universität wien

MASTERARBEIT /MASTER'S THESIS

Titel der Masterarbeit / Title of the Master's Thesis

„Validation of an off-line enrichment and fractionation method for ferritin by centrifugal ultrafiltration“

verfasst von / submitted by

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angestrebter akademischer Grad / in partial fulfilment of the requirements for the degree of

Master of Science (MSc)

Wien, 2019 / Vienna 2019

Studienkennzahl lt. Studienblatt / degree programme code as it appears on the student record sheet:	A 066 862
Studienrichtung lt. Studienblatt / degree programme as it appears on the student record sheet:	Masterstudium Chemie UG2002
Betreut von / Supervisor:	Univ.-Prof. Dr. Gunda Köllensperger

Abstract

Ferritin is an iron storage protein with a molecular weight of about 450 kDa. The iron bound to ferritin is suspected to be involved in the formation of plaques found in the brain of patients with Alzheimer´s disease. Ferritin is present in blood serum at levels of about 100 ng mL^{-1}. Consequently, methods to enrich and fractionate ferritin from the sample matrix along with sensitive analytical instruments are required in order to quantify iron loading of ferritin.

Within this presented work, a method for the off-line enrichment and fractionation for ferritin by centrifugal ultrafiltration was validated. This methodology was applied to samples from animal models such as fetal calf serum and pig brain as well as for the separation of free iron from the ^{57}Fe-isotopically enriched ferritin spike. Therefore, membrane filters consisting of regenerated cellulose and polyethersulfone were investigated. The cut-off value of the filters accounted to 100 kDa and 300 kDa. Thus, ferritin should be retained in the filter, while smaller metal containing proteins such as transferrin (80 kDa) and albumin (66 kDa) should be recovered in the filtrate. For the separation into a protein and small molecule fraction regenerated cellulose filter with a cut-off value of 3 kDa were used. Thus, the metal containing proteins such as ferritin, transferrin and albumin should be retained in the filter, while molecules with a molecular weight of below 3 kDa should be recovered in the filtrate. Subsequently, the samples before and after fractionation were analyzed by flow injection-size exclusion chromatography-inductively coupled plasma quadrupole mass spectrometry (FI-SEC-ICP-QMS). Iron and sulphur were quantified via external calibration using well characterized ferritin standards.

The proposed method using regenerated cellulose filter with a cut-off value of 100 kDa is fit for purpose for the enrichment and quantification of Fe and S in ferritin. However, polyethersulfone filters with a cut-off value of 300 kDa are not suitable for the enrichment of ferritin. Neither filters with a cut-off value of 100 kDa nor of 300 kDa are suitable for the fractionation of ferritin from transferrin and albumin. The proposed method using regenerated cellulose filters with a cut-off value of 3 kDa is fit for fractionation of synthetic and fetal calf serum into a protein and a small molecule fraction. In contrast, the method is not useful for fractionation of pig brain. Furthermore, the method using regenerated cellulose filters with a cut-off value of 100 kDa is fit for separation of free iron from the ^{57}Fe-isotopically enriched ferritin spike.

Zusammenfassung

Ferritin ist ein Eisenspeicherprotein mit einem Molekulargewicht von etwa 450 kDa. Es wird vermutet, dass das an Ferritin gebundene Eisen bei der Entstehung von Plaques im Gehirn von Patienten mit Alzheimer Erkrankung eine Rolle spielt. Die Konzentration von Ferritin im Blutserum beträgt in etwa 100 ng mL^{-1}. Infolgedessen, sind Methoden für die Anreicherung und Fraktionierung von Ferritin aus der Probenmatrix mit empfindlichen analytischen Instrumenten nötig, um die Eisenbeladung in Ferritin zu quantifizieren.

Im Zuge dieser Arbeit wurde eine Methode für die Anreicherung und Fraktionierung für Ferritin mittels zentrifugaler Ultrafiltration validiert. Diese Methode wurde an Proben tierischen Ursprungs wie etwa fetales Kälberserum und Schweinehirn als auch für die Trennung von freiem Eisen von einem mit Eisen-57 angereicherten Ferritins verwendet. Deshalb wurden Membranfilter aus regenerierter Zellulose und Polyethersulfon untersucht. Die Molekulargewichtsgrenze der Filter betrug 100 kDa und 300 kDa. Somit soll Ferritin im Filter zurückgehalten werden, während kleinere metallhältige Proteine wie etwa Transferrin (80 kDa) und Albumin (60 kDa) im Filtrat wiedergefunden werden sollen. Für die Trennung in eine Protein- und kleine Molekülfraktion wurden 3 kDa Filter aus regenerierter Zellulose verwendet. Somit sollen Ferritin, Transferrin und Albumin im Filter zurückgehalten werden, während Moleküle mit einem Molekulargewicht von unter 3 kDa im Filtrat wiedergefunden werden sollen. Anschließend wurden die Proben vor und nach der Fraktionierung mittels Fließinjektion-Größenauschlusschromatographie gekoppelt an Massenspektrometrie mit induktiv gekoppeltem Plasma analysiert. Eisen und Schwefel wurden mittels externer Kalibrierung unter der Verwendung von gut charakterisierten Ferritinstandards quantifiziert.

Die vorgeschlagene Methode unter der Verwendung von 100 kDa regenerierter Zellulose Filtern ist für die Anreicherung und Quantifizierung von Fe und S in Ferritin geeignet. Jedoch sind 300 kDa Polyethersulfon Filter nicht für die Anreicherung von Ferritin geeignet. Weder 100 kDa noch 300 kDa Filter sind für die Fraktionierung von Ferritin von Transferrin und Albumin geeignet. 3 kDa regenerierte Zellulose Filter sind geeignet für die Fraktionierung von synthetischem Serum und fetalem Kälberserum in eine Protein-und kleine Molekülfraktion. Dagegen ist die Methode nicht geeignet für die Fraktionierung von Schweinehirn. Des Weiteren ist die Methode unter der Verwendung von 100 kDa regenerierter Zellulose Filtern geeignet für die Trennung von freiem Eisen von einem mit Eisen-57 angereicherten Ferritins.

Table of Contents

Abstract I

Zusammenfassung II

Table of Contents III

Acknowledgements VI

Abbreviations VII

1 Objectives 1

2 Introduction 2

2.1 The demand of reference measurement procedures for clinically relevant metalloproteins 2

2.2 Role of ferritin in human iron metabolism 3

2.3 Iron incorporation into ferritin 6

2.4 Quantification strategies used for ferritin and ferritin-bound iron 6

2.5 Inductively coupled plasma mass spectrometry 11

2.5.1 The measurement of iron and sulphur 12

2.5.2 Quantification 13

2.6 Size exclusion chromatography 13

2.7 Filter 14

2.8 Hypothesis 15

3 Experimental 17

3.1 Chemicals and standards 17

3.2 Samples 18

3.3 Instrumentation 18

3.4 Centrifugal ultrafiltration 20

3.4.1 Method adaptation and validation 20

3.4.2 Application of the methodology to biologically relevant matrixes 21

3.5 Washing, drying and conditioning of filters 21

3.6 Fractionation by centrifugal ultrafiltration 21

3.7 Removal of residual $^{57}FeCl_2$ by centrifugal ultrafiltration 22

4 Results and discussion 24

4.1 Size ladder 24

4.2 The enrichment of ferritin by centrifugal ultrafiltration 27

4.2.1 Effect of washing and drying of filters on the procedural limit of quantification 27

4.2.2 Effect of washing, drying and conditioning of filters on the enrichment of ferritin 30

4.2.3 Comparison of the enrichment of ferritin using regenerated cellulose and polyethersulfone filters 50

4.3 The fractionation of ferritin from transferrin and albumin by centrifugal ultrafiltration 55

4.4 The fractionation of a synthetic serum by centrifugal ultrafiltration 60

4.5 Application of the methodology to fetal calf serum and pig brain 64

4.6 Application of the methodology for the separation of free iron from the ^{57}Fe-isotopically enriched ferritin spike 71

5 Summary and Conclusion 74

6 Outlook 75

7 References 77

8 Appendix 83

A. Standard Operating Procedure 83

B. Report 91

C. Abstract of poster 102

D. List of figures 103

E. List of tables 107

Acknowledgements

First of all, I would like to express my sincere gratitude to Gunda Köllensperger for her supervision and the opportunities she gave me to be involved in such an exciting project and to improve my skills.

I would like to extend my gratitude to Anastassiya Tchaikovsky for her kind support and constant guidance to successfully complete my thesis.

Furthermore, I would like to express my special thanks to Sophie Neumayer, Anna Schöberl and Luis Galvez for all their support regarding the instrumentation and the explanations how to behave in a clean room.

Additionally, I am very grateful to the other members in the working group for the nice atmosphere.

Last but not least I want to thank my parents and friends for her vital support and encouragement in challenging situations.

Abbreviations

Aß	beta-amyloid peptide
AD	Alzheimer´s disease
Ar	Argon
CE	Capillary electrophoresis
CH_3COONH_4	Ammonium acetate
CRC	Collision-reaction cell
CSF	Cerebrospinal fluid
ECLIA	Electrochemiluminescence immunoassay
ELISA	Enzyme-linked immunoadsorbent assay
ESI-MS	Electrospray ionization-mass spectrometry
FBI	Ferritin-bound iron
FCS	Fetal calf serum
Fe	Iron
Fe_2O_3*0.5 H_2O	Ferrihydrite mineral
FI-ICP-QMS	Flow injection-inductively coupled plasma-quadrupole mass spectrometry
GE	Gel electrophoresis
GFC	Gel filtration chromatography
Gln	Glutamin
Glu	Glutamic acid
GPC	Gel permeation chromatography
H	Heavy
H_2	Hydrogen
H_2O_2	Hydrogen peroxide
He	Helium
His	Histidine

HMF	High molecular weight fraction
HNO_3	Nitric acid
HPLC	High pressure liquid chromatography
HPLC-SEC-ICP-QMS	High pressure liquid chromatography-size exclusion chromatography-inductively coupled plasma-quadrupole mass spectrometry
IAC	Immunoaffinity chromatography
ICP-MS	Inductively coupled plasma-mass spectrometry
ICP-QMS	Inductively coupled plasma-quadrupole mass spectrometry
ICS	Ion Chromatography system
IF	Isoelectric focusing
ISO	International Organization for Standardization
IVD	In vitro diagnostic medical devices
kDa	Kilo Dalton
L	Light
LC-ESI-MS	Liquid chromatography-electrospray ionization-mass spectrometry
LMF	Low molecular weight fraction
LOQ	Limit of quantification
MALDI-MS	Matrix-assisted laser desorption ionization-mass spectrometry
MALDI-TOF-MS	Matrix-assisted laser desorption ionization-time of flight-mass spectrometry
MS	Mass spectrometry
m/z	mass-to-charge ratio
NH_3	Ammonia
NTI-MS	Negative thermal ionization-mass spectrometry
O_2	Oxygen
OH*	Hydroxyl radical

RBC	Red blood cell
PEEK	Polyether ether ketone
PFA	Perfluoroalkoxy
ROS	Reactive oxygen species
RSD	Relative standard deviation
RT	Retention time
S	Sulphur
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEC	Size exclusion chromatography
SEC-ICP-QMS	Size exclusion chromatography-inductively coupled plasma-quadrupole mass spectrometry
SELDI-MS	Surface enhanced laser desorption ionization-mass spectrometry
SELDI-TOF-MS	Surface enhanced laser desorption ionization-time of flight-mass spectrometry
SF-ICP-MS	Sector field-inductively coupled plasma-mass spectrometry
SI	International System of Units
SOP	Standard Operating Procedure
TBS	TRIS-buffered saline
TOF	Time of flight
TRIS	Tris(hydroxymethyl)aminomethane
Tyr	Tyrosine
USA	United States of America
2-DGE	Two-dimensional gel electrophoresis

1 Objectives

In this study a method for the off-line enrichment and fractionation for ferritin by centrifugal ultrafiltration was validated. This methodology was applied to samples from animal models such as fetal calf serum and pig brain. Furthermore, this method was applied for the separation of free iron from the ^{57}Fe-isotopically enriched ferritin spike.

2 Introduction

2.1 The demand of reference measurement procedures for clinically relevant metalloproteins

Metalloproteins which cover around 30 % of the whole proteome have become important in clinical diagnosis because they are often considered as important biomarker for distinguishing of diseased patients from healthy ones. For proteins which are routinely analysed in clinical laboratories, the comparability and reliability of the results obtained with different measurement procedures in different laboratories still poses a problem leading to a considerable number of misdiagnoses [20, 22]. Measurement methods usually used in routine laboratories are mainly immunoassays such as enzyme-linked immunoadsorbent assay (ELISA), immunoturbidimetry and immunonephelometry [20]. However, due to a lack of selectivity of antibodies used for such assays, these methods do not qualify as reference measurement procedures. For most clinically relevant metalloproteins neither reference measurement procedures nor a reference material exists [20]. Therefore, an establishment of a reference system including such primary reference measurement procedures for these biomarkers with results traceable to the International System of Units (SI) will ensure reliable and comparable results [20, 22]. The EC-directive (In vitro diagnostic medical devices (IVD) directive 98/79/EC) which covers all in vitro medical devices demands to assure “the traceability of the values assigned to calibrators and control materials […] through reference measurement procedures and/or available reference materials of higher order” [20]. Another important standard is the International Organization for Standardization (ISO) 17511:2003 which demands reference measurement systems including reference measurement procedures for the determination of analytes in samples of human origin [20, 25].

2.2 Role of ferritin in human iron metabolism

Iron (Fe) is essential for life because it plays an important role in maintaining several physiological functions in all living organisms such as oxygen transport, mitochondrial respiration, cell growth and differentiation [2, 9]. In the brain, iron is important for maintaining the metabolic requirements of neuronal tissues and is involved in myelin and neurotransmitter synthesis. The biological function of iron is the redox potential allowing the transition of ferrous iron (Fe^{2+}) to the ferric (Fe^{3+}) state [9]. Under physiological conditions iron is bound within metalloproteins. However, iron is toxic in its free form and in excess amounts [2, 9]. Fe^{2+} promotes the formation of highly reactive oxygen species (ROS) which can cause DNA and protein damage, lipid peroxidation and cellular death. The most common pathway is the Fenton reaction, in which hydroxyl radicals (OH*) are produced by the reaction of hydrogen peroxide (H_2O_2) and ferrous iron ($H_2O_2 + Fe^{2+} \rightarrow OH^* + Fe^{3+} + OH^-$) [6, 9].

The iron-containing proteins can be separated into three major classes. The first class includes haemoproteins or iron-protoporphyrins where iron is bound to a porphyrin ring structure like haemoglobin and myoglobin. Both proteins play an important role in the transport and storage of oxygen. The second class represents the iron-transport and storage proteins transferrin/lactoferrin and ferritin/haemosiderin which contain iron mostly as Fe^{3+}. The third class represents the iron-sulfur enzymes or ferroflavinic proteins like cytochrome C reductase, succinate dehydrogenase and xanthine oxidase which need iron for their biological function [2].

Figure 1 shows the iron metabolism in the human organism. The amount of total body iron in healthy adults is normally in the range between 3.5 and 5.0 g, which is mostly stored in the liver (1800 mg), in red blood cells (RBC; 1800 mg), in macrophages (600 mg), in bone marrow (300 mg) and in muscles (300 mg). Low amounts of iron can also be found in kidney and brain. The daily amount of iron that is absorbed from food by intestinal uptake in the form of ferrous and heme iron is around 1-2 mg per day. Around 1-2 mg of iron per day is lost through urine, feces, sweat, desquamation and menstruation [2, 9].

The plasma iron turnover is around 20 mg per day. About 65 % of the plasma iron which is bound to transferrin is transported to the bone marrow where the synthesis of red blood cells (erythropoiesis) takes place [2]. Due to the lack of an excretion system, most of the iron is

recycled from red blood cells in the spleen and bone marrow and the rest of iron is stored in ferritin and hemosiderin which is a degradation product of ferritin [2, 9].

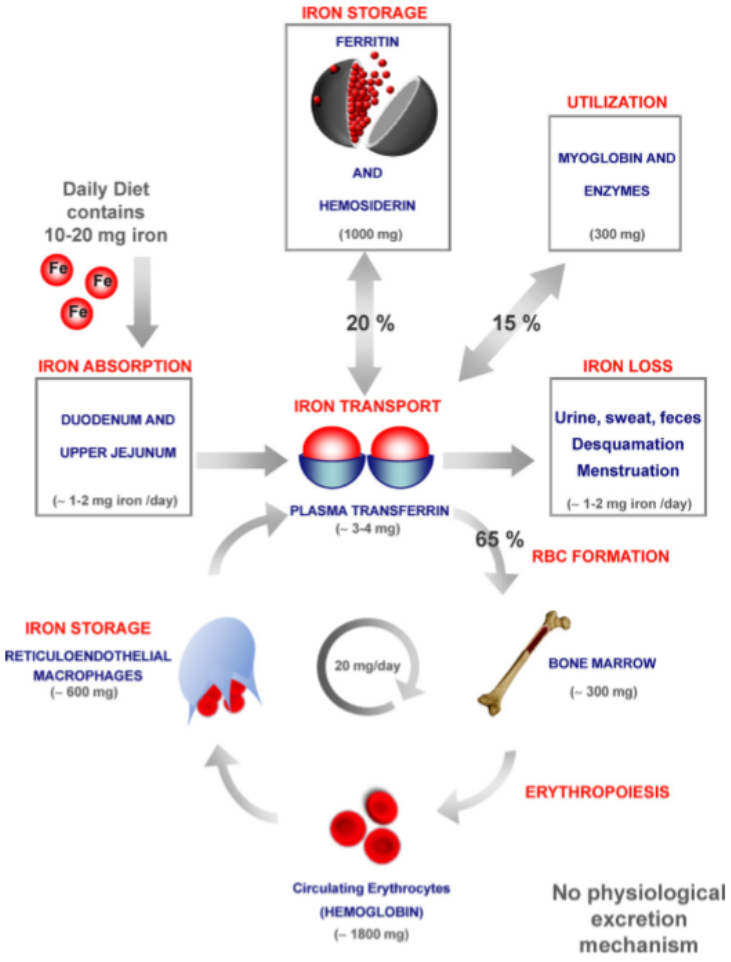

Figure 1 Iron metabolism in the human organism [2]

Ferritin is the predominant iron storage protein in all living organisms. Primary it is considered as an intracellular iron storage protein, but it is also present in extracellular compartments such as in serum and cerebrospinal fluid (CSF) [2, 9]. Ferritin is a hollow sphere protein (apo-ferritin) composed of 24 subunits of heavy chain ferritin (H-ferritin) and light chain ferritin (L-ferritin). It has a total protein molecular weight of 450 to 702 kDa depending on the iron filling grade of the shell. The apo-ferritin shell has an outer diameter of 12 nm and a cavity of 8 nm [7, 9, 24]. It can store up to 4500 iron atoms in a bioavailable and non-toxic form as a ferrihydrite mineral [$(Fe_2O_3*0.5\ H_2O)$] [7, 9, 15]. Ferritin is normally not completely saturated and contains around 2000 iron atoms [2]. While the L monomer contains 174 amino acids with

a molecular weight of 19 kDa, the H monomer has 183 amino acids with a molecular weight of 21 kDa [7, 15]. The proportion of the H-chain and L-chain varies widely depending on the tissue source and function and can be modified in inflammatory and infectious conditions [5, 24]. The H-subunit shows ferroxidase activity and is associated with the oxidation of Fe^{2+} to Fe^{3+} for uptake of iron into its cavity and is mainly found in heart and brain. Whereas the L-subunit is responsible for Fe^{3+} nuclei formation within the ferritin cavity and promotes long-term iron storage and is mainly present in iron storage organs like liver and spleen [2, 7, 16]. L-rich ferritins usually have a high iron content (1500 Fe atoms/molecule), whereas H-rich ferritins have relatively low iron contents (less than 1000 Fe atoms/molecule) [3]. Ferritin tends to form oligomers which are considered to be the first step in the conversion of ferritin to hemosiderin [23].

The amount of ferritin in serum of healthy adults varies from 15 to 300 ng mL^{-1}, but its concentration increases under inflammatory conditions including chronic kidney disease, rheumatoid arthritis and other autoimmune disorders [2, 8]. In the absence of inflammation and/or chronic disease, serum ferritin and ferritin-bound iron (FBI) are clinically considered as robust biomarkers in assessing body iron stores. Ferritin is a useful parameter for the evaluation of iron deficiency anaemia and diseases related to iron overload caused by hereditary hemochromatosis and chronic transfusion therapy [7, 8]. The concentration of ferritin-bound iron in serum is around 20 µg L^{-1} which makes its analysis a challenging task regarding instrument sensitivity [7]. Therefore, an enrichment and fractionation procedure for ferritin is needed.

Nowadays, ferritin (and FBI) is being recognized as an important molecule in some neurological disorders such as Alzheimer´s disease (AD) [16]. Roberts et al. reviewed that iron and other metals such as copper and zinc might contribute to Alzheimer´s disease. These metals are highly enriched within amyloid plaques in the brain of patients with Alzheimer´s disease and are known to cause oxidative damage through radical formation by inducing aggregation of the beta-amyloid peptide (Aß) which is the major constituent of plaques [9, 10].

2.3 Iron incorporation into ferritin

In mammals two types of small channels are formed between the H- and L-subunits being the 3-fold symmetry and 4-fold symmetry channels which are rising from the outside into the cavity of the ferritin protein [24]. The H-chain of ferritin has a ferroxidase centre of seven amino acids (Glu-27, Tyr34, Glu-61, Glu-62, His-65, Glu-107 and Gln-141) [14]. The catalytic ferroxidase center is located inside the 4-fold symmetry channel [13]. The sphere consisting of 24 subunits has eight hydrophilic pores along the three-fold symmetry axis and six hydrophobic and impermeable pores on the four-fold symmetry axis [7, 13]. Iron in the oxidation state of 2+ enters ferritin via the threefold hydrophilic channels and binds to the ferroxidase centre, where the oxidation of Fe^{2+} to Fe^{3+} takes place [3, 13]. During the oxidation process a peroxide forms which decays into H_2O_2. The oxidized iron (Fe^{3+}) then moves inside the cavity for hydrolysis and nucleation [3, 13]. Compared to the H-chain, the L subunit has no ferroxidase activity and therefore L-rich ferritins like those from liver and spleen are more efficient in iron nucleation and promote iron storage [4, 5, 9].

2.4 Quantification strategies used for ferritin and ferritin-bound iron

Ferritin is routinely measured in clinical laboratories because it is one of the most sensitive biomarkers for iron metabolic disorders [15]. **Table 1** shows a brief summary of the techniques used for the determination of ferritin and ferritin-bound iron.

Table 1 Techniques used for the determination of ferritin and ferritin-bound iron

Analytical technique	Main principle	References
immunochemical methods (enzyme-linked immunoadsorbent assay (ELISA), electrochemiluminescence immunoassay (ECLIA))	methods based on selective binding using antibodies specific for an analyte ELISA: uses an enzyme linked to an antibody or antigen as a marker for the detection of an analyte of interest ECLIA: uses the principle of chemiluminescence	[2, 15, 36]
matrix-assisted laser desorption ionization-time of flight-mass spectrometry (MALDI-TOF-MS)	sample within a matrix on a MALDI plate; laser-induced desorption and ionization of sample molecules; produced singly positively charged ions are detected by a TOF mass analyzer by measuring their mass-to-charge ratio	[28, 38]

surface-enhanced laser desorption ionization-time of flight-mass spectrometry (SELDI-TOF-MS)	protein chip array with a chemically pre-activated surface or a protein specific surface for selective binding of proteins; retained proteins on the surface are ionized and produced ions are detected by a TOF mass analyzer	[19, 32]
liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS)	the intact protein is nebulized into the ESI source and sample molecules are ionized by a capillary discharge producing charged analyte ions which are detected by a mass analyzer by measuring their mass-to-charge ratio	[2, 30, 40]
two-dimensional gel electrophoresis (in combination with mass spectrometry, e.g. MALDI-MS, SELDI-MS, LC-ESI-MS)	separation by charge by use of isoelectric focusing and molecular mass by use of sodium dodecyl sulphate polyacrylamide gel electrophoresis resulting in a distinct pattern of protein spots that can be identified using MS methods	[19, 29, 30, 31]
immunoaffinity chromatography (IAC; coupled with mass spectrometry)	type of liquid chromatography in which the stationary phase consists of an antibody or antibody-related agent which is used for the selective purification of a compound	[27, 33]
negative thermal ionization-mass spectrometry (NTI-MS)	thermal atomization and ionization of an element which is loaded as a sample solution on to a heated metal filament; by applying a voltage to the filament, the accelerated ions are separated in a magnetic field according to their mass-to-charge ratio; requires a trace-matrix separation before isotope ratio measurements	[7, 17, 34, 35]
high pressure liquid chromatography-size exclusion chromatography-inductively coupled plasma mass spectrometry (HPLC-SEC-ICP-MS)	separation of molecules by their molecular size and subsequent quantification of their atomic constituents using a high-temperature plasma discharge as an ionization source	[11, 16, 37, 39]

The techniques currently used in routine clinics for the determination of ferritin are immunochemical methods such as enzyme-linked immunoadsorbent assay (ELISA) and electrochemiluminescence immunoassay (ECLIA) [7, 15]. All these methods are based on

selective binding using antibodies specific for an analyte [44]. In the case of ELISA which uses an enzyme linked to an antibody, the detection of the target compound is accomplished by assessing the conjugated enzyme activity via the incubation with a substrat [36]. ECLIA uses the principle of chemiluminescence for measurement. This method uses two antibodies specific for ferritin, one of them biotinylated and the other one labeled with a ruthenium chelate. By using magnetic microparticles that enable the complex to bind to the surface of an electrode, an application of a voltage to the electrode leads to a chemiluminescent emission which is measured by a photomultiplier. The concentration of ferritin can be determined based on a calibration function using a reference ferritin standard of known concentration [15]. A main drawback of these immunochemical methods is the lack of selectivity and low antibody specificity which can lead to high differences in results and to a poor comparability [7, 20]. These assays only allow the determination of the ferritin protein but not the iron bound to ferritin which is variable due to the iron filling grade of the shell [7]. Therefore, these methods do not qualify as reference measurement procedures [20].

For establishing a reference measurement system, mass spectrometry (MS) combined with efficient chromatographic separation techniques such as high-pressure liquid chromatography (HPLC), size exclusion chromatography (SEC), affinity chromatography, capillary electrophoresis (CE) or gel electrophoresis (GE) is used [20, 22]. The method mainly used for the analysis of metalloproteins such as ferritin is molecular mass spectrometry including electrospray ionization-mass spectrometry (ESI-MS) and matrix-assisted laser desorption ionization-mass spectrometry (MALDI-MS) which are typically combined with different protein separation methods [19, 20]. Another relevant method in this field which is a variation of MALDI is surface enhanced laser desorption ionization-mass spectrometry (SELDI-MS). SELDI is a type of protein chip array which has a chemically preactivated surface (ionic, hydrophobic and chelating metal, etc.) or a surface specific for a protein (antibody) for the selective binding of proteins from complex matrixes. The proteins bound to the surface are then ionized and detected by a mass analyser [19]. In MALDI-MS the sample within a matrix is immobilized on a MALDI plate following by a laser induced desorption and ionization of sample molecules. The produced singly charged ions are separated by their mass-to-charge ratio (m/z) and measured by a mass analyser [38]. In ESI-MS the intact protein is nebulized into the ESI source. The sample molecules are ionized by a capillary discharge producing charged analyte ions which are detected by a mass analyzer by measuring their m/z [2, 40].

One of the most widely used mass analyzer is the time of flight (TOF) analyzer [19]. Prior to protein identification and determination, a high-resolution separation technique is required [37]. One of the most common approaches for the separation of proteins is two-dimensional gel electrophoresis (2-DGE) due to its increased sensitivity and high resolving power [19]. 2-DGE separates proteins according to charge and molecular mass by using isoelectric focusing (IF) and sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) resulting in a distinct pattern of protein spots which can be analysed by MS techniques such as MALDI-MS, SELDI-MS, SELDI-TOF-MS and LC-ESI-MS [19, 29, 30, 31, 32].

Most of these methods are qualitative (allow the identification of proteins) and provide quantitative information of a biomarker [2, 19]. However, main drawbacks of molecular MS are the poor linearity for calibration and lack of available standards for quantification. Furthermore, in case of metalloproteins which contain the metal in a non-covalently bound form, ionization sources such as MALDI and ESI lead to the destruction of the metal-bound protein complex [20]. In case of SELDI, main drawbacks are the reproducibility and that proteins with a high molecular weight are analyzed less efficiently [19].

Immunoaffinity chromatography (IAC) is a powerful technique for the selective isolation of a given compound from complex samples such as biological samples [33]. Passaniti and Roth for example used immunoaffinity chromatography for the purification of ferritin from chicken liver [27]. For further quantification of the target compound it can be coupled with analytical techniques such as mass spectrometry. IAC is a type of liquid chromatography in which the stationary phase consists of an antibody or antibody-related agent which is used for the selective purification of a target compound. The elution of the target analyte is often carried out by lowering the effective strength of antibody binding to the target for example by changing the pH value of the mobile phase [33]. However, the main drawback of this method is the choose of appropriate buffer solutions for an efficient binding of the desired analyte to the immobilized antibody as well as for an optimum elution of the target compound [33]. Equal to immunochemical techniques, lack of selectivity and low antibody specificity are highly problematic.

The methods described in literature for the determination of ferritin-bound-iron are negative thermal ionization-mass spectrometry (NTI-MS) and inductively coupled plasma-mass spectrometry (ICP-MS) [7, 16]. Ren and Walczyk for example used NTI-MS for the iron isotope

analysis of ferritin in human serum [7]. The principle of NTI-MS is based on the thermal atomization and ionization of an element which is loaded as a sample solution on to a heated metal filament. By applying an acceleration voltage to the filament, the accelerated ions are separated in a magnetic field according to their m/z which is detected by a multicollector system [34, 35]. The main advantage of TIMS compared to ICP-MS is that only few interferences occur. A reason for that is that the ionization is thermally and that ions such as Ar^+ or O^- are not used. Furthermore, the target element is already separated from matrix elements by a chemical procedure [17, 34]. However, a major drawback of NTI-MS is that it is not able to measure directly an element in a matrix. Therefore, it requires extended sample preparation procedures such as a trace-matrix separation by chemical separation techniques such as ion exchange chromatography before isotope ratio measurements [7, 17].

Inductively coupled plasma-mass spectrometry (ICP-MS) is considered as the state-of-the-art hyphenated detection technique for the quantification of elements and elemental species (e.g. metalloproteins) in complex biological samples [2, 11]. Konz, Montes- Bayón and Sanz-Medel for example used HPLC-SEC-ICP-MS for the quantification of ferritin-bound iron in human serum [16]. The principle of ICP-MS is described in more detail in the following chapter. For absolute protein quantification by ICP-MS, the heteroelement sulphur plays an important role. A reason for that is that sulphur which is incorporated into methionine and cysteine is almost present in all natural proteins [18]. The main advantages of ICP-MS are its versatility and easy coupling to chromatographic separation techniques such as HPLC and SEC as well as its capability of highly sensitive and isotope-specific analysis of metalloproteins [2, 11, 37]. In comparison to NTI-MS, ICP-MS is a faster technique because it requires no trace-matrix separation. However, due to no matrix separation interferences occur frequently [17].

2.5 Inductively coupled plasma mass spectrometry

Introduced in the 1980s, inductively coupled plasma-mass spectrometry (ICP-MS) is the state-of-the-art hyphenated element-specific detection technique which offers extremely low detection limits in the sub ng L^{-1} range up to high mg L^{-1} levels covering a wide dynamic detection range [11, 39].

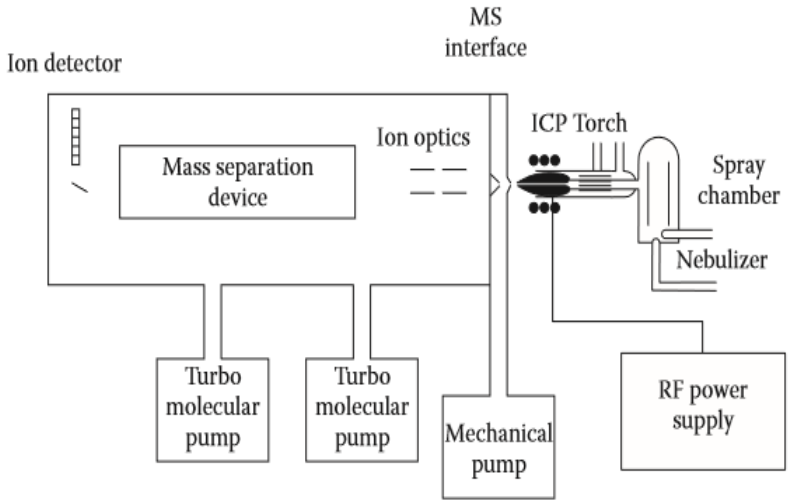

Figure 2 Graphical representation of the instrumental components of an ICP-MS spectrometer [39]

Figure 2 shows the basic components of an ICP-MS spectrometer such as a spray chamber, nebulizer, plasma torch, mass separation device and detector. The sample is usually in liquid form and is pumped with a peristaltic pump into a nebulizer, where it is converted with argon gas into a fine aerosol. In the spray chamber the fine droplets of the aerosol, which represent only 1-2% of the sample, are separated from larger droplets. The fine aerosol is then transported via a sample injector into the plasma torch. The plasma torch consists of three concentric quartz tubes, an outer tube, a middle tube and a sample injector. The argon gas which is used for the formation of the plasma is passed between the outer and middle tube, whereas the auxiliary gas passes between the middle tube and the sample injector. The nebulizer gas transports the fine aerosol through the sample injector. The plasma is formed by the interaction of a magnetic field (produced by radio frequency passing through a copper coil) in combination with argon gas flow through the plasma torch. This induces the ionization of the argon gas leading to the formation of a very-high-temperature plasma discharge (at

about 6000-10 000 Kelvin) mainly consisting of singly, positively charged ions. This plasma discharge serves as an ionization source which leads to desolvation, vaporization, atomization and ionization of sample constituents. The generation of positively charged analyte ions by the plasma discharge is based on the excitation of an outer electron of a ground-state atom. After the production of the analyte ions in the plasma which is at atmospheric pressure (760 torr), they are directed into the mass spectrometer via the interface region. The interface region is maintained at vacuum of 1-2 torr with a mechanical pump and consists of two metallic cones. These cones are called the sampler and skimmer cone and are usually made from copper, nickel or platinum and have a small orifice in the range of 0.6-1.2 mm. After the extraction of ions from the interface region, they are directed into the main vacuum chamber (maintained at about 10^{-3} torr with a turbomolecular pump) by electrostatic lens which are also called ion optics. The main function of these ion optics is to focus the ion beam towards the mass separation device which is kept at vacuum of about 10^{-6} torr with a second turbomolecular pump. The most common mass separation devices are quadrupole, magnetic sector and time-of-flight analyzer. These mass separation devices allow the analyte ions of a particular mass-to-charge ratio to pass through to the detector and to filter out interfering ions. In the end the ions are converted into an electrical signal by an ion detector such as a discrete dynode detector [11, 34, 39].

2.5.1 The measurement of iron and sulphur

ICP-MS was chosen to be the best approach for the measurement of iron and sulphur. However, the major drawbacks of ICP-MS are spectral interferences that can be generated by the conditions in the Argon plasma gas hampering the determination of many elements. The interferences that occur most frequently and are most problematic are argon-based polyatomic ions such as $^{40}Ar^{35}Cl^+$ and $ArOH^+$. Being the elements of interest, iron (^{56}Fe) mainly suffers from interferences such as $^{40}Ar^{16}O^+$, whereas sulphur (^{32}S) mainly suffers from O-based polyatomic ions such as $^{16}O_2^+$. For overcoming of spectral interferences, a high-resolution spectrometer such as sector-field ICP-MS (SF-ICP-MS) or a collision-reaction cell (CRC) system can be used. Apart from that, quadrupole-based ICP-MS (ICP-QMS) with a CRC system enables various options to avoid spectral interferences. Despite offering different reaction gases including hydrogen (H_2), helium (He), ammonia (NH_3) and oxygen (O_2), it also includes

different approaches such as the on-mass and mass-shift approach. In the mass-shift approach the gas reacts with the analyte ion leading to a product ion that can be measured free from any interference at another m/z. One of the most frequently used reaction gases in ICP-QMS is oxygen which has a mass shift of +16 because many elements react with O_2. The mass-shift approach provides more versatility to avoid spectral interferences [1, 17]. Therefore, the mass-shift approach using O_2 as reaction gas was chosen for all measurements of iron and sulphur. The isotopes of iron (^{54}Fe, ^{56}Fe, ^{57}Fe and ^{58}Fe) and sulphur (^{32}S) were measured as shifted species ($^{54}Fe^{16}O$, $^{56}Fe^{16}O$, $^{57}Fe^{16}O$, $^{58}Fe^{16}O$and $^{32}S^{16}O$).

2.5.2 Quantification

The iron and sulphur elemental content in all samples before and after centrifugal ultrafiltration in the low (i.e. filtrate, LMF) and high molecular weight fraction (i.e. retentate, HMF) was determined via external calibration using Fe-S standards by flow injection-inductively coupled plasma-quadrupole mass spectrometry (FI-ICP-QMS).

2.6 Size exclusion chromatography

Size exclusion chromatography (SEC) is considered as the most frequently used separation method [12]. Size exclusion chromatography is a type of liquid chromatography that separates molecules according to size. The principle of SEC by the example of a solution consisting of two sizes of molecules is shown in **figure 3**. The sample solution is injected onto the column which is packed with porous particles with a defined pore size that serves as a stationary phase. The sample is then transported through the column by a mobile phase. The distribution according to size is based on the exchange of sample molecules between the bulk solvent of the mobile phase and the liquid phase within the pores of the packing material. The molecular size range within the separation takes place is determined by the pore size of the packing material. Depending on the type of the mobile phase, SEC is also called gel permeation chromatography (GPC) and gel filtration chromatography (GFC). GPC uses organic solvents as mobile phase, whereas in GFC usually aqueous solvents are employed. While the smaller molecules penetrate the pores of the packing material and pass through the column, larger molecules are not able to reside inside the pores and remain in the interstitial space.

Therefore, these molecules flow more rapidly through the column than smaller ones, thus high molecular weight components elute first from the SEC column. For each molecule whose size is proportional to the concentration a SEC chromatogram in form of a signal peak is obtained [41, 42, 43].

The iron and sulphur elemental content in ferritin was determined via external calibration using well characterized ferritin standards by size exclusion chromatography-inductively coupled plasma-quadrupole mass spectrometry (SEC-ICP-QMS). The protein pattern of the original sample as well as the low and high molecular weight fraction was determined by SEC-ICP-QMS.

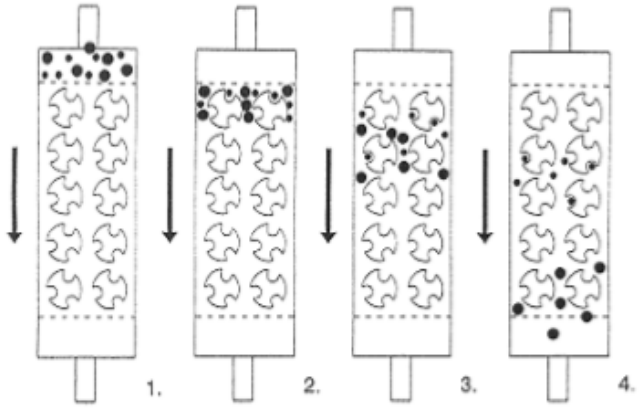

Figure 3 The principle of size exclusion chromatography [43]

2.7 Filter

Based on the papers from Konz et al. and Ren and Walczyk two different filter materials consisting of polyethersulfone (cut-off value of 300 kDa; Sartorius Stedim Biotech GmbH, Germany) and regenerated cellulose (cut-off value of 100 kDa; UFC5100 Amicon Ultra-0.5 mL Centrifugal Filter Device; Wagner and Munz GmbH Austria, Germany) were used [7, 15]. The cut-off value of the filters accounted to 100 kDa and 300 kDa. Thus, ferritin which has a molecular weight of 450 kDa should be retained in the filter, while smaller metal containing proteins such as transferrin (80 kDa) and albumin (66 kDa) should be recovered in the low molecular weight fraction [37].

For the separation into a protein and a small molecule fraction, regenerated cellulose filters with a cut-off value of 3 kDa (UFC5003 Amicon Ultra-0.5 mL Centrifugal Filter Device; Wagner

and Munz GmbH Austria, Germany) were used. The cut-off value of the filters is chosen to be well below the molecular weight of ferritin, transferrin and albumin. This metal containing proteins should be recovered in the high molecular weight fraction, while molecules with a molecular weight of below 3 kDa should be recovered in the low molecular weight fraction.

2.8 Hypothesis

Centrifugal ultrafiltration using membrane filters should allow an enrichment of ferritin as well as a fractionation of ferritin from other metal containing biomolecules such as transferrin and albumin. This hypothesis is based on the papers from Konz et al. and Ren and Walczyk who dealed with the analysis of ferritin-bound iron in human serum [7, 15]. As reviewed by Ren and Walczyk, ferritin-bound iron is present in serum at trace levels (nanograms per milliliter) which makes its analysis a challenge in terms of instrument sensitivity [7]. Therefore, an enrichment of ferritin was needed which was performed by centrifugal ultrafiltration using membrane filters.

3 Experimental

3.1 Chemicals and standards

Nitric acid (HNO_3, 69% *w/w,* Rotipuran Supra), hydrochloric acid (35% *w/w*, Rotipuran Supra) and acetic acid (100% *w/w*, suprapure) were achieved from Carl Roth (Germany). Ammoniac (25% *w/w*, suprapure) was achieved from Avantor (United States of America (USA)). An iron single-element standard (LK1-00260201, 1000 mg L^{-1} ± 0.3% in 2% HNO_3) and a sulphur single-element standard (LK1-00162101, Lot No. 1103036, 1000 mg L^{-1} ± 0.3% in 2% HNO_3) were achieved from LabKings B.V. (Netherlands). A ferritin standard from equine spleen (F4603-100MG, Lot No. SLBM0474V, 54 mg mL^{-1} ferritin; F4503-100MG, Lot No. SLBW3977, 61 mg mL^{-1} ferritin), a transferrin standard from human blood plasma (90190-100MG, Lot No. BCBJ0304, ≤ 95% grade) and an albumin standard from human serum (lyophilized powder, essentially fatty acid free, A3782-10G, CAS No. 70024-90-7, ≤ 97% grade) were achieved from Sigma-Aldrich (United States of America). Tris(hydroxymethyl)aminomethane (TRIS, 121.14 g mol^{-1}, 648311, CAS 77-86-1, ≤ 99% ultrol grade), sodium chloride (58.44 g mol^{-1}, CAS 7647-14-5, ≤ 99.5% purity), hydrochloric acid (36.46 g mol^{-1}, 258148-M, CAS 7647-01-0, ACS reagent 37%) and polyethylene glycol sorbitan monolaurate (Tween 20 BioXtra, viscous liquid, P7949, CAS 9005-64-5) were achieved from Merck (KGaA, Germany). For mobile phase and buffer preparation only suprapure chemicals were used. Laboratory water type I (18 MΩ cm, Veolia Water Solutions, France) was used for all dilutions, for buffer preparation and for rinsing the filter. An ammonium acetate buffer (CH_3COONH_4) 50 mM pH 6.8 was prepared from laboratory water type I (18 MΩ cm, Veolia Water Solutions and Technologies, France), ammoniac (25% *w/w*, suprapure, Avantor, USA) and acetic acid (100% *w/w*, suprapure, Carl Roth, Germany) and used as solvent and mobile phase. A 5% HNO_3 solution was prepared from nitric acid (69% *w/w,* Rotipuran Supra) and laboratory water type I (18 MΩ cm, Veolia Water Solutions and Technologies, France) and used for rinsing the filter. All laboratory consumables (polyethylene bottles, tubes, pipette tips) were double acid washed with nitric acid (10% and 1% HNO_3 *w/w*) before use. For the size ladder a ferritin standard from equine spleen (F4503-100MG, Lot No. SLBW3977, 61 mg mL^{-1} ferritin), a transferrin standard from human blood plasma (90190-100MG, Lot No. BCBJ0304, ≤ 95% grade) and an albumin standard from human serum (lyophilized powder, essentially fatty acid free , A3782-10G, CAS No. 70024-90-7, ≤ 97% grade) were used (all achieved from Sigma-Aldrich, USA). For the size ladder the standards

were diluted gravimetrically in 50 mM ammonium acetate to a final concentration of 20 µg mL^{-1} ferritin, 10 mg mL^{-1} transferrin and 1 mg mL^{-1} albumin.

3.2 Samples

A fetal calf serum (FCS) was obtained from Merck KGaA (Germany). FCS was delivered frozen, thawed and stored at 4 – 8 °C before use. A fresh pig brain was achieved from butcher´s Ringl (Austria) and was immediately kept on ice. During the whole sample preparation procedure, the pig brain and all brain samples prepared from it were kept on ice. The extraction of cytosolic proteins from pig brain using TBS buffer (TRIS-buffered saline) was conducted out following the Standard Operating Procedure (SOP) of Raab [45].

3.3 Instrumentation

Preparatory work and elemental analysis were performed at the University of Vienna, Department of Analytical Chemistry in an ISO class 8 clean room according to ISO 14644-1 for a low iron and sulphur background and to avoid any contamination. All measurements were performed with an Agilent 8800 ICP-QMS (Agilent Technologies, USA) (operated in reaction mode (30% O_2)) hyphenated to a 1260 Infinity Bioinert High Pressure Liquid Chromatography system (Agilent Technologies, Germany) or to a Dionex ICS-5000+ Ion Chromatography system (Thermo Fisher Scientific, USA). The sample introduction system consisted of a glass nebulizer (MicroMist, 400 µL min^{-1} sample uptake, AHF Analysentechnik AG, Germany) or a perfluoroalkoxy nebulizer (PFA-ST Micro-Flow, 20, 50, 100, 200 and 400 µL min^{-1} self-aspiration, Elemental Scientific Inc., USA) and a Scott double-pass spray chamber (Agilent Technologies, Germany). In order to achieve maximum intensity the instrument was tuned daily based on the $^{59}Co^{+}$, $^{89}Y^{16}O^{+}$ and $^{205}Tl^{+}$ signal, low oxide formation ($^{140}Ce^{16}O^{+}/^{140}Ce^{+}$ below 1.5%) and a doubly charged ratio of Ce^{2+}/Ce^{+} (70/140) below 2.5%. The used cones consisted of nickel. The integration time for each registered isotope accounted to 0.1 seconds. The sample volume accounted to 5 µL and the flow rate to 200 µL min^{-1} or 250 µL min^{-1}. The run time for flow injection was set to 1.5 minutes per single run, whereas the run time for size exclusion was set to 15 min per single run. Ammonium acetate 50 mM (pH 6.8) was employed as mobile phase. The isotopes of iron and sulphur were registered as shifted species ($^{54}Fe^{16}O$,

$^{56}Fe^{16}O$, $^{57}Fe^{16}O$, $^{58}Fe^{16}O$ and $^{32}S^{16}O$). The separation of ferritin from other iron containing biomolecules such as transferrin and albumin was performed with size exclusion chromatography using a MabPac SEC column (Thermo Fisher Scientific, USA), as it is the most frequently used separation method [12]. The instrumental acquisition parameters were based on Theiner et al. [21]. The instrumental conditions are summarized in **Table 2.**

Table 2 ICP-MS operation and chromatographic conditions used

ICP RF power [W]	1550
Cones	Ni
Plasma gas flow [L min^{-1}]	5
Auxiliary gas flow [L min^{-1}]	15
Nebulizer gas flow [L min^{-1}]	0.90
Reaction gas [L min^{-1}]	1.07
Nebulizer	MicroFlow PFA-ST/MicroMist
Spray chamber	Scott double-pass
Sample introduction	flow injection
Flow rate [µL min^{-1}]	200, 250
Sample volume [µL]	5
Mobile phase	50 mM CH_3COONH_4, pH 6.8
Column	MabPac SEC, PEEK, 4 x 300 mm, 5 µm, 10 - 1000 kDa
Reaction gas	Oxygen (30 %)
Analytes	$^{32}S^{16}O$, $^{54}Fe^{16}O$, $^{56}Fe^{16}O$, $^{57}Fe^{16}O$, $^{58}Fe^{16}O$
Integration time	0.1 s
Autosampler temperature [°C]	4
Flow Injection run time [min]	1.5
Size Exclusion run time [min]	15

During the preparation of all samples and calibration standards piston pipette (0.10-10 µL, 10-100 µL, 100-1000 µL, 1000-10000 µL; Eppendorf, Germany), a microbalance CPA225D (Sartorius Weighing Technology GmbH, Germany) and a vortex mixer Vortex V-1 plus (Biosan SIA, Latvia) were used. To separate the precipitate and the supernatant solution as well as to fractionate the samples into a low and high molecular weight fraction a microliter centrifuge Z 216 MK (Hermle Labortechnik GmbH, Germany) was used. For the preparation of the ammonium acetate buffer (50 mM, pH 6.8) a pH meter 16461276 (SI analytics GmbH, Germany), a magnetic stirring bean (Labxperts, Austria) and a magnetic stirrer RSM-02HP (PHOENIX Instrument, Germany) were used.

3.4 Centrifugal ultrafiltration

3.4.1 Method adaptation and validation

The method for the off-line enrichment and fractionation for ferritin by centrifugal ultrafiltration was adapted to laboratory conditions. During the course of method validation, not washed and not dried filters as well as different washing procedures in combination with drying and not drying of the filters were tested regarding the limit of quantification (LOQ) of iron and sulphur. Additionally, not washed and not dried filters in combination with conditioning and not conditioning as well as different washing procedures with drying and not drying in combination with conditioning and not conditioning of filters were tested regarding the enrichment of ferritin. Furthermore, regenerated cellulose and polyethersulfone filters were compared with each other in terms of the enrichment of ferritin. Following filter preparation procedures were investigated:

1. not washed, not dried and not conditioned/conditioned with 500 µL 50 mM ammonium acetate pH (6.8) (regenerated cellulose and polyethersulfone filters)
2. washed (3 x with 500 µL 5% HNO_3 and 2 x with 500 µL ultrapure water, 1 x centrifuged with 500 µL ultrapure water (10 min, 15000 g)), not dried and conditioned with 500 µL 50 mM ammonium acetate pH (6.8) (regenerated cellulose and polyethersulfone filters)
3. washed (3 x with 500 µL 5% HNO_3, 1 x centrifuged with 500 µL ultrapure water (5 min, 15000 g), 1 x centrifuged without ultrapure water (5 min, 15000 g), 1 x centrifuged with 500 µL ultrapure water (10 min, 15000 g)), not dried and conditioned with 500 µL 50 mM ammonium acetate pH (6.8) (regenerated cellulose and polyethersulfone filters)
4. washed (3 x with 500 µL 5% HNO_3 and 3 x with 500 µL ultrapure water), dried (15 min) and conditioned with 500 µL 50 mM ammonium acetate pH (6.8) (regenerated cellulose and polyethersulfone filters)
5. washed (3 x with 500 µL 5% HNO_3 and 3 x with 500 µL ultrapure water), dried (1 hour) and not conditioned (regenerated cellulose and polyethersulfone filters)
6. washed (3 x with 500 µL 5% HNO_3 and 3 x with 500 µL ultrapure water), dried (1 hour) and conditioned with 500 µL 50 mM ammonium acetate pH (6.8) (regenerated cellulose and polyethersulfone filters)

3.4.2 Application of the methodology to biologically relevant matrixes

Serum and brain are important sources for identifying of biomarkers for example for neurodegenerative diseases [19]. While ferritin in serum serves as an important clinical marker for iron status, ferritin-bound iron plays a role in the formation of plaques found in the brain of patients with Alzheimer´s disease [5, 10]. Thus, the method for the fractionation by centrifugal ultrafiltration was applied to fetal calf serum and pig brain.

3.5 Washing, drying and conditioning of filters

For an optimum enrichment of ferritin and to obtain a low limit of quantification of iron and sulphur the filters were prepared according to the following procedure. The filters were washed 3 times with 500 µL 5% HNO_3 and 3 times with 500 µL laboratory water type I. After each washing step the remaining solution was removed from the filters by knocking 5-10 times. After the washing procedure the filters were dried at room temperature for about one hour. Subsequently, the filters were conditioned with 500 µL ammonium acetate buffer (50 mM pH 6.8) at 15000 g at 4 °C for 10 min (5 acc/dec). The filter was turned upside down into the same microcentrifuge tube and centrifuged for a second time at 1000 g at 4 °C for 2 min (5 acc/dec). The pooled fractions were used as procedural blank for the FI-ICP-QMS measurement.

3.6 Fractionation by centrifugal ultrafiltration

The fractionation was performed by centrifugal ultrafiltration. Therefore, regenerated cellulose filters with a cut-off value of 3 kDa and 100 kDa and polyethersulfone filters with a cut-off value 100 kDa and 300 kDa were used. For the fractionation of sample solutions containing only ferritin, only transferrin and only albumin regenerated cellulose filters with a cut-off value of 100 kDa and polyethersulfone filters with a cut-off value of 100 kDa and 300 kDa were used. Therefore, the ferritin, transferrin and albumin standards were diluted gravimetrically in 50 mM ammonium acetate to a final concentration of 10.8 µg mL^{-1} ferritin or 5.0 µg mL^{-1} ferritin, 1 mg mL^{-1} transferrin and 0.5 mg mL^{-1} albumin. For the fractionation of a synthetic serum regenerated cellulose filter with a cut-off value of 3 kDa were used. For preparing the synthetic serum, a ferritin, transferrin and albumin standard were used.

Therefore, the standards were diluted gravimetrically in 50 mM ammonium acetate to a final concentration of 10.8 µg mL^{-1} ferritin, 1 mg mL^{-1} transferrin and 0.4 mg mL^{-1} albumin shortly before fractionation. The fractionation of FCS was carried out using regenerated cellulose filters with a cut-off value of 3 kDa. The FCS sample was centrifuged at 15000 g at 4 °C for 10 min (5 acc/dec) to separate the precipitate and the supernatant solution. The supernatant FCS solution was diluted gravimetrically 1:5 using 50 mM ammonium acetate shortly before fractionation. The pig brain extracts obtained after the extraction procedure with TBS-buffer were used undiluted for the fractionation using regenerated cellulose filters with a cut-off value of 3 kDa.

Each sample (500 µL) was applied onto the washed, dried and conditioned filters and centrifuged at 15000 g at 4 °C for 10 min (5 acc/dec). The filtrate was collected as low molecular weight fraction. To obtain the high molecular weight fraction, the filter was placed upside down into a new microcentrifuge tube and centrifuged at 1000 g at 4 °C for 2 min (5 acc/dec). The original sample as well as the low and high molecular weight fraction were used for the FI- and SEC-ICP-QMS measurement.

3.7 Removal of residual $^{57}FeCl_2$ by centrifugal ultrafiltration

In order to remove the residual $^{57}FeCl_2$, the ^{57}Fe-isotopically enriched ferritin spike solution was subjected to centrifugal ultrafiltration using regenerated cellulose filters with a cut-off value of 100 kDa. 500 µL of the ^{57}Fe-ferritin spike solution was applied onto the washed, dried and conditioned filter and centrifuged at 15000 g at 4 °C for 10 min (5 acc/dec). Afterwards 400 µL of the ^{57}Fe-ferritin spike solution was applied a second time onto the filter and centrifuged two times at 15000 g at 4 °C for 10 min (5 acc/dec). For the last step, 400 µL of the ^{57}Fe ferritin spike solution was applied a third time onto the filter and centrifuged at 15000 g at 4 °C for 10 min (5 acc/dec). The purity of the ^{57}Fe-ferritin spike solution (1:20 diluted gravimetrically in 50 mM ammonium acetate pH 6.8) was confirmed by SEC-ICP-QMS.

4 Results and discussion

4.1 Size ladder

For size exclusion chromatography a size ladder was performed using ferritin (20 µg mL^{-1}), transferrin (10 mg mL^{-1}), and albumin (1 mg mL^{-1}) standards and was measured with SEC-ICP-QMS operated in reaction mode (30% O_2). The flow rate accounted to 200 µL min^{-1} and 250 µL min^{-1}. **Figure 4** and **5** show the SEC chromatogram of the size ladder measured at a flow rate of 200 µL min^{-1}. The iron trace in **figure 4** indicated that ferritin which has a molecular weight of 450 kDa showed predominantly a monomer peak at a retention time (RT) of 9.9 min and oligomer peaks at a retention time of 7.2-8.6 min. Transferrin which has a molecular weight of 80 kDa showed a monomer peak at a retention time of 12.0 min. The iron signal of albumin was too low, thus showing no monomer peak. The sulphur trace in **figure 5** indicated that transferrin and albumin (66 kDa) were predominantly present in monomer form at a retention time of 12.0 min and 11.8 min, but also showed oligomer peaks at a retention time of 10.5 min and 10.4 min. The retention time and molecular weight of the used markers for the size ladder are shown in **table 3**.

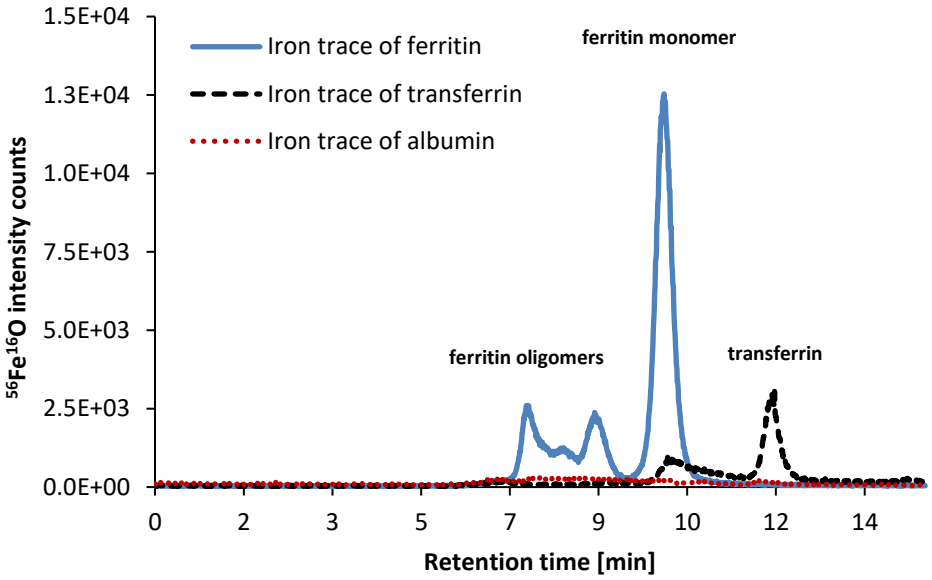

Figure 4 SEC chromatogram of a size ladder. Iron trace of ferritin (20 µg mL^{-1}, blue trace), transferrin (10 mg mL^{-1}, black trace) and albumin (1 mg mL^{-1}, red trace) standards in 50 mM ammonium acetate measured consecutively using an IC Dionex 5000 sample introduction system and a SEC MabPac column coupled to a Agilent 8800 ICP-QMS operated in reaction mode (30% oxygen).

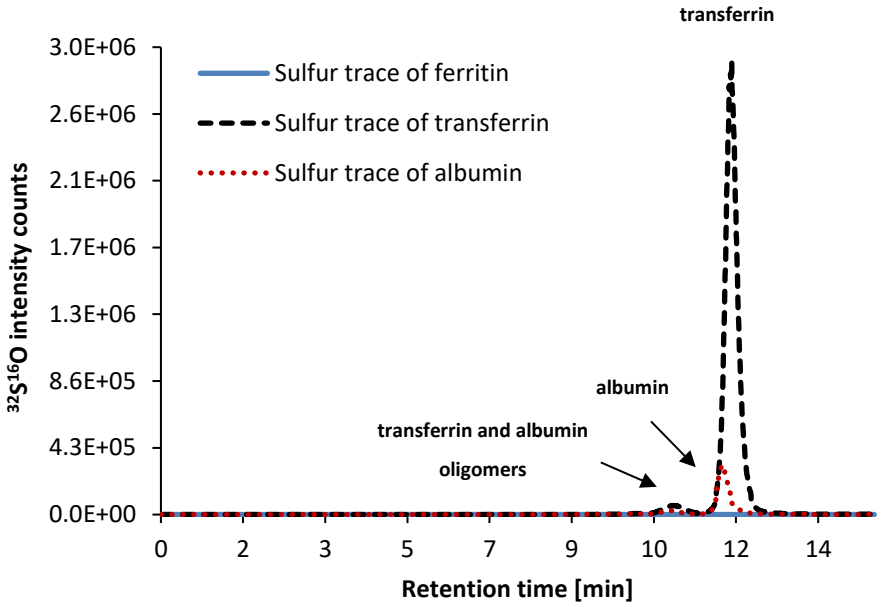

Figure 5 SEC chromatogram of a size ladder. Sulphur trace of ferritin (20 µg mL^{-1}, blue trace), transferrin (10 mg mL^{-1}, black trace) and albumin (1 mg mL^{-1}, red trace) standards in 50 mM ammonium acetate measured consecutively using an IC Dionex 5000 sample introduction system and a SEC MabPac column coupled to a Agilent 8800 ICP-QMS operated in reaction mode (30% oxygen).

Table 3 Size ladder. A size ladder using ferritin (450 kDa), transferrin (80 kDa) and albumin (66 kDa) standards was measured at a flow rate of 200 µL min^{-1} with corresponding retention time.

	MW [kDa]	RT [min]
Ferritin	450	9.9
Transferrin	80	12.0
Albumin	66	11.8

Figure 6 and **7** show the SEC chromatogram of the size ladder measured at a flow rate of 250 µL min^{-1}. The iron trace in **figure 6** indicated that ferritin (450 kDa) showed predominantly a monomer peak at a retention time of 7.9 min and oligomer peaks at a retention time of 5.8-6.8 min. Transferrin (80 kDa) showed a monomer peak at a retention time of 9.6 min. The iron signal of albumin was too low, thus showing no monomer peak. The sulphur trace in **figure 7** indicated that transferrin and albumin (66 kDa) were predominantly present in monomer form at a retention time of 9.6 min and 9.4 min, but also showed oligomer peaks at a retention time of 9.0 min and 8.9 min. The retention time and molecular weight of the used markers for the size ladder are shown in **table 4**.

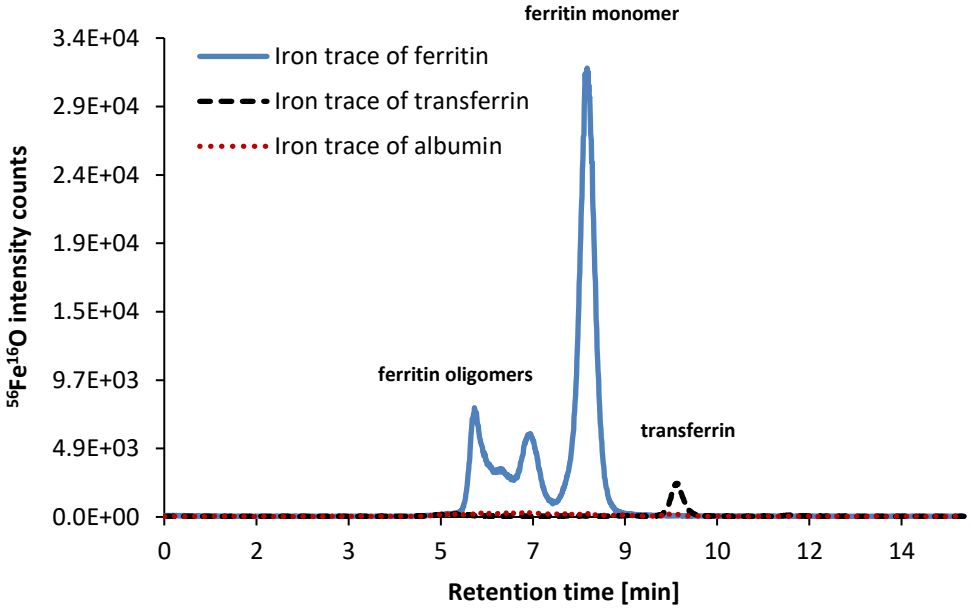

Figure 6 SEC chromatogram of a size ladder. Iron trace of ferritin (20 µg mL^{-1}, blue trace), transferrin (10 mg mL^{-1}, black trace) and albumin (1 mg mL^{-1}, red trace) standards in 50 mM ammonium acetate measured consecutively using an IC Dionex 5000 sample introduction system and a SEC MabPac column coupled to a Agilent 8800 ICP-QMS operated in reaction mode (30% oxygen).

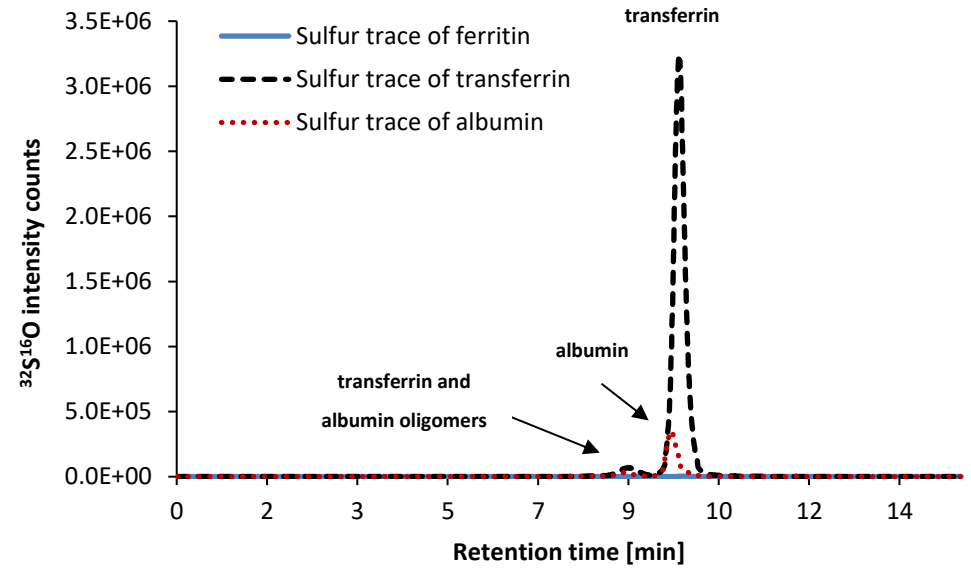

Figure 7 SEC chromatogram of a size ladder. Sulphur trace of ferritin (20 µg mL^{-1}, blue trace), transferrin (10 mg mL^{-1}, black trace) and albumin (1 mg mL^{-1}, red trace) standards in 50 mM ammonium acetate measured consecutively using an IC Dionex 5000 sample introduction system and a SEC MabPac column coupled to a Agilent 8800 ICP-QMS operated in reaction mode (30% oxygen).

Table 4 Size ladder. A size ladder using ferritin (450 kDa), transferrin (80 kDa) and albumin (66 kDa) standards was measured at a flow rate of 250 µL min^{-1} with corresponding retention time.

	MW [kDa]	RT [min]
Ferritin	450	7.9
Transferrin	80	9.6
Albumin	66	9.4

Comparing the SEC chromatograms of the size ladder measured at a flow rate of 200 µL min^{-1} and 250 µL min^{-1}, it can be observed that at a flow rate of 250 µL min^{-1} the used size ladder markers elute sooner. The distance between the peak maxima of ferritin and transferrin or transferrin and albumin account to 2.1 min and 0.2 min measured at a flow rate of 200 µL min^{-1}. Whereas the distance between the peak maxima of ferritin and transferrin or transferrin and albumin account to 1.7 min and 0.2 min measured at a flow rate of 250 µL min^{-1}.

4.2 The enrichment of ferritin by centrifugal ultrafiltration

4.2.1 Effect of washing and drying of filters on the procedural limit of quantification

Different filter preparation procedures were conducted out and their effect on the procedural limit of quantification (LOQ) of Fe and S were investigated. **Table 5** shows the values of the limit of quantification of Fe and S obtained with different filter preparation procedures for regenerated cellulose and polyethersulfone filters. The procedural LOQ_{Fe} and LOQ_S were calculated as equivalent to ten times the standard deviation of procedural blank measurements, respectively.

Table 5 The procedural limit of quantification (LOQ) of iron and sulphur obtained with different filter preparation procedures for regenerated cellulose and polyethersulfone filters

Filter preparation procedure	Analyte	Unit	Replicates	Procedural LOQ (regenerated cellulose filter)	Procedural LOQ (polyethersulfone filter)
1.	Fe	ng mL^{-1}	n = *3*	25.04	25.96
	S	ng mL^{-1}	n = *3*	69.18	81.35
2.	Fe	ng mL^{-1}	n = *3*	1.36	84.54
	S	ng mL^{-1}	n = *3*	9.45	487.13
3.	Fe	ng mL^{-1}	n = *5*	93.63	1.88
	S	ng mL^{-1}	n = *5*	97.47	18.59
4.	Fe	ng mL^{-1}	n = *3*	9.11	36.16
	S	ng mL^{-1}	n = *3*	20.61	51.67
6.	Fe	ng mL^{-1}	n = *3*	12.21	74.93
	S	ng mL^{-1}	n = *3*	32.97	34.95

The procedural LOQ_{Fe} and LOQ_S of not washed and not dried regenerated cellulose filters (1.) accounted to 25.04 ng mL^{-1} and 69.18 ng mL^{-1}, respectively. Whereas the procedural LOQ_{Fe} and LOQ_S of not washed and not dried polyethersulfone filters (1.) accounted to 25.96 ng mL^{-1} and 81.35 ng mL^{-1}, respectively.

The procedural LOQ_{Fe} and LOQ_S of washed (3 x with 5% HNO_3 and 2 x with ultrapure water, 1 x centrifuged with ultrapure water (10 min, 15000 g)) and not dried regenerated cellulose filters (2.) accounted to 1.36 ng mL^{-1} and 9.45 ng mL^{-1}, respectively. Whereas the procedural LOQ_{Fe} and LOQ_S of washed (3 x with 5% HNO_3 and 2 x with ultrapure water, 1 x centrifuged with ultrapure water (10 min, 15000 g)) and not dried polyethersulfone filters (2.) accounted to 84.54 ng mL^{-1} and 487.13 ng mL^{-1}, respectively.

The procedural LOQ_{Fe} and LOQ_S of washed (3 x with 5% HNO_3, 1 x centrifuged with ultrapure water (5 min, 15000 g), 1 x centrifuged without ultrapure water (5 min, 15000 g), 1 x centrifuged with ultrapure water (10 min, 15000 g)) and not dried regenerated cellulose filters (3.) accounted to 93.63 ng mL^{-1} and 97.47 ng mL^{-1}, respectively. Whereas the procedural LOQ_{Fe} and LOQ_S of washed (3 x with 5% HNO_3, 1 x centrifuged with ultrapure water (5 min, 15000 g), 1 x centrifuged without ultrapure water (5 min, 15000 g), 1 x centrifuged with ultrapure water (10 min, 15000 g)) and not dried polyethersulfone filters (3.) accounted to 1.88 ng mL^{-1} and 18.59 ng mL^{-1}, respectively.

The procedural LOQ_{Fe} and LOQ_S of washed (3 x with 5% HNO_3 and 3 x with ultrapure water) and dried (15 min) regenerated cellulose filters (4.) accounted to 9.11 ng mL^{-1} and

20.61 ng mL^{-1}, respectively. Whereas the procedural LOQ_{Fe} and LOQ_S of washed (3 x with 5% HNO_3 and 3 x with ultrapure water) and dried (15 min) polyethersulfone filters (4.) accounted to 36.16 ng mL^{-1} and 51.67 ng mL^{-1}, respectively.

The procedural LOQ_{Fe} and LOQ_S of washed (3 x with 5% HNO_3 and 3 x with ultrapure water) and dried (1 hour) regenerated cellulose filters (6.) accounted to 12.21 ng mL^{-1} and 32.97 ng mL^{-1}, respectively. Whereas the procedural LOQ_{Fe} and LOQ_S of washed (3 x with 5% HNO_3 and 3 x with ultrapure water) and dried (1 hour) polyethersulfone filters (6.) accounted to 74.93 ng mL^{-1} and 34.95 ng mL^{-1}, respectively.

The lowest procedural LOQ_{Fe} and LOQ_S for regenerated cellulose filters was achieved with the second filter preparation procedure followed by the fourth, sixth, first and third filter preparation procedure. Washing (3 x with 5% HNO_3 and 3 x with ultrapure water) and drying (15 min, 1 hour) of regenerated cellulose filters allowed to achieve a lower procedural LOQ_{Fe} and LOQ_S compared to not washed and not dried filters. Comparing the second and third filter preparation procedure, it can be implicated that centrifugation of regenerated cellulose filters with ultrapure water for several times without rinsing the filter with ultrapure water before leads to a considerably higher procedural LOQ_{Fe} and LOQ_S in comparison to 2 x rinsing and 1 x centrifugation of filters with ultrapure water.

Regarding polyethersulfone filters, centrifugation with ultrapure water for several times without rinsing the filter with ultrapure water before leads to a considerably lower procedural LOQ_{Fe} and LOQ_S in comparison to 2 x rinsing and 1 x centrifugation of filters with ultrapure water. Washing (3 x with 5% HNO_3 and 3 x with ultrapure water) and drying (15 min, 1 hour) of polyethersulfone filters allowed to achieve a lower procedural LOQ_S compared to not washed and not dried filters. Not washed and not dried polyethersulfone filters allowed to achieve a lower procedural LOQ_{Fe} compared to washed (3 x with 5% HNO_3 and 3 x with ultrapure water) and dried (15 min, 1 hour) filters.

4.2.2 Effect of washing, drying and conditioning of filters on the enrichment of ferritin

Different filter preparation procedures were conducted out and their effect on the enrichment of ferritin were investigated. **Table 6-8** shows the total Fe and S content in ferritin as well as the Fe/S ratio of ferritin before and after centrifugal ultrafiltration using 100 kDa regenerated cellulose filters (not washed, not dried and not conditioned/conditioned) in the low and high molecular weight fraction. The Fe and S elemental content in ferritin was determined via external calibration using Fe-S standards by FI-ICP-QMS operated in reaction mode (30% O_2). The ferritin standard was diluted gravimetrically to a final concentration of 10.8 µg mL^{-1} ferritin with 50 mM ammonium acetate (pH 6.8) shortly before measurement. The ferritin sample was measured three times and the average Fe and S concentration was determined. The Fe elemental content in the original sample accounted to 1035 ng mL^{-1} with a RSD of 10%. The S elemental content in the original sample accounted to 39 ng mL^{-1} with a RSD of 4%. The Fe and S content in the low molecular weight fraction using not washed, not dried and not conditioned/conditioned 100 kDa regenerated cellulose filters was below the procedural LOQ_{Fe} and LOQ_S of 25.04 ng mL^{-1} and 69.18 ng mL^{-1}. The Fe and S content in the high molecular weight fraction using not washed, not dried and not conditioned 100 kDa regenerated cellulose filters was above the procedural LOQ, accounting to 11821 ng mL^{-1} Fe with a RSD of 16% and 600 ng mL^{-1} S with a RSD of 10%. The Fe and S content in the high molecular weight fraction using not washed, not dried and conditioned 100 kDa regenerated cellulose filters was above the procedural LOQ, accounting to 14228 ng mL^{-1} Fe with a RSD of 26% and 741 ng mL^{-1} S with a RSD of 18%. Centrifugal ultrafiltration using not washed, not dried and not conditioned/conditioned 100 kDa regenerated cellulose filters enabled a 11 ± 2-fold and 14 ± 4-fold enrichment of ferritin **(figure 8)**. The Fe/S ratio of ferritin before and after the enrichment using not washed, not dried and not conditioned/conditioned 100 kDa regenerated cellulose filters indicated significant Fe loss due to sticking of ferritin-bound iron to the filter membrane **(figure 9)**.

Table 6 The Fe and S elemental content in a ferritin standard before centrifugal ultrafiltration was determined via external calibration using Fe-S standards by FI-ICP-MS (30% O_2) and the Fe/S ratio was calculated. The concentration values are given with the relative standard deviation of *n = 3* measurements.

original sample	S [ng mL^{-1}]	Fe [ng mL^{-1}]	Fe/S ratio
1	41	1147	28
2	38	1024	27
3	38	933	24
Average	39	1035	26
SD	2	108	2
RSD [%]	4	10	7

Table 7 The Fe and S elemental content in a ferritin standard after centrifugal ultrafiltration using not washed, not dried and not conditioned 100 kDa regenerated cellulose filters in the low (LMF) and high molecular weight fraction (HMF) was determined via external calibration using Fe-S standards by FI-ICP-MS (30% O_2) and the Fe/S ratio was calculated. The concentration values are given with the relative standard deviation of *n = 3* measurements.

LMF	S [ng mL^{-1}]	Fe [ng mL^{-1}]	HMF	S [ng mL^{-1}]	Fe [ng mL^{-1}]	Fe/S ratio
1	< LOQ	< LOQ	1	533	9993	19
2	< LOQ	< LOQ	2	617	11737	19
3	< LOQ	< LOQ	3	650	13733	21
Average	< LOQ	< LOQ	Average	600	11821	20
SD	-	-	SD	61	1871	1
RSD [%]	-	-	RSD [%]	10	16	7

Table 8 The Fe and S elemental content in a ferritin standard after centrifugal ultrafiltration using not washed, not dried and conditioned 100 kDa regenerated cellulose filters in the low (LMF) and high molecular weight fraction (HMF) was determined via external calibration using Fe-S standards by FI-ICP-MS (30% O_2) and the Fe/S ratio was calculated. The concentration values are given with the relative standard deviation of *n = 3* measurements.

LMF	S [ng mL^{-1}]	Fe [ng mL^{-1}]	HMF	S [ng mL^{-1}]	Fe [ng mL^{-1}]	Fe/S ratio
1	< LOQ	< LOQ	1	852	17780	21
2	< LOQ	< LOQ	2	594	10308	17
3	< LOQ	< LOQ	3	777	14596	19
Average	< LOQ	< LOQ	Average	741	14228	19
SD	-	-	SD	133	3750	2
RSD [%]	-	-	RSD [%]	18	26	9

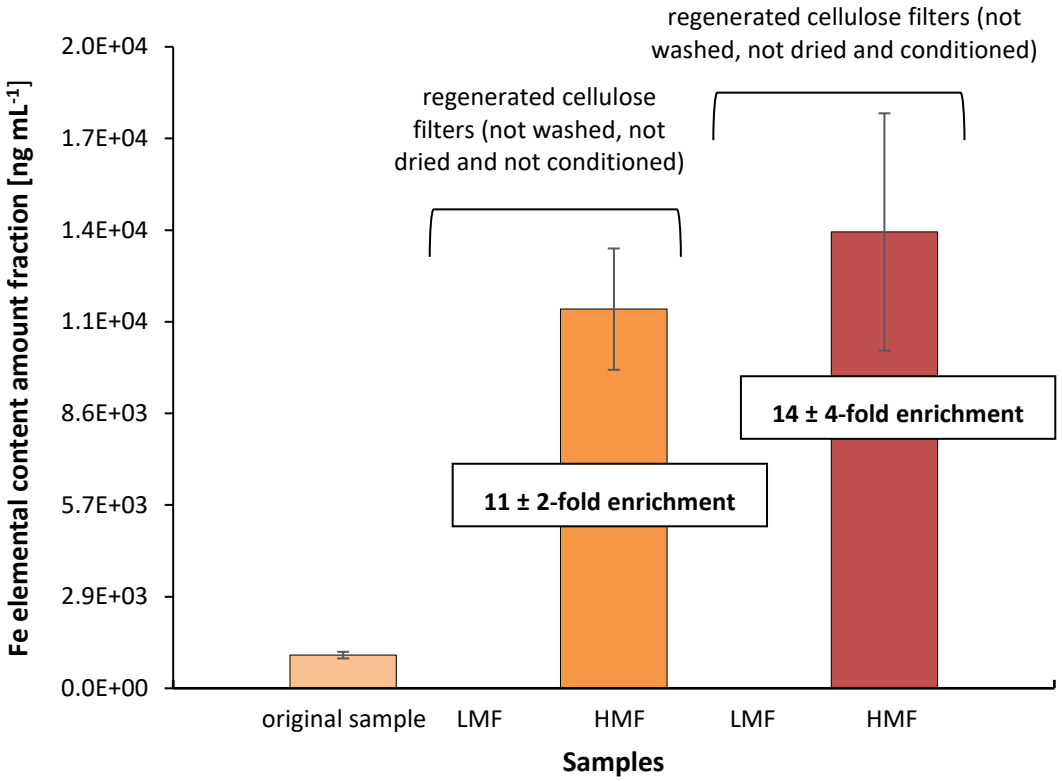

Figure 8 Fe elemental content in ferritin before (light orange bar) and after enrichment in the low (LMF) and high molecular weight fraction (HMF) using not washed, not dried and not conditioned (dark orange bar)/conditioned (dark red bar) 100 kDa regenerated cellulose filters; error bars equal to 1 *SD (n = 3)*

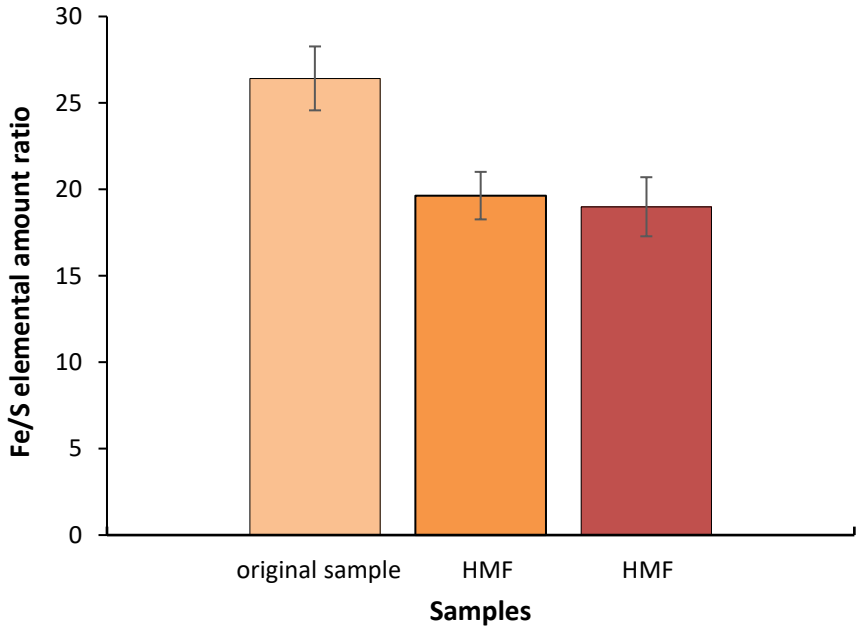

Figure 9 Fe/S elemental ratio in ferritin before (light orange bar) and after enrichment using not washed, not dried and not conditioned (dark orange bar)/conditioned (dark red bar) 100 kDa regenerated cellulose filters; error bars equal to 1 *SD (n = 3)*

Table 9-11 shows the total Fe and S content in ferritin as well as the Fe/S ratio of ferritin before and after centrifugal ultrafiltration using washed (3 x with 5% HNO_3 and 2 x with ultrapure water, 1 x centrifuged with ultrapure water (10 min, 15000 g)), not dried and conditioned 100 kDa regenerated cellulose and polyethersulfone filters in the low and high molecular weight fraction. The Fe and S elemental content in ferritin was determined via external calibration using Fe-S standards by FI-ICP-QMS operated in reaction mode (30% O_2). The ferritin standard was diluted gravimetrically to a final concentration of 5.0 µg mL^{-1} ferritin with 50 mM ammonium acetate (pH 6.8) shortly before measurement. The ferritin sample was measured three times and the average Fe and S concentration was determined. The Fe and S elemental content in the original sample accounted to 449 ng mL^{-1} with a RSD of 8% and 19 ng mL^{-1} with a RSD of 2%. The Fe and S content in the low molecular weight fraction using 100 kDa polyethersulfone filter was below the procedural LOQ_{Fe} and LOQ_S of 84.54 ng mL^{-1} and 487.13 ng mL^{-1}. The S content in the low molecular weight fraction using 100 kDa regenerated cellulose filter was below the procedural LOQ_S of 9.45 ng mL^{-1}, whereas the Fe content in the low molecular weight fraction was above the LOQ_{Fe} of 1.36 ng mL^{-1}. The Fe and S elemental content in the high molecular weight fraction using 100 kDa regenerated cellulose and polyethersulfone filters was above the procedural LOQ. Centrifugal ultrafiltration using washed (3 x with 5% HNO_3 and 2 x with ultrapure water, 1 x centrifuged with ultrapure water (10 min, 15000 g)), not dried and conditioned 100 kDa regenerated cellulose filters enabled a 9 ± 4-fold enrichment of ferritin. Whereas, 100 kDa polyethersulfone filters enabled a 16 ± 3-fold enrichment of ferritin **(figure 10)**. The Fe/S ratio of ferritin before and after the enrichment using washed (3 x with 5% HNO_3 and 2 x with ultrapure water, 1 x centrifuged with ultrapure water (10 min, 15000 g)), not dried and conditioned 100 kDa regenerated cellulose and polyethersulfone filters indicated no significant Fe loss **(figure 11)**.

Table 9 The Fe and S elemental content in a ferritin standard before centrifugal ultrafiltration was determined via external calibration using Fe-S standards by FI-ICP-MS (30% O_2) and the Fe/S ratio was calculated. The concentration values are given with the relative standard deviation of *n = 3* measurements.

original sample	S [ng mL^{-1}]	Fe [ng mL^{-1}]	Fe/S ratio
1	20	410	18
2	20	479	21
3	19	458	24
Average	19	449	21
SD	0.5	35	3
RSD [%]	2	8	13

Table 10 The Fe and S elemental content in a ferritin standard after centrifugal ultrafiltration using washed (3 x with 5 % HNO_3 and 2 x with ultrapure water, 1 x centrifuged with ultrapure water (10 min, 15000 g)), not dried and conditioned 100 kDa regenerated cellulose filters in the low (LMF) and high molecular weight fraction (HMF) was determined via external calibration using Fe-S standards by FI-ICP-MS (30% O_2) and the Fe/S ratio was calculated. The concentration values are given with the relative standard deviation of *n = 3* measurements.

regenerated cellulose filter						
LMF	**S [ng mL^{-1}]**	**Fe [ng mL^{-1}]**	**HMF**	**S [ng mL^{-1}]**	**Fe [ng mL^{-1}]**	**Fe/S ratio**
1	< LOQ	3	1	287	3671	13
2	< LOQ	-	2	235	2672	11
3	< LOQ	3	3	215	6001	28
Average	< LOQ	3	Average	246	4115	17
SD	-	0.03	SD	37	1708	9
RSD [%]	-	1	RSD [%]	15	42	53

Table 11 The Fe and S elemental content in a ferritin standard after centrifugal ultrafiltration using washed (3 x with 5 % HNO_3 and 2 x with ultrapure water, 1 x centrifuged with ultrapure water (10 min, 15000 g)), not dried and conditioned 100 kDa polyethersulfone filters in the low (LMF) and high molecular weight fraction (HMF) was determined via external calibration using Fe-S standards by FI-ICP-MS (30% O_2) and the Fe/S ratio was calculated. The concentration values are given with the relative standard deviation of *n = 3* measurements.

polyethersulfone filter						
LMF	**S [ng mL^{-1}]**	**Fe [ng mL^{-1}]**	**HMF**	**S [ng mL^{-1}]**	**Fe [ng mL^{-1}]**	**Fe/S ratio**
1	< LOQ	< LOQ	1	457	8586	19
2	< LOQ	< LOQ	2	362	5946	16
3	< LOQ	< LOQ	3	348	6426	18
Average	< LOQ	< LOQ	Average	389	6986	18
SD	-	-	SD	60	1407	1
RSD [%]	-	-	RSD [%]	15	20	7

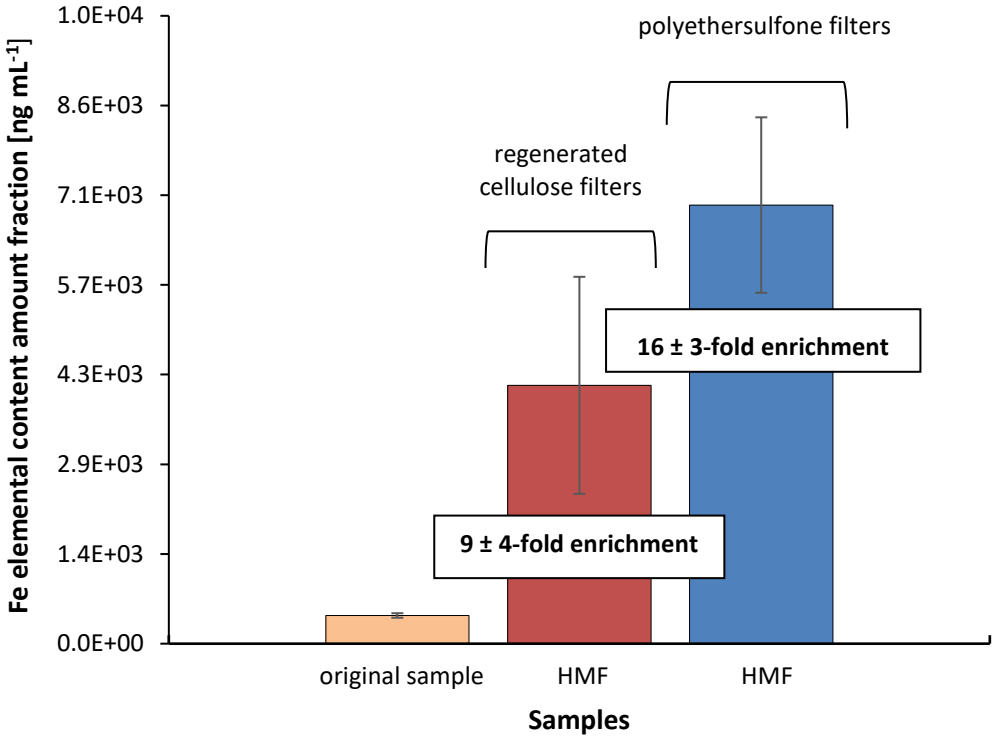

Figure 10 Fe elemental content in ferritin before (light orange bar) and after enrichment in the high molecular weight fraction (HMF) using washed (3 x with 5% HNO_3 and 2 x with ultrapure water, 1 x centrifuged with ultrapure water (10 min, 15000 g)), not dried and conditioned 100 kDa regenerated cellulose (dark red bar) and polyethersulfone filters (dark blue bar); error bars equal to 1 *SD (n = 3)*

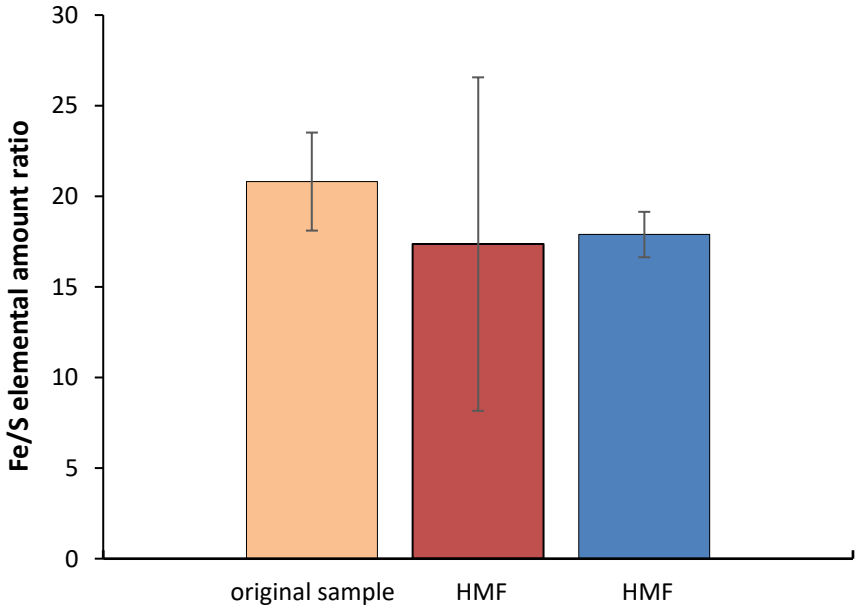

Figure 11 Fe/S elemental ratio in ferritin before (light orange bar) and after enrichment using washed (3 x with 5% HNO_3 and 2 x with ultrapure water, 1 x centrifuged with ultrapure water (10 min, 15000 g)), not dried and conditioned 100 kDa regenerated cellulose (dark red bar) and polyethersulfone filters (dark blue bar); error bars equal to 1 *SD (n = 3)*

Table 12-14 shows the total Fe and S content in ferritin as well as the Fe/S ratio of ferritin before and after centrifugal ultrafiltration using washed (3 x with 5% HNO_3, 1 x centrifuged with ultrapure water (5 min, 15000 g), 1 x centrifuged without ultrapure water (5 min, 15000 g), 1 x centrifuged with ultrapure water (10 min, 15000 g)), not dried and conditioned 100 kDa regenerated cellulose and polyethersulfone filters in the low and high molecular weight fraction. The Fe and S elemental content in ferritin was determined via external calibration using Fe-S standards by FI-ICP-QMS operated in reaction mode (30% O_2). The ferritin standard was diluted gravimetrically to a final concentration of 10.8 µg mL^{-1} ferritin with 50 mM ammonium acetate (pH 6.8) shortly before measurement. The ferritin sample was measured five times and the average Fe and S concentration was determined. The Fe elemental content in the original sample accounted to 1135 ng mL^{-1} with a RSD of 4%. The S elemental content in the original sample accounted to 58 ng mL^{-1} with a RSD of 7%. The Fe and S content in the low molecular weight fraction using 100 kDa regenerated cellulose filters was below the procedural LOQ_{Fe} and LOQ_S of 93.63 ng mL^{-1} and 97.47 ng mL^{-1}. The S content in the low molecular weight fraction using 100 kDa polyethersulfone filters was below the procedural LOQ_S of 18.59 ng mL^{-1}, whereas the Fe content in the low molecular weight fraction was above the LOQ_{Fe} of 1.88 ng mL^{-1}. The Fe and S content in the high molecular weight fraction using 100 kDa regenerated cellulose filters was above the procedural LOQ, accounting to 7760 ng mL^{-1} Fe with a RSD of 25% and 497 ng mL^{-1} S with a RSD of 29%. The Fe and S content in the high molecular weight fraction using 100 kDa polyethersulfone filters was above the procedural LOQ, accounting to 8741 ng mL^{-1} Fe with a RSD of 21% and 823 ng mL^{-1} S with a RSD of 49%. Centrifugal ultrafiltration using washed (3 x with 5% HNO_3, 1 x centrifuged with ultrapure water (5 min, 15000 g), 1 x centrifuged without ultrapure water (5 min, 15000 g), 1 x centrifuged with ultrapure water (10 min, 15000 g)), not dried and conditioned 100 kDa regenerated cellulose filters enabled a 7 ± 2-fold enrichment of ferritin. Whereas, 100 kDa polyethersulfone filters enabled an 8 ± 2-fold enrichment of ferritin **(figure 12)**. The Fe/S ratio of ferritin before and after the enrichment using washed (3 x with 5% HNO_3, 1 x centrifuged with ultrapure water (5 min, 15000 g), 1 x centrifuged without ultrapure water (5 min, 15000 g), 1 x centrifuged with ultrapure water (10 min, 15000 g)), not dried and conditioned 100 kDa regenerated cellulose and polyethersulfone filters indicated significant Fe loss due to sticking of ferritin-bound iron to the filter membrane **(figure 13)**.

Table 12 The Fe and S elemental content in a ferritin standard before centrifugal ultrafiltration was determined via external calibration using Fe-S standards by FI-ICP-MS (30% O_2) and the Fe/S ratio was calculated. The concentration values are given with the relative standard deviation of *n = 3* measurements.

original sample	S [ng mL^{-1}]	Fe [ng mL^{-1}]	Fe/S ratio
1	55	1087	20
2	62	1165	19
3	57	1154	20
Average	58	1135	20
SD	4	43	1
RSD [%]	7	4	4

Table 13 The Fe and S elemental content in a ferritin standard after centrifugal ultrafiltration using washed (3 x with 5 %HNO_3, 1 x centrifuged with ultrapure water (5 min, 15000 g), 1 x centrifuged without ultrapure water (5 min, 15000 g), 1 x centrifuged with ultrapure water (10 min, 15000 g)), not dried and conditioned 100 kDa regenerated cellulose filters in the low (LMF) and high molecular weight fraction (HMF) was determined via external calibration using Fe-S standards by FI-ICP-MS (30% O_2) and the Fe/S ratio was calculated. The concentration values are given with the relative standard deviation of *n = 5* measurements.

regenerated cellulose filter						
LMF	S [ng mL^{-1}]	Fe [ng mL^{-1}]	HMF	S [ng mL^{-1}]	Fe [ng mL^{-1}]	Fe/S ratio
1	< LOQ	< LOQ	1	361	5554	15
2	< LOQ	< LOQ	2	498	8224	17
3	< LOQ	< LOQ	3	356	6266	18
4	< LOQ	< LOQ	4	590	8368	14
5	< LOQ	< LOQ	5	682	10387	15
Average	< LOQ	< LOQ	Average	497	7760	16
SD	-	-	SD	143	1910	1
RSD [%]	-	-	RSD [%]	29	25	8

Table 14 The Fe and S elemental content in a ferritin standard after centrifugal ultrafiltration using washed (3 x with 5% HNO_3, 1 x centrifuged with ultrapure water (5 min, 15000 g), 1 x centrifuged without ultrapure water (5 min, 15000 g), 1 x centrifuged with ultrapure water (10 min, 15000 g)), not dried and conditioned 100 kDa polyethersulfone filters in the low (LMF) and high molecular weight fraction (HMF) was determined via external calibration using Fe-S standards by FI-ICP-MS (30% O_2) and the Fe/S ratio was calculated. The concentration values are given with the relative standard deviation of *n = 5* measurements.

polyethersulfone filter						
LMF	S [ng mL^{-1}]	Fe [ng mL^{-1}]	HMF	S [ng mL^{-1}]	Fe [ng mL^{-1}]	Fe/S ratio
1	< LOQ	10	1	530	7997	15
2	< LOQ	16	2	523	7052	13
3	< LOQ	3	3	658	8678	13
4	< LOQ	6	4	933	11914	13
5	< LOQ	2	5	1472	8066	5
Average	< LOQ	7	Average	823	8741	12
SD	-	6	SD	399	1866	4
RSD [%]	-	75	RSD [%]	48	21	31

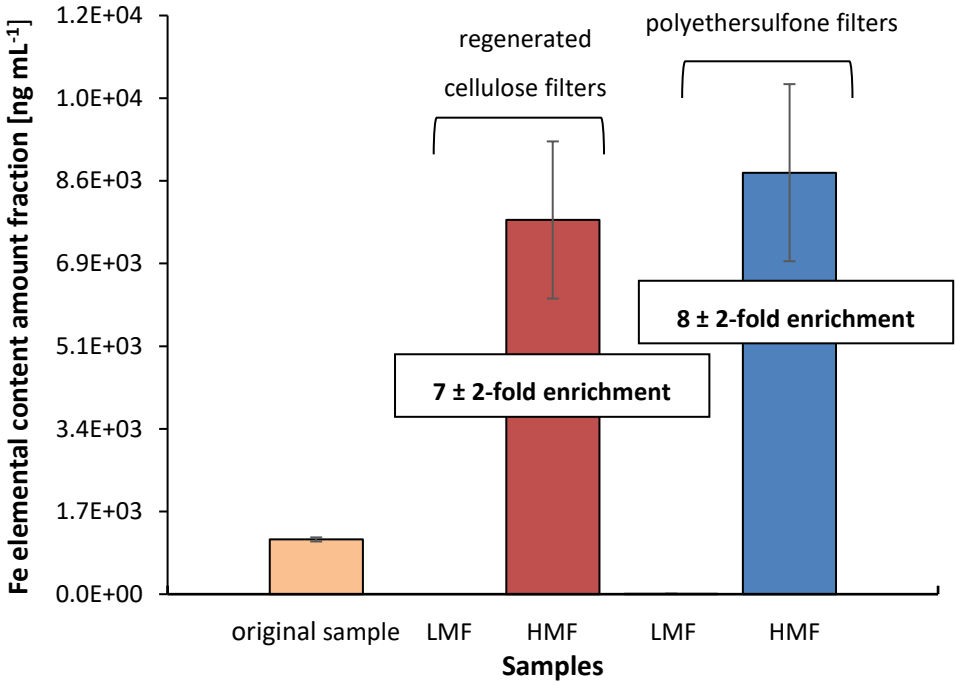

Figure 12 Fe elemental content in ferritin before (light orange bar) and after enrichment in the low (LMF) and high molecular weight fraction (HMF) using washed (3 x with 5% HNO_3, 1 x centrifuged with ultrapure water (5 min, 15000 g), 1 x centrifuged without ultrapure water (5 min, 15000 g), 1 x centrifuged with ultrapure water (10 min, 15000 g)), not dried and conditioned 100 kDa regenerated cellulose (dark red bar) and polyethersulfone filters (dark blue bar); error bars equal to 1 *SD (n = 5)*

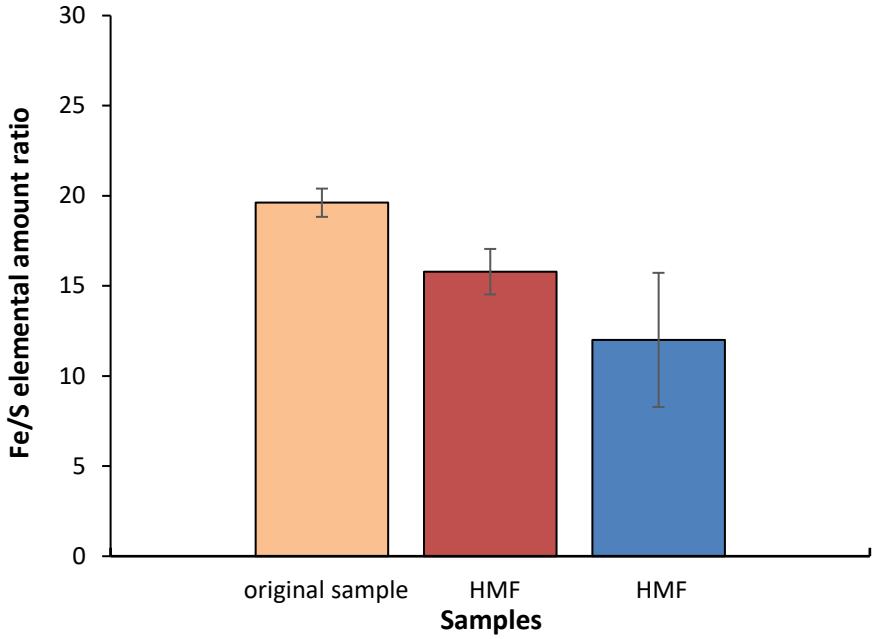

Figure 13 Fe/S elemental ratio in ferritin before (light orange bar) and after enrichment using washed (3 x with 5% HNO_3, 1 x centrifuged with ultrapure water (5 min, 15000 g), 1 x centrifuged without ultrapure water (5 min, 15000 g), 1 x centrifuged with ultrapure water (10 min, 15000 g)), not dried and conditioned100 kDa regenerated cellulose (dark red bar) and polyethersulfone filters (dark blue bar); error bars equal to 1 *SD (n = 5)*

Table 15-**17** shows the total Fe and S content in ferritin as well as the Fe/S ratio of ferritin before and after centrifugal ultrafiltration using washed (3 x with 5% HNO_3 and 3 x with ultrapure water), dried (15 min) and conditioned 100 kDa regenerated cellulose and polyethersulfone filters in the low and high molecular weight fraction. The Fe and S elemental content in ferritin was determined via external calibration using Fe-S standards by FI-ICP-QMS operated in reaction mode (30% O_2). The ferritin standard was diluted gravimetrically to a final concentration of 5.0 µg mL^{-1} ferritin with 50 mM ammonium acetate (pH 6.8) shortly before measurement. The ferritin sample was measured three times and the average Fe and S concentration was determined. The Fe elemental content in the original sample accounted to 486 ng mL^{-1} with a RSD of 3%. The S elemental content in the original sample accounted to 24 ng mL^{-1} with a RSD of 7%. The Fe and S content in the low molecular weight fraction using 100 kDa regenerated cellulose filters was below the procedural LOQ_{Fe} and LOQ_S of 9.11 ng mL^{-1} and 20.61 ng mL^{-1}. The Fe and S content in the low molecular weight fraction using 100 kDa polyethersulfone filters was below the procedural LOQ_{Fe} and LOQ_S of 36.16 ng mL^{-1} and 51.67 ng mL^{-1}. The Fe and S content in the high molecular weight fraction using 100 kDa regenerated cellulose filters was above the procedural LOQ, accounting to

3065 ng mL^{-1} Fe with a RSD of 19% and 250 ng mL^{-1} S with a RSD of 14%. The Fe and S content in the high molecular weight fraction using 100 kDa polyethersulfone filters was above the procedural LOQ, accounting to 2316 ng mL^{-1} Fe with a RSD of 18% and 243 ng mL^{-1} S with a RSD of 14%. Centrifugal ultrafiltration using washed (3 x with 5% HNO_3 and 3 x with ultrapure water), dried (15 min) and conditioned 100 kDa regenerated cellulose filters enabled a 6 ± 1-fold enrichment of ferritin. Whereas, 100 kDa polyethersulfone filters enabled a 5 ± 1-fold enrichment of ferritin **(figure 14)**. The Fe/S ratio of ferritin before and after the enrichment using washed (3 x with 5% HNO_3 and 3 x with ultrapure water), dried (15 min) and conditioned 100 kDa regenerated cellulose and polyethersulfone filters indicated significant Fe loss due to sticking of ferritin-bound iron to the filter membrane **(figure 15)**.

Table 15 The Fe and S elemental content in a ferritin standard before centrifugal ultrafiltration was determined via external calibration using Fe-S standards by FI-ICP-MS (30% O_2) and the Fe/S ratio was calculated. The concentration values are given with the relative standard deviation of *n = 3* measurements.

original sample	S [ng mL^{-1}]	Fe [ng mL^{-1}]	Fe/S ratio
1	26	475	19
2	24	504	21
3	22	478	22
Average	24	486	20
SD	2	16	2
RSD [%]	7	3	8

Table 16 The Fe and S elemental content in a ferritin standard after centrifugal ultrafiltration using washed (3 x with 5% HNO_3 and 3 x with ultrapure water), dried (15 min) and conditioned 100 kDa regenerated cellulose filters in the low (LMF) and high molecular weight fraction (HMF) was determined via external calibration using Fe-S standards by FI-ICP-MS (30% O_2) and the Fe/S ratio was calculated. The concentration values are given with the relative standard deviation of *n = 3* measurements.

regenerated cellulose filter						
LMF	S [ng mL^{-1}]	Fe [ng mL^{-1}]	HMF	S [ng mL^{-1}]	Fe [ng mL^{-1}]	Fe/S ratio
1	< LOQ	< LOQ	1	276	3645	13
2	< LOQ	< LOQ	2	210	2465	12
3	< LOQ	< LOQ	3	265	3083	12
Average	< LOQ	< LOQ	Average	250	3065	12
SD	-	-	SD	35	590	1
RSD [%]	-	-	RSD [%]	14	19	7

Table 17 The Fe and S elemental content in a ferritin standard after centrifugal ultrafiltration using washed (3 x with 5% HNO_3 and 3 x with ultrapure water), dried (15 min) and conditioned 100 kDa polyethersulfone filters in the low (LMF) and high molecular weight fraction (HMF) was determined via external calibration using Fe-S standards by FI-ICP-MS (30% O_2) and the Fe/S ratio was calculated. The concentration values are given with the relative standard deviation of *n = 3* measurements.

polyethersulfone filter						
LMF	**S [ng mL^{-1}]**	**Fe [ng mL^{-1}]**	**HMF**	**S [ng mL^{-1}]**	**Fe [ng mL^{-1}]**	**Fe/S ratio**
1	< LOQ	< LOQ	1	279	2769	10
2	< LOQ	< LOQ	2	213	2263	11
3	< LOQ	< LOQ	3	236	1917	8
Average	< LOQ	< LOQ	Average	243	2316	10
SD	-	-	SD	34	428	1
RSD [%]	-	-	RSD [%]	14	18	14

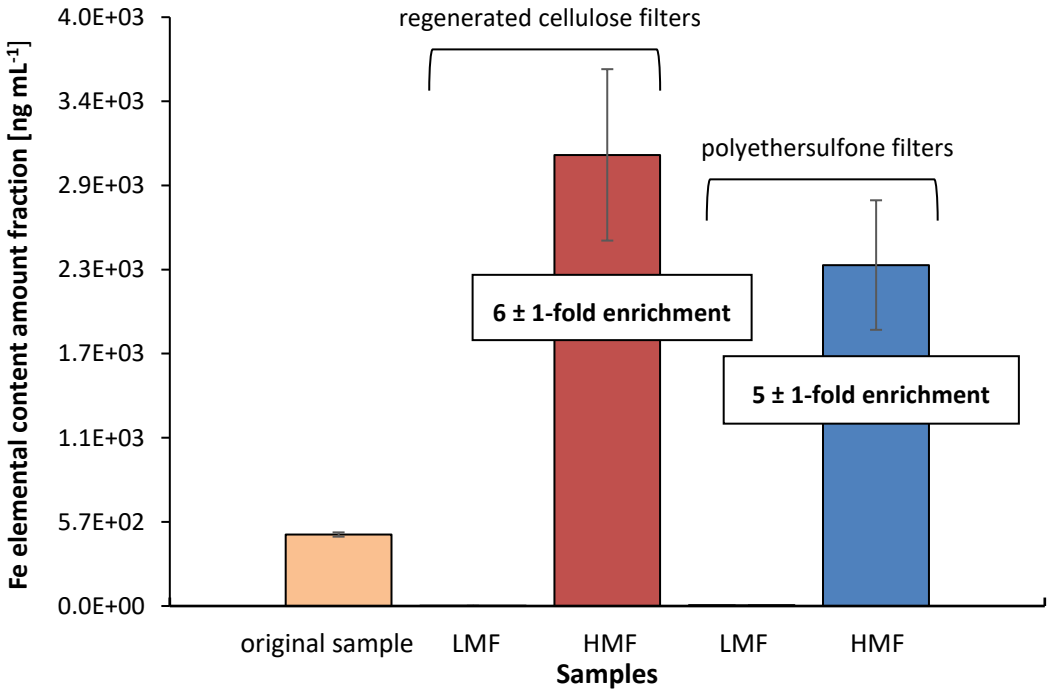

Figure 14 Fe elemental content in ferritin before (light orange bar) and after enrichment in the low (LMF) and high molecular weight fraction using washed (3 x with 5% HNO_3 and 3 x with ultrapure water), dried (15 min) and conditioned 100 kDa regenerated cellulose (dark red bar) and polyethersulfone filters (dark blue bar); error bars equal to 1 *SD (n = 3)*

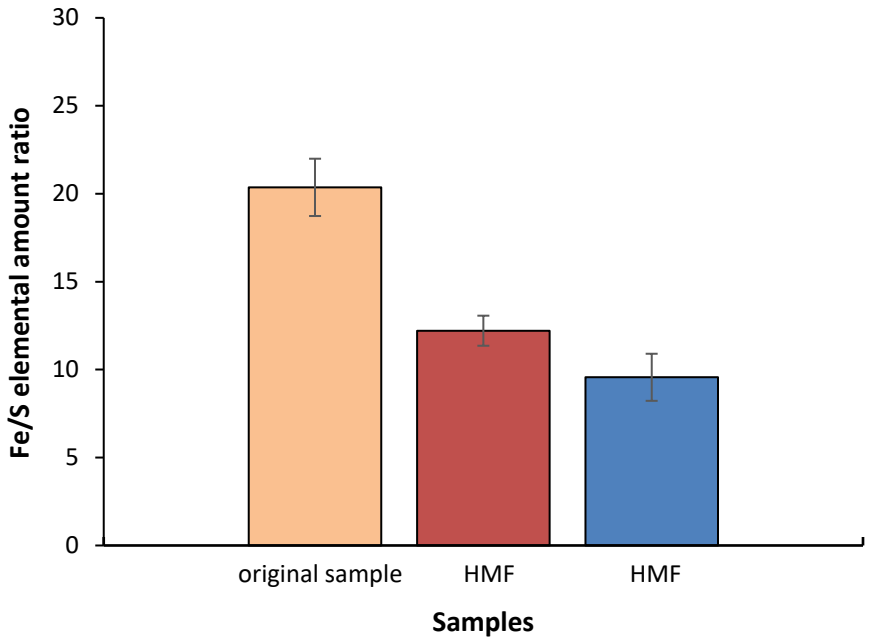

Figure 15 Fe/S elemental ratio in ferritin before (light orange bar) and after enrichment using washed (3 x with 5% HNO_3 and 3 x with ultrapure water), dried (15 min) and conditioned 100 kDa regenerated cellulose (dark red bar) and polyethersulfone filters (dark blue bar); error bars equal to 1 *SD (n = 3)*

Table 18-**24** shows the Fe and S content in ferritin as well as the Fe/S ratio of ferritin before and after centrifugal ultrafiltration using washed (3 x with 5% HNO_3 and 3 x with ultrapure water), dried (1 hour) and not conditioned/conditioned 100 kDa regenerated cellulose and polyethersulfone filters as well as not washed and conditioned 100 kDa polyethersulfone filters in the low and high molecular weight fraction. The Fe and S elemental content in ferritin was determined via external calibration using well characterized ferritin standards by FI-SEC-ICP-QMS operated in reaction mode (30% O_2). The ferritin standard was diluted gravimetrically to a final concentration of 10.8 µg mL^{-1} ferritin and 5.0 µg mL^{-1} ferritin with 50 mM ammonium acetate (pH 6.8) shortly before measurement. The ferritin sample was measured three times and the average Fe and S concentration was determined. The Fe elemental content in the original samples accounted to 532 ng mL^{-1} with a RSD of 4% and 855 ng mL^{-1} with a RSD of 2%. The S elemental content in the original samples accounted to 34 ng mL^{-1} with a RSD of 5% and 46 ng mL^{-1} with a RSD of 11%. The Fe and S content in the low molecular weight fraction using washed (3 x with 5% HNO_3 and 3 x with ultrapure water), dried (1 hour) and not conditioned/conditioned 100 kDa regenerated cellulose filters was below the procedural LOQ_{Fe} and LOQ_S of 12.21 ng mL^{-1} and 32.97 ng mL^{-1}. The Fe and S content in the

low molecular weight fraction using washed (3 x with 5% HNO_3 and 3 x with ultrapure water), dried (1 hour) and not conditioned/conditioned 100 kDa polyethersulfone filters was below the procedural LOQ_{Fe} and LOQ_S of 74.93 ng mL^{-1} and 34.95 ng mL^{-1}. The Fe and S content in the low molecular weight fraction using not washed and conditioned polyethersulfone filters was below the procedural LOQ_{Fe} and LOQ_S of 25.96 ng mL^{-1} and 81.35 ng mL^{-1}. The Fe and S content in the high molecular weight fraction using washed (3 x with 5% HNO_3 and 3 x with ultrapure water), dried (1 hour) and not conditioned 100 kDa regenerated cellulose filters was above the procedural LOQ, accounting to 9945 ng mL^{-1} Fe with a RSD of 24% and 575 ng mL^{-1} S with a RSD of 18%. The Fe and S content in the high molecular weight fraction using washed (3 x with 5% HNO_3 and 3 x with ultrapure water), dried (1 hour) and conditioned 100 kDa regenerated cellulose filters was above the procedural LOQ, accounting to 10777 ng mL^{-1} Fe with a RSD of 23% and 603 ng mL^{-1} S with a RSD of 22%. The Fe and S content in the high molecular weight fraction using washed (3 x with 5% HNO_3 and 3 x with ultrapure water), dried (1 hour) and not conditioned 100 kDa polyethersulfone filters was above the procedural LOQ, accounting to 3971 ng mL^{-1} Fe with a RSD of 76% and 358 ng mL^{-1} S with a RSD of 72%. The Fe and S content in the high molecular weight fraction using washed (3 x with 5% HNO_3 and 3 x with ultrapure water), dried (1 hour) and conditioned 100 kDa polyethersulfone filters was above the procedural LOQ, accounting to 32184 ng mL^{-1} Fe with a RSD of 5% and 1772 ng mL^{-1} S with a RSD of 5%. The Fe and S content in the high molecular weight fraction using not washed and conditioned 100 kDa polyethersulfone filters was above the procedural LOQ, accounting to 19232 ng mL^{-1} Fe with a RSD of 5% and 1192 ng mL^{-1} S with a RSD of 7%.

Centrifugal ultrafiltration using washed (3 x with 5% HNO_3 and 3 x with ultrapure water), dried (1 hour) and not conditioned/conditioned 100 kDa regenerated cellulose filters enabled a 19 ± 5-fold and 20 ± 4-fold enrichment of ferritin **(figure 16)**. Whereas, centrifugal ultrafiltration using washed (3 x with 5% HNO_3 and 3 x with ultrapure water), dried (1 hour) and not conditioned/conditioned 100 kDa polyethersulfone filters enabled a 5 ± 4-fold and 38 ± 2-fold enrichment of ferritin. Not washed and conditioned 100 kDa polyethersulfone filters enabled a 23 ± 1-fold enrichment of ferritin **(figure 17)**. The Fe/S ratio of ferritin before and after the enrichment using washed (3 x with 5% HNO_3 and 3 x with ultrapure water), dried (1 hour) and not conditioned/conditioned 100 kDa regenerated cellulose filters indicated no significant Fe loss **(figure 18)**. The Fe/S ratio of ferritin before and after the enrichment using washed (3 x with 5% HNO_3 and 3 x with ultrapure water), dried (1 hour) and conditioned as

well as not washed and conditioned 100 kDa polyethersulfone filters indicated no significant Fe loss. However, the Fe/S ratio of ferritin before and after the enrichment using washed (3 x with 5% HNO_3 and 3 x with ultrapure water), dried (1 hour) and not conditioned 100 kDa polyethersulfone filters indicated significant Fe loss due to sticking of ferritin-bound iron to the filter membrane **(figure 19)**.

Table 18 The Fe and S elemental content in a ferritin standard before centrifugal ultrafiltration was determined via external calibration using well characterized ferritin standards by FI-SEC-ICP-MS (30% O_2) and the Fe/S ratio was calculated. The concentration values are given with the relative standard deviation of *n = 3* measurements.

original sample	S [ng mL^{-1}]	Fe [ng mL^{-1}]	Fe/S ratio
1	33	508	16
2	36	537	15
3	34	550	16
Average	34	532	16
SD	2	22	1
RSD [%]	5	4	5

Table 19 The Fe and S elemental content in a ferritin standard after centrifugal ultrafiltration using washed (3 x with 5% HNO_3 and 3 x with ultrapure water), dried (1 hour) and not conditioned 100 kDa regenerated cellulose filters in the low (LMF) and high molecular weight fraction (HMF) was determined via external calibration using well characterized ferritin standards by FI-SEC-ICP-MS (30% O_2) and the Fe/S ratio was calculated. The concentration values are given with the relative standard deviation of *n = 3* measurements.

regenerated cellulose filter						
LMF	S [ng mL^{-1}]	Fe [ng mL^{-1}]	HMF	S [ng mL^{-1}]	Fe [ng mL^{-1}]	Fe/S ratio
1	< LOQ	< LOQ	1	469	7571	16
2	< LOQ	< LOQ	2	574	9947	17
3	< LOQ	< LOQ	3	681	12317	18
Average	< LOQ	< LOQ	Average	574	9945	17
SD	-	-	SD	106	2373	1
RSD [%]	-	-	RSD [%]	18	24	6

Table 20 The Fe and S elemental content in a ferritin standard after centrifugal ultrafiltration using washed (3 x with 5% HNO_3 and 3 x with ultrapure water), dried (1 hour) and conditioned 100 kDa regenerated cellulose filters in the low (LMF) and high molecular weight fraction (HMF) was determined via external calibration using well characterized ferritin standards by FI-SEC-ICP-MS (30% O_2) and the Fe/S ratio was calculated. The concentration values are given with the relative standard deviation of *n = 3* measurements.

regenerated cellulose filter						
LMF	**S [ng mL^{-1}]**	**Fe [ng mL^{-1}]**	**HMF**	**S [ng mL^{-1}]**	**Fe [ng mL^{-1}]**	**Fe/S ratio**
1	< LOQ	< LOQ	1	539	8905	17
2	< LOQ	< LOQ	2	756	13589	18
3	< LOQ	< LOQ	3	515	9836	19
Average	< LOQ	< LOQ	Average	603	10777	18
SD	-	-	SD	133	2480	1
RSD [%]	-	-	RSD [%]	22	23	7

Table 21 The Fe and S elemental content in a ferritin standard before centrifugal ultrafiltration was determined via external calibration using well characterized ferritin standards by FI-SEC-ICP-MS (30% O_2) and the Fe/S ratio was calculated. The concentration values are given with the relative standard deviation of *n = 3* measurements.

original sample	**S [ng mL^{-1}]**	**Fe [ng mL^{-1}]**	**Fe/S ratio**
1	52	844	16
2	43	870	20
3	43	851	20
Average	46	855	19
SD	5	14	2
RSD [%]	11	2	12

Table 22 The Fe and S elemental content in a ferritin standard after centrifugal ultrafiltration using washed (3 x with 5% HNO_3 and 3 x with ultrapure water), dried (1 hour) and not conditioned 100 kDa polyethersulfone filters in the low (LMF) and high molecular weight fraction (HMF) was determined via external calibration using well characterized ferritin standards by FI-SEC-ICP-MS (30% O_2) and the Fe/S ratio was calculated. The concentration values are given with the relative standard deviation of *n = 3* measurements.

polyethersulfone filter						
LMF	**S [ng mL^{-1}]**	**Fe [ng mL^{-1}]**	**HMF**	**S [ng mL^{-1}]**	**Fe [ng mL^{-1}]**	**Fe/S ratio**
1	< LOQ	< LOQ	1	643	730	11
2	< LOQ	< LOQ	2	147	1345	9
3	< LOQ	< LOQ	3	283	3271	12
Average	< LOQ	< LOQ	Average	358	3970	11
SD	-	-	SD	256	3037	1
RSD [%]	-	-	RSD [%]	72	76	13

Table 23 The Fe and S elemental content in a ferritin standard after centrifugal ultrafiltration using washed (3 x with 5% HNO_3 and 3 x with ultrapure water), dried (1 hour) and conditioned 100 kDa polyethersulfone filters in the low (LMF) and high molecular weight fraction (HMF) was determined via external calibration using well characterized ferritin standards by FI-SEC-ICP-MS (30% O_2) and the Fe/S ratio was calculated. The concentration values are given with the relative standard deviation of *n = 3* measurements.

polyethersulfone filter						
LMF	**S [ng mL^{-1}]**	**Fe [ng mL^{-1}]**	**HMF**	**S [ng mL^{-1}]**	**Fe [ng mL^{-1}]**	**Fe/S ratio**
1	< LOQ	< LOQ	1	1670	30834	18
2	< LOQ	< LOQ	2	1819	34054	19
3	< LOQ	< LOQ	3	1827	31665	17
Average	< LOQ	< LOQ	Average	1772	32184	18
SD	-	-	SD	88	1672	1
RSD [%]	-	-	RSD [%]	5	5	4

Table 24 The Fe and S elemental content in a ferritin standard after centrifugal ultrafiltration using not washed and conditioned 100 kDa polyethersulfone filters in the low (LMF) and high molecular weight fraction (HMF) was determined via external calibration using well characterized ferritin standards by FI-SEC-ICP-MS (30% O_2) and the Fe/S ratio was calculated. The concentration values are given with the relative standard deviation of *n = 3* measurements.

polyethersulfone filter						
LMF	**S [ng mL^{-1}]**	**Fe [ng mL^{-1}]**	**HMF**	**S [ng mL^{-1}]**	**Fe [ng mL^{-1}]**	**Fe/S ratio**
1	< LOQ	< LOQ	1	1120	18752	17
2	< LOQ	< LOQ	2	1275	18592	15
3	< LOQ	< LOQ	3	1180	20352	17
Average	< LOQ	< LOQ	Average	1192	19232	16
SD	-	-	SD	78	973	1
RSD [%]	-	-	RSD [%]	7	5	9

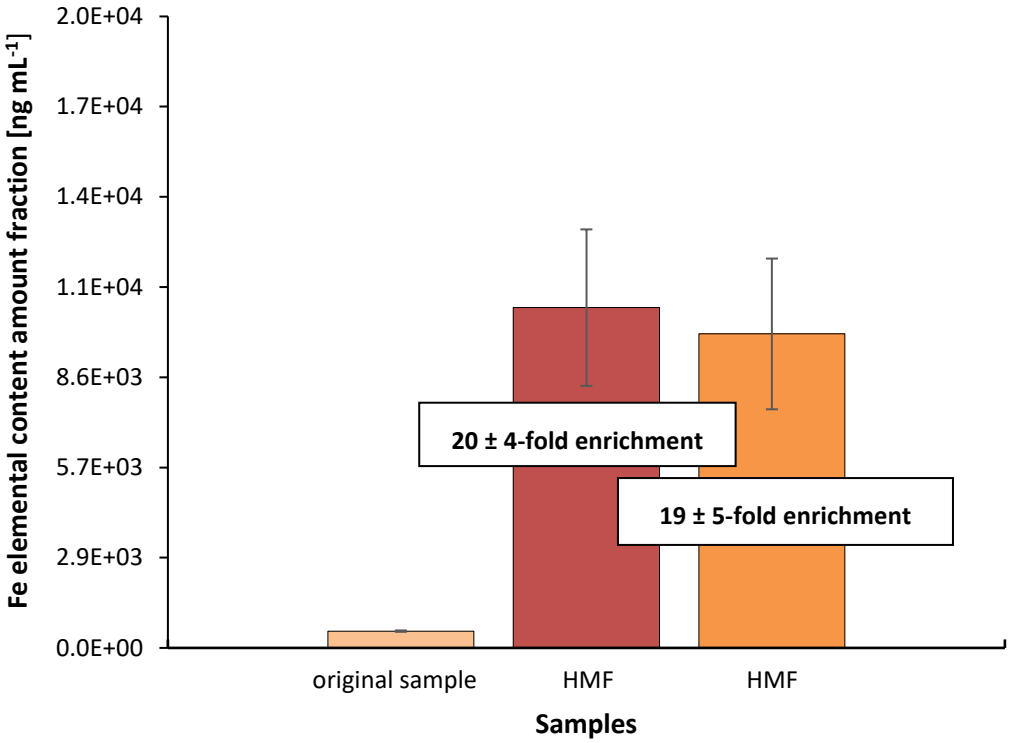

Figure 16 Fe elemental content in ferritin before (light orange bar) and after enrichment in the high molecular weight fraction (HMF) using washed (3 x with 5% HNO_3 and 3 x with ultrapure water), dried (1 hour) and not conditioned (dark orange bar)/conditioned (dark red bar) 100 kDa regenerated cellulose filters; error bars equal to 1 *SD (n = 3)*

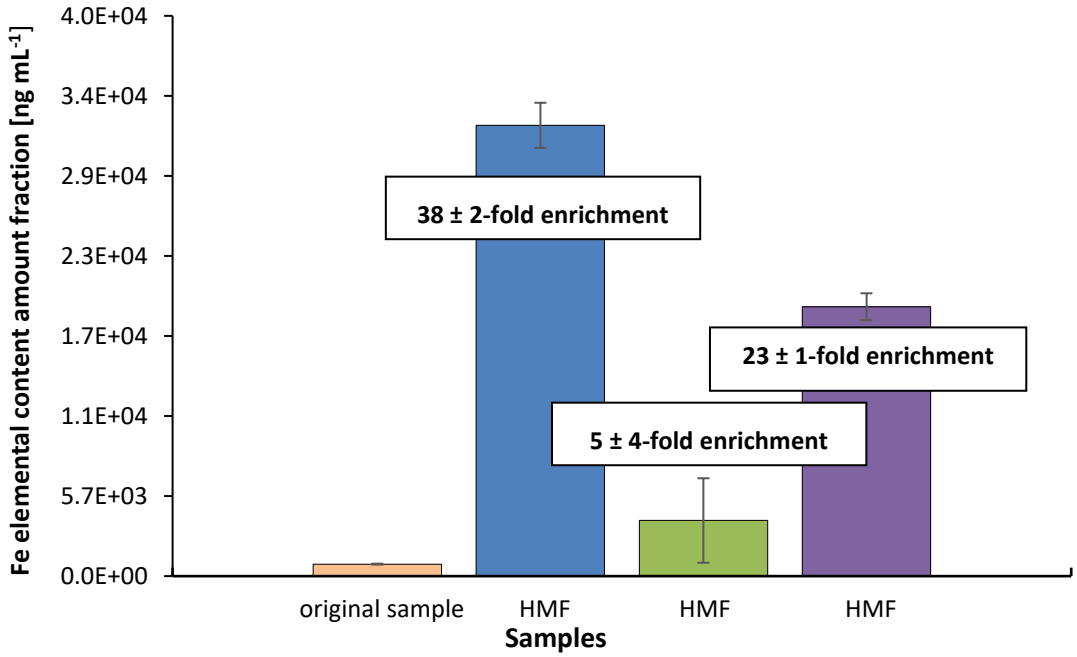

Figure 17 Fe elemental content in ferritin before (light orange bar) and after enrichment in the high molecular weight fraction (HMF) using washed (3 x with 5% HNO_3 and 3 x with ultrapure water), dried (1 hour) and not conditioned (green bar)/conditioned (dark blue bar) as well as not washed and conditioned (violet bar) 100 kDa polyethersulfone filters; error bars equal to 1 *SD (n = 3)*

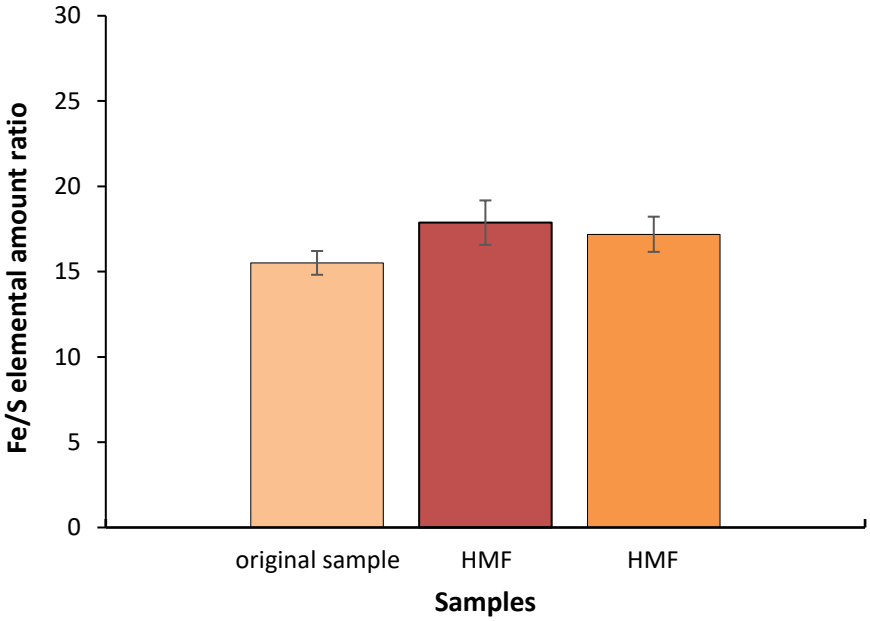

Figure 18 Fe/S elemental ratio in ferritin before (light orange bar) and after enrichment using washed (3 x with 5% HNO_3 and 3 x with ultrapure water), dried (1 hour) and not conditioned (dark orange bar)/conditioned (dark red bar) 100 kDa regenerated cellulose filters; error bars equal to 1 *SD (n = 3)*

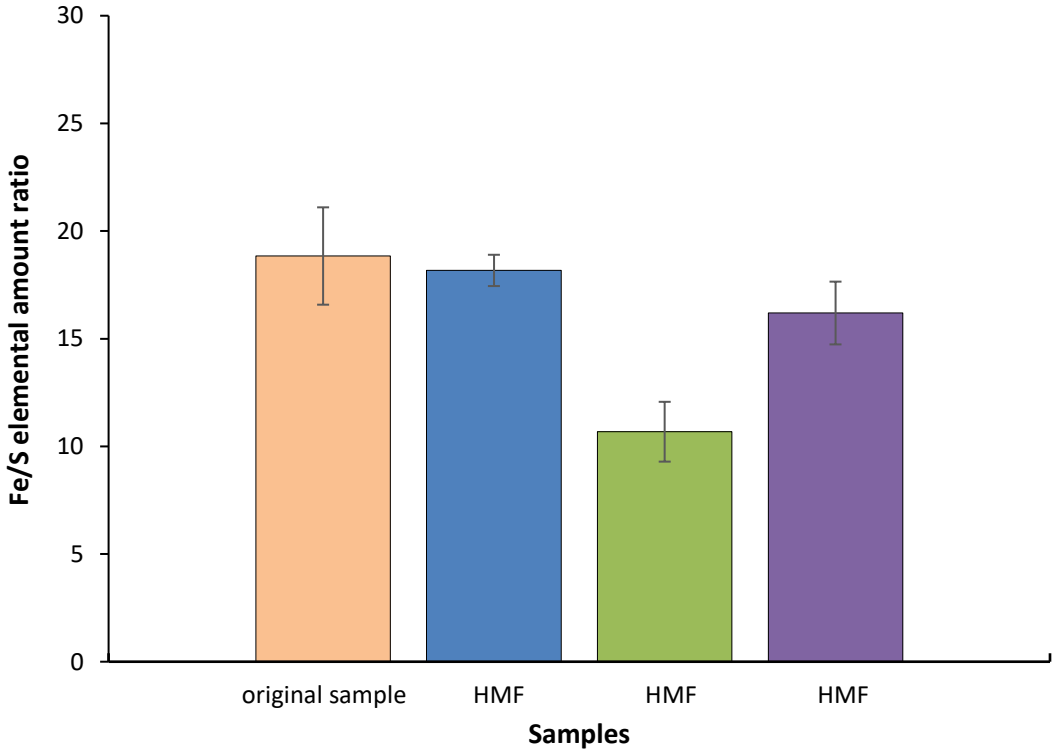

Figure 19 Fe/S elemental ratio in ferritin before (light orange bar) and after enrichment using washed (3 x with 5% HNO_3 and 3 x with ultrapure water), dried (1 hour) and not conditioned (green bar)/conditioned (dark blue bar) as well as not washed and conditioned (violet bar) 100 kDa polyethersulfone filters; error bars equal to 1 *SD (n = 3)*

Table 25 Summary of the procedural limit of quantification (LOQ) of iron and sulphur obtained with different filter preparation procedures for regenerated cellulose and polyethersulfone filters with the corresponding enrichment of ferritin

Filter preparation procedure	Analyte	Unit	Procedural LOQ (regenerated cellulose filter)	Procedural LOQ (polyethersulfone filter)	Enrichment of ferritin (regenerated cellulose filter)	Enrichment of ferritin (polyethersulfone filter)
1.	Fe	ng mL^{-1}	25.04	25.96	14 ± 4; 11 ± 2	23 ± 1
	S	ng mL^{-1}	69.18	81.35		
2.	Fe	ng mL^{-1}	1.36	84.54	9 ± 4	16 ± 3
	S	ng mL^{-1}	9.45	487.13		
3.	Fe	ng mL^{-1}	93.63	1.88	7 ± 2	8 ± 2
	S	ng mL^{-1}	97.47	18.59		
4.	Fe	ng mL^{-1}	9.11	36.16	6 ± 1	5 ± 1
	S	ng mL^{-1}	20.61	51.67		
5.	Fe	ng mL^{-1}	-	-	19 ± 5	5 ± 4
	S	ng mL^{-1}	-	-		
6.	Fe	ng mL^{-1}	12.21	74.93	20 ± 4	38 ± 2
	S	ng mL^{-1}	32.97	34.95		

Table 25 shows a summary of the procedural LOQ_{Fe} and LOQ_S obtained with different filter preparation procedures for regenerated cellulose and polyethersulfone filters with the corresponding enrichment of ferritin. To sum up, washing the filters 3 x with 5% HNO_3 and 3 x with ultrapure water in combination with drying (1 hour) and conditioning (with 50 mM ammonium acetate pH 6.8) of the filters has proven to be the best approach regarding a low procedural LOQ_{Fe} and LOQ_S and an optimum enrichment of ferritin. Therefore, this filter preparation procedure was applied to all the experiments.

4.2.3 Comparison of the enrichment of ferritin using regenerated cellulose and polyethersulfone filters

Regenerated cellulose and polyethersulfone filters were compared with each other in terms of the enrichment of ferritin. **Table 26**-**28** shows the total Fe and S content in ferritin as well as the Fe/S ratio of ferritin before and after centrifugal ultrafiltration using 100 kDa regenerated cellulose and polyethersulfone filters in the low and high molecular weight fraction. The Fe and S elemental content in ferritin was determined via external calibration using Fe-S standards by FI-ICP-QMS operated in reaction mode (30% O_2). The ferritin standard was diluted gravimetrically to a final concentration of 10.8 µg mL^{-1} ferritin with 50 mM ammonium acetate (pH 6.8) shortly before measurement. The ferritin sample was measured five times and the average Fe and S concentration was determined. The Fe and S elemental content in the original sample accounted to 1221 ng mL^{-1} Fe with a RSD of 5% and 57 ng mL^{-1} S with a RSD of 3%. The Fe and S content in the low molecular weight fraction using 100 kDa regenerated cellulose filter was below the procedural LOQ_{Fe} and LOQ_S of 12.21 ng mL^{-1} and 32.97 ng mL^{-1}. The Fe and S content in the low molecular weight fraction using 100 kDa polyethersulfone filters was below the procedural LOQ_{Fe} and LOQ_S of 74.93 ng mL^{-1} and 34.95 ng mL^{-1}. The Fe and S content in the high molecular weight fraction using 100 kDa regenerated cellulose filters was above the procedural LOQ, accounting to 12650 ng mL^{-1} Fe with a RSD of 21% and 621 ng mL^{-1} S with a RSD of 12%. The Fe and S content in the high molecular weight fraction using 100 kDa polyethersulfone filters was above the procedural LOQ, accounting to 23773 ng mL^{-1} Fe with a RSD of 19% and 1329 ng mL^{-1} S with a RSD of 9%. Centrifugal ultrafiltration using 100 kDa regenerated cellulose filters enabled a 10 ± 2-fold enrichment of ferritin. Whereas, 100 kDa polyethersulfone filters enabled a 19 ± 4-fold enrichment of ferritin **(figure 20)**. The Fe/S ratio of ferritin before and after the enrichment using 100 kDa regenerated cellulose filters indicated no significant Fe loss. However, a weak permeability of the 100 kDa polyethersulfone membrane for ferritin could be observed **(figure 21)**.

Table 26 The Fe and S elemental content in a ferritin standard before centrifugal ultrafiltration was determined via external calibration using Fe-S standards by FI-ICP-MS (30% O_2) and the Fe/S ratio was calculated. The concentration values are given with the relative standard deviation of *n = 3* measurements.

original sample	S [ng mL^{-1}]	Fe [ng mL^{-1}]	Fe/S ratio
1	56	1151	20
2	56	1235	22
3	59	1277	22
Average	57	1221	21
SD	2	64	1
RSD [%]	3	5	4

Table 27 The Fe and S elemental content in a ferritin standard after centrifugal ultrafiltration using 100 kDa regenerated cellulose and polyethersulfone filters in the low molecular weight fraction (LMF) was determined via external calibration using Fe-S standards by FI-ICP-MS (30% O_2) and the Fe/S ratio was calculated. The concentration values are given with the relative standard deviation of *n = 5* measurements.

regenerated cellulose filter			polyethersulfone filter		
LMF	S [ng mL^{-1}]	Fe [ng mL^{-1}]	LMF	S [ng mL^{-1}]	Fe [ng mL^{-1}]
1	< LOQ	< LOQ	1	< LOQ	< LOQ
2	< LOQ	< LOQ	2	< LOQ	< LOQ
3	< LOQ	< LOQ	3	< LOQ	< LOQ
4	< LOQ	< LOQ	4	< LOQ	< LOQ
5	< LOQ	< LOQ	5	< LOQ	< LOQ
Average	< LOQ	< LOQ	Average	< LOQ	< LOQ
SD	-	-	SD	-	-
RSD [%]	-	-	RSD [%]	-	-

Table 28 The Fe and S elemental content in a ferritin standard after centrifugal ultrafiltration using 100 kDa regenerated cellulose and polyethersulfone filters in the high molecular weight fraction (HMF) was determined via external calibration using Fe-S standards by FI-ICP-MS (30% O_2) and the Fe/S ratio was calculated. The concentration values are given with the relative standard deviation of *n = 5* measurements.

regenerated cellulose filter				polyethersulfone filter			
HMF	S [ng mL^{-1}]	Fe [ng mL^{-1}]	Fe/S ratio	HMF	S [ng mL^{-1}]	Fe [ng mL^{-1}]	Fe/S ratio
1	613	12226	20	1	1273	22808	18
2	528	9810	19	2	1443	26551	18
3	726	16668	23	3	1421	27824	20
4	637	13678	21	4	1180	17909	15
5	599	10869	18	5	-	-	-
Average	621	12650	20	Average	1329	23773	18
SD	71	2675	2	SD	125	4452	2
RSD [%]	12	21	10	RSD [%]	9	19	10

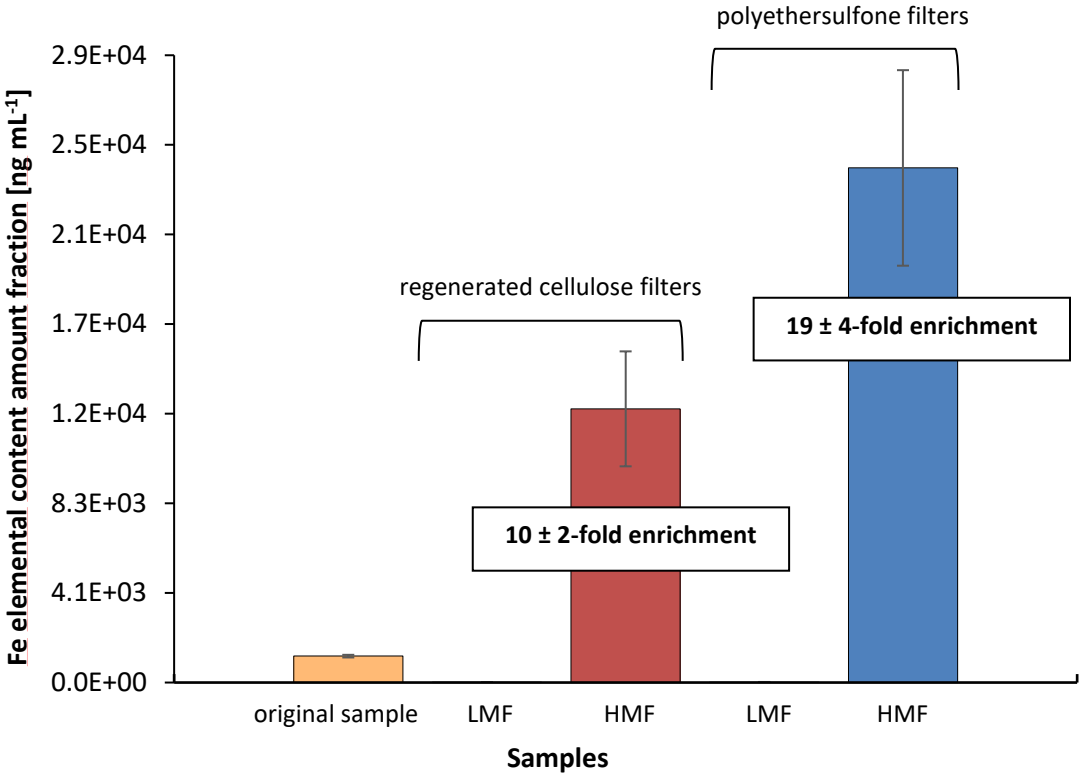

Figure 20 Fe elemental content in ferritin before (light orange bar) and after enrichment in the low (LMF) and high molecular weight fraction (HMF) using 100 kDa regenerated cellulose (dark red bar) and polyethersulfone filters (dark blue bar); error bars equal to 1 *SD (n = 5)*

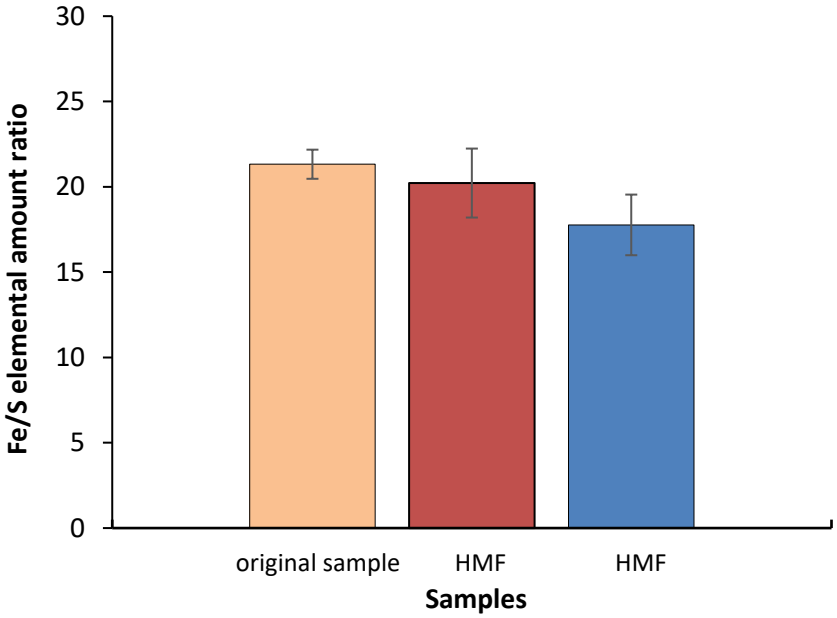

Figure 21 Fe/S elemental ratio in ferritin before (light orange bar) and after enrichment using 100 kDa regenerated cellulose (dark red bar) and polyethersulfone filters (dark blue bar); error bars equal to 1 *SD (n = 5)*

Table 29 and 30 shows the total Fe and S content in ferritin as well as the Fe/S ratio of ferritin before and after centrifugal ultrafiltration using 300 kDa polyethersulfone filters in the low and high molecular weight fraction. The Fe and S elemental content in ferritin was determined via external calibration using Fe-S standards by FI-ICP-QMS operated in reaction mode (30% O_2). The ferritin standard was diluted gravimetrically to a final concentration of 10 µg mL^{-1} ferritin with 50 mM ammonium acetate (pH 6.8) shortly before measurement. The ferritin sample was measured three times and the average Fe and S concentration was determined. The Fe and S elemental content in the original sample accounted to 330 ng mL^{-1} Fe with a RSD of 25% and 100 ng mL^{-1} S with a RSD of 34%. The Fe and S content in the low molecular weight fraction using 300 kDa polyethersulfone filters was above the procedural LOQ_{Fe} and LOQ_S of 32.83 ng mL^{-1} and 9.70 ng mL^{-1}. The Fe and S content in the high molecular weight fraction using 300 kDa polyethersulfone filters was above the procedural LOQ, accounting to 279 ng mL^{-1} Fe with a RSD of 51% and 155 ng mL^{-1} S with a RSD of 132%. Centrifugal ultrafiltration using 300 kDa polyethersulfone filters revealed that only 31 ± 8% of ferritin remained in the high molecular weight fraction and about 69 ± 8% of ferritin remained in the low molecular weight fraction (**figure 22**).

Table 29 The Fe and S elemental content in a ferritin standard before centrifugal ultrafiltration was determined via external calibration using Fe-S standards by FI-ICP-MS (30% O_2) and the Fe/S ratio was calculated. The concentration values are given with the relative standard deviation of *n = 3* measurements.

original sample	S [ng mL^{-1}]	Fe [ng mL^{-1}]
1	125	273
2	76	388
3	-	-
Average	100	330
SD	34	81
RSD [%]	34	25

Table 30 The Fe and S elemental content in a ferritin standard after centrifugal ultrafiltration using 300 kDa polyethersulfone filters in the low (LMF) and high molecular weight fraction (HMF) was determined via external calibration using Fe-S standards by FI-ICP-MS (30% O_2) and the Fe/S ratio was calculated. The concentration values are given with the relative standard deviation of *n = 3* measurements.

LMF	S [ng mL^{-1}]	Fe [ng mL^{-1}]	HMF	S [ng mL^{-1}]	Fe [ng mL^{-1}]
1	14	599	1	57	169
2	17	684	2	390	440
3	13	654	3	17	229
Average	15	646	Average	155	279
SD	2	43	SD	205	143
RSD [%]	13	7	RSD [%]	132	60

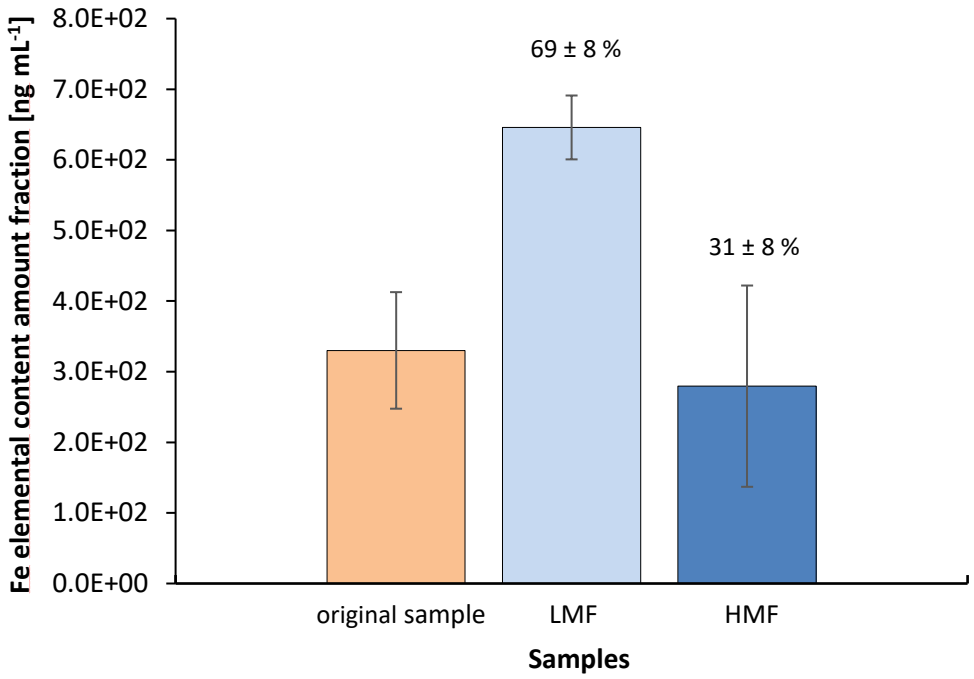

Figure 22 Fe elemental content in ferritin before (light orange bar) and after enrichment in the low (light blue bar) and high molecular weight fraction (dark blue bar) using 300 kDa polyethersulfone filters; error bars equal to 1 *SD (n = 3)*

To sum up, the proposed method using regenerated cellulose filters with a cut-off value of 100 kDa is fit for purpose for the enrichment of ferritin. However, polyethersulfone filters with a cut-off value of 300 kDa are not suitable for the enrichment of ferritin.

4.3 The fractionation of ferritin from transferrin and albumin by centrifugal ultrafiltration

The fractionation of ferritin from other iron containing biomolecules such as transferrin and albumin was performed by centrifugal ultrafiltration using 100 kDa regenerated cellulose and 300 kDa polyethersulfone filters. Therefore, the ferritin, transferrin and albumin standards were diluted gravimetrically in 50 mM ammonium acetate to a final concentration of 10.8 µg mL^{-1} ferritin, 1 mg mL^{-1} transferrin and 0.5 mg mL^{-1} albumin. The Fe and S elemental content in ferritin was determined via external calibration using well characterized ferritin standards by FI-SEC-ICP-QMS operated in reaction mode (30% O_2). The percentage of transferrin and albumin in the low and high molecular weight fraction was determined by integrating the iron trace of the transferrin and the sulphur trace of the albumin monomer peak in the high molecular weight fraction and comparing these obtained peak areas (counts) with the integrated iron trace of transferrin (counts) and sulphur trace of albumin (counts) in the low molecular weight fraction. **Table 31 and 32** shows the Fe and S content in ferritin before and after centrifugal ultrafiltration using 100 kDa regenerated cellulose filters in the low and high molecular weight fraction. The Fe content in ferritin before centrifugal ultrafiltration accounted to 1000 ng mL^{-1} with a RSD of 12%. The Fe content in ferritin in the low molecular weight fraction was below the procedural LOQ_{Fe} of 12.21 ng mL^{-1}. Whereas the Fe content in ferritin in the high molecular weight fraction accounted to 17296 ng mL^{-1} with a RSD of 22%.

Table 31 The Fe and S elemental content in ferritin before centrifugal ultrafiltration was determined via external calibration using well characterized ferritin standards by FI-SEC-ICP-MS (30% O_2). The concentration values are given with the relative standard deviation of *n = 3* measurements.

original sample	S [ng mL^{-1}]	Fe [ng mL^{-1}]
1	40	907
2	56	1131
3	55	963
Average	50	1000
SD	9	116
RSD [%]	17	12

Table 32 The Fe and S elemental content in ferritin after centrifugal ultrafiltration using 100 kDa regenerated cellulose filters in the low (LMF) and high molecular weight fraction (HMF) was determined via external calibration using well characterized ferritin standards by FI-SEC-ICP-MS (30% O_2). The concentration values are given with the relative standard deviation of *n = 3* measurements.

LMF	S [ng mL^{-1}]	Fe [ng mL^{-1}]	HMF	S [ng mL^{-1}]	Fe [ng mL^{-1}]
1	< LOQ	< LOQ	1	592	20322
2	< LOQ	< LOQ	2	443	18462
3	< LOQ	< LOQ	3	343	13102
Average	< LOQ	< LOQ	Average	459	17296
SD	-	-	SD	125	3749
RSD [%]	-	-	RSD [%]	27	22

Figure 23 shows the SEC chromatogram of ferritin (10.8 µg mL^{-1}) in the low and high molecular weight fraction. Ferritin showed a monomer peak at a retention time of 8.20 min and oligomer peaks. Centrifugal ultrafiltration using 100 kDa regenerated cellulose filter showed that about 99.6 ± 0.1% of ferritin remained in the high molecular weight fraction.

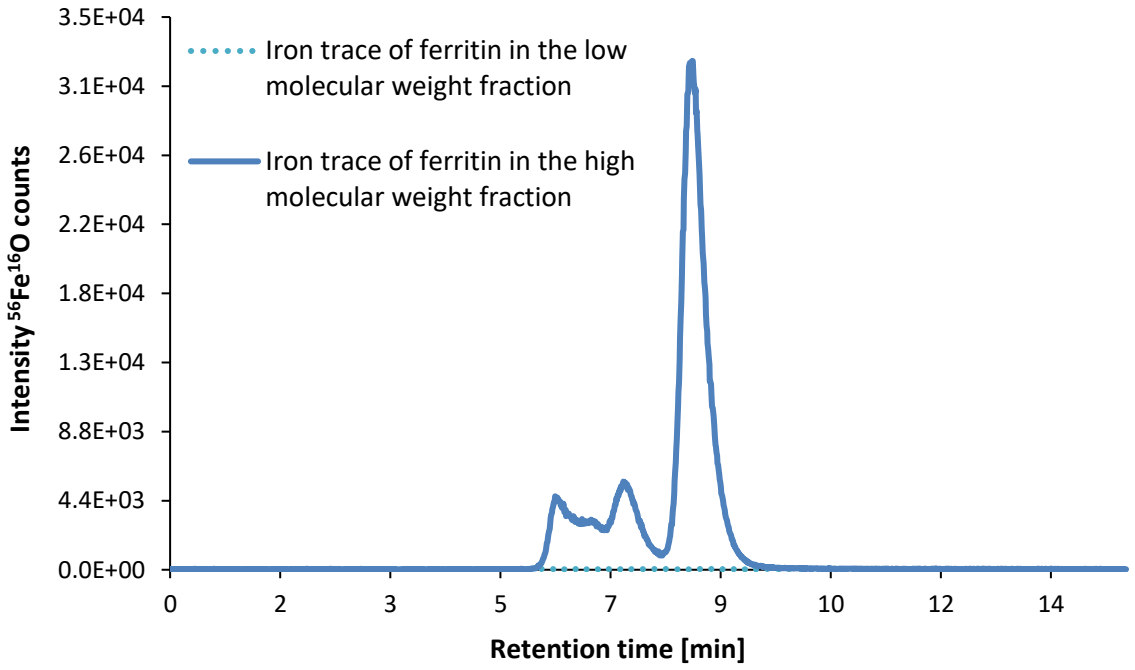

Figure 23 SEC chromatogram of ferritin (10.8 µg mL^{-1}) in the low (dotted blue line) and high molecular weight fraction (blue line); Fe was monitored at $^{56}Fe^{16}O$ (blue trace)

Figure 24 shows the SEC chromatogram of transferrin (1 mg mL^{-1}) in the low and high molecular weight fraction. Transferrin showed a monomer peak at a retention time of 9.86 min and an oligomer peak (RT 9.25 min). Centrifugal ultrafiltration using 100 kDa regenerated cellulose filters showed that about 94 ± 1% of transferrin remained in the high molecular weight fraction.

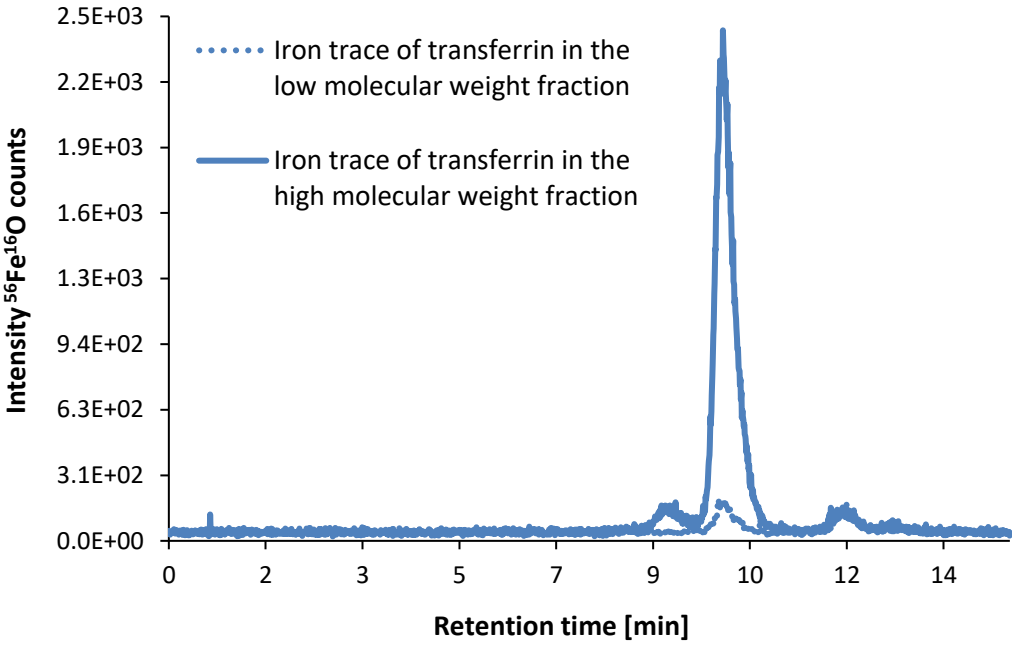

Figure 24 SEC chromatogram of transferrin (1 mg mL^{-1}) in the low (dotted blue line) and high molecular weight fraction (blue line); Fe was monitored at $^{56}Fe^{16}O$ (blue trace)

Figure 25 shows the SEC chromatogram of albumin (0.5 mg mL^{-1}) in the low and high molecular weight fraction. Albumin showed a monomer peak at a retention time of 9.70 min and oligomer peaks. Centrifugal ultrafiltration using 100 kDa regenerated cellulose filters showed that about 90 ± 1% of albumin remained in the high molecular weight fraction.

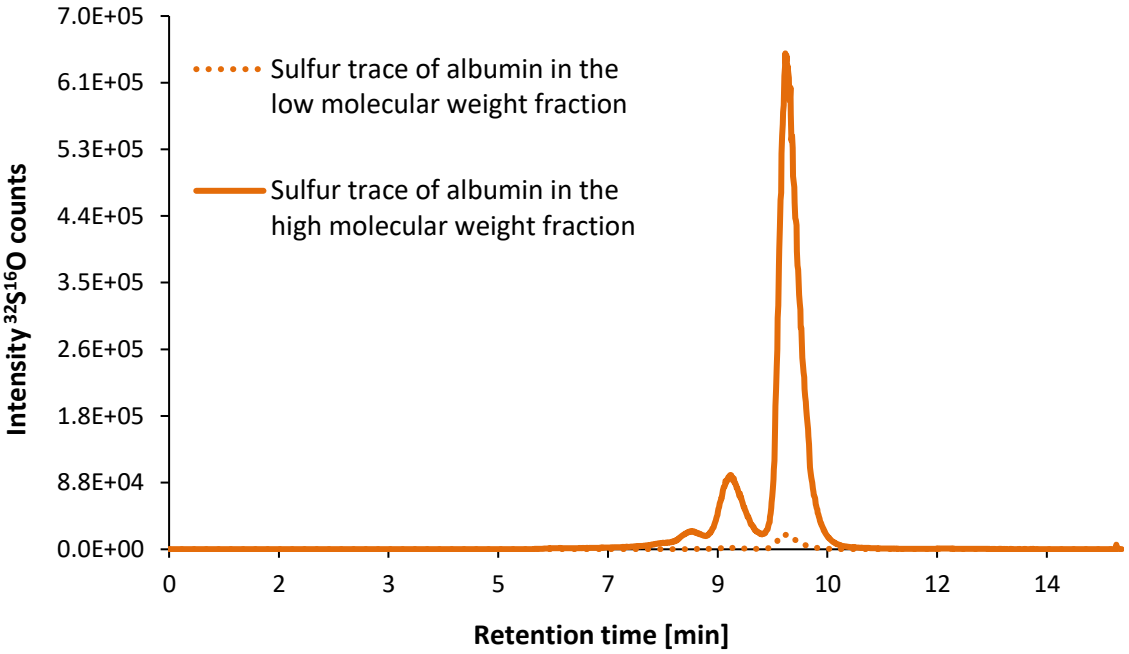

Figure 25 SEC chromatogram of albumin (0.5 mg mL^{-1}) in the low (dotted orange line) and high molecular weight fraction (orange line); S was monitored at $^{32}S^{16}O$ (orange trace)

Figure 26 and **27** shows the Fe intensity counts of transferrin and S intensity counts of albumin after centrifugal ultrafiltration using 300 kDa polyethersulfone filters in the low and high molecular weight fraction. Centrifugal ultrafiltration using 300 kDa polyethersulfone filters showed that about 85 ± 9% of transferrin remained in the high molecular weight fraction, whereas about 32 ± 12% of albumin remained in the high molecular weight fraction.

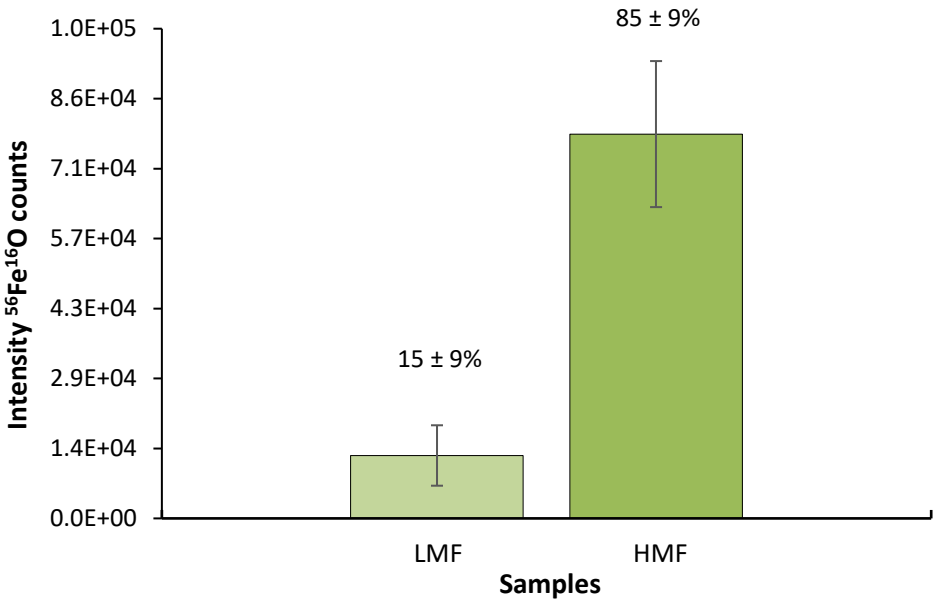

Figure 26 Fe intensity counts of transferrin after centrifugal ultrafiltration using 300 kDa polyethersulfone filters in the low (LMF, light green bar) and high molecular weight fraction (HMF, dark green bar); error bars equal to 1 *SD (n = 3)*

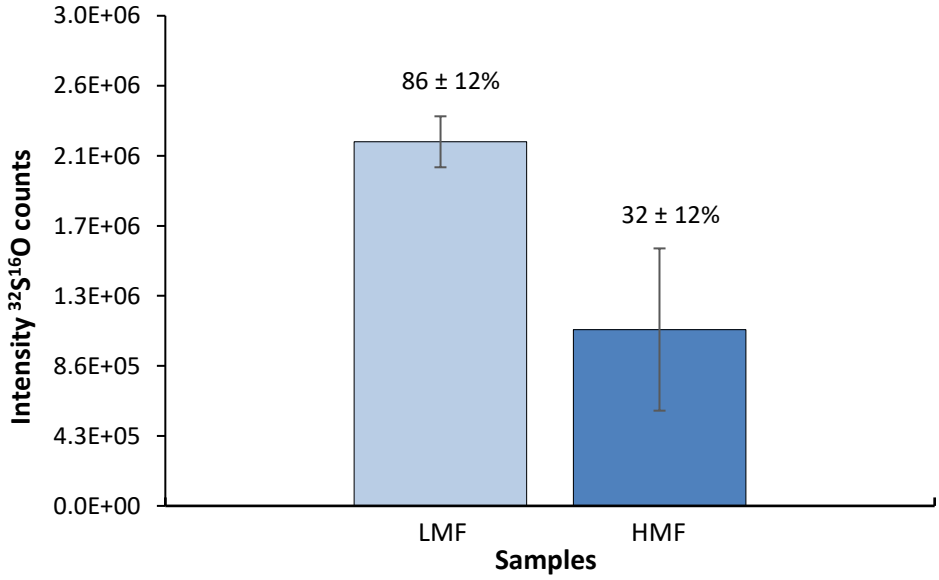

Figure 27 S intensity counts of albumin after centrifugal ultrafiltration using 300 kDa polyethersulfone filters in the low (LMF, light blue bar) and high molecular weight fraction (HMF, dark blue bar); error bars equal to 1 *SD (n = 3)*

To sum up, regenerated cellulose filters with a cut-off value of 100 kDa and polyethersulfone filters with a cut-off value of 300 kDa proved to be not suitable for the fractionation of ferritin from other iron containing biomolecules such as transferrin and albumin.

4.4 The fractionation of a synthetic serum by centrifugal ultrafiltration

The fractionation of a synthetic serum containing ferritin, transferrin and albumin was performed by centrifugal ultrafiltration using 3 kDa regenerated cellulose filters. Therefore, the ferritin, transferrin and albumin standards were diluted gravimetrically in 50 mM ammonium acetate to a final concentration of 10.8 µg mL^{-1} ferritin, 1 mg mL^{-1} transferrin and 0.4 mg mL^{-1} albumin. The Fe elemental content in ferritin was determined via external calibration using well characterized ferritin standards by FI-SEC-ICP-MS operated in reaction mode (30% O_2). The percentage of transferrin and albumin in the low and high molecular weight fraction was determined by integrating the iron trace of the transferrin and the sulphur trace of the albumin monomer peak in the high molecular weight fraction and comparing these obtained peak areas (counts) with the integrated iron trace of transferrin (counts) and sulphur trace of albumin (counts) in the low molecular weight fraction. **Table 33** shows the Fe content in ferritin before and after centrifugal ultrafiltration in the low and high molecular weight fraction. The Fe content in ferritin before centrifugal ultrafiltration accounted to 1669 ng mL^{-1} with a RSD of 8%. The Fe content in ferritin in the low molecular weight fraction was below the procedural LOQ_{Fe} of 12.21 ng mL^{-1}. Whereas the Fe content in ferritin in the high molecular weight fraction accounted to 3665 ng mL^{-1} with a RSD of 3%.

Table 33 The Fe elemental content in ferritin in a synthetic serum before and after centrifugal ultrafiltration in the low (LMF) and high molecular weight fraction (HMF) was determined via external calibration using well characterized ferritin standards by FI-SEC-ICP-MS (30% O_2). The concentration values are given with the relative standard deviation of *n = 3* measurements.

original sample	Fe [ng mL^{-1}]	LMF	Fe [ng mL^{-1}]	HMF	Fe [ng mL^{-1}]
1	1523	1	< LOQ	1	3790
2	1761	2	< LOQ	2	3657
3	1722	3	< LOQ	3	3547
Average	1669	Average	< LOQ	Average	3665
SD	128	SD	-	SD	122
RSD [%]	8	RSD [%]	-	RSD [%]	3

Figure 28 shows the SEC chromatogram of a synthetic serum containing ferritin (10.8 µg mL^{-1}), transferrin (1 mg mL^{-1}) and albumin (0.4 mg mL^{-1}) standards in 50 mM ammonium acetate before centrifugal ultrafiltration. The iron trace indicated that ferritin was well separated from other iron containing biomolecules such as transferrin and albumin and was present in the form of ferritin monomer (RT 7.8 min) and oligomers (RT 5.8-7.5 min), allowing the quantification of ferritin-bound iron. However, the determination of the sulphur content in ferritin was hampered by other iron containing biomolecules such as transferrin and albumin. This can be observed by the higher sulphur signal at retention time of 7.9-9.2 min, thus indicating the co-elution of ferritin with other iron containing biomolecules such as transferrin and albumin. The iron and sulphur trace indicated that transferrin and albumin coeluted, thus showing a monomer peak at a retention time of 9.5 min.

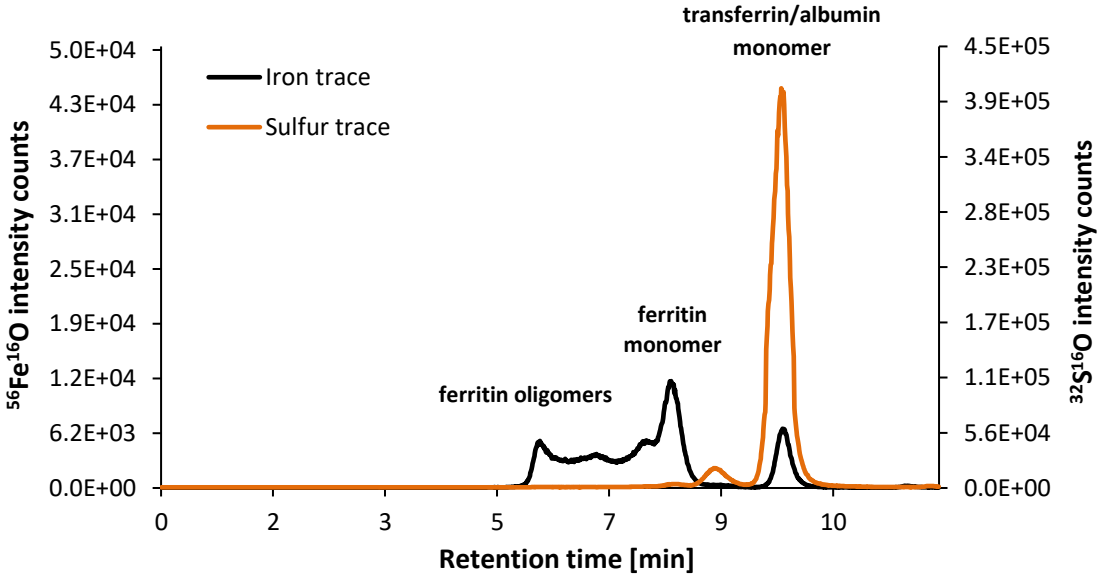

Figure 28 SEC chromatogram of a synthetic serum containing ferritin (10.8 µg mL^{-1}), transferrin (1 mg mL^{-1}) and albumin (0.4 mg mL^{-1}) standards in 50 mM ammonium acetate before centrifugal ultrafiltration; Fe and S were monitored at $^{56}Fe^{16}O$ (black trace) and $^{32}S^{16}O$ (orange trace), respectively

Figure 29 shows the SEC chromatogram of a synthetic serum containing ferritin (10.8 µg mL^{-1}), transferrin (1 mg mL^{-1}) and albumin (0.4 mg mL^{-1}) standards in 50 mM ammonium acetate after centrifugal ultrafiltration in the low molecular weight fraction. The iron trace indicated that about 0.59% of ferritin was found in the low molecular weight fraction. The sulphur trace indicated that a weak permeability of the regenerated cellulose

filter membrane for transferrin and albumin could be observed. Nevertheless, only 0.41% of transferrin and 0.34% of albumin was found in the low molecular weight fraction, indicating that the fractionation of the synthetic serum into a protein and a small molecule fraction by centrifugal ultrafiltration using regenerated cellulose filters with a cut-off value of 3 kDa was successful.

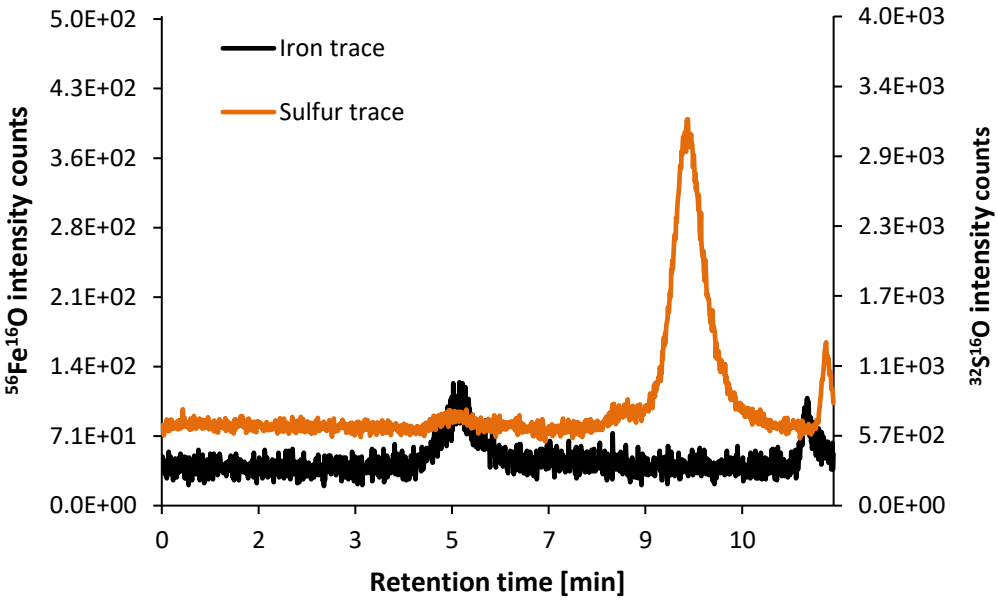

Figure 29 SEC chromatogram of a synthetic serum containing ferritin (10.8 µg mL^{-1}), transferrin (1 mg mL^{-1}) and albumin (0.4 mg mL^{-1}) standards in 50 mM ammonium acetate after centrifugal ultrafiltration in the low molecular weight fraction; Fe and S were monitored at $^{56}Fe^{16}O$ (black trace) and $^{32}S^{16}O$ (orange trace), respectively

Figure 30 shows the SEC chromatogram of a synthetic serum containing ferritin (10.8 µg mL^{-1}), transferrin (1 mg mL^{-1}) and albumin (0.4 mg mL^{-1}) standards in 50 mM ammonium acetate after centrifugal ultrafiltration in the high molecular weight fraction. By comparing the SEC chromatograms before and after centrifugal ultrafiltration, it can be observed that both SEC chromatograms look like as the same. After centrifugal ultrafiltration a higher iron and sulphur signal was obtained. Centrifugal ultrafiltration using 3 kDa regenerated cellulose filter showed that about 99.41% of ferritin, 99.59% of transferrin and 99.66% of albumin remained in the high molecular weight fraction. Consequently, the fractionation of the synthetic serum into a protein and a small molecule fraction by centrifugal ultrafiltration using regenerated cellulose filters with a cut-off value of 3 kDa proved to be

successful. Furthermore, centrifugal ultrafiltration using regenerated cellulose filters with a cut-off value of 3 kDa allowed a 2.4 ± 0.1-fold enrichment of ferritin (**figure 31**).

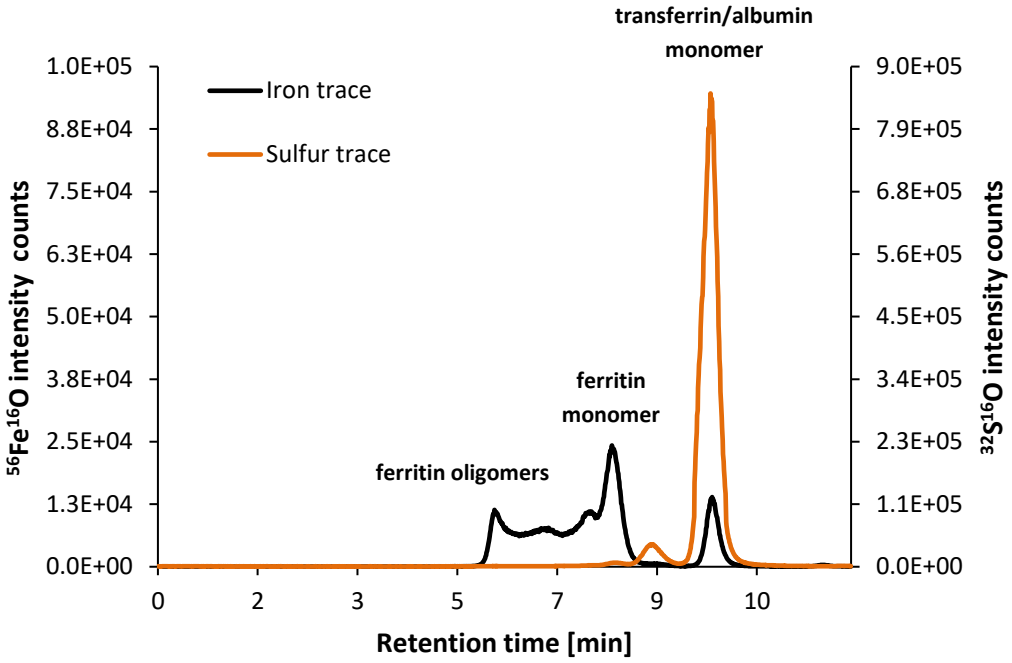

Figure 30 SEC chromatogram of a synthetic serum containing ferritin (10.8 µg mL^{-1}), transferrin (1 mg mL^{-1}) and albumin (0.4 mg mL^{-1}) standards in 50 mM ammonium acetate after centrifugal ultrafiltration in the high molecular weight fraction; Fe and S were monitored at $^{56}Fe^{16}O$ (black trace) and $^{32}S^{16}O$ (orange trace), respectively

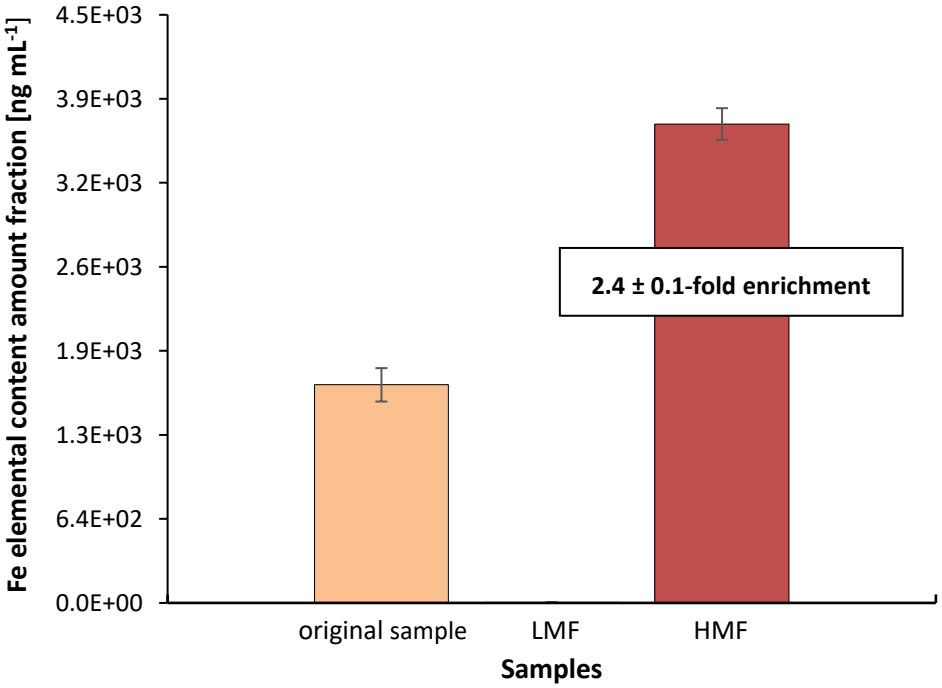

Figure 31 Fe elemental content in ferritin before (light orange bar) and after enrichment in the low and high molecular weight fraction (dark red bar) using 3 kDa regenerated cellulose filters; error bars equal to 1 *SD (n = 3)*

4.5 Application of the methodology to fetal calf serum and pig brain

The proposed method for the fractionation into a protein and a small molecule fraction using regenerated cellulose filters with a cut-off value of 3 kDa was applied to fetal calf serum and pig brain. **Figure 32** shows the SEC chromatogram of FCS (diluted 1:5 in 50 mM ammonium acetate pH 6.8) before centrifugal ultrafiltration. Transferrin and albumin showed a monomer peak (RT of 9.6 min and 9.4 min) and oligomer peaks (RT 5.8-8.5 min). The determination of the iron and sulphur content in ferritin was hampered by other iron containing biomolecules present in FCS such as transferrin and albumin. This can be observed by the higher iron and sulphur signal at retention time of 5.8-8.5 min, thus indicating the co-elution of ferritin with other iron containing biomolecules present in FCS.

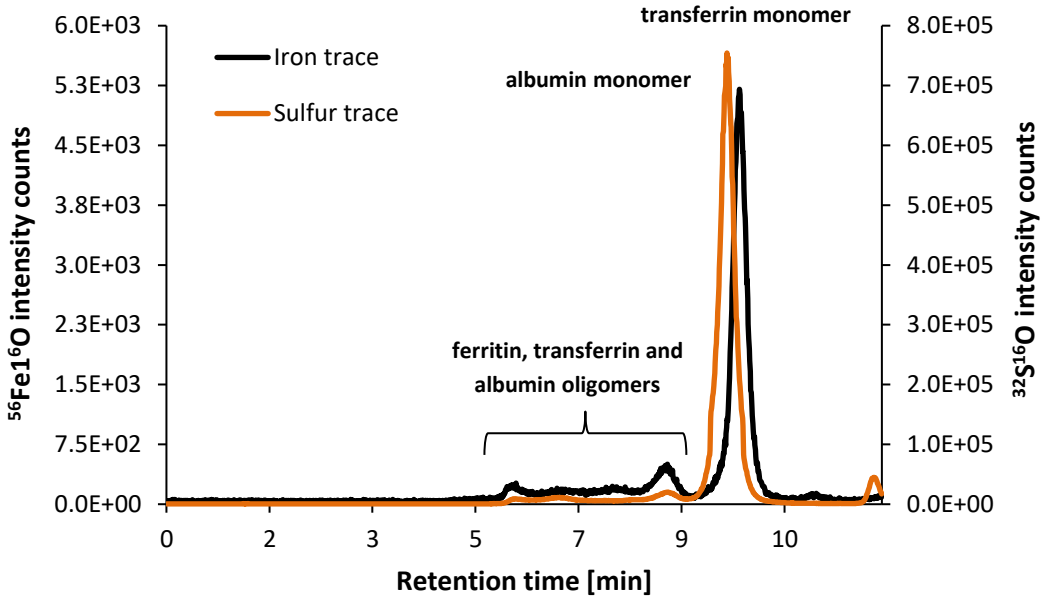

Figure 32 SEC chromatogram of FCS (diluted 1:5 in 50 mM ammonium acetate pH 6.8) before centrifugal ultrafiltration; Fe and S were monitored at $^{56}Fe^{16}O$ (black trace) and $^{32}S^{16}O$ (orange trace), respectively

Figure 33 shows the SEC chromatogram of FCS after centrifugal ultrafiltration in the low molecular weight fraction. The slight increase of the iron trace indicated a weak permeability of the regenerated cellulose filter membrane for ferritin and other iron containing biomolecules in FCS such as transferrin. The sulfur trace indicated that no albumin was found in the low molecular weight fraction.

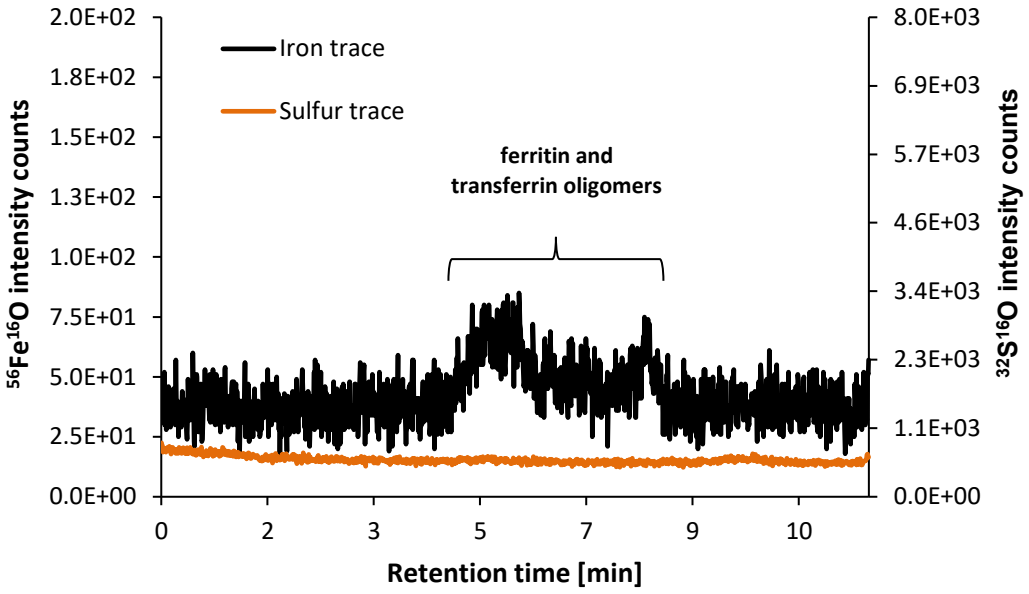

Figure 33 SEC chromatogram of FCS after centrifugal ultrafiltration in the low molecular weight fraction; Fe and S were monitored at $^{56}Fe^{16}O$ (black trace) and $^{32}S^{16}O$ (orange trace), respectively

Figure 34 shows the SEC chromatogram of FCS after centrifugal ultrafiltration in the high molecular weight fraction. As expected, by comparing the SEC chromatograms of FCS before and after centrifugal ultrafiltration, it can be observed that both chromatograms look like as the same. After centrifugal ultrafiltration a higher iron and sulphur signal was obtained, taking into account a dilution factor of 5 of FCS before and a dilution factor of 10 of FCS after centrifugal ultrafiltration. Centrifugal ultrafiltration using 3 kDa regenerated cellulose filters showed that about 99.78% of transferrin and 99.98% of albumin remained in the high molecular weight fraction.

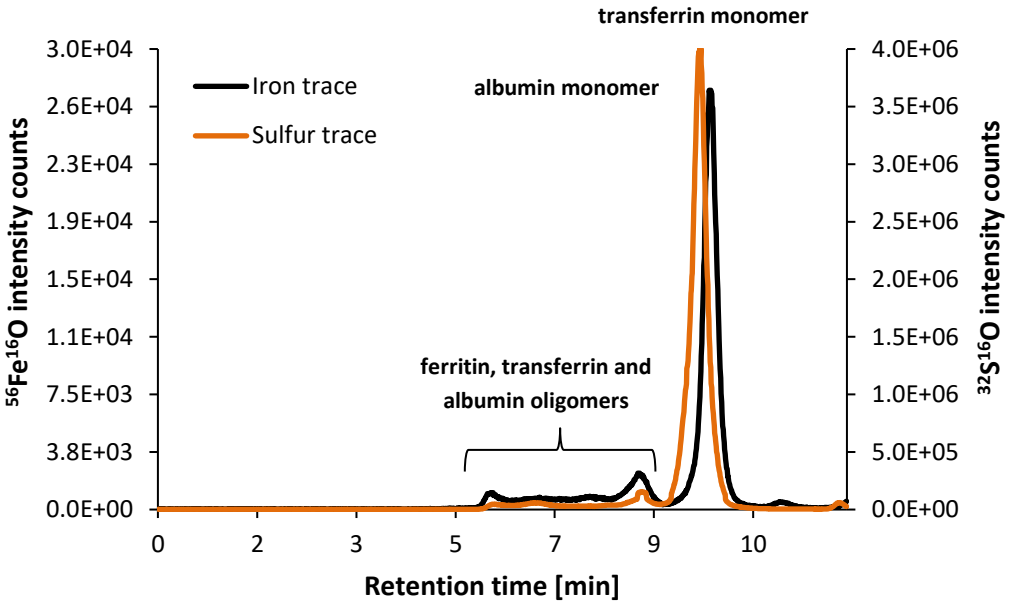

Figure 34 SEC chromatogram of FCS (diluted 1:10 in 50 mM ammonium acetate pH 6.8) after centrifugal ultrafiltration in the high molecular weight fraction; Fe and S were monitored at $^{56}Fe^{16}O$ (black trace) and $^{32}S^{16}O$ (orange trace), respectively

This study showed that the fractionation of fetal calf serum by centrifugal ultrafiltration using regenerated cellulose filters with a cut-off value of 3 kDa was successful. Chromatograms obtained by SEC-ICP-QMS showed that more than 99% of transferrin and albumin were found in the high molecular weight fraction. Consequently, the proposed method is fit for separation of serum into a protein and a small molecule fraction.

Table 34 shows the Fe content in ferritin in pig brain before and after centrifugal ultrafiltration in the low and high molecular weight fraction. The Fe elemental content in ferritin in pig brain was determined via external calibration using well characterized ferritin standards by FI-SEC-ICP-MS operated in reaction mode (30% O_2). The Fe content in ferritin before centrifugal ultrafiltration accounted to 83 ng mL^{-1} with a RSD of 1%. The Fe content in ferritin in the low molecular weight fraction was above the procedural LOQ_{Fe}, accounting to 75 ng mL^{-1} with a RSD of 27%. Whereas the Fe content in ferritin in the high molecular weight fraction accounted to 555 ng mL^{-1} with a RSD of 27%.

Table 34 The Fe elemental content in ferritin in pig brain before and after centrifugal ultrafiltration in the low (LMF) and high molecular weight fraction (HMF) was determined via external calibration using well characterized ferritin standards by FI-SEC-ICP-MS (30% O_2). The concentration values are given with the relative standard deviation of *n = 3* measurements.

original sample	Fe [ng mL^{-1}]	LMF	Fe [ng mL^{-1}]	HMF	Fe [ng mL^{-1}]
1	82	1	98	1	694
2	84	2	69	2	393
3	83	3	59	3	580
Average	83	Average	75	Average	555
SD	1	SD	20	SD	152
RSD [%]	1	RSD [%]	27	RSD [%]	27

Figure 35 shows the SEC chromatogram of pig brain before centrifugal ultrafiltration. The iron trace indicated that ferritin was well separated from other iron containing biomolecules and predominatly present in the form of ferritin monomer (RT 9.9 min). While ferritin-bound iron could be quantified, the determination of the sulphur content in ferritin was hampered by other iron containing biomolecules present in pig brain such as transferrin and albumin. This can be observed by the higher sulphur signal at retention time of 6.3-10.5 min, thus indicating the co-elution of ferritin with other iron containing biomolecules present in pig brain. The iron and sulphur trace indicated that neither transferrin nor albumin showed monomer and oligomer peaks. Transferrin showed only a small peak at a retention time of 12.0 min. The iron trace at a retention time of 13.7 min showed the monomer peak of myoglobin. This can be verified on the basis of the SEC chromatogram of the size ladder as reviewed by Schöberl which is shown in **figure 36** [46].

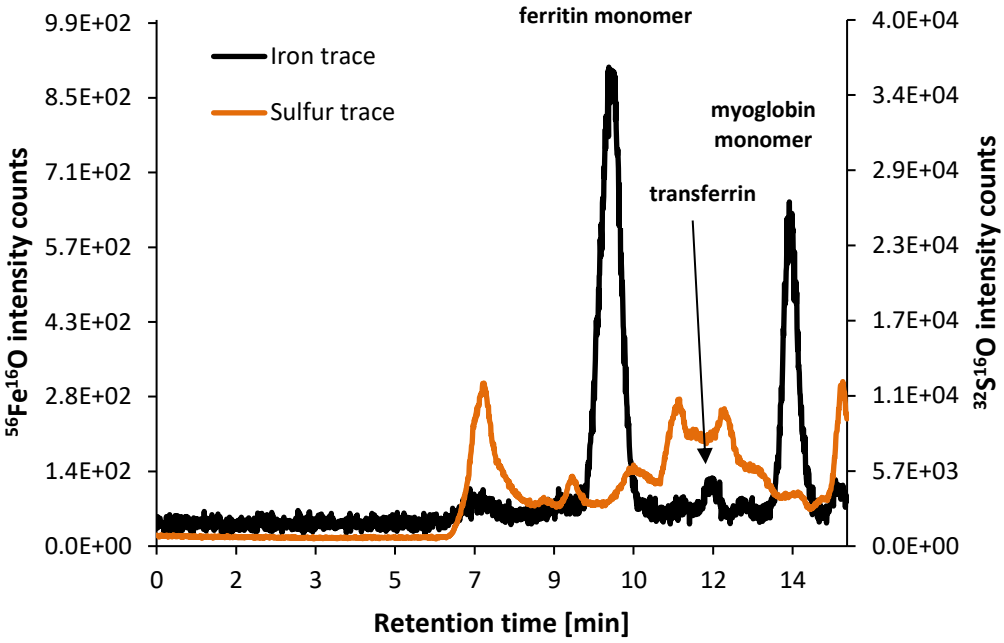

Figure 35 SEC chromatogram of pig brain before centrifugal ultrafiltration; Fe and S were monitored at $^{56}Fe^{16}O$ (black trace) and $^{32}S^{16}O$ (orange trace), respectively

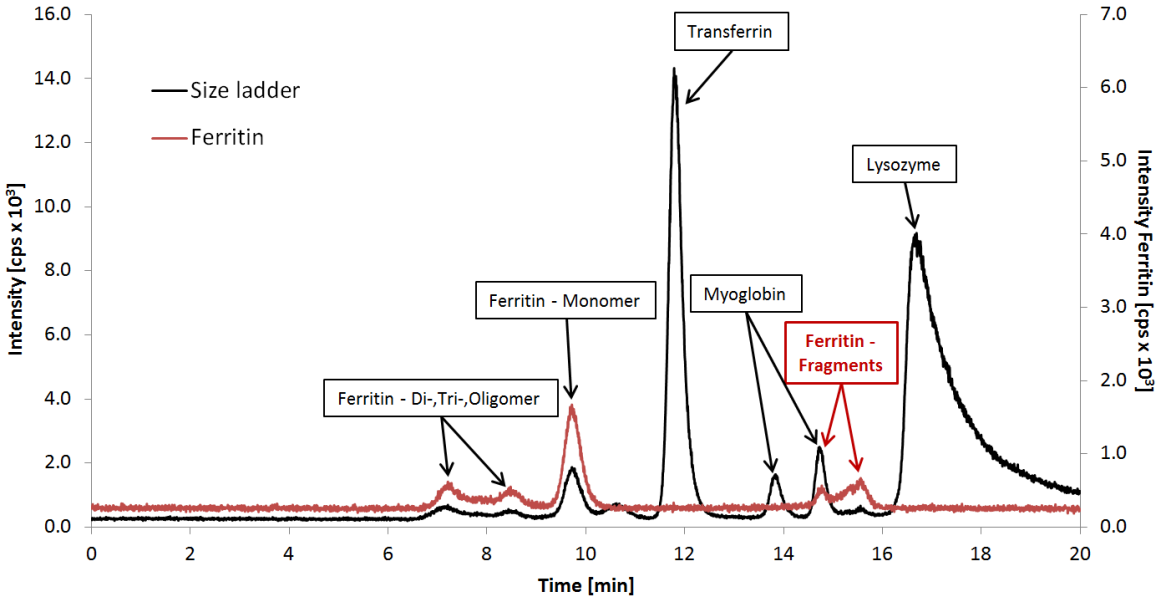

Figure 36 Size ladder. The $^{32}S^{16}O$ signal of ferritin (1 mg mL^{-1}), transferrin (1 mg mL^{-1}), myoglobin (1 mg mL^{-1}) and lysozyme (1 mg mL^{-1}) was measured and a size ladder was established. The molecular weight of the ferritin fragments was estimated [46].

Figure 37 shows the SEC chromatogram of pig brain after centrifugal ultrafiltration in the low molecular weight fraction. The slight increase of the iron trace indicated a weak permeability of the regenerated cellulose filter membrane for ferritin. About 18% of ferritin was found in the low molecular weight fraction. The sulfur trace indicated that no other iron containing biomolecules such as transferrin and albumin were found in the low molecular weight fraction.

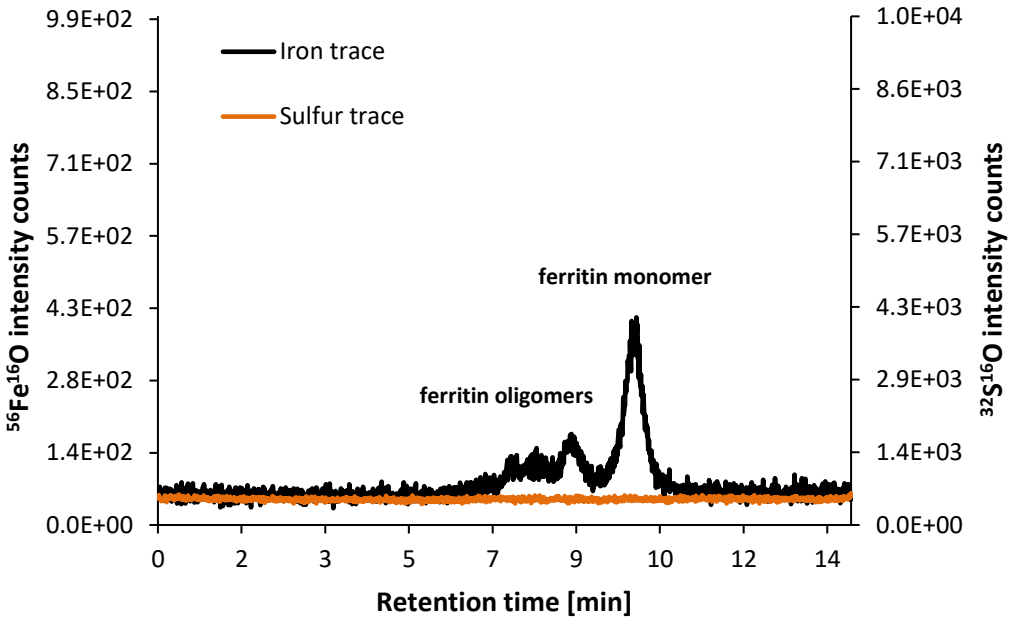

Figure 37 SEC chromatogram of pig brain after centrifugal ultrafiltration in the low molecular weight fraction; Fe and S were monitored at $^{56}Fe^{16}O$ (black trace) and $^{32}S^{16}O$ (orange trace), respectively

Figure 38 shows the SEC chromatogram of pig brain after centrifugal ultrafiltration in the high molecular weight fraction. As expected, by comparing the SEC chromatograms of pig brain before and after centrifugal ultrafiltration, a higher iron and sulphur signal was obtained. When comparing the SEC chromatogram before and after centrifugal ultrafiltration, it can be observed that all other iron containing biomolecules such as transferrin and albumin remained in the high molecular weight fraction. Centrifugal ultrafiltration using 3 kDa regenerated cellulose filter showed that about 82% of ferritin remained in the high molecular weight fraction.

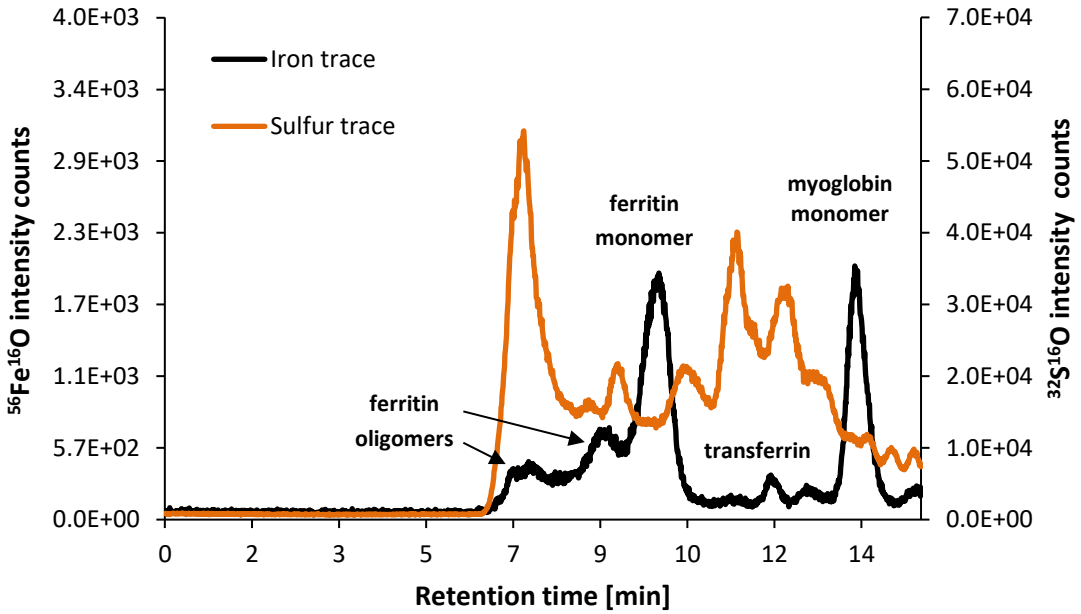

Figure 38 SEC chromatogram of pig brain after centrifugal ultrafiltration in the high molecular weight fraction; Fe and S were monitored at $^{56}Fe^{16}O$ (black trace) and $^{32}S^{16}O$ (orange trace), respectively

This study showed that the fractionation of pig brain by centrifugal ultrafiltration using regenerated cellulose filters with a cut-off value of 3 kDa was not successful. Chromatograms obtained by SEC-ICP-QMS showed that no iron containing biomolecules such as transferrin and albumin were found in the low molecular weight fraction, thus indicating that these biomolecules remained in the high molecular weight fraction. However, around 18% of ferritin was found in the low molecular weight fraction. Consequently, the proposed method is not fit for separation of pig brain into a protein and a small molecule fraction.

4.6 Application of the methodology for the separation of free iron from the ^{57}Fe-isotopically enriched ferritin spike

The proposed method using regenerated cellulose filters with a cut-off value of 100 kDa was applied for the separation of free iron from the ^{57}Fe-isotopically enriched ferritin spike. **Figure 39** shows the SEC chromatogram of the ^{57}Fe-isotopically enriched ferritin spike (diluted 1:20 in 50 mM ammonium acetate pH 6.8) after centrifugal ultrafiltration. The results indicate that the separation of free iron from the ^{57}Fe-isotopically enriched ferritin spike by centrifugal ultrafiltration using regenerated cellulose filters with a cut-off value of 100 kDa was successful. This can be seen on the basis of the very low ^{57}Fe background in the SEC chromatogram between 10 to 15.5 minutes (see below for more details).

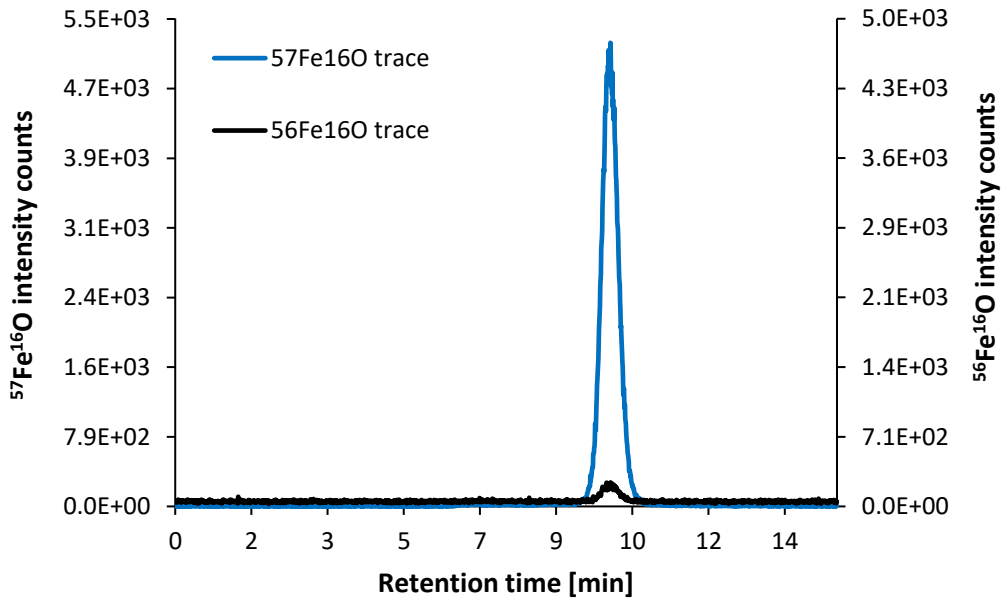

Figure 39 SEC chromatogram of the ^{57}Fe-isotopically enriched ferritin spike (diluted 1:20 in 50 mM ammonium acetate pH 6.8) after separation of free iron by centrifugal ultrafiltration using regenerated cellulose filters with a cut-off value of 100 kDa; Fe was monitored at $^{56}Fe^{16}O$ (black trace) and $^{57}Fe^{16}O$ (blue trace), respectively

In comparison to **figure 40** which shows the SEC chromatogram of the isotopically enriched ^{57}Fe-ferritin spike before centrifugal ultrafiltration, it can be observed that a part of iron is present in free form and is not bound at all to the ^{57}Fe-ferritin spike. This can be seen on the basis of the increased ^{57}Fe background in the SEC chromatogram between 12 to 20 minutes [46].

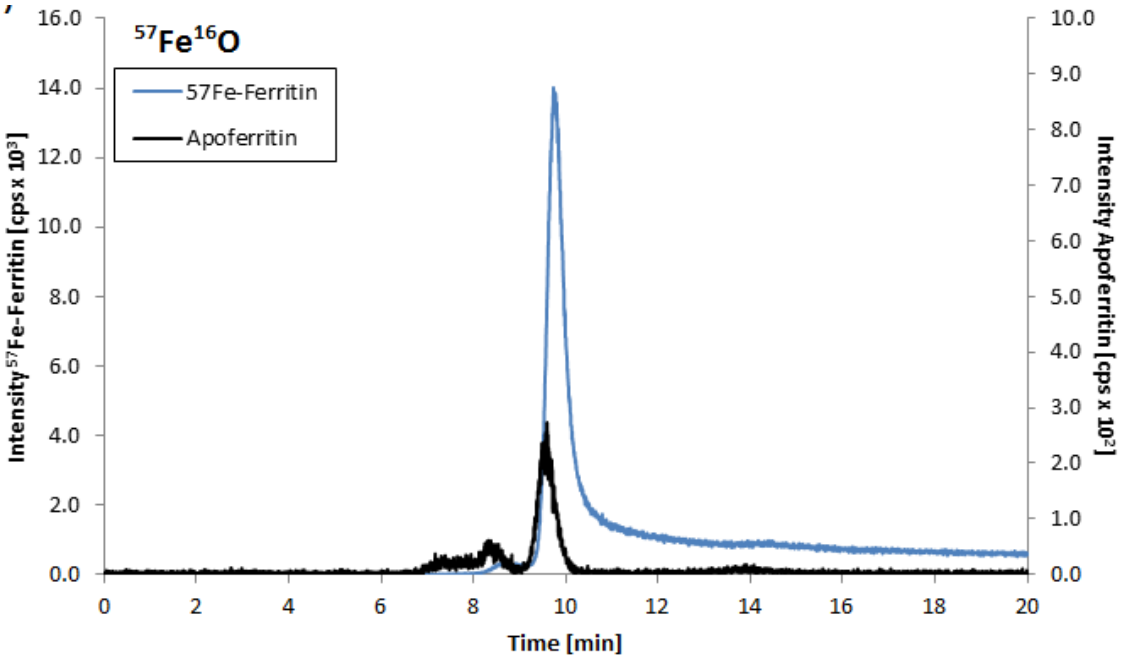

Figure 40 SEC chromatogram of the ^{57}Fe-isotopically enriched ferritin spike before centrifugal ultrafiltration; the $^{57}Fe^{16}O$ trace of the ^{57}Fe-isotopically enriched ferritin spike (blue trace) was monitored [46]

5 Summary and Conclusion

This work demonstrated that washing (3 x with 5% HNO_3 and 3 x with ultrapure water), drying (1 hour) and conditioning of filters has proven to be the best approach regarding a low procedural LOQ_{Fe} and LOQ_S and an optimum enrichment of ferritin. Polyethersulfone filters allowed a two-fold enrichment of ferritin in comparison to regenerated cellulose filter. The proposed method using filters with a cut-off value of 100 kDa is fit for purpose for the enrichment of ferritin. In contrast, polyethersulfone filters with a cut-off value of 300 kDa proved to be not suitable for the enrichment of ferritin. Neither filters with a cut-off value of 100 kDa nor 300 kDa were suitable for the fractionation of ferritin from other iron containing biomolecules such as transferrin and albumin.

The fractionation of a synthetic serum and fetal calf serum using regenerated cellulose filters with a cut-off value of 3 kDa was successful. Consequently, the proposed method is fit for separation of serum into a protein and a small molecule fraction. However, the fractionation of pig brain using regenerated cellulose filters with a cut-off value of 3 kDa proved to be not successful. Consequently, the proposed method is not fit for separation of pig brain into a protein and a small molecule fraction. Furthermore, centrifugal ultrafiltration using regenerated cellulose filters with a cut-off value of 100 kDa allowed a successful separation of free iron from the ^{57}Fe-isotopically enriched ferritin spike.

6 Outlook

Further research should focus on the development of an off-line enrichment and fractionation method for ferritin by immunoaffinity chromatography.

7 References

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8 Appendix

A. Standard Operating Procedure

17/10/2019 **15HLT02**

DELIVERABLE – SOP

Grant Agreement number	15HLT02 ReMIND
Project short name	ReMind
Project full title	**Role of metals and metal containing biomolecules in neurodegenerative diseases such as Alzheimer's disease**
Version numbers of latest contracted Annex 1 and Annex 2 against which the assessment will be made	Annex 1: V# Annex 2: V#
Period covered (dates)	From 01July 2016 To 26 July 2019

For JRPs:

Project start date and duration:	1 July 2016, 36 months	
Coordinator: Dr. Claudia Swart, PTB, Bundesallee 100, DE-38116 Braunschweig, Germany, +49 531-5923150, claudia.swart@ptb.de Project website address: *https://www.ptb.de/emrp/remind-home.html*		
Internal Funded Partners: 1 PTB, Germany 2 BAM, Germany 3 LGC, UK 4 TUBITAK, Turkey	External Funded Partners: 5 Charité, Germany 6 UGent, Belgium 7 UNIABDN, UK 8 UNIVIE, Austria	Unfunded Partners: 9 10 11 12

Standard Operating Procedure
Off-line fractionation of serum using centrifugal ultrafiltration

Abbreviations

CH_3COONH_4	Ammonium acetate
HNO_3	Nitric acid
ICP-QMS	Inductively coupled plasma quadrupole mass spectrometry
PFA	Perfluoroalkoxy alkanes
SEC	Size exclusion chromatography
SOP	Standard operating procedure

1. Scope and applicability

This SOP describes the fractionation of serum by centrifugal ultrafiltration using regenerated cellulose filter with a cut-off value of 3 kDa. The cut-off value of the filters is chosen to be well below the molecular weight of ferritin (> 450 kDa), transferrin (80 kDa) and albumin (66 kDa). This metal containing proteins should be recovered in the retentate (i.e. high molecular weight fraction), while molecules with a molecular weight of < 3 kDa should be recovered in the filtrate (i.e. low molecular weight fraction). The obtained low and high molecular weight fraction after centrifugal ultrafiltration are used for determination of the metal and heteroelement content via external calibration by flow injection inductively coupled plasma spectrometry (FI-ICP-QMS).

2. Important considerations

The sample preparation and measurements need to be conducted in a clean room. All consumables have to be double acid washed before use (10 % HNO_3 *w*/*w*, 1 % HNO_3 *w*/*w*).

3. Materials

3.1. Apparatus

Tab. 1: Instrumentation

Instrumentation	Manufacturer
ICS-5000+ Ion Chromatography system	Thermo Fisher Scientific, USA
8800 ICP-QMS	Agilent, USA
Vortex mixer Vortex V-1 plus	Biosan SIA, Latvia
Microbalance CPA225D	Sartorius Weighing Technology GmbH, Germany
pH meter 16461276	SI analytics GmbH, Germany
Magnetic stirrer RSM-02HP	PHOENIX Instrument, Germany
Microliter Centrifuge Z 216 MK	HERMLE Labortechnik GmbH, Germany
Water purification system	Veolia Water Solutions and Technologies, France
Piston pipette (0.10-10 µL, 10-100 µL, 100-1000 µL, 1000-10000 µL)	Eppendorf, Germany
Magnetic stirring bean	Labxperts, Austria

3.2. Consumables

- Disposable pipette tips (0.5-20 µL, 2-200 µL, 50-1000 µL, 1000-10000 µL) (e.g. Eppendorf, Germany, cat code 10370842, 10248621, 10708972, 10333682)
- Manual disposable pestles (e.g from VWR, cat code 231-0106)
- Polypropylen tubes, 10 mL, with caps (e.g. from Labxperts, cat code BIOL-109151)
- Cetra beakers (e.g. from VWR, 26 mL, PP, cat code SARS75.568)
- 1000 mL PFA bottle (e.g. from chemoline, 1000 mL, cat code 50-1970)
- 50 mL PP bottle (e.g. from AHF, Analysentechnik, Germany, cat code T61-050)
- UFC 5003 BK Amicon Ultra-0.5 mL Centrifugal Filter Device (equipped with 1.5 mL microcentrifuge tubes, filter material: regenerated cellulose, 3 kDa, e.g. from Wagner and Munz GmbH Austria, Germany, cat code 6269410)

4. Reagents

4.1. Chemicals

- Nitric acid (Rotipuran Supra 69 %, e.g. from VWR, USA, cat code 1.00441.1000, Hazards: H272-H290-H314-H331, EUH071, Precautions: P280-P301+P330+P331-P304+P340-P305+P351+P338-P308+P310)
- Acetic acid (Rotipuran Supra 100 %, e.g. from Lactan, cat code RHN55.1, Hazards: 226-290-314, Precautions: 210-280-301+330+331+305+351+338-308+310)
- Ammoniac (supra pure 25 %, e.g. from VWR, USA, cat code 1.05428.0500)
- Ultrapure water (18.2 MΩ cm Elga,Veolia Water Solutions and Technologies, France)

4.2. Safety considerations

During all preparation steps the wearing of safety coat, safety glasses and gloves is required. The safety data sheets have to read before starting of experimental work. When handling with biological samples from human/animal origin, contaminated surfaces have to be disinfected immediately with disinfection solutions (e.g. Isopropanol, Merck, Germany) and contaminated clothes and gloves have to be removed. Contaminated waste has to be discarded in containers for this kind of waste. Nitric acid and acetic acid have to handled in a fume hood.

4.3. Preparation of reagent solution

Preparation of 50 mM ammonium acetate : For the preparation of 50 mM ammonium acetate (pH 6.8) fill a 1000 mL PFA bottle to 800 mL with laboratory water type 1, insert a magnetic stirring bean and place the bottle on a magnetic stirrer (260 rpm). Add 7.76 mL of ammonia and 2.89 mL of acetic acid and mix. Adjust the pH to 6.8 with acetic acid by pipetting continuously 50 µL of acetic acid to the buffer solution until the desired pH is reached. For the detection of the pH use a pH meter. When the desired pH is reached, fill the bottle to 1000 mL with ultra-pure water and mix the buffer solution well by shaking it manually up and down.

Preparation of 5 % HNO_3: For the preparation of the 5 % HNO_3 solution, mix 42.40 mL of laboratory water type 1 and 3.60 mL of a 69 % supra pure nitric acid in a 50 mL PP bottle.

5. Transport and storage of samples

The serum sample has to be kept at 2-8 °C in the fridge. (Do not freeze the serum samples for ferritin analysis). Supra pure nitric acid, hydrochloric acid and acetic acid can be kept at room temperature. Ammoniac supra pure have to be kept at 2-8 °C in the fridge. The 50 mM ammonium acetate buffer and the 5 % HNO_3 solution can be kept at room temperature. While the 5 % HNO_3 solution is stable for many months, the 50 mM ammonium acetate buffer should be prepared freshly every week.

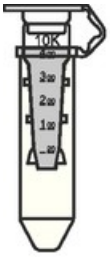
6. Procedure

6.1. Schematic of the procedure

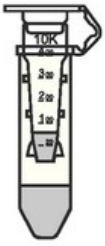
Preparation of filters for fractionation

1. Wash filter 3 x with 5 % HNO_3 and 3 x with high purity deionized water → 2. Let filter dry for 1 hour → 3a. Centrifuge 500 µL of 50 mM CH_3COONH_4 buffer (pH 6.8) at 15000 g for 10 min at 4 °C in order to condition the filter.
3b. Use filtrate as procedural blank

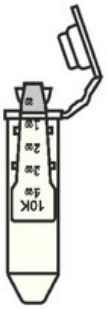
Fractionation



4. Fill 500 µL of the serum sample in the clean filter

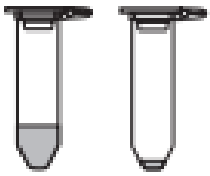


5. Centrifuge sample at 15000 g for 10 min at 4 °C



6. Turn filter upside down in a clean 1.5 mL microcentrifuge tube

7. Centrifuge at 1000 g for 2 min at 4 °C (5 acc/dec)



8. Collect the low and high molecular weight fraction

9. Analyse collected fractions with FI-ICP-QMS for metal/heteroelement content

6.2. Experimental

Washing and conditioning of filters

In the first step, wash the filters 3 times with high purity (18 MΩ cm) deionized water (with a 1 mL pipette tip). After washing 3 times with 5 % HNO_3 rinse the filter 3 times with high purity deionized water. After each washing step turn the filters upside down and remove the rest solution by knocking onto the device 5-10 times. Place the filter upright into a 1.5 mL microcentrifuge tube and let the filter dry at room temperature for about an hour.
For the conditioning step, pipette 500 µL of the 50 mM CH_3COONH_4 buffer (pH 6.8) into the filter and centrifuge at 15000 g 4 °C for 10 min (5 acc/dec). Then turn the filter upside down into the same 1.5 mL microcentrifuge tube and centrifuge for a second time at 1000 g 4 °C for 2 min (5 acc/dec). Use the collected low and high molecular weight fraction as procedural blank for the measurement by mixing both fractions.

Fractionation of serum

In the first step, weigh a 10 mL tube with cap and add 320 µL of a serum sample (with a 1000 µL pipette tip). Weigh the tube inclusive the serum sample again. Add 1.280 mL of 50 mM CH_3COONH_4 buffer (with a 10 mL pipette tip) and weigh the tube inclusive the 1:5 diluted serum sample a second time. Mix the serum sample well.

Transfer 500 µL of the prepared serum sample (with a 1 mL pipette tip) into the cleaned and conditioned filter equipped with a 1.5 mL microcentrifuge tube and subject it to centrifugal ultrafiltration by centrifuging at 15000 g at 4 °C for 10 min (5 acc/dec). Take out the filter of the tube, turn it upside down and place it into a new 1.5 mL microcentrifuge tube and centrigure at 1000 g for 2 min (4 °C, 5 acc/dec). Use the low and high molecular weight fraction for further analysis.

The determination of the total metal (magnesium, iron, aluminum, copper, zinc, cadmium and lead) content is performed by FI-ICP-MS using an ICS-5000+ Ion Chromatography system coupled to an ICP-QMS operated in no gas mode and reaction mode (30 % O_2) following the procedure of Theiner et al. [1].

7. Documentation

Write all sample preparation steps (reagents, weighting, …), method parameter (frequency of washing the filter, drying time of filter, centrifugation force/time ,…) as well as all observations in the laboratory journal.

8. References

[1] S. Theiner et al., ´FI-ICP-TOFMS for high-throughput and low volume multi-element analysis in environmental and biological matrices´, J. Anal. At. Spectrom., DOI: 10.1039/c9ja00022d, 2019.

B. Report

17/10/2019 **15HLT02**

REPORT - Task A2.2.1

Grant Agreement number	15HLT02 ReMIND
Project short name	ReMind
Project full title	**Role of metals and metal containing biomolecules in neurodegenerative diseases such as Alzheimer's disease**
Version numbers of latest contracted Annex 1 and Annex 2 against which the assessment will be made	Annex 1: V# Annex 2: V#
Period covered (dates)	From 01July 2016 To 26 July 2019

For JRPs:

Project start date and duration:	1 July 2016, 36 months	
Coordinator: Dr. Claudia Swart, PTB, Bundesallee 100, DE-38116 Braunschweig, Germany, +49 531-5923150, claudia.swart@ptb.de Project website address: *https://www.ptb.de/emrp/remind-home.html*		
Internal Funded Partners: 1 PTB, Germany 2 BAM, Germany 3 LGC, UK 4 TUBITAK, Turkey	External Funded Partners: 5 Charité, Germany 6 UGent, Belgium 7 UNIABDN, UK 8 UNIVIE, Austria	Unfunded Partners: 9 10 11 12

17/10/2019 15HLT02

HTL02 ReMiND WP2

Task A2.2.1(UNIVIE).

Report - Off-line fractionation of serum by centrifugal ultrafiltration

1 Introduction

This report describes the off-line fractionation of fetal calf serum by centrifugal ultrafiltration using regenerated cellulose filter with a cut-off value of 3 kDa. It provides the metal and heteroelement content in the low and high molecular weight fraction, which was determined by flow injection size exclusion chromatography inductively coupled plasma quadrupole mass spectrometry (FI-SEC-ICP-QMS) using external calibration.

2 Materials and methods

Preparatory work and elemental analysis were performed at the University of Vienna, Department of Analytical Chemistry in an ISO class 8 clean room according to ISO 14644-1. An ammonium acetate buffer 50 mM pH 6.8 was prepared from laboratory water type I (18 MΩ cm, Veolia Water Solutions, France), ammoniac (25 %, supra pure, Avantor, USA) and acetic acid (100 %, suprapure, Carl Roth, Germany) and used as solvent and mobile phase. All laboratory consumables (polyethylene bottles, tubes, pipette tips) were double acid washed (10 % and 1 % HNO_3 *w*/*w*) before use. For the size ladder a ferritin standard from equine spleen (F4503-100MG, Lot# SLBW3977, 61 mg mL^{-1} ferritin), a transferrin standard from human blood plasma (≤ 95 % grade, 90190-100MG, Lot# BCBJ0304) and an albumin standard from human serum (lyophilized powder, essentially fatty acid free , ≤ 97 % grade, A3782-10G, CAS No. 70024-90-7) were used (all achieved from Sigma-Aldrich, USA). For the size ladder the standards were diluted in 50 mM ammonium acetate to a final concentration of 20 µg mL^{-1} ferritin, 10 mg mL^{-1} transferrin and 1 mg mL^{-1} albumin.

Off-line fractionation by centrifugal ultrafiltration was performed on the example of fetal calf serum (FCS) obtained from Merck KGaA, Germany. FCS was delivered frozen,

thawed and stored at 4 – 8 °C before use. The FCS sample was centrifuged (15000 g, 4 °C, 10 min; Z 216 MK, Hermle, Germany) to separate the precipitate and the supernatant solution. The supernatant FCS solution was diluted gravimetrically 1:5 using ammonium acetate 50 mM shortly before fractionation.

Fractionation of serum was performed by centrifugal ultrafiltration. Therefore, membrane filters (regenerated cellulose, Amicon, Wagner and Munz GmbH Austria, Germany) with a cut-off value of 3 kDa were washed 3 times with 5 % HNO_3 and 3 times with high purity deionized water. After each washing step the remaining solution was removed from the filter by knocking 5-10 times. After the washing procedure the filters were dried at room temperature for about one hour. Subsequently, the filters were conditioned with 500 µL ammonium acetate buffer 50 mM pH 6.8 (15000 g, 4 °C for 10 min, 5 acc/dec, Hermle). The filter was turned upside down into the same microcentrifuge tube and centrifuged for a second time at 1000 g, 4 °C for 2 min (5 acc/dec, Hermle). The pooled fractions were used as procedural blank for the FI-ICP-QMS measurement.

Serum samples (500 µL) were applied onto the conditioned filters and centrifuged at 15000 g at 4 °C for 10 min (5 acc/dec, Hermle). The filtrate was collected as low molecular weight fraction. To obtain the high molecular weight fraction, the filter was placed upside down into a new microcentrifuge tube and centrifuged at 1000 g, 4 °C for 2 min (5 acc/dec, Hermle).

The total magnesium, aluminium, calcium, iron, copper, zinc, cadmium and lead elemental content in the serum sample before centrifugation as well as the low and high molecular weight fraction was determined via external calibration using Seronorm L-1 and multielemental standards by FI-ICP-QMS (ICP-QMS 8800, Agilent, USA; 5000+ Ion Chromatography system, Thermo Fisher Scientific, USA) in no gas mode and in gas mode using oxygen as reaction gas (30 % O_2). The protein pattern of the original sample as well as the low and high molecular weight fraction were determined by SEC-ICP-QMS (SEC MabPac, Thermo Scientific, USA; ICP-QMS 8800 and 1260 Infitinty bioinert, both from Agilent, USA) using oxygen as reaction gas (30 % O_2). The sample volume accounted to 5 µL and the flow rate to 250 µL min^{-1}. Instrumental conditions are summarized in table 1.

The percentage of transferrin and albumin in the low and high molecular weight fraction was determined by integrating the iron trace of the transferrin and the sulphur trace of the albumin monomer peak in the high molecular weight fraction and comparing these

obtained peak areas (counts) with the integrated iron trace of transferrin (counts) and sulphur trace of albumin (counts) in the low molecular weight fraction.

Table 1 ICP-MS operation and chromatographic conditions used

Sample introduction	flow injection
Sample volume	5 µL
Flow rate	250 µL min^{-1}
Mobile phase	50 mM CH_3COONH_4, pH 6.8
Column	MabPac SEC, PEEK, 4 x 300 mm, 5 µm, 10 - 1000 kDa
Nebulizer	MicroFlow PFA-ST
Spray chamber	Scott double-pass
Nebulizer gas flow	1.07 L min^{-1}
Auxiliary gas flow	0.90 L min^{-1}
Plasma gas flow	15 L min^{-1}
ICP RF power	1550 W
Cones	Ni
Reaction gas	Oxygen (30 %)
Analytes	$^{32}S^{16}O$, $^{54}Fe^{16}O$, $^{56}Fe^{16}O$, $^{57}Fe^{16}O$, $^{58}Fe^{16}O$
Integration time	0.1 s

3 Results

Table 2 shows the total Mg, Al, Ca, Fe, Cu, Zn, Cd and Pb content in fetal calf serum (FCS) before and after centrifugal ultrafiltration in the low and high molecular weight fraction determined in the no gas mode. The Al, Fe, Zn, Cd and Pb content in the low molecular weight fraction was below the LOQ. The Al and Fe content in the high molecular weight fraction was below the LOQ. The Al, Cd and Pb content in FCS before centrifugal ultrafiltration was below the LOQ.

Table 2 The metal (Mg, Al, Ca, Fe, Cu, Zn, Cd and Pb) content in fetal calf serum (FCS) before and after centrifugal ultrafiltration in the low and high molecular weight fraction was determined via external calibration using Seronorm L-1 standards by FI-ICP-QMS (no gas mode).

Sample	Analyte	Unit	LOQ	Replicates	Measured value	SD	RSD
low molecular weight fraction	Mg	µg g^{-1}	2.50E-01	*n* = 3	22.1	1.6	7 %
	Al	ng g^{-1}	7.20E+01	*n* = 3	< LOQ	< LOQ	< LOQ
	Ca	mg g^{-1}	3.56E-03	*n* = 3	0.098	0.004	5 %
	Fe	ng g^{-1}	3.72E+03	*n* = 3	< LOQ	< LOQ	< LOQ
	Cu	µg g^{-1}	1.13E-02	*n* = 3	0.138	0.007	5 %
	Zn	ng g^{-1}	2.75E+01	*n* = 3	< LOQ	< LOQ	< LOQ
	Cd	ng g^{-1}	2.00E-01	*n* = 3	< LOQ	< LOQ	< LOQ
	Pb	ng g^{-1}	1.22E+00	*n* = 3	< LOQ	< LOQ	< LOQ
high molecular weight fraction	Mg	µg g^{-1}	2.50E-01	*n* = 3	35	7	21 %
	Al	ng g^{-1}	7.20E+01	*n* = 3	< LOQ	< LOQ	< LOQ
	Ca	mg g^{-1}	3.56E-03	*n* = 3	0.196	0.007	4 %
	Fe	ng g^{-1}	3.72E+03	*n* = 3	< LOQ	< LOQ	< LOQ
	Cu	µg g^{-1}	1.13E-02	*n* = 3	0.85	0.38	45 %
	Zn	µg g^{-1}	2.75E-02	*n* = 3	64	33	51 %
	Cd	ng g^{-1}	2.00E-01	*n* = 3	0.42	0.22	53 %
	Pb	ng g^{-1}	1.22E+00	*n* = 3	2.9	2.6	89 %
original sample	Mg	µg g^{-1}	2.50E-01	*n* = 3	28	5	17 %
	Al	ng g^{-1}	7.20E+01	*n* = 3	< LOQ	< LOQ	< LOQ
	Ca	mg g^{-1}	3.56E-03	*n* = 3	0.111	0.019	18 %
	Fe	µg g^{-1}	3.72E+00	*n* = 3	5.3	0.9	17 %
	Cu	µg g^{-1}	1.13E-02	*n* = 3	0.49	0.19	40 %
	Zn	µg g^{-1}	2.75E-02	*n* = 3	2.08	0.33	16 %
	Cd	ng g^{-1}	2.00E-01	*n* = 3	< LOQ	< LOQ	< LOQ
	Pb	ng g^{-1}	1.22E+00	*n* = 3	< LOQ	< LOQ	< LOQ

Table 3 shows the total Fe and Mg content in fetal calf serum (FCS) before and after centrifugal ultrafiltration in the low and high molecular weight fraction determined in the oxygen mode.

Table 3 The iron and magnesium content in FCS before and after centrifugal ultrafiltration in the low and high molecular weight fraction was determined via one-point external calibration using multielemental standards by FI-ICP-QMS (30 % O_2).

Sample	Analyte	Unit	LOQ	Replicates	Measured value	SD	RSD
low molecular weight fraction	Fe	ng g^{-1}	4.54E+00	*n* = 3	22	5	22 %
	Mg	µg g^{-1}	2.58E-01	*n* = 3	32.6	1.6	5 %
high molecular weight fraction	Fe	µg g^{-1}	4.54E-03	*n* = 3	13.2	1.7	13 %
	Mg	µg g^{-1}	2.58E-01	*n* = 3	59	5	9 %
original sample	Fe	µg g^{-1}	4.54E-03	*n* = 3	2.2	0.8	38 %
	Mg	µg g^{-1}	2.58E-01	*n* = 3	34	10	30 %

Figure 1 shows the iron trace of ferritin, transferrin and albumin used for the determination of a size ladder. The iron trace indicated that ferritin showed predominantly a monomer peak (t_r 7.9 min). Furthermore, oligomer peaks of ferritin could be observed (t_r 5.8 min and 6.8 min). Transferrin showed a monomer peak at a retention time of 9.6 min. The iron signal of albumin was too low.

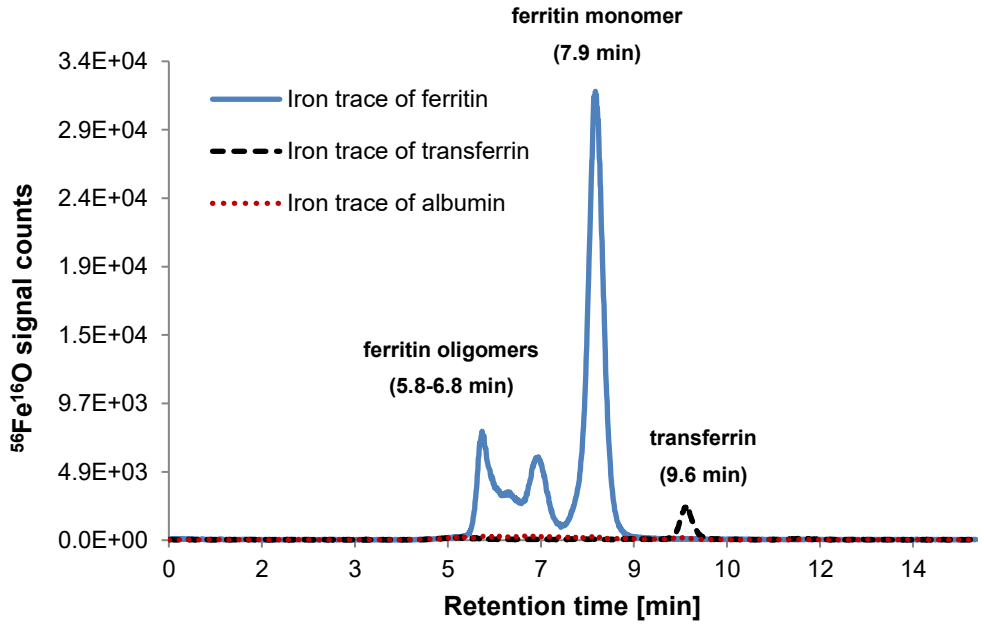


Figure 1 SEC chromatogram of a size ladder. Iron trace of ferritin (20 µg g^{-1}, blue trace), transferrin (10 mg g^{-1}, black trace) and albumin (1 mg g^{-1}, red trace) standards in 50 mM ammonium acetate measured consecutively using an IC Dionex 5000 sample introduction system and a SEC MabPac column coupled to a Agilent 8800 ICP-QMS operated in reaction mode (30 % oxygen)

Figure 2 shows the sulfur trace of the SEC chromatogram of the size ladder. The sulfur trace indicated that transferrin and albumin were predominantly present in monomer form (t_r 9.6 min and t_r 9.4 min), but also showed oligomer peaks (t_r 9.0 min and t_r 8.9 min).

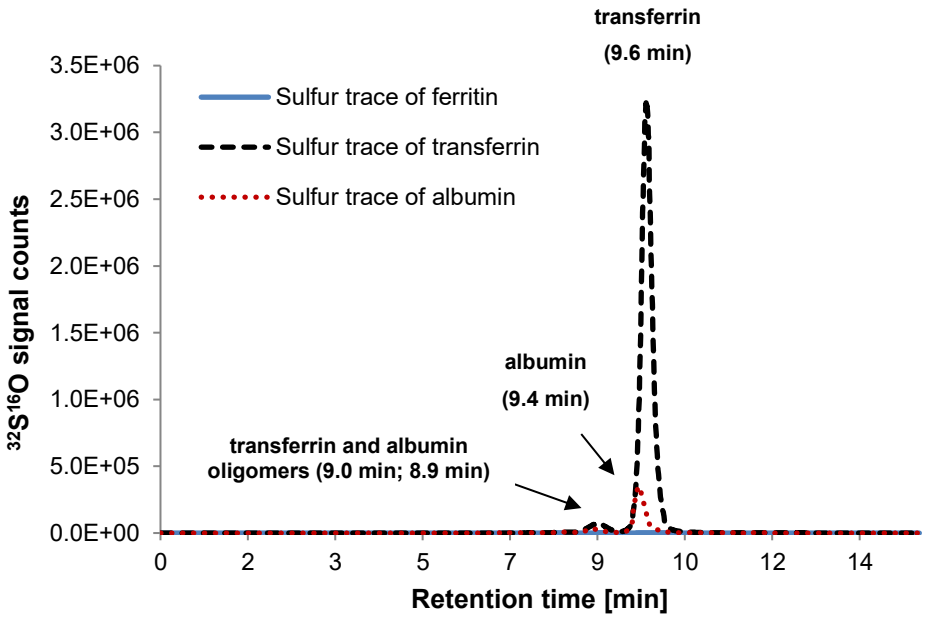


Figure 2 SEC chromatogram of a size ladder. Sulphur trace of ferritin (20 µg g^{-1}, blue trace), transferrin (10 mg g^{-1}, black trace) and albumin (1 mg g^{-1}, red trace) standards in 50 mM ammonium acetate measured consecutively using an IC Dionex 5000 sample introduction system and a SEC MabPac column coupled to a Agilent 8800 ICP-QMS operated in reaction mode (30 % oxygen)

Figure 3 shows the SEC chromatogram of FCS before centrifugal ultrafiltration. Transferrin and albumin showed a monomer peak (t_r 9.6 min and 9.4 min) and oligomer peaks (t_r 5.8-8.5 min). The determination of the iron and sulphur content in ferritin was hampered by other metal containing biomolecules present in FCS such as transferrin and albumin. This can be observed by the higher iron and sulphur signal at retention time of 5.8-8.5 min, thus indicating the co-elution of ferritin with other metal containing biomolecules present in FCS.

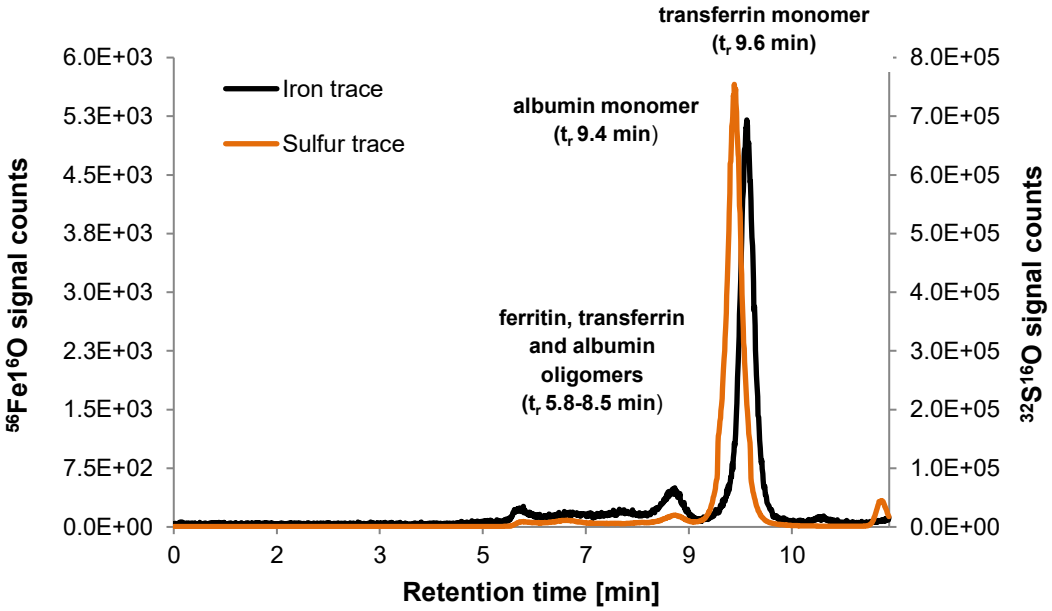


Figure 3 SEC chromatogram of fetal calf serum before centrifugal ultrafiltration; Fe and S were monitored at $^{56}Fe^{16}O$ (black trace) and $^{32}S^{16}O$ (orange trace), respectively

Figure 4 shows the SEC chromatogram of FCS after centrifugal ultrafiltration in the low molecular weight fraction. The slight increase of the iron trace indicated a weak permeability of the regenerated cellulose filter membrane for ferritin and other metal containing biomolecules in FCS such as transferrin. The sulfur trace indicated that no metal containing biomolecules such as transferrin and albumin were found in the low molecular weight fraction.

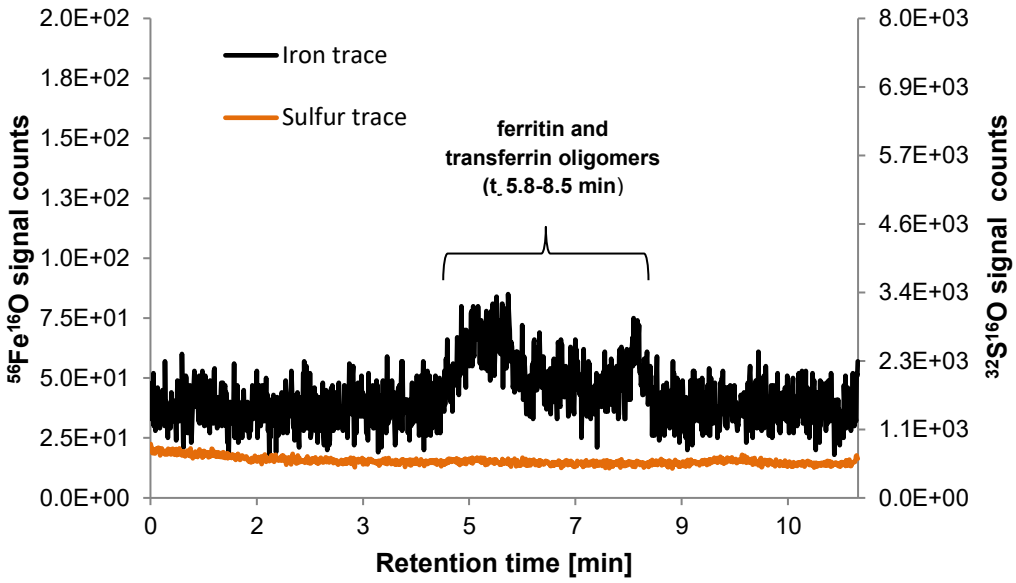


Figure 4 SEC chromatogram of fetal calf serum after centrifugal ultrafiltration in the low molecular weight fraction; Fe and S were monitored at $^{56}Fe^{16}O$ (black trace) and $^{32}S^{16}O$ (orange trace), respectively

Figure 5 shows the SEC chromatogram of FCS after centrifugal ultrafiltration in the high molecular weight fraction. As expected, by comparing the SEC chromatograms of FCS before and after centrifugal ultrafiltration, it can be observed that both chromatograms look the same. After centrifugal ultrafiltration a higher iron and sulphur signal was obtained, taking into account a dilution factor of 5 of FCS before and a dilution factor of 10 of FCS after centrifugal ultrafiltration. Centrifugal ultrafiltration using 3 kDa regenerated cellulose filter showed that 99.80% of transferrin and 99.98% of albumin remained in the high molecular weight fraction.

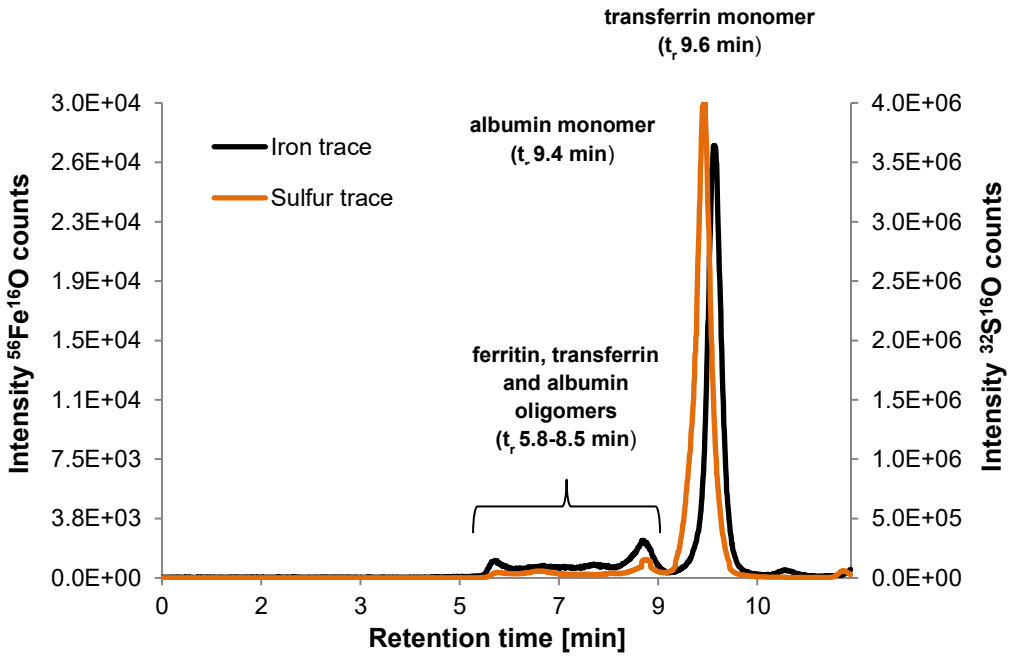


Figure 5 SEC chromatogram of fetal calf serum after centrifugal ultrafiltration in the high molecular weight fraction; Fe and S were monitored at $^{56}Fe^{16}O$ (black trace) and $^{32}S^{16}O$ (orange trace), respectively

Conclusion

This study showed that the fractionation of fetal calf serum (FCS) by centrifugal ultrafiltration using regenerated cellulose filter with a cut-off value of 3 kDa was successful. Chromatograms obtained by SEC-ICP-QMS showed that more than 99% of transferrin and albumin were found in the high molecular weight fraction. Consequently, the proposed method is fit for separation of serum into a protein and a small molecule fraction.

C. Abstract of poster

Investigation of enrichment and fractionation of ferritin by centrifugal ultrafiltration

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The aim of the ReMiND 15HLT02 project is the investigation of the role of metals and metal containing biomolecules in neurodegenerative diseases such as Alzheimer´s disease. One particular objective is the investigation of the relationship between ferritin bound iron and formation of plaques found in the brain of patients with Alzheimer´s disease. This requires the development of analytical methods capable of traceable quantification of ferritin bound iron in biological samples, such as blood serum, cerebrospinal fluid and brain homogenates.

In this pilot study, we investigated offline enrichment and fractionation methods of ferritin by centrifugal ultrafiltration. For the study samples containing only ferritin, only transferrin and only albumin were consulted. Therefore, two different filter materials consisting of regenerated cellulose and polyethersulfone were investigated. The cut-off value of the filters accounted to 100 kDa and 300 kDa. Thus, ferritin (molecular weight of 450 kDa) should be retained in the filter, while smaller metal containing proteins such as transferrin and albumin (molecular weight of < 80 kDa) should be recovered in the filtrate. The original and fractionated samples were analysed by flow injection size exclusion chromatography coupled to an inductively coupled plasma triple quadrupole mass spectrometer (FI-SEC-ICP-QQQ-MS; Thermo Scientific ICS Dionex 5000 and MabPac SEC, Agilent 8800, 30 % oxygen as reaction gas). The iron and sulfur content of the samples was determined by external calibration using well characterised ferritin standards.

The preliminary results obtained by testing a solution containing only ferritin showed that the Fe and S content of the filtrate was < the procedural BLK-LOQ_{Fe} of 14 ng g^{-1} and -LOQ_S of 44 ng g^{-1} indicating that all ferritin remained in the retentate (100 kDa regenerated cellulose filter). The Fe and S content in the filtrate obtained with 100 kDa polyethersulfone filters was < the procedural BLK-LOQ_{Fe} of 68 ng g^{-1} and -LOQ_S of 31 ng g^{-1}, but a weak permeability of the filter membrane for ferritin could be observed. Regenerated cellulose filters allowed to achieve a 12-fold enrichment of the Fe and S elemental content by centrifugal ultrafiltration, whereas polyethersulfone filters revealed a 23-fold enrichment of the Fe and S content. The Fe/S elemental ratio of ferritin before and after the centrifugal ultrafiltration using regenerated cellulose filter overlapped within limits of relative standard deviation indicating for no significant loss of ferritin. Regarding the other two experiments carried out with transferrin and albumin, only 6 ± 1 % of transferrin and 10 ± 1 % of albumin were found in the filtrate indicating that the cut-off value of 100 kDa is insufficient to eliminate the smaller metal containing proteins from the retentate. The preliminary results obtained with 300 kDa polyethersulfone filter showed that only 31 ± 8 % of ferritin was found in the retentate and only 62 ± 9 % of transferrin and 68 ± 12 % of abumin was found in the filtrate indicating that the cut-off value of 300 kDa is not suitable for the enrichment of ferritin as well as for the elimination of smaller metal containing proteins from the retentate.

The proposed method using filter with a cut-off value of 100 kDa is fit for purpose for the enrichment and quantification of Fe and S in ferritin. Neither filter with a cut-off value of 100 kDa nor that of 300 kDa are suitable for the fractionation of ferritin from transferrin and albumin.

D. List of figures

Figures		Page No.
Figure 1	Iron metabolism in the human organism	4
Figure 2	Graphical representation of the instrumental components of an ICP-MS spectrometer	11
Figure 3	The principle of size exclusion chromatography	14
Figure 4	SEC chromatogram of a size ladder. Iron trace of ferritin (20 µg mL^{-1}, blue trace), transferrin (10 mg mL^{-1}, black trace) and albumin (1 mg mL^{-1}, red trace) standards in 50 mM ammonium acetate measured consecutively using an IC Dionex 5000 sample introduction system and a SEC MabPac column coupled to a Agilent 8800 ICP-QMS operated in reaction mode (30% oxygen)	24
Figure 5	SEC chromatogram of a size ladder. Sulphur trace of ferritin (20 µg mL^{-1}, blue trace), transferrin (10 mg mL^{-1}, black trace) and albumin (1 mg mL^{-1}, red trace) standards in 50 mM ammonium acetate measured consecutively using an IC Dionex 5000 sample introduction system and a SEC MabPac column coupled to a Agilent 8800 ICP-QMS operated in reaction mode (30% oxygen)	25
Figure 6	SEC chromatogram of a size ladder. Iron trace of ferritin (20 µg mL^{-1}, blue trace), transferrin (10 mg mL^{-1}, black trace) and albumin (1 mg mL^{-1}, red trace) standards in 50 mM ammonium acetate measured consecutively using an IC Dionex 5000 sample introduction system and a SEC MabPac column coupled to a Agilent 8800 ICP-QMS operated in reaction mode (30% oxygen)	26
Figure 7	SEC chromatogram of a size ladder. Sulphur trace of ferritin (20 µg mL^{-1}, blue trace), transferrin (10 mg mL^{-1}, black trace) and albumin (1 mg mL^{-1}, red trace) standards in 50 mM ammonium acetate measured consecutively using an IC Dionex 5000 sample introduction system and a SEC MabPac column coupled to a Agilent 8800 ICP-QMS operated in reaction mode (30% oxygen)	26
Figure 8	Fe elemental content in ferritin before (light orange bar) and after enrichment in the low (LMF) and high molecular weight fraction (HMF) using not washed, not dried and not conditioned (dark orange bar)/conditioned (dark red bar) 100 kDa regenerated cellulose filters; error bars equal to 1 *SD (n = 3)*	32
Figure 9	Fe/S elemental ratio in ferritin before (light orange bar) and after enrichment using not washed, not dried and not conditioned (dark orange bar)/conditioned (dark red bar) 100 kDa regenerated cellulose filters; error bars equal to 1 *SD (n = 3)*	32
Figure 10	Fe elemental content in ferritin before (light orange bar) and after enrichment in the high molecular weight fraction (HMF) using washed (3 x with 5% HNO_3 and 2 x with ultrapure water, 1 x centrifuged with ultrapure water (10 min, 15000 g)), not dried and conditioned 100 kDa regenerated cellulose (dark red bar) and polyethersulfone filters (dark blue bar); error bars equal to 1 *SD (n = 3)*	35

Figure 11 Fe/S elemental ratio in ferritin before (light orange bar) and after enrichment using washed (3 x with 5% HNO_3 and 2 x with ultrapure water, 1 x centrifuged with ultrapure water (10 min, 15000 g)), not dried and conditioned 100 kDa regenerated cellulose (dark red bar) and polyethersulfone filters (dark blue bar); error bars equal to 1 *SD (n = 3)* 35

Figure 12 Fe elemental content in ferritin before (light orange bar) and after enrichment in the low (LMF) and high molecular weight fraction (HMF) using washed (3 x with 5% HNO_3, 1 x centrifuged with ultrapure water (5 min, 15000 g), 1 x centrifuged without ultrapure water (5 min, 15000 g), 1 x centrifuged with ultrapure water (10 min, 15000 g)), not dried and conditioned 100 kDa regenerated cellulose (dark red bar) and polyethersulfone filters (dark blue bar); error bars equal to 1 *SD (n = 5)* 38

Figure 13 Fe/S elemental ratio in ferritin before (light orange bar) and after enrichment using washed (3 x with 5% HNO_3, 1 x centrifuged with ultrapure water (5 min, 15000 g), 1 x centrifuged without ultrapure water (5 min, 15000 g), 1 x centrifuged with ultrapure water (10 min, 15000 g)), not dried and conditioned 100 kDa regenerated cellulose (dark red bar) and polyethersulfone filters (dark blue bar); error bars equal to 1 *SD (n = 5)* 39

Figure 14 Fe elemental content in ferritin before (light orange bar) and after enrichment in the low (LMF) and high molecular weight fraction using washed (3 x with 5% HNO_3 and 3 x with ultrapure water), dried (15 min) and conditioned 100 kDa regenerated cellulose (dark red bar) and polyethersulfone filters (dark blue bar); error bars equal to 1 *SD (n = 3)* 41

Figure 15 Fe/S elemental ratio in ferritin before (light orange bar) and after enrichment using washed (3 x with 5% HNO_3 and 3 x with ultrapure water), dried (15 min) and conditioned 100 kDa regenerated cellulose (dark red bar) and polyethersulfone filters (dark blue bar); error bars equal to 1 *SD (n = 3)* 42

Figure 16 Fe elemental content in ferritin before (light orange bar) and after enrichment in the high molecular weight fraction (HMF) using washed (3 x with 5% HNO_3 and 3 x with ultrapure water), dried (1 hour) and not conditioned (dark orange bar)/conditioned (dark red bar) 100 kDa regenerated cellulose filters; error bars equal to 1 *SD (n = 3)* 47

Figure 17 Fe elemental content in ferritin before (light orange bar) and after enrichment in the high molecular weight fraction (HMF) using washed (3 x with 5% HNO_3 and 3 x with ultrapure water), dried (1 hour) and not conditioned (green bar)/conditioned (dark blue bar) as well as not washed and conditioned (violet bar) 100 kDa polyethersulfone filters; error bars equal to 1 *SD (n = 3)* 47

Figure 18 Fe/S elemental ratio in ferritin before (light orange bar) and after enrichment using washed (3 x with 5% HNO_3 and 3 x with ultrapure water), dried (1 hour) and not conditioned (dark orange bar)/conditioned (dark red bar) 100 kDa regenerated cellulose filters; error bars equal to 1 *SD (n = 3)* 48

Figure 19 Fe/S elemental ratio in ferritin before (light orange bar) and after enrichment using washed (3 x with 5% HNO_3 and 3 x with ultrapure water), dried (1 hour) and not conditioned (green bar)/conditioned (dark blue bar) as well as not washed and conditioned (violet bar) 100 kDa polyethersulfone filters; error bars equal to 1 *SD (n = 3)* 48

Figure 20 Fe elemental content in ferritin before (light orange bar) and after enrichment in the low (LMF) and high molecular weight fraction (HMF) using 100 kDa regenerated cellulose (dark red bar) and polyethersulfone filters (dark blue bar); error bars equal to 1 *SD (n = 5)* 52

Figure 21 Fe/S elemental ratio in ferritin before (light orange bar) and after enrichment using 100 kDa regenerated cellulose (dark red bar) and polyethersulfone filters (dark blue bar); error bars equal to 1 *SD (n = 5)* 52

Figure 22 Fe elemental content in ferritin before (light orange bar) and after enrichment in the low (light blue bar) and high molecular weight fraction (dark blue bar) using 300 kDa polyethersulfone filters; error bars equal to 1 *SD (n = 3)* 54

Figure 23 SEC chromatogram of ferritin (10.8 µg mL^{-1}) in the low (dotted blue line) and high molecular weight fraction (blue line); Fe was monitored at $^{56}Fe^{16}O$ (blue trace) 56

Figure 24 SEC chromatogram of transferrin (1 mg mL^{-1}) in the low (dotted blue line) and high molecular weight fraction (blue line); Fe was monitored at $^{56}Fe^{16}O$ (blue trace) 57

Figure 25 SEC chromatogram of albumin (0.5 mg mL^{-1}) in the low (dotted orange line) and high molecular weight fraction (orange line); S was monitored at $^{32}S^{16}O$ (orange trace) 58

Figure 26 Fe intensity counts of transferrin after centrifugal ultrafiltration using 300 kDa polyethersulfone filters in the low (LMF, light green bar) and high molecular weight fraction (HMF, dark green bar); error bars equal to 1 *SD (n = 3)* 59

Figure 27 S intensity counts of albumin after centrifugal ultrafiltration using 300 kDa polyethersulfone filters in the low (LMF, light blue bar) and high molecular weight fraction (HMF, dark blue bar); error bars equal to 1 *SD (n = 3)* 59

Figure 28 SEC chromatogram of a synthetic serum containing ferritin (10.8 µg mL^{-1}), transferrin (1 mg mL^{-1}) and albumin (0.4 mg mL^{-1}) standards in 50 mM ammonium acetate before centrifugal ultrafiltration; Fe and S were monitored at $^{56}Fe^{16}O$ (black trace) and $^{32}S^{16}O$ (orange trace), respectively 61

Figure 29 SEC chromatogram of a synthetic serum containing ferritin (10.8 µg mL^{-1}), transferrin (1 mg mL^{-1}) and albumin (0.4 mg mL^{-1}) standards in 50 mM ammonium acetate after centrifugal ultrafiltration in the low molecular weight fraction; Fe and S were monitored at $^{56}Fe^{16}O$ (black trace) and $^{32}S^{16}O$ (orange trace), respectively 62

Figure 30 SEC chromatogram of a synthetic serum containing ferritin (10.8 µg mL^{-1}), transferrin (1 mg mL^{-1}) and albumin (0.4 mg mL^{-1}) standards in 50 mM ammonium acetate after centrifugal ultrafiltration in the high molecular weight fraction; Fe and S were monitored at $^{56}Fe^{16}O$ (black trace) and $^{32}S^{16}O$ (orange trace), respectively 63

Figure 31 Fe elemental content in ferritin before (light orange bar) and after enrichment in the low (light red bar) and high molecular weight fraction (dark red bar) using 3 kDa regenerated cellulose filters; error bars equal to 1 *SD (n = 3)* 63

Figure 32 SEC chromatogram of FCS (diluted 1:5 in 50 mM ammonium acetate pH 6.8) before centrifugal ultrafiltration; Fe and S were monitored at $^{56}Fe^{16}O$ (black trace) and $^{32}S^{16}O$ (orange trace), respectively 64

Figure 33 SEC chromatogram of FCS after centrifugal ultrafiltration in the low molecular weight fraction; Fe and S were monitored at $^{56}Fe^{16}O$ (black trace) and $^{32}S^{16}O$ (orange trace), respectively 65

Figure 34 SEC chromatogram of FCS (diluted 1:10 in 50 mM ammonium acetate pH 6.8) after centrifugal ultrafiltration in the high molecular weight fraction; Fe and S were monitored at $^{56}Fe^{16}O$ (black trace) and $^{32}S^{16}O$ (orange trace), respectively 66

Figure 35 SEC chromatogram of pig brain before centrifugal ultrafiltration; Fe and S were monitored at $^{56}Fe^{16}O$ (black trace) and $^{32}S^{16}O$ (orange trace), respectively 68

Figure 36 Size ladder. The $^{32}S^{16}O$ signal of ferritin (1 mg mL^{-1}), transferrin (1 mg mL^{-1}), myoglobin (1 mg mL^{-1}) and lysozyme (1 mg mL^{-1}) was measured and a size ladder was established. The molecular weight of the ferritin fragments was estimated [46]. 68

Figure 37 SEC chromatogram of pig brain after centrifugal ultrafiltration in the low molecular weight fraction; Fe and S were monitored at $^{56}Fe^{16}O$ (black trace) and $^{32}S^{16}O$ (orange trace), respectively 69

Figure 38 SEC chromatogram of pig brain after centrifugal ultrafiltration in the high molecular weight fraction; Fe and S were monitored at $^{56}Fe^{16}O$ (black trace) and $^{32}S^{16}O$ (orange trace), respectively 70

Figure 39 SEC chromatogram of the ^{57}Fe-isotopically enriched ferritin spike (diluted 1:20 in 50 mM ammonium acetate pH 6.8) after separation of free iron by centrifugal ultrafiltration using regenerated cellulose filters with a cut-off value of 100 kDa; Fe was monitored at $^{56}Fe^{16}O$ (black trace) and $^{57}Fe^{16}O$ (blue trace), respectively 71

Figure 40 SEC chromatogram of the ^{57}Fe-isotopically enriched ferritin spike before centrifugal ultrafiltration; the $^{57}Fe^{16}O$ trace of the ^{57}Fe-isotopically enriched ferritin spike (blue trace) was monitored [46] 72

E. List of tables

Tables		Page No.
Table 1	Techniques used for the determination of ferritin and ferritin-bound iron	6
Table 2	ICP-MS operation and chromatographic conditions used	19
Table 3	Size ladder. A size ladder using ferritin (450 kDa), transferrin (80 kDa) and albumin (66 kDa) standards was measured at a flow rate of 200 uL min^{-1} with corresponding retention time.	25
Table 4	Size ladder. A size ladder using ferritin (450 kDa), transferrin (80 kDa) and albumin (66 kDa) standards was measured at a flow rate of 250 uL min^{-1} with corresponding retention time.	27
Table 5	The procedural limit of quantification (LOQ) of iron and sulphur obtained with different filter preparation procedures for regenerated cellulose and polyethersulfone filters	28
Table 6	The Fe and S elemental content in a ferritin standard before centrifugal ultrafiltration was determined via external calibration using Fe-S standards by FI-ICP-MS (30% O_2) and the Fe/S ratio was calculated. The concentration values are given with the relative standard deviation of *n = 3* measurements.	31
Table 7	The Fe and S elemental content in a ferritin standard after centrifugal ultrafiltration using not washed, not dried and not conditioned 100 kDa regenerated cellulose filters in the low (LMF) and high molecular weight fraction (HMF) was determined via external calibration using Fe-S standards by FI-ICP-MS (30% O_2) and the Fe/S ratio was calculated. The concentration values are given with the relative standard deviation of *n = 3* measurements.	31
Table 8	The Fe and S elemental content in a ferritin standard after centrifugal ultrafiltration using not washed, not dried and conditioned 100 kDa regenerated cellulose filters in the low (LMF) and high molecular weight fraction (HMF) was determined via external calibration using Fe-S standards by FI-ICP-MS (30% O_2) and the Fe/S ratio was calculated. The concentration values are given with the relative standard deviation of *n = 3* measurements.	31
Table 9	The Fe and S elemental content in a ferritin standard before centrifugal ultrafiltration was determined via external calibration using Fe-S standards by FI-ICP-MS (30% O_2) and the Fe/S ratio was calculated. The concentration values are given with the relative standard deviation of *n = 3* measurements.	33
Table 10	The Fe and S elemental content in a ferritin standard after centrifugal ultrafiltration using washed (3 x with 5 % HNO_3 and 2 x with ultrapure water, 1 x centrifuged with ultrapure water (10 min, 15000 g)), not dried and conditioned 100 kDa regenerated cellulose filters in the low (LMF) and high molecular weight fraction (HMF) was determined via external calibration using Fe-S standards by FI-ICP-MS (30% O_2) and the Fe/S ratio was calculated. The concentration values are given with the relative standard deviation of *n = 3* measurements.	34

Table 11 The Fe and S elemental content in a ferritin standard after centrifugal ultrafiltration using washed (3 x with 5 % HNO_3 and 2 x with ultrapure water, 1 x centrifuged with ultrapure water (10 min, 15000 g)), not dried and conditioned 100 kDa polyethersulfone filters in the low (LMF) and high molecular weight fraction (HMF) was determined via external calibration using Fe-S standards by FI-ICP-MS (30% O_2) and the Fe/S ratio was calculated. The concentration values are given with the relative standard deviation of *n = 3* measurements. 34

Table 12 The Fe and S elemental content in a ferritin standard before centrifugal ultrafiltration was determined via external calibration using Fe-S standards by FI-ICP-MS (30% O_2) and the Fe/S ratio was calculated. The concentration values are given with the relative standard deviation of *n = 3* measurements. 37

Table 13 The Fe and S elemental content in a ferritin standard after centrifugal ultrafiltration using washed (3 x with 5 %HNO_3, 1 x centrifuged with ultrapure water (5 min, 15000 g), 1 x centrifuged without ultrapure water (5 min, 15000 g), 1 x centrifuged with ultrapure water (10 min, 15000 g)), not dried and conditioned 100 kDa regenerated cellulose filters in the low (LMF) and high molecular weight fraction (HMF) was determined via external calibration using Fe-S standards by FI-ICP-MS (30% O_2) and the Fe/S ratio was calculated. The concentration values are given with the relative standard deviation of *n = 5* measurements. 37

Table 14 The Fe and S elemental content in a ferritin standard after centrifugal ultrafiltration using washed (3 x with 5% HNO_3, 1 x centrifuged with ultrapure water (5 min, 15000 g), 1 x centrifuged without ultrapure water (5 min, 15000 g), 1 x centrifuged with ultrapure water (10 min, 15000 g)), not dried and conditioned 100 kDa polyethersulfone filters in the low (LMF) and high molecular weight fraction (HMF) was determined via external calibration using Fe-S standards by FI-ICP-MS (30% O_2) and the Fe/S ratio was calculated. The concentration values are given with the relative standard deviation of *n = 5* measurements. 37

Table 15 The Fe and S elemental content in a ferritin standard before centrifugal ultrafiltration was determined via external calibration using Fe-S standards by FI-ICP-MS (30% O_2) and the Fe/S ratio was calculated. The concentration values are given with the relative standard deviation of *n = 3* measurements. 40

Table 16 The Fe and S elemental content in a ferritin standard after centrifugal ultrafiltration using washed (3 x with 5% HNO_3 and 3 x with ultrapure water), dried (15 min) and conditioned 100 kDa regenerated cellulose filters in the low (LMF) and high molecular weight fraction (HMF) was determined via external calibration using Fe-S standards by FI-ICP-MS (30% O_2) and the Fe/S ratio was calculated. The concentration values are given with the relative standard deviation of *n = 3* measurements. 40

Table 17 The Fe and S elemental content in a ferritin standard after centrifugal ultrafiltration using washed (3 x with 5% HNO_3 and 3 x with ultrapure water), dried (15 min) and conditioned 100 kDa polyethersulfone filters in the low (LMF) and high molecular weight fraction (HMF) was determined via external calibration using Fe-S standards by FI-ICP-MS (30% O_2) and the Fe/S ratio was calculated. The concentration values are given with the relative standard deviation of *n = 3* measurements. 41

Table 18 The Fe and S elemental content in a ferritin standard before centrifugal ultrafiltration was determined via external calibration using well characterized ferritin standards by FI-SEC-ICP-MS (30% O_2) and the Fe/S ratio was calculated. The concentration values are given with the relative standard deviation of *n = 3* measurements. 44

Table 19 The Fe and S elemental content in a ferritin standard after centrifugal ultrafiltration using washed (3 x with 5% HNO_3 and 3 x with ultrapure water), dried (1 hour) and not conditioned 100 kDa regenerated cellulose filters in the low (LMF) and high molecular weight fraction (HMF) was determined via external calibration using well characterized ferritin standards by FI-SEC-ICP-MS (30% O_2) and the Fe/S ratio was calculated. The concentration values are given with the relative standard deviation of *n = 3* measurements. 44

Table 20 The Fe and S elemental content in a ferritin standard after centrifugal ultrafiltration using washed (3 x with 5% HNO_3 and 3 x with ultrapure water), dried (1 hour) and conditioned 100 kDa regenerated cellulose filters in the low (LMF) and high molecular weight fraction (HMF) was determined via external calibration using well characterized ferritin standards by FI-SEC-ICP-MS (30% O_2) and the Fe/S ratio was calculated. The concentration values are given with the relative standard deviation of *n = 3* measurements. 45

Table 21 The Fe and S elemental content in a ferritin standard before centrifugal ultrafiltration was determined via external calibration using well characterized ferritin standards by FI-SEC-ICP-MS (30% O_2) and the Fe/S ratio was calculated. The concentration values are given with the relative standard deviation of *n = 3* measurements. 45

Table 22 The Fe and S elemental content in a ferritin standard after centrifugal ultrafiltration using washed (3 x with 5% HNO_3 and 3 x with ultrapure water), dried (1 hour) and not conditioned 100 kDa polyethersulfone filters in the low (LMF) and high molecular weight fraction (HMF) was determined via external calibration using well characterized ferritin standards by FI-SEC-ICP-MS (30% O_2) and the Fe/S ratio was calculated. The concentration values are given with the relative standard deviation of *n = 3* measurements. 45

Table 23 The Fe and S elemental content in a ferritin standard after centrifugal ultrafiltration using washed (3 x with 5% HNO_3 and 3 x with ultrapure water), dried (1 hour) and conditioned 100 kDa polyethersulfone filters in the low (LMF) and high molecular weight fraction (HMF) was determined via external calibration using well characterized ferritin standards by FI-SEC-ICP-MS (30% O_2) and the Fe/S ratio was calculated. The concentration values are given with the relative standard deviation of *n = 3* measurements. 46

Table 24 The Fe and S elemental content in a ferritin standard after centrifugal ultrafiltration using not washed and conditioned 100 kDa polyethersulfone filters in the low (LMF) and high molecular weight fraction (HMF) was determined via external calibration using well characterized ferritin standards by FI-SEC-ICP-MS (30% O_2) and the Fe/S ratio was calculated. The concentration values are given with the relative standard deviation of *n = 3* measurements. 46

Table 25 Summary of the procedural limit of quantification (LOQ) of iron and sulphur obtained with different filter preparation procedures for regenerated cellulose and polyethersulfone filters with the corresponding enrichment of ferritin 49

Table 26 The Fe and S elemental content in a ferritin standard before centrifugal ultrafiltration was determined via external calibration using Fe-S standards by FI-ICP-MS (30% O_2) and the Fe/S ratio was calculated. The concentration values are given with the relative standard deviation of *n = 3* measurements. 51

Table 27 The Fe and S elemental content in a ferritin standard after centrifugal ultrafiltration using 100 kDa regenerated cellulose and polyethersulfone filters in the low molecular weight fraction (LMF) was determined via external calibration using Fe-S standards by FI-ICP-MS (30% O_2) and the Fe/S ratio was calculated. The concentration values are given with the relative standard deviation of *n = 5* measurements. 51

Table 28 The Fe and S elemental content in a ferritin standard after centrifugal ultrafiltration using 100 kDa regenerated cellulose and polyethersulfone filters in the high molecular weight fraction (HMF) was determined via external calibration using Fe-S standards by FI-ICP-MS (30% O_2) and the Fe/S ratio was calculated. The concentration values are given with the relative standard deviation of *n = 5* measurements. 51

Table 29 The Fe and S elemental content in a ferritin standard before centrifugal ultrafiltration was determined via external calibration using Fe-S standards by FI-ICP-MS (30% O_2) and the Fe/S ratio was calculated. The concentration values are given with the relative standard deviation of *n = 3* measurements. 53

Table 30 The Fe and S elemental content in a ferritin standard after centrifugal ultrafiltration using 300 kDa polyethersulfone filters in the low (LMF) and high molecular weight fraction (HMF) was determined via external calibration using Fe-S standards by FI-ICP-MS (30% O_2) and the Fe/S ratio was calculated. The concentration values are given with the relative standard deviation of *n = 3* measurements. 54

Table 31 The Fe and S elemental content in ferritin before centrifugal ultrafiltration was determined via external calibration using well characterized ferritin standards by FI-SEC-ICP-MS (30% O_2). The concentration values are given with the relative standard deviation of *n = 3* measurements. 55

Table 32 The Fe and S elemental content in ferritin after centrifugal ultrafiltration using 100 kDa regenerated cellulose filters in the low (LMF) and high molecular weight fraction (HMF) was determined via external calibration using well characterized ferritin standards by FI-SEC-ICP-MS (30% O_2). The concentration values are given with the relative standard deviation of *n = 3* measurements. 56

Table 33 The Fe elemental content in ferritin in a synthetic serum before and after centrifugal ultrafiltration in the low (LMF) and high molecular weight fraction (HMF) was determined via external calibration using well characterized ferritin standards by FI-SEC-ICP-MS (30% O_2). The concentration values are given with the relative standard deviation of *n = 3* measurements. 60

Table 34 The Fe elemental content in ferritin in pig brain before and after centrifugal ultrafiltration in the low (LMF) and high molecular weight fraction (HMF) was determined via external calibration using well characterized ferritin standards by FI-SEC-ICP-MS (30% O_2). The concentration values are given with the relative standard deviation of *n = 3* measurements. 67