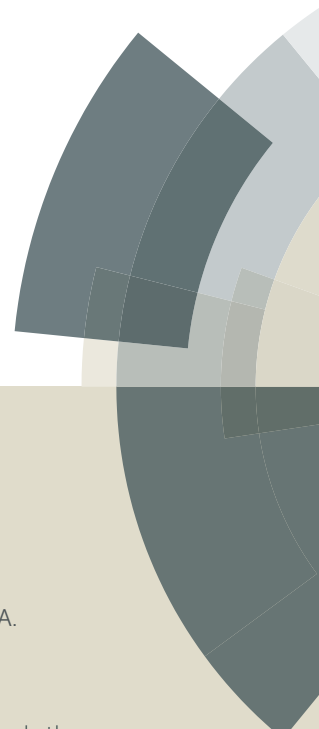


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## Accurate and precise quantification of Cu,Zn-SOD in human red blood cells using species-specific double and triple IDMS

Julia Gleitzmann,<sup>\*a</sup> Andrea Raab,<sup>b</sup> Dirk Schulze,<sup>a</sup> Hermann Wätzig,<sup>c</sup> Jörg Feldmann<sup>b</sup> and Claudia Swart<sup>a</sup>

<sup>a</sup>Physikalisch Technische Bundesanstalt, Bundesallee 100, 38116 Braunschweig, Germany

<sup>b</sup>University of Aberdeen, Trace Element Speciation Laboratory, Meston Walk, Meston Building, Aberdeen AB24 3UE, United Kingdom

<sup>c</sup>Technical University of Braunschweig, Institute of Medical and Pharmaceutical Chemistry, Beethovenstr. 55, 38106 Braunschweig, Germany

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Cu,Zn-superoxide dismutase (SOD1) is a protein involved in the antioxidant defense system of the body responsible for the dismutation of the superoxide anion. It contains two Cu and two Zn ions per molecule. As this protein is also involved in several diseases it is used in clinical diagnostics as biomarker, which requires the accurate and reliable determination of SOD1. Therefore, a candidate reference measurement procedure for the quantification of this protein in human erythrocytes was developed using species-specific isotope dilution mass spectrometry (IDMS), a method giving results traceable to the International System of Units (SI). The measurement procedure was validated with regard to a metrological point of view. Commercially available SOD1 was thoroughly characterized to be used as pure protein calibration standard in the IDMS approaches. Furthermore, <sup>65</sup>Cu and <sup>67</sup>Zn labeled SOD was produced to be used as spike material required for species-specific IDMS. Finally, SOD1 was quantified in human erythrocytes using both double and triple IDMS and a complete uncertainty budget for both approaches was estimated according to the Guide for the Expression of Uncertainty in Measurement (GUM). A calculated mass fraction of SOD1 with its associated expanded uncertainty of (63.94 ± 0.93) µg/g (*n* = 30) for double and (64.02 ± 0.96) µg/g (*n* = 30) for triple IDMS was obtained.

### Introduction

Cu,Zn-superoxide dismutase (SOD1)<sup>1</sup> is an homodimeric enzyme which contains a closely spaced non-covalently bound Cu and Zn ion pair<sup>2,3</sup> and has a molecular mass of about 31800 g/mol for the human species. It catalyzes the disproportionation of the superoxide anion (O<sub>2</sub><sup>-</sup>) into oxygen (O<sub>2</sub>) and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>).<sup>4</sup> Responsible for the enzymatic activity is the Cu ion as the disproportionation of O<sub>2</sub><sup>-</sup> is driven by the [Cu(II)/Cu(I)] ion redox cycle.<sup>3</sup> Besides a single disulfide bond and a complex network of hydrogen bonds, the Zn ion accounts for the great stability of the SOD1. The enzymatic activity is linked to the Cu bound to the protein. The involvement of SOD1 in preventing oxidative stress can be used as bioindicator for environmental impact assessments.<sup>5</sup> Additionally, the protein plays a role in medical diagnostics. SOD1 activity is known to decrease in malignant lymphoma,<sup>6</sup> chronic alcoholism<sup>7</sup> and medullar carcinoma,<sup>8</sup> its activity is increased in leukemia.<sup>9</sup> It can be used as prenatal marker for Down's syndrome<sup>10</sup> and is involved in neurodegenerative diseases such as Alzheimer's disease.<sup>11</sup> Furthermore, it is a potential marker for gastric cancer.<sup>12</sup> When results from the quantitative determination of SOD1 are used for clinical diagnostics, it is of great importance that the results of the protein quantification are comparable and reliable (inter- and intralaboratory).<sup>13</sup> In routine or clinical laboratories immunoassays such as enzyme-linked immunosorbent assay (ELISA)<sup>14</sup> or spectrophotometric assays<sup>15,16</sup> are used to quantify SOD1, but there are no reference methods or materials available yet. Comparison of some of these methods led to large differences in the results, the limits of quantification (LODs) and the reproducibility.<sup>17</sup> This shows that reference methods and materials are urgently required. None of these clinical assays is based on metrological principles and results are not traceable to the International System of Units (SI) as demanded by directives such as Directive 98/79/EC.<sup>18</sup> Furthermore, no uncertainty budget according to the Guide for the Expression of Uncertainty in Measurement (GUM) was estimated for the quantification of SOD1 for these methods. Within the project HLT05, a reference method traceable to the SI<sup>19</sup> was developed for the first time using species-specific double and triple isotope dilution mass spectrometry (IDMS) for the quantification via the Cu content of SOD1. By measuring the Cu containing portion of the SOD1, only the amount of active protein is quantified. Thus, the method can

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3 45 serve as reference method of higher order for the routine measurements. The basic principle of IDMS is the change of the  
4 natural isotopic composition of a chosen element in the sample by the addition of a substance enriched in a minor isotope of  
5 the chosen element, called spike. For the double IDMS approach,<sup>20</sup> a blend of a reference material with the spike, the  
6 reference spike blend, is prepared and both sample spike and reference spike blends are measured with ICP-MS. In the  
7 reference material, the analyte is well characterized and acts as back spike. A further approach recently developed is triple  
8 IDMS,<sup>21,22,23</sup> in which additionally a second reference spike blend is prepared. This is especially recommendable when a  
9 spike material is used with a comparable low enrichment leading to a high uncertainty in the measurement of the isotope ratio  
10 of the spike material as this isotope ratio is canceled from the equation. A more detailed description of the advantages of  
11 triple IDMS is given by Vogl.<sup>21</sup> Although the IDMS approach is well-established and accepted for elemental analysis,<sup>24</sup> the  
12 application in elemental speciation, especially for metalloproteins, is still challenging.<sup>25</sup> For the use of IDMS in speciation  
13 analysis there are two basic approaches: The first one is the species-unspecific IDMS also referred to as post-column  
14 IDMS,<sup>26,27</sup> where the elemental spike is added to the analyte after sample preparation and separation. The second one is the  
15 species-specific IDMS.<sup>28</sup> Here, the species-specific spike material is added to the sample as soon as possible before any  
16 sample preparation and separation which has the advantage that any losses or changes of the analyte during the sample  
17 preparation process can be compensated for.  
18  
19 Commercially available SOD1 was characterized for the use as reference material and species-specific spike material was  
20 prepared from this. As proof of concept SOD1 was quantified in a human erythrocyte sample by species-specific double and  
21 triple IDMS and the results were compared.  
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## Experimental

### 65 Material and Reagents

All chemicals used were of analytical grade or better unless stated otherwise. High-purity deionized water (18 M $\Omega$ cm, Merck Millipore, Darmstadt, Germany) was used for the preparation of buffers and solutions. The chemicals for the preparation of buffer and solvents include ammonia acetate (NH<sub>4</sub>Ac), tris(hydroxymethyl)methylamine (Tris, both purchased from Sigma-Aldrich, St. Louis, USA), acetic acid, nitric acid, hydrochloric acid (HCl), potassium hydroxide (KOH, all purchased from Merck, Darmstadt, Germany), ethylenediaminetetraacetic acid (EDTA), guanidinium chloride (GndCl, both purchased from Carl Roth, Karlsruhe, Germany), sodium chloride (NaCl, J.T. Baker, Center Valley, USA) and potassium chloride (KCl, Fisher, Loughborough, UK). Furthermore, ethanol (Carl Roth) and chloroform (AppliChem, Darmstadt, Germany) were used. For the activity assay pyrogallol and diethylenetriaminepentaacetic acid (DTPA), purchased from BDH (Dorset, UK), were used. Polyacrylamide gel electrophoresis (PAGE) was performed with hand-cast native gels and with native tris-glycine buffer. Reagents were purchased from Carl Roth. SOD1 from bovine erythrocytes as well as from human erythrocytes were purchased from Sigma-Aldrich. External calibration was performed using Cu and Zn standard solutions (AccuTrace, Bryn Mawr, USA). <sup>65</sup>Cu and <sup>67</sup>Zn, which were used for isotopic labelling of the bovine SOD1 were from CK Gas Products (Hampshire, UK) with a certified isotopic abundance of <sup>63</sup>Cu 0.8 % and <sup>65</sup>Cu 99.2 % and <sup>64</sup>Zn 0.38 %, <sup>66</sup>Zn 0.33 % and <sup>68</sup>Zn 99.0 %. For labelling the human SOD1, <sup>65</sup>Cu and <sup>67</sup>Zn were obtained from Chemotrade (Leipzig, Germany) with a certified isotopic abundance of <sup>63</sup>Cu 0.3 % and <sup>65</sup>Cu 99.7 % as well as <sup>64</sup>Zn 0.67 %, <sup>66</sup>Zn 1.19 %, <sup>67</sup>Zn 97 %, <sup>68</sup>Zn 1.11 % and <sup>70</sup>Zn < 0.02 %. Stock solutions of isotopically enriched Cu and Zn were prepared by dissolving the metal in concentrated subboiled nitric acid and diluted gravimetrically to an appropriate working solution. Human erythrocytes were obtained from the German Blood Bank (Braunschweig, Germany), the bovine erythrocytes were obtained from a local abattoir (Aberdeen, UK).

### 85 Instrumentation

The IDMS measurements as well as the characterization of the potential reference material were accomplished with a hyphenated high performance liquid chromatography (HPLC)-ICP-MS system. The chromatographic separation was performed with an Agilent 1200 (Agilent Technologies, Santa Clara, USA) equipped with a degasser, a binary pump, a thermostated autosampler, a column oven and a diode array detector (DAD). The separation was achieved on a strong anion

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3 90 exchange column (MonoQ 5/50 GL, GE Healthcare, Uppsala, Sweden). The coupling of the HPLC to the ICP-MS was  
4 realized with a PEEK capillary. The isotopes of interest were detected with a quadrupole ICP-MS system (Agilent 7700cx) in  
5 pulse-counting mode. Typical instrumental working conditions for HPLC and ICP-MS are summarized in *ESI* table 1. For the  
6 post-column IDMS measurements the mass flow of the isotopically enriched spike was monitored continuously using the  
7 balance Genius ME215S (Sartorius, Göttingen, Germany) applying a method improved by Swart et. al.<sup>29</sup> The balance sent the  
8 mass reading to the data acquisition software SartoConnect provided by the manufacturer of the balance. To determine the  
9 molecular mass of the native protein electrospray ionisation (ESI)-MS was used. The measurements were performed on the  
10 quadrupole time-of-flight (TOF) MS micrOTOF-Q (Bruker Daltonics, Billerica, USA). It was used in a positive detection  
11 mode with an extended mass range from 300 - 3000 Da. The obtained spectra were deconvoluted with the Data Analysis  
12 software provided by the manufacturer.

#### 100 **Characterization of commercial available SOD1 for the use as reference for double IDMS**

13 The identity of the purchased protein was verified with molecular MS (ESI-TOF-MS). The sample was separated on a  
14 reversed phase column (Jupiter300-C4, Phenomenex, Torrance, USA) using an Agilent 1200 HPLC system. To determine the  
15 purity of the prepared protein solutions concerning the Cu amount post-column IDMS was used. The isotopic composition of  
16 the used spike material was fully characterized with a sector-field ICP-MS in high resolution. Furthermore, the protein was  
17 determined by UV-Vis, which is integrated in the HPLC, at 259 nm and 280 nm.

#### 105 **Preparation and characterization of isotopically enriched SOD1**

18 Three published methods were compared regarding their demetallation efficiency and the possibility to remetalate the  
19 apo-protein to produce the isotopically labelled SOD1. In method A, the protein was dialyzed against NH<sub>4</sub>Ac buffer at pH  
20 2.8<sup>30</sup> and in method B against NH<sub>4</sub>Ac buffer containing EDTA at pH 3.8,<sup>31</sup> whereas in method C the demetallation was  
21 performed using GndCl.<sup>32</sup> For remetalation to the produced apo-SOD1, <sup>68</sup>Zn was added in surplus, firstly, and <sup>65</sup>Cu,  
22 secondly. Both the apo- and remetalated SOD1 as well as the native protein were characterized regarding metal content,  
23 protein integrity and activity.<sup>15</sup> Further information about instrumentation and methods are given in the *ESI*.

#### 110 **Determination of SOD1 in human erythrocytes using IDMS**

24 For homogenization all solutions, erythrocytes, reference stock solution and spike stock solution, were vortexed at r.t. for at  
25 least one hour before use. The stock solutions of reference material and spike material were diluted to a final concentration of  
26 0.025 mg/g (reference material) and 0.1 mg/g (spike material), respectively. The reference spike blends as well as the  
27 erythrocyte spike blends for the IDMS measurements were prepared gravimetrically and homogenized for about an hour at  
28 r.t. before further use. To precipitate the hemoglobin, a mixture of ethanol/chloroform (3:1) was added to the blends in the  
29 ratio of 1:1. The blends were shaken for 20 min and then centrifuged at 12000 rpm and 4 °C for 15 min. The supernatant was  
30 transferred to another tube and the residue was discarded. To remove the organic solvents, the blends were concentrated  
31 using MWCO centrifugal filters and the residues were brought to their final volume with water. Finally, the prepared blends  
32 were measured with HPLC-ICP-MS. Peak areas were integrated with MassHunter software from Agilent. The SOD amount  
33 in the erythrocytes was calculated using the equations for double IDMS and triple IDMS (equations are given in the results  
34 and discussion section). The expanded uncertainty for both approaches was estimated with the GUM workbench (Metrodata  
35 GmbH, Weil am Rhein, Germany).

### 49 **Results and discussion**

#### 50 **Characterization of commercial available SOD1 for the use as reference for double IDMS**

51 The identity of the purchased human SOD1 was checked by means of ESI-TOF-MS (data given in *ESI* figure 1). To exclude  
52 the occurrence of protein impurities the protein was determined after separation by UV-vis. The column recovery was  
53 calculated to be (68.62 ± 0.90) % for SOD1 (*n* = 10) on the SAX column regarding the Cu content. A purity of > 80 % of the  
54 protein material by Biuret is stated by the manufacturer. To check this, the purity of the prepared protein solutions was  
55 determined by performing post column IDMS as developed by Heumann et. al.<sup>26</sup> and optimized by Swart et. al.<sup>29</sup>, in which  
56 the isotopically enriched spike material is added continuously to the effluent from the HPLC with a peristaltic pump via a T-  
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3 135 piece. With the SartoConnect software the mass flow of the spike was recorded continuously over the whole time of the  
4 chromatogram. The  $^{63}\text{Cu}$  isotope serves as reference isotope. In all measurements, the isotope ratios were corrected for mass  
5 bias by the determination of the correction factor  $K$  calculated by setting the natural isotope ratio of a measured sample in  
6 relation to the “true” isotope ratio according to the International Union of Pure and Applied Chemistry (IUPAC). Protein  
7 solutions and spike solutions, which were added to the sample post-column, were prepared gravimetrically. The deployed  
8  
9 140 masses were corrected for air buoyancy. To take into account the sample loss during the separation procedure the column  
10 recovery is considered in the calculation of the Cu mass fraction of the sample. The used equation is given in the *ESI*.<sup>29</sup> For  
11 the used SOD1 material, a Cu mass fraction of  $(0.290 \pm 0.014) \mu\text{g/g}$  was calculated ( $n = 20$ ), which corresponds to a SOD1  
12 amount of  $(0.073 \pm 0.004) \text{mg/g}$ . The purity of the purchased material and its estimated uncertainty was found to be  
13  $(72.80 \pm 4.9) \%$  with regard to the Cu amount. The uncertainty budget was estimated with the GUM workbench as extended  
14 uncertainty ( $k = 2$ ).  
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#### 16 Production of enriched SOD1

17 The first step of the SOD1 spike production is the demetallation followed by remetallation with isotopically enriched metals  
18 (a general scheme is given in figure 1). Due to the high stability of the Cu and Zn bonds in SOD1, relatively harsh conditions  
19 are required for demetallation, which may lead to irreversible changes of the secondary structure. Three methods are used  
20 predominantly in the literature and these were tested for the preparation of bovine SOD1 spike material (\*SOD1) and  
21 compared to each other to get a spike fit for purpose in species-specific IDMS. One of the first methods for the removal of  
22 both Cu and Zn, was the treatment of the protein with EDTA at low pH (called method B in the present work).<sup>33,34,35</sup> Another  
23 method includes the use of even lower pH but omits the use of EDTA (method A).<sup>30</sup> One method which does not expose the  
24 enzyme to low pH, uses the denaturing ability of GndCl and EDTA as complexing agents (method C).<sup>36,37,32</sup> Demetallation  
25 and remetallation efficiency as well as isotope enrichment was determined by SEC-ICP-MS (*ESI* table 3 and *ESI* figures 3  
26 and 4). The correct incorporation of the metals into their native sites is checked by performing native gel electrophoresis and  
27 activity assay (*ESI* table 3 and *ESI* figure 2). To ensure that the produced \*SOD1 is stable in the biological matrix, the spike  
28 was added to bovine erythrocytes and the isotope ratios were measured (*ESI* figures 5 and 6). Compared to the other two  
29 methods \*SOD1 produced according to method B has the highest remetallation efficiency and isotopic enrichment. Dimeric  
30 protein structure as well as activity is restored nearly completely. Furthermore, \*SOD1 is stable when added to biological  
31 samples, which shows that method B is the most suited method for producing species-specific spike material.<sup>38,39</sup>  
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#### 165 Quantification of SOD1 in human erythrocytes using species-specific double and triple IDMS

166 Bovine SOD1 spike showed different stability in samples of human origin and a different retention time compared to human  
167 SOD and is, therefore, not suitable as spike for human erythrocyte lysate as shown by Nuevo-Ordonez et. al.<sup>39</sup> Hence, for the  
168 determination of SOD1 in human erythrocyte lysate, a spike was prepared from commercial human SOD1 following method  
169 B. A chromatogram of the produced human \*SOD1 is shown in figure 1. To determine if the human \*SOD1 is applicable for  
170 the use in species-specific IDMS, the stability of the spike in human erythrocytes was tested over a time period of 48 h and  
171 found to be satisfactory (*ESI* figure 7), which makes it fit for use in double and triple IDMS experiments. The advantage in  
172 performing double species-specific IDMS is that, compared to single species-specific IDMS,<sup>39</sup> the purity of the used species-  
173 specific spike has not to be known as it is canceled from the equation when using exact matching IDMS. However, a key step  
174 in double IDMS measurements is the characterization of the purchased protein to be used as reference. The sample spike  
175 blends (bx) and the reference spike blends (bz) were prepared three times each independently on three different days. Each  
176 blend was measured ten times in a bracketing sequence. For the double IDMS experiments, the reference as well as the  
177 erythrocyte sample were mixed with the isotopically enriched spike material to result in an isotope ratio of  $^{65}\text{Cu}/^{63}\text{Cu}$  of  
178 approximately 1:1 (figure 2). For all measurements, the isotope ratios were corrected for mass bias. Equation 2<sup>20</sup> was used for  
179 calculation of the SOD1 amount. Meanings of the used symbols are described in table 1.

$$180 \quad W_x = W_z * \frac{m_{yx}}{m_x} * \frac{m_z}{m_{yz}} * \frac{R_y - R_{bx}}{R_{bx} - R_x} * \frac{R_{bz} - R_z}{R_y - R_{bz}} \quad (1)$$

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4 180 The deployed masses of reference solution, spike solution and samples were corrected for air buoyancy. The calculated  
5 SOD1 mass fraction in the erythrocyte sample as well as the estimated expanded uncertainty is listed in table 2. As shown in  
6 table 3, the main contributions to the uncertainty were found to be the uncertainty of the purity of the used reference ( $w_{\text{pur}}$ )  
7 and the measured isotope ratios in the sample spike blend ( $R_{\text{bx}}$ ) and the reference spike blend ( $R_{\text{bz}}$ ). For the double IDMS  
8 measurements the variation of the individual results and the average are shown in figure 3A. For triple IDMS the same  
9 erythrocyte sample was mixed with \*SOD1 to result in an approximated 1:1 ratio. Unlike for double IDMS, the triple IDMS  
10 approach requires two reference spike blends, both with a slightly different final isotope ratio but both close to 1:1. The final  
11 isotope ratio  $^{65}\text{Cu}/^{63}\text{Cu}$  was chosen to be approximately 0.9 for the first reference spike blend ( $R_{\text{bz1}}$ ) and approximately 1.1  
12 for the second reference spike blend ( $R_{\text{bz2}}$ ). In this approach the sample spike blend ( $\text{bx}$ ) and reference spike blends ( $\text{bz1}$ ,  
13  $\text{bz2}$ ) were prepared three times independently and on three different day. While  $\text{bx}$  is measured ten times,  $\text{bz1}$  and  $\text{bz2}$  were  
14 measured six and five times, respectively, in a bracketing sequence (means  $\text{bz1}_1, \text{bx}_1, \text{bz2}_1, \text{bx}_2, \text{bz1}_2, \text{bx}_3, \text{bz2}_2,$   
15  $\text{bx}_4, \text{bz1}_3, \text{bx}_5, \text{bz2}_3, \dots$ ). The deployed masses during the preparation of the blends were corrected for air buoyancy.  
16 Equation 3<sup>23</sup> was used to calculate the SOD1 amount in the sample following triple IDMS approach. Meanings of the used  
17 symbols are described in table 1.

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$$195 \quad w_x = w_z * \frac{m_{yx}}{m_x} * \frac{1}{R_x - R_{\text{bx}}} * \left[ \frac{m_{z1}}{m_{yz1}} * \frac{R_{\text{bz2}} - R_{\text{bx}}}{R_{\text{bz2}} - R_{\text{bz1}}} * (R_x - R_{\text{bz1}}) + \frac{m_{z2}}{m_{yz2}} * \frac{R_{\text{bx}} - R_{\text{bz1}}}{R_{\text{bz2}} - R_{\text{bz1}}} * (R_x - R_{\text{bz2}}) \right] \quad (2)$$

The calculated SOD1 amount in erythrocytes and the appropriate expanded uncertainty given by the triple IDMS approach is shown in table 2. Major contributions to the uncertainty were found to be the measured isotope ratios in  $\text{bx}$  followed by the measured isotope ratios in the reference spike blends and the purity of the reference used as shown in table 3. The variation of the individual results and the average of the triple IDMS measurements are shown in figure 3B. In comparison to double IDMS the advantage of triple IDMS is, that the isotopic composition of the spike ( $R_y$ ) has not to be determined as it is cancelled from the equation. This is offset by a slightly higher workload due to the preparation of a second reference spike blend and an increased measurement time. The results obtained from the double IDMS and triple IDMS approach for the determined human erythrocyte sample fit together very well within the estimated uncertainty which is also confirmed by the  $t$ -test as the empirical  $t$ -value is smaller than the critical  $t$ -value ( $0.502 < t < 2.776$ ,  $\alpha = 0.05$ ). The high precision of the applied methods is pointed out by the achieved estimated expanded uncertainty of 1.45 % and 1.50 %, respectively. This demonstrates the usefulness of species-specific double and triple IDMS to provide reference values for clinical laboratories as there are no reference methods or materials for SOD1 available yet.

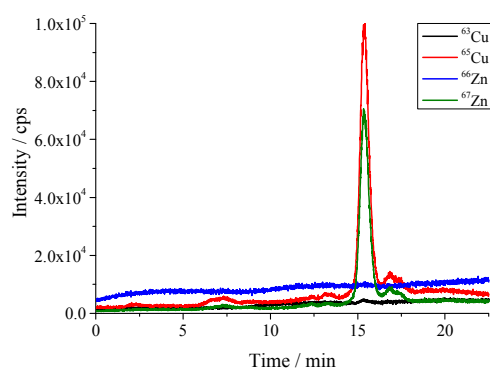
## 210 Conclusion

Two methods based on double and triple IDMS were applied for the precise quantification of SOD1 in human erythrocytes. Purchased human SOD1 was characterized using single post-column IDMS, UV-Vis and ESI-TOF-MS before use as a reference, which is the key component in IDMS. Furthermore, species-specific spike material was produced and characterized. A detailed comparison study of three methods published in the literature showed that using low pH and EDTA is the most suitable method for demetallation in the SOD1 spike preparation procedure. Final quantification of SOD1 in a real sample of human erythrocytes via double and triple IDMS shows a good agreement for the results of both methods within the expanded uncertainty. A complete uncertainty budget was estimated using the GUM workbench. The expanded uncertainty (with  $k=2$ ) of below 2 % for both approaches demonstrates the high precision of the applied methods. The developed methods enable traceability of the results to the SI and can, therefore, be used as reference methods of higher order for routine analysis methods in clinical laboratories as required by the European *in vitro* diagnostica directive (Directive 98/79/EC) for the use of *in vitro* diagnostica in samples of human origin.<sup>18</sup> Furthermore, there is the chance to characterize SOD1 in a biological matrix for the use as reference material for validating quantification methods in medical routine

laboratories. Thus, providing reference values to clinical laboratories will help to make the results of different routine measurement procedures more comparable. As SOD1 is an important marker amongst others for neurodegenerative diseases such as Alzheimer's disease or amyotrophic lateral sclerosis, where the concentration is much lower (0.5  $\mu\text{g/g}$  in cerebrospinal fluid<sup>40</sup>) than in erythrocytes, in the future perspectives strategies for the enrichment of SOD1 have to be developed so that quantification by species-specific IDMS can be applied for this purpose.

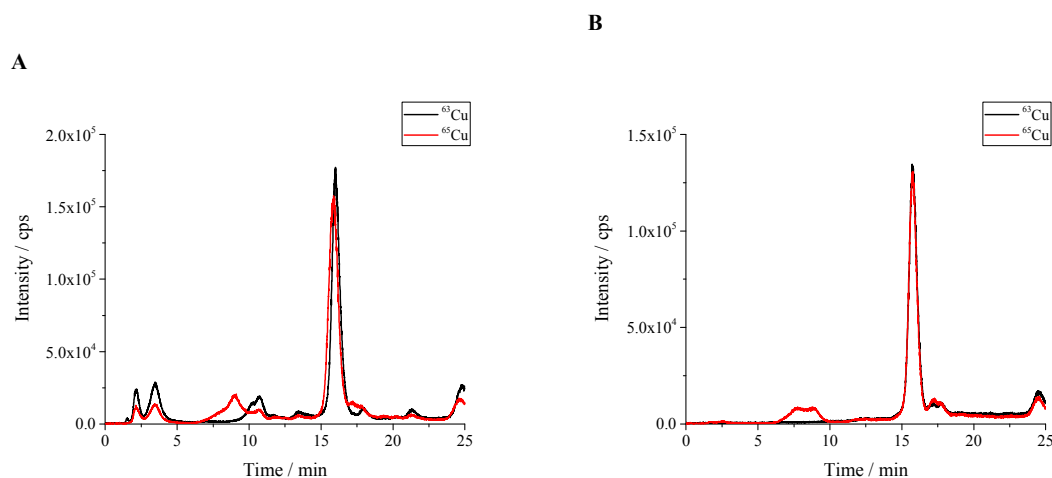
### Acknowledgement

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**Figure 1.** Chromatogram of produced human SOD1 spike material.



**Figure 2.** A) sample spike blend and B) reference spike blend prepared for the use in double IDMS measurements.

**Table 1.** Meaning of the symbols used in the equations (1) – (3).

symbol	unit	definition
$w_x, w_z$	g/kg	mass fraction of Cu in sample x and reference z, resp.

$R_x, R_y, R_z$	mol/mol	isotope amount ratio of spike and reference isotope ( $^{65}\text{Cu}/^{63}\text{Cu}$ ) in sample x, spike y and reference z, resp.
$R_{bx}, R_{bz}, R_{bz1}, R_{bz2}$	mol/mol	isotope amount ratio of spike and reference isotope ( $^{65}\text{Cu}/^{63}\text{Cu}$ ) in blends bx (sample x + spike y) and blends bz, bz1 and bz2 (reference z, z1, z2 + spike y), resp.
$m_x, m_z, m_{z1}, m_{z2}, m_{yx}, m_{yz}, m_{yz1}, m_{yz2}$	g	masses of sample x, references z, z1, z2 and spike y solutions, resp.

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**Table 2.** Calculated SOD1 amount in a human erythrocyte sample and the appropriate expanded uncertainty for species-specific double and triple IDMS approach.

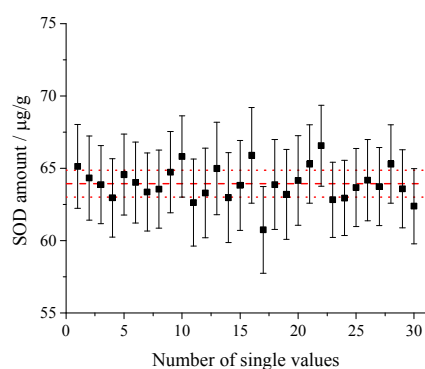
	SOD1 amount in erythrocytes	expanded uncertainty ( $k = 2$ )	expanded uncertainty ( $k = 2$ )
	in $\mu\text{g/g}$	in $\mu\text{g/g}$	in %
double IDMS	63.94	0.93	1.45
triple IDMS	64.06	0.96	1.50

**Table 3.** Major contributions to the uncertainty of the amount of SOD1 in erythrocytes using species-specific double and triple IDMS, resp.

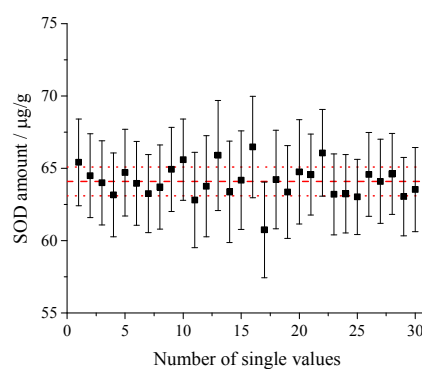
245

	double IDMS	triple IDMS
	in %	in %
$w_{\text{pur}}$	71.7	70.4
$R_{bx}$	15.3	15.6
$R_{bz}$	10.6	-
$R_{bz2}$	-	11.4

A



B



**Figure 3.** Variation of the individual results for A) double IDMS measurements and B) triple IDMS measurements. The error bars represent the expanded uncertainty ( $k = 2$ ) of the single values. The average with its estimated uncertainty is illustrated in red.

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