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# PRACTICAL PROCEDURE FOR METHOD VALIDATION IN INAA – A TUTORIAL

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## ABSTRACT

This paper describes the procedure employed by the Neutron Activation Laboratory at the Nuclear and Energy Research Institute (LAN, IPEN - CNEN/SP) for validation of Instrumental Neutron Activation Analysis (INAA) methods. According to recommendations of ISO/IEC 17025 the method performance characteristics (limit of detection, limit of quantification, trueness, repeatability, intermediate precision, reproducibility, selectivity, linearity and uncertainties budget) were outline in an easy, fast and convenient way. The paper presents step by step how to calculate the required method performance characteristics in a process of method validation, what are the procedures, adopted strategies and acceptance criteria for the results, that is, how to make a method validation in INAA. In order to exemplify the methodology applied, obtained results for the method validation of mass fraction determination of Co, Cr, Fe, Rb, Se and Zn in biological matrix samples, using an internal reference material of mussel tissue were presented. It was concluded that the methodology applied for validation of INAA methods is suitable, meeting all the requirements of ISO/IEC 17025, and thereby, generating satisfactory results for the studies carried at LAN, IPEN – CNEN/SP.

**keywords:** method validation, uncertainty, simulated method, mussel, INAA

## 1. INTRODUCTION

The Neutron Activation Analysis Laboratory (LAN) is a subdivision of the Research Reactor Center (CRPq) at IPEN - CNEN/SP. Since 2005's, quality policies have been implemented aiming to obtain full compliance with the requirements of ISO/IEC 17025 in the laboratories. The implementation of the quality system is essential to LAN, aiming to produce reference materials for environmental studies. In the context, the method validation is essential for suitability of the laboratory in this quality system.

According to ISO/IEC 17025, the method validation is defined as "*the confirmation by examination and the provision of objective evidence that the particular requirements for a specific intended use are fulfilled*". Thus the validation process shall be as extensive as is necessary to meet the needs of the application field. Furthermore, the laboratory shall record the results obtained, the procedure used to validation and issue a statement as to whether the method is fit for the intended use [1].

The objective of this study was to present how to make the process of method validation in Instrumental Neutron Activation Analysis (INAA) for the determination of Co, Cr, Fe, Rb, Se and Zn mass fractions in biological matrix samples. Investigated method performance characteristics were: accuracy (trueness and precision), linearity, selectivity, limit of detection (*LOD*), limit of quantification (*LOQ*) and uncertainty. The estimate of uncertainty sources was carried out of according to recommendations of the Guide to the Expression of Uncertainty in Measurement, using the simulated method [2].

## 2. METHOD VALIDATION

The process of method validation consists in performing and documenting a series of experiments in order to generate evidence that the results obtained by a measurement method are suitable for the intended use, by means of a study about the performance characteristics of the method. But, how do make the method validation in INAA?

1- Define the method (technique, principle of method, measurands, application, intended use and additional information);
2- Define the method performance characteristics to be investigated (validation scope);
3- Define how to perform the experiments required by validation scope;
4- Define how to calculate the results and uncertainties (statistically treatment);
5- Define the acceptance criteria of the results;
6- Define the materials, reagents and equipments to be used;
7- Execute the validation planning;
8- Register and report the results.

### 2.1. Define the method

*Technique:* INAA on the comparative method.

*Principle of method:* INAA is employed for determination of several elements, based on the conversion of a non radioactive nuclide, in a radioactive nuclide, that is, a radionuclide. This process occur through neutron bombardment in the material to be analyzed resulting in nuclear reactions characteristic of each element. In INAA, the reaction product to be measured is the gamma radiation arising from the radionuclide obtained by the neutron bombardment process [3]. Mass fractions of the measurands were obtained by comparing the photopeak area of the interest element in the spectrum of the sample with that of the multi-element standard (comparative method).

*Measurands:* the measurands were the mass fractions of Co, Cr, Fe, Rb, Se and Zn in biological matrix samples.

*Application and intended use*: determination of the mass fractions of the measurands by INAA for studies carried in the laboratories of LAN, CRPq at IPEN - CNEN/SP.

*Additional information*: Depending on the characteristics of the measurement method, different configurations may generate more or less favorable results. Thus, in a process of method validation it is necessary to make the definition of the method characteristics, taking into account prior knowledge about the technique and measurands. Table 1 present the set information on the validation process.

**Table 1. Configuration of measurement method to the validation process**

| | |
|---|---|
| Method name | Método BIO-2 |
| Technique | INAA |
| Detection | Gamma-ray spectrometry |
| Measurands | Co, Cr, Fe, Rb, Se and Zn mass fraction |
| Matrix sample | Biological matrix samples |
| Sample mass | 150 ± 10 mg |
| Sample-Standard geometry | 10 mm x 8 mm x 1 mm |
| Mass of measurand in the standard | Co (2.5µg); Cr (5.0µg);Fe (500µg); Rb (7.5µg);Se (2.5 µg); Zn (25 µg) |
| Measurands analytical peaks (keV) | $^{60}$Co (1173.23); $^{51}$Cr (320.08);$^{59}$Fe (1099.25); $^{86}$Rb (1077.00);$^{75}$Se (264.66); $^{65}$Zn (1115.54) |
| Irradiation time | 8 hours |
| Thermal neutron flux | 0.5 to 1.0 x $10^{13}$ $cm^{-2}$ $s^{-1}$ |
| Sample-Standard decay time to measurement | 21 to 24 days |
| Sample measurement time | 360 minutes (real time) |
| Standard measurement time | 20 minutes (real time) |
| Sample-Standard distance to detector | 3 mm |
| Gamma ray detector | HPGe - Canberra GC 2018 |
| Resolution of detector (FWHM) | 1.0 keV for the 122 keV peak of $^{57}$Co; and 1.78 keV for 1332 keV peak of $^{60}$Co |
| Software | Genie 2000 – Gamma Acquisition & Analysis, version 3.1 |
| Calculations | Microsoft Office Excel 2010 Spreadsheet |
| Multichannel analyzer | DSA 1000 |

## 2.2. Define the method performance characteristics to be investigated

The method performance characteristics investigated in this step of method validation were limit of detection (*LOD*), limit of quantification (*LOQ*,), trueness, limit of repeatability (*r*), intermediate precision (*IP*), limit of reproducibility (*R*), selectivity, linearity and the estimative of uncertainties budget. The EURACHEM Guide to Method Validation and Related Topics [4] and the International Vocabulary of Metrology [5] presents the general

concepts to the method validation parameters. Heydorn *et. al.* [6] and Bode and Blaauw [7] define, describe and comment about each of these parameters with regards to INAA.

### 2.3. Define how to perform the experiments required by validation scope

Experiments were carried according to definitions described in the Table 1. Table 2 present the sample used, number of experiments required and complementary information.

**Table 2.Experimental planning to make the validation of INAA methods**

| Parameter | Sample(s) | *n* | Complementary information |
|---|---|---|---|
| *LOD* | *Mussel* RM | 1 | calculated for one typical sample |
| *LOQ* | *Mussel* RM | 1 | calculated for one typical sample |
| trueness | *Mussel* RM | a.e.* | calculated by means of *En* score |
| *r* | *Mussel* RM | 7 | under the *repeatability* conditions [5] |
| *IP* | *Mussel* RM | 15 | under to *reproducibility* conditions (inside the laboratory) [5] |
| *R* | *Mussel* RM | 15 | estimated by means of the results obtained to *IP* |
| selectivity and linearity | *Mussel* RM;<br>*Mussel tissue* NIST SRM 2976;<br>*Oyster tissue* NIST SRM 1566b;<br>*Fish tissue* IAEA 407;<br>*Dogfish Muscle* NRCC DORM 2 | 1<br>1<br>1<br>1<br>1 | according to experimental conditions described in the Table 1 |
| uncertainty | *Mussel* RM | a.e.* | presented as the mean result obtained for relative uncertainty (*U* %) to *n* measurements |

*a.e. means "*all experiments*"

### 2.4. Define how to calculate the results and uncertainties

Results were calculated using the Microsoft Excel 2010 Spreadsheet. Equations and considerations about the calculations are presented below.

***INAA main equation* :** results for the analysis carried by INAA were calculated according to equation (1):

$$C = \frac{(A_s w_{st} C_{st})\ \mathrm{e}^{\lambda\,(ts\,-\,tst)}}{A_{st} w_s} \qquad (1)$$

where:
$C$ is the element mass fraction in the sample (mg kg$^{-1}$);
$C_{st}$ is the standard element concentration (mg kg$^{-1}$);
$A_s$ is the activity of the element in the sample (cps);
$A_{st}$ is the activity of the element in the standard (cps);

$w_s$ and $w_{st}$ are the weights of the sample and standard, respectively;
$\lambda$ is the decay constantof the radionuclide;
and ($t_s - t_{st}$) is the difference of the counting time between the sample and standard.

***LOD* and *LOQ*:** were calculated according to (2) for *LOD* and (3) for *LOQ* [8]. Results obtained for LOD and LOQ are converted to mass fraction by means of the INAA main equation (1).

$$LOD = 3{,}29\frac{\sqrt{b}}{t} \tag{2}$$

$$LOQ = 10\frac{\sqrt{b}}{t} \tag{3}$$

where:
*LOD* is the *limit of detection* (cps);
*LOQ* is the *limit of quantification* (cps);
*b* is the number of counts to background radiation for the analytical peak (cps);
and *t* is the live time of measurement.

***Trueness:*** were determinated according to *En* score (4).

$$En = \frac{x - x_v}{\sqrt{U^2 + U_v^2}} \tag{4}$$

where:
*En* is the value to *En* score;
x is the result of measurement;
$x_v$ is the reference value to RM;
*U* is the expanded uncertainty of the measurement, $k = 2$;
and $U_v$ is the uncertainty of the reference value to RM, $k = 2$.

***Precison:***
*Limit of repeatability*, *intermediate precision* and *limit of reproducibility* were calculated according to (5), (6) and (7), respectivelly.

$$r = 2{,}8\ s \tag{5}$$

where:
*r* is the *limit of repetability* (mg $kg^{-1}$);
and*s* is the standard deviation to mean results of experiments (under *repeatability* condition) ($n = 7$).

$$IP = s \tag{6}$$

where:
*IP* is *intermediate precision* (mg $kg^{-1}$);
and*s* is the standard deviation to mean results of experiments (under *reproducibility* condition) ($n = 15$).

$$R = 2{,}8\ IP \tag{7}$$

where:
*R* is the *reproducibility limit* (mg $kg^{-1}$);
and *IP* is the value obtained to *intermediate precision* ($n = 15$).

***Selectivity and linearity:***
The selectivity of the method was evaluated according the trueness results obtained to analysis of 5 different RM and/or CRM. The linearity was estimated by means of the angular coefficient value to linear regression of the results obtained in the analysis of 5 different RM and/or CRM. Acceptance criteria were presented below.

***Uncertainty:***
Estimate of the measurement uncertainty was performed using the simulated method [2, 9]. This method is based on the relationship between the uncertainty associated to each input component *f* with the measurement result. Generally it has to be (8):

$$C = \frac{f_1 f_2}{f_3} \tag{8}$$

where:
*C* is the output component, i.e, mass fraction of measurand (mg $kg^{-1}$);
and $f_x$ is the factor associated to each input component, x = {1, 2, 3}

The uncertainty $uf_x$ related to each input component $f_x$ it was estimated according to the way that $f_x$ defines the result (output component). Calculation is made by means of the variation to each input component with your respectively uncertainty. Thus, we have that (9):

$$C_{f1} = \frac{(uf_1 f_1)\, f_2}{f_3}$$
$$C_{f2} = \frac{f_1\, (uf_2 f_2)}{f_3} \tag{9}$$
$$C_{f3} = \frac{f_1 f_2}{(uf_3 f_3)}$$

Thus, each input component $f_x$ of the result it have your uncertainty easily calculated according to (10).

$$\Delta f_x = \left| C - C_{fx} \right| \tag{10}$$

where:
$\Delta f_x$ is the uncertainty assigned to the input component $f_x$;
*C* is the measurement result (mass fraction);
and $Cf_x$ is the value obtained for the result taking into account the uncertainty assigned to $f_x$.

The expanded uncertaintyof the result (*U*; $k = 2$; $\alpha = 0.05$) it is calculated according to (11).

$$U = 2\sqrt{\sum (\Delta f_x)^2} \tag{11}$$

For the estimate of the uncertainty results were considered the input components described in the INAA main equation (1).

### 2.5. Define the acceptance criteria of the results

Acceptance criteria of the results was defined as:

- According to *trueness* results:

$|En| \leq 1$ as satisfactory result;
$|En| > 1$ as unsatisfactory result.

- According to relative *uncertainty* value ($U$ %):

$|U\ \%| \leq 10$ % as satisfactory result;
$|U\ \%| > 10$ % as unsatisfactory result.

### 2.6. Define the materials, reagents and equipment to be used

***Samples*:** sub-samples of *mussel* RM and sub-samples of several CRM of similar matrix.

***Reagents*:** Spex CRM element solutions and deionized water.

***Equipaments*:** nuclear reactor, gamma-ray spectrometer, computer, analytical balance and sealing machine.

***Glassware and others*:** volumetric flasks, micropipettes, paper filter and polyethylene packing.

## 3. EXPERIMENTAL

### 3.1. Sampleand standard preparation

Subsamples of *mussel* RM [9] of 150 ± 10 mg were weighed in polyethylene packing previously cleaned, using a calibrated Shimadzu AEM-5200 analytical balance. The *working solution* was prepared by dilution of measurand Spex CRM element solutions in volumetric flask. The multielemental standards were prepared by pipetting of the *working solution* onto Whatman paper filters using calibrated Eppendorf micropipette. After drying, paper filters were folded, placed in polyethylene packaging with the same sample geometry and sealed.

### 3.2. Method validation

Experiments of method validation were performed according to conditions prescribed in the item 2 of this paper.

## 4. RESULTS AND DISCUSSION

Table 3 present the results obtained to *LOD*, *LOQ*, *r*, *IP*, *R* and relative uncertainty for each measurand.

**Table 3. Results to *LOD, LOQ, r, IP, R* and relative uncertainty for each measurand**

| **Measurand** | **Results (mg kg$^{-1}$)** | | | | | |
|---|---|---|---|---|---|---|
| | *LOD* | *LOQ* | *r* | *IP* | *R* | *U* % (*k* = 2) |
| Co | 0.0083 | 0.025 | 0.037 | 0.045 | 0.13 | 6.2 % |
| Cr | 0.066 | 0.14 | 0.066 | 0.14 | 0.40 | 8.0 % |
| Fe | 4.3 | 20 | 22 | 28 | 78 | 7.1 % |
| Rb | 0.31 | 0.95 | 0.16 | 0.39 | 1.1 | 7.4 % |
| Se | 0.18 | 0.56 | 0.12 | 0.26 | 0.73 | 9.0 % |
| Zn | 0.36 | 1.1 | 4.0 | 5.4 | 15 | 8.5 % |

Results obtained for *LOD* and *LOQ* ranged from 0.0083 mg kg$^{-1}$ and 0.025 mg kg$^{-1}$ (Co) to 4.3 mg kg$^{-1}$ and 20 mg kg$^{-1}$ (Fe), respectively. These results are very dependent on the measurands mass fraction, sample matrix, sample mass, irradiation condition, measurement condition and others factors characteristic for the measurement method. Similar values for *LOD* and *LOQ* were reported by Moreira [10].

The evaluation of the *IP*, i.e., the natural variability of the results within LAN, was performed considering four main factors: different irradiation processes, different gamma-ray spectrometers, different operating days and different analysts. Aiming to evaluate the significance of the factors, a *t*-test was performed for mean results obtained to each condition at the 5 % significance. Results did not present any significant factors for *t*-test analyses.

Values to estimate of *r* (minimum natural deviation expected for two or more independent results), *IP* (maximum variation expected for two or more independent results obtained at LAN) and *R* (maximum variation expected for two or more independent results obtained from two more different laboratories) were: 0.037 mg kg$^{-1}$, 0.045 mg kg$^{-1}$ and 0.13 mg kg$^{-1}$ for Co; 0.066 mg kg$^{-1}$, 0.14 mg kg$^{-1}$ and 0.40 mg kg$^{-1}$ for Cr; 22 mg kg$^{-1}$, 28 mg kg$^{-1}$ and 78 mg kg$^{-1}$ for Fe; 0.16 mg kg$^{-1}$, 0.39 mg kg$^{-1}$ and 1.1 mg kg$^{-1}$ for Rb; 0.12 mg kg$^{-1}$, 0.26 mg kg$^{-1}$ and 0.73 mg kg$^{-1}$ for Se; and 4.0 mg kg$^{-1}$, 5.4 mg kg$^{-1}$ and 15 mg kg$^{-1}$ for Zn, respectively.

By means of the *r*/*IP* ratio it is possible to make an interpretation about the percentage corresponding to the natural variation of the results for the method, i.e, how much of the *IP* value can be explained by the natural variability of the results (*r*).

According to *r/IP* ratio, 82 % (Co), 47 % (Cr), 78 % (Fe), 41 % (Rb), 46 % (Se) and 74 % (Zn) of the variability of the results obtained to *IP* can be explained by the natural variability

of the results. In this context, the interpretation is how the higher value found for *r*/*IP* ratio, greater robustness of the measurement method.

Trueness results ranged from -1.0 and 1.0 according to *En* calculations and were considered suitable results for all mensurands.Trueness results are presented in the Figure 1.

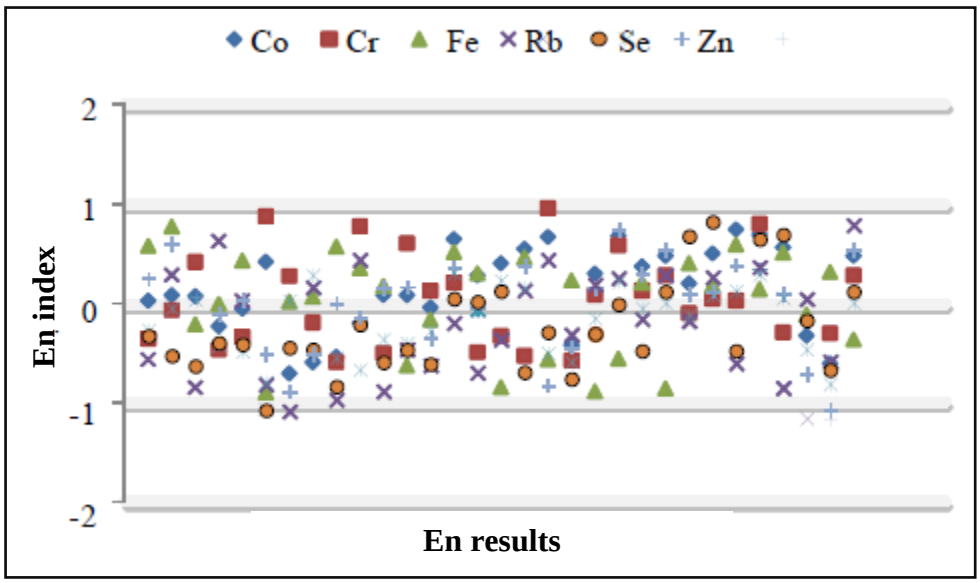


**Figure 1.Trueness results for all measurands**

In the Figure 1 it is possible observe that En results obtained in the different experiments required by the scope of method validation (except repeatability experiments that were not included in this graph) presented suitable results for all measurands, that is, were obtained values in accordance to acceptance criteria between -1.0 to 1.0 for En results (trueness results).

Relative uncertainty of the results were 6.2 % (Co), 8.0 % (Cr), 7.1 % (Fe), 7.4 % (Rb), 9.0 % (Se) and 8.5 % (Zn), considering the mean results for all experiments. Moreira, *et al*. [11] using a similar method reported values obtained to relative uncertainty for analysis of biological samples between 6.0 % to 13 % (Co), 4.4 % to 36 % (Cr), 5.9 % to 9.0 % (Fe), 7.1 % to 14% (Se) and 3.5 % to 4.8 % (Zn). Thus, it is possible observed that the results obtained in this study are very similary those found to Moreira, *et al*. França, *et al*. [12] investigated the concentration of some measurands in samples of *Marlierea tomentosa* by means use of Quantu Software obtaining values to relative uncertainty of 0.7 % (Co), 1.5 % (Fe) and 2.6 % (Rb). These values are much lowest than those found in this study, suggesting that a different method for uncertainty calculation was utilized by the authors.

According to the results, three main sources of uncertainty in INAA were observed: radionuclide activity in the sample, radionuclide activity in the standard and mass of measurand in the standard. These uncertainties sources are between 96% and 99% of the total uncertainty on the results. Mass of measurand in the standard was the most important source of uncertainty for Cr, Fe, Rb and Se, while that radionuclide activity in the standard was most important source of uncertainty for Co and Zn. Figure 2 present main sources of uncertainty for each measurand in INAA.

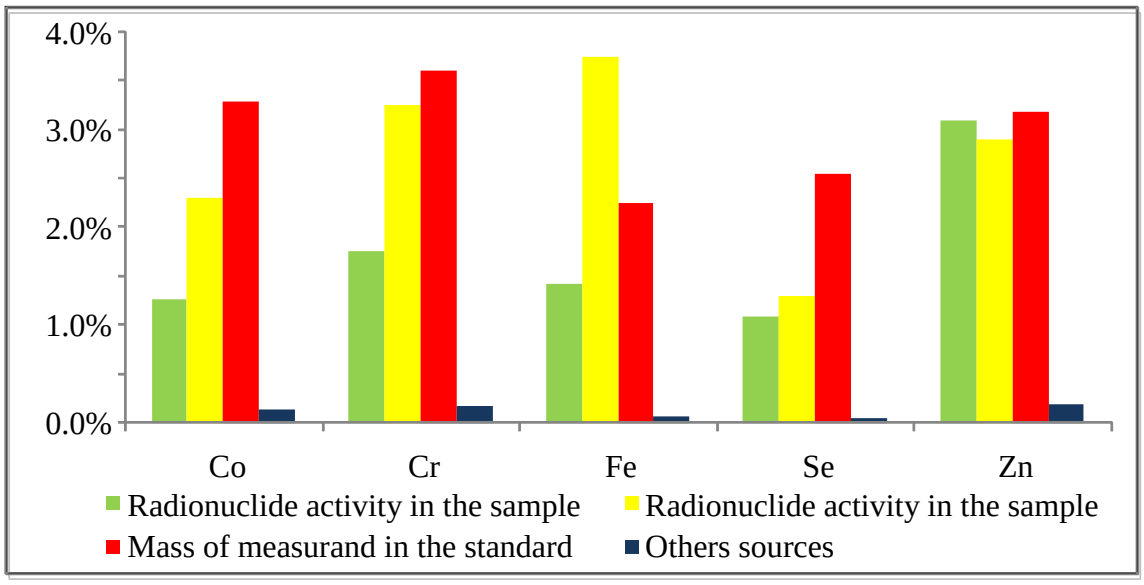


**Figure 2.Main sources of uncertainty on the results**

Table 4 present the results obtained for selectivity and linearity.

**Table 4.Selectivity and linearity results in INAA method**

| | Co | Cr | Fe | Rb | Se | Zn |
|---|---|---|---|---|---|---|
| *Mussel* RM | 0.833 ± 0.032 (0.829 ± 0.077) | 1.37 ± 0.12 (1.24 ± 0.28) | 577 ± 23 (593 ± 53) | n.a.[a] (n.c.)[b] | 4.55 ± 0.34 (4.42 ± 0.45) | 110.3 ± 8.3 (118.5 ± 9.5) |
| NIST SRM 2976 | 0.66 ± 0.053 (0.61 ± 0.02) | 0.48 ± 0.024 (0.5 ± 0.16) | 189 ± 16 (171 ± 4.9) | 4.02 ± 0.12 (4.14 ± 0.09) | 1.81 ± 0.16 (1.8 ± 0.15) | 125 ± 15 (137 ± 13) |
| NIST SRM 1566b | 0.356 ± 0.019 (0.371 ± 0.009) | n.a.[a] (n.c.)[b] | 206 ± 11 (205.8 ± 6.8) | 3.52 ± 0.18 (3.26 ± 0.14) | 1.95 ± 0.11 (2.06 ± 0.15) | 1480 ± 106 (1424 ± 46) |
| IAEA 407 | 0.2 ± 0.011 (0.182 ± 0.031) | 40.8 ± 3.6 (34.7 ± 5.5) | 161 ± 15 (142 ± 10) | n.a.[a] (n.c.)[b] | 1.389 ± 0.081 (1.4 ± 0.09) | 22.9 ± 1.7 (25.6 ± 2.3) |
| NRCC DORM 2 | 0.0965 ± 0.0023 (0.1 ± 0.01) | 0.811 ± 0.052 (0.73 ± 0.06) | 158.5 ± 9.3 (146 ± 3) | 3.15 ± 0.13 (2.86 ± 0.41) | 2.89 ± 0.11 (2.83 ± 0.13) | 64.3 ± 2.1 (67.1 ± 0.8) |
| | | | | | | |
| Linearity | 0.997 | 0.999 | 0.999 | 0.992 | 0.999 | 0.999 |

[a] (n.c.) means "*not certified*" value for the measurand;
[b] n.a. means "*not analysed*"

The selectivity of method was evaluated according to the results obtained for trueness from 5 different RM and/or SRM. Suitable results were obtained for all measurands, i.e, were obtained $|En| \leq 1$ values. Linearity of the method was estimated by means of the angular coefficient to linear regression for each measurand. Results obtained were 0.997 (Co), 0.999 (Cr), 0.999 (Fe), 0.992 (Rb), 0.999 (Se) and 0.999 (Zn) representing good linearity to INAA.

## 5. CONCLUSION

In this study it was proposed a standardized procedure for method validation and calculation of the measurement expanded uncertainty of result in INAA. It was concluded that suitable results of method validation were obtained for mass fraction determination of Co, Cr, Fe, Rb, Se and Zn in biological matrix samples. The estimate of the uncertainty sources was performed according to the recommendations of the ISO GUM, using the simulated method. Relative uncertainty values were 6.2 % (Co), 8.0 % (Cr), 7.1 % (Fe), 7.4 % (Rb), 9.0 % (Se) and 8.5 % (Zn), considering the mean results for all experiments. According to the results, the main sources of uncertainty were radionuclide activity in the sample, radionuclide activity in the standard and mass of measurand in the standard. This study presented in a easily, fast and convenient way, the step by step to performing of method validation in INAA. The whole process was conducted in accordance with the guidelines ISO/IEC 17025.

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