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Short Communication

Quantification of ferritin-bound iron in murine samples for Alzheimer's disease studies using species-specific isotope dilution mass spectrometry

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Abstract

We have investigated species-specific isotope dilution mass spectrometry (IDMS) for the quantification of ferritin-bound iron in murine serum and brain. Therefore, fresh samples were analyzed using size exclusion chromatography inductively coupled plasma mass spectrometry (SEC-ICP-MS). Isotopically labeled (⁵⁷Fe)ferritin was used as calibrant for the quantification of ferritin-bound iron in murine samples. Assessment of the iron load of serum ferritin was impaired by concomitant iron-containing proteins of similar size and shape, which could not be separated by SEC nor centrifugal ultra-filtration. In contrast, ferritin was the main iron-containing protein in cytosolic extracts of murine brain, which showed a total ferritin-bound iron content of $(1.05 \pm 0.12) \mu\text{g g}^{-1}$ ($n = 10$; U , $k = 2$). The relative expanded uncertainty achieved for a mass fraction of ca. $1 \mu\text{g g}^{-1}$ ferritin-bound iron was 11% (U_{rel} , $k = 2$). The relative expanded uncertainty of the iron mass fraction of the (⁵⁷Fe)ferritin spike was 5.7% and represented the major contributing factor to the overall uncertainty. Statistical tests suggested no significant difference in ferritin-bound iron content between mouse brain hemispheres. The presented analytical tool provides low limits of quantification (2.2 ng g^{-1}) and uncertainties (11%, U_{rel} , $k = 2$), thus enables the quantification of ferritin-bound iron in murine brain extracts with high sensitivity and accuracy. Furthermore, this analytical workflow assures comparability of measurement results across research laboratories. This provides the basis for investigation of the iron loading of ferritin in brain tissue of healthy and Alzheimer's disease mouse models, which may help answering the question if iron regulation is impaired in Alzheimer's disease.

Keywords: neurodegenerative diseases, metalloproteins, iron metabolism, measurement uncertainty budget

(Some figures may appear in colour only in the online journal)



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1. Introduction

The Alzheimer's disease (AD) is a neurodegenerative disease, which affects millions of people worldwide [1]. The symptoms of AD are well described. However, the fundamental mechanisms of AD as well as reliable diagnostic tools are still subject of active research [1]. One hypothesis suggests that AD is a result of dysregulation of iron homeostasis in brain [2, 3]. In particular, it is believed that ferritin (FER), the major iron storage protein in cells [4], cannot fulfill its regulatory function. This leads to an excess of free iron (Fe^{2+}), a chemical form which can cause oxidative stress and cell damage [5]. To investigate this hypothesis, solid knowledge on FER-bound iron levels in healthy and AD brain are required. In addition, it is suggested that damaged cells release FER to serum [6]. Thus, when the iron contents of brain and serum FER correlate, the latter might represent a highly promising new biomarker for AD.

Quantification of FER-bound iron in brain and serum is not trivial. FER needs to be extracted from brain in its intact form to avoid disruption of the protein shell and loss of FER-bound iron. Furthermore, the amount of FER varies across tissues and fluids [7, 8]. Finally, FER shows variable iron loading, which can range from no to several thousand iron atoms per molecule [9]. Consequently, analysis of FER-bound iron in brain and serum requires an instrumental technique, which provides sensitivity, selectivity and a large dynamic range. Moreover, to draw conclusions as to the role of FER in AD, measurement results have to be as accurately as possible and comparable across measurement systems, location and time.

These specific requirements are fulfilled by the combination of inductively coupled plasma mass spectrometry (ICP-MS) and species-specific isotope dilution mass spectrometry (IDMS). ICP-MS is an instrumental technique, which offers detection limits down to fg g^{-1} levels and a dynamic range of more than nine orders of magnitude [10]. Furthermore, an important feature of ICP-MS is its capability to be coupled to liquid chromatographic (LC) systems [11], which allows online separation of FER from concomitant iron containing proteins prior to elemental analysis. Species-specific IDMS is the most accurate analytical tool for the quantification of the elemental content of proteins [12]. It uses an isotopically labeled analogue of the protein of interest as calibrant, which is mixed with the sample at the start of the sample preparation. Subsequently, the analyte-calibrant mixture is purified and mass spectrometrically analyzed. An additional feature of this calibration technique is that the analyte and calibrant are equally affected by e.g. instrumental drifts, species instabilities or sample loss. Accordingly, these effects are inherently corrected for, thus do not influence the final results [12]. Furthermore, when a certified reference material is used as spike material or as calibration material in double IDMS, species-specific IDMS ensures that the measurement results are traceable to an SI unit (e.g. kg) though an unbroken calibration chain [13, 14]. This guarantees that analytical results are comparable independently of time, location and operator [14].

This procedure was successfully applied for the quantification of FER-bound iron in human serum [15]. However, the

most common model organisms for AD research are mice. They provide only a few 100 μl of serum compared to mL amounts available from humans. Furthermore, to the best of our knowledge, no species-specific IDMS quantification of FER-bound iron in murine brain has been performed yet.

Thus, the aim of this pilot study was to investigate the applicability of species-specific IDMS for the quantification of FER-bound iron in murine serum and brain tissue. Furthermore, we examined if the FER-bound iron content differs between brain hemispheres and evaluated the uncertainty of the analytical procedure.

2. Experimental

2.1. Reagents and methods

Preparatory work and elemental analysis were carried out at the University of Vienna, Department of Analytical Chemistry in an ISO class 8 clean room according to ISO 14644-1. Aqueous solutions were prepared using ultrapure water (18 $\text{M}\Omega$ cm, Veolia Water Solutions, France). Per analysis nitric acid (HNO_3 , 69% w/w) was purchased from Merck, Germany. Supra pure-quality HNO_3 (69% w/w) and hydrochloric acid (HCl , 35% w/w) were purchased from Carl Roth, Germany. Equine spleen FER and apo-FER, human plasma transferrin and equine skeletal muscle myoglobin were purchased from Sigma Aldrich, USA. An ammonium acetate buffer 50 mM was prepared by addition of ammonia (25%, supra pure, Avantor, USA) to ultrapure water and adjusting the pH to 6.8 using acetic acid (100%, suprapure, Carl Roth, Germany). A 20-fold concentrated extraction buffer (TRIS buffered saline) containing 1 M TRIS-HCl (100%, ultrapure, Gerbu, Germany; HCl: Carl Roth; pH 7.6) with 3.08 M sodium chloride (NaCl , 99.99%, supra pure, Merck, Germany) in ultrapure water was prepared. Tween 20 (Bio-Rad, USA) was diluted tenfold. Final lysis buffer contained 0.5 ml of TRIS buffer saline, 0.05 ml of diluted Tween 20 and 9.45 ml of ultrapure water. All laboratory consumables (polyethylene tubes, pipette tips, etc) were washed using 10% HNO_3 (w/w, Merck) followed by 1% HNO_3 (w/w, Carl Roth) and ultrapure water before use. All dilutions were performed gravimetrically.

2.2. Preparation of calibrants for (reverse) isotope dilution mass spectrometry

Isotopically enriched iron metal powder with a ^{57}Fe isotopic abundance of 95.08% (Eurisootope, France) was dissolved using conc. HCl in a PFA beaker on a hot plate. The solution was evaporated to dryness and diluted using 5% HCl (w/w) to obtain a $^{57}\text{FeCl}_2$ solution of ca. 2000 $\mu\text{g g}^{-1}$ total Fe content. A native FER standard (equine spleen, Sigma Aldrich, Lot Num. SLBMO474 V) was divided into five aliquots of ca. 50 mg and microwave-assisted (Multiwave Pro, Anton Paar, Austria) acid digested using a reaction mixture of 2 ml of 20% HNO_3 (w/w) and 0.1 ml of H_2O_2 (30% w/w, ultrapure, Merck, Germany). Digested samples were diluted to 4% HNO_3 (w/w)

using ultrapure water. Isotopically labeled FER was synthesized using a modified version of the protocol by Konz *et al* [15]. Apo-FER (Sigma Aldrich) was diluted with ammonium acetate buffer 50 mM pH 6.8 to a volume of 2 ml resulting in a 500 $\mu\text{g g}^{-1}$ protein solution and mixed with $^{57}\text{FeCl}_2$ solution (125 μl , 2000 $\mu\text{g g}^{-1}$ total Fe content in 5% HCl *w/w*). The solution was then vortexed for 1 min (Vortex V-1 plus, Biosan SIA, Latvia) and incubated at room temperature for 1 h. Centrifugal ultra-filtration facilitated the purification of synthesized (^{57}Fe)FER from residual free ^{57}Fe . Therefore, membrane filters with a cut-off value of 100 kDa (Amicon Ultra-0.5 ml, Merck, Germany) were washed ($3 \times 5\%$ HNO_3 *w/w*, 3x ultrapure water) and conditioned by centrifugation of 500 μl of ammonium acetate buffer 50 mM pH 6.8 (15 000 g, 10 min, 4 °C, Microliter Centrifuge, HERMLE Labortechnik GmbH, Germany) prior to use. The mixture was loaded on the filter, centrifuged (15 000 g, 30 min, 4 °C, Microliter Centrifuge) and washed three times using ammonium acetate buffer 50 mM pH 6.8. The purified (^{57}Fe)FER was stored in the fridge at 4 °C until use. (For the analysis of the purity and the iron content of the (^{57}Fe)FER spike see paragraph 'Instrumentation' and 'Quantification of iron content by (reverse) isotope dilution mass spectrometry' below.)

2.3. Sample collection

Two male 18-weeks old Balb/c mice (purchased from Envigo, Italy) were anesthetized with rompun/ketavet and blood was collected by heart puncture followed by sacrifice via cervical dislocation. The blood samples were allowed to clot in conventional Eppendorf tubes at room temperature (~25 min) and separated into serum and pellet by centrifugation (2x 900 g for 10 min at 4 °C). In addition, the brain of one mouse was collected during dissection, cooled on ice and (together with the serum) immediately (within 10 min) used for the experiments without further processing.

2.4. Sample preparation

Murine serums were vortexed (Biosan SIA). The serum sample of one mouse (ca. 100 μl) was used as such. The serum sample of the second mouse (ca. 500 μl) was divided into two aliquots: (1) One aliquot of 30 μl was used as such. (2) The second aliquot of ca. 470 μl was subjected to centrifugal ultra-filtration. Ren and Walczyk proposed this procedure for off-line FER fractionation from other iron containing proteins [16]. Therefore, 470 μl of undiluted murine serum were applied on preconditioned membrane filters (100 kDa, Amicon; see above), centrifuged (15 000 g, 1 h, 4 °C, Microliter Centrifuge) and washed three times using ammonium acetate buffer 50 mM pH 6.8. The murine brain was placed in a disposable plastic cup and divided in two hemispheres using a plastic spatula. Brain extracts were prepared following a modified procedure for the extraction of cytosolic proteins developed by the University of Aberdeen, Department of Chemistry. Each hemisphere (ca. 200 mg) was transferred into a separate 2 ml low binding tube, mixed with 1 ml of freshly prepared ice-cold lysis buffer (see above) and homogenized manually using

a plastic spatula for 5 min on ice. The extracts were centrifuged (15 000 g, 20 min, 4 °C, Microliter Centrifuge) and the supernatant solutions transferred into separate tubes stored on ice, respectively. The extraction procedure was repeated two times. All samples were measured within 24 h after harvest.

2.5. Instrumentation

Digested samples and native proteins were diluted as required and analyzed for their iron content using a triple quadrupole ICP-MS (Agilent 8800, Agilent Technologies, USA) equipped with a Scott double-pass spray chamber (Agilent, USA). Iron isotopes were assessed in reaction mode (30% oxygen) at mass-to-charge ratios of $^{54}\text{Fe}^{16}\text{O}$, $^{56}\text{Fe}^{16}\text{O}$, $^{57}\text{Fe}^{16}\text{O}$ and $^{58}\text{Fe}^{16}\text{O}$, respectively. An autosampler (ASX-500, Agilent, USA) facilitated direct injection of mineralized solutions (Fe single element standard, $^{57}\text{FeCl}_2$ solution, digested equine spleen FER) into the ICP-MS. Analysis of the iron content of intact proteins (native equine spleen FER, native (^{57}Fe)FER spike, native murine serum and brain extracts) was accomplished by size exclusion chromatography ICP-MS (SEC-ICP-MS). For this a LC system (1260 Infinity Bio-Inert, Agilent Technologies, USA) was used as sample introduction system. The sample volume accounted to 5 μl and the flow rate of the ammonium acetate buffer 50 mM pH 6.8 used as mobile phase to 200 $\mu\text{l min}^{-1}$. Subsequently, a SEC column (MabPac, Thermo Fisher Scientific, USA) separated iron-containing proteins according to their hydrodynamic volume followed by elemental analysis with ICP-MS (Agilent 8800). Table 1 summarizes the instrumental conditions used for the determination of the total iron content of mineralized samples and the iron load of intact FER (Fe:FER; quantification of the iron content was performed by (reverse) IDMS, see paragraph 'Quantification of iron content by (reverse) isotope dilution mass spectrometry' below). The limit of quantification (LOQ) of the direct injection method accounted to ten times the standard deviation of the process blank quantified using a 5-point calibration (Fe single element standard, LabKings, The Netherlands). The LOQ of the SEC-ICP-MS method was determined following EURACHEM guidelines [17] as equivalent of ten times the standard deviation of the lowest detectable measurement standard of a 5-point equine spleen FER calibration (Sigma Aldrich). The quantification of the sulphur content of FER was focus of the Physikalisch-Technische Bundesanstalt (PTB) and will be discussed in a follow-up publication.

2.6. Quantification of iron content by (reverse) isotope dilution mass spectrometry

IDMS is an established primary method of highest metrological order, which facilitates the accurate and traceable quantification of analyte content at ng g^{-1} levels [13]. Theoretical considerations are described in detail elsewhere [18]. The IDMS workflow in this study comprised four steps:

- (a) Quantification of the total Fe content of the isotopically enriched $^{57}\text{FeCl}_2$ solution by direct injection reverse

Table 1. Instrumental and method parameters for the determination of the iron content of digested samples and native proteins by ICP-MS operated in reaction mode.

	Direct injection ICP-MS	SEC-ICP-MS
Sample introduction	peristaltic pump	LC system
Sample volume	2 ml	5 μ l
Flow rate	pump speed 0.3 rps	200 μ l min^{-1}
Mobile phase	—	50 mM $\text{CH}_3\text{COONH}_4$, pH 6.8
Column	—	MabPac SEC, PEEK, 4×300 mm, 5 μ m, 10–1000 kDa
Nebulizer	MicroFlow PFA-ST	MicroFlow PFA-ST
Spray chamber	Scott double-pass	Scott double-pass
Nebulizer gas flow	1.07 l min^{-1}	1.07 l min^{-1}
Auxiliary gas flow	0.90 l min^{-1}	0.90 l min^{-1}
Plasma gas flow	15 l min^{-1}	15 l min^{-1}
ICP RF power	1550 W	1550 W
Cones	Ni	Ni
Reaction gas	Oxygen (30%)	Oxygen (30%)
Analytes	$^{54}\text{Fe}^{16}\text{O}$, $^{56}\text{Fe}^{16}\text{O}$, $^{57}\text{Fe}^{16}\text{O}$, $^{58}\text{Fe}^{16}\text{O}$	$^{54}\text{Fe}^{16}\text{O}$, $^{56}\text{Fe}^{16}\text{O}$, $^{57}\text{Fe}^{16}\text{O}$, $^{58}\text{Fe}^{16}\text{O}$
Integration time	0.30 s	0.10 s
LOQ(Fe)	0.05 ng g^{-1}	2.2 ng g^{-1}

IDMS using an Fe standard (LabKings) with a certified elemental content traceable to NIST SRM 3126;

- Quantification of the total Fe content of digested equine FER (Sigma Aldrich) by direct injection IDMS using the isotopically enriched $^{57}\text{FeCl}_2$ solution characterized in step 1;
- Quantification of FER-bound iron in intact isotopically labeled (^{57}Fe)FER by species-specific reverse IDMS using intact equine FER (Sigma Aldrich) characterized in step 2;
- Quantification of FER-bound iron in intact murine FER by species-specific IDMS using intact isotopically labeled (^{57}Fe)FER characterized in step 3.

This unbroken calibration chain links the measured iron content of intact murine FER to the certified iron content of the NIST SRM 3126. This certified reference material represents a primary calibration standard and its certified mass fraction is metrologically traceable to the SI unit of mass. As the aim of this pilot study was to evaluate if species-specific IDMS is suitable for quantification of FER-bound iron in murine samples relevant to study the mechanisms of AD, this calibration hierarchy was considered fit-for-intend-use. If this method is used for future provision of calibration services the NIST SRM 3126 or a material certified by a National Metrology Institute should be used.

Equation (1) enables the calculation of the Fe content w_y in the isotopically enriched calibrants (step 1 and 3 above). The Fe content w_x of equine and murine FER was assessed by equation (2) (step 2 and 4 above). Equations are from [18].

$$w_y = w_x \cdot \frac{m_x}{m_y} \cdot \frac{M_y}{M_x} \cdot \frac{A_x^{56}}{A_y^{57}} \cdot \left(\frac{1 - {}^{56}\text{Fe}/{}^{57}\text{Fe}_{\text{mix}} \cdot {}^{57}\text{Fe}/{}^{56}\text{Fe}_x}{{}^{56}\text{Fe}/{}^{57}\text{Fe}_{\text{mix}} - {}^{56}\text{Fe}/{}^{57}\text{Fe}_y} \right) \quad (1)$$

$$w_x = w_y \cdot \frac{m_y}{m_x} \cdot \frac{M_x}{M_y} \cdot \frac{A_y^{57}}{A_x^{56}} \cdot \left(\frac{{}^{56}\text{Fe}/{}^{57}\text{Fe}_{\text{mix}} - {}^{56}\text{Fe}/{}^{57}\text{Fe}_y}{1 - {}^{56}\text{Fe}/{}^{57}\text{Fe}_{\text{mix}} \cdot {}^{57}\text{Fe}/{}^{56}\text{Fe}_x} \right) \quad (2)$$

Here, x denotes samples with a natural isotopic composition of iron, y samples with an isotopically enriched iron composition and mix the sample-calibrant blend. The masses m were assessed using a microbalance (CPA225D, Sartorius, Germany). All stable isotope ratios of iron, ${}^i\text{Fe}/{}^j\text{Fe}$ where $i > j$, were determined experimentally. Mass bias correction was omitted as all isotope ratios were measured under the same experimental conditions [19] and used for the calculation of the isotopic abundances A of iron in the samples. The atomic weights M were calculated using experimentally assessed isotopic abundances A and atomic masses of iron according to Berglund and Wieser [20].

2.7. Uncertainty evaluation

The uncertainty indicates the quality of analytical results and enables their comparison independent of the applied analytical method, time and location [21]. Thus, the measurement uncertainty goes hand in hand with metrological traceability. All uncertainties in this study were determined in a Kragten approach [22] following EURACHEM guidelines [21]. Each sample solution was analyzed five-times. The assessed mean and the standard deviation (SD) were used as input parameters in the model equation (equations (1) and (2), see above) for the determination of the combined measurement uncertainty. Evaluation of sample homogeneity was performed by preparing and measuring five individual sample-calibrant blends. The expanded uncertainty (U , $k = 2$) in this work corresponds to the combination of the contribution of the measurement uncertainty and the sample homogeneity multiplied by a coverage factor k of 2.

2.8. Statistical analysis

A boxplot visualized the content of FER-bound iron in mouse brain hemispheres. A Kolmogorov-Smirnov test showed normal distribution and a Levene test equal variances of

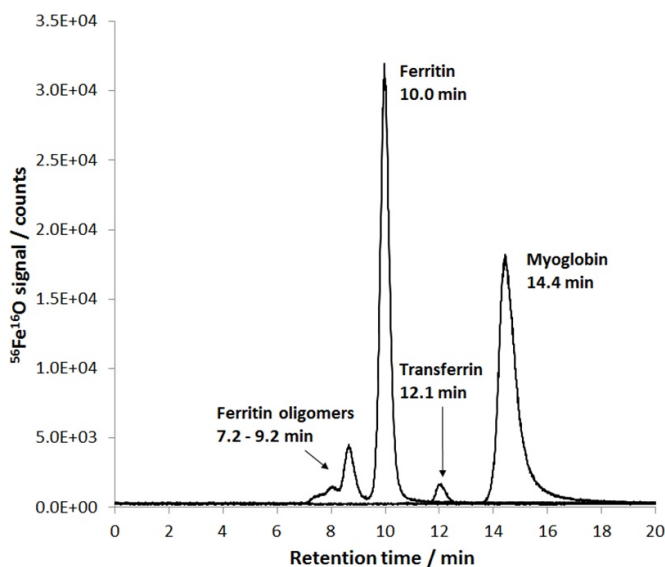


Figure 1. Equine spleen ferritin 0.010 mg g^{-1} (weight of protein shell ca. 440 kDa), human plasma transferrin 1.0 mg g^{-1} (ca. 72 kDa) and equine skeletal muscle myoglobin 0.10 mg g^{-1} (ca. 17 kDa) protein standards separated by SEC; iron was measured as $^{56}\text{Fe}^{16}\text{O}$ using ICP-MS.

FER-bound iron content in mouse brain extracts. Consequently, an independent sample t-test was used to determine significant differences of FER-bound iron content in extracts of mouse brain hemispheres. The statistical significance level corresponded to $\alpha = 0.05$. Statistical data evaluation was performed using SPSS© (IBM SPSS Statistics 24, Armonk, USA).

3. Results and discussion

3.1. Method suitability for ferritin-bound iron quantification

Species-specific IDMS is an accurate analytical method, which can determine the iron loading of FER in complex biological matrixes. Therefore, it requires: (i) The separation of intact FER from concomitant iron-containing proteins, (ii) the measurement of the isotopic composition of iron in FER and (iii) a well characterized isotopically labeled FER spike used as calibrant for the quantification of FER-bound iron.

Figure 1 shows FER, transferrin and myoglobin standards separated by SEC and eluting Fe measured by ICP-MS. Two thirds of FER were present in the monomeric form (peak at retention time of 10.0 min) and one third in the form of oligomers (peaks at retention time of 7.2–9.2 min). These findings were in accordance with studies by Konz *et al* [15]. They suggested that oligomerization occurs as a result of high FER content and the presence of free Fe^{2+} , which might reduce disulphide bonds on the surface of the protein monomers [15]. Consequently, to account for the entire iron load of FER, the combined peak areas of FER monomers and oligomers were used for further data evaluation hereafter.

The LOQ of FER-bound iron accounted to 2.2 ng g^{-1} (table 1). FER protein levels in serum of healthy mice range from

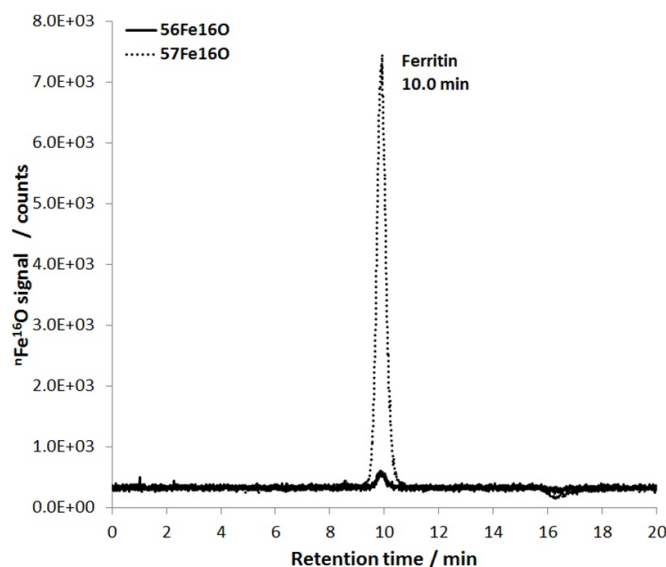


Figure 2. SEC chromatogram of the isotopically labeled (^{57}Fe)FER spike (ca. $4 \mu\text{g g}^{-1}$ FER-bound iron); black line corresponds to the $^{56}\text{Fe}^{16}\text{O}$ trace and dotted line to the $^{57}\text{Fe}^{16}\text{O}$ trace.

170 to 438 ng ml^{-1} [23, 24]. This is comparable to human serum FER protein levels of $1\text{--}800 \text{ ng ml}^{-1}$, which showed FER-bound iron content of $3\text{--}77 \text{ ng g}^{-1}$ [16, 25]. Assuming similar iron loading of murine and human FER, the low LOQ of the presented protocol facilitates FER-bound iron analysis of murine samples without enrichment.

The chromatographic profile of the isotopically labeled (^{57}Fe)FER spike shows that FER was solely present in the monomeric form (figure 2). Repeated measurements of the spike showed that it was stable for over 6 months after production.

Species-specific reverse IDMS using an intact equine spleen FER standard, with a well characterized iron load of the native protein, as calibrant (see paragraph ‘Quantification of iron content by (reverse) isotope dilution mass spectrometry’, step 3, above) revealed that FER-bound iron content of the synthesized isotopically enriched (^{57}Fe)FER amounted to $(3.87 \pm 0.32) \mu\text{g g}^{-1}$ ($n = 5$; $U, k = 2$; table 2). Calculation of the relative isotopic abundances of the synthesized (^{57}Fe)FER showed that ^{57}Fe accounted for about 95% of FER-bound iron. This result was in accordance with the isotopic composition of the isotopically enriched iron used for apo-FER loading and suggested successful isotopic labeling of FER.

These results indicated that the proposed analytical methodology is fit-for-purpose of FER-bound iron quantification in murine serum and brain samples.

3.2. Method application to murine serum

Figure 3 shows the SEC chromatogram of fresh murine serum. Surprisingly, no characteristic FER peaks could be observed. Instead, the chromatogram shows unspecific signals at retention time of 8–12 min. This observation was confirmed by SEC-ICP-MS analysis of the second serum sample ($500 \mu\text{l}$;

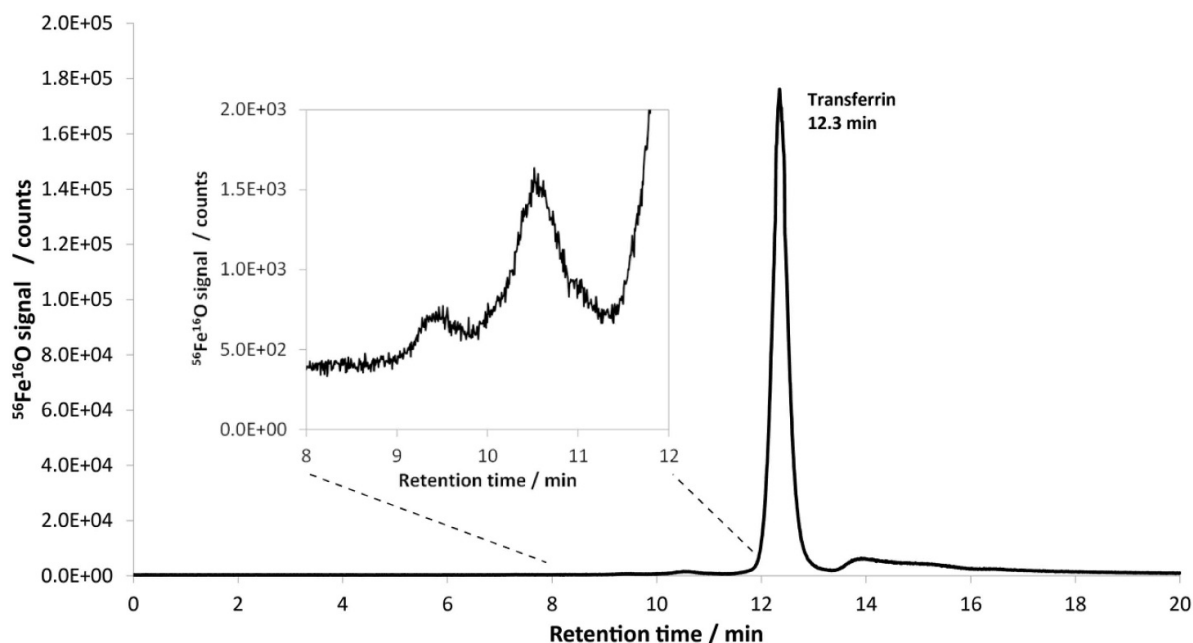


Figure 3. SEC chromatogram of fresh mouse serum shows unspecific peaks at retention times of 8–12 min; iron was monitored as $^{56}\text{Fe}^{16}\text{O}$.

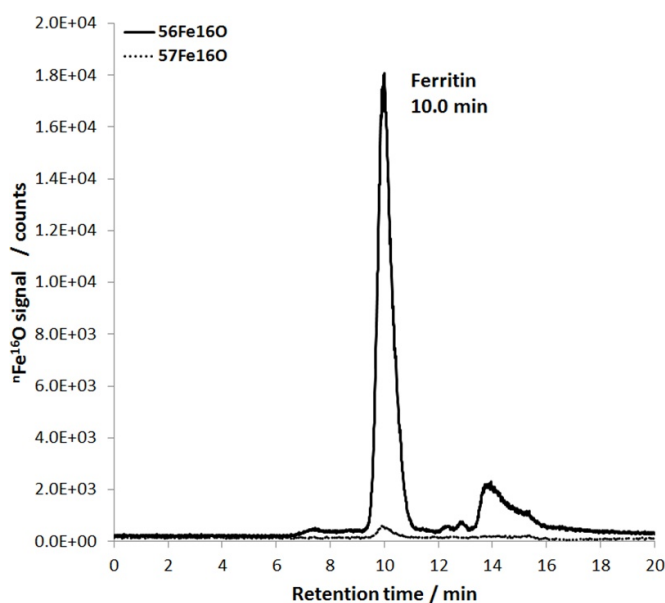


Figure 4. SEC chromatogram of fresh undiluted mouse brain extract; black line corresponds to the $^{56}\text{Fe}^{16}\text{O}$ trace and dotted line to the $^{57}\text{Fe}^{16}\text{O}$ trace.

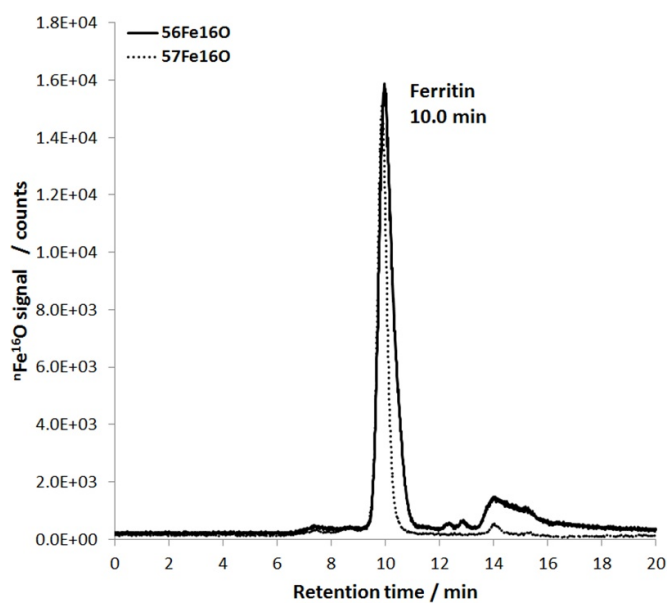


Figure 5. Fresh mouse brain extract was mixed with isotopically enriched (^{57}Fe)FER from equine spleen (ca. $4\ \mu\text{g g}^{-1}$ FER-bound iron) to achieve equal height of FER-bound iron peaks; black line corresponds to the $^{56}\text{Fe}^{16}\text{O}$ trace and dotted line to the $^{57}\text{Fe}^{16}\text{O}$ trace.

see chapter ‘Samples’ above). These findings suggested co-elution of FER and concomitant iron containing proteins, which were present in the serum sample. A subsequent iron and sulfur SEC-ICP-MS analysis of a FER and transferrin standard mixture suggested that these species might be transferrin oligomers.

To remove interfering iron containing proteins, we subjected an aliquot of the second serum sample to centrifugal ultra-filtration prior to SEC-ICP-MS analysis. However, this purification attempt failed as neither centrifugal ultra-filtration

nor size exclusion chromatography can separate proteins of similar size and shape.

This problem could be overcome by a combination of chemical and size-based separation techniques. For example, Konz *et al* showed effective purification of intact FER from human serum by methanol and heat precipitation prior to centrifugal ultra-filtration and SEC-ICP-MS analysis [25]. However, this multi-step purification procedure is time consuming and FER recoveries accounted for 40%. Furthermore, murine

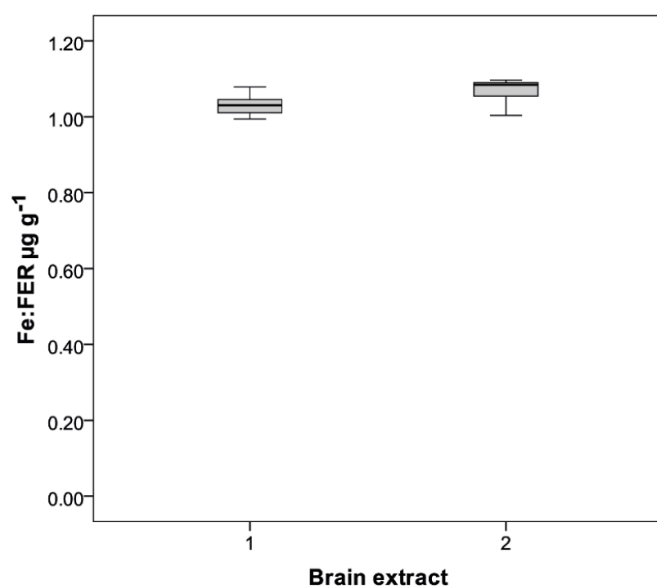


Figure 6. Boxplot of FER-bound iron (Fe:FER) content of extracts of mouse brain hemispheres determined by species-specific IDMS ($n = 5$); whiskers correspond to minimum and maximum FER-bound iron values.

serum is available only in limited amount of a few 100 μl in comparison to 1.5 ml human serum used by Konz *et al* [25]. Consequently, major loss of FER might result in FER-bound iron levels being below LOQ. Thus, quantification of FER-bound iron in murine serum by species-specific IDMS was not considered further.

3.3. Method application to murine brain extract

The SEC chromatogram of fresh murine brain extract showed a pronounced FER peak at retention time of 10.0 min (figure 4). Furthermore, in contrast to murine serum, iron containing matrix proteins did not interfere with the FER signal. Thus, it was possible to apply species-specific IDMS for FER-bound iron quantification in murine brain extracts.

Figure 5 shows the $^{56}\text{Fe}^{16}\text{O}$ and $^{57}\text{Fe}^{16}\text{O}$ trace of murine brain extract spiked with $(^{57}\text{Fe})\text{FER}$, which represented the calibrant for FER-bound iron quantification. Species-specific IDMS revealed a FER-bound iron content of $(1.03 \pm 0.11) \mu\text{g g}^{-1}$ and $(1.07 \pm 0.12) \mu\text{g g}^{-1}$ in extracts of mouse brain hemispheres ($n = 5$; $U, k = 2$), respectively. These results overlapped within limits of uncertainty. Thus, according to EURACHEM guidelines they have to be considered equal [21]. This result was additionally confirmed by an independent t-test and illustrated by a boxplot diagram (figure 6). Consequently, these findings suggested no significant difference in FER-bound iron content between extracts of brain hemispheres of the investigated control mouse.

These results represent a first step towards the application of species-specific IDMS for FER-bound iron quantification in murine samples relevant for AD studies. It is also of interest to note that FER seems to be the major iron containing protein in brain cytosol extracts, which is in strong contrast to serum.

Table 2. Content of total Fe and FER-bound iron in calibrants and mouse brain extracts determined by (reverse) IDMS; uncertainties correspond to $U, k = 2$; significant numbers of digits are given according to EURACHEM guidelines.

Solution	Replicates	Unit	Fe	U	U_{rel}
Fe standard	$n = 5$	mg g^{-1}	1.0000	0.0030	0.30%
$^{57}\text{FeCl}_2$ solution	$n = 5$	mg g^{-1}	1.952	0.026	1.3%
FER standard	$n = 5$	mg g^{-1}	10.8	0.6	5%
$(^{57}\text{Fe})\text{FER}$	$n = 5$	$\mu\text{g g}^{-1}$	3.87	0.32	8%
Brain extract	$n = 10$	$\mu\text{g g}^{-1}$	1.05	0.12	11%

3.4. Uncertainty contributors to ferritin-bound iron content

In this study, the reported uncertainty comprised the combined measurement uncertainty and the sample homogeneity. The first component takes all sources of error of the measurement procedure into account. The second component corresponds to the reproducibility, *i.e.* the variation of FER-bound iron content in murine brain extracts.

The gross mean of FER-bound iron content in murine brain extracts accounted for $(1.05 \pm 0.12) \mu\text{g g}^{-1}$ ($n = 10$; $U, k = 2$; table 2). Thus, species-specific IDMS facilitated to achieve a relative expanded uncertainty of 11% ($U_{\text{rel}}, k = 2$). The uncertainty of the iron elemental content of the $(^{57}\text{Fe})\text{FER}$ spike represented the major contribution to the combined uncertainty. It accounted to a relative expanded uncertainty of 5.7% and was a consequence of the applied multi-step calibration procedure (table 2; Note: The relative uncertainty of the result is always greater than the uncertainty of the calibrant used for quantification.). The contribution of the sample homogeneity to the expanded uncertainty accounted for 4.7% ($U_{\text{rel}}, k = 2$). This value corresponds to 2 RSD of the determined FER-bound iron content in mouse brain extracts. Other uncertainty sources such as the weights of the sample and the spike, the atomic weights, the isotope ratios, etc contributed to 0.6% of the relative expanded uncertainty of FER-bound iron content in mouse brain extracts.

4. Conclusion

The investigation of fundamental mechanisms of the Alzheimer's disease calls for sophisticated methods capable of delivering accurate and comparable results. Species-specific IDMS facilitated the determination of FER-bound iron in cytosolic extracts of murine brain, which accounted for $(1.05 \pm 0.12) \mu\text{g g}^{-1}$ ($n = 10$; $U, k = 2$). The achieved low LOQ of 2.2 ng g^{-1} and relative expanded uncertainty of 11% ($U_{\text{rel}}, k = 2$) obtained for a mass fraction of ca. $1 \mu\text{g g}^{-1}$ iron in FER are suitable for determining differences in FER-bound iron content of brain extracts of healthy and AD mice. Furthermore, using this method analytical results are traceable to the SI unit kg through an unbroken calibration chain. This ensures comparability of FER-bound iron levels across research centers. This methodology provides the basis for deepening our understanding of the role of FER in the onset

and progress of Alzheimer's disease. In future, it could potentially serve as a reference method in (pre)clinical Alzheimer's disease studies. These metrological measures will enable to combine and compare large numbers of analytical results from different research laboratories all over the world.

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Conflict of interest

There is no conflict of interest.

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