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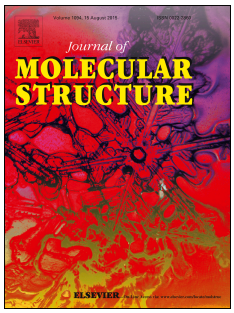

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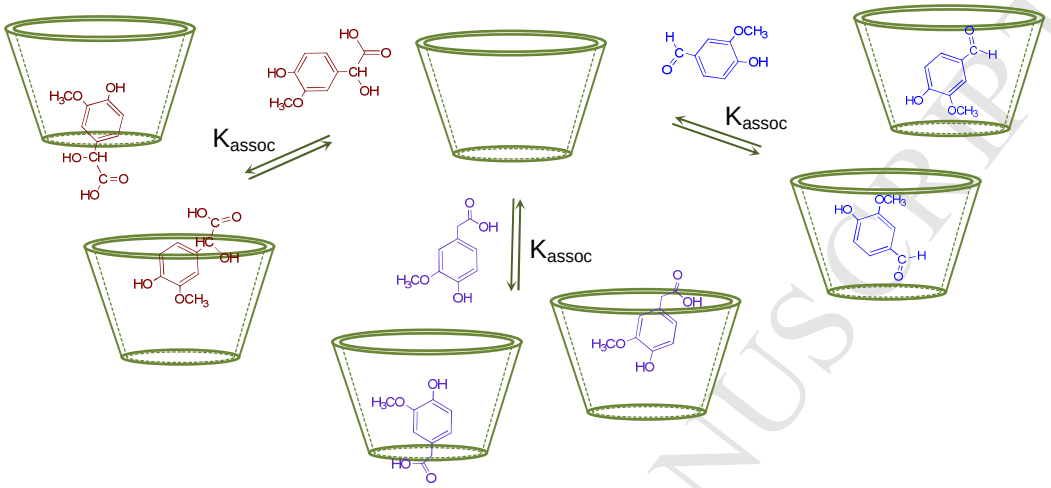

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Spectroscopic study on the inclusion complexes of β-cyclodextrin with selected metabolites of catecholamines

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Abstract

Inclusion complexes formed between β-cyclodextrin (β-CD) and metabolites of catecholamines, i.e. vanillylmandelic acid (VMA), homovanillic acid (HVA) as well as vanillin (VA) were studied using NMR spectroscopy. Due to the importance of these compounds for the diagnosis tumours of the sympathoadrenal system, hydrogels containing β-CD moieties for enhancing entrapping metabolites of catecholamine

from aqueous solutions are located in the area of our interest. Stoichiometry and association constants of the complexes of β-CD with VMA, HVA and VA respectively were determined by using continuous variation and ^{1}H NMR titration methods. Significant discrepancies were pointed out depending on used referencing method. In this study water solution of 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid sodium salt as an external reference was used to avoid errors in the determination of association constants. β-CD formed the most stable complexes with VA and HVA molecules whilst smallest value of association constant was determined for the VMA/β-CD complex. Two-dimensional rotating-frame Overhauser effect spectroscopy (2D ROESY) allowed to establish definite information on the molecular structures of the complexes formed. Geometry of the latter was proposed basing on contour plots of the 2D ROESY spectra, which also indicated two possibilities of complexed molecule arrangement into β-cyclodextrin interior. The values of determined association constants are in good agreement with postulated geometry of the complexes. Value of association constant determined for inclusion complexes of β-cyclodextrin with homovanillic acid an vanillin indicates the strongest binding of molecules among investigated complexes, so it was finally concluded that β-CD moiety introduced into hydrogel network could be effective for homovanillic acid and vanillin entrapping.

Keywords:

β-cyclodextrin; metabolite of catecholamine; inclusion complex; association constant; ROESY

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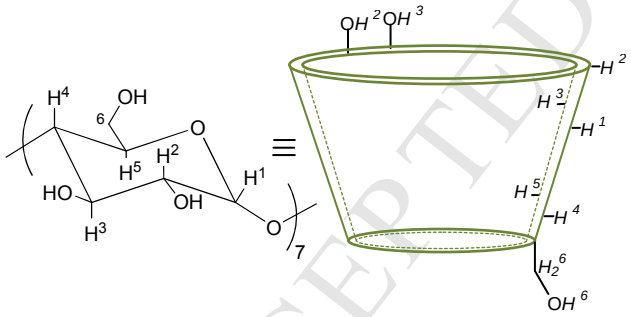
1. Introduction

Cyclodextrins (CDs) are known to form inclusion complexes with variety of guest compounds, having hydrophobic moieties of appropriate size. Inclusion complexes of cyclodextrins with 1:1 or 1:2 guest-host stoichiometry are the most extensively studied but non-inclusion complexes have also been described [1].

An inclusion phenomenon of CDs originates from their structure. Cyclodextrins are a group of cyclic oligomers, composed of six or more α-D-glucopyranose units. In this study, we have used β-cyclodextrin (β-CD) containing 7 units (Scheme 1). Due to the chair conformation of the glucopyranose units, the CDs have truncated cone shape. The primary hydroxyl groups are oriented towards the narrower cone exterior whilst secondary hydroxyl groups towards the wider edge. Interior of cyclodextrins is somewhat hydrophobic thus water molecules in CD cavity are energetically unfavoured and therefore can be readily substituted by less polar guest molecules.

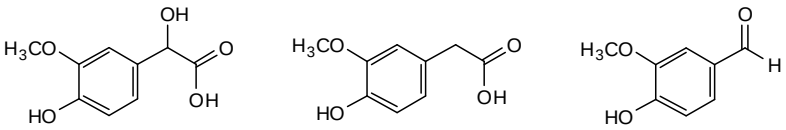


Scheme 1

A wide variety of analytical methods was employed for characterization of inclusion complexes formed between the guest and cyclodextrin molecules both in the solid and solution state. The most direct evidence for the inclusion of a guest into a CD cavity in solution is obtained by NMR spectroscopy [2]. The NMR techniques enable determining stoichiometry, association constants, structural characterization of CDs complexes and allow to analysis of their dynamics (for review, see [3,4]).

Cyclodextrins, due to their ability to form inclusion complexes with selective molecules, achieved great significance in molecular recognition processes. Moreover, CDs are chiral and exhibit chiral recognition [5]. Native, modified CDs or CDs moieties introduced into polymers have been used as masking agent and stabilizer in food and cosmetics [6], in chromatographic separations, drug delivery systems (as drug solubilizing agent), hydrogels formed *in situ* [7], molecularly imprinted polymers [8].

We have considered the use of hydrogels with β-CD moieties for enhancing entrapping metabolites of catecholamine from aqueous solutions. Catecholamines are biogenic amines which play an important role in the nervous systems. Some catecholamines as well as their metabolites are used as markers of certain types of tumours. Exemplary quantification of catecholamines or their metabolites [dopamine, norepinephrine, epinephrine, vanillylmandelic acid (VMA) (I, Scheme 2) and homovanillic acid (HVA) (II, Scheme 2)] in biological fluids is important in the diagnosis of neuroblastoma as well as the other catecholamine secreting malignancy such as pheochromocytomas. Neuroblastoma is a cancer of the symphathetic nervous system and is the most common solid tumor in childhood. More than 90 % of patients afflicted with neuroblastoma exhibit elevated levels of dopamine, VMA and HVA in serum and urine [9]. Pheochromocytomas are endocrine tumours and are characterized by elevated levels of urinal metanephrines (norepinephrine and epinephrine) and vanillylmandelic acid [10]. Moreover, excretion rate of VMA with urine is also informative and of great interest in characterization the effects of many drugs.



I **II** **III**

Scheme 2

Materials composed of *N*-isopropylacrylamide, methacrylic acid or itaconic acid and crosslinking agent were demonstrated recently as materials for dopamine recognition [11]. Designing hydrogels with β-CD moieties for entrapping VMA and HVA is the subject of our present study. In this paper we report a NMR study of the β-CD inclusion complexes with VMA and HVA, formed in water. Additionally, inclusion complexation of vanillin (VA) (III, Scheme 2) by β-CD was studied because of that vanillylmandelic acid readily undergoes oxidation in mild condition to vanillin. In the case of obtaining effective materials for binding vanillin, indirect determination of VMA could be also possible.

The inclusion of vanillin by β-cyclodextrin in water was confirmed by NMR by Divakar [12] and Pîrnău group [13]. Complexes of β-cyclodextrin with homovanillic acid and other natural polyphenols were also investigated by NMR, thermodynamic and molecular modeling studies by A. Rescifina group [14]. Association constants were determined from NMR analysis in all abovementioned cases. L. A. Kartsova et al. used Capillary Zone Electrophoresis for association constants determination of complexes of i.a. β-CD with VMA and HVA [15].

In this article complexes of β-CD with selected metabolites of catecholamines and vanillin were characterized. Their geometry and stability in water solution were studied in details in order to evaluate the usefulness of the β-CD moieties in binding process of the VMA, HVA and VA molecules in aqueous environment.

2. Experimental

2.1. Materials

Vanillylmandelic acid, homovanillic acid and vanillin were supplied by Acros Organics (Chemiatrade, Gliwice, Poland). Deuterium oxide was obtained from The Radioisotope Production and Distribution Centre (Świerk, Poland). β-cyclodextrin and 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid sodium salt (TSP) were purchased from Sigma-Aldrich Co. (Poznań, Poland).

All chemicals and solvents were of commercial grade and used as received.

2.2. NMR experiments

NMR spectra were recorded at 25 ℃ with the aid of UNITY/*INOVA* 300 MHz or 600 MHz spectrometers (Varian). All tested solutions were prepared one day prior the measurements.

2.2.1. Job's plot

Equimolar solutions (10 mM) of the β-CD and one of complexed molecule (CM): VMA, HVA or VA in D_2O were prepared respectively and distributed among 11 NMR tubes in such a way that the molar fractions of β-CD varied between 0:1 and 1:0 (with a constant sample volume of 0.6 mL). Solution of 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid sodium salt (TSP) in D_2O placed in melt-point capillary was used as an external reference. Standard ^{1}H NMR spectra were acquired with 48 scans and the relaxation delay of 3s. The widths at half-height of TSP signals were less than 1 Hz. Values of the molar fractions were recalculated from appropriate ^{1}H NMR signals integration.

2.2.2. Titration studies for evaluation of association constants

Two stock solutions were prepared in D_2O: first containing the β-CD (10 mM), second containing the β-CD (10 mM) and the VMA, HVA or VA respectively (50 mM). These

stock solutions were mixed to a constant volume (6 mL) keeping the β-CD concentration equal to 10 mM and ranging the CM/β-CD nominal ratio from 1.0 to 5.0. Solution of TSP in D_2O placed in capillary was used as an external reference. Standard ^1H NMR spectra were acquired with 48 scans and the relaxation delay of 3s. The widths at half-height of TSP signals were less than 1 Hz. Experiments were carried out in triplicate. Values of molar ratio of CM to β-CD were recalculated from appropriate ^1H NMR signals integration.

2.2.3. 2D ROESY

For two-dimensional rotating-frame Overhauser effect spectroscopy (2D ROESY) measurements solutions of β-CD (15 mM) and the VMA, HVA or VA (60 mM) were prepared. No reference was added and chemical shifts were set based on the residue water signal at 4.790 ppm [16]. ROESY experiments were carried out on 600 MHz spectrometer using a mixing time of 500 ms.

3. Results and Discussion

3.1. NMR chemical shift referencing

One of the fundamental and most important factor for a correct analysis of β-cyclodextrin complexes in aqueous solution by the NMR technique is the selection and properly using chemical shift reference. This aspect has been discussed in the literature [17,18]. More than twenty years ago Q.X. Guo et. al. signalized that tetramethylsilane (TMS) and sodium salt of 3-(trimethylsilyl)propane-1-sulfonic acid (DSS) cannot be used as the internal reference, and suggested to use DSS water solution rather than TMS as the external reference for cyclodextrin species in aqueous solution for NMR measurement [17]. Meanwhile one can meet up with practice of using DSS, TSP or TMS as internal reference, despite the fact that β-CD

can form an inclusion complexes with all these standards. In the case of TMS molecule complexation by β-CD increase the solubility of TMS in water threefold [18]. Frequently chemical shifts are referenced to the residual water signal which is very temperature depended and sensitive to acidic impurities [19]. We decided to use external reference (TSP solution in D_2O) rather than residual water signal referencing for stoichiometry and association constant determination. The reason is that we have observed upfield shift of the HOD signal relative to TSP solution as external reference with increasing complexed molecule content. In our case the sample and reference are both diluted solutions in the same solvent, so the correction for the change of bulk magnetic susceptibility is negligible [20].

3.2. Stoichiometry and association constants of the complexes

The inclusion complexes: VMA/β-CD, HVA/β-CD and VA/β-CD were characterized in D_2O by ^1H NMR spectroscopy. In these systems, a fast exchange regime between the complexed and non-complexed species was observed in the NMR time-scale. In this case observed chemical shift (δ_{obs}) is the mole fraction weighted average of the shifts observed in the free and complexed molecule [21]. Stoichiometry of complexes of β-CD with VMA, HVA and VA respectively were determined by the Job's method of continuous variation method. This is commonly used procedure for determining the composition of complexes in solutions which requires preparation of series of solution containing host and guest molecules. The mole ratios of total concentration of complexed molecule (CM), i.e.: xVMA, xHVA and xVA respectively, is setting between 0 and 1. Job's plots prepared on the basis of chemical shifts of the signals of the β-CD's H^3 are presented in Figure 1. The maximum visible for equimolar composition of β-CD and appropriate complexed molecule indicated 1:1 stoichiometry in all cases.

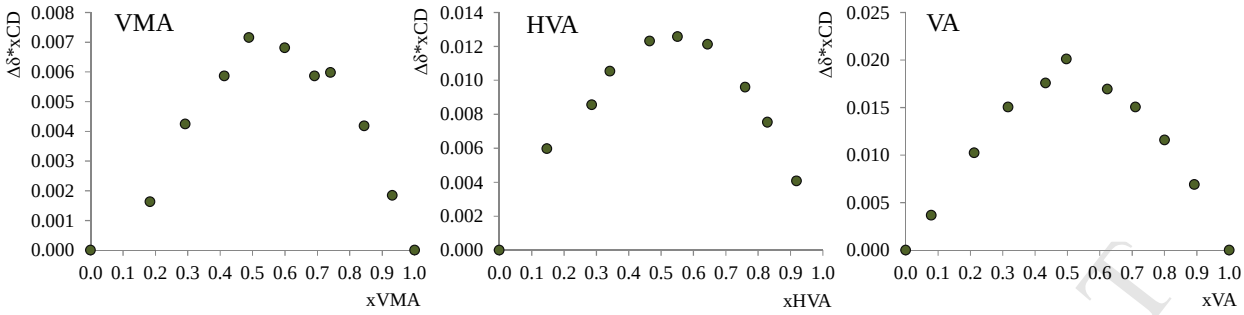


Figure 1: Job's plots for the interaction between β-CD and VMA, HVA and VA respectively in D_2O by monitoring chemical shifts of the H^3 signal of the β-CD

Consequently, for the determination of the association constants (K_{assoc}) the following equilibrium was assumed:

CM + β-CD = CM/β-CD $\qquad K_{assoc} = \frac{[CM/\beta-CD]}{[CM][\beta-CD]}$ (1)

Vanillylmandelic acid, homovanillic acid and vanillin have better solubility in water than β-cyclodextrin. This is why the concentration of the β-CD was keeping constant during experiments whilst VMA, HVA or VA was varied. Complexation induced chemical shift δ_{obs} was traced and K_{assoc} values were calculated basing on the H^3 and H^5 protons of β-CD signal in ^1H NMR spectra respectively. The aforementioned signals were selected because of the highest change of the chemical shift among all protons. In our study we have assumed similarly to L. Fielding [21] that the guest molecule is the NMR observed species. Therefore in such an approach, commonly used terms for guest and host are switched and β-CD is treated as guest molecule (G) whilst symbol (H) denotes to the one of the complexed molecules (VMA, HVA or VA).

The association constants of the β-CD with VMA, HVA or VA were calculated by using curve fitting method, which requires no approximations and allow unrestricted distribution of reagents. The data obtained from the titration experiments were fitted to a 1:1 binding model, using WinEQNMR computer program [22]. Exemplary data

fitting for the ^{1}H NMR titration of β-CD with VMA, HVA and VA respectively with residuals showing deviation from the best fit are collected figure 2.

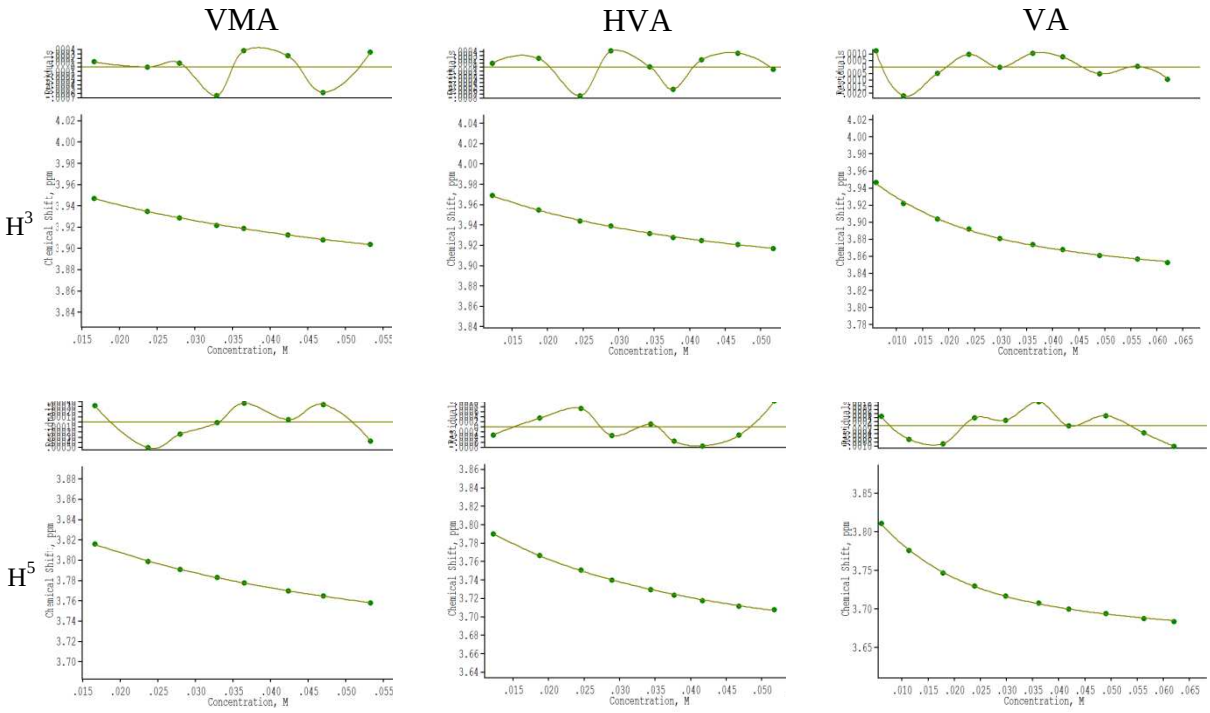


Figure 2: Exemplary plots with experimental data and calculated chemical shifts of signals of the H^3 (upper) and H^5 (bottom) of the β-CD together with the residues in magnified form (WinEQNMR program)

The corresponding values of association constants (K_{assoc}) of the β-CD with VMA, HVA and VA respectively, calculated by WinEQNMR are summarized in Table 1.

Table 1: The association constants for VMA, HVA and VA 1:1 complexes with β-CD

complexed molecule (H)	K_{assoc} ± SD [M^{-1}]	
	based on δ_{obs} of H^3 of CD (G)	based on δ_{obs} of H^5 of CD (G)
VMA	29.3 ± 1.8	30.0 ± 2.9
HVA	43.1 ± 4.1	54.7 ± 3.7
VA	73.7 ± 5.1	105.0 ± 3.6
VA*	152.0 ± 3.6	171.8 ± 3.3

*values determined using HOD proton signal at 4.790 ppm as an internal reference

Each value of K_{assoc} is expressed as the mean ± standard deviation (SD) from three independent experiments (in which association constant was determined with errors below 10%). Values of association constants are arranged in series: K_{assoc} of VMA/β-CD < K_{assoc} of HVA/β-CD < K_{assoc} of VA/β-CD. From presented data it can be observed that the type of substituent at C1 position affects the inclusion of the complexed molecule. VMA having largest group at C1 is bound least. At this position vanillin has group which is smallest and with less tendency to forming hydrogen bonds thus VA forms the most stable complex with β-CD.

As it was mentioned earlier, upfield chemical shift of residual water signal was observed with increasing CM content. Presumably these chemical shift changes are related to the release of water molecules included in CD cavity upon the β-CD/CM complexes formation. Table 1 shows supplementary values of K_{assoc} evaluated for VA when HOD signal was used as internal reference. It is seen that values of K_{assoc} evaluated for VA/β-CD differ significantly depending on method of referencing.

In the Table 2 values of association constants for VMA/β-CD, HVA/β-CD and VA/β-CD complexes reported in the literature are collected. Values of association constant determined under similar conditions should be independent of the applied methodology [23,24]. Meanwhile differences between association constants given for the same complexes are significant even when the same method is used. Available literature data for the association constant of VA/β-CD complex are derived from NMR analysis. Our value of K_{assoc} for β-cyclodextrin complex with vanillin is about 50% smaller than literature values whilst association constants for VMA/β-CD and HVA/β-CD determined in this study are in agreement with those obtained by L. A. Kartsova group that used Capillary Zone Electrophoresis [15].

Table 2: The literature data concerning determination of K_{assoc} of VMA, HVA or VA 1:1 complexes with β-CD

analytical method /conditions [ref.]	VMA	HVA	VA
Capillary Zone Electrophoresis/ borate buffer solution (25 ℃) [15]	27 ± 2	56 ± 6	
NMR titration / D_2O solution (25 ℃), in the presence of DSS as internal standard [14]		137 [M^{-1}]	
NMR titration / D_2O solution (20 ℃), no information about applied referencing method [12]			1.11 x 10^4 ± 1800 [M^{-1}]
NMR titration /D_2O solution (25 ℃), chemical shifts measured relative to TMS [13]			170.2 [M^{-1}]

3.3. Structure of the complexes

Two-dimensional rotating-frame Overhauser effect spectroscopy experiments were performed for characterization of the geometry of β-CD complexes formed with the VMA, HVA and VA respectively. The fourfold excess of complexed molecule relative to β-CD was used in order to shift the equilibrium of the reaction (1) toward formation of the complex. 2D ROESY experiments detect both intra- and intermolecular spatial proximity among protons.

Partial contour plot of 2D ROESY spectrum for β-CD/VMA complex is shown in the Figure 3. Strong ^1H-^1H cross signals of the methoxy group protons with aromatic proton $H^{2'}$ within VMA molecule is observed. Much less intense signals came from intermolecular spatial coupling of aromatic protons of VMA with β-CD protons. NOE cross-peaks of protons $H^{5'}$ and $H^{2'}$ with H^3 of β-CD indicates binding VMA molecule via the wider rim of cyclodextrin. Additionally, weak spatial coupling between α-CH of VMA and H3 of β-CD (data not shown) proves formation the complex of proposed structure. Simultaneously from the cross-peaks $H^{5'}/H^5$ and $H^{2'}/H^5$ and especially $H^{6'}/H^6$ we concluded that the partial inclusion occurs also by the inclusion into

cyclodextrin cone through its narrower rim. Presumably in both cases only partially inclusion takes place due to steric hindrance and additionally owing to hydrogen bonding between carboxylic group of VMA and hydroxyl groups of the β-CD.

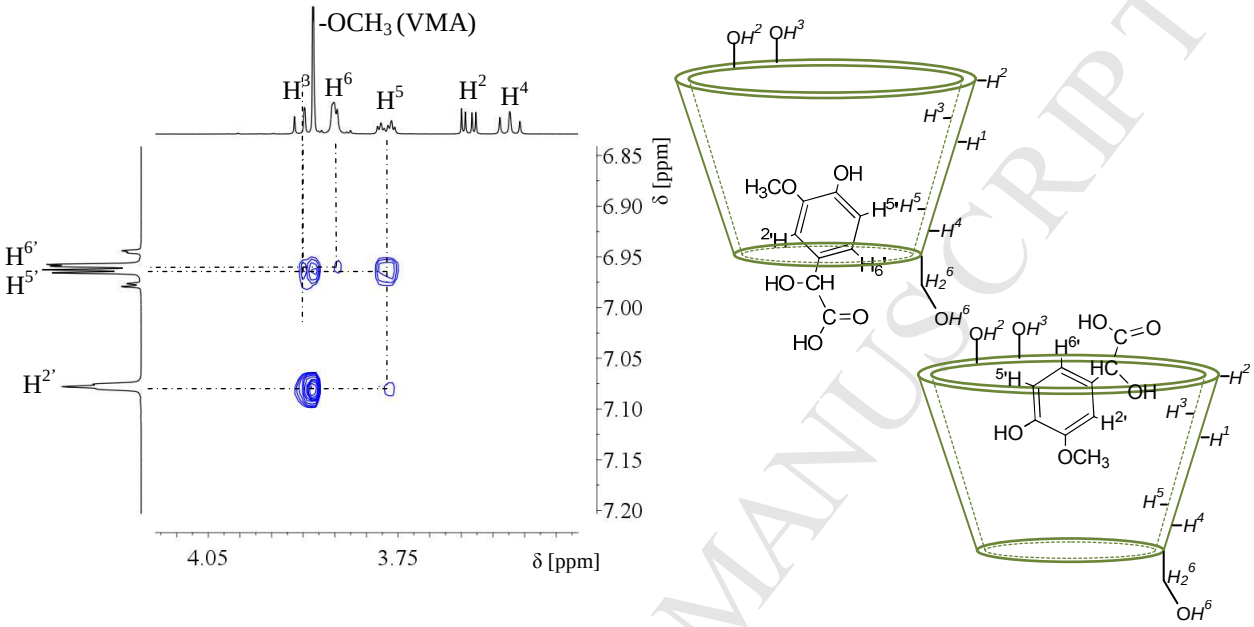


Figure 3: Partial contour plot of the two-dimensional ROESY spectrum of 4:1 VMA/β-CD ratio and postulated geometry of the complexes

Substituent in the 1-position of the phenyl ring in HVA is smaller than that of VMA therefore one can expect that HVA molecule will entered the interior of cyclodextrin deeper than VMA molecule. However analogical to VMA/ß-CD, hydrogen bonding can also slightly hindrance the inclusion of HVA into cyclodextrin cavity. Indeed all aromatic protons ($H^{6'}$, $H^{5'}$, $H^{2'}$) of VMA show more intensive intermolecular NOE cross-peaks with H^5 and H^3 protons of β-CD and concurrently there is not observed any spatial coupling with proton H^6 (Figure 4). Presented ROESY cross-peak pattern for characterized complex HVA/β-CD proves both modes of inclusion of the complexed molecules. For HVA molecule strong intramolecular

NOE cross-peaks are also observed which correspond to the spatial coupling of protons of $–OCH_3$ and $–CH_2$- groups with aromatic protons.

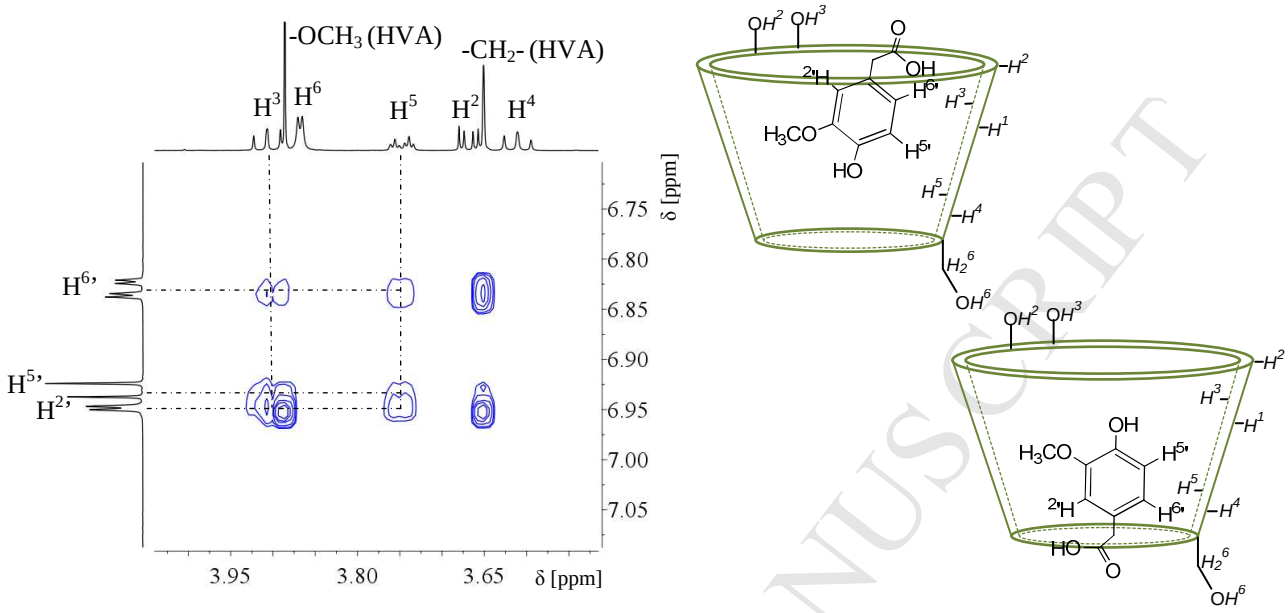


Figure 4: Partial contour plot of the two-dimensional ROESY spectrum of 4:1 HVA/β-CD ratio and postulated geometry of the complexes

In the case of both: VMA/β-CD and HVA/β-CD complexes, values of K_{assoc} determined basing on chemical shifts of H^3 and H^5 (Table 1) are almost the same and suggest similar contacts between aromatic protons of the complexed molecules and hydrogens of the β-CD located inside the cavity (H^3, H^5).

The spatial coupling of aldehyde proton of vanillin with H^5 and H^3 of β-CD (Figure 5) shows that VA molecule enters most deeply into the cyclodextrin interior among the three tested complexed molecules. Furthermore, vanillin/β-CD complex can occur with two different inclusion modes. Visible cross-peaks: $H^{5'}$, $H^{6'}$ and probably $H^{2'}$ with H^3 (superimposing with intramolecular cross-peak $–OCH_3/H^{2'}$) and $H^{5'}$, $H^{2'}$ of VA with H^5 of β-CD, additionally prove the concomitance of two complexes VA/β-CD of different geometry. Values of K_{assoc} determined from δ of H^5 signal are

slightly higher than determined from chemical shift of the H_3 proton and indicate that the bottom structure presented in the Figure 5 is somewhat probable.

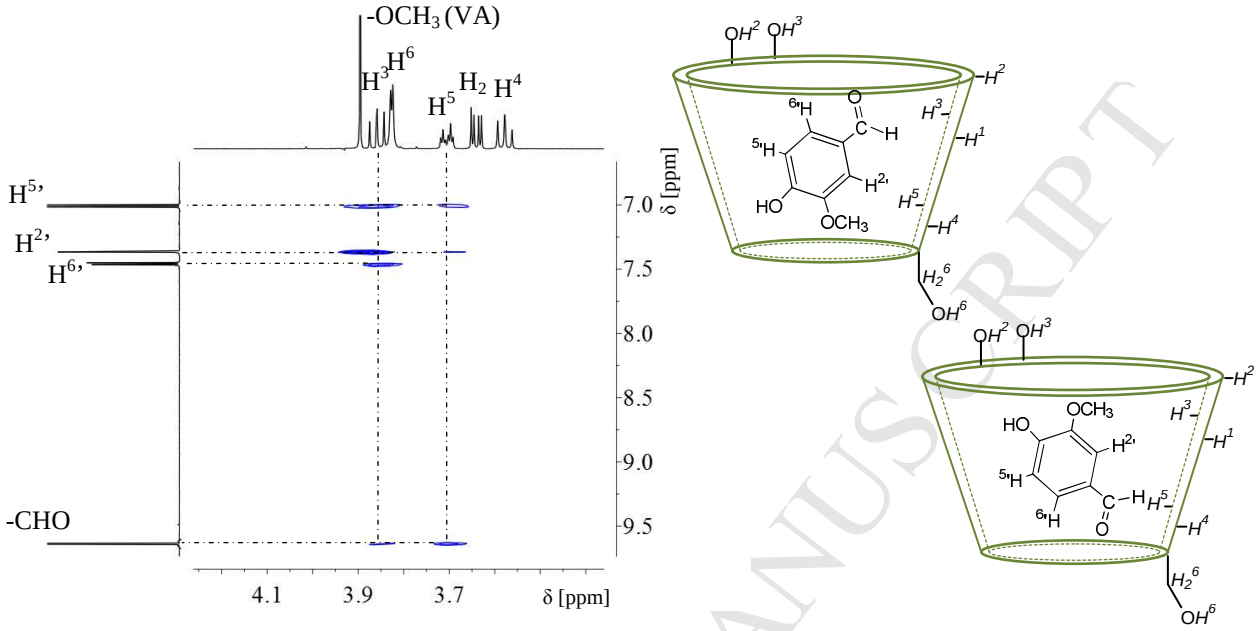


Figure 5: Partial contour plot of the two-dimensional ROESY spectrum of 4:1 VA/β-CD ratio and postulated geometry of the complexes

4. Conclusions

The synthesis of polymeric materials with ability of effective binding of selected molecules can be rationalized by cognition of the interaction between the functional monomer and the targeted molecule. In this study NMR technique was chosen for stoichiometry and association constant determination as well as for finding arrangement of VMA/β-CD, HVA/β-CD and VA/β-CD complexes. The use of external standard allows for elimination the errors associated with interaction of the chemical shift reference with β-cyclodextrin.

2D ROESY spectra showed two binding modes between β-CD and complexed molecules which can exist simultaneously in the aqueous solution.

The values of association constants are consistent with a depth of penetration of complexed molecules into β-cyclodextrin. The smallest value of K_{assoc} exhibits a

complex formed between β-CD and VMA where only partially inclusion takes place. Inclusion complex stability in this case may be insufficient for effective binding of vanillylmandelic acid from aqueous solutions. For this complexed molecule presumably more favourably will be to use complexing agent with basic moieties. Alternatively vanillylmandelic acid may be subjected oxidation to vanillin for the indirect indication of VMA.

2D ROESY spectra showed that vanillin molecules are completely tucked into the β-cyclodextrin cavities. Value of K_{assoc} determined for VA/ β-CD indicates the strongest binding of molecules among investigated complexes. The HVA/ β-CD complex characterizes slightly lower K_{assoc}, so we finally conclude that β-CD moiety introduced into hydrogel network could be effective for homovanillic acid and vanillin entrapping.

Acknowledgments

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Highlights

Vanillylmandelic and homovanillic acid have importance in medical diagnosis

β-cyclodextrin forms inclusion complexes with these compounds as well with vanillin

Stability constant of complexes were calculated and geometry was established from NMR

Water solution of TSP as an external reference ensures more reliable values of K_{assoc}

β-cyclodextrin is able for effective entrapping of homovanillic acid and vanillin

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