Flavonoid mixture analysis by matrix-assisted diffusion-ordered spectroscopy

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The structural similarity of flavonoids, often present in natural product mixtures, makes their analysis by NMR less than straightforward. This similarity is a dual problem for one of the most powerful NMR methods for mixture analysis, diffusion-ordered spectroscopy (DOSY), which relies both on well-resolved peaks and on differences in hydrodynamic radius for separating the signals from different components in a mixture. To overcome these limitations, we use a matrix-assisted DOSY (MAD) approach that exploits differential chemical interactions with a slow diffusion matrix (here micellar sodium dodecyl sulphate SDS) to resolve flavonoid mixtures in mixed solvents.

Phenolic compounds like flavonoids have received considerable attention because of their potent antioxidant properties, mainly as scavengers of free oxygen species.[1] They are widely distributed in nature, are present in a large number of foods of plant origin, and have a wide range of recognized biological activities including hypoglycaemic,[2] anticancer,[3] antimalarial,[4] anti-inflammatory and antiviral.[5]

Flavonoids such as flavone, fisetin, catechin and quercetin have considerable biological significance[6] and are usually consumed in natural foods from plants;[7, 8] they can also be found in medicinal plants such as ginger varieties.[9] Efficient analysis of these components is clearly important. They belong to a family of compounds that have in common a benzopyran (A and C rings) and a phenyl group (B ring) and differ mainly in the type and number of substituents.[1] Analyses of mixtures containing flavonoids commonly employ HPLC, but are time-consuming, complicated and expensive.[10] It is often necessary to couple HPLC with mass spectrometry and/or NMR for identification of the compounds separated.[10, 11]

NMR is one of the most powerful tools in the elucidation of structures, and diffusion-ordered NMR spectroscopy (DOSY) has become an important tool used in the analysis of mixtures.[12, 13] However, the method struggles when diffusion coefficients are very similar and/or when spectra are highly overlapped. Recently, it has been shown that performing DOSY in a matrix with which the analytes interact differentially can resolve signals from similar compounds that would otherwise show the same diffusion. In such a matrix-assisted DOSY (MAD) experiment the interaction of the analytes with the matrix modulates the average diffusion coefficients as different mixture components bind to the matrix to different extents; it also sometimes helps to resolve the spectral overlap by causing differential chemical shift changes.[14, 15] MAD methods have great potential for the analysis of complex mixtures, such as those common in natural product chemistry; [16-17] here we have employed sodium dodecyl sulphate (SDS) micelles to aid an analysis of flavonoids.

In a MAD experiment the analytes interact differentially with the micelles, depending *inter alia* on polarity, amphiphilicity, and structure, resulting in altered diffusion coefficients allowing the separation

of the individual component spectra.[14, 15] The solute diffusion coefficients D_{av} are a weighted average of the bound and free coefficients, and are given by Lindman's Law:

$$D_{av} = D_u p_u + D_b p_{b,}$$

where D_u and p_u and D_b and p_b are the diffusion coefficients and the fractions of unbound and bound solute respectively.[18]

SDS readily forms micelles in aqueous solution;[16] there are numerous studies on pure and mixed micelle formation in aqueous solutions, and also some reports of micelles in binary solvent systems.[19-22] Above the critical micellar concentration (CMC), aqueous SDS forms micelles, and as concentration increases these micelles first change shape and then form entangled polymer-like superstructures.[23] Many flavonoids, however, have low solubility in water, so it would be advantageous to perform MAD in other solvents. It has previously been shown that SDS can form micelles in DMSO,[24] a much better solvent for flavonoids. We therefore demonstrate here the first application of matrix-assisted DOSY using mixed water-DMSO solutions of SDS, enhancing the solubility of the analytes fisetin, flavone, catechin and quercetin.

Results and Discussion

DOSY spectra of the flavonoids in the mixture A (Figure 1, flavonoids 1,2,3) in aqueous solution showed that flavone diffused at a higher rate, as expected from its smaller size, but that hydrodynamic radii of fisetin and catechin were too similar for their signals to be separable, suggesting the desirability of a matrix-assisted experiment. However, the low solubility made acquisition times prohibitive; instead, the use of the binary system DMSO-d_6:D_2O was explored.

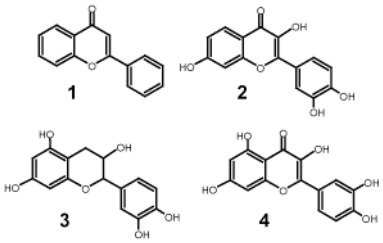

Figure 1. Structures of the flavonoids, flavone(1), fisetin (2), catechin (3) and quercetin (4)

The DOSY spectra of the flavonoid mixtures in DMSO-d_6:D_2O mixtures (Figure 2a, b) show that the flavone signals can be separated from those of catechin and fisetin; this is unsurprising, as flavone is expected to have a smaller hydrodynamic radius. As the mole fraction of fraction of DMSO-d_6 increases (Figure 2b), the higher viscosity causes all compounds to diffuse more slowly. Adding SDS completely changes the situation, as shown in Figure 3a and 3b; now, due to its weaker interaction with the micelles, the signals of catechin are easily separated from those of flavone and fisetin. This allows the identification of each of the three components by comparing experiments with and without SDS. The source of the specificity in the interactions between flavonoids and SDS micelles is not obvious; it is possible that the role of the carbonyl group is important and as a result, causes catechin to have a different conformation and to interact only weakly with the micelles.

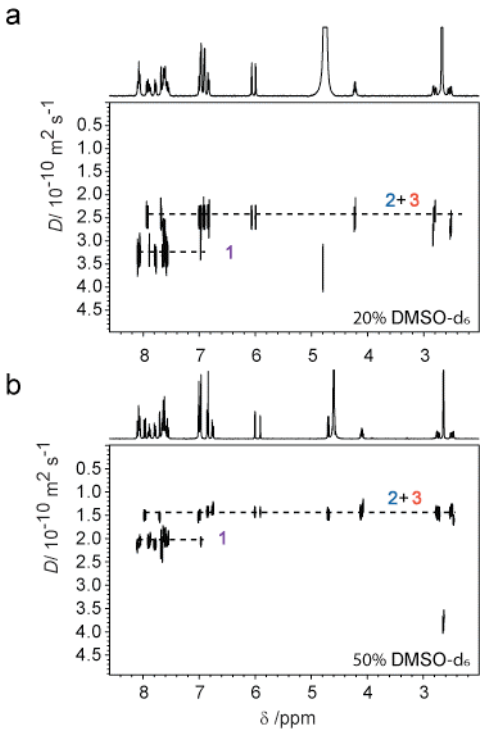

Figure 2. Oneshot DOSY experiments for mixture A, for two different percentages of DMSO-d_6:D_2O: 20% (a) and 50% (b)

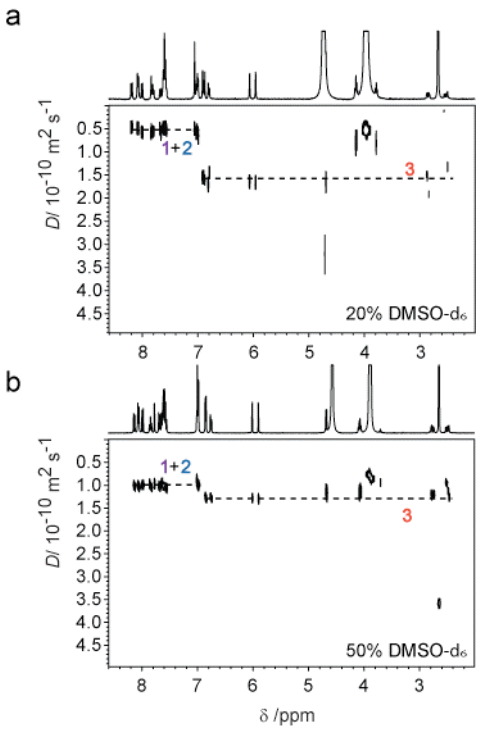

Figure 3. Oneshot DOSY experiments after the addition of SDS (100 mM) to the mixture A and two different percentages of DMSO-d_6:D_2O: 20% (c) and 50% (d)

The presence of micelles in the binary solvent was confirmed for the two mixtures of solvent selected (DMSO-d_6 20% and 50%) by investigating the dependence of SDS diffusion coefficient on concentration. Figure 4, which plots the measured SDS diffusion coefficient against the inverse of the total SDS concentration in 20% v/v DMSO-d_6, shows that micellisation in this solvent is well-described by the associative model. The CMC was found to change from 7 mM for pure D_2O to 11 mM in 20% v/v DMSO-d_6 and 25 mM in 50% DMSO-d_6, consistent with the literature.[17]

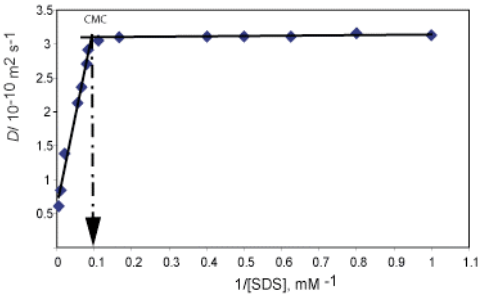

Figure 4. Plot of the measured diffusion coefficient, *D*, against the inverse of the total SDS concentration for 20% of DMSO-d_6, CMC value was found as the intersection of the two slopes.

The power of this MAD approach, using SDS micelles in binary solvents, is further demonstrated on a second mixture of flavonoids, fisetin (**2**), catechin (**3**) and quercetin (**4**). Figure 5 shows the DOSY spectrum of this mixture in 50:50 v/v DMSO-d_6:D_2O before and after the addition of SDS. Before adding SDS (Figure 5a), the three flavonoids were not resolved in the diffusion dimension; with 80 mM SDS, as shown in Figure 5b, flavonoids **2** and **4** interact differentially with the micelles and as a result show different diffusion coefficients. Although there is some overlap of signals between compounds **2** and **4**, they are sufficiently well resolved in the presence of SDS to allow adequate characterization.

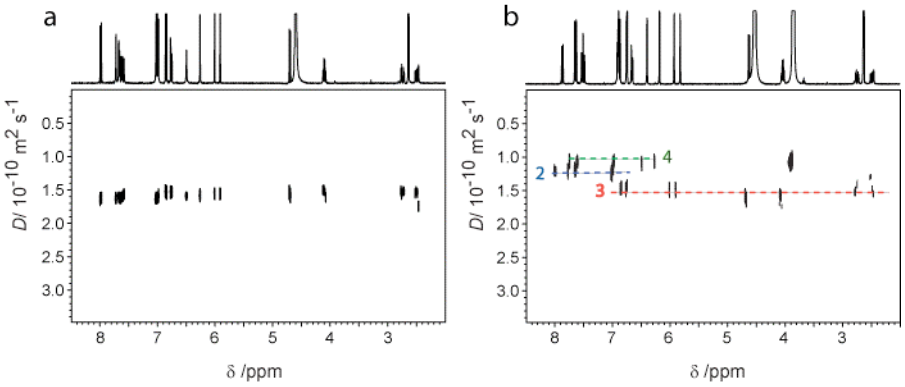

Figure 5. Oneshot DOSY spectra of flavonoids **2**,**3** and **4** (mixture B), before and after addition of SDS (80 mM) in 50% v/v DMSO-d_6:D_2O 50% v/v

In this investigation, we have demonstrated an efficient methodology for NMR analysis of flavonoids, combining binary solvent mixtures and SDS in a novel matrix-assisted DOSY experiment. Solvent systems of 20% and 50% v/v DMSO-d_6:D_2O were used to increase the solubility of flavonoids and, by addition of the surfactant SDS, to exploit differential binding to separate the NMR signals of compounds with very similar sizes. Further investigation on the nature of the interactions between flavonoids and SDS micelles is in progress. Understanding such interactions should in principle give structural information, and would also make it easier to predict the best conditions (solvent composition, surfactant concentration) for signal resolution. This should simplify the analysis of more complex flavonoid mixtures, as well as increasing the information obtainable by NMR.

Experimental methods

NMR experiments were carried out non-spinning on a Varian Inova 400 MHz spectrometer. A 5 mm indirect detection probe equipped with a gradient coil with a rated maximum gradient of 30 G cm^{-1} was used with the sample temperature control regulated at 25 ºC. The Oneshot pulse sequence[25] was used for DOSY experiments with a total diffusion-encoding pulse width (δ) of 3 ms, a diffusion delay (Δ) of 150 ms, and 20 nominal gradient amplitudes ranging from 5 to 27 G cm^{-1} in equal steps of gradient squared.

All processing was done using a modified version of the manufacturers's VnmrJ software, and for all DOSY processing the data were corrected for the effects of non-uniform pulsed field gradients.[26]

Flavone, fisetin, catechin and quercetin, deuterated solvents, sodium dodecyl sulphate (SDS) and sodium trimethylsilyl propionate-d_4 (TSP) were purchased from Sigma-Aldrich (UK) and were used without further purification. Two mixtures of flavones were prepared: flavone, fisetin and catechin mixture A, and fisetin, catechin and quercetin (mixture B). Stock solutions of the flavones in DMSO-d_6 were diluted either with D_2O or with a solution of SDS in D_2O, as appropriate, to yield a final concentration of 5 mM for each flavonoid. TSP was used as an internal reference for reference deconvolution[27] in all the samples. Solutions for the experiments of Figure 4 were prepared by dilution of a stock solution of SDS in D_2O with D_2O and DMSO-d_6 as appropriate.

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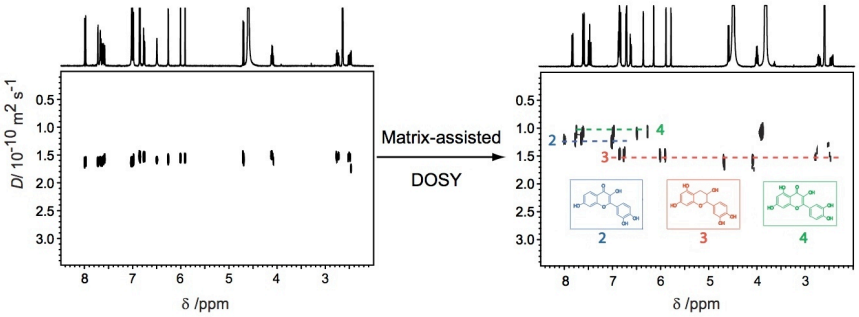
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