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SMoESY: An Efficient and Quantitative Alternative to On-Instrument Macromolecular $^1$H-NMR Signal Suppression†

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SMoESY impact on time and cost for the NMR metabolomics pipeline

Figure S1. The experimental scheme of NMR based metabolomics pipeline for biofluids with macromolecular content (e.g. proteins, lipoproteins, lipids etc.) – SMoESY contribution. The total amount of experimental time is up to 16 minutes. The 1D-NOESY experiment [containing both macromolecules and low molecular weight (MW) metabolites signals] is followed by a one-dimensional spin-echo experiment (e.g. CPMG), which suppresses the broad signals of macromolecules by $T_2$ relaxation times filtering. Finally, a pseudo-2D experiment is acquired, J-res, employed for the assignment facilitation of low MW metabolites overlapped signals. As shown, spin-echo experiments (given the limited acquisition time) do not efficiently attenuate broad signals of macromolecules, and they cannot be employed for absolute quantification of metabolites as they are highly modulated by $T_2$ values (the same stands for the projections of J-res experiments that are highly modulated by the J-coupling constant values). On the other hand, SMoESY product requires < 1 sec per spectrum, supersedes spin-echo experiments in broad signals attenuation as well as maintains its quantitative ability for low MW metabolites absolute quantification. Consequently, a substitution of a spin-echo experiment by SMoESY could lead to up to 30% decrease of acquisition time per spectrum (or up to 30% increase of acquired spectra/samples), whereas it could speed up assignment/quantification of small MW metabolites. Consequently, SMoESY could significantly reduce the cost of NMR analyses for large scale epidemiological studies and provide more details for the low MW metabolites content.
SMoESY approach in its simplest form

**Figure S2.** Examples of enhanced spectral resolution by the imaginary NMR spectral part differentiation. (A) The real spectral data (i.e. doublet, d) of the spin system from the -CH$_3$ group of L-alanine in a typical plasma/serum matrix (upper panel). The 1$^\text{st}$ numerical derivative of the real data from the L-alanine -CH$_3$ $^1$H-NMR signal (after Fourier transform and phase correction) (bottom panel), produces an antisymmetric signal (positive on one side and negative on the other). (B) The imaginary spectral data of the spin system from the -CH$_3$ group of L-alanine in a typical plasma/serum matrix (upper panel). In contrary to the real data, the 1$^\text{st}$ derivative of the imaginary data, due to its gradient (namely positive-negative maxima per signal) (bottom panel), produces a positive transformed signal. (C) Overlaid real and 1$^\text{st}$ derivative of the imaginary part of the L-alanine -CH$_3$ doublet spectral regions, show no chemical shifting, without the need of applying any symmetrisation algorithms. The transformed signal from the imaginary spectral data could be immediately employed for any NMR-based metabolomics or analytical study. (D) Comparison between the 2$^\text{nd}$ derivative of the real data of the NMR spectrum multiplied by -1 (this could be the same for the 2$^\text{nd}$ power derivative) and the 1$^\text{st}$ derivative of the imaginary part of the same spectral region, taken from a $^1$H-NMR plasma spectrum. It is immediately appreciated that the signal-to-noise ratio of the 2$^\text{nd}$ derivative of the real spectral data is decreased compared to the 1$^\text{st}$ derivative of the imaginary part.
Testing metabolites intra-reproducibility by artificial mixtures of metabolites

A

Citric acid

- Citric acid1 ($R^2 = 0.984$)
- Citric acid2 ($R^2 = 0.988$)

B

Benzonic acid

- Benzonic acid1 ($R^2 = 0.993$)
- Benzonic acid2 ($R^2 = 0.992$)
C

Caprylic acid
- Caprylic acid1 ($R^2 = 0.991$)
- Caprylic acid2 ($R^2 = 0.992$)

D

L-isoleucine
- L-isoleucine1 ($R^2 = 0.989$)
- L-isoleucine2 ($R^2 = 0.983$)
E

L-tryptophan

- L-tryptophan1 (R² = 0.981)
- L-tryptophan2 (R² = 0.99)
- L-tryptophan3 (R² = 0.986)

F

L-phenylalanine

- L-phenylalanine1 (R² = 0.980)
- L-phenylalanine2 (R² = 0.979)
- L-phenylalanine3 (R² = 0.980)
Figure S3. Validation of SMolESY intra-metabolites signals reproducibility. Plots of the fitted linear regression curves ($R^2 \geq 0.98$) based upon the SMolESY versus 1D-NOESY integrals from various $^1$H spin systems (highlighted by light blue circles) with different multiplicities (horizontal/vertical error bars point at ±1% error in integration) of (A) Citric acid, (B) Benzoic acid, (C) Caprylic acid, (D) L-isoleucine, (E) L-tryptophan and (F) L-phenylalanine in 9 concentrations in the artificial metabolites mixtures. All slope, intercepts as well as one-way ANOVA tests for the confirmed all intercepts/slopes coincidence for each metabolite (see below Table S1). These results highlight the intra-metabolites SMolESY signals reproducibility.
Table S1. Statistical analyses data for SMoESY intra-metabolites signals reproducibility tests. Statistical data of the fitted linear regression curves for the SMoESY versus 1D-NOESY integrals of NMR signals from various $^1$H spin systems from several metabolites in 9 concentrations in the artificial metabolites mixtures. All linear curves statistically pass through the zero point and according to the one-way ANOVA tests both slopes and intercepts of all curves for each metabolite coincide.

| Best-fit values | Cytidine 1 | Cytidine 2 | Cytidine 3 | Cytidine 4 | Cytidine 5 | one-way ANOVA results |
|-----------------|-----------|-----------|-----------|-----------|-----------|----------------------|
| Slope           | 65.41     | 66.63     | 68.62     | 67.53     | 65.03     |                      |
| Y-intercept     | 36.72     | 30.63     | -36.76    | -11.33    | -230.3    | For the slopes      |
| X-intercept     | -56.14    | -0.4597   | 53.57     | 16.77     | 25.41     | $F = 0.265$         |
| U/slope         | 0.01529   | 0.01501   | 0.01457   | 0.01481   | 0.01538   | $F = 2.191$         |
| Std. Error      | 95%       |           |           |           |           | $F = 0.8984$        |
| Slope           | 18.54     | 2.873     | 2.827     | 2.406     | 3.347     | The pooled slope equals 66.59 |
| Y-intercept     | 2413      | 4415      | 3544      | 3079      | 4310      |                      |

95% Confidence Intervals

| Slope           | 61.02 to 69.79 | 57.94 to 75.32 | 61.93 to 75.31 | 61.84 to 73.22 | 57.12 to 72.94 |                      |
| Y-intercept     | -9049 to 9377  | -102056 to 4704 | -8413 to 6148  | -12493 to 7887 |                      |                      |
| X-intercept     | -151.62 to 29.54 | -175.8 to 142   | -74.53 to 163.1 | -97.89 to 116.7 | -134.9 to 175.3   |                      |

Best-fit values

| Citric acid 1 | Citric acid 2 |
|---------------|---------------|
| Slope         | 14.15         | 12.78         |
| Y-intercept   | -5.23         | -28.75        |
| X-intercept   | 36.98         | 22.35         |
| U/slope       | 0.07068       | 0.07772       |

95% Confidence Intervals

| Slope           | 12.53 to 15.77 | 11.61 to 14.13 |
| Y-intercept     | -1577 to 530.6 | -1096 to 520.9 |
| X-intercept     | -41.41 to 102.3 | -44.08 to 78.99 |

Best-fit values

| Benzoic acid 1 | Benzoic acid 2 |
|----------------|----------------|
| Slope          | 146.2          | 142.3          |
| Y-intercept    | 11191          | 11549          |
| X-intercept    | -5.655         | -81.15         |
| U/slope        | 0.00684        | 0.00727        |

95% Confidence Intervals

| Slope           | 139.7 to 152.6 | 135.2 to 149.4 |
| Y-intercept     | -1285 to 23667 | -2371 to 25468 |
| X-intercept     | -167.9 to 8.492 | -186.6 to 16.02 |

Best-fit values

| Caprylic acid 1 | Caprylic acid 2 |
|-----------------|----------------|
| Slope           | 5.566          | 5.273          |
| Y-intercept     | -378.0         | -631.1         |
| X-intercept     | 67.92          | 121.3          |
| U/slope         | 0.1797         | 0.1896         |

95% Confidence Intervals

| Slope           | 5.090 to 6.042 | 4.801 to 5.746 |
| Y-intercept     | -1932 to 1176  | -1795 to 453.3 |
| X-intercept     | -227.2 to 325.2 | -92.74 to 318.1 |

Best-fit values

| L-Isocitric 1   | L-Isocitric 2   |
|-----------------|-----------------|
| Slope           | 203.0           | 197.0          |
| Y-intercept     | 21257           | 207108         |
| X-intercept     | -104.7          | -105.1         |
| U/slope         | 0.004927        | 0.005076       |

95% Confidence Intervals

| Slope           | 183.5 to 222.4 | 173.7 to 220.3 |
| Y-intercept     | -6147 to 48661 | -9975 to 31441 |
| X-intercept     | -2060 to 2312  | -137.8 to 46.09 |

Best-fit values

| L-Tryptophan 1 | L-Tryptophan 2 | L-Tryptophan 3 |
|----------------|----------------|----------------|
| Slope          | 48.99          | 52.14          |
| Y-intercept    | 508.7          | 1243           |

95% Confidence Intervals

| Slope           | 48.99          | 52.14          |
| Y-intercept     | 508.7          | 1243           |
|                        | L-phenylalanine 1 | L-phenylalanine 2 | L-phenylalanine 3 | one-way ANOVA results |
|------------------------|-------------------|-------------------|-------------------|-----------------------|
| Best-fit values        |                   |                   |                   |                       |
| Slope                  | 0.004732          | 0.004916          | 0.004833          |                       |
| V-intercept            | 131.5             | 29.58             | 31.80             |                       |
| X-intercept            | -229.94           | -60.16            | -65.80            |                       |
| Std. Error             | 211.3             | 203.4             | 206.9             |                       |
| 95% Confidence Intervals |                  |                   |                   |                       |
| Slope                  | 0.0004053 to 0.005411 | 0.003859 to 0.005973 | 0.003914 to 0.005751 |                       |
| V-intercept            | -77.10 to -340.1  | -143.2 to 202.4   | -161.0 to 224.6   |                       |
| X-intercept            | -8.150 to 14.700  | -50.122 to 25.000 | -55.897 to 28.945 |                       |

1DFn: degrees of freedom numerator.
2DFd: degrees of freedom denominator.
Figure S4. 3D scores plots of the first three components from the PCA of a urine dataset for both 1D-NOESY and SMoIESY spectra. From the analysis, it is observed that both NOESY and SMoIESY spectra capture similar cumulative variability, 93.4% and 89.4% respectively, and provide similar discrimination of sample groups.
Correlation of SMoESY vs. CPMG spectra for 994 plasma-EDTA samples

A

1D-NOESY spectrum of 994 plasma-EDTA samples/spectra

CPMG spectrum of 994 plasma-EDTA samples/spectra

SMoESY spectrum of 994 plasma-EDTA samples/spectra
mean 1D-NOESY spectrum of 994 plasma-EDTA samples/spectra

mean CPMG spectrum of 994 plasma-EDTA samples/spectra

mean SMoESY spectrum of 994 plasma-EDTA samples/spectra
mean 1D-NOESY spectrum of 994 plasma-EDTA samples/spectra

mean CPMG spectrum of 994 plasma-EDTA samples/spectra

mean SMoESY spectrum of 994 plasma-EDTA samples/spectra
D

mean 1D-NOESY spectrum of 994 plasma-EDTA samples/spectra

mean CPMG spectrum of 994 plasma-EDTA samples/spectra

mean SMoESY spectrum of 994 plasma-EDTA samples/spectra
mean 1D-NOESY spectrum of 994 plasma-EDTA samples/spectra

mean CPMG spectrum of 994 plasma-EDTA samples/spectra

mean SMoESY spectrum of 994 plasma-EDTA samples/spectra
mean 1D-NOESY spectrum of 994 plasma-EDTA samples/spectra

mean CPMG spectrum of 994 plasma-EDTA samples/spectra

mean SMoESY spectrum of 994 plasma-EDTA samples/spectra
mean 1D-NOESY spectrum of 994 plasma-EDTA samples/spectra

mean CPMG spectrum of 994 plasma-EDTA samples/spectra

mean SMoESY spectrum of 994 plasma-EDTA samples/spectra
mean 1D-NOESY spectrum of 994 plasma-EDTA samples/spectra

mean CPMG spectrum of 994 plasma-EDTA samples/spectra

mean SMoESY spectrum of 994 plasma-EDTA samples/spectra
mean 1D-NOESY spectrum of 994 plasma-EDTA samples/spectra

mean CPMG spectrum of 994 plasma-EDTA samples/spectra

mean SMolESY spectrum of 994 plasma-EDTA samples/spectra
mean 1D-NOESY spectrum of 994 plasma-EDTA samples/spectra

mean CPMG spectrum of 994 plasma-EDTA samples/spectra

mean SMoESY spectrum of 994 plasma-EDTA samples/spectra
mean 1D-NOESY spectrum of 994 plasma-EDTA samples/spectra

mean CPMG spectrum of 994 plasma-EDTA samples/spectra

mean SMoESY spectrum of 994 plasma-EDTA samples/spectra
mean 1D-NOESY spectrum of 994 plasma-EDTA samples/spectra

mean CPMG spectrum of 994 plasma-EDTA samples/spectra

mean SMoESY spectrum of 994 plasma-EDTA samples/spectra
mean 1D-NOESY spectrum of 994 plasma-EDTA samples/spectra

mean CPMG spectrum of 994 plasma-EDTA samples/spectra

mean SMolESY spectrum of 994 plasma-EDTA samples/spectra
**Figure S5.** SMoLESY performance in 994 plasma-EDTA samples. (A-N) Mean spectrum of 994 plasma-EDTA 1D-NOESY, CPMG and SMoLESY ¹H-NMR spectra (0.55–8.7 ppm) zoomed at ~0.5-0.7 ppm windows. SMoLESY is colored according to the Pearson coefficients from SMoLESY versus CPMG signals correlation in 994 spectra. The majority of highly resolved SMoLESY signals are linearly correlated to the CPMG and > 99.5 % of CPMG features of low molecular weight metabolites are maintained, while successfully suppressing the broad signals of macromolecules in contrast to CPMG (examples of unsuppressed CPMG broad signals are highlighted by red dashed boxes in panels A-I, L, M). It is noted that broad signal of urea along with 3 broad signals of very low abundance metabolites (< 1.5 times the signal-noise-ratio) are highly suppressed and low correlated to the CPMG (black dashed boxes in panels K, J), even though being recovered by the SMoLESY.
Examples of SMoIESY vs. CPMG and NOESY signals

**Figure S6.** Mean spectrum of 994 plasma-EDTA samples spectra focusing on the 3.5-4.0 $^1$H-NMR ppm region. Mean spectrum is calculated by the (A) 1D-NOESY, (B) CPMG and (C) SMoIESY 994 spectra. SMoIESY is colored according to the Pearson coefficients from SMoIESY versus CPMG signals correlation for the 994 spectra. The majority of highly resolved (e.g. pointed by grey arrows) SMoIESY signals are linearly correlated to the CPMG. Few signals are zero or anti-correlated to the CPMG spectra (light blue arrows), due to partial overlapping with other signals and/or baseline distortions in the CPMG (i.e. ineffective suppression of broad signals).
SMoESY vs CPMG spectral bins correlation – spiking experiments in real plasma matrix
Figure S7. SMoIESY application and reproducibility validation to spectra binning. Comparison between CPMG (left panel) and SMoIESY (middle panel) spectral bins including various $^1$H-NMR signals of 13 spiked metabolites (11 concentrations) in a plasma sample: (A) L-valine, (B) L-glutamine, (C) 2-hydroxybutyric acid, (D) L-histidine, (E) Citric acid, (F) Formic acid, (G) Glucose, (H) Glycerol, (I) Acetone, (J) Acetic acid (K) L-lactic acid, (L) L-isoleucine and (M) L-threonine. Linear regression curves (right panel) exhibit $0.98 < R^2 < 1$ for all metabolites, indicate that SMoIESY is highly reproducible while superseding CPMG in broad signals suppression resulting in zero baseline distortions. Due to ~0 error in bin integration, horizontal and vertical error bars are not plotted in all linear regression plots. Light blue circles indicate the proton spin system of each metabolite included in each spectral bin which might include signals of other (not spiked) metabolites.
Comparison between SMoESY and signal deconvolution approaches for absolute quantification

Figure S8. The relative root mean square errors (RRMSE) of SMoESY and deconvolution algorithms used for 12 spiked plasma metabolites absolute quantification were calculated based upon the regression analyses (see Fig. 5). RRMSE values show that absolute quantification via SMoESY signals integration provides less errors compared to the fitting procedures followed by deconvolution algorithms.
An overview of the SMoLESY_platform graphical user interface (GUI) toolbox.

![GUI Toolbox](image)

**Figure S9.** (A) The development of SMoLESY_platform provides the opportunity for any user to load 1D $^1$H-NMR raw spectra, so as to transform them into SMoLESY and export them into a .txt file. In addition, the user has the opportunity to calibrate the SMoLESY spectra to the doublet of the anomic proton of glucose (~5.25 ppm) in case of plasma/serum/CSF etc. acquired spectra. (B) It offers the possibility to plot both 1D $^1$H-NMR and SMoLESY spectra for a synchronized zoom in both panels, (C) as well as to align into specific reference peaks a set of spectral bins or individual signals so as to integrate SMoLESY features either for qualitative (i.e. option of variable-size SMoLESY spectra binning) or quantitative purposes (i.e. option of SMoLESY signals integration for quantification). The alignment of the signals could be performed both manually or in a semi-automated way upon users experience and request. More details and user guidelines of the software could be found at: [https://github.com/pantakis/SMoLESY_platform](https://github.com/pantakis/SMoLESY_platform).
Computer code for the calculation of the Pearson correlation coefficients.

```matlab
function [SMolESY_ints,CPMG_ints,Corv,Pv] = pearson_cor_SMolESYvsCPMG(ppm_SMolESY,ppm_CPMG,SMolESY_data,CPMG_data)
    % Calculating the integrals of each feature/point of SMolESY for a
    % comparison to CPMG equal width of ppm.
    % NOTE: SMolESY - CPMG spectral data should be aligned to the same
    % reference peak (e.g. glucose, TSP etc.)
    % Inputs
    % ppm_SMolESY, PPM data of all SMolESY data (one vector)
    % ppm_CPMG, PPM data of all CPMG data (one vector)
    % SMolESY_data, SMolESY data (intensities) matrix, rows: spectra, columns:
    % intensities
    % CPMG_data, CPMG data (intensities) matrix, rows: spectra, columns:
    % intensities
    % Outputs
    % SMolESY_ints, Integral of each SMolESY feature
    % CPMG_ints, Integral of each CPMG feature (describing the same ppm region
    % as the corresponding SMolESY feature)
    % Corv: calculated Pearson's Linear Correlation Coefficient
    % Pv: p-value

    num_of_spectra = size(SMolESY_data,1);
    XAXIS_CPMG_rounded = round(ppm_CPMG,4);
    XAXIS_SMolESY_rounded = round(ppm_SMolESY,4);
    % equal Xaxis buckets for both CPMG and SMolESY/NOESY
    for l = 2:length(ppm_SMolESY)
        [~,ii] = find(ppm_CPMG <= ppm_SMolESY(:,l-1) & ppm_CPMG >= ppm_SMolESY(:,l));
        KL(l-1).aa = ii;
        clearvars ii
        for i = 1:num_of_spectra
            SMolESY_ints(i,l-1) = trapz(fliplr(XAXIS_SMolESY_rounded(1,l-1:l)),fliplr(SMolESY_data(i,l-1:l)));
        end
        if length(KL(l-1).aa) > 1
            CPMG_ints(i,l-1) = trapz(fliplr(XAXIS_CPMG_rounded(1,min(KL(l-1).aa):max(KL(l-1).aa))),fliplr(CPMG_data(i,min(KL(l-1).aa):max(KL(l-1).aa))));
        else
            CPMG_ints(i,l-1) = CPMG_data(i,KL(l-1).aa);
        end
    end
    [rho,pval] = corr(CPMG_ints(:,l-1),SMolESY_ints(:,l-1), 'Type', 'Pearson');
    Corv(l-1,:) = rho;
    Pv(l-1,:) = pval;
end
```

Data S1 The described MATLAB code was followed to correlate each SMoLESY feature intensity (i.e. integral) versus its corresponding CPMG feature for more than 3000 plasma spectra from different individuals as depicted in Fig. 2 and Fig. S5.
17 spiked metabolites 11 different concentrations spiked in real plasma sample

### Table S2

| Metabolites                  | Concentration (mM)* |
|------------------------------|---------------------|
| Lactic acid                  | 0  0.333  0.666  0.999  1.332  1.665  1.998  2.331  2.664  2.997  3.330 |
| 2-hydroxybutyric acid sodium salt | 0  0.016  0.032  0.048  0.063  0.079  0.095  0.111  0.127  0.143  0.159 |
| Acetone                      | 0  0.034  0.069  0.103  0.138  0.172  0.207  0.241  0.275  0.310  0.344 |
| Citric acid                  | 0  0.078  0.156  0.234  0.312  0.390  0.469  0.547  0.625  0.703  0.781 |
| D-glucose                    | 0  0.611  1.221  1.832  2.442  3.053  3.663  4.274  4.885  5.495  6.106 |
| Ethanol                      | 0  0.043  0.087  0.130  0.174  0.217  0.261  0.304  0.347  0.391  0.434 |
| Glycerol                     | 0  0.054  0.109  0.163  0.217  0.271  0.326  0.380  0.434  0.489  0.543 |
| L-aspartic acid              | 0  0.038  0.075  0.113  0.150  0.188  0.225  0.263  0.300  0.338  0.376 |
| L-glutamine                  | 0  0.082  0.164  0.246  0.328  0.411  0.493  0.575  0.657  0.739  0.821 |
| L-histidine                  | 0  0.045  0.090  0.135  0.181  0.226  0.271  0.316  0.361  0.406  0.451 |
| L-isoleucine                 | 0  0.023  0.046  0.069  0.091  0.114  0.137  0.160  0.183  0.206  0.229 |
| L-phenylalanine              | 0  0.049  0.097  0.145  0.194  0.242  0.291  0.339  0.387  0.436  0.484 |
| L-threonine                  | 0  0.025  0.050  0.076  0.101  0.126  0.151  0.176  0.201  0.227  0.252 |
| L-tryptophan                 | 0  0.073  0.147  0.220  0.294  0.367  0.441  0.514  0.588  0.661  0.735 |
| L-valine                     | 0  0.085  0.170  0.256  0.341  0.427  0.512  0.598  0.683  0.768  0.854 |
| Sodium acetate               | 0  0.024  0.049  0.073  0.098  0.122  0.146  0.171  0.195  0.219  0.244 |
| Sodium formate               | 0  0.044  0.088  0.132  0.176  0.221  0.265  0.309  0.353  0.397  0.441 |

*Zero values correspond to the non-spiked plasma sample.
Example of a smoothing filter for denoising SMolESY

Figure S10. SMolESY (upper panel), noise filtered SMolESY (middle panel) and the CPMG (lower panel) spectral regions, focusing on the $^1\text{H}$-NMR signal of the proton from Formic acid (at ~0.02 mM) in a plasma sample. The selected singlet resonates in usually noisy spectral region of a plasma $^1\text{H}$-NMR profile, consequently a quite large s/n decrease is expected after its transformation from the 1D-NOESY spectrum. Indeed, SMolESY spectrum shows the lowest s/n (~27% decrease compared to CPMG), whereas the application of a simple lowpass filter (i.e. filter coefficients equal to the reciprocal of the window span) results in ~18% increase of s/n compared to pure SMolESY signal, and exhibits quite similar s/n with CPMG (~10% lower than CPMG).