

# NMR studies of complex formation between natural cyclodextrins and benzene

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## Research Article

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# Abstract

Inclusion complexes of benzene (Bz) with cyclodextrins (CD) have been investigated so far using non-NMR techniques resulting in conflicting data. Here, the first application of NMR spectroscopy in combination with rigorous statistical analysis of the results has allowed us to determine accurately the stoichiometry of complexes and their association constants. Titration measurements have been performed by  $^1\text{H}$  NMR spectroscopy in  $\text{D}_2\text{O}$  at a magnetic field  $B_0$  of 14.1 T.  $\alpha\text{CD}$  and  $\gamma\text{CD}$  host molecules form weak 1 : 1 complexes with Bz. In contrast, Bz and  $\beta\text{CD}$  build 1 : 1 and 2 : 1 complexes coexisting in solution with large binding constants. Binding of second benzene molecule is strongly cooperative.

## Introduction

Components of supramolecular complexes studied in this research have been known in chemistry for a very long time. Benzene (Bz) is a hydrocarbon that was first isolated in its pure form in 1825 by Michael Faraday [1]. Its six-membered cyclic structure was proposed in 1865 by August Kekulé and confirmed in 1929 using X-ray diffraction methods by Kathleen Lonsdale [2]. Cyclodextrins (CD), carbohydrates obtained from starch, were first mentioned in scientific reports in 1891 by Antoine Villiers. In the late 1950s, Dexter French and co-workers determined the molecular weight, exact chemical structure, dimensions and bond types of the three naturally occurring basic cyclodextrins:  $\alpha\text{CD}$ ,  $\beta\text{CD}$  and  $\gamma\text{CD}$ . At this time, the first studies on the formation of inclusion complexes by cyclodextrins accepting small molecules into their cavity were also described [3].

Cyclodextrins (CDs) are macrocyclic oligosaccharides composed of a number of glucopyranoside units bound together by  $\alpha$ -1,4 bonds. The naturally occurring  $\alpha$ ,  $\beta$  and  $\gamma$  cyclodextrins ( $\alpha\text{CD}$ ,  $\beta\text{CD}$ , and  $\gamma\text{CD}$ ) consist of six, seven, and eight glucopyranose units, respectively [4]. They are obtained by enzymatic starch degradation [4–6]. CDs, whose shape remains a truncated cone, contain a lipophilic central cavity paved with apolar glucopyranose H3 and H5 hydrogens and endocyclic oxygens suitable for entrapping nonpolar molecules. Outer CD surface is hydrophilic owing to hydroxyl groups orientated outwards [6, 7]. The size of CD cavity allows for accommodating many low and medium molecular weight compounds (approximately  $\leq 1000$  daltons).  $\alpha$ - and  $\gamma$ -cyclodextrins dissolve well in water in contrary to  $\beta\text{CD}$  exhibiting an order of magnitude smaller solubility. However, at high pH, ionization of hydroxyl groups increases solubility of  $\beta\text{CD}$  in water [8]. It is also noteworthy that solubilities of all three CDs in  $\text{D}_2\text{O}$  are considerably diminished in comparison with those in  $\text{H}_2\text{O}$  [9]. CDs are also soluble in other polar solvents, dimethylsulfoxide or dimethylformamide, but insoluble in nonpolar solvents. In aqueous solutions, CDs can form host–guest inclusion complexes with many partially or fully lipophilic molecules often increasing a guest solubility. Hence their wide application in chemistry, pharmacy, or food industry as drug or fragrance carriers [4, 6, 10, 11]. Their complexes with harmful chemicals are regarded as one of the methods allowing to remove pollutants from the environment [12–15]. Besides  $\alpha\text{CD}$ ,  $\beta\text{CD}$ , and  $\gamma\text{CD}$  larger cyclodextrins built up of dozens of glucopyranose units exist [16, 17]. One has to be aware, however, that large cyclodextrins display strong internal mobility of macrocycle [18] resulting in their

irregular shape [19] and are prone to the macrocycle ring opening owing to hydrolysis of glycosidic bond not only under acidic conditions [20] but also at room temperature and neutral pH. This makes questionable the use of large CDs as host molecules.

Benzene is currently produced from fossil resources. It has long been classified as a highly carcinogenic compound and therefore its toxicity has been the subject of much research [21]. Despite this, its production is increasing rapidly worldwide [22] because the compound is used to obtain numerous precursors of commonly used polymers and resins. Benzene at room temperature under atmospheric pressure is a colorless liquid with high volatility (94.8 mm Hg – 12.64 kPa at 25°C) [23] slightly soluble in water (1.79 g/dm<sup>3</sup>) [24].

The association constants of the complexes formed between benzene (Bz) and cyclodextrins in water have been determined by several methods, such as volatilization or transport methods, UV absorption, fluorescence or isothermal titration calorimetry. However, the reported results concerning stoichiometry and association constants are contradictory. They have been collected in the Table 1.

Table 1  
Stoichiometries, association constants and experimental conditions reported in the literature for Bz/CD inclusion complexes in aqueous solutions

CD	stoich	$K$ [dm <sup>3</sup> /mol]	method	t [°C]	c(Bz) [mmol/dm <sup>3</sup> ]	Ref.
α	1:1	28.6 (1.4)	UV absorption & fluorescence	25	0.02–0.2 ??	[28]
α	1:1	31.6 (0.1)	vapor pressure/solubility	25	0–16	[29]
	2:1	a) 97 (4)				
	1:2	a) 325 (11)				
α	1:1	17 (2)	volatilization	25		[30]
	1:2	17 (3)				
	1:1	19 (1)	static HSGC	25	var.	[33]
	1:2	16 (13)				
β	1:1	194 (9)	UV absorption & fluorescence	25	20–200 ??	[28]
β	1:1	169 (1)	vapor pressure/solubility	25	0–16	[29]
	2:1	a) 2270 (30)				
β	1:1	120 (10)	volatilization	25		[30]
β	1:1	196 (10)	UV absorption & fluorescence	28		[31]
β	1:1	86	UV absorption	25	0.3	[32]
β	1:1	107 (31)	ITC	25	22.3 (sat)	[34]
	2:1	1000 (3000)				
γ	1:1	9.1 (0.1)	vapor pressure/solubility	25	0–16	[29]
γ	1:1	12 (2)	volatilization	25		[30]

It has to be pointed out that the method best suited for studying molecular complexes, the nuclear magnetic resonance (NMR), has not yet been exploited due to poor benzene solubility in water and inherently low NMR sensitivity. Recently, NMR sensitivity has significantly increased due to technical progress in NMR equipment making feasible the NMR titration experiments of Bz/CD complexes. Thus, we reinvestigated inclusion complexes formation of benzene and natural cyclodextrins examining a range of possible stoichiometries.

## Experimental

### Materials and sample preparation

Benzene (Bz) p.a., from OBRPCh (Płock, Poland) was distilled from  $P_2O_5$  before use.  $\alpha$ -,  $\beta$ - and  $\gamma$ -cyclodextrins were from Sigma-Aldrich (St. Louis, MO) and were used without further purification. Dimethyl sulfone was manufactured by Alfa Aesar.  $D_2O$  from Armar Chemicals (Dottingen, Switzerland) contained 99.8% D. A solution of Bz in  $D_2O$  was prepared by stirring for 5 minutes. A part of the Bz solution was separated from the rest and cyclodextrin was added to it in excess over Bz. In the case of  $\beta$ CD the excess was limited since its solubility in  $D_2O$  is lower than those of  $\alpha$ CD and  $\gamma$ CD [9].

Cyclodextrins contain an imprecisely known amount of water because they crystallize from water as hydrates of variable composition [19, 27, 35, 36]. Therefore, the concentration of cyclodextrin was determined by integrating the  $^1H$  NMR signal of its anomeric protons relative to the six protons of dimethylsulfone of known concentration. Dimethylsulfone is considered the standard for quantitative NMR studies [37, 38]. Since the longitudinal  $T_1$  relaxation time of dimethylsulfone is long ( $T_1 = 6.17 \pm 0.01$  s at  $T = 298$  K,  $B_0 = 14.1$  T, concentration  $10$  mM/dm<sup>3</sup>), an extended relaxation time between scans was used.

Basic solutions, containing either Bz or Bz and cyclodextrin, were mixed afterwards together in order to prepare eight to eleven NMR samples ( $0.65$  cm<sup>3</sup> total volume) of various CD/Bz molar ratios,  $R$ , so that the concentrations of Bz remained constant during the titrations;  $1.0$ ,  $0.3$ , and  $0.4$  mmol/dm<sup>3</sup> for  $\alpha$ CD,  $\beta$ CD, and  $\gamma$ CD experiments, respectively. Four groups of CD protons which do not superpose one another ( $H1$ ,  $H3$ ,  $H2 + H4$ ,  $H5 + H6,6$ ) can be integrated accurately. Therefore, integration of Bz/CD  $^1H$  NMR spectra delivers four values of molar ratios  $R$ .  $R$  values from six independent measurements have been averaged allowing to estimate their accuracies which are better than 4%. Precaution has to be undertaken concerning the Bz concentration. Benzene is very volatile and easily evaporates from a solution. Therefore, NMR tubes with Bz/CD solutions were flame sealed immediately after filling to avoid the slow evaporation of benzene.

## NMR measurements

All measurements have been performed at a magnetic field of  $14.1$  T, using a Varian DDR2 600 MHz spectrometer equipped with a triax probe. NMR spectra of each sample have been measured six times at a temperature carefully adjusted to  $298.1$  K with an accuracy of  $0.1$  K and checked by an ethylene glycol reference sample [39]. All chemical shifts in  $^1H$  NMR spectra are reported with respect to external TSP-d4.  $^1H$  chemical shifts have been determined from 1D  $^1H$  NMR spectra measured with a  $60$  s relaxation delay and a  $1.5$  s acquisition time. A long repetition time allows avoiding the partial saturation of benzene protons displaying extensive longitudinal relaxation time  $14.6 \pm 0.4$  s. Acquisition parameters are as follows: sweep width,  $9615.38$  Hz, number of acquired data points,  $28\ 846$ , and  $32$  scans. FIDs have been processed with  $1.5$  Hz line broadening and zero-filled to  $128$  k points, resulting in a  $0.07$  Hz spectrum digital resolution. The accuracy of the chemical shift readout is not better than  $0.0001$  ppm. Stability of benzene resonance frequency in the series of spectra is not worse than  $0.22$  Hz ( $0.00037$  ppm). Hence

error introduced by the accuracy of benzene resonance frequencies is negligible in comparison with the determination of host/guest molar ratios.

## Analysis of titration data

In the case of several competing reactions of complex formation the best approach is to fit the titration data to different stoichiometry models and choose the best among them on the basis of statistical tests [40–43]. This approach has been used in the present analysis of Bz/CD complexes. Changes in the  $^1\text{H}$  chemical shifts of protons in benzene, as a function of CD/Bz molar ratios,  $R = [\text{H}]_0/[\text{G}]_0$ , have been analyzed assuming relevant stoichiometry.  $[\text{H}]_0$  and  $[\text{G}]_0$  denote the initial molar concentrations of the host (in this work cyclodextrin) and guest (in this work benzene), respectively. For all three complexes (Bz/ $\alpha$ CD, Bz/ $\beta$ CD, Bz/ $\gamma$ CD) four stoichiometry models have been compared: 1 : 1, 2 : 1, 1 : 2, and 2 : 2. All the pertinent equations defining macroscopic association constants for each model are given explicitly in Supplementary Materials.

Since one signal of Bz protons is observed, the exchange between the free and complexed Bz molecules is fast on the chemical shift timescale. Therefore, the observed chemical shifts are the mole fraction weighted averages of the chemical shifts specific for the free and complexed Bz molecules [41, 44]. The explicit equations for the discussed stoichiometry models are given in Supplementary Materials.

The model parameters, association constants  $K_i$  and complexation shifts  $\text{CS}_i$  are determined by fitting the chemical shifts calculated for the specific model to the experimental ones. The least-squares procedure uses a Fortran routine written in-house optimizing the model parameters that consists of minimization through a grid search of the target function RSS (the sum of squares of residuals), where  $N$  is the number of different molar ratios  $R$ .

$$RSS = \sum_{i=1}^N (\delta_{i,obs} - \delta_{i,calc})^2$$

The confidence limits of fitted parameters are estimated by the use of F–statistics [45, 46].

## Results and discussion

As mentioned above, four stoichiometry models (1 : 1, 2 : 1, 1 : 2, and 2 : 2) were fitted to all three Bz/CD complexes. Selection of the best stoichiometry based on the comparison of the target function values,  $RSS$ , and/or determination of Fisher-Snedecor F–statistics, if applicable.

## Bz/ $\alpha$ CD and Bz/ $\gamma$ CD inclusion complexes

The stoichiometry 1 : 1 describes two complexes, Bz/ $\alpha$ CD and Bz/ $\gamma$ CD, in the best manner as can be concluded from the data given in Table 2. Appropriate plots of experimental data titration curves and calculated for 1 : 1 stoichiometry are shown in Figs. S1 and S2.

Table 2

Values of target function  $RSS$ , in  $10^{-5}\text{ppm}^2$ , and parameters characterizing 1 : 1 complexes: association constant  $K_1$  [ $\text{dm}^3/\text{mol}$ ] and complexation shift  $CS_1$  [ppm]

G : H	Bz/ $\alpha$ CD	Bz/ $\gamma$ CD
1 : 1	3.8982	0.7317
1 : 2	3.8981 <sup>a</sup>	0.7336 <sup>a</sup>
2 : 2	3.8971 <sup>a</sup>	0.7277 <sup>c</sup>
2 : 1	3.8563 <sup>b</sup>	0.7323 <sup>a</sup>
$K_1$ (1 : 1)	$25.1 \pm 0.5$	$2.1 \pm 0.2$
$CS_1$ (1 : 1)	$0.164 \pm 0.002$	$0.093 \pm 0.007$
<sup>a</sup> $K_2 = 0$ . Calculations converge back to the 1 : 1 stoichiometry		
<sup>b</sup> comparison of $RSS$ values for 2 : 1 and 1 : 1 stoichiometries gives statistics $F = 0.24$ corresponding to the probability $p = 0.79$ which is much larger than commonly accepted rejection probability $p \leq 0.05$		
<sup>c</sup> comparison of $RSS$ values for 2 : 2 and 1 : 1 stoichiometries gives statistics $F = 0.17$ corresponding to the probability $p = 0.84$ which is much larger than commonly accepted rejection probability $p \leq 0.05$		

Results of numerical analyses performed with initial assumption of 1 : 2 or 2 : 2 stoichiometry for Bz/ $\alpha$ CD complex always resulted in  $K_2 = 0$  thus converging back to the 1 : 1 stoichiometry. The same was true for Bz/ $\gamma$ CD complex and stoichiometries 1 : 2 or 2 : 1.

Experimental data fit slightly better to 2 : 1 than to 1 : 1 Bz/ $\alpha$ CD complex. The  $RSS$  improvement, however, is statistically insignificant. Determination of confidence limits of  $K_1$  and  $K_2$  association constants (2 : 1) are shown in Figs. S3 and S4. Moreover, association constant  $K_2$  displays absurd confidence limits (cf. Fig. S4). Similar situation took place during comparison of 2 : 2 and 1 : 1 Bz/ $\gamma$ CD complexes. Therefore, one can safely conclude that Bz/ $\alpha$ CD and Bz/ $\gamma$ CD form 1 : 1 complexes.

Both, association constant and complexation shift for Bz/ $\gamma$ CD 1 : 1 complex are smaller than the corresponding parameters of Bz/ $\alpha$ CD complex. In much more spacious cavity of  $\gamma$ CD the benzene molecule can probably freely rotate inside the cyclodextrin. In consequence association constant is an order of magnitude smaller. Smaller complexation shift ensues from weaker interactions between benzene molecule and  $\gamma$ CD cavity.

## Bz/ $\beta$ CD inclusion complex

In contrast, the parameters obtained for the Bz/ $\beta$ CD complex clearly differ from those discussed above. Among four stoichiometries used to describe titration experiment for Bz/ $\beta$ CD complex the best fit and the smallest  $RSS$  value were obtained when two molecules of benzene were located in the  $\beta$ CD inner space; 2 : 1 stoichiometry (Table 3) resulting in the association constants  $K_1 = 405 \pm 18 \text{ dm}^3/\text{mol}$ ,  $K_2 = 6000 \pm 1100 \text{ dm}^3/\text{mol}$ , and complexation shifts  $CS_1 = 0.080 \pm 0.002 \text{ ppm}$ ,  $CS_2 = -0.038 \pm 0.004 \text{ ppm}$ .

Table 3  
Values of target function  $RSS$ , in  $10^{-5}\text{ppm}^2$  for Bz/ $\beta$ CD inclusion complex

G : H	$RSS$
1 : 1	1.1865
1 : 2	0.7201
2 : 2	0.8201
2 : 1	0.2439

Titration curve calculated applying the best fit parameters and experimental data are shown in Fig. 3. Determination of parameter confidence limits are presented in Figs. S5 - S8.

Binding of a second benzene molecule to the 1 : 1 complex is strongly cooperative. The cooperativity factor,  $c$ , has been delimited from the macroscopic association constants  $K_1$  and  $K_2$  applying formula  $c \geq 4 \cdot K_2 / K_1$  [47]. The obtained value,  $c \geq 59 \pm 11$ , is markedly larger than the cooperativity factors for parent complex naphthalene/ $\beta$ CD,  $5.2 \pm 0.3$  and  $25.4 \pm 1.7$  for 2 : 1 and 2 : 2 stoichiometries, respectively [43]. One can safely conclude that two benzene molecules fit better to the  $\beta$ CD cavity than two larger naphthalene molecules. It should be also recalled that the cooperativity factors for 1 : 2 inclusion complexes of (+)-fenchon/ $\alpha$ CD and (-)-fenchone/ $\alpha$ CD are  $42.6 \pm 3.0$  and  $9.9 \pm 0.7$ , respectively [42].

While  $K_1$  association constant and corresponding complexation shifts  $CS_1$  are determined with good accuracy, parameters of the step binding,  $K_2$  and  $CS_2$ , are calculated much less precisely. Several studies have reported 1 : 2 stoichiometry and large overall association constant for camphor/ $\alpha$ CD complexes [48–50]. Dodziuk et al. [51] reported that analyzing titration data of (+)-camphor/ $\alpha$ CD system were not able to partition overall association constant into stepwise constants. because such partitioning becomes equivocal for  $K_1 < K_2$ . In the case of Bz/ $\beta$ CD complex the separation of stepwise association constants was possible, but accuracy of determined parameters suffered seriously. It ensues from the fact that concentration of  $G_2H$  complex is virtually constant from  $\beta$ CD/Bz molar ratio  $R = [H]_0/[G]_0 \approx 4$  (Fig. 4). Therefore, the observed chemical shift of benzene becomes effectively decoupled from further  $R$  changes.



Generally, the protection of Bz molecules from solvent in the CD cavity results in the deshielding effect of guest protons. Therefore,  $CS_1$  values in all studied complexes are positive. On the other hand, the interaction of two  $\pi$  electron systems in 2 : 1 Bz/ $\beta$ CD complex causes a shielding effect of the involved protons [52]. These two effects interplay and their combined effect depends on the mutual orientation of complex components. Hence, it is not surprising that  $CS_2$  in Bz/ $\beta$ CD complex is negative.

## Conclusion

NMR experiments carried out at a magnetic field of 14.1 T, coupled with rigorous statistical analysis of the results, allowed the exact determination of the stoichiometry and corresponding association constants of the benzene complexes with the three natural cyclodextrins. Furthermore, the parameter values obtained provide insights into the structures of the complexes studied based on chemical intuition. The supramolecular complex of benzene with  $\beta$ CD differs from the association schemes with  $\alpha$ CD and  $\gamma$ CD. Bz/ $\beta$ CD displays well-defined 2 : 1 stoichiometry and a considerable cooperativity coefficient. Complexes Bz/ $\alpha$ CD and Bz/ $\gamma$ CD are described by 1 : 1 stoichiometry. Both complexes are weak but the latter is extremely weak ( $K_1 = 2.1 \text{ dm}^3/\text{mol}$ ) owing to large  $\gamma$ CD cavity which allows for unrestricted motion of benzene inside the host molecule.

## Declarations

## Author Contribution

AE and MN: conceptualization; GS, MN and JW: samples preparation; GS and MN: measurements; GS and AE: data analysis; GS, JW, AE and MN: manuscript preparations.

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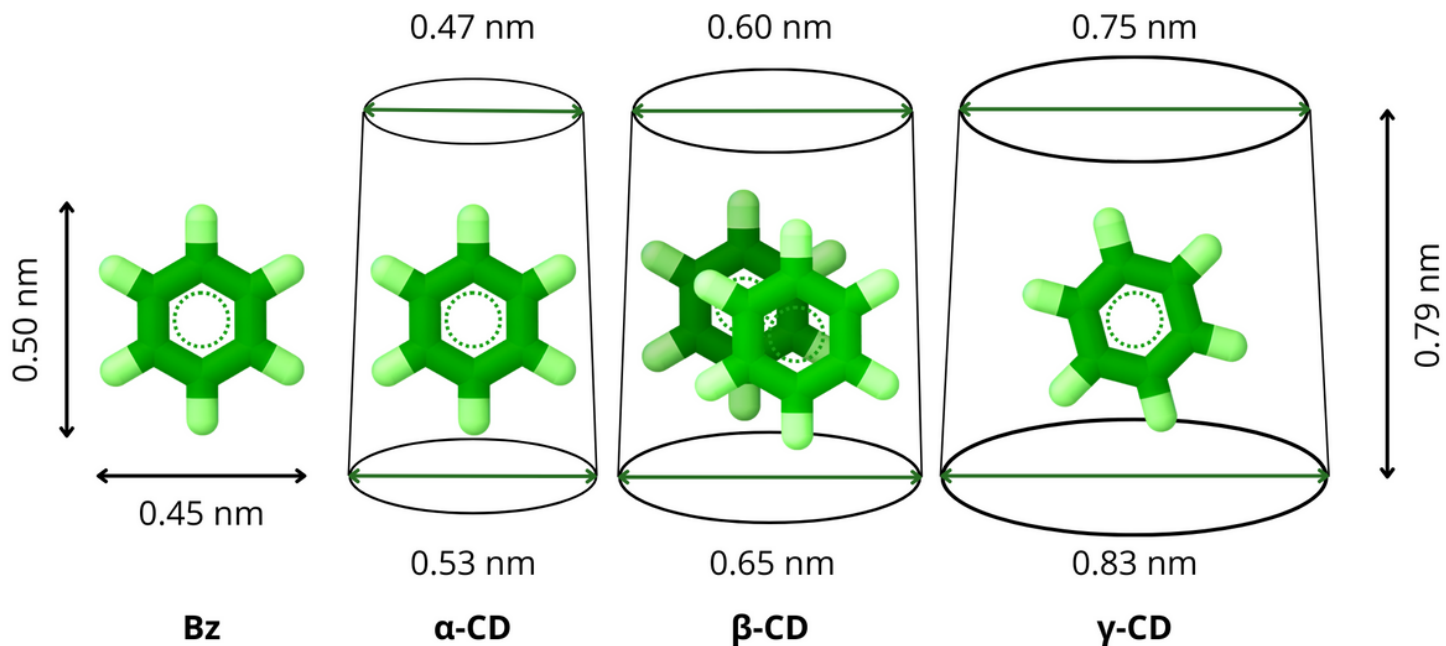
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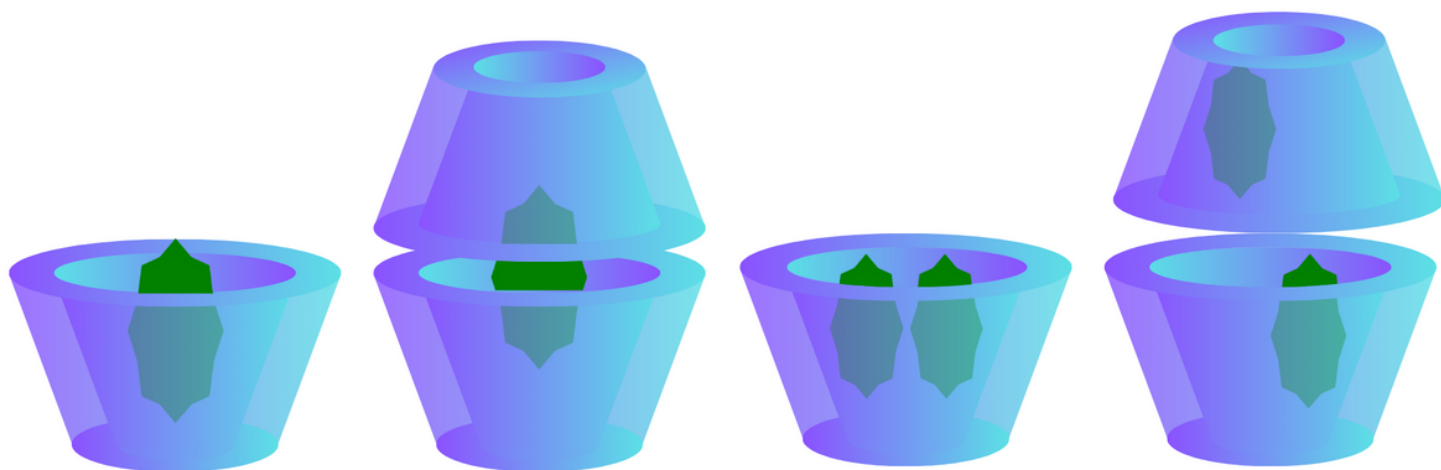
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## Figures



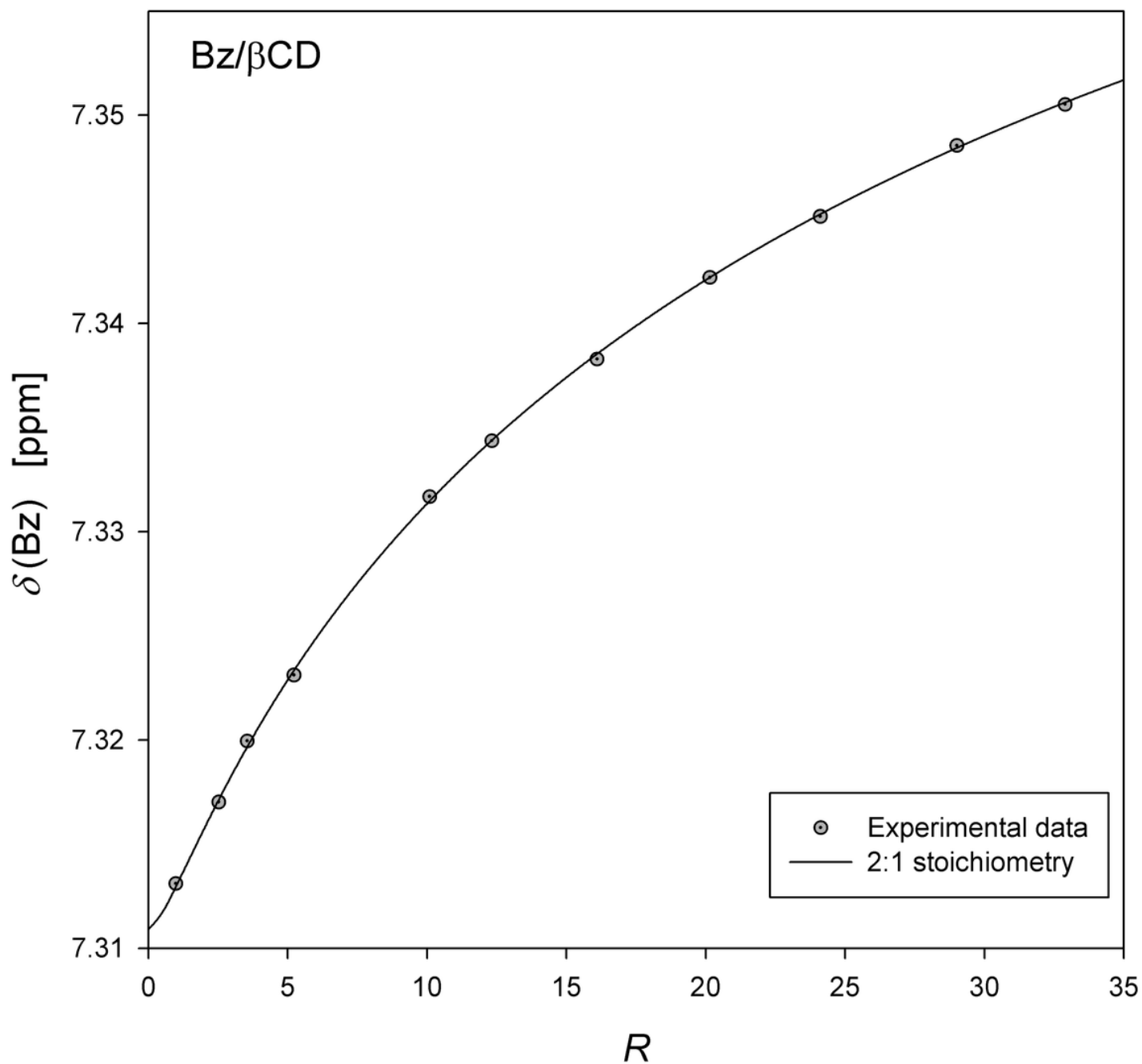
**Figure 1**

Molecular dimensions of benzene molecule and inner cavities in cyclodextrins. The diagrams are drawn to scale. Benzene molecule was built using accepted bond lengths [25]. For cyclodextrins, data the most frequently quoted selected from inconsistent literature data are used [4,6,19,26,27]



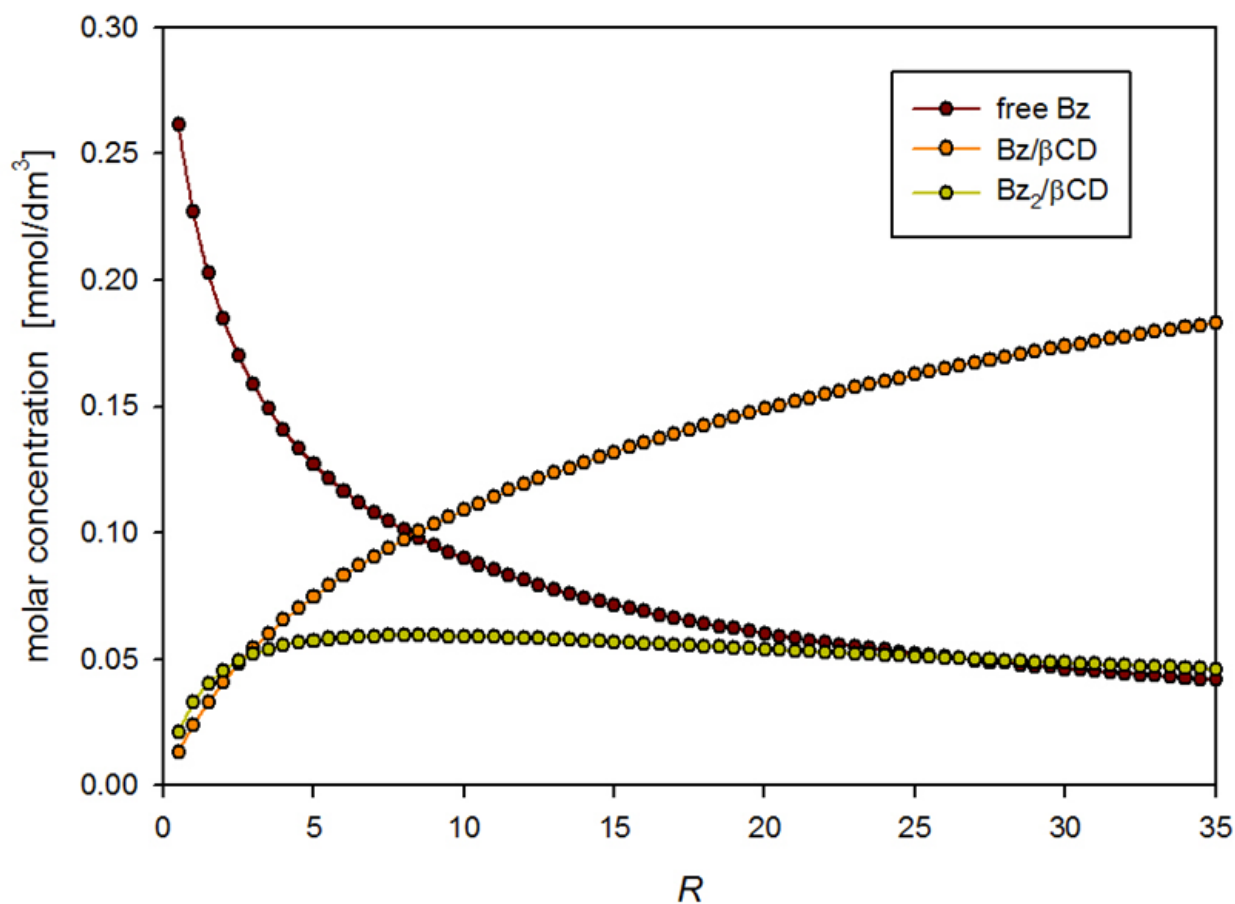
**Figure 2**

Visualization of stoichiometries used in the present study. G:H from left to right: 1:1, 1:2, 2:1, 2:2



**Figure 3**

Experimental data measured for Bz/bCD complex and the titration curve calculated using the best fit parameters: 2 : 1 stoichiometry,  $K_1 = 405 \pm 18 \text{ dm}^3/\text{mol}$ ,  $K_2 = 6000 \pm 1100 \text{ dm}^3/\text{mol}$ ,  $CS_1 = 0.080 \pm 0.002 \text{ ppm}$ ,  $CS_2 = -0.038 \pm 0.004 \text{ ppm}$



**Figure 4**

Molar concentrations of species containing benzene molecules in Bz/bCD solution. Calculation have been performed using the best fit parameters for 2 : 1 stoichiometry. Experimental range of  $R$  values: (0.0, 32.9)

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