**High resolution quantitative metabolome analysis of urine by automated flow injection NMR**

Laeticia Da Silva[1,2], Markus Godejohann[3], François-Pierre J. Martin[1,2], Sebastiano Collino[1,2], Alexander Bürkle[4], María Moreno-Villanueva[4], Jürgen Bernhardt[5], Olivier Toussaint[6], Beatrix Grubeck-Loebenstein[7], Efstathios S. Gonos[8], Ewa Sikora[9], Tilman Grune[10,11], Nicolle Breusing[10], Claudio Franceschi[12], Antti Hervonen[13], Manfred Spraul[3], Sofia Moco[1,14]*

1 BioAnalytical Science, Nestle Research Center, Vers-chez-les-Blanc, PO Box 44, 1000 Lausanne 26, Switzerland

2 present address: Molecular Biomarkers, Nestle Institute of Health Sciences, EPFL campus, Innovation square, H, 1015 Lausanne, Switzerland

3 Bruker-Biospin GmbH, Am Silberstreifen 4, D-76287 Rheinstetten, Germany

4 Molecular Toxicology Group, Department of Biology, University of Konstanz, 78457 Konstanz, Germany

5 BioTeSys GmbH, 73728 Esslingen, Germany

6 Research Unit on Cellular Biology (URBC), University of Namur (FUNDP), 5000 Namur, Belgium

7 Institute for Biomedical Aging Research, University of Innsbruck, 6020 Innsbruck, Austria

8 Institute of Biological Research and Biotechnology, National Hellenic Research Foundation, Athens 11635, Greece

9 Nencki Institute of Experimental Biology, 02-093 Warsaw, Poland

10 Institute of Biological Chemistry and Nutrition, University of Hohenheim, 70593 Stuttgart, Germany

11 present address: Chair of Food Toxicology, University of Jena, 07743 Jena, Germany

12 Department of Experimental Pathology, University of Bologna, 40126 Bologna, Italy

13 School of Public Health, University of Tampere, 33014 Tampere, Finland

14 present address: Natural Bioactives and High Throughput Screening, Nestle Institute of Health Sciences, EPFL campus, Innovation square, H, 1015 Lausanne, Switzerland

*corresponding author: sofia.moco@rd.nestle.com; telephone: +41 21 632 6165; fax: +41 21 632 6499

**SUPPLEMENTARY EXPERIMENTAL INFORMATION**

**Sample preparation.**

Urine samples were randomised before proceeding with sample preparation. All samples (urine and QC) were prepared for analysis using an eight-channel MICROLAB STAR Liquid Handling Workstation (Hamilton), able to perform aliquot tasks using discardable tips (Conductive/filtered/clean unstacked framed Hamilton CO-RE tips), among other operations. The biological samples (provided in 15 mL-cryogenic tubes) were placed in adapted carrier trays and 400 µL of sample were dispensed into 96-deep well (DW) riplate plates 1 mL (Ritter, Germany, note: these plates are known to be compliant with the FIA system cooling tray design). Sample extraction buffer, placed in reagent tubs (Hamilton), was added to each well in a 1:2 proportion (buffer : sample). The plate was sealed with a peelable aluminium seal PP/PS (Agilent) using the robot's embedded plateLoC thermal microplate sealer (Agilent). Samples were prepared immediately prior to analysis. This automated sample preparation was done using tailor-made routines implemented under the control of Hamilton Method Manager, version 5 (Hamilton).

**Flow injection analysis (FIA).**

A septum-piercing bevelled stainless steel needle (dimensions, mm, L x OD x ID: 221 x 2 x 0.8 with tip: 2.1 x 1.5 x 0.8) was used to perform sample injection. Low pressure tubing (ID x L, mm) for dilutor inlet (0.5 x 2550), injection port (0.5 x 90), sample loop (1 x 3,000) and inlet and outlet to the NMR probe was used.
The infusion method was optimised for aqueous solutions ($D_2O$ and $H_2O$) and the transfer volumes and transfer speeds parameters. The dilutor, by using push solvent, aspirated the sample (360 µL - this volume should be typically three times the active cell volume) at 3.5 mL/min speed and infused it via the injector into the flow probe by means of a "sample train" where the injected sample is surrounded by a series of leading (100 µL gas + 150 µL eluent + 30 µL gas + 30 µL eluent + 30 µL gas) and trailing air gaps (30 µL gas + 20 µL eluent + 30 µL gas + 20 µL eluent + 30 µL gas). In this way, the sample is isolated from the push eluent. After NMR analysis, the sample is pushed out (6 s of gas pulse, 1 s of gas intermission and 2 pulse counter) at a speed of 2.0 mL/min to waste. This process was optimized after set-up, and unchanged throughout the whole analysis. Before injection of a sample, a cycle of washing was implemented: 800 µL wash line flush and 1800 µL probe flushing, followed

by drying: 6 s of gas pulse, 1 s of gas intermission and 2 pulse counter, using 20 mL/min speed for aspiration at 10 mL/min for dispensing.

In the case of obstruction, cleaning was performed after disconnecting the inlet and outlet capillaries from the probe and closing the system with a plastic union. Rinsing of the whole system with push-eluent, at both injector valve positions inject and load, at least for 5 minutes, was performed by using the 215 Priming Utility, version 1.02 (Gilson). When needed, FIA cleaning solution was injected into the FIA-NMR system, followed by extensive rinsing with push eluent, before re-initiating analyses.

**Nuclear magnetic resonance (NMR).**

*The temperature calibration*. Fresh deuterated methanol-d4 was directly infused into the flow cell, using a flat needled-syringe, and a one-scan $^{1}$H NMR 30$^{o}$ excitation pulse experiment (zg30) was acquired. The spectrum was Fourier transformed with exponential multiplication, followed by phase and baseline correction. The line broadening was set to 3.0 Hz. An embedded macro was used to calculate the real temperature of the sample, by a one-point verification of the calibration.

*Standard lineshape test* (1% $CHCl_3$ in acetone-$d_6$) was acquired using a one-scan $^{1}$H NMR 90$^{o}$ excitation pulse experiment (zg), with no dummy scans, 65k data points, a 2 s relaxation delay, 3.3331 ppm of spectral width, 16.38 s of acquisition time, by placing the offset on 7.7 ppm. The spectrum was Fourier transformed with exponential multiplication, followed by phase and baseline correction, and the implemented AU program humpcal was used to determine the hump (width of the $CHCl_3$ signal at the height of the carbon satellites (0.55%) and at one fifth thereof (0.11%) of the $CHCl_3$ signal).

*Sensitivity and water suppression tests* were applied using a QC and the following parameters were set for NMR analysis 1D version of Nuclear Overhauser effect spectroscopy ($^{1}$H NOESY-1D; noesygppr1d) with pre-saturation, during relaxation delay and mixing time, and spoil gradients. Eight scans with 4 initial dummy scans were recorded where a 90$^{o}$ pulse is applied with a relaxation delay of 10 s and a acquisition time of 2.66 s with spectral width 20.6 ppm with 65k data points. The offset was optimised around 4.7 ppm by applying a saturation power. The spectrum was Fourier transformed with exponential multiplication, followed by phase and baseline correction, and the implemented AU sino and suppcal programs were used to obtain the signal-to-noise ratio (noise: 7.00-6.00 ppm, and signal: 5.45-5.25 ppm) of the anomeric proton of sucrose and the water suppression (by determining the width at 50% and 10% height of the water signal), respectively.

*Flow cell profiles* of all samples were obtained by acquiring a one-scan 1D Gradient Echo for the gradshim-procedure experiment (imgegp1d) using 2 µs flip angle pulse and pre-processed on-the-fly by a macro including Fourier transformation, followed by magnitude calculation.

The $^{1}$H NMR experiment used for acquiring metabolomics data was *$^{1}$H NOESY-1D* (noesygppr1d; 90$^{o}$ pulse is applied with a relaxation delay of 10 s and a acquisition time of 2.68 s; the 90$^{o}$ pulse and the pre-saturation power is adjusted automatically for each sample using the pulsecal algorithm; receiver gain 128; spectral width 30 ppm; 98k time domain). The transmitter frequency offset was manually optimized on the first sample for optimum pre-saturation. The number of scans was tested and optimized to 32, with 16 initial dummy scans, leading to a total of 10 min 11 s of acquisition time per experiment. $^{1}$H NMR NOESY-1D spectra were processed using the AU program apk0.noe which includes Fourier transformation with exponential multiplication, zero order phase correction, baseline correction, and calibration of the chemical shift axis to TSP = 0 ppm.

For a sub-set of spectra, a homonuclear $^{1}$H *J*-Resolved 2D correlation was acquired with pre-saturation during relaxation delay (*J-Res-2D*, jresgpprqf) recording 16 scans with 16 initial dummy scans, 4k data points for 48 increments, a spectral width 12.66 ppm (F2: chemical shift axis) and 0.0868 ppm (F1: spin-spin coupling axis). The pre-processing was done automatically after spectral acquisition including spectral referencing, apodization and transformation in 2 dimensions, tilting, symmetrisation, and baseline correction.

**SUPPLEMENTARY FIGURE**

**Supplementary figure 1** Linearity of NMR signals acquired with the present protocol, over a range of concentrations. Standard compounds were added to urine in 1:3 dilutions ($n$ = 8): hippuric acid ($\delta_H$ = 7.750 +/- 0.050 ppm), succinic acid ($\delta_H$ = 2.335 +/- 0.035 ppm), alanine ($\delta_H$ = 1.400 +/- 0.050 ppm), and (±)-glucose (alpha-, $\delta_H$ = 5.150 +/- 0.050 ppm; beta-, $\delta_H$ = 4.563 +/- 0.037 ppm).