of animals and humans gives rise to two different diastereoisomers, namely α- and β-zearalenol (ZOLs). The oestrogenic effect of α-ZOL is approximately three times stronger than that of ZEN and β-ZOL. Only α-ZOL has been observed to occur in naturally contaminated cereal grains, probably produced *via* metabolization of ZEN by competitive microflora [2]. Although suspected, the carcinogenic properties of ZEN are not yet well defined and are currently under study (IARC class III carcinogen).

The levels of ZEN contamination frequently found in cereals are largely variable up to 2,900 ppb [3], depending on climate, harvest, and storage conditions. Several countries have established guidelines or maximum tolerable levels for ZEN, ranging from 0 ppb in the Netherlands and in Hungary to 1,000 ppb in Russia. The European Union has very recently set legal limits for the Member Countries, in particular to regulate the presence of this mycoestrogen in cereal-based foods for human consumption and in baby foods (Commission Regulation (EC) No 1126/2007 of 28 September 2007; Official Journal of the European Union, L 255/14, 29.9.2007).

For ZEN detection in foods several analytical methods are currently available, including chromatographic techniques [4], hyphenated techniques [5], and immunoassays [6]. According to the fluorescent properties of ZEN, the most commonly used method for its analytical determination in food is based on HPLC with fluorescence detection, although extensive clean-up of the sample is usually

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required. Excitation is carried out at 275–280 nm and fluorescence emission is measured at 450–470 nm, allowing to reach limit of detection down to 2 ng/g.

Methods able to enhance the native fluorescence response of the mycotoxin could be very helpful in lowering the detection limits and in simplifying the analytical procedures.

One of the most promising tools is the use of cyclodextrins (CDs), cyclic oligosaccharides formed by six (α-CD), seven (β-CD) or eight (γ-CD) glucose residues linked by α-(1→4) glycosidic bonds, as supramolecular host. Indeed, CDs have the shape of a hollow truncated cone with an hydrophobic cavity and an hydrophilic external part which affords their water solubility and their ability to form host–guest inclusion complexes with a variety of organic and inorganic molecules. Upon inclusion, the guests increases its solubility and stability as well as modifies its physical and chemical properties, effects particularly useful in the food sector both from the analytical and technological point of view [7–9]. In particular, CDs have successfully been used as fluorescence enhancers to increase sensitivity in fluorescence-based food diagnostic for many compounds which are usually found at trace level, as the fluorophore included into the cavity is protected by the quenching effect exerted by the solvent. In this field, the study of complex stability and its formation mechanism is very important for the development of reliable analytical applications [10–13]. Very recently, the application of cyclodextrins in the field of mycotoxin analysis has been reviewed by Maragos et al. and Galaverna et al. [14, 15].

Although electrophoretic and chromatographic separations of ZEN and other mycotoxins were obtained by using cyclodextrins as additives in order to improve the efficiency of separation [16–18], in many cases UV detection was used and the potential effect of the inclusion complex formation on ZEN fluorescence was not described. Instead, very recently, the fluorescence enhancement of zearalenone in the presence of cyclodextrins was used by Maragos and Appell [19] for improving its analytical determination in maize by capillary electrophoresis with laser-induced fluorescence detection (CE-LIF). The enhancement effect was ascribed to inclusion complex formation, but no data have been produced so far to characterize the complex. Thus, the aim of this work was to investigate which factors affect the ZEN fluorescence enhancement in the presence of β-CDs and to characterize the host–guest ZEN–CD inclusion complex. In particular, we studied the effect of unmodified and functionalized β-CDs on the fluorescence properties of zearalenone and its main metabolites α- and β-zearalenol (ZOLs) in aqueous solution, as well as the nature of the ZEN–CD and ZOLs–CD interactions by spectroscopic experiments, such as NMR and fluorescence measurements. Stability constants of the ZEN–CD and ZOLs–CD complexes were evaluated by performing fluorescence titrations with β-CD.

Materials and methods

Reagents

Zearalenone (ZEN), α- and β-zearalenol (α- and β-ZOL) standards were from Sigma Biochemicals (St. Louis, MO, USA). All solvents were LC grade from Carlo Erba (Milan, Italy) and bidistilled water was produced in our laboratory utilising an Alpha-Q System Millipore (Marlborough, MA, USA). β-CD was purchased from ACROS (Carlo Erba, Italy). 2-Hydroxypropyl-β-CD (HP-β-CD), 2,6-dimethyl-β-CD (DIMEB) and deuterium oxide (99.96 atom %D) were purchased from ALDRICH (Steinheim, Germany).

Standard solution of zearalenone and zearalenols: preparation and decontamination

Standard stock solution of zearalenone and its metabolites α- and β-zearalenol were prepared by dissolving the powder (1 mg) in 2.5 mL of acetonitrile. Working solutions of zearalenone and zearalenols (10^{-6} M) were prepared from standard stock solutions by evaporating the organic phase under nitrogen and dissolving the residue in water. These solutions are stable at +4 °C for one month. Decontamination of waste solutions and glassware was performed with sodium hypochlorite (10% aqueous solution) for 12 h.

Fluorescence measurements

Fluorescence spectra were recorded on a PERKIN ELMER LS50 instrument (Perkin Elmer, Waltham, Massachusetts, USA) in a 0.2 × 0.2 cm quartz microcuvette (type: 105.251-QS, light path: 3 mm, centre: 15 mm) (Hellma, Müllheim, Germany). Due to the solvent effects on the absorption and luminescence spectra, the proper excitation wavelengths were selected by scanning the excitation spectra of the different zearalenone and zearalenols solutions (10^{-6} M). In particular, the best excitation wavelenght for zearalenone and zearalenols aqueous solutions (10^{-6} M) was found to be 274 nm. The emission scan was performed in the wavelength range 380–600 nm. Both emission and excitation slits were set at 15 nm. Each spectrum was recorded in triplicate.

In the conditions used for this study, no light scattering phenomena were recorded for cyclodextrin solutions (1 mM), which have been treated in a sonicator bath for 5 min after preparation and filtered just before fluorescence monitoring using a 0.2 μm nylon filter.

Measurements of fluorescence enhancements by CDs

The solutions of ZEN or ZOLs (10^{-6} M) and cyclodextrins (1 mM) were prepared in bidistilled water and the spectra of each analyte alone (blank) and in the presence of cyclodextrin (molar ratio Guest:CD = 1:10^3) were recorded; the F/F_0 ratio was calculated, where F and F_0 are the fluorescence intensities at 454 nm recorded in the presence (sample) or in the absence (blank) of the CDs, respectively.

Determination of stoichiometry and association constants by fluorescence measurements

The stoichiometry of inclusion complexes was determined by the continuous variation method (Job's Plot) [20]. Equimolar 3.0×10^{-6} M solutions of ZEN and CD were mixed to a standard volume varying the molar ratio but keeping the total concentration of the species constant. After stirring for 2 h, the fluorescence at 450 nm was measured for all solutions. The collected data were reported by plotting ΔF[ZEN], where $\Delta F = F - F_0$ is the difference in emission in the presence and in the absence of CDs, against r, where r = [ZEN]/{[ZEN] + [CD]}is the molar fraction of the guest.

For the determination of binding constants (K_c), aqueous solutions of β-CD (1 mM) were prepared and diluted to the desired concentrations. The concentration of ZEN or ZOLs was fixed at 1×10^{-6} M and the concentration of β-CD was changed from 1×10^{-4} M to 1×10^{-3} M.

Suitable aliquots of the CD solutions were added to the zearalenone or zearalenol solutions and the fluorescence spectra were recorded, measuring the variation of the F/F_0 ratio at 454 nm as a function of the aliquots of CD added. The fluorescence intensities were corrected for dilution effects. The experimental data were used to calculate the binding constants [21–23].

The fluorescence intensity at any wavelength (F) can be related to the CDs concentration by a non-linear regression described by the following equation [22]:

$$F = F_0 + \frac{(F_\infty - F_0)K_c[CD]_i}{1 + K_c[CD]_i} \quad (1)$$

where F_∞ is the fluorescence intensity when the complex mycotoxin:CD is the only species present at the equilibrium and F_0 is the fluorescence of ZEN or ZOLs in the absence of CD. Experimental data of F as a function of [CD] can be fitted to Eq. 1, using as initial parameters K_c and F_∞ those obtained from the analysis of the experimental data using the Benesi-Hildebrand equation for 1:1 complexes (double reciprocal plot) [21]:

$$\frac{1}{(F_i - F_0)} = \frac{1}{(F_\infty - F_0)K_c[CD]_i} + \frac{1}{(F_\infty - F_0)} \quad (2)$$

where F_i and F_0 are the fluorescence intensities of ZEN in the presence and in the absence (blank) of CD, respectively; F_∞ is the fluorescence intensity of the complex and $[CD]_i$ is the CD concentration after each addition.

NMR experiments

^{1}H-NMR spectra were recorded on a NMR Varian Inova 600 MHz at 25°C (Varian, Palo Alto, California, USA). The measurement were carried out with a solution of ZEN and ZEN:β-CD (molar ratio 1:1). A stock solution of zearalenone was prepared by dissolving 10 mg of the mycotoxin into 1.5 mL of acetonitrile. The ZEN working solutions (10^{-3} M in deuterium oxide) were prepared daily as follows: a proper amount of stock solution was dried under nitrogen flow and then under vacuum overnight, the residue was dissolved in 750 µL of deuterium oxide, degassed, spun and transferred into the NMR tube. The working solution of ZEN:β-CD 1:1 was prepared in the same way, by dissolving the dried ZEN residue in β-CD solution (10^{-3} M in deuterium oxide). The chemical shift and the ^{1}H–^{1}H correlation were obtained from the proton spectra (^{1}H-NMR) and from the homonuclear correlation spectra (gCOSY); the NOESY spectra were recorded to evaluate the interaction between ZEN and β-CD. All the experiments were performed using the default programs available on the instrument. NMR spectra were processed with MestReC 4.9.9.9 (Mestrelab Research SL, Santiago de Compostela, Spain) and the coupling constants were determined by line fitting and simulation of the real spectra.

ESI-MS experiments

Mass spectrometry experiments were performed on a Waters Acquity SQ (single quadrupole) mass spectrometer using electrospray ionization (Waters Co., Milford, MA, USA). The source temperature was set at 180 °C and the probe voltage was 3.2 kV in positive ion mode. Nitrogen was applied as both the nebulizer and desolvation gas. Data were acquired in a mass range of 200–2,000 m/z. Each working solution (1 mM β-cyclodextrin, 1 mM zearalenone and 1 mM β-CD:ZEN 1:1 complex) was infused in the equipment at an infusion rate of 10 µL/min and the mass spectra were recorded in the continuous mode for 2 min.

Results and discussion

ZEN fluorescence enhancement obtained with different CDs

In order to evaluate which cyclodextrin induced the highest fluorescence enhancement for zearalenone and its derivatives, a spectroscopic screening of native β-CD and of the substituted hydroxypropyl-β-CD (HP-β-CD) and dimethyl-β-CD (DIMEB) were carried out in aqueous solutions. These three CDs were chosen on account of their commercial availability and their specific properties: in particular, β-CD as reference compound, DIMEB as one of the most efficient enhancer of fluorescence intensity on the base of Maragos' results [19] and HP-β-CD for its high solubility in water. The aim was that of investigating the role played by the CD cavity and by the nature of the substituents on the fluorescence enhancement.

The recorded fluorescence enhancements due to the addition of CDs at a molar ratio ZEN (ZOLs):CD = $1{:}10^5$ are reported in Table 1. All the tested CDs induced a high enhancement in the fluorescence emission spectra of the analytes and a blue shift in the emission maximum wavelength (from 458 nm to 454 nm), both phenomena suggesting a strong interaction between the mycotoxins and CD. In particular, blue shifts of 3–6 nm in the emission maximum were similar to those recorded for the analytes in media less polar than water, as methanol or ethanol, and were consistent with the lower polarity of the environment experienced by the fluorophore due to the inclusion into the CD cavity.

As reported, the highest enhancement was recorded for zearalenone, whereas α- and β-zearalenol showed lower enhancement. Concerning CDs, the most effective enhancement was obtained with DIMEB, followed by HP-β-CD and the native β-CD, for all the three analytes. Fluorescence enhancement was in agreement with the hypothesis of the inclusion of the phenolic moiety, which is responsible for the analyte fluorescence. Moreover, on the base of these data, it may be inferred that the mycotoxin C6 moiety influences the inclusion mechanism: indeed, the highest enhancement is recorded for zearalenone, which present a sp^2 carbonyl group in C6. The isomeric forms α-zearalenol and β-zearalenol showed lower fluorescence enhancement in the presence of CDs, suggesting that the position of the hydroxyl-group on C6, inducing different conformation of the molecule, may influence the stability of the inclusion complex.

Table 1 Fluorescence enhancements of zearalenone (ZEN), α-zearalenol and β-zearalenol (ZOL) in the presence of CDs expressed as F/F_0, where F is the emission intensity recorded for the guest in the presence of CDs and F_0 is the emission intensity of the guest in the absence of CDs

	F/F_0		
	β-CD	DIMEB	HP-β-CD
ZEN	19.9 ± 0.5	24.3 ± 0.8	20.9 ± 1.1
α-ZOL	13.0 ± 0.8	18.0 ± 0.6	14.8 ± 1.4
β-ZOL	8.7 ± 0.9	11.9 ± 0.6	10.1 ± 0.7

NMR experiments

The nature of the ZEN:β-CD complex was investigated by NMR spectroscopy. 1D and 2D NMR spectra of zearalenone were performed in D_2O in the presence or in the absence of β-cyclodextrin. ^1H and ^{13}C NMR spectra for ZEN were already reported in DMSO and methanol [24, 25]. Since the aqueous medium is necessary for complexation of zearalenone with β-CD, the NMR spectrum of pure zearalenone was acquired in D_2O. The data obtained are in agreement with those already published in the literature which support a planar structure with a slight bending of the aliphatic part respect to the aromatic moiety [24]. The solution structure is also in agreement with the solid state structure of the molecule [24].

By comparison of the ^1H-NMR spectra of the toxin alone with that of the toxin in the presence of β-cyclodextrin, large variations of the chemical shifts of the toxin protons were measured in the presence of CD: in particular, significant downfield shifts were observed for H-5, H-1′, H-10′, and an upfield shift for H-2′ (Fig. 1a).

The downfield shift is common for guest molecules in the presence of CDs as hosts: in particular, a downfield shift of the aromatic proton H5, and those close to it (H1′, H10′ and H11′), is due to the deshielding effect exerted by the hydrophobic cavity on the aromatic ring. The upfield shift of the proton H2′ is probably due to the effect of the shielding cone of the aromatic ring, whereas the alternate upfield and downfield shifts of some aliphatic protons (H4′, H5′, H7′) apparently indicate an interaction of only one side of the macrocyclic moiety of ZEN molecule with the rim of the β-CD. Correspondingly, a large shielding of the cyclodextrin protons was observed. In particular, significant upfield shifts of the H-3 and H-5 protons, which are directed inwards the cavity, were observed (Fig. 1b). Moreover, modification of the coupling constants observed for ZEN and CD are indicative of conformational changes in the host as well as in the guest as a consequence of the formation of the inclusion complex.

The formation of the inclusion complex was confirmed by ROESY experiment: this NMR experiment based on the NOE effect is particularly useful to study interactions between host and guest as correlation spots are present in a bidimensional map when two protons are close to each

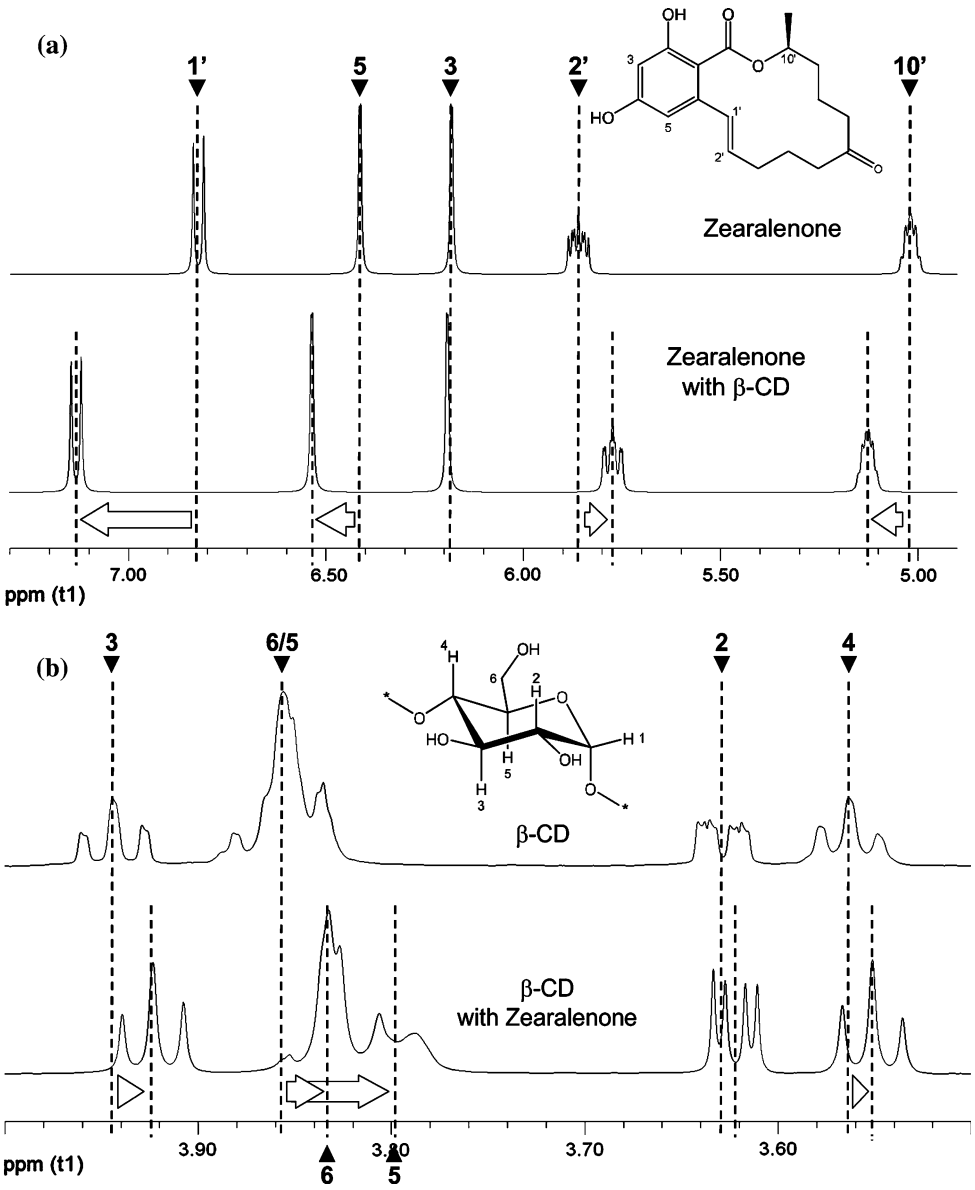

Fig. 1 Comparison between ^{1}H-NMR spectrum of ZEN in the absence and in the presence of β-CD in D_2O (2a) and between ^{1}H-NMR spectrum of β-CD in the presence and in the absence of ZEN in D_2O (2b)

other in space: indeed, correlation peaks between the cyclodextrin protons inside the cavity and the aromatic and one double bond proton of zearalenone were observed (see Fig. 2).

In particular, strong correlation peaks were found between H-2′ of zearalenone and H-3 of β-CD and between H-5 of zearalenone and H-5 (stronger peak) and H-3 (weaker peak) of β-CD: these correlations strongly support the inclusion of the aromatic moiety into the cavity. The stronger correlation between the H-5 proton of ZEN with H-5 proton of β-CD and the correlation peak between the aromatic H-3 proton of the toxin with the H-6 protons of β-CD is indicative of the deep insertion of the phenyl ring inside the cavity from the lower rim, with the H-3 proton protruding from the upper rim close to the H-6 of β-CD.

Electrospray-mass spectrometry experiments

In order to obtain further evidences of the formation of inclusion complexes between CD and zearalenone ESI-MS experiments were performed: indeed, several papers report the use of this technique to study host–guest interactions [26].

The identification of major peaks recorded for zearalenone and β-cyclodextrin solutions are reported in Table 2 and the mass spectrum obtained for ZEN-β-CD complex is reported in Fig. 3.

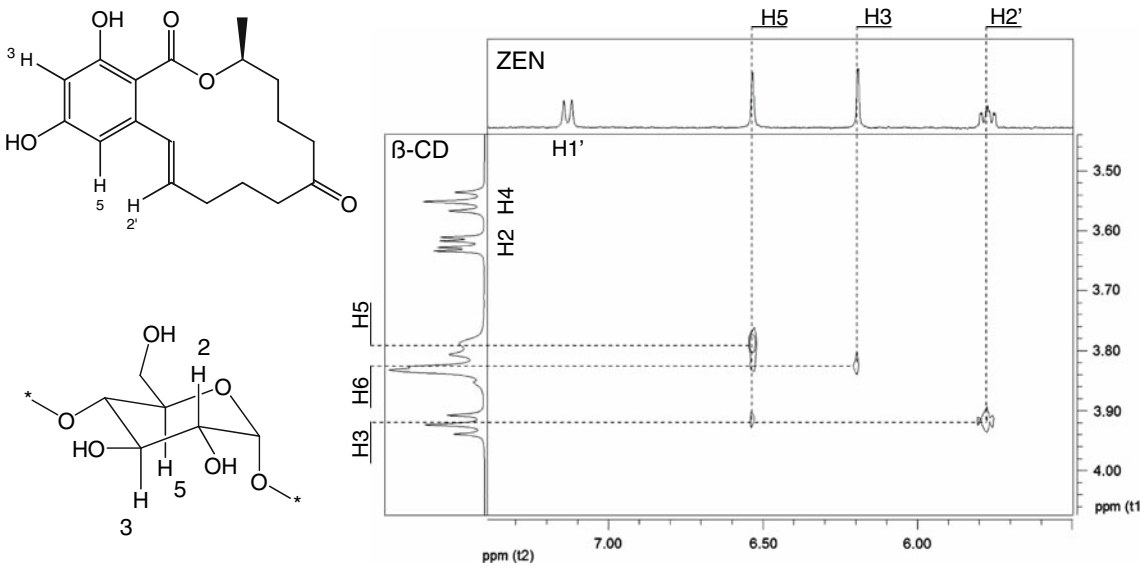

Fig. 2 Portion of the ^{1}H–^{1}H 2D ROESY spectrum for a solution of ZEN:β-CD complex in D_2O

Table 2 Identification of the main ions recorded in the ESI mass spectra of zearalenone, β-cyclodextrin and of the complex ZEN:β-CD

β-cyclodextrin (1 mM)		Zearalenone (1 mM)		Zearalenone: β-cyclodextrin (1 mM)	
Mass *m/z*	Identification	Mass *m/z*	Identification	Mass *m/z*	Identification
579	$[CD+H+Na]^{2+}$	283	$[ZEN\text{-}2H_2O+H]^{+}$	**492**	**$[ZEN+CD+2H+Na]^{3+}$**
587	$[CD+H+K]^{2+}$	301	$[ZEN\text{-}H_2O+H]^{+}$	**497**	**$[ZEN+CD+2H+K]^{3+}$**
1,135	$[CD+H]^{+}$	319	$[ZEN+H]^{+}$	579	$[CD+H+Na]^{2+}$
1,157	$[CD+Na]^{+}$	341	$[ZEN+Na]^{+}$	587	$[CD+H+K]^{2+}$
		357	$[ZEN+K]^{+}$	**738**	**$[ZEN+CD+H+Na]^{2+}$**
		637	$[2ZEN+H]^{+}$	**746**	**$[ZEN+CD+H+K]^{2+}$**
		659	$[2ZEN+Na]^{+}$	897	$[2ZEN+CD+H+Na]^{2+}$
				905	$[2ZEN+CD+H+K]^{2+}$
				1,135	$[CD+H]^{+}$
				1,157	$[CD+Na]^{+}$
				1,475	**$[ZEN+CD+Na]^{+}$**

The fragments due to the ZEN:CD 1:1 complex are typed in bold

The mass spectrum of the complex is characterized by the presence of several multicharged adducts between CD, zearalenone and cations. In particular, the cationization involved mainly sodium and potassium: this behaviour is typical for cyclodextrins, as recently reported by Sforza et al. [27]. The most intense peaks of the depicted range at *m/z* 492 and 498 are the triple charged ion adducts $[CD:ZEN + 2H + Na]^{3+}$ and $[CD:ZEN + 2H + K]^{3+}$, while the peaks at *m/z* 738 and 746 refer to the double charged adducts of the 1:1 complex (Fig. 3). Moreover, the sodium adduct $[CD\text{-}ZEN + Na]^{+}$ also occurred, although at lower intensity. The signals due to uncomplexed cyclodextrin are also present in the spectrum: in particular, $[CD + H + Na]^{2+}$ and $[CD + H + K]^{2+}$ have a good intensity with the same cationization pattern. Finally, the signals at *m/z* 897 and 905 are attributable to the formation of the doubly charged 1:2 CD:ZEN complex: the presence of this species, which probably occurs only in the gas phase, is a phenomenon already observed for ESI-MS investigation and related to the ionization conditions passing from the liquid to the gas phase: the so called "false positives" [28]. However, to further confirm the hypothesis of a 1:1 complex formation, we performed fluorescence titration experiments.

Determination of the complex stoichiometry

In order to verify the stoichiometry of the complex formation, we used the method of continuous variations, often

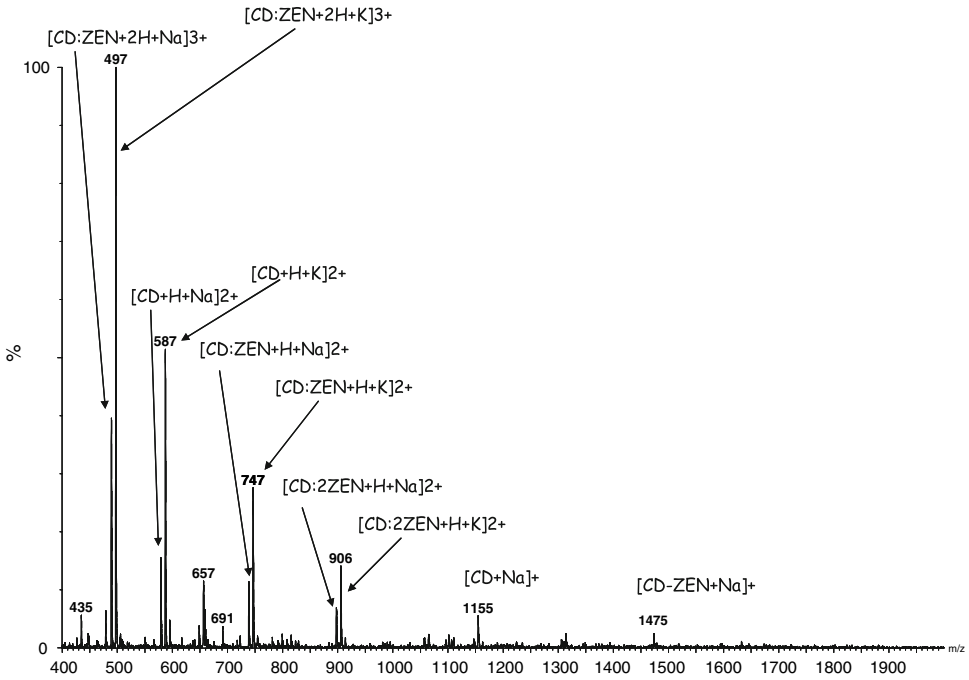

Fig. 3 Mass spectrum of the ZEN:β-CD complex

known as the Job's Plot [20], commonly used for determining the stoichiometry of two interacting components. With this method, the total molar concentration of the two components is held constant while their molar ratios are continuously varied. A measurable parameter that is linearly proportional to the complex formed is plotted against the mole fractions to generate a curve. The binding stoichiometry is then determined from the ratio of the mole fractions of the two components found at the maximum of the curve.

According to this method, ΔF values were calculated by measuring the emission intensity of ZEN solutions in the presence and in the absence of β-CD. In these standard solutions, the total molar concentration of the two species was constant ($M = 1 \times 10^{-6}$ M), but the ratio of the initial concentrations, expressed by r varied between 0 ([ZEN]/[β-CD] = 0:10) and 1 ([ZEN]/[β-CD] = 10:0). After the mixing, the working solution were stirred for 5 min to allow equilibration before fluorescence emission monitoring. The resulting continuous variation plots (Fig. 4) demonstrate that since the ΔF[ZEN] maximum value corresponds to a r value of 0.5, ZEN:β-CD complex has a 1:1 stoichiometry.

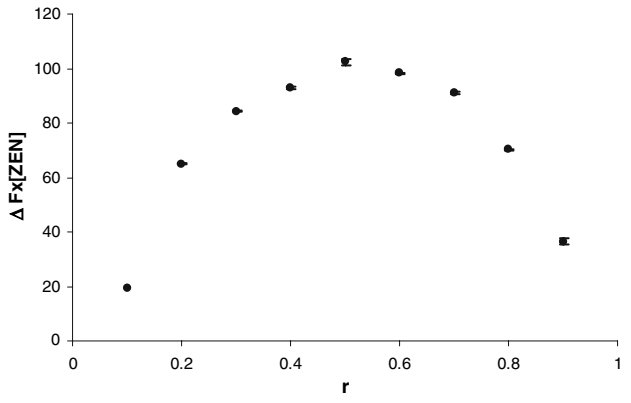

Fig. 4 Continuous variation plot (Job Plot) for the ZEN:β-CD complex from fluorescence measurements

Determination of the binding constants K_c

Many studies have been reported in the literature about the calculation of binding constants (K_c) between cyclodextrins and different compounds, using different methods, such as fluorescence, nuclear magnetic resonance, enzymatic methods, conductometry, calorimetry and affinity capillary electrophoresis. In our case, fluorescence measurements have been chosen as experimental data for binding constants calculation, since these technique provides reliable results even when small amounts of toxin are used, as required on account of its toxicity. Moreover, experiments are fast and simple to perform and thus, a large number of data can be easily collected and elaborated.

The binding constant calculation based on fluorescence data started from the observation that the increase in fluorescence intensity is a function of toxin complexation by CD. When increasing concentrations of CD were added to the reaction medium, the fluorescence intensity increased until a maximum was reached.

The representation of $1/(F - F_0)$ versus 1/[CDs] (double reciprocal plot), known as Benesi–Hildebrand plot, leads to a straight line for the three guests used. This linear

Table 3 Data obtained for the calculations of the binding constants of ZEN, α-ZOL and β-ZOL with β-CD considering a stoichiometry 1:1

	Fitting model	R^2	log K
ZEN:CD	Benesi–Hildebrand equation	0.9929	4.27 ± 0.21
	Non-linear fitting	0.9974	4.33 ± 0.18
α-ZOL:CD	Benesi–Hildebrand equation	0.9978	4.45 ± 0.17
	Non-linear fitting	0.9995	4.44 ± 0.11
β-ZOL:CD	Benesi–Hildebrand equation	0.9928	4.27 ± 0.12
	Non-linear fitting	0.9970	4.43 ± 0.19

relation agrees with that described by the Benesi–Hildebrand Equation (Eq. 2, Experimental Section). The linear plots can be used to determine K_c values by simply dividing the intercepts by the slopes (Table 3). Since in the Benesi–Hildebrand plots correlation come out using the reciprocal of the different variables, the slope of the line is more sensitive to the ordinate values of the points for the lowest CD concentrations. In order to avoid this effect, a non-linear regression analysis of the plots was carried out by applying the Eq. 1 and using as the initial parameter values those estimated by the Benesi–Hildebrand plot. The K_c values obtained by the non-linear regression (Table 3) were in very good agreement with those calculated by the Benesi–Hildebrand plot.

All the regression coefficients calculated for complexes of ZEN and ZOLs with β-CD showed very good correlation values (>0.99) for the 1:1 stoichiometry, in agreement with the results obtained from the Job's plot experiment. The calculated binding constants are reported in Table 3.

The experimental fluorescence titration data obtained for the considered guests have been fitted with theoretical curves obtained using the data calculated by the non-linear regression model. The fitting was very good, as reported in Fig. 5.

Since substituted CDs such as DIMEB and HP-β-CD seem to have a stronger effect on the ZEN fluorescence intensity, the corresponding binding constants were calculated using the same approach previously described. As reported in Table 4, logK values obtained for ZEN with HP-β-CD and DIMEB are in agreement with the stronger enhancement reported in Table 1. On the other hand, the standard deviations reported for these experiments are also higher: the CV% obtained for ZEN: β-CD binding constant calculation was 4.2%, whereas those obtained for ZEN:HP-β-CD and ZEN:DIMEB were 15.7 and 16.3%, respectively. Since these cyclodextrins are commercial, they are a mixture of different isomers with a substitution degree that may vary between different batches, thus the logK values should be considered as an average value and can be affected by a larger variance.

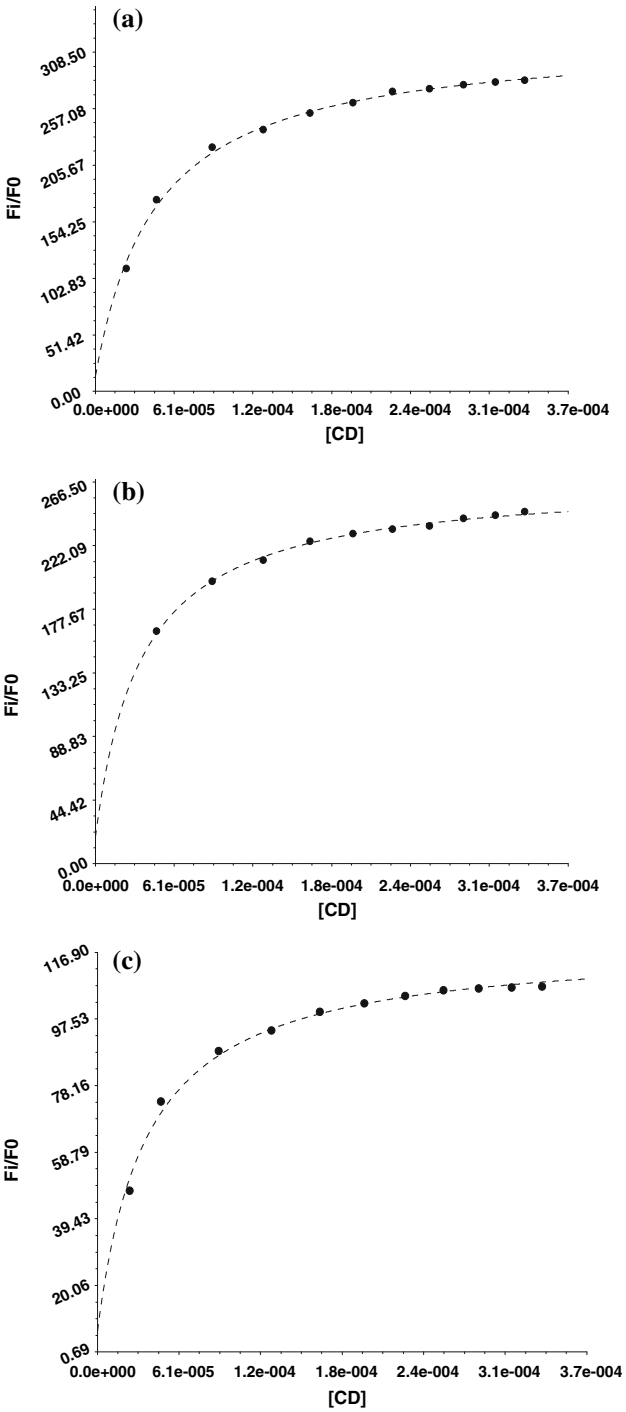

Fig. 5 Fluorescence titrations (●) and theoretical curves (- - -) obtained using the non-linear regression method according to Eq. 1 for zearalenone (**a**), α- zearalenol (**b**), and β-zearalenol (**c**) with β-cyclodextrin

Conclusion and perspectives

In conclusion, all the data obtained confirm that zearalenone, α- and β-zearalenols and cyclodextrins give rise to a host–guest complexation, based on the inclusion of the

Table 4 Data obtained for the calculations of the binding constants of ZEN with β-CD, HP-β-CD and DIMEB considering a 1:1 stoichiometry

	log K
ZEN: β-CD	4.33 ± 0.18
ZEN: HP-β-CD	4.98 ± 0.78
ZEN: DIMEB	5.21 ± 0.85

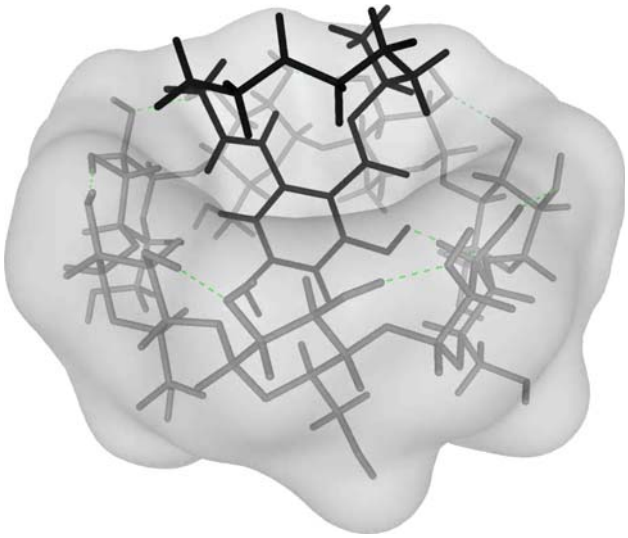

Fig. 6 Model of the inclusion complex between ZEN and β-CD

phenolic moiety inside the CD cavity from the lower rim. The inclusion into the cavity is responsible for the high fluorescence enhancement observed. On the base of these data the proposed structure of the complex between zearalenone and β-cyclodextrin is reported in Fig. 6. The model is obtained using Spartan™ 04 (ver. 1.0.0) by Wavefunction Inc. (Irvine, CA), using Molecular Mechanics MMFF algorithm in absence of solvent.

An analogous complexation mode may be envisaged in the case of the two metabolites α-ZOL and β-ZOL.

The understanding of the inclusion mechanism and of the complexation mode may actually allow to design and prepare selectively modified CDs in order to increase the affinity of the guest. These tailored selectors with enhanced selectivity may be used in the near future to set up very efficient detection methods, also suitable for rapid screening of food and feed products. Cyclodextrins are currently tested as fluorescence enhancers for zearalenone and zearalenols detection in HPLC or with spectrofluorimetric instrumentation, in order to achieve lower detection limits and simplified sample preparation. Moreover, chemosensors based on a luminescence response may be integrated into microarray systems, which could be applied for early detection of post-harvest contamination, providing for an easy-to-use control tool for mycotoxin analysis.

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