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ARTICLE

Standard sample preparing and calibration procedure of imaging zinc and magnesium in rats' brain tissue by laser ablation - inductively coupled plasma - time of flight - mass spectrometry.

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A calibration procedure consists of several steps, each of which has a significant impact on the final result of the analysis. The preparation of standard samples for analytical calibration is a far more important step in the analytical procedure than it might seem. In this paper, we have discussed a new, innovatory calibration procedure, which is itself a development of one previously published by us concerning a calibration strategy in the determination of trace elements in rat brain tissues by the Laser Ablation Inductively Coupled Plasma Time of Flight Mass Spectrometry (LA-ICP-TOF-MS) method. Moreover, the article has described an important step in the preparation of standard samples, which is both an innovation and exclusive to this work. The linearity of calibration function analysis is fully acceptable (for zinc R = 0.944 and for magnesium R = 0.989), and the applied calibration method, the conventional extrapolative method (CEM), known more generally as the "standard addition method", makes it possible to avoid interferences from the sample matrix. The results show the usefulness of the procedure developed in the presented analytical problem related to the analysis of solid biological samples. The developed research methodology enabled the distribution maps of zinc and magnesium in the rat's hippocampus, which is a frontier providing unique research in the pathophysiology of a rat brain.

Introduction

A laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) technique with multi-element determination capability and high spatial resolution is a progressive and modern analytical tool for imaging metals in biological tissue[1]. This method plays a suitable role as a micro-destructive technique, enabling trace element imaging and isotope ratio measurements at the trace and ultra-trace level in solid samples[1,2,3,4] because LA-ICP-MS has a detection limit of approximately 0.01 $\mu g \cdot g^{-1}$ [5]. From the standpoint of the analysis of biological samples, this method irreparably damages samples, leaves small spots, and has an analytical volume (craters) of approximately 100-200 µm diameter[6].

The LA ICP-MS method is difficult to quantify due to a lack of standard reference materials. A quantitative (bio)image of metals can be made using laboratory prepared "standard samples"[7]. The concept of preparing standard samples is a very important step in the entire calibration procedure. In this paper we describe the procedure of preparing a standard sample which leads to the making a distribution map of zinc and magnesium.

Depending on the kind of sample (biological material), a calibration strategy can be distinguished substantially[8]; usually the conventional extrapolative method (CEM), or extrapolative method (generally known as the "standard addition method" - SAM).

Analytical calibration is a process that is focused on establishing of the actual (theoretical / real) dependence of the analytical signal on the concentration of the analyte (calibration function) in an empirical form (the calibration function), and then using a plot to determine the concentration of the analyte in the sample under examination (i.e. obtaining the analytical results)[7,9].

The concept of the calibration procedure ought to be understood as a detailed mode of performing particular stages (laboratory, measurement and mathematical) of the calibration. Any such procedure should be performed strictly in accordance with the established rules, which define a more general way of the proceeding, except in the leading to the main tasks of calibration

where there are some additional analytical purposes. In turn, the *calibration method* can be defined as the realization of the analytical calibration procedure of a certain specificity, leading to the achievement of (apart from the main task of calibration) preconceived, additional analytical purposes[7,10].

The aim of this study was to evaluate the method of standard matrix matched samples preparation and the calibration procedure for the quantitative imaging of zinc and magnesium in rat brain tissue for farther use in neuropharmacological studies.

Experimental

A. Apparatus

A Cryostat Leica CM 1850 - Leica MICROSYSTEMS (Poland) was used to prepare the standard samples in the form of slices on a microscope slide (cryo-cutting), The LA-ICP-TOF-MS measurements of ^{25}Mg, ^{66}Zn and ^{13}C were performed using an OptiMass 9500 (GBC, Australia) mass spectrometer coupled to a laser ablation system (New Wave UP 266 Macro, New Wave, Fremont, CA, USA) with New Wave Research, which is Ablation System software. The laser ablation (in line mode for standard samples and in raster mode for mapping) of the thin rat brain tissue sections was performed using a repetition frequency of 5 Hz; spot diameter, 100 μm, scan speed 100 μm·sec^{-1} and an interline distance of 100 μm. The ablated material was transported by argon as a carrier gas into the inductively coupled plasma (ICP). The software for ICP-TOF-MS was Optimass 9500 ver. 2.11, Windows. For the real mean concentration of zinc and magnesium evaluation in the brain, subsamples of non-spiked and doped material were analyzed after wet microwave assisted digestion in nitric acid. Mineralization of brain homogenate was performed with the use of a Mars x5 CEN (USA). For determination of zinc and magnesium Optima 2100 (PerkinElmer, USA) atomic emission spectrometer was used.

B. Optimization of the determination procedure.

In the performed examination, some of the parameters of determination were chosen following those in the previous study[7,11,12,13], while three others were optimized to improve sensitivity. These included: the nebulizer gas flow rate, auxiliary gas flow rate and the radio frequency (RF) coil power set.

Nebulizer (carrier) gas flow rate

The three values of the nebulizer flow rates: 1.0 L·min^{-1}, 1.1 L·min^{-1}, and 1.2 L·min^{-1} were examined to find the best one. For this optimization, signals of Mg and Zn were measured for an un-spiked sample slide on a 2 mm long ablation line. In both cases, the highest signals were recorded for the carrier gas flow equal to 1.2 L·min^{-1}, and this value was chosen for further studies.

Auxiliary gas flow rate

In this case, two different values of flow rate were checked: 0.4 L·min^{-1} and 0.5 L·min^{-1} with a constant carrier gas flow rate equal to 1.2 L·min^{-1}. Due to the fact that the higher values of analytical signals for zinc and magnesium do not correspond with the common value of the auxiliary gas flow rate, adopting the value producing a higher signal for the element of lower concentration in brain tissue and in standard sample (zinc) – 0.5 L·min^{-1} was decided.

RF coil power

The third optimized parameter was the RF coil power set. The three different values of the RF coil power were examined: 1.0 kW, 1.1 kW and 1.2 kW, with a constant carrier gas flow rate of 1.2 L·min^{-1}, and auxiliary gas flow rate of 0.5 L·min^{-1}. Due to the fact that the maximum values of the analytical signal for zinc and magnesium corresponds with the same RF coil power set, it was decided that the optimal value of the parameter describing the generator coil power is the value of 1.1 kW. The finally reached signals for a non-spiked sample were of about $2.6 \cdot 10^4$, $5.2 \cdot 10^3$, $1.2 \cdot 10^4$ cps for ^{25}Mg (94 μg g^{-1}), ^{66}Zn (11 μg g^{-1}) and ^{13}C , respectively. The obtained precision (RSD) was at the level of 8.3% for ^{25}Mg, 5.2% for ^{66}Zn and 2.8% for ^{13}C. To evaluate the blank, signals were measured for a chamber containing an examined slice in but without the ablation active and were of about 61, $3.4 \cdot 10^2$, $6.1 \cdot 10^3$, cps for ^{25}Mg, ^{66}Zn and ^{13}C, respectively.

Parameters of LA-ICP–TOF-MS and ICP-OES analysis

Table 1 lists all of the experimental parameters applied in the analysis.

Laser ablation system	**NewWave Research UP-266 Macro**
Wavelength of Nd:YAG laser [nm]	266
Laser fluence [J·cm^{-2}]	4.29
Ablation mode	scanning - line by line/mapping
Repetition frequency [Hz]	5
Laser beam diameter [μm]	100
ICP-TOF-MS system	**OptiMass 9500**
Nebulizer (carrier) gas flow rate [L·min^{-1}]	1.2
Auxiliary gas flow rate [L·min^{-1}]	0.5
RF coil power set [kW]	1.1
Plasma gas flow rate [L·min^{-1}]	10
ICP-Q-MS system	**Optima 2100**
Nebulizer gas flow rate [L·min^{-1}]	0.8
Auxilary gas flow rate [L·min^{-1}]	0.2
RF coil power set [kW]	1.3

Table 1. The selected[14] and optimized operating conditions of LA-ICP-TOF-MS in imaging zinc and magnesium in rat brain tissue and analysis by ICP--OES of mineralizates of non-spiked standard sample homogenate and standard sample homogenate on highest level.

C. Biological material

Rat brains were collected from seven male Sprague–Dawley rats (Charles River, Germany), weighing 300–350 g. The animals were singly housed with food and water freely available, and were maintained on a 12 h light/dark cycle with constant temperature (22 ± 2 °C) and humidity (50 ± 5%) conditions. The rats were euthanized and decapitated according to a protocol approved by the Bioethics Commission of the Institute of Pharmacology, Polish Academy of Science, Krakow, Poland. The isolated brain was then immediately frozen and stored for further operations at -70 °C.

D. Standard samples preparation.

The rat brains were homogenized and spiked with a two-element standard solution prepared by dissolving in ultrapure water two hydrated salts: $Zn(NO_3)_2 \cdot 6\ H_2O$ and $Mg(NO_3)_2 \cdot 6\ H_2O$, to give a solution of a concentration, 0.72 g·L^{-1} of zinc and 8.8 g·L^{-1} of magnesium, respectively (the real concentrations were determined after adequate dilution by a flame atomic absorption spectrometry (FAAS). Carefully mixing of different masses of a homogenized brain with the above-mentioned standard solution, and with a small addition of water to keep the volume of solution added constant

(100 µL), a series of standard samples was prepared. The concentrations of spikes for standard samples preparation were chosen on the basis of the previously determined physiological concentration of the elements in the rat brain, to covering the potential range of changes. The assumed concentrations of Zn and Mg spiking in the standard samples are shown in **Table 2**.

standard sample	concentration of the analyzed element added into the standard samples [µg·g^{-1}]	
	Zn	Mg
non-spiked	0*	0*
1	5.7	70
2	9.8	120
3	19.8	242
4	43.1	527

** Concentration of the zinc and magnesium in non-spiked standard sample, represents a point of reference in determining the actual concentration of the analyte in the sample.*

Table 2. Concentrations of spikes standard samples of the analyzed elements into the standard samples.

The obtained standard samples were homogenized and then immediately frozen in liquid nitrogen in order to preserve their homogeneity. In the next step, 30 µm thin slices of the standard sample (frozen homogenates) were prepared by cryo-cuttings in cryostat and fixed on a microscopic slide. So prepared standard samples were stored until analysis at -70 °C.

E. Brain slices preparation

The brain specimens to be mapping were prepared from frozen rat brain by cryo-cuttings in cryostat to 30 µm thin slices. The slices of the sample were fixed on a microscopic slide. The slice used for analysis of Mg and Zn distribution came from the area of the brain characterized by: interaural 5.20 mm and Bregma -3.80 mm. The slides were kept frozen until analysis.

F. Calibration strategy

The basic workflow of the proposed calibration strategy in imaging/mapping zinc and magnesium by the LA-ICP-TOF-MS method is illustrated in **Fig. 1.** The procedure consists of the following steps:

- euthanasia and decapitation of Sprague-Dawley rats brains fed a normal diet;
- rat brains homogenization in agate mortar;
- transfer of homogenate portions into several Eppendorf vials;
- preparation of two element standard solutions by dissolving hydrated nitrates of considered elements and mixing solutions;
- spiking of rat brain tissue homogenates by previously prepared two element standard solutions, and carefully mixing;
- immediately freezing the standard homogenate in liquid nitrogen;
- cryo-cutting solid standard samples by cryostat to thin section on microscope slides;
- standard samples analysis by LA-ICP-TOF-MS;
- preparing distribution map by graphics program.

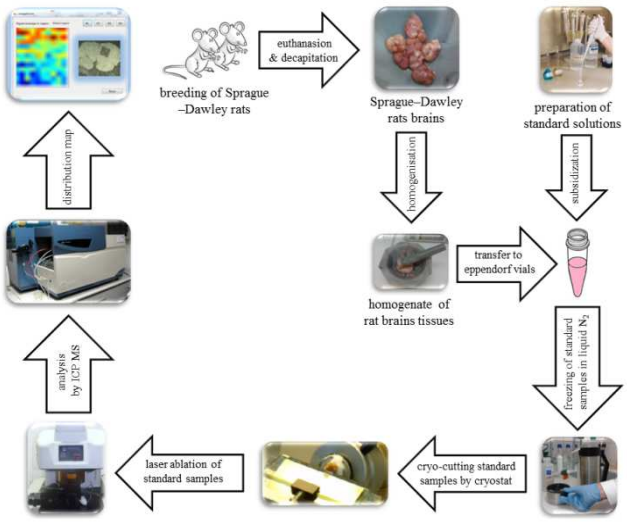

Fig. 1 Schedule of preparing standard samples for analytical calibration of LA-ICP-MS and imaging zinc and magnesium elements in rat brain tissue.

In our new strategy - compared to previously developed, three innovations were introduced: 1) spiking with a standard solution obtained by the dissolving of adequate solid salt, meaning it is therefore not acidified; 2) reducing the number of analytes spiked into a standard samples; 3) immediately freezing the homogenized standard with liquid nitrogen. Noteworthy is the fact that for the preparation of standard samples we use the same biological material, resulting in the elimination of interferences arising from the sample matrix.

G. Determination of Zn and Mg in sliced standard samples for proofing of the analytes concentration.

To examine the prepared standards first, a real concentration of analytes in the particular standard samples resulting from initial brain contents and the spiked amount were determined. Analysis was performed on a standard sample spiked with 43.1 µg·g^{-1} of zinc and 527 µg·g^{-1} of magnesium, respectively, and a non-spiked sample. For this analysis, portions of the standards were digested and then the Mg and Zn were determined by ICP-OES. The frozen homogenate of the standard samples was immediately cut using a ceramic knife for three pieces which were placed in teflon vessels. Next, 6 mL of 65% nitric acid were added and the samples were left for 30 minutes in room temperature (solubilization). After this, the microwave-assisted acid digestion procedure was carried out according to a 7 step program: (1) ramp time - 4 min., 50% of enter power (~700 W); (2) holding time - 4 min., 50% of enter power (~700 W); (3) ramp time - 4 min., 70% of enter power (840 W); (4) holding time - 4 min., 70% of enter power (~840 W); (5) ramp time - 4 min., 90% of enter power (~1080 W); (6) holding time - 4 min., 90% of enter power (~1080 W); (7) cooling and ventilation -30 min. The digests were transferred quantitatively to 10 mL measuring flasks and filled with super pure water to the mark. The concentrations of zinc and magnesium were determined by the means of ICP-OES at 279.077 nm for Mg and 213.857 nm for Zn.

Results and discussion

A. Quality of the standard samples

Figure 2 presents a comparison of the appearance of the edges of standard samples (homogenate) obtained both in the procedure described previously by Jurowski et al.[7] and in the current research.

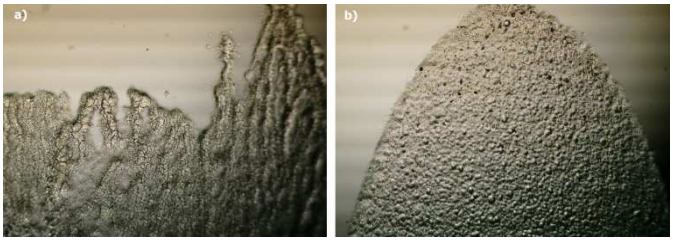

Fig. 2 A **microscope photos of** standard samples **slices** obtained in the previously developed procedure (a), and after (these studies) the introduction of new steps in the calibration procedure (b).

The Fig. 2 a) and b) demonstrated that the changes in the samples standard preparation procedure improved the quality of the obtained standard samples. There was no formation of air bubbles and free spaces in the frozen samples resulting from uncontrolled secondary reactions.

B. Proofing of the analytes concentrations in the standard samples.

For the comparison of results with the CEM calibration method, the concentration of zinc and magnesium in a non-spiked standard sample and highest standard sample mineralizated by the ICP-OES method were measured. The table 2. Presents the concentration of zinc and magnesium in a non-spiked standard sample, plus standard characterized by the highest level concentration by LA-ICP-TOF-MS and ICP-OES analysis**.**

Analyte	Concentration [$\mu g \cdot g^{-1}$]			
	analysis by LA-ICP-MS		analysis by ICP-OES	
	A	B	C	D
	non-spiked homogenate sample	highest standard sample	non-spiked homogenate sample	highest standard sample
zinc	11±5	50±5	5±1	52±20
magnesium	94±27	634±27	121±5	781±15

Table 3. The comparison of the concentration of zinc and magnesium in a non-spiked standard sample, plus standard characterized by the highest level concentration by LA-ICP-TOF-MS and ICP-OES analysis.

In the table 3, column B contains the results obtained using calibration lines established assuming spiked concentration as a true value, but in the column A, the concentration in a non-spiked standard was calculated by extrapolation of this line. In this case concentrations were obtained by far extrapolation of calibration lines therefor they could be considered only as informative values. The concentration in columns C and D were calculated based on the digests' analysis with external calibration (interpolative). As such, data in column D expresses the sum of initial concentration and a spike. Therefore for a comparison, the relevant concentrations from columns A and C should be taken into account.

C. Analysis of the standards homogeneity

In order to check the homogeneity of prepared standards for every preparation, signals of magnesium and zinc were measured at a distance of 4 mm for 3 parallel lines. The RSDs within each line were in the range of 6.5 -11.2% for magnesium and 4.2 – 6.1% for zinc, but the RSDs between the lines were 6.8% for ^{25}Mg, 4.9% for ^{66}Zn, respectively.

D. Calibration strategy to imaging of magnesium and zinc in hippocampus part of rat brain and its applications.

For each line, 22 measurements were made, while for reliability of the result the first and last four measurements were rejected. Every single repetition result obtained for the element was exported by GBC Optimass 9500 Ver 2.11 software to Microsoft Excel 2010, where the individual results for each analyte were divided into tables. In order to standardize the results, each analytical signal was divided by the value of the corresponding carbon isotope ^{13}C signal. Carbon is the main component of tissues, therefore its spatial concentration within a sample may be assumed as constant. Owing to that, as well as due to the fact that the signal values for ^{13}C are closer to the values of analyte isotopes, this isotope may be considered as an internal standard in tissue analysis[15]. In the case of the (bio)imagining, signal standardization allows for the distinguishing of the natural variability of signals related to the concentration of local analytes concentration from its variability, which is caused both by different interaction of the laser with the surface of a sample and by local differences in tissue thickness (differently ablated sample masses). The obtained relative signals for a non-spiked standard were as follow: for magnesium 0.214 with RSD 6.3% and for zinc 0.043 with RSD 5.0% (table 4). Table 4 shows mean signals obtained for standard samples, described earlier in the table 2.

Standard sample	Mean relative signals	
	Mg	Zn
non-spiked	0.214 ± 0.003	0.0429 ± 0.0001
1	0.300 ± 0.019	0.0522 ± 0.0001
2	0.292 ± 0.009	0.1369 ± 0.0001
3	0.532 ± 0.029	0.1892 ± 0.0010
4	1.074 ± 0.213	0.2563 ± 0.0052

Table 4. Mean relative signals obtained for standard samples by LA-ICP-MS analysis.

Based on the obtained data a coordinate system relating to the analytical signals, and the concentration of the analytes spikes, expressed in [$\mu g \cdot g^{-1}$] of the rat brain tissue, were prepared. In the system signals for a non-spiked standard sample cross the axis 0y. The resulting functions were extrapolated to the intersection with the axis 0x allowing for the determination of the analyte concentration in a non-spiked standard sample according to the conventional extrapolative method (or CEM, which is more popularly known as a standard addition method). Due to the fact that the analytes are in the natural environment of the sample, and therefore in the presence of possible interferents with an ideal relation to their concentration and type, the applied conventional extrapolative method (CEM) is a mean of compensating for multiplicative interferences from the sample matrix. The analytical result that has been calculated ought to be accurate because the calibration plot should also ideally represent the calibration function. The theoretical predictions confirm the correlation coefficient ranging from 0.994 to 0.999[16]. The results showing the calibration functions and linear correlation coefficients for the determined elements are as follows:

- for zinc $0.0051x + 0.0560$ ($R = 0.944$)
- for magnesium $0.0017x + 0.1593$ ($R = 0.989$)

In this part of the examination, while using relative signals, the blank was not considered. When the chamber is empty or the laser is switched off, the relation of the signals' analyte/carbon is not

consistent with other data, because the signals depends on the carrier gas path contamination only. Due to the lack of certified reference materials, it was not possible to calculate the accuracy of this method. However, prepared standard samples and the corresponding calibration functions allow for the obtaining of distribution maps of a given elements in the rat brain sample.

The optimized procedure for the preparation of the standard samples and calibration was used for the imaging distribution of the zinc and magnesium in a rat brain region including the hippocampus (*cornus ammonis* – CA1, CA2 and CA3 structure; red dotted line), as in **Fig. 3A**. This area (4 mm x 4 mm) was ablated by a laser system in the raster mode. The resulting tracks of the laser ablation are illustrated in **Fig. 3B**.

To imagine the distribution of zinc and magnesium in the analyzed sample, a graphic program was used, which transferred signals obtained for the sample onto colors, based on the previously calculated calibration function where signals are measured against the concentration of analytes concentration in the standard samples. The quantitative distribution maps are presented in the form of the intensity of colors, with red being assigned to the highest levels and blue to the lowest. **Fig. 3C and D** presents a distribution maps of zinc and magnesium in a control sample of rat brain tissue.

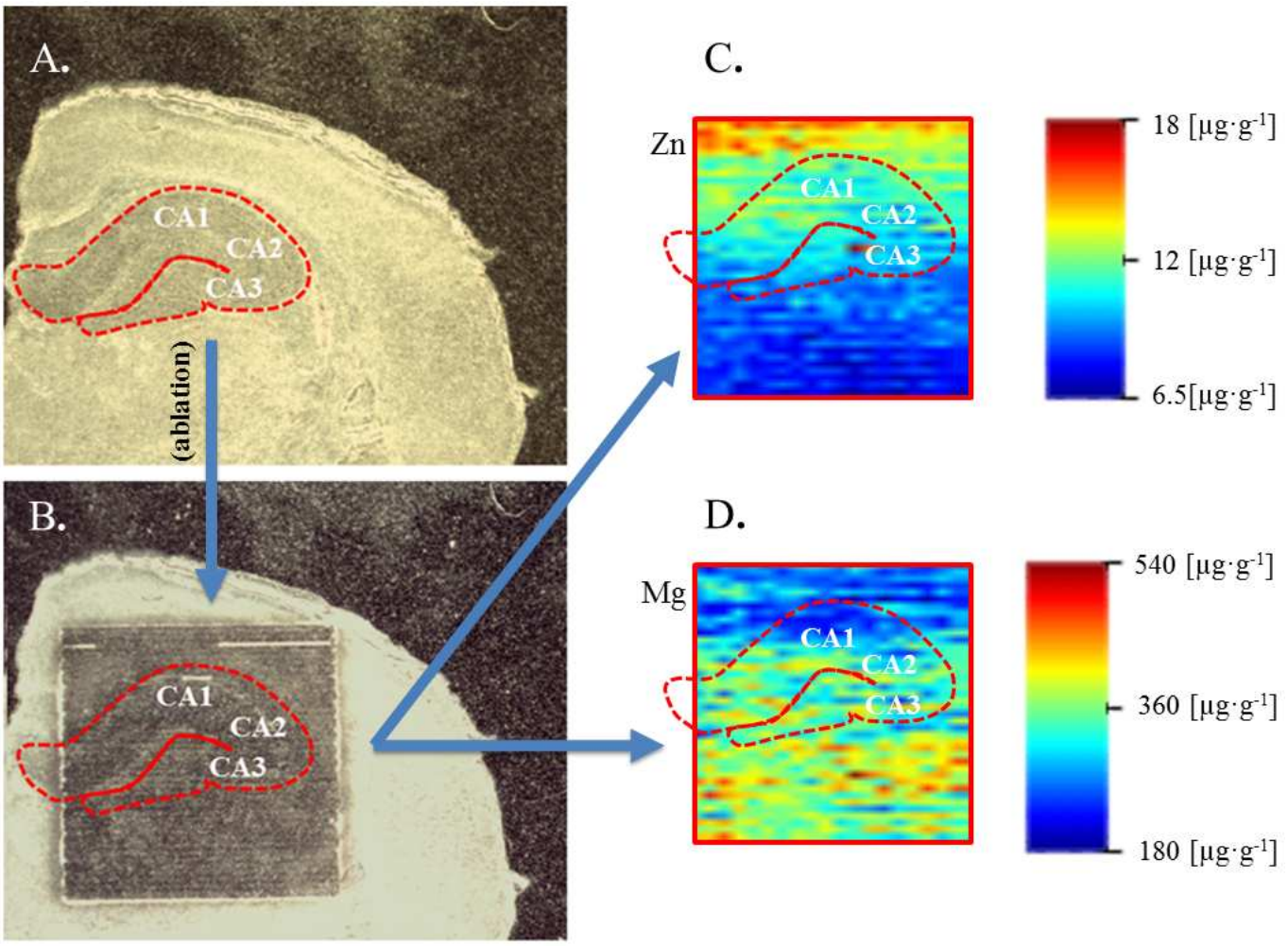

Fig. 3. A microscope photo of the analyzed sample of the hippocampus; **A.**- before laser ablation with the marked hippocampus region (red dotted lines) and B. – after; A quantitative distribution map of zinc – **C.** and magnesium **- D**. in the hippocampus region in a brain sample.

Conclusions

For the LA-ICP-MS calibration different strategies are applicable depending on type of sample, but for imaging it is very popular to the base this on a matrix-matched standard[4,17,18,19].

Applied modification of standard sample preparation like a) spiking with a standard solution not acidified obtained by the dissolving of adequate solid salt; b) reducing the number of analytes spiked into a standard samples and thus the added volume and c) immediately freezing the homogenized standard with liquid nitrogen, significantly improved general appearance of standards. Moreover obtained standards looks homogenous in respect to analytes distribution.

Obtained standards, although applied to not only to imagining of element distribution, but also to concentration determination by CEM method of calibration, in the last case not fulfill all requirements. In proposed methodology for LA-ICP-MS for non-spiked sample analytes are determined using extrapolation of obtained line. Because in our case we focused on better evaluation of the physiological range of concentrations (resulting in small values

of spikes), to determined concentration in non-spiked tissue we have to apply far extrapolation. This can explain the difference between LA-ICP-MS and ICP-OES results.

As for the methodological point of view the applied calibration method (CEM) makes it possible to avoid interferences from the sample matrix. It could be also acceptable towards to the accuracy of the zinc determination.

Developed calibration procedure allows to maintain the homogeneity and very good quality of the standard samples. Moreover, calibration strategy is characterised by good linearity (correlation coefficients equal to 0.944 for zinc and 0.989 for magnesium) and wide range of concentration included physiological level of analysed elements.

LA-ICP-MS is a modern tool for toxicological and pharmaceutical research thanks to its micro-sampling characteristic allowing us to imagine lateral or in depth (slide by slide) distribution elements of interest, although it however still suffers from difficulties in the calibration process.

The proposed calibration strategy can be applied for the quantitative study of metal distribution in different biological samples. At this stage of investigation, the presented data for a rat brain are only to illustrate the calibration methodology and does not claim to solve any pathophysiological problem of concerning rats.

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