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Use of INAA in the homogeneity evaluation of a bovine kidney candidate reference material

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Abstract Instrumental neutron activation analysis was used to evaluate the between bottle homogeneity and the minimum sample intake of a bovine kidney candidate reference material. The mass fractions of ten inorganic constituents were determined, obtaining satisfactory homogeneity results for all of them. Statistical analysis of the results was performed using a one way analysis of variance and multivariate techniques were applied as complementary techniques, confirming the usefulness of these techniques for homogeneity assessment.

Keywords Bovine kidney reference material · Homogeneity study · Neutron activation analysis · Principal component analysis · Hierarchical cluster analysis

Introduction

The use of reference materials is a fundamental tool in the achievement of comparability and traceability of chemical measurements. They can be used in many of the analytical process steps needed to ensure the quality of measurements, such as validation of analytical methods, estimation of measurement uncertainties, personnel training, in proficiency testing and internal quality controls using statistical process control with control charts [1–3]. Reference materials should be as similar as possible (matrix and mass fraction) with the sample to be analyzed for covering all analytical problems that may cause measurement errors [4].

Despite its great importance, the production of reference materials in the South American region is still incipient, especially in complex matrices, such as may be those of biological origin. This fact affects the food production and consumption field where a huge amount of chemical measurements are performed in this type of materials. In particular, analyses regarding meat products are very relevant. Brazil is the main exporter country of bovine meat in the world, and on the other hand, the consumption of bovine meat per capita in Brazil is in the second place in the South American region [5]. An increase in the amount of locally produced reference materials with meat matrix is needed, to ensure the quality and safety of the products consumed by the population, to support the national industry and also to facilitate trade within the region. In this context, a new local reference material of bovine kidney has been prepared.

Evaluation of the homogeneity is a critical step in the preparation process of any reference material. The material batch must be sufficiently homogeneous for the intended use and this must be reflected in the value assigned to the material uncertainty and by the minimum sample intake for which the assigned values and their uncertainties are valid. To ensure the representativeness of the value assigned to the certificate parameters and their uncertainties, the assessment of the homogeneity of the material must be performed very carefully. Instrumental neutron activation analysis (INAA) has proved to be a method of election for the homogeneity evaluation because its intrinsic characteristics such as high sensitivity, accuracy and precision [6] and it has been widely used for this purpose [7–10].

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Table 1 Description of the main steps in preparation of the bovine kidney reference material

Preparation of the material	
Pre treatment of the material	Removal of connective and fatty tissue using ceramic knives. Preparation of pieces for freeze-drying
Freeze-drying	24 h freeze-drying to achieve residual water content of approximately 5 %
Grinding	Planetary ball mill RETSCH, PM 400. Grinding jars: tungsten carbide. Grinding balls: zirconium oxide
Sieving	Vibratory sieve shaker Fritsch, Analysette 3. Sieves 160, 140, 125 and 100 μm. The minor fraction was the only one chosen as candidate reference material
Homogenization	For 72 h using Y shape mixer Marconi, MA 201/10 MO/E
Bottling	In amber glass bottles previously cleaned and decontaminated. Final batch: 175 flasks containing approximately 12, 6 g each one
Sterilization	Performed with a dose of 10 kGy of gamma radiation using a ^{60}Co source

The present work describes the use of INAA for the between homogeneity evaluation and the minimum sample intake of the prepared bovine kidney material.

Given that the intended use of the reference material is the quality control of meat products, some elements with nutritional relevance or that could constitute potential food contaminants were chosen for being determinate. The elements selected for the present study were As, Cl, Co, Cr, Fe, Mg, Mn, Na, Se and Zn. In the selection of these elements it was also taken into account the nutritional food labeling regulations from several South American countries, in which some elements must appear compulsorily (Na and Fe) and others are usually added as additional information to the population (Cl, Mg, Se and Zn).

Statistical analysis of the results was performed using a one way analysis of variance (ANOVA) and multivariate techniques such as principal component analysis (PCA) and hierarchical cluster analysis (HCA) were applied to complement the traditional univariate analysis. Multivariate techniques take into account the correlation among many variables at the same time, and for this reason, they can provide much more information than univariate techniques, for example, by showing an underlying structure of data not visible by other means [11]. On the other hand, they also make possible a graphical representation of a larger amount of information allowing a simpler visualization of the data set and facilitating its evaluation [12]. Despite its usefulness there are not many precedents in using these statistical techniques in the evaluation of reference materials homogeneity. Some exceptions are the studies performed on wheat and corn flour [13, 14] and in pharmaceutical products by the Brazilian National Institute of Metrology, Quality and Technology (INMETRO) [15, 16].

Experimental

Preparation of the candidate reference material

The candidate reference material was prepared using 35 kg of fresh bovine kidney from cattle reared under controlled feeding conditions. The kidneys were grinded, lyophilized and sieved to achieve a material with particle size less than 100 μm. The description of each step is shown in Table 1.

Table 2 Certified values, measured values and E_n score calculated for analyzed CRMs

Element	SRM 1577b	Obtained value	E_n	SRM 1577c	Obtained value	E_n
As (mg kg^{-1})	0.05[a]	<0.34	–	0.0196 ± 0.0014	<0.34	–
Cl (%)	–	–	–	0.287 ± 0.013[b]	0.285 ± 0.024	−0.07
Co (mg kg^{-1})	0.25[a]	0.241 ± 0.008	–	0.300 ± 0.018	0.309 ± 0.008	0.5
Cr (mg kg^{-1})	–	–	–	0.053 ± 0.014	<0.14	–
Fe (mg kg^{-1})	184 ± 15	194.3 ± 6.3	0.6	197.94 ± 0.95	195.6 ± 5.6	−0.4
Mg (mg kg^{-1})	–	–	–	620 ± 42	621 ± 50	0.01
Mn (mg kg^{-1})	–	–	–	10.46 ± 0.47	10.3 ± 1.2	−0.1
Na (%)	0.242 ± 0.006	0.246 ± 0.011	0.3	0.2033 ± 0.0064	0.195 ± 0.008	−0.8
Se (mg kg^{-1})	0.73 ± 0,06	0.79 ± 0.11	0.5	2.031 ± 0.045	2.13 ± 0.11	0.8
Zn (mg kg^{-1})	127 ± 16	124.7 ± 2.9	−0,1	181.1 ± 1.0	177.3 ± 5.1	−0.7

[a] Informative value

[b] Reference value

Table 3 Mass fraction obtained by INAA for each analyzed bottle of the candidate reference material

Bottle	Cl (%)	Co (mg kg^{-1})	Fe (mg kg^{-1})	Mg (mg kg^{-1})	Mn (mg kg^{-1})	Na (%)	Se (mg kg^{-1})	Zn (mg kg^{-1})
1	0.907 ± 0.046	1.76 ± 0.13	252 ± 17	720 ± 131	5.28 ± 0.72	0.825 ± 0.085	6.81 ± 0.46	90.5 ± 4.2
37	0.942 ± 0.079	1.706 ± 0.095	243 ± 14	712 ± 91	5.18 ± 0.90	0.829 ± 0.031	6.70 ± 0.31	88.3 ± 5.0
55	0.945 ± 0.046	1.661 ± 0.085	247 ± 15	730 ± 68	4.86 ± 0.72	0.814 ± 0.034	6.63 ± 0.30	89.6 ± 2.7
62	0.958 ± 0.082	1.703 ± 0.060	252 ± 11	652 ± 82	4.84 ± 0.81	0.822 ± 0.046	6.83 ± 0.19	90.8 ± 2.7
76	0.904 ± 0.101	1.652 ± 0.081	246 ± 8	665 ± 121	4.84 ± 0.66	0.814 ± 0.035	6.77 ± 0.16	89.6 ± 1.8
106	0.888 ± 0.093	1.653 ± 0.074	247 ± 24	689 ± 75	5.24 ± 0.65	0.795 ± 0.045	6.70 ± 0.39	89.6 ± 5.5
117	0.889 ± 0.074	1.618 ± 0.082	250 ± 21	721 ± 120	5.09 ± 0.83	0.826 ± 0.077	6.69 ± 0.40	88.7 ± 6.1
123	0.903 ± 0.128	1.686 ± 0.098	245 ± 13	716 ± 71	4.70 ± 0.76	0.798 ± 0.034	6.74 ± 0.33	90.8 ± 5.0
156	0.878 ± 0.058	1.659 ± 0.079	248 ± 13	665 ± 58	4.61 ± 0.42	0.804 ± 0.036	6.84 ± 0.27	90.4 ± 4.6
171	0.957 ± 0.135	1.644 ± 0.036	252 ± 13	690 ± 118	4.92 ± 0.99	0.804 ± 0.066	6.75 ± 0.16	90.1 ± 1.5

Mean result and uncertainty at 95 % confidence level for $n = 5$

Experimental design

For the between bottle homogeneity evaluation, ten bottles from the total batch of 175 were chosen using a random stratified scheme. Five test portions of each bottle were randomly measured by instrumental neutron activation analysis for the determination of As, Cl, Co, Cr, Fe, Mg, Mn, Na, Se and Zn in concomitance with certified reference materials (CRM) Bovine Liver NIST SRM 1577b and 1577c. CRMs were used as quality control materials.

For the minimum sample intake one bottle was chosen. The concentration of the same elements was determined in test portions with masses of 20, 50, 100, 150 and 250 mg. Five test portions from each one of these masses were measured.

Preparation of element calibration standards

Calibration standards were prepared from high purity standard solutions (SPEX Certiprep Inc., USA and LGC Standards, UK) or appropriate dilutions of these standard solutions using Milli-Q water (Millipore Corporation, USA). Appropriate aliquots of these solutions were pipetted onto Whatman 40 filter papers and dried inside a laminar flow hood. After drying, filter papers were transferred to polyethylene bags, previously cleaned with 10 % nitric acid and Milli-Q water.

Table 4 ANOVA results for the between bottle homogeneity study

Element	$MS_{between}$	MS_{within}	F	P_{value}	u_{bb} (%)
Cl	464,813	709,284	0.65	0.74	2.8
Co	0.0080	0.00466	1.72	0.11	1.5
Fe	52.0	152.8	0.34	0.96	1.5
Mg	3852	6053	0.64	0.76	3.4
Mn	0.266	0.380	0.70	0.70	3.8
Na	68,123	179,859	0.38	0.94	1.7
Se	0.023	0.063	0.37	0.94	1.1
Zn	3.6	11.4	0.31	0.96	1.2

$F_{critic} = 2.12$ is the F distribution critical value for the level of significance $\alpha = 0.05$, and degrees of freedom $\upsilon_1 = 9$; $\upsilon_2 = 40$

Irradiation and element determination

All the measured test portions or CRMs were weighed in polyethylene bags, previously cleaned with 10 % nitric acid and Milli-Q water. Test portions, element calibration standards and CRMs were irradiated under a thermal neutron flux of 4.6×10^{12} cm^{-2} s^{-1} at the research nuclear reactor IEA-R1 of the Nuclear and Energy Research Institute, IPEN—CNEN/SP, São Paulo, Brazil.

To perform the determination of short lived radionuclides (^{27}Mg, ^{56}Mn and ^{38}Cl), test portions, element calibration standards and CRM were irradiated simultaneously for 20 s. Mg and Cl radionuclides were measured for 300 s, immediately after irradiation. Mn radionuclide was measured for 300 s, after a 30-min decay period.

To perform the determination of ^{76}As, ^{60}Co, ^{51}Cr ^{59}Fe, ^{24}Na, ^{75}Se and ^{65}Zn radionuclides, test portions, element calibration standards and CRMs were irradiated for 6 h. ^{76}As and ^{24}Na radionuclides were measured for 1 h, after a 7-day decay period. ^{60}Co, ^{51}Cr ^{59}Fe, ^{75}Se and ^{65}Zn radionuclides were measured for 6 h, after a 21-day decay period.

Gamma ray measurements were performed using a GC2018 Canberra HPGe detector coupled to a Canberra DSA-1000 digital spectral analyser. The resolution (FWHM) of the system was 1.8 keV at the 1332 keV gamma peak of ^{60}Co and the relative efficiency was 20 %. Gamma ray spectra were collected and processed using a Canberra Genie 2000 version 3.1 spectroscopy software and all element content calculations were carried out using a Microsoft Excel spreadsheet.

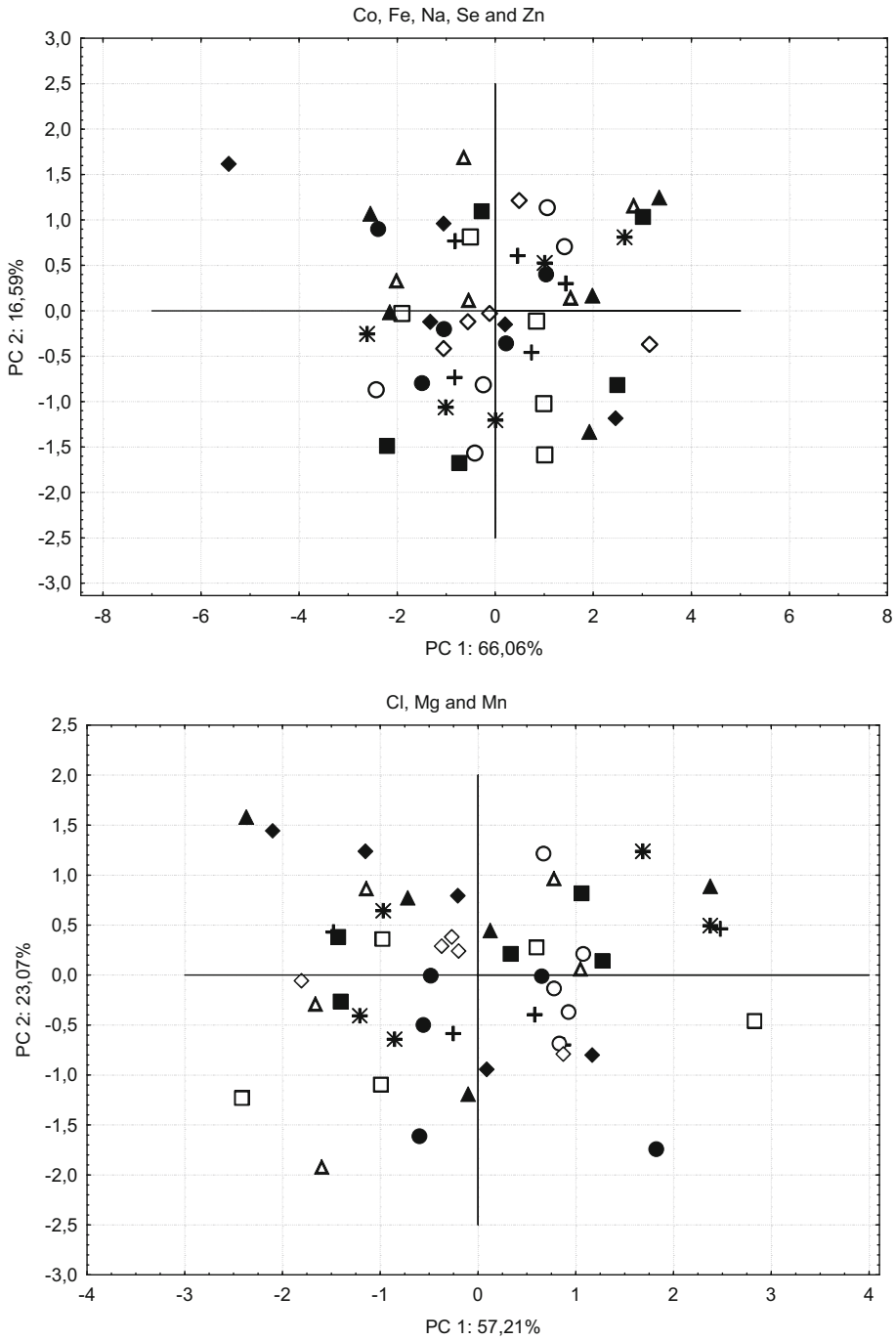

Fig. 1 Score plot of the first two principal components for the between bottle homogeneity study

Results and discussion

Data quality control

In order to evaluate the trueness of the measurements, two certified reference materials (Bovine Liver NIST SRM 1577b and 1577c) were irradiated simultaneously with the test portions to be analyzed. In the long time irradiation, ten aliquots of each CRM were measured. In the short time irradiation, eight aliquots of SRM 1577c were analyzed.

The values obtained were evaluated using E_n score [17] described as follows:

$$E_n = \frac{(x_i - x_{ref})}{\sqrt{(U_i^2 + U_{ref}^2)}}$$

where x_i is the value obtained experimentally, x_{ref} is the certified value of the CRM, U_i is the measurement expanded uncertainty and U_{ref} is the expanded uncertainty of the certified value. One result is considered satisfactory when $|E_n| < 1$. Table 2 presents the certified values and

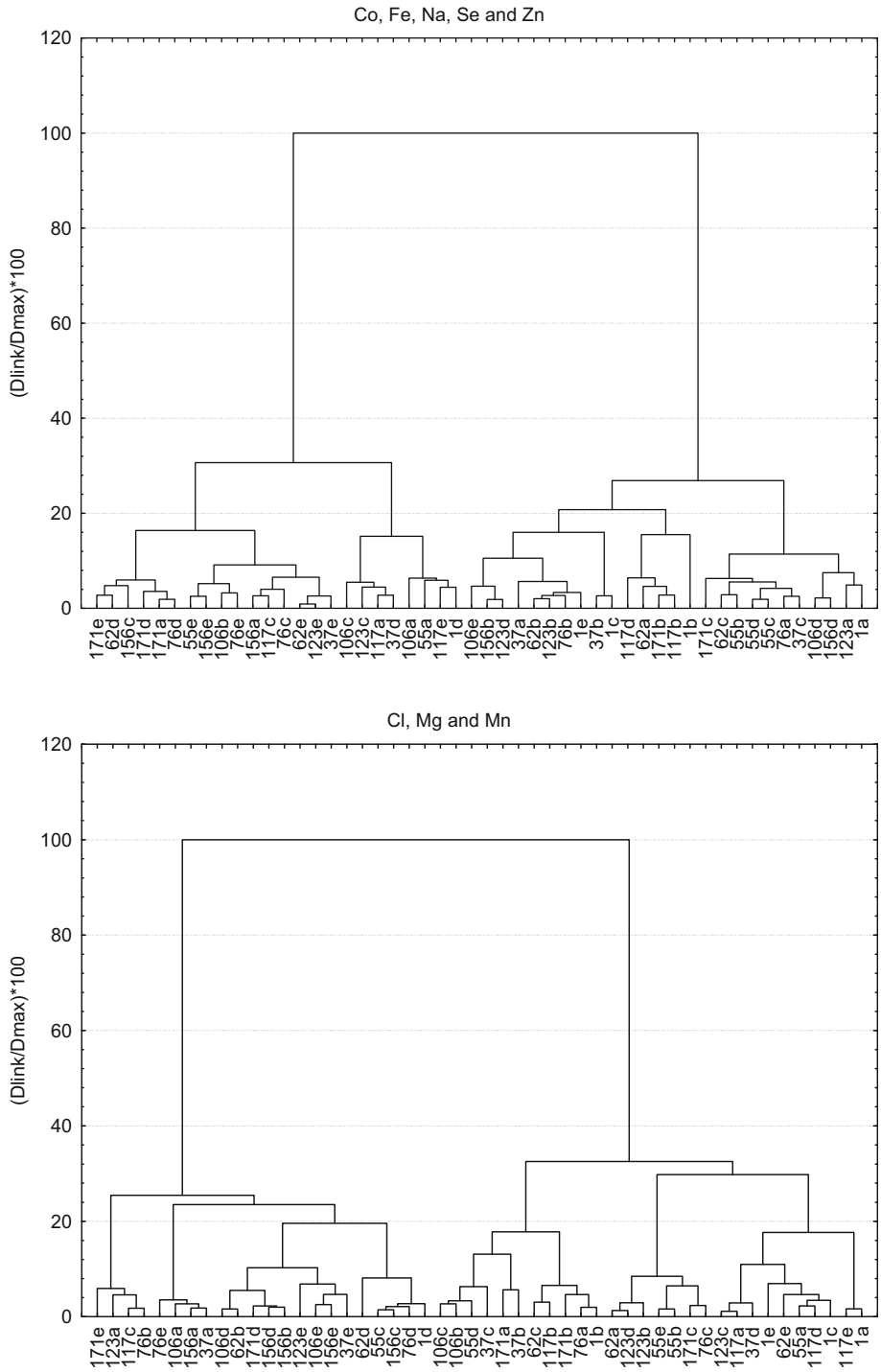

Fig. 2 Dendogram for the HCA results using Ward's clustering method with Euclidean distances for the between bottle homogeneity study

expanded uncertainties for both CRMs measured, along with the mean values and expanded uncertainties obtained in this study and the calculated E_n. The standard uncertainty was calculated using the standard deviation of the mean and the coverage factor, k, used to expand the uncertainty, was chosen based on the two-tailed value of Student's t distribution for $(n - 1)$ degrees of freedom and 95 % confidence.

As it may be seen in the table, all the results obtained were satisfactory.

Between bottle homogeneity study

Table 3 shows the mean value for each element obtained in the bottles analyzed in the between bottle homogeneity study, expressed in mass fraction, and the estimated

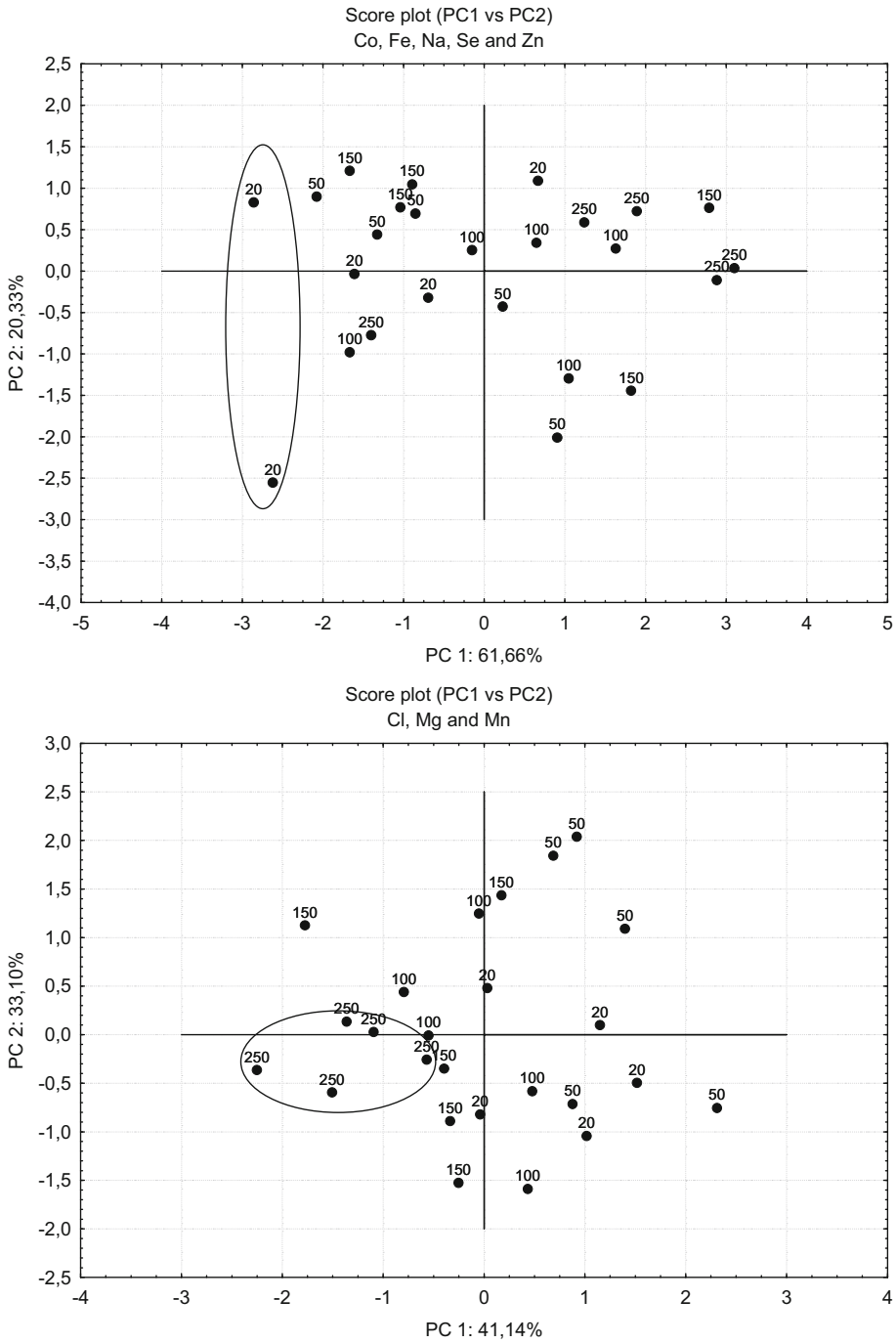

Fig. 3 Score plot of the first two principal components for the minimum sample intake study

expanded uncertainty. The standard uncertainty was calculated using the standard deviation of the mean and the coverage factor, k, used to expand the uncertainty, was chosen based on the two-tailed value of Student's t distribution for four $(n - 1)$ degrees of freedom and 95 % confidence ($k = 2.8$).

In all the analyzed test portions, As and Cr were below the detection limit (0.34 mg kg^{-1} for As and 0.14 mg kg^{-1} for Cr), for this reason, no results are presented for this two elements.

For each analyzed element, a one way analysis of variance (ANOVA) was performed. Table 4 presents the obtained values for F calculated and p values, along with mean square values (MS), obtained by ANOVA. In all cases the obtained results were satisfactory, since no statistically significant differences were observed between test

Table 5 Results for the minimum sample intake study

Element	p_{value} ($\alpha = 0.05$)	Data set used in ANOVA	Minimum sample intake (mg)
Cl	0.27	20, 50, 100 and 150 mg test portions	50
Co	0.30	100, 150 and 250 mg test portions	100
Fe	0.23	20, 50, 100, 150 and 250 mg test portions	50
Mg	0.10	20, 50, 100 and 150 mg test portions	50
Mn	0.24	20, 50, 100, 150 and 250 mg test portions	50
Na	0.76	20, 50, 100, 150 and 250 mg test portions	50
Se	0.42	20, 50, 100, 150 and 250 mg test portions	50
Zn	0.23	20, 50, 100, 150 and 250 mg test portions	50

portions of each bottle. Mean square values obtained were also used to estimate the contribution of the residual heterogeneity of the material (u_{bb}) to the combined uncertainty of the assigned values, as is recommended in ISO Guide 35 [4]. Calculated u_{bb} for each component, relative to the mass fraction, is also shown in Table 4.

To confirm and complement this univariate analysis a principal component analysis (PCA) was performed on the standardized data using Statistica 7.0 software program [18]. The standardization consisted in substracting the mean value from each data and then dividing it by the standard deviation. The data were separated in two groups for analysis because of the differences in the irradiation conditions used to measure the elements in each of them. The first group consisted of the elements Co, Fe, Na, Se and Zn, that were measured by the long time irradiation, and the second one, consisted of the elements Cl, Mg and Mn that were measured by the short time irradiation. Figure 1 shows, for each group of elements, the score plots for the first two principal components which explain approximately 80 % of the total variability. Each analyzed bottle is represented by a different type of mark. Results of all the analyzed test portions are shown in the graph, so each bottle mark appears five times. As it may be seen, no evident group or tendency can be observed, indicating the homogeneity of the samples in all cases.

To confirm this result a hierarchical cluster analysis (HCA) using Wards clustering method was performed, using Statistica 7.0 software program [18]. As shown in Fig. 2, results for the different test portions are separated into two major groups at an approximate linkage distance of 30, showing a high similarity between them. On the other hand, all the analyzed sub-samples are scattered in all the subgroups indicating an homogeneous distribution. These results are in agreement with that obtained by PCA results.

Minimum sample intake evaluation

For each analyzed element, ANOVA was performed. For Fe, Mn, Na, Se and Zn no statistically significant differences were observed between the analyzed test portions with different masses.

In the case of Cl, Co and Mg statistically significant differences were found and so it was investigated which one of the test portions was responsible for this differences. A PCA was applied obtaining the patterns shown in Fig. 3. In the case of short lived radionuclides it can be seen a grouping tendency in 250 mg test portions, due probably to gamma ray self absorption and the elevation of dead times, a limitation of the technique. For Cl and Mg the ANOVA was re applied without taking into account the 250 mg test portions data. The results obtained showed no significant differences between the data, confirming that lower mass test portions were statistically equivalent.

In the case of long lived radionuclides it can be seen a higher dispersion in 20 mg test portions. This higher dispersion was observed for all the analyzed elements. For the case of Co, satisfactory results were obtained by applying ANOVA when 20 and 50 mg test portions were omitted from the analysis. Taking all these facts into account it was decide to choose 50 mg as the minimum sample intake for all the elements, except Co, despite this value is probably an overestimation for most of the analyzed elements. Table 5 summarizes the obtained results.

Conclusions

The between bottle homogeneity for the bovine kidney candidate reference material was assessed, at a 95 % confidence level, by analysis of variance and confirmed by multivariate analysis for Cl, Co, Fe, Mg, Mn, Na, Se and Zn. Multivariate techniques proved to be useful tools in homogeneity evaluation of samples, as simplifies graphical representation and visualization of the data set. Values obtained for u_{bb} are considered low and will not significantly affect the expected final uncertainty.

As and Cr were below the detection limit in all the analyzed test portions.

The estimated minimum sample intake was 50 mg for all the elements with the exception of Co, where the estimated minimum sample intake was set at 100 mg even though these values are probably an overestimation for most of the analyzed elements.

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