# The use of diffusion-ordered spectroscopy and complexation agents to analyze mixtures of catechins†

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Mixtures of catechins, (+)-catechin (C), (−)-epicatechin (EC), (−)-epigallocatechin (EGC) and (−)-epigallocatechin gallate (EGCG) have been analyzed by diffusion-ordered spectroscopy (DOSY) using liquid and high-resolution magic angle spinning (HR-MAS) NMR probes. Beta-cyclodextrin (β-CD) and bovin serum albumin (BSA), often used as ligands in affinity chromatography, were added to the mixture of catechins to mimic chromatographic conditions and modify the average mobility. The influence of the solvent, water, dimethyl sulfoxide, methanol, acetone and acetonitrile, was also investigated. The best separation of the components was achieved with β-CD in the liquid probe using a 15% $CD_3CN$–85% $D_2O$ solution, and this was applied to the analysis of catechins from green tea extract. The parts of the catechin molecules having the closest contact to the BSA protein were also determined by saturation transfer difference (STD) NMR experiments.



## Introduction

The preventive activity of tea against cancer is a topic of intensive investigation. The active components and the molecular mechanisms responsible are not clearly understood but most mechanistic studies have focused on green tea catechins.[1–5] The six major catechins displaying biological activity, are (−)-epigallocatechin-3-gallate (EGCG), (−)-gallocatechingallate (GCG), (−)-epigallocatechin (EGC), (−)-epicatechin-3-gallate (ECG), (−)-epicatechin (EC) and (+)-catechin (C). Of these, EGCG is the most abundant and most active catechin. It is a strong antioxidant and the polyphenolic structure also allows binding to many proteins such as plasma proteins, fibronectin and the CD4 receptor at the attachment site of the glycoprotein gp120 present at the surface of the HIV virus.[6,7] Thus, because of the possible use of catechins as chemotherapeutic agents, there is a strong need of developing highly sensitive and selective methods for their isolation and characterization.

The identification of molecules in a mixture usually requires a separation step and a structural characterization step often achieved by chromatographic techniques and by NMR or mass spectrometry. The chromatographic procedures frequently used for the separation of catechins are based on derivatized silica adsorbents and good separation is achieved with reversed-phase liquid chromatography using C18 columns.[8–11] The use of soft chromatographic media, such as Sephadex LH-20[12,13] and partition chromatography such as high-speed counter-current chromatography (HSCCC)[14,15] has also been reported.

Diffusion-ordered spectroscopy (DOSY) is an NMR technique used to separate signals from different species according to their diffusion rates and has sometimes been described as "in tube chromatography" or "NMR chromatography". It has been used for identification of metabolites, characterization of aggregates and hydrogen bonding and for affinity NMR.[16–23] The latter technique is based on the fact that ligands bound to a receptor will have reduced translational diffusion rates allowing their signals to separate from those of non-binding molecules. It has also been shown that using high-resolution magic-angle spinning (HR-MAS) NMR spectroscopy, the separation properties of DOSY can be enhanced upon addition of a solid chromatographic phase such as silica or C18 gels used in HPLC.[24–27] The method mimics a part of the chromatographic process in that the molecular average mobility in the mixture is modified according to the individual affinities for the stationary phase. This technique has been applied to the separation of benzene, naphthalene and anthracene[26] as well as to the separation of acetone, butanone and pentanone.[25]

We have recently investigated the complexation between EGCG and different cyclodextrins (CDs),[28] as well as the adsorption mechanism of EGCG on CD-substituted agarose[29] and on the silica gel medium CYCLOBOND.[28] The interaction between EGCG and β- or γ-CD bonded to silica beads was studied by $^1$H HR-MAS NMR spectroscopy and it was demonstrated that the chromatographic retardation of EGCG was due to interaction of EGCG with the CDs. Non-specific interactions with the silica gel were not observed.[28] The NMR data obtained from hydroxy protons suggested that for the complex, intermolecular hydrogen bonding in addition to hydrophobic interaction stabilized the β-CD–EGCG complex.[28]

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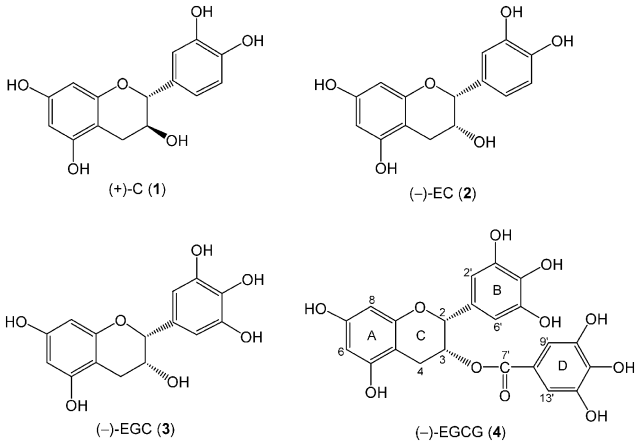


**Chart 1** The structure of (+)-catechin (**1**), (−)-epicatechin (**2**), (−)-epigallocatechin (**3**) and (−)-epigallocatechin-3-gallate (**4**).

The absence of non-specific interactions between catechins and the silica gel, together with the fact that different catechins bind to β-CD with different affinities[30] open the possibility of achieving mixture analysis of catechins using DOSY but without solid-phase chromatographic support. Indeed, DOSY has been used to investigate the complexation of cyclodextrins with diverse host molecules[31–33] and recently with (+)-catechin[34] but to our knowledge DOSY and β-CD have not been used to separate mixture of catechins. Thus, in the present work, four of the major catechins in green tea, C, EC, EGC and EGCG (Chart 1), were chosen to study how HR-DOSY or HR-MAS-DOSY affinity experiments can be used to analyze a mixture of similar compounds. This was further applied on the catechin components from green tea extract. Since the diffusion properties also can be manipulated by changes in solvent just as changes in mobile phase are used to control retention time in chromatography, different solvents were investigated. The diffusion behavior of catechins in the presence of bovine serum albumin (BSA), widely used as a chiral recognition ligand[35] and in affinity chromatography[36] was also studied. Fluorescence quenching,[37,38] Fourier transform infrared, circular dichroism,[39] gel electrophoresis[40] and isothermal titration calorimetry[41] experiments have demonstrated binding of some catechins to BSA. The aim of this work was thus two-fold: first to study how the diffusion properties of mixtures of catechins can be modified upon addition of β-CD or BSA as complexation agents in different solvents; secondly, to investigate if and how catechins from green tea extract can be analyzed using this technique.

## Experimental

β-CD, BSA (purity 99%, fatty acid free and essentially globulin free) and the catechins, C, EC, EGC and EGCG were purchased from Sigma-Aldrich. Crude extracts of green tea (GTE) were supplied by Shenglong Bioproduct Co., Guangxi, China.

The HR-NMR experiments were performed on a Bruker 400 MHz spectrometer using a 5-mm $^{1}H/^{13}C/^{15}N/^{31}P$ inverse detection probe equipped with z-gradient, while the HR-MAS NMR experiments were performed on a Bruker 600 MHz spectrometer using a 4-mm HR-MAS SB BL4 $^{1}H/^{13}C$ inverse detection probe equipped with z-gradient.

### Saturation transfer difference (STD) NMR

The NMR samples were prepared in 50 mM sodium phosphate buffer (pH 6.8) and 99.96% $D_2O$. The concentration of catechin and BSA was 5 mM and 0.05 mM, respectively, unless otherwise stated. The chemical shifts for $^{1}H$ NMR signals were referenced by setting the residual HDO signal to $\delta_H$ = 4.70 ppm at 25 °C. Water suppression was achieved by the WATERGATE pulse sequence.[42]

The STD NMR spectra were acquired using a series of 40 equally spaced 50 ms Gaussian-shaped pulses for selective saturation, with 1 ms delay between pulses. The on-resonance saturation frequency of the protein was set at $\delta$ −3 ppm. The off-resonance saturation frequency was set at $\delta$ 30 ppm. Subtraction of FIDs with on- and off-resonance saturation was achieved by phase cycling. Investigation of the time dependence of the saturation transfer with saturation times from 0.5 to 5 s showed that 2 s was sufficient for efficient transfer of saturation from the protein to the catechins. 2048 scans were collected. Relative STD values were calculated by dividing the STD signal intensities by the intensities of the corresponding signals in the one-dimensional $^{1}H$ NMR reference spectrum of the same sample similarly processed. Optimization of the experimental conditions was achieved using samples without BSA. Several on-resonance irradiation frequencies were tested (0, −1, −2, −3 and −4 ppm) and with the irradiation frequency set at $\delta$ −3 ppm, no signals were present in the STD NMR spectrum, indicating that the effects observed in the presence of the protein were due to true saturation transfer.

### Catechin/β-CD interaction

A 1 : 1 molar ratio of catechins and β-CD in three different solvents ($D_2O$, DMSO-$d_6$ and $CD_3CN$) was used to study the complex formation between catechins and β-CD, unless otherwise stated. The concentration of each catechin was kept below 7 mM to avoid self-association.[43,44]

### DOSY experiments

Data acquisition and analysis were performed using the Bruker TOPSPIN software (version 1.3). The DOSY experiments were performed using the ledbpgp2s pulse sequence from the Bruker library, with stimulated echo, longitudinal eddy current compensation, bipolar gradient pulses, and two spoil gradients using 16 different gradient values varying from 2 to 95% of the maximum gradient strength. Diffusion time ranging from 50 to 250 ms and gradient length from 1 to 5 ms were tested. 100 ms diffusion time was chosen for samples in $D_2O$ and 200 ms for samples in DMSO-$d_6$ and $CD_3CN$. The gradient length was set to 2.2 ms for all solvent systems. Processing was achieved using 4096 points in the F2 dimension and 256 points in F1. An exponential window function with 1 Hz line broadening was applied in the F2 dimension prior to Fourier transformation. After baseline correction, the diffusion dimension was processed with the DOSY processing program (Bruker TopSpin software 2.0). A logarithmic scaling was applied in the diffusion axis, and a noise sensitivity factor of 4 and line width factor of 2 were used. The fitting of the diffusion dimension in the 2D-DOSY spectra was obtained



 

using a single exponential fit (Nexp = 1). Each DOSY experiment was carried out two or three times and for at least two different sample preparations and the reported values for the diffusion coefficients are the mean and mean deviation. The error ranges given indicate the range of values obtained from the experiments.

Prior to the DOSY, for experiments with $CD_3CN$ as a co-solvent, the content of $CD_3CN$ in $D_2O$ ranging from 0% to 20% (v%) was measured by liquid chromatography using a column (300 mm × 5 mm i.d.) packed with CYCLOBOND I 2000.

### HR-MAS DOSY

β-CD bonded to silica gel (CYCLOBOND I 2000) was used and obtained from ASTEC. The amount of β-CD bonded to the silica beads has been quantified previously.[28] Prior to analysis, the gels were submerged in $D_2O$ for 1 h, and carefully inserted into zirconia rotors (4 mm outer diameter, spherical sample volume of 20 μL, Bruker, Karlsruhe, Germany). The rotor was subsequently sealed with the rotor spacer, sealing screw and finally the rotor cap. During the experiments recorded at 25 °C, the samples were spun at 3 kHz at the magic angle (54.7°). The optimized diffusion time and gradient length were 150 ms and 2.2 ms, respectively.

### Preparation of green tea samples

Green tea extract (100 mg) was dissolved in deuterated solvent (2 mL), the solution obtained was then clarified by micro-filtration (0.45 μm) and 0.6 mL of a clear solution was used for NMR experiments. The concentrated samples were prepared as follows: 10 mL of 50 mg $mL^{-1}$ GTE solutions in different solvents were filtered and dried using freeze drying for the water solution and $N_2$ for the organic solvents methanol, acetone and acetonitrile. The dried sample was re-dissolved in 2 mL deuterated solvents for deuterium exchange and then dried, the procedure repeated, and finally dissolved in 1.5 mL solvent for further NMR experiments.

## Results and discussion

### Binding of catechins to β-CD and BSA

Before analyzing mixtures of catechins by DOSY NMR experiments, their binding to β-CD and BSA were studied. The NMR techniques used to study complexation or binding between two molecules are dependent on the size of the molecules involved in the interaction. For the small molecules β-CD and catechins, the formation of inclusion complexes was studied by measuring $^1$H NMR chemical shift changes and by 2D ROESY experiments, whilst the binding of the catechins to the large BSA receptor was investigated by saturation transfer difference (STD) NMR.[45–49] In this experiment, magnetization is transferred from the protein to the ligand thereby identifying the binding of the ligand. The degree of saturation of individual ligand protons reflects their proximity to the protein surface and precise binding epitope on the ligand can be obtained.

**Binding to β-CD.** The chemical shift changes observed upon addition of equimolar amount of β-CD and the intermolecular ROEs (see ESI†) were consistent with previous reports[30,34,50] showing that the catechins **1–4** form complexes with β-CD. The B ring of **1**, **2**[50] and **3**[30] and the A and C rings of **4**[28] are inserted into the cavity of β-CD from the wide secondary hydroxyl group side. The different mode of inclusion of **4** is attributed to the additional gallate part. Both hydrogen bonding and hydrophobic interactions were shown to be involved in the complex formation.[28,51]

**Binding to BSA.** The STD NMR spectra of catechins **1–4** in the presence of BSA show large effects (Fig. 1) indicating binding to the protein. STD effects are observed for all protons suggesting that all parts of the molecules are involved in the interaction with the protein. Stronger STDs are seen for H2′, H5′, H6′ of **1** and **2**, and H2′, H6′ of **3**, showing that the B ring in **1**, **2** and **3** has closest contact to the protein. In **4**, H9′ and H13′ from the D ring in addition to H2′ and H6′ from the B ring yield the most intense signals. This is to our knowledge the first study providing direct experimental evidence on the part of the catechins that have stronger interaction with BSA. From docking studies with **4**,[39] it has been suggested that the galloyl ring is very important for the interaction with BSA. From the present work, it appears that the galloyl ring has no closer contact to the protein than the B ring has, and the increase in size of the catechin is probably responsible for the higher binding affinity.

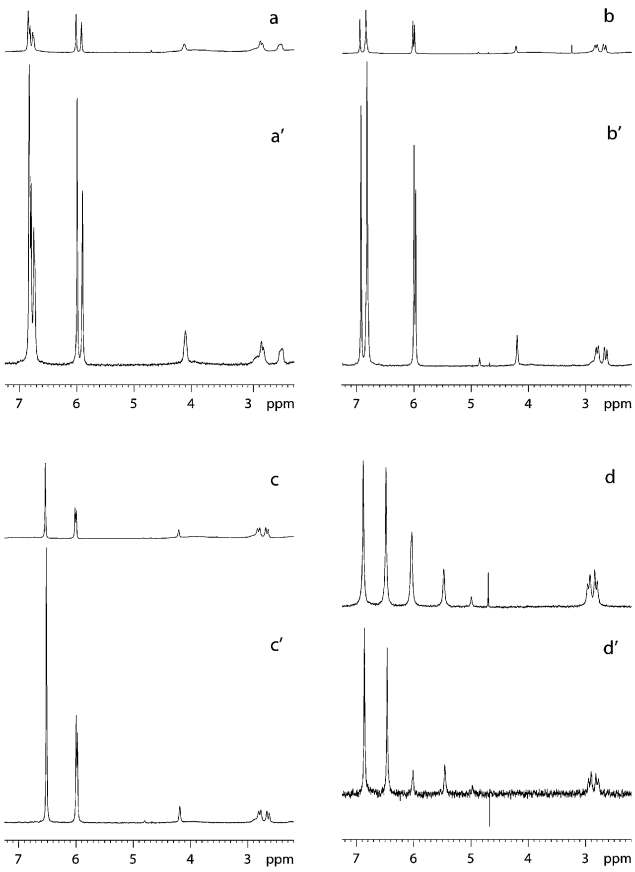


**Fig. 1** $^1$H reference and STD NMR spectra of catechins (5 mM) and BSA (0.05 mM): a, a′ (C), b, b′ (EC), c, c′ (EGC) and d, d′ (EGCG).

### DOSY spectra of catechins

DOSY spectra for each of the catechins **1–4** and for a mixture of them were recorded for $D_2O$, DMSO-$d_6$ and $CD_3CN$ solutions. The diffusion coefficients ($D$) measured for samples in $D_2O$ are reported in Table 1. Fig. 2(a) shows that the signals from **4** were separated from those from the other three catechins due to slower diffusion caused by the higher molecular mass of EGCG. **1**, **2** and **3** had similar diffusion coefficients in the three solvent systems. The complexation agents β-CD and BSA were then added to the mixture of catechins in order to see if and how the separation properties of the DOSY experiment were changed.

**DOSY spectra of catechins in the presence of β-CD.** The observed diffusion coefficients are the weighted average of those of the free and bound molecules, due to the fast exchange of free and bound species on the NMR timescale (eqn (1)). The association constant $K$ is determined by eqn (2)[52] on the premise of known mole fraction $\chi$ of the bound guest $\chi_b$.

$$D_{obs} = \chi D_{bound} + (1 - \chi)D_{free} \quad (1)$$

$$K = \frac{\chi_b}{(1 - \chi_b)([H]_0 - \chi_b[G]_0)} \quad (2)$$

**Table 1** Diffusion coefficients (log $D$) in $D_2O$ (catechins alone 5 mM, diffusion/$m^2$ $s^{-1}$), log $D_{+CD}$ (catechins 5 mM with β-CD 5 mM), log $D_{+BSA}$ (catechins 5.0 mM with BSA 0.05 mM) and log $D_{free\text{-}DMSO}$, log $D_{free\text{-}ACN}$ in DMSO and acetonitrile, respectively

| | C (**1**) | EC (**2**) | EGC (**3**) | EGCG (**4**) |
|---|---|---|---|---|
| log $D_{free}$ | −9.38 | −9.37 | −9.39 | −9.47 |
| log $D_{+CD}$ | −9.57 | −9.51 | −9.50 | −9.63 |
| log $D_{+BSA}$ | −9.53 | −9.52 | −9.44 | −9.85 |
| log $D_{free\text{-}DMSO}$ | −9.76 | −9.76 | −9.78 | −9.85 |
| log $D_{free\text{-}ACN}$ | −8.89 | −8.89 | −8.89 | −8.98 |

Here $D_{obs}$ is the observed diffusion coefficient, and $D_{free}$ and $D_{bound}$ are diffusion coefficient of free and bound guest, respectively. $[H]_0$ and $[G]_0$ are the total concentrations of the host and guest, respectively. The association constants were derived using single-point procedure[53,54] in which it is assumed that the diffusion coefficient of the host–guest complex is the same as that of the host molecule. The binding strength of the four catechins **1–4** to β-CD is in the order C > EGCG > EC > EGC with $K$ values for the β-CD/C, β-CD/EC, β-CD/EGC and β-CD/EGCG inclusion complexes at 25 °C around 21 000, 1000, 600 and 18 000 $M^{-1}$, respectively. These values should only be taken as an indication of the relative affinity of the different catechins for β-CD due to the large uncertainty introduced by the single-point approximation method. The values for the β-CD/EC, β-CD/EGC complexes are similar to those obtained with other

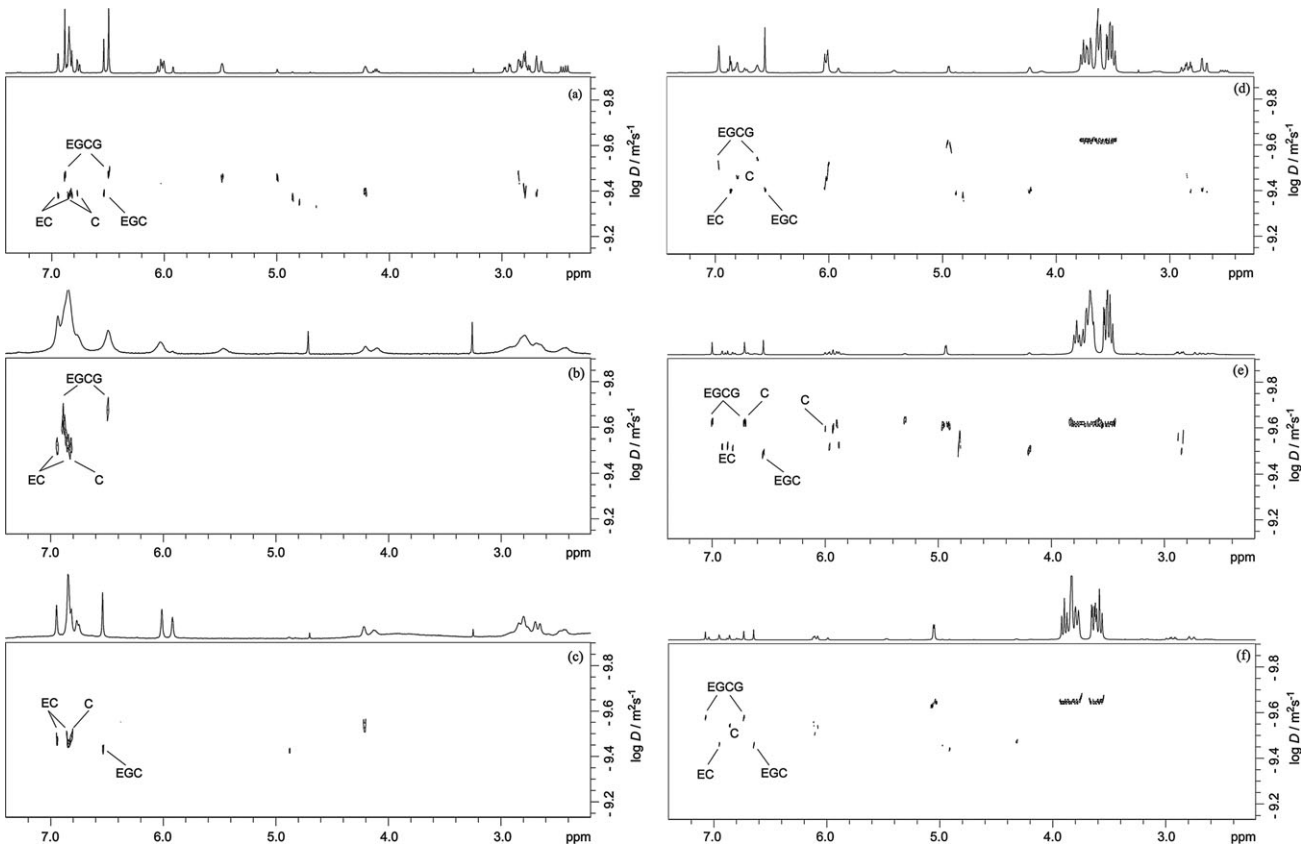


**Fig. 2** DOSY spectra of catechins **1–4** (a: 2.0 mM for each); in the presence of BSA (b: BSA 0.05 mM, catechins **1**, **2** and **4** 2.0 mM for each; c: BSA 0.05 mM, catechins **1–3** 2.0 mM for each); in the presence of β-CD (d: β-CD 2.0 mM, catechins 2 mM for each in $D_2O$; e: β-CD 8 mM, catechins 2 mM for each in $D_2O$; f: β-CD 8 mM, catechins 2 mM for each in 15% $CD_3CN$–85% $D_2O$). Only some protons are marked but the NMR signals for all protons in each catechin were identified.

 

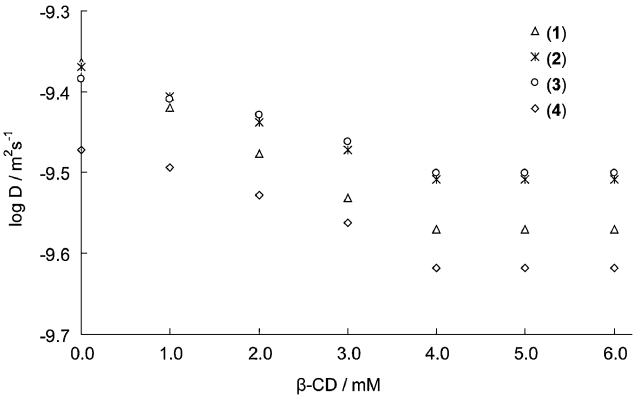


**Fig. 3** Plot of the diffusion coefficients of catechins **1–4** in $D_2O$ at 25 °C as a function of the concentration of β-CD. The catechin concentration was kept constant at 4 mM.

methods such as NMR chemical shifts or calorimetry, while those for β-CD/C and β-CD/EGCG are significantly larger.[30,50] The value for the β-CD/C complex is however very similar to the *K* value obtained by Julian *et al.*[34] using the same procedure. A plot of the apparent diffusion coefficient of the catechins **1–4** as a function of the concentration of β-CD (Fig. 3) shows a successive decrease in the value of log *D* until an equimolar amount of β-CD and catechin is reached. No changes are observed when β-CD is in excess indicating a 1 : 1 stoichiometry for the cyclodextrin–catechin complexes.

Upon addition of β-CD (2.0 mM) into the solution of catechins (2.0 mM each), **1** exhibits a decreased motion because of its favored complexation with β-CD, leading to the separation from **2** and **3** (Fig. 2(d)). The diffusion coefficients of **1** and **4** become similar when the amount of β-CD is increased and for a 1 : 1 molar ratio of β-CD and catechins, the separation between the two compounds is lost (Fig. 2(e)).

Due to differences in binding affinity, organic solvents will affect the equilibrium of the various catechin/β-CD complexes to a different extent. Thus, the best separation of catechins was obtained with acetonitrile as the co-solvent in a 15% $CD_3CN$–85% $D_2O$ (v/v) ratio (Fig. 2(f)).

**DOSY spectra of catechins in the presence of BSA.** The diffusion of catechins **1–4** in the presence of BSA was studied in aqueous solution using a 40 molar ratio excess of catechins and Fig. 2(b) shows that **4** still has the lower diffusion coefficient. Also, the changes in the apparent diffusion coefficients (Table 1) indicate that **4** is binding most strongly to BSA, in good agreement with results obtained from other types of studies[37,38,40] and confirming the importance of the gallate moiety in the interaction. Addition of BSA resulted in the separation of **3** from **2** and **1** (Fig. 2(c), Table 1). Despite being a larger molecule, **3** has a higher diffusion coefficient than that of **1** or **2**, demonstrating its weaker binding to BSA. The only difference between **2** and **3** is the presence of a third hydroxyl group in ring B, this ring being in close proximity to BSA as shown by STD NMR experiments (Fig. 1). It is thus possible that the additional OH is not favorable for the interaction. Since the B ring is identical in **3** and **4**, the latter showing the strongest binding to BSA, it is thus most likely that the two compounds bind to BSA in different ways.

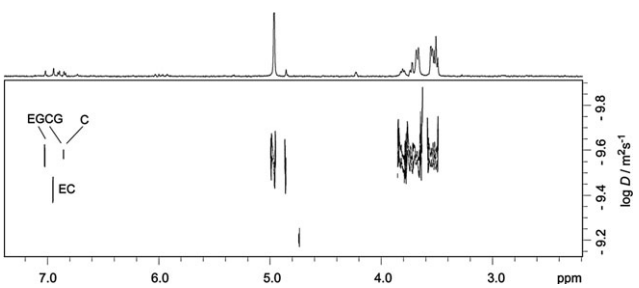


**Fig. 4** DOSY spectra of catechins **1**, **2** and **4** (2.0 mM for each) and β-CD bonded to silica gel recorded with an HR-MAS probe in $D_2O$.

Although the concentration of BSA was much less than that of catechins, the aggregation of BSA due to the presence of catechins resulted in poor spectral resolution. Better resolution was obtained in the DOSY spectra using β-CD which was therefore chosen as the complexation agent for the analysis of green tea extract.

**HR-MAS DOSY of β-CD and mixture of catechins.** Fig. 4 shows the HR-MAS DOSY spectrum of a mixture of catechins **1**, **2** and **4** and β-CD bonded to silica gel in $D_2O$. Comparison with the DOSY spectrum obtained with the liquid probe (Fig. 2(e)) shows that the MAS probe produced a spectrum with broader diffusion peaks. The lower quality of DOSY spectra obtained with the HR-MAS probe has been previously observed and attributed to larger statistical error due to the vortexing sample.[55] Extensive signal averaging was shown to eliminate partially the observed erratic behavior.[55]

Since we have previously shown that there was no interaction between the silica gel column and the catechins and the apparent diffusion coefficient leading to component identification is entirely based on the affinity of a given component for the β-CD ligand, all subsequent experiments were performed with the liquid NMR probe.

## DOSY of green tea extract (GTE)

Water, methanol, acetonitrile and acetone were used to investigate the effect of the solvent on the extraction of compounds from green tea. As shown in Fig. 5, the water and methanol extractions on the one hand and the acetonitrile and acetone extractions on the other hand produce relatively similar proton NMR spectra. The low solubility of carbohydrates in acetonitrile and acetone leads to relatively simpler NMR spectra (*e.g.*, the 2.5–4.5 ppm region) for extractions performed with these solvents. Major metabolites such as caffeine and theanine could be readily assigned from the one-dimensional $^1$H NMR spectra by comparing the resonances to those of well-defined standard compounds as well as to those reported for green tea.[56] The presence of other compounds such as quinic acid, alanine, sucrose and 2-*O*-(β-L-arabinopyranosyl)myoinositol was confirmed from 2D COSY, TOCSY and HSQC experiments. Since the main goal of this study was to assess the use of DOSY to identify and separate catechin components from green tea, no further effort was made to assign the other metabolites present in the NMR spectra of the green tea extract.

The GTE samples were concentrated in order to increase the signal intensity. For concentrated samples in water or in

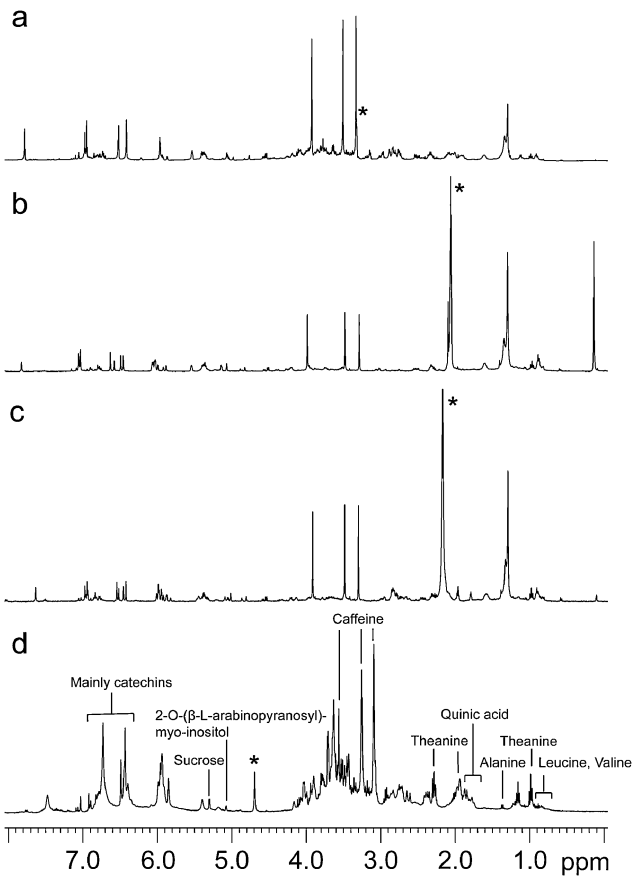


**Fig. 5** $^{1}$H NMR spectra of GTE in different solvents (a) methanol-$d_4$, (b) acetone-$d_6$, (c) acetonitrile-$d_3$, (d) $D_2O$. * Indicates solvent peak.

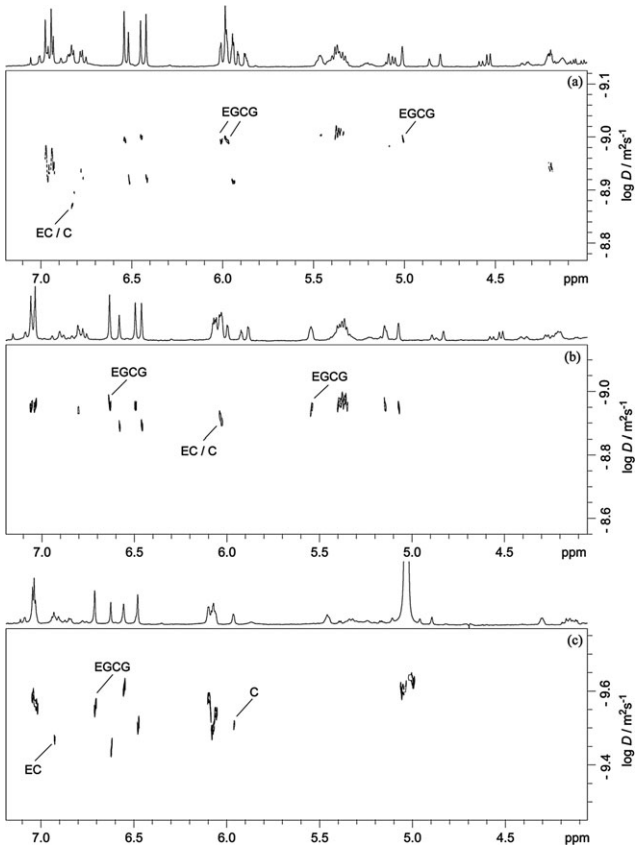


**Fig. 6** DOSY spectra of the concentrated samples in (a) acetonitrile, (b) acetone and (c) with β-CD in 15% $CD_3CN$–85% $D_2O$ (sample 2).

methanol, greater shielding and lower diffusion coefficients due to higher viscosity[57] were observed (see ESI†). Thus, for methanol and water extractions, concentrations above 15 and 50 mg mL$^{-1}$, respectively, lead to a decrease in diffusion rate due to an increase of the medium viscosity. For the acetonitrile and acetone extracts, the highly concentrated sample of 300 mg mL$^{-1}$ showed the same diffusion as the 10 mg mL$^{-1}$ GTE extraction because of the absence of sugars. Therefore, the use of acetonitrile and acetone as solvents was preferred in the study of catechins from green tea extract, while water and methanol can be used to investigate compounds with higher polarity such as carbohydrates. The catechins EGCG, EC and C were clearly identified and separated in the DOSY spectra of the concentrated sample (Fig. 6(a) and (b)). A better resolution in the diffusion dimension was obtained with acetonitrile making the assignment of catechins in the mixture easier.

Thus, the sample obtained from acetonitrile extraction was after solvent evaporation, dissolved in $D_2O$ (sample 1) and in 15% $CD_3CN$–85% $D_2O$ (sample 2) and β-CD was added to each sample. Due to the fact that β-CD forms inclusion complexes with many of the small molecules present in green tea, the amount of β-CD was increased until no chemical shift changes were observed in the $^{1}$H NMR spectrum (Fig. 6(c)). It was then assumed that full complexation between β-CD and the binding molecules from the green tea extract were reached. With sample 2, the diffusion coefficients of EGCG, EC and C in complex with β-CD were consistent with those shown in Fig. 2(f), confirming the identification of these catechins in green tea extract. The signals of C and EC were weak if compared to those from EGCG due to their lower amounts in green tea (Fig. 6(c)). EGCG could also be assigned in the DOSY spectra of sample 1 (data not shown) but signals from EC and C were not observed. In addition to changing the equilibrium of the cyclodextrin–catechin inclusion complexes, the use of acetonitrile as a co-solvent to water (sample 2) improved the resolution of the DOSY spectra.

## Conclusions

β-CD and BSA, coupled to solid chromatographic supports such as silica gel or agarose, are widely used in chromatography to separate mixtures on the basis of molecular interaction. In the present work, it is shown that they can be used to enhance or modify the spectral separation of the DOSY spectra of catechins. Thus, while EGCG could be separated from a mixture of four catechins due to its higher molecular size, separation of EGC from C and EC on the basis of their apparent diffusion coefficient was achieved with BSA while C, EC and EGC could be differentiated using β-CD and different solvent systems. A better knowledge of the binding interaction between small metabolites and different types of receptors should increase the ability to identify compounds in a mixture by this type of experiment. A drawback is that chemical shift changes are often induced upon binding, their amplitude being dependant on the ligand and on the strength of binding.

 

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