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NMR for sample quality assessment in metabolomics

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ABSTRACT

The EU Framework 7 project SPIDIA was the occasion for development of NMR approaches to evaluate the impact of different pre-analytical treatments on the quality of biological samples dedicated to metabolomics. Systematic simulation of different pre-analytical procedures was performed on urine and blood serum and plasma. Here we review the key aspects of these studies that have led to the development of CEN technical specifications, to be translated into ISO/IS in the course of the EU Horizon 2020 project SPIDIA4P. Inspired by the SPIDIA results, follow-up research was performed, extending the analysis to different sample types and to the different effects of long-term storage. The latter activity was in conjunction with the local European da Vinci Biobank. These results (which partially contributed to the ANNEX of CEN/TS 16945"MOLECULAR IN VITRO DIAGNOSTIC EXAMINATIONS - SPECIFICATIONS FOR PRE-EXAMINATION PROCESSES FOR METABOLOMICS IN URINE, VENOUS BLOOD SERUM AND PLASMA") are presented in detail.

Introduction

Metabolomics deals with the -omic analysis of low molecular weight metabolites present in biological samples [1,2]. Metabolomic studies applied to biomedical research generally employ biofluids, tissues and cells of human or animal origin. Among them, urine and blood are relatively easy to obtain and thus become particularly relevant in studies aimed at following disease diagnosis, progression and individual response to treatment [3]. According to their nature, the chemical composition and chemical variability of urine and blood are widely different. The urine metabolome is strongly influenced by environmental factors such as food and drug intake [4], while the molecular content of blood is strongly controlled by homeostasis [5].

The preservation of the chemical composition (in terms both of nature and concentration of metabolites) of the 'original' metabolome of a sample during the entire workflow that ends with metabolomic analyses represents a key factor in ensuring highly accurate and reproducible analyses. A typical metabolomic workflow is made up of the pre-analytical phase, including the steps of sample collection, processing, transport and storage, and the analytical phase, including sample preparation, according to the requirements of the specific technique, usually Mass Spectrometry (MS) or Nuclear Magnetic Resonance (NMR), and instrument measurements, (Fig. 1). Both phases comprise several steps that may influence the composition of the sample metabolome, and validated and detailed procedures are required to reduce chemical changes in the metabolome as far as possible. This aspect has received particular attention in recent years [6,7] and has led to the development of validated Standard Operating Procedures (SOPs) for the collection, handling, storage and analysis of the most commonly used biofluid samples dedicated to metabolomic applications.

In particular, within the EU 7th Framework Programme (FP7) SPIDIA project, NMR approaches to evaluate the impact of different pre-analytical treatments on the quality of urine and blood serum and plasma samples for metabolomics were developed, leading to the production of European Committee for Standardization (CEN) technical specifications (TS). Here, we summarize the key aspects of that study and illustrate follow-up systematic simulations that we have performed to extend the range of possible pre-analytical practices, including the role of long-term storage. All these results are based on NMR analyses, which provide highly reproducible data and permit fast acquisition, two essential features in order to compare results obtained on several different aliquots undergoing different pre-analytical treatments [1,8].

Abbreviations: CEN, European Committee for Standardization; CPMG, Carr-Purcell-Meiboom-Gill; D, Double; EDTA, Ethylenediaminetetraacetic acid; HDL, high-density lipoproteins; HMDB, human metabolome database; LDL, low-density lipoproteins; m, multiple; MS, mass spectrometry; NMR, nuclear magnetic resonance; NOESY, nuclear Overhauser effect spectroscopy; s, singlet; SMDB, serum metabolome database; t, triplet; TMSP, sodium trimethylsilyl propionate; TS, technical specifications; UMDB, urine metabolome database; VLDL, very low-density lipoproteins

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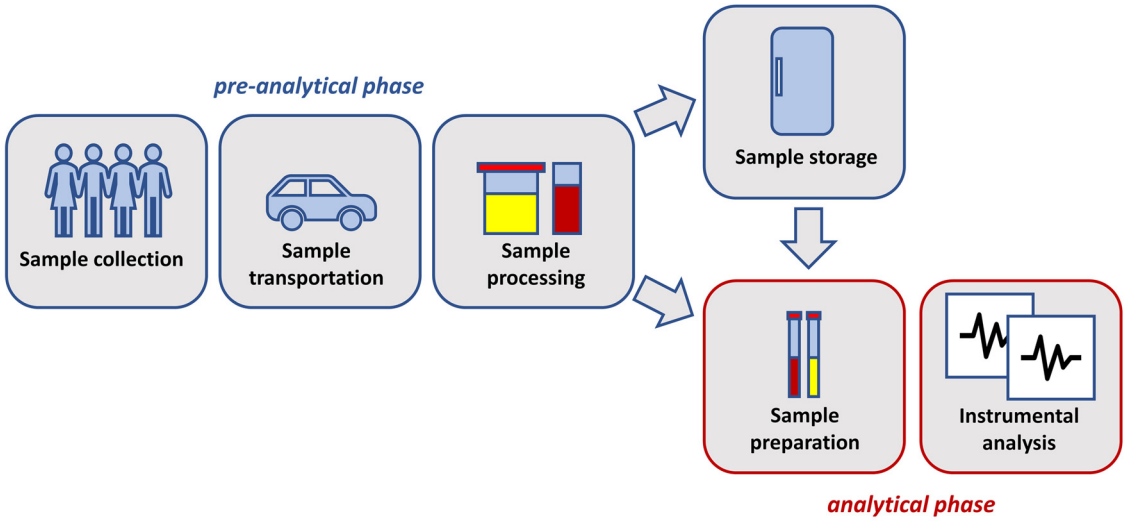


Fig. 1. Typical metabolomic workflow scheme. The blue and red boxes show the main steps of the pre-analytical and analytical phase, respectively. The storage step is avoided whenever samples are immediately analyzed.

Material and methods

Pre-analytical treatment of urine and blood

Urine samples were obtained from healthy subjects. Donors collected the first urine of the morning under fasting conditions. Each sample was kept no more than 2 h at 2–8 °C[1] before further analysis (see Results section). Every urine sample was divided into different aliquots for independent treatment. The processing phase, made up of mild centrifugation (2500 × *g*, 5 min, 4 °C) followed by filtration, using 0.2 µM filters, was used to remove cells and large particles, according to CEN document CEN/TS 16945:2016 "Molecular in vitro diagnostic examination - Specifications for pre-examination processes for metabolomics in urine, venous blood serum and plasma" [9]. The choice of the vial for sample collection/storage and, of the filter, is critical due to the risk of contamination by exogenous molecules (e.g. filters are often coated with compounds such as glycerol). Therefore, plastics and filters should be pre-tested with waste biofluids or physiological solutions, to exclude the possibility of contaminant release. Sarsted Filtropur S 0.2 filters were employed.

The resulting samples are here indicated as processed or P urine. They were also analyzed in their untreated form, without applying any processing method before the NMR sample preparation (raw, R urine).

Serum samples were taken from the repository of the da Vinci European Biobank [10]. Blood was collected fasting in the morning into sterile tubes and was allowed to clot in an upright position for 30–60 min at room temperature (RT). Each sample was centrifuged at 1500 xg for 10 min at RT. Serum was recovered, divided into different aliquots and immediately frozen at −80 °C [6]. The data on plasma samples refer to previously published work [11].

NMR sample preparation

Samples for NMR analysis were prepared according to standard procedures [1,12]. Frozen samples were thawed at RT and shaken before use. A volume of 750 µL of each urine aliquot was centrifuged at 14,000 xg for 5 min, and 630 µL of the supernatant were added to 70 µL of potassium phosphate buffer (1.5 M K_2HPO_4, 100% (v/v) ^2H_2O, 2 mM NaN_3, 5.8 mM sodium trimethylsilyl [2,2,3,3-$^2H_4$] propionate (TMSP). The pH was adjusted to the final value of 7.4 using 1 M HCl). A volume of 350 µL of each aliquot of serum or plasma was added to 350 µL of sodium phosphate buffer (70 mM Na_2HPO_4, 20% (v/v) ^2H_2O, 6.1 mM NaN_3, 4.6 mM TMSP. The pH was adjusted to the final value of 7.4 using 1 M HCl). The mixtures were homogenized by vortexing for 30 s and a total of 600 µL of each mixture was transferred into 5.00 mm NMR tube (Bruker BioSpin srl) for analysis.

NMR spectra acquisition

^{1}H NMR spectra for all samples were acquired using a Bruker 600 MHz spectrometer (Bruker BioSpin srl) operating at 600.13 MHz proton Larmor frequency and equipped with a 5 mm PATXI ^{1}H-^{13}C-^{15}N and ^{2}H-decoupling probe including a z axis gradient coil, an automatic tuning-matching (ATM) and an automatic and refrigerated sample changer (SampleJet). A BTO 2000 thermocouple served for temperature stabilization at the level of approximately 0.1 K at the sample. Before measurement, samples were kept for 5 min inside the NMR probehead for temperature equilibration, at 300 K for urine or 310 K for plasma/serum samples, according to established procedures.

For each urine sample, a monodimensional ^{1}H NMR spectrum was acquired with water peak suppression using a standard NOESY (Nuclear Overhauser Effect Spectroscopy) pulse sequence [13] with 32 scans, 65,536 data points, spectral width of 12,019 Hz, an acquisition time of 2.7 s, a relaxation delay of 4 s and a mixing time of 0.01 s.

For each blood serum/plasma sample, two monodimensional ^{1}H NMR spectra were acquired with water peak suppression and different pulse sequences that allowed the selective observation of different molecular components. (i) A standard NOESY pulse sequence [13], using 32 scans, 98,304 data points, a spectral width of 18,028 Hz, an acquisition time of 2.7 s, a relaxation delay of 4 s and a mixing time of 0.01 s. This pulse sequence was designed to obtain a spectrum in which both signals of metabolites and high molecular weight molecules (lipids and lipoproteins) are visible. (ii) A standard CPMG (Carr-Purcell-Meiboom-Gill) pulse sequence [14], using 32 scans, 73,728 data points, a spectral width of 12,019 Hz and a relaxation delay of 4 s. This pulse sequence was designed for the selective observation of free small molecule components in solutions containing macromolecules.

[1] The temperature values concerning the pre-analytical phase are provided in Celsius rather than in Kelvin for consistency with the developed standards [9].

Spectral processing

Free induction decays were multiplied by an exponential function equivalent to a 0.3 Hz line-broadening factor before applying Fourier transform. Transformed spectra were automatically corrected for phase and baseline distortions and calibrated. All urine and blood derivative spectra were calibrated to the reference signal of TMSP at 0.00 ppm, and to the glucose doublet at 5.24 ppm, respectively, using TopSpin 3.5 (Bruker BioSpin srl).

Each spectrum in the region 10.00 – 0.2 ppm was segmented into 0.02 ppm chemical shift bins, and the corresponding spectral areas were integrated using the AMIX software. The area of each bin was normalized to the total spectral area, calculated with exclusion of the water regions (4.50–5.20 ppm).

Spectral analysis

The relative concentrations of the various metabolites in different spectra were calculated by integrating the corresponding signal areas in defined spectral range. Signal identification was performed using an in-house NMR spectral library of pure organic compounds, public databases such as the Human Metabolome Database (HMDB, www.hmdb.ca), stored reference NMR spectra of metabolites, spiking NMR experiments and literature data. Matching between the present NMR data and databases was performed using the AMIX software (Bruker BioSpin srl). Signals that could not be assigned were labeled as unknown (Unk). Significance in metabolite concentration changes was assessed using the non-parametric Wilcoxon-Mann-Whitney test; a p-value < 0.05 was considered significant.

Results and discussion

Urine

Urine is one the most frequently used biofluids in metabolomic studies. As well as being easy to obtain in large volumes and almost free of interfering macromolecules (proteins or lipids), its chemical complexity makes urine particularly suitable for metabolomic investigations. As a liquid waste material of the organism, urine typically contains breakdown products from foods, beverages, drugs, cosmetics, environmental contaminants, endogenous waste metabolites and microbial by-products [4]. Thus, its composition is strongly affected by daily variations. The Urine Metabolome Database [15–17] (UMDB: http://www.urinemetabolome.ca) currently lists about 4,200 metabolites found in human urine identified using all current technologies. About 2,600 of these molecules are endogenous while about 3,300 are exogenous metabolites, with a number of compounds in common between the two classes. Among all these metabolites, about 400 small molecules have been detected by ^{1}H NMR in urine, although not simultaneously in one sample.

In Fig. 2, a few representative ^{1}H NOESY NMR spectra of urine samples from healthy subjects are reported. As clearly visible, the NMR spectra are highly complex, comprising thousands of resolved signals coming from about 200 different main metabolites detected simultaneously within a few minutes of experimental acquisition. Despite the strong daily variability, the presence of an individual metabolomic phenotype, i.e. a chemical signature typical for each individual [18,19], which is stable over a long period of time [20,21], makes urine analysis extremely suitable as an approach to precision medicine with the possibility of monitoring and following individual variations during disease progression or in response to drug administration [22,23] or nutrition and lifestyle interventions [24]. Moreover, in the past years urinary metabolomics has contributed to the characterization of the metabolic signature of diseases for diagnostic and prognostic purposes and to the understanding of its biochemical cause [25–28].

Background information

In 2011, the presence of human or bacterial cells was identified as a potential source of alteration of the urine metabolic profiles during the various steps of the pre-analytical phase (Fig. 1), leading to the development of ad hoc SOPs concerning samples for metabolomic studies [6]. Cells present in urine may lyse, especially upon sample freezing (due to the formation of water crystals), or high-speed centrifugation (required to remove particulate matter during NMR sample preparation), thus releasing their soluble constituents, including metabolites and proteins, into the samples and affecting their metabolomic profiles. The removal of cells in fresh urine samples was strongly suggested through the combined use of mild pre-centrifugation and filtration. These results are in agreement with those of other groups and are reviewed in a joint publication, which summarizes the main procedures to be adopted [7]. Moreover, these procedures are beginning to be implemented in some biobanks [29] and constitute a key part of the CEN document CEN/TS 16945:2016 “Molecular in vitro diagnostic examination - Specifications for pre-examination processes for metabolomics in urine, venous blood serum and plasma” [9].

To reiterate the importance of cell removal for the stability of the metabolomic profiles of urine samples, an independent validation of the proposed pre-analytical procedures was performed. To this end, three urine samples, characterized by different cellular contents (i.e. UR-03 - very high; UR-09 – modest; UR-07 – low) were collected and treated as summarized in Fig. 3A. The cell content was estimated from visual inspection of the pellet present after mild pre-centrifugation in P samples. The behavior of P and R urine metabolic profiles over time was followed by acquiring NMR spectra at different time points after sample collection (0, 1 h, 2 h, 4 h, 6 h, 8 h), up to a maximum of 20–24 hours, indicated as overnight (ON). Samples were kept at 4 °C in between spectra. As shown in Fig. 3B, the profile of UR-07, with low cell content, was very stable over time, both in P and R urine; in contrast, the profiles of UR-03 and UR-09 in R samples were progressively unstable over time compared to their P counterparts. The importance of the processing step clearly depends on the cellular component content of the analyzed samples and may therefore be very different from one individual to another.

It was confirmed that the extent of changes in metabolic profile between P or R samples are essentially attributable to marked chemical shift variations over time in signals that reflect drifts in pH, as monitored via the Hε1 signal of histidine, Fig. 3C, a very sensitive indicator of pH changes [6]. For samples containing a large number of cells, the pH changes occur earlier and are larger.

New results

Sources of alteration of the urinary metabolomic profiles

In addition to the presence of cells, two other sources of alteration of the urine metabolic profiles were identified, namely the occurrence of chemical and enzymatic reactions [6]. Indeed, although the protein content of urine is low in healthy subjects, non-negligible enzymatic activity due to human and bacterial enzymes has been measured [6,30]. A detailed investigation of the consequences of the presence of these influencing factors in urine was therefore performed.

In the independent experiment summarized in the background section above, in addition to the marked chemical shift variation occurring in R samples, changes in intensity of some signals as well as appearance of resonances attributable to new metabolites, were detected over time in the spectra of both P and R urine (Fig. 4). To interpret the detected metabolite concentration changes, urine samples from 6 different subjects were collected and processed, and then used to prepare NMR samples. The metabolic profile of each was followed for 24 h, acquiring an NMR spectrum every 2 h; samples were kept at 4 °C in between spectra. Table 1 summarizes the observed variations in metabolite concentrations in the analyzed spectra. These variations

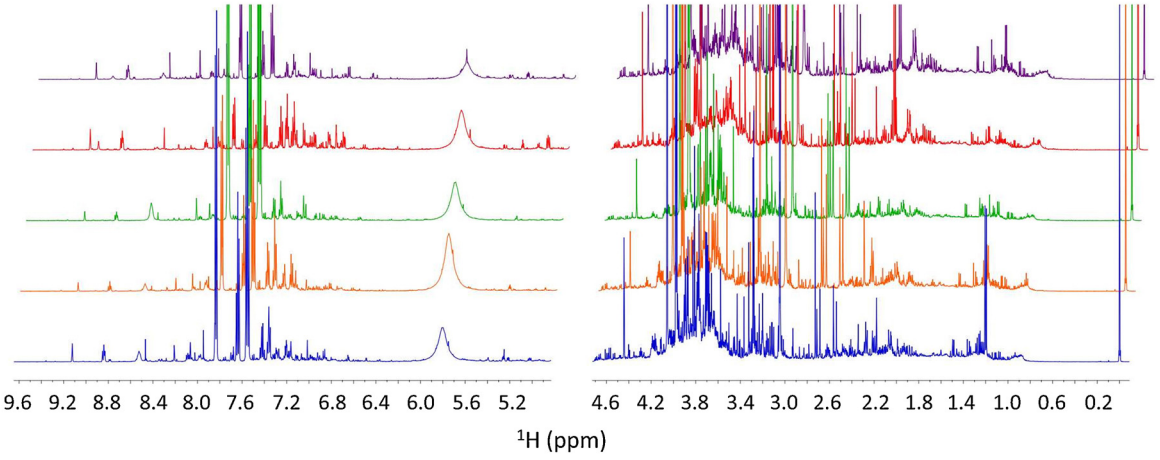


Fig. 2. ^{1}H NOESY NMR spectra of urine samples from 5 different healthy subjects. Left panel: Downfield (9.6–5.0 ppm); right panel: upfield (4.6–0.0 ppm).

involved metabolites that are always present in urine (100% occurrence) of healthy subjects; in particular, acetate, succinate and creatine increased over time, while pyruvate, creatinine and 2-oxoglutarate decreased. In addition, other changes were strongly individual-dependent, involving metabolites (e.g. ascorbate and other unassigned signals) whose occurrence depended on the individual and' daily' chemical composition of the different urine samples.

Moreover, patterns of changes were detected that correlated with the presence or absence of certain urinary metabolites. In particular, the presence/absence of ascorbate strongly influenced the changes over time (Fig. 5), as detailed below:

a) High ascorbate concentration: these samples were characterized by a decrement of ascorbate signal intensities over time and the appearance/increment of new signals in the spectra (collectively indicated as Unk6, 4.48 (t), 4.53 (s), 4.56 (s), 4.59 (s), 4.63 (s) ppm).
b) Absence of ascorbate: these samples were characterized by the appearance and strong increment of a new unknown signal, Unk5, at 4.05 ppm (t).
c) Medium-low ascorbate concentration: these samples were characterized by the joint presence of both types of change (a and b), i.e. decrement of ascorbate levels, slight increments of the signals associated to Unk6, and slight increment of Unk5 signal at 4.05 ppm.

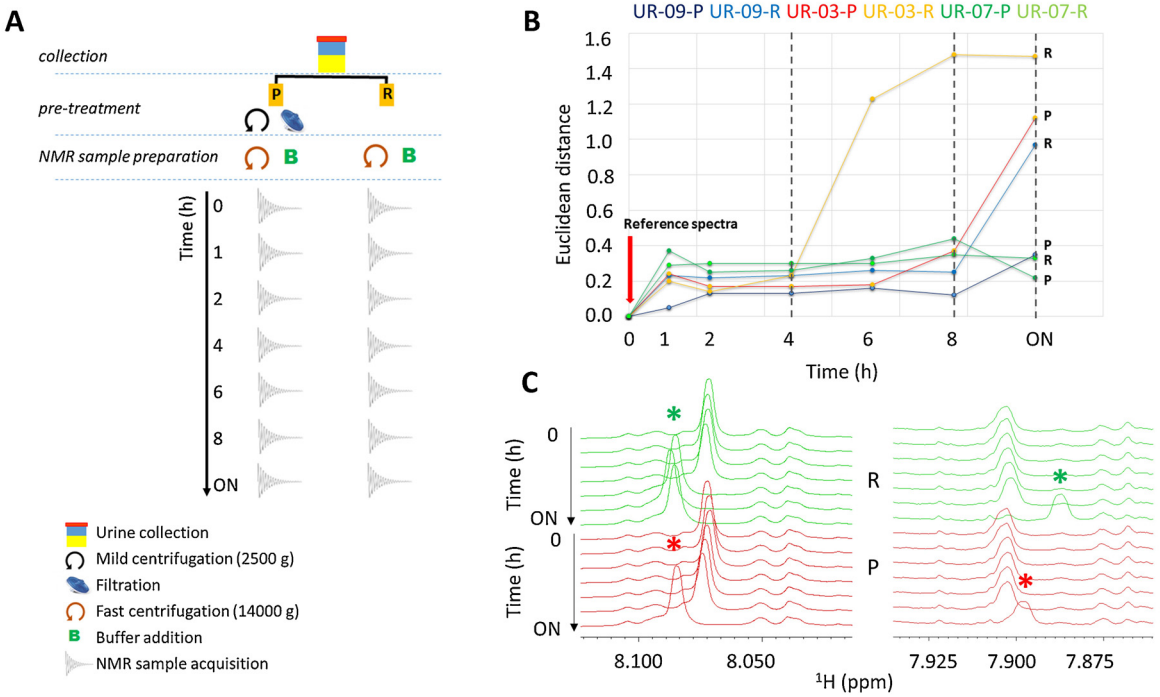


Fig. 3. Stability of the urine molecular profiles over time: the impact of the processing phase. A) Experimental design; B) The Euclidean distance between the spectra obtained at each time point and the spectrum at time T0 (i.e. T1 vs T0, T2 vs. T0, …) for each sample. A larger distance value indicates a low similarity between the compared spectra; C) Hε1 and Hδ1 signals of histidine; * indicates the position of the signal after pH variation. Processed urine samples with a mild centrifugation and a filtration are indicated as P (red traces); raw urine samples as R (green traces). ON, overnight.

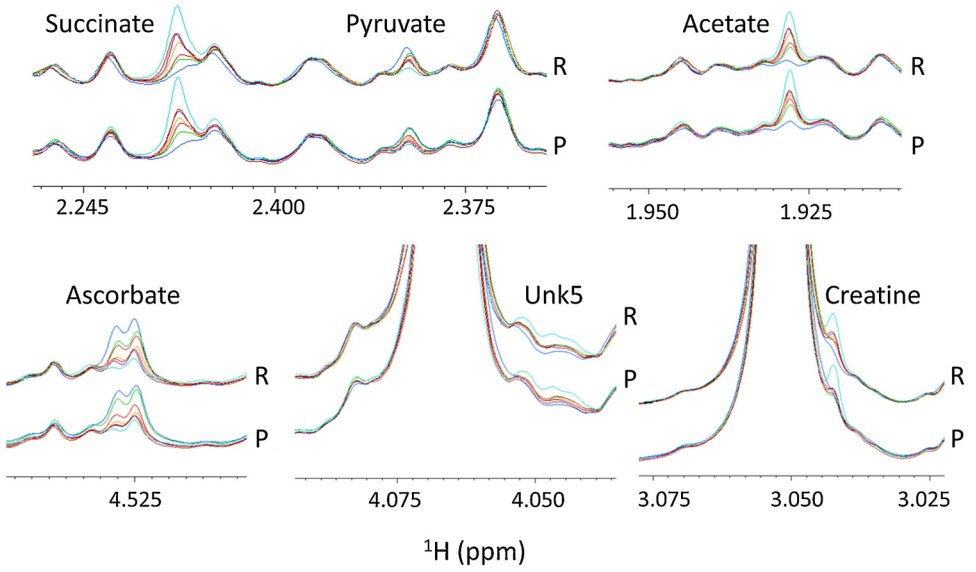


Fig. 4. Stability of the R and P urine molecular profiles over time: effects independent from the processing phase. Blue traces: T0; green traces: T = 1 h; red traces: T = 2 h; orange traces: T = 4 h; dark red traces: T = 6 h; purple traces: T = 8 h; cyan traces: T = ON.

Table 1
List of metabolites for which concentration levels changed over time in urine NMR spectra. The metabolite name along with the corresponding HMDB code is reported, except for those signals corresponding to unassigned molecules, which are labeled as Unk. Most of the signals have an occurrence of 100% in the metabolomic urine NMR profiles [4]. The occurrence of unknown degradation products cannot be defined (*). Signal chemical shift and multiplicity (singlet, s; doublet, d; multiplet, m; triplet, t) of all detectable signals for a given metabolite are also listed together with the observed trend in concentration changes over time: ↑ increment, ↓ decrement.

Metabolite	HMDB	Occurrence (%) presence/ absence in the spectra	Chemical shift (ppm)	Changes
Acetate	HMDB0000042	100	1.92 (s)	↑
Unk2	–	70	2.17 (s)	↑
Unk3	–	70	2.18 (s)	↓
Pyruvate	HMDB0000243	100	2.38 (s)	↓
Succinate	HMDB0000254	100	2.40 (s)	↑
2-Oxoglutarate	–	100	2.44 (t)	↓
			3.00 (t)	
Creatine	HMDB0000064	100	3.04 (s)	↑
			3.93 (s)	
Creatinine	HMDB0000562	100	3.05 (s)	↓
			4.06 (s)	
Unk4		-*	3.35 (m)	↑
Ascorbate	HMDB0000044	14	3.74 (m)	↓
			4.01 (m)	
			4.51 (d)	
Unk5		-*	4.05 (t)	↑
Unk6	–	-*	4.48 (t)	↑
ascorbic acid oxidation by-products			4.53 (s)	
			4.56 (s)	
			4.59 (s)	
			4.63 (s)	
Unk7	–	100	5.61 (d)	↓
Urea	HMDB0000294	100	5.80 (s)	↓

To investigate the nature of these changes, variations in the urine metabolome of four subjects were followed over time both under an aerobic atmosphere and under an inert atmosphere (N_2), as described in Fig. 6. The latter condition was achieved by bubbling through the solution a nitrogen flux for 30 min. During the intervals between experiments, the samples were kept at 4 °C and NMR spectra for each sample were acquired at 300 K every 2 h for a total of 24 h. The results are shown in Fig. 6. Under N_2, the urine metabolic profiles were more stable over time. Under these conditions, most of the metabolite concentration changes were completely quenched or strongly reduced. In particular, the changes in acetate, pyruvate, succinate, 2-oxoglutarate, ascorbate and Unk6 were found to be strongly oxygen-dependent. In contrast, creatine and creatinine levels were not influenced by oxygen depletion. The results can be explained by considering urine as a highly complex mixture of metabolites, free to react with one another and with atmospheric dioxygen, their reactivity dependent on their chemical properties. Under aerobic conditions, oxidation by dioxygen occurs, leading to the accumulation of the reaction products and to the depletion of the reagents over time, strongly affecting the original metabolome of the samples.

Changes associated with the presence of ascorbate can be attributed to its antioxidant properties; when present in urine, it exerts a protective action against dioxygen, being oxidized to dehydroascorbic acid, Table 2 (not detected in the spectrum), and further oxidative products up to oxalic acid [31]. The Unk5 discussed above appears to be an indicator of the reactivity associated to the presence of ascorbic acid, although it does not correspond to any of its oxidation products, but may correspond to the reduction products of other metabolites. Pyruvate can undergo an oxidative decarboxylation leading to the formation of acetate. In the same way, 2-oxoglutarate can react to produce succinate, Table 2. Succinate increases with time (average increase of about 70%). This variation can be only partially attributed to the oxidation of 2-oxoglutarate, whose signal showed an average decrease of only 7.5%. Succinate can be also the final product of other reactions occurring in urine, including enzymatic reactions. Indeed, a number of changes in metabolite levels can also be explained as a direct consequence of the enzymatic actions of proteins present in urine [6]. The increase of creatine and decrease of creatinine is at least partially due to a hydrolytic conversion of creatinine (creatine kinase activity or pH variation over time); a residual activity of bacterial urease and of isocitrate lyase could cause the decomposition of urea and the increase in succinate, respectively [6].

The list of changes is far from exhaustive, due to the inherent

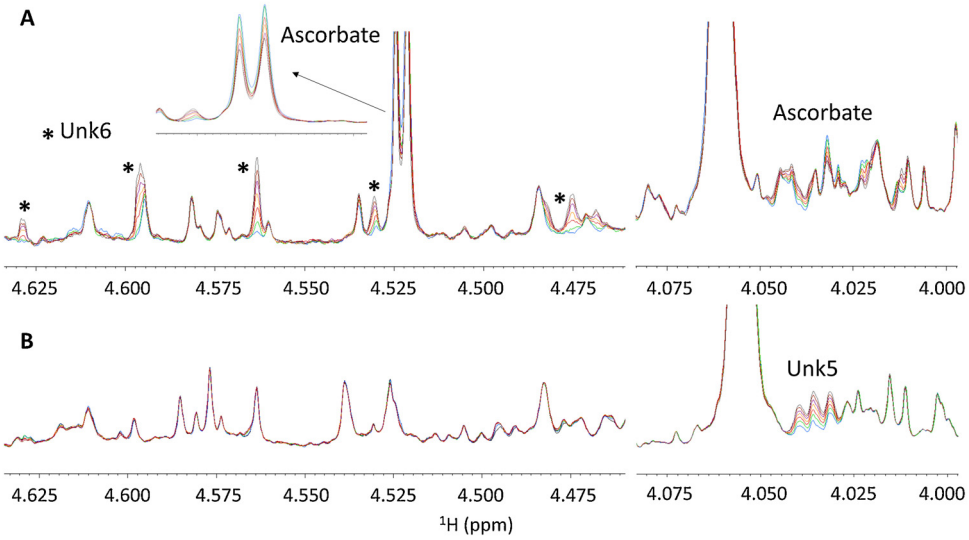
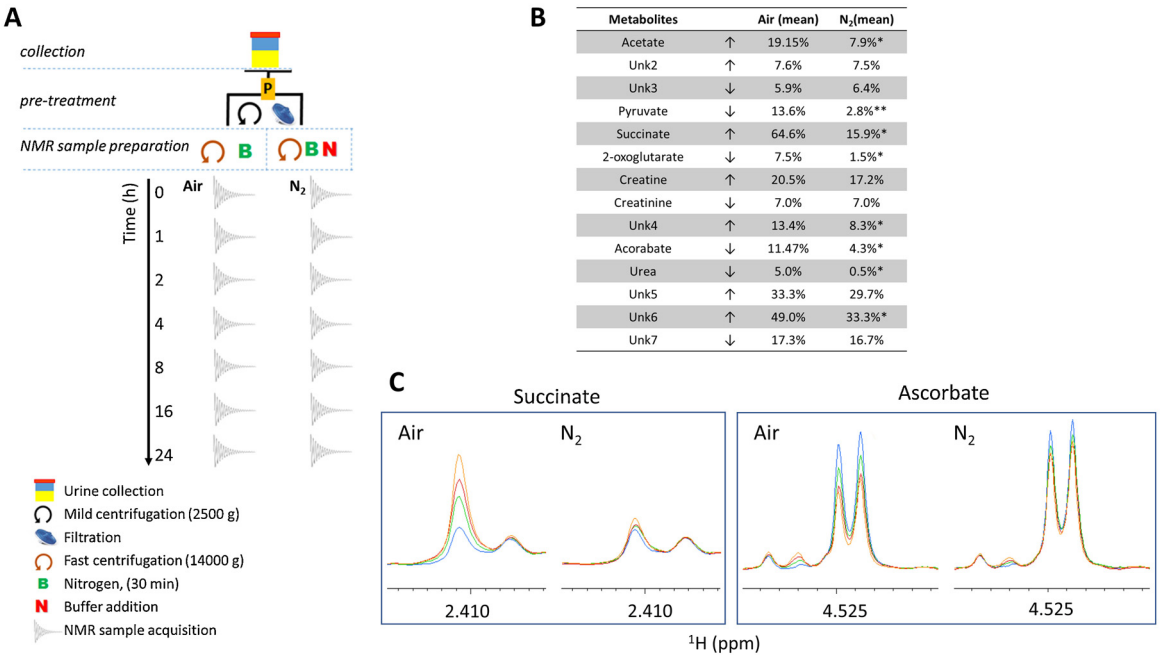


Fig. 5. Patterns of changes visible in the NMR spectra over time according to the presence/absence of ascorbic acid. A) High ascorbic acid concentrations. B) Absence of ascorbic acid. Blue traces: T0; green traces: T = 2 h; red traces: T = 4 h; orange traces: T = 8 h; purple traces: T = 12 h; dark red traces: T = 12 h; gray traces: T = 20 h.



Metabolites		Air (mean)	N_2(mean)
Acetate	↑	19.15%	7.9%*
Unk2	↑	7.6%	7.5%
Unk3	↓	5.9%	6.4%
Pyruvate	↓	13.6%	2.8%**
Succinate	↑	64.6%	15.9%*
2-oxoglutarate	↓	7.5%	1.5%*
Creatine	↑	20.5%	17.2%
Creatinine	↓	7.0%	7.0%
Unk4	↑	13.4%	8.3%*
Acorabate	↓	11.47%	4.3%*
Urea	↓	5.0%	0.5%*
Unk5	↑	33.3%	29.7%
Unk6	↑	49.0%	33.3%*
Unk7	↓	17.3%	16.7%

Fig. 6. A) Experimental design; B) List of metabolites for which the concentration levels change over time (over 24 h) in urine NMR spectra in aerobic (Air) and inert atmosphere (N_2). C) Succinate and ascorbate changes over time (24 h) in normal and inert atmospheres. The changes of both metabolites were strongly quenched under inert atmosphere. Blue traces: T0; green traces: T = 8 h; red traces: T = 16 h; orange traces: T = 24 h.

Table 2
Midpoint Reduction Potential at pH 7 ($E_{m,7}$) for some key reactions. In urine, we observe the corresponding oxidation reaction.

Reaction	Midpoint Reduction Potential at pH 7 ($E_{m,7}$)
Acetate + CO_2 → Pyruvate	−0.7 V
Succinate + CO_2 + $2H^+$ → 2-Oxoglutarate + H_2O	−0.6 V
Dehydroascorbic acid→ Ascorbic acid	+0.06 V

chemical variability of urine composition and the intrinsic low sensitivity of NMR that does not allow one to observe metabolites present in low concentration. Nevertheless, it is possible to conclude that chemical and enzymatic redox reactions are the main factors inducing changes in concentration levels of urinary metabolites. These effects cannot be avoided but can be reduced by maintaining the samples at low temperatures throughout the analytical and pre-analytical processes and reducing exposure to air, as proposed in [6]. The presence of azide in the NMR buffer does not influence the extent of changes in the metabolic profiles of P urine.

The observed changes also operate during the analytical phase, which occurs at RT. NMR spectra are generally acquired in automatic mode, using a sample changer that can manage hundreds of samples; the time lapse between the acquisition of the first and the last sample can be up to a few days, making the effects of both reactions very relevant. To minimize these changes the use of a refrigerated sample changer where the samples can be stored at 4–6 °C before acquisition is strongly recommended. Instead, the total effective measurement time is relatively short (5–15 min) and no major changes occurred during it.

Effect of storage

The contribution of the chemical and enzymatic content of P urine to changes in the metabolic profiles was also investigated during short- (4 weeks) and long-term storage (5 years). In general, fresh urine samples are collected and stored at −80 °C. They may be stored from few weeks up to several years before NMR sample preparation and spectra acquisition. It is very important for the accuracy of the analysis that the metabolic profiles of the samples are maintained during storage.

To evaluate the metabolic changes occurring during storage, urine samples from different healthy subjects were collected and processed to remove cellular components. Each P urine sample was divided into several aliquots which were stored at −80 °C. NMR spectra for each were acquired at different time points using different aliquots: time 0 (fresh urine) and after 1, 2, 3 and 4 weeks of storage for short-term analysis, and after 3, 4 and 5 years of storage for long-term analysis. The results showed that during both short and long-term storage urine samples are stable over time, Fig. 7. At −80 °C, both chemical and enzymatic reactions are strongly slowed down leading to a very good preservation of the samples even after 5 years. When the 5-year storage-aliquots are kept at 4–6 °C for 48 h after thawing, the spectra again showed the redox changes described above due to oxygen exposure and enzymatic activity (Fig. 7). Importantly, the profiles of R urine samples were less stable over long-term storage than those of P urine. These samples, besides showing concentration changes in some metabolites, displayed signal shifts due to pH variation induced by the presence of cells and thus for metabolomics purposes their analysis was more problematic.

The effects of freeze/thaw cycles on the metabolomic profiles were also characterized. Freeze/thaw cycles induce the same types of degradation effects described above for air exposure and enzymatic activity when the samples are brought and kept at RT. Thus, it is imperative to divide the samples into different small volume aliquots in order to avoid repeated opening and thawing of the stored samples.

In summary, the long-term storage experiment demonstrated the strong reproducibility of the NMR method for urine samples that have been properly handled, and further confirm the part of the Annex of CEN where it was generically demonstrated that stability during long-term cryo-conservation is strongly related to appropriate pre-analytical treatments [9].

Blood serum and plasma

Both plasma and serum are aqueous solutions containing metabolites and macromolecules, including proteins, lipids and lipoproteins. As a primary carrier of small molecules, blood plasma composition is strictly regulated and less affected by daily variations compared to urine. Of course, blood composition is altered by the presence of organism disfunctions or pathological states [5]. As a consequence, both blood derivatives are among the preferred biofluids for metabolomic studies aimed at disease characterization, early diagnosis, prognosis or patient stratification [32–36]. A complete list of metabolites that have been detected in human serum is available in the Serum Metabolome Database (http://www.serummetabolome.ca/) [15–17], which currently contains 25,373 detectable metabolites, most of which are endogenous molecules (23,916 endogenous and 3,380 exogenous, with some metabolites in common). Among all these, the NMR detectable fraction of the serum/plasma metabolome generally consists of about

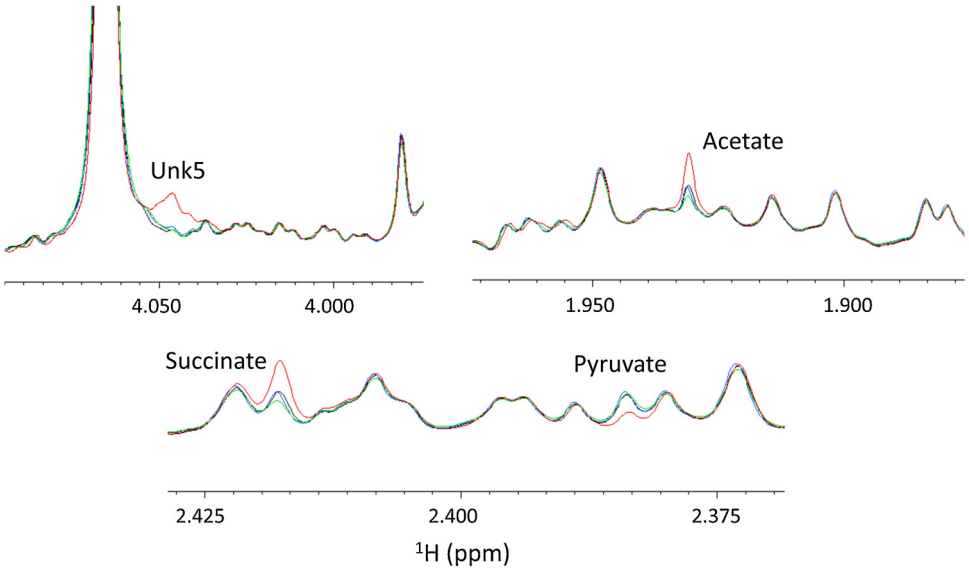


Fig. 7. Long-term storage at −80 °C. The metabolic phenotype of urine samples was followed over 5-year storage. Black trace: T0; green trace: 4 year-storage; blue trace: 5 year-storage; red traces: 5 year-storage + 48 h at 4–6 °C.

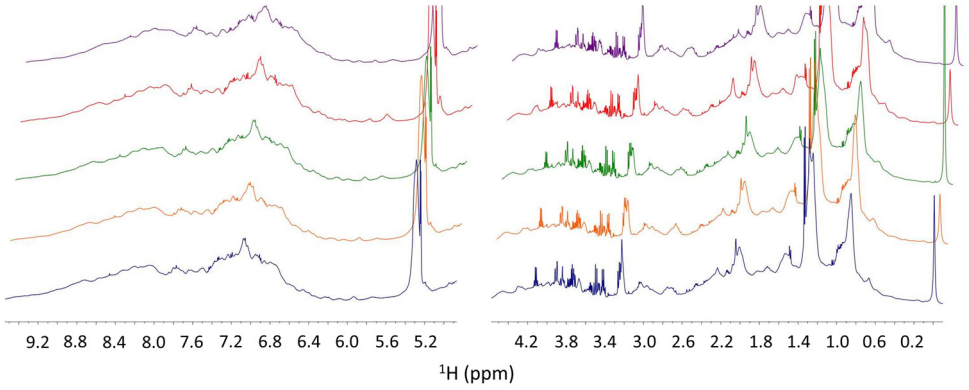


Fig. 8. ^{1}H NOESY NMR spectra of serum samples from five different healthy subjects. Left panel: Downfield (9.5–5.0 ppm); right panel: upfield (4.5–0.0 ppm). The broad features detected below the sharp metabolite signals are due to the high mass protein and lipid components. For better peak visualization, a 4x vertical expansion is used for the downfield region.

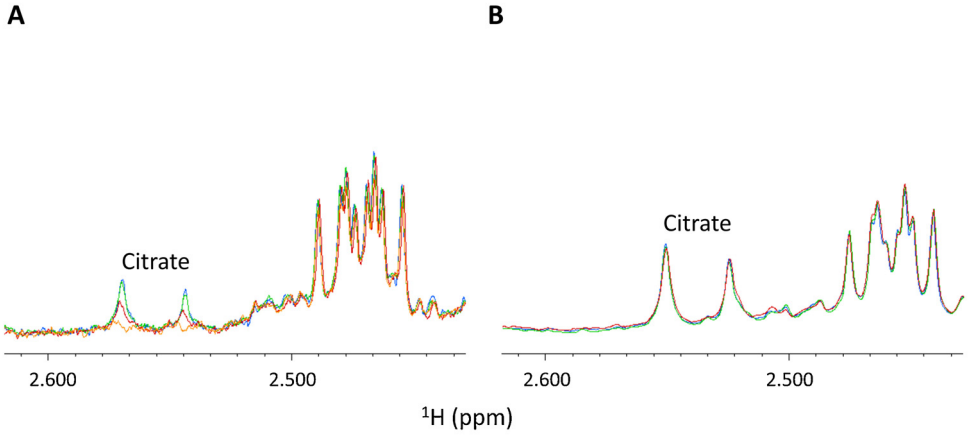


Fig. 9. A) Citrate levels over time (12 h). The metabolic phenotype of serum samples was followed over 12 h; blue trace: T0; green trace: T = 4 h; red trace: T = 8 h; orange trace: T = 12 h. B) Citrate levels over long-term storage. The metabolic phenotype of serum samples was followed over 5-year storage; blue trace: T0; green trace: 4 year-storage; red trace: 5 year-storage.

50 main compounds that can be observed and quantified (Fig. 8).

Human blood is also rich in lipids of chemically distinct classes, mainly fatty acids, cholesterol and triglycerides, that are additional potential candidate biomarkers. The lipid fraction is the subject of systematic study in another -omics science, lipidomics [37]. Beyond the scope of this review, but worthy of note, the Bruker IVDr (B.I) tool for automatic analysis of metabolites present in biofluids has been recently released by Bruker BioSpin. This platform also generates a detailed lipoprotein subclass analysis (B.I.-LISA) of plasma or serum samples, providing more than 100 lipoprotein-related parameters in one measurement under full automation.

In terms of small molecules, the compositions of plasma and serum are very similar; however, for both NMR- and MS-based metabolomics, serum is preferred over plasma as the anticoagulant agents interfere with the small molecule composition [38]. Nevertheless, many samples stored in biobanks are plasmas. The pre-analytical SOPs for plasma must include the selection of the best anticoagulant, each of which has its own drawbacks. Heparin might give rise to broad resonances underneath the sharp metabolite signals [39]. EDTA and citrate cause intense although sharper signals that obscure some spectral areas [38]. EDTA signals overlap with those of a number of metabolites including choline and its derivatives [6,38]; endogenous citrate cannot be quantified in citrate-plasma. Sodium fluoride and potassium oxalate do not give rise to any ^{1}H signals but may influence the biofluid composition due to the low solubility of some of their salts. In fact, plasma-EDTA is much preferred and remains one of the most used biofluids in NMR-based metabolomics.

Background information

As for urine, in blood the presence of cellular activity in the pre-processing phase is the major source of alterations. In particular, erythrocyte activity has been identified as mainly responsible for the concentration changes in important metabolites, such as glucose, lactate and pyruvate. After the removal of the cellular component, the concentrations of those metabolites remain stable over time. Thus, serum and plasma processing should be initiated within 30 min after blood collection in order to minimize these alterations attributable to changes in biochemical pathways [6,40].

After blood processing, the sources of variation are attributable to oxidation reactions occurring under aerobic conditions that cause concentration changes of other metabolites, such as proline and citrate, as well as in lipoproteins. In the latter case, LDL/VLDL conversion into HDL over time has been well characterized [6,41].Nevertheless, the

degradation processes observed in plasma or serum are weaker and the metabolomic profiles are more stable over time that those of urine samples. Particularly evident is only the decrement of citrate over time, for which NMR signals completely disappear from the spectrum within 12 h when samples are left at 4–6 °C (Fig. 9). To minimize these changes, immediate analysis after processing, keeping the samples in a refrigerated sample changer or immediate storage at −80 °C are advisable. As suggested for urine, it is good practice to create sample aliquots before storage to avoid freeze/thaw cycles.

New results

The use of other types of plasma

The need to develop SOPs to obtain high quality metabolomic data has been extensively demonstrated above. On the other hand, the possibility of using the same sample for multiple *in vitro* approaches may offer advantages in studies combining the results from different techniques (genomics, transcriptomics, proteomics and metabolomics) [42]. Finally, the possibility to use samples already available in biobanks, although not collected for the specific purpose of metabolomics, is extremely important to extend the number of possible samples in large-scale multicenter studies.

We evaluated the feasibility of NMR metabolomic analyses on plasma collections obtained using Ficoll gradient centrifugation [11], commonly employed for lymphocyte separation. It was demonstrated that for NMR analysis, plasma collections obtained using Ficoll as a separation medium should be avoided because it strongly contaminates the sample metabolomic profiles. Ficoll introduces several signals into the NMR spectra, some of which are very intense and have different linewidths (and cannot be filtered out by CPMG probably due to internal dynamics of the polymer), thus severely reducing the active spectral area and the number of metabolites that can be included in the subsequent analysis. In practice, almost 30% of the active spectral area becomes useless due to the need to exclude the region between 3.33 and 6.00 ppm, which includes important metabolites such as glucose, glycine, lactate, proline, serine and threonine. For comparison, in plasma-EDTA only a few non-contiguous areas accounting for a 1 ppm in total need to be excluded from the analysis (Fig. 10).

Effect of storage on serum samples

Given the complications related to the use of anti-coagulants or other types of plasma extraction procedures, we strongly prefer and recommend the use of sera for NMR-based studies. In order to evaluate the suitability of biobanked sera samples, and in analogy with what described above for urine, a long-term storage experiment was performed. The metabolic changes occurring in serum samples during storage were evaluated in different samples collected and processed according to SOPs [6] and stored at −80 °C. NMR spectra were acquired for each at different time points using different aliquots: time 0 (collection time) and after 4 and 5 years of storage. The metabolomic profiles of the samples were stable and none of the detected metabolites, including citrate, showed significant changes in concentration over time (Fig. 9). In addition, the oxidation processes involving the lipoprotein fractions were slowed down, with a consequent very good preservation of the biological samples. These results further confirm the validity of the CEN recommendation document [9].

Conclusion

Overall, SPIDIA and SPIDIA4P activities have demonstrated that NMR spectra of biofluids are a fast and reliable method for the evaluation of sample quality and validation of sample handling and storage procedures. One of the main weaknesses of available metabolomics studies is the lack of comparability when using samples collected from different centers, due to the different pre-analytic procedures. Here, the developed guidelines (CEN TS) are demonstrated to provide a valuable tool towards standardized pre-analytical procedures that ensure long-term stability of urine and blood derivatives. Combining this with SOPs for the available well-standardized NMR platforms will be a key step towards multicenter large-scale metabolomics, possibly including the use of samples stored in biobanks.

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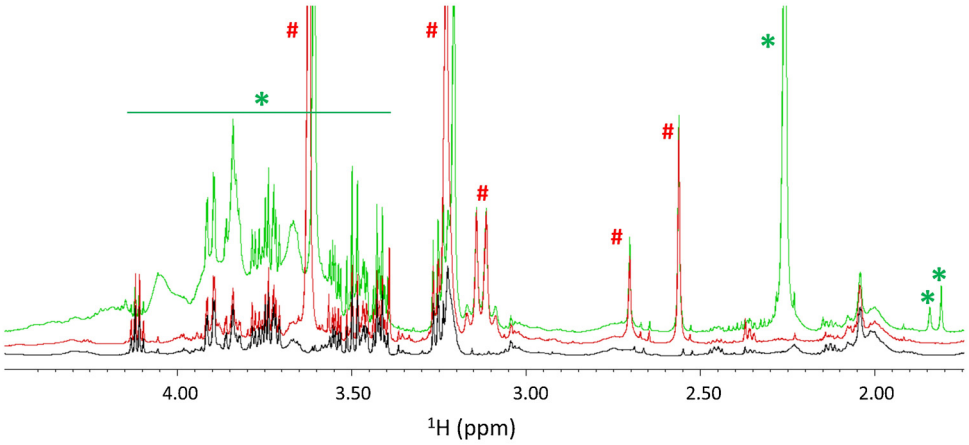

Fig. 10. ^{1}H NMR CPMG spectra. The figure underlines the differences among a Ficoll-obtained EDTA-plasma (green trace), a' normal' EDTA-plasma (red trace) and a serum (black trace). EDTA and Ficoll resonances are marked # and *, respectively.

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