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A novel calibration strategy for the quantitative imaging of iron in biological tissues by LA-ICP-MS using matrix-matched standards and internal standardisation

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CONTENTS ENTRY

This paper describes a novel and straightforward procedure for the preparation of matrix-matched calibration standards for the quantitative imaging of iron (Fe) in biological tissues by laser ablation (LA)-ICP-MS with on-tissue internal standard addition. It enabled on-tissue addition of Rh as internal standard to both calibrants and samples without altering the original Fe distribution of the sample. Assessment of the accuracy of the method for the quantitative imaging of Fe in brain tissue sections was undertaken by comparison of the LA-ICP-MS data with that obtained by micro-XRF; the average Fe concentrations in selected tissue regions obtained by using XRF fell within the window defined by the LA-ICP-MS values and their associated standard deviations.

ABSTRACT

The development of a novel and straightforward procedure for the preparation of matrix-matched calibration standards for the quantitative imaging of iron (Fe) in biological tissues by laser ablation (LA)-ICP-MS with on-tissue internal standard addition is described. This simple approach enabled on-tissue addition of Rh as internal standard to samples (with heterogeneous Fe distribution) and calibrants (with homogeneous Fe distribution). This is achieved without altering the original Fe distribution of the sample. Calibration standards were prepared by full horizontal immersion of slides with mounted homogenised sheep brain tissue section into the corresponding solution containing 0.5, 0.75, 1, 5, 10 and 20 mg/kg Fe (each also containing 250 µg/kg Rh as IS) in pure methanol for 30 minutes (6 immersions, each for 5 minutes). Subsequent air-drying (bench drying at room temperature) for approximately 5 minutes was undertaken in between consecutive immersions, to prevent long-term exposure of the tissue to lipid degradation. Tissue-matched standards were characterised *in-house* for Fe composition, homogeneity and stability (at storage temperatures of -80^{o}C, -20^{o}C, 4^{o}C and 25^{o}C for up to 2 months) in order to investigate their suitability as calibrants for quantitative LA-ICP-MS. The homogeneity data suggested that the materials are homogeneous in terms of Fe and Rh distribution with RSDs (n = 30) of 8.3% and 4.7%, respectively. The Fe measurement precision was improved by approximately a factor of 2 when normalising ^{56}Fe intensities to ^{103}Rh intensities; the RSD (n = 30) for ^{56}Fe/^{103}Rh was 3.6%. The produced calibration standards were found to be stable when stored at room temperature for approximately 50 days, suggesting that they can be reused for multiple batches. Using LA coupled to double-focusing sector field ICP-MS in medium resolution mode (m/Δm =

4000), linear calibration over a range of 107 to 1519 mg/kg Fe (R^2 = 0.99) was achieved with a limit of detection of 1.84 mg/kg Fe. Assessment of the accuracy of the method for the quantitative imaging of Fe in tissues was undertaken by comparison of the LA-ICP-MS data with that obtained by micro-XRF; the average Fe concentrations in selected tissue regions obtained by using XRF fell within the window defined by the LA-ICP-MS values and their associated standard deviations.

Keywords

Alzheimer's disease (AD); beta (β)-amyloid; quantitative imaging of iron, laser ablation ICP-MS; MRI, matrix-matched calibration standards, internal standardisation.

INTRODUCTION

The impact of neurodegenerative disorders, and predominantly Alzheimer's disease (AD), is escalating rapidly within the changing demographics of our society.[1] Accurate quantification, rather than qualitative assessment of the metal distribution in diseased brain tissues, compared to healthy brain, is required to validate iron-sensitive Magnetic Resonance Imaging (MRI) acquisitions,[2] needed for predicting clinical outcome, and for potential development of therapies.

Amongst the numerous imaging techniques that have been established for elemental mapping of biological tissues, laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) [3-7] and secondary ionisation mass spectrometry (SIMS) [8,9] are sensitive imaging analytical techniques that possess the capability to determine metals and metalloids distribution in biological tissues in a selected area of interest or in a complete thin tissue section. In comparison with SIMS, significantly lower matrix effects were observed in LA-ICP-MS.[10] Moreover, the advantages of LA-ICP-MS in terms of relatively high sample throughput and limits of detection at the sub mg/kg level have helped to make it increasingly popular for biological tissue imaging.[10]

Various calibration strategies have been reported for quantitative elemental imaging of biological tissues by LA-ICP-MS.[3,11-14] On-line solution based calibration [11,12] has been shown to produce 'fit for purpose' quantitative data in the absence of solid calibration standards or reference materials. However, with this approach the use of internal standardisation is mandatory to compensate for the different mass transport rates of the solid sample (introduced by LA) and aqueous standard (introduced post LA). This

requires having knowledge of the exact concentration of the element, to be used as an internal standard, which should be homogeneously distributed in the solid tissue. Although carbon has frequently been used as an internal standard due to its availability and apparent homogeneity in biological tissues, its feasibility for quantitative LA-ICP-MS tissue imaging has been argued by some groups [15]. More recently, data have been reported suggesting its lack of suitability for LA-ICP-MS internal standardisation.[15]

Efforts have been made to produce solid standards prepared *in-house*. These include pressed pellets of biological certified reference materials [12], metal spiked polymer films [13], sol-gel tissue pellets [14] and metal spiked tissue homogenates (matrix-matched standards) [7,12,16], the latter (true matrix matching of standards and samples) being the most accurate approach.[3] Although there has been some success in the preparation and characterisation of true matrix-matched standards, this has been found to be a challenging task. The biggest difficulties are to ensure standard homogeneity, ease-of-use and sufficient stability for routine clinical applications and the possibility to correct for matrix induced interferences by having a suitable internal standard, which is homogeneously distributed and present at the same concentration in the matrix-matched calibrants and samples. To the authors' knowledge, no work describing an easy-to-follow protocol for the preparation and systematic characterisation of matrix-matched standards for accurate quantitative LA-ICP-MS tissue imaging has been reported so far.

This work describes the development of a calibration strategy for quantitative imaging of Fe in biological tissues using laser ablation (LA) coupled to ICP-MS with internal standardisation. It involves the use of a simple and straightforward approach for preparation of matrix-matched calibration standards, enabling on-tissue addition of an internal standard (Rh) to samples and calibration standards without altering the original Fe heterogeneous distribution of the sample. This involves the immersion of mounted sheep brain tissue sections in methanol matrix solutions containing increasing concentrations of Fe, along with a known amount of the internal standard. A range of elements (Sc, Ga, Ge, Y, Rh) were investigated as possible internal standards on the basis of their low natural abundance in the brain (low background), similar behaviour to Fe in the ICP and absence of potential interferences. The tissue-matched Fe standards were characterised *in-house* for Fe composition, homogeneity and stability (at storage temperatures of -80^{o}C, -20^{o}C, 4^{o}C and 25^{o}C for up to 2 months) in order to investigate their suitability as calibrants to be re-used for multiple batch LA-ICP-MS analyses. The methodology developed was applied to quantitative Fe imaging of thin slices of mouse

brain tissue. Efforts were made to validate this calibration approach, intended for use in routine elemental imaging analysis, by micro-XRF analysis of the same sample.

EXPERIMENTAL

Materials and reagents

Methanol (Promochem, LGC Standards, Middlesex, UK) was used to prepare the matrix-matched calibration standards. Methanolic solutions of Fe and Rh were prepared by dilution of elemental stock solutions (Ultra Scientific, LGC Standards, Middlesex, UK). Deionised water (18.2 MΩ cm) from an ELGA purelab flex system (ELGA, Veolia Water, Marlow, UK) was used.

Instrumentation

A double-focusing sector field ICP-MS (Element2, Thermo Fisher Scientific, Bremen, Germany) operated in time resolved analysis (TRA) mode was used throughout. All measurements were performed in medium mass resolution ($m/\Delta m = 4000$), to eliminate polyatomic ion interferences at *m/z* 56 and 57. A dual introduction system using wet and dry aerosol was utilized for this work. Wet plasma conditions were achieved [11] by nebulisation of a 1% (v/v) HNO_3 solution *via* a MicroMist™ concentric nebuliser. The optimal ICP-MS operating conditions are listed in Table 1.

For laser ablation analysis, a commercially available UP-213 Nd:YAG laser ablation unit (New Wave Research Inc., Huntingdon, Cambridgeshire, UK) operating in the deep UV (λ = 213 nm) was configured to perform multiple line scanning for 2D profiling of tissue sections (Table 1). Operating conditions for laser ablation ensured efficient removal of standard/sample (i.e. total consumption of thin section incident to the laser) irrespective of section thickness. Coupling of the LA and ICP-MS instruments was achieved using Tygon® tubing (1 m x 4 mm i.d.) between the ablation cell and the ICP-MS spray chamber. The tissue slides (calibrants and samples) were mounted on an *xyz* translation stage, which was controlled using a computer and monitored by a charge-coupled device (CCD) camera. Optimisation of the LA-ICP-MS was carried out daily using single shot ablation of a prepared pressed pellet of NCS ZC81001 pork muscle (LGC Standards, Middlesex, UK) with a certified value of 43.6 mg/kg for Fe. Multiple parallel line scans were performed to generate 2D distribution maps. Scanned lines were spaced at 100 μm intervals (*y*-direction) to prevent contamination of adjacent tissue with ablation debris from previous runs. A typical sample area of 12 x 7 mm equated to a run

time of approximately 5 hrs. For imaging analysis of thin sections of mouse brain, standards were placed alongside the samples in the ablation chamber. Instrumental drift checking was carried out at regular intervals throughout the run by repeat analysis of calibration standards.

In order to confirm the LA-ICP-MS data, an EDAX Eagle III Micro X-Ray Fluorescence Spectrometer (EDAX Inc., New Jersey, USA) equipped with a rhodium X-ray tube was utilised for this aspect of the study. The instrument was operated with a 40kV/500μA excitation potential/current. Optimal instrumental parameters can be seen in Table 2. XRF measurements were performed on six specific selected areas of a 50 μm thick cryo-sliced mouse brain section, mounted onto 3 μm-thick Mylar film. This XRF working method was verified by further mapping two of the selected tissue areas since it could be anticipated that the sample heterogeneity might have some influence on the XRF results in the case that point measurements are performed. The same tissue section was then mapped by LA-ICP-MS by using tissue sample features for which their location coordinates acted as reference points between the two techniques. Quantification by XRF was performed on the basis of measurements of a known amount of the reference material SRM 1577c bovine liver (NIST, Gaithersburg, USA) with a certified Fe value of 197.94 ± 0.65 mg/kg. No iron was found to be present in the Mylar film following background analysis.

Procedures

Preparation of matrix-matched calibration standards

Whole sheep brains were obtained from a local halal butcher and stored at -80^{o}C until tissue homogenisation was carried out. Prior to homogenisation, sheep brain samples were allowed to defrost at room temperature, before being rinsed with de-ionised water and transferred to a pestle and mortar. Brain tissue was thoroughly blended in the mortar until a smooth consistency was achieved. The homogenised material was split into agar plates, capped and stored at -80^{o}C. Portions of the frozen homogenised sheep brain were taken from the plate when required (e.g. for cryo-slicing) using a ceramic knife.

Homogenised sheep brain (HSB) samples were prepared, frozen and cryo-sectioned at -20^{o}C (Leica Cryostat CM1850, Milton Keynes, UK) into 50 μm sections for all method development work. CD1 mouse brain model samples fixed in 4% paraformaldehyde solution (supplied courtesy of King's College London, UK) were cryo-sectioned sagitally (anterior to posterior of the brain) at a thickness of 50 μm. All

sections were mounted onto 1 mm thick glass microscope slides (Thermo Fisher Scientific, Loughborough, UK) prior to LA-ICP-MS analysis. Glass slides (55 x 26 mm) were washed successively in acetone, 1% (v/v) nitric acid (Romil, UpA Grade, Cambridge, UK) and ultrapure water (18.2 MΩ cm; Elga PURELAB flex, Marlow, UK) prior to mounting of the tissue sections.

Calibration standards were prepared by full horizontal immersion of the mounted HSB section slides into the corresponding solution containing 0.5, 0.75, 1, 5, 10 and 20 mg/kg Fe (each also containing 250 µg/kg Rh as IS) in pure methanol for 30 minutes (6 immersions, each for 5 minutes). Subsequent air-drying (bench drying at room temperature) for approximately 5 minutes was undertaken in between consecutive immersions, to prevent long-term exposure of the tissue to lipid degradation. This helped to maintain the structural integrity of the tissue. The newly produced matrix-matched calibrants were stored in a box at room temperature until analysis for homogeneity and stability was carried out using LA-ICP-MS.

Total Fe determination of matrix-matched calibration standards

Calibration standards were characterised *in-house* for total elemental concentration by ICP-MS analysis of digested tissue. To achieve this, tissue sections were carefully removed from the microscopic slide, accurately weighed and subjected to microwave digestion with HNO_3 using a Multiwave 2000 system (Anton Paar, Hertfordshire, UK). The obtained Fe concentrations (expressed on a dry weight basis) plotted against the LA-ICP-MS Fe/Rh signals were used for the determination of the Fe distribution in the mouse brain section by LA-ICP-MS. The reference material SRM 1577b bovine liver (NIST, Gaithersburg, USA) with a certified Fe value 184 ± 15 mg/kg was used for quality control of the total Fe measurements.

Data processing

Individual text output files generated by LA-ICP-MS analysis were exported/collated in Microsoft® Excel using a customised macro. The final compiled dataset was copied into IgorPro (version 6.3.4.1) for processing and modelling of 2D colour contour plots.

For each line scan, the relative standard deviation of the data (RSD, %) was calculated as the ratio of the standard deviation (SD) of raw counts to the mean of raw counts of the line scan data. Average signal intensities as well as variations across individual line rasters were assessed. Average ion-responses of individual rasters were

plotted against calibration concentrations to yield a linear calibration graph, enabling distribution maps to be displayed in concentration units (mg/kg).

RESULTS AND DISCUSSION

Characterisation of matrix-matched standards prepared in-house

For preparation of matrix-matched Fe standards, different factors including the concentration of methanol, the immersion time, the drying time, the number of immersion steps and the choice of internal standard were investigated. The matrix-matched standards, prepared using the optimal conditions described in the procedures above, were analysed for their content of Fe using ICP-MS after microwave acid digestion of removed tissue sections. They were found to contain Fe in the range of 107 to 1519 mg/kg. A recovery of 100.2 ± 4.9% (n = 3) was obtained for the NIST 1577b bovine liver reference material analysed using the same procedure.

The use of Ga, Ge, Rh, Sc as internal standards was investigated by determining the RSDs (%) of Fe-to-internal standard ratio to rank the relative quality of each Fe-internal standard pair and their response to changing conditions. The best measurement precision on the tissue-matched calibration standards was obtained when using Rh as IS (3.6%) compared to the other investigated elements, for which RSDs ranged between 7.9 and 12.1%. Also, for total Fe measurements in the reference material SRM 1577b bovine liver tissue, most accurate data was obtained when using Rh in comparison with other candidate internal standards. Therefore, Rh was selected as IS for all further work.

Figure 1 shows the calibration graph obtained by plotting the measured total Fe concentrations against the Fe/Rh signals obtained by LA-ICP-MS after ablating each standard five times along a path length of approximately 5 mm. As shown, a linear correlation ($R^2 > 0.99$) was achieved. The instrumental limit of detection (LOD) for Fe using the proposed LA-ICP-MS method was 1.84 mg/kg (3σ criterion, using the glass slide mounted brain tissue with no Fe spike as the 'blank' standard). Typical relative standard deviations of the normalised raw intensity across line scans for the matrix-matched standards were lower than 20%.

Homogeneity of matrix-matched calibrants

The homogeneity of the analyte distribution (^{56}Fe, ^{57}Fe and ^{103}Rh) in the HSB tissue standard containing 0.75 mg/kg Fe was investigated. Laser ablation conditions were optimised to achieve maximum depth profiling of the tissue whilst minimising penetration of the glass slide. A total of 30 line scan analyses were performed.

Multiple line scans were carried out over a tissue area of approximately 5 mm^2, which gave a good representation of the entire standard section. Each individual line scan was performed over a two minute run (incorporating a 20 second laser warm-up) at a scan rate of 40 µm/sec. Figure 2 shows typical ^{56}Fe and ^{103}Rh signal intensities obtained for the ablated calibrant section by LA-ICP-MS. The isotope signal intensities were found to be significantly higher than those of the background signal (baseline contribution ranged from 3.3 – 7.9% of total signal output for all analytes monitored). Relative standard deviations of 8.3% and 4.7% were obtained for ^{56}Fe and ^{103}Rh, respectively. For ^{56}Fe/^{103}Rh, an RSD of 3.6% (n = 30) was obtained. Data were collated from multiple line scans to create elemental distribution plots of the sample surface, which are shown in Figure 3.

The results shown in Figures 2 and 3 suggest that both Fe and the internal standard Rh are distributed homogeneously across the matrix-matched calibrant section investigated by LA-ICP-MS. This was achieved with the simple and straightforward approach developed in this work for the preparation of matrix-matched Fe calibrants.

Stability of matrix-matched calibrants

Slide-mounted HSB sections immersed in methanolic solutions containing 5 mg/kg and 20 mg/kg Fe were stored at temperatures of -80^{o}C, -20^{o}C, 4^{o}C and 25^{o}C for up to 58 days to investigate their stability in terms of Fe content and distribution. Samples were routinely analysed by LA-ICP-MS between storage intervals and the ^{56}Fe data was normalised to ^{103}Rh. Figure 4 shows the variation of signal intensity with the storage temperature over time for the investigated Fe calibrants.

Calibrants stored at -80^{o}C displayed deteriorated physical integrity due to constant freezing-thawing and, therefore, these storage conditions were ruled out. Calibrants stored at -20^{o}C displayed the largest signal variability (RSD of 21% for 5 mg/kg Fe and 13% for 20 mg/kg Fe).

The lowest RSDs (10% for 5 mg/kg Fe and 3% for 20 mg/kg Fe) were obtained by LA-ICP-MS for calibrants stored at room temperature. The results in Figure 4 suggest

that the Fe content does not vary significantly between standards stored at -20°C, 4°C and room temperature during the same time period. Therefore, storage at room temperature, for which the standards were found stable up to 50 days, was selected as optimal. This has the major advantage that the standards can be re-used in multiple analyses.

Use of matrix-matched Fe calibrants for quantitative tissue imaging by LA-ICP-MS and internal standardisation

Effect of addition of an internal standard

The effect of addition of Rh (as internal standard) on the original Fe distribution of sample was investigated by LA-ICP-MS with a 50 μm thick mounted mouse brain section, selected as the model sample. To do this, the Fe distribution of the sample (without Rh) was compared with that after addition of Rh, using the same immersion procedure as for preparation of matrix-matched Fe calibrants. The same tissue area was measured by LA-ICP-MS for the sample before and after Rh addition using a different line offset in the y-axis. This was achieved by multiple line scanning at 200 μm spacing using the operating conditions summarised in Table 1. Figure 5 shows comparative images obtained for Fe in the mouse brain section by using LA-ICP-MS before and after the addition of Rh. As can be seen in this figure, the addition of Rh did not significantly alter the original iron distribution of the tissue section; only approximately 5% difference in the ^{56}Fe signal intensity was observed between the untreated and treated (addition of IS) mouse brain section. Moreover, no Fe was detected in the immersion methanolic solution after the addition of Rh. Therefore, the developed method provides an easy and straightforward approach to the addition of a selected IS to calibration standards and samples without altering the sample Fe distribution, which is invaluable for the correction of matrix induced and transportation effects in quantitative elemental tissue imaging by LA-ICP-MS. This, combined with the simplicity of matrix-matched Fe spiked standard preparation, makes this approach advantageous over methods previously reported, which involve steps such as tissue spiking, re-homogenisation and re-freezing of the spiked tissue, thus making calibrant preparation time consuming and more prone to contamination. The use of internal standardisation using these previous methods has also represented a very difficult task.

Quantitative metal mapping of mouse brain tissue

The Fe distribution of a sagital section of mouse brain was determined by LA-ICP-MS using the newly developed quantitative method. ^{56}Fe intensities (normalised to ^{103}Rh intensities) were converted into concentration values (mg/kg Fe dry weight) as described above to produce a quantitative surface map. Quantitative Fe data obtained by using normalised ^{56}Fe intensities were compared with those obtained using non-normalised ^{56}Fe data and the corresponding quantitative surface maps are compared in Figure 6. A clear disagreement was observed between data generated by using normalised and non-normalised ^{56}Fe intensities with concentration ranges of 191 – 328 mg/kg Fe (for normalised ^{56}Fe data) and 40 – 182 mg/kg Fe (for non-normalised ^{56}Fe data). This could be explained by differences in ablation and/or transportation efficiency between calibrants and sample and/or matrix-induced effects.[17] These may affect the accuracy of the quantitative Fe data in the absence of internal standardisation. This possible explanation is also supported by the fact that the precision (as RSD) of the Fe imaging data (0.1 – 13%) obtained with normalisation to ^{103}Rh on the matrix-matched standard sections is much better than that obtained without internal standard correction (1 – 49%). It is important to note that relative ^{103}Rh signal intensities of sample sections agreed with those of matrix-matched standards within approximately 10%, showing average values (n ~ 35) of 28380 cps and 31475 cps, respectively.

Confirmation of LA-ICP-MS measurements using μ-XRF

In order to obtain confirmatory data by using an independent technique, a mouse brain section (50 μm thick), was mounted onto Mylar film before being stretched across the opening of a "Superfrost" glass slide and treated with the internal standard (Rh). The sample underwent μ-XRF analysis at Ghent University prior to analysis by LA-ICP-MS.

Semi-quantitative analysis was carried out by μ-XRF using a pressed pellet of NIST SRM 1577c bovine liver (approximate diameter 13mm; 20.9 mg; 100 μm thickness). The standard operating conditions can be found in Table 2. A series of six point measurements (e11_1 to e16_1) were taken on specific selected areas across the entire brain section to determine areas of potential interest (i.e. high Fe content). This working method was verified by carrying out a more extensive aerial mapping (during approximately 24 h) of two of the selected areas (measurement points e11_1 and e12_1)

for spatial Fe determination. Reported Fe concentrations (see Table 3) were based on average measurements over a clustering of pixels, identifying "hot spot" Fe areas.

Due to the lack of structural integrity of the sample on the Mylar film, laser ablation analysis was only performed on half of the brain section since an increasing amount of sample debris was produced. Laser ablation analysis was carried out in order to match the μ-XRF resolution settings as closely as possible. Correlation of the two techniques allowed for a matching of two of the measured areas (point measurement e16_1 and aerial map e12_1). The comparative results obtained for the same sample by μ-XRF and LA-ICP-MS are summarised in Table 3. It is important to notice that the point measurements generated by μ-XRF are single data points. Therefore, in order to produce a suitable match from LA, the immediate area under investigation was identified and a series of 50 μm LA data points were averaged in order to obtain data which is representative of the spatial region corresponding with the 100 μm XRF beam spot size.

Table 3 shows that the average Fe concentrations in selected tissue regions obtained by XRF fell within the window defined by the LA-ICP-MS values and their associated standard deviations.

CONCLUSIONS

A novel calibration approach that involves a simple, robust and straightforward method for the preparation of matrix-matched biological tissue standards (as calibrants) has been developed for quantitative elemental imaging in biological tissues using LA-ICP-MS. Application of the proposed method to Fe in brain tissue sections provided quantitative data on the spatial distribution of this element with a limit of detection for Fe at the μg/kg level. The approach used for calibrant preparation enabled addition of a suitable internal standard (Rh) to both calibrants and samples without altering their original Fe distribution. The prepared calibration standards have been shown to be stable for approximately 50 days when stored at room temperature, enabling long-term use over multiple batch analyses. The distribution of Fe within the calibrants showed good homogeneity with typical RSDs of < 10% for ^{56}Fe (n = 30), and 5 – 10% for ^{103}Rh (n = 30). The use of μ-XRF as a confirmatory technique provided imaging data, which is in good agreement with the LA-ICP-MS data.

Future studies will be pursued to investigate the feasibility of the developed method for multi-element quantitative analysis of clinical tissues relevant to health/disease. Efforts will also be put into the development of primary methodology

based on isotope dilution mass spectrometry calibration. Such analytical developments will be invaluable for further validation of the high throughput calibration method described in this work.

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Legends of Figures

Figure 1: Correlation between normalised ^{56}Fe intensities measured by LA-ICP-MS and Fe concentrations of HSB matrix-matched calibration standards determined by ICP-MS after microwave acid digestion

Figure 2: LA-ICP-MS ^{56}Fe, ^{57}Fe and ^{103}Rh signal intensities obtained for a matrix-matched Fe calibrant (0.75 mg/kg Fe) section

Figure 3: LA-ICP-MS spatial distribution plots for (a) ^{56}Fe and (b) ^{103}Rh (internal standard) obtained for a 0.75 mg/kg Fe HSB calibration standard. Over 30 lines scan was performed.

Figure 4: Normalised ^{56}Fe signal intensity plots obtained by LA-ICP-MS for 5 mg/kg Fe (A) and 20 mg/kg Fe (B) HSB calibration standards stored at different temperatures for over a 2 month period. Error bars are standard deviations (n = 5). RT means room temperature.

Figure 5: Comparative images obtained for ^{56}Fe intensities in the mouse brain section by using LA-ICP-MS before (A) and after (B) the addition of Rh.

Figure 6: Comparison of quantitative LA-ICP-MS Fe data obtained by using normalised ^{56}Fe intensities (A) with that obtained using non-normalised ^{56}Fe data (B) for a sagitally sectioned mouse brain.

Table 1 Operating conditions for LA-ICP-MS, using a dual introduction of wet and dry plasma

Laser ablation system (New Wave UP-213, solid state Nd:YAG)	
Fluence	4 mJ/cm^2
Repetition rate	20 Hz
Laser energy	50%
Spot size / sampling mode	50 µm line raster
Pulse energy	0.2 mJ at surface
Sample translation rate	40 µm/s
Carrier gas flow	0.45 L/min Ar
*No make up gas was used through ablation cell	
ICP-MS (Thermo Fisher Scientific Element2)	
Plasma RF power	1250 W
Nebuliser	MicroMist™
Spray Chamber	Quartz Peltier
Torch position (mm)	x: 3.0; y: 1.6; z: -3.9
ICP cones	Ni
Cooling gas flow	15.5 L/min
Auxiliary gas flow	0.94 L/min
Nebuliser gas flow	0.85 L/min
Isotopes monitored	^{56}Fe, ^{57}Fe,^{103}Rh
Detector mode	Dual range
Dwell time	150 ms

Table 2 Operating conditions for µ-XRF

X-Ray Fluorescence (Eagle III µ-XRF)		
X-ray tube	Target material: Rh	
HV generator	Max. voltage: 50 kV; max. power: 50 W	
X-ray optic	XOS polycapillary	
	Incident angle: 60^o	
	Spot size: Varispot 300 µm, 100 µm, 25 µm	
Shutter	Closed (Pb), open, filters (thin Al, Ti, Ni, Nb, thick Al, Rh)	
Detector	Si(Li) 80mm^2; collimated; LN2-cooled	
Vacuum pump		
Optical system	High mag. (x100 = 1.6 x 1.2 mm)	
	Low mag. (x10 = 15 x 11 mm)	
Sample chamber	Axis:	X, Y, Z
	Step size:	X: 5 µm; Y: 5 µm; Z: 5 µm
	Max. sample size:	250 x 200 x 120 mm^3
	Flat samples:	250 x 200 x 10 mm^3
	Max. load:	5 kg
Aperture	50 µm x 50 µm	
Working conditions	20 – 25^{o}C	

Table 3 Comparative Fe concentrations (in mg/kg) obtained by LA-ICP-MS and μ-XRF for mouse brain tissue

Measurement	**μ-XRF**	**LA (± Std Dev.)**
Point measurement: e16_1 (n=1)	1438	1647
Map f: e12_1 hot region (6 pixels) (n=6)	481	528 ± 56
Map f: e12_1 surrounding (25 pixels) (n=25)	349	400 ± 135

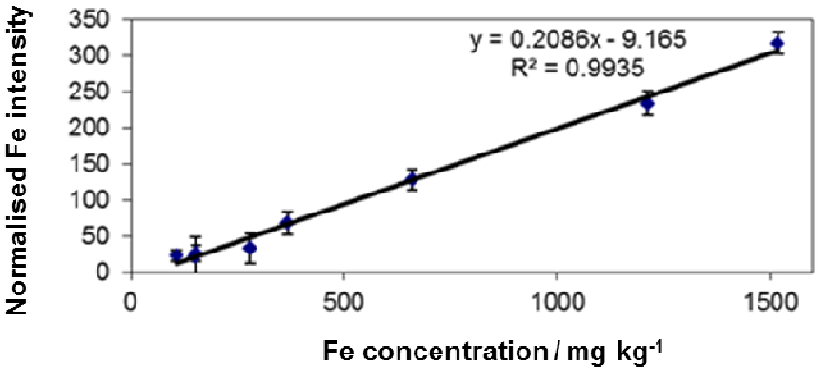


Figure 1

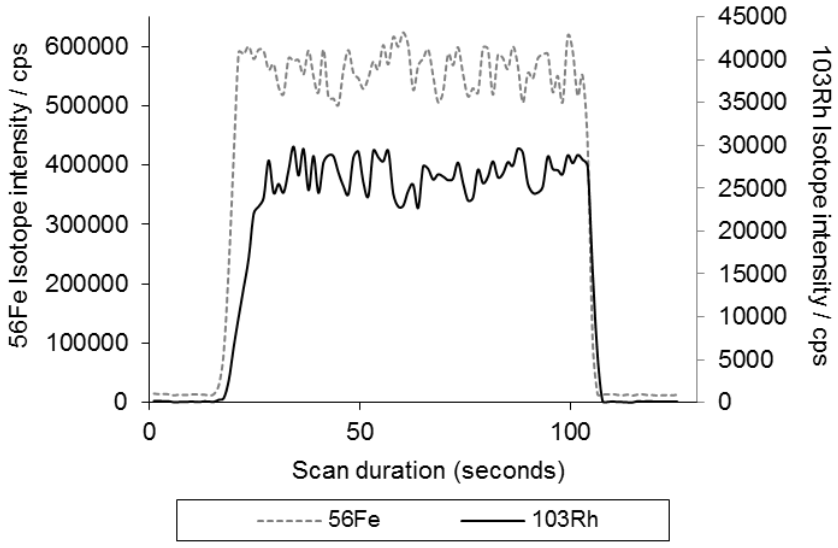


Figure 2

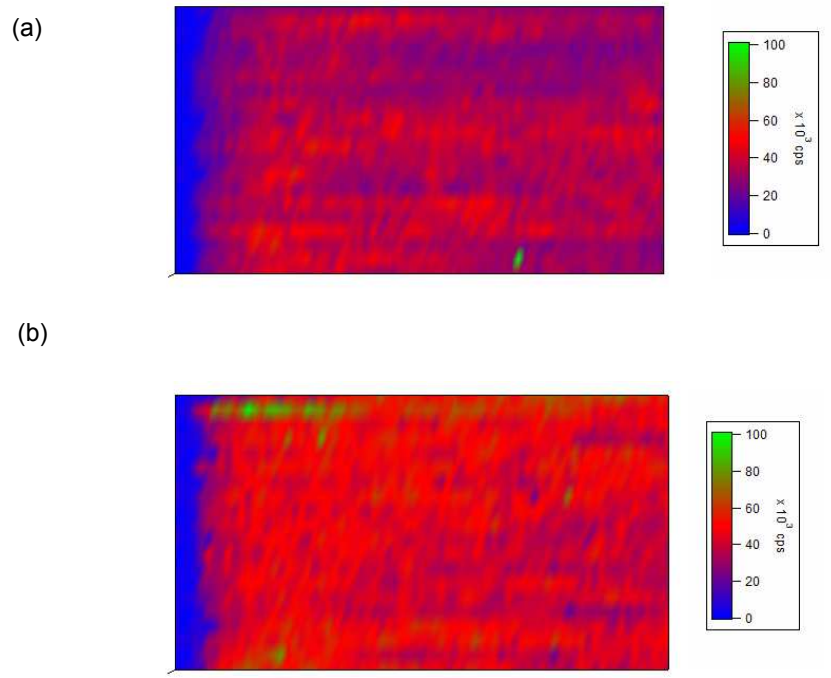


Figure 3

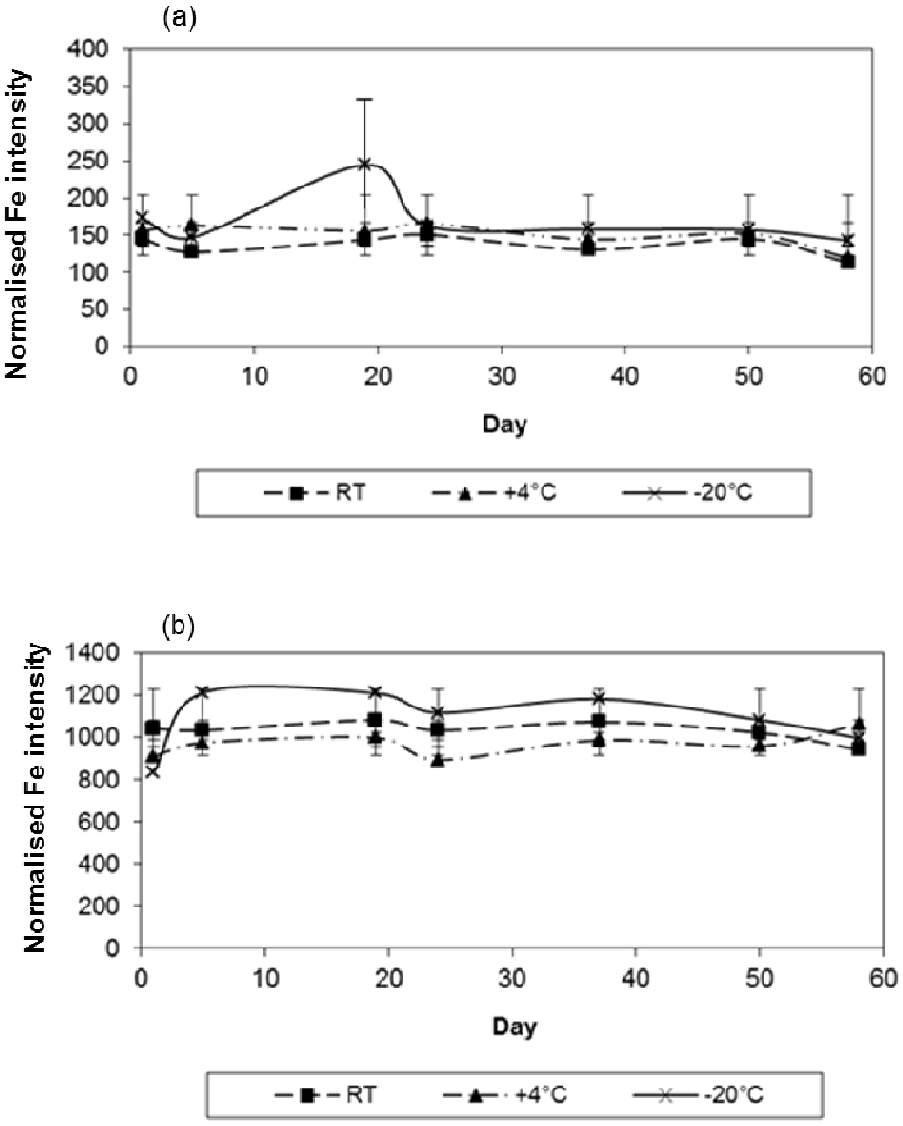
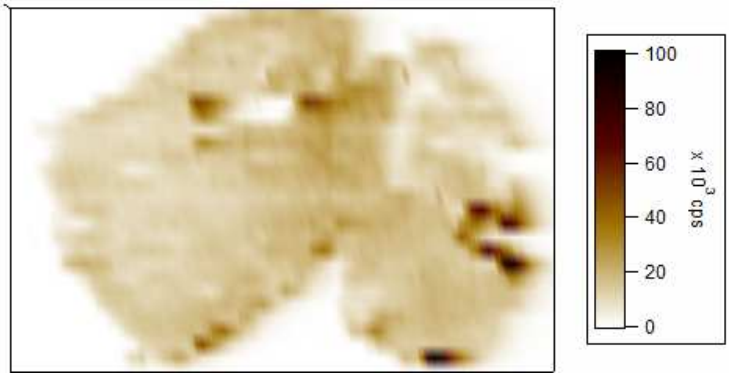


Figure 4

(a)



(b)

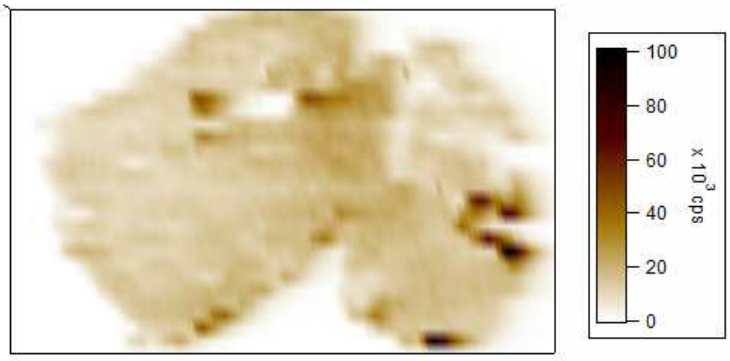
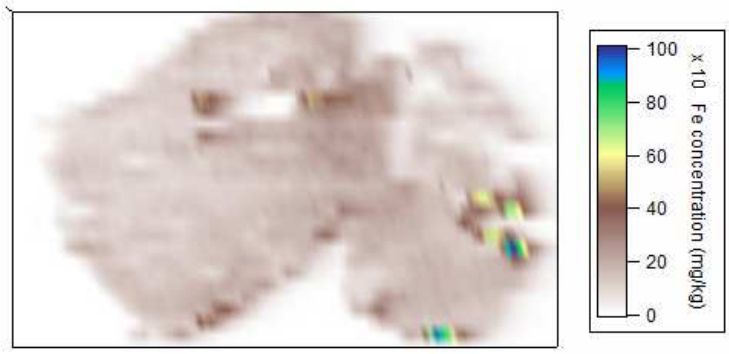


Figure 5

(a)



(b)

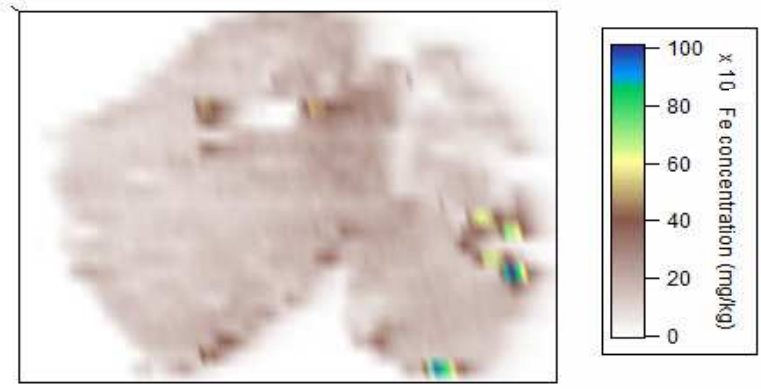


Figure 6