

Enhanced extract preparation of native manganese and iron species from brain and liver tissue

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Abstract To date, no reference method for the extraction of labile Mn species from biological tissues is published which provides sufficient extraction efficiency combined with monitoring speciation. Here, an extraction method is reported using cryogenic conditions (+N) under inert gas atmosphere. Fresh brain and liver tissues were used, then stored either 1 day (+N) or 1 month in N₂liq (+N 1 m) to evaluate degradation effects during long-term storage. Both attempts were compared to a previous extraction method (−N) using neither N₂liq nor storage ability. Mn and Fe concentrations in extracts and pellets were determined with inductively coupled plasma (ICP)-atomic emission spectroscopy (AES) and compared to acid digests of the same sample. Element ratios of extracts/digest indicated the extraction efficiency, which was increased from 17% (−N) to 26% (+N) for Mn in brain or from 28% (−N) to 44% (+N) in liver extracts. For Fe species, the increase was only from 40% (−N) to 44% (+N) in brain but from 64% (−N) to 74% (+N) in liver. Size exclusion chromatography (SEC)-ICP-mass spectrometry (MS) was employed to screen for Mn and

Fe species pattern in extracts. In brain, surplus extracted Mn (+N, +N 1 m) was assigned to organic Mn species, mainly from the 0.7–4 kDa fraction, while in the liver, it was seen in the 70–80 kDa fraction. Fe speciation was similar for −N and +N methods in brain extracts. In liver, higher amounts of Fe species were extracted from the 140–160 kDa fraction. Storage at −196 °C for 1 month did neither affect Mn speciation in brain nor in liver extracts. Fe species pattern showed a negligible shift (≤5%) from 140–160 to 70–80 kDa fraction in liver extracts stored 1 month in N₂liq.

Keywords Manganese · Iron · Extraction · Speciation · Rat · Brain

Introduction

Mn speciation is of paramount importance to understand the metabolism of Mn species and to elucidate still unknown pathways of Mn transport across the blood–brain barrier (BBB) into the brain, with Mn accumulation in basal ganglia (especially globus pallidus, putamen, hypophysis) [1–3]. Methods for the sophisticated Mn speciation analysis in biological fluids already exist [3–9]. However, an efficient but species-preserving extraction of Mn species is required for analyzing Mn speciation in tissues, most notably brain tissue. To the authors best knowledge, only few extraction methods were employed where species stability of labile organometal compounds was of major concern, and probable changes in speciation were checked.

A previously published method from Nischwitz et al. [10] using Ar atmosphere preventing species oxidation was quality controlled. However, this approach appeared less practical when handling a higher number of low mass tissue samples in parallel at one time. Also, the extraction

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efficiency was partly too low for subsequent speciation, as only small rat organs with low mass were extracted. Therefore, a method was developed for improved extraction efficiency, maintaining species stability and comfortable handling of an increased sample number at one time. The method was checked by determination of extraction efficiency and monitoring Mn speciation by size exclusion chromatography (SEC)-inductively coupled plasma (ICP)-mass spectrometry (MS). Results from $+N$ and $-N$ methods were compared proving the superiority of the new $+N$ approach. Analysis of $+N$ extracts after 1 day or 1 month ($+N$ 1 m) proved the applicability of long-term storage.

Materials and methods

Chemicals

The chemical list consists of the following: Tris (99.9%) from Roth, Karlsruhe, Germany; bovine γ -globulin from Serva Electrophoresis, Heidelberg, Germany; human serum albumin (99%), bovine apo-transferrin (98%), manganese(II)chloride tetrahydrate (99.99%), sodium chloride (99.999%) metallothionein, citric acid, reduced and oxidized glutathione, histidine, and methionine, each from Sigma–Aldrich, Steinheim, Germany; NaCl (p.a.), NaOH (1 M), HCl (30%, suprapur), and HNO₃ (65%, sub-boiling distilled), each from Merck, Darmstadt, Germany; iron(II)chloride tetrahydrate (98%), from Applichem, Darmstadt, Germany; certified reference materials (CRM) 184, 185, and 186, each from the Institute for Reference Materials and Measurements, Geel, Belgium; certified custom assurance standard solutions XGLEN-1686/7, from Spex Certiprep, Metuchen, NJ, USA; Ar, Ar/H₂, and NH₃, each from Air Liquide, Gröbenzell, Germany; and N_{2liq} from Linde, Unterschleißheim, Germany.

Manganese–citrate/transferrin standards

Solutions of 1,000 mg/L citrate or apo-transferrin and 100 μ g/L Mn (0.36 mg/L MnCl₂·4H₂O) in eluent A (see “SEC” section, adjusted to pH=7.4) were incubated for 24 h at 37 °C and stored at 8 °C. A sufficient formation of Mn–citrate and Mn–transferrin complexes was detected after 1 week of storage using online coupling of SEC-ICP-MS.

Animals/sample collection

Three-month-old male Sprague–Dawley rats (450–550 g, Charles River WIGA GmbH, Sulzfeld, Germany) were sacrificed painlessly by blood removal after 4 days of adaption. Brain and liver were dissected with ceramic knives and PTFE-coated forceps (Neolab, Heidelberg, Germany) to avoid contact with Mn-containing stainless

steel instruments. The organ samples were frozen in N_{2liq} for the $+N$ method immediately after the section or kept at <8 °C (\leq 2 h storage) until prompt extraction ($-N$). Three rat brains were pooled for $+N$ and $-N$ approaches each to minimize intra-individual variations.

Sample preparation

Figure 1 gives an overview track scheme of consecutive steps for homogenization and extraction with ($+N$) and without ($-N$) liquid nitrogen.

Homogenization

“ $+N$ ” method The deep-frozen brain and liver samples were coarsely homogenized under liquid nitrogen using a PTFE pestle and mortar. The low temperature of N_{2liq} supported a complete mechanical destruction of the tissues and provided inert gas atmosphere above the samples by vaporized N₂. This “coarse” homogenate was stored in N_{2liq} for 1 day ($+N$) or 1 month ($+N$ 1 m) until further extraction (see “Tissue extraction” section) aimed for speciation.

“ $-N$ ” method For comparison, a reference method without N_{2liq} ($-N$) was applied to a second set of tissue [10]. Coarse homogenizations of freshly dissected brain and liver samples were prepared on ice under inert Ar atmosphere in a glove bag (Sigma–Aldrich, Steinheim, Germany) with PTFE pestle and mortar. Aliquots were stored on ice until their prompt extraction (see “Tissue extraction” section) aimed for speciation.

Aliquots of both approaches were taken for freeze drying, subsequent pressure digestion and, finally, total element determination.

Tissue extraction

“ $+N$ ” method Extracts of the above “coarse” homogenized frozen samples were prepared either after 1 day ($+N$) or 1 month of storage in N_{2liq} ($+N$ 1 m) to evaluate species stability during long-term storage.

“ $-N$ ” method Extracts of aliquots from fresh tissue samples were prepared immediately (\leq 1 h on ice) after “coarse” homogenization [10].

Further steps apply to each of the three approaches ($+N$, $-N$, and $+N$ 1 m) Aliquots of coarse homogenates were weighed (brain, 0.5–1 g; liver, 1–2 g) into 15 mL Greiner tubes with 2 mL (brain) or 4 mL (liver) 10-mM Tris–HCl buffer (0 °C, pH=7.4, flushed with Ar for at least 3 h). Thus, optimum extraction conditions (extractant/tissue ratio=3,

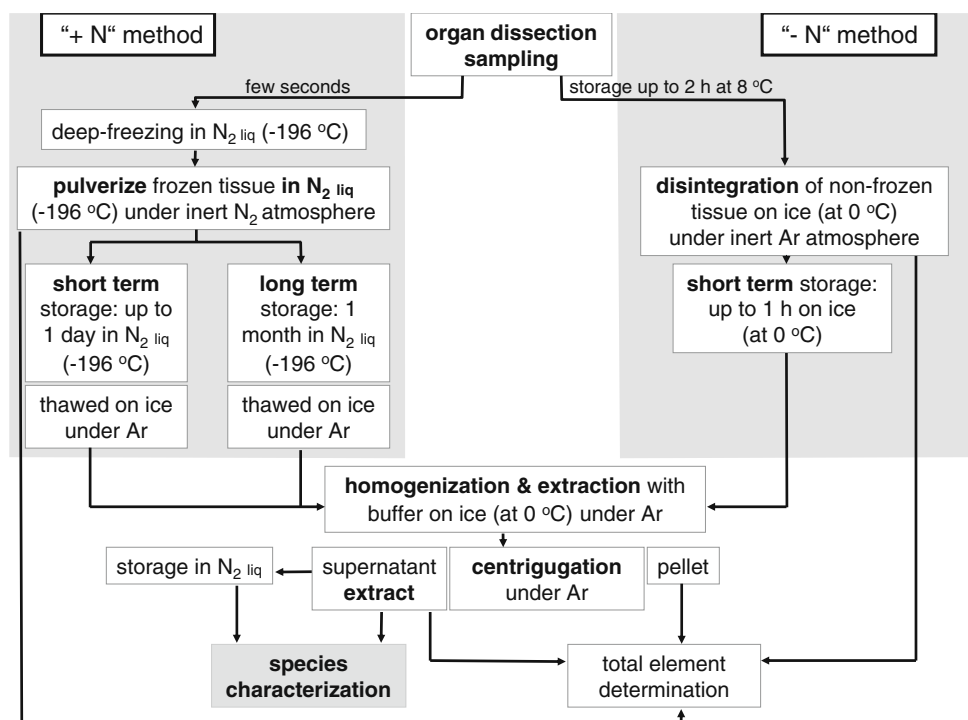


Fig. 1 An overview track scheme of consecutive steps for homogenizations/extractions with and without N_{2liq} is shown. “+N method”: The pulverization of tissue in liquid N_2 provided a more thorough grinding and thus enabled increased extraction efficiency. The immediate deep-freezing of organs and storage at -196 °C after dissection made the parallel handling of a high number of samples possible. Inert gas atmosphere and very low temperatures throughout

improved Mn and Fe species stability even during long-term storage for at least 1 month in N_{2liq} “-N method”: This method was used as a reference [10]. Species stability was maintained due to low temperature and inert Ar atmosphere. However, only short-term storage was allowed for preserving species stability. Therefore, only few samples could be handled at one time. The disintegration of non-frozen tissue was less efficient compared to the pulverization from the “+N method”

10-mM Tris-HCl extraction buffer) were achieved [10]. An extractant at pH=7.4 was found to stabilize native Mn proteins [9] and low molecular mass (LMM) Mn species (e.g., Mn^{2+} , Mn^{2+} -citrate $^-$, and Mn -citrate $_2^{4-}$) [11]. Each sample was put into a 10-mL hand homogenizer with PTFE plunger (Neolab, Heidelberg, Germany) by flushing the remaining sample with additional 1 mL extractant. Extraction and “fine” homogenization was carried out on ice under inert Ar atmosphere by at least ten times, squishing the cells between homogenizer and plunger and mixing with extractant. Extracts were stored in capped Greiner tubes (≤ 2 h on ice) until centrifugation: 2,000 $\times g$, 5 min, (Sigma-2D-centrifuge, Osterode am Harz, Germany), followed from 8,500 $\times g$, 45 min at 2 °C, (Heraeus Sepatech Biofuge 17RS, Hanau, Germany). The supernatant was collected into a 15-mL Greiner tube, while the pellet was resuspended with 1 mL extractant and centrifuged (30 min, 8,500 $\times g$, 2 °C). Supernatants of both centrifugation steps were unified and filled up to the mark (5 mL for brain and 10 mL for liver extract) with extraction buffer. Aliquots (1–2 mL) were stored at 8 °C for prompt analysis (pressure digestion and species analysis), at -20 °C for analysis, within 14 days and in N_{2liq} for long-time storage. The remaining pellets were kept at $<8\text{ °C}$ for microwave digestion.

Pressure digestion

Aliquots of each homogenized and freeze-dried tissue sample (100 mg), dry CRM (BCR 184, 185, and 186), and each extract (1 mL) were digested with 1 mL HNO_3 in a Seif digestion apparatus (Seif-Aufschlusstechnik, Unterschleißheim, Germany) according to Schramel et al. [12].

Pellets from centrifugation were digested with a Multiwave 3000 microwave digestion system (Anton Paar, Graz, Austria). Pellets were transferred into 50 mL Paar quartz tubes with 10 mL HNO_3 . The microwave digestions used an 80-min program (0–500 W in 5 min, 500 W for 10 min, 500–1,000 W in 5 min, 1,000 W for 45 min, and 0 W and cooling for 15 min). The clear digestion solutions were passed into 50-mL flasks (Merck, Darmstadt, Germany) and filled up to the mark with Milli-Q H_2O .

ICP-AES

For total Mn and Fe determination, a “Spectro Ciros Vision” ICP-AES system (SPECTRO Analytical Instruments GmbH & Co. KG, Kleve, Germany) was used. Sample introduction was accomplished by a peristaltic pump and a Meinhard nebulizer/cyclon spray chamber.

Measurement parameters were spectral lines Mn 257.611 nm, Fe 259.941 nm, RF power 1,300 W, plasma gas 12 L Ar/min, and nebulizer gas 700 mL/min (550 mL Ar/min plus 150 mL 7.5% H₂ in Ar/min). Determinations of blanks and certified control Mn and Fe standards (Spex multi-element standard XGLEN-1686/7) were performed periodically after ten samples each.

SEC

Two PEEK columns (250×4 mm I.D., Dionex, Idstein, Germany) packed with Toyopearl HW55S (TosoHaas, Stuttgart, Germany) or with the Toyopearl HW40S were used for separation of high molecular mass (HMM), e.g., Mn–transferrin and Mn–albumin, from LMM compounds, e.g., Mn–citrate. Two eluents were used for the isocratic chromatography by a metal-free Knauer Smart-line high performance liquid chromatography system (Knauer, Berlin, Germany) at a flow rate of 200 µL/min: 10 mM Tris–HCl, 20 mM NaCl at pH=7.4 as eluent A for chromatography between 0 and 45 min and eluent B (eluent A+HCl, pH=2) for cleaning from 45 to 55 min. The cleaning ensured high reproducibility and quantitative elution of metals from the columns. For quality control, the columns were purged overnight (100 µL/min, eluent A) and back-flushed at weekend (50 µL/min, eluent B). Blank runs were performed daily before first sample analysis. No Mn species formation on the stationary phase was observed. For SEC mass calibration, bovine γ-globulin (150 kDa), bovine apo-transferrin (79 kDa), human serum albumin (66 kDa), metallothionein (6.5 kDa), oxidized (612 Da) and reduced (307 Da) glutathione, histidine (155 Da), and methionine (150 Da) were analyzed. The separation characteristic (molecular size/elution time) is described by two equations: $y = -6.22 \times 10^5 \ln(x) + 1.6 \times 10^6$ for HMM compounds eluting before 13.5 min and $y = 5.4 \times 10^3 e^{-0.152x}$ for LMM species eluting after 13.5 min.

SEC-ICP-MS (online)

Online SEC-ICP-dynamic reaction cell (DRC)-MS was applied for species characterization The column effluent (200 µL/min) was mixed (PEEK “T”) with internal standard (IS, 10 µg/L Rh in 1% HNO₃, 450 µL/min) and introduced to ICP-DRC-MS (Perkin-Elmer/Sciex, Toronto, ON, Canada).

ICP-DRC-MS parameter Plasma gas 15 L Ar/min, nebulizer gas 840 mL Ar/min, DRC gas 0.55 mL NH₃/min, band pass $q=0.45$, and DRC optimization (NH₃ flow rate, band pass) were done according to manufacturers' instrument operation instructions; monitored isotopes were ⁵⁵Mn, ⁵⁶Fe,

and ¹⁰³Rh; dwell time was 100 ms. Chromatograms were exported to Microsoft Excel where ⁵⁵Mn and ⁵⁶Fe were normalized due to IS ¹⁰³Rh. Peaks of normalized chromatograms were integrated with Clarity Chrom 2.6 (Knauer) and Peakfit v4.11 (Systat, Erkrath, Germany). Daily DRC conditioning was performed for low baselines (⁵⁵Mn typically 10–30 cps and ⁵⁶Fe typically 100–400 cps). The five-point calibration was performed by flow injection of appropriate dilutions of a multi-element stock standard (1 mg Mn/L and 10 mg Fe/L). Eluent B was used as buffer, being mixed at the T-piece with the IS and directed to the nebulizer (see above).

Statistics

All data were analyzed by Microsoft Excel one-sample T^2 test (* $p \leq 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.005$).

Results and discussion

Quality control

CRM analysis of bovine muscle (BCR 184), bovine liver (BCR 185), and pig kidney (BCR 186) was used to check the accuracy of the digestion and element determination. The determined Mn and Fe concentrations met the certified values [13] in all CRM (Table S1 in Electronic Supplementary Material). ICP-AES apparently provided accurate Mn and Fe measurements and was used for total element determinations.

For online hyphenation, the sensitivity of ICP-AES was insufficient. Therefore, ICP-DRC-MS was employed with a limit of detection (LOD=3· s_0) <1 pg Mn and <50 pg Fe and a limit of quantification (LOQ=10· s_0) <10 pg Mn and <100 pg Fe.

Comparability between the ICP-AES and -MS was checked by analyzing identical rat brain and liver extracts ($n=3$) with both detectors. Concurrent results were found from ICP-AES and -MS in both tissue extracts (Table S2 in Electronic Supplementary Material). ICP-MS showed a comparable Fe level in liver extracts and slightly higher Fe values (increase <7%) in brain, which may be explained by interferences. ⁵⁶Fe is mostly interfered by ⁴⁰Ar-¹⁶O⁺, ⁴⁰Ca-¹⁶O⁺ and, to a lesser extent, by ³⁷Cl-¹⁸O-¹H⁺ [14]. As well as argon, chlorine from buffer is abundantly present and may contribute to the slightly elevated Fe levels detected by ICP-MS. Assuming that these interferences do not affect the size characterized (SC) Fe composition, the concentration measured by ICP-AES related to the peak area ratio from SEC-ICP-MS was used to quantify the SC Fe species in rat extracts.

The column recovery of Mn species (summed amount from species/injected amount) ranged from 77% to 100% for brain and 90–94% for liver extracts.

Total element concentration in extracts, pellets, and tissue

Table 1 shows total Mn and Fe concentrations of the three approaches (–N, +N, and +N 1 m). When using the +N (1 day storage) or +N 1 m (1 month storage) method, the Mn concentration was considerably higher in extracts of rat brain (increased by a factor of 1.5 related to the value from –N) and liver tissue (factor 1.58). Iron showed higher (factor 1.14) concentration in +N or +N 1 m extracts of rat liver tissue, while in turn, Mn and Fe being lower in the pellet fractions, both, compared to the –N method. The low (≤5%) standard deviation (SD) of the total Fe and Mn concentrations in extracts, pellets, and tissues (total concentration) proved the high reproducibility of the different methods. Importantly, the sum of the Fe or Mn concentrations of extracts and pellets from each of the extraction methods was nearly meeting the respective total element

concentrations in digests, proving ca. 100% recovery of Fe and Mn by each of the methods. This also proved that the higher element concentration in extracts after +N/+N 1 m sample preparation was not related to contamination but to a higher extraction efficiency.

Mn species characterization with SEC-ICP-MS

In speciation investigations, it is of paramount importance that speciation of a sample is not impaired during extraction [15, 16]. The online coupling of SEC to ICP-MS provides a mild method for analyzing labile Mn and Fe species. Characterizing their molecular size by SEC allows subsequent comparison of species peak pattern in extracts from a reference method (–N) with that one of the enhanced method (+N). Changes could show species transformations and thus indicate impaired species stability.

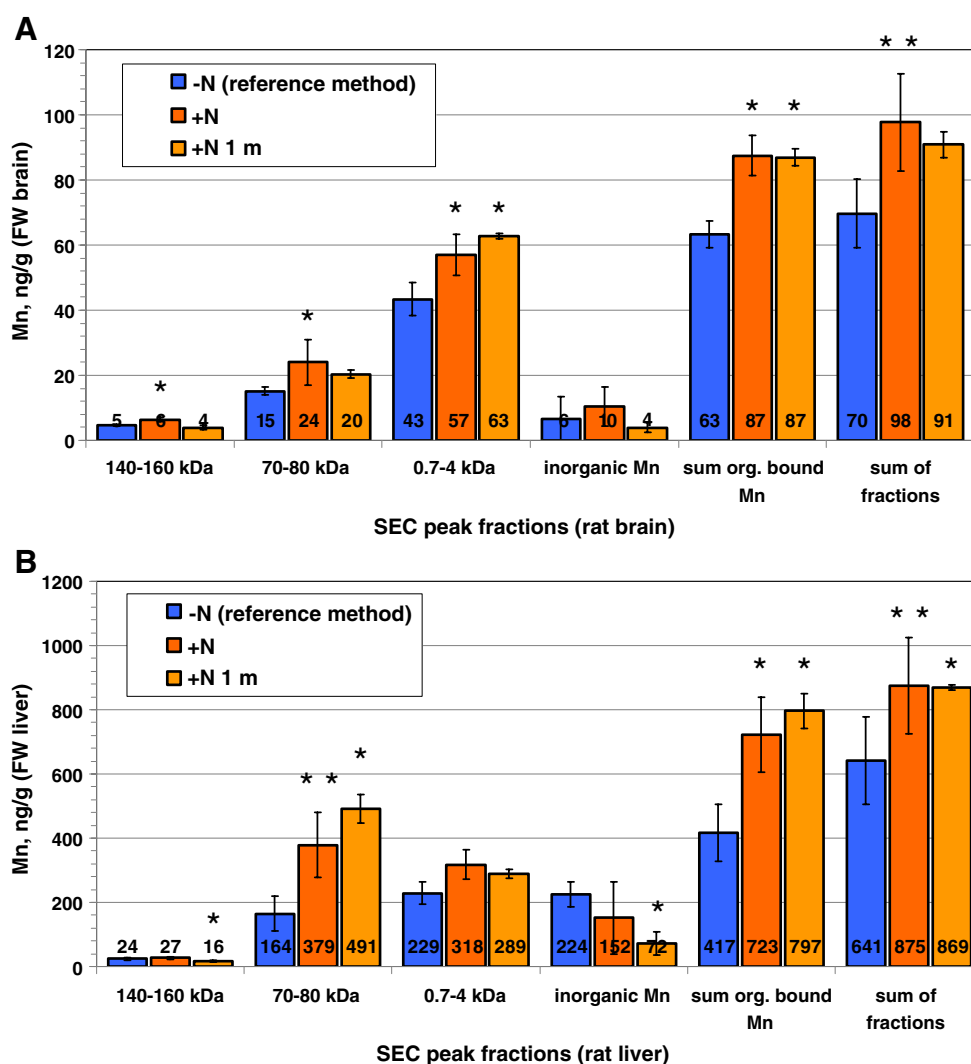
Figure 2 shows the Mn species pattern of brain and liver tissue extracts. For some fractions, relatively high SD was observed. This was explained by the fact that the low physiological Mn concentration in tissues resulted in ultra

Table 1 Mn and Fe concentration in extracts, pellets, and tissues of rat brain and liver measured by ICP-AES after pressure digestion

	“–N” (reference method)		+N		+N 1m	
	Mean	SD	Mean	SD	Mean	SD
Brain						
Manganese						
Extract (%)	17.4	0.8	26.6***	1.5	25.0*	2.5
Pellet (%)	82.8	3.7	79.8	1.3	71.8	0.0
Extract+pellet (%)	100.2	3.9	106.3	0.7	96.8	2.5
Total conc. (%)	100.0	4.4	100.0	0.9	100.0	1.3
Total conc. (µg/g FW)	0.400	0.018	0.368	0.003	0.365	0.005
Iron						
Extract (%)	40.2	2.3	44.6	0.6	42.3	0.4
Pellet (%)	50.7	1.5	56.1	0.5	52.8	0.1
Extract+pellet (%)	90.8	2.8	100.7	0.2	95.2	0.6
Total conc. (%)	100.0	3.7	100.0	2.6	100.0	4.6
Total conc. (µg/g FW)	19.6	0.7	17.3	0.4	18.2	0.8
Liver						
Manganese						
Extract (%)	27.7	2.6	43.8*	0.3	43.8**	2.7
Pellet (%)	78.7	2.1	58.3**	5.2	59.0*	2.1
Extract+pellet (%)	106.4	3.9	102.1	5.0	102.8	0.6
Total conc. (%)	100.0	0.2	100.0	1.1	100.0	2.8
Total conc. (µg/g FW)	2.314	0.004	1.996	0.023	1.984	0.055
Iron						
Extract (%)	63.9	1.6	72.8*	1.3	74.8*	1.4
Pellet (%)	34.3	1.8	24.9*	3.7	28.9	1.0
Extract+pellet (%)	98.2	3.3	97.7	3.6	103.7	0.4
Total conc. (%)	100.0	1.4	100.0	1.7	100.0	2.8
Total conc. (µg/g FW)	104.9	1.5	132.7	2.2	130.4	3.6

The rows “extract+pellet” show the summed percentage of both fractions related to the total element concentration (set 100%) by ICP-AES. Data are shown as percentage of total concentration (conc.) of the tissue and total concentration as µg/g FW. One-sample T^2 test related to “–N (reference method)”
 * $p \leq 0.05$; ** $p \leq 0.01$;
 *** $p \leq 0.005$

Fig. 2 Size characterized Mn species in rat brain (a) and liver (b) tissue extracts. Extracts were prepared either within 2 h after organ dissection without pulverization in N_{2liq} ($-N$ =reference method, $n=3$), after pulverization of deep-frozen tissue in N_{2liq} ($+N$, $n=3$), or again after pulverization of deep-frozen tissue in N_{2liq} but after 1 month storage in N_{2liq} ($+N$ 1 m, $n=2$). Mn concentrations of the 140–160-kDa fraction showed values near LOQ (<10 pg Mn) or partially ($+N$ 1 m) near LOD (<1 pg Mn). Data are shown as ng Mn/g FW. One-sample T^2 test related to $-N$ ($*p\leq 0.05$, $**p\leq 0.01$)



low Mn species concentrations after SEC separation, being partly close to LOD and thus contributing to the elevated SD of respective fractions. Statistical evaluation however showed differences to be significant (cf. respectively, p values) even when SD was high.

Brain This increased extraction efficiency was seen also in the summed concentrations of SEC fractions, which were considerably higher compared to the summed fractions of the $-N$ method (c.f. Fig. 2a). Apparently, this increase is caused by higher extracted amounts of organic Mn species, predominantly associated to the 0.7–4-kDa fraction, where also Mn–citrate complexes are eluting. The 70–80-kDa fraction (e.g., Mn–transferrin) contributed to the concentration increase of organic Mn species, too, but to a far lesser extent. Inorganic Mn was detected in very low amounts and was found in the same range as with the “ $-N$ ” method. This is important as an increased inorganic Mn concentration could indicate degradation of organic Mn species into Mn-free ligands and inorganic Mn. However, no decrease in

concentration for organic Mn species and vice versa nor increase of inorganic Mn was found. Mn species remained stable during “ $+N$ ” extraction.

Fe concentration in brain extracts was nearly identical for all extraction approaches with 7.9 ($-N$) or 7.7 $\mu\text{g/g}$ ($+N$, $+N$ 1 m). The Fe species pattern showed a slight shift of 0.4 $\mu\text{g/g}$ from the 70–80-kDa fraction to the 140–160-kDa fraction between $+N$ approaches and $-N$. This is not explained by degradation, as the additionally found compounds have even higher masses (140–160 kDa) compared to the compounds from the reference method (70–80 kDa), and no increase in inorganic Fe was seen. In turn, the $+N$ approach probably prevented a degradation of 140–160 kDa compounds to smaller ones.

No significant changes were found for Mn and Fe species pattern in brain extracts after 1 month storage in N_{2liq} .

Liver In liver extracts, some differences in Mn speciation compared to brain were seen. The increased extraction

efficiency of $+N$ and $+N$ 1 m is reflected in the higher Mn amount of summed SEC fractions and an increased Mn concentration associated to the 70–80-kDa fraction in particular, where Mn–transferrin elutes and, to a small extent, in the 0.7–4-kDa fraction (c.f. Fig. 2b). This improved Mn–transferrin extraction underlines the enhanced extraction potential of our new “ $+N$ ” method from Mn-rich liver tissue because liver is known for the highest physiological Mn tissue concentration, and Mn–transferrin is the main native Mn carrier, which is present only in minute amounts in brain due to the action of BBB [3]. No change in speciation from organic bound Mn to inorganic Mn could be observed after 1 month in N_{2liq} .

Fe species extraction efficiency was improved from 64% ($-N$) to 73% ($+N$), which is attributed to an increase in the 140–160-kDa fraction. No significant differences were seen in other size fractions compared to the $-N$ extract. Storage of 1 month in N_{2liq} had negligible influences on Fe species pattern of liver extracts resulting in a slight shift of ≤ 7 μg Fe/g (5% of total Fe concentration) from the 140–160 to the 70–80-kDa fraction.

Although, the degradation of labile Mn protein species to Mn-free-protein and inorganic Mn is well known and was specifically observed previously for Mn–transferrin [5], no changes of the Mn species pattern could be assumed for brain and liver extracts from $+N$ method during 1 month storage in N_{2liq} .

Conclusion

An enhanced extraction method for labile species was developed for simple parallel handling of a high number of samples. It is based on thorough grinding and pulverization of deep-frozen tissue and subsequent homogenization and extraction. All steps were performed at low temperatures (-196 °C or 0 °C) and entirely under inert gas atmosphere. Extraction efficiency and Mn or Fe species pattern were compared to results from a reference method [10].

Extraction efficiency could be increased significantly for Mn in brain and Mn or Fe in liver, but only little for Fe in brain. Notably, in brain, the 0.7–4-kDa fraction (containing Mn–citrate), but in liver, the 70–80-kDa fraction (containing Mn–transferrin), contributed to the increased Mn concentration of extracted organic Mn species. This finding supports the conclusion that major Mn carriers in brain and liver tissue are different. Quality control-motivated species characterization of all extracts with SEC-ICP-MS showed that the new $+N$ approach did not impair species stability. Fe species showed only negligible degradation ($\leq 5\%$) of HMM Fe species in liver, but no increase of inorganic Fe was observed after 1 month storage in N_{2liq} .

In summary, this new $+N$ extraction method supports a quality controlled standard extraction protocol for Mn and Fe species. It showed higher extraction efficiency, and species stability was improved even after storage for 1 month. A comfortable preparation of numerous low weight samples in parallel was now possible.

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