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Abbreviations

AD:	Alzheimer's disease
ALB:	albumin
Aß:	beta amyloid
Ca:	calcium
CER:	ceruloplasmin
CSF:	cerebrospinal fluid
Cu:	copper
DNA:	deoxyribonucleic acid
DTNB:	5,5'-disulfanediylbis-2-nitrobenzoic acid
EDTA:	ethylenediaminetetraacetic acid
FCS:	fetal calf serum
Fe:	iron
Fe-IMAC:	iron immobilised affinity chromatography
FER:	ferritin
Fe_xO_y:	iron oxide
HGB:	haemoglobin
$HGBA_0$:	non-glycated haemoglobin A
HPLC:	high performance liquid chromatography
ICP-MS/MS:	inductively coupled plasma tandem mass spectrometry
ICP-MS:	inductively coupled plasma mass spectrometry
ID:	isotope dilution
IDMS:	isotope dilution mass spectrometry
IVDR:	*in vitro* diagnostic regulation
KCN:	potassium cyanide
LC-MS/MS:	liquid chromatographic tandem mass spectrometry
Mg:	magnesium
P:	phosphorous
PICAA:	peptide impurity corrected amino acid
P-tau:	phosphorylated tau-protein
QC:	quality control
RNA:	ribonucleic acid
S:	sulphur
SDS:	sodium dodecyl sulphate
SDS-PAGE:	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEC:	size exclusion chromatography
SERS:	surface enhanced Raman spectrometry
SOD1:	Cu, Zn-superoxide dismutase
SOP:	standard operating procedure
SPE:	solid phase extraction
SS-IDMS:	species specific isotope dilution mass spectrometry
TBS:	Tris-buffered saline
TRF:	transferrin
Tris:	tris(hydroxymethyl)aminomethane
T-tau:	total tau-protein
UPLC:	ultra performance liquid chromatography
XRD:	X-ray diffraction

1 Overview

Due to an ageing population, neurodegenerative diseases such as Alzheimer's disease (AD) are a major challenge facing the health care system, currently affecting over 6 million people in the European Union. Diagnostic methods used for the identification and quantification of relevant AD biomarkers require improved accuracy in order to help treatment in early stages of the disease. This project focussed on the development of potential reference measurement procedures for these biomarkers based on isotope dilution to improve the reliability and comparability of the results in clinical laboratories.

2 Need

Due to an ageing population, neurodegenerative diseases are one of the major challenges facing the health care system, currently affecting over 6 million people in the European Union and 44.4 million people worldwide. The most common cause of dementia is Alzheimer's disease (AD), representing 70 % of all cases. Studies have shown that treatment for the disease is most promising in the early stages, However, due to a lack of accuracy in diagnostic methods used for identification and quantification of relevant biomarkers only half of the patients suffering from AD are currently identified, and they are often in the advanced stages of the disease.
The most established biomarkers for AD are β-amyloid peptide 1-42 (A β1-42), β-amyloid peptide 1-40 (A β1-40), total tau-protein (T-tau), hyperphosphorylated tau-protein (P-tau), and ratios thereof, measured in cerebrospinal fluid (CSF). As these markers are also formed to some extent during normal ageing, cut-off values are needed by clinicians to distinguish between healthy and diseased individuals. These biomarkers are commonly determined using immunoassays or optical methods but often give inconsistent results and high variabilities, of up to 25 % for one specific method between laboratories prevents these universal cut-off values being established.

3 Objectives

The aim of this project was to provide reference measurement procedures for the identification and quantification of relevant biomarkers for the diagnosis of neurodegenerative diseases such as AD.
This project addressed the following scientific and technical objectives:

1. **To develop methods for the traceable quantification of metals and metal containing biomolecules** of neurodegenerative diseases at µg/L levels or below and in small µL sample volumes.
2. **To produce and characterise isotopically labelled spike materials** for metals and metal containing biomolecules of neurodegenerative diseases.
3. **To develop new and accurate methods for measuring peptide and protein biomarkers** from onset and through progression of neurodegenerative diseases, at µg/L levels and below in small µL sample volumes.
4. **To characterise the uptake, metabolism and transport to the brain of metals and metal containing biomolecules** related to neurodegenerative diseases using the developed methods and spike materials. In addition, to develop accurate methods for the quantification of metals and the co-localisation of metals with biomarkers relevant for neurodegenerative diseases in biological samples.
5. **To facilitate the uptake of the technology and measurement infrastructure** developed by the project by the measurement supply chain (accredited laboratories, instrumentation manufacturers), standards developing organisations (ISO, CEN) and end users (medical practitioners, medical (academic) hospitals and industry).

4 Results

4.1 Introduction

The main goal of this project was to provide reference measurement procedures for the quantification of relevant biomarkers for the diagnosis of neurodegenerative diseases such as Alzheimer's disease (AD). The targeted markers included the well-established biomarkers beta amyloid 1-40 (Aß1-40), beta amyloid 1-42 (Aß1-42), total tau-protein (T-tau) and phosphorylated tau-protein (P-tau), as well as various metals and metalloproteins, which are suspected to play a role in the development of AD. These biomarkers were investigated in serum, cerebrospinal fluid (CSF) and brain tissue. The development of traceable and accurate methods will ensure reliable and comparable results between laboratories.

Isotope dilution mass spectrometry (IDMS) is recognised by the Consultative Committee on Amount of Substance (CCQM) as a primary measurement procedure, i.e. a method which is completely described and understood, having the highest metrological qualities and for which the results can be given with a complete uncertainty statement. The combination of IDMS with inductively coupled plasma (ICP) as ionisation source can lead to results with the highest accuracy, precision and smallest combined uncertainty. Therefore, IDMS is the method commonly used for reference material certification or used as a reference method for elemental and speciation analysis (species specific approach). In this sense, most of the candidate reference measurement procedures developed within the project were based on IDMS.

The basic principle of IDMS (shown in Figure 1) is the exchange of the natural isotopic composition of the element of interest (e.g. Fe) in the sample by the addition of a substance enriched in a minor isotope of this element (e.g. ^{57}Fe), called spike. Once the analyte and spike isotopes are equilibrated in the mixture to ensure identical behaviour, the ratio of the two isotopes in the sample spike blend (sample + spike, bx) is measured using ICP-MS. Since the weights of sample, reference and spike, the isotopic composition and the mass fraction of the element in the spike are known, the amount of the element in the sample (w_x) can be determined from the isotopic ratio in the sample blend (single IDMS approach). 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 at the earliest stage of the analytical procedure.

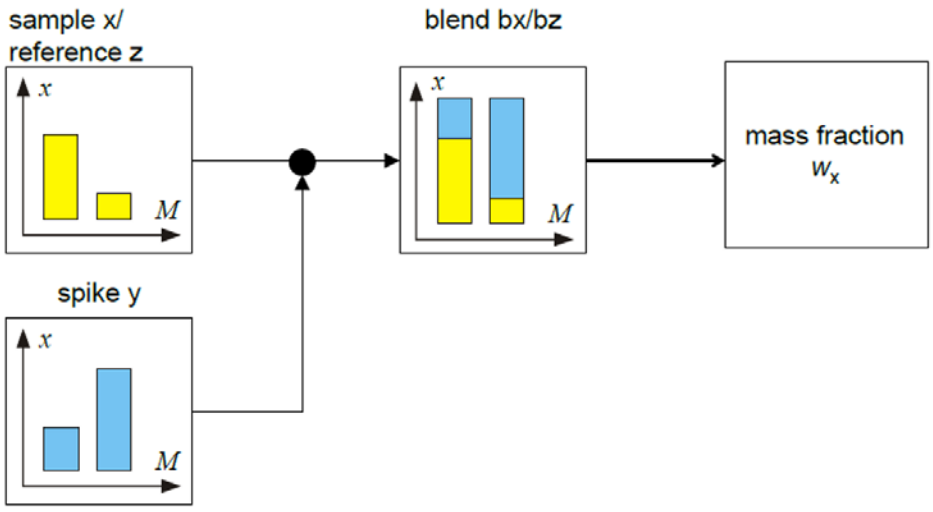


Figure 1: Single and Double IDMS approach

The single IDMS method is reliant on the accurate quantification of the spike, which is hard to establish since isotopically-enriched spikes are usually available in small quantities with uncertain purity. As an alternative, double IDMS can be applied. Here, a second blend is prepared using a reference material (calibration spike blend, bz) of high purity, well characterised in terms of isotopic composition (R_z) and mass fraction (w_z). The main advantage of double IDMS is that the mass fraction of the spike is not required for the calculation of the mass fraction of the analyte (w_x) in the sample, as is shown below:

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

where,

w_z (g/kg)	mass fraction of analyte (Fe) 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 ($^{57}Fe/^{56}Fe$) in reference z
R_{bz} (mol/mol)	isotope ratio of the analyte ($^{57}Fe/^{56}Fe$) in blend bz (reference z + spike y)

A further approach used within the project was triple IDMS in which additionally a second calibration spike blend is prepared. This is especially recommendable when a spike material is used with a comparable low isotopic enrichment leading to a high uncertainty in the measurement of the isotope ratio in the spike material since this parameter is cancelled from the equation in triple IDMS.

A similar approach was used for the quantification of tau-protein with Raman spectrometry only that in this case a shift in the spectra is observed for the isotopically enriched material instead of mass shift.

4.2 Methods for the traceable quantification of metals and metal containing biomolecules

To investigate the influence of metals and metalloproteins in the development of AD, sensitive and accurate measurement procedures were developed within the project, mainly using inductively coupled plasma spectrometry (ICP-MS). ICP-MS provides excellent capabilities for the determination of metal concentrations in biological samples and also for the elemental detection of proteins containing a metal or heteroatom (metalloproteins or metal-containing proteins). ICP-MS is also characterised by its high sensitivity and specificity, its multielement capability to monitor simultaneously the different metals or heteroatoms associated/bound to a protein. It provides direct isotopic information (which allows isotopic ratio and isotopic dilution analysis) and it is versatile and easy to be coupled to separation methods such as high-performance liquid chromatography (HPLC) or field-flow fractionation (FFF). Therefore, the development of hyphenated separations with ICP-MS elemental detection (mainly HPLC-ICP-MS) has been the analytical tool for conducting speciation studies to underpin clinical measurements in this project.

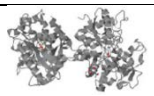
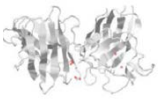
Methods for high throughput accurate quantification of multiple trace elements in biological fluids and tissues meeting the demands of the highly regulated clinical environment are of utmost importance. The careful balance of trace element concentrations in human body fluids and tissues is essential for maintaining the optimal homeostasis to avoid adverse side effects of excess metal exposure and/or depletion. While for serum and blood, reference levels are fairly well established for most trace elements and serve as important diagnostic parameters, limited data is available for elemental concentrations in CSF. CSF plays an important role in the homeostasis and metabolism of the central nervous system. The consortium implemented, compared and presented different ICP-MS methods for low sample volume, high-throughput multielement analysis in biological fluids including CSF. For this latter sample matrix, due to the lack of certified reference material, open vessel digestion in combination with sectorfield-ICP-MS analysis was used as gold standard to establish a reference data set. Owing to the low sample volume and trace element concentrations of CSF, flow injection methods with 5 µL sample intake were developed integrating matrix matched calibration concepts and ID (Theiner et al.; Analyst 2019, Theiner et al.; JAAS 2019).

The inorganic quantification approach of IDMS for the analysis of metalloproteins is based on the determination of the metal or heteroatom bound to the protein by ICP-MS coupled to a separation technique (e.g HPLC). There are two different groups of metalloproteins: the ones where the metal or heteroatom is covalently bound to the protein, normally present in the amino-acid sequence, and the ones where the metal is not covalently bound to the backbone (e.g. Copper (Cu) bound to albumin (ALB)). Sulphur (S) and Phosphorus (P) are examples of heteroatoms frequently found in proteins (e.g. T-tau or P-tau) which are covalently bound. However, the detection of S and P by conventional quadrupole ICP-MS is compromised by their high ionisation potential and by the presence of polyatomic interferences. The recent introduction of a triple quadrupole as

mass analyser in ICP technology (referred to as tandem ICP-MS or ICP-MS/MS) into the market has extended its multielemental capability to S and P at lower detection limits and expanded its use in proteomics.

When IDMS is applied to speciation studies it is called species specific IDMS (SS-IDMS) and in this case, it makes use of a spike compound identical to the compound under investigation but isotopically labelled in the target element. This concept, described previously for covalently bound organic compounds (e.g. tributyltin), was extended to the following metalloproteins: transferrin (TRF), ceruloplasmin (CER), Cu, Zn superoxide dismutase (SOD1) and haemoglobin (HGB) in the previous EMRP project HLT05 *Metalloproteins*, where candidate reference measurement procedures were developed for TRF and CER in serum and for SOD1 and HGB in erythrocytes. However, the concentration of these proteins in CSF and brain tissues (the matrices under study here) are expected to be lower. Table 1 summarises the metalloproteins or metal-containing proteins that have been pursued in this project alongside with its biological functions, the measured parameters and the tested biological matrices:

Table 1. Potential clinical markers for AD

Name	Structure	Functions	Measured Parameters	Matrices
Ferritin (FER)		- Fe storage protein - able to bind other metals (including toxic ones) with similar properties - potential shuttles for metals to avoid oxidative stress	Fe bound to FER (Fe-FER) FER via S content	Serum Pig brain
Transferrin (TRF)		- Fe transport protein - same as FER	TRF via Fe content	Serum CSF Bovine brain
Albumin (ALB)		- maintenance of colloid osmotic pressure - multiple ligand-binding capacities - free radical-trapping properties	Cu bound to ALB (Cu-ALB)	Serum CSF
Cu, Zn-Superoxide dismutase (SOD1)		- oxidative stress prevention - acute phase protein - indication of inflammation processes	SOD1 via Cu	CSF Pig brain
Ceruloplasmin (CER)		- Cu storage protein - ferroxidase - potential shuttle, since Cu is suspected to be involved in plaque formation	CER via Cu Cu isotope ratio in CER	Serum CSF

Due to their potential involvement in the progression of AD, two extra proteins (FER and ALB) were included in this project, which have required the development of new separation and quantification methods. As it was mentioned before, the metalloprotein is quantified via its metal or heteroatom by ICP-MS (e.g. Fe in TRF, Cu in ALB or CER). And only if stoichiometric ratio of metal-protein and the molecular weight of the protein are known, the protein mass fraction can be calculated (e.g. 2 moles Fe bound to 1 mol TRF). Therefore, different measurands have been pursued within the project: metal bound to the protein (e.g. Fe bound to FER or Cu bound to ALB) and the protein content (e.g. FER via S and CER via Cu).

Model samples of the three different matrices, selected by the consortium at the beginning of the project, were used for method development. Examples of commercially available sample sources used here are given below:

- Serum: ERM DA470k/IFCC, quality control (QC) Seronorm Trace Level 1 (Seron, Norway), QC Human Seronorm (Sero), fetal calf serum (Merck), etc.
- CSF: QC Liquid CSF Control 2 Randox (Randox Laboratories Ltd), artificial CSF (Tocris, Harvard Apparatus), etc.
- Brain: locally purchased animal brains from different sources such as pig and bovine.

A standard operating procedure (SOP) about the preparation of artificial CSF as model sample was created and shared between the partners to be used for method development. Two different procedures were used: a protein free solution based on glucose and different electrolytes in a tris(hydroxymethyl)aminomethane (Tris) buffer solution and the second one contaning bovine serum ALB at different concentrations in order to simulate protein content in CSF.

The preservation of the biomolecules under study (metal bound to the protein, protein activity and monomer form, among others) is a requirement and a special challenge in any analytical method and particularly for speciation analysis. Special attention was paid to sample handling and storage conditions of biomolecules for which no sample handling SOPs were previously developed or published. In this sense, protein stability in the matrices under study was evaluated under different storage conditions for SOD1, TRF, FER and Cu-ALB.

In general, samples should be stored cooled or frozen in low protein binding tubes. Freeze/thaw cycles should be avoided, so it is always recommended to prepare aliquots of the protein solutions and samples and store them at -80 °C for single use. In the particular case of SOD1, for short-term storage (up to two weeks) a storage temperature of 4 °C was found suitable, whereas for longer periods (up to six weeks) the samples should be stored at -20 °C. For long-term storage (several months) a storage temperature of -80 °C is recommended. Brain tissue samples should be stored at -80 °C. Futhermore, it was found that human SOD1 is less stable in pig brain matrix compared to the artificial CSF samples.

Moreover, the storage temperature was found to have a significant effect on the formation of FER oligomers in commercially available FER and fetal calf serum (FCS). Freezing of FCS led to the highest FER oligomer formation. On the other hand, lowest formation was observed when the serum sample was stored at 4 – 8 °C. Therefore, a sample storage temperature in this range is recommended when analysing FER to ensure it is in its monomeric form and to achieve a good separation from other Fe containing biomolecules.

In order to carry out metalloprotein speciation studies in tissue samples, the first required step is the extraction of the metallo-species into a liquid phase without any alteration of its chemical form. Sodium dodecyl sulfate (SDS), an ionic detergent widely used for the rapid disruption of biological membranes, should be avoided for speciation analysis of non-covalently bound metalloproteins. It breaks the non-covalent bonds and S-S bridges of the proteins, denaturing them and the protein loses its structure. Therefore, the use of SDS is not recommended when the structure and the activity of the proteins have to be preserved.

A dedicated SOP for the extraction of cytosolic proteins from brain tissues using a Tris buffer saline (TBS-buffer) was also developed and sucessfully applied for the extraction of SOD1 and FER from pig brain tissue. The extract can be used for the determination of either proteins *per se* or for further enzymatic digestion of proteins. This buffer is especially useful for tau-protein and determination of native proteins or their tryptic digests by ICP-MS, since it is relative low in S or P containing compounds. Moerover, a heat denaturation protocol of the extracted proteins was used for semi-purification of heat-stable proteins such as SOD1, FER and tau-protein.

The development of SS IDMS methods requires several steps:

- the separation of the analyte protein from interfering components in the biological matrix
- the production and characterisation of the species-specific spike material (see section 4.2 for more detail) and calibrant (reference material)
- both analyte and spike isotopes should be free of spectral interferences. These are most likely to occur in the sample blend due to the sample matrix

Therefore, isotopically labelled spike and standard reference materials were produced and characterised for the target metalloproteins (see section 4.2 for more details). After establishing appropriate separation and detection methods in the different biological matrices under study, the whole procedure of SS ICP-IDMS was carefully validated according to metrological principles and a comprehensive uncertainty budget was

estimated. Potential reference measurement procedures based on SS-IDMS for the determination of TRF, Cu-ALB and SOD1 at low concentrations in body fluids (serum, CSF and brain homogenates) were developed achieving the target uncertainties of < 15%.

Transferrin

The method previously developed in the EMRP project HLT05 for the quantification of TRF in serum was adapted to lower protein concentrations expected in CSF (around 100 times lower) and in brain tissue samples. The separation of TRF from other biomolecules in the three matrices under study (human serum, a pooled CSF from a clinical laboratory and bovine brain homogenates) was achieved using a strong anion exchange column (MonoQ GL 5/50 HPLC column, Pharmacia) with an ammonium acetate gradient for elution. In the case of tissue samples, TRF was spiked at the concentration expected in CSF. During sample preparation, neither lipid precipitation nor ALB removal were applied. The determination of the TRF via its Fe content was conducted by collision cell ICP-MS/MS using hydrogen. Triple SS-IDMS was applied and TRF mass fraction values with target expanded uncertainties < 15 % were achieved: ~ 6 % for ERM-DA470k/IFCC and CSF (TRF value 100 times lower than in serum) and ~ 12 % for the spiked bovine brain matrix.

Copper bound to albumin

First experiments were focused on the separation of Cu-containing proteins (main target Cu-ALB) and Fe-TRF by complementary monolithic liquid chromatography and FFF coupled to ICP-MS/MS using standards and/or matrix matched standards. When the complementary monolithic liquid chromatography separation method was applied to serum samples, a poor resolution of the main Cu-containing proteins (ALB and CER) was obtained, hampering the accurate quantification of Cu bound to ALB. Finally, a good separation was achieved by strong anion exchange HPLC in serum and CSF (QC Liquid CSF Control 2). The method was later applied to Wilson's disease serum samples to get a better understanding of the distribution of Cu species in patient samples. The validation of the developed double SS-IDMS approach for the determination of Cu-ALB in a serum matrix was performed on three independent days obtaining an expanded uncertainty of ~5 %.

Cu, Zn-Superoxide dismutase

The quantification method previously developed for SOD1 using strong anion exchange chromatography was successfully transferred from erythrocytes to CSF and brain matrix. The QC Liquid CSF and pig brain were used as CSF and brain model samples, respectively. Since no native human protein was present, native SOD1 was spiked at the beginning of the sample preparation for proof of concept. Double SS-IDMS was used in this candidate measurement procedure for the quantification of SOD1 via its Cu content. By measuring the Cu containing part of the SOD1, only the amount of active protein is quantified, which is the important quantity. The quantification of SOD1 in CSF was successfully applied to the stability measurements mentioned previously. The method could not be applied to pig brain matrix since the human SOD1 calibrant and spike materials were different from the porcine SOD1 present, eluting at different retention times due to different isoelectric points (6.8 and 5.0, respectively).

Ferritin

Two different quantification approaches were pursued in this project for the quantification of FER: via its a) Fe content and b) S content. Size exclusion chromatography (SEC) is a state-of-the-art separation technique, which has the potential to fractionate FER from other Fe containing biomolecules according to their molecular weight. The hyphenation of SEC to ICP-MS, and particularly to a ICP-MS/MS using oxygen as reaction gas, has allowed the simultaneous determination of Fe and S content in FER.

a) *FER bound Fe*

The Fe content is directly related to the amount of Fe atoms stored in the FER cage and S present in its backbone. Consequently, FER bound Fe can be determined via the Fe to S ratio of FER. A SS-IDMS method for the quantification of FER bound Fe in pig brain homogenates was developed using the isotopically enriched ^{57}Fe-FER spike (see section 4.2). Measurements were performed using an on-line fractionation method for FER from other metal containing proteins such as TRF and ALB using ultra performance liquid chromatography (UPLC) SEC-ICP-MS. The quantification of FER was not possible when the samples were frozen or lyophilised due to changes in the native form of FER (oligomerisation) or the binding to other proteins.

b) FER via S

A complementary approach is the quantification of FER at the whole protein level via the S content in the protein backbone without the need of peptide digestion using HPLC-ICP-MS/MS. Strategies including SEC, heat treatment, ALB depletion, desalting, ultrafiltration and immune affinity chromatography were tested for the separation of FER from interfering matrix compounds in serum without success due to the binding of FER to other proteins. As alternative, a quantification of FER via specific peptides is now attempted.

The determination of FER presented the biggest challenge among all the metalloproteins. FER oligomerisation was observed to increase over time and depending on the storage temperature conditions. Although special attention was paid to convert FER oligomers into monomers, a decrease in the protein content was observed. Moreover, the presence of other proteins, mainly ALB, in the serum matrix hampered the quantification of FER. Therefore, development of ALB depletion protocols was required for the determination of FER. This challenge was also common in the quantification of CER in CSF. Furthermore, it was observed that FER is bound to other FER binding proteins in serum and CSF, the more heavier chains the protein contains (FER is a complex of 24 chains containing a varying amount of light and heavy chains). This leads to a different behaviour of FER spike/calibration material and FER originally contained in serum /CSF.

Ceruloplasmin

The method for the quantification of CER in CSF was adapted from the previous one developed in serum in the project HLT05. Different kits were tested to achieve a complete separation of CER from ALB on a SEC column. The use of an ALB depletion kit from Agilent turned out to be the most successful. However, the CER spike production method previously developed was not reproducible. Therefore, its procedure had to be modified by replacing potassium thiocyanide (KSCN) with potassium cyanide (KCN) as demetallation agent. First SS-IDMS measurements of CER in CSF proved the feasibility of the developed method in this type of matrix.

A new CER immunoprecipitation method was also developed, based on protein-coated nanodiamonds, for the production of enriched CER fractions from human serum and CSF samples for Cu isotope ratio analysis. The proposed immunoprecipitation method was applied to human serum and CSF for the final Cu IR ratio by multicollector ICP-MS (see section 4.5).

Haemoglobin

The determination of HGB is used for the control of blood contamination in CSF. Therefore, PTB tried to transfer the method previously developed for its determination in blood. However, the quantification of HGB was not possible since the protein is not present in a free form in CSF but bound to haptoglobin which made its quantification with the developed species spike material for HGB impossible.

In order to show the capabilities of the methods developed for metals and metal containing proteins using isotopically labelled spike materials, an interlaboratory comparison was conducted at the end of the project. The sample consisted of a lyophilised human serum (Seronorm Human, Sero) used as a QC material to be used to monitor precision and trueness of laboratory measurement procedures, providing reference values of the following analytes under study: TRF, Fe, Zn and Cu. The material was distributed between the partners together with a preparation and stability protocol based on manufacter's instructions.

Various partners contributed to the comparison with the measurement of different parameters. A good agreement between partners' data and manufacture's certificate was found for TRF and for Fe, Cu and Zn. The only common parameter among the participants was Cu and Zn isotope ratio in bulk serum. For the isotope ratio measurements, Cu values were comparable, however quite large uncertainties reported for Zn renders a comparison difficult. Mass fraction of Cu bound to ALB, S and Cu isotope ratio in the CER immunoprecipated fraction of the serum were also measured in this material.

Collaboration:

The cooperation within this consortium enabled the development of methods for a lot more metalloproteins than one institution alone would be able to deliver within this timeframe and with available resources. So was the method for TRF developed by TUBITAK, Cu bound to ALB by LGC, SOD1, CER and HGB by PTB and FER by both PTB and UNIVIE. Methods for the latter were investigated for the quantification via Fe by UNIVIE and via S by PTB, thus looking at the protein from different perspectives.

Key outputs and conclusions:

This objective was successfully completed, potential reference measurement procedures based on SS-IDMS were developed for metals and metal containing biomolecules. Target uncertainties < 15 % were achieved in clinical samples (serum, CSF and brain tissue homogenates) for the determination of SOD1, TRF and Cu bound to ALB. Feasibility of the developed SS-IDMS was proven also for CER and Fe bound to FER. Their validation could not be carried out within the lifetime of the project. In general, the determination of FER via S or Fe presented the biggest challenge. The preservation of FER in its native form and the presence of other interfering proteins, mainly ALB, hampered its quantification in serum and CSF. To address this, PTB is currently investigating a completely different approach using FER specific peptides after tryptic digestion.

4.3 Production and characterisation of isotopically labelled spike materials

To perform SS IDMS the analytes under investigation have to be available in an isotopically enriched form (here the metalloprotein labelled with isotopically enriched metal). Since no spike materials of the proteins under study are currently commercially available, procedures for their production were developed within this project. Two different strategies were investigated and applied to the production of isotopically labelled metalloproteins, depending on the type of binding between the metal/heteroatom and the protein:

1. Exchange of natural metal ions/prosthetic groups:

When the natural metal is not covalently bound to the protein, the metal with the natural isotopic abundance can be exchanged by a metal enriched in one isotope as shown in Figure 2. The starting point is the pure protein (commercially available) containing the metal with a natural abundance (e.g. SOD1). Then, a demetallation procedure, for example dialysis, is applied to remove all natural metal ions (in this case ^{63}Cu and ^{66}Zn) in order to generate the apo-form of the protein (protein without metal). Afterwards, isotopically enriched metal ions of high purity (^{65}Cu, ^{67}Zn) are incorporated into the apo-form to generate the spike material (^{65}Cu, ^{67}Zn-SOD1), which should behave the same way as the natural one (calibrant material).

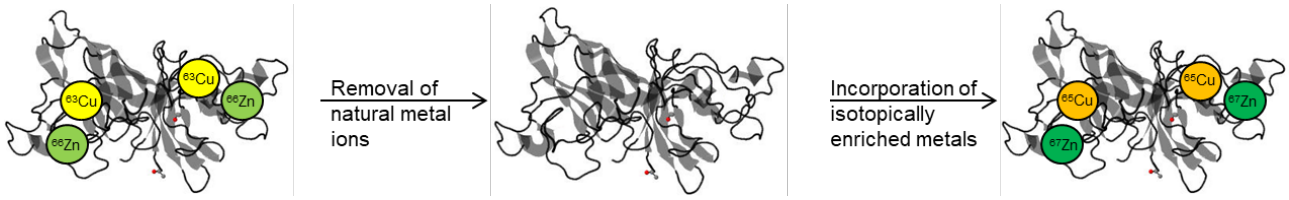


Figure 2: General steps of the production of SOD1 spike enriched in ^{65}Cu and ^{67}Zn. The procedure for other metalloproteins containing non-covalently bound metals is similar.

This procedure was applied to generate most of the spike materials within the project: ^{57}Fe-TRF, ^{65}Cu-ALB, ^{65}Cu/^{67}Zn-SOD1, ^{65}Cu-CER, ^{57}Fe-FER. Not only metal ions can be exchanged, also prosthetic groups. That is especially the case for HGB, where the natural haeme group of the protein is exchanged by a synthesised ^{54}Fe- haeme produced from protoporphyrin IX (haemin without Fe) and $^{54}FeCl_3$.

The main challenge of these kinds of spikes is to reconstitute a folding state similar to the natural proteins as usually some kind of denaturing conditions have to be applied to remove the metal. Therefore, it is sometimes difficult to obtain 'like for like' behaviour between the natural and the spike material.

2. Enrichment at the protein/peptide level:

In this case, the enrichment of the heteroatom (e.g. S, Se) has to be achieved in the amino acid backbone of the protein. It can be done through the synthesis of enriched peptides or amino acids or via the production of recombinant proteins using the *E. coli* protein production system (e.g. ^{34}S-FER).

Here the main challenges are the likelihood of the synthesis of the spike material and its cost and it is limited to covalently bound proteins, i.e. proteins where the heteroatom is mainly located in the amino acid chain.

The following isotopically labelled spike and corresponding calibrant materials have been produced and characterised within this project:

<u>Isotopically enriched Fe-transferrin spike material</u>

Two TRF spike materials isotopically enriched in ^{57}Fe (^{57}Fe-TRF) were synthesised according to the method developed in HLT05. Briefly, human apo-TRF from Sigma (T2036, purity ≥ 98%) was used as the common starting material and the complete saturation of the protein was performed using a ^{57}Fe enriched solution in the presence of sodium carbonate as synergistic anion. In parallel, the natural Fe-TRF standard (^{nat}Fe-TRF) was prepared following the same procedure using natural $FeCl_3$. In both cases, an excess of Fe was used in order to assure the complete saturation of the protein (1 mol TRF can bind 2 mol Fe).

The synthesised spike materials (^{57}Fe-TRF) were characterised regarding isotopic composition, total Fe mass fraction, storage stability at different temperatures and isotope exchange. TRF solution at a concentration of 0.6 g/kg was found to be stable at -20 °C for at least 4 months. The blends prepared using ERM-DA470k/IFCC and ^{57}Fe spike material were stable at +4 °C for at least 7 days and the samples can be frozen and thawed at least 6 times according to the stability study results.

Cu-albumin spike material

For the first time, a species-specific ^{65}Cu(II)-ALB spike material was synthesised within this project, proving the feasibility of its production in order to be used for the accurate determination of Cu bound to ALB in biological matrices by SS-IDMS.

In this case, a Cu demetallation procedure was carried out to generate apo-Cu(II)-ALB from the native protein. The efficiency of around 80 % was confirmed by anion exchange complementary monolithic liquid chromatography. The generated apo-protein was remetalled using a solution enriched in ^{65}Cu (~99.6 %). The synthesised ^{65}Cu(II)-ALB spike was further characterised regarding isotopic and elemental composition, obtaining an enrichment ≥ 98%. The production of enriched Fe-ALB spike was not pursued, due to its low concentration compared to the major metal-protein species (Cu-ALB) of relevance to the project.

Ferritin spike material

The production of the FER spike material was carried out using two different approaches: A) integration of enriched Fe (^{57}Fe) into apo-FER, and B) enrichment of ^{34}S in the protein backbone of a recombinant FER.

a) Synthesis of ^{57}Fe-FER spike:

A commercially available standard of apo-FER from equine spleen (Sigma) was selected as the starting point. Then, repeated addition of $^{57}Fe^{2+}$ solution in small amounts to the apo-form was conducted to avoid aggregation and/or precipitation of Fe, which can occur when large amounts are added simultanously. A FER spike material (^{57}Fe-FER) with a Fe load of about 1200 Fe atoms/molecule was achieved.

b) Recombinant ^{34}S-FER spike:

Recombinant native and spike FER materials, containing both light and heavy chains, were produced for the first time in an *E. coli* expression system and further purified by ion exchange and additionally SEC. Recombinant native FER (^{32}S-FER) and spike (^{34}S-FER) were analysed by LC-MS/MS after tryptic digestion to confirm protein identity and the integration ^{34}S into the S-containing amino acids in the spike material (^{34}S-FER) could be proven.

Ceruloplasmin spike material

The isotopically enriched CER spike was prepared from native CER following the general procedure described in Figure 2. Firstly, the native protein was dialysed against KCN in an imidazole buffer followed by a second dialysis to remove KCN. Afterwards, under anaerobic conditions, a ^{65}Cu solution (99.7 %) was added to the generated apo-CER and the mixture was incubated overnight at room temperature. The excess of Cu was removed by dialysis against a phosphate buffer. KCN was found the most suitable demetallation agent for the production of the CER spike material (^{65}Cu-CER) and an enrichment of around 90 % was obtained.

Cu,Zn-Superoxide dismutase spike material

Following a similar approach to the one described in Figure 2 SOD1, spike material from the human native protein was produced. In this case, the demetallation step consisted of a dialysis against ethylenediaminetetraacetic acid (EDTA) in ammonium acetate buffer followed by two extra dialysis steps against ammonium acetate buffer to remove EDTA. The remetallation of the apo-SOD1 protein was performed using isotopically enriched ^{65}Cu and ^{67}Zu (99.7 % ^{65}Cu and 97 % ^{67}Zn). The order of the spikes' addition (firstly Zn and afterwards Cu) was essential as Cu can occupy Zn binding sites irreversibly.

The produced SOD1 spike and the native protein showed the same chromatographic and enzymatic behaviour, and the generated spike contained a high enrichment of ≥ 99 % ^{65}Cu and ≥ 96 % ^{67}Zn.

Haemoglobin spike material

The isotopically labelled $HGBA_0$ spike material (^{54}Fe-$HGBA_0$) was synthesised in 3 steps: (1) the native human $HGBA_0$ protein was stirred in an acidic acetone media at -20 ˚C in order to remove the haeme group and to obtain apo-$HGBA_0$, (2) the incorporation of ^{54}Fe into the protoporphyrin IX (haemin without Fe) to generate the ^{54}Fe-haeme group and (3) finally the assembly of the haeme group and the apo-protein for the production of the spike material (^{54}Fe-$HGBA_0$).

The native protein and the generated spike were derivatised with KCN in order to obtain the same species with the same Fe oxidation state (cyanmetahaemoglobin) and, therefore, make them suitable for IDMS experiments. Afterwards, the synthesised spike material was characterised regarding ^{54}Fe enrichment (≥ 99%), protein structure and folding by anion exchange chromatography ICP-MS, HPLC-MS and Raman spectrometry, respectively.

Collaboration:

Again, the cooperation within this consortium enabled the production of spike materials for a lot more metalloproteins than one institution alone would be able to deliver within this timeframe and with available resources. Isotopically enriched metals are very expensive and the production of protein spike material can be very time consuming. For HGB, for example, the production at PTB takes about two weeks before the isolated spike material is available and characterised. Furthermore, spike material for SOD1, CER and ^{34}S-FER was produced and characterised at PTB. ^{57}Fe-FER was produced and characterised at UNIVE, while TUBITAK achieved the same for TRF and LGC for Cu-ALB.

Key outputs and conclusions:

This objective was successfully completed, and all natural and isotopically labelled materials proposed were prepared and fully characterised to be further used for the development of the SS-IDMS methods (see section 4.2 for more details). Particularly, three isotopically labelled metalloproteins were produced for the first time: recombinant FER labelled in ^{34}S (^{34}S-FER), native FER in ^{57}Fe (^{57}Fe-FER) and native Albumin with ^{65}Cu (^{65}Cu-ALB). Furthermore, the protocols developed previously in the EMRP project HLT05 *Metallomics* were reproduced or adapted successfully for the production of new spikes of ^{57}Fe-TRF, ^{65}Cu/^{66}Zn-SOD1, ^{65}Cu-CER and ^{54}Fe-HGB.

4.4 New and accurate methods for measuring peptide and protein biomarkers

For the important biomarkers T-tau, P-tau and Aß, not only the concentration of every single analyte is important but also their ratios. Up to now, no reliable, comparable and traceable quantification methods exist for theses markers, although this is demand, for example, in the “Regulation of the European Union on *in vitro* diagnostic medical devices” (EU 2017/746). The considered proteins and peptides are found at low concentration levels in the brain and CSF. In the literature disagreement prevails about the concentrations of such biomarkers in healthy patients (ca. <400 pg/mL for T-tau, ca. <60 pg/mL for P-tau and ca. >600 pg/mL for Aß 1-42, in CSF) (Llorens et al. 2017, Sjören et al. 2001, Sobów et al. 2004). Besides these relatively low concentrations in complex matrices, only small sample amounts from patients are available in a clinical setting. Therefore, quantification methods need to be developed which are selective for such AD biomarkers and sensitive enough to measure significantly lower concentrations than those stated above.

Tau protein quantification with Raman Spectrometry

Metallic and magnetic nanoparticles provide versatile sensing platforms for biological and biomedical applications such as the detection and quantification of large biomolecules serving as diagnostic markers in human medicine. A new sandwich immuno-assay for a surface-enhanced Raman spectrometry (SERS) based determination in combination with the ID approach of tau-protein has been developed within this project.

The developed assay is used to separate the target analyte from the matrix with the help of magnetic iron oxide (Fe_xO_y) nanoparticles as well as to quantify the tau protein by utilisation of a sensitive SERS active marker coupled to gold nanoparticles. The linkage of both nanoparticles to the protein will be ensured by immunoreaction with specific antibodies (Figure 3). Due to the high Raman cross section of the marker 5,5'-

disulfanediylbis(2-nitrobenzoic acid) (DTNB), this method is suitable for very small sample volumes and low analyte concentrations typically found in CSF samples for tau-protein while highest accuracy is achieved through the ID approach (Zakel et al. 2013). Therefore, an isotopically enriched form of the marker was used as perfect internal standard (spike). Due to the higher molecular weight of the spike, a specific Raman shift can be observed and used for quantification. For this quantification method a set of calibration mixtures is necessary containing different ratios of the assay in its natural and its isotopic enriched form. Figure 4 shows the Raman spectra of those measurements.

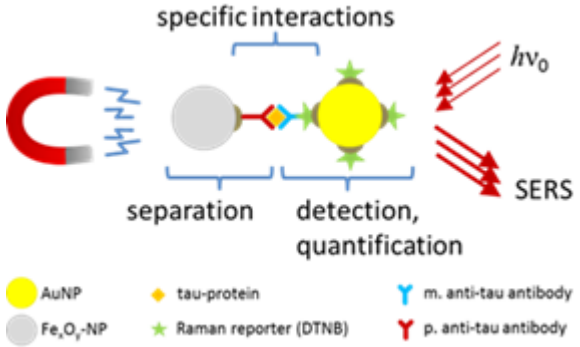


Figure 3: Schematic depiction of the magnetic and SERS active nanoparticle sandwich assay for the detection of tau-protein in its natural composition. For the isotopic enriched version, the DTNB must be exchanged by ^{15}N-DTNB (AuNP: gold nanoparticles).

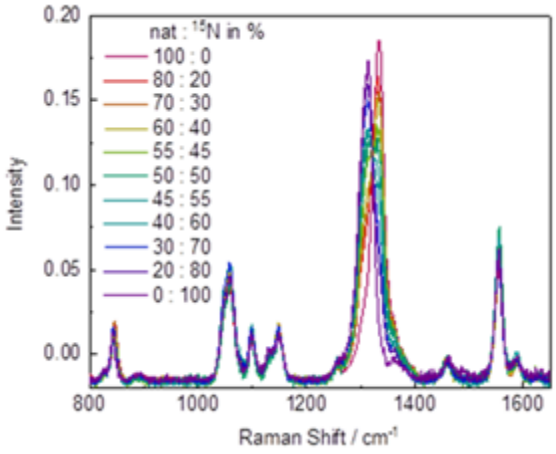


Figure 4: Normalised Raman spectra of the sandwich assay for different ratios of the natural (nat) and the isotopic enriched form (^{15}N).

To control the characteristics and to ensure a batch to batch comparability, both gold and Fe_xO_y nanoparticles were produced and functionalised inhouse. The production of the gold nanoparticles includes a nucleation step and a growth step according to Tainuchi *et al*. (Tainuchi et al. 2007). The size and the Ostwald ripening of the particles were monitored over time to ensure repeatable Raman intensities. The Fe_xO_y nanoparticles were produced using a top-down process. Both the build-up of the gold and the Fe_xO_y nanoparticle side were carefully characterised using techniques like UV/Vis spectrometry, X-ray diffraction, transmission electron microscopy, dynamic light scattering, thermogravimetric analysis, single-angle X-ray scattering and zeta potential measurements.

Measurements of tau protein in artificial CSF and mouse brain tissue show the possibility to reach the low concentrations which comply with the natural biological range in humans.

Total tau-protein and phosphorylated tau-protein quantification with ICP-MS

Another accurate quantification method for peptides and proteins is via their intrinsic S- and/or P-containing amino acids by ICP-MS/MS after separation of the analyte from the matrix. In the literature several tryptic digestion methods are described, sometimes only partially diverting from each other. Considering that S and P were used to determine the concentration, methods which included the use of S- and/or P-containing chemicals are to be avoided where possible. Additionally, methods which require desalting the tryptic digest (due to the use of e.g. urea) were avoided due to the inherent risk of losing the analyte. The most promising methods were adapted from Percy *et al.* (Percy et al. 2013) and Proc *et al.* (Proc et al. 2010). They allowed reproducible digestion of standard proteins, like casein and albumin, and their quantification via S- and/or P-containing peptides. The transfer of this method to biological matrices, such as cytosolic brain proteins, HeLa-cells or other cell types, showed that the tryptic digest of the proteins is reproducible. Using ICP-MS/MS for analysis, it is obvious that different tissues contain a range of non-proteogenic P-containing compounds, which need to be removed before the peptide analysis. These compounds were identified as deoxyribonucleic acid (DNA) and/or ribonucleic acid (RNA) oligomers. It was possible to remove them successfully by a benzonase digestion before the tryptic digest. A further challenge when working with biological tissues is the high concentration of low molecular S- and P-containing compounds, which can overshadow especially hydrophilic peptides eluting early from the separation column. A desalting of the proteins after benzonase treatment, denaturation and reductive alkylation of the cysteine residues with a customary desalting column was found to be the best way to overcome this issue without the loss of proteins and/or peptides.

For a successful quantification of the proteins using ICP-MS/MS, it is essential to achieve a baseline separation of the peptides in the chromatographic separation. Biological matrices typically contain hundreds if not thousands of proteins, each single one capable of generating more than one S and/or P-containing peptide. Therefore, a purification of the sample is unavoidable. Several possibilities are available for this purpose. At the protein level the use of specific antibodies or simply separating heat stable from instable proteins by heat precipitation is possible. At the peptide level, after the tryptic digest, purification can be achieved, for example, by using a cation exchange column with high pH-reversed phase separation and subsequently collecting the fractions of interest. Another possibility is the use of specific affinity materials to enrich peptides with specific functional groups such as phosphate groups.

Affinity materials for the enrichment of P-containing proteins and/or peptides are widely used in “standard” proteomics research. Due to the fact that protein phosphorylation has a huge impact on cellular processes, there is a high interest in P-containing proteins in medical and biochemical research. Enrichment of phosphorylated proteins and/or peptides is important for the identification and quantification of these proteomics, since phosphorylated forms are generally present at much lower concentration than the non-phosphorylated ones. Furthermore, they have a lower ionisation efficiency in ICP and a worse detection limit in molecular MS.

In contrast to molecular MS, the detection of peptides with ICP-MS does not have such severe problems of compound specific signal intensity, but it requires the presence of either S or P in the peptide which could be detected. Nevertheless, to improve the separation efficiency a purification of the analyte from the matrix by specifically enriching phosphorylated peptides is necessary, assuming these are the target compounds. The most common methods for phosphorylated peptide enrichment are the use of titanium dioxide nanoparticles and Fe immobilised affinity chromatography (Fe-IMAC). In this study, both were evaluated regarding the reproducibility of peptide recovery and additionally a third method, using Mullite instead of titanium dioxide nanoparticles, was tested. The reproducibility of all three methods was investigated using a mix of casein and ALB and was found to be not as good as expected from the literature. Measurements showed relative standard deviations of 5 to 50 % depending on the peptide. The best reproducibility and recovery of only one phosphate containing peptides was found after enrichment with titanium dioxide nanoparticles followed by Fe-IMAC. The recovery of peptides containing multiple phosphate groups is lower than that of mono-phosphorylated peptides for all three methods. Applying either of the methods to the standard tryptic digest of cellular origin showed that DNA and/or RNA oligomers present in these extracts are enriching extremely well on all materials and, therefore, must be removed in advanced.

Another possibility to quantify tau-protein was attempted by biochemical means using commercially available tau-protein standards as calibrants. But this approach has been proven futile because the concentrations of tau-protein standards were not accurately indicated by the manufacturers. The comparison of different standards showed strong deviations in measured signals when same concentration, according to

manufacturer's information, were used. Therefore, a method for reliable, accurate and traceable protein quantification of pure proteins using ICP-IDMS was developed. Using this method, the amount of S is accurately quantified and can be used to calculate the amount of protein in solution, as S is present in the two amino acids cysteine and methionine and the amino acid sequence of the target proteins is known. By measuring well characterised S standards and samples in one measurement sequence, the results are traceable to the standard and are comparable between laboratories. To correct for S containing impurities in the protein solution, an offline separation method was developed to quantify and correct for free S in the sample.

Beta-amyloid quantification by amino acid analysis

Two different approaches for the quantification of Aß1-40 and Aß1-42 were developed. The first one was with ICP-MS/MS detection after chromatographic separation. The method was developed using the ID approach through the analysis of the S-containing amino acids methionine and cysteine within the Aß peptides. For the method development two protein standards (myoglobin and lysozyme) as well as the standard reference material NIST 2389a (amino acids in hydrochloric acid) were used. ^{34}S-enriched yeast hydrolysates containing L-methionine and L-cystic acid was the isotopic enriched standard. Samples were spiked with the yeast and treated with performic acid to hydrolyse and oxidise the amino acids or peptides. Both Aß peptides contain a methionine, the only S-containing amino acid, in their peptide sequence, which is shown below in the one letter abbreviation notation.

DAEFRHDSG YEVHHQKLVF FAEDVGSNKG AIIGL**M**VGGV VIA

Aß1-42 is represented by the complete sequence while Aß1-40 is the same without the last two amino acids.

Applying this method to the standard reference material 2389a results in a mass fraction for cysteine of (0.297 ± 0.005) mg/g (certified value: (0.295 ± 0.013) mg/g) and for methionine of (0.368 ± 0.012) mg/g (certified value: (0.373 ± 0.011) mg/g). Two myoglobin and lysozyme samples each were used to establish this method for proteins and peptides. For myoglobin recoveries of (96.4 ± 3.4) % via cysteine and (100.7 ± 2.9) % via methionine could be achieved, while the recoveries for lysozyme were (97.44 ± 0.89) % via cysteine and (100.0 ± 1.6) % via methionine. However, the recoveries achieved when analysing Aß from rPeptide were (126.78 ± 0.005) % for Aß1-40 and (52.56 ± 0.01) % Aß1-42 compared to the manufacture's amount indication. Since the entire content of the vial has been solved and the differences between the individual fractions are small, it is to be assumed that there is a problem with the weight specifications of the manufacturer. Weighing in fact was problematic due to electrostatic charging, which could not be overcome by the usual discharge procedures. Thus, the manufacture's indication was used as refence value.

The second method is an SI traceable method for the accurate quantification of Aß which is highly specific and flexible. Therefore, a liquid chromatographic separation with LC-MS/MS using an Orbitrap MS detection was combined with a selective sample preparation for the simultaneous measurement of multiple Aß peptides in CSF. This study focuses on the development of methods for the Aß 1-40 and Aß 1-42 peptides as preclinical and clinical biomarkers. A solid phase extraction (SPE) sample preparation protocol was used to extract Aß fractions in CSF to eliminate matrix interferences. As strategies for disease modification in AD emerge, it may be necessary to identify other types of Aß that may correlate with AD pathology. The developed method can be used for other types of Aß peptides as well.

The Aß peptides used for calibration were purchased from rPeptide and analysed with peptide impurity corrected amino acid (PICAA) analysis. The mass fractions were (517.1 ± 72.8) mg/g for Aß 1-40 and (440.4 ± 27.1) mg/g for Aß 1-42. ^{15}N-Aß 1-40 and ^{15}N-Aß 1-42 were used as internal standards and supplied from rPeptide as well.

Reference dilutions for standard and quality control (QC) samples with different concentrations of Aß 1-40 and Aß 1-42 between 2 and 30 ng/mL were prepared. Further working control solutions were prepared between 12.5 and 400 ng/mL and spiked with labelled internal standards. The same set of solutions were also prepared by spiking artificial CSF containing 4 mg/mL bovine serum ALB. These were subjected to SPE cleaning before LC-MS/MS analysis.

For the chromatographic separation, a Phenomenex Jupiter 5µm C18 column was used with a mobile phase containing (A) 0.075 % ammonia and 5 % acetonitrile and (B) 0.075 % ammonia and 95 % acetonitrile. Table 2 summarises the gradient program.

Table 2: Gradient program for the chromatographic separation

Time min:s	% A	% B	Flow rate µL/min
00:00 to 01:00	90	10	200
01:00 to 10:00	90 to 55	10 to 45	200
10:00 to 10:30	55 to 0	45 to 100	200
10:30 to 14:30	0	100	200
14:30 to 15:00	0 to 90	100 to 10	200
15:00 to 17:30	90	90	200

For the ID LC-MS/MS quantification method a six-point calibration mode using the PICAA analysed peptides in artificial CSF was used. The calibration curves are shown in Figure 5. Linearity was found in the range of 500 – 4000 pg/mL for Aß 1-42 and 500 – 20000 pg/mL for Aß 1-40. With a correlation coefficient of 0.995. The recovery of the analysed quality controls was between 90.03 % and 109.58 %. Afterwards this method was applied on a pooled CSF sample with an expanded uncertainty of 9.5 % for Aß 1-40 and 17 % for Aß 1-42.

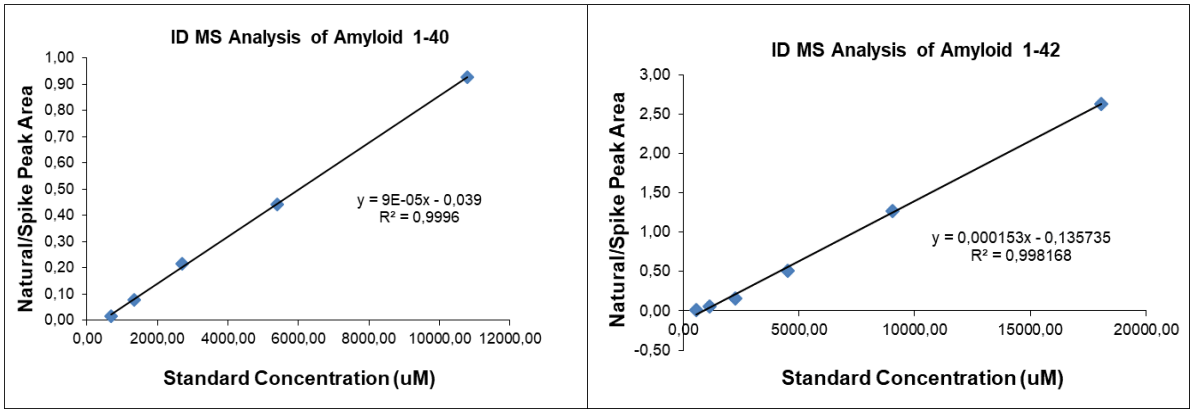


Figure 5: Calibration curves obtained in artificial CSF for aβ 1-40 and aβ 1-42.

Collaboration:

The cooperation within the consortium enable the investigation of different aspects and forms of tau protein. While PTB developed a method for tau protein based on SERS BAM and Charité worked on a method for immunoprecipitation followed by quantification via S using ICP-MS/MS. UNIABDN on the other hand focused on the identification and quantification of phosphorylated peptides to determine the quosphorylation state of the protein which is deemed important for the proggress of AD.

UNIVIE and TUBITAK developed complementary methods for the second established biomarker: aβ. While UNIVIE determined the peptide via S, TUBITAK validated a IDMS method using ^{13}C labeled peptides. As stakeholders made us aware during the course of the project that current clinical trials showed that the ratio of aβ1-42/aβ1-40 seems to be a more significant biomarker, TUBITAK agreed to additionallly developed a method for the quantification aβ1-40, so that this ratio could also be measured in the according mouse model.

Key outputs and conclusions:

This objective was successfully completed, methods for two aβ peptides and protein biomarker, T-tau, were developed. For the two clinically relevant aβ peptides methods could be developed and validated that enables the determination of these peptides in clinical samples such as CSF at the relevant physiological µg/L levels and in small µL sample volumes. A new measurement procedure for T-tau based on a SERS immunoassay sandwich approach could be developed with a sensitivity sufficient for clinical samples. However, as the

method development took longer than expected the method validation in CSF at physiological levels is still in progress. For P-tau there were too many interferences in clinical samples to quantify this biomarker reliable via phosphorylated peptides.

4.5 Uptake, metabolism and transport to the brain of metals and metal containing biomolecules

Whether trace elements contribute to AD or not is a hotly debated subject within parts of the AD research community (Adlard et al. 2018). In some studies, differences in element concentrations in the brain or certain regions of the brain between AD patients and healthy controls were found, other could not confirm these difference (Schrag et al. 2011). Another reason for the debate is that Fe, Zn and Cu can at least *in vitro* contribute to aβ agglomeration (Ha et al. 2007) and that Fe is influencing the aggregation status of tau-protein (Yamamoto et al. 2002).

Genetically modified mice are wide spread models for specific aspects of AD, with the models generally either displaying amyloid plaque or Tau fibril pathology. The consortium studied the concentration of essential elements (Fe, Cu, Zn, calcium (Ca) and magnesium (Mg)) in the brains of mouse models either overexpressing human APP or Tau protein, and their non-modified controls. The elements were determined using ICP-MS/MS after complete digestion of the sample matrix. Brain samples were not dissected into different brain regions for this experiment. Very little differences between the models and wildtypes were found. From the results it seems, in contrast to expectations, that the chosen mouse models do not display significant changes in Fe, Cu and Zn concentrations. Whether this is a problem of sampling (whole brains were analysed instead of specific brain regions) or whether the mouse models genetically modified to overexpress a specific human protein (either APP or Tau) do not show the shift in element distribution seen sometimes in human brains is yet unknown. Another factor might be also the age of the mice. For example, Ciavardelli et al. (Ciavardelli et al. 2012) used a similar model of amyloid overexpressing mouse as the one in this project, but the mice were considerably older than the ones studied here (18 months vs. 5 months). They noticed small differences in element concentrations in brains. Others found significant changes in specific brain regions and specific metal-plaque associations in old mice (>14 months). Whether there are changes in element concentration and/or distribution might well depend on the mouse model and its age used for the study.

In addition to multi-elemental patterns, the project explored isotopic ratios as potential tools for preclinical AD diagnosis and further research. High-precision isotopic analysis has recently emerged as a valuable tool for studying the metabolism of essential elements. Additionally, a systematic variation in the isotopic composition of an essential element accompanying a disease condition can potentially be exploited as a biomarker (Lauwens et al. 2018, Costas-Rodríguez at al. 2016).The contribution of this research project consisted of the development of novel methods (sample preparation and measurement protocols) for accurate and precise isotope ratio measurements of Fe, Cu and Zn in biofluids and brain tissue using multi-collector ICP-MS. Subsequently, it was verified whether or not systematic variation of the isotope ratios of these elements is occurring in AD. Figure 6 illustrates the occurrence of measurable variations in the isotopic composition of Fe, Cu and Zn between serum, CSF and brain tissue. Methodical efforts focused on (i) ensuring the accuracy of the isotope ratio measurements using a small amount of biological material only and (ii) the traceability of the results. To ensure the quality of the measurement results, the method was validated using ‘model’ serum and CSF samples. Adequate uncertainty ranges have been obtained.

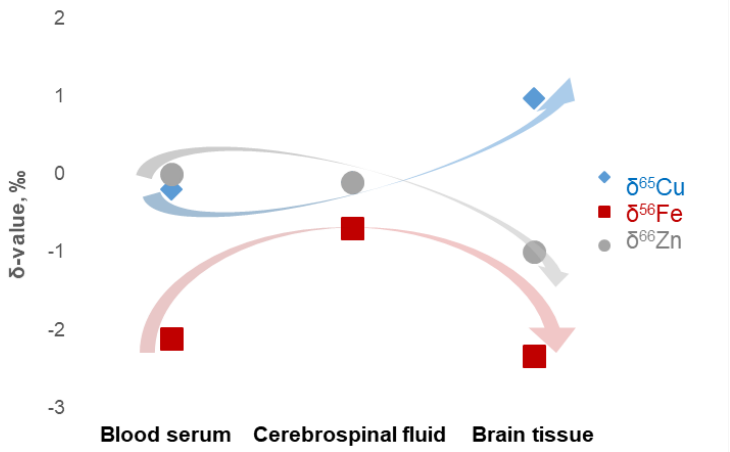


Figure 6: Isotopic composition of Fe, Cu and Zn in serum, cerebrospinal fluid and brain tissue.

Fe, Cu and Zn are potentially involved in the process or are even the cause of pathological processes in AD, such as redox dyshomeostasis and protein misfolding. To test the hypothesis that the isotopic composition reflects their involvement in such processes, the isotopic compositions of Cu, Fe, and Zn in brain tissue and biofluids have been investigated in two different AD murine models, representing two pathological processes occurring in AD – the tau- and amyloid-pathology (Kitazawa et al. 2012, Melis et al. 2015). Relevant changes in the isotopic composition of Fe and Cu were observed in the brain tissue of mice exhibiting tau-pathology. This may indicate increased metal transport and/or accelerated turnover in the brain of the affected animals.

To study this, the isotope ratio of the metals in the according metalloproteins should also be investigated. Measuring isotope ratios in key metalloproteins requires enrichment and selective separation (isolation) of these otherwise too low abundant biomolecules. In order to avoid isotopic fractionation during sample preparation, a 100 % mass balance has to be achieved for all steps, which posed severe challenges. For FER, the conflicting goals of selective separation from other (high abundant) Fe proteins and enrichment without loss could not be fulfilled. For CER promising experiments could be carried out paving the way to future metalloprotein specific isotopic studies. In the following the enrichment of the Cu-containing protein CER by immunoaffinity chromatography from biological samples is briefly described.

A particularly powerful method for the isolation of a specific protein is affinity chromatography, which is often based on the use of specialised binding molecules, called antibodies (Bernevic et al 2018). In order to enable an easy separation step, these antibodies need to be firmly connected to a carrier material, such as cellulose, magnetic particles or other materials. In this work, nanoparticles based on diamonds were used, which are very pure in terms of metal contamination and are stable against many kinds of harsh acid treatment, which is necessary for the sample preparation preceding most ICP-MS measurements of isotope ratios. One advantage of nanodiamonds is their extremely large surface, which facilitates the efficient immobilisation of bio-chemical reagents.

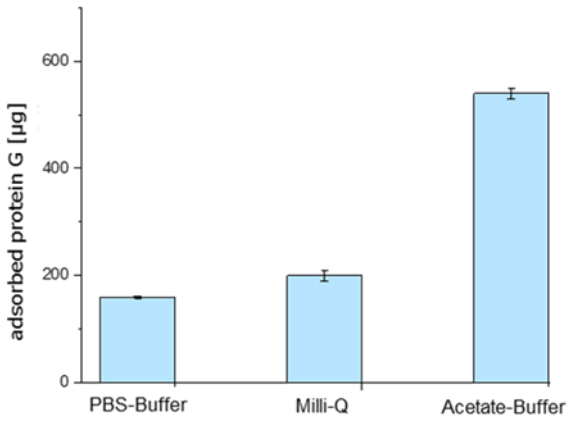


Figure 7: Amount of adsorbed protein G on 1 mg of nanodiamonds. By using a slightly acidic acetate buffer, the highest capacity of protein adsorption was achieved.

The nanodiamonds were then coated with recombinant protein G, which forms an orienting interlayer for the antibodies against our target CER. This protein G selectively traps the antibodies on the nanodiamond surface, without hampering their activity. Activity losses are very frequent problems in protocols using selective antibodies, which have to be treated very cautiously not to be inactivated. In a first step, the adsorptive coating of the nanodiamonds was examined. It could be shown that the isoelectric point of the reagent should be considered. When the pH of the coating solution is adjusted to the isoelectric point of the protein, a significant improvement of the surface coating was obtained (Figure 7). In a further step, a polyclonal antibody solution was incubated with the protein G coated nanodiamonds. The antibodies and the protein G were firmly linked with a cross-linker chemical to avoid that the antibodies are lost in the subsequent handling steps. Then the human sample, such as serum containing CER was mixed with the nanodiamonds. This step should selectively bind CER and wash out other irrelevant but potentially Cu-containing molecules. This selective separation step was examined with sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), separating different proteins in distinguishable, blue bands, which have been made visible with a special dying procedure using Coomassie Brilliant Blue. The results can be seen in Figure 8.

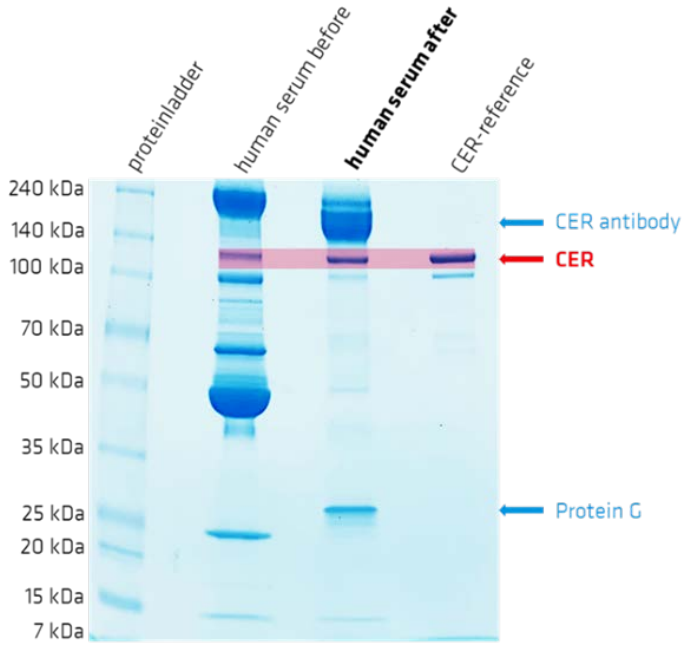


Figure 8: SDS-PAGE separation of human serum before (2nd lane) and after (3rd lane) the enrichment on nanodiamonds.

The gel shows that CER could be selectively enriched from serum at a mass of about 135 kDa. Finally, the CER bound to the nanodiamonds was digested by a strong acid to make the Cu available for ICP-MS measurement. Due to the extreme chemical stability of the nanodiamonds, they are not destroyed and remain in the centrifugation pellet.

Collaboration:

Both UGent and BAM are well known in the community for their work in IR measurements. UGent is especially experienced in IR measurements in the clinical context. Besides IR measurements BAM has a lot experience in the isolation of biomolecules using immunoreactions. Within this project both partners jointly developed methods for the IR meaurements of Zn, Cu and Fe and compared their results in the mouse models provided by Charité and UNIABDN.

Key outputs and conclusions:

This objective could only be partly achieved. The methods for metals and IR of the metals could developed and were applied to the mouse models indicating differences between wildtype and diseased mouse lines. However, further investigations for the determination of the metal IR in specific metalloproteins such as FER or CER are still required as the complete isolation of these proteins from the biological matrix, which is required for a reliable measurement, could not yet be achieved.

Besides the determination of metalloproteins and metal ion contents, metrologically validated procedures for the determination of the isotopic composition of the metals in plasma, CSF and brain tissue have been developed. The special challenge, beside the separation from the complex matrix, are the low sample volumes available. A method for the determination of Fe and Cu isotope ratios at ultra-trace level could be developed that requires only very small sample volumes. The methods for the determination of metals and isotope ratios were then applied to the mouse models for tau and aβ pathology and were compared to the according wild-type mouse lines. The data revealed some statistically significant differences in isotopic compositions of Fe, Cu and Zn in the brains and serum of the tested mice lines and an indication of differences in the metal content as well, indicating a change in the processes of metal homeostasis. This proof-of-concept study opens the way for investigating whether isotopic information can be used for diagnostic purposes and/or to achieve a more profound understanding of the disease conditions. If changes are revealed this method can serve as a novel method for medical diagnosis for AD at a much earlier stage than is possible with current biomarkers and could even enable a prognosis for people at risk of developing AD.

5 Impact

The project partners regularly interacted with relevant stakeholders (hospitals, clinical laboratories, reference laboratories) and the project results have been disseminated through the following activities:

- Initial project results were presented at the JCTLM stakeholder workshop in December 2017 with attendees from the metrological community, reference laboratories, clinicians and IVD industry. Results from the project will be presented at the 2019 stakeholder workshop in December.
- An advisory board, consisting of researchers, a clinical reference laboratory and a member of the society Alzheimer's Research UK (ARUK) amongst others was established to ensure that the research also meets the needs of clinicians as well as clinical researchers in the field of neurodegenerative diseases.
- A stakeholder workshop was held in conjunction with the kick-off meeting in 2016 with attendees from reference laboratories, hospitals and caretakers for the consortium to understand all aspects involved in the development of AD and to discuss planned activities with the stakeholders. In addition, the project held a dedicated session during the 6th International Symposium on Metallomics in Vienna in 2017 for a mainly scientific audience.
- The ReMiND 2019 - Biomolecules in Neurodegenerative Diseases conference was organised at PTB in June 2019. 28 participants, including some high-profile speakers, from all over Europe contributed to the conference with presentations, posters and lively discussions. Participants came from research institutes, hospitals and vendors. As part of the conference, the project partners presented the

measurement procedures developed within the project and also discussed future activities with the stakeholders for their implementation within the wider community and also with colleagues involved in the EMPIR project NEUROMET, another project concerned with the diagnosis of neurodegenerative diseases.

- Training material on the developed measurement procedures will be published as soon as they have been published in peer-reviewed journals. The first SOPs have already been published on the project website and some other training material is currently in production and will be available soon.
- Raising the awareness of "future" researchers about metrology and the need for it by presenting the project at several RSC organised general student and early career researcher activities (ao. Twitter conference, Analytical Science Network: Bright spark Symposium) and at conferences and meetings of student and early career researcher activities connected with ARUK (Alzheimer Research UK). This is important since metrology does not feature in the normal curriculum of either chemistry students or medicinal / neurological orientated studies.

Impact on industrial and other user communities

A European Network on Traceability in Laboratory Medicine (TraceLabMed) has been set-up. As this network brings together all relevant stakeholders in the field of laboratory diagnostics including IVD producers, clinical laboratories and medical societies, the partners will use this network for further dissemination. Furthermore, the involved NMIs and DIs will offer services based on the methods developed within this project through the network.

Producers of calibration and matrix reference materials are now able to benefit from the project. As many of them are partners of both JCTLM and TraceLabMed, they have been made aware of the methods developed in this project via both JCTLM and TraceLabMed meetings. The developed reference measurement procedures allow them to provide reference materials with values directly traceable to the SI. Those materials are urgently needed for quality control in clinical laboratories. LGC, a potential producer of such materials, is one of the project partners.

The reliable and comparable measurement procedures developed in this project are now able to support physicians and pharmaceutical companies in the development and testing of potential treatments for AD in clinical studies. A pharmaceutical company currently working on the development of such a treatment was associated with this project providing advice as well as the stock for breading the tau mouse model. The work has already attracted the interest of a German and a Canadian university who are interested in a future collaboration in this field. They currently investigate reasons for the onset of neurodegenerative diseases and will need measurements of the biomarkers developed within this project. Besides providing measurement capabilities also training and student exchanges is currently discussed. The procedures developed within this project will not only support research in the field of AD. LGC supported a clinical study on Wilson's disease, rare genetic disorder that causes copper poisoning in the body, by applying the procedures for Cu-ALB developed within this project.

The ability to measure changes in the concentration of metals and metalloproteins as well as in their isotopic composition has provided physicians with the ability to understand the biological processes leading to AD in more detail and help to identify the right time to start treatment. Furthermore, the determination of trace element distribution between plasma and brain tissue, on the basis of isotope ratios, can provide information about uptake rate and trans-localisation of trace elements into the brain and will significantly contribute to the understanding of how the metabolism of essential trace elements is influenced during AD progression.
The activities on the quantification of FER in serum have attracted attention of a European consortium including research institutes and IVD producers currently formed to establish a more reliable determination of FER and Fe load of FER in serum in clinical routine laboratories. The first stage was positively evaluated, and a full project proposal is currently compiled.

Impact on the metrology and scientific communities

The project has provided the metrological basis for the traceable, reliable and comparable determination of established and novel AD biomarkers in biological samples. In particular, reference laboratories can benefit

from the availability of SI-traceable reference values provided by the NMIs/DIs involved. These reference laboratories play a significant role as they can now use the reference values in their interlaboratory comparisons for routine clinical laboratories, thereby providing traceability for patients' samples. This makes results of the routine clinical laboratories more reliable and provides a basis for establishing universal cut-off values for the diagnosis of AD, rendering it unnecessary for every laboratory to define their own cut-off value and control group. One of the German reference laboratories was a collaborator of this project ensuring the dissemination to this community. To make other NMIs/DIs in Europe aware of the project, it was presented at the EURAMET TC-MC and CCQM meetings. PTB already offers a measurement service for SOD1.
PhD students involved in the project participated in the activities of the Alzheimer's Society funded Doctoral Training centre at UNIABDN and the Alzheimer Research UK conference. Furthermore, results of this project were presented at various key scientific conferences such as the 6th International Symposium on Metallomics and the Winter Conference on Plasma Spectrochemistry and published in key peer-reviewed journals.

Impact on relevant standards

The reference measurement procedures developed within this project will enable the implementation of the EU regulation 2017/746 on *in vitro* diagnostic medical devices which states clearly that "the traceability of values assigned to calibrators and/or control materials must be assured through available reference measurement procedures and/or available reference materials of a higher order". Moreover, the standard EN ISO 17511:2003 demands reference measurement systems including reference measurement procedures for the determination of analytes in samples of human origin. However, neither reference measurement procedures nor reference materials of higher order for analytes related to AD currently exist. One of the aims of the network TraceLabMed is to assist the parties involved in the implementation of the new EU regulation 2017/746. The results achieved in this project will serve as examples of how traceability in laboratory diagnostics can be established.

Longer-term economic, social and environmental impacts

As the risk of getting AD increases with age and people are tending to live longer, so the number of AD patients is expected to increase in the future, with estimates of up to 90 million dementia patients worldwide within the next 20 years, making dementia one of the greatest health issues today.

Besides the importance of early diagnosis to potentially develop a future cure for AD, reliable early diagnosis is important as early intervention can delay the on-set of severe symptoms of dementia, resulting in a better quality of life for AD patients and their carers. However, for such an early diagnosis, universal cut-off values to distinguish between healthy and diseased people and reliable routine measurement procedures are a prerequisite. The reference measurement procedures developed in this project will help to improve the reliability of routine measurement kits and reduce the variations between the results of different kits thus enabling the establishment of the required universal cut-off values.

Dementia is already burdening health care systems with total estimated costs of € 32.8 billion in Europe in 2008. Included in these costs are those attributed to informal care such as unpaid care provided by family and others, direct costs of social care such as community care professionals and in residential homes, and the direct costs of medical care. Direct medical care costs account for roughly 20 % of global dementia costs, while direct social sector costs and informal care costs each account for roughly 40 %. Sensitive and reliable measurements will contribute to a reduction of these health care costs because they will enable early detection of AD and, therefore, allow earlier intervention resulting in a delayed on-set of severe impairments requiring hospitalisation. As well as improving the quality of life for both patients and their families, delayed hospitalisation is estimated by some studies to save around US$ 10,000 per person with dementia over the course of the disease.

6 List of publications

Bogdan Bernevic, Ahmed H. El-Khatib, Norbert Jakubowski and Michael G. Weller, *Online immunocapture ICP-MS for the determination of the metalloprotein ceruloplasmin in human serum*, BMC Research Notes 2018, 11:213. https://doi.org/10.1186/s13104-018-3324-7

Sara Lauwens, Marta Costas-Rodríguez, Frank Vanhaecke, *Ultra-trace Cu isotope ratio measurements via multi-collector ICP-mass spectrometry using Ga as internal standard: an approach applicable to micro-samples*, Analytica Chimica Acta 1025, Pages 69-79. https://doi.org/10.1016/j.aca.2018.05.025

Ahmed H. El-Kathib, *Gadolinium in human brain sections and colocalization with other elements,* Neurology-Neuroimmunology Neuroinflammation, 2019, 6. https://doi.org/10.1212/NXI.0000000000000515

Theiner S, Schoeberl A, Fischer L, Neumayer S, Hann S, Koellensperger G, *FI-ICP-TOFMS for quantification of biologically essential trace elements in cerebrospinal fluid – high-throughput at low sample volume*, The Analyst, 2019, https://doi.org/10.1039/C9AN00039A

Theiner S, Schoeberl A, Neumayer S, Koellensperger G, *FI-ICP-TOFMS for quantification of biologically essential trace elements in cerebrospinal fluid – high-throughput at low sample volume*, J. Anal. At. Spectrom., 2019, Advance Article, https://doi.org/10.1039/C9JA00022D

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