
**Molecular in vitro diagnostic
examinations — Specifications
for pre-examination processes in
metabolomics in urine, venous blood
serum and plasma**

*Analyses de diagnostic moléculaire in vitro — Spécifications relatives
aux processus préanalytiques pour l'analyse du métabolome dans
l'urine et le sang veineux (sérum et plasma)*



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Contents

Page

Foreword	iv
Introduction	v
1 Scope	1
2 Normative references	1
3 Terms and definitions	1
4 General considerations	3
5 Urine	4
5.1 Outside the laboratory	4
5.1.1 Urine collection	4
5.1.2 Transport requirements	5
5.2 Inside the laboratory	5
5.2.1 Specimen reception	5
5.2.2 Storage requirements	6
5.2.3 Urine sample processing	6
5.2.4 Long-term storage requirements for urine samples	6
5.2.5 Urine thawing	6
6 Blood	7
6.1 Outside the laboratory	7
6.1.1 Primary collection	7
6.1.2 Transport of pre-processed specimens to laboratory	8
6.2 Inside the laboratory	8
6.2.1 Specimen reception	8
6.2.2 Sample processing	9
6.2.3 Transport of processed samples to a laboratory for metabolomics analysis or transport to a biobank	9
6.2.4 Long-term storage requirements	9
6.2.5 Serum and plasma thawing and use	10
Annex A (informative) Instability of the metabolome	11
Bibliography	17

Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

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This document was prepared by Technical Committee ISO/TC 212, *Clinical laboratory testing and in vitro diagnostic test systems*, in collaboration with the European Committee for Standardization (CEN) Technical Committee CEN/TC 140, *In vitro diagnostic medical devices*, in accordance with the Agreement on technical cooperation between ISO and CEN (Vienna Agreement).

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Introduction

Metabolomics is the "-omic" science that deals with the characterization of the metabolome, in turn defined as the whole set of small molecules (molecular mass <2 000 Da) in a certain biological system such as a cell, a tissue, an organ, or an entire organism^[1]. The analyses are mainly performed via two major analytical techniques, namely mass spectrometry (MS) and nuclear magnetic resonance (NMR)^{[2][3][4]}. The former has a sensitivity that can be as low as picomolar, requires sample separation and multiple experimental runs targeted to specific classes of compounds. The latter measures metabolites present at concentration above 1 µM and is mainly used for untargeted analyses, where all metabolites above the detection limit are observed simultaneously, independent of their chemical nature, without any separation procedure.

The metabolome is dynamic and quite sensitive to perturbations. The metabolome can change drastically during primary sample collection, transport, storage, and processing. As a result, the outcome from the diagnostic and research measurements could become an unreliable representation of the specific targeted physiological state or point in time, but instead describes an artificial profile generated during the pre-examination process. Pre-analytical variations have been identified to originate from two main sources:

- a) enzymatic activity in the samples, mainly related to the presence of cells;
- b) chemical reactions (e.g. redox reactions) among metabolites or between metabolites and oxygen, see References ^[5] to ^[11].

Moreover, the analyses can be influenced by the use of additives or by the introduction of contaminants, and therefore the selection of appropriate collection tubes and plasticware is also a critical aspect of the pre-examination phase.

Studies have been undertaken to establish the best pre-examination procedures in terms of maintenance of the original sample metabolome by identifying the critical steps and parameters affecting the metabolome composition. Additionally, standardization of the entire pre-examination workflow is needed to ensure comparability in multicentre studies. At the present state of the art, there are no defined pre-examination procedures for metabolomic samples. As a consequence, the procedures adopted by the various centres differentially influence the metabolome of the samples, making their comparison unreliable. The adoption of the present requirements for the pre-examination phase make it possible to compare and evaluate the results obtained from metabolic analysis.

This document draws upon such studies to codify and standardize the steps for urine, serum and plasma metabolomics analysis in what is referred to as the pre-analytical phase.

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Molecular in vitro diagnostic examinations — Specifications for pre-examination processes in metabolomics in urine, venous blood serum and plasma

1 Scope

This document specifies requirements and gives recommendations for the handling, documentation and processing of urine, venous blood plasma and serum intended for metabolomics analysis in the pre-examination processes. This document is applicable to metabolomics examinations and can be used by biomedical laboratories, customers of laboratories, in vitro diagnostics developers and manufacturers, institutions and companies performing biomedical research, biobanks, and regulatory authorities.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 15189, *Medical laboratories — Requirements for quality and competence*

ISO 15190, *Medical laboratories — Requirements for safety*

3 Terms and definitions

For the purposes of this document, the terms and definitions given in ISO 15189 and the following apply.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

- ISO Online browsing platform: available at <https://www.iso.org/obp>
- IEC Electropedia: available at <http://www.electropedia.org/>

3.1

biofluid

biological fluid which can be excreted (such as urine or sweat), secreted (such as breast milk, saliva or bile), obtained with a needle (such as blood or cerebrospinal fluid), or produced as a result of a pathological process (such as blister or cyst fluid)

3.2

examination

set of operations having the object of determining the value or characteristics of a property

Note 1 to entry: Processes that start with the isolated analyte and include all kinds of parameter testing or chemical manipulation for quantitative or qualitative examination.

Note 2 to entry: For metabolomic analysis, analyte isolation is not necessarily required.

[SOURCE: ISO 20166-1:2018, 3.10, modified — admitted term “analytical test” has been deleted and Note 2 entry has been added.]

3.3

fasting

abstinence from any solid or liquid food excluding water for at least 8 hours

3.4
mass spectrometry
MS

method used to analyse chemical compounds on the basis of their mass to charge ratio

3.5
metabolic profiling

use of analytical platforms to simultaneously measure the ensemble of *metabolites* (3.6) in biological systems that can be measured by the employed (or selected) technique

EXAMPLE Examples for such techniques are NMR and MS.

3.6
metabolites

small molecules ($\leq 2\ 000$ Da) that are intermediates and/or products of metabolism of the host organisms, of its microflora, deriving from food, drinks, drugs or pollutants.

Note 1 to entry: For further information see Reference [1].

3.7
metabolome

complete set of *metabolites* (3.6) to be found within an organism or a biological sample

Note 1 to entry: For further information see Reference [1]

3.8
metabolomics

comprehensive analysis of the *metabolome* (3.7) of a biological *specimen* (3.14) (e.g., organism, cell, tissue or *biofluids* (3.1))

3.9
MS-based metabolomics

use of *mass spectrometry* (3.4) to measure *metabolites* (3.6) in biological samples

3.10
nuclear magnetic resonance spectroscopy
NMR

method based on the selective absorption of high frequency radio waves by atomic nuclei subjected to a stationary magnetic field

Note 1 to entry: NMR provides chemical and structural properties of molecules.

3.11
NMR-based metabolomics

use of *NMR spectroscopy* (3.10) to measure *metabolites* (3.6) in biological samples

3.12
plasma

liquid part of unclotted blood

Note 1 to entry: Plasma samples can contain anti-coagulants.

3.13
pre-examination processes
preanalytical phase
preanalytical workflow

processes that start, in chronological order, from the clinician's request and include the *examination* (3.2) request, preparation and identification of the patient, collection of the primary sample(s), temporary storage, transportation to and within the analytical laboratory, aliquoting, retrieval

Note 1 to entry: The preanalytical phase can include preparative processes that can influence the outcome of the intended *examination* (3.2).

[SOURCE: ISO 15189:2012, 3.15, modified — An additional term was added, and more details were included.]

3.14

primary sample specimen

discrete portion of a body fluid, breath, hair or tissue taken for *examination* (3.2), study or analysis of one or more quantities or properties assumed to apply for the whole

[SOURCE: ISO 15189:2012, 3.16, modified — The term and definition are used here without the original notes.]

3.15

room temperature

temperature which is defined as 18 °C to 25 °C for the purpose of this document

3.16

serum

liquid that can be separated from clotted blood

3.17

stability

characteristic of a reference material, when stored under specified conditions, to maintain a specified property value within specified limits for a specified period of time

[SOURCE: ISO Guide 30:2015, 2.1.15 — The term and definition are used here without the original note.]

4 General considerations

For general statements on medical laboratory quality management systems and in particular on specimen collection, reception, and handling (including avoidance of cross contaminations) see ISO 15189 or ISO/IEC 17020. The requirements on laboratory equipment, reagents, and consumables according to ISO 15189 shall be followed; ISO/IEC 17020 can also apply.

All steps of a diagnostic workflow can influence the final analytical test result and a risk assessment shall be performed (see also ISO 14971). Mitigation measures for eliminating or reducing identified risks shall be established where required for ensuring the performance of the examination. It shall especially be investigated and ensured that the metabolites intended to be analysed are not compromised in a manner impacting the examination performance. This can be done, e.g. by applying the intended examination to specimens/samples which underwent time course studies reflecting the individual pre-examination process steps such as transport and storage and by implementing measures to prevent or reduce impacts by the identified pre-analytical variables.

In the absence of suitable specimen stabilization technologies, regarding the metabolome, the specimen collection shall be carried out in hospital premises or institutions where there are immediate suitable biofluid processing procedures available.

Specifically, for specimens intended to be analysed by metabolomics, the following steps shall be considered:

- a) patient pre-treatment (fasting, therapy, etc.);
- b) the specimen collection from the patient;
- c) the selection of collection containers and packages (e.g. collection tubes, cooling box, box for storing and transportation);
- d) the selection of stabilization procedures (e.g. any compounds added for stabilizing the specimen);
- e) the recording of any additions or modifications to the specimen;

f) the recording of types and quantity as well as description of specimens.

Safety requirements for facilities, transport and handling shall be considered in accordance with ISO 15189 and ISO 15190. WHO Guidelines for the Safe Transport of Infectious Substances and Diagnostic Specimens^[14] should be followed.

5 Urine

5.1 Outside the laboratory

5.1.1 Urine collection

5.1.1.1 General

For the collection of the specimen the requirements (e.g. disease condition, specimen size) for the intended molecular examination shall be considered.

See also ISO 15189.

5.1.1.2 Information about the specimen donor/patient

The documentation shall include the ID of the specimen donor/patient, which can be in the form of a code.

The documentation should include, but is not limited to:

- a) the health status and relevant lifestyle factors of the urine donor [e.g. healthy, disease type, concomitant disease(s)];
- b) demographics (e.g., age, sex);
- c) the information about medical treatment and any treatment prior to urine collection (e.g. anaesthetics, medications, diagnostic procedures);
- d) the collection time, including information about fasting, previous activities.
- e) the appropriate consent from the specimen donor/patient.

5.1.1.3 Selection and labelling of collection containers

The laboratory shall define the container intended for urine collection.

Additives are usually not used, because they can interfere with the analytical method. If they are required for specific purposes, their impact on the analytical performance and outcome shall be analysed. Some additives in collection tubes could present a risk to patients (e.g. toxic or corrosive).

For the labelling (specimen identification) of the urine collection tube a routine procedure (see also ISO 15189) or a procedure with additional information (e.g. 2D-barcode) shall be used.

5.1.1.4 Urine collection and reception from the specimen donor

5.1.1.4.1 General

Instruction for the urine collection shall be given to the donor, including any safety measures that need to be followed while handling collection containers containing harmful additives. All urine collection

devices should be checked for compatibility with metabolomics, e.g. avoiding any interference with the metabolomics profile.

NOTE For non-toilet-trained children, the most popular non-invasive method used is the clean catch, which National Institute for Health and Clinical Excellence (NICE), 2007 defines as a gold standard. This involves catching a sample by holding a sterile specimen bottle in the urine stream. Urine collection bags and urine collection pads can also be used to collect urine. NICE suggests urine collection pads as the next best option to clean catch.

The first midstream urine of the morning should be collected after a minimum of 8 h fasting. Drinking can influence urine metabolite concentrations. This requires a normalization. Specify, if collected at different times, or for 24 h collection. Any variations to instructions shall be validated. Fasting enables to perform the metabolomics analysis of urine where donors are synchronized having similar metabolic conditions. Research or dedicated analytical tests can require different patient conditions.

A sufficient volume of urine shall be collected according to the requirements of the preanalytical preparation steps and the analytical test.

Any clinical procedure affecting the specimen collection shall be documented. The total collected volume shall be documented.

5.1.1.4.2 Information on the urine specimen and storage requirements at the urine collection site

As metabolic profiles can change after urine collection and can thereby affect the validity and reliability of the analytical test result, the documentation on the primary urine specimen shall include the time and date of urine collection.

The whole urine specimen should be kept refrigerated at 2 °C to 8 °C for a maximum of 2 h and shall not be frozen prior to centrifugation and/or filtration to avoid cell disruption upon ice crystal formation, unless specified differently by the analytical test.

The allowed urine specimen total storage duration includes the time for storage at the point of urine collection, transportation to the testing laboratory and further storage at the testing laboratory or other institutions.

5.1.2 Transport requirements

During transport, the specimen should be kept cool (temperature range 2 °C to 8 °C).

Appropriate measures shall be taken to secure temperature specifications and to reduce time for the delivery, which should be completed within 2 h from collection.

The conformity with the protocol for the transport procedure shall be documented. Any deviations from the protocol shall be described and documented.

WHO Guidelines for the Safe Transport of Infectious Substances and Diagnostic Specimens^[14] should be followed.

5.2 Inside the laboratory

5.2.1 Specimen reception

The urine specimen reception time and conditions (e.g. labelling, transport conditions, volume, leaking and precipitation) of the received specimens shall be documented. Nonconformities of labelling, transport conditions and urine volume differences to specifications described for the urine collection or specimen preparation requirements shall be documented.

Where there are nonconformities in transport conditions, overall storage and transport time or urine volume that could affect the validity and reliability of the analytical test result^{[7][8][9]}, a new specimen should be obtained.

If required for the analytical test, specimen properties should be assessed (e.g. pH-value, creatinine concentration, blood and/or bacterial contaminations).

5.2.2 Storage requirements

The storage temperature and time interval between specimen receipt and sample processing for urine shall be documented.

The storage temperature should be according to [5.1.1.4.2](#).

The urine specimen total storage duration shall include the time for storage at the urine collection site (see [5.1.1.4.2](#)), transportation to the laboratory (see [5.1.2](#)) and further storage at the laboratory or other institutions.

Some examinations need special urine storage/archiving conditions. Manufacturers'/providers' instructions shall be followed. Appropriate measures shall be taken to ensure compliance with temperature recommendations.

5.2.3 Urine sample processing

Centrifugation (recommended: 1 000 g to 3 000 g for 5 min at 2 °C to 8 °C) followed by filtration (e.g. with a 0.20 µm cut-off filter) to remove particulate matter and cells.

Alternatively, only filtration can be used and documented.

Filter material and devices shall be proven neither to absorb nor to release metabolites or interfere with their analyses.

NOTE The above mentioned centrifugation/filtration is important to avoid cell disruption that would contaminate the specimen^{[7][8][9]}.

Alternative processing procedures shall be validated.

5.2.4 Long-term storage requirements for urine samples

The temperature and durations between sample receipt, sample processing and freezing of the processed sample shall be documented.

If the processed sample is intended to be stored frozen, the impact on the metabolomics analyses should be validated. The processed sample should be aliquoted into cryo-vials in the suitable volume needed for the metabolic profile testing. The minimum aliquot volume is determined by the analytical test. The vials shall be tested to avoid sample contamination (e.g. from phthalates).

Before freezing, cells should be removed, following [5.2.3](#). Controlled-rate freezing via slow programmable cooling can be applied.

Provided centrifugation/filtration procedures have been strictly followed (see [5.2.3](#)), storage at -70 °C is sufficient to ensure stability of the NMR-detectable part of the metabolome for at least 5 years^[9].

For MS-based metabolomics, in case of metabolites that are not routinely measured, in absence of specific recommendations, temperatures below -130 °C are recommended to ensure longer stability^[15]. For specific metabolites, the long-term stability should be checked.

5.2.5 Urine thawing

Thawing on ice is recommended. The thawing duration shall be documented. The time elapsing after the thawing until the analysis shall be documented.

The thawing procedure and duration until commencing the subsequent analysis shall be validated.

6 Blood

6.1 Outside the laboratory

6.1.1 Primary collection

6.1.1.1 General

For the collection of the specimen the requirements (e.g. disease condition, specimen size) for the intended molecular examination shall be considered.

See also ISO 15189.

6.1.1.2 Information about the specimen donor/patient

The documentation shall include the ID of the specimen donor/patient, which can be in the form of a code.

The documentation should include, but is not limited to:

- a) the health status and relevant lifestyle factors of the blood donor [e.g. healthy, disease type, concomitant disease(s)];
- b) demographics (e.g., age, sex);
- c) the information about medical treatment and any treatment prior to blood collection (e.g. anaesthetics, medications, diagnostic procedures);
- d) the collection time, including information about fasting, previous activities.
- e) the appropriate consent from the specimen donor/patient.

6.1.1.3 Selection of the blood collection tube

A blood collection tube permitting to draw a sufficient amount of venous blood shall be selected. The size of blood collection tube is determined by the amount of venous blood required to perform both preanalytical and analytical procedures.

The choice of the specific tube suitable for harvesting plasma or harvesting serum will depend on the requirements of the subsequent metabolic profile analysis.

For plasma, EDTA, sodium fluoride or citrate can be used as anticoagulants. EDTA is preferred for NMR-based metabolomics as well as for most of MS studies^{[2][7][9]}. In NMR-based metabolomics, the use of heparin tubes is problematic. Although the NMR spectra are not obviously altered, NMR heparin signals could complicate the analysis of the other resonances including those coming from lipoprotein subclasses. Also for MS, the anticoagulant used for sampling should be suitable for the subsequent analysis. The use of high molecule weight molecules such heparin could interfere with the measurement.

Commonly used collection tubes can also contain additional additives, such as clot activators for serum, or polymer gels (gel separators) that facilitate the separation of plasma/serum from the cellular component.

The use of the anticoagulant and any other additive shall be documented because their presence could interfere with the measurements (see [Annex A](#) for some examples)^{[9][16]}.

6.1.1.4 Blood collection from the specimen donor

The following are requirements associated with collecting blood from a patient for metabolomic investigations:

- a) The time of collection according to ISO 15189 shall be documented.
- b) For the labelling (specimen identification) of the blood collection tube a routine procedure (see also ISO 15189) or a procedure with additional information (e.g. 2D-barcode) shall be used.
- c) Blood should be collected after a minimum of 8 h fasting. Specify and document if collected at different or under non-fasting conditions.
- d) Standard venipuncture technique should be used. Steps for preventing possible backflow can be required. The manufacturers' instructions for using the blood collection tubes shall be followed. In certain circumstances, different techniques can be appropriate.
- e) Blood collection tubes shall be filled in accordance to the manufacturers' instructions and attention should be drawn to the correct positioning of the collection tube during the blood draw as well as the required volume.
- f) Blood collection tube manufacturers' instructions for mixing or inverting the tube immediately after blood collection shall be followed.
- g) Blood collection tubes shall be proven neither to absorb nor to release metabolites or interfere with their analyses.

NOTE Unless additives are homogeneously mixed with the blood sample, processes can occur that further affect the metabolic profile, thereby impacting the validity and reliability of the analytical test results.

6.1.1.5 Pre-processing of blood specimen

The documentation on the blood specimen shall include the time of blood collection.

Processing (see 6.2.2) of the blood specimen shall start within 30 min from collection (when specimens are kept at room temperature)^[9]. If a different time is specified by the vendor of the collection tube, the time should be documented.

If the analyses are targeted to specific metabolites, longer times may be used, if validated.

All the steps in the specimen pre-processing shall be documented and validated.

6.1.2 Transport of pre-processed specimens to laboratory

If primary blood is not processed at the collection site, the blood specimen shall be transported and validated in accordance to (6.1.1.5).

The use of a pneumatic tube transport system should be validated, as it can impact specimen quality due to high acceleration/deceleration forces^[17].

Appropriate measures shall be taken to secure temperature specifications and to reduce time for the delivery.

6.2 Inside the laboratory

6.2.1 Specimen reception

The blood specimen reception time and conditions (e.g. labelling, transport conditions, volume, and leaking/broken tube) of received specimen shall be documented. Nonconformities of labelling, transport conditions and blood volume differences to specifications described for the blood collection or specimen preparation requirements shall be documented.

Where there are nonconformities in transport conditions, overall storage and transport time or blood volume that could affect the validity and reliability of the analytical test result, a new specimen should be obtained, whenever possible.

6.2.2 Sample processing

The processing should start within 30 min (at room temperature) from blood collection (see also [6.1.1.5](#)). Serum and plasma shall be prepared according to documented standard procedures. To remove all circulating cellular contaminants, specific procedures can be used for plasma preparation.

NOTE 1 An example of such a procedure is a two-step centrifugation. The second centrifugation is usually an ultracentrifugation.

The impact of the applied documented procedures should be investigated and shall be validated.

NOTE 2 Application of ultracentrifugation changes e.g. the lipoprotein profile significantly and thereby influences, for instance, the NMR metabolomics significantly.

For serum, the processing time starts with the clotting, which requires about 30 min at room temperature before centrifugation. If clotting problems occur, these should be annotated.

If the processed sample is intended to be stored frozen (see [6.2.4](#)), it should be aliquoted into cryovials in the suitable volume needed for the metabolic profile test before freezing. The minimum aliquot volume is determined by the analytical test. The vials shall be tested to avoid sample contamination (e.g. from phthalates).

6.2.3 Transport of processed samples to a laboratory for metabolomics analysis or transport to a biobank

If samples are transported to a laboratory for immediate analysis, the transport conditions should be validated.

If applicable, specimens should be transported to and from a biobank as frozen (see [6.2.4](#)).

Upon receipt, record the serum or plasma arrival time and conditions (e.g. labelling, transport conditions, sample volume, leaking) of the received samples. Nonconformities of labelling, transport conditions and obvious sample volume differences to specifications described for the collection tubes and any variations from assay shall be documented.

Where there are nonconformities in transport conditions, overall storage and transport time or other factors that could affect the validity and reliability of the analytical test result^{[7][9][10][11]}, a new sample should be obtained, whenever possible.

WHO Guidelines for the Safe Transport of Infectious Substances and Diagnostic Specimens^[14] should be followed.

6.2.4 Long-term storage requirements

The temperature and durations between sample receipt, sample processing and freezing of the processed sample shall be documented.

Controlled-rate freezing can be applied.

Provided centrifugation procedures have been strictly followed (see [6.2.2](#)), storage at -70°C is sufficient to ensure stability of the NMR-detectable part of the metabolome for at least 5 years^[9].

For MS-based metabolomics, in case of metabolites that are not routinely measured, in absence of specific recommendations, temperatures below -130°C are recommended to ensure longer stability^[16]. For specific metabolites, the long-term stability should be checked.

6.2.5 Serum and plasma thawing and use

For the analytical testing, the sample shall be thawed. Thawing on ice is recommended. The thawing procedure and time period for commencing the subsequent analysis shall be validated.

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Annex A (informative)

Instability of the metabolome

A.1 General

As it emerges from the literature, there are two main critical aspects for the stability of the metabolome of biofluids ex-vivo:

- a) Post-collection changes induced by the presence of cells, mainly related to enzymatic reactions.
- b) Post-collection changes due to chemical reactions occurring in the complex mixture of small molecules and dissolved dioxygen.

Here we provide examples of the observed changes in urine and blood serum/plasma, which can serve as a tool to assess internal procedures.

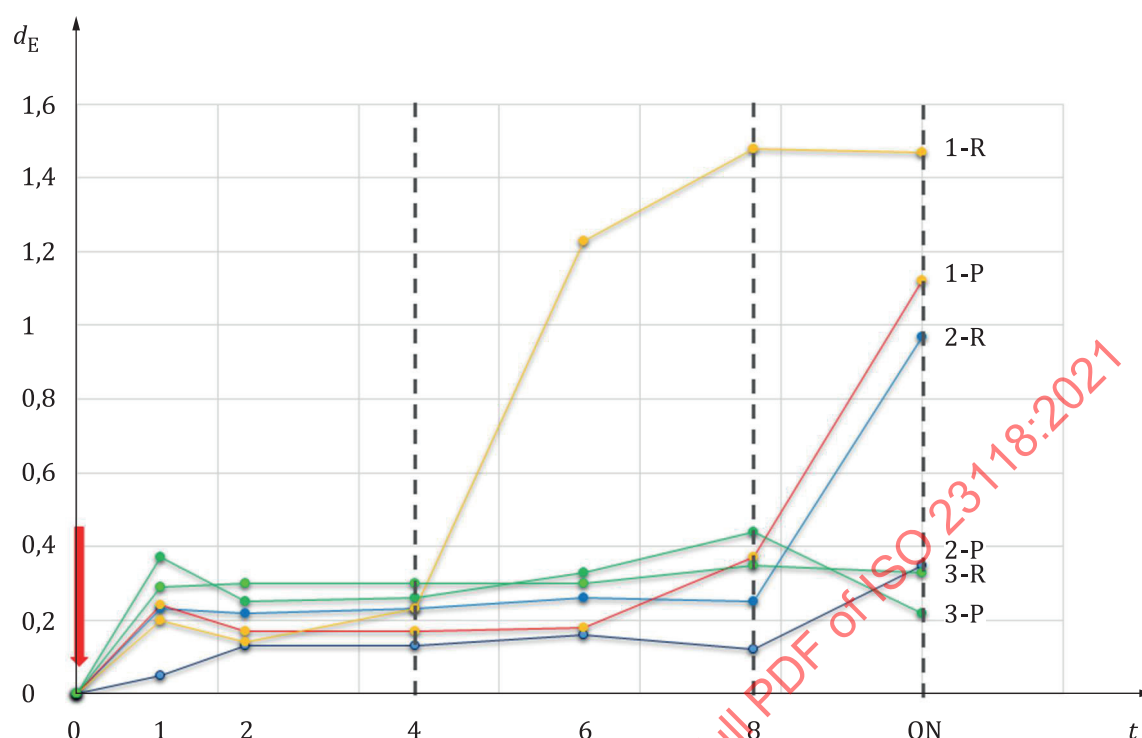
A.2 Urine

- a) Post-collection changes induced by the presence of cells.

Three urine samples (1, 2 and 3), characterized by different cellular contents (i.e. 1-very high; 2-modest; 3-low) were collected, split into 2 different aliquots, one (labelled R) was analysed as such and the other (labelled P) was subjected to mild centrifugation prior to analysis. The cell content was estimated from visual inspection of the pellet present after mild pre-centrifugation in P samples.

The stability over time of the metabolome in P and R urine was monitored by acquiring NMR spectra (^1H NOESY experiments at 600 MHz, 300 K) at different time points after sample collection (0 h, 1 h, 2 h, 4 h, 6 h, 8 h), up to a maximum of 20 h to 24 h, indicated as overnight (ON) (see [Figure A.1](#)). Samples were kept at 4 °C in between spectra acquisition.

The deviation from the original metabolome was calculated as the Euclidean distance between the spectra obtained at each time point and the spectrum at time 0. The Euclidean distance between two spectra is calculated as the distance in an n-dimensional space where n corresponds to the number of points constituting each spectrum.



Key

t time, in h

d_E euclidean distance

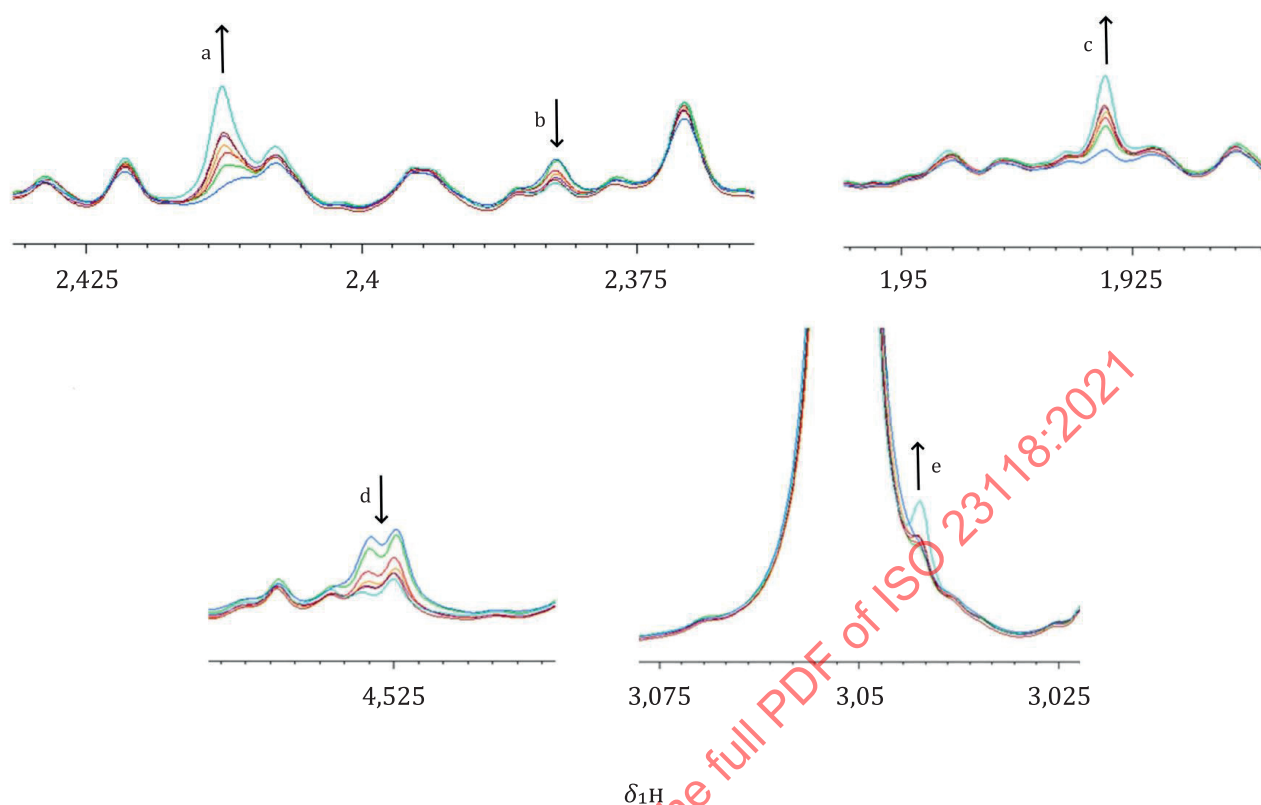
NOTE The red arrow indicates time 0.

Figure A.1 — Deviation from the original metabolome in samples with different cell content

The profile of sample 3 is very stable over time both in P and R urine. In contrast, the profiles of samples 2-R and 1-R were progressively unstable over time compared to their P counterparts. These data show that the use of mild centrifugation and/or filtration to remove cells in fresh urine ensures sample consistency and stability. The importance of this processing step depends on the cell content in each sample, which varies from patient to patient. The higher the number of cells, the larger is the extent of observed changes. Freezing before cell removal shall be avoided to prevent cell breaking and release of intracellular content.

b) Post-collection changes due to chemical reactions.

The stability of the metabolome in pre-processed (P) urine was followed for 24 h, acquiring an NMR spectrum every 2 h; samples were kept at 4 °C in between data acquisition. ¹H NOESY experiments were recorded at 600 MHz, 300 K at different time points after pre-processing (0 h, 1 h, 2 h, 4 h, 6 h, 8 h), up to a maximum of 20 h to 24 h, indicated as overnight (ON).



Key

blue traces: $t = 0$ h;
 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: $t = \text{ON}$ (overnight);

- a Succinate.
- b Pyruvate.
- c Acetate.
- d Ascorbate.
- e Creatine.

δ_{1H} : proton chemical shift, in Hz/MHz (The chemical shift here provided as Hz/MHz is commonly referred to as ppm.)

NOTE The arrows indicate the direction of change with time.

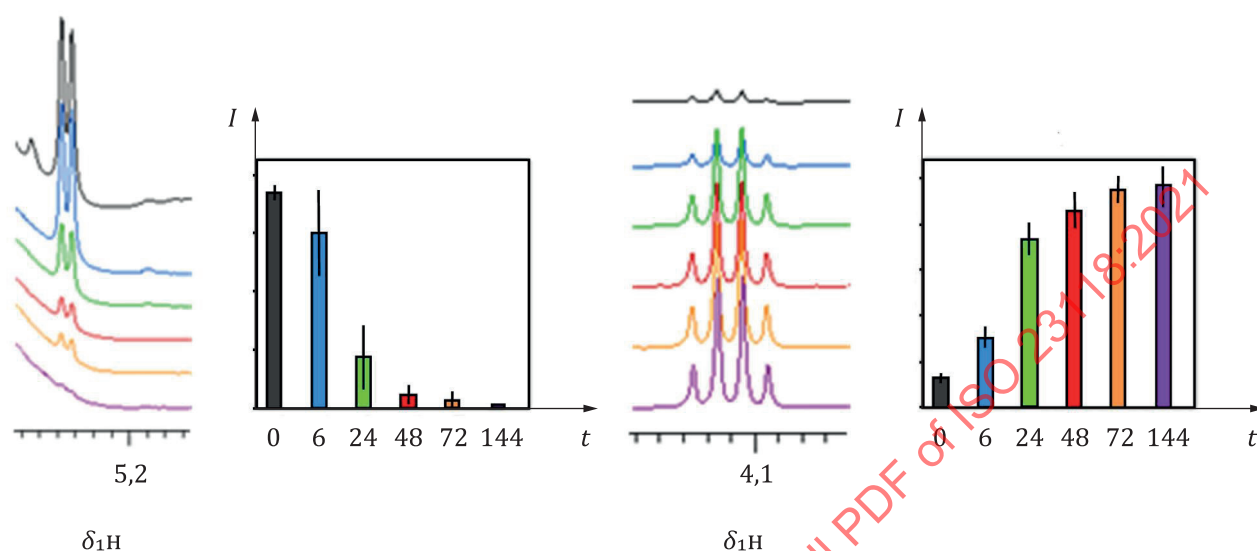
Figure A.2 — Effects independent from the processing phase in urine samples

There are a number of changes that occur independent of the presence of cells and can be attributed to (enzymatic or non-enzymatic) redox reactions. Succinate, pyruvate, acetate, ascorbate and creatine result among the most affected metabolites (see [Figure A.2](#)). To maintain the levels of the most sensitive metabolites, samples should be kept on ice before processing and frozen at -80 °C immediately. Similar effects are observed as a consequence of repeated freeze/thaw cycles, that shall therefore, be avoided.

A.3 Blood serum/plasma

- a) Post-collection changes induced by the presence of cells.

Plasma-EDTA samples were collected from 5 healthy donors. For each donor, samples were processed at 6 time points (0 h, 6 h, 24 h, 48 h, 72 h and 144 h). Samples were kept at room temperature before processing. Processed samples were frozen immediately and thawed just before analysis. ^1H NOESY experiments at 600 MHz and 310 K were acquired. The changes in metabolite levels were determined by integrating representative ^1H NMR signals.



Key

t time, in h

I relative intensity (arbitrary units)

$\delta_{1\text{H}}$ proton chemical shift, in Hz/MHz

Figure A.3 — Pre-processing variations in glucose (left panels) and lactate (right panels) levels in plasma samples

Between blood collection and processing, the overall metabolomic profile undergoes changes in the concentration of several metabolites. The most striking variations, attributable to red blood cells, involve glucose, which decreases, and lactate, which increases (see [Figure A.3](#)). Thus it is essential to process the samples as fast as possible.

b) Post-collection changes due to chemical reactions.

The stability of serum metabolome was followed for 12 h, acquiring an NMR spectrum every 4 h (0 h, 4 h, 8 h, 12 h). Samples were kept at 4 °C in between data acquisition. ^1H NOESY experiments were recorded at 600 MHz and 310 K.