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Quantitative Metabolic Profiling of Human Serum by Nonlinear Sampling and Forward Maximum Entropy Reconstruction of Two-dimensional ^{1}H-^{13}C HSQC

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Abstract: *This work developed a 2-dimensional (2D) ^{1}H-^{13}C heteronuclear single quantum coherence (HSQC) approach to directly quantify metabolites in chemical mixtures without using a calibration curve. Using non-linear sampling (NLS) and forward maximum (FM) entropy reconstruction, this method is fast enough to be used for routine quantitative metabonomics. Here, the use of this method to analyse complex human serum samples was demonstrated. This method resulted in 24-fold reduction in the nuclear magnetic resonance (NMR) data collection time compared to previously existing methods.*

Keywords: Non-linear sampling, metabolic profiling, forward maximum entropy, human serum samples, HSQC

1. INTRODUCTION

Nuclear magnetic resonance is a powerful tool for the identification and quantification of various metabolites in biological fluids, tissue extracts and complex mixtures. The ^{1}H nuclear magnetic resonance (NMR) spectrum is an excellent method of obtaining quantitative information for simple mixtures of low molecular weight metabolites. The quantification of various metabolites from a 1-dimensional (1D) ^{1}H NMR spectrum can be achieved because NMR spectral intensities are directly proportional to metabolite concentrations.[1,2] However, as the number of small molecules increases in mixtures, the quantification of 1D ^{1}H NMR data is often impaired due to spectral overlapping. This occurs even when the highest available magnetic field is used, which can affect accurate quantification.

Spectral overlapping can easily be removed using 2-dimensional (2D) NMR spectroscopy techniques.[3] However, the quantification of various metabolites from 2D NMR spectroscopy data is difficult because various experimental parameters depend on the measured cross-peak intensities. These

experimental factors include relaxation times, mixing time, evolution time and uneven excitation profiles, etc.[4–7] Recently, a variety of 2D methods have been developed to quantify various metabolites in complex mixtures, serum, urine and other biological fluids.[4,5,7–10] The aim of these studies was to address these issues and allow for quantification from the 2D heteronuclear single quantum coherence (HSQC) NMR spectrum. These methods are broadly based on 2 criteria: the first utilises calibration curves,[11–13] and the second compensates for various experimental parameters using pulse sequence modifications or theoretical calculations.[4–9]

The authors previously published a method based on calculating the effect of various experimental parameters on cross-peak intensity and measuring concentration by calculating correction factors.[5] For other Q-HSQC variants, suppression of J_{CH}-dependence is achieved by modulating the Insensitive Nuclei Enhanced by Polarisation Transfer (INEPT) polarisation transfer delays of HSQC and using a total relaxation delay sufficient to avoid the ^{1}H T_1 (5 times the longest T_1 of interest) relaxation effect.[7,8,14,15] However, the long acquisition time of Q-HSQC is impractical for metabonomics studies.[5] A recent approach reported by Hu et al. directly measures the extrapolated time zero signal intensity using 2D ^{1}H-^{13}C HSQC ($HSQC_0$), which is linearly proportional to the sample concentration.[6] This approach sequentially records 3 HSQC spectra with incremented repetition times (basic HSQC block repeated once [i = 1], twice [i = 2], and optionally, thrice [i = 3]).[6]

The attenuation factor associated with each cross-peak can be directly measured using the log-linear regression of the integrated cross-peak intensities, and this value can be used to calculate the unattenuated intensity at time zero. The applicability of this method is limited by strong t_1 noise from high concentration metabolites.[6] To avoid this problem, gradient selective version $HSQC_i$ ($gsHSQC_i$) with "fast maximum likelihood reconstruction" (FMLR) has been implemented in the Newton software package to improve the precession and accuracy of metabolite quantification. The only drawback of this new protocol is that 3 HSQC spectra must be recorded for quantification.[4,16] These approaches all work well, but they require substantial experimental time for NMR data collection, making them inappropriate for routine metabonomics studies. Moreover, because large sample sizes are required to obtain statistically significant results, the NMR experiment time for each sample must be as small as possible.

Recently, the authors developed a quantitative ^{1}H-^{13}C HSQC method based on non-linear sampling (NLS) with forward maximum (FM) entropy reconstruction.[10,17] The modulation of cross-peak intensity in the 2D HSQC experiment was compensated using heteronuclear J-coupling by modulating

delays in INEPT polarisation transfer.[14] The modulation of cross-peak intensity in HSQC due to relaxation parameters was calculated using a previously described method.[5] The method-based NLS with FM reconstruction provides a linear correlation between linearly and non-linearly sampled NMR cross-peak intensity with minimal offset. This method was successfully applied to analyse mixtures of amino acids in solution as well as urine samples. Here, the application of NLS with FM reconstruction we described to analyse human serum samples, which is an important biological fluid for various metabonomic studies.

2. EXPERIMENTAL

2.1 Sample Preparation

Serum samples were collected from the blood of healthy control patients. 2 mm blood samples were collected from fasting subjects. Samples were incubated for 30 min at room temperature and centrifuged (at 10,000 rpm, 5°C for 5 min). The golden yellow supernatant was collected and stored at –80°C until NMR analysis. Serum samples were thawed just before acquiring the NMR spectra, and 450 µl of serum was added to each NMR tube. A co-axial insert containing 290 µM TSP solution (sodium salt of trimethylsilyl-2,2,3,3-tetradeuteropropionic acid) was used as a deuterium lock and as an external standard reference.

2.2 NMR Spectroscopy

All NMR spectra were recorded using a Bruker 800-MHz NMR spectrometer equipped with a triple-resonance TCI (^{1}H, ^{13}C, ^{15}N and ^{2}H lock) cryogenic probe. For the NLS sampling schedule generated using the Poisson gap–sampling schedule generator from Hyberts et al., 84 t_1 points out of 256 t_1 points were selected using the above approach.[18]

For the serum samples, spectra were collected using 2048 × 256 points and 8 scans. 84 t_1 points were collected using the Poisson gap-sampling schedule with relaxation delays of 15.5 s (5 times the longest T_1 of interest) and 2 s. To estimate the longitudinal relaxation parameter T_1 of proton resonances, inversion recovery experiments were performed with pre-saturation of the water signal. The transverse relaxation rate (T_2) of the proton resonances was determined using the Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence with a variable echo time and pre-saturation during the relaxation delay. The relaxation parameters, T_1 and T_2, were measured for all standard mixture solutions and serum samples. The values of the relaxation parameters are listed in Table 1. A correlation analysis was performed to determine the similarity between the measured concentration

using a Q-HSQC relaxation delay of 15.5 s and Q-HSQC with NLS and a relaxation delay of 2 s. Computer Aided Resonance Assignment (CARA) software was used to calculate the peak volume.

Table 1: Values of relaxation parameters.

		A	B	C	D	E	F	G	H
1	TSP CH_3	0/0	4.534	1.16	3739.31	14016558	6572800	–	–
2	Valine CH_3	1.04/ 19.45	1.17	0.42	2932	2001573	2167843	124.23	134.62
3	Isoleucine CH_3	0.99/ 17.41	0.63	0.23	3018.67	1707956	1831560	106.0119	97.88
4	Lactate CH_3	1.33/ 20.89	1.12	0.25	2696.79	26758310	25224238	1650.52	1632
5	Glutamate CH_2	2.13/ 29.87	0.95	0.31	1975.19	1805242	2168437	168.07	188.54
6	Glutamine CH_2	2.44/ 33.36	0.90	0.25	1810.7	4837429	4492294	450.22	402.22
7	Acetate CH_3	1.92/ 26.20	0.85	0.20	2130.56	1246848	1271820	77.38	70.65
8	Choline CH_3	3.21/ 56.6	1.40	0.44	1191.84	22667812	21805713	466.9	480.84
9	Alanine CH_3	1.48/ 19	1.03	0.42	2582.9	8054403	7857003	499.728	466.56
10	Glycine CH_2	3.58/ 44.46	1.35	0.56	917.38	2932014	2814743	272.87	280.65

Notes:

1. *A = Chemical shift $^1H/^{13}C$ ppm; B = T^1 (s); C = T^2 (s); D = Offset of metabolite (Hz); E = Volume (relaxation 15 s); F = Volume (relaxation 2 s with NLS); G = Concentration of metabolite (relaxation 15.5 s); and H = Concentration of metabolite (relaxation 2 s with NLS and FM).*
2. *T_1, T, and offset, cross peak volumes have been measured from serum for the verification of results from spectra recorded with different sampling schedule (data used for preparing Figure 2 and 3). Recycle delay = 15.5 and 2 s.*

2.3 Data Processing and FM-reconstruction

An FM-reconstruction program was used to construct the 2D HSQC data and the NMRPipe format for data handling.[19]

3. THEORY AND DETAILS OF METHOD

3.1 Theory

The quantification of various metabolites from a 1D NMR spectra is straightforward because the signal intensity is proportional to the concentration of the metabolite.[3] Thus, using the peak intensity ratio between a standard of known concentration (usually TSP) and a metabolite, we can estimate the concentration of the metabolite.[2] This is true if the 1D spectrum is recorded in a fully-relaxed state (repetition time $\geq 5 \times$ T1). If the repetition time is less than $5 \times$ T1, the signal intensity correction can be calculated using the Bloch equation. This phenomenon is applied to calculate metabolite concentration. Quantitative analysis of biological fluids using 1D NMR suffers from spectral overlap, making quantitative analysis challenging. 2D methods can be used to quantify bodily fluids and complex mixtures, but the quantification of various metabolites using 2D NMR spectroscopy is not straightforward, as the cross-peak volume in 2D NMR is not directly proportional to the metabolite concentration. Cross-peak volume is affected by several experimental factors, including relaxation time, mixing time, evolution time, uneven excitation profiles, etc.[20]

The authors recently reported that resonance-specific signal attenuation factors that occur during coherence transfer periods due to relaxation, imperfect pulses, and different transfer efficiencies of inept transfer for different spin systems can be back calculated for each individual metabolite and that those correction factors can be used to evaluate their concentration from adjusted cross-peak intensities (Figure 1).[5] The correction factor for J_{CH} coupling efficiency was calculated for the different INEPT transfer of each heteronuclear spin pair (C-H) in ^{1}H-^{13}C HSQC and the relaxation correction factor due to proton resonances (T_{1H}, T_{2H}). However, at high magnetic fields, imperfect pulses and ^{13}C offset effects are more pronounced, requiring recalculation of the correction factors.

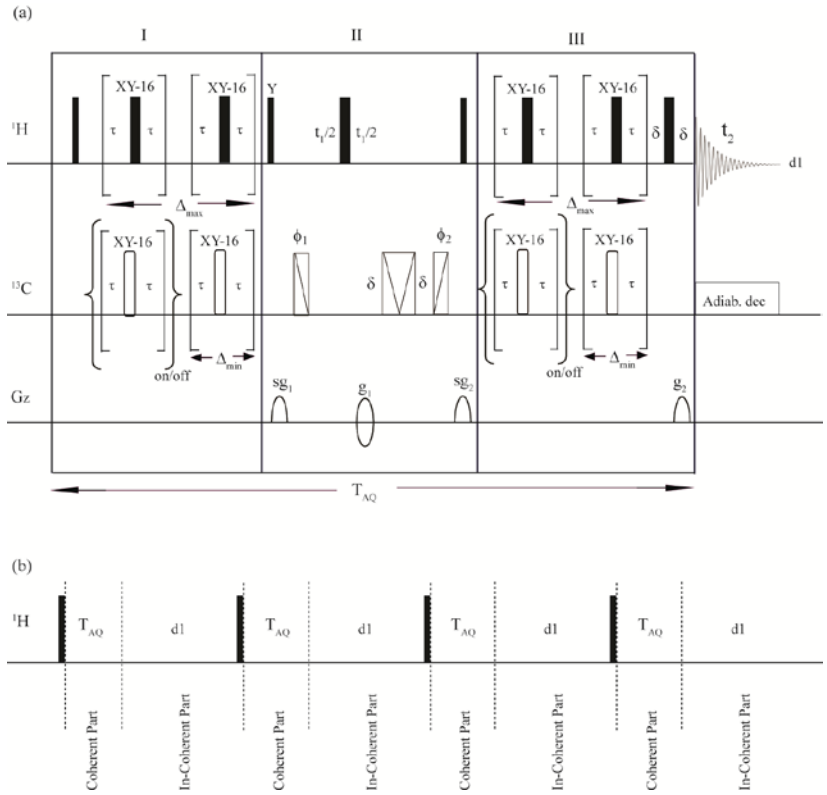


Figure 1: Pulse sequence for the (Q-OCCAHSQC) HSQC used for the FM-Reconstruction (Figure 1[a]). Narrow and wide filled rectangles represent the rectangular 90° and 180° pulses, respectively. Detailed description of the pulse sequence is explained in H. Koskela et al.[7] A simplified version of the same pulse sequence was used to calculate equilibrium ^{1}H Z magnetisation. The time (TAQ) represents the total time of pulse sequence, and (d1) represents the relaxation delay.

Hari et al. demonstrated that the HSQC (Q-OCCAHSQC) ^{13}C offset performance can be dramatically improved using broadband pulses (adiabatic and phase-modulated).[14] Thus, the pulse sequence used for quantitative analysis in this report accounts for the *J*-coupling effect of the heteronuclear (C-H) pair during different INEPT polarisation transfer. Therefore, we modified the correction factor for HSQC (Q-OCCAHSQC) as follows:

For 2D CPMG-adjusted HSQC (Q-OCCAHSQC) version, the cross-peak intensity (V_0) is calculated using the following equation:

$$V_0 \propto \eta^0(T_1^H, T_2^H, d) \times C_0$$

where η^0 is the correction factor that depends on the relaxation parameters of the proton resonances (T_1^H, T_2^H) and the recycle delay (d) used for recording the spectra. C_0 represents the molar concentration of the metabolite. By measuring the ratio of the cross-peak intensity from the 2D NMR spectra between a standard peak of known concentration (V_0) and the test metabolite (V_m) and calculating various correction factors, we can estimate the molar concentration of the metabolite.

$$\frac{V_0}{V_m} = \frac{\eta^0}{\eta^m} \times \frac{C_0}{C_m} \tag{1}$$

Here,

$$\eta = \frac{\left(1 - \mathrm{E}_1\right)\ \left(1 - \mathrm{E}_2 \cos\varphi\right)}{\left(1 - \mathrm{E}_1\right)\ \left(1 - \mathrm{E}_2 \cos\varphi\right) - \left(\mathrm{E}_1\right)\ \left(\mathrm{E}_2 - \cos\varphi\right)\mathrm{E}_2} \tag{2}$$

where

$$E_1 = \exp\left(-\frac{d}{T_1}\right)$$

$$E_2 = \exp\left(-\frac{d}{T_2}\right)$$

and ϕ = offset of the proton × d, with d the recycle delay used to record the spectra. The Carr-Purcell-Meiboom-Gill (CPMG)-adjusted HSQC (Q-OCCAHSQC) pulse sequence is designed as Q-CAHSQC, which gives a uniform intensity response over a large range of J_{CH} (115–220 Hz) couplings, and for the suppression of J_{HH} coupling evolution distortions during constant-time CMPG-INEPT periods.[14]

4. RESULTS AND DISCUSSION

The quantification method used here was previously verified using a solution containing a mixture of amino acids, including valine alanine, glycine and methionine.[10] In this study, the authors verify this method using human serum samples. It is difficult to obtain quantitative information from serum

samples because broad protein resonances hamper metabolite signals in simple 1D NMR (Figure 2[a]). However, it is possible to eliminate broad protein resonances using the CPMG sequence. Once we acquire CPMG spectra, we can easily obtain quantitative information using the Bharti et al. method[21] (Figure 2[b]). For serum samples, several peaks significantly overlapped (Figure 3[a]), hampering the quantification of 1D NMR spectra. In those cases, it becomes necessity to use 2D NMR techniques for quantification.

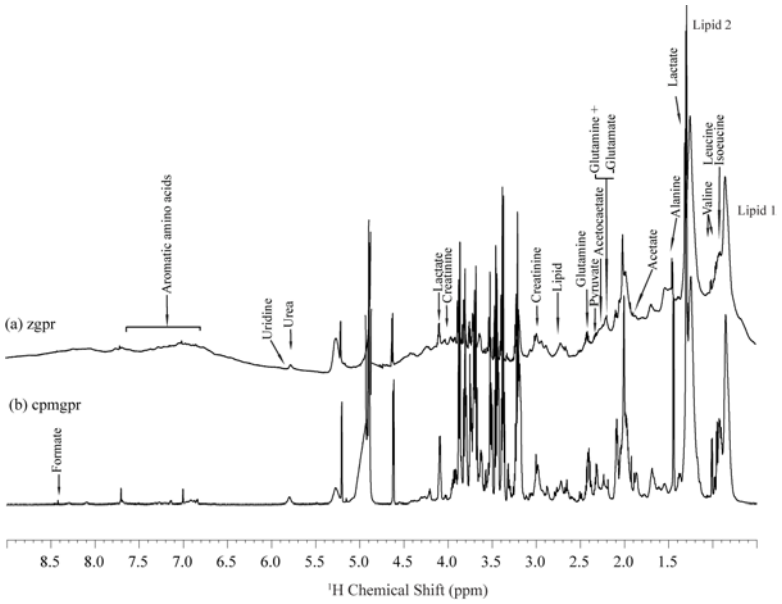


Figure 2: ^{1}H NMR spectra of serum samples: (a) Simple one-pulse NMR with water pre-saturation, and (b) ^{1}H NMR spectra using the CPMG sequence with 400 ms T_2 filtering to remove broad protein signals.

NMR Quantitative ^{1}H-^{13}C Q-OCCAHSQC with a 15.5 s relaxation delay (repetition time $\geq 5 \times$ T1) is shown in Figure 3(a). Various resonances can be unambiguously assigned based on ^{1}H and ^{13}C chemical shifts. It took almost 18 h and 46 min to record this spectrum. The various 2D NMR spectra were recorded using NLS and FM reconstruction with different numbers of t_1 points to assess the reproducibility of our data. Serum is a unique bodily fluid that contains lipoproteins and small molecular metabolites at varying concentrations. Thus, it is very important to choose the minimum number of NLS sample points needed to consistently reconstruct the concentration.

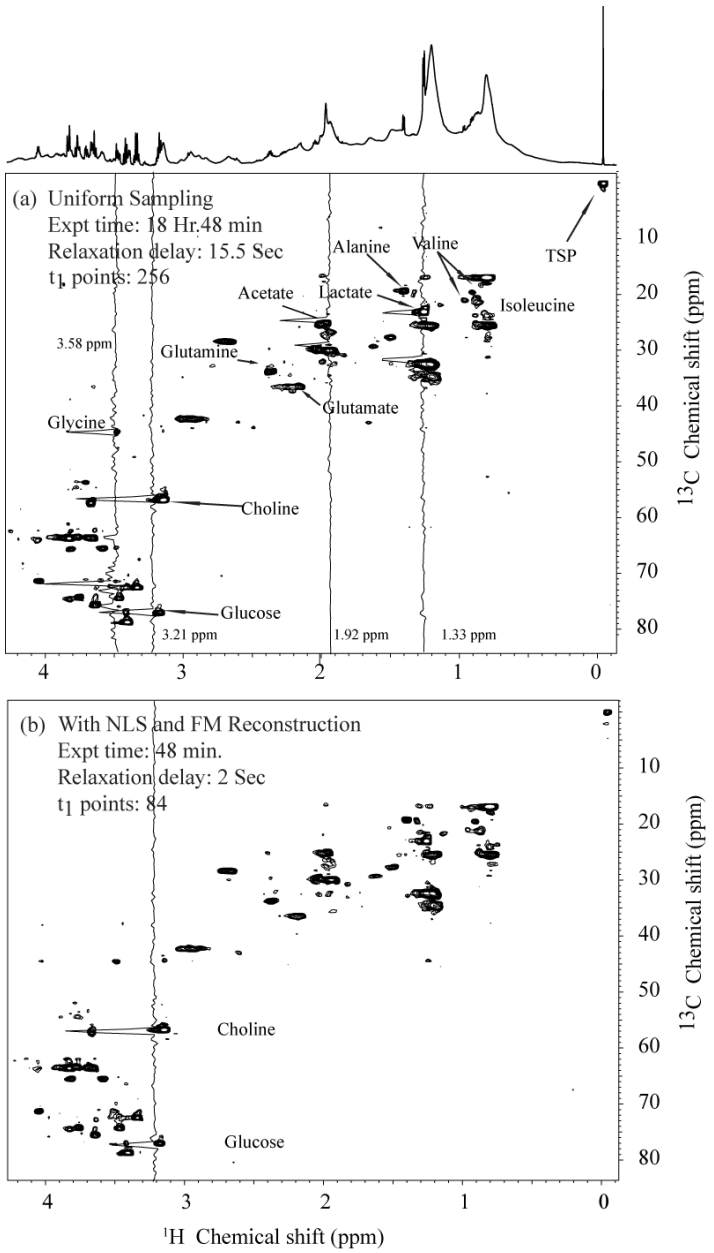
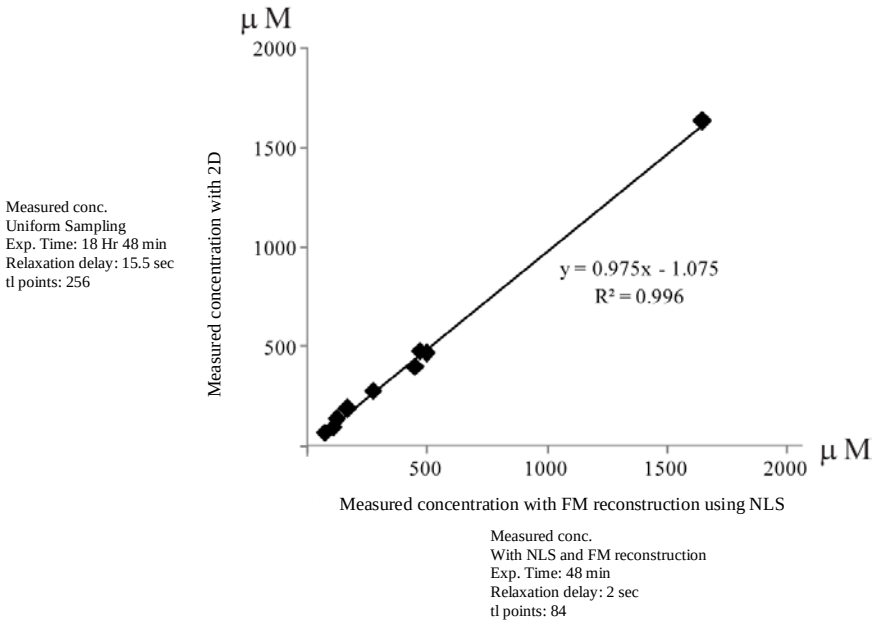


Figure 3: 2D ^{1}H-^{13}C HSQC spectrum of a human serum sample recorded with (a) a relaxation delay of 15.5 s and cross sections at different ^{1}H chemical shifts, and (b) 2D ^{1}H-^{13}C HSQC spectrum of a human serum sample recorded with NLS and FM reconstruction with a relaxation delay of 2 s and cross sections at different ^{1}H chemical shifts.

Out of 256 points, the authors used a 32, 64, 84 and 128 t_1-point Poisson gap-sampling schedule. It was challenging to reconstruct the HSQC cross-peak volume to allow for quantification using 32 and 64 t_1 points, but it was easily reconstructed using 84 and 128 t_1 points. NLS with FM reconstruction with 84 t_1 recorded took 48 min, as shown in Figure 3(b). Numerous metabolites, including valine alanine, isoleucine, glycine, choline, acetate and lactate cross-peaks, were clearly identified, and their concentrations were measured from the 2D spectrum. We measured the concentration using both Quantitative ^{1}H-^{13}C Q-OCCAHSQC with a relaxation delay of 15.5 s and NLS with FM reconstruction with a relaxation delay of 2 s. All cross-peak volumes with the respective relaxation delay and concentrations of individual metabolites are presented in Table 1. Regression analysis was performed between uniform sample data and NLS and FM reconstruction data (Figure 4). Good correlation was observed for the spectra recorded with NLS and FM reconstruction. However, the experimental time of the spectra recorded with NLS was dramatically shorter than those of the spectra recorded with uniform sampling, representing an approximately 24-fold reduction in NMR experimental time.



Notes: Curves were plotted for the measured concentrations from 2D ^{1}H-^{13}C HSQC recorded with uniform sampling and a relaxation delay of 15.5 s, as well as the HSQC spectra recorded with NLS and FM reconstruction with a relaxation delay of 2 s. The best-fit, straight-line equation and R^2 value are shown in the figure.

Figure 4: Regression curves of the different amino acids in a human serum sample.

Here, the authors demonstrated the application of a newly developed NLS with FM reconstruction for the quantification of bodily fluids, such as serum. It has been proposed that 2D spectra allow for better characterisation of statistically relevant changes in the low-abundance metabolites compared to 1D NMR.[22] The proposed method has the potential to make quantitative metabolic profiling by 2D NMR a routine method.

5. CONCLUSION

We have shown that present method of measuring concentration of various metabolites with 2D 1H−13C HSQC NMR spectra with NLS and FM reconstruction is efficient for human urine sample. The time reduction in the case of human urine sample is 24-fold and does not affect the concentration measurement. The efficiency of this method can be improved if we incorporate "fast maximum likelihood reconstruction" (FMLR) for the peak intensity calculation.[22] Use of two internal standards for the higher and lower abundance metabolite separately will further improve quantification results.

6. ACKNOWLEDGEMENT

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