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

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National foreword

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

Tests 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)

Molekularanalytische in-vitro-diagnostische Verfahren - Spezifikationen für präanalytische Prozesse für Metabolomuntersuchungen in Urin, venöses Blutserum und -plasma

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European foreword

This document (CEN/TS 16945:2016) has been prepared by Technical Committee CEN/TC 140 “In vitro diagnostic and medical devices”, the secretariat of which is held by DIN.

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Introduction

Molecular *in vitro* diagnostics has enabled a significant progress in medicine. Further progress is expected by new technologies analysing signatures of nucleic acids, proteins, and metabolites in human tissues and body fluids. However, the profiles of these molecules can change drastically during primary sample collection, transport, storage, and processing thus introducing biases and making the outcome from diagnostics or research unreliable or even impossible because the subsequent analytical assay will not determine the situation in the patient but an artificial profile generated during the pre-examination process. Therefore, a standardization of the entire process from sample collection to metabolomics analysis is needed. Studies have been undertaken to determine the important influencing factors. This Technical Specification draws upon such work to codify and standardize the steps for urine, serum and plasma metabolomics analysis in what is referred to as the preanalytical phase.

Metabolomics, the global profiling of metabolites (namely molecules with a molecular weight MW ≤ 2 000 Da [3]) in biological samples, is the determination of the dynamic multi-parametric metabolic response of living systems to pathophysiological stimuli and/or genetic modification. Metabolomics studies, which can be semiquantitative or quantitative, help in identifying metabolic profiles that are characteristic for given pathological conditions, for disease prognosis, for the evaluation of the individual response to medical intervention and pharmaceutical treatments. Metabolites are physically and chemically different, and include e.g. sugars, acids, bases, and lipids [3]. This diversity of metabolites and the dynamic range of their concentration in biological samples complicate the separation and detection methods and make it impossible to identify all the metabolites in a single experiment. However, new high-throughput technologies based on NMR (nuclear magnetic resonance) spectroscopy and MS (mass spectrometry) hold great potential due to their ability to look at large parts of the whole metabolome, although with different sensitivity. These two main analytical platforms are now well standardized. Equally well established are the statistical approaches needed to extract information from the huge amount of data resulting from metabolomic analysis.

The metabolic profiles are very sensitive to preanalytical variations that can result from enzymatic activity in the samples and chemical reactions (e.g. oxidation, [4], [5]). This Technical Specification series provides guidelines arising from systematic studies conducted on the most commonly employed biofluids: urine and blood derivatives, serum and plasma.

1 Scope

This Technical Specification covers the preanalytical phase and recommends the handling, documentation and processing of urine, venous blood plasma and serum intended for metabolomics analysis. This Technical Specification is applicable to metabolomics examinations and is of importance to biomedical laboratories, customers of laboratories, *in vitro* diagnostics developers and manufacturers, institutions and companies performing biomedical research, biobanks, and regulatory authorities.

The adoption of the described procedures for the preanalytical phase make it possible to compare and evaluate the results obtained from metabolic profiling analysis.

2 Normative references

The following documents, in whole or in part, are normatively referenced in this document and are indispensable for its application. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

EN ISO 15189:2012, *Medical laboratories - Requirements for quality and competence (ISO 15189:2012, Corrected version 2014-08-15)*

ISO 15190, *Medical laboratories — Requirements for safety*

3 Terms and definitions

For the purposes of this document, the terms and definitions given in EN ISO 15189:2012 and the following apply.

3.1
analytical phase
processes that start with the isolated analyte and include all kind of parameter testing or chemical manipulation for quantitative or qualitative analysis

Note 1 to entry: For metabolomic analysis, analyte isolation is not necessarily required.

3.2
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)

3.3
fasting
abstinence from any solid or liquid food excluding water

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 that are accessible to the employed (or selected) technique

EXAMPLE Examples for such techniques are NMR and MS.

3.6
metabolites
small molecules (≤ 2000 Da) that are intermediates and/or products of metabolism

Note 1 to entry: For further information see [3].

3.7
metabolome
complete set of metabolites to be found within an organism or a biological sample

Note 1 to entry: For further information see [3].

3.8
metabolomics
scientific study of the whole metabolome present within a biological sample (e.g., organism, cell, tissue or biofluids) under a given set of conditions

3.9
MS-based metabolomics
use of mass spectrometry to measure metabolites in biological samples

3.10
Nuclear magnetic resonance spectroscopy
NMR
method where the resonance magnetic properties of atomic nuclei are used to determine physical and chemical properties of atoms and molecules

[SOURCE: ISO/TS 80004-6:2013, 4.26]

3.11
NMR-based metabolomics
use of NMR spectroscopy to measure metabolites 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 request, preparation and identification of the patient, collection of the primary sample(s), temporary storage, transportation to and within the analytical laboratory, aliquotting, retrieval, isolation of analytes, and end when the analytical examination begins

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

[SOURCE: EN 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, study or analysis of one or more quantities or properties assumed to apply for the whole

[SOURCE: EN 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
ability of a sample material, when stored under specified conditions, to maintain a stated property value within specified limits for a specified period of time

Note 1 to entry: The analytes for the purpose of this document are metabolites.

[SOURCE: ISO Guide 30:1992, 2.7]

4 General Considerations

For general statements on specimen collection and handling (including avoidance of cross contaminations) see EN ISO 15189:2012, 5.2.6, 5.4.4. Consumables including kits shall be verified before use in examination (see EN ISO 15189:2012, 5.3.2.3); EN ISO 15189:2012, 5.5.1.2 and 5.5.1.3 can also apply.

As all steps of a diagnostic workflow can influence the final analytical performance, the entire workflow comprising the preanalytical steps, including information on specimen stability and storage conditions, and analytical steps should be verified and validated (see EN ISO 15189).

In the absence of suitable specimen stabilization technologies, regarding the metabolome, the specimen collection should 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) the specimen collection from the patient;

b) the selection of collection containers and packages (e.g. cooling box, box for storing and transportation);

c) the selection of stabilization procedures (e.g. any compounds added for stabilizing the specimen);

d) the recording of any additions or modifications to the specimen;

e) the recording of types and quantity and description of specimens.

Safety regulations on facilities, transport and handling shall be considered (see EN ISO 15189:2012, 5.2.3 and 5.4.5, and ISO 15190).

5 Urine

5.1 Outside the laboratory

5.1.1 Urine collection manual

5.1.1.1 Information on the primary specimen donor

The documentation should include, but is not limited to:

a) the specimen donor/patient ID, which can be in the form of a code;

b) the health status and relevant lifestyle factors of the urine donor (e.g. healthy, disease type, diet, gender, age);

c) the information about medical treatment and special treatment prior to urine collection (e.g. anaesthetics, medications);

d) the collection time, including information about fasting, previous activities.

See also EN ISO 15189:2012, 5.4.4.

5.1.1.2 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, their impact on the analytical performance and outcome shall be analysed. Additives can be harmful (e.g. toxic or corrosive).

A sufficient minimum volume of urine should be collected according to the requirements of the preanalytical preparation steps and the analytical test. For the labelling (specimen identification) of the urine collection tube a routine procedure (EN ISO 15189:2012, 5.4.4.3, e)) or a procedure with additional information (e.g. 2D-barcode) shall be used.

5.1.1.3 Urine collection and reception from the specimen donor

Instruction for the urine collection shall be given to the donor, including any safety measures concerning additives in the collection container.

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 standard instructions shall be validated.

NOTE This 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.

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

The identity of the person receiving the specimen from the patient and the time of urine collection according to EN ISO 15189, 5.4.4.3, f) shall be documented.

5.1.1.4 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 use of a pneumatic tube transport system should be validated, as it can impact specimen quality due to high acceleration/deceleration forces [12].

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 [6], [7], 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.

The urine specimen total storage duration shall include the time for storage at the urine collection site (5.1.1.4), transportation to the laboratory (5.1.2) and further storage at the laboratory or other institutions.

Some analytical assays need special urine storage/archiving conditions – therefore follow assay manufacturers'/assay providers' instructions. Appropriate measures shall be taken to ensure 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.

NOTE The above mentioned centrifugation is important to avoid cell disruption [6], [7] that would contaminate the specimen.

Alternatively, only filtration can be used. Filter material and devices should be proven neither to absorb nor to release metabolites or interfere with their analyses by increasing the blank.

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 [8]. As recommendation, the processed sample should be aliquoted into cryo-vials in the suitable volume needed for the metabolic profile test. The minimum aliquot volume is determined by the analytical test.

Before freezing, cells should be removed, following (5.2.3). Controlled-rate freezing can be applied.

Provided centrifugation/filtration procedures have been strictly followed (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 (for an example see Annex A).

In absence of recommendations, for MS-based metabolomics temperatures below −130 °C are recommended to ensure stability of metabolites [9].

5.2.5 Urine thawing

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 blood collection manual

6.1.1.1 Information on the specimen donor

The documentation should include, but is not limited to:

a) the specimen donor/patient ID, which can be in the form of a code;

b) the health status and relevant lifestyle factors of the blood donor (e.g. healthy, disease type, diet, gender, age);

c) the information about medical treatment and special treatment prior to blood collection (e.g. anaesthetics, medications);

d) the instructions for the preparation of the patient for the blood draw procedure (e.g. fasting status);

e) the type and purpose of the analytical test requested.

See also EN ISO 15189:2012, 5.4.4.

6.1.1.2 Selection of the blood collection tube

A blood collection tube permitting to draw a sufficient amount of venous blood according to the requirements of the preanalytical preparation steps and the analytical test shall be selected.

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 [6], [10] as well for most of MS studies. Heparin-based anticoagulants shall be avoided for NMR analysis.

The anticoagulant shall be documented.

6.1.1.3 Blood collection from the specimen donor

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

1. The identity of the person collecting the specimen and the time of collection according to EN ISO 15189:2012, 5.4.4.3, f) shall be documented.

2. For the labelling (specimen identification) of the blood collection tube a routine procedure (EN ISO 15189:2012, 5.4.4.3, e)) or a procedure with additional information (e.g. 2D-barcode) shall be used.

3. Blood should be collected after a minimum of 8 h fasting. Specify and document if collected at different or under non-fasting conditions.

4. Standard venepuncture technique should be used. Steps for preventing possible backflow can be required. Follow the manufacturers' instructions for using the blood collection tubes. In certain circumstances, different techniques may be appropriate.

5. 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.

6. Blood collection tube manufacturers' instructions for mixing or inverting the tube immediately after blood collection shall be followed.

NOTE Unless additives are homogenously mixed with the blood sample, the metabolic profile can be significantly compromised, thereby impacting the validity and reliability of the analytical test results.

6.1.1.4 Pre-processing of blood specimens

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

Processing (6.2.2) of the blood specimen shall start within 30 min from collection (when specimens are kept at room temperature) [11]. For specific metabolites, longer times may be appropriate, 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 (6.1.1.4).

The use of a pneumatic tube transport system should be validated, as it can impact specimen quality due to high acceleration/deceleration forces [12].

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) 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.

6.2.2 Sample processing

The processing should start within 30 min (at room temperature) from blood collection (see also 6.1.1.4).

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 for 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.

Serum requires about 30 min clotting time at room temperature before centrifugation. If clotting problems occur, these should be annotated.

If the processed sample is intended to be stored frozen (6.2.4), it should be aliquoted into cryo-vials in the suitable volume needed for the metabolic profile test. The minimum aliquot volume is determined by the analytical test.

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 [12].

If applicable, specimens should be transported to and from a biobank as frozen (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. Report nonconformities of labelling, transport conditions and obvious sample volume differences to specifications described for the collection tubes and any variations from assay requirements.

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 [6], a new sample should be obtained.

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.

If the processed sample is intended to be stored frozen, it should be aliquoted into cryo-vials in the suitable volume needed for the metabolic profile test. The minimum aliquot volume is determined by the analytical test.

Controlled-rate freezing can be applied.

Provided centrifugation procedures have been strictly followed (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 (for an example see Annex A).

In absence of recommendations, for MS-based metabolomics temperatures below −130 °C are recommended to ensure stability of metabolites [9].

6.2.5 Serum and plasma thawing and use

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

Annex A
(informative)

Long-term stability of urine and serum ^{1}H NMR metabolic profiles

A.1 General

The long-term stability of the ^{1}H NMR metabolic profiles of urine and serum samples stored at −70 °C was checked by comparing the ^{1}H NMR spectra of two aliquots of the same samples, one collected before storage and the other collected after 5 years at −70 °C. The obtained results demonstrated that, provided the proposed preanalytical procedures are used, the profiles are stable during storage and can safely be used for metabolomics studies after the storage.

A.2 Urine ^{1}H NMR measurement result

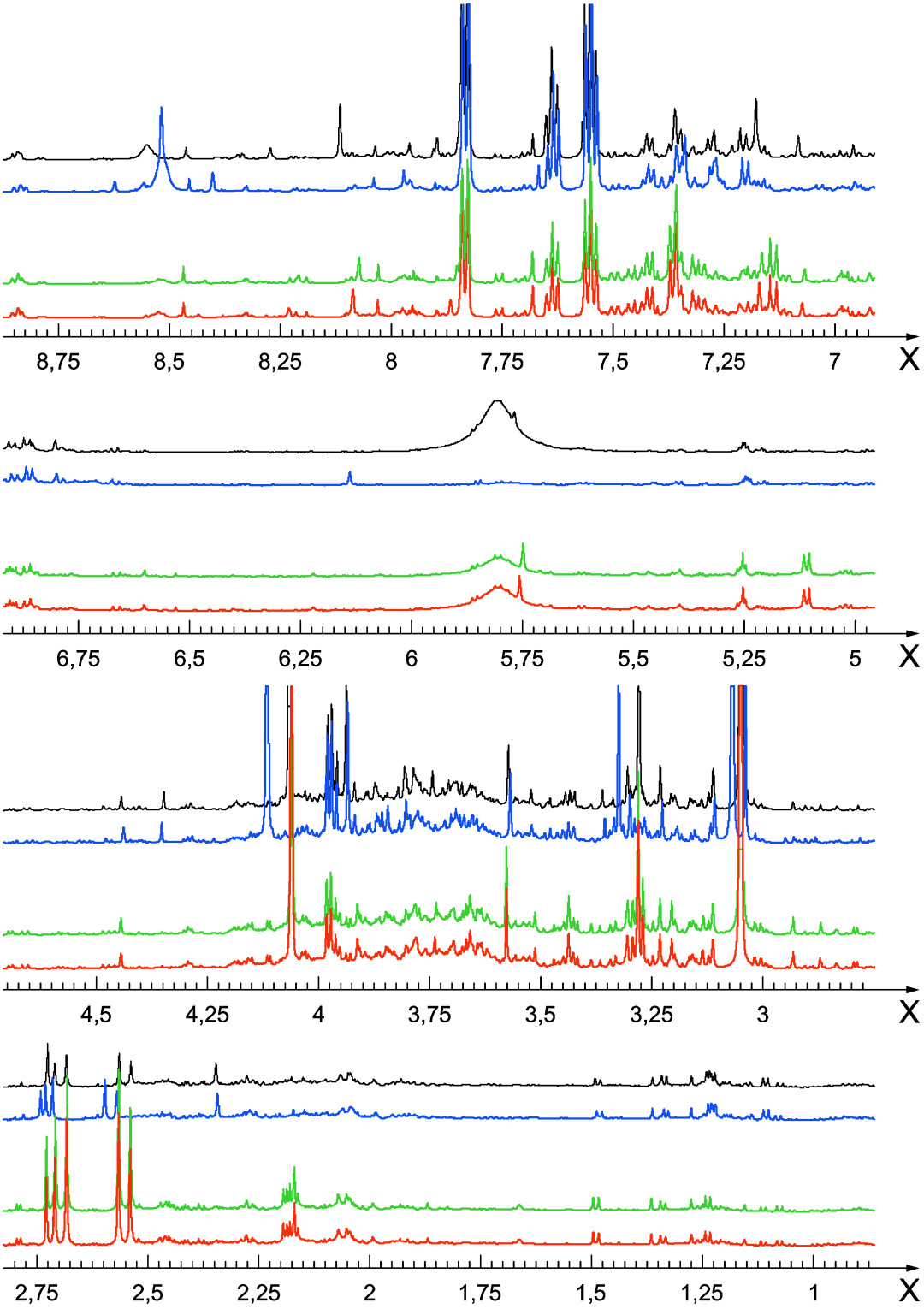
In the case of urine, a comparative stability analysis was performed between

— samples collected according to the proposed preanalytical procedures and

— samples that did not undergo centrifugation or filtration before storage.

The spectral changes over time in the latter group of samples were much larger than those for the former group, as it can be observed in two pairs of representative spectra in Figure A.1. The median value of the Euclidean distances calculated on 11 samples of the former group is 0,52; the corresponding value for 5 samples of the latter group is 1,74.

As an example for the described results, Figure A.1 depicts the different spectral regions of the ^{1}H NMR profiles of two aliquots from two independent urine samples (A and B). The two top traces of each panel represent the spectrum acquired on the fresh aliquot (black) and on an aliquot of sample A after 5-years storage at −70 °C (blue); these two aliquots come from a sample which didn't undergo centrifugation or filtration before storage. The two bottom traces of each panel represent the fresh aliquot (green) and the aliquot after 5-years storage at −70 °C (red) of sample B handled according to the proposed procedures, which include centrifugation at 1 000 g to 3 000 g for 5 min at 2 °C to 8 °C followed by filtration.



Key

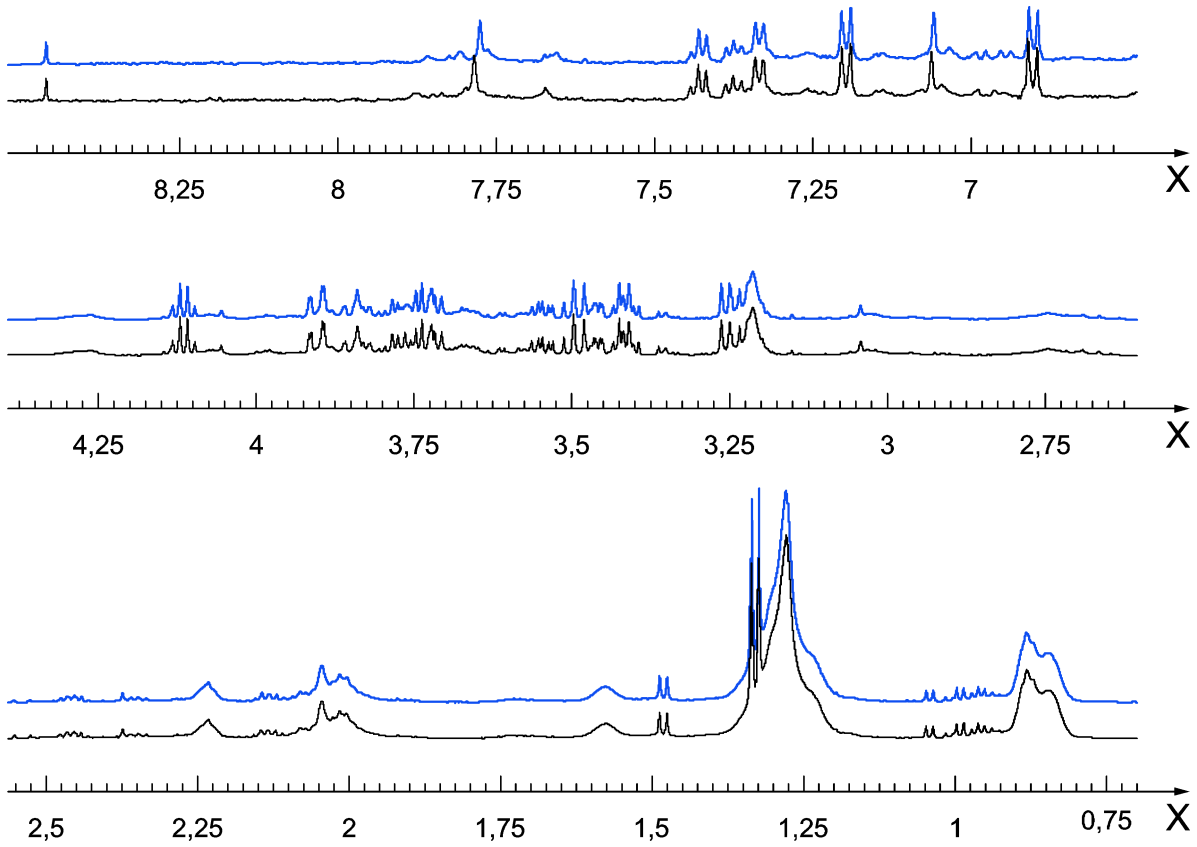
X	^{1}H chemical shift (in ppm)
black	sample A – without centrifugation and/or filtration, fresh aliquot
blue	sample A – without centrifugation and/or filtration, aliquot after 5-years storage at −70 °C
green	sample B – with centrifugation and filtration, fresh aliquot
red	sample B – with centrifugation and filtration, aliquot after 5-years storage at −70 °C

Figure A.1 — Different spectral regions of the ^{1}H NMR profiles of two aliquots from two independent urine samples

A.3 Serum ^{1}H NMR measurement result

In the case of serum, the analysis was only conducted on samples collected and handled according to the proposed preanalytical procedures. The corresponding spectral profiles resulted very stable (see Figure A.2 for a pair of representative spectra). The median value of the Euclidean distances calculated on 5 independent samples is 0,72.

In Figure A.2, different spectral regions of the ^{1}H NMR profiles of two aliquots from the same serum sample (sample C) are shown. The top trace was acquired on the fresh aliquot (blue), the bottom trace on an aliquot of the same sample after 5-years storage at −70 °C (black). The sample was collected, handled and stored according to the proposed procedures.



Key

X	^{1}H chemical shift (in ppm)
black	sample C – with centrifugation and filtration, fresh aliquot
blue	sample C – with centrifugation and filtration, aliquot after 5-years storage at −70 °C

Figure A.2 — Different spectral regions of the ^{1}H NMR profiles of two aliquots from the one serum sample

A.4 NMR methods for urine and serum

A.4.1 NMR samples preparation

Frozen urine and serum samples were thawed at room temperature and shaken before use. All urine NMR samples were prepared centrifuging 630 µL of each sample at 14 000 g for 5 min. 540 µL of the supernatant were added to 60 µL of phosphate potassium buffer (1,5 M K_2HPO_4, 100 % (v/v) $^{2}H_2O$,

10 mM sodium trimethylsilyl [2,2,3,3-$^{2}H_4$]propionate (TMSP) pH 7,4). All serum NMR samples were prepared adding 300 µL of serum to 300 µL of phosphate sodium buffer (70 mM Na_2HPO_4; 20 % (v/v) $^{2}H_2O$; 0,025 % (v/v) NaN_3; 0,8 % (w/v) sodium trimethylsilyl [2,2,3,3-$^{2}H_4$]propionate (TMSP) pH 7,4). A total of 450 µL of each of these mixtures was transferred into a 4,25 mm NMR tube for analysis.

A.4.2 NMR analysis

Monodimensional ^{1}H NMR spectra for all samples were acquired using a 600 MHz spectrometer operating at 600,13 MHz proton Larmor frequency and equipped with a 5 mm CPTCI ^{1}H-^{13}C-^{31}P and ^{2}H-decoupling cryosonde including a z-axis gradient coil, an automatic tuning-matching (ATM) and an automatic sample changer. A thermocouple served for temperature stabilization at the level of approximately 0,1 K at the sample. Before measurement, samples were kept for at least 3 min inside the NMR probehead, for temperature equilibration (300 K for urine samples, 310 K for serum samples).

^{1}H NMR spectra were acquired with water peak suppression and different pulse sequences [13]:

— for urine, a standard NOESY (Nuclear Overhauser effect spectroscopy) pulse sequence [14], using 64 scans, 64 000 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 0,1 s was used;

— for serum, a standard Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence [15], using 32 scans, 73 728 data points, a spectral width of 12 019 Hz and a relaxation delay of 4 s was used.

A.4.3 NMR spectra processing

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 (3-Trimethylsilylpropionate, TMSP, singlet at 0,00 ppm for urine samples and Glucose doublet at 5,24 ppm for serum spectra). Each spectrum in the range between 0,2 ppm and 10,00 ppm was segmented into 0,02-ppm chemical shift buckets, and the corresponding spectral areas were integrated. Regions between 6,0 ppm and 4,5 ppm containing residual water and urea signals were excluded in subsequent statistical analyses. Total area normalization was carried out on the urine and serum data, prior to pattern recognition.

A.4.4 Euclidean distance

Euclidean distances were calculated on the data matrix. The distances were calculated between the couple of samples X and X', where X is a spectrum acquired before the sample was frozen and stored in the biobank and X' is the spectrum of the same sample acquired after 5-years storage.

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