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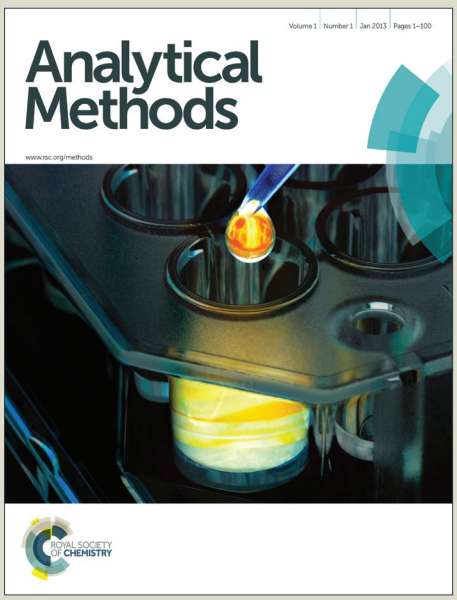

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The application of high resolution diffusion NMR for the characterisation and quantification of small molecules in saliva/dentifrice slurries.

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Abstract

The application of DOSY (Diffusion Ordered SpectroscopY) NMR as a technique for the virtual separation of toothpaste adjuvants in model saliva is reported for the first time. In addition, the scope and limitations of DOSY NMR are considered using the DOSY Tool Box processing software, as is the quantification of the adjuvants and components of saliva by quantitative NMR (qNMR). These techniques represent a new and powerful tool for the evaluation of complex mixtures of natural products with a view to identifying biomarkers for disease within the oral cavity.

Keywords

Diffusion NMR

qNMR

Natural products

Anti-microbial

Dental plaque

Saliva

Main text

1. Introduction

Within the field of oral care research Nuclear Magnetic Resonance (NMR) techniques have barely been considered as a technique for characterising the small molecule components of saliva or those anti-microbial adjuvants in a dentifrice slurry. The main references to NMR in the oral sciences focus on solid state (MAS) ^{19}F and ^{31}P NMR spectroscopy and the potential application of advanced 2D and pseudo 2D NMR techniques have largely been ignored.[1]

The direct application of Diffusion Ordered Spectroscopy (DOSY) NMR and quantitative NMR (qNMR) for the characterisation and quantification of small molecules, natural products and biomarkers in saliva/dentifrice slurry models has until now gone unreported.

To augment the effectiveness of toothpaste a number of actives are added to improve the health of both hard and soft tissues found in the oral cavity. These fall into three broad categories: anti-caries actives (e.g. sodium fluoride, sodium monofluorophosphate and amine fluoride), sensitivity actives (e.g. potassium nitrate, potassium chloride, strontium acetate, stannous fluoride and bioactive glass), gum health actives, comprising mainly antimicrobial agents such as triclosan, zinc citrate, stannous, essential oils, isopropylmethylphenol plus a wide range of other natural products).[2,3,4,5] Despite a considerable number of publications on dental plaque pathogenicity and biofilm formation, there are relatively few specific studies looking at the biological mode of action/activity of natural products.[6,7] Many of the actives evaluated are well-known secondary plant metabolites of the polyphenol class; however, the majority of agents are non-specific anti-microbial agents , having an impact on a variety of oral microorganisms in a range of ways. The antimicrobial effects of such agents are generally measured as the minimum bactericidal concentration (MBC) which kills particular microbes, or the minimum inhibitory concentration (MIC) which prevents bacterial growth. However, in a flow through environment such as the mouth. the longevity and retentivity in the oral cavity (often termed substantivity) of agents are also crucial to their efficacy. For example during toothbrushing, antimicrobial agents may be present at greater than the MBC or MIC for short periods. However, at sub-MIC concentrations, agents may have a range of more subtle, but still important effects, such as reducing microbial (re)growth, inhibiting key metabolic processes such as acid production, protease

activities or polysaccharide synthesis interfering with bacterial adhesion.[8,9] Understanding where agents reside in the oral cavity, for how long, and to which components they bind are therefore crucial to improving oral care product efficacy. Such understanding could then lead to more rational approaches to optimising product formulation and active delivery. The reported difficulties when attempting to evaluate the efficacy of a natural product are predicated on the complexity of the often crude mixtures of natural products available and the technical challenges of time-consuming purification steps.[10] The importance of basic chemical characterisation of small/natural extract molecules cannot be overstated, particularly where the synergistic effects of more than one component are being investigated.[6]

Recent improvements in high resolution NMR instrumentation, coupled with the exceptional work conducted in the improvement of flexible processing software has made DOSY NMR an increasingly valuable tool in complex mixture analysis,[11,12,13] including biofluids and drug preparations, simultaneously identifying and in part quantifying their components.[14,15,16] The variation in isolation and derivitisation methods has led to often inconsistent data and, despite the excellent review by Koo *et al*.[6] there is clearly scope for the evaluation of DOSY NMR as a non-destructive analytical method, which requires little standardisation. The NMR experiments are efficient, with data being acquired in just over one hour from sample collection. This, coupled with the ability to discriminate NMR signals on the basis of size (hydrodynamic radius), can obviate the need for 1D NMR spectral “binning” of multiple chemical shift regions when attempting to identify principal components. Through internal standardisation, approximate data regarding the mass of a component can be achieved. The aims of this communication are primarily to consider the scope and limitation of DOSY NMR as tools for the characterisation of natural product adjuvants in saliva models and saliva/dentifrice slurries and to demonstrate the potential for internally standardised qNMR to be used to quantify individual component concentrations, without the need for protracted separation and purification steps. For the purpose of evaluating the application of DOSY NMR/qNMR the organic antimicrobial preservative 4-isopropyl-3-methyl phenol (IPMP) was initially used as a reference compound in different matrices: These included a simple surfactant solution, model saliva and saliva/dentifrice slurry.

Whilst MS methods have been used to analyse complex mixtures, the sample preparation and purification processes influence how the molecules of interest interact withother components in solution.[17] MS^n methods have been shown to be useful in these scenarios bu the technique is still destructive. Use of conventional 2D techniques for molecular identification have been used to verify ambiguous signals and should be used in tandem with a technique such as DOSY. Whilst it is possible to use for example HSQC to separate 1H overlaps and aid in signal assignment, the possible interactions between the various components meant we were just as interested in characterising their environment as much as identifying components individual molecules. This will prove of particular value for our current research, which looks at the interactions of such molecules with proteins found in saliva in gingivitis. All of the above mentioned techniques were used in the cases of ambiguity. The interaction between SDS and the IPMP, whilst predictable, illustrates the merit of this technique in a simple system such as that discussed. The application of DOSY to identify these environments and also individual molecules has been previously reported for other complex mixtures and this work aimed to extend this methodology further.

2. Materials and Methods

Being phenolic and having similar chemical and physical motifs to other naturally occurring phenolic compounds, IPMP provided a convenient model for DOSY NMR/qNMR method development. Using IPMP, DOSY NMR was used to observe the influence of pH, sodium dodecyl sulphate (SDS) and salt concentration on the diffusion characteristics of different adjuvants and also their impact on the accuracy of the qNMR data at different concentrations of IPMP. . It is worthy of note that the proportional change in diffusion for TSP was not significantly greater than the observed change in diffusion for other internal standards owing to increased viscosity of saliva. Interactions with proteins seem fast on the NMR timescale and therefore not particularly strong. We acknowledge, however, that this may be a limitation in this study.

Simple Surfactant Solution

Unless otherwise stated, all chemicals were obtained from Sigma-Aldrich. Measurement of solutes was carried out on a Fisherbrand MH-214 balance. PBS Buffer solution was prepared (100mM NaCl)

and corrected to pH 6, 7, 8 & 9. The SDS was added to a concentration of 0.75% w/w and IPMP was dissolved directly in the buffer and made up to the concentration range 0.005%, 0.01%, 0.025%, 0.05% and 0.1% w/w. Each of these sample concentrations was prepared separately five times and 3 qNMR experiments run on each to validate the precision of concentration calculations. Deuterated sodium trimethylsilylpropionate (TSP) was used as an internal reference standard at 14.4mM (DOSY) and 7.4mM (qNMR) concentrations.

2.1 Model Saliva

Fresh model saliva solutions were prepared using the recipe as stated by Klimek *et al* and used on the same day of preparation.[18] The solutions were kept at 25°C and out of direct sunlight.

2.2 Model Saliva/Dentifrice Slurry

The slurry was prepared by stirring 5 g of Aquafresh Ultimate Toothpaste (GSK, Brentford, UK) slurried in a solution of model saliva (8 ml) prepared as per section 2.2. Five samples were prepared at each concentration (0.005%, 0.01%, 0.025%, 0.05% and 0.1% w/w wrt IPMP). The slurry was centrifuged at 4500 rpm (3089 grams) for 30 minutes to remove the solid-components of toothpaste such as silicas and titanium dioxide, and the supernatants only used for analysis. NMR experiments were undertaken in triplicate for qNMR on the supernatant.

2.3 Qualitative/DOSY NMR analysis

A Bruker Avance III 600MHz NMR spectrometer with 5 mm TXI Probe and temperature control unit was used for all ^{1}H NMR experiments. 5mm Bruker Single Use NMR tubes (Product Code Z117777) were used. All spectra were acquired on Topspin 3.0 (Bruker, Germany) and 64 000 complex data points were acquired over a sweep width of 10.3112 ppm using a stimulated echo bipolar pulsed field gradient with 1 spoil gradient and 3-9-19 WATERGATE sequence (STEBPGP1S19). This was used to obtain the diffusion series with δ = 2.4 ms and Δ = 100 ms. The relaxation delay was set to 7s and the WATERGATE pulse duration was 1000 μs with 64 linear gradient steps, from 2-95% gradient intensity, each consisting of 16 scans.

Each sample was allowed to equilibrate within the NMR spectrometer for 5 minutes. All NMR experiments were carried out at 25°C. A sine bell shaped window function phase was applied over all data points prior to Fourier transformation (16 384 points) using Topspin 3.0 (Bruker, Germany). Diffusion data were processed using DOSY Toolbox, (Mathias Nilsson, Manchester University) and TSP was used for Lorentzian reference deconvolution. Individual peaks were fitted exponentially after a 2nd order polynomial baseline correction was employed.[15] Errors in diffusion coefficient were calculated based on the Standard Deviation for each diffusion curve and are in line with the estimated error as reported for a similar mixture of *ca* 0.1 $\times 10^{-10}$ m^2s^{-1}.[19] The Residual Sum of Squares for each of the diffusion curves was less than 5 $\times 10^{-3}$ in all cases.

^{1}H-^{1}H correlation spectroscopy (COSY) was used in addition to predicted M_r data from the diffusion correlation to correctly assign the ^{1}H signals relating to the mixture of components for the commercial toothpaste/artificial saliva samples.

A 2D homonuclear shift correlation pulse sequence using gradient pulses for selection and multiple quantum filtering was used (cosygpmfqf). Size of F1 FID was 2048 with a sweep width of 7.7692 ppm and a dwell time of 161 μs and relaxation delay of 1.861s to give an acquisition time of 0.3297 s. A sine bell shaped window function was applied over all data points prior to Fourier transformation, phasing and baseline correction using Topspin 3.0 (Bruker, Germany).

Matrix matched samples were spiked with reference standards to confirm the identity of the components where there was ambiguity. All reference standards were obtained from Sigma-Aldrich Ltd and used without further purification.

2.5 Quantitative NMR (qNMR)

The quantification of components in natural product mixtures through comparison with the internal standard TSP has already been reported.[10,11] A Bruker Avance III 600MHz NMR spectrometer with 5mm TXI Probe and temperature control unit was used for all experiments. 5mm Single Use NMR tubes (Bruker, Product Code Z117777) were used. All spectra were acquired on Topspin 3.0 and 65 536 complex data points were acquired over a sweep width of 12.9909 ppm using a 90° pulse angle

and an acquisition time of 4.2030 s. A sine bell shaped window function phase shifted by 90° was applied over all data points prior to Fourier transformation, phasing and baseline correction using Topspin 3.0 . The chemical shift of all data was referenced to the TSP reference 0 ppm. All spectra were acquired at 25 °C. Three replicates of the qNMR experiments described were carried out for each of the five IPMP samples and the 5 different concentrations. The average integral of all IPMP signals was used for quantification and all IPMP signals had a s/n ratio greater than 200 making them acceptable for qNMR processing. Quantitative data obtained using this method has been shown to compare well to traditional LC-MS and LC-UV techniques.[20]

3. Results

3.1 DOSY NMR

3.1.1 Internal calibration

For globular molecules of similar density, it has been shown that the diffusion coefficient is proportional to cube root of the relative molecular mass. However, this assumes an even, spherical distribution of density and the Stokes-Einstein equation, on which this principle is based, assumes free diffusion, necessitating a coefficient be determined to infinite dilution. As the diffusion coefficient varies with concentration and viscosity change, internal references can be used to eliminate the complications of these effects when determining molecular mass. This technique has been reported by Li, *et al* [21] and is reviewed by Macchioni, Ciancaleoni, Zuccaccia & Zuccaccia[22] and allows the determination of the molecular mass of an unknown component by using internal reference standards. In the case of IPMP in PBS, IPMP (170 g mol^{-1}), water (18 g mol^{-1}) and TSP (150 g mol^{-1}) were used to generate a calibration graph based on Eq 1, where D = diffusion coefficient (m^2 s^{-1}) and Mr is relative molecular mass.

Eq 1. $\log D = a \log Mr + b.$

Where the viscosity and density remain constant between samples, corrections to a and b can be avoided, however diffusion correlation was determined for each of the samples as such variables cannot be taken for granted. The correlation of LogD and M_r for IPMP in simple solution ($r^2 = 1$) values for a and b constants of -0.37and + 1.49 respectively. The value of a agrees well with the literature

value but the value of b is substantially lower owing to the relatively high viscosity of water when compared to common organic solvents. This has been reported previously by our group.[10]

Figure 1 shows a generally good correlation of mass *vs* diffusion for all components with the exception of IPMP in the presence of sodium dodecyl sulphate (SDS). As reported by Nilsson *et al.* [23] association of lipophillic molecules to SDS micelles substantially decreases the apparent diffusion of these molecules and can actually be used to resolve compounds of similar mass in carefully controlled Matrix Assisted Diffusion experiments. The deviation from linearity indicated the association of IPMP with a molecule/aggregate of substantially higher Mr.

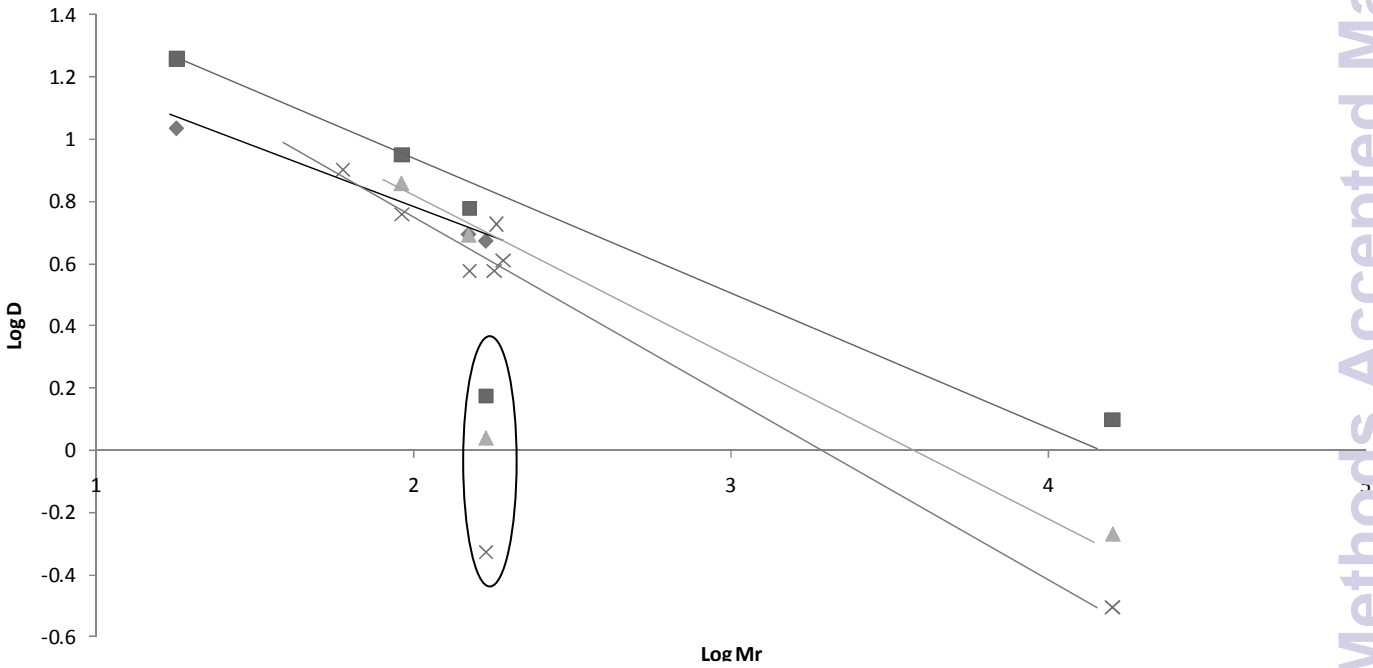

Figure 1. Correlation of LogD values of components to Log M_r in ♦ Phosphate Buffered saline (pH 9) , ■Glycerol and sodium dodecyl sulphate (pH 8)▲Glycerol, sodium dodecyl sulphate and Artificial Saliva, and X Toothpaste and Artificial Saliva (pH 8). IPMP was dosed in all cases aside from the toothpaste. R^2 value for all range from 0.95-1. Elipsoid indicates IPMP outliers due to binding to sodium dodecyl sulphate Micelles. Decreasing trend line gradient is shown with viscosity.

3.1.2 Characterisation in simple buffer

The control solution of IPMP in PBS shows uniform diffusion for those signals correlating to IPMP and the TSP control (Figure 2). Standard deviation of the diffusion coefficients ranges from 0.1-0.2 x 10^{-10} m^2s^{-1}. It should be noted that the lipophillic nature of IPMP demanded a solution of higher pH than

would normally be encountered in saliva in order to ensure complete dissolution. This is reflected in some of the qNMR data discussed in section 4. The DOSY spectrum for IPMP in the presence of SDS has been overlaid in Figure 2 and highlights the substantial shift in diffusion coefficient for IPMP when compared to TSP. TSP is used as both a chemical shift standard and diffusion standard to enable compensation for changes in viscosity and therefore the disproportionately low diffusion coefficient for IPMP suggests association with SDS micelles. The change in diffusion rate cannot be ascribed to an increase in overall viscosity as correction for this with TSP still results in a statistically significant reduction of the diffusion coefficient for IPMP.

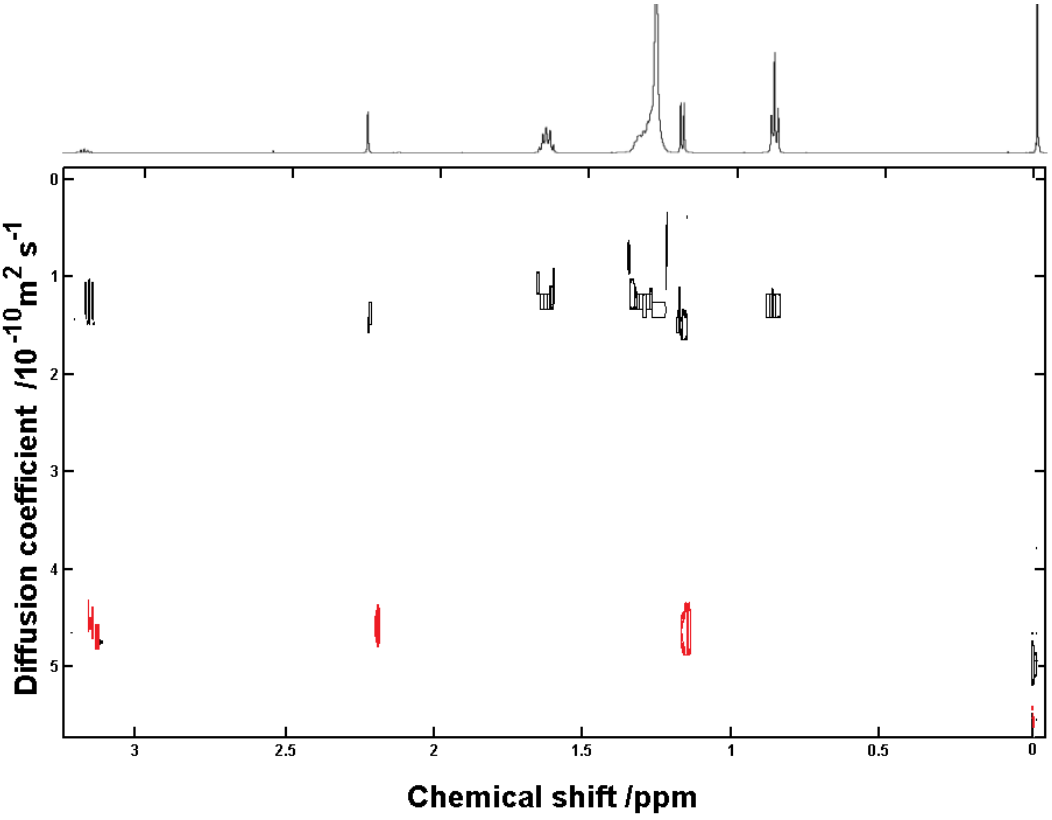

Figure 2. Aliphatic expansion of 2D-DOSY plot for IPMP in PBS solution (shown in red) and in the presence of SDS (shown in black). TSP as an internal chemical shift and diffusion reference is evident at 0.0ppm.

3.1.3 Characterisation of saliva

The impact of artificial saliva, as a complex mixture of salts and protein, on diffusion was analysed and the pseudo 2D DOSY plot (Figure 3) provided similar diffusion coefficients for IPMP signals to those found for SDS alone. The standard deviations for the determined diffusion coefficients and the signal resolution are similar to those observed in the absence of artificial saliva. Despite a high ionic strength, which has the potential to significantly attenuate proton signal strengths, it was possible to obtain a good quality DOSY map of the components IPMP, SDS and TSP in artificial saliva. TSP has a similar diffusion value in both cases, implying that the overall viscosity changes due to the introduction of artificial saliva are relatively small.

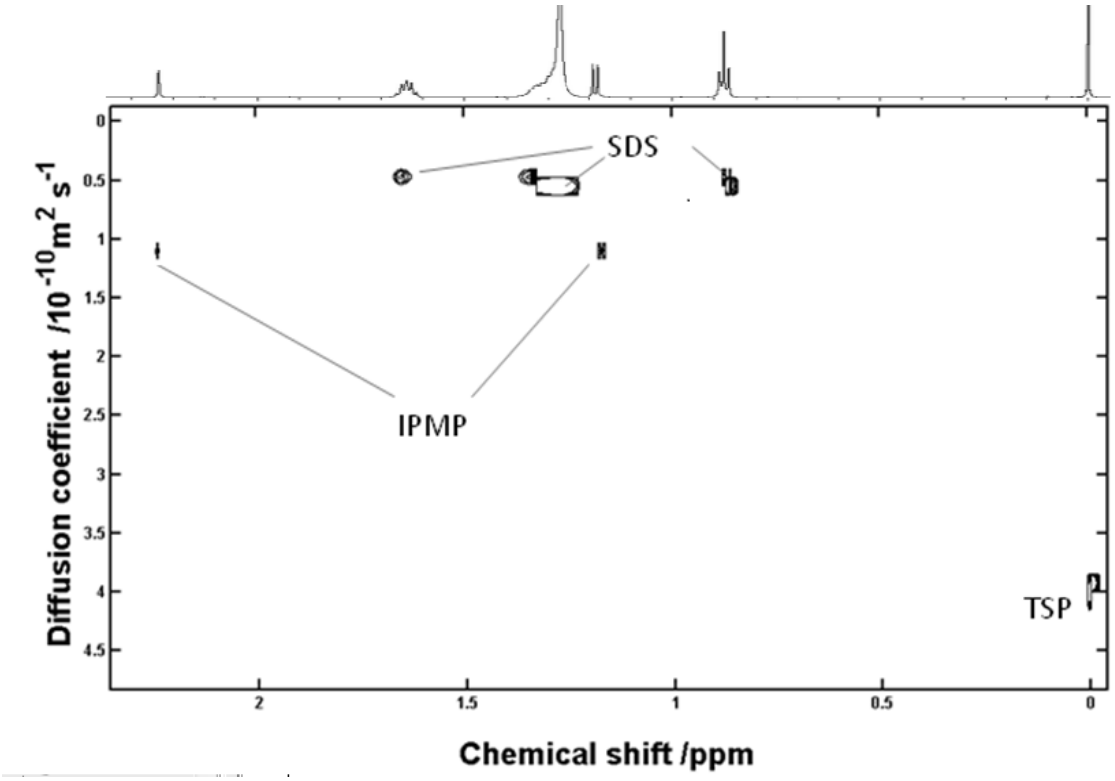

Figure 3. Pseudo 2D DOSY plot of aliphatic region for IPMP in the presence of SDS, glycerol and artificial saliva. Short T_2 relaxation times for ^{1}H resonances on the mucin protein and the comparatively long acquisition time effectively filter the protein signals from the spectrum.

3.1.4 Characterisation of dentifrice slurry

Having characterised IPMP in the presence and absence of a surfactant and established TSP as an internal reference for viscosity and verified a correlation between LogMr and LogD for different systems, samples of commercial toothpaste (n=3) were analysed to validate the capacity of DOSY NMR for virtual separation of components of dentifrice slurry. Figures 4, 5 & 6 show aliphatic, carbohydrate and aromatic regions of the averaged pseudo 2D DOSY plots. By determining the constants a and b for the LogD/Log M_r correlation it was possible to directly extract approximate mass data for lipophillic components with reasonable accuracy (±4%). These predicted mass values coupled with chemical shift and coupling data from ^{1}H and ^{1}H ^{1}H COSY experiments were used to assign the signals in the DOSY plots as shown.

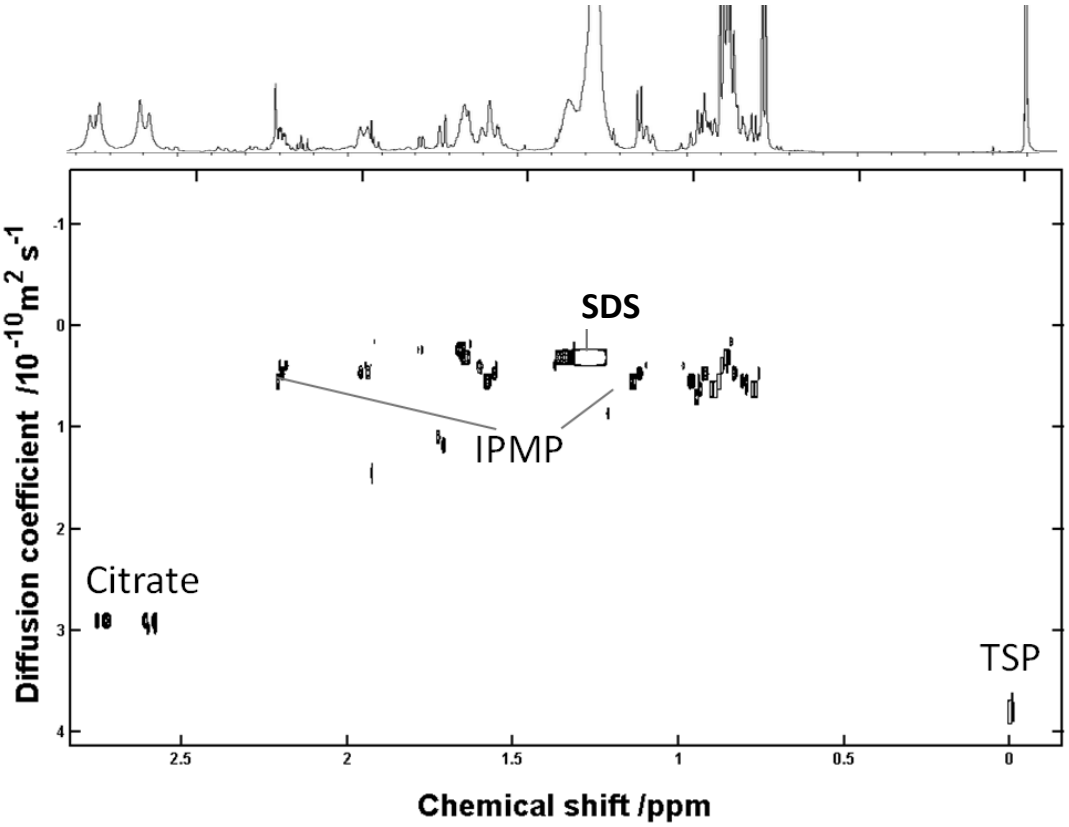

Figure 4 Aliphatic region of 2D DOSY plot of Commercial toothpaste and Artificial Saliva

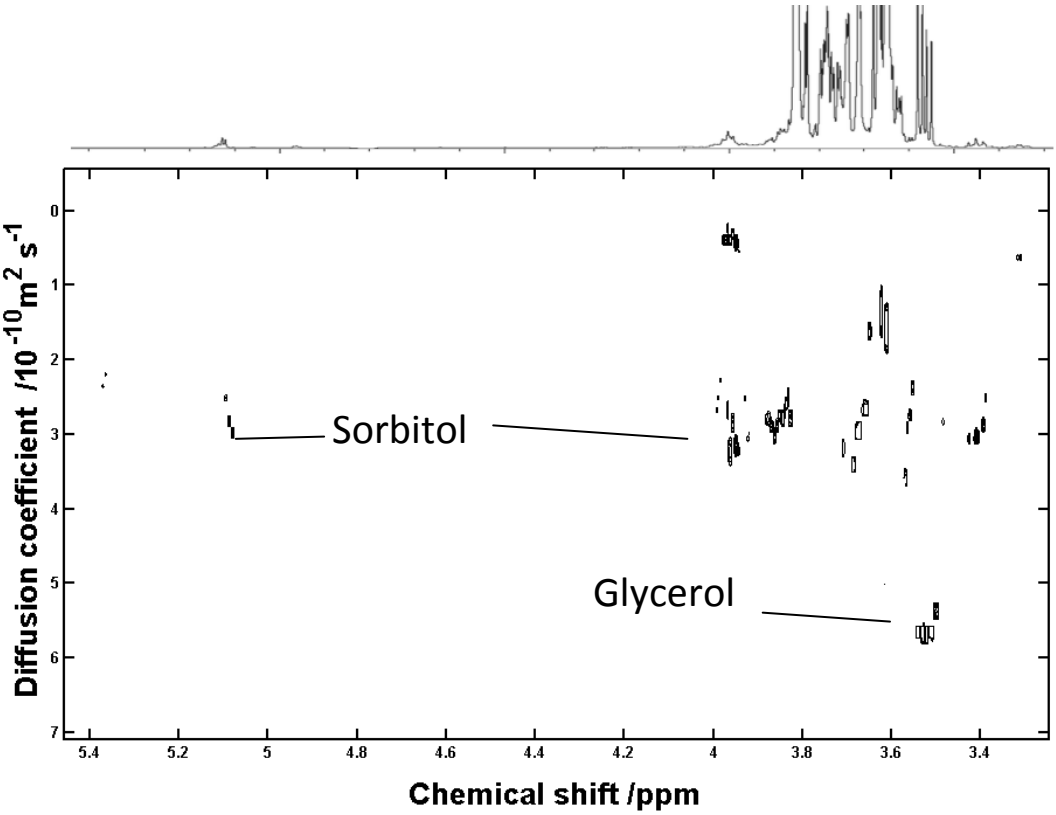

Figure 5. Carbohydrate region of 2D DOSY plot of commercial toothpaste and artificial saliva, showing the distinct separation of glycerol from sorbitol.

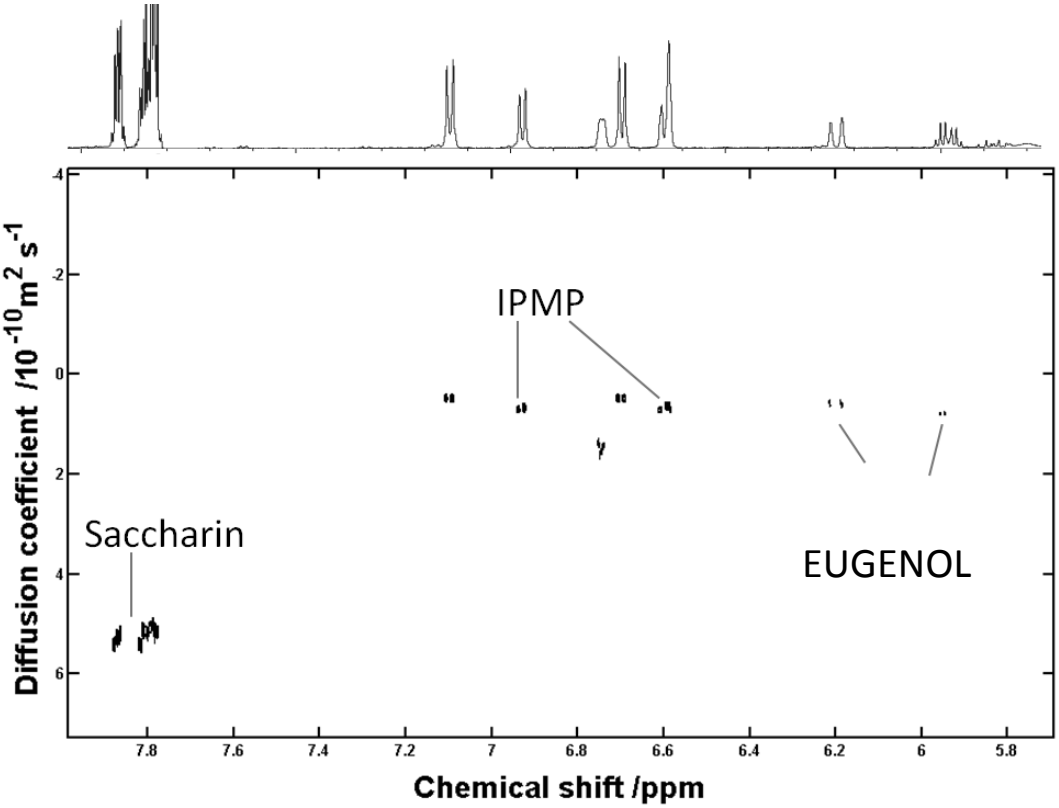

Figure 6. Aromatic region of 2D DOSY plot of Commercial toothpaste and Artificial Saliva, showing distinct separation of IPMP from Saccharin and Eugenol.

3.2 qNMR

Quantitative NMR (qNMR) was first described in 1963 and is increasingly regarded as a powerful non-destructive technique with traceability to SI units, providing advantages over many other analytical methods.[24,25] The development of affordable high field NMR instruments coupled with more elegant pulse sequences has prompted a recent flurry of activity in the field of NMR analysis, including human and plant metabolomic studies.[26,27] A recent review of the applications of qNMR over the last eight years highlights its growing importance in the context of metrology and supports the statement that qNMR can be regarded as a primary method of purity analysis for organic compounds.[28] Using TSP as the internal standard, not only for diffusion but also for quantification, enables the calculation of unknown analytes by comparing the integral of known and unknown signals. Despite growing acceptance of this technique, there are acquisition and processing factors which can contribute to uncertainty when calculating concentrations of analytes. When dealing with complex mixtures with often high ionic strength (Q-factor), accuracy can be substantially reduced[29] and it is important that the matrix in which the analytes appear is properly characterised. A key part of the current work is to consider the impact of high ionic strength, viscous matrices and the direct impact on the ease with which qNMR experiments can be efficiently and accurately undertaken. Figure 7 shows the effect of pH and co-solvents/surfactants on the accuracy with which IPMP can be quantified. The impact of viscosity and high salt content from the artificial saliva on the validity of this technique to quantify IPMP is shown in Figure 8. The direct application of qNMR in dentrifrice slurry is evaluated in Figure 9. The range of concentrations was deliberately reduced in size to highlight the limits of quantification using this method with potential applications in real-world saliva samples. The impact of pH on the correlation graphs is shown in all cases.

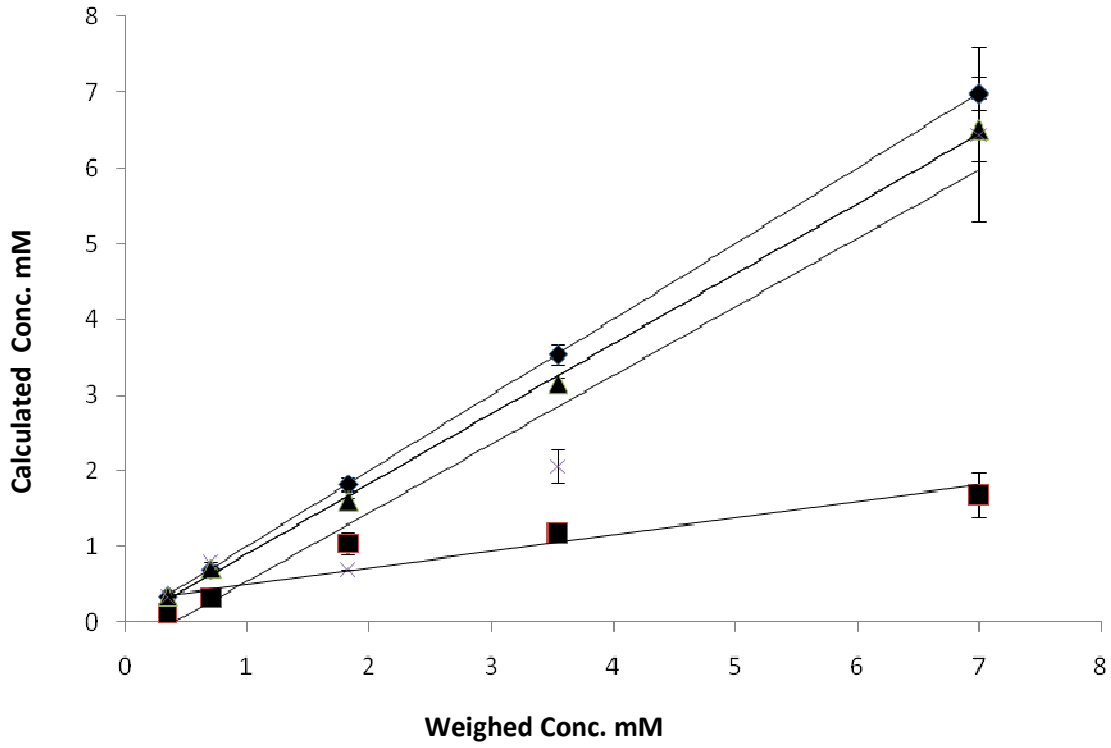

Figure 7. Calculated concentration of IPMP from qNMR experiments when compared to weighed samples (n=15). Error bars are standard deviation of 5 replicates of n=3 independently prepared samples at each concentration. ♦ Concentration calculated from mass IPMP R^2 = 1 ■ Phosphate Buffered Saline (pH 9) R^2 = 0.84. ▲ Glycerol and sodium dodecyl sulphate (pH 8) R^2 = 0.99. X Glycerol, $ZnCl_2$, sodium dodecyl sulphate (pH 7) R^2 =0.94 – 3rd trendline from top. IPMP was dosed in all cases.

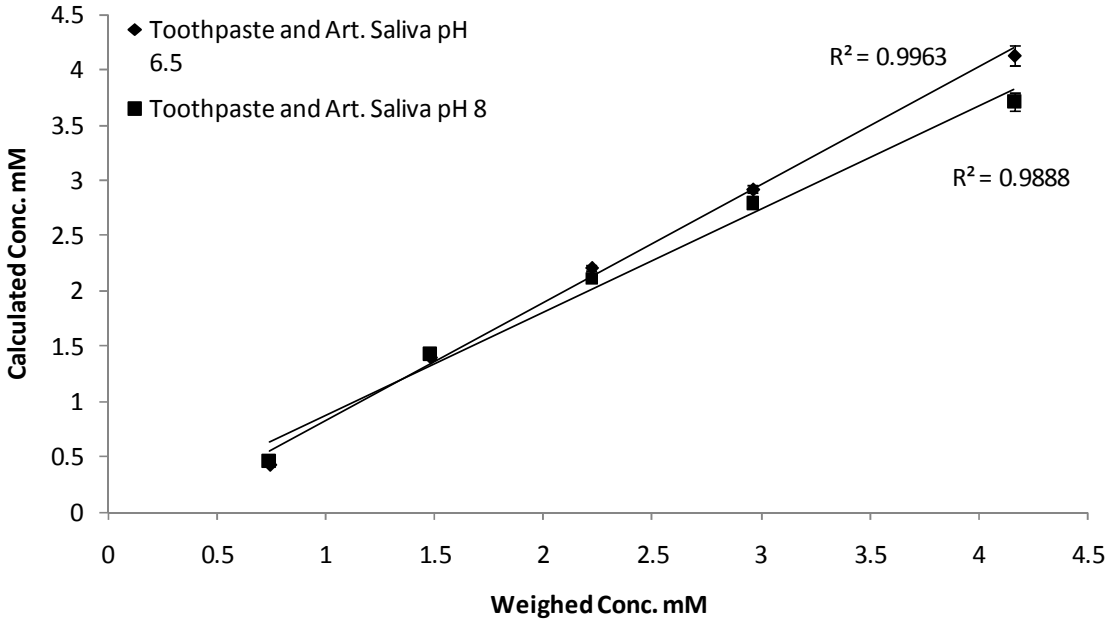

Figure 8. Commercial toothpaste and artificial saliva - calculated concentration of IPMP from qNMR experiments when compared to weighed samples at different pH (n=15). Error bars are standard de*via*tion of 5 replicates of n=3 independently prepared samples at each concentration. Equations: ♦ y = 1.04x-0.49 ■ y = 0.94x-0.04

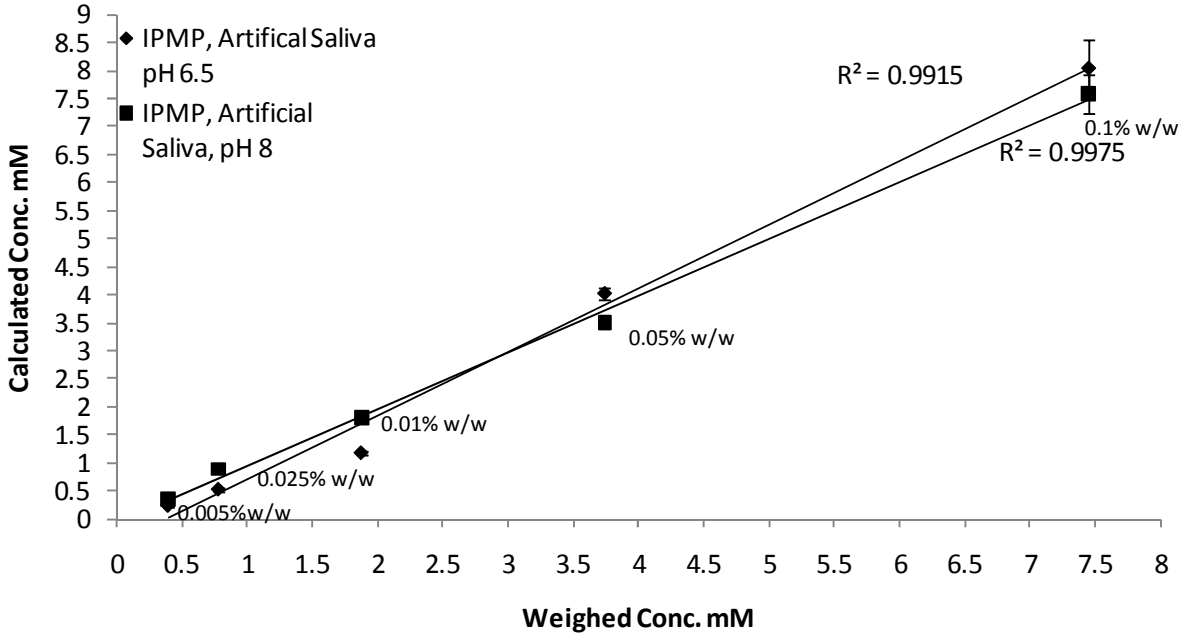

Figure 9. IPMP and Artificial Saliva - Calculated concentration of IPMP from qNMR experiments when compared to weighed samples at different pH (n=15). Error bars are standard de*via*tion of 5

replicates of n=3 independently prepared samples at each concentration. Equations: ♦ y = 1.07x-0.25 ■ y = 0.93x-0.05

4. Discussion

4.1 Virtual separation of the components

Whilst the virtual separation of small molecules in mixtures using DOSY NMR is established,[14] we report the analysis of dentifrice slurry using DOSY NMR for the first time. Use of TSP as an internal diffusion standard enables the association of the IPMP to SDS micelles to be clearly observed (Figure 2) and for simple systems where there is no competition it is possible to extract information as to what extent association is occurring between Host and Guest based on Eq 2, discussed in the review by Fielding.[16]

Eq 2. $D_{obs} = X_I D_I + X_{IS} D_{IS}$

Where D_{obs} = Observed diffusion coefficient, X_I = Mole fraction of free IPMP, X_{IS} = Mole fraction of bound IPMP, D_I = Diffusion of Free IPMP, D_{IS} = Diffusion of Bound IPMP

Using the drop in diffusion coefficient for TSP upon addition of SDS as a marker of increased viscosity, it is possible to correct the unbound Diffusion coefficient and calculate the approximate mole fraction for bound and unbound fractions of IPMP. The extent to which the diffusion coefficient is reduced as a consequence of association with SDS micelles (Mr ~ 18000), suggests that 60% IPMP is bound to SDS and 40% unbound. It should be stated that the lack of additional signals for IPMP in the presence of SDS suggests fast exchange on the NMR timeframe. This finding is in good agreement with the measured exchange rates of sparingly soluble fluorescent probes in SDS micelles, which has been previously measured on the microsecond time frame.[13]

Whilst the inclusion of artificial saliva does little to impact the separation of the organic components, the limitation of DOSY NMR becomes more apparent when commercial toothpaste is included. It has previously been reported[10,14] that it is possible to correlate Mr with D and this appears to be independent of viscosity. From Figure 1 it is possible to observe a trend for Citrate, TSP, and glycerol and obtain a and b constant values of -0.803 and 2.332 respectively. This enabled the prediction of M_r from LogD values and this correlation coupled with ^{1}H ^{1}H COSY data enabled the identification of

Saccharrin, sorbitol and urea in the dentifrice slurry (Figure 5). A linear trend for the LogD *vs* LogMr exists, and this can be observed in this case for the suitably hydrophilic components.

However, the lipophillic components, which are associated with SDS micelles, appear to deviate from the linear trend, rendering prediction of M_r with any accuracy difficult. The attempt to extrapolate approximate Mr values for unknown components using a LogD vs LogMr would appear limited to those components that do not associate with SDS micelles. Some of the components, which appeared micellised, could be identified through ^1H ^1H COSY and included Eugenol (Figure 6) and (-) Menthol.

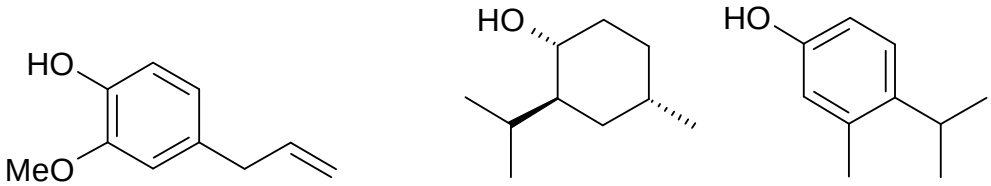

Figure 10 Eugenol, (-) Menthol and IPMP

Recent work on the virtual separation of regioisomers of methoxyphenol using SDS implies that differences in diffusion can be ascribed to different relative affinity for the SDS micelles, LogP values of the compounds in Figure 10 were evaluated using ChemOffice Chemdraw Ultra 12.[22] It could be hypothesised that a greater LogP could result in greater affinity for the non-polar component of SDS micelles and result in a reduced observed diffusion coefficient (as originally observed for IPMP). The variation in diffusion coefficient between these three molecules should, therefore correlate approximately to their individual LogP values, however, the diffusion coefficients observed by this group actually show a correlation (R^2 = 0.98 of Log D to Log Mr for these three molecule series. Whilst further work will be necessary to determine the factors which contribute the most to the observed change in diffusion coefficient of a lipophillic molecule when in the presence of a surfactant, this preliminary data suggests it may still be possible to predict M_r values based on LogD data in the presence of surfactants. In mixtures such as these, not only are a small series of known hydrophilic diffusion standards necessary for the establishment of a LogD *vs* Log Mr correlation, but also that a series of lipophillic diffusion standards may be necessary for a reliable extrapolation of Mr values for unknown compounds based on LogD data. It should be stressed that further studies will need to be

conducted to look at the effects of residency time in the micelles and the potential impact of any complexed lipophilic components on shape and thus apparent apparent diffusion.

4.2 Reliability of qNMR

One of the initial hypothesis was that qNMR was a valid technique for the accurate quantification of organic species in a complex, viscous mixture. Reference to figure 7 indicates that pH has a substantial impact on the accuracy of the technique and that proper dispersion of the analyte is essential for correlation. This is reflected in the discrepancy between data shown in Figure 7 compared to samples containing SDS. Even at pH 9, the IPMP phenoxide is insoluble at high concentrations >0.05%w/w. It should be mentioned that other groups have focused on instrumental uncertainties (type B)[30] to account for the observed precision, however for this work, they are largely dwarfed by the inherent error in the weighing balance and the type A uncertainties, so their relevance in this case was questionable. In light of the lipophilicity of the IPMP, SDS was essential for its dispersion and for correlation of the calculated concentration to the amounts added. The accuracy of the qNMR method was 98-99% based on the weighed amounts of IPMP for each % concentration for an SDS/Glycerol mixture in PBS, but in the presence of $ZnCl_2$, the accuracy fell to 85% at higher concentrations of IPMP with an increase in error to +/- 1mM, suggesting insoluble Zn^{2+} salt formation over time. The exchange of Zn^{2+} with Na^{+} could also affect the size and shape of the SDS micelle, this may result in the promotion of longer rods, rather than spheroids; however, if this is a uniform occurrence throughout the solution it should have equal impact on lipophillic species. Surprisingly the qNMR correlation for IPMP in artificial saliva (Figure 9) and dentifrice slurry (Figure 8) showed a good correlation at all concentrations. The accuracy was affected by pH with a lower pH favouring accuracy of 98-99% for IPMP in the dentifrice slurry and a higher pH favouring accuracy of 96-98% in the artificial saliva, SDS, Glycerol model. There was no statistically significant difference in the error margins for the qNMR calculation of concentration of IPMP for either of the two systems at low concentration.

5. Conclusion

We have demonstrated that DOSY NMR is a useful tool for the characterisation and quantification of natural products as represented by IPMP in systems of variable viscosity and in the presence of surfactants and high salt concentration. Whilst it is important that multiple diffusion standards should be used when attempting to identify unknown components based on predicted Mr from LogD data, the use of qNMR to determine accurately the concentration of dissolved organic shows considerable promise for analysing complex mixtures of natural products. Through the non-destructive identification and quantification of these components, some of the anti-plaque or antimicrobial effects of polyphenolic and terpinoid derivatives could potentially be investigated directly *in situ* and without the need for separation and the potential loss of key components, which may contribute to this activity. Consistent and accurate quantitative data is possible for these systems and this will facilitate the investigation of the combined impact of individual components with the potential to assist in the development of a combination of natural products with enhanced anti-plaque activity.

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References

[1] C. J. Silwood, E. Lynch, A. W. Claxson and M. C. Grootveld, *J. Dent. Res.* 2002, **81**, 422-427.

[2] I. Chinou, Primary and secondary metabolites and their biological activity; in Waksmundzka-Hajnos M, Sherma J, Kowalska T (eds): Thin Layer Chromatography in Phytochemistry. New York, CRC, 2008, pp 59–76.

[3] S. Duarte, S. Gregoire, A. P. Singh, N. Vorsa, K. Schaich, W. H. Bowen and H. Koo, *FEMS Microbiol. Lett.*, 2006, **257**, 50-56.

[4] H. Koo, S. Duarte, R. M. Murata, K. Scott-Anne, S. Gregoire, G. E. Watson, A. P.Singh, N. Vorsa N., *Caries Res.,* 2010, **44**, 116–126.

[5] S. Shibata, T. Suge, T. Kimura, K. Ishikawa, T. Matsuo, *Journal of Dentistry*, 2012, **25**, 31-34.

[6] J. G. Jeon, P. L. Rosalen, M. L. Falsetta, H. Koo, *Caries Res.,* 2011, **45**, 243-263.

[7] L. Cheng, R. A. Exterkate, X. Zhou, J. Li, J. M. Cate, *Caries Res.* 2011, **45**, 87-92.

[8] S. Twetman, *Caries Res.* 2004, **38**, 223-229.

[9] P. D. Marsh, *British Dental Journal*, 2012, **212**, 601-606.

[10] A. Le Gresley, J. Kenny, C. Cassar, A. Kelly, A. Sinclair, M. Fielder, *Food Chem*., 2012, **135**, 2879-

2886.

[11] M. Nilsson, P. Aman, H. Harkonen, G. Hallmans, K. E. B. Knudsen, W. Mazur, H. Adlercreutz, *Journal of the Science of Food and Agriculture*, 1997, **73**, 143-148.

[12] M. Nilsson, *J. Magnet. Res.,* 2009, **200**, 296-302.

[13] L. Fielding, *Tetrahedron*, 2000, **56**, 6151-6170.

[14] J. A. Donarski, D. P. T. Roberts, A. J. Charlton, *Anal. Methods*, 2010, **2**, 1479-1483.

[15] R. Consonni, L. R. Cagliani, *J. Agri. Food Chem*., 2008, **56**, 6873-6880.

[16] J. S. McKenzie, J. A. Donarski, J. C. Wilson, A. J. Charlton, A. J., *Progress in Nuclear Magnetic Resonance Spectroscopy*, 2011, **59**, 336-359.

[17] R. Jaiswal, L. Jayasinghe, N. Kuhnert, *J. Mass Spectrom.* 2012, **47**, 502-515.

[18] J. Klimek, E. Hellwig, G. Ahrens, G., *Caries Res.* 1982, **16**, 156-1616.

[19] M. Nilsson, I. F. Duarte, C. U. Almeida, I. Delgadillo, B. J. Goodfellow, *J. Agri. Food Chem*., 2004, **52**, 3736-3743.

[20] M. H. Gehlen and F. C. De Schryver, *Chem. Rev*. 1993, **93**, 199-221.

[21] D. Li, G. Kagan, R. Hopson, P. G. Williard, *JACS*, 2009,**131**, 5627-5634.

[22] A. Macchioni, G. Ciancaleoni, C. Zuccaccia, D. Zuccaccia, *Chem. Soc. Rev*. 2008, **37**, 479-489.

[23] R. W. Adams, J. A. Aguilar, J. Cassani, G. A. Morris, M. Nilsson, *Organic and Biomolecular Chemistry*, 2011, **9**, 7062-7064.

[24] C. F. Tormena, R. Evans, S. Haiber, M. Nilsson, G. A. Morris, *Magnet. Reson. Chem*., 2010, **48**, 550-553.

[25] S. Malz, H. Janke, *Journal of Pharmaceutical and Biomed. Analysis*, 2005, **38**, 813-823.

[26] N. J. Serkova and M. S. Brown, *Bioanalysis*, 2012, **4**, 321-341.

[27] G. F. Pauli, T. Godecke, B. U. Jaki, D. C. Lankin, *Journal of Natural Products*, 2012, **75**, 834-851.

[28] M. Weber, C. Hellriegel, A. Ruck, R. Sauermoser, J. Wuthrich, *Accred. Qual. Assur*., 2013, **18**, 91 -98.

[29] H. Kovacsa, D.Moskaua, M. Spraulb, *Progress in Nuclear Magnetic Resonance Spectroscopy*, 2005, **46**, 131-155.

[30] T. Schoenberger, *Anal. Bioanal Chem*. 2012, **403**, 247-254.