

This is the peer reviewed version of the following article: Robertson, Cameron, Lucas, Robert A. and Le Gresley, Adam (2020) Scope and limitations of nuclear magnetic resonance techniques for characterisation and quantitation of vitamin D in complex mixtures. *Skin Research and Technology*, 26(1), pp. 112-120., which has been published in final form at <https://doi.org/10.1111/srt.12773>. This article may be used for non-commercial purposes in accordance with Wiley Terms and Conditions for Use of Self-Archived Versions.

Scope and limitations of Nuclear Magnetic Resonance techniques for characterisation and quantitation of vitamin D in complex mixtures.

Pureshift NMR for characterisation/quantitation of vitamin D

Cameron Robertson^a, Robert A. Lucas^b, Adam Le Gresley^{a*}

^aKingston University, Penryn Road, Kingston Upon Thames, UK.

^bGlaxoSmithKline Consumer Healthcare, Weybridge, UK.

*Corresponding author: Tel: +44 (0)20 84177432 a.legresley@kingston.ac.uk

Acknowledgement

This work was supported by GlaxoSmithKline.

Abstract

Background

The accurate determination of vitamin D in skin is of considerable importance in evaluating penetration of skin health products through the different layers of the skin.

Objective

We report on the characterisation and quantitation of vitamin D in an idealised sample and in complex mixtures which mimic that of a typical skin cream, using qNMR, 2D NMR and DOSY techniques.

Methods

The characterisation and quantitation conditions were acquired over several heterogeneous samples, allowing for analysis of how the dynamic range and complexity of the different sample mixtures affect the Limits of Detection (LOD) and Limits of Quantitation (LOQ) of vitamin D.^{1,2} NMR is of particular value to this task as it is non-destructive, uses a primary ratio method for quantification, and tolerates a wide variety of hydrophilic and hydrophobic components within a given matrix.

Results

In this investigation we have attained a trueness level <10%, repeatability values of <1% and brought the limit of quantitation down to 100nM (\approx limit of baseline range of vitamin D₂ and D₃ per litre seen *in vivo*³), commenting on the limitations observed, such as peak overlap and sensitivity limits.

Conclusions

Pure shift optimised sequences allow us to reduce peak overlapping, allowing further characterisation of individual compounds and the separation of complex mixtures using NMR.

Keywords:

Vitamin D, NMR, Skin cream, Diffusion

1. Introduction

The photo-production of vitamin D takes place exclusively in the skin and once formed in the epidermis, diffuses to the dermal capillary bed, where it is

transported to the liver by vitamin D binding protein.^{4,5,6} Sufficient UVB (255-350nm) levels to produce vitamin D₃ from its provitamin are typically only available during short periods of the day. Absorption by ozone, which varies depending on latitude, decreases the amount of UVB radiation that the skin is exposed to. In addition, the relationship between skin cancer and sun exposure has inadvertently caused a large decrease in vitamin D production because of UVB being absorbed by sun screens and reduced sun exposure. There is also a significant effect from skin pigmentation, which leads, for example, to a much greater rate of vitamin D deficiency in dark-skinned people living at higher latitudes.^{7,8,9} Ageing has also been shown to decrease the amount of 7-dehydrocholesterol in the skin.¹⁰

Roles for vitamin D, in immunological functions, include an induction effect of Toll like receptor 2 and its co-receptor CD14, which initiates the innate immune response in the skin, leading to CYP27B1 and cathelicidin production.^{11,12,13,14}

With greater awareness of the detrimental effects of vitamin D deficiency, it is of significant importance to be able to determine vitamin D levels in skin and ascertain the amount of available vitamin D in skin creams.^{15,16,17,18,49}

NMR is of particular value as a non-destructive analytical method; its capacity to be used as a primary method for quantification, its tolerance of a wide variety of hydrophilic and hydrophobic elements within a given matrix and its large sample throughput by automation make it very promising for mixture analysis.²⁰ In this manuscript, we investigate the potential of different NMR techniques to achieve physiologically relevant characterisation and quantitation of free vitamin D₂/D₃ in

solution and skin cream formulation. Owing to simple sample preparation *and in-situ* nature of NMR, it is possible to use models for the composition of skin creams and even the *Dermis* extracellular matrix capillary beds that vitamin D₃ is diffused into.^{5,6}

NMR has primarily been used as a characterisation tool, but recently it is being explored more and more for quantitation of metabolites, pharmaceuticals and compounds commercial products and mixtures.^{21,22} The use of quantitative NMR has primarily been through the calculation of concentrations through direct integral ratio determination in 1D spectra's, however 2D quantitative techniques are being validated for more complex mixture quantitation (qqHSQC, HSQC0).²³

We see from table 1 that two issues exist for qNMR that limit its applications, low sensitivity compared to that of Mass spectrometry and overlapping of resonances. The first issue is being addressed mainly through the development of new hardware, larger magnets and Dynamic Nuclear Polarization techniques which increase the sensitivity of NMR acquisition by nearly a thousand fold. The second issue, of overlapping peaks, can be addressed through the use of more advanced acquisition sequences, such as Pureshift Yielded by Chirp Excitation (PSYCHE), which involves homonuclear decoupling, along with improved processing techniques.

2. Materials and Methods

2.1 Chemicals and materials for project

All chemicals were obtained from Sigma-Alrich or GSK and were used without further purification. Quantification targets: vitamin D₂ (Cas Number: 50-14-6); vitamin D₃ (Cas number: 67-97-0) were ordered from Sigma-Alrich.

Emulsion compounds: Glycerol (CAS number: 56-81-5); Caprylic Triglyceride (CAS number: 73398-61-5); Isostearyl Isoterate (CAS number: 41669-36-1); Niacinamide (CAS number: 98-92-0); Phospholipon (CAS number 92128-87-5); Vegetable Oil/Corn Oil (CAS number: 8001-30-7); Panthenol (CAS number: 81-13-0); Pentylene Glycol (CAS number: 111-29-5) were provided by GSK.

A high shear mixer (SCILOGEX D160 Homogenizer 850101019999) was used for the preparation of cream emulsions to allow for immiscible components to be dispersed into the liquid phase of the mixture.

Volume measurements were done using VWR *Single-channel pipettes, mechanical, variable volume, Ergonomic High Performance (EHP)*, alongside a Mettler MT5 scale used for solid state mass measurements.

A range of vitamin D₂ and vitamin D₃ concentrations from 10mM to 100nM with concentration changes in increments of $[N_{n-1}] = \frac{[N_n]}{10}$ were prepared.

2.2 NMR sample preparation

NMR tubes used were Wilmad 5mm, thin walled tubes obtained from GPE Scientific (Product No: 502-7). Samples used for DOSY and PSYCHEiDOSY were prepared in 3mm tubes (Product No: S-3-HT-7) to reduce convection effects within the tube during the acquisition.

NMR samples were spiked with Sodium d₄-trimethylsilylpropionate (TSP). In preliminary experiments, 10mM of TSP was spiked into the sample, in optimised experiments the concentration was kept consistent with that of the vitamin D_{2/3} concentration.

2.4 NMR Experiments

2.4.1 Instrumentation and software

All Spectra of vitamin D₂ and D₃ were recorded in D₂O or DMSO-d₆ solutions (10mM to 100nM) at 300K using a Bruker Avance III 600MHz FT-NMR.

For each sample quantitative conditions were set with D1 at > 5x T₁ (10s, as D₂/D₃ T₁ shown to be around 1s)

Absolute concentration determination was determined via comparison of vitamin D₂ and D₃ integrals to a known concentration of TSP.

Bruker Topspin 3.5 pl7 was used for assignment of peaks, manual phasing, baseline correction and manual integration. Manual integration was done to ensure bias and slope was correct and that the integrals were all of the same width.

2.4.2 Quantitation

Quantitation was done through the primary ratio method analysis of integrals.

For this we used an internal standard, TSP, at concentrations relevant to that of the target analyte (vitamin D_{2/3}). Concentrations were calculated from the integral

values in the 1D spectrum. Triplicate and interleaved experiments for each sample were run to allow for time discrepancies and repeatability of experiments to be accounted for.

The optimisation of 1D experiments involved the implementation of Non uniform sampling (NUS), reduced spectral width (making sure integrals are not affected), calibrating TSP concentration to that of the required limits of detection/quantitation of the target analyte, calibrating the D1 relaxation time through acquisition of T₁ relaxation data for vitamin D₂/D₃ and TSP.

2.4.3 DOSY

2.4.3.1 Calibration of DOSY

DOSY was calibrated through 1D survey scans to reduce the error seen in the calculated diffusion coefficients through setting the diffusion encoding parameters appropriately for the peaks of interest.

2.4.3.2 Determining Diffusion coefficient's and molecular weights

Dynamics centre (Bruker UK Ltd) was used for determining the diffusion coefficients using the diffusion fit function.

$$f(g) = I_0 * e^{-\gamma^2 * g^2 * \delta^2 * (\Delta - \frac{\delta}{3}) * D}$$

From the diffusion coefficients of each peak, molecular weights were extrapolated through the use internal references (TSP, DMSO-6, water) and a calibration curve plotting log(mw) against log(diffusion coefficient).

2.4.5 Pure Shift Experiments

Pure shift yielded by chirp excitation (PSYCHE) was used for all pure shift experiments (1D proton, TOCSY and Diffusion ordered spectroscopy) for maximum sensitivity and artefact suppression. Pulse sequences, processing macros and guidance on parameter set-ups was acquired from the University of Manchester NMR methodology group on their pure shift workshop page.²⁴

3. Results

3.1 Characterisation and Quantitation with 1D NMR techniques

3.1.1 Characterisation using 1D Proton NMR

Complex samples and compounds often suffer greatly in NMR through spectral crowding. To investigate these limitations 1D ¹H spectra were acquired and peaks assigned through the use of integral ratio calculation, peak picking, prediction software and online databases for vitamin D₂ and D₃;

FIGURE 1

Figure 1 shows the specificity of assignment achieved by standard 1D ¹H acquisition techniques. Assignment of methyl groups, alkenes and alcohol groups is unambiguous and specificity is high. The assignment of aliphatic hydrogens however is ambiguous and the specificity is low, especially in the range of $\delta=2.5\text{ppm}$ to 1ppm where CH and CH₂ peaks are present. The overlap of peaks which causes interpretation of multiplets to be hindered.

Despite these limitations we can identify candidate peaks for quantitation. The peak at 0.5ppm, assigned to the methyl group numbered 16, is a good signal to choose owing to: singlet multiplicity, high signal to noise, and separation from other peaks allowing for an integral to cover 64x the full width at half maximum.

3.1.2 1D Pure Shift for complete assignment of vitamin D₂ and D₃

The limitations addressed from assignment of the 1D 1H spectra can be rectified with pure shift techniques to remove the multiplicity from the standard spectrum, which allows for greater specification through the reduction in peak overlap. Figure 2 demonstrates the effectiveness of this technique in separating the overlapping peaks and giving a clearer indication of how many proton environments exist in the spectra. It also allows for a much greater specificity when assigning the structure of vitamin D₂ and D₃ to the spectra and differences between the two structures can be seen more clearly in the respective spectrums.

FIGURE 2

This is exemplified by the successful assignment of all aliphatic protons within the spectra, validated with HSQC/COSY experiments and prediction software.²⁵

3.1.3 Quantitation using standard 1D NMR techniques

Results from table 2 show that with standard 1D 1H NMR spectra we can obtain a limit of quantification (LOQ) of 100µM, with RSD's of 1.73 and 2.63 for vitamin D₂ and D₃ respectively at this concentration. However the trueness values vary

between D₂ and D₃ with D₂ having a trueness value of 1.294 (RSD = 0.479) and D₃ trueness of 1.354 (RSD=1.14294).

3.1.4 Quantitation using optimised 1D NMR techniques

By optimising certain parameters (Table 3) we were able to lower the limit of quantitation to 10µM for both vitamin D₂ and D₃ with LOD down to 50nM for D₃ and 500nM for D₂.

Vitamin D₃ trueness values down to 100nM are 50% of the gravimetric concentration consistently with RSD values all below 20% (Table 4). Whereas with D₂ trueness values we see 0.09-0.08 down to 10µM with RSD below 10% however the trueness increases to 0.135 when the concentration drops down to 1µM with an RSD value of 52.2%.

3.1.5 Quantitation of D₂ and D₃ within cream formulations

Within cream formulations the peaks used for quantitation in the vitamin D samples are overlapped by the more concentrated cream formulation components. We are limited, therefore, to selecting peaks at $\delta = 6\text{ppm}$, doublets rather than singlets, necessitating the integration of both peaks. Another issue is the homogeneity of the sample, even after high shear mixing, we see a distinct loss of free vitamin D concentration within the cream. With concentrations of 1.91mM and 4.47mM detected from 10mM D₂ and D₃ samples respectively.

FIGURE 3

It is also possible that in our cream formulation micellisation of the vitamin D is taking place, thus reducing the amount of free vitamin D in solution. This has implications in terms of skin permeability and vitamin D penetration into the *Dermis*. It is also the case that the large ratio of other analytes to the small detectable vitamin D causes a great loss in S/N for smaller concentration peaks. The micellation of vitamin D can be investigated through the acquisition of Methanol-d4 (MeOD) solvent samples alongside that of the D2O samples, which allows for the collapse of any micelles and all vitamin D being free in solution. The reason peaks are not visible when micellised is because the Vitamin D is incorporated with much larger structures and their tumbling rate is slowed therefore causing broadening of peaks. This is not seen in MeOD samples as the micelles cannot be formed. Because of this we see sharp line shapes for the vitamin D peaks. From the formulation made we can use NMR to measure the capacity of micellation and with this the optimal amount of drug product or analyte of interest to add into our respective cream formulations. An example is shown in fig. 4 for vitamin D3 at a concentration of 10mM.

FIGURE 4

3.2 Complex mixture separation using NMR techniques

The main limitation encountered when investigating complex mixtures with NMR is the overlapping of peaks between the different components, alongside the previously discussed issues with receiver gain when the dynamic range is large.

To address the first limitation the effectiveness of separating compounds based upon their diffusion characteristics was investigated.

3.2.1 Diffusion Ordered Spectroscopy (DOSY)

Conventional Diffusion Ordered Spectroscopy separates NMR signals based on their apparent diffusion and this can be related to the volume of a molecule. This technique, however, was of limited use in analysing cream formulations owing to overlapping of peaks from compounds with different diffusion characteristics, causing large errors to appear in the calculated diffusion coefficients for the respective peaks.

3.2.2 PSYCHEiDOSY

Based on a recently developed pureshift pulse sequence, a diffusion element was added to this proton-decoupling sequence (PSYCHEiDOSY). This reduced the error in the calculated diffusion coefficient from the overlap of peaks, as multiplets are collapsed to singlets. For simpler compounds in a mixture this is very useful; peaks with their approximate molecular weight can give a robust method for selecting peaks to specific compounds.

FIGURE 5

Fig. 5 shows the successful separation of peaks in respect to their compounds relative molecular weight using the PSYCHEiDOSY experiment. With reference peaks in place we can test the trueness of the estimated molecular weight from diffusion coefficient values.

DOSY followed by selective TOCSY experiments have been shown to be effective in achieving these goals through acquisition however with PSYCHEiDOSY projection's it is possible to get a selective pure shift spectra based on the diffusion characteristics rather than selective pulses and magnetisation transfer, shown in figure 6.

FIGURE 6

Discussion

In this paper, we report, for the first time, the whole structure of vitamin D₂ and D₃ by 1H NMR and the precise assignment of signals on the steroid ring system through the use of 1D pure shift acquisitions. This will allow easier analysis of vitamin D metabolism through the qualitative and quantitative tracking of D₂/D₃ in its ergocalciferol, cholecalciferol, calcifedol, calcitriol (Active form) and 24, 25-Dihydroxycholecalciferol (Inactive form) forms, which all have very similar chemical structures. The separation of formulation components through pure shift diffusion ordered spectroscopy experiments (PSYCHEiDOSY) gives a non-destructive and automated method for complex mixture analysis and separation. We demonstrated that NMR can be used to obtain LODs at physiological concentrations of vitamin D_{2/3} in simple samples. When this was applied to skin cream formulation models, free vitamin D was not observed at the expected concentration in D₂O samples. This suggests that in skin cream, precipitation or micellisation of the vitamin D is occurring, reducing the apparent amount of freely available vitamin D in solution. This result can be demonstrated by the concurrent running of experiments in D₂O with critical micelle concentrations of surfactants,

alongside samples run in MeOD, which does not allow the formation of micelles. Whilst it has also been reported that Vitamin D₃ can be photodegraded at relatively low intensity of UV, no evidence of this was observed in simple solution and it is unlikely that this is the cause of the lower concentrations observed in the skin cream formulations.²⁶

Free vitamin D is observed at 15-40% of the initial concentration, meaning 15-40% of vitamin D is unable to permeate skin in the same way as micellised Vitamin D within the cream formulations. There is also an issue with overlapping of target peaks by emulsion peaks. Whilst we do observe considerable signal overlap, we were able to identify olefin peaks of vitamin D_{2/3} at 6ppm and 0.55ppm that are resolved well enough to be detected within the mixtures.

Conclusion

Looking forward, the implementation of real time acquisition through shortening of the pulse sequence time to fit within the dwell time would reduce the time required for pure shift experiments, alternatively post-processing methods, such as covariance analysis can make experiments possible in a suitably short time period. We have evidenced the application of NMR for detecting physiologically relevant concentrations of vitamin D and highlighted the effects of the traditional skin cream matrix on the amount of free vitamin D available. We are currently working on adapting this technique to consider models for the *Dermis* and assess vitamin D penetration using the optimised techniques reported here for both characterisation and quantification.

References

1. Fardus-Reid F, Warren J, & Le Gresley A. Validating heteronuclear 2D quantitative NMR. *Analytical Methods* 2016;8(9): 2013-2019.
2. Le Gresley A, Fardus F, & Warren J. Bias and uncertainty in non-ideal qNMR analysis. *Critical reviews in analytical chemistry* 2015;45(4):300-310.
3. Holick MF. Biological effects of sunlight, ultraviolet radiation, visible light, infrared radiation and vitamin D for health. *Anticancer research* 2016;36(3): 1345-1356.
4. Holick MF. Resurrection of vitamin D deficiency and rickets. *The Journal of clinical investigation* 2006;116(8):2062-2072.
5. Tian XQ, Chen TC, Lu ZHIREN, Shao QING, & Holick MF. Characterization of the translocation process of vitamin D₃ from the skin into the circulation. *Endocrinology* 1994;135(2):655-661.
6. Haddad JG, Matsuoka LY, Hollis BW, Hu YZ, & Wortsman, J. Human plasma transport of vitamin D after its endogenous synthesis. *The Journal of clinical investigation* 1993;91(6):2552-2555.

7. Matsuoka LY, Ide L, Wortsman J, Maclaughlin JA, & Holick M. F. *Sunscreens suppress cutaneous vitamin D3 synthesis. The Journal of Clinical Endocrinology & Metabolism* 1987;64(6):1165-1168.
8. Aloia JF, Talwar SA, Pollack S, & Yeh J. *A randomized controlled trial of vitamin D3 supplementation in African American women. Archives of internal medicine* 2005;165(14):1618-1623.
9. Clemens TL, Henderson SL, Adams JS, & Holick MF. *Increased skin pigment reduces the capacity of skin to synthesise vitamin D3. The Lancet* 1982;319(8263):74-76.
10. Holick M, Matsuoka L, & Wortsman J. *Age, vitamin D, and solar ultraviolet. The Lancet* 1989;334(8671):1104-1105.
11. Hawker NP, Pennypacker SD, Chang SM, & Bikle DD. *Regulation of human epidermal keratinocyte differentiation by the vitamin D receptor and its coactivators DRIP205, SRC2, and SRC3. Journal of Investigative Dermatology* 2007;127(4):874-880.
12. Bikle DD, & Pillai S. *Vitamin D, calcium, and epidermal differentiation. Endocrine Reviews* 1993;14(1):3-19.

13. Liu PT, Stenger S, Li H, Wenzel L, Tan BH, Krutzik SR., & Kamen DL. *Toll-like receptor triggering of a vitamin D-mediated human antimicrobial response. Science* 2006;311(5768):1770-1773.
14. Adams JS, Ren S, Liu PT, Chun RF, Lagishetty V, Gombart AF, & Hewison M. *Vitamin D-directed rheostatic regulation of monocyte antibacterial responses. The Journal of Immunology* 2009;182(7):4289-4295.
15. Holick MF, *Vitamin D: a D-Lightful health perspective. Nutrition reviews* 2008;66(suppl_2):S182-S194.
16. Raulio S, Erlund I, Männistö S, Sarlio-Lähteenkorva S, Sundvall J, Tapanainen H, & Virtanen SM. *Successful nutrition policy: improvement of vitamin D intake and status in Finnish adults over the last decade. The European Journal of Public Health* 2016;27(2):268-273.
17. Norman AW, & Bouillon R. *Vitamin D nutritional policy needs a vision for the future. Experimental Biology and Medicine* 2010;235(9):1034-1045.
18. Ogan D, & Pritchett K. *Vitamin D and the athlete: risks, recommendations, and benefits. Nutrients* 2013;5(6):1856-1868.

19. Annweiler C, Karras SN, Anagnostis P, & Beauchet O. Vitamin D supplements: a novel therapeutic approach for Alzheimer patients. *Frontiers in pharmacology* 2014;5:6.
20. Le Gresley A, Simpson E, Sinclair AJ, Williams N, Burnett GR, Bradshaw DJ, & Lucas RA. The application of high resolution diffusion NMR for the characterisation and quantification of small molecules in saliva/dentifrice slurries. *Analytical Methods* 2015;7(6):2323-2332.
21. Ramakrishnan V, & Luthria DL. Recent applications of NMR in food and dietary studies. *Journal of the Science of Food and Agriculture* 2017;7(1):33-42.
22. Simmler C, Napolitano JG, McAlpine JB, Chen SN, & Pauli GF. Universal quantitative NMR analysis of complex natural samples. *Current opinion in biotechnology* 2014;25:51-59.
23. Holzgrabe U, Deubner R, Schollmayer C, & Waibel B. Quantitative NMR spectroscopy—applications in drug analysis. *Journal of pharmaceutical and biomedical analysis* 2005;38(5):806-812.
24. <https://nmr.chemistry.manchester.ac.uk/?q=node/42>; last date accessed: 05/11/2018

26. Binev Y, Marques MM, & Aires-de-Sousa J. Prediction of ^1H NMR coupling constants with associative neural networks trained for chemical shifts. *Journal of chemical information and modeling* 2007;47(6):2089-2097.

26. Webb AR, DeCosta BR, & Holick MF. Sunlight regulates the cutaneous production of vitamin D3 by causing its photodegradation. *The Journal of Clinical Endocrinology & Metabolism* 1989;68(5):882-887.

Table 1 Comparison of considerations for quantitative scope and limitations for NMR and Mass Spectrometry, highlighted considerations are addressed here.

Step of Analysis	Mass Spectrometry	qNMR
Sample Preparation	Weight/dilutions/filtrations. No Recovery after analysis	Recovery after analysis
Detection	Physical restrictions	Universal Structural properties
Calibrant	Need structurally identical reference	CRM's available
Sensitivity	Low nM-pM	Low uM
Selectivity and specificity	Chromatographic separation	Overlapping of resonances
Reproducibility	Mainly instrument dependent	Instrument dependent

Table 2 Results for unoptimised experiments

Vitamin	Determined Concentration (mM)	S/N	RSD	Trueness
D₂	16.7	4880	0.155	2.01
D ₂	0.777	755	2.27	0.936
D ₂	0.078	107	1.73	0.938
D ₂	0.003	8.03	13.6	0.404
D ₂	0.001	4.43	32	1.46
D₃	26.1	1460	0.652	3.14
D ₃	0.4	397	1.17	0.482
D ₃	0.0364	51.7	2.63	0.439
D ₃	0.00339	5.11	90.6	0.408

Table 3 Parameters for non-sample optimised and sample optimised experiments

Parameter	Standard qNMR	Optimised qNMR
TSP Concentration	10mM	≈ [Analyte]
D1 (Dwell time)	3s	10s
NS (Number of Scans)	1024	1024
SW (Sweep width)	10	20
TD (Data points)	64K	64K

Table 4 *Results for optimised experiments*

Vitamin	Determined Concentration (mM)	S/N	RSD	Trueness
D₂	0.0733	2499	1.9	0.147
D ₂	0.00696	213	1.07	0.139
D ₂	0.000710	54.8	7.53	0.142
D ₂	0.000112	29.5	52.2	0.0224
D₃	0.461	11027	0.217	0.922
D ₃	0.0462	1594.08	0.450	0.924
D ₃	0.00462	230.5	1.15	0.924
D ₃	0.000425	4.62	19	0.85
D ₃	0.0000421	2.03	9.91	0.842

Figure Legends

Figure 1 (Top) Partially assigned structure of Vitamin D3 with partially assigned 1D 1H spectrum; (Bottom) Partially assigned structure of Vitamin D2 with partially assigned 1D 1H spectrum.

Figure 2 (Left) Structure of Vitamin D2, with a 1H PSYCHE NMR spectrum of Vitamin D2 in DMSO-d6; (Right) Structure of Vitamin D3, with a 1H PSYCHE NMR spectrum of vitamin D3 in DMSO-d6

Figure 3 (Top) 1H NMR spectrum of cream mixture (Glycerol, Caprylic Triglyceride, Isostearylisoterate, Niacinamide, Vitamin D2, Vegetable Oil, Pentylene Glycol, Panthenol); (Bottom) Zoomed in region covering Vitamin D2 Alkene peaks of 1H NMR of above spectrum

Figure 4 Micellised and free vitamin D3 analysis from 1D 1H qNMR spectra

Figure 5 (Left) 2D 1H PSYCHEiDOSY Spectrum of cream formulation; (Right) Table of diffusion coefficients and their comparison to actual molecular weights calculated with UCC Polymer Predictor (<http://www2.ual.es/NMRMBC/downloads/UCCPolymerPrediction.rar>)

Figure 6 1D projections of Figure 4 PSYCHEiDOSY spectra; extracting individual pure shift spectra for each individual component of mixture.