**The importance of speciation analysis in neurodegeneration research**

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**Abstract**

Element speciation offers deeper insight into the molecular mechanisms of disease by determining element species pattern. Thus, having great potential for investigating neurodegeneration in Alzheimer’s disease, Parkinson’s disease, amyotrophic lateral sclerosis, and mild cognitive impairment speciation is increasingly considered in epidemiological or clinical neurological studies. This review analyses recent speciation findings in neurodegeneration research, concentrating on measurements in cerebrospinal fluid and brain. Elements considered are aluminum, arsenic, copper, iron, mercury, manganese, and selenium. Typically, hyphenated techniques are used in neurodegeneration speciation studies. The results allow sorting-out less important species from compounds significant for the disease, with subsequent use of molecular biology methods to uncover the exact mechanisms. This review indicates the trend of combining speciation and neuroscience and provides a sketch about data and outcomes. For brain research we recommend using modern, powerful techniques throughout which provide advanced validity and information in a chemical sense.

**Keywords**:

Speciation; neurodegeneration; cerebrospinal fluid; brain; hyphenated techniques; inductively coupled plasma mass spectrometry; high pressure liquid chromatography; trace element; redox stability; quality control

**Abbreviations**

AAS atomic absorption spectrometry

Aß amyloid beta

AD Alzheimer´s disease

AFS atomic fluorescence spectroscopy

ALS amyotrophic lateral sclerosis

BBB blood brain barrier

CE capillary electrophoresis

CNS central nervous system

CSF cerebrospinal fluid

CV cold vapor

CZE capillary zone electrophoresis

DNA deoxyribonucleic acid

DMA dimethylarsinic acid

DRC dynamic reaction cell

ESI electrospray ionization

FI flow injection

FT Fourier transformation

GABA gamma aminobutyric acid

GFAAS graphite furnace atomic absorption spectrometry

GPX glutathione peroxidase

GPX4 phospholipid hydroperoxidase glutathione peroxidase 4

HG-CT-AAS hydride generation-cryotrapping-atomic absorption spectrometry

HPLC high pressure liquid chromatography

HMW high molecular weight

HSA human serum albumin

IC ionic chromatography

ICP inductively coupled plasma

ICR ion cyclotron resonance

ID isotope dilution

i.v. intra venous

LMW low molecular weight

MCI mild cognitive impairment

Mn-Tf Mn-transferrin

MRT magnetic resonance tomography

MeSeCys methylselenocysteine

MMA monomethylarsonic acid

MS mass spectrometry

MS/MS tandem mass spectrometry

MSA multiple system atrophy

MT metallothionein

NB neural barrier

NMR nuclear magnetic resonance

QC quality control

PAGE polyacrylamide gel electrophoresis

PD Parkinson´s disease

RM reference material

RPLC reversed phase liquid chromatography

ROS reactive oxygen species

SAX strong anion exchange chromatography

SCX strong cation exchange chromatography

SEC size-exclusion chromatography

SeCys selenocysteine

SeMet selenomethionine

SELENOP selenoprotein P

sf sector field

*SN substantia nigra*

TMA trimethylarsenic acid

TXNRD thioredoxin reductase

UF ultra-filtration

**1. Introduction**

Element speciation is an important field within analytical chemistry since already more than about thirty years. For definitions of speciation related terms the reader is referred to IUPAC regulations or the paper of Templeton et al. [1]. Speciation analysis offers deeper insight into molecular mechanisms and pathways of disease by determining the speciation of an element – the pattern of distinct element binding forms. Speciation research can compare such species pattern between healthy controls and patients for disease-related changes or shifts. Having such valuable potential for investigating mechanisms of neurodegenerative conditions, speciation research is increasingly considered in neurological studies within clinical or epidemiological context. Elemental speciation nowadays is providing an important bridge between powerful techniques of analytical chemistry and neurodevelopment or brain degeneration research [2]. Neurological disorders and age-related dementia are pressing problems in an aging society. Alzheimer´s disease (AD) is the most common age-related disorder, affecting two percent of total population, and it is expected to increase above fifty percent in elderly over sixty-five years in the USA. Parkinson´s disease (PD) and mild cognitive impairment (MCI) are further neurodegenerative disorders with strongly increasing prevalence [3]. The etiology of AD, PD, and MCI is closely linked to oxidative stress followed from cascades of protein misfolding and other metabolic changes. Aside from further reasons, oxidative stress, in turn, is also caused by misbalances of essential metals with redox capability, such as iron, copper, and manganese, or by exchange or exposure to adverse elements like aluminum, mercury or arsenic. Another neurodegenerative condition is amyotrophic lateral sclerosis (ALS), a motor neuron disease, whose etiology remains substantially unknown, except for few cases with gene mutations [4]. Environmental factors are, however, suspected causative. Epidemiological studies pointed to selenium as a risk factor for ALS, which however is somewhat contradictory to molecular biology studies based on knockout animal models, showing mainly that Se species are neuroprotective, especially, SELENOP and GPX [5, 6].

Element speciation has matured to provide key-knowledge by investigating changes in both species concentration and pattern of essential elements (e.g. Mn-Tf vs. Mn-citrate; SELENOP vs. Se (IV)) or shifts in their redox pairs (e.g. Fe (II) vs. Fe (III)).

An important issue is samples and sample matrices for speciation studies. Due to simplicity in sample availability, blood and serum are still used in most studies. However, according to the selective permeability of NB, element species composition, species concentration and pattern in the brain or CSF – or more general speaking, beyond NB – are typically independent and may be completely different from cycling fluids in the body [7]. Apart from different information gained by imaging techniques like MRT, CSF offers the closest chemical analysis view on the brain, while it is operating in its actual physiological or diseased condition. CSF is an excretion of the *choroid plexus* and is in permanent close contact to brain in the extraparenchymal cave [8]. The use of CSF as specimen for speciation also circumvents the need for extrapolation to humans from results gained by animal models. Consequently, the sample type of choice from living humans is CSF. However, limitations also have to be considered: by law, drawing CSF is only allowed after a strict medical indication. The primary importance for sampling is the sake of the patient. Sampling procedures have to follow medical needs and standards (disinfection = danger of contamination), using stainless steel needles (= contact to Mn and Fe). Analytical demands stand behind such medical constraints.

Importantly, it should be stressed that for the case-control studies, involving CSF analysis, the control CSF samples originate from “neurological healthy persons”. This means, that such persons initially seemingly had unspecific neurological complaints, provoking CSF sampling, but subsequent clinical chemistry excluded neuronal adverse conditions. CSF from healthy controls without any complaints is unavailable according to ethical standards and legal regulations. Also, it must be considered that samples from CSF banks could show limited suitability for speciation as samples were initially intended for different use. Sampling or storage history may be unclear or even unsuitable for later speciation studies. Due to the above limitations, animal studies are inevitable. These are performed as a compliment to the CSF studies, specifically for speciation investigations after defined exposure to metals or other chemicals.

Speciation techniques in use for neurobiology research are in general the same as applied for other research fields. Such methods are summarized already in various reviews, including their suitability for different matrices or element species, and the reader is referred to those articles for the basic technical information [9]. Overall, techniques used in neurodegeneration studies, too, consist of hyphenated methods, mostly HPLC coupled to ICP-MS. For QC and species identification orthogonal 2D-approaches are employed, using e.g. CE as independent separation device. Complementary species information is partly achieved by ESI-MS/MS or ESI-ICR-MS. The overview of the general workflow for speciation analysis in neurobiology, with the QC stressed, is presented in Fig. 1.



Fig. 1 General workflow for the speciation analysis in neurobiological studies. \* – gels may be analyzed by laser ablation technique or after digestion with standard elemental techniques.

A matter of concern is species- and redox-stability during extraction procedures from the brain (experimental animals or human post mortem studies) and/or during species separation of brain extracts or CSF samples. Covalently bound stable element species, like selenium in SeMet, tolerate reversed-phase or ion-exchange chromatography for species separation, whereas currently most interesting element species, regarding AD or PD, e.g. Mn-citrate, Mn-Tf, are typically at danger to be transformed during sample preparation and species separation. Therefore, during sample preparation low-temperature and inert-gas atmosphere are typically necessary and smooth separation techniques with often only limited chromatographic resolution can be used [10].

In molecular biology studies, which mostly are based on knockout animal models [11, 12], “single-species assays” commonly are applied to follow changes of one specific element species. Then, specific single species like SELENOP are determined after e.g. relevant genes were knocked out and the related metabolic pathways are changed [13]. In such approaches, the specificity of the assay is of paramount importance. The subsequent sections will indicate that analytical techniques of speciation research have started to be introduced into neurological research and during recent years this changed from a beginning initiative to an established application trend resulting in fruitful cooperation between neurologists, epidemiologists, molecular biologists, and analytical chemists.

**2. Speciation of iron, copper, and zinc**

Iron (Fe) as well as copper (Cu) and zinc (Zn) are essential trace elements in human body. Many enzymes and proteins, which are required for proper function of the body and specifically the brain, employ these three elements as prosthetic group. Any disturbance of the tightly regulated balances between different species can lead to neurodegeneration. Both metals are implicated in AD and PD. Fe additionally seems to be involved in Huntington´s disease and Friedreich´s ataxia and Cu is implicated in ALS, Wilson´s disease, and Menkes disease [14]. Fe and Cu share similar transporters into the CNS and both can have redox potential and induce oxidative stress via Fenton reaction.

Zinc is also well-recognized as an element relevant to the brain pathology [15]. Multiple studies addressed its essential role in neuronal signaling [16-18] and potential involvement into protein aggregation in AD and other neurodegenerative disorders [15, 19, 20]. At this point, the interplay between Zn and Cu is often being discussed [21].

Especially for Fe, there are numerous studies quantifying the total content in different human tissues, e.g. serum, CSF, and brain [22-26]. But the results are partially contradictory; therefore, a speciation analysis could give deeper insights and show more specific alterations. A defined redox balance of iron in brain is mandatory, because ferrous iron (Fe(II)) appears to be much more toxic than ferric (Fe(III)) ions; ferrous iron can form reactive oxygen species (ROS) via Fenton reaction. Recently developed speciation methods differentiate Fe(II) from Fe(III) [10] (and Mn(II)/Mn(III), Cu(I)/Cu(II)) using SCX-ICP-MS [27]. However, early speciation studies used simple spectrophotometric iron determination methods. Sofic et al. [28] (1988) found significantly increased Fe(III) in *SN* of PD-patients, resulting in an altered Fe(II)/Fe(III)-ratio in favor of Fe(III). Riederer et al. [29] (1989) confirmed this shift towards Fe(III) in *SN* of PD-patients also, using a spectrophotometric technique, and found a significant increase of ferritin, which is the major ferric iron storage protein. QC measures were not reported in both references. Such alteration in favor of Fe(III) reduces the risk for ROS formation via Fenton reaction, counteracting mechanisms of neurodegeneration. These early results are in contradiction to more recent findings based on modern speciation methods and are not in concordance with the investigations, which strongly suggest a connection between neuropathogenesis and oxidative stress. Recent studies indicate shifts of iron homeostasis towards Fe(II). Quintana et al. [30] found increased Fe-concentrations in AD-brains, but without equivalent increase in ferritin, being associated with Fe(III). As a conclusion, they suggested an increase of toxic Fe(II)-ions in brain and an increased ROS formation as a consequence. In line with this finding is an increase in ferrous ions in AD brain reported by Salvador [31]. A recent study showed a connection between Mn-exposure, Mn-species and iron homeostasis [10]: after low-dose Mn-exposure, Mn-species increased in brain paralleled by a shift from Fe(III) towards Fe(II). The experiments were ruled by strict QC management for avoiding any changes in redox species. The observed Fe(II)/Fe(III)-dyshomeostasis was followed by multiple changes in metabolites, including increased markers for oxidative stress, inflammation and lipid peroxidation [10, 32].

In an AD mouse model, Fe-speciation in the brain tissue was performed using X-ray absorption near edge spectroscopy, a non-destructive speciation technique [33]. The results showed increased total iron-concentration but Fe-protein-species remained unaffected. The brain tissue composition was determined indirectly by linear combination fitting of previously measured standards and was found to consist of 60 % ferritin and 40 % myoglobin. Visanji et al. [34] investigated ferritin-levels by Western-blotting and ferritin-related iron by superconducting quantum interference device magnetometry, whereas total iron-content was analyzed by GFAAS. They performed these measurements in extracts of different brain regions in PD- and multiple system atrophy (MSA)-patients as well as matched controls (three patients each). Blanks and certified RMs were analyzed for QC. The authors found an increase in ferritin in *basis pontis* and *putamen* for MSA, but in *SN* ferritin-levels remained unchanged. Additionally, Fe-concentrations changed according to ferritin-levels. Unlike in MSA, ferritin level was unaffected in *basis pontis* and *SN* from PD-samples, but was slightly increased in *putamen*. In this study, too, *basis pontis* and *putamen* Fe-concentration changed along with ferritin, whereas in *SN* iron was increased. The results are in line with the ferritin-determination of Bourassa et al. [33] and seem to go in the same direction as the Fe(II)/Fe(III)-speciation studies, which showed an increase in Fe(II).

Iron can also form complexes with peptides, which are known to be misfolded or aggregated in neurodegeneration (AD: Aß; PD/MSA: α-synuclein). The Fe(II)/Fe(III)-differentiation is also important in this respect. Peng et al. [35] investigated the binding abilities of both iron-redox states to α-synuclein using ESI-MS. The stability of the complexes was checked by voltammetry. The results show a weak binding of α-synuclein and Fe(II) with 1:1 stoichiometry. In the presence of O2 this complex oxidizes to an α-synuclein-Fe(III)-complex, and in turn, O2 is reduced to H2O2, a highly mobile ROS.

Similar to Fe, total Cu and Zn were often quantified in CSF and brain compartments [36-38]; however, speciation studies are scarce, specifically in brain or CSF. Nevertheless, few interesting studies contributing to the frame of this review (speciation and “beyond NB”: brain or CSF) are summarized. Cu(II) is known to bind to proteins and peptides related to neurodegeneration, whereas no complexes of Cu(I) are known, except ceruloplasmin, a mixed Cu(I)-Cu(II)-protein. A comprehensive review about Cu and Zn binding to proteins was already published [20], so we will concentrate only on Cu and Zn speciation studies, related to neurobiology.

Becker et al. [39] successfully identified phospho-, Cu, and Zn-metalloproteins and partially quantified Zn, Cu, and uranium (U) as well as protein phosphorylation in a protein mixture from human *somatomotor cortex*. For this challenging purpose, the authors used a combination of two-dimensional electrophoresis with laser ablation ICP-sf-MS (directly from the gel) and MALDI-FT-ICR-MS (after excision from gel and tryptic digestion) [39].

Nischwitz et al. [40] investigated Zn distribution in paired serum/CSF samples aside from other elements. In this study, from 2008 the first aim was an improved quality control management during SEC separation, species stability and recovery. As a second aim element distributions, e.g. for Zn in CSF, and CSF/serum ratios were determined in 29 control samples. Total CSF zinc was measured at 19±15 µg/L (range 4-142 µg/L), being slightly less than literature values 32.9±8.9 from [36]. The ratio CSF/serum for Zn was 0.03±0.02. In serum, zinc was found only in protein fractions, whereas in CSF three peaks could have been resolved, with 56 % being attributed to Zn-LMW compounds. It was concluded that LMW species, being dominant for e.g. Mg and Ca, could permeate NB more easily than HMW species, which were predominate for Fe, Cu, and Zn in serum. Nevertheless, in CSF slightly more than half of Zn was bound to LMW species, which were not further investigated.

To study the role of the metals (Zn, Fe, nickel – Ni, Cu, and lead – Pb) in the cerebrovascular diseases, Ellis et al. [41] proposed a CSF speciation approach, based on RP- and anion exchange-HPLC-ICP-MS combined with nano-LC-ESI-MS. In the follow-up study from the group of Prof. J. Caruso, Zhang et al. [42] used SEC-ICP-MS to investigate the role of Zn, Fe, Ni, Cu, and Pb species in the developing cerebral vasospasm (CV), a complication of subarachnoid hemorrhagic stroke (SAH). Three groups of CSF samples (from healthy controls, n=2; SAH patients, n=4; SAH patients with CV complication, n=4) have been screened for protein-associated metal fractions, which have been further analyzed on nano-LC-Chip-ESI-IT-MS after tryptic digestion. CSF samples revealed four Zn containing SEC fractions, with the LMW Zn-fraction being the most prominent. Similarities were found in the first protein fraction for “Zn-finger proteins 524” between SAH patients and CV- patients and in a mixed collected SEC fraction of third and fourth Zn peak, for “Zn-finger proteins 513”. These similarities were not monitored for controls. Also, for Fe, Cu, and, interestingly, Pb different SEC patterns have been observed. The authors critically mentioned that SEC fractions, while collected based on a metal target or cohort, included non-metalloproteins as well, when considering the relatively wide peaks. Unfortunately, at the time of their paper, the major databases available did not specifically indicate such metal association(s) [42]. This made it difficult to sort out detected compounds, which were not connected to Zn or other metals of interest.

An important domain of Zn and some toxic metals (Hg, cadmium – Cd) biomedical speciation is related to the determination of metallothioneins (MT) [43, 44], specific metal-binding cysteine-rich proteins, secreted by the liver. For instance, Prange et al. [44] examined Cu-Zn-metallothionein isoforms, which may be affected in AD-brain. They used CE for separation of three isoforms and ICP-MS for on-line detection of respective metal ions. The hyphenated device was appropriate to separate efficiently the metallothionein isoforms without interfering species stability. The results showed a significant decrease in Cu bound to metallothionein-1 and -3. Gellein et al. [45] presented the study on offline SEC-ICP-sf-MS profiling of Cd, Mn, Fe, Pb, Cu, and Zn with a focus on MT, expectedly finding major association of Zn and Cd with MT-fraction. The QC included the recovery vs. total Zn concentration and RM analysis.

Gonzalez-Dominguez et al. [46] investigated serum, but not CSF, of AD and MCI patients by carefully performed SEC-ICP-MS. Inter-element and inter-fraction ratios were computed. Aluminum (Al) and labile forms of Fe and Cu were increased in demented patients, while Mn, Zn, and Se were reduced. Interestingly, levels of Fe, Al, and Mn, were closely inter-related, suggesting a complex interplay between the homeostasis of these metals. Imbalances in Cu, Zn, and Se metabolism could be associated to abnormal redox status. Therefore, these results may contribute to better understanding of the pathological mechanisms related to metals in AD. This study shows the emerging trend of combining speciation and neurology. It provides a valuable set of data about element concentrations and inter-fraction ratios changes in AD based on careful analytical performed work. With respect to brain metabolism, however, the evaluation is somewhat limited since serum and no samples beyond NB like CSF were available for the study.

**3. Manganese speciation**

Manganese (Mn) is an essential trace element, which is indispensable for proper brain function, like for the enzyme glutaminesynthetase. However, Mn overexposure from environmental or occupational sources leads to neurotoxic effects causing a series of symptoms, such as adynamia/fatigability, sialorrhea, cephalalgia, sleep disturbances, muscular pain and hypertonia, masklike face, gait changes, reduced coordination, hallucinations, and mental irritability [47], finally leading to a Mn-induced Parkinsonism-like disease, called manganism [48]. Further, Mn has been shown to increase prevalence of PD and, therefore, it may contribute to this neurodegenerative condition. Additionally Mn is implicated in ALS [14].

The speciation of Mn is difficult due to unstable species and fast degradation [49]. A pH-value of 7.4 was found stabilizing native proteins and LMW-species of Mn. For the determination of the species only mild separation techniques can be applied, like CE or SEC, to be sure that species are not altered.

An important issue, connecting speciation with neurodegeneration, is how Mn crosses the NB and accumulates in the CNS. The three most relevant species to enter the brain are Mn(II), Mn(II)/(III)citrate, and Mn(III)-transferrin. Crossgrove et al. [50] investigated the brain influx rates of unbound Mn(II), the LMW-complex Mn-citrate and the protein-bound complex Mn-Tf in nine different brain regions and the *choroid plexus* by in situ brain perfusion technique in the rat brain. The influx values did not significantly differ between different regions, but the influx rate of Mn-citrate was higher than for unbound and protein-bound Mn. With these results, it seems that Mn-citrate is the major Mn-related species entering the brain. Additional experiments by Yokel [51] showed a 3-fold higher uptake of Mn-citrate compared to unbound Mn-ions. This uptake is higher than diffusion rates would yield, which supports the suggestion of Mn-citrate as the main species entering the brain. These experiments on animal models are supported by speciation studies in human CSF. In a series of related studies we found Mn from CSF correlating to Mn-Tf, the physiological Mn-carrier in serum, as long as total Mn concentration was below 1.5 µg/L. Notably, above 1.9 µg/L, a switch from Mn-Tf towards Mn-citrate appeared. Mn-citrate was identified in 2-dimensional orthogonal speciation schemes, including SEC-CE-ICP-MS and analysis by ESI-FT-ICR-MS [52]. This carrier-switch is of primary importance since passage of Mn-Tf across the NB is limited by Tf-receptor shuttle, whereas Mn-citrate was shown to be enriched five-fold beyond the NB [40]. Thus, it was concluded that at elevated Mn-levels an even accelerated Mn-influx across the NB appeared.

Diederich et al. [49] investigated Mn-speciation beyond the NB after defined exposure. Mn-species formation and distribution was measured by SEC-ICP-MS in a rat model after i.v. injection of Mn. In serum, Mn-Tf was the main Mn-fraction, being strongly increased compared to control animals, aside from slightly increased formation of LMW-species. After 4 days Mn-species in serum reached baseline levels again, but in the rat brain (=beyond NB) Mn-citrate was significantly elevated, while Mn-Tf remained at the control level. These results are in line with the results of Yokel [51] and Crossgrove et al. [50], that Mn-citrate seems to be the major species entering the brain. It supports also findings from Yokel [14], that transferrin-dependent uptake upon NBs is strictly regulated. In a follow-up studies, Neth et al. analyzed Mn-speciation by SEC-ICP-MS and in parallel monitored the changes in rat serum and brain metabolism by ESI-FT-ICR-MS after long-term low-dose Mn feeding [53, 54] and after single i.v. MnCl2 injection [32, 54]. The feeding study showed increase in LMW-compounds (citrate and amino acids) but not in Mn-Tf. Brain metabolites were differently affected accordingly to the different Mn-species. Oxidative stress markers and prostaglandins (inflammation marker) were strongly positively correlated with increased Mn and LMW-Mn-compounds in the brain but negatively correlated with Mn-proteins [53]. In the i.v. injection approach, major alterations were observed for amino acid, fatty acid, glutathione, glucose, and purine/pyrimidine metabolisms [32]. The power of this study was the combination of speciation with a metabolomic approach providing a broad and detailed overview of affected brain metabolic pathways related to distinct Mn-species.

In contrast to this increased uptake of Mn-species to the brain, Mn efflux is only diffusion-driven. Summarizing these results, the brain has neither an effective protection against increased Mn-uptake, due to the Mn-carrier switch, nor an appropriate efflux-mechanism, which prevents Mn-accumulation. No studies are reported about defined Mn-exposure of humans after occupational accidents with subsequent speciation of respective CSF. Thus, results from animal models have to be extrapolated. Similarities of symptoms from PD and Mn-dependent PD suggest Mn-speciation to be a promising tool also for PD, continuing the trend using analytical chemistry methods in neurology.

**4. Selenium speciation**

Selenium (Se) is a metalloid, which attracts a high degree of interest in respect of speciation analysis. The role of Se in the CNS is rather sophisticated as it is essential for the brain and also can be highly neurotoxic, depending on the intake and speciation [2, 55]. In the body, Se is covalently bound to the specific SeCys-containing proteins, the so-called selenoproteins, which are mainly responsible for the biological functions of Se [55, 56]. For more details on Se biological outcomes, please, see focused reviews [2, 57, 58].

In comparison to transition metals for instance, speciation of metalloids such as Se and As seems somewhat easier task, owing to the covalent nature of their species (so, “stronger” separation approaches – IC, RPLC – may be implemented), less risks of species redistribution at the stage of analysis and simpler availability of the standards. However, when it comes to speciation of selenoproteins, more problems may arise, since species degradation is more probable then and there are no standards for certain Se-proteins of importance, first of all, SELENOP [59]. Nevertheless, the current introduction of ID-ICP-MS for the accurate quantification [60] of selenoproteins will probably overcome the standardization and QC complications [61, 62].

Although Se speciation is rather widely used in neurobiological research, total Se quantification in the CSF still remains much more frequent. Tondo et al. [63] assessed Se CSF levels in 212 pediatric patients with varied neurological disorders. Mild Se deficiencies were reported for the cases with epileptic encephalopathies and hypoxic-ischemic encephalopathy, whereas elevated levels were attributed to several patients with hypoxic-ischemic encephalopathy, epileptic encephalopathy, and mitochondrial disorders. Quantification was performed by ICP-MS, though QC data were not presented. As a single species determination, GPX activity was also assayed in the CSF. Strong positive correlation between GPX and Se level was found. Interestingly, HSA in the CSF also had a strong correlation with Se level, which the authors attributed to Se transport via NB. Our speciation results [59] showed elevated CSF/serum ratios for GPX and TXRND. Additionally, the content of SELENOP was confirmed to be independent in CSF and serum [64]. To obtain these results, we assessed Se-species distribution in 24 paired samples of serum and CSF from neurologically healthy individuals using SAX-ICP-DRC-MS. 2D-separation, recovery of the total Se (measured by FI-ICP-DRC-MS), and RMs were employed for QC. We proposed that GPX and TXRND either were additionally produced in the brain or had facilitated diffusion mechanism, which partially supports the hypothesis that Se transport across the BBB may not be limited by the endocytosis of SELENOP [65].

Important results on a role of Se-species in the brain came from “single-species assays” such as Western blot, immunolabelling, and immunohistochemistry. For instance, in *medial temporal gyrus* of AD individuals, SELENOP-immunoreactive cells were shown to be co-localized with Aβ-plaques and neurofibrillary tangles; thus, direct interplay between SELENOP and the molecules involved in AD pathogenesis may be assumed [66]. In further studies, the same research group observed elevated levels of SELENOP in *choroid plexus* and CSF of AD patients, using Western blot and immunohistochemistry, respectively [67]. Bellinger et al. [68, 69] also investigated selenoprotein (GPX4 and SELENOP) expression pattern in post mortem PD brain. While overall GPX4 was reduced in *SN* of PD brains vs. healthy control (12 cases, 11 matched controls), its relative content to the cell density of the nigral cells was found to be increased; for *putamen* no difference in GPX4 was found between normal and PD brains. Also, GPX4 was found to co-localize with neuromelanin in the *midbrain* and to co-express with dopamine-synthesizing enzyme tyrosine hydroxylase in the dopaminergic terminals [68]. As for SELENOP, it was reduced in the *SN* of PD brains (12 cases, 11 matched controls) but also had relative increase to the total number of the cells. SELENOP expression was concentrated within the PD brain lesions – the Lewy bodies; however, contrary to GPX4, no specific co-localization of SELENOP with neuromelanin or tyrosine hydroxylase was observed [69]. The authors proposed that altered GPX4 and SELENOP distributions may be related to pathological PD changes.

Coupling-based studies are currently emerging for the studying of Se biological functions and its role in brain pathology. Sánchez-Martínez et al. [70] studied Se metabolism in male rats after feeding them a diet with 20% fermented cabbage, enriched in [77Se]-MeSeCys (94% MeSeCys of total Se). The speciation of Se in liver, kidney, brain, testes, and heart was performed by isotope dilution SAX-ICP-MS and C8-RPLC-ESI-MS/MS. Two RMs were analyzed for QC. Also, control of total Se in both animal feed and tissues under study was performed. The authors reported an increase of SeCys content in the rat brain (from 22 to 39% of total Se content) as well as appearance of unidentified Se-compound after supplementation with MeSeCys-enriched diet, showing that dietary Se-compounds may penetrate the BBB.

Cardoso et al. [71] studied Se status of AD patients. They have assessed Se content in serum, erythrocytes and CSF of AD patients, MCI individuals and age- and gender-matched healthy controls. The authors used ICP-MS to measure total Se content and SEC-ICP-MS to assess the profiling of Se in serum. The authors reported SELENOP rather than HSA to be the main protein-associated fraction of Se. However, the SEC-profiling data for the CSF samples was not reported, which unfortunately diminishes the value of the study with respect to neurological assessment. However, the study was supported by QC of total Se determination by RM. CSF and serum showed insignificant differences between AD, MCI, and control individuals, whilst erythrocytes of AD were reported to contain decreased Se.

Our own speciation results showed that, whilst certain Se-species, e.g. SELENOP, were protective to the brain, other species might promote neurodegeneration. In the first project, we used SAX-ICP-DRC-MS, following the previously established method [59], to quantify Se species in the CSF of 38 patients with newly diagnosed sporadic ALS vs. 38 matched controls [7]. The recoveries of Se species were calculated vs. total Se, measured by FI-ICP-DRC-MS. The validation was performed by RMs analysis and standard spiking. Epidemiological evaluation of the speciation data demonstrated that elevated levels of Se(IV) and HSA-bound Se were associated with higher risk of sporadic ALS, whereas higher organic Se level, and SELENOP, in particular, corresponded to lower risks of neurodegeneration. In the recent case study, a similar approach was used for familial ALS patients [4]. Nine ALS patients with varied gene mutations (C9ORF72, SOD1, FUS, TARDBP, ATXN2, and TUBA4A) were compared to 42 controls. Species quantification was performed using SAX-ICP-sf-MS. We have found that levels of inorganic Se (Se(IV) and Se(VI)), GPX, and SELENOP were increased in a patient with the tubulin-related TUBA4A mutation, whereas in the remaining ALS individuals elevated SeMet was observed. Finally, in our most recent study we investigated a possible correlation between certain Se-species and the risk for MCI patients to develop AD. The previously established and QC checked method was employed here again. For the patients with MCI, elevated levels of CSF Se(VI)were found to be associated with higher risk of developing AD later in life. We concluded that higher levels of some Se species in the CNS do not protect and may even increase the risk of AD in subjects with MCI (unpublished results by Vinceti et al., under review). Thus, our studies showed that Se transport and metabolism in the brain was considerably impaired under neurodegenerative states. We are sure that further speciation studies will provide an insight whether the found alterations in Se distribution are an innocent bystander to the neurodegeneration pathology or alternatively they play an independent and relevant role in the etiopathogenesis of the brain disorders [4].

**5. Arsenic speciation**

Arsenic (As) is also a metalloid, which seems to be involved into the brain pathology. As and Se have somewhat similar chemical properties, however, their biological relevance differs considerably [57]. Since As being considered non-essential, the speciation studies, related to this element, mainly concentrate on environmental tasks, *e.g.* exposure assessment of the endemic regions, and model studies. Pre-natal exposure to As may cause toxic effects to the CNS, which is especially vulnerable at the stage of development [72]. Inorganic As (As(III) and As(V)) is especially hazardous to the brain cells [73]; however, later findings showed that methylated species might also be cytotoxic [74, 75].

As speciation studies related to brain pathology are attracting growing interest either after environmental exposure or with As compounds used as pharmaceuticals. Kiguchi et al. [76] tested As2O3 as an antitumor agent against acute promyelocytic leukemia, evaluating its ability to pass through the BBB. For this purpose, the authors assessed As2O3 metabolites in the CSF of 3 patients, undergoing acute promyelocytic leukemia treatment. Samples were analyzed by C18-RPLC-ICP-MS. The proteins were preliminary removed by ultrafiltration with 10 kDa molecular weight cutoff. The authors reported that all main As species (As(III), As(V), MMA, DMA, and arsenobetaine) were found in CSF after administration of As2O3. Importantly, after the As2O3 treatment an increase of all As-species in CSF was observed, except for arsenobetaine, which was attributed to the background dietary As exposure. Unfortunately, a limited data on the QC (peak assignment only) is available from the previous publication of the same group on As speciation in serum samples [77].

Xi et al. [78] modeled As toxicity in weanling rat pups after transplacental and early life exposure to sodium arsenite. The arsenicals (inorganic As – As(III) + As(V), MMA, DMA, and TMA) were determined in brain and liver. Arsenicals showed dose-dependent increase in the tissues, whilst DMA was reported to be the predominant species in both liver and brain. TMA levels in liver exceeded those of the brain 2-4 folds. This partially supported previous finding of Jin et al. [72] for the newborn mice. Arsenicals were shown to pass both the placental barrier and BBB, being transported from dams to pups. The authors followed their previous method of As speciation by hydride generation of volatile arsines with cryogenic separation and AAS detection [72]. HG-CT-AAS is a comparatively new method, which seems to be uniquely suited for the oxidation state specific speciation analysis of As in complex bio-matrices. The method offers preservation of methylation state of unstable MMA(III) and DMA(III) [79]. Alkaline digestion was used to extract As-compounds from the tissues. The assessment of total As and inorganic As was verified vs. RMs, whereas As background level in the animals’ chow was also reported. In another study of the same group, As speciation in the offspring rat brain was investigated in relation to brain oxidative stress and enzymatic activity of glutamine synthetase and acetylcholinesterase [80]. These findings showed As exposure to cause alterations in the cholinergic and GABAergic neurotransmission and reduced antioxidant defense, which may lead to cognitive changes. HG-CT-AAS also was applied to characterize the retention of tri- and pentavalent arsenicals in tissues, including the brain, of wild-type and As-methyltransferase-knockout (As3mt-KO C57/BL6) mice after exposure to As(III) [81]. In this case, the aim of the study was to evaluate the adverse effects of individual arsenicals, owing to the low capacity of As3mt-KO-mice to methylate inorganic As. Unfortunately, analytical information (method optimization and QC) as well as description of the animals’ phenotype were not presented.

From our literature search it appears that As speciation is widely used in various research fields except those with a clear link to neuropathology. From that we conclude, that more As speciation studies, especially combined with molecular biology approaches, first of all, proteomics, will be helpful to better understand the mechanisms of As neuro- and cytotoxicity. This may lead to both improving the As-based chemotherapy and will possibly provide new diagnostic and risk assessment approaches for the As excessive endemic regions and highly polluted environments.

**6. Mercury speciation**

Mercury (Hg) is a well-known neurotoxicant and attracts attention worldwide due to its high environmental mobility, bioaccumulation, and its implementation in certain technological processes [82]. Being a non-essential element, Hg is highly toxic for humans and the environment in general. The two main groups of Hg species are inorganic (Hg(I) and Hg(II)) and organic (MeHg, EtHg, and PhHg) forms. Inorganic species are present in water, soil, and sediments, while organic ones are typical for biota and the atmosphere. It was clearly shown that Hg species have different toxicity mechanisms. The most toxic form is MeHg, due to its high capacity to bind sulfhydryl groups and low polarity, which increases BBB permeability. Neurotoxicity of organic Hg-species is attributed to ROS-production, impaired Ca2+- and protein kinases-associated signaling, alterations in gene expression, and affected GABAergic and glutamatergic neurotransmission [83]. So, Hg toxicity is not limited solely to unspecific protein binding. Interestingly, MeHg was shown to influence the expression of genes, altered in 1-methyl-4-phenylpyridinium PD model [84]. This indicates that MeHg exposure may be relevant to PD pathogenesis.

The ability of MeHg to penetrate via placenta barrier leads to high accumulation rates in the developing CNS of the fetus. In case of Hg exposure of pregnant women, MeHg may cause cerebral palsy-like symptoms in feti, even if maternal organism does not show any symptoms of intoxication [85]. This effect is attributed to both high Hg accumulation in the fetal brain and the ability of organic Hg-species to produce DNA single-strand breaks in neuronal cells. The model study by Glover et al. [86] showed alteration pattern of the gene expression in mice dams and post-partum pups. The dams were exposed to MeHgCl or MeHg-cysteine (corresponding to the naturally occurring MeHg) through diet. In MeHgCl-exposed animals, immunoglobulin and transmembrane disposition genes were affected, while for MeHg-cysteine methylation and protein metabolism genes showed some alteration. However, Zn-finger transcription factor cluster and genes related to the cytoskeleton/microtubules and multicellular development were affected for both MeHgCl and MeHg-cysteine groups. The author also studied the behavioral patterns and total Hg distribution in pup and maternal liver and pups’ brain (with no actual difference between MeHgCl and MeHg-cysteine groups) [86], but no actual speciation has been performed in this study, unfortunately.

The speciation studies e.g. from Rodrigues et al. [87] showed, that primary route of detoxification of organic Hg is demethylation [88]. Conversion of organic Hg into inorganic form reduces its mobility and binding, preventing crossing the NBs. In this respect, MeHg is also the most dangerous species, since comparing to closest homolog EtHg it provides not only higher total Hg levels in the brain under single exposure, but also have much smaller conversion rate into inorganic Hg [87]. A recent work, based on Hg species exposure to human neuron (LUHMES) and astrocyte (CCF-STTG1) cell cultures, was presented by Lohren et al. [89]. They compared in vitro toxicity (cytotoxicity and apoptosis) of MeHg, thiomersal (EtHg), and HgCl2. Cellular bioavailability of Hg-compounds was evaluated using ICP-MS. However, speciation analysis within the cells was not performed. Treatment with organic Hg-species resulted in considerably higher concentration of Hg within neurons (140-fold higher than in astrocytes), whereas significant cytotoxic and pro-apoptotic effect for organic Hg was observed for much lower concentrations than that of HgCl2. Interestingly, in astrocytes, exposure by equal concentrations of all Hg-species did not induce cytotoxicity or caspase-3 activation. Thus, the authors confirmed different toxicity mechanisms in neurons and astrocytes for inorganic and organic Hg-species and proposed that neurons are more susceptible to organic Hg-species owing to high accumulation of organic Hg. Also recently, Chan et al. [90] reported that inorganic Hg affected retinoic acid induced cellular differentiation in SH-SY5Y human neuroblastoma cells, reducing the expression of the proteins, involved in cellular differentiation (tubulin-βIII, neuronal nitric oxide synthase, tyrosine hydroxylase, and amyloid precursor protein). So, all Hg-species seem to impair neuronal development.

As most Hg species of interest contain alkyl groups, the most common speciation techniques are C8- or C18-RPLC [85, 87] and SCX chromatography [91]. Coupling to ICP-MS usually provides necessary sensitivity and allows avoiding preconcentration or pre-oxidation/reduction steps [85, 87], like in CV-AAS/AFS. Moreover, ICP-MS sensitivity can be improved by application of isotopic dilution or isotopic tracer techniques, which are widely used for accurate Hg measurements [85]. Also, in a methodical study of Gáspár and Páger [92], CZE was used for Hg speciation in CSF, urine, and saliva. The authors successfully separated primary Hg species (Hg(II), MeHg, EtHg, and PhHg) as their complexes with cysteine, applying UV detection. CZE looks a promising technique for Hg speciation even in CSF, especially when coupled to a sensitive detector like ICP-MS. In future, CZE-ICP-MS, thus, may be applied more frequently in neurodegeneration research characterizing Hg (and other species) in PD, AD or ALS.

Wang et al. [93] have presented important results on brain Hg speciation in a model study in Sprague-Dawley rats. The authors used SEC, coupled to post-column isotope dilution ICP-MS, to quantify Hg-containing protein fractions from the brain cytosol of female rats, exposed to MeHg as well as in the offspring 20 days post-partum. Also, Cu and Zn distribution has been monitored. The QC of the measurement was performed through the protein standard recoveries assessment (87-98%), however total Hg content in the brain extracts as well as QC of extraction procedure was not presented. There were five Hg-associated protein fractions in maternal brain (ca. 10, 17, 25, 96, and 480 kDa), whereas for the pups only a single 17 kDa fraction was observed. At the same time, the patterns of Cu and Zn were similar in both dams and pups [93].

Since Minamata Incident (1956), a high amount of data on Hg speciation in different environmental media were designed and validated and Hg-species RMs are also available. Environmental speciation studies are mostly conducted for varied ecosystems, first of all, aquatic ones, to evaluate Hg-exposure and/or pollution level. For example, using C18-RPLC-ICP-MS, Krey et al. [94] quantified Hg-species in the brain regions (*cerebellum*, *frontal lobe*, and *brain stem*) of polar bears. The same analytical approach was used in another environmental study [95], providing data on Hg speciation in beluga whales CNS (*frontal lobe*, *temporal lobe*, *cerebellum*, *brain stem*, and *spinal cord*). Though, both studies report Hg speciation in the brain tissue and are supported by the QC, those generally ascertain the fact of Hg presence, rather than discussing possible health outcomes, especially in respect of neurodegeneration. To conclude, Hg toxicity, biotransformation, and metabolism still remain an important problem, since actual pathways of Hg-species in the brain compartments are not studied properly up to now. We hope that Hg speciation, related to neurobiological research, including measurement of Hg-species in CSF of affected individuals would become a powerful tool of such studies.

**7. Aluminum speciation**

Aluminum (Al) chronic neurotoxicity and its possible role in neurodegeneration pathology are well documented. However, the underlying cause and biochemical mechanisms are not yet clear [96]. Al received the highest attention after ascertaining its role in dialysis encephalopathy in 1976. This discovery triggered investigations on relationship between Al exposure and dementia. Despite the similarity of Al-induced encephalopathy and AD in many symptoms, there still exists controversial linkage between Al exposure and cognitive decline. Especially considering the fact, that not all AD patients have elevated Al levels [96] while familial AD patients show Al concentrations higher than all previous measurements of brain Al [97], the discussion on Al being cause or consequence of AD is not decided.

In comparison with heavy metals (e.g. Hg) and metalloids (As and Se), Al does not tend to form strong bonds with proteins. However, it exhibits its toxic effect via the substitution of calcium at specific binding sites in e.g. calmodulin, protein kinase C etc. Also, ability of Al to bind to DNA with changing its conformation was observed by fluorescence and circular dichroism using short synthetic polynucleotides [98]. Another conformational changes promoted by Al are racemization of aspartate. Interestingly, D-forms of amino acids are known to have age-related accumulation in the brain, whereas Aβ treated with Al in vitro was also shown to have elevated content of D-amino acids [99].

Nowadays it is established, that Al is mainly transported in bloodstream and through the BBB as a citrate complex (similar to Mn transport) or bound to transferrin. Therefore, Al-citrates are currently the primary target of Al speciation. For example, the toxicity of Al-citrates was assessed in hippocampus glial and neuronal cell cultures [100]. A distinction between citrate-complexes and Al(III) was observed: the most toxic form appeared to be Al(III). Also, different time dependence of toxic effects among the species was revealed. Thus, the transformation of citrate-complexes to more toxic Al(III) seems to be an important issue.

Generally, Al does not virtually have acute toxicity, which hinders laboratory modeling [101]. Al species stability is highly dependent on pH of the media. And as Al is a ubiquitous element, without special care to avoid contamination during sampling and analysis procedures, results will be surely compromised. All these facts are making the studies of Al biochemical pathways and health outcomes utterly complicated. Owing to the aforementioned complications as well as to difficulties on Al-species identification and separation, the data on Al speciation is notably scarce [102]. First works, dedicated to Al speciation, was related to fractionation of serum samples. Application of ultrafiltration with 30 kDa cutoff allowed for separation of LMW and HMW Al-forms. Later the same results were obtained using SEC, with only 2 fractions observed in serum. It was confirmed, that about 10% of Al was ultrafiltrable and 90% protein-bound [103]. Later, RPLC and SAX allowed identifying the protein, carrying Al in HMW fraction of blood serum. It appeared to be transferrin. LMW fraction consisted of Al-citrate, Al-phosphate, and, possibly, mixed complexes of both ligands. Linkage of Al transportation with pathways of iron absorption is also exhibited in accumulation of Al in brain regions with the highest density of transferrin receptors; interestingly, in some studies, Fe content in AD brain was shown to be considerably increased [101].

At the same time, speciation of Al in CSF by weak anion exchange HPLC with off-line coupling to GFAAS [104] indicated the presence of Al-citrate/-silicate and unidentified HMW compound, which was not attributed to the transferrin complex. Additionally, Konoha et al. [105] modeled the neurotoxicity of Al in rat *cerebral cortex* cell culture. Using 27Al-NMR, they modeled the formation of Al13-cluster ([AlO4Al12(OH)24(H2O)12]7+) under physiological conditions in vitro. The exposure to Al13-cluster for 1 hour resulted in delayed decrease in cells viability after 2 weeks. Thus, although main Al-species in serum are well-described as citrate and transferrin complexes, which were shown to penetrate the BBB, there might be some unknown Al pathway in the brain, which substantially changes Al-species, secreted by *choroid plexus* into CSF.

Current situation in Al speciation related to brain research is rather complicated. On the one hand, there is much data on Al content in different brain regions and even some information on Al-species is present. On the other hand, the speciation data lacks proper QC and verified analytical procedures. Anyway, the data available strongly indicate a global role of Al in neurodegeneration pathology and the necessity of further studies.

**8. Interactions of trace element species**

Numerous facts are known from literature about trace element interactions regarding total element concentrations [106]. This wide and important field deserves a comparative, updated survey, which, however, is outside the scope of this review, concentrating on element speciation. Nevertheless, some interactions of element species are found in the literature, of which some are shortly described.

Trace elements affect the homeostasis of each other since in part they share the same protein transporters or bind to the same proteins. At such organic molecules those elements are competitors for binding sites and, consequently, the resulting organometal complexes are considered as different element species. Divalent metal species like Fe (II), Mn(II) or Zn(II) are using DMT1 at membranes or NB [107]. As a consequence, changes in the concentration of one of the competing divalent metal ion species influence the concentration and action of the other ones. In brain extracts from Mn-exposed rats an increased Mn-concentration was monitored, being paralleled from reduced total iron, but even more important, also paralleled by a shift of the Fe(II)/(III) ratio towards Fe(II). This finding proposed a shift towards an increase of Fenton reactions with subsequent elevation of oxidative stress. Actually, an increase in oxidative stress- and lipid peroxidation- markers was detected in those brains using ESI-FT-ICR-MS [10].

Further interactions are known for Cu and Zn, which are competitors for binding to metallothioneins [108, 109]. Additionally, mercury – a known neurotoxicant – and cadmium can bind to MTs after exposure, which is considered as a detoxification mechanism. Aside from inducing MT transcription after Cd and/or Hg exposure also the distribution of Cu-Zn-MTs is affected. In the already mentioned speciation study from Prange et al. [44], it is reported that in brains affected by AD-pathology the ratio of Cu vs. Zn is shifted at Cu-Zn-MTs since Cu-MT-1 and Cu-MT-3 are decreased. Anyway, the field of species interactions seems somewhat underexplored, currently. So we may expect more studies in this domain, owing to the progress in analytical techniques, enabling realization of multi-element speciation studies.

**Conclusion**

Apart from the typically applied methods and strategies to elucidate neurodegenerative conditions, element speciation is able to offer a bridge to powerful methods from analytical chemistry, which additionally should be applied in neurodegeneration research. The results from hyphenated techniques give basically a “species screening”, which allows to sort-out less important species from the important compounds with high significance for the disease. Molecular biology methods (histochemistry, blotting, miRNA) can then be applied for elucidating the exact molecular mechanisms. This review indicates the new trend of combining speciation and neuroscience and provides a short sketch about the data and fruitful outcomes. Critically, we have to mention that, contrary to the promising results, the potential of speciation is still not fully recognized in the neurological field. Still, biologists and physicians often implement outdated techniques in a chemical sense, regarding validity and advanced information content. Therefore, since relevant speciation techniques are established, we recommend a closer cooperation between neurologists/ neuroscientists and analytical chemists.

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**Conflict of interest statement**

None

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