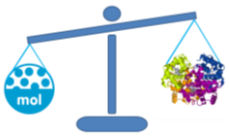


# Publishable JRP Summary for HLT05 - Metallomics
# Metrology for metalloproteins

## Overview

This project developed reference methods for measuring concentrations of metalloproteins in patient samples. 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.

## Need for the project

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 (see box opposite), can indicate inflammation, deficiency disorders such as anaemia, and Down's syndrome in prenatal testing, whilst selenoproteins 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 proteins under investigation:**

**Metalloproteins**; proteins that contain metal atoms as cofactors. Such as:

- Transferrin (Tf)
- Haemoglobin (Hb)
- Superoxide dismutase (SOD)
- Ceruloplasmin (Cp)

**Selenoproteins**; metalloproteins that contain the amino acid selenocysteine. Such as:

- Glutathione peroxidase (GPx)
- Selenoprotein P (SEPP)

**Pt-adducts**; anti-cancer drugs based on platinum-containing small organic molecules which attach to serum proteins (which is an unwanted side effect)

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.

## Scientific and technical objectives

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).



EMRP
European Metrology Research Programme
Programme of EURAMET

The EMRP is jointly funded by the EMRP participating countries within EURAMET and the European Union

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.

## Results

1. Development of separation methods for metalloproteins:

As a first step towards identifying and quantifying metalloproteins, methods were needed for separating the different metalloproteins in a sample. A range of different separation approaches were tested:

- Ion exchange chromatography, using ammonium acetate buffer with a gradient in ion strength, was found to be the most successful at separating SOD, Hb or Tf in blood samples and serum, respectively.
- However, an additional separation method was developed for Hb using SEC, for the quantification of total Hb as this method does not distinguish between the different Hb variants.
- The ion exchange chromatography method did not successfully separate Cp from serum, thus a SEC method was also developed for Cp in serum.
- A method based on double affinity chromatography was devised for separating SEPP and GPx from serum.
- Separation using FFF was attempted for Pt-adducts, however, complete separation could not be achieved. Therefore, another separation procedure using monolithic columns was developed and was used for further research within the project.

The objective was achieved, as separation methods were successfully developed for the metalloproteins Tf, Hb, SOD and Cp, the selenoproteins GPx and SEPP, as well as for Pt-adducts.

2. Preparation and characterisation of isotopically labelled spike materials:

Isotopically labelled spike materials are molecules that have had some of their atoms replaced by heavier or lighter versions of that atom (isotopes). Here, metal atoms with a natural isotopic abundance have been replaced by metal atoms enriched in one isotope to produce the metalloprotein spike materials , i.e. iron-56 in Tf is replaced with the heaver iron-57 isotope. The isotopically labelled spike materials are added to the clinical samples and help determine the concentration of metalloproteins in these samples.

The objective was achieved, as isotopically enriched spike materials were successfully produced for the metalloproteins Tf, Hb, SOD, and Cp, the selenoproteins GPx and SEPP as well as for the Pt-adductsto serum proteins. Additionally, isotopically labelled spike materials based on peptides were produced for the selenoproteins, allowing them to be quantified via these peptides rather than the whole proteins. The advantage of this approach is that, in case of selenoproteins, it is easier and cheaper to produce isotopically labelled peptides instead of whole proteins. The reason is that selenium is strongly bound in the proteins and cannot easily be removed and replaced. Therefore, the selenium enriched in one isotope has to be introduced into the protein while the protein is formed in a biological system.

For all metalloprotein spikes, special attention was paid to the proteins regaining their natural conformation after introducing the metal in its isotopically enriched form so that their behaviour during sample preparation and separation is similar to the native protein.

3. A multimodal approach for the quantification of metalloproteins:

Based on the outcomes of the previous two objectives, primary measurement procedures were successfully developed for quantifying the metalloproteins Tf, Hb, SOD, and Cp, and the selenoproteins GPx and SEPP, using species-specific double isotope dilution mass spectrometry (IDMS). In addition to double IDMS, triple IDMS was used to quantify Tf and SOD, which has the advantage that neither the concentration nor the isotopic enrichment in the spike material has to be known to produce results. As the result for both approaches were in good agreement, it could be shown that the influence of the metal blank in double IDMS is low and the isotopic abundances in the spike materials were determined correctly.

Besides the quantification of Tf containing iron, a species-specific exact matching double IDMS for the accurate quantification of complexes of vanadium with human serum Tf at relevant clinical levels was developed. Vanadium compounds are used in diabetes mellitus treatment. In blood it binds to Tf creating the pharmaceutical active compound. Furthermore, a method for the quantification of Hb and Hb variants using MS was developed. Performance parameters were evaluated by quantifying “total” Hb and haemoglobin A2 (HbA2) in spiked plasma. Samples with various ratios of HbA2 to total Hb were prepared covering both reference ranges of healthy patients and of β-thalassaemia carriers. For method demonstration anonymised blood samples from different patients were used.

An inter-laboratory comparison for the quantification of Tf in serum was conducted within the project. Four international project partners were involved as well as a clinical laboratory. The results revealed significant differences between the methods quantifying this protein via its iron and sulphur content, respectively, in samples with the same levels of Tf. Further research is needed to understand and remove this discrepancy.

In addition to IDMS, an ID Raman method was developed for quantifying total Hb. The Raman spectra of a reference material and the spectra of two human Hb samples (one in whole blood and one in red blood cells) showed good agreement, demonstrating the method is fit-for-purpose. The isotopic shifts within the spike spectrum depicted a confidence basis for Hb quantification and calibration was achieved through multivariate data analysis. Furthermore, studies on the quantification of Hb in control blood samples and fresh blood samples were conducted to compare the conversion protocols and methods used routinely in laboratories with the methods developed in this project. All results were in good agreement within the expanded uncertainty.

Particular highlights include the validation of a method for the quantification of whole SEPP, using affinity chromatography and species-specific single ICP-IDMS. In addition to using whole proteins as spike material, an alternative method was also developed using just spike peptides specific to the protein under investigation. Another highlight was the completion and validation of a method for quantifying adducts of carboplatin with plasma proteins such as albumin and Tf, using monolithic chromatography with species-specific double ICP-IDMS. Both the method for the quantification of SEPP and of carboplatin-plasma protein adducts are used to define optimal cancer treatments for patients, and both methods were applied in a clinical study for the treatment of cancer at a hospital in the UK.

## Actual and potential 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.

## List of publications


- Claudia Swart, Paola Fisicaro, Heidi Goenaga-Infante and Sabine Zakel; Metalloproteins - a new challenge for metrology. Metallomics (2012), 4 (11), 1137-1140.
- Claudia Frank, Olaf Rienitz, Reinhard Jährling, Detlef Schiel and Sabine Zakel; Reference measurement procedures for the iron saturation in human transferrin based on IDMS and Raman scattering. Metallomics (2012), 4 (12), 1239-1244

- E. Moreno-Gordaliza, D. Esteban-Fernández, C. Giesen, K. Lehmann, A. Lázaro, A. Tejedor, C. Scheler, B. Cañas, N. Jakubowski, M. Linscheid and M. Gomez; LA-ICP-MS and nHPLC-ESI-LTQ-FT-MS/MS for the analysis of cisplatin-protein complexes separated by two dimensional gel electrophoresis in biological samples. J. Anal. At. Spectrom (2012), 27, 1474-1483.
- J Heroult, T Harvard and H Goenaga-Infante; Asymmetric Flow Field Flow Fractionation Coupled with ICP-MS for Speciation Analysis of Plasma Proteins. LCGC The Column (2012).
- A. Konopka, C. Wild, M. E. Böhm and W. D. Lehmann; Proteomics standards with controllable trueness - Absolute quantification of peptides, phosphopeptides and proteins using ICP- and ESI-MS. In S. Gaskell, editor, of Quantitative Proteomics: Techniques and Applications, Publisher: RSC Publishing, Cambridge, 2013.
- Claudia Frank, Olaf Rienitz, Claudia Swart and Detlef Schiel; Improving species-specific IDMS: the advantages of triple IDMS. Analytical and Bioanalytical Chemistry (2013), 405 (6), 1913-1919.
- Claudia Swart; Metrology for metalloproteins - where are we now, where are we heading?.Analytical and Bioanalytical Chemistry 405 (17), 5697-5723.
- K. Witt, H. U. Wolf, C. Heuck, M. Kammel, A. Kummrow and J. Neukammer; Establishing traceability of photometric absorbance measurements for accurate determination of the haemoglobin concentration in blood. Metrologia (2013), 50 (5), 539-548.
- Lisa M. Munter, Holger Sieg, Tobias Bethge, Filip Liebsch, Frank S. Bierkandt, Michael Schleeger, Heiko J. Bittner, Norbert Jakubowski, Peter W. Hildebrand and Gerd Multhaup; Model peptides uncover the role of the β-Secretase transmembrane sequences in metal-ion mediated oligomerization. J Am Chem Soc (2013), 135 (51), 19354 – 19361.
- A. Konopka, N. Zinn, C. Wild and W. D. Lehmann; Preparation of heteroelement-incorporated and stable isotope-labeled protein standards for quantitative proteomics, Meth Mol Biol (2014), 1156, 337-63.
- Larissa Müller, Heike Traub, Norbert Jakubowksi, Daniela Drescher and Janina Kneipp; Trends in single-cell analysis by use of ICP-MS. Anal Bioanal Chem (2014), 406 (27), 6963-6977
- Christian Lutz Deitrich, Susana Cuello-Nuñez, Diana Kmiotek, Frank Attila Torma, Maria-Estela Del Castillo Busto, Paola Fisicaro, and Heidi Goenaga-Infante, Accurate quantification of Selenoprotein P (SEPP1) in plasma using isotopically enriched seleno-peptides and speciesspecific isotope dilution with HPLC coupled to ICP-MS/MS, Anal. Chem. (2016), DOI: 10.1021/acs.analchem.6b00715.
- Claudia Swart and Norbert Jakubowski, Update on the status of metrology for metalloproteins, J. Anal. At. Spectrom. (2016), 31, 1756-1765.
- M. Estela del Castillo Busto, Caroline Oster, Susana Cuello-Nuñez, Christian L. Deitrich, Andrea Raab, Anna Konopka, Wolf D. Lehmann, Heidi Goenaga-Infante and Paola Fisicaro, Accurate quantification of selenoproteins in human plasma/serum by isotope dilution ICP-MS: focus on selenoprotein P, J. Anal. At. Spectrom. (2016), 31, 1904-1912
- C. Brauckmann, C. Frank, D. Schulze, P. Kaiser, R. Stosch and C. Swart, Preparation and characterisation of an 57Fe enriched haemoglobin spike material for species-specific isotope dilution mass spectrometry, J. Anal. At. Spectrom. (2016), 31, 1846-1857.
- Gerrit Hermann, Laura Hyrup Møller, Bente Gammelgaard, Jonas Hohlweg, Diethard Mattanovich, Stephan Hann and Gunda Koellensperger, In vivo synthesized 34S enriched amino acid standards for species specific isotope dilution of proteins, J. Anal. At. Spectrom. (2016), 31, 1830-1835.
- Anna Konopka, Dominic Winter, Witold Konopka, M. Estela del Castillo Busto, Susana Nunez, Heidi Goenaga-Infante, Paola Fisicaro and Wolf D. Lehmann, [Sec-to-Cys]selenoprotein – a novel type of recombinant, full-length selenoprotein standard for quantitative proteomics, J. Anal. At. Spectrom. (2016), 31, 1929-1938.
- Julia Gleitzmann, Andrea Raab, Dirk Schulze, Hermann Wätzig, Jörg Feldmann and Claudia Swart , Accurate and precise quantification of Cu,Zn-SOD in human red blood cells using species-specific double and triple IDMS, J. Anal. At. Spectrom. (2016), 31, 1922-1928.
- Julia Gleitzmann, Development of primary measurement procedures for the determination of Cu-containing proteins with clinical relevance, PhD thesis at Technical University of Braunschweig, 2016.

| | |
|---|---|
| JRP start date and duration: | 2012-05-01, 36 months |
| JRP-Coordinator:<br>Dr. Claudia Swart, PTB, Tel: +49-(0)531-5923150 E-mail: Claudia.swart@ptb.de<br>JRP website address: http://www.ptb.de/emrp/metallomics.html: | |
| JRP-Partners:<br>JRP-Partner 1 PTB, Germany<br>JRP-Partner 2 BAM, Germany<br>JRP-Partner 3 LGC, United Kingdom<br>JRP-Partner 4 LNE, France<br>JRP-Partner 5 TUBITAK UME, Turkey | |
| REG-Researcher 1<br>(associated Home Organisation):<br>REG-Researcher 2<br>(associated Home Organisation):<br>REG 3<br>(institutional REG Home Organisation):<br>REG 4<br>(institutional REG Home Organisation): | Anna Konopka, Poland<br>DKFZ, Germany<br>Andrea Raab, Germany<br>UNIABDN, UK<br>University of Loughborough, UK<br>LU, UK<br>Universität für Bodenkultur, Austria<br>BOKU, Austria |

***The EMRP is jointly funded by the EMRP participating countries within EURAMET and the European Union***