Analytical Methods

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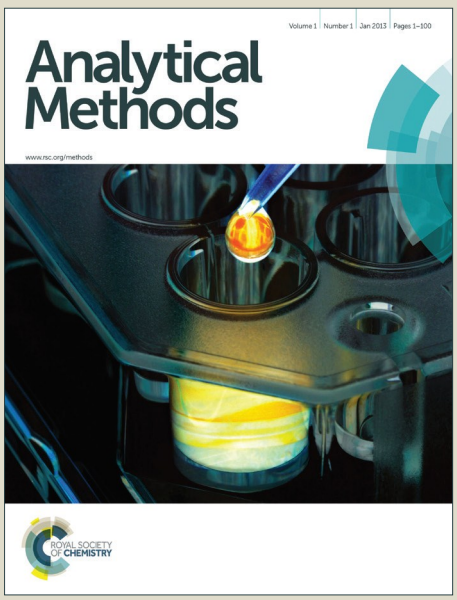

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Methodologies for the rapid determination of the diffusion interaction parameter using Taylor Dispersion Analysis.

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Taylor Dispersion Analysis (TDA) is a fast and simple method for determining diffusion coefficients of molecules in solution. This is achieved by measuring the dispersion of a small quantity of solute after its injection into a mobile phase. Due to repulsive or attractive molecular interactions, such as self-association in the latter case, the diffusion coefficient of a solute may vary with concentration. Typically, the analysis of such concentration dependent behavior requires a concentration titration experiment. In this paper, we present a method for extracting concentration-dependent diffusion coefficients and, hence, the diffusion interaction parameter, k_D, in a single measurement. In TDA, this is achieved by injecting a slug of the solute into the mobile phase and measuring its dispersion as a function of concentration along the resulting front. Three ways of applying the method are presented and applied to aqueous caffeine (negative interaction parameter) and Bovine Serum Albumin prepared in two different buffers (positive interaction parameters). The results were found to be in good agreement with literature values and Dynamic Light Scattering (DLS) measurements.

Introduction

Taylor Dispersion Analysis (TDA) is an absolute method for rapidly determining the mutual diffusion coefficients of molecules. The method, sometimes referred to as Taylor-Aris dispersion, was first described by Taylor in his classic paper[1] and involves the injection of a small plug of the solute into a mobile phase in a capillary and measuring its subsequent dispersion as a function of time. The mutual diffusion coefficient of the injected solute can then be deduced by a variety of methods ranging from fitting Taylor's solution to the concentration profile or taylorgram of the solute to the calculation of moments[2-15].

Whilst there are numerous methods for the determination of intradiffusion (or self-diffusion) coefficients, Taylor dispersion analysis is one of a few methods[16, 17] that can be used to determine mutual diffusion coefficients which are the appropriate measures for bulk ion transport. Although theoretical relationships between intradiffusion and mutual diffusion coefficients, these have met with limited success[17, 18]. Hence, there is special interest in the direct measurement of mutual diffusion coefficients.

Furthermore, the concentration dependence of mutual diffusion coefficients is widely used to characterise the behavior of molecules in solution and, in particular, to identify conditions where molecular interactions are most favourable in terms of stability. This is because typically, the strengths of these interactions become more pronounced with increasing solute concentration as the solution tends to non-ideality[19], thereby leading to a dependence of the diffusion coefficient on solute concentration. This is of great importance in the development of biopharmaceutical drugs[20, 21, 22] where it can be used to determine the second virial coefficient (B_2), which is a measure of the strength of protein-protein interactions for example.

The diffusion interaction parameter, k_D, is a metric that describes the variation of a binary diffusion coefficient with solute concentration in a given medium and is defined by:

$$D = D_0(1 + k_D C) \qquad (1)$$

where D is the measured mutual diffusion coefficient at a solute concentration C and D_0 is the value of D at infinitesimal concentration. Hence the value of k_D can be determined by measuring the mutual diffusion coefficient at a series of solute concentrations and calculating the slope from a plot of D against C. Furthermore, D_0 can be determined from the intercept. It is generally accepted that a negative value for k_D is indicative of an increase in molecular self-association with concentration, whilst a positive value for k_D is indicative of an increase in the strength of repulsive molecular interactions with concentration. Presently, the most widely used technique for determining k_D is Dynamic Light Scattering (DLS).

A titration-style approach can also be undertaken with TDA. Here, the diffusion coefficient is determined at each concentration by injecting a short pulse of the solute into a mobile phase, which comprises the sample solvent as well as a slightly lower concentration of the solute; typically about 0.5 mg/mL lower[23]. Studies of the concentration dependence of binary diffusion coefficients using the TDA titration method have been previously reported for Insulin[24], caffeine[25], polymers[8] and 2-butoxyethanol/water mixtures[23]; although the extension of such data to the determination of the diffusion interaction parameters have yet to be published. There have also been successful studies into the determination of the multicomponent diffusion coefficients of caffeine in ternary[26] and quaternary[27] systems. These involved fitting the relevant ternary and quaternary Taylor dispersion solutions to the taylorgrams obtained from titration measurements.

In this paper, we present an alternative method, based on Boltzmann-Matano analysis[28], which exploits the inherent concentration profile that is generated during Taylor Dispersion and allows the concentration-dependence of the diffusion coefficients and hence the diffusion interaction parameter to be determined in a single measurement. The method, which involves the measurement of the diffusion distance of solute molecules in a diffusion couple as a function of concentration, was discovered by Ludwig Boltzmann and applied by Chijuro Matano to metal alloys[28].

We find that by injecting a long sample plug, rather than a short plug, into a flow-stream of the mobile phase within a microcapillary, a diffusion couple in the shape of a front can be set up between the solute and solvent molecules. In TDA terms, this amounts to a dispersion couple since the solute disperses into the solvent according to Fick's law via a combination of axial convection and radial diffusion[1]. The dispersion distance from the position of the original front is measured as a function of the solute concentration and the corresponding dispersion coefficients, d, calculated with the aid of the Boltzmann-Matano method. Three different applications of the method are considered and the diffusion coefficients, D, computed from the relation:

$$D = \frac{r^2 v^2}{48d} \qquad (2)$$

where *r* is the capillary radius and *v* is the average flow speed. This analysis therefore enables the determination of diffusion coefficients along the sample front (which represents a series of concentrations) in a single measurement.

The paper is arranged as follows. First, the Boltzmann-Matano method for determining concentration-dependent binary diffusion coefficients and techniques for its application are described. Next, its extension to Taylor Dispersion Analysis is discussed. The Boltzmann-Matano method is then applied to aqueous caffeine and Bovine Serum Albumin dissolved in iodide and sulfate salt solutions and the results compared to literature values and DLS measurements respectively.

Theoretical Methods

Boltzmann-Matano analysis

The Boltzmann- Matano method is used to convert the partial differential equation resulting from Fick's law of diffusion into an ordinary differential equation. If the diffusion coefficient *D* is assumed to be a function of concentration *C*, Fick's law of diffusion is:

$$\frac{\partial C}{\partial t} = \frac{\partial}{\partial x}\left[D(C)\frac{\partial C}{\partial x}\right] \tag{3}$$

where *x* is the distance diffused in the time *t.* By defining the variable λ as:

$$\lambda = \frac{x}{\sqrt{t}} \tag{4}$$

Eq. (3) can be re-written as

$$-\frac{\lambda}{2}\frac{dC}{d\lambda} = \frac{d}{d\lambda}\left[D(C)\frac{dC}{d\lambda}\right] \tag{5}$$

which gives $D(C^*)$ as

$$D(C^*) = -\frac{1}{2}\frac{d\lambda}{dC}_{C=C^*}\int_0^{C=C^*}\lambda dC \tag{6}$$

Fig. 1 is an example of a concentration-λ curve where the starting left and right concentrations have been defined as C_L and C_R with the implicit assumption that $C_R > C_L$.

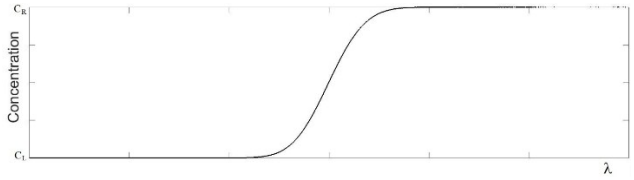

FIG. 1. A typical concentration-λ curve.

In these terms, Eq. (6) becomes:

$$D(C^*) = -\frac{1}{2}\frac{\int_{C=C_L}^{C=C^*}\lambda dC}{\frac{dC}{d\lambda}_{C=C^*}} \tag{7}$$

It can be observed that Eq. (7) also holds for $\lambda \rightarrow \lambda_M$ where λ_M is any arbitrary constant. This is because the Boltzmann transformation in Eq. (4) is independent of the reference point it is measured from. The physically meaningful value of λ_M, known as the Matano interface, can however be determined by an examination of Eq. (7). Since the denominator goes to zero as *C* tends to C_L, the integral in the numerator must also tend to zero, otherwise $D(C_L)$ will tend to infinity. Hence, by imposing the following condition

$$\int_{C_L}^{C_R}(\lambda - \lambda_M)dC = 0 \tag{8}$$

λ_M can be determined from

$$\lambda_M = \frac{1}{C_R - C_L}\int_{C_L}^{C_R}\lambda dC \tag{9}$$

In a constant volume system, Eq. (9) is a conservation of mass condition and in this case λ_M corresponds to the initial position of the interface at time $t = 0$. The transformation $\lambda \rightarrow \lambda - \lambda_M$ is then performed on λ.

Methods of Application

There are a number of ways in which the diffusion coefficient may be calculated from Eq. (7). Three methods will be considered in this report. The first is the graphical method[29] which involves the numerical computation of the integral in the numerator and the differential in the denominator. This amounts to the respective calculations of the area of the grey region and the gradient of the tangent in the concentration-λ curve in Fig. 2. $D = D_0(1 + k_D C)$

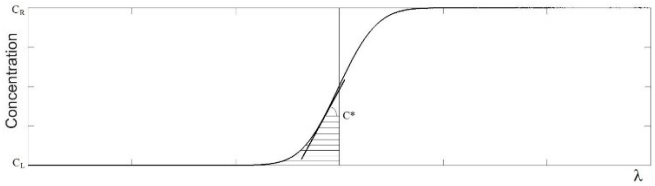

FIG. 2. Graphical method for estimating differentials and integrals.

Likewise, the Matano interface can be determined from Eq. (9) and amounts to the determination of the value of λ_M for which the two areas, A_L and A_R in Fig. 3 are equal.

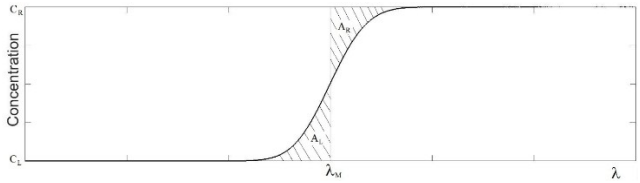

FIG. 3. The Matano interface.

Due to the difficulty in estimating the gradients and areas at low and high values of the concentration, this method may be prone to errors. Therefore alternative but more approximate methods have been proposed for this analysis. One, which shall be termed the analytical method[30, 31], involves the parameterization of the concentration profile by the following expression:

$$C = \frac{C_R - C_L}{2}(1 + \mathrm{erf}(u)) \tag{9}$$

where

$$u = h\lambda + m \tag{10}$$

and *h* and *m* are parameters that vary with *C*.

This may be contrasted with the solution to the diffusion equation when the diffusion coefficient is independent of concentration:

$$C = \frac{C_R - C_L}{2}\left(1 + \mathrm{erf}\left(\frac{\lambda}{2\sqrt{k}}\right)\right) \quad (11)$$

where *k* is the dispersion coefficient. Substituting Eq. (11) into Eq. (7) gives the following expression for *D*:

$$D(C^*) = \frac{1}{4h^2} + \frac{m\sqrt{\pi}}{2h^2} e^{u^2} \frac{C^*}{C_R - C_L} \quad (12)$$

At each value of the concentration along the front, the value of *u* is determined by inverting Eq. (9) and the corresponding values of *h* and *m* are determined by fitting a straight line locally at each value of the concentration. These can then be inserted into Eq. (12) to determine *D* as a function of *C*.

Typically *m* is close to zero in which case Eq. (12) reduces to

$$D(C^*) = \frac{1}{4h^2} \quad (13)$$

We propose an alternative method for determining *h* which involves the numerical differentiation of the concentration profile with respect to λ at each value of the concentration and equating it to the differential of the expression in Eq. (9). The value of *h* at each concentration *C* is then given by

$$h = \sqrt{\pi}(C_R - C_L)\frac{dC}{d\lambda} e^{u^2} \quad (14)$$

D can then be estimated from Eq. (13). This method shall be termed the explicit differentiation method.

Extension to Taylor Dispersion Analysis

Fig. 4 shows an example of a taylorgram obtained from a 2 minute injection of a slug of solute of 2 mg mL^{-1} of caffeine into a mobile phase of water at 140 mbar. The solute is driven at a run pressure of 140 mbar and its absorbance (which is proportional to its concentration) was measured as a function of time as it flowed past a fixed detection point.

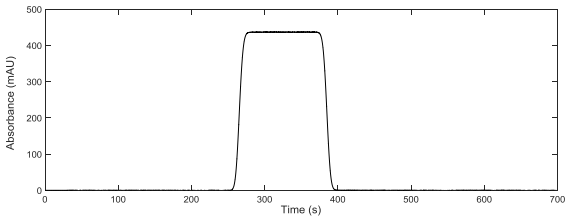

FIG. 4. Taylogram from a slug of aqueous caffeine.

As mentioned earlier, the dispersion of the solute obeys Fick's law and hence *x* in the equations above is the dispersion distance and *t* is the time of dispersion. Hence, it is the dispersion coefficient as a function of concentration that is determined from the measurement. Furthermore, the dispersion is relative to a plane which moves with the average velocity of the flow. This plane is the Matano interface from which the dispersion distances *x* are to be measured. If we denote the time at which the plane arrives at the detection window as t_M, the dispersion distance *x* at a given time *t* is given by

$$x = v(t - t_M) \quad (15)$$

where *v* is the average velocity of the flow and λ_M is equal to $\frac{vt_M}{\sqrt{t}}$.

From Eq. (4), λ can be computed and the transformation $\lambda \rightarrow \lambda - \lambda_M$ is performed. A concentration-λ curve can then be obtained for the solute from Fig. 4. From this curve, the dispersion coefficients as a function of concentration can then be computed by the methods described earlier. An alternative way to determine t_M would be to use Eq. (9). This is more rigorous as it circumvents any errors in the buffer viscosity, capillary radius and the run pressure, all of which are required to determine the average flow velocity. The concentration-dependent diffusion coefficients *D* can be determined from the dispersion coefficients *d* via Eq. (2). The interaction parameter k_D can then be determined from a fit of Eq. (1) to the results.

There are a few points worth noting about this extension of the Boltzmann-Matano method to TDA. The first is the assumption that the molecular interactions have reached an equilibrium at each measured concentration. This is strictly true when the timescales of the interactions are significantly less than the timescale of the dispersion which is the case for short-range interactions. There is a further assumption that the average flow velocity of the slug is constant over the duration of its dispersion. This is true after the slug has been injected into the capillary but only true during the injection if the solute viscosity is the same as the viscosity of the mobile phase. Hence, this extension is more appropriate for dilute solutions or solutions with viscosities that do not differ much from the viscosity of the mobile phase. Another point worth noting is that it has been assumed that the original interface between the sample and the mobile phase is a plane perpendicular to the capillary axis. This is true at the start of the injection, and hence the time *t* in Fig. 4 should be measured from this point. In practice, the injection step and the run step of the measurement are usually separate and hence, there is a time gap between the end of the injection and the beginning of the run step when the flow velocity is zero. If this time gap is too long, the concentration profile might not be preserved due to the influence of axial diffusion. Hence it is advisable to keep this gap as short as possible and ensure that it is not included in the measured time for the flow. For the measurements reported in this paper, this time gap was measured to be about 12 s which is sufficiently short. Finally, the constraints on the flow rate and capillary dimensions required for TDA to be feasible[32] also apply to this analysis.

Experimental Methods

All buffers, salts and other reagents were purchased from (Sigma-Aldrich, Poole, UK). Aqueous caffeine (purity 99%) was prepared at a concentration of 2 mg mL^{-1} and data were acquired using the Viscosizer TD (Malvern Instruments Ltd., Worcestershire, UK) fitted with a standard uncoated capillary (ID 75 µm, OD 360 µm, L = 1.3 m, Malvern Instruments Ltd., Worcestershire, UK) at 25 °C using a 254 nm wavelength filter. The UV absorbance of the solute was measured at the first detection window, 0.45m away from the inlet, and assumed to be linearly proportional to its concentration as predicted by Beer-Lambert's law. Furthermore, corrections for the level of stray light within the instrument[33] were applied to the measured UV absorbance in order to obtain the true sample absorbance. The delivery of the sample slugs into the capillary containing the deionised water was achieved by a pressure-driven injection at 140 mbar for 2 minutes. The sample slugs were then driven at a run pressure of 140 mbar. Three replicates of the measurement were performed. Details of the analyses are contained within the next section. The literature value for the k_D was calculated from a straight-line fit of published concentration-dependent diffusion coefficients for aqueous caffeine determined by

Least and Hui[21]. The diffusion coefficients used for this fitting spanned the concentration range 0-2 mg mL^{-1}.

Bovine Serum Albumin (BSA, purity 96%) was prepared at a concentrations of 30 mg mL^{-1} in two buffered salt solutions:

(1) 20 mM sodium acetate (purity 99%), 50 mM sodium iodide (purity 99.5%) and

(2) 50 mM sodium acetate, 50 mM sodium sulfate (purity 99%),

both at pH 7.4.

Data were acquired using the Viscosizer 200 (Malvern Instruments Ltd., Worcestershire, UK) fitted with a standard uncoated capillary (ID 75 µm, OD 360 µm, L = 1.3m, Malvern Instruments Ltd., Worcestershire, UK) at 22 °C using a 280 nm wavelength filter. Corrections for stray light were applied. The sample slugs were injected into a buffer-filled capillary at 140 mbar for 3 minutes and a subsequent run pressure of 140 mbar was applied. The UV absorbance of the solute was measured at the first detection window, 0.45m away from the inlet. Five replicates of each measurement were performed.

The diffusion interaction parameter was also determined for these BSA solutions by Dynamic Light Scattering (DLS). The diffusion coefficients were measured on a Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK; laser wavelength 633 nm) for a series of 6 concentrations in the range 0-30 mg mL^{-1} and the interaction parameter determined from a straight-line fit to the plot of D against concentration C.

Results and Discussion

Fig. 4 shows an example of the taylorgrams obtained for the aqueous caffeine whilst Figs. 5 and 6 show examples of the taylrograms obtained for BSA in iodide salt and sulfate salt solutions respectively.

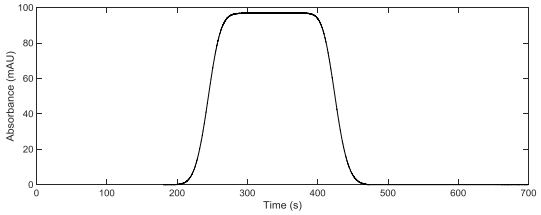

FIG. 5. Taylorgram from a slug of BSA in iodide salt solution.

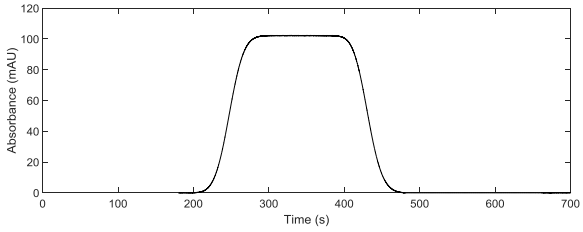

FIG. 6. Taylorgram from a slug of BSA in sulfate salt solution.

Subsequent analyses of these taylorgrams were performed at the leading edges only. This is due to uncertainties in the shape of the sample plug at the trailing edge immediately after the injection step. At this point it is not certain that the plane of the trailing edge is initially perpendicular to the capillary axis, which is a required starting condition for the analysis.

The time of arrival t_M of the Matano interface, and hence λ_M, was determined from Eq. (9). Figs. 4-6 were then converted to the concentration-λ curves in Figs. 7-9 respectively.

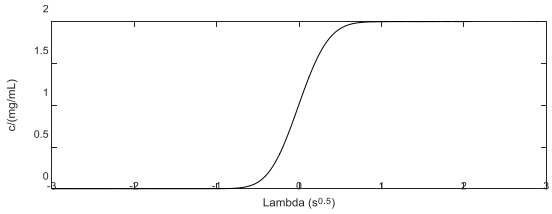

FIG. 7. Concentration-λ curve for aqueous caffeine.

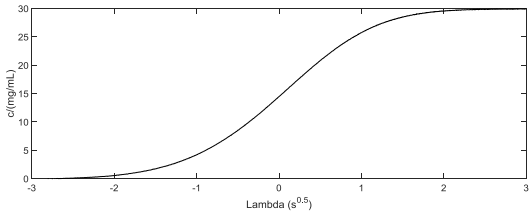

FIG. 8. Concentration-λ curve for BSA in iodide salt solution.

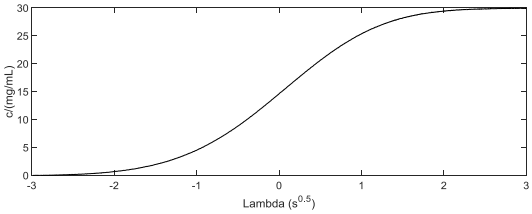

FIG. 9. Concentration-λ curve for BSA in sulfate salt solution.

From these curves, the dispersion coefficients were estimated using the three methods described earlier. The differentials in Eq. (7) for the graphical method and Eq. (14) for the explicit differentiation method were performed with a Savitzky-Golay filter[34]. Representative results obtained from one measurement of each solution are shown in Figs. 10-12 for the three different methods. Values for D_0 and k_D were obtained by fitting a straight line to the plots and the average values are shown in Tables I-III. Also shown are the averaged regression coefficients (R^2) for the fits. Note, that the concentration ranges considered for the straight-line fits have been restricted to between 20% and 80% of the maximum concentrations because the slopes of the profiles are difficult to define accurately near the concentration maxima and minima. As the slopes approach zero any errors in the estimates in these regions will be magnified in Eq. (7).

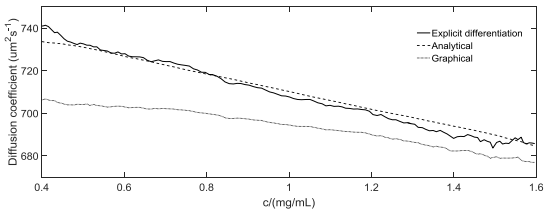

FIG. 10. Diffusion coefficients of aqueous caffeine.

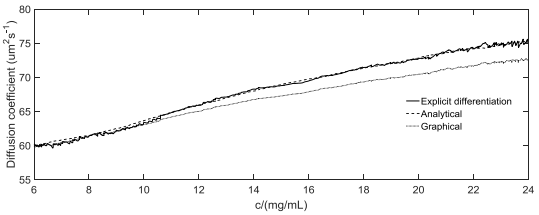

FIG. 11. Diffusion coefficients of BSA in iodide salt solution.

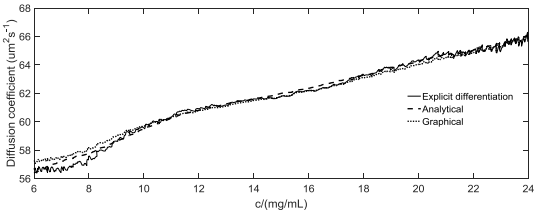

FIG. 12. Diffusion coefficients of BSA in sulfate salt solution.

TABLE 1. Results for aqueous caffeine.

Aqueous caffeine	k_D (10^{-2} mL mg^{-1})	D_0 ($\mu m^2 s^{-1}$)	R^2
Explicit differential method	-6.4 +/- 0.2	767 +/- 9	0.990
Analytical method	-5.7 +/- 0.2	761 +/- 9	0.999
Graphical method	-1.8 +/- 1.6	727 +/- 8	0.982
Literature value	-4.0	766.1	

TABLE 2. Results for BSA in Iodide salt solution.

BSA in Iodide Salt	k_D (10^{-2} mL mg^{-1})	D_0 ($\mu m^2 s^{-1}$)	R^2
Explicit differential method	1.61 +/- 0.05	54.4 +/- 0.2	0.998
Analytical method	1.59 +/- 0.04	54.7 +/- 0.2	0.999
Graphical method	1.24 +/- 0.01	55.6 +/- 0.4	0.995
DLS	1.30 +/- 0.01	59.7 +/- 0.4	0.999

TABLE 3. Results for BSA in Sulfate salt solution.

BSA in Sulfate Salt	k_D (10^{-2} mL mg^{-1})	D_0 ($\mu m^2 s^{-1}$)	R^2
Explicit differential method	0.93+/- 0.04	54.3 +/- 0.3	0.985
Analytical method	0.93 +/- 0.04	54.4 +/- 0.3	0.991
Graphical method	0.84 +/- 0.01	54.5 +/- 0.5	0.989
DLS	0.75 +/- 0.01	59.7 +/- 0.4	0.993

For comparison, the literature k_D values for aqueous caffeine and the experimental k_D values obtained from DLS measurements of the BSA solutions have also been provided in Tables I-III. For all solutions under study, the order of magnitude and sign of k_D values predicted by the three Boltzmann-Matano methods are in good agreement with literature and DLS results, where applicable. For aqueous caffeine, the explicit differential and analytical methods give much closer and repeatable estimates for k_D and D_0 compared to the literature values. These small differences between experimental and literature k_D values may be ascribed to one or more factors, including measurement temperature differences; differences introduced into the literature k_D value by the fitting method; uncertainties in stray light correction at higher absorbances or a result of the different detection methods used. The underestimation of D_0 and the greater degree of uncertainty of k_D observed in the graphical method is most likely a consequence of the inherent difficulties in estimating the slopes and areas, as described earlier. For both BSA solutions, the k_D and D_0 values determined are concordant between Boltzmann-Matano methods and highly complementary with DLS results.

Conclusions

The Boltzmann-Matano method for the computation of concentration-dependent mutual diffusion coefficients has been extended to the dispersion of solutes into a mobile phase and hence, to the determination of the diffusion interaction parameter, k_D, by Taylor Dispersion Analysis. By analysing the front that arises from the dispersion of the solute around a Matano interface that moves at the average flow speed of the solvent, the diffusion coefficients of the solute as a function of concentration are determined. Three ways of applying the method are described and applied to an aqueous caffeine solution and BSA dissolved in iodide and sulfate salt solutions. Whilst the explicit differential and analytical methods were generally found to be more robust than the graphical method, all three methods successfully predicted the expected signs and relative strengths of the interaction parameters (negative for aqueous caffeine and positive for BSA in iodide and sulfate salt solutions). Since alternative methods require the construction of a titration curve from measurements undertaken at separate concentrations, this report demonstrates the potential of TDA as a method for evaluating the strength of molecular interactions in a single measurement using only a few microliters of sample. Further work in this area would involve the application of this method to understanding the relative stability of a wider range of dispersed solutes.

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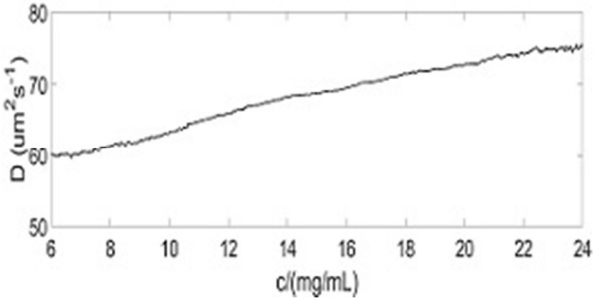
Using Taylor Dispersion Analysis determine diffusion coefficients as a function of concentration in a single measurement.



78x39mm (96 x 96 DPI)