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A calibration strategy in bioimaging trace elements in rat brain tissue by LA ICP-TOF-MS method

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ABSTRACT

A calibration step in an analytical procedure is often not adequately treated, although it is a very important step in the analysis. Also, the approach to the nomenclature seems to be disrespectful. In order to resolve this problem we chose a new classification based on both how the calibration dependence is reconstructed, and how the measurement data is then transformed. In this paper we discussed the steps of a developed calibration procedure 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. The developed calibration procedure uses the long established calibration method – the method of standard addition – although the standard samples are in this case the rat brain tissue samples. The results show the usefulness of the procedure developed in the presented analytical problem related to the analysis of solid samples, which is where the work is original.

1. Introduction

It is well known that analytical calibration is a very important step during an analytical procedure. Nevertheless, there is a lack of literature concerning this step, which would then be recommended by the IUPAC or commonly and correctly used by analytical chemists. In addition, there are misunderstandings and obscurities, which can cause improper or wrong analytical proceedings and can create didactic difficulties, themselves sometimes leading to an improper or wrong analytical proceeding.

However, there is a proposed definition, which in a very accurate and specific way reflects the ideas of the analytical calibration. The concept of analytical calibration ought to be understood as denoting a process which consists of representing the actual (whether real or theoretical) dependence of the analytical signal on the concentration of the analyte (which in this context is called a calibration function) in an empirical form (calibration plot/graph or, more precisely, 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) [1,2].

The calibration plot/graph is constructed under specified and constant conditions (instrumental and physicochemical), with the use of already established up to several standard solutions (or, more precisely, a standard sample); i.e. solutions of known, precisely determined, concentrations of at least one component (usually an analyte) [1].

The detailed procedure for the performance of each stage creates a so-called calibration procedure. Any such procedure should be performed strictly in accordance with the established rules, which themselves define a more general way of the proceeding, except in the leading to the main tasks of a calibration where there are some additional analytical purposes. Thus, a specific calibration procedure can be considered to be a method of calibration. All the known calibration methods in analytical chemistry can be divided into three categories: interpolation, extrapolation, and indicator [1,3,4].

In each case the analytical result is determined by using a calibration plot, which takes into account the analytical signal having been measured using a sample.

For the analysis of samples that are solutions, it is reasonable to use the term: standard solution. However, for the analysis of rat brain tissue samples, for example, by LA ICP-TOF-MS, we suggest the most appropriate term a “standard sample”. This concept is universally suitable for the application of each of the samples (physical state).

Another important issue in addition to the nomenclature for the analysis of using the LA ICP-TOF-MS method is the selection of a suitable calibration method for obtaining the most reliable analytical result and the possibility of its use. There is a problem regarding the lack of certified reference materials which should be

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used to prepare standard samples. In this case, in order to prepare standard samples we used samples prepared in an analogous way as the sample for analysis. Hence, in the case of trace elements, bioimaging in selected brain structures should use as many separate representative samples, if required, for a calibration method.

The most commonly used calibration methods for the analysis of selected rat brain structures include the conventional extrapolative method (CEM), or extrapolative method; known popularly as the "standard addition method" [4,5]. This method is a typical application (liquid samples) that involves preparing a series of sample solutions with successive additions of standard solution of different concentration and performance measurements for each of these solutions separately [1]. Such a calibration procedure is not feasible for the analysis of brain samples because they are solid samples. Obviously, there is a possibility for applying this method to properly prepared liquid brain samples by mineralization, but due to the direction of research (bioimaging/mapping), it would be pointless.

In this case, the CEM method should be modified in order to permit its use while maintaining the reliability of the results. This problem has received no attention before. There are only a few papers on this subject although they usually do not treat it in a specific way [6–10].

The aim of this study was to develop a calibration strategy procedure in bioimaging trace elements in rat brain tissue by the LA ICP-TOF-MS method.

The basic workflow of elemental bioimaging/mapping trace elements by the LA ICP-TOF-MS method is illustrated in Fig. 1 [14,15].

The first step in this analytical procedure is the collecting of a rat's brain. The next step is cryo-cuttings by cryostat into thin slices. The thin (15–20 μm) sections of the rat brain tissue (samples) are ablated by a focused Nd:YAG laser and then transported by argon as a carrier gas into the inductively coupled plasma. After ion formation in the inductively coupled plasma ion source, the positively charged ions are extracted from the argon plasma via the differentially pumped interface between the sampler and skimmer cones into the high vacuum of the Time of Flight (TOF) analyzer, and are separated with respect to their mass-to-charge ratios, and detected by the ion detector [6–9].

2. Experimental

2.1. Apparatus

To prepare the samples (cryo-cutting), we used cryostat Leica CM 1850 – Leica Microsystems (Poland). The LA ICP-TOF-MS measurements used were an OptiMass 9500 (GBC, Poland) coupled to a laser ablation system (New Wave UP 266, New Wave, Fremont, CA, USA). The laser ablation (line by line) of the thin rat brain tissue sections was performed using a Nd:YAG laser (wavelength, 266 nm, repetition frequency, 20 Hz; spot diameter, 155 μm). The measurement time for one sample depends on the size of the area analyzed (2 × 2 mm), the laser scan speed applied (50 μm s^{-1}) and the interline distance (110 μm). The software for the laser ablation system was New Wave Research – Ablation System. The ablated material was transported by carrier gas (argon) into the inductively coupled plasma (ICP). The software for ICP TOF-MS was Optimass 9500 Ver 2.11, Windows 7. The optimized experimental parameters are summarized in Table 1 [11–13].

2.2. Biological material

Brains were collected from three male Sprague–Dawley rats (Charles River, Germany), weighing 250–270 g. The animals were kept under standard laboratory conditions of lighting (light phase 7:00–19:00) and temperature (19–21 °C). Food (Standard RM1A (P), Merazet; Poznan, Poland) and water were freely available.

The rats were euthanized according to a protocol approved by the Bioethics Commission of the Institute of Pharmacology, Polish Academy of Science, Krakow, Poland, and decapitation was conducted by a special guillotine, ensuring the rapidly removed head would then be taken to a suitable place.

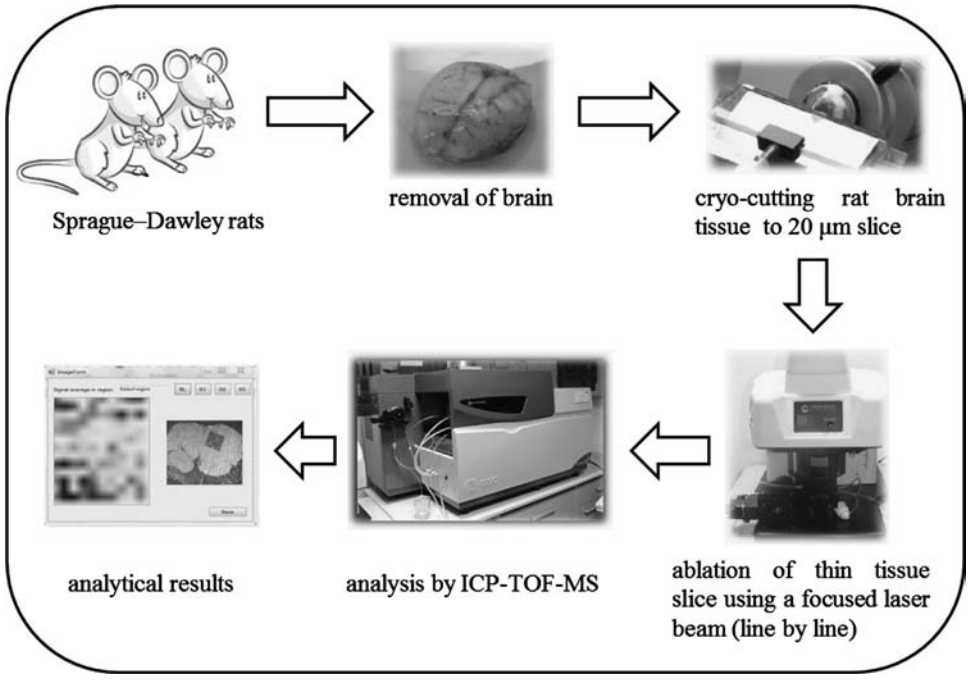

Fig. 1. The basic workflow of elemental bioimaging/mapping trace elements by LA ICP-TOF-MS method.

Table 1
Optimized operating conditions of LA ICP-TOF-MS in bioimaging trace elements in rat brain tissue.

Laser ablation system	**New Wave Research UP-266 Macro**
Wavelength of Nd:YAG laser (nm)	266
Laser power density (W cm^{-2})	10^9
Ablation mode	Scanning—line-by-line
Repetition frequency (Hz)	5
Laser beam diameter (μm)	155
Energy output (%)	50
ICP TOF-MS	**OptiMass 9500 (GBC, Poland)**
RF power (W)	1000
Cooling gas flow rate (L min^{-1})	10

Table 2
Concentrations of elements in multielement intermediate in ultrapure distilled water.

Element	Concentration in 100 mL multielement intermediate solution (mg L^{-1})
^{66}Zn	0.15
^{25}Mg	1.50
^{44}Ca	0.50
^{57}Fe	0.25
^{65}Cu	0.05
^{55}Mn	0.5×10^{-2}
^{77}Se	0.25×10^{-2}

The rat brain samples were homogenized in an agate mortar and were used for the preparation of standard samples preparation as described below.

2.3. Calibration strategy

2.3.1. Standard solution and standard sample preparation

The analysis was performed for the elements: ^{44}Ca, ^{65}Cu, ^{57}Fe, ^{25}Mg, ^{66}Zn, ^{55}Mn, and ^{77}Se.

For the preparation of the standard samples the homogenate of the brain samples was spiced with (the expected concentration in the brain [6,16–18]) the appropriate volume of standard solution of multielement solution (TITRISOL®, standard solution – concentration: 1 g L^{-1} Merck, Darmstadt, Germany) dissolved in ultrapure distilled water (Table 2).

The concentration of microelements in standard samples is shown in Table 3.

The first step in the cryo-cutting sample was affixed by resinous glue to a special element from the cryostat chamber. The prepared specimen was placed in the cryostat chamber at a temperature of −14 °C, and cut into slices of a thickness of 20 μm.

The resulting slices were shifted from the cryostat with a brush, and placed on a microscope's slide [7–9].

The obtained specimens of the samples were placed in signed boxes and stored in a refrigerator until analysis at −14 °C for no longer than one week [19].

The prepared samples were analyzed by the LA ICP-TOF-MS mapping mode (line-by-line) with the parameters of the apparatus presented earlier.

3. Results and discussion

The analytical results obtained from the element of the crater were exported by the Laser Ablation System to the file format. xml. Then the data was transferred to Microsoft Excel 2010, where the individual results for each analyte were divided into tables. In order to refer to the component of the results of the sample, which in each case has a constant concentration (carbon isotope ^{12}C), each analytical signal was divided by the value of the analyte signal to the carbon isotope ^{12}C for the measurements (the crater).

Table 3
The concentrations of the analyzed elements in the standard samples.

Standard sample	Concentration of the analyzed element in the standard sample (μg g^{-1})						
	Zn	Mg	Ca	Fe	Cu	Mn	Se
0 (blank sample)	0[a]	0[a]	0[a]	0[a]	0[a]	0[a]	0[a]
1	5.92	59.17	19.72	9.86	1.97	0.20	0.10
2	10.05	100.53	33.51	16.76	3.35	0.34	0.17
3	23.27	232.70	77.57	38.78	7.76	0.78	0.39

[a] Concentration of the analyzed elements in the blank sample is not in fact equal to 0 μg g^{-1}, but from the point of view of the analytical calibration standard addition method should be unknown concentration of the blank as zero, as it represents a point of reference in determining the actual concentration of the analyte in the sample.

The primary objective of this procedure was to determine the analytical signal with increased precision. It is understood that if the "internal standard" (in the form of a carbon isotope ^{12}C) and analyzed element have similar chemical properties, and the concentration of the "internal standard" is maintained at a constant level in the sample, the size and direction of the random variation of the measured analytical signals of both components are also similar. Hence, there is a good chance that the ratio of the two signals will have much smaller fluctuations than the same signal measured for the analyte. As a result, the method of standardization by measurement (an analytical signal) decreases the chance of a random error in the analytical result.

The results were then obtained and characterized by a similar value due to the fact that they were standard samples in order to give the corresponding calibration functions (curves reference/calibration charts), meaning it was possible to determine the concentration of a non-subsidized—blank sample. For this purpose, we prepared a coordinate system relating to the analytical signal (counts rate/intensity) and the concentration of the analyte subsidized expressed in μg g^{-1} of the rat brain tissue. The coordinate system thus obtained is applied to the measurement points representing the mean values of the signals for each of the standard sample with a given concentration of a doped element. Measurement points for non-doped standard sample (blank sample) crosses the axis 0*y*. The resulting functions were extrapolated to the intersection with the axis 0*x*, thus creating a new coordinate system. The resulting calibration function for the analyzed standard samples for example zinc are shown in Eq. (1).

$$y = 0.0065x + 0.0575 \quad (1)$$

The obtained calibration function to determine the concentration allowed no fortified sample (blank sample), which in turn formed the basis of the designation of element concentrations in the analyzed samples for analysis. The standard addition method was used, and the concentration of the blank values was obtained by calculating the concentration of the analytical signal for zero (the extrapolated intersection with the axis of the calibration function o*X*).

The conventional extrapolative method (CEM) is a mean of compensating for interferences from what sample matrix effects represents. This is 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 analytical result that has been calculated ought to be accurate because the calibration graph should also ideally represent the calibration function [20]. The theoretical predictions

confirming the correlation coefficient equal between 0.994 and 0.999.

Analysis of the results to be obtained can start a discussion about calibration function because with the analysis of the obtained results they would benefit the direct impact, using Graph 1 as a basis.

It can be concluded that it is almost linear in the entire concentration range. The signal from the standard sample number 2 (10.05 μg g^{-1}) significantly deviates from a linear course of it in relation to the other points on the calibration function. The reason for this observation may be because, in the preparation of standard samples in the form of histological specimens, number 2 was characterized in the presence of an air bubble inside the frozen homogenate. It can be assumed that the standard sample preparation procedure at the stage of subsidizing the analyte has been incorrectly carried out. Due to the fact that the other functions of the calibration points form a line graph, and a reference signal for the sample number 2 differs very significantly from the rest of the graph, it was decided that the point should be rejected due to our recognizing it as a gross error. Because of the aforementioned results, it was concluded that it is necessary to remove a deviated point without resorting to chemometric appropriate calculations.

In turn, this was obtained by analyzing the calibration function that was based on three experimental points and it should be noted that Eq. (1) has an acceptable correlation coefficient (R) of 0.999.

Results showing calibration functions and linear correlation coefficients for all of the determined elements are presented in Table 4.

Due to the lack of certified reference materials it is not to possible calculate the accuracy of the analytical method.

A blatant issue is the result of the measurement for the standard sample, number 2. All standard samples were prepared in the same laboratory and using the same preparation procedure. On the other hand, in the preparation of homogenates by cutting the cryostat, the sample was characterized by its individual morphology when compared to the other samples. The observed characteristic of the sample was probably linked to form the inner layers of the brain homogenate of the sample of the air bubble, which occurs in the processes during the storage time of the sample in the cryostat cut created space. Hypothetically, a created space should not affect the distribution of analytes in the standard sample, and the minimum possible quantitative changes are made as such because the homogeneity of the sample does not conduct. However, such a large difference between the major and the other specimen samples under discussion is impossible for physical reasons. It is therefore likely to lead to a bad performance standard sample of number 2. In order to confirm the hypothesis, it compares with the results of the analysis for the same standard samples under similar conditions while optimizing the physicochemical analytical methods. Using the data obtained as a basis, it is possible to eject the same conclusion.

Fig. 2 present a used computer program with an example of obtained maps such as zinc distributions in standard samples (left) on the surface of the evaporated standard sample (right) with the

Table 4
Calibration functions and linear correlation coefficients for all determined elements.

Analyte	Calibration function	Correlation coefficient (R)
^{44}Ca	$y=0.0009x+0.0375$	0.998
^{65}Cu	$y=0.0197x+0.0626$	0.999
^{57}Fe	$y=0.0543x+0.5803$	0.997
^{25}Mg	$y=7\times10^{-5}x+0.0064$	0.995
^{66}Zn	$y=0.0065x+0.0575$	0.999
^{55}Mn	$y=4.9826x+1.0295$	0.998
^{77}Se	$y=2.651x+0.5202$	0.999

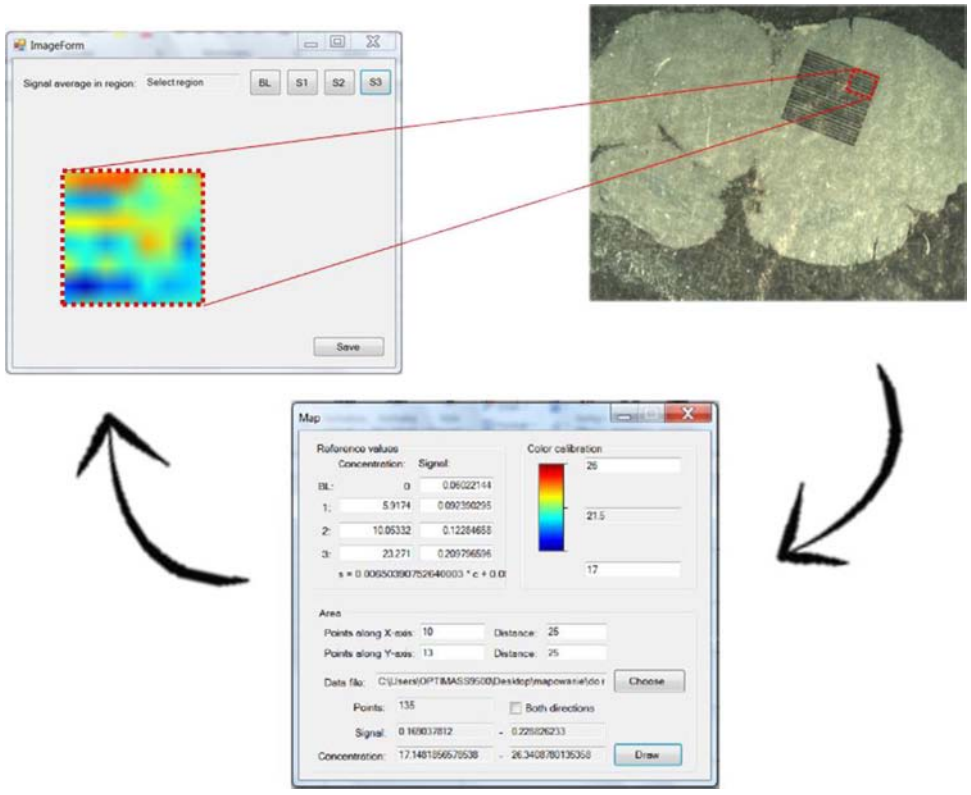

Fig. 2. Zinc distributions map (left) and the surface of the evaporated standard sample (right)—spiked concentration of zinc was 23.27 μg g^{-1}.

highest concentrations level 23.27 μg g^{-1} (17–26 μg g^{-1}) when the expected concentration of zinc in homogenate was 23.27 μg g^{-1}.

4. Conclusions

The developed calibration procedure consists of several steps, each of which has a significant impact on the final result of the analysis. A closer look at the various stages, provides far-reaching conclusions

- The value of the correlation coefficient (r) leads to the conclusion that the steps of the calibration were carried out correctly (except standard sample number 2);
- It should be noted that with the high complexity of the analytical steps in the analytical procedure we obtained a linear correlation coefficient equal to 0.999;
- In addition to the accuracy of the analysis, this proposed calibration procedure is fully acceptable (R=0.999);
- The calibration method used is considered to be akin to extrapolative methods and, as such, when compared, so to the standard series method it is not possible to fully detect the non-linearity.
- It is possible to obtain a result carrying a higher error than via the standard series method;
- The applied calibration method (CEM) provides the possibility to avoid the interferences from the sample matrix;
- The results for the prepared samples were calculated relative to the internal standard, which served as a ^{12}C carbon isotope because the synthetic sample weight was different;
- When served as an internal standard the mentioned carbon isotope ^{12}C is due to the fact that in biological samples of the element concentration it is high and constant for a sample matrix of this type;

Most of the authors have published material on the mapping of trace elements preparing samples for the calibration procedure in a different way. However, the present strategy improves the reliability of the calibration due to the distribution of the analytes in rat brain tissue as demonstrated by the correlation coefficient. We also hope that the term “standard sample” will be adopted for common use as a universal term referring to all types of samples

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