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ARTICLE

Standard operating procedures for pre-analytical handling of blood and urine for metabolomic studies and biobanks

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Abstract ^{1}H NMR metabolic profiling of urine, serum and plasma has been used to monitor the impact of the pre-analytical steps on the sample quality and stability in order to propose standard operating procedures (SOPs) for deposition in biobanks. We analyzed the quality of serum and plasma samples as a function of the elapsed time ($t = 0-4$ h) between blood collection and processing and of the time from processing to freezing (up to 24 h). The stability of the urine metabolic profile over time (up to 24 h) at various storage temperatures was monitored as a function of the different pre-analytical treatments like pre-storage centrifugation, filtration, and addition of the bacteriostatic preservative sodium azide. Appreciable changes in the profiles, reflecting changes in the concentration of a number of metabolites, were detected and discussed in terms of chemical and enzymatic reactions for both blood and urine samples. Appropriate procedures for blood derivatives collection and urine preservation/storage that allow maintaining as much as possible the original metabolic profile of the fresh samples emerge, and are proposed as SOPs for biobanking.

Keywords Metabolomics · NMR spectroscopy · Serum · Plasma · Urine · Biobanks

Introduction

Metabolomic research is an emerging area focused on measuring the ensemble of the metabolites in biospecimens (Fiehn 2002; Nicholson et al. 1999). The objects of interest of metabolomic researches are the low-molecular weight compounds (MW $<$ 1,500 Da) that serve as substrates and/or products in various metabolic pathways. These small molecules include compounds such as lipids, sugars, amino acids, nucleotides, and a number of different organic molecules, that are the reactants, intermediates or products of biochemical reactions as well as building blocks for all other biochemical species including proteins, nucleic acids and cell membranes (German et al. 2005). The number of different metabolites in humans is unknown; estimates range from a minimum of 2,000–3,000 to a maximum of around 20,000 metabolites (browsing the Human Metabolome Database, HMBD, www.hmbd.ca), to be compared with estimated 30,000 genes (from www.ensembl.org) and about 40,000 proteins (browsing the protein database of the National Center for Biotechnology Information, NCBI, www.ncbi.nih.gov and disregarding splicing variants and post-translational modifications). Most metabolomic studies involve common biofluids as urine and serum/plasma that are obtainable from mammals, especially from humans, in a non- or minimally-invasive way and are easily available because commonly used for many other clinical analyses. A standard approach in metabolomics is

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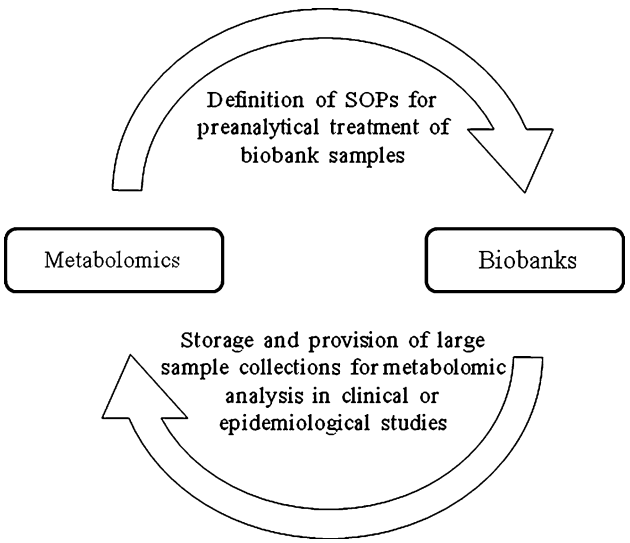
that of measuring monodimensional ^{1}H NMR spectra at medium/high magnetic fields. Such spectra, often called metabolic profile, constitute a "fingerprint" of the NMR detectable part of the whole metabolome. The potential of metabolomics for disease diagnosis, prognosis and, in a clinical trial setting, for monitoring drug therapy relies on its ability to extract a disease signature from the multivariate analysis of the metabolic profiles of statistically relevant ensembles of samples derived from different donors (Oakman et al. 2010; Sreekumar et al. 2009; Bertini et al. 2009; Akira et al. 2008; Holmes et al. 2008; Bartsch et al. 2008; Gao et al. 2008; Fearnside et al. 2008; Makinen et al. 2008; Teichert et al. 2008; Coolen et al. 2008; Claudino et al. 2007; Schnackenberg et al. 2007; Marchesi et al. 2007; Griffin et al. 2007; Constantinou et al. 2007; Coen et al. 2005; Lindon et al. 2004; Brindle et al. 2002). Clearly, the reliability of the approach requires that the chemical nature and the relative concentration of all the metabolites present in the biofluids are neither affected by the preanalytical treatment used to store the samples nor by the analytical methodology. Conversely, as depicted in Scheme 1, metabolomics itself is assuming a growing importance in the definition of operating procedures aimed at collecting and preserving biological samples and for standardizing protocols (Barton et al. 2008; Dunn et al. 2008; Jackson et al. 2008; Peakman and Elliott 2008; Saude and Sykes 2007). The definition of suitable Standard Operating Procedures (SOPs) is therefore essential to allow data comparison worldwide. Biobanks, that are infrastructures devoted to the collection, cataloguing and storing of biological samples in order to make them available for medical and clinical research, represent an irreplaceable support for all those studies in which the impact of the results is linked to the large number of the collected samples. At the same time, they have to guarantee that the quality of the stored biological samples remains as close as possible to the fresh sample for any possible future studies, including metabolomics.



Scheme 1 Biobanks/metabolomics interplay

Among the different metabolomic tools, NMR represents the technique of choice (Nicholson and Lindon 2008) for the definition of pre-analytical procedures. Indeed, NMR is a high-throughput methodology requiring only minimal sample handling before spectra acquisition and allowing collection of the whole metabolic profile, i.e. the simultaneous detection of a large number of metabolites with different physiochemical properties e.g., hydrophobicity/hydrophilicity, acidity/basicity, redox reactivity. The variety of detectable molecules allows the monitoring of residual enzymatic activities and/or chemical reactions that may alter the NMR profile of the analyzed sample, which becomes not representative any longer of the metabolome before collection. These alterations could seriously bias the results of studies based on samples having different collection, treatment and storage histories.

Standardization and improvement of pre-analytical tools and procedures for in vitro molecular diagnostics is the main goal of the European Union FP7 project SPIDIA (www.spidia.eu), which involves a consortium of public research organizations and private companies, and a standards organization. As a partner of the consortium, we performed the present research focusing on the most commonly studied biofluids: urine, serum and plasma.

Urine might be regarded as a biospecimen with little scientific value, but it is assuming an increasing importance as a bio-bankable sample thanks to metabolomics studies. Browsing PubMed for metabolomics/metabonomics publications resulted in 250 publications based on urine, 147 publications on serum, 185 on plasma and 185 on tissues. Use of urine as a first choice object of study is justified by the fact that the collection method is simple and is the least invasive one: these characteristics permit multiple collection schemes that greatly improve the reliability of the statistical analysis. Indeed, some of us have recently shown that NMR fingerprinting of multiple urine samples from the same donors is able to reveal individual metabolic phenotypes (Assfalg et al. 2008; Bernini et al. 2009). Several methods of urine sample processing (centrifugation, filtration, addition of preservatives) as well as sample storage temperature (at −80°C or in liquid nitrogen) were tested in several combinations and found to have significant effects on the metabolome, as detected by ^{1}H-NMR fingerprinting. The presence of host cells or bacterial cells in urine is identified as a potential major source of alteration of the metabolic profile, and appropriate countermeasures emerge.

Blood derivatives like serum and plasma are also very common biofluids in metabolomics studies. The disadvantage with respect to urine is that the collection of blood samples is slightly more invasive. Therefore, it may be more difficult to obtain multiple collections from patients, and even more from healthy volunteers. On the other hand, blood is less affected by daily variations and daily diet than urine. We analyzed here the quality of serum and plasma samples as a function of the time delay (0–4 h) and storage temperature (25 and 4°C) before processing. Moreover, changes in the NMR profiles of these two biofluids after serum and plasma preparation were monitored for a further 24 h, to simulate the effect of the time between processing and freezing. A number of metabolites which are often used as disease biomarkers in metabolomics studies are heavily affected by the tested variation factors, and again, appropriate countermeasures emerge.

The present results, taken together, contribute to the definition of standard operating procedures for specimen collection that represent a step forward with respect to the available recommendations for biobanking procedures (Yuille et al. 2010). These SOPs are being implemented at the local da Vinci European Biobank (http://www.davincieuropeanbiobank.org) and proposed for adoption by the European Biobanking and Biomolecular Resources Research Infrastructure (BBMRI, www.bbmri.eu) presently in the preparatory phase.

Materials and methods

Sample collection

Urine samples were collected from 6 healthy donors (3 females and 3 males) following the standard procedure for urine culture and kept not more than a half hour at 2–8°C before further processing (as detailed in the Results section). Urine from each donor was aliquoted in 1.0 mL fractions, and each aliquot was treated independently. Different centrifugation speeds were tested (see "Results and discussion"); during centrifugation samples were kept at 4°C. For filtration a 0.20 μm filter was used; such a cut-off is small enough to ensure removal of cells and large particles still avoid problems of obstruction of the pores that may slow down urine processing and lead to sample loss. Sodium azide was added to the sample until a final concentration of 3 mM, as described in our previous metabolomics studies (Weckwerth 2007; Assfalg et al. 2008; Bertini et al. 2009; Bernini et al. 2009).

Blood samples were withdrawn from healthy donors using different BD Vacutainers® for plasma or serum isolation: K2E 5.4 mg and SST™ II *Advance* respectively. Multiple 5 mL aliquots were taken at the same time from each patient and treated in parallel afterwards. We tested different time delays (0–4 h) between blood withdrawal and processing for serum/plasma preparation; during these time intervals the vacutainers were incubated at 4 and 25°C. In the case of serum $t = 0$ was considered to be 30′ at room temperature after blood collection, as required for clotting. After the incubation, vacutainers for plasma collection were centrifuged at 820 RCF (Relative Centrifugal Force) for 10′ at 4°C, while vacutainers for serum collection were centrifuged at 1,500 RCF for 10′ at 25°C. In both cases, after centrifugation, the supernatants were collected and used for the preparation of NMR samples.

NMR sample preparation

According to a commonly used protocol (Assfalg et al. 2008; Bernini et al. 2009), urine samples were shaken before use and 630 μL were centrifuged at 14,000 RCF for 5 min. 540 μL of the supernatant were added to sixty microliters of potassium phosphate buffer (buffer 1: 1.5 M K_2HPO_4 and 10 mM sodium trimethylsilyl [2,2,3,3-2H4] propionate, TMSP, in 100% ^2H_2O, pH 7.4). 540 μL of the mixture were pipetted into 4.25 mm NMR tubes (Bruker BioSpin srl).

Blood-derivative NMR samples were prepared adding 300 μL of phosphate sodium buffer (buffer 2: 70 mM Na_2HPO_4, 38 mM NaN_3 and 55 mM TMSP in 20% ^2H2O, pH 7.4) to 300 μL of plasma or serum. A total of 450 μL of this mixture was transferred into a 4.25 mm NMR tube (Bruker BioSpin srl). In the follow-up of blood derivates over time, we used a buffer with the same composition and concentration described above.

NMR spectra

All ^{1}H-NMR spectra were acquired using a Bruker 600 MHz spectrometer operating at 600.13 MHz proton Larmor frequency and equipped with a 5 mm CPTCI ^{1}H-^{13}C/^{31}P-^{2}H cryo-probe including a *z*-axis gradient coil, an automatic tuning-matching (ATM) and an automatic sample changer. A PT 100 thermocouple served for temperature stabilization at the level of approximately ±0.1 K at the sample. Before measurement, samples were kept for 3–5 min inside the NMR probehead, for temperature equilibration i.e., 300.0 K for urine samples, 310.0 K for serum/plasma samples; the highest temperature for blood derivatives is used for better detection of lipidic profiles (Suna et al. 2006).

For each urine sample, a one-dimensional (1D) NMR spectrum was acquired with water peak suppression using a standard pulse sequence (NOESYpresat; Bruker), 64 scans, 64 k data points, a spectral width of 12,019 Hz, an acquisition time of 2.7 s, a relaxation delay of 4 s and a mixing

time of 100 ms. ^{1}H-^{1}H J-resolved (J-res) spectra were also acquired in order to get more information about signal multiplicity and coupling patterns. For each serum/plasma sample 1D NOESYpresat spectra were recorded; 1D spectra were acquired also with the Carr-Purcell-Meiboom-Gill (CPMG; Bruker) spin-echo sequence to suppress signals arising from high molecular weight molecules. In this case NOESYpresat spectra consisted of 64 scans, 98 k 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 100 ms. CPMG spectra consisted of 64 scans, 74 k data points, a spectral width of 12,019 Hz, an acquisition time of 3.1 s, a relaxation delay of 4 s and a mixing time of 100 ms.

Spectral processing and analysis

Free induction decays were multiplied by an exponential function equivalent to a 1.0 Hz line-broadening factor before applying Fourier transform. Transformed spectra were automatically corrected for phase and baseline distortions and calibrated using TopSpin 2.1 (Bruker Biospin srl). In the case of serum and plasma, spectra were calibrated assigning a value of 1.5 ppm to the alanine peak. Calibration of urine spectra was achieved by aligning the signal of the pH-invariant resonance of trigonelline at 9.12 ppm; with this approach, the other signals of trigonelline and those of other pH-insensitive metabolites such as 1-methylnicotinammide and hippurate do not shift with pH. Each 1D spectrum in the range between 0.02 and 10.00 ppm was segmented into 0.02-ppm chemical shift bins, and the corresponding spectral areas were integrated using AMIX software (Bruker BioSpin). Regions between 6.0 and 4.5 ppm containing residual water and urea signals were removed. The total spectral area was calculated on the remaining bins and normalization was carried out on the data prior to pattern recognition.

All resonances of interest were assigned on template one-dimensional NMR profiles by using matching routines of AMIX 3.8.4 (Bruker BioSpin) in combination with the BBIOREFCODE (Version 2-0-0; Bruker BioSpin) reference database and published literature when available. All calculations were made using MATLAB 7.6.0 and AMIX 3.8.4.

Statistical analysis

Various kinds of multivariate statistical techniques were applied on the obtained buckets: PLS (Partial Least Squares)-regression and PCA (Principal Component Analysis) for data analysis, manova (One Way analysis with Multiple Responses) for data reduction, kNN (k-Nearest Neighbour) as classification method, Kruskal–Wallis test (for the determination of the meaningful metabolites) (Madsen et al. 2010).

Urine culture tests

Urine culture tests were performed by using Linearcount6® Urinocoltura: this is an integrated system that allows the bacterial count, the bacterial isolation and the presumed identification of bacteria present in the urine.

Results and discussion

Urine

The starting point for this research are the Recommendations on Biobanking Procedures for urine processing and management recently published by the European Consensus Expert Group Report (Yuille et al. 2010). According to this document, biobanking procedures for urine should consider the following general consensus recommendations: (i) cells and particulate matter should be removed (e.g. by centrifugation); (ii) samples should be stored at −80°C or below; (iii) time limits for the processing should have been defined experimentally and should be appropriate to the analytes to be measured; (iv) unless specified for a particular downstream analysis, urine samples should be stored without additives. As the authors pointed out, advances in new downstream analytical platforms may impose specific requirements related to the nature of the targeted analytes, thus leading to specific revision/elaboration of the general procedures. Here we address the above critical issues in relation to the consequences for downstream metabolomics applications.

Fresh urine is characterized by the presence of human cells (erythrocytes, leucocytes, urothelial cells, epithelial cells), bacteria, fungi, sperm counts, non cellular components (mucus filaments, cylinders, cylindroids, pseudocylindres, crystals, urates). The protocol for the analysis of urinary sediment proposes a speed around 1,600 RCF for the centrifugation (Koukoulaki et al. 2008) in order to avoid the breaking of the frailest elements like cylinders. On the other hand, to avoid the presence of any particulate components in the NMR samples that will affect the spectral quality, standard protocols (Beckonert et al. 2007) for the immediate NMR analysis of urine require a centrifugation of the sample at 14,000 RCF for 5 min at 4°C. Such a high speed may induce cell breaking and release of cellular components into the biofluid. We thus analyzed the advantage of applying a mild centrifugation (pre-centrifugation) to fresh urine before the high-speed centrifugation needed by the preparation of NMR samples for immediate use. Multiple aliquots from two different donors were

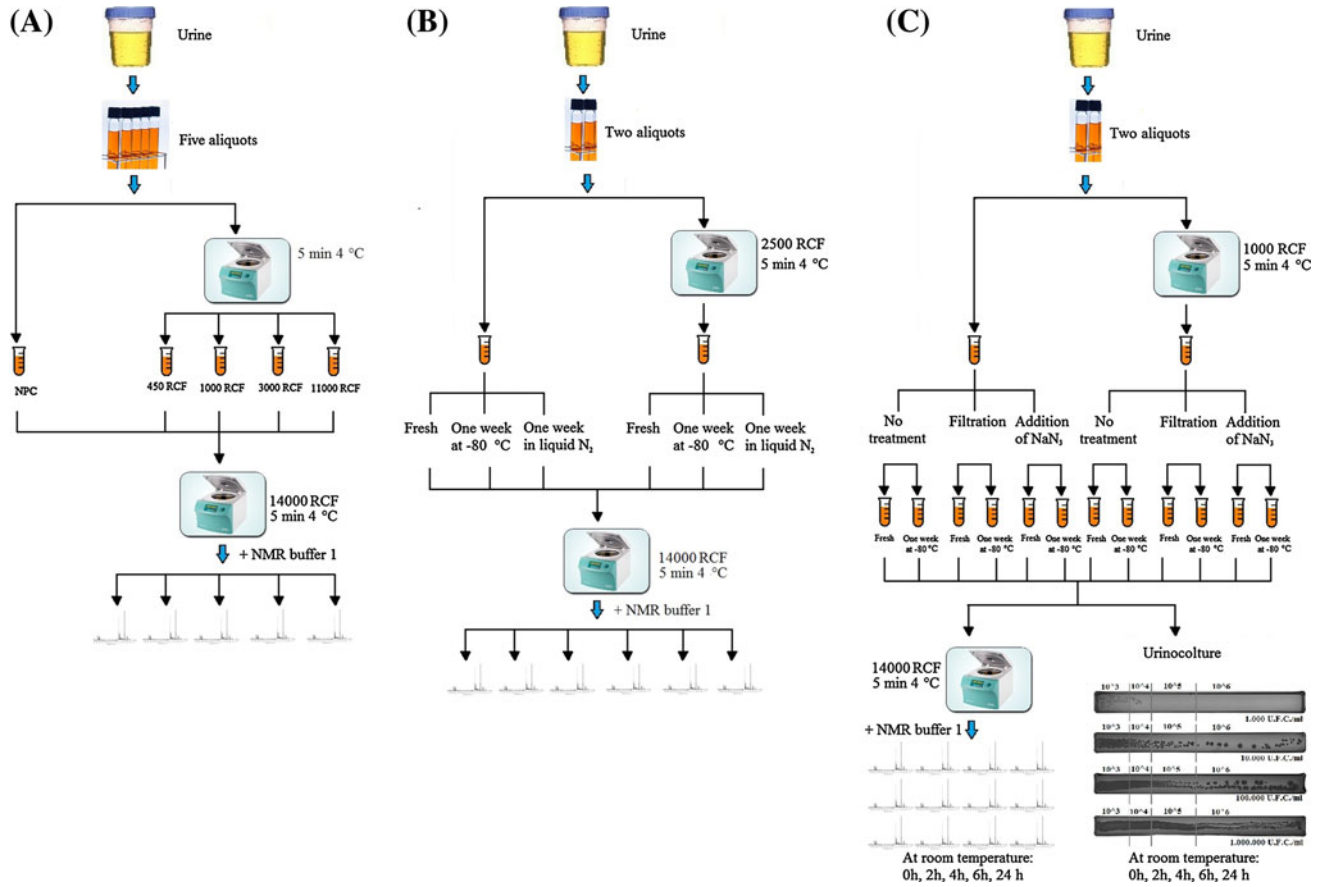


Fig. 1 Experimental design for: **a** the evaluation of the impact of pre-centrifugation speed on the NMR metabolomic fingerprint, **b** the evaluation of the effect of the use of pre-centrifugation as a function of the temperature used for sample storage, **c** the comparison of the efficiency of pre-centrifugation, filtration and addition of sodium azide in reducing bacterial content

collected and treated, as detailed below and summarized in Fig. 1: one aliquot per donor had no pre-centrifugation, the other aliquots were pre-centrifuged at 450, 1,000, 3,000 and 11,000 RCF (Fig. 1a). After pre-centrifugation, the supernatant was recovered and analyzed immediately, following the protocol described in the section "Materials and methods" for the preparation of the NMR samples. PLS (Partial Least Squares)-regression coupled with CA (Canonical Analysis) was performed on the obtained spectra as a function of the pre-centrifugation speed. To visualize the distance between the spectra of differently treated samples, the value of the first component for the non pre-centrifuged sample was taken equal to zero and the absolute value of the difference between the value of the first component in the pre-centrifuged samples and in the non pre-centrifuged sample was calculated. The plot of these values as a function of the pre-centrifugation speed (Fig. 2) shows an interesting behavior. The most distant from non pre-centrifuged samples are the aliquots pre-centrifuged at a speed in the 1,000–3,000 RCF range, while aliquots pre-centrifuged at >11,000 RCF are slightly closer to the samples that do not undergo any pre-centrifugation. Apparently, the proposed 5-min 14,000 RCF-centrifugation of the NMR protocol is too harsh; speeds ≥11,000 RCF at the pre-centrifugation level induce at least partial breaking of the cellular components and release of their soluble constituents, thus affecting the NMR profile. If a mild pre-centrifugation (between 1,000 and 3,000 RCF, Fig. 2) is applied, the cellular components are spun down and the subsequent 14,000 RCF centrifugation of the supernatant is useful for the elimination of other suspended, mainly inorganic, particles. On the other hand, a too mild pre-centrifugation (450 RCF) is not effective in eliminating the cellular components. The importance of the pre-centrifugation step of course depends upon the content in cellular components of the analyzed samples, and may therefore be very different from one individual to another (Fig. 2).

The differences in metabolic profile between pre-centrifuged or non pre-centrifuged samples are essentially ascribable to chemical shift changes in signals that are extremely sensitive to pH variations such as the Hε1 signal of histidine and of N^{τ}- and N^{π}-methyhistidine (Fig. S1A). For example, using a pre-centrifugation speed in the range defined by us as mild pre-centrifugation, namely 2,500

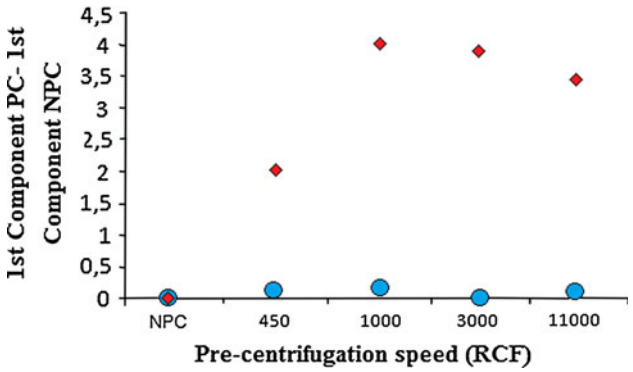


Fig. 2 Effect of the pre-centrifugation speed on the NMR spectra obtained with the experimental design of Fig. 1a. The absolute value of the difference in the first component of the PLS-CA analysis for spectra of samples pre-centrifuged (PC) at different speeds and that of the non pre-centrifuged sample (NPC) is plotted as a function of the centrifugation speed. *Diamonds* and *circles* are used to indicate urine samples from two different individuals, the former containing a larger number of cells. The comparison of the two plots shows the importance of the pre-centrifugation step in samples with high cellular components

RCF, the largest chemical shift variation was observed for the well resolved (in this pH range) Hε1 signal of N^{τ}-methyhistidine and found to be statistically relevant by Kruskal–Wallis test. The observed changes are consistent with a pH increase (Fig. S1B). The extent of the observed pH variation is different from one donor to another and is larger for samples containing larger amount of cells. However, no resonances attributable to new metabolites or increase in intensity of existing signals could be observed. This suggests that soluble components released by cells in fresh urine are below the detection limit of the method (because they are low in concentration and/or are high molecular weight components) but their presence is still able to alter the physiochemical properties of the solution.

The presence of cellular components has an impact on the NMR profiles even if non-fresh urine samples are analyzed, and the importance of the observed change depends upon the storage temperature (Fig. 1b). NMR metabolic profiles of non pre-centrifuged samples stored for a week at −80°C do differ from those that have undergone a mild (600–2,500 RCF) pre-centrifugation before freezing at the same temperature. The effect is less severe if urine samples are stored in liquid nitrogen. This result is not unexpected because rapid freezing in liquid nitrogen i.e., below the critical ice crystal temperature (−130°C) avoids crystal formation which may cause cell breaking. For example, from a PLS analysis of the spectral shift variation for samples not pre-centrifuged and pre-centrifuged at 2,500 RCF and analyzed either (i) fresh, or (ii) after 1 week at −80°C and (iii) after 1 week at liquid nitrogen (Fig. 1b), it results that the largest difference occurs for the samples stored at −80°C. Assigning a value of 100% to the changes observed between pre-centrifuged and not pre-centrifuged samples stored at −80°C for a week, the corresponding changes between pre-centrifuged and not pre-centrifuged samples analyzed fresh or after a week of storage in liquid nitrogen are much smaller and of comparable extent (9 and 8%, respectively).

PLS analysis of the discussed samples shows that the recognition capability of the model, i.e. the accuracy in assigning different aliquots to the same sample, is not seriously affected by any of these treatments (Fig. S2). Nevertheless, reducing any possible external sources of variations is vital for specific studies whose results depend on revealing differences in the relative concentration of a limited set of metabolites.

Finally, we evaluated the two most commonly used preservation methods (Saude and Sykes 2007): filtration and addition of sodium azide. They were used as such or in combination with a pre-centrifugation step at 1,000 RCF, as summarized in Fig. 1c. Differently treated aliquots of the same sample were analyzed either fresh or after storage for a week at −80°C, i.e. at the temperature that is most commonly used in biobanking. Spectral variations were followed for 24 h after urine processing or thawing after a week of storage at −80°C. Changes over time are observed for pH-sensitive metabolites that undergo chemical shift variations. In particular, the shift variations for the Hε1 N-methylhistidine (Fig. S1) are consistent with an alkalinisation of the samples; the chemical shifts of the resonances of xantine also change with time. Changes in the relative concentration of some molecules are also observed: succinate and acetate increase with time; urea, lactate and glutamate/glutamine decrease (Fig. S3). The extent of their variations depends upon the preservation method, pre-centrifugation and storage. Consistent with available literature data (Saude and Sykes 2007), filtration is the method that makes the NMR spectra more stable over time.

In order to evaluate the origin of the changes that affect the NMR spectra of samples over time, we tested three working hypothesis:

- The chemical hypothesis: reactions (e.g. oxidations) occur with time.
- The bacterial hypothesis: the bacteria present in urine grow over time with the consequent production of bacterial metabolites.
- The enzymatic hypothesis: the enzymatic activities of urine cause the consumption of certain metabolites and the increase of others.

In order to test the chemical hypothesis, as far as possible oxidation reactions are concerned, the samples were kept under inert atmosphere for the entire duration of the

experiment and compared with the corresponding aliquots kept under normal atmosphere. The effect of the inert atmosphere over the 24 h in which the samples were followed is modest. The only difference is a slight reduction in the increase of succinate concentration.

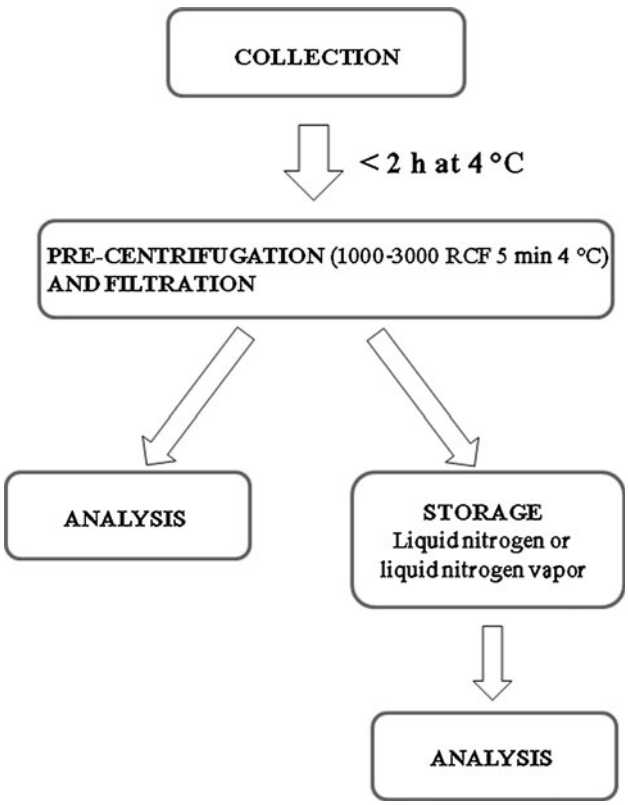
In order to test the bacterial hypothesis, in parallel with the analysis by NMR, we performed urine culture tests. The results of these tests (Tables S1 and S2) show that pre-centrifugation, addition of sodium azide and storage at −80°C have a disinfectant effect, but they do not eliminate completely the bacterial load. The effect of sodium azide is more clearly visible after 6 h at room temperature, whereas the effect of storage is more clearly visible when temperature-sensitive bacteria like coliforms are present. The fact that non-precentrifuged samples have the highest decrease in glutamate concentration suggests that degradation of this metabolite is mainly attributable to bacteria. The reduced degradation of lactate in samples stored at −80°C can be explained considering that coliforms bacteria, sensitive to the low temperatures, are the main source of its transformation. Filtration, alone or in combination with pre-centrifugation, completely eliminates bacteria from urine; nevertheless NMR spectral changes are not fully quenched in filtered samples. Therefore, the bacterial growth is not the only source of the observed spectral changes occurring with temporal progression.

The enzymatic hypothesis is justified by the notion that some activities due to human enzymes such as γ-glutamyltransferase, alkaline phosphatase and N-acetylglucosaminidase are present in the urine of healthy adults (Jung and Pergande 1983). The presence of γ-glutamyltransferase is a good proof of the disappearance of glutamate/glutamine in urine with time. On the other hand, a residual activity of urease and of isocitrate lyase, two metalloenzymes of bacterial origin, could be the cause of the decomposition of urea and of the increase of succinate, respectively. In order to test this hypothesis, we tried two different inhibitors. A 50 mM concentration of acetohydroxamic acid (AHA), a known inhibitor of urease, does inhibit the decrease of urea. A similar concentration of the bivalent metal chelator EDTA is also effective in inhibiting urea decomposition and partially inhibits the increase of succinate, acting on both urease and isocitrate lyase. The increase of acetate is also inhibited by EDTA. Because acetate is the main product of several bacterial fermentations, this result suggests that also the increase of acetate is at least in part due to metal-dependent bacterial enzymatic activities.

The three working hypotheses suggested above help us in understanding the observed variations of the NMR signals, but it is also important to highlight that all three mechanisms are present and their effects may partially overlap.

On the basis of the results of our experiments, we propose the following procedures for the optimal processing and management of urine samples to maintain as much as possible their original metabolome (Scheme 2): (i) removal of cells and particulate matters through the combined use of a mild pre-centrifugation 1,000–3,000 RCF (5′ at 4°C) and filtration; (ii) long-term storage of samples in liquid nitrogen (or liquid nitrogen vapour) to avoid breaking of residual cells; (iii) fast processing (within 2 h for collection); (iv) storage at 4°C between collection and processing. Recommendations at points (iii) and (iv) aim at reducing the effects of any possible enzymatic/cellular activities. Addition of additives (like enzyme inhibitors) should be avoided because the required concentrations will introduce signals in the NMR spectra covering the resonance of metabolites and may also induce changes in pH, ionic strength, etc. thus further affecting the original NMR profiles.

With respect to the general recommendations in Yuille et al., our studies permitted the definition of the best practices for the removal of cells and particulate matter. Nevertheless, as complete removal of cells is never achieved, long-term storage temperatures below the critical ice crystal temperature would be advisable. Changes in metabolite concentrations at room temperatures are relatively fast (Fig. S3); reduction of time between collection



Scheme 2 Optimal operating procedures for urine samples

and processing and the temperature at which urine samples are kept during this time delay is critical for the maintainance of the metabolome of the fresh samples. Annotation of time delays and temperatures for the entire history of each sample will add statistical value.

Blood

According to the same recommendations document mentioned in the urine section (Yuille et al. 2010), in the plasma/serum processing and management one should consider the following aspects: (i) use of EDTA or citrate as anticoagulant, not heparin; (ii) sample storage at a temperature of −80°C, or below if e.g. critical ice crystal formation is problematic; (iii) recording of the time from collection through processing; (iv) experimental definition of time limits that are appropriate to the analytes to be measured. These guidelines for blood derivatives biobanking focus on the use of stored samples for future extraction of DNA for genetic analysis. Although the latter still represents the main application for biobanked serum and plasma, new analytical platforms are assuming increasing importance and specific requirements for appropriate samples for new biomarker analysis have to be defined. Here we test the validity of best practices proposed for DNA analysis in the context of metabolomics and evaluate whether additional procedures may be needed for the optimal maintainance of the original metabolome.

The time limits for processing whole blood were examined (Fig. 3a) by evaluating the effect on the NMR metabolic profile of serum and plasma-EDTA samples, prepared according to the standard protocols described in Materials and Methods. PLS-CA analysis was again used at the descriptive level and showed that the spectral patterns of serum and plasma samples processed at different times carry meaningful differences (Fig. 4a, b and d, e). The PLS analysis with information of time delays from collection through processing was performed assuming the mean of the value of the first component as zero at time zero and consequently calculating the values corresponding to the following tested times. Plots c and f in Fig. 4 show the changes in the main component of PLS for serum and plasma samples at 4°C and at 25°C. Degradation processes are time-dependent and temperature-dependent both for serum and plasma samples: in both cases incubation at 25°C causes deeper changes in the NMR profile. In order to unravel the main mechanisms at the basis of the degradation processes occurring from collection through processing, we singled out the significant buckets ($P \leq 0.05$) by Kruskal–Wallis test. They correspond to the resonances of glucose, lactate and pyruvate. The intensities derived from integrals of the NMR peaks of these metabolites were normalized with respect to the alanine signal at 1.5 ppm, which has a constant intensity under the tested conditions. Average values are reported in Fig. S4. As a general trend, a decrease in glucose concentration is observed. Glycolysis is expected to represent about 90% of glucose consumption by erythrocytes (Baynes and Dominiczak 2010). The decrease in glucose concentration is more important in serum, most probably because EDTA in plasma tubes

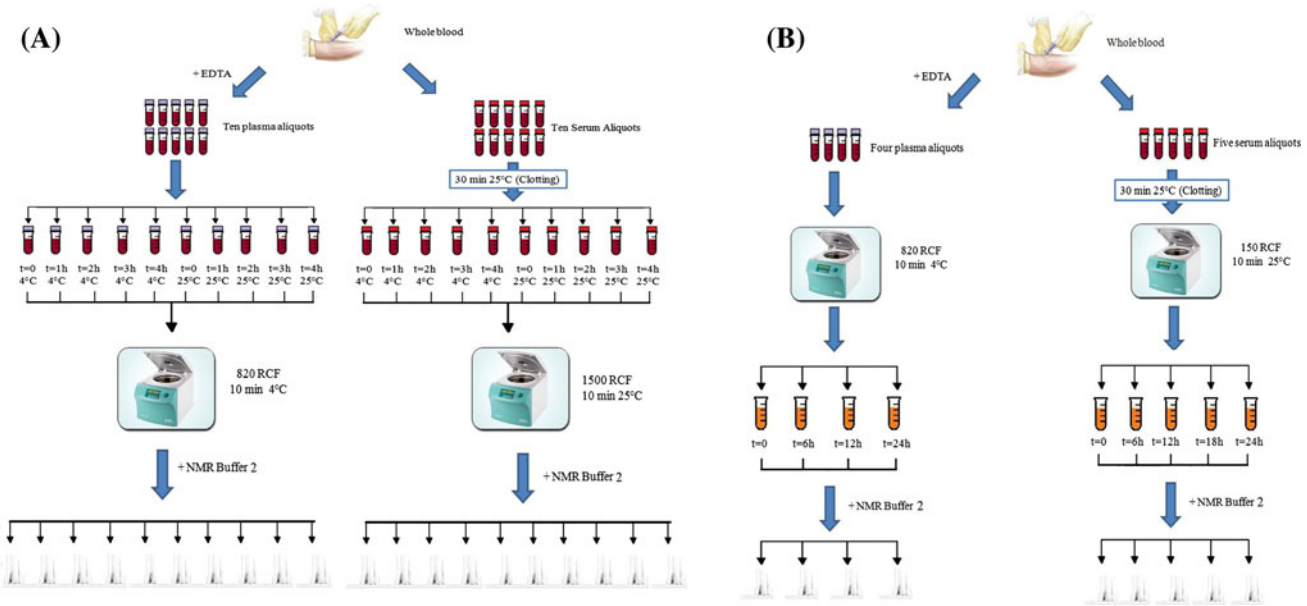


Fig. 3 Experimental design for: **a** the evaluation of the effect of the time between blood collection and processing on serum and plasma NMR spectra; **b** the evaluation of the effect of the time between processing and freezing on the quality of NMR spectra of plasma and serum

exerts an inhibitory effect on metalloenzymes and metal-dependent enzymes involved in glycolysis. An increase in lactate, the end product of the anaerobic glycolysis is observed, which is steeper than the decrease in glucose, roughly in agreement with the expected stoichiometry of the reaction. More complex is the trend in the concentration of pyruvate that is an intermediate metabolite of glycolysis: in plasma it is slightly decreasing/constant at 4°C and it increases at 25°C; in serum, it decreases at 4°C and is almost constant at 25°C. In clinical analyses, the standard for the measurement of glycaemia is represented by fluoride/oxalate coated vacutainers. It has been reported that glucose concentrations in plasma from blood collected into EDTA coated vacutainers showed no significant differences with glucose concentrations in plasma from fluoride/oxalate coated vacutainers up to 36 h (Peakman and Elliott 2008; Jackson et al. 2008). Comparison of the changes in concentration of glucose and its derivatives during the time from collection through processing of citrate, EDTA and fluoride/oxalate plasma in the present work did not show meaningful differences. Therefore, none of these three anticoagulants solves the problem of glucose degradation and we can just suggest that for a reliable evaluation of glucose, lactate and pyruvate whole blood should be processed within 2 h from collection, keeping it at 4°C, although the latter may still not be the best procedure for pyruvate.

The choice of the anticoagulant for metabolomic studies of plasma via NMR is not so obvious and represents a study-specific additional procedure. Citrate and EDTA are commonly used but have some drawbacks. Citrate is by itself a metabolite, identified as a relevant biomarker as it is involved in the energy metabolism and found to undergo changes in our degradation studies. On the other hand EDTA is not present in blood, but its presence as an anticoagulant covers a number of metabolite resonances (choline, dimethylamine and one signal of citrate). From the point of view of NMR metabolomics, use of fluoride/oxalate coated vacutainers as plasma collection tubes would have the advantage that the anticoagulant does not introduce any additional signal in the ^{1}H NMR spectra (however, see additional discussion below).

Possible degradation processes occurring in blood derivatives after processing were analyzed using 5 plasma and 5 serum samples of different patients (Fig. 3b). Each sample was split in multiple aliquots and NMR spectra of each aliquot were acquired in the time range 0–24 h keeping the samples at room temperature.

A PLS-CA analysis of the CPMG spectra of plasma and serum samples acquired over time provided the clustering reported in Fig. 4g and h; a better discrimination is observed for serum. Although the PLS-CA first component of the spectra gradually shift in the same direction during the 24 h of monitoring time, the effects are larger for serum (Fig. 4i). In order to single out the significant buckets ($P \leq 0.05$), and thus the corresponding metabolites that are responsible of this time progression, we performed the Kruskal–Wallis test. From this procedure we identified the following metabolites as markers of the time changes (Fig. S5): triglycerides, proline, choline, citrate and histidine. The Hε1 signal of His shifts with time, consistently with a pH variation increase of about 0.1 units. The signals of the other metabolites, but citrate, decrease in intensity. Albumin and LDL/VLDL (low-density-lipoproteins/very-low-density lipoproteins) also contribute to the variations of the NMR profile in NOESY spectra; although an accurate quantitation of the observed effect for such broad signals is not possible, a clear decreasing trend is observed. The decrease in concentration for fatty acids and LDL/VLDL is attributable to oxidation reactions, as the effect is attenuated if samples are kept under inert atmosphere. The chemical shift variations observed for the signals of citrate (Fig. S6) are consistent with literature data about saliva (Silwood et al. 2002) where the observed behavior has been rationalized in terms of a competition between calcium(II) and magnesium(II) binding to this chelator. Consistently, no chemical shift changes are detected in the present work for citrate in plasma EDTA-samples. The decrease in concentration observed in serum of all these molecules, but proline and choline, parallels that observed in plasma. No meaningful decrease in proline concentration is visible in plasma EDTA and citrate samples, whereas proline decreases sizably in plasma fluoride/oxalate samples. The latter observation indicates that EDTA or citrate are still preferable with respect to fluoride/oxalate-containing samples. The signals of choline is covered by EDTA resonances and its behavior cannot be followed. In summary, plasma appears to be slightly more stable than serum when kept at room temperature.

Collection, transport, handling, storage and analysis of serum and plasma samples may imply different times of exposure to light for different samples. In order to verify whether this translates into meaningful changes in the ^{1}H NMR metabolic profiles, spectra have been acquired to monitor possible differences between samples kept in the dark or exposed to light. A set of 7 serum samples was used: after addition of the buffer, each sample was split into two aliquots. One of them was utilized to follow the degradation mechanisms at room temperature in the light and the other while kept in the dark. Multiple and consecutive NMR spectra were acquired over a total period of 24 h. Exposure to light was found not to induce any difference in the degradation processes that occur with time.

Based on the above findings, we propose the following procedures for the optimal processing and management of

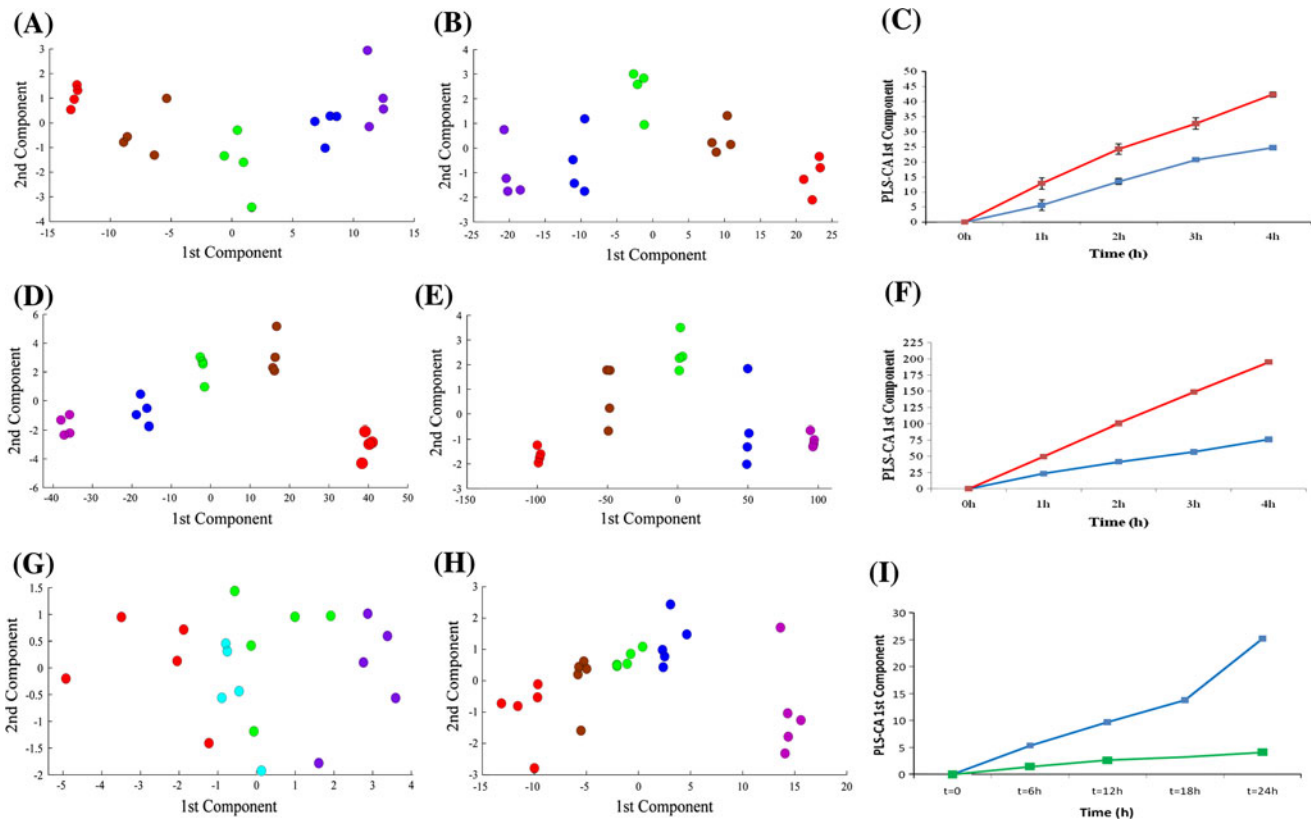


Fig. 4 PLS-CA clustering as a function of the time delay between blood collection and processing for plasma at **a** 4 and **b** 25°C and serum at **d** 4 and **e** 25°C. *Dots colour* coding is: *red* 0 h; *yellow* 1 h; *green* 2 h; *blue* 3 h; *purple* 4 h. Change of the average value of the first PLS-CA component as a function of time at 4°C (*blue line*) and 25°C (*red line*) for plasma (**c**) and serum (**f**). PLS-CA clustering as a function of time delay between processing and analysis on plasma (**g**) and serum (**h**). *Dots color* coding is *red* 0 h; *yellow* 6 h; *green* 12 h; *blue* 18 h; *purple* 24 h. **i** Change of the average value of the first component as a function of time for plasma (*green line*) and serum (*blue line*)

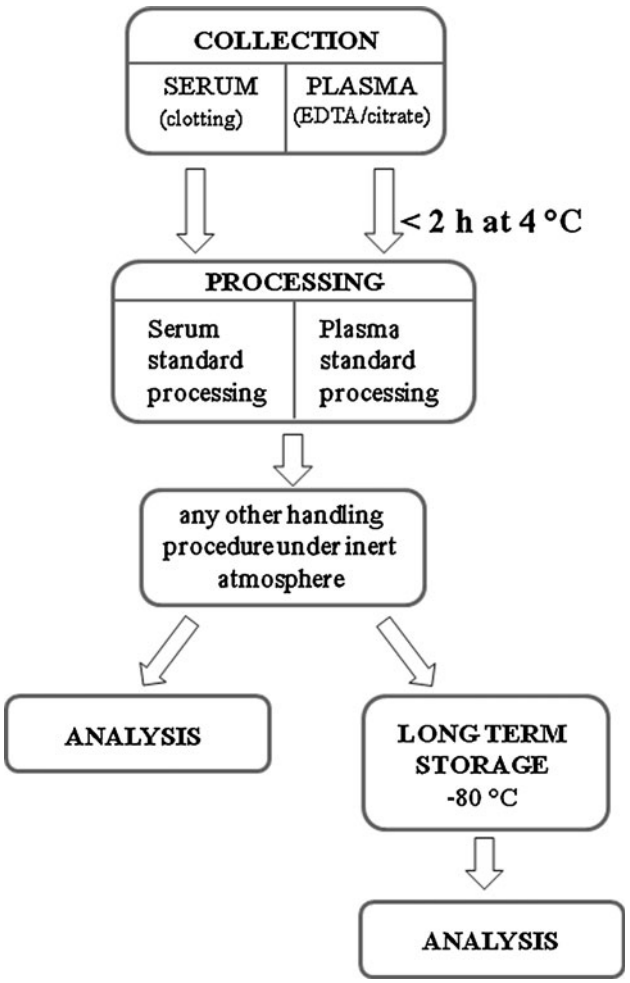
blood and its derivatives when they have to be used for future metabolomic NMR studies (Scheme 3): (i) either EDTA or citrate can be used as anticoagulant for plasma samples; (ii) time between collection and processing should not exceed 2 h; (iii) during this time delay samples should be kept at 4°C; (iv) any handling procedure of plasma and serum should be performed under inert atmosphere; (v) samples should be frozen immediately after processing; (vi) long term storage should be done at −80°C (no need of lower temperature once erythrocytes have been removed).

Conclusions

The primary objective of biobanks is not merely archiving but also distributing conserved and documented biological samples for research. The quality of biological samples is crucial for the outcome of subsequent studies. Sample quality is directly related to pre-analytical variations, whose impact depends on its end use. The molecules constituting the metabolome are generally more sensitive to handling and storage procedures than e.g. nucleic acids; in addition changes in metabolites due to residual enzymatic activity in biofluid samples can be extremely fast. The objective is to ensure that the analyzed sample is representative as much as possible of the metabolome before collection, i.e. is as close as possible to the freshly collected sample.

Validation of methods for sample collection/handling of human biofluids is essential. As samples are generally not collected in the confines of well-regulated academic laboratories for research purposes but typically in clinics or even in a domestic environment (urine) where organization and priorities are not those of future possible non-standard analytical approaches like metabolomics, time and temperature from collection through processing should be carefully recorded.

Urine and whole blood result to be “living fluids” even after collection from the patient: presence of cells and



Scheme 3 Optimal operating procedures for serum and plasma

residual enzymatic activities bring about significant changes in the specimen from the point of collection through the time of analysis.

In order to quench bacterial/enzymatic activity in urine a mild pre-centrifugation combined with filtration results to be the safest way to avoid contamination of the metabolome with soluble molecules derived from cellular components. This is particularly important when urine samples need to be stored in biorepositories at −80°C; storage in liquid nitrogen would be preferred but may become too expensive in a biobanking environment, if it has to be applied for a the long-term storage of large collections of urine samples. Urine did not result to be sensitive to the presence of oxygen, but a number of enzymatic reactions occur in this biofluid. Addition of inhibitors is able to quench/slow-down such activities but invariably alters the metabolic profile as it introduces in the spectra signals of the added molecules, whose presence may also induce changes in pH, ionic strength, further altering the whole spectral properties. In this sense, keeping the sample at the lowest possible temperature from collection throughout the analysis is extremely important.

Whole blood is generally collected in vacutainers that protect it from oxygen. The effect of time and temperature from blood collection through processing are revealed by the alterations in the relative concentration of three very important metabolites i.e. glucose, lactate and pyruvate, which are attributable to erythrocyte activity. These metabolites often play a key role in determining the signature of a disease in metabolomics studies (Warburg 1956; Garber 2004; Shaw 2006; Sreekumar et al. 2009), for example low glucose and enhanced lactate in cancer are indicators of the enhanced glycolysis expected on the basis of the Warburg effect (Qiu et al. 2009). The variability of these metabolites thus represents a real pitfall in metabolomic studies, as blood is usually collected in clinics and then transferred to analytical departments where it is processed: samples from different patients are collected the same day at different times and then delivered all together to the processing. It is also important that samples are all kept at a controlled temperature.

For serum, the processing time has to be reduced to a minimum (after the canonical 30' at room temperature needed for clotting) and must never to be longer than 2 h; storage for any time after clotting and before processing should be at 4°C. The same holds for plasma.

After blood processing, glucose, lactate and pyruvate concentrations remain stable over time, but the presence of oxygen causes changes in a number of other metabolites (albumin, triglycerides, LDL/VLDL, proline, citrate and histidine). Processing and sample handling under inert atmosphere and storage and analysis under inert atmosphere would be advisable.

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