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Technical note

A quick and simple method for the determination of six trace elements in mammalian serum samples using ICP-MS/MS

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ABSTRACT

In order to assess the individual trace element status of humans for either medical or scientific purposes, amongst others, blood serum levels are determined. Furthermore, animal models are used to study interactions of trace elements. Most published methods require larger amounts (500–1000 µL) of serum to achieve a reliable determination of multiple trace elements. However, oftentimes, these amounts of serum cannot be dedicated to a single analysis and the amount available for TE-determination is much lower. Therefore, a published ICP-MS/MS method for trace element determination in serum was miniaturized, optimized and validated for the measurement of Mn, Fe, Cu Zn, I and Se in as little as 50 µL of human and murine serum and is presented in this work. For validation, recoveries of multiple LOTs and levels from commercially available human reference serum samples were determined, intra- and inter-day variations were assessed and limits of detection and quantification determined. It is shown, that the method is capable of giving accurate and reproducible results for all six elements within the relevant concentration ranges for samples from humans living in central Europe as well as from laboratory mice. As a highlight, the achieved limits of detection and quantification for Mn were found to be at 0.02 µg/L serum and 0.05 µg/L serum, respectively, while using an alkaline diluent for the parallel determination of iodine.

1. Introduction

Trace elements (TE) play a role in a myriad of bodily functions, such as enzymes, hormones and other transmitters. Therefore, almost any disease will interact with TE-homeostasis. While most research into TE is focused on a single element or sometimes the interaction of two, very few studies consider the interactions of multiple TE at the same time. However, the homeostasis of one TE is often influenced by the abundance of another within the body. One well established example is thyroid hormone homeostasis. The iodination step during thyroid hormone synthesis is catalyzed by the iron (Fe) dependent thyroid peroxidase [1], while the active thyroid hormone triiodothyronine (T3) is made by deiodinases, which have selenocysteine incorporated in their active sites [2]. Furthermore, selenium (Se), manganese (Mn), copper (Cu) and zinc (Zn) also influence the thyroid function as an essential constituent of enzymes which protect against reactive oxygen species, such as superoxide dismutases 1 (Cu/Zn) and 2 (Mn) and glutathione peroxidases (Se) [3]. The DFG research unit #2558 TraceAGE aims to investigate this issue by establishing TE-fingerprints, based on more comprehensive TE-profiles for six different TE and other TE-related markers with regards to aging and age-related diseases. The status for these TE, namely Mn, Cu, Fe, Zn, Se and iodine (I), will be determined in large human cohorts as well as in samples originating from *in vivo* experiments with for example *Mus musculus*. For both human and murine serum samples, the amount of available sample can be very limited. This is either due to the high number of other analyses being performed on a single sample from a large study cohort in the case of human serum or to the low volume of total available serum from a single specimen in the case of murine samples. Therefore, to determine the total amount of the six TE in question in these samples, a method is required that is capable of giving reliable results for all six elements in very minute amounts of serum.

For multi-elemental analysis of trace elements, ICP-MS has been the method of choice in recent years, due to its wide dynamic range, general robustness and high sensitivity. However, for the six TE in question, a few considerations have to be made. First and foremost, the expected concentrations in serum originating from people living in central Europe need to be assessed. For Cu, Fe and Zn, the expected concentrations are within 0.4–1.6 mg/L [4], which can be easily detected with ICP-MS. However, Se, I and Mn occur at much lower

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Table 1
Method parameters for the ICP-MS/MS.

ICP-MS/MS parameters		
instrument	Agilent 8800 ICP-QQQ-MS, equipped with integrated autosampler	
plasma RF power	1550 W	
nebulizer	Glass Expansion MicroMist	
spray chamber	Scott-type	
plasma gas flow	15.0 L/min	
nebulizer gas flow	1.20 L/min	
spray chamber temperature	2 °C	
cones	Ni	
sample depth	8.0 mm	
gas modes	**He**	**O_2**
cell gas flow	3.0 mL/min He	0.6 mL/min O_2
stabilization time	18 s	18 s
m/z Q1	55 (Mn), 56 (Fe), 63 (Cu), 66 (Zn), 103 (Rh), 127 (I)	77 (Se), 80 (Se), 103 (Rh)
m/z Q2	55 (Mn), 56 (Fe), 63 (Cu), 66 (Zn), 103 (Rh), 127 (I)	93 (Se), 96 (Se), 103 (Rh)
integration time	0.5 s	0.5 s
replicates	3	3
target tuning parameters (optimized daily)		
sensitivity	maximum sensitivity on m/z $^{59}Co^+ \rightarrow {}^{59}Co^+$, $^{89}Y^+ \rightarrow {}^{89}Y^+$, $^{205}Tl^+ \rightarrow {}^{205}Tl^+$	maximum sensitivity on m/z $^{59}Co^+ \rightarrow {}^{59}Co^+$, $^{89}Y^+ \rightarrow (^{89}Y^{16}O)^+$, $^{205}Tl^+ \rightarrow {}^{205}Tl^+$
oxide ratio	< 1.5% $(^{140}Ce^{16}O)^+/{}^{140}Ce^+$	NA
doubly charged ratio	< 2% $^{140}Ce^{2+}/{}^{140}Ce^+$	< 2% $^{140}Ce^{2+}/{}^{140}Ce^+$
background	< 0.1 CPS	< 0.1 CPS

concentrations ranging around 70–150 µg/L, 40–92 µg/L, and < 1–3 µg/L, respectively [4,5]. Considering dilution due to sample preparation, these concentrations can borderline the quantification capabilities of the ICP-MS, especially in the case of Mn [6,7]. Regarding Se, another issue arises due to the very prevalently occurring and argon (Ar) and chlorine (Cl) based polyatomic interferences on most of its isotopes [8]. This can be elegantly solved by using more sophisticated ICP-MS instruments with a collision-/reaction cell, which eliminate interferences with their higher diameter as compared to the analyte by collision with He or H_2. Another option is the addition of a reactive gas, such as O_2 and thereby transforming Se^+ quantitatively to $[SeO]^+$, which effectively adds 16 amu to the analyte's mass. This separation can be further improved by using an ICP-MS/MS instrument. Furthermore, isotope dilution analysis (IDA) can be applied to enhance the robustness of the method [9,10]. As a final obstacle, iodine cannot be reliably quantified under any kind of acidic conditions, since iodine species can undergo comproportionation reactions to form I_2, which is volatile and would therefore be removed during sample preparation or cause very long wash out times in the sample introduction system [11]. However, most conventional methods for elemental determination rely on acidic digestions for matrix removal as sample preparation step. In this case, an alkaline work-up is necessary to capture iodine along with the other five elements, since two separate sample preparation procedures were not feasible due to constraints on both, sample volume as well as time.

Konz et al. [12] developed and validated a comprehensive method, capable of measuring 29 elements in human serum without the need for digestion. Instead, they employ a "dilute-and-shoot"-approach, based on a 1 + 9 dilution in an alkaline diluent containing ammonia, EDTA, non-ionic surfactant (Triton™ X100) and butanol (for signal enhancement). All six elements relevant for this work are covered by this method. Therefore, the method was adapted and further optimized for analysis in very little amounts of serum. Furthermore, for Se, instead of using an external calibration approach, an isotope dilution approach was adopted from Marschall et al. [13] to further improve the robustness and precision of the method regarding this element.

2. Method miniaturization and optimization

We aimed for a sample volume of 50 µL, as this is an amount of serum to be reliably acquired from a single mouse in an animal experiment or to be reasonably requested for a single analysis from an epidemiological study cohort.

The given dilution factor by Konz et al. [12] of 1 + 9 and 50 µL of sample gives a total volume of 500 µL. The task of introducing such small sample amounts to the ICP (Agilent 8800 ICP-QQQMS, Agilent Technologies, Waldbronn, Germany) usually calls for a flow-injection approach. However, brief testing of the latter resulted in high and unstable Mn-backgrounds when introducing the EDTA-containing diluent to the flow-injection system (HPLC 1200 infinity, Agilent Technologies, Waldbronn, Germany). Furthermore, in serum from large cohorts, sample quality may vary with samples occasionally containing high amounts of particulate matter from protein precipitation or fatty residues. This can become an issue for HPLC-based flow-injection approaches, where the sampling system uses low inner diameter capillaries and needles, which can easily get clogged by particulate matter. A classic autosampler uses wider tubing and is thus less affected by these issues. Therefore, we modified the sample tray of an integrated autosampler system (Agilent Technologies, Waldbronn, Germany) for use with ICP-MS instruments to support 1.5 mL plastic vessels. This was achieved by adding a mineral filled acrylic polymer disc on top of the original sample tray, which was custom-cut for purpose. In addition, the uptake tubing was shortened to minimize the liquid volume of the uptake system and peristaltic pump tubing with an inner diameter of 0.89 mm was employed. The uptake timing was minimized to allow for a variance of 5 s over the course of the measurement from detoriating peristaltic pump tubing as well as viscosity variances. After uptake, stabilization of the signal was reliably achieved after 15 s for all relevant isotopes. Using 500 µL of diluted sample, the signal was found to be stable for a minimum of 60 s. The next time- (and thus sample) consuming step was stabilization of the collision cell when switching gas modes. The method developed by Konz et al. [12] applies no-gas mode for I, He-mode for Mn, Fe, Cu and Zn, as well as O_2-mode for Se. Applying a standard stabilization time of 30 s per gas mode is therefore

Table 2
Method validation parameters. LOD and LOQ of Mn, Fe, Cu, Zn and I are calculated as equivalent concentrations of 3σ or 10σ of 6 blank signals, respectively. For Se, LOD and LOQ were calculated according to Yu et al. [18].

Element	Mn	Fe	Cu	Zn	I	Se
Calibration	external	external	external	external	external	isotope dilution analysis
calibration range [µg/L]	0.05–10	5–500	1–500	5–500	0.5–100	NA
calibration correlation coefficient (r^2)	0.9999	0.9993	0.9993	0.9996	0.9998	NA
limit of detection (LOD); [µg/L]	0.018	2.9	2.3	2.0	0.37	0.019
limit of quantification (LOQ); [µg/L]	0.050	3.4	2.5	7.6	2.1	0.099
Reference Material: RECIPE ClinChek Serum (Ref. 8880-8882, LOT 544)						
Level I						
reference concentration [µg/L]; (range)	2.26 (1.81–2.72)	1090 (874–1310)	1060 (902–1220)	737 (626–848)	105 (83.9–126)	123 (98.1–147)
mean experimental concentration [µg/L]; (SD, n = 9)	2.34 (0.23)	1140 (84)	1058 (85)	658 (41)	94.9 (7.5)	101 (8.2)
mean recovery of reference value	103.7%	104.6%	99.8%	89.2%	90.4%	82.3%
RSD intraday (n = 3)	7.2%	2.6%	2.8%	3.9%	4.6%	2.2%
RSD interday (n = 3)	8.5%	8.3%	8.2%	6.1%	4.2%	8.1%
Level II						
reference concentration [µg/L]; (range)	6.65 (5.32–7.98)	1730 (1380–2070)	1390 (1180–1600)	1090 (926–1250)	149 (119–179)	158 (126–189)
mean experimental concentration [µg/L] ; (SD, n = 9)	7.21 (0.45)	1842 (77)	1428 (50)	1046 (103)	146 (9)	140.4 (11)
mean recovery of reference value	108.5%	106.5%	102.7%	96.0%	92.4%	94.2%
RSD intraday (n = 3)	7.1%	3.1%	2.9%	2.9%	2.3%	5.1%
RSD interday (n = 3)	6.2%	4.2%	3.5%	9.8%	7.6%	6.4%

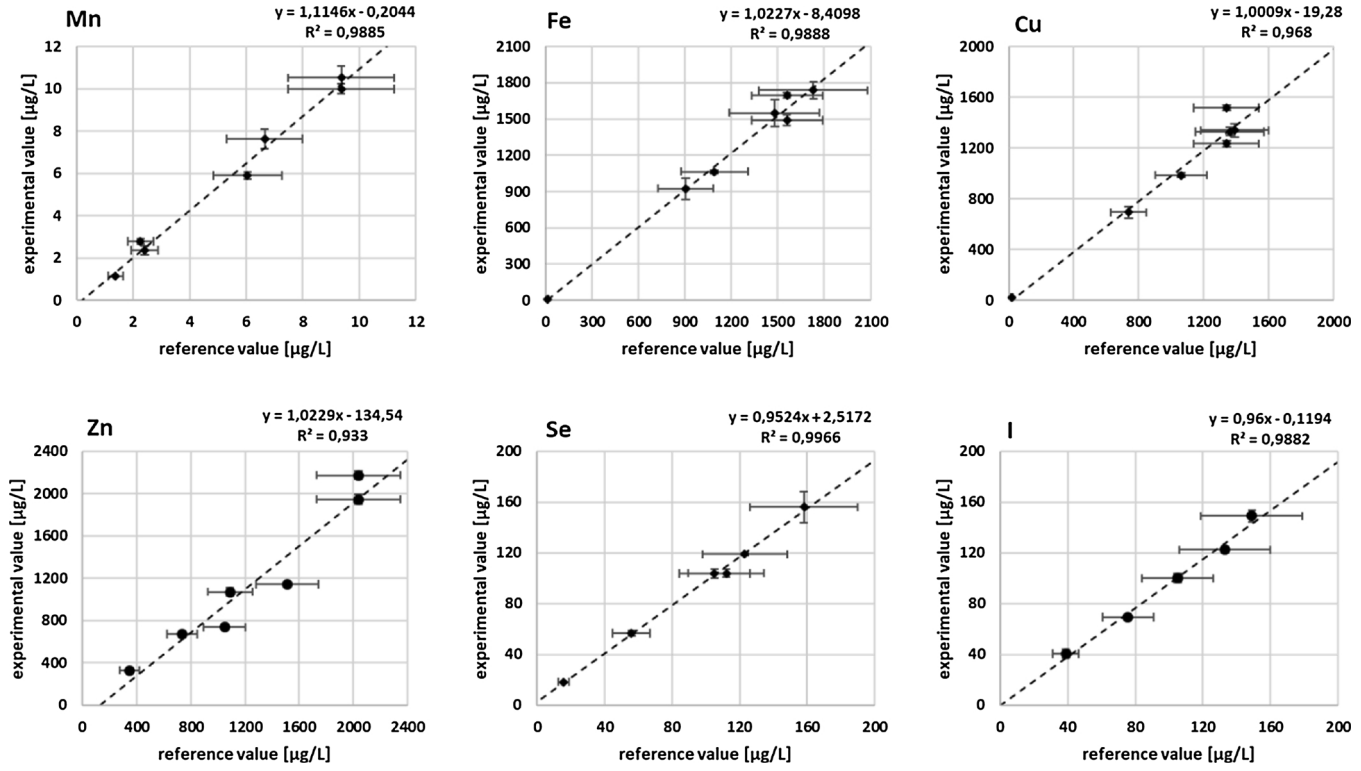

Fig. 1. Comparison of concentration values for multiple levels of serum and one urine reference material. Values obtained using the presented method (mean ± SD) are compared with the reference value ± uncertainty budget as specified by the manufacturer.

not feasible within the time of signal stability. Since it has been shown in multiple publications and was confirmed for this case as well (data not shown), O_2-mode for the determination of Se is highly beneficial for interference removal and should thus not be forgone [14,15]. Additionally, the employed IDA method also relies on using this mode [9]. However, measuring I in He-mode had been reported elsewhere [16,17] and was found to be feasible and give sufficiently low detection limits. Thus, the number of necessary gas-modes could be reduced to two: ^{55}Mn, ^{56}Fe, ^{63}Cu, ^{66}Zn and ^{127}I were measured in He-mode (on-mass), while IDA for ^{80}Se/^{77}Se was carried out in O_2-mode (mass-shift to ^{96}[SeO]/^{93}[SeO]), compare Table 1. ^{103}Rh as internal standard was monitored in both modes.

Further optimization of the gas-mode stabilization time showed reliable reproducibility for stabilization times ≥18 s across multiple days. This left 24 s for signal acquisition, which was ample time to analyze one isotope of each externally calibrated element in addition to an internal standard, as well as 2 isotopes for Se IDA. The final parameters for the method are shown in Table 1.

Table 3
Comparison of mouse serum concentrations for six trace elements measured in this work and data found in literature. The data in the table represents the mean of serum samples from multiple mice.

	Mn [µg/L]	Fe [µg/L]	Cu [µg/L]	Zn [µg/L]	I [µg/L]	Se [µg/L]
this study mean (SD), n = 3	6.5 (2.0)	3496 (257)	490 (67)	764 (110)	124 (34)	353 (11)
Frisk et al. [19] median (IQR), n = 6	5.2 (4.2)	4000 (300)	465 (79)	805 (180)	NA	547 (145)
Edvinsson et al. [20] mean (SD), n = 6	14.2 (3.5)	14,800 (5700)	632 (84)	983 (157)	NA	581 (186)

3. Method validation

For external calibration, standards were prepared in the diluent solution using 1000 mg/L single element stock solutions, purchased from Carl Roth (Karlsruhe, Germany). For Se IDA, a 10,000 mg/L stock solution was prepared from isotopically enriched ^{77}Se (97.20 ± 0.20% ^{77}Se; 0.10% ^{74}Se; 0.40 ± 0.10% ^{76}Se; 2.40 ± 0.10% ^{78}Se; 0.10% ^{80}Se; 0.10% ^{82}Se as certified by Trace Sciences International (Ontario, Canada)), purchased from Eurisotop SAS (Saarbrücken, Germany).

For method validation, linearity of the calibration curve, limits of detection (LOD), limits of quantification (LOQ) as well as intra- and interday precision were determined. LODs and LOQs were calculated using the 3σ- and 10σ-criterion. Therefore, the equivalent concentration of 3 (LOD) or 10 (LOQ) standard deviations (σ) of the signal from 6 independent blank determinations was calculated. For Se-determination using IDA, LOD and LOQ were calculated according to Yu et al. [18]. For accuracy and inter- and intraday precision, both concentration levels of the reference material RECIPE® ClinChek® serum control lyophilized (Ref. 8880-8882, LOT 347) were analysed as independent duplicates or triplicates on 7 different days. 50 µL of serum reference material were mixed with 10 µL of a solution containing 50 µg/L ^{77}Se and 5 µg/L Rh as internal standard and diluted with 440 µL of the diluent solution to give 500 µL of analysis solution (10 µg/L ^{77}Se and 1 µg/L Rh). For the diluent solution, ammonium hydroxide (puriss. p.a. plus, 25% in H_2O) was purchased from Fluka (Buchs, Germany), Na-EDTA (Titriplex® III, pro analysi) from Merck (Darmstadt, Germany), 1-Butanol (99%) from Alfa Aesar (Karlsruhe, Germany) and Triton™ X-100 (10% in H_2O) from Merck Sigma-Aldrich (Steinheim, Germany). The diluent solution was prepared to contain 5 vol.-% butanol, 0.05 m.-% Na-EDTA, 0.05% vol.-% Triton™ X-100 and 0.25 vol.-% ammonium hydroxide. Prepared samples were kept at 4 °C no longer than a day prior to analysis to prevent butanol evaporation.

The concentrations obtained were within the reference range of the material for all elements analysed. Across the six elements, intra-day relative standard deviation ranged from 2.2% to 7.3% considering both available concentration levels of the reference material. Inter-day relative standard deviation ranged from 4.2% to 9.5%. The achieved validation parameters can be found in Table 2.

4. Application of the method to human and murine serum samples

After evaluation of the main validation parameters, we expanded the range of serum concentrations to cover as much of the expected spectrum as possible. Therefore, we measured both levels of additional LOTs of the reference material RECIPE® ClinChek® serum control lyophilized (Ref. 8880-8882), namely LOT 544 and 1497. Furthermore, to include very low concentrations, we added a urine reference material (Seronorm™ Urine LOT 1403080, Level 1), which was prepared in the same way as the serum samples. Fig. 1 shows the obtained values (mean ± SD of at least 2 replicates) as compared to the reference values given by the manufacturer (mean ± uncertainty). The obtained values for the correlation coefficient were Mn: 0.9885, Fe: 0,9888, Cu: 0.968, Zn: 0.933, Se: 0.9966 and I: 0.9882. The slopes, which can be compared to the average recovery of the reference materials, were found to be as follows: Mn: 1.11, Fe: 1.02, Cu: 1.00, Zn: 1.02, Se: 0.95 and I: 0.96. Therefore, it is shown, that the method is in good agreement with and shows good correlation across the entire spectrum of analyzed reference concentration levels.

Furthermore, the method was applied to 3 serum samples from an animal experiment, approved by and conducted following national guidelines of the Ministry of Environment, Health and Consumer Protection of the federal state of Brandenburg (Germany, 2347-44-2017) and institutional guidelines of the German Institute of Human Nutrition (Potsdam-Rehbrücke). Therefore, C57BL/6JRj mice were sacrificed at an age of 10–12 weeks after being fed with a standard diet (V1534, Ssniff, Soest, Germany) *ad libitum*. The concentrations obtained match well with previously published data by Frisk et al. [19] and Edvinsson et al. [20], see Table 3.

5. Conclusions

A method suitable for the determination of the six trace elements manganese, iron, copper, zinc, selenium and iodine in very small amounts of mammalian serum has been developed, validated and applied. The method is capable of accurately detecting all six trace elements in the relevant concentration ranges with good precision. This was shown for samples from both human and murine origin. Due to an alkaline workup, iodine can be simultaneously determined with the other elements, without the need for a separate workup. Furthermore, due to a simple dilute-and-shoot approach, a work- and background-intensive microwave-assisted acid digestion is avoided. Thus, the method can be applied to determine trace element concentrations in large cohorts.

Conflicts of interest

There are no conflicts to declare.

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