Research Article

Differing cytotoxicity and bioavailability of selenite, methylselenocysteine, selenomethionine, selenosugar 1 and trimethylselenonium ion and their underlying metabolic transformations in human cells

Talke Anu Marschall¹, Julia Bornhorst¹, Doris Kuehnelt², Tanja Schwerdtle¹

¹ Institute of Nutritional Science, University of Potsdam, Nuthetal, Germany

² Institute of Chemistry - Analytical Chemistry, University of Graz, Graz, Austria

Correspondence: Professor Tanja Schwerdtle, University of Potsdam, Arthur-Scheunert-Allee 114-116, 14558 Nuthetal, Germany (Tanja.Schwerdtle@uni-potsdam.de)

Keywords: Cellular bioavailability, ICP-QQQ-MS, selenosugar 1, small selenium species, speciation

Abbreviations: **SeMet**, selenomethionine; **MeSeCys**, methylselenocysteine; **TMSe**, trimethylselenonium ion; **selenosugar 1**, methyl-2-acetamido-2-deoxy-1-seleno- β -D-galactopyranoside; **ICP-QQQ-MS**, inductively coupled plasma tandem mass spectrometry; **HPLC**, high performance liquid chromatography; **HepG2**, human hepatoma cell line; **UROtsa**, human urothelial cell line; **CCF-STTG1**, human astrocytoma cell line; **CRC**, collision and reaction cell; ***m/z***, mass to charge ratio; **Q1**, quadrupole mass filter 1; **Q2**, quadrupole mass filter 2; **LOD**, limit of detection; **ERM**, European Reference Material

Abstract

Scope: The trace element selenium (Se) is an integral component of our diet. However, its metabolism and toxicity following elevated uptake are not fully understood. Since the either adverse or beneficial health effects strongly depend on the ingested Se species, five low molecular weight species were investigated regarding their toxicological effects, cellular bioavailability and species-specific metabolism in human cells.

Methods and results: For the first time, the urinary metabolites methyl-2-acetamido-2-deoxy-1-seleno- β -D-galactopyranoside (selenosugar **1**) and trimethylselenonium ion (TMSe) were toxicologically characterised in comparison to the food relevant species methylselenocysteine (MeSeCys), selenomethionine (SeMet) and selenite in human urothelial, astrocytoma and hepatoma cells. In all cell lines selenosugar **1** and TMSe showed no cytotoxicity. Selenite, MeSeCys and SeMet exerted substantial cytotoxicity, which was strongest in the urothelial cells. There was no correlation between the potencies of the respective toxic effects and the measured cellular Se concentrations. Se speciation indicated that metabolism of the respective species is likely to affect cellular toxicity.

Conclusion: Despite being taken up, selenosugar **1** and TMSe are non-cytotoxic to urothelial cells, most likely because they are not metabolically activated. The absent cytotoxicity of selenosugar **1** and TMSe up to supra-physiological concentrations, support their importance as metabolites for Se detoxification.

1 Introduction

The ambivalent role of the ubiquitously occurring trace element selenium (Se) has been an important subject of ongoing research in the last decades. Se is essential for various systemic functions in the form of selenoproteins on the one hand [1, 2] and can induce adverse effects at elevated uptake on the other hand [3, 4]. Adequate Se supply and supplementation as well as its antioxidant and pro-oxidant properties have been examined in numerous studies [5, 6]. Concerning its cancer-preventing or cancer-promoting effects, contradictory results have been obtained and intensively discussed [7–9].

The specific effects of Se largely depend on the ingested species, as Se occurs in various forms in our food and in nutritional supplements. Selenomethionine (SeMet), which is ubiquitously incorporated into proteins instead of methionine as well as selenocysteine containing proteins belong to the major forms of Se in food. *Allium* and *Brassicaceae* plants are rich in methylselenocysteine (MeSeCys). Se supplements consist of SeMet, MeSeCys, inorganic selenite, selenate or selenized yeast, which provides a mixture of various organic Se species [1, 10, 11].

The organ-specific effects and metabolism of these Se species have not yet been fully understood. However, it is well known that excess Se is mainly excreted *via* urine as trimethylselenonium ion (TMSe) and methyl-2-acetamido-2-deoxy-1-seleno- β -D-galactopyranoside (selenosugar **1**). Depending on a genetic polymorphism, some humans excrete Se as TMSe in high amounts, while it is quantitatively of minor importance for others [12, 13]. A first cytotoxicity screening of selenosugar **1** indicated no influence on the cell viability of human liver cells up to 1000 μ M [14]. TMSe has been shown to exert low toxicity *in vivo*, e.g. an oral LD50 in rat of 49.4 mg Se/kg body weight was reported [15–18]. Therefore, current research on selenosugar **1** and TMSe focusses mainly on their quantification in urine and blood as biomarkers for Se intake and metabolism [12, 19]. Data on the cellular bioavailability and possible further metabolism routes in order to elucidate potential risks or benefits for cellular systems are still missing, especially for

selenosugar **1**. Lunøe et al. investigated the influence on apoptosis and cell death (assessed by PI/annexin V flow cytometry) of small Se species occurring in food. From the most toxic to the least toxic substance, they obtained the sequence selenite > methylselenic acid > SeMet, MeSeCys > selenate in three cancer cell lines (HT-29, PC3 and Jurkat) revealing no correlation to total cellular Se concentrations and metabolites [20]. Consequently, the question arose if this also proves to be the case for other cellular systems and the urinary metabolites.

This study aimed to investigate the cytotoxicity and cellular bioavailability of MeSeCys, SeMet, selenosugar **1** and TMSe in comparison to selenite as inorganic reference compound (figure 1). As cellular model systems human hepatoma cells (HepG2) and for the first time human urothelial (UROtsa) and astrocytoma cells (CCF-STTG-1) were examined because these cell lines represent potential target organs: The liver plays a central role in Se metabolism [21]. Since excess Se is mainly excreted *via* urine small Se species are likely to get into contact with urothelial cells. The transfer of these species across brain barriers has not been studied yet, but as they are potential mimics for sugars and amino acids their transfer might be possible [22].

In order to assess the cellular bioavailability of the Se species a reliable quantification method for total Se in very small cell pellets was needed. Inductively coupled plasma mass spectrometry (ICP-MS) presents a valuable tool for highly sensitive multi element analysis. However, Se quantification by ICP-MS faces spectral overlaps, e.g. caused by interfering salt ions and argon (Ar) clusters [23]. For example, ^{80}Se , the most abundant Se isotope, is massively affected by omnipresent argon dimers from the plasma gas [24]. Moreover, the ionization yield of Se in the plasma is low due to its high first ionization potential (9.75 eV). Consequently, carbon from sample matrix may intermediately enhance Se ionization rates leading to non-reproducible results [25, 26]. To overcome these problems, the present work describes the development of selective methods to

quantify total Se with a low LOD and to detect Se species based on ICP tandem mass spectrometry (ICP-QQQ-MS).

2 Materials and Methods

2.1 Materials

For the *in vitro* cultivation of UROtsa, HepG2 and CCF-STTG1 cells minimal essential medium Eagle (MEM), non-essential amino acids, RPMI 1640 medium and culture dishes were purchased from Biochrom (Berlin, Germany). PAA Laboratories (Pasching, Austria) provided trypsin, penicillin-streptomycin and fetal calf serum (FCS). Sigma-Aldrich (Steinheim, Germany) provided resazurin dye (cell culture tested). All other chemicals were pro-analysis products of Sigma-Aldrich (Steinheim, Germany), Merck (Darmstadt, Germany), Alfa Aesar (Karlsruhe, Germany) or Roth (Karlsruhe, Germany). The UROtsa cell line was kindly provided by Prof. M. Stýblo (University of North Carolina, USA). It originates from a primary culture of a normal human urothelium which has been immortalized with the SV-40 large T antigen. The HepG2 cell line was purchased from the European Collection of Cell Cultures (ECACC; number 85011430, Salisbury, UK). The CCF-STTG1 cell line (CCL-185TM) was from the American Type Culture Collection (Bethesda, MD, USA).

Selenite ($\text{Na}_2\text{SeO}_3 \cdot 5 \text{H}_2\text{O} \geq 99.0\%$) was obtained from Sigma-Aldrich (Steinheim, Germany). The organic Se species selenosugar **1** and TMSe were synthesized at the University of Graz, Institute of Chemistry, Analytical Chemistry according to published protocols [27, 28]. Identity and purity of the synthesized compounds were assured by molecular MS, NMR, and elemental analysis [14, 27] and selenium purity was demonstrated by HPLC/ICP-MS. MeSeCys was from Sigma-Aldrich (Karlsruhe, Germany) and D/L SeMet from Fluka (Buchs, Switzerland). The elemental standards Se (1 g/L) and germanium (Ge) (1 g/L) used for ICP-MS analysis were from Merck (Darmstadt, Germany) and from High Purity Standards (Charleston, SC, USA), respectively. Isopropanol was supplied by Carl Roth (Rotisolv $\geq 99.95\%$ for LC-MS, Carl Roth, Karlsruhe, Germany). Nitric acid (65%, suprapur) was obtained from Merck (Darmstadt, Germany). As reference material for the verification of the Se quantification method the certified fish muscle reference material

ERM® - BB422 (Joint Research Centre, European Commission, Geel, Belgium) was applied. For all experiments Milli-Q water (10 MΩcm, Elix® 15 water purification system, Merck Millipore) was used.

2.2 Preparation of Se species stock solutions for cell culture cytotoxicity testing

Stock solutions (10-40 mM) of the Se species were prepared in sterile water and further diluted to the desired concentrations. Selenite stock solutions were freshly prepared every week while the organic Se stock solutions were prepared only once due to a limited amount of the organic standards. They were stored in the dark at 4 °C up to one year. A stability study carried out for selenosugar **1** indicated no significant degradation during up to 28 weeks of storage at 4 °C [29]. All stock solutions were measured regularly by ICP-QQQ-MS and HPLC-ICP-QQQ-MS to verify their concentration and to exclude degradation processes (data not shown).

2.3 Culture of human cells and incubation with Se species

UROtsa cells were cultured in plastic dishes as a monolayer with MEM which was complemented with 10% FCS, penicillin (100 U/mL) and streptomycin (100 µg/mL). For the cultivation of HepG2 cells additionally non-essential amino acids (1% v/v) were added to the medium mixture. Both sub-confluent cultures (70-80%) were split every 2-3 days removing the cells from the plates with 0.25% trypsin. For incubation experiments, cells were seeded and after one cell cycle (24 h) the logarithmically growing cells were incubated with the respective Se species for 48 h. In all experiments selenite served as reference substance. CCF-STTG1 cells were cultured in RPMI 1640 medium supplemented with 10% FCS, penicillin (100 U/mL), streptomycin (100 mg/mL) and L-glutamine (1.4 mM). Sub-culturing was carried out once a week and fresh medium was applied every 2-3 days. For incubation experiments, cells were seeded and after one cell cycle (48 h) the logarithmically growing cells were incubated with the respective Se species for 48 h. All cells were cultured under standard conditions for human cell culture at 37 °C in 5% CO₂ and 100% atmospheric humidity.

2.4 Cytotoxicity testing

The cytotoxic effects of SeMet, MeSeCys, TMSe, selenosugar **1** and selenite were studied by quantitatively investigating their influence on cell number and cellular dehydrogenase activity as described before [30, 31]. After 48 h of incubation with the respective Se species, cell number testing was carried out by washing and detaching the cells with trypsin. Afterwards, an automated cell counting system (Casy TTCs, Roche Innovatis AG) was used to simultaneously determine cell number and cell volume. As a second endpoint cell viability was measured using the dye resazurin (Alamar Blue) which is reduced to fluorescent resorufin in the presence of cellular dehydrogenases [32]. For these experiments the cells were incubated for 48 h with the respective compounds in 96-well culture plates. Subsequently, medium was replaced by medium mixed with resazurin (5 µg/mL). After 2 h at 37 °C the fluorescent intensity of each well was detected with a plate reader (Fluostar Optima, BMG Labtech, Ortenberg, Germany) at 590 nm following excitation at 540 nm.

2.5 Cellular bioavailability of Se in UROtsa cells

After incubation of the UROtsa cells for 48 h with the Se species, the cell layer was washed twice with warm PBS (37 °C) and subsequently detached by trypsinizing. Cell volume and number were measured in the cell suspension using the automatic cell counting system. Following centrifugation (5 min, 300 x g, 4 °C), extensive washing of the residue with ice-cold PBS in order to completely remove non-covalently bound Se and centrifugation (5 min, 2200 x g, 4 °C), the cell pellet was obtained. For assessing the total Se content, a method for selective and sensitive Se quantification by ICP-QQQ-MS was developed.

2.6 Total Se quantification in cell pellets, cell culture media and reference material

Briefly, cell pellets were digested with nitric acid in a closed microwave digestion system (Mars 6, CEM, Kamp-Lintfort, Germany) and Se quantification was carried out with an ICP-QQQ-MS

8800 mass spectrometer (Agilent, Waldbronn, Germany). For microwave digestion a procedure modified from Juresa et al. was used [29]. Either 500 μ L of cell culture medium or cell pellets resuspended in 500 μ L water were transferred into 20 mL TFA microwave vessels with additional 500 μ L of water. 500 μ L of suprapur nitric acid (65%) and 25 μ L internal standard solution of Ge (50 μ g/L) were added. Digestion was carried out in closed vessels by heating to 200 °C within 15 min applying 650 W and maintaining this temperature for another 20 min. After cooling down, samples were completely transferred into 15 mL polyethylene tubes and filled up to a total volume of 2.5 mL. For the analysis of reference material a portion of ca. 10 mg weighed to 0.1 mg was digested with 2 mL nitric acid (65%), 2 mL water, 100 μ L internal standard solution and the digested solution was subsequently diluted to 10 mL. Prior to ICP-QQQ-MS quantification, 2.5 mL of the sample solutions and the calibration standards (0.1 – 10 μ g/L Se) were mixed with 75 μ L (3%) of isopropanol to enhance Se ionization in the plasma. ICP-QQQ-MS parameters are shown in table 1. Total Se was measured with a reaction and collision cell gas mixture of oxygen (0.4 mL/min) and hydrogen (1 mL/min) in the CRC using the mass shift of isotope $^{80}\text{Se}^+$ (m/z 80) to $^{80}\text{Se}^{16}\text{O}^+$ (m/z 96). Additionally, isotope $^{78}\text{Se}^+$ (m/z 78) shifting with oxygen to $^{78}\text{Se}^{16}\text{O}^+$ (m/z 94) as well as both Se isotopes on mass were monitored in the same gas mode to screen for interferences. As a commonly used internal standard, Ge was detected on m/z 72 [12, 13].

2.7 Se speciation in the water-soluble fraction of UROtsa cells

Cell pellets prepared as described above (see 2.5) were sonicated (15 sec, 0.8 cycles, 100% amplitude) on ice in 200 μ L of water. Following centrifugation (10 min, 21000 x g, 4 °C) 20 μ L of the supernatants representing the water-soluble cell fraction, were applied to HPLC-ICP-QQQ-MS analysis without further dilution. For separation reversed phase chromatography was performed on a YMC-TriartPFP column (3 μ m, 3x250 mm) from YMC (Dinslaken, Germany) with 20 mM ammonium formate (pH 3, 3% methanol) as mobile phase [33] at a flow rate of 250 μ L/min and a column temperature of 30 °C. The eluate was transferred online into the ICP-QQQ-MS operated as described above (see 2.6). For quantification $^{80}\text{Se}^{16}\text{O}^+$ (m/z 96) was detected in

combination with $^{80}\text{Se}^+$ (m/z 80) as qualifier transition with 0.5 sec integration time, respectively. Se speciation in the water-soluble cell fractions was confirmed in two independent *in vitro* experiments.

3 Results and Discussion

ICP-QQQ-MS method development

In contrast to conventional ICP-Q-MS, ICP-QQQ-MS comprises an additional quadrupole mass filter (Q1) before the collision and reaction cell (CRC) in addition to the standard quadrupole mass filter behind the CRC (Q2). This set-up enables enhanced control of the ions entering the CRC avoiding the need of mathematical interference correction.

Nevertheless, optimization of the applied gases in the CRC is mandatory for selective Se detection in order to exclude various interfering species owing the same m/z as the most abundant Se isotopes ^{80}Se (49.8%) and ^{78}Se (23.5%). Since hydrogen and oxygen reactions with interferences or selenium are energetically favored [24], these gases were compared separately and in combination regarding their ability to remove interferences. Hydrogen might eliminate interferences by reacting with interfering species shifting them to a higher m/z which is rejected by Q2. Respectively, Se^+ ions are filtered on equal m/z in Q1 and Q2 (on mass detection). In contrast, oxygen may selectively react with Se^+ ions and the analyte is subsequently filtered as SeO^+ on a shifted m/z ($m/z + 16$; mass shift detection). Gas flows were optimized to maximize Se signal intensity resulting in 0.4 mL/min for O_2 , 6 mL/min for H_2 and for the mixture of O_2 and H_2 in 0.4 and 1 mL/min, respectively (data not shown).

To increase Se signal intensity, the carbon enhancement effect can be exploited by constantly adding a specific amount of carbon to the plasma [25]. Signal enhancement by acetonitrile, methanol and isopropanol was compared for a 2.5 $\mu\text{g/L}$ Se standard solution. Addition of 3% isopropanol to standards and sample solutions directly before measurement resulting in a 6.5 to 8.8 fold increase of the Se signal (data not shown) was chosen for further experiments. Carbon

from the sample matrix leading to unspecific signal enhancement was eliminated by sample preparation with microwave-assisted acid digestion in closed vessels.

Due to the lack of reference materials in the field of cell culture matrices, ready to use cell culture medium, which naturally contains about 1 µg/L total Se, was digested and analyzed for total Se to screen for interference-free measuring modes. Since the method aims at low detection limits the most abundant Se isotopes ^{78}Se and ^{80}Se were chosen for comparison in on mass and mass shift mode. Good agreement of Se concentrations quantified with both mass shift modes and with the on mass quantification mode on ^{80}Se using O_2/H_2 as cell gas mixture was found (figure 2). For on mass quantification, the addition of H_2 seems to be decisive since the sole use of O_2 could not prevent ^{80}Se from being intensively overlapped by Ar interferences (data not shown). On the contrary, ^{78}Se on mass seems to be interfered in all tested gas modes. The medium was also analyzed by the method of Kokarnig et al. [19] revealing 1.19 ± 0.08 µg/L which nicely fits to the results obtained with O_2/H_2 for ^{80}Se on mass and for the mass shift transitions of ^{80}Se and ^{78}Se . These observations are in accordance with Jackson et al., who identified mass shift modes as especially effective in removing interferences and recommended a O_2/H_2 cell gas mixture for Se detection [34]. As a possible reason the combined gases might eliminate interferences more effectively by shifting Se ions + m/z 16 ($1/2 \text{ O}_2$) as well as interferences + m/z 1 ($1/2 \text{ H}_2$) [24]. As discussed in the literature, Se detection on m/z 80 by ICP-Q-MS suffers from $^1\text{H}^{79}\text{Br}$ interferences when using H_2 as cell gas [24, 35]. However, in mass shift mode $^1\text{H}^{79}\text{Br}$ clusters are rejected due to the first quadrupole mass filter, which prevents $^{79}\text{Br}^+$ to reach the CRC. Comparing total Se concentrations measured in cell culture media with the results obtained from cell pellets, results for the latter matched for ^{78}Se and ^{80}Se both, on mass and in mass shift modes, indicating a less complex matrix of cell pellets in contrast to cell culture media (data not shown). As a result, both on mass and mass shift transitions of ^{78}Se and ^{80}Se were chosen for validation using a cell gas mixture of O_2 and H_2 .

In table 2 basic characteristics of the developed method are presented. ^{78}Se and ^{80}Se show high linearity for both, on mass and mass shift transitions. Since there are no commercially available

reference materials for cell culture matrices, small portions (10 mg) of certified fish reference material (ERM®–BB422) were digested to simulate comparable conditions as for small and protein-rich cell pellets. Recoveries for the fish material as well as spiking recoveries were well within the certified and expected range, respectively, for all validated transitions (table 2). For quantification the mass shift mode m/z 80 $\rightarrow$ 96 was selected because it offered the lowest LOD and was effective in interference removal. The other three m/z were used to screen for interferences during measurements. LODs in the ng/L range for Se determination by ICP-QQQ-MS are comparable to those recently reported [26, 34].

Cytotoxicity

The developed method was used to determine total Se in human cells after incubation with MeSeCys, SeMet, TMS₂Se and selenosugar **1** in comparison to selenite as reference substance in order to characterize their toxicity in cells of potential target organs. As a first step, cell viability was assessed by the colorimetric Resazurin assay in HepG2, UROtsa and CCF-STTG1. Additionally, cell number was investigated in HepG2 and UROtsa cells. The results differ greatly among the tested species (figures 3 and 4). Similar tendencies in the sequence from the most toxic to the least toxic substance in all cell lines and from the most sensitive to the most robust cell line were obtained. For all species which induced toxic effects (selenite, MeSeCys and SeMet) the UROtsa cell line represented the most sensitive target followed by HepG2 cells, while the CCF-STTG1 cells were quite resistant towards the Se species (figures 3 and 4). The inhibitory concentrations (IC) and effect concentrations (EC) detected by cell number in the UROtsa cells (table 3) were generally lower as determined by cell viability (Supporting Information, table 2 and 3: IC₅₀ and EC₃₀ values for HepG2 and CCF-STTG1 cells). Selenite was by far the most toxic substance in all tested cell lines with IC₅₀ values in the low μ M concentration range (table 3). As a frequently used reference substance and a common constituent of Se supplements, selenite has already been examined in various *in vivo* and *in vitro* studies, resulting in more pronounced cytotoxic effects as compared to organic Se species [20, 36, 37]. Hoefig et al. determined for

HepG2 cells after 48 h of incubation using the MTT test an IC_{50} value of 5.5 μM , which is close to the value determined here by cell number (IC_{50} 7.6 μM) [38]. In the urothelial cell line the IC_{50} values of MeSeCys were about 20-fold higher as compared to selenite for both tested endpoints (table 3). MeSeCys was about 10-times more toxic as compared to SeMet, which induced toxic effects only in high μM concentrations. Similar sequences were observed by Lunøe et al. in HT-29, PC-3 and Jurkat E6-1 cells and by Kipp et al. and Hoefig et al. in HepG2 cells [20, 38, 39]. Selenosugar **1** and TMSe induced no toxic effects up to 1000 μM in all cell lines. These high and still non-toxic concentrations, which are unlikely to occur in the brain, urothelium or liver, provide further evidence that both substances present no inherent risk for human health regarding the investigated organ types. The results are in line with a previous cytotoxicity testing of selenosugar **1** showing no effects in HepG2 cells up to 1000 μM as well [14]. With human brain and bladder cells being included in this study, the diversified results support the assumption that TMSe and selenosugar **1** are detoxification products after (excess) Se uptake [40].

Cellular bioavailability in UROtsa cells

As a possible explanation for the lacking cytotoxicity of selenosugar **1** and TMSe, missing bioaccessibility was hypothesized for these substances in the tested cell lines. As the most sensitive cell line with highlighted relevance for the urinary metabolites, urothelial cells were chosen to investigate the cellular bioavailability of the Se species. Selenite and the seleno-amino acids displayed increasing total cellular Se concentrations with higher incubation concentrations (table 4). Generally, total cellular Se concentrations did not seem to be decisive for the cytotoxic effects of the respective compounds. When comparing the ratio between cellular and incubated Se concentrations (Se accumulation), incubation with the most toxic species selenite (2- till 4-fold Se accumulation) caused much lower cellular Se concentrations than less toxic SeMet (12- till 22-fold Se accumulation) incubation. Selenite pellets contained roughly as much total Se as the pellets incubated with much less toxic MeSeCys in comparable incubation concentrations. These results are in accordance with the observations of Lunøe et al. who reported no obvious

correlation of cytotoxicity and total Se uptake in cancer cells incubated with selenite, MeSeCys, SeMet, methylselenic acid and selenate [20]. Species-specific cellular uptake and metabolism are likely to account for the missing correlation. SeMet is non-specifically incorporated into various proteins in place of methionine and serves as Se storage. In contrast, selenite and MeSeCys are not non-specifically incorporated but have to be metabolized to be used for selenoprotein formation or excretion [41]. Thus, after cellular overload these two compounds by themselves but also their potential metabolites, e.g. selenide, are likely to cause cytotoxicity.

Furthermore, the results disprove a missing cellular bioaccessibility of TMSe and selenosugar **1** since about 13-22% of the incubated Se were able to enter the cells (table 4). However, cellular Se concentrations were low compared to the incubated concentrations of the urinary metabolites. *In vivo* experiments with rats also showed low Se amounts in the organs of animals exposed to TMSe and selenosugars via drinking water suggesting low *in vivo* bioavailability of these substances [42].

Speciation

Because TMSe and selenosugar **1** did not exert any cytotoxic effects, even though their incubation resulted in significant cellular Se concentrations, it was supposed that the urothelial cells are not able to metabolize those species in contrast to the more toxic ones e.g. due to a lack of enzymatic competence. This theory is supported by the general assumption of TMSe and selenosugar **1** being excretion products without contributing to the Se pool or forming potentially toxic intermediates [43]. In order to screen for potentially formed Se species as indication for a cellular metabolism of the small Se species, the water-soluble fractions of the incubated cells were analysed by HPLC-ICP-QQQ-MS. For detection, the developed ICP-QQQ-MS method was used. For chromatographic separation a polar-modified reversed phase column (phenyl and pentafluorophenyl phase) was applied in combination with the eluent system of Kokarnig et al. [33]. Even though accurate quantification was not feasible in the presence of selenite, which yielded in a broad peak partially overlapping with MeSeCys (Supporting Information, figure 1),

metabolic patterns could be obtained for all five investigated Se species (figure 5). The water-soluble fractions of the cells incubated with selenite exhibited an additional Se species (figure 5, peak I) with higher intensity as compared to selenite itself. Even though its retention time resembles the retention time of SeMet, its actual identity has to be clarified by electrospray ionization (ESI) MS/MS experiments. Since traces of an unknown species with similar retention time were also detected in the cell culture medium (Supporting Information, figure 2, **B**), cellular metabolism with subsequent efflux processes might be a possible source. In accordance with the results of Gabel-Jensen & Gammelgaard in hepatocytes, selenite was transformed to a large extent to unknown metabolites [44].

In addition, MeSeCys and SeMet incubations caused the formation of additional Se species to a lower percentage as compared to selenite in the water-soluble fraction (figure 5, peaks II, III and IV). Spiking experiments (data not shown) showed that none of the unknown peaks co-eluted with the available Se species standards. Contrary to these results, previous studies did not detect any metabolites of MeSeCys in HepG2 cells after 24 h, in PC-3, Jurkat E6-1 and HT-29 cells after 24 h and in rat hepatocytes after 4 h, respectively [20, 44, 45]. However, non-toxic concentrations were incubated in these earlier studies, whereas in this study a cytotoxic MeSeCys concentration was applied. Following SeMet incubation an additional cellular Se species (figure 5, peak IV) eluting under similar separation conditions as chosen here has been detected before and was suspected to be selenomethionine-oxide, an oxidation artefact from sample preparation [20]. However, in this study it was not detected in cell culture medium (Supporting Information, figure 2 **D**).

In contrast, the cytosols gained from TMSe and selenosugar **1** experiments did not show substantial transformation of the incubated species (figure 5 **E** and **G**). Surprisingly, in the water-soluble fraction of the cells incubated with selenosugar **1** traces of some minor compounds (figure 5 **H**, peaks V and VI) were detected suggesting that cellular processes might have caused a biotransformation to a very limited extent. Control pellets spiked with the five selenium species of interest showed spiking recoveries between 93 and 119% (Supporting Information, table 1)

indicating no significant species degradation during sample preparation. Spiking of the sample with MeSeCys (6.5 min) and SeMet (8.3 min) indicated retention time matching for MeSeCys (Supporting Information, figure 3), which has to be confirmed by additional analytical methods. According to Suzuki et al., isotope-labelled selenosugar **1** fed to rats was partially metabolized contributing to the selenium pool in slight extent [46]. These results indicate that the organism as well as certain cellular systems might be able to recycle selenosugar **1** sparsely.

Taken together, the speciation analysis of the incubated cells resulted in the detection of transformation products to a notable extent in the cases of selenite, SeMet and MeSeCys while TMSe and selenosugar **1** were not significantly metabolized. This observation points to no metabolic activation of TMSe and selenosugar **1** and might explain why they do not induce any cytotoxic effects even though entering the cells. The water-soluble fractions of UROtsa cells following MeSeCys, SeMet and selenite incubation did not show any selenosugar **1** or TMSe indicating that the urothelial cell line is not capable of this detoxification route.

4 Concluding remarks

The developed and validated ICP-QQQ-MS method offered a convenient and reliable tool for total Se quantification as well as for detecting chromatographically separated Se species in cellular matrices. It enabled easy interference recognition by the detection of four independent m/z transitions and proved to provide adequate recoveries and limits of quantification for the accomplished *in vitro* study.

The cytotoxicity testing revealed UTOtsa cells as the most sensitive cell line followed by HepG2 cells and CCF-STTG1 astrocytes. Intensive variances were obtained among the small Se species in all three cell lines with selenite inducing pronounced toxic effects in low μM concentrations followed by MeSeCys and SeMet which caused distinct effects in high μM concentrations. In contrast, the urinary metabolites selenosugar **1** and TMSe did not disturb cell viability up to 1000 μM . These differences could not be explained with the cellular bioavailability data of the UROtsa cells, because total cellular Se following selenite incubation was much lower as for highly bioavailable and less toxic SeMet. However, speciation studies in the water-soluble fraction of the incubated cells indicated intense conversion in the case of selenite as the most toxic substance, partial transformation of less toxic MeSeCys and SeMet, and no measurable or very limited metabolism of the non-toxic urinary metabolites TMSe and selenosugar **1**, respectively. These observations confirm the general assumption that both, TMSe and selenosugar **1**, function as detoxification and excretion metabolites for Se metabolism. Interestingly, the water-soluble fractions of cells incubated with selenosugar **1** exhibited traces of additional Se species pointing to currently unknown breakdown routes for selenosugar **1** within the UROtsa cells. In order to identify the unknown species HPLC-ESI-MS/MS experiments and an accurate quantification method combined with additional chromatographic separations (anion exchange chromatography) will help to fathom the effects of small Se species in cellular systems.

We thank the “Fonds der Chemischen Industrie” for financial support. This project was supported by the DFG grant number SCHW 903/9-1 and the Austrian Science Fonds (FWF), project number I 2262-N28. Moreover, we thank Professor Dr. K. A. Francesconi from the University of Graz for providing the organic selenium compounds and Dr. A. P. Kipp from the German Institute of Human Nutrition (DIfE) for her conceptional contribution to this work.

The authors have declared no conflicts of interest.

5 References

- [1] Rayman, M.P., Selenium and human health. *Lancet* 2012, 379, 1256–68.
- [2] Hatfield, D.L., Tsuji, P.A., Carlson, B.A., Gladyshev, V.N., Selenium and selenocysteine: roles in cancer, health, and development. *Trends Biochem. Sci.* 2014, 39, 112–20.
- [3] European Commission, Opinion of the Scientific Committee on Food on the Tolerable Upper Intake Level of Selenium. 2000.
- [4] U.S. Department for Health and Human Services, Toxicological Profile for Selenium. 2003.
- [5] Misra, S., Boylan, M., Selvam, A., Spallholz, J.E., et al., Redox-active selenium compounds-from toxicity and cell death to cancer treatment. *Nutrients* 2015, 7, 3536–3556.
- [6] Wrobel, J.K., Power, R., Toborek, M., Biological activity of selenium: Revisited. *IUBMB Life* 2016, 68, 97–105.
- [7] Lippman, S.M., Klein, E.A., Goodman, P.J., Lucia, M.S., et al., Effect of Selenium and Vitamin E on Risk of Prostate Cancer and Other Cancers: The Selenium and Vitamin E Cancer Prevention Trial (SELECT). *J. Am. Med. Assoc.* 2009, 301, 39–51.
- [8] Kristal, A.R., Darke, A.K., Morris, J.S., Tangen, C.M., et al., Baseline selenium status and effects of selenium and vitamin E supplementation on prostate cancer risk. *J. Natl.*

Cancer Inst. 2014, 106.

- [9] Jablonska, E., Vinceti, M., Selenium and Human Health: Witnessing a Copernican Revolution? *J. Environ. Sci. Heal. Part C-Environmental Carcinog. Ecotoxicol. Rev.* 2015, 33, 328–368.
- [10] Gammelgaard, B., Jackson, M.I., Gabel-Jensen, C., Surveying selenium speciation from soil to cell-forms and transformations. *Anal. Bioanal. Chem.* 2011, 399, 1743–63.
- [11] Weekley, C.M., Harris, H.H., Which form is that? The importance of selenium speciation and metabolism in the prevention and treatment of disease. *Chem. Soc. Rev.* 2013.
- [12] Jäger, T., Drexler, H., Göen, T., Human metabolism and renal excretion of selenium compounds after oral ingestion of sodium selenite and selenized yeast dependent on the trimethylselenium ion (TMSe) status. *Arch. Toxicol.* 2015.
- [13] Kuehnelt, D., Engström, K., Skröder, H., Kokarnig, S., et al., Selenium metabolism to the trimethylselenonium ion (TMSe) varies markedly because of polymorphisms in the indolethylamine. *Am. Clin. Nutr.* 2015, 102, 1406–15.
- [14] Kuehnelt, D., Kienzl, N., Traar, P., Le, N.H., et al., Selenium metabolites in human urine after ingestion of selenite, L-selenomethionine, or DL-selenomethionine: a quantitative case study by HPLC/ICPMS. *Anal. Bioanal. Chem.* 2005, 383, 235–46.
- [15] Obermeyer, B.D., Palmer, I.S., Olson, O.E., Halverson, A.W., Toxicity of trimethylselenonium chloride in the rat with and without arsenite. *Toxicol. Appl. Pharmacol.* 1971, 20, 135–146.
- [16] Palmer, I.S., Arnold, R.L., Carlson, C.W., Toxicity of various selenium derivatives to chick embryos. *Poult. Sci.* 1973, 52, 1841–1846.
- [17] Ip, C., Ganther, H., Biological activities of trimethylselenonium as influenced by arsenite. *J. Inorg. Biochem.* 1992, 46, 215–222.
- [18] Olson, O.E., Selenium Toxicity in Animals with Emphasis on Man. *Int. J. Toxicol.* 1986, 5, 45–70.

- [19] Kokarnig, S., Tsirigotaki, A., Wiesenhofer, T., Lackner, V., et al., Concurrent quantitative HPLC-mass spectrometry profiling of small selenium species in human serum and urine after ingestion of selenium supplements. *J. Trace Elem. Med. Biol.* 2014, 29, 83–90.
- [20] Lunøe, K., Gabel-Jensen, C., Stürup, S., Andresen, L., et al., Investigation of the selenium metabolism in cancer cell lines. *Metallomics* 2011, 3, 162–8.
- [21] Kato, T., Read, R., Rozga, J., Burk, R.F., Evidence for intestinal release of absorbed selenium in a form with high hepatic extraction. *Am. J. Physiol.* 1992, 262, 854–858.
- [22] Wolfram, S., Berger, B., Grenacher, B., Scharrer, E., Transport of selenoamino acids and their sulfur analogues across the intestinal brush border membrane of pigs. *J. Nutr.* 1989, 89, 706–712.
- [23] Goossens, J., Vanhaecke, F., Moens, L., Dams, R., Elimination of interferences in the determination of arsenic and selenium in biological samples by inductively coupled plasma mass spectrometry. *Anal. Chim. Acta* 1993, 280, 137–143.
- [24] D’Illo, S., Violante, N., Majorani, C., Petrucci, F., Dynamic reaction cell ICP-MS for determination of total As, Cr, Se and V in complex matrices: Still a challenge? A review. *Anal. Chim. Acta* 2011, 698, 6–13.
- [25] Gammelgaard, B., Jøns, O., Determination of selenium in urine by inductively coupled plasma mass spectrometry : interferences and optimization. *J. Anal. At. Spectrom.* 1999, 14, 867–874.
- [26] Bolea-Fernandez, E., Balcaen, L., Resano, M., Vanhaecke, F., Interference-free determination of ultra-trace concentrations of arsenic and selenium using methyl fluoride as a reaction gas in ICP–MS/MS. *Anal. Bioanal. Chem.* 2015, 407, 919–929.
- [27] Traar, P., Belaj, F., Francesconi, K.A., Synthesis of methyl 2-acetamido-2-deoxy-1-seleno- β -d-glucopyranoside and galactopyranoside: Selenium metabolites in human urine. *Aust. J. Chem.* 2004, 57, 1051–1053.
- [28] Hoffman, J.L., Ion chromatographic analysis of the purity and synthesis of sulfonium

- and selenonium ions. *J. Chromatogr. A* 1991, 588, 211–216.
- [29] Juresa, D., Darrouzès, J., Kienzl, N., Bueno, M., et al., An HPLC/ICPMS study of the stability of selenosugars in human urine: implications for quantification, sample handling, and storage. *J. Anal. At. Spectrom.* 2006, 21, 684–690.
- [30] Ebert, F., Weiss, A., Bültemeyer, M., Hamann, I., et al., Arsenicals affect base excision repair by several mechanisms. *Mutat. Res.* 2011, 715, 32–41.
- [31] Lohren, H., Bornhorst, J., Fitkau, R., Pohl, G., et al., Effects on and transfer across the blood-brain barrier in vitro - Comparison of organic and inorganic mercury species. *Neurometals* 2015.
- [32] O'Brien, J., Wilson, I., Orton, T., Pognan, F., Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. *Eur. J. Biochem.* 2000, 267, 5421–5426.
- [33] Kokarnig, S., Kuehnelt, D., Stiboller, M., Hartleb, U., et al., Quantitative determination of small selenium species in human serum by HPLC/ICPMS following a protein-removal, pre-concentration procedure. *Anal. Bioanal. Chem.* 2011, 400, 2323–7.
- [34] Jackson, B., Liba, A., Nelson, J., Advantages of reaction cell ICP-MS on doubly charged interferences for arsenic and selenium analysis in foods. *J. Anal. At. Spectrom.* 2015, 30, 1179–1183.
- [35] Sucharová, J., Optimisation of DRC ICP-MS for determining selenium in plants. *J. Anal. At. Spectrom.* 2011, 26, 1756.
- [36] Flores Villavicencio, L.L., Cruz-Jiménez, G., Barbosa-Sabanero, G., Kornhauser-Araujo, C., et al., Human lung cancer cell line A-549 ATCC is differentially affected by supranutritional organic and inorganic selenium. *Bioinorg. Chem. Appl.* 2014, 2014, 923834.
- [37] Ammar, E., Couri, D., Acute toxicity of sodium selenite and selenomethionine in mice after ICV or IV administration. *Neurotoxicology* 1981, 2, 383–386.
- [38] Hoefig, C.S., Renko, K., Köhrle, J., Birringer, M., et al., Comparison of different

- selenocompounds with respect to nutritional value vs. toxicity using liver cells in culture. *J. Nutr. Biochem.* 2011, 22, 945–55.
- [39] Kipp, A.P., Frombach, J., Deubel, S., Brigelius-Flohé, R., Selenoprotein w as biomarker for the efficacy of selenium compounds to act as source for selenoprotein biosynthesis. *Methods Enzymol.* 2013, 527, 87–112.
- [40] Byard, J., Trimethyl a urinary metabolite of selenite. *Arch. Biochem. Biophys.* 1969, 130, 556–560.
- [41] Rayman, M.P., Infante, H.G., Sargent, M., Food-chain selenium and human health: spotlight on speciation. *Br. J. Nutr.* 2008, 100, 238–53.
- [42] Juresa, D., Blanusa, M., Francesconi, K.A., Kienzl, N., et al., Biological availability of selenosugars in rats. *Chem. Biol. Interact.* 2007, 168, 203–210.
- [43] Suzuki, K.T., Doi, C., Suzuki, N., Metabolism of ⁷⁶Se-methylselenocysteine compared with that of ⁷⁷Se-selenomethionine and ⁸²Se-selenite. *Toxicol. Appl. Pharmacol.* 2006, 217, 185–195.
- [44] Gabel-Jensen, C., Gammelgaard, B., Selenium metabolism in hepatocytes incubated with selenite, selenate, selenomethionine, Se-methylselenocysteine and methylseleninic acid and analysed by LC-ICP-MS. *J. Anal. At. Spectrom.* 2010, 25, 414.
- [45] Cuello, S., Ramos, S., Mateos, R., Martín, M.A., et al., Selenium methylselenocysteine protects human hepatoma HepG2 cells against oxidative stress induced by tert-butyl hydroperoxide. *Anal. Bioanal. Chem.* 2007, 389, 2167–78.
- [46] Suzuki, K.T., Somekawa, L., Suzuki, N., Distribution and reuse of ⁷⁶Se-selenosugar in selenium-deficient rats. *Toxicol. Appl. Pharmacol.* 2006, 216, 303–8.

Tables

Table 1: ICP-QQQ-MS parameters for total Se quantification

Nebulizer type	MicroMist
Spray chamber type	Scott-type
Sampling and skimmer cone	nickel
RF power	1550 W
Reflected power	< 5 W
Plasma gas flow	15 L/min
Carrier gas flow	1 L/min
Auxiliary gas flow	0.9 L/min
Collision/reaction gas mixture in the CRC	O ₂ + H ₂ : 0.4 + 1 mL/min (Purity 99.999%)
Quadrupole 1 → 2	<i>m/z</i> 80 → 96 for quantification (reaction with 1/2 O ₂) <i>m/z</i> 78 → 94 for monitoring (reaction with 1/2 O ₂) <i>m/z</i> 78 → 78 for monitoring <i>m/z</i> 80 → 80 for monitoring <i>m/z</i> 72 → 72 internal standard
Integration time	0.99 s
Number of sweeps	100
Internal standard	Ge (0.5 µg/L)
Sample flow	0.33 mL/min

Table 2: Characteristics of the optimized method. Limits of detection (LOD) and quantification (LOQ) were calculated according to DIN 32 645 (Reference norm of the German Institute for Standardization) by calibration between 0.1 – 10 µg/L in 20% nitric acid.

	Certified/ spiked concentration	<i>m/z</i> 78 → 78	<i>m/z</i> 80 → 80	<i>m/z</i> 78 → 94	<i>m/z</i> 80 → 96
Linearity		0.99999	0.99998	0.99998	0.99999
LOD [ng/L]		24	22	30	20
LOQ [ng/L]		90	85	114	75
Results for certified fish muscle ERM®–BB422 [mg/kg]	1.33 ± 0.13	1.33 ± 0.09	1.33 ± 0.08	1.33 ± 0.09	1.34 ± 0.06
Results for 1 µg/L Se spiked to cell culture medium [µg/L]	1.00	1.07 ± 0.07	0.97 ± 0.06	1.02 ± 0.05	0.99 ± 0.04

Table 3: Effect concentrations 30 (EC₃₀) and inhibitory concentrations 50 (IC₅₀) after 48 h of incubation with the respective Se species in UROtsa cells. EC₃₀ values indicate a decrease of cell viability or cell number by 30% as compared to the controls. IC₅₀ values indicate a decrease by 50%, respectively.

	Cell viability		Cell number	
	EC ₃₀ [μM]	IC ₅₀ [μM]	EC ₃₀ [μM]	IC ₅₀ [μM]
Selenite	3.0	4.3	2.2	2.35
MeSeCys	59.9	90.0	24.6	39.5
SeMet	743	>1000	265	443
Selenosugar 1	>1000	>1000	>500	>500
TMSe	>1000	>1000	>500	>500

Table 4: Cellular total Se after 48 h of incubation with the respective Se species in UROtsa cells. *Cytotoxic concentrations defined as $\geq$ effect concentration 30 (EC₃₀) according to cell number as the most sensitive endpoint.

	Incubation [μ M Se]	Cellular total Se [μ M]	Accumulation $\frac{\text{Cellular Se } [\mu\text{M}]}{\text{Incubated Se } [\mu\text{M}]}$
Control	0	1.2 ± 0.6	
Selenite	1.5	2.5 ± 0.7	1.7
	2.5	$11 \pm 1.9^*$	4.4
MeSeCys	2.5	8.6 ± 3.2	3.4
	25	$23 \pm 4.3^*$	0.9
SeMet	25	553 ± 77	22.1
	250	$3194 \pm 399^*$	12.8
Selenosugar 1	500	111 ± 12	0.2
TMSe	500	66 ± 10	0.1

Figure legends

Figure 1. Structures of the Se species investigated in this study: Selenite (**A**), methylselenocysteine (MeSeCys) (**B**), trimethylselenonium ion (TMSe) (**C**), selenomethionine (SeMet) (**D**) and selenosugar **1** (**E**).

Figure 2: Comparison of ICP-QQQ-MS Se quantification in non-spiked cell culture medium for various gas modes for the most abundant ^{80}Se and ^{78}Se .

Figure 3: Cell viability assessed by cellular dehydrogenase activity after 48 h of incubation with Se species: In HepG2 cells (**A**), in UROtsa cells (**B**) and in CCF-STTG1 cells (**C**). Shown are mean values of at least three independent determinations + SD. Cell viability of selenite is shown enlarged. Selenite $\blacksquare$, MeSeCys $\blacksquare$, SeMet $\blacksquare$, TMSe $\blacksquare$, selenosugar **1** $\blacksquare$.

Figure 4: Cell number in HepG2 cells (**A**) and in UROtsa cells (**B**) after 48 h of incubation with Se species. Shown are mean values of at least two independent determinations + SD. Cell number of selenite is shown enlarged. Selenite $\blacksquare$, MeSeCys $\blacksquare$, SeMet $\blacksquare$, TMSe $\blacksquare$, selenosugar **1** $\blacksquare$.

Figure 5: RP-HPLC-ICP-QQQ-MS chromatograms of a 2 $\mu\text{g/L}$ standard mix (**A**) and of the water-soluble fraction of UROtsa cells incubated for 48 h with 2.5 μM selenite (**B**), 25 μM MeSeCys (**C**), 25 μM SeMet (**D**), 500 μM TMSe (**E, F**) and 500 μM selenosugar **1** (**G, H**). YMC-TriartPFP column (3 μm , 3x250 mm) at 30 $^{\circ}\text{C}$ with 250 $\mu\text{L/min}$ 20 mM ammonium formate (pH 3, 3% methanol) as mobile phase. m/z 80 $\rightarrow$ 80 $\blacksquare$; m/z 80 $\rightarrow$ 96 $\blacksquare$.

Figure 1. Structures of the Se species investigated in this study: Selenite (**A**), methylselenocysteine (MeSeCys) (**B**), trimethylselenonium ion (TMSe) (**C**), selenomethionine (SeMet) (**D**) and selenosugar **1** (**E**).

Figure 2: Comparison of ICP-QQQ-MS Se quantification in non-spiked cell culture medium for various gas modes for the most abundant ^{80}Se and ^{78}Se .

Figure 3: Cell viability assessed by cellular dehydrogenase activity after 48 h of incubation with Se species: In HepG2 cells (A), in UROtsa cells (B) and in CCF-STTG1 cells (C). Shown are mean values of at least three independent determinations + SD. Cell viability of selenite is shown enlarged. Selenite —, MeSeCys —, SeMet —, TMSe —, selenosugar 1 -.-.

Figure 4: Cell number in HepG2 cells (**A**) and in UROtsa cells (**B**) after 48 h of incubation with Se species. Shown are mean values of at least two independent determinations + SD. Cell number of selenite is shown enlarged. Selenite —, MeSeCys —, SeMet —, TMSe —, selenosugar 1 —.

Figure 5: RP-HPLC-ICP-QQQ-MS chromatograms of a 2 µg/L standard mix (A) and of the water-soluble fraction of UROtsa cells incubated for 48 h with 2.5 µM selenite (B), 25 µM MeSeCys (C), 25 µM SeMet (D), 500 µM TMS₂Se (E, F) and 500 µM selenosugar 1 (G, H). YMC-TriartPFP column (3 µm, 3x250 mm) at 30 °C with 250 µL/min 20 mM ammonium formate (pH 3, 3% methanol) as mobile phase. m/z 80 $\rightarrow$ 80 $-$; m/z 80 $\rightarrow$ 96 $-$.

Electronic supplementary information

Supplementary table 1: ICP-QQQ-MS parameters.

RF power	1550 W
Reflected power	< 5 W
Plasma gas flow	15 L/min
Carrier gas flow	1 L/min
Auxiliary gas flow	0.9 L/min
Collision and reaction gas mixture in the CRC	Oxygen (99.999%) 0.4 mL/min Hydrogen (99.999%) 1 mL/min
m/z (Q1 → Q2)	80 → 96 used for quantification 80 → 80 interference monitoring 78 → 94 interference monitoring 78 → 78 interference monitoring 77 → 93 spike 77 → 77 spike
Integration time	0.1 s
Number of sweeps	100
Sample flow	0.33 mL/min
Isopropanol flow	0.01 mL/min

Supplementary table 2: Quantitative investigation of the degradation of unknown species I and II applying HPLC-ICP-QQQ-MS analysis every 120 min with a 50 μ M MeSeCys HepG2 cell lysate (4 d), which was stored at room temperature between the runs. As the volatile species DMSe shows enhanced response,⁴⁰ it was not included. (See also figure 3)

Run #	Concentration in the measured solution [μ g Se/L]			Sum [μ g Se/L]
	MeSeCys	Unknown species I	Unknown species II	
1	12.9	2.7	21.4	37.0
2	27.2	1.4	7.1	35.7
3	34.0	0.7		34.7
4	34.5			34.5

Supplementary table 3: Percentual comparison of total cellular Se concentrations to the amount of speciated Se compounds.

Incubation [μM] time			total cellular Se [μM]	Sum of small species [% of total cellular Se]	Amount of small Se species [% of total cellular Se]			Gap of sum of small species to total Se [% of total cellular Se]
MeSeCys	25	48 h	9	29	13 % MeSeCys	0,0 % unknown species I	15 % unknown species II	71
		4 d	12	40	22 % MeSeCys	0,0 % unknown species I	18 % unknown species II	60
	50	48 h	14	36	18 % MeSeCys	0,0 % unknown species I	18 % unknown species II	64
		4 d	20	42	20 % MeSeCys	2,5 % unknown species I	19 % unknown species II	58
	100	48 h	24	32	16 % MeSeCys	2,0 % unknown species I	14 % unknown species II	68
		4 d	65	62	27 % MeSeCys	6,0 % unknown species I	29 % unknown species II	38
SeMet	150	48 h	963	9,2	5,8 % SeOMet?	2,6 % SeMet	0,7 % unknown species III	91
		4 d	1664	9,7	6,6 % SeOMet?	2,6 % SeMet	0,5 % unknown species III	90
	200	48 h	1066	13	8,2 % SeOMet?	3,8 % SeMet	0,9 % unknown species III	87
		4 d	1664	14	8,2 % SeOMet?	4,7 % SeMet	0,8 % unknown species III	86
TMSe	500	4 d	99	94	94 % TMSe			6,2
SeSugar 1	500	4 d	91	89	89 % SeSugar 1			11

Supplementary figure 1: Instrumental set-up for Se speciation and total Se quantification using ID(-HPLC-)ICP-QQQ-MS.

Supplementary figure 2: Standard reference chromatogram of 5 $\mu\text{g/L}$ MeSeCys (7.3 min), TMSe (8.4 min), SeMet (9.6 min) and SeSugar 1 (11.1 min) (A) and the corresponding mass flow chromatogram (B). Chromatographic separation was performed on a YMC-TriartPFP column (3 μm , 3x250 mm) at 30 $^{\circ}\text{C}$ with 250 $\mu\text{L/min}$ 20 mM ammonium formate (pH 3, 3% methanol) as mobile phase.