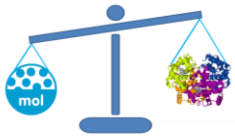

FINAL PUBLISHABLE JRP REPORT

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JRP-Coordinator	
Name, title, organisation	Claudia Swart, Dr., Physikalisch-Technische Bundesanstalt
Tel:	+49-(0)531-592-3150
Email:	claudia.swart@ptb.de
JRP website address	http://www.ptb.de/emrp/metallomics.html
Other JRP-Partners	
Short name, country	PTB, Germany BAM, Germany LGC, United Kingdom LNE, France TUBITAK, Turkey

REG-Researcher (associated Home Organisation)		
Researcher name, title	Anna Konopka, Dr.	Start date: 01/05/2012
(Home organisation Short name, country)	DKFZ, Germany	Duration: 18
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1 Executive Summary

Introduction

Metalloproteins are molecules used in clinical diagnostic to determine the medical condition of patients. Some of the measurement methods developed here are already being used by clinical laboratories, e.g. in cancer trials, and are supporting the development of new international standards. Ultimately, the project's results will support the development of more reliable diagnostic techniques, leading to more effective patient care and reductions in healthcare costs throughout Europe

The Problem

The costs of healthcare are rising rapidly throughout Europe as populations' age and the prevalence of chronic disease increases. The World Bank estimates public healthcare spending in the EU could rise to 14% of GDP by 2030, up from 8% in 2000, and continuing to rise thereafter. Clinical techniques are needed that can provide rapid and reliable diagnosis, to provide faster and more effective patient care, and to address rising healthcare costs.

The concentration of particular proteins in patient samples can be used to identify and monitor health conditions. Metalloproteins [such as Transferrin (Tf), Haemoglobin (Hb), Superoxide dismutase (SOD), Ceruloplasmin (Cp)] can indicate inflammation, deficiency disorders such as anaemia, and Down's syndrome in prenatal testing, whilst selenoproteins [such as Glutathione peroxidase (GPx) and Selenoprotein P (SEPP)] and Pt-drugs are widely used in cancer treatments. These metal-containing organic molecules are associated with some of the most serious health issues in Europe. SOD and Cp are markers for rheumatoid arthritis (around 4 million patients in the EU) as well as ischemic myocardium, a cardiovascular disease which causes around 820,000 deaths per year. Whilst approximately 3.2 million people are diagnosed with cancer each year in the EU.

The Solution

The ability to measure concentrations of these proteins reliably is crucial for effective diagnosis and treatment. However, before this project there were few standardised techniques available, let alone primary reference methods, for doing so, and results of inter-laboratory comparisons and evaluations of different diagnostic kits for measuring metalloprotein concentrations were inconsistent. To address this issue, the project developed primary reference measurement procedures for the reliable quantification of metalloproteins, traceable to international measurement standards, including methods to separate, identify, and detect the proteins.

Impact

Dissemination of results

To increase awareness and promote the uptake of the methods developed within this project, results have been shared through the publication of 17 papers in international journals (listed in the next section), and 48 contributions to relevant conferences and workshops. The results of the project were included as six manuscripts in the special issue "Speciation Analysis" in the peer-reviewed Journal of Analytical Atomic Spectrometry (JAAS). The results for the Raman measurements were presented at the 2015 HORIBA symposium to Raman users and instrument manufacturers. The results aroused interest and a researcher from Jena has already visited a project partner to discuss further cooperation. A stakeholder workshop was held at the 2015 European Winter Conference on Plasma Spectrochemistry, the largest global event for users of plasma based spectroscopy. Organised by the research consortium and project stakeholders, the workshop shared the project's results and started a wide discussion within the user community.

Impact on standardisation

The project's work on the measurement of HbA_2 has been presented to the International Federation of Clinical Chemistry and Laboratory Medicine's (IFCC) working group on the standardisation of HbA_2 measurement. Based on the project's alkaline haematin detergent (AHD) approach and an improved version of the German national standard DIN 58931, the approach was proposed to the European Committee for Standardization (CEN). The proposal, "*Haematology - Determination of the concentration of total haemoglobin in blood - Reference methods*" has been discussed at CEN, and it is intended to include the AHD method besides the current approach, which uses potassium cyanide a substance banned in many countries.

Early impact

The primary reference methods developed in the project provide traceability to measurement standards and establish compliance with EU regulations, such as EC-directive 98/79/EC which requires *in vitro* diagnostic devices to be traceable to measurement standards and control materials. The methods will improve the accuracy and reliability of results obtained from patient samples in clinical laboratories, ensuring improved diagnoses and patient treatment, and supporting the development of new therapies. The research consortium are dedicated to ensuring the reference methods are accepted by the BIPM's Joint Committee for Traceability in Laboratory Medicine (JCTLM), to be used in clinical reference laboratories throughout Europe.

Examples of early adoption and impact of the project's results include:

- Two reference laboratories were involved in the project, and have compared results from their clinical techniques to results gained using the project's techniques, allowing results from their routine methods to be traced to the SI for the first time.
- Furthermore, staff from the two clinical reference laboratories in Germany have been trained in the use of the AHD method optimised in the project and are now implementing the method in their laboratories.
- The methods developed for the quantification of Pt-adducts and selenoproteins have been used in clinical cancer trials to help understand differences in response of leukaemia patients to similar Pt-drug doses.
- The project consortium is in contact with the BIPM's Joint Committee for Traceability in Laboratory Medicine (JCTLM), the Hospital of the University of Munich, and research groups concerned with end-user needs, including the Rowett Institute of Nutrition and Health, to discuss the application of the project's results.
- The project's methods were presented at the JCTLM meeting in December 2015 in Paris, with the intention of having them registered in the JCTLM database as reference measurement procedures.

Potential future impact

The techniques developed in this project have a great potential to become the first pimary reference measurement procedures for determining concentrations of metalloproteins in patient samples. These techniques will lead to improved patient diagnoses in a wide range of medical conditions, from arthritis to cancer, but will also support the development of new therapies. For instance, the more effective binding of metallodrugs (such as Pt-containing chemotherapy agents) to DNA is thought to be an important step in the optimisation of cancer therapies. Reference methods that allow for the accurate quantification of Pt-drugs and their adducts to proteins and DNA will support the investigation into improved biding mechanisms. Ultimately, through improved diagnoses and treatment, the techniques developed by this project will improve patient care and outcomes, and can contribute to a reduction in healthcare costs throughout Europe.

2 Project context, rationale and objectives

Metallomics, the science of metal containing proteins, is a very recent and rapidly growing research field, although this kind of proteins is presumed to account for around 30 % of all proteins. They are amongst others relevant in such important processes as respiration and photosynthesis (L. Stryer 1995). Metalloproteins are proteins that need one or more metal ions to perform its specific function in the body. Thereby the metal can either be covalently bound in the protein structure or serve as a so called co-factor. Many of them can serve as important biomarkers for certain diseases such as rheumatoid arthritis with

around 4 million patients in the EU as well as ischemic myocardium which cause around 820000 deaths per year (for example SOD and Cp). Additionally, Cp is discussed as a marker for Alzheimer's disease which affects over 6 million people in the EU. Transferrin (Tf) and haemoglobin (Hb), are amongst others, markers for anaemia caused by iron (Fe) deficiency. According to the French Health Insurance (Sécurité Sociale) refund statistics, haematology laboratory tests account for 32 million of tests per year with a total cost of 237 million Euros.

The quantification of these biomarkers becomes increasingly important in medical diagnostic. This requires reliable and comparable results throughout different measurement kits and different laboratories. Mandatory interlaboratory comparisons, however, show a different picture. In interlaboratory comparisons, as required e.g. in Germany by the Directive of the German Medical Assembly, the different kits were assigned different target values calculated as the mean of all results from laboratories using the same kit. The target values of the various kits can vary significantly. Furthermore, Infusino et al. (2010) analysed various proteins in the reference material BCR-470 at different concentration levels with two different kits and found a significant deviation of the results for Cp of up to 39 %. As important clinical decisions are based on these results, reliable reference values are urgently required to validate and improve the performance of the different test kits based on immunoassays, immunoturbidimetry, immunonephelometry or fluorometry used in clinical laboratories and to render more reliable and comparable results in the daily routine of clinical laboratories. However, a traceable reference value is necessary to achieve comparable results and, thus, increase the reliability of clinical measurements. Furthermore, for some analytes e.g. glycosylated Hb (HBA1c) different calibrators and reference systems are used in different countries which are linked via conversion equations (W. Hoelzel et al. 2004). This renders also the judgement of results from clinical laboratories and the decision about necessary treatment steps by the responsible physician difficult.

To improve the situation, the EC-directive covering *in vitro* diagnostic medical devices (Directive 98/79/EC) requires assurance of "the traceability of the values assigned to calibrators and control materials [..] through reference measurement procedures and/or available reference material of higher order". Additionally, the standard EN ISO 17511:2003 demands reference measurement systems including reference measurement procedures for the determination of analytes in samples of human origin. It is particularly important for medical laboratory measurements concerned with both patient care and health screening to give adequately comparable, reproducible and accurate results (ISO 15193:2009). However, such primary reference measurement procedures are only available for electrolytes, some metabolites, glucose, cholesterol, steroid hormones, some thyroid hormones and some pharmaceuticals. For the majority of clinically relevant proteins no such procedures exist. Therefore, the aim of this project was to develop potential reference measurement procedures for these important metalloproteins. As the metal or small compound containing a metal can often be removed from the protein without damaging the primary protein structure, it opens an easy way to produce isotopically labelled metalloproteins enabling the application of IDMS, which is considered to be a primary measurement procedure. In a first step the metal with a natural isotope composition is removed from the protein and replaced by an isotopically enriched one (*see objective 2*). This protein, the so called spike, is then added to the sample to act as an ideal internal standard. By separating the analyte and the spike from the matrix and detecting the isotope ratio, the concentration of the protein in the sample can be calculated. For the quantitative determination of metalloproteins such as SOD, Cp or selenoproteins, measurement procedures are needed that are sensitive enough to detect proteins in the µg/L to ng/L range and enable the separation of the protein under investigation from possibly interfering proteins (*s. objective 1*). Such procedures could be developed within the project for the named metalloproteins and are now available as references in interlaboratory comparisons.

Biological samples such human serum or erythrocytes represent a complex matrix containing some hundred thousands of different proteins, peptides and electrolytes, interferences of the protein under investigations with other constituents in the sample have to be investigated thoroughly. Furthermore, many proteins exist in different variants which not all have the same clinical significance. Therefore, complimentary methods such as ID Raman and IDMS based on characteristic peptides of the proteins were developed and the results were compared with the results obtained with ICP-IDMS (*s. objective 3*).

For the determination of Hb, one of the most frequently determined analytes in clinical laboratories, the WHO has recommended the so called HiCN method quantifying Hb after the conversion of the protein to cyanmethaemoglobin (HiCN) with potassium cyanide (KCN). As the HiCN method is not applicable in all countries due to the toxicity of KCN and the cost associated with the method, which is mainly a problem in

developing countries, alternative methods are urgently needed. Therefore, Heuck et al. published an alternative in 2008 using the alkaline haematin detergent (AHD_{575}) to convert Hb and measure it at the haematin maximum at 574 nm as well as at its minimum at 534 nm. This method was improved within the project and the calibration material (haematin chloride) was carefully characterised regarding purity and photometric properties. The method was then validated against the ID methods developed within this project (*s. objective 3*). The AHD_{575} method will replace the HiCN method in the future and is already included in the new version of the DIN 58931, proposed also as an ISO norm.

Oral drugs against diabetes such as vanadium (V) containing drugs are of increasing importance as the expenditure for insulin is almost 2-fold higher than that for oral drugs. However, to verify the successful uptake of the drug and for treatment control sensitive methods are required to quantify for example V containing Tf (V-Tf) in blood. Within this project isotopically labelled ^{50}V-Tf could successfully be produced (s*. objective 2*) and a method for the separation and quantification of V-Tf in serum could be developed (*s. objective 1*) and successfully applied to clinical samples.

With 3.2 million people diagnosed with cancer in the EU each year, cancer remains a key health concern. The formation of desoxyribonucleic acid (DNA) adducts with metallodrugs is thought to be a pharmacokinetic parameter in the optimisation of cancer therapy. The efficacy of platinum (Pt) containing drugs (used as chemotherapy agents) to bind to DNA, which is the main reason for these drugs to function as anti-cancer drug, has been proven to be hampered by Pt binding to intracellular glutathione or specific plasma proteins (e.g. human serum albumin (HSA)). Therefore, the search for mechanisms by which Pt containing drugs can be made more available for binding DNA, for example by co-administration of Pt and selenium (Se) containing drugs which have strong affinity for glutathione, are also of particular interest. The interaction between Se and Pt based drugs is still not well studied. Therefore, on the one hand, the accurate quantification of plasma selenoproteins after supplementation of Se containing drugs could be used as a valuable indicator of cancer treatment by Se and, on the other hand, Pt adducts to DNA and plasma proteins have to be studied to investigate the behaviour of Pt containing drugs in the body and understand their severe side effects. In both cases, IDMS was intended to be applied. However, as Se is covalently bound in selenoproteins, the production of spike material was not as straight forward as with the metalloproteins named above. A new cell-free approach to produce ^{76}Se containing selenoproteins as well as the production of ^{76}Se containing peptides specific for the selenoproteins under investigations was developed (*s. objective 2*) and the spike materials were used for the quantification of selenoproteins in human serum (*s. objective 1*). Besides, spike material for adducts of Pt containing anti-cancer drugs with proteins enriched in ^{194}Pt was produced (*s. objective 2*) and applied in IDMS quantification of the adducts in human samples after separation from the matrix (*s. objective 1*). Two alternative separation methods were developed based on field flow fractionation (FFF) and reversed phase chromatography, respectively (*s. objective 3*).

The potential reference measurement procedures developed within this project are necessary both for providing reference values in interlaboratory comparisons as well as clinical studies and for the development and certification of reference materials, which are important in the quality management of each clinical laboratory.

The goal of this project was to develop **primary reference measurement procedures** for identifying and quantifying metalloproteins in patient samples. To achieve this, the project developed methods to separate different proteins in the samples (objective 1), to produce isotopically labelled spike materials to help identify metalloproteins (objective 2), and a range of complementary methods to quantify metalloprotein concentrations (objective 3).

1. Development of separation methods for metalloproteins:

To provide methods for the complete separation of metalloproteins from interfering matrix components in body fluids, such as serum or haemolysates, using size exclusion chromatography (SEC), reversed phase high performance liquid chromatography (RP-HPLC), field flow fractionation (FFF) or a combination of those methods.

2. Preparation and characterisation of isotopically labelled spike materials:

To develop a procedure for the preparation and characterisation of isotopically labelled spike materials by replacing the metal contained in Tf, Hb, SOD and Cp with metal ions from metal enriched in one isotope, as well as synthesising isotopically labelled spike material for selenoproteins such as GPx and SEPP1, and adducts of Pt containing drugs and biomolecules.

3. A multimodal approach for the quantification of metalloproteins:
To establish complementary methods for the identification and quantification of metalloproteins using organic mass spectrometry (EI-MS, EI-MS/MS, MALDI-MS), Raman spectrometry, and inductively coupled plasma mass spectrometry (ICP-MS) for elemental detection, in combination with separation methods such as chromatography or FFF.

3 Research results

For the most part IDMS was used for the development of candidate reference measurement procedures within this project. IDMS is a method with high precision and comparability. In short: The natural isotopic composition of one element in the sample (e.g. Cu) is changed by adding a so called spike material, in which the minor isotope (e.g. ^{65}Cu) is enriched. As the isotopic composition and the concentration of the element in the spike are known, the amount of the element in the sample can be determined from the isotopic ratio in the blend of sample and spike (sample spike blend). The spike serves as an ideal internal standard during the measurement. Any sample loss during sample preparation and separation can be compensated as long as the spike is added before any sample preparation. However, one problem of this method is that the purity of the spike material is often not characterized well enough. Therefore, double IDMS was developed. In double IDMS a second blend is prepared using a well characterized reference material (reference spike blend). The reference material acts as a kind of back spike and is characterized precisely regarding its purity. The advantage is that the mass fraction of the spike is not included in the equation 1 for the amount of mass of the analyte in the sample.

$$w_x = w_z \cdot \frac{m_{yx}}{m_x} \cdot \frac{m_z}{m_{yz}} \cdot \frac{(R_y - R_{bx})}{(R_{bx} - R_x)} \cdot \frac{(R_{bz} - R_z)}{(R_y - R_{bz})} \quad (1)$$

with:

w_z (g/kg)	mass fraction of Cu in reference z
m_x, m_y (g)	mass of solutions of sample x and reference z
m_{yx}, m_{yz} (g)	added mass of spike y solution to sample x and reference z
R_z (mol/mol)	isotope ratio of the analyte (^{65}Cu/^{63}Cu) in reference z
R_{bz} (mol/mol)	isotope ratio of the analyte (^{65}Cu/^{63}Cu) in blend bz (reference z + spike y)

Another problem could be the exact determination of the isotope ratio Ry in the spike material. This factor can be cancelled from the equation by using a second reference spike blend with a different isotope ratio in the second blend as in the first reference spike blend. This approach is called triple IDMS (eq. 2).

$$w_x = w_z \cdot \frac{m_{yx}}{m_x} \cdot \frac{1}{R_x - R_{bx}} \cdot \left[\frac{m_{z1}}{m_{yz1}} \cdot \frac{R_{bz2} - R_{bx}}{R_{bz2} - R_{bz1}} \cdot (R_x - R_{bz1}) + \frac{m_{z2}}{m_{yz2}} \cdot \frac{R_{bx} - R_{bz1}}{R_{bz2} - R_{bz1}} \cdot (R_x - R_{bz2}) \right] \quad (2)$$

with:

m_{z1}, m_{z2} (g)	mass of solutions of reference z1 and reference z2
m_{yz1}, m_{yz2} (g)	added mass of spike y solution to reference z1 and reference z2
R_{bz1}, R_{bz2} (mol/mol)	isotope ratio of the analyte (^{65}Cu/^{63}Cu) in blend bz1 (reference z1 + spike y) and blend bz2 (reference z2 + spike y)

To use species-specific IDMS (the analyte contains the element under investigation in the same chemical form as the spike) in speciation analysis three major requirements have to be fulfilled:

- the separation of the analyte protein from interfering components in the biological matrix
- very precisely characterized reference material
- species-specific spike material.

For the characterisation of the spike material post-column IDMS was applied to determine the metal content in the isotopically enriched proteins. In post-column IDMS the spike is added in inorganic form to the sample after separation via a T-piece. The mass fraction of the metal is calculated from the measured isotope ratio according to:

$$w_x = \frac{m_y \cdot w_y}{\rho_x \cdot V_{inj}} \cdot \frac{M_x}{M_y} \cdot \frac{\sum R_x}{\sum R_y} \cdot \int_{t_1}^{t_2} \frac{R_y - R_{bx}}{R_{bx} - R_x} \, dt \qquad (3)$$

with:

w_x, w_y (g/kg)	mass fraction of Cu in sample x and spike y
m_y (g)	added mass of solution of spike y
ρ_x (g/cm^3)	density of sample x
V_{inj} (mL)	injection volume of sample x
M_x, M_y (g/mol)	molecular masses of sample x and spike y
R_x (mol/mol)	isotope ratio of the analyte (^{65}Cu/^{63}Cu) in sample x
R_{bx} (mol/mol)	isotope ratio of the analyte (^{65}Cu/^{63}Cu) in blend bx (sample x + spike y)

For an easier understanding, the results are composed according to their classification as non-covalently bound metalloproteins, non-covalently bound metallloenzymes and covalently bound metalloproteins and the results are then described according to the objectives named above.

3.1 Non-covalently bound metalloproteins

Introduction

Tf and Hb, are two well-known metalloproteins with non-covalently bound metals, which are used as biomarkers in routine analysis to guide clinical decisions every day. Most routine methods are based on immunoassays or spectrophotometric measurements, which are not considered as primary methods. Due to their clinical impact and in order to assess metrological traceability in clinical biochemistry, these two Fe-containing proteins have been selected as protein models to show the feasibility of a new way to develop reference measurement procedures.

Tf is one of the major proteins that controls the Fe metabolism and is responsible for the Fe transport whereas Hb is responsible for the oxygen transport and storage. Together with the Fe storage protein ferritin those two proteins contain almost all Fe present in the body. The blood concentrations of these two proteins are important indicators in clinical diagnostics. Tf is a biomarker for congenital disorders of glycosylation, cerebrospinal fluid leakage as well as for certain cancers. In case of Hb, higher and lower level might induce diseases such as heart attacks, strokes or fatigue. Therefore, accuracy and reliability in the quantification of Tf and Hb is essential.

Beside the Fe containing form of Tf (Fe-Tf), the vanadium (V) containing variant (V-Tf) is important to quantify due to the ability of V to mitigate deficient insulin response in *Diabetes Mellitus.* A reference method for the quantification of V species in relevant clinical samples will be invaluable to understand the mechanisms by which V supplementation benefits *Diabetes* treatment. It is also needed to provide reference values to clinical diabetes trials and for the production of more efficacious drugs. Therefore, a reference IDMS method for the accurate quantification of V complexes with human serum transferrin (hTf) at a clinical relevant level has been developed by LGC.

Objective 1: Development of separation methods for metalloproteins

One important step in the reliable quantification of proteins is their separation from the biological matrix to avoid interferences. For the separation of Tf from human serum, strong anion exchange (AE) chromatography using the MonoQ GL 5/50 HPLC column (50 x 5 mm i.d., Pharmacia) was selected. The determination of the target protein via Fe was carried out by collision-cell ICP-MS (PTB, TÜBITAK) and sector field ICP-MS (LNE) at medium and high resolution to eliminate spectral interferences on the Fe masses such as ArO.

In order to confirm the separation of the target protein, the Tf peak was collected and analysed with different identification methods: molecular MS such as matrix assisted desorption ionisation time-of-flight MS (MALDI-TOF-MS) (TÜBITAK), LC-ESI-TOF-MS and LC-ESI-Orbitrap-MS (both PTB), gel electrophoresis (GE) at TÜBITAK and an immunosubstraction method (LNE) using a human antibody against Tf. The results of all these techniques confirmed that the Fe peak was indeed caused only by Tf.

Furthermore, a complete interference study was performed, considering exogenous and endogenous interferences. Several blood collection tubes were tested by LNE for exogenous interferences. Only tubes containing ethylene diamine tetra acetic acid (EDTA), used for whole blood haematology determinations, interfere with Tf separation due to the presence of an artefact peak with the same retention time as Tf. Regarding endogenous interferences, other Fe containing proteins such as Hb, bilirrubin and ferritin showed no interference on Tf at the concentrations evaluated (1 g/L, 0.2 gl/L and 50 ng/mL, respectively). However, the accurate determination of Tf was not warranted at higher concentrations of C-reactive protein (> 250 mg/L) due to the co-elution of this protein with Tf. Also, the analysis of ERM-DA470k/IFCC with and without lipid precipitation, showed no statistically differences. Several serum CRMs (e.g. SRM 1950, SRM 909c, BCR 637) were also analysed using the developed separation method and lipoprotein precipitation. An albumin depletion kit was tested by PTB, which is only required for the separation of Tf in a pool serum samples provided by the collaborator Instand e.V.

The developed HPLC-ICP-MS method was validated by LNE in terms of linearity, limit of detection, precision and trueness by external calibration. A limit of detection of (0.036 ± 0.006) g/L Tf was obtained; intra and day-to-day peak area RSDs were ≤ 5 % and RSDs of migration times were ≤ 0.6 % at the three levels evaluated (0.1, 0.4 and 0.7 g/L Tf). The trueness of the method was performed by the analysis of the ERM-

DA470k/IFCC at three different days, obtaining a value of (2.38 ± 0.09) g/L (k = 2), in good agreement with the certified value of (2.36 ± 0.08) g/L.

Finally species-specific IDMS approaches were developed by PTB, LNE and TÜBITAK to determine total Tf mass fraction (g/kg Tf) or mass concentration (g/L Tf) in matrix serum applying the developed separation procedure. To perform IDMS ($R_{56/57}$), the ^{57}Fe-Tf spike was added to the samples after Fe saturation to account for possible sample changes or losses. Double and triple SS IDMS was performed using the two synthesised spikes (C. Frank et al. 2013).

For method comparison and validation, the reference material ERM-DA470k/IFCC, certified for total Tf ((2.36 ± 0.08) g/L k=2; density of 1.0221 g/mL), was used. For the metrological validation of the species-specific IDMS procedures, each NMI analysed 3 to 6 replicates of the CRM at 3 different days using the two different spike materials provided by PTB and TÜBITAK. A good agreement between the results of the NMIs and with the certified value was achieved, indicating the validation of the developed procedures (Table 1).

Table 1: Total Tf mass fractions (g/kg Tf) of the CRM ERM-DA 470k/IFFC obtained with the developed species-specific IDMS procedures.

Tf, g/kg (k=2)	Double (LNE)	Double (TÜBITAK)	Triple (PTB)
SPIKE Batch 1	2.37 ± 0.11	2.38 ± 0.07	
SPIKE Batch 2	2.14 ± 0.11	2.38 ± 0.07	2.43 ± 0.11
Certified value	2.31 ± 0.08 (ρ = 1.0221 g/mL)		

Uncertainty budgets were estimated by each NMI. Relative expanded uncertainties (k=2) were lower than 5.0 % in all cases. In this regard, the objective fixed at the beginning of the project to get an uncertainty lower than 10 % was successfully achieved by the three NMIs for the determination of Tf in serum using species-specific ICP-IDMS.

For the separation, detection and identification of V-Tf isoforms in serum fast protein liquid chromatography (FPLC) using an anion exchange column and on-line ICP-MS detection was selected. Using FPLC, Tf was separated from HSA, which is advantageous because both are plasma proteins that may bind V. A double species-specific IDMS method was developed for the accurate quantification of the major isoform of V-Tf in serum. The target sample was serum spiked with natural V-hTf.

First, V-Tf calibrant with natural isotopic V composition and ^{50}V enriched-Tf spike were prepared by LGC. Media solution includes sodium bicarbonate as synergistic anion which is required for an adequate binding of V ions to Tf and ascorbic acid to prevent V(IV) oxidation. Afterwards, the optimal molar ratio between V and apo-Tf was studied in order to minimize unbound free V and avoid the formation of more than one main V-Tf species, which is not desirable for IDMS. Finally, V-Tf calibrant was fully characterised regarding isotopic composition and concentration with total IDMS using ^{50}V spike solution previously characterized for total V content with ICP-OES. Both V-Tf calibrant and ^{50}V-Tf spike were prepared and stored at -80 °C. Molecular identification of the calibrant and spike was confirmed using ESI-Q-TOF-MS/MS.

Once the synthesis and full characterization of both natural V-hTf and ^{50}V-hTf spike standards was accomplished, characterisation and validation of the IDMS method (e.g. in terms of measurement uncertainties, spike recovery, etc) for the accurate quantification of clinical relevant level of V attached to hTf was carried out. For this purpose, double “approximate” matching species-specific IDMS approach was selected using the previously optimized FPLC-ICP-MS method. Two different blends were prepared: (i) Sample blend (SB) which consists of a commercial human serum sample (SIGMA H4522) fortified with the natural ^{51}V-hTf calibrant. This step was necessary due to the extremely low amount of V present in real samples from clinical trials. Then, the ^{50}V-hTf spike solution was added to this fortified model sample. Blank serum was also prepared in the same dilution to correct for the possible, but rare, presence of native V-hTf. (ii) Calibration blend (CB) which contains both natural V-hTf and ^{50}V-hTf spike mixed in order to match the concentrations used for the SB. These two blends were accurately weighed to give a ^{51}V/^{50}V ratio of

approximately 4. Higher ratios resulted in poor results due to the low enrichment of the available inorganic ^{50}V spike. Within each single measurement batch, each sample blend was analysed three times and bracketed by the mass-bias CB. All the area ratios ^{51}V / ^{50}V between CB and SB were equivalent within < 5 % mass bias deviation. An average V mass fraction of 2.4948 ng/g (n = 3) with an associated expanded uncertainty of 0.3233 ng/g (12.96 %) were obtained for the representative sample. Recovery of V-Tf calibrant added to the serum matrix turned out to be quantitative (99 ± 3) %, demonstrating the reliability of the species-specific IDMS method. As a matter of fact, two independent quantifications (within and between days) provided matching quantitative results. This is clear evidence that degradations occurring to the V-hTf complex during the analytical separation process, independently of its extent, could be completely corrected and so accurate and reliable results are achieved with this method even at the particularly low V concentration levels used.

For the quantification of Hb four different approaches were investigated: species-specific ICP-IDMS (PTB), post-column ICP-IDMS (PTB), external calibration / standard addition and quantification via total Fe content (LNE).

Triple species-specific ICP-IDMS was developed by PTB for the determination of HbA_0 in haemolysates. The separation of the protein was conducted by anion exchange chromatography using a MonoQ 5/50 GL FPLC column and a TRIS/ammonium acetate gradient. The detection was carried out by measuring the Fe of the haeme group using a collision-cell ICP-MS. Characterisation of the HbA_0 standard was carried out using double HPLC-IDMS against a highly purified HbA_0 reference material (IRMM/IFCC-467), certified for total Hb content and traceable to the ICSH (International Council for Standardization in Haematology) reference method. The validation of the triple species-specific ICP-IDMS approach was performed by analysing the reference material JCCRM 912-2, certified for total Hb. A good correlation between the experimental and the certified value was observed, showing the great potential of the developed method.

For the determination of Hb via total Fe analysis, double ICP-IDMS was applied by LNE. This off-line quantification approach is based on the determination of Fe content with double ICP-IDMS using an inorganic spike (^{57}Fe) and closed vessel acid digestion. Via the known number of Fe atoms in Hb (one Fe per subunit) the content of Hb can be calculated. The method was successfully validated by the analysis of the CRM JCCRM 912-1, certified for total Hb, after confirmation of the absence of free Fe. Relative error and expanded uncertainty of 2.7 % and 2.4 %, respectively, were achieved. The quantification of Hb using double Fe IDMS is a promising approach for the determination of Hb in haemolysate matrix when a spike of Hb is not available or difficult to synthesise. It is also a traceable method through the use of a high purity Fe standard. However, it is applicable and gives accurate results only after verifying the absence of free Fe and other Fe-containing proteins.

As a further approach, external calibration and standard addition in combination with HPLC-ICP-MS was investigated by LNE. The separation of Hb was performed by LC using cation and anion exchange columns (Mono S™ 5/50 GL, GE Healthcare; Source 15Q 4.6/100 PE, GE Healthcare). The detection was conducted by measuring the Fe contained in the haeme-group of the protein, using a sector field ICP-MS at medium resolution. At the same time, visible spectroscopy (Vis) detection was performed at 415 nm (characteristic wavelength of the haeme-group). First of all, a characterisation of the Hb standard (H7379, Sigma Aldrich) was carried out via total Fe analysis using double ICP-IDMS. This Hb standard was further characterised regarding water content and protein identification with Karl Fisher and nanoLC-MS/MS, respectively, in order to be used as a primary calibration solution. Only the anion exchange HPLC-ICP-MS method using the standard addition approach provides result for the certified reference material comparable to the certified value within the uncertainty. It seems that the standard addition approach overcomes the matrix effect, which on the other hand leads to biased results for external calibration.

Furthermore, a species-unspecific approach using post-column ID-ICP-MS was used for the Hb quantification at PTB. The on-line ID approach was developed adding post-column an isotopically enriched solution of ^{57}Fe. To assure SI traceability, several parameters were evaluated and determined: the flow of the spike, the mass of the sample loop and the concentration of the spike solution, determined using reverse IDMS against a high purity Fe natural standard. Lower recoveries were obtained with this approach, confirming the need of a species-specific IDMS method able to compensate and correct any losses during the analytical procedure, for example the column recovery.

In the light of these results, the developed and validated species-specific ICP-IDMS procedures are well suited to be used as potential reference measurement procedures for the determination Hb in blood and for the determination of Tf in serum, both Fe containing and V containing Tf. The method for V-hTF provided reference levels of V for the clinical trials which have been carried out recently using new V-based insulin-mimetic drugs such as BMOV or BEOV.

Objective 2: Preparation and characterisation of isotopically labelled spike materials

To perform species-specific IDMS the analytes under investigation have to be available in isotopically enriched form. As for most proteins such form does not exist, procedures for their production were developed within this project.

The synthesis of the isotopically enriched Tf spike material (^{57}Fe-Tf) were synthesised using human apo-Tf obtained from Sigma (T2036) as the common starting material. This material was characterised by LNE, PTB and TÜBITAK regarding water content as well as organic and Fe containing impurities. Organic (LC/ESI-MS) and Fe (LC/ICP-MS) containing impurities were not detected, for that reason only the water content was considered when determining the purity of this Tf starting material.

In Fig. 1 the procedure for the saturation of apo-Tf with ^{57}Fe enriched spike solution is shown. The pH value is very important during and after the saturation because Tf complexes Fe only at a pH value above 4; at pH < 2 the denaturation of the protein can be observed. For this reason at first buffer, sodium carbonat as synergistic anion and acetic Fe solution were well homogenized before the apo-Tf solution was added.

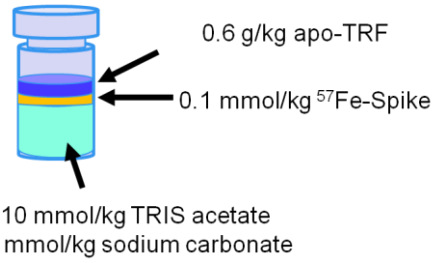


Fig. 1: Saturation of apo-Tf with ^{57}Fe enriched spike solution.

After successfully producing the spike material in small quantities, the production of the isotopically enriched spike was up-scaled (~ 100 mg of ^{57}Fe-Tf) for conducting ID experiments along the project, following two different approaches. Within the up-scaling procedure purification steps were included. Two different approaches were tested:

- Batch 1 is purified 48 h with dialysis against different low concentrated buffer solutions and then lyophilised by PTB.
- Batch 2 was ultra-filtrated at TÜBITAK using a centrifugation filter with a cut-off of 50 kDa for buffer exchange followed by a lyophilisation step.

Several characterisation studies regarding isotopic composition, saturation degree, isotope exchange and storage stability were performed for both spike materials:

a) The Fe isotopic composition, determined with collision-cell ICP-MS (4.0 mL/min H_2) at PTB and TÜBITAK and sector field ICP-MS (at medium resolution), at LNE has been proved to remain stable over the studied 16 months. Results showed an enrichment ≥ 95 % on ^{57}Fe isotope.

b) The metal loading, calculated with species-speicific ICP-IDMS at PTB and LNE, revealed a saturation degree of about 100 % for both spike materials.

c) The possibility of Fe isotope exchange is evaluated by LNE, PTB and TÜBITAK in the spikes and also in serum samples. It has been proven that the isotope ratio ($R_{57/56}$) remained stable in the sample blend within one month and also in the spike after the addition of an excess of natural Fe. In this regard, no Fe isotope exchange could be observed in the presence of natural Fe.

d) An isochronous study has been carried out at TÜBITAK to assess the stability of the spikes under different storage temperatures (4 °C and -20 °C) during 6 months. The most suitable storage condition is at -20 °C due to higher changes in $R_{57/56}$ at 4 °C.

e) The stability of the various sialoforms has also been investigated regarding the influence of storage time, storage temperature and spike preparation. The ratio of sialoforms in the spike material stored at -20 °C as lyophilised powder or in solution has been proven to be stable over a 5 month period (PTB, TÜBITAK).

f) Further characterization studies were carried out by TÜBITAK based on native PAGE analysis, showing that the synthesized spike (^{57}Fe-Tf) exhibits the same electrophoresis behaviour than the natural ^{56}Fe-Tf standard.

For the quantification of V-Tf using IDMS spike material containing ^{50}V was was produced. Isotopically enriched vanadyl sulfate was incubated with hTf to produce ^{50}V-enriched Tf. After partial isolation / purification, using on-line and off-line separation procedures, this spike was characterised for total V content (with ICP-OES). Also, the V isotopic composition of this material (to check whether isotopic fractionation occurred during synthesis) was checked using double focusing magnetic sector field ICP-MS. The material was found to be stable in aqueous buffer solution for at least 6 months when stored at -80 °C.

The production of isotopically labelled Hb was not as straight forward. Hb is an Fe-containing oxygen transport-metalloprotein consisting of two α, two β chains and four complexed haemegroups. Each haemegroup consists of an Fe ion strongly bound to a protoporphyrin ring. Therefore, it was chosen to exchange not only the metal but the whole metal containing subgroup. HbA_0, the most abundant isoform of Hb, was chosen as target protein. The isotopically labelled HbA_0 spike material was synthesised by PTB in three steps: (1) ^{57}Fe enriched Fe was introduced into the protoporphyrin IX (haemin without Fe) to result in protohaeme, (2) secondly an Fe-free HbA_0 (apo-HbA_0) was prepared from a HbA_0 standard (Sigma-Aldrich) and (3) finally the ^{57}Fe enriched protohaeme group was incorporated into the apo-HBA_0.

The synthesised spike (^{57}Fe-HbA_0) was characterised at PTB regarding ^{57}Fe enrichment, protein folding and oxidation state by Raman spectrometry, ESI-TOF-MS and HPLC-UV/VIS-ICP-MS. It was observed that the starting material (HbA_0) contained predominantly Hb with Fe(II), while the spike material consisted of methaemoglobin (Fe (III)). To successfully apply IDMS, it is necessary to have both natural analyte and spike material in the same oxidation state. Several oxidizing and reducing agents were tested; however, only the derivatisation with KCN, a highly reactive agent, worked successfully formatting one stable species (cyanmethaemoglobin), suitable for IDMS experiments.

In this project, it could be to demonstrate that it is feasible to prepare isotopically enriched Hb spike material. The transfer of the HbA_0 spike preparation procedure to other Hb variants is very promising due to protein structure similarity. However, the procedure had to be altered considerable compared to Tf. This shows clearly that the procedure for the exchange of metals in metalloproteins varies greatly due to different binding conditions and conformations of the protein.

For Tf an up-scaling of the production of two different isotopically enriched Tf spike materials (~ 100 mg of ^{57}Fe-Tf) could be achieved, which have been thoroughly characterised, showing stability of it for the duration of this project. This opens the way to a commercial production of the material in order to make it available to reference laboratories. This simplifies the installation of the procedures into reference laboratories as it renders it unnecessary for these laboratories to produce their own spike material in a time consuming process.

3.2 Proteins with non-covalently bound metalloenzymes

Introduction

The Cu containing proteins SOD and Cp were investigated as examples for metalloenzymes with non-covalently bound metal ions.

SOD is a homodimeric enzyme, which contains one Cu and one zinc (Zn) ion per subunit. It is known to catalyse the disproportionation of the superoxide anion to oxygen and hydrogen peroxide preventing the organism from oxidative stress. It is an acute phase protein and changes in its activity indicate some diseases such as myelogenous leukemia or renal failure. Furthermore, it is used as a drug for the treatment of rheumatoid arthritis in China.

Cp is an α_2-glycoprotein and is the Cu storage protein in the body. It binds about 95 % of the Cu amount in the body, carries 6 to 8 Cu ions and occurs in blood serum in a concentration range of (0.2 - 0.6) g/L. It can be used in clinical diagnostics to verify various Cu related diseases, e.g. Wilson's disease in which the Cu metabolism is disturbed. The Cu is not secreted by the bile anymore, but is enriched in the liver, brain and the eyes. Affected patients have decreased Cp levels in their blood (< 0.2 g/L).

As both proteins are relevant in clinical diagnostics, reliable and traceable determination in human blood is of great importance. Nowadays, the quantification of proteins in clinical laboratories is performed with commercially available immuno-based measurement kits, which often use different calibrators or antibodies. The results might not be reliable and are often not comparable. A good example to elucidate the problem is the determination of Cp. When different measurement kits were compared their results are inconsistent with a variation of up to 30 % between the kits. As no reference material or reference measurement procedure exists, no one could state which kit delivers the correct results. Therefore, it is important to develop a primary reference measurement procedure with reliable, comparable and traceable results. Double and triple IDMS used detecting Cu and Zn with ICP-MS are a very promising approach.

Objective 1: Development of separation methods for metalloproteins

As there is also no SOD reference material available, the native protein purchased from Sigma-Aldrich was characterised very precisely by PTB. To exclude protein impurities, the native SOD was measured with HPLC-ESI-TOF-MS and native GE. The water content was determined by constant weighing. Furthermore, the metal content was measured using single post-column IDMS to verify the purity of the purchased material. From the Cu mass fraction the SOD mass fraction could be calculated. A purity of the purchased native SOD was found to be 68 %.

Separation of SOD from other Cu containing components in human erythrocytes was achieved at PTB and UNIABDN using HPLC-ICP-MS. Red blood cells were obtained from the German Blood Bank. Right before the measurement, Hb, contained in the blood in high quantities, was precipitated to reduce its concentration in the erythrocytes as Hb can unspecifically bind a variety of metals. The separation was achieved using an anion exchange column. The operating conditions are summed up in Tables 2 and 3.

Table 2: HPLC operating conditions for the separation of SOD from interfering matrix developed and optimised by PTB and UNIABDN.

HPLC	**Agilent 1200**
Column	MonoQ 5/50 GL (GE Healthcare)
Column oven	30 °C
Flow rate	0.5 mL/min
Sample loop	100 µL
Injection volume	25 µL
Eluent A	12.5 mM Tris pH 7.1
Eluent B	A + 125 mM NH_4Ac pH 7.1
Gradient	7 % B 0 – 5 min 7 – 50 % B 5 – 10 min 50 % B 10 – 12 min 50 – 86 % B 12 – 17 min 86 % B 17 – 19 min 86 – 100 % B 19 – 20 min

	100 % B 20 – 25 min

Table 3: ICP-MS operating conditions for the detection of Cu and Zn optimised by PTB and UNIABDN.

ICP-MS	**Agilent 7700x**
Plasma power	1500 W
Lenses	cx
Reaction mode	no gas
Mass analyzer	quadrupol
Acquisition mode	time resolved analysis
Monitored isotopes	^{63}Cu, ^{65}Cu, ^{66}Zn, ^{67}Zn
Integration time	0.1 s

With the developed gradient it is possible to separate SOD from all other Cu carrying proteins in erythrocytes. To verify the successful separation of the SOD peak in the erythrocyte sample from interferences, this fraction was collected and measured with ESI-TOF-MS. The MS spectrum showed no other proteins coeluting with the SOD.

For the quantification of SOD IDMS was applied. The reference spike blend as well as the erythrocyte spike blend, required for the IDMS measurements, were prepared gravimetrically. Hb was precipitated with a mixture of ethanol/chloroform. Finally the prepared blends were measured using HPLC-ICP-MS. To determine the precision, repeatability and reproducibility of the method the samples were prepared threefoldly at PTB and measured on three different days. Every sample was measured ten times. The measured isotope ratio in the blends was entered into the equation for double IDMS (eq. 1) and the mass fraction of SOD in the sample was calculated to be 65 µg/g was calculated with an expanded uncertainty of 3.2 % (k=2). The uncertainty budget was estimated using the GUM workbench.

To verify the results of the double IDMS and to reduce the uncertainty, triple IDMS was performed by PTB. In this case, two reference spike blends with different isotope ratios were prepared. Here, again, the samples were prepared threefoldly and measured ten times on three different days. The mass fraction of SOD in the sample was calculated according to equation 2. The results of double IDMS were confirmed with triple IDMS. It was possible to reduce the expanded uncertainty to 1.8 %.

It was intended to transfer the methods for SOD to the determination of Cp. However, it turned out that this was not feasible and a different procedure had to be developed using a size exclusion column. Because of the difficulties to separate Cp from HSA, this protein had to be precipitated using an albumin depletion kit before the chromatographic separation. The chromatographic conditions for the separation of Cp from serum developed at PTB are compiled in table 4 and 5.

Table 4: HPLC conditions for the separation of Cp from interferring components in serum.

HPLC	**Agilent 1200**
Column	SEC (Supelco)
Column oven	30 °C

Flow rate	0.35 mL/min
Sample loop	100 µL
Injection volume	15 µL
Eluent A	10 mM Tris + 125 mM NH_4Ac pH 7.1
Eluent B	A + 125 mM NH_4Ac pH 7.1

Table 5: ICP-MS operating conditions for the detection of Cu.

ICP-MS	**Agilent 7700**
Plasma power	1500 W
Lenses	cx
Reaction mode	He
Mass analyzer	quadrupol
Acquisition mode	time resolved analysis
Monitored isotopes	^{63}Cu, ^{65}Cu
Integration time	0.1 s

As IDMS was applied, pure Cp was used as reference after carefully characterising the protein purity at PTB using the single post-column IDMS approach as described above. Additionally, BOKU determined the proteins purity by quantifying the Cp via its S containing amino acids. For this purpose ^{34}S enriched yeast (Pichia pastoris) was produced. The enriched biomass was hydrolyzed and characterised for its S proteinogenic amino acid content by reversed species-specific IDMS using commercially available methionine sulfone and cysteic acid standards. Finally, Cp standards (as well as lysozym standards) were quantified with IDMS using the produced spike material. The protein concentration was calculated based on the known number of cysteines and methionines in the amino acid sequence. The quantitative values obtained considering cysteine and methionine were in good agreement. The experimental repeatability of completely independent measurements (two laboratories, two different ICP-MS instruments) was about 10 %. Method validation comprised cross validation with two different ICP-MS concepts and uncertainty budget calculations.

Both SOD and Cp could successfully be separated from the matrix of human erythrocytes and quantified via their Cu content using IDMS. These measurement procedures are now available to be used as reference measurement procedures. However, in case of Cp the stability of the protein is still an issue and has to be monitored carefully. After publication of the procedures, it is intend to submit them to JCTLM in order to be registered as reference measurement procedures.

Objective 2: Preparation and characterisation of isotopically labelled spike materials

As there is no species-specific SOD spike material available on the market, the SOD spike had to be prepared from native SOD in two steps. The first step was the demetallation of the native protein. The protein's tertiary structure has to be denatured reversibly so that the metals with natural isotopic composition are released. In the second step, the isotopically enriched metals were incorporated into the protein structure.

For the spike preparation, different methods were tested by UNIABDN. In Fig. 2 the three most successful demetallation methods for SOD are shown. In method A, the native protein was demetallated by dialysis against an acidic ammonium acetate (NH_4Ac) buffer. In method B, the SOD was also dialysed against an

acidic NH_4Ac buffer, but in the presence of EDTA to complex the released metals. To remove the EDTA further dialysis steps were required. In method C, the native SOD was demetallated with guanidinium chloride (GndCl) also in the presence of EDTA. A dialysis against neutral NH_4Ac buffer was performed to remove the GndCl and the EDTA. The apo-SOD solutions prepared with the different methods were analysed and compared according to their demetallation efficiency. The spike was then prepared from the produced apo-SOD. All three produced apo-SODs were remetallated in the same way. The spikes were analysed and the remetallation efficiency as well as the isotopic enrichment and the stability of the spike were compared for all methods by UNIABDN and PTB, revealing that demetallation of method B is the most suitable method for SOD spike production.

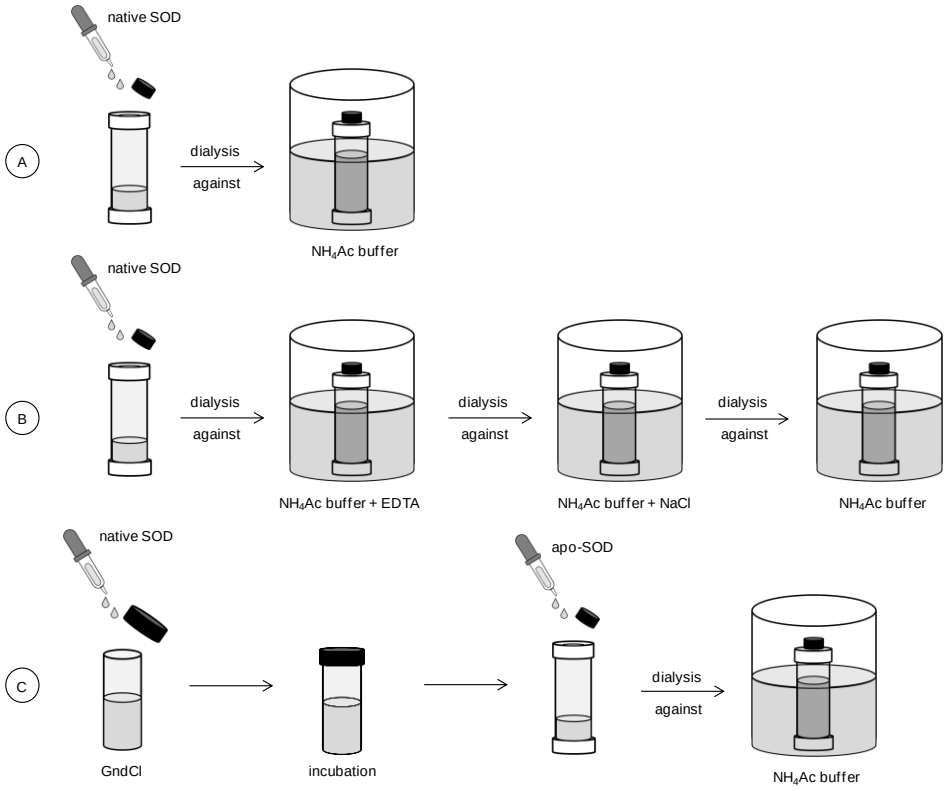


Fig. 2: Different demetallation methods A, B and C for the removal of Cu and Zn from SOD to produce apo-SOD.

Before remetallation (Fig. 3) the apo-SOD solution was neutralised. ^{67}Zn was added in surplus and the mixture was incubated. Afterwards the ^{65}Cu was added, also in surplus, followed again by incubation. The order of the addition of ^{67}Zn and ^{65}Cu is essential as Cu can occupy the Zn binding sites irreversibly. To get rid of the excess metals, the spike material was centrifuged using a mass weight cut-off (MWCO) filter with a cut-off of 3 kDa.

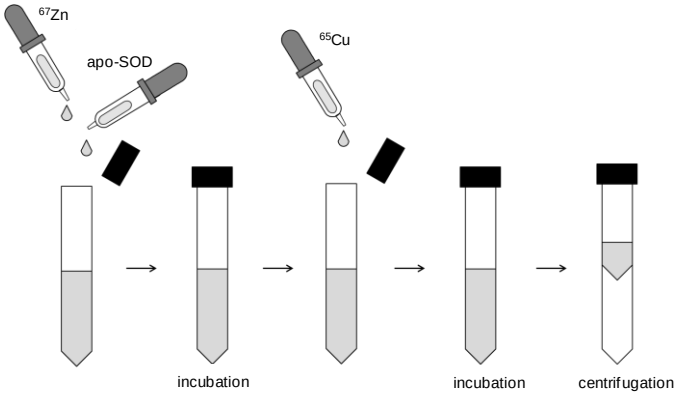


Fig. 3: Remetallation of the produced apo-SOD with isotopically enriched ^{68}Zn and ^{65}Cu to produce the necessary species specific spike material.

As Cu has a comparable environment in Cp as in SOD, it was intended to transfer the methods for SOD to the determination of Cp. However, it turned out that the transfer of the spike preparation from SOD to Cp was not possible. All three demetallation methods, which were tested for SOD, could successfully remove the metal from Cp. However, the incorporation of the isotopically labelled metals into the protein structure was not possible. Supposedly the protein structure is denatured irreversibly when subjected to the conditions of the demetallation step, thus preventing the refolding necessary to incorporate the enriched metals. Therefore, other demetallation methods for Cp were tested (Fig. 4) by PTB. In method D, the native Cp was dialysed against KCN in an imidazole acetate buffer followed by the dialysis against a pure imidazole acetate buffer to remove the KCN. In method E, the native protein was dialysed against potassium thiocyanide (KSCN) in an NH_4Ac buffer followed by the dialysis against pure NH_4Ac buffer to remove the KSCN.

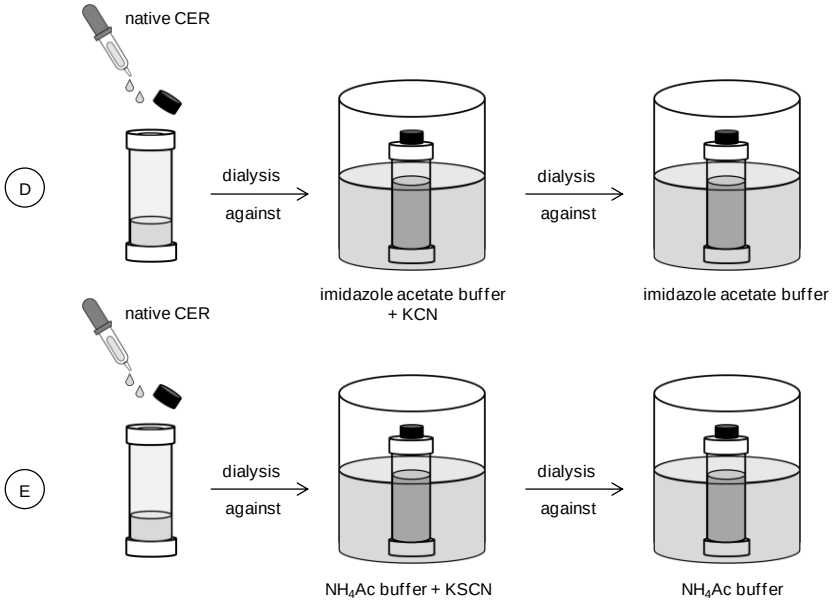


Fig. 4: Demetallation method D and E to remove Cu reversibly from Cp.

The remetallation (Fig. 5) was performed under anaerobic conditions. ^{65}Cu solution was added in surplus to apo-Cp over a time range of an hour. The mixture was then allowed to incubate over night. The excess metals are removed by dialysis against phosphate buffer. The prepared Cp spike solutions were concentrated using a spin-filter with 3 kDa MWCO.

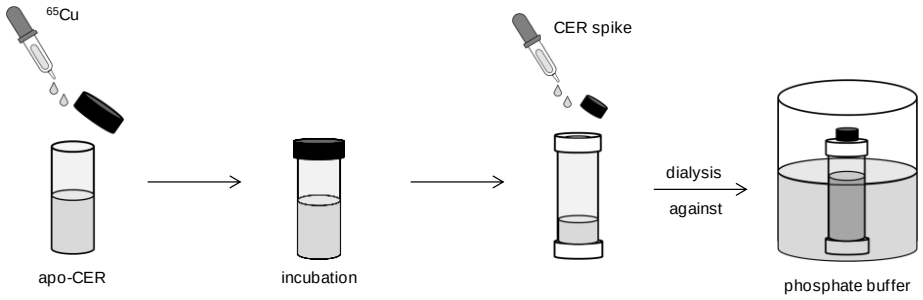


Fig. 5: Remetallation of produced apo-Cp.

Both demetallation methods D and E resulted in the apo-form of Cp. However, the remetallation following method D was only partially successful. Less than 50 % of the protein incorporated ^{65}Cu correctly. The rest remained in its unfolded state carrying no Cu. The remetallation following method E showed a lower isotopic enrichment, but the main portion of the protein was remetallated correctly. Just a tiny fraction of the protein remained in its unfolded form. This might be optimised further by shortening the incubation time and replacing the final dialysis step with a desalting step using a SPE column. Altogether, method E is a very promising approach for the preparation of Cp spike material.

For both Cu containing proteins under investigation, SOD and Cp, isotopically labelled spike material could be produced and is now available to be used in IDMS. However, further investigations regarding long-term stability have to be conducted before they can be produced on a larger scale.

3.3 Covalently bound metalloproteins, metallodrugs and mediating metabolites in cancer chemoprevention and treatment

Introduction

Evidence has indicated an association between Se, reduction of DNA damage and oxidative stress together with data showing an effect of selenoprotein genotype on cancer risk, implying that selenoproteins are indeed implicated in cancer chemoprevention.

The formation of DNA adducts with metallodrugs is thought to be a pharmacokinetic parameter which could be used in clinics to optimise cancer therapy. The search for mechanisms by which Pt containing drugs can be made more available for binding DNA with minimal side effects are also of particular interest. This requires methods able to measure the unbound and protein-bound fractions of the Pt containing drug to elucidate protein-drug interactions and their respective kinetics.

Objective 1: Development of separation methods for metalloproteins

To separate Se species in serum an off-line fractionation method using ultra-microfiltration centrifugal devices with a cut-off of 3 kDa (Amicon Ultra -0.5 mL 3 K, regenerated cellulose, Sigma) were developed by LNA and TÜBITAK. These devices enabled a selective and quantitative separation of inorganic Se, free selenomethionine (Se-Met) / selenocysteine (Se-Cys), chlorine (Cl) and bromine (Br) from selenoproteins and/or Se-containing proteins (SEPP1 43.174 Da; glutathione peroxidase 3 (GPX3) 25.552 Da and ALB 69.367 Da) in human sera. Besides, off line ultrafiltration of serum provided by the UK Precise pilot study (from individuals supplemented with Se-yeast) were performed using ultrafiltration cellulose membranes of 30 kDa to determine the Se content in high molecular weight serum fraction and that associated to low molecular weight Se metabolites. The Se-Met content of the low molecular weight fraction, as obtained using enzymatic hydrolysis, was used to determine the content of Se associated mostly with HSA. By subtracting this Se value from the total Se in the high molecular weight fraction, the amount of Se associated with selenoproteins (SEPP1 and GPX3) could be determined. Most Se associated to proteins could be recovered from the filters (average recoveries ranging from (98 – 101) %).

For the separation of metalloproteins, a separation technique for the simultaneous speciation analysis of Se-proteins and/or Se-containing proteins (particularly, GPX3, SEPP1 and HSA) in serum samples was developed at LNE, based on double affinity-HPLC (AF-HPLC) hyphenated to ICP-MS with collision/reaction cell system. Double-column AF-HPLC was carried out using custom AF columns (5 x 0.4 cm i.d.) packed with stationary phases of commercial columns (GE Healthcare), namely Heparin-Sepharose and Blue-Sepharose. The Heparin-Sepharose column is able to retain selectively SEPP1, while the Blue-Sepharose is able to retain both SEPP1 and ALB. On the other hand, GPX3 is not retained due to the lack of affinity for both columns. The separation of GPX3 in serum by double AF-HPLC is hampered by the co-elution of non-retained species, such as Cl, Br, inorganic Se (e.g. selenite, selenate) and/or other low molecular weight Se-species (e.g. free Se-Met/Se-Cys). In order to eliminate them, serum samples were passed through centrifugal devices with a cut-off of 3 kDa. Quantification of GPX3, SEPP1 and HSA in commercially human serum samples was performed by on-line external calibration using Se-Met standards. A chromatogram of such a separation is presented in Fig. 6.

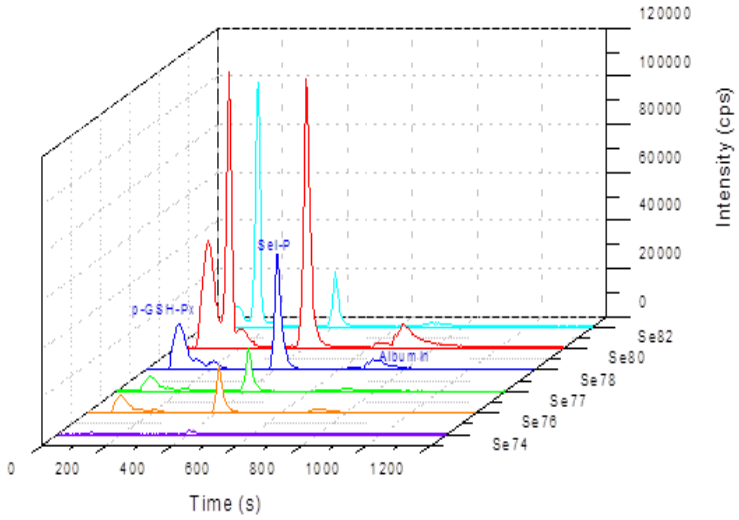


Fig. 6: Separation of different Se species in human serum samples using double AF-HPLC-ICP-MS after filtration with a 3 kDa cut off filter.

Based on the use of AF chromatography on line with ICP-MS and species-specific single IDMS a procedure for quantification of whole SEPP1 was developed and validated jointly by LGC, LNE, TÜBITAK and UNIABDN. The method accuracy was verified by analysis of BCR-637 and the SRM 1950, for which certified values for SEPP1 are not available but an indicative value is given for SRM 1950 and the literature contains a large number of SEPP1 data for BCR-637. A SEPP1 mass fraction of (55.52 ± 3.16) ng/g Se was obtained for BCR-637, which is in good agreement with values reported in the literature using post-column IDMS or on-line external calibration. A relative expanded uncertainty (k=2) of 5.6 % was achieved; the main contribution to the total uncertainty budget come from the measurement precision (78.3 %). In case of the SRM 1950, a SEEP1 mass fraction of (63.87 ± 4.93) ng/g Se was obtained with a relative expanded uncertainty of 7.7 %. The SEPP1 value obtained by species-specific IDMS is higher than the reference value given by NIST ((50.2 ± 4.3) ng/g Se) obtained by post-column IDMS. Note that although recoveries of total Se as the sum of Se species from serum have been reported using AFchromatography and post-column IDMS calibration, this calibration approach does not account for effects of losses or species transformation that may occur during sample preparation or analysis on the accuracy of the speciation data. The main contributing factors to the overall measurement uncertainty were the inter- and intra-day standard deviation, the serum between-batch variability and the use of different home-packed AF columns.

In addition to the quantification using the whole selenoproteins, a procedure for the quantification of selenoproteins via the peptides was developed as the production of isotopically labelled peptides is easier than for the whole proteins and might serve as a low cost alternative. For this the fractions containing the peptides characteristic for the protein under investigation were collected and concentrated using 30 kDa spin filters and subjected to tryptic digestion with the protocol optimised for serum proteins (trypsin/protein ratio

1:25, pH=7, incubation time of 2 h). Four peptides specific for SEPP1 were identified: S**U**CCHCR, TGSAIT**U**QCK, ENLPSLCS**U**QGLR and AEENITESCQ**U**R where U = L-Sec. Due to a lack of stability of the peptides S**U**CCHCR and TGSAIT**U**QCK, only the peptides ENLPSLCS**U**QGLR and AEENITESCQ**U**R were selected for further investigations. Unfortunately, analysis of the tryptic digests by nano-LC with ESI-TOF-MS/MS did not enable peptide identification, due to the low peptide concentration and the high complexity of the digests. Using the selected separation method, SEPP1 was quantified by LGC via the two peptides with double exact matching species-specific IDMS. The chosen sample was a serum sample from NIST (SRM 1950) which has a reference value for SEPP1 of (50.2 ± 4.3) µg/kg. Spikes and calibrants were characterised by total IDMS to obtain the amount of total Se. The impurities of both, calibrant and spike, were quantified using HPLC-ICP-MS. For quality control purposes, a standard addition blend was included in each of the IDMS batches. In addition to that, the serum sample BCR-637, which is a serum sample certified for total Se and with values reported in the literature for SEPP1, was measured within the batches as well. The recoveries for these samples were close to 100 %. Using the proposed method an expanded uncertainty of 3.24 µg/kg (k=2) or 5.35 % (relative) was found for Se in the SEPP1 of the SRM 1950. The main contributors to the uncertainty budget were the measured isotope ratios either in the calibration or sample blend, concentration of the calibrant and the blend to blend variation.

Different approaches for the quantification of SEPP1 in NIST SRM 1950 were compared. Within the expanded uncertainty the value for the mass fraction obtained with species-specific double IDMS (at the Se peptide level) of (60.56 ± 3.24) µg/kg Se (LGC) agrees well with the one obtained using species-specific single IDMS for the whole protein ((63.87 ± 4.93) µg/kg Se) (LNE).

Fig. 7 shows a chromatogram obtained for the target Se peptides in the IDMS sample blend as using double HPLC-ICP-IDMS.

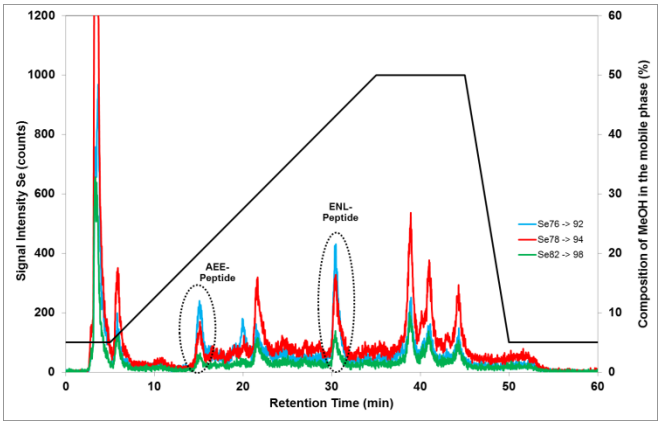


Fig. 7: Separation of Se peptides in a blend of sample and spike obtained by LGC.

Beside selenoproteins, which play a role in cancer treatment, adducts of Pt-containing drugs, used in cancer treatment, and plasma proteins as well as nucleotides was investigated. Different approaches for the separation, detection and quantification of the Pt-protein adducts were developed. A fractogram obtained by LGC with FFF-ICP-MS for a mixture of adducts of Pt containing drugs with Tf and HSA in 30 mM Tris-HCl (pH 7) is presented in Fig. 8. Although FFF was found to be useful to provide an insight into the formation and stability of adduct spikes and standards, it was not used for further work with the real matrix since the chromatographic selectivity between target adducts required for IDMS quantification could not be achieved with this method.

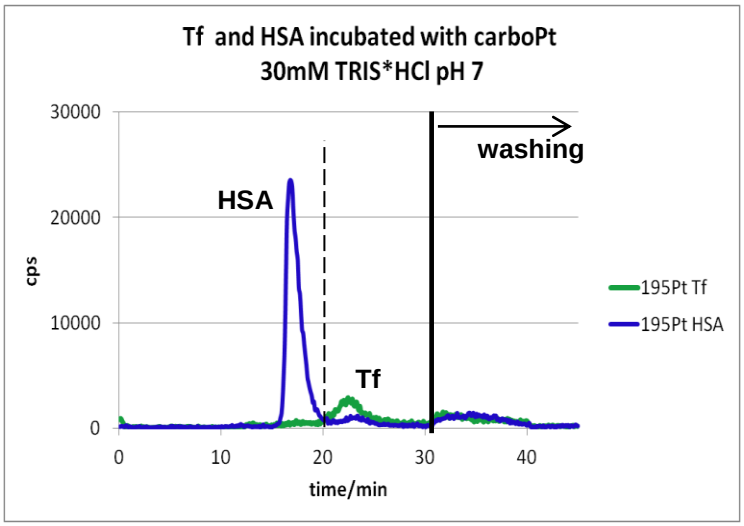
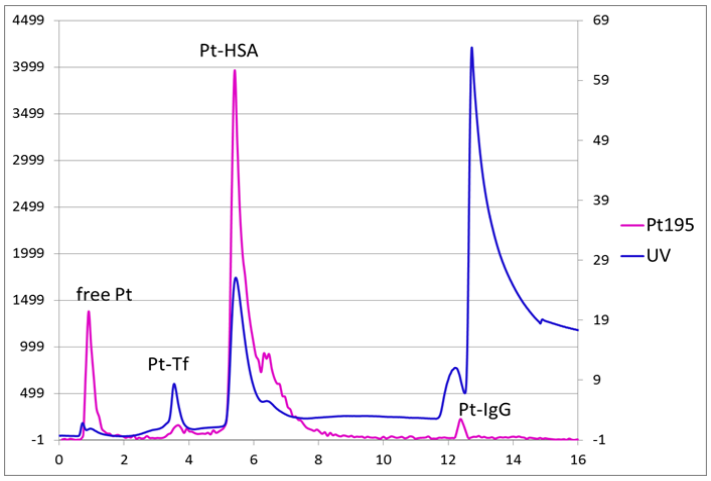


Fig. 8: Chromatogramme for the separation of Pt-drug adducts to serum proteins such as HSA and Tf obtained by LGC using FFF

BAM used a method based on the separation with native polyacrylamide GE and detection of Pt-protein adducts with laser ablation (LA) ICP-MS directly applied to the gels. For simultaneous S/metal detection, alternative membranes were investigated since the S content of the nitrocellulose membranes was found to be quite high, thus hampering S detection of the target proteins. Another approach is the separation/detection of adducts of carboplatin with HSA, Tf and IgG using monolithic chromatography with ICP-MS detection (LGC). Two different discs, protein G with an affinity to IgG and a weak anion exchange (DEAE) discs, were used. The mass chromatogram for Pt and the according UV chromatogram are presented in Fig. 9, showing baseline separation of the investigated adducts. Incubation experiments of HSA, Tf and IgG with carboplatin at clinical levels demonstrated that approximately 84 % of the total Pt is associated with HSA. Therefore, further work focused on the HSA-carboplatin adducts using only the DEAE disks.



HSA: 37.2 g/L

Tf: 2.36 g/L

IgG: 9.17 g/L

Carbo-Pt: 1 µg/g of Pt

In serum matrix (fraction <10 kDa) after incubation at 37 °C for 10 days

Fig. 9: Chromatogram of Pt and the according UV for samples of Pt-protein adducts obtained using carboplatin with HSA, Tf and IgG.

Afterwards, a method for the accurate quantification of the adducts of HSA with carboplatin was developed by LGC using species-specific double IDMS calibration with HPLC-ICP-MS. Calibrants and spikes were produced and characterised as described in the next section. The selected sample was the serum ERM®-DA470k/IFCC certified in protein content (HSA: 37.2 g/L, Tf: 2.36 g/L, IgG: 9.17 g/L), incubated with 1 µg/g Pt (as ^{nat}carboPt). An expanded uncertainty of 24.86 mg/kg (k=2) or 4.71 % (relative) was found with species-specific double IDMS for a level of Pt drug adduct with HSA of 527.63 mg/kg. The overall uncertainty

budget estimation was carried out and the main sources of uncertainty were identified; they include the uncertainty contribution of the concentration of natural adduct calibrant, the blend to blend variation and the isotope ratios of calibration and sample blends measured with HPLC-ICP-MS. In the absence of a certified reference material for method validation, the method accuracy was assessed by recovery measurements on spiked serum ERM®- DA470k/IFCC; the average recovery was (101.6 ± 3.5) % (n=3).

The effect of Pt containing drugs in cancer treatment is due to the binding of those drugs to the DNA with adducts to the protein causing the side effects often observed. Besides the Pt-proteins adducts also the adducts to DNA was investigated within this project. A method based on the use of microflow reversed phase HPLC with double focusing magnetic sector field ICP-MS and ESI-MS/MS was developed by LGC for the selective separation/detection of the major adduct of DNA (Pt-GG) with the free carboplatin drug. Isocratic elution was performed on a Zorbax Rx-C_8 column (250 × 4.6 mm, particle size 5 µm) (Agilent, Palo Alto, CA, USA) with 10 mM NH_4Ac with 3.5 % methanol (pH 6.9) at a flow rate of 1.5 ml/min. The Pt-GG standard for method development was produced by incubation of a Sigma GG standard with natural carboplatin. The adduct formation was verified by microflow HPLC-ESI-MS/MS. The limit of detection was estimated to be 0.2 ng Pt/mg DNA (based on the amount of 14 µg DNA). The quantification of the adducts formed by carboplatin with DNA nucleotides GG was achieved using species-specific IDMS. The Pt limit of detection obtained using µ-HPLC with sector field ICP-MS is 0.1 ng Pt/mg DNA (85 µg DNA). An expanded uncertainty (k=2) lower than 10 % was achieved for a Pt-GG mass fraction of 5.5 ng Pt/mg DNA. For the first time a reference sample of calf thymus Pt-DNA was prepared and characterised by LGC for use as a quality control sample in batches of real samples and in particular as spike material to determine the recovery from enzymatic cleavage in the presence of the matrix of a real sample.

The parent ion was found at *m/z* 824. Main fragment ions were detected at m/z 789, 513 and 497. These data are consistent with MS/MS data reported for Pt-GG adducts from other drugs (e.g. cisPt) in the literature. The ESI MS/MS mass spectrum of the generated Pt-GG adducts (*m/z* 824) is presented in Fig. 10.

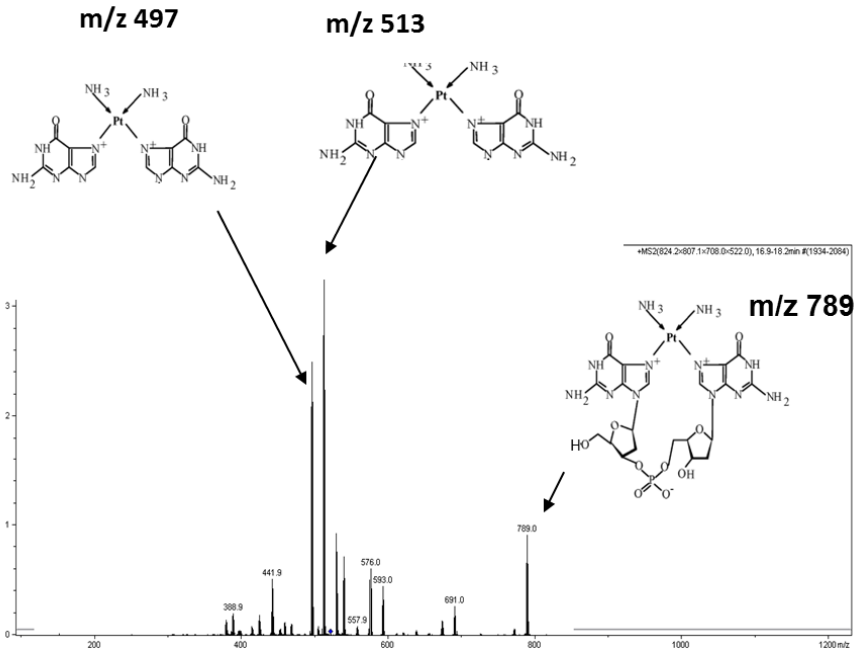


Fig. 10: ESI MS/MS mass spectrum of the generated Pt-GG adducts at *m/z* 824.

Additionally, LU investigated the behaviour of Pt-drugs directly in cells. Species-unspecific IDMS was used to quantify Pt in cells. Dosing experiments of A549 cell culture, a human lung carcinoma cell line, with oxaliplatin were performed. Samples were fractionated into cytosolic, membrane/organelle, nucleic protein and cytoskeletal fractions using an existing LU cell fractionation method. Pt concentrations in these fractions as well as in the corresponding whole-cell digestions have been determined with ICP-MS. To aid the understanding of Pt-drug binding to cytosolic proteins, a series of short S-rich peptide sequences (4-5

peptides) from hypothesised digestions of proteins that are up-regulated in certain cancer cell lines, were identified, reacted with oxaliplatin and analysed.

Additionally experiments involving single cell LA-ICP-MS were performed using the A549 cells dosed with oxaliplatin. In-house preparation of cells was performed by LU using a refrigerated centrifuge and cytofuge accessories. Cells were mounted onto glass slides and analysed with LA-ICP-MS. Method developments have focused on cell suspension volume and media composition, as well as the centrifuge parameters used to mount cells.

Primary and well-characterised methods for the accurate quantification of plasma selenoproteins, adducts of Pt containing drugs with proteins (Pt-protein adducts) and DNA adducts with the new generation of Pt containing drugs was developed and validated. Such methods will be invaluable to help validate measurements in the clinic and to provide reference values to clinical trials in the EU. They were applied, for example, in collaboration with the University of Leicester where LU accessed patient derived squamous lung tumour biopsies which have been grown on in the laboratory and dosed with cisplatin. Furthermore, a researcher of LU in collaboration with LGC performed distribution mapping experiments with another kind of tumor cells. The samples in this case were derived from a cisplatin resistant tumour and results indicated a relative decrease in Pt levels in areas corresponding to tumour cells, when compared to surrounding healthy stromal cells.

Objective 2: Preparation and characterisation of isotopically labelled spike materials

As described above species specific spikes are necessary for accurate IDMS analysis. For selenoproteins the synthesis of isotopically labelled compounds is more challenging than for the other metalloproteins investigated in this project because Se is covalently bound in the protein back-bone. For the first time [Sec-to-Cys]SEPP1 and [Sec-to-Cys]GPx3 spikes containing ^{76}Se-enriched Se-Met, needed for IDMS quantification of the entire proteins (plasma selenoprotein P and glutathione peroxidase), was synthesised and characterised in close cooperation of LGC and DKFZ. In these isotopically labelled proteins Se-Cys is replaced by Cys ([Sec-to-Cys]) because the system used at DKFZ for the synthesis (cell-free *Escherichia coli* (*E.coli*)) cannot “read” the DNA code for Se-Cys as it is identical with the stop codon and only encoded with an addition to this sequence. Instead, the labelling was realised using ^{76}Se-Met, incorporated instead of Met. The resulting protein synthesis mixture was provided with Se-Met or ^{76}Se-enriched Se-Met and the expression vector pEXP2-dest/SEPP1_all_mut with modified nucleotide sequence was applied as DNA template for protein synthesis. Different purification procedures including electro-elution from polyacrylamide gel and subsequent dialysis with a cut-off of 12-14 kDa and the Ni^{2+} IMAC technique were investigated at DKFZ. Incorporation of ^{76}Se-enriched and/or natural Se-Met was verified by tryptic digestion of the protein standard followed by LC-ESI-MS analysis of the obtained peptides mixture. To estimate the content of other proteins in the standard sample (coming from the *E.coli* extract), the total protein concentration was also determined. Furthermore, co-elution of synthesised proteins with native proteins as isolated from NIST serum SRM 1950 was investigated at LGC by using affinity HPLC and field flow fractionation (FFF) with ICP-MS detection. The determination of the Se content in the [Sec-toCys]SEPP1 spike was carried out using post-column IDMS (using a natural Se standard), on-line external calibration (using Se-Met standards) and total IDMS after an ultrafiltration step with 30 kDa cut off filter to remove excess ^{76}Se-Met used in the spike production. Most Se in the spike is present as SEPP1 ((91.7 ± 0.5) %). However, in case of the [Sec-toCys]SEPP1 standard, containing Se with natural abundances, only (32.6 ± 0.3) % is present as SEPP1 and the rest of Se elutes in the non-retained AF fraction (0-5 min). Batches of these materials, after using the different purification procedures, were tested and distributed to the project partners to be used in IDMS protein quantification. For single IDMS, the characterisation of the protein and the spike is the most critical step.

For the first time, a method for the quantification of selenoproteins via Se peptides has been developed by LGC. To achieve this, a suitable separation method for the selenopeptides was selected and the Se signal was detected by triple quadrupole ICP-MS in oxygen mode. Baseline separation of the target peptides was achieved on a C_8 column using a methanolic concentration gradient in the presence of formic acid at a flow rate of 0.2 mL/min. Four peptides specific for SEPP1 were identified, which then were produced with a ^{76}Se enrichment of 99.8 %. Synthesis of both natural and ^{76}Se-enriched peptides including S**U**CCHCR, TGSAIT**U**QCK, ENLPSLCS**U**QGLR and AEENITESCQ**U**R where U = L-Sec was undertaken. Due to the very poor purity and lack of stability of the peptides S**U**CCHCR and TGSAIT**U**QCK, only the peptides ENLPSLCS**U**QGLR and AEENITESCQ**U**R were selected for further investigations. Using ESI QTOF MS/MS,

the structural composition of the peptides ENLPSLCS**U**QGLR and AEENITESCQ**U**R, which are selective to SEPP1, was successfully characterised/verified and these peptides were then used for protein quantification.

For the quantification of Pt-drug adducts to serum proteins and DNA with IDMS ^{194}Pt-enriched carboplatin was produced by LGC, which was characterised in house and distributed to the project partners. The isotopic composition of ^{194}Pt-enriched carboplatin was confirmed by double focusing magnetic sector field ICP-MS. The total Pt content of this spike has been undertaken using ICP-optical emission spectroscopy (OES). A highly concentrated solution of this material was analysed by HPLC-ICP-MS to check whether most Pt is present as one Pt species. A method for the synthesis of ^{194}Pt-enriched carboplatin adducts to plasma proteins (e.g. HSA, Tf and immunoglobuline G (IgG)) using incubation protocols and a FFF-ICP-MS method were developed at LGC. Best adduct yields were achieved when using protein/carboPt ratios of 68 (HSA) and 97 (Tf) and 10 h incubation time.

3.4 Objective 3: Multimodal approach for the quantification of metalloproteins

Introduction

Proteins are very often complex structure and for most of them a variety of different modifications exist. To ensure that only the target analyte is included in the determination, complementary methods were developed and applied to the determination of the same proteins. The methods used different properties of the proteins such as optical emission, Raman emission or the mass spectrometry of characteristic peptides / amino acids and elements, respectively.

Raman Spectrometry

Hb is an Fe containing protein in the erythrocytes of vertebrates and consequently in humans as well. It is responsible for the oxygen transport in the body. The German Medical Association demands for this molecule traceable and reliable measurement procedures in clinical laboratories. Therefore, a procedure for total Hb quantification on basis of double ID Raman spectrometry (ID Raman) was developed by PTB. Initially the Raman spectra of a recombinant HBA_0 reference material and the spectra of human Hb and erythrocyte lysate were compared. All spectra showed good agreement. In comparison with the reference spectrum, isotopic shifts occur in the spike spectrum of ^{15}N-HBA_0. The good agreement and the observed isotopic shifts depict a confident basis for Hb quantification.

Based on these results, a set of calibration mixtures of Hb reference and spike material was prepared and Raman spectra of these mixtures were acquired after droplet deposition. Fig. 11 shows a drop of haemolysed and dried erythrocytes under a microscope. From these spectra a PLS model was created based on multivariate data analysis. The prediction of the Hb concentration in artificial serum using this model showed good agreement with the gravimetrically prepared value. Afterwards a real blood sample was analysed with ID Raman which was also analysed in a clinical laboratory. Both results were in good agreement within the uncertainties of the ID Raman method. The clinical laboratory did not indicate their uncertainties. In a last step the method was compared to other methods for Hb quantification developed within this project like IDMS and ICP-IDMS. The comparison was realized by measuring a reference material which is certified in total Hb concentration. While the results of ID Raman and IDMS are in good agreement both results differ from the certified value of the reference material and the ICP-IDMS results. Further investigations are necessary to solve this discrepancy.

Fig. 11: Haemolysed and dried drop of erythrocyte lysate under a microscope with 10 x enlargement. The Hb is concentrated in the fringe.

Affinity Chromatography

One of the most powerful separation methods for complex samples such as human serum is affinity chromatography, also known as affinity extraction or immunoaffinity extraction. A highly selective binder (often an antibody) is used to separate the analyte of interest from all other matrix compounds. The major limitation is the poor availability of good antibodies and the relatively high costs. On the other hand, the extremely good selectivity leads to one-step-separations, which are perfectly suitable for many kinds of hyphenations. The approach of affinity chromatography was developed by BAM for the detection of the Cu-containing protein Cp in human serum. First of all, a suitable antibody had to be found on the market. Some of the tested antibodies were suitable for the development of sensitive immunoassays for the detection of Cp. The characterization and further validation of the selected enzyme linked immunosorbent assay (ELISA) showed a sensitivity much better than required for the respective application. Nevertheless, none of the antibodies was found to meet all the requirements for the production of affinity columns, particularly the availability in larger amounts.

Therefore, a new antibody was produced by BAM. To achieve higher amounts, the production was performed in chicken. Chicken antibodies have significant advantages in terms of animal welfare, since they can be directly collected from the eggs, and no blood samples are necessary. Furthermore, chicken antibodies (IgY) are relatively economic to produce. Pure Cp was used for immunization. The IgY were affinity purified themselves on immobilized Cp. Subsequently, the purified IgY were immobilized on a NHS-activated Sephadex column, which was considered to be optimal for this purpose.

To characterize the affinity columns, elution curves were prepared. Here, defined amounts of Cp were loaded on the IgY affinity column and subsequently eluted with acidic buffer. It was shown that the elution experiments led to distinct elution peaks of Cp, which were verified with ELISA experiments. Finally, BAM applied the respective anti-ceruloplasmin affinity column to a novel affinity ICP-MS hyphenation. The liquid flows were supplied with syringe pumps, delivering washing buffer, distilled water, elution buffer and samples. The elution curves (Fig. 12) show a perfect accumulation of Cp on the column and a good correlation between the elution curves based on Cu (ICP-MS) and Cp (off-line ELISA). This means that the concept of affinity chromatography ICP-MS was implemented successfully.

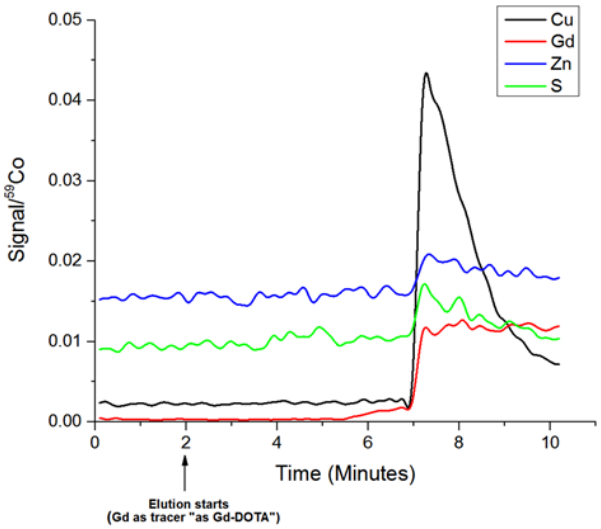


Fig. 12: Elution of Cp from the affinity column prepared by BAM monitored with ICP-MS. The Cu peak correlates with the presence of the metalloproteins validated by ELISA. Gadolinium (red line) was used as monitor for the elution buffer. Under optimal conditions, a S and a Zn peak can be detected.

Absorption Spectrometry

The procedure recommended by the WHO and used to derive the total Hb concentration is based on the conversion of the different Hb variants into cyanmethaemoglobin and the absorbance measurement at the wavelength of 540 nm. PTB investigated as alternative the AHD (alkaline haematin and non-ionic dispersant) method where the Hb concentration is derived from the absorbance value at the wavelength of 574 nm. For reliable comparison of absorbance values, a procedure was established to warrant traceability of photometric absorbance values in the respective wavelength regions.

In total, 82 control or fresh blood samples, covering pathological and normal concentrations from 60 g/L to 180 g/L were analysed. Good agreement for both procedures was observed. On the average, the AHD procedure yielded slightly higher values by 0.6 % and the distribution of differences between both protocols shows a standard deviation of 0.5 %.

Single wavelength absorbance measurements are susceptible towards perturbations, since contributions arising from other constituents in blood will result in an additional background and hence in an overestimation of the Hb concentration. To improve the accuracy of total Hb concentration measurements, a protocol was established for the HiCN and AHD conversion method based on the preparation of six dilutions and the measurement of absorption spectra.

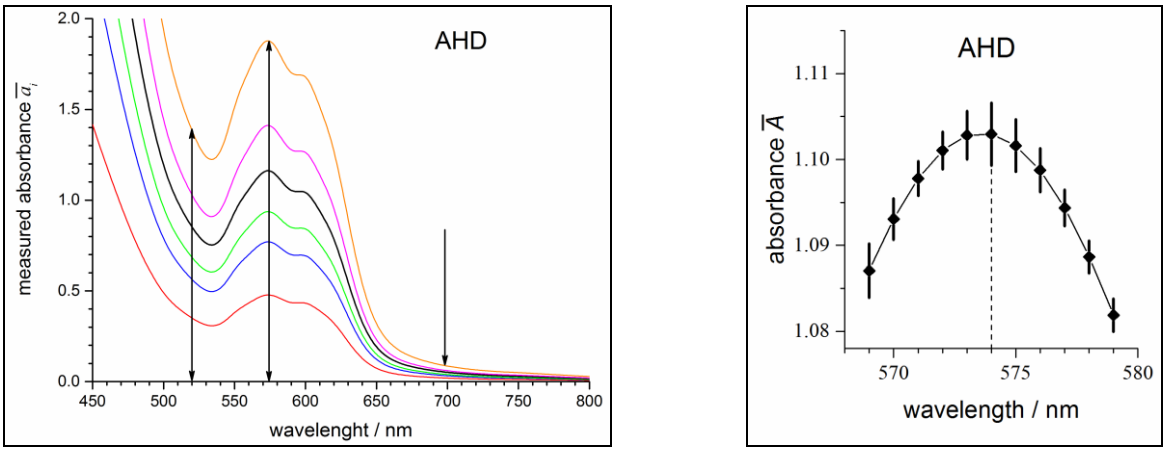


Fig. 13: Absorption spectra for six different preparations of a blood sample in AHD conversion solutions (left) and enlarged section depicting the absorption maximum at 574 nm (right).

In Fig. 13 typical results of a dilution series are shown. The three vertical arrows indicate the absorbance value at the relative maximum of the spectrum (λ = 574 nm), used to derive the total Hb concentration, and absorbance values at 520 nm and 700 nm to identify perturbations due to high bilirubin concentration and high concentration of vesicles. Our results indicate that the relative absorbance values for each dilution shall be $\left(\overline{A}_{520nm}/\overline{A}_{574nm}\right)<0.75$ and $\left(\overline{A}_{700nm}/\overline{A}_{574nm}\right)<0.045$ to ensure negligible influence of such perturbations.

Solutions of chlorohaemin were characterised with respect to purity and identified as candidate reference material by comparison of the expected target value (180 g/L) of the gravimetrically prepared solution and the value of (180.12 ± 1.00) g/L determined from a dilution series.

A first comparison experiment between absorbance measurements and double Fe ICP-MS demonstrated good agreement, indicating that in future method independent assignment of concentration values for total Hb reference materials is feasible.

Molecular IDMS

As a candidate reference measurement procedure for the determination of HbA_2 fraction in human blood an IDMS-based method was developed by PTB employing recombinant human Hb unlabelled and labelled with ^{15}N. It includes a direct tryptic digest of whole blood samples. After tryptic digestion, signature peptides of α- and δ-globin are quantified related to values for total- Hb and HbA_2, respectively. As part of the method validation the certified reference material JCCRM 912-2L, certified for total- Hb, was analysed.

IDMS-results for total- Hb were compared to certified values obtained using spectrophotometry.

Recovery of known amounts (fractions) of HBA_2 was investigated both with IDMS and different commercial ion exchange-high pressure liquid chromatography systems.

Comparison of the results of complementary measurement procedures for Hb

As the final step, the results of the different approaches for the quantification of Hb were compared. The Japanese reference material JCCRM 912-2M, which is certified for total Hb with an amount of mass fraction of (132.1 ± 1.5) g/kg, was used as the common sample. The content of total Hb was determined using three different optical methods (HiCN, AHD and ID-Raman), two species specific ICP-IDMS methods (double and triple IDMS), a molecular IDMS and an elemental IDMS method focused on total Fe. All results agreed with the certified value within the expanded uncertainties.

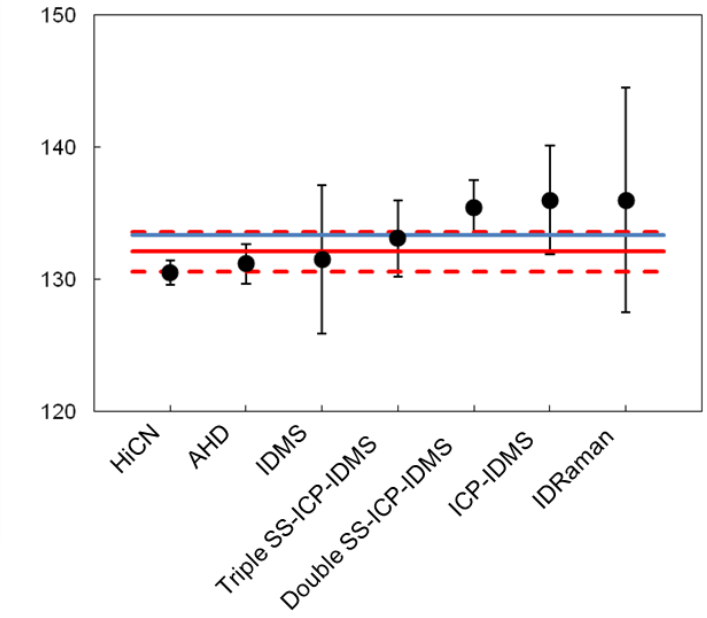


Fig 14: Comparison of the results with their expanded uncertainties for the quantification of total Hb in JCCRM 912-2M using different measurement procedures. The solid red line represents the certified value and the dashed line the according expanded uncertainty. The solid blue line represents the mean of the various results.

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4 Actual and potential impact

4.1 Direct and early impact

4.1.1 Dissemination to the scientific and stakeholder community

The direct impact of the project was predominantly achieved through publications in relevant peer reviewed journals and presentations on conferences. Up to now, 17 publications and 50 contributions to relevant conferences and workshops demonstrate the great interest in the work of this consortium. In short: A critical review about the recent developments regarding traceability, available reference materials and reference measurement procedures was published by the coordinator at the beginning of the project to evaluate the state of the art. The preparation and characterisation as well as the quantification of the investigated metalloproteins was and is currently published by all partners in peer-reviewed journals. The achievements for the accurate quantification of selenoproteins and, in particular of SEPP1, which is a known biomarker of several diseases such as cancer, Alzheimer's disease and diabetes, go far beyond the state of the art. Especially the production of the spike material necessary for the application of species-specific IDMS was challenging. The results have been summarised in several scientific papers. Another focus was the quantification of Pt-drug adducts to proteins as presented above. For the first time a reference sample of calf thymus Pt-DNA was prepared and characterised by LGC for use as a quality control sample in batches of real samples and in particular as spike material to determine the recovery from enzymatic cleavage in presence of the real sample matrix. The results have also been summarised in peer-reviewed publications. A special issue regarding metalloproteins is currently prepared by all partners for the peer-reviewed journal *Metallomics* (publisher RSC). The special issue will mainly summarize the results of the project and present it to a wide scientific community. Additionally, a PhD thesis with the title "Development of primary measurement methods for metalloproteins with non-covalently bound Cu" is about to be finished within the project.

Besides publications in peer-reviewed journals the partners furthered the dissemination of the results from this project by actively participating in international conferences with 31 oral and 29 poster presentations as well as organising sessions and a workshop at large international conferences. The partners contributed to such important conferences as two European Winter Conference on Plasma Spectrochemistry, the International Conference on Raman Spectroscopy, the International Symposium on Metallomics, Euroanalyis and Euromedlab. Two sessions on Metallomics were organized by researchers from BAM and PTB at the SciX14 meeting in Reno (USA), the meeting of the largest societies in Analytical Chemistry. Besides the project partners, who presented the results of this JRP there, internationally recognised scientists in the field from the University of Cincinnati and from NIST as well as stakeholders from important companies such as Agilent Technologies, Perkin Elmer and Thermo Fisher Scientific contributed with interesting talks to the success of these sessions. The opportunity was also used by the partners to further discuss the outcomes of the project and future activities with the stakeholders. Additionally, the results for the Raman measurements were presented at the HORIBA symposium where end-user and instrument producers were participating. The results aroused interest and a researcher from Jena visited the JRP partner already to discuss further cooperation.

A stakeholder workshop was organised by the partners in the framework of the Winter Conference on Plasma Spectrochemistry. The aim of the project was to present the outcome of the project to the relevant stakeholders and get input from them about future challenges in clinical chemistry. The workshop was open to all participants of the conference to further broaden its impact. Besides speakers from the consortium and members from the associated researcher excellent grant institutions, representatives of important stakeholders gave talks:

- A colleague from NIST (USA) presented the approach to achieve traceability in clinical chemistry in the US
- A colleague from the Rowett Institute of Nutrition and Health at the University of Aberdeen (UK) showed the metal containing analytes that might become an important issue for the development and treatment of neurodegenerative diseases

- A colleague from BIPM (France) and also a JCTLM representative presented the work of JCTLM to the interested audience and emphasised the importance of traceability and comparability of clinical laboratory results achieved for example by measurement procedures such as were developed within this project.

Furthermore, a physician and professor from the University Hospital in Munich, who is also chairman of INSTAND e.V. (Germany), participated in the workshop and the partners could discuss in detail the implementation of the reference measurement procedures for the provision of reference values into the mandatory interlaboratory comparisons via the reference laboratories.

The achievements of the project and the upcoming events could also be followed on the project homepage, which was set-up at the beginning of the project and was updated regularly. This homepage will continue to be available even after the end of the project to update stakeholders on activities of the consortium in the field of uptake and dissemination.

4.1.2 Impact on stakeholder community

Two reference laboratories were involved in the project as stakeholders. AQura GmbH, a laboratory which is a daughter of Evonik Industries, was amongst others participating in the interlaboratory comparison for Tf in human serum to compare their routine measurement procedures with the potential reference measurement procedures developed in this project in order to enable traceability of the results of the routine methods to the SI. However, in the course of the comparison it turned out that there is a significant difference between the quantification of Tf via Fe compared to the quantification via sulphur in the routine laboratory. This discrepancy could not be solved yet and will be the subject of further studies. But this example shows how important well characterised reference measurement procedures are.

Instand e.V., an IFCC reference laboratory for clinical diagnosis, supported PTB in the production and characterisation of the Hb spike material. Beside Instand e.V., PTB is in close contact with DGKL, also an IFCC reference laboratory for clinical analysis, to implement the AHD_{575} method for the quantification of total Hb. This method was improved in this project and the traceability of its results was established by comparison with the ID measurement procedures developed as reference measurement procedures within this project. Both laboratories participated in training's courses on the new method and are about to implement this method in their laboratories already this year to provide reference values for the mandatory interlaboratory comparisons for all clinical laboratories in Germany which provide this measurement service.

PTB also presented the project to the blood bank in Braunschweig who was very interested in the project and provided the partners with serum samples and erythrocyte concentrates for the method development. They are well aware of the problem of deviating results for the same analytes between laboratories and measurement kits. As they are obligated to test all blood donations, they are aware that they will also directly profit of more reliable and comparable results once reference measurement procedures are available to provide reference values in interlaboratory comparisons provided by the clinical reference laboratories involved in this project.

The method developed in this project for the quantification of Pt adducts with DNA and proteins as well as the reference measurement procedures for selenoproteins have been and will be used in clinical cancer trials. This helps to elucidate the mechanisms why there are such different and patient-specific responses of leukemia patients to similar Pt-drug doses. In this vein, it was looked at the competition between serum albumin and DNA for the Pt-drug binding and the effect of Se supplementation on the treatment success. The reference methods for selenoproteins developed by LGC is used to provide reference values for an ongoing cancer trial organised by the Oncology department of the Waikato Hospital, New Zealand. The trial will incorporate different Se-species (selenite, methylselenocysteine and selenomethionine) which is administered at different doses combined with chemotherapy drugs to patients with lymphoma. Se incorporation into selenoproteins is one of the biomarkers measured in response to Se species/dose. The protein levels between healthy individuals and patients with cancer will be determined. The use of the reference measurement procedures developed within this project is ensuring the comparability of the results over the duration of the study and between patients handing the physicians a reliable base for the comparison of different treatment approaches. Hopefully, the trial outcome will support the development of improved combined therapies for cancer treatment.

The reference methods for Pt-adducts with plasma proteins and/or DNA developed within this project wa used by LCG in collaboration with Leicester Cancer Research Centre in UK to provide reference values to a clinical trial using real blood samples of patients receiving chemotherapy to assess the competition between DNA and proteins for the Pt-drugs administered to patients intravenously at different dose/time. Evaluation of dose-dependence response to treatment for every patient aims at finding more effective therapies at lower cost. In this vein, LGC also looked at the competition between HSA and DNA for the Pt drug binding. Again the use of IDMS as reference measurement procedure is ensuring the comparability of the results over the duration of the study and between patients and, thus, provides a reliable base for the comparison of different treatment approaches The evaluation is still on-going.

4.1.3 Impact on European and international metrology and standardisation

The partners successfully developed and used their complementary expertise for the traceable quantification of metalloproteins. This can act as a basis for a common platform for protein analysis within the European metrology landscape which renders it unnecessary for every NMI/DI to develop an expertise for the whole spectrum of protein analysis. The methods developed within this project can provide for the first time a way to receive results for the determination of metalloproteins traceable to the SI, thus improving the comparability of the results for these proteins and increasing the reliability of the determination of proteins important in clinical diagnosis.

The potential reference measurement procedures were presented at a JCTLM meeting with the intension to have them registered in the JCTLM database as reference measurement procedures as soon as they are published in a peer-reviewed journal.

The results of the work were presented at technical meetings of EURAMET and CCQM. Furthermore, the results for the complementary Hb measurement procedures were presented at the IFCC meeting of the working group on the standardisation of HbA_2. Based on an improved version of the national German standard DIN 58931 and the publication in Metrologia (Witt et al. 2013) for the determination of total Hb using the photometric methods improved by members of this consortium and validated with the reference measurement procedures developed in this project, the Haematology working group of the German Standardisation Body (DIN) has decided to make a new working item proposal for CEN standardisation. This proposal is entitled "Haematology - Determination of the concentration of total haemoglobin in blood - Reference methods" and was discussed at the CEN meeting in February. On the long term, it is intended to replace the HiCN method using KCN, which is banned in many countries, by the improved AHD_{575} method.

Based on the results obtain in this and also former projects, the German partners are presently discussing a cooperation to produce traceable metal standards based on the BAM “Primary Calibration Standards“ for application in clinical diagnosis. The initiative for this project is coming from the European Directorate for the Quality of Medicines & HealthCare (EDQM), Council of Europe.

A EURAMET (Ref. 1351) international intercomparison for Tf was organised by PTB in which also a clinical laboratory was involved enabling them to compare their routine method to the candidate reference measurement procedures developed within this project.

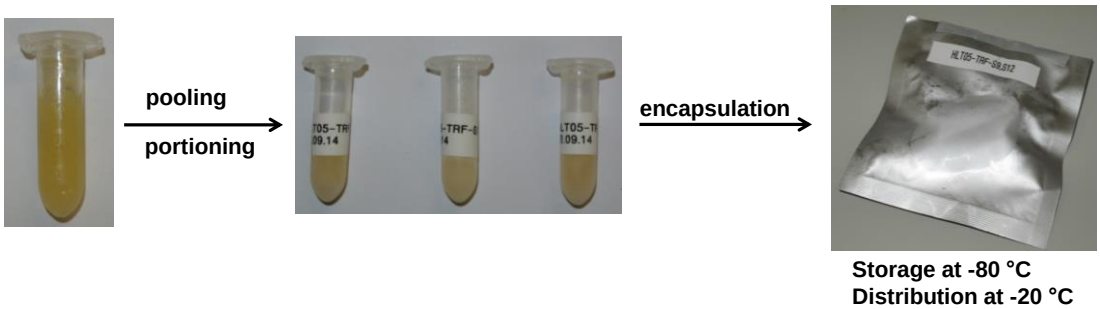

Fig. 15: Preparation and distribution of the serum samples for the interlaboratory comparison for Tf organised by the project partners.

Based on the experiences gained in this interlaboratory comparison it is planned to submit a proposal for a CCQM interlaboratory comparison in order to establish the measurement procedures as reference measurement procedures, after some minor issue, arisen during the comparison, were solved.

4.1.4 Cooperation between the project partners and researchers

The expertise and instrumentation available at the various partner institutes enabled the successful production and characterisation of isotopically labelled spike materials for the determination of metalloproteins. Especially the contribution of DKFZ is here to be highlighted as none of the partners from the participating NMIs and DIs was able to synthesize labelled selenoproteins at the beginning of the project. The procedure was successfully transferred to LGC who produced the spike material for selenoproteins afterwards and provided it to the partners involved in the development of a measurement procedure for these proteins. The various instrumentations available at the partner institutes enabled the development and comparison of measurement procedures targeting at different properties of the analytes. As biological matrices are especially complicated and can create different interferences in the various methods, the variety of measurement procedures ensured an unambiguous identification of the target proteins and the validation of the methods.

All technical activities have been successfully completed thanks to the commitment and contributions of all project partners and researchers. Mutual trainings for researchers from the partner institutes such as the transfer of the platform for production of selenoprotein spike material from DKFZ to LGC, the training of a researcher from PTB at UNIABDN in the use of GE in protein separation as well as in separation of the analytes from the matrix using HPLC and an introductory training in species-unspecific IDMS given to LU as well as an one week training course for an employee from BAM on FFF-ICP-MS, both organized by partner LGC, were essential for the success of the work in the project. Additionally, during a visit of a PTB researcher at BOKU Cp was jointly characterised using the instrumentation available at BOKU and compared to the results achieved with the equipment at PTB. Furthermore, through trainings of non-NMI/DI partners in the project in the requirements to achieve traceability of the results and in uncertainty estimations, the metrological approach for measurements in these institutes could be established.

4.2 Longer term impacts

4.2.1 Social impact

The work in this project was focusing on the development of primary measurement procedures for metalloproteins using Tf, Hb, SOD and Cp as model proteins which were chosen due to their importance in clinical diagnosis. Diseases related to this analytes are serious health issues all over Europe. Thus, the project will, on the long term, mainly have social impact as these diseases affect the quality of life of the patients and their social environment and also burden the national health care systems. SOD and Cp, for example., are markers for rheumatoid arthritis (around 4 million patients in the EU) as well as ischemic myocardium which cause around 820000 deaths per year. With 3.2 million people diagnosed with cancer in the EU each year, cancer remains a key health concern. The formation of DNA adducts with metallodrugs is thought to be a pharmacokinetic parameter in the optimisation of cancer therapy. The efficacy of Pt containing drugs (used as chemotherapy agents) to bind to DNA has been proven to be hampered by Pt binding to intracellular glutathione or specific plasma proteins (e.g. human serum albumin). Therefore, the search for mechanisms by which Pt containing drugs can be made more available for binding DNA e.g. by co-administration of Pt and Se containing drugs, which have strong affinity for glutathione, are of particular interest. The methods for the reliable quantification of adducts of Pt containing drugs and proteins are already and will in the future support this important research in clinical studies.

Traceability to the reference measurement procedures developed within this project will directly improve the quality of the measurement results obtained for patient samples in clinical laboratories. They are reliable and comparable and, therefore, establish compliance with the EU regulations. As the improved and traceable measurements in medical laboratories and improvement of rapid diagnostic tests will render the results more reliable, they will also enable a faster and more specific treatment of individual patients. This will enable an earlier diagnosis and, thus, will help to improve the quality of life of chronically ill patients allowing them to stay a productive part of society for a longer time. Furthermore, the method for the determination of adducts of Pt containing drugs with proteins and DNA will help hospitals to devise a more efficient and personal treatment of cancer, thus reducing the severe side-effects often observed in cancer therapy. To achieve the broadest dissemination possible to the clinical and reference laboratories, the partners strive to have the

potential reference measurement procedures developed within this project to be accepted by JCTLM as reference measurement procedures so that they can be used in clinical reference laboratories throughout Europe.

5 Website address and contact details

Project public website: http://www.ptb.de/emrp/metallomics.html

Contact details:

Dr. Claudia Swart, PTB, Bundesallee 100, 38108 Braunschweig, Germany, Email: claudia.swart@ptb.de, Phone: +49-(0)531-5923150

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