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# Experimental design and *z*-score results are useful tools for optimizing instrumental neutron activation analysis procedures

**Uanda P. M. Santos[1] · Felipe R. Lourenço[2] · Edson G. Moreira[1]**

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**Abstract**
In this study $2^k$ experimental designs were used for the optimization of an instrumental neutron activation analysis method for the determination of bromine, chlorine, magnesium, manganese, potassium, sodium and vanadium in biological matrix materials. Depending on radionuclide half-lives, $2^3$ or $2^4$ experimental designs were used with irradiation time, sample–detector distance, counting time and sample decay time as factors. Gamma ray spectrometer, comparator mass fraction and sample mass were kept constant. Optimized irradiation and measurement conditions were established based on the experimental design results that used *z*-scores as response on a reference material for the various elements and experimental conditions.

**Keywords** Neutron activation analysis · Experimental design · Factorial design · *z*-Score · Method validation

## Introduction

Design of experiments (DOE) or experimental design is a multivariate methodology of experiment planning used for the optimization of methods and processes. Regardless of the model used, DOE presents a number of advantages over the use of a univariate (conventional) methodology, such as the possibility of reduction in the number of rounds or experiments; indication of the major or crucial variable or variables of the investigated process and the way the most diverse variables correlate to each other [1]. Experimental design, also called factorial design, represents a set of tests established with scientific and statistical criteria in order to determine the influence of several variables on the results of a given system or process [2].

In a previous study at our institute, a $2^3$ experimental design was carried out with biological and geological samples, aiming the analysis of As, Co, Cr, Sb, Sc and Zn by instrumental neutron activation analysis (INAA), using the corresponding long lived radionuclides [3]. The idea was to optimize the experimental conditions in order to carry out the formal process of method validation at the laboratory. As far as we know, DOE was not applied in any other nuclear analytical technique up to now and the purpose of this study was to apply the $2^3$ experimental design and other types of planning, such as $2^4$, in the optimization of an INAA method using short time irradiations at a pneumatic system of the IEA-R1 reactor at IPEN—CNEN/SP. In this case, Br, Cl, K, Mg, Mn, Na and V mass fractions were determined by INAA in a biological matrix reference material and commercial software was used to evaluate the experimental design output, also with method validation in mind.

## Theory

### Instrumental neutron activation analysis

INAA is used to determine mass fractions of elements present in several matrices. The technique has the advantage of being multielemental and determining a wide range of elements in small samples (usually 50–200 mg) [4]. Recently, the character of INAA as a primary method of analysis has been proven, a fact that is of great relevance for its application in the certification of reference materials [5]. The comparative method of INAA consists of simultaneously subjecting samples and elemental standards with known mass

✉ Edson G. Moreira
emoreira@ipen.br

[1] Research Reactor Center, Nuclear and Energy Research Institute, Av. Prof. Lineu Prestes, 2242, São Paulo, SP 05508-000, Brazil

[2] School of Pharmaceutical Sciences, University of São Paulo, Av. Prof. Lineu Prestes, 580, São Paulo, SP 05508-000, Brazil

fractions (comparators) to the thermal neutron flux produced in a nuclear reactor and, after an appropriate decay period, performing element mass fraction determination by comparing the photopeak area of the samples with the photopeak area of the standard, obtained by gamma spectrometry [4].

## Experimental design

Experiment design techniques are basically used to improve product quality characteristics of manufacturing processes, reduce the number of tests and optimize the use of company resources (material, time of employees, availability of equipment etc.) [6]. This general objective can be divided into other secondary objectives:

- identify the variables or control *factors* of the process that most influence the *response* parameters of interest;
- assign values to influential variables of the process so that the variability of the response of interest is minimal or that the value of the result (quality parameter) is close to the nominal value;
- assign values to the influential variables of the process so that the *effect* of the non-controllable variables is reduced.

The most appropriate method to treat several factors is a *factorial experiment* or *factorial design* and hence these names have been used as synonym to *experimental design* [6]. In this approach, the factors vary together, rather than one at a time. If we have $k$ factors, each with two *levels*, the experiment will be $2^k$ factorial. For experiments with four, five or more factors, it is not necessary to use all possible combinations. In such cases, the technique to be used will be the fractional factorial experiment, which is a variation of the complete factorial experiment.

The three basic principles of experiment planning are randomization, replication, and blocking. Through randomization, the allocation of the experiment material and order in which the observations of the experiment are performed and determined at random. Replication is the independent repetition of each combination of factors. Replication has two important properties: it allows obtaining an estimate of experimental error; this estimate is used to evaluate the difference between the statistically significant data. Blocking is a planning technique to improve the accuracy of how the comparison of factors of interest is done. Generally, blocking is used to decrease or eliminate the variability transmitted by factors that may influence the response [6].

Factorial designs are more efficient to study the effects of two or more factors in an experiment as in each replication, all combinations of *factor levels* are investigated. The variation of the response produced by a factor variation is what we call the *effect of a factor*. By often referring to the *primary factors of interest*, we call it the *principal effect*. The high and low levels are denoted respectively by "level + 1 "and "level − 1". Some specific models may also require information on the factor configuration that is assigned to the default level, which is called "level 0" [7].

A factorial experiment with $k$ factors, each with two levels is called a $2^k$ experiment. The experimental process of this technique consists on performing tests with each of the combinations of the *experimental matrix*, to then determine and interpret the *main effects* and *interactions* of the investigated factors of the product, manufacturing process or system under investigation [7].

The major advantage of using a DOE is the realization of a multivariate optimization; through the calculations of the *main effect* and *interaction effects* it is possible to understand how the different factors are correlated and the importance that a factor carries in the final result. In addition, the method requires a smaller number of experiments when compared to the univariate optimization methodology. This implies lower expenses and less time required for the execution of the planning [8–10].

Complete $2^3$ and $2^4$ experimental designs were used in this study. The respective designs were investigated in two different levels, in which one expects to obtain more favorable results (level + 1) and the other one less favorable results (level − 1), for the measurement result accuracy in INAA.

The factors evaluated in the $2^3$ design for the INAA method optimization study were: irradiation time (A), sample–detector distance (B) and counting time (C). This experimental design was used to investigate the determination of Mg and V, elements with the shorter half-lives in this study ($^{27}$Mg: 9.458 min; $^{52}$V: 3.75 min [11]). For the determination of Br, Cl, K, Mn and Na, with longer lived analytical radionuclides ($^{80}$Br: 17.68 min; $^{38}$Cl: 37.24 min; $^{42}$K: 12.360 h; $^{56}$Mn: 2.58 h; $^{24}$Na: 14.96 h [11]), the $2^4$ experimental design was used, where besides A, B and C, decay time (D) was also evaluated.

# Experimental

## $2^3$ and $2^4$ experimental designs

Table 1 presents the assigned levels for the investigated factors used for the $2^3$ and $2^4$ experimental designs for the INAA method used. The different sample to detector distances (factor B) were obtained using fixed shelf positions over the detector window (shelf 0 at 3 mm and shelf 2 at 60 mm). It should be noted that for factor C, shorter ($2^3$) and longer ($2^4$) counting times were used due to the different half-lives between the two experiments.

**Table 1** Factors and levels for the $2^3$ and $2^4$ experimental designs

| Factor | | Level + 1 | Level − 1 | Level 0 |
|---|---|---|---|---|
| A | Irradiation time, s | 60 | 15 | 30 |
| B | Sample–detector distance, mm | 3 (Shelf 0) | 60 (Shelf 2) | 30 (Shelf 1) |
| C | Counting time, s | 300 | 100 | 600 |
| | | 2700 | 1800 | 2100 |
| D | Decay time, s | 3600 | 1800 | 2700 |

## Instrumental neutron activation analysis

Approximately 0.2 g test samples of a mussel tissue certified reference material (CRM) produced at IPEN—CNEN/SP were taken to perform the experimental designs [12].

Multielemental standards for Mg and V (MV standards), prepared from Spex CRM solutions, along with test samples of the mussel tissue CRM were assembled in eight experiments, i.e., in eight polyethylene irradiation vials, for the $2^3$ factorial design as presented in Table 2. Multielemental standards for Na, K and Mn (NKM standards) and for Cl and Br (CB standards) were prepared from Spex CRM solutions and assembled with test samples of the mussel tissue CRM in 16 irradiation vials, for the $2^4$ factorial design, presented in Table 3. Both designs were subjected to element determination of 3 test samples ($n=3$) for each experiment, performed at random. Standards and test samples were irradiated at the IEA-R1 research reactor at 4.5 MW, under a neutron flux of 0.5 to $1.0\times10^{12}$ $cm^{-2}$ $s^{-1}$ attained at the pneumatic station facility.

After irradiation, gamma radiation measurements were performed using a Canberra GC2018 HP Ge detector coupled to a Canberra DSA-1000 digital spectrum analyzer. Gamma-ray spectra were collected and processed using the Genie 2000 program, version 3.1 (Canberra). The calculation of the element mass fractions was done using a Microsoft Excel spreadsheet.

Data interpretation for the statistical treatment of the factorial experimental design was performed with the aid of the Minitab 18 software for *t* test and the analysis of variance (ANOVA) studies as well as for graphical representations. In a complementary way, *z*-scores were calculated on the obtained results, using the CRM certified values for the element mass fractions and their uncertainties as reference.

## Results and discussion

The $2^3$ factorial design results for Mg and V are presented in Table 2, while the results for the $2^4$ factorial design are presented in Table 3 for Br, Cl, Mn, Na and K. All results were randomly obtained and Mg and V results were obtained on the same day. From a first screen on the results presented in Table 2, it could be verified that, except for some V results, there was no great variability in the mean mass fraction results over the several experiments performed. Apparently the variations in the factors were not high enough to cause changes in the obtained mass fractions. Similar response was observed for the elements evaluated in the $2^4$ factorial design.

**Table 2** Mass fraction results obtained by INAA at a $2^3$ factorial design in biological matrix sample (mean value ± standard deviation, $n=3$) [12]

| Experiment | Factor and level | | | Mass fraction | |
|---|---|---|---|---|---|
| | A | B | C | Mg, % | V, mg $kg^{-1}$ |
| 1 | −1 | −1 | −1 | 0.29 ± 0.10 | 2.80 ± 0.21 |
| 2 | +1 | −1 | −1 | 0.31 ± 0.04 | 2.89 ± 0.11 |
| 3 | −1 | +1 | −1 | 0.32 ± 0.05 | 2.91 ± 0.06 |
| 4 | +1 | +1 | −1 | 0.32 ± 0.13 | 2.11 ± 0.03 |
| 5 | −1 | −1 | +1 | 0.32 ± 0.06 | 2.49 ± 0.03 |
| 6 | +1 | −1 | +1 | 0.35 ± 0.21 | 2.74 ± 0.04 |
| 7 | −1 | +1 | +1 | 0.31 ± 0.08 | 2.43 ± 0.03 |
| 8 | +1 | +1 | +1 | 0.38 ± 0.52 | 2.76 ± 0.10 |
| Reference value[a] | | | | 0.36 ± 0.04 | 2.89 ± 0.82 |

[a]Certified value and expanded uncertainty, $k=2$

For a better visualization of the results and to verify the influence of the factors on the observed variability, the factorial experiment analysis function of the design of experiment (DOE) statistical tool of the Minitab 18 software was applied [13]. Among these tools, we highlighted the effect normal probability plot and the Pareto chart as presented for Mg and V in Fig. 1 [14].

The normal probability plot evaluates the fit of a distribution to the data, estimates percentiles, and compares sample distributions. For this purpose, the plot presents dots showing the distribution in the graph and the graph is a straight line passing through the sample means. The Pareto chart analyzes the sample percentages presented as bars. Bars crossing the sample mean represent significant results.

Irradiation and counting times (factor A and factor C, respectively) and the AC interaction seem to be significant for Mg while for V, factor B (sample–detector distance) and the interactions AB, AC, BC and ABC were significant.

The main effect plot explores the influence of each effect on the response of the experiment, with the central line being the mean of all results. The inclination of the Interaction matrix plots present how strong is the influence of changing one variable to the others and how they interact. Main effect an interaction matrix plots are presented for Mg and V concentration responses at the $2^3$ factorial design in Figs. 2 and 3, respectively.

**Table 3** Mass fraction results obtained by INAA at a $2^4$ factorial design in biological matrix sample (mean value ± standard deviation, $n=3$) [12]

| Exp. | Factor and level | | | | Mass fraction | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | A | B | C | D | % | | | mg kg$^{-1}$ | |
| | | | | | Cl | K | Na | Br | Mn |
| 1 | −1 | −1 | −1 | −1 | 3.87 ± 0.46 | 0.73 ± 0.16 | 2.48 ± 0.69 | 268 ± 0.4 | 26.46 ± 0.53 |
| 2 | +1 | −1 | −1 | −1 | 3.45 ± 0.78 | 0.86 ± 0.56 | 2.54 ± 0.31 | 265 ± 0.8 | 27.26 ± 1.32 |
| 3 | −1 | +1 | −1 | −1 | 3.46 ± 0.73 | 0.76 ± 0.55 | 2.61 ± 0.34 | 255 ± 1.6 | 27.46 ± 0.54 |
| 4 | +1 | +1 | −1 | −1 | 3.52 ± 0.99 | 0.76 ± 0.83 | 2.75 ± 0.29 | 258 ± 1.2 | 25.73 ± 0.40 |
| 5 | −1 | −1 | +1 | −1 | 3.65 ± 0.86 | 0.81 ± 0.81 | 2.69 ± 0.38 | 261 ± 0.8 | 27.73 ± 0.89 |
| 6 | +1 | −1 | +1 | −1 | 3.61 ± 0.93 | 0.77 ± 0.31 | 2.50 ± 0.28 | 266 ± 0.8 | 22.90 ± 0.65 |
| 7 | −1 | +1 | +1 | −1 | 3.54 ± 0.99 | 0.66 ± 0.07 | 2.64 ± 0.24 | 277 ± 1.3 | 23.63 ± 0.77 |
| 8 | +1 | +1 | +1 | −1 | 3.57 ± 0.77 | 0.70 ± 0.12 | 2.62 ± 0.24 | 274 ± 0.8 | 23.51 ± 0.81 |
| 9 | −1 | −1 | −1 | +1 | 3.73 ± 0.48 | 0.86 ± 0.14 | 2.63 ± 0.33 | 274 ± 1.7 | 23.26 ± 0.90 |
| 10 | +1 | −1 | −1 | +1 | 3.44 ± 0.63 | 0.87 ± 0.85 | 2.53 ± 0.59 | 276 ± 0.8 | 24.27 ± 0.69 |
| 11 | −1 | +1 | −1 | +1 | 3.44 ± 0.68 | 0.69 ± 0.57 | 2.59 ± 0.60 | 265 ± 1.6 | 24.04 ± 0.16 |
| 12 | +1 | +1 | −1 | +1 | 3.33 ± 0.56 | 0.99 ± 0.35 | 2.43 ± 0.52 | 266 ± 0.8 | 23.28 ± 0.53 |
| 13 | −1 | −1 | +1 | +1 | 3.65 ± 1.08 | 0.82 ± 0.33 | 2.62 ± 0.56 | 276 ± 0.9 | 24.62 ± 0.86 |
| 14 | +1 | −1 | +1 | +1 | 3.22 ± 0.62 | 0.88 ± 0.25 | 2.63 ± 0.29 | 263 ± 1.2 | 24.16 ± 0.38 |
| 15 | −1 | +1 | +1 | +1 | 3.20 ± 0.34 | 0.86 ± 0.95 | 2.64 ± 0.43 | 259 ± 2.1 | 22.50 ± 0.74 |
| 16 | +1 | +1 | +1 | +1 | 3.48 ± 1.20 | 0.70 ± 0.73 | 2.61 ± 0.27 | 257 ± 0.8 | 26.16 ± 0.75 |
| Reference value[a] | | | | | 3.62 ± 0.43 | 0.81 ± 0.11 | 2.27 ± 0.30 | 250 ± 42 | 23.4 ± 3.1 |

[a]Certified value and expanded uncertainty, $k=2$

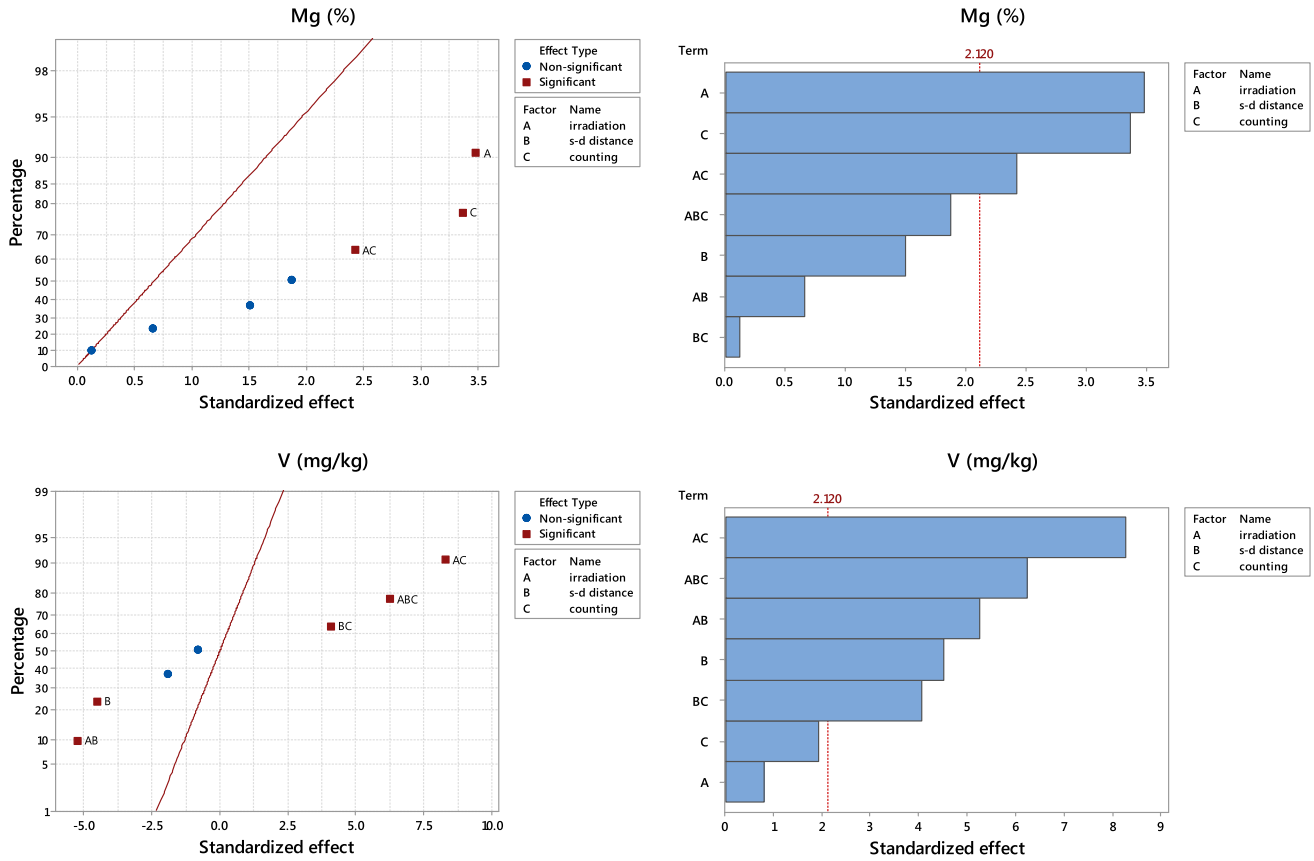


**Fig. 1** Effect normal probability plots and Pareto charts for the mass fractions of Mg and V at the $2^3$ factorial design

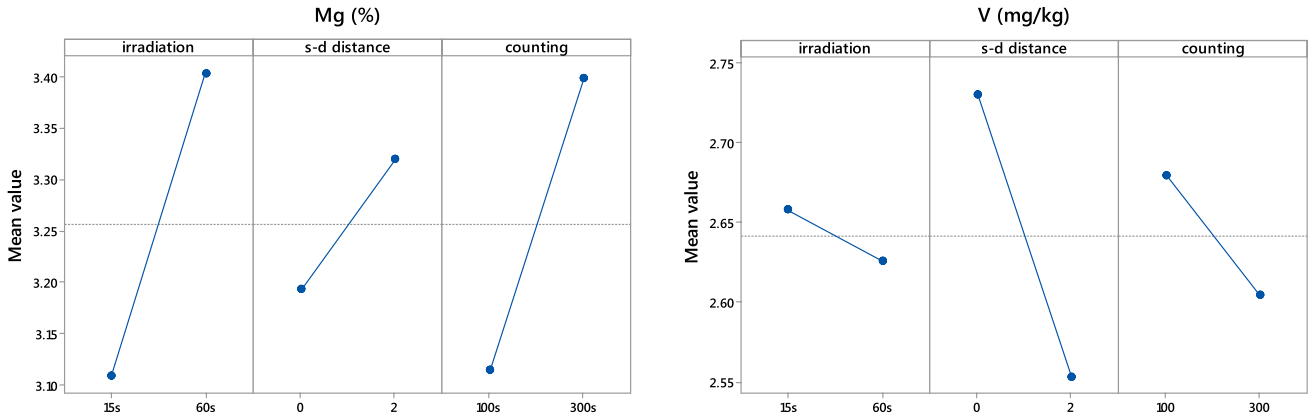


**Fig. 2** Main effect plots for the mass fractions of Mg (%) and V (mg $kg^{-1}$) obtained at the $2^3$ factorial design

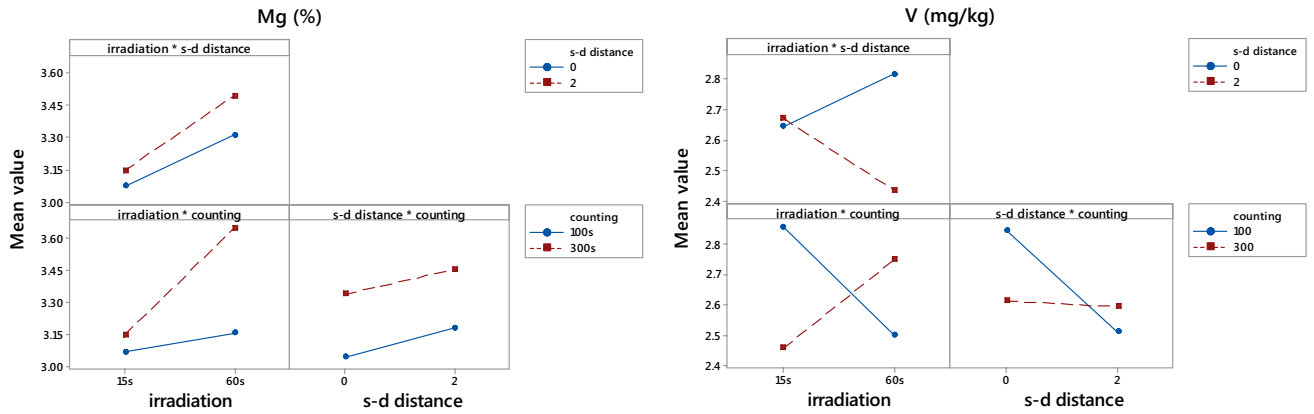


**Fig. 3** Interaction matrix plots for the mass fractions of Mg (%) and V (mg $kg^{-1}$) obtained at the $2^3$ factorial design

From Fig. 2, it was observed that with the exception of sample–detector distance, fixing one parameter for Mg, mass fraction results were higher changing factors from level – 1 (irradiation time = 15 s; counting time = 100 s and Shelf 2) to level + 1 (irradiation time = 60 s; counting time = 300 s and Shelf 0), however, V presented opposite behavior, i.e., mean mass fraction values were lower changing factors from level – 1 to level + 1, also with the exception of sample–detector distance. Coinciding information was obtained for Mg in Fig. 3 in the interaction matrix plot and the situation was more complex for the effect interactions for V.

Similar results were obtained for the other elements at the $2^4$ factorial design, making it difficult to decide for the best irradiation and measurement conditions for the analyzed elements in biological materials of marine origin.

It has been hypothesized that since mass fractions are calculated from the induced activities in the samples and standards, one may be compensating the possible variations caused by the factorial design levels and therefore the influence of the factors and their interactions cannot be clearly defined. Thus, $2^3$ and $2^4$ factorial designs were performed directly with the raw data (induced activities) instead of mass fractions as a response of the study (output quantity) and the Minitab 18 variability analysis function was used. Instead of investigating the response itself, the function investigates the degree of variability of the response by changing the factors from level – 1 to level + 1.

Observed results were no more promising to define the factors that most influence the results in short irradiation INAA. Nevertheless, a better fit of the linear model applied by Minitab was observed when the variability analysis was performed. We chose not to present such results in this text as they were not decisive for the development of the project. It was concluded that it was not possible to adjust a model that could explain the observed variability from the selected factors or that the intrinsic robustness of the INAA method made it little subject to variations in the levels.

Of the many possible uses for DOE, one of the main and most widely used is the selection of key factors that affect the response. The method can also be used to maximize or

**Table 4** Mg and V $z$-scores at the $2^3$ factorial design

| Experiment | $z$-score | | \|z\| |
|---|---|---|---|
| | Mg | V | |
| 1 | −1.75 | −0.11 | 0.93 |
| 2 | −1.25 | 0.00 | 0.63 |
| 3 | −1.00 | 0.02 | 0.51 |
| 4 | −1.00 | −0.95 | 0.98 |
| 5 | −1.00 | −0.49 | 0.74 |
| **6** | −0.25 | −0.18 | **0.22** |
| 7 | −1.25 | −0.56 | 0.91 |
| 8 | 0.50 | −0.16 | 0.33 |

minimize a response or to achieve a goal or objective [14]. In the specific case of this study, rather than verifying which of the factors most affects the obtained mass fraction, what really matters was which of the experimental conditions makes element mass fraction results simultaneously more accurate, since INAA is a multi-element analytical technique, in other words, that is the goal. As the objective, in this case, is the greater accuracy of the results and as a certified reference material was used as a sample, the $z$-score was taken as the response for the $2^3$ and $2^4$ factorial designs, as it is a normalization of the mass fraction values as a function of the certified values. Here $z$-score values were defined as the difference between obtained and reference value over the expanded uncertainty of the reference value. The closer the $z$-score modulus (absolute value) is to 0, the more accurate the analysis is, since the obtained value is closer to the expected result, i.e., the certified value.

The $z$-score values in the experiments for Mg, V and Br, Cl, K, Mg and Na are presented in Tables 4 and 5, respectively. Also, the tables introduce the parameter |$z$|, calculated as the mean of the $z$-score absolute values for each experiment. This parameter is used in order to provide a general quality score for the various factorial design experiments, showing which of the experiments are more favorable with regard to obtaining results with lower $z$-score in modulus and therefore more accurate results. All obtained $z$-score results were considered adequate at the 95% confidence level for the INAA method used since they were within ±2 for all elements [15].

From Tables 4 and 5, the smallest values of |$z$| were observed for Experiment number 6 (|$z$| = 0.22 for the $2^3$ factorial design and |$z$| = 0.34 for the $2^4$ factorial design). Thus, by only analyzing the $z$-scores, the condition of choice to be worked with in the validation of the INAA method applied to marine biological materials is the 60-s irradiation time, the 60-mm distance sample–detector (Shelf 2) and 300-s counting time for Mg and V and the same irradiation conditions and sample–detector distance, with 2700-s counting time and 30-min decay time for the other elements.

The factorial design models were used with $z$-scores as response parameter, in order to confirm (or not) this evaluation of $z$-scores as described below.

Figure 4 presents the normal probability plots of effects and the Pareto plots for Mg and V, obtained for the $2^3$ factorial design and using the $z$-score as the study response. It was observed that for Mg, the BC interaction became significant, while for V factor B and all interactions between factors were significant. It was also observed that the

**Table 5** Br, Cl, Mn, Na, and K $z$-scores at the $2^4$ factorial design

| Experiment | $z$-score | | | | | \|z\| |
|---|---|---|---|---|---|---|
| | Br | Cl | K | Mn | Na | |
| 1 | 0.43 | 0.58 | −0.73 | 0.32 | 0.43 | 0.63 |
| 2 | 0.36 | −0.40 | 0.45 | 1.25 | 0.90 | 0.67 |
| 3 | 0.12 | −0.37 | −0.45 | 1.31 | 1.13 | 0.68 |
| 4 | 0.19 | −0.23 | −0.45 | 0.75 | 1.60 | 0.65 |
| 5 | 0.26 | 0.07 | 0.00 | 1.40 | 1.40 | 0.63 |
| **6** | 0.38 | −0.02 | −0.36 | −0.16 | 0.77 | **0.34** |
| 7 | 0.64 | −0.19 | −1.36 | 0.70 | 1.23 | 0.70 |
| 8 | 0.57 | −0.12 | −1.00 | 0.04 | 1.17 | 0.58 |
| 9 | 0.57 | 0.26 | 0.45 | −0.05 | 1.20 | 0.51 |
| 10 | 0.62 | −0.42 | 0.55 | 0.28 | 0.87 | 0.55 |
| 11 | 0.36 | −0.42 | −1.09 | 0.21 | 1.07 | 0.63 |
| 12 | 0.38 | −0.67 | 1.64 | −0.04 | 0.53 | 0.65 |
| 13 | 0.62 | 0.07 | 0.09 | 0.39 | 1.17 | 0.47 |
| 14 | 0.31 | −0.93 | 0.64 | 0.25 | 1.20 | 0.66 |
| 15 | 0.21 | −0.978 | 0.45 | −0.29 | 1.23 | 0.63 |
| 16 | 0.17 | −0.33 | −1.00 | 0.89 | 1.13 | 0.70 |

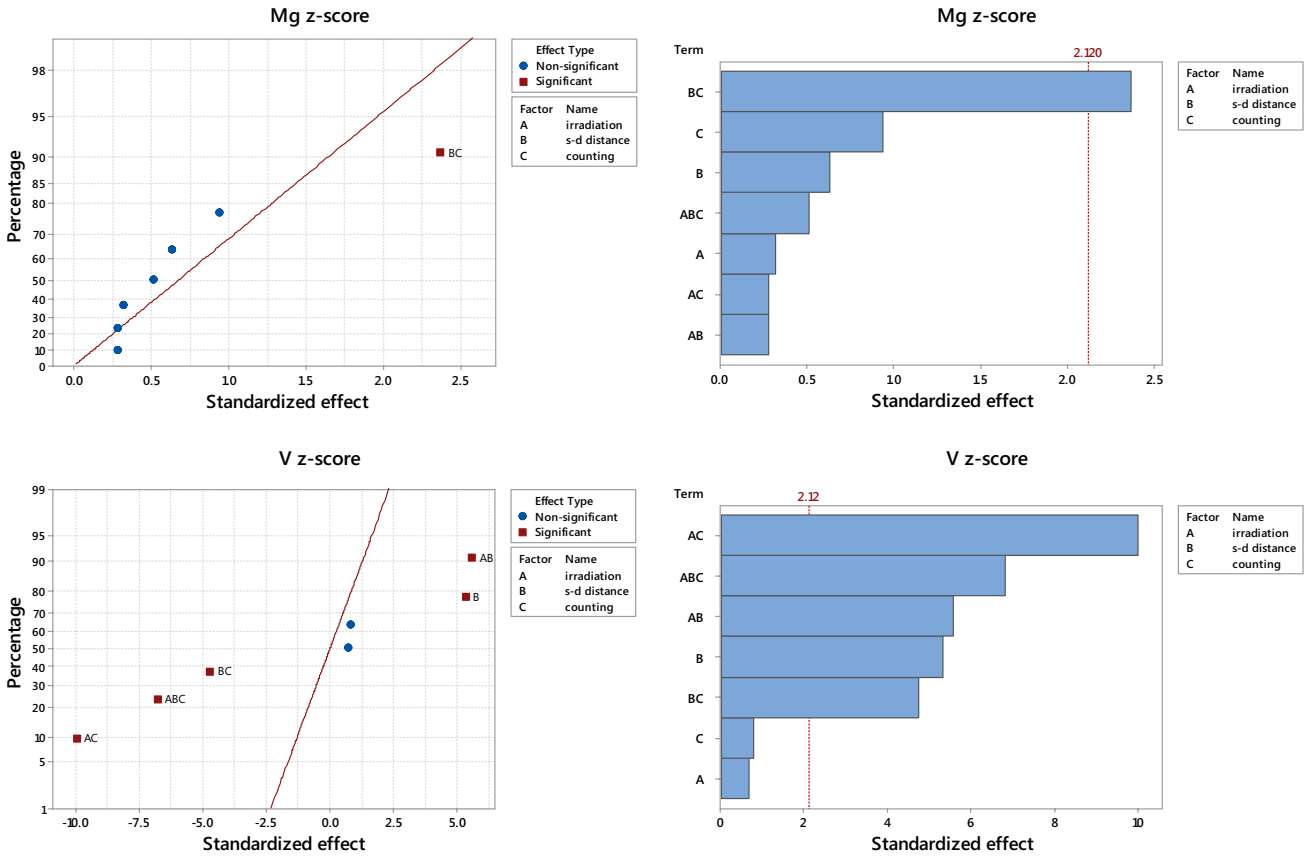


**Fig. 4** Effect normal probability plots and Pareto charts for the *z*-scores of Mg and V at the $2^3$ factorial design

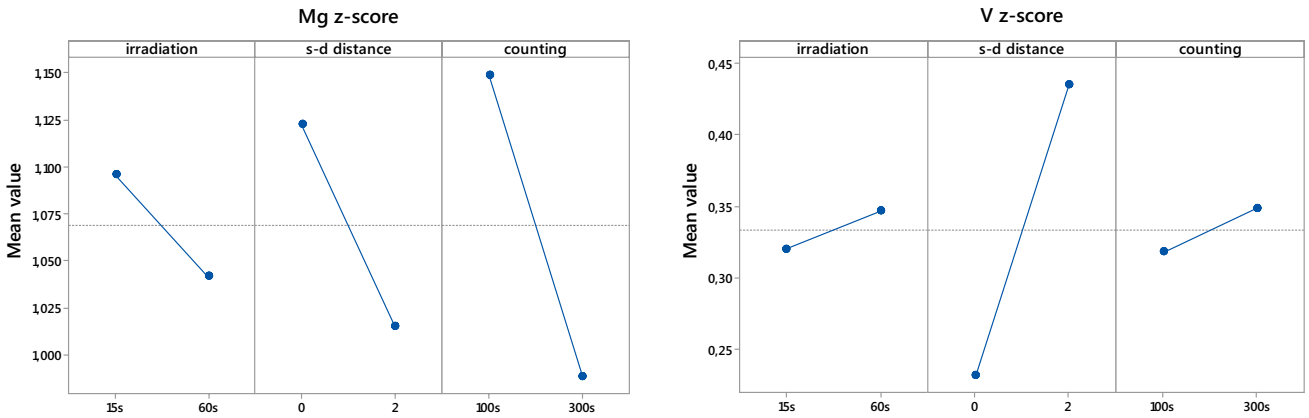


**Fig. 5** Main effect plots for the Mg and V *z*-scores obtained at the $2^3$ factorial design

adjustment of the residuals to the linear model was not adequate, especially in the case of V.

Figures 5 and 6 show the obtained adjusted mean values for the main effects and the interactions, respectively, for Mg and V in the $2^3$ factorial design using *z*-scores as the response.

According to Fig. 5, except for the sample–detector distance, conditions + 1 are the most favorable for Mg, since under these conditions the lowest values for the *z*-score for this element are observed. For V alone, there was smaller variation in the *z*-score when changing irradiation and counting time levels, but the use of Shelf 0 greatly favors

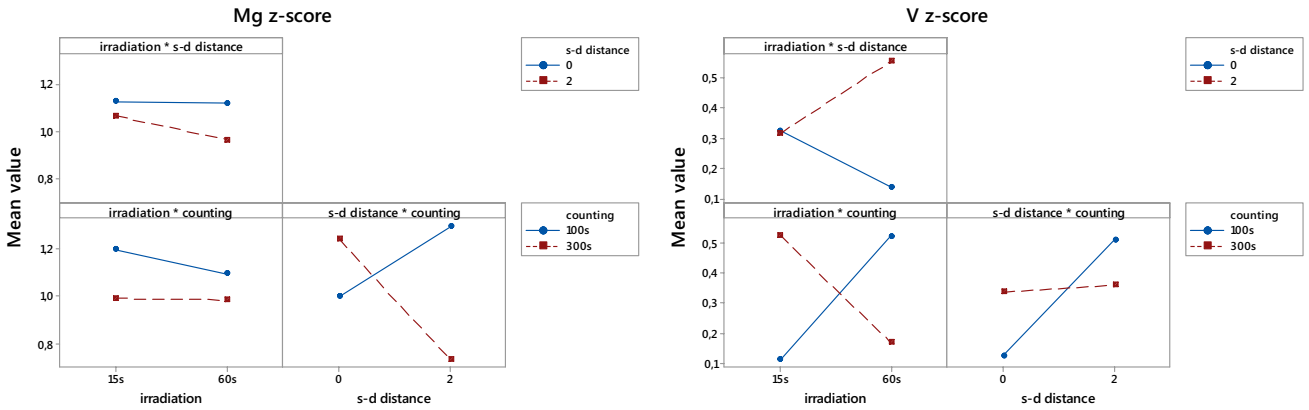


**Fig. 6** Interaction matrix plots for the *z*-scores of Mg and V obtained at the $2^3$ factorial design

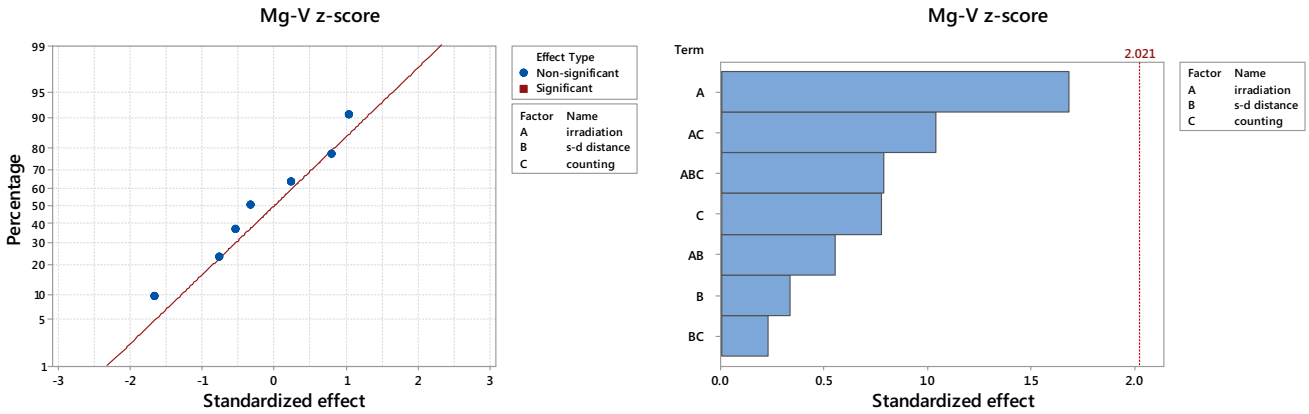


**Fig. 7** Effect normal probability plot and Pareto chart obtained simultaneously for the *z*-scores of Mg and V at the $2^3$ factorial design

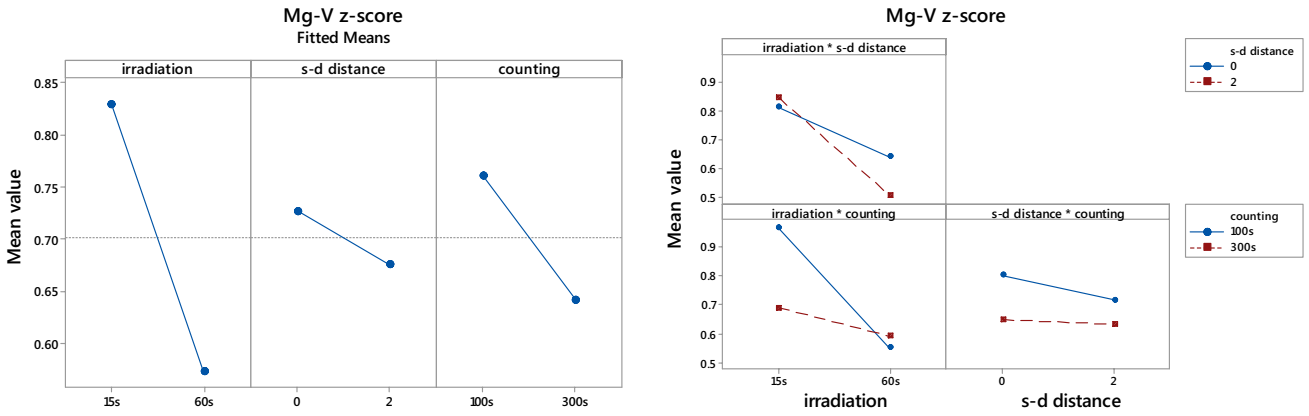


**Fig. 8** Main effect plots and interaction matrix plots obtained simultaneously for Mg and V *z*-scores at the $2^3$ factorial design

the obtaining of a lower *z*-score. Figure 6 presented factor interaction results with similar complexity to the ones obtained with the study using mass fractions as response of the experiments.

As the *z*-score is a standardized parameter, it was also possible to study the factorial design of Mg and V simultaneously to verify the simultaneous irradiation and measurement conditions of the samples, as shown in Figs. 7 and 8.

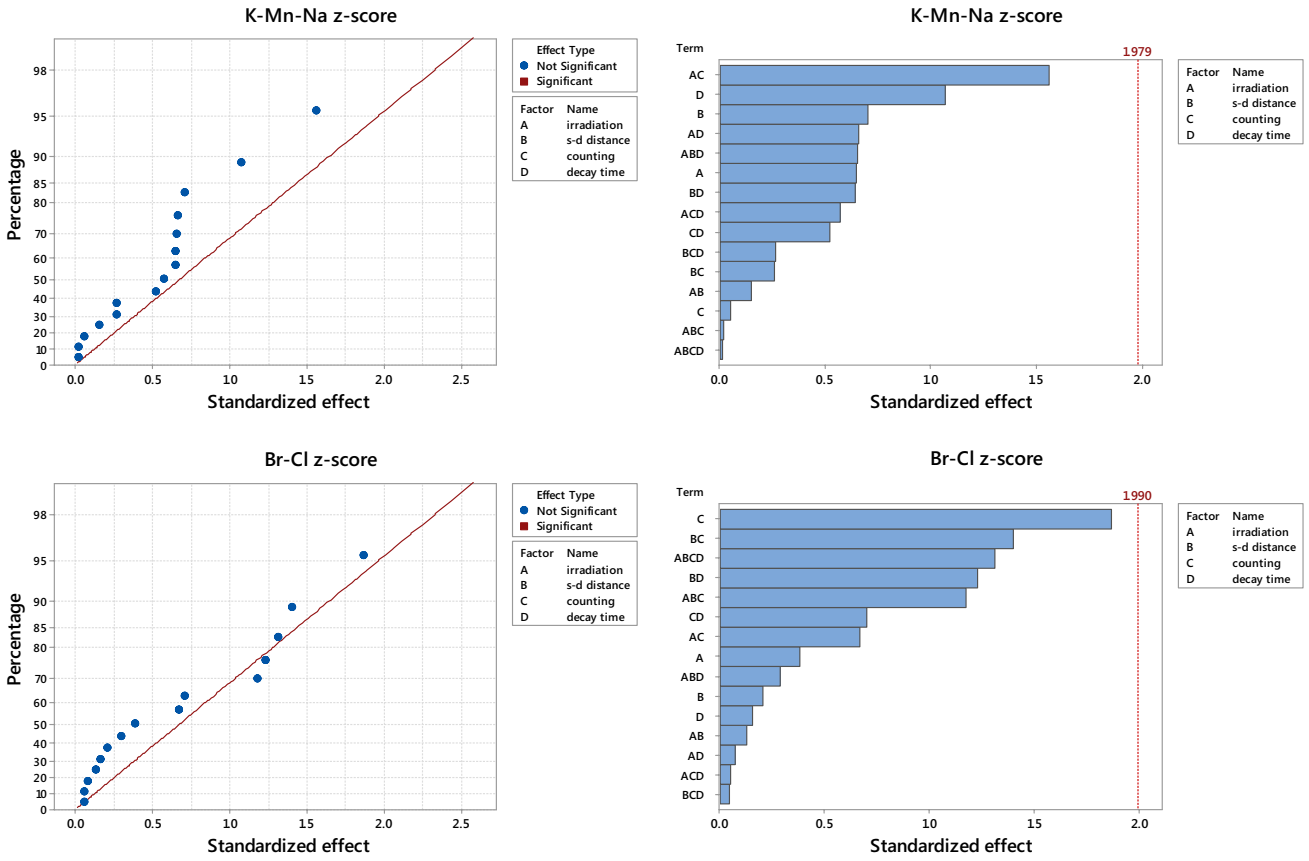


**Fig. 9** Effect normal probability plots and Pareto charts obtained simultaneously for **a** K, Mn and Na and **b** Br and Cl *z*-scores at the $2^4$ factorial design

By evaluating the two elements simultaneously, a good fit to the factorial design model was observed from Fig. 7, but there was no discrimination of any factor as significant in the variability. Moreover, it was observed in Fig. 8 that the conditions of experiment number 6 (irradiation time = 60 s, counting time = 300 s and Shelf 2) are the most recommended for the simultaneous determination of the two elements by INAA in biological matrix and that the irradiation time provides the greatest variability, so it is the most critical factor for the measurement process for these elements.

For the $2^4$ factorial design, an analogous *z*-score analysis was performed for elements one by one, but also grouped. Only the results for the analysis of the elements in two groups are presented: K, Mn and Na in the first group and Br and Cl in the second group. This was done because these elements were determined with multi-element standards pipetted with these sets of elements and because the Minitab 18 software did not support the analysis of all data simultaneously (240 results: 5 elements, 16 experiments for each element and in triplicate). The output is presented by Figs. 9 and 10.

It was observed in Fig. 9 that there were no significant factors for the evaluated data sets. As observed before for Mg and V, it is possible that the conditions used were not sufficient to cause very large changes in the obtained responses with the experiments. In addition, it can be argued that INAA is a fairly robust technique for irradiation and measurement conditions, provided they are the same for the sample and the elemental standard in the comparative method.

Regarding the optimal conditions for the INAA (lowest *z*-score in modulus), it was observed that the Experiment 6 conditions for irradiation time, sample–detector distance and counting time was simultaneously the most favorable for all elements at the $2^4$ experiment. In the case of decay time, Experiment 6 conditions favored the analysis for Cl and Br (decay time = 1800 s). We opted to maintain the conditions for Experiment 6 for the validation study, although decay time + 1 (3600 s) was the most favorable according to the results of the $2^4$ factorial design for K, Mn and Na.

To complement the $2^3$ and $2^4$ factorial design analyses in identifying possible significant effects, numerical indicators were used to test the linear model adjusted to the

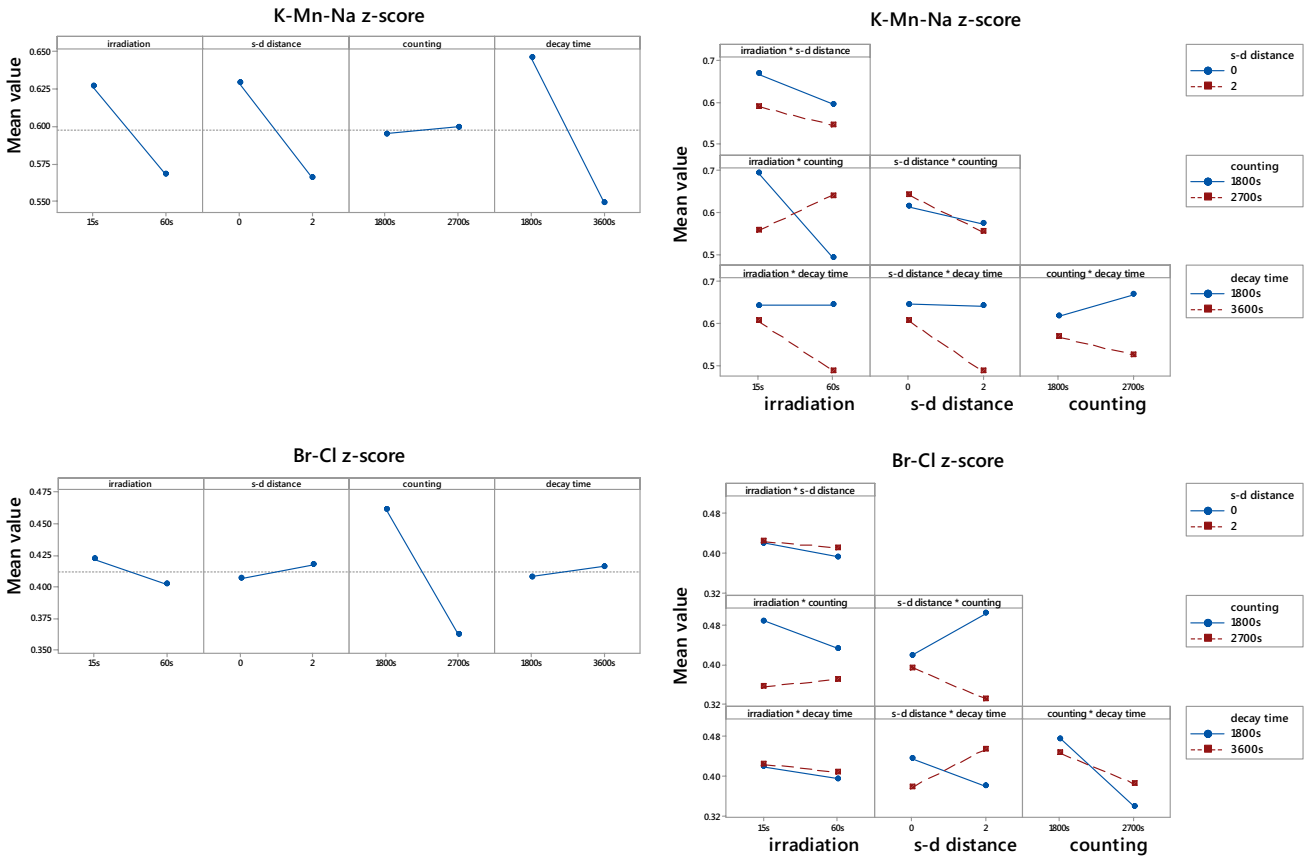


**Fig. 10** Main effect plots and interaction matrix plots obtained simultaneously for **a** K, Mn and Na and **b** Br, and Cl *z*-scores at the $2^4$ factorial design

**Table 6** ANOVA output for the $2^3$ and $2^4$ factorial designs for groups of elements

| Factor/interaction | $2^3$ factorial design | | $2^4$ factorial design | | | |
|---|---|---|---|---|---|---|
| | Mg–V | | K–Mn–Na | | Br–Cl | |
| | *F* | *p* value | *F* | *p* value | *F* | *p* value |
| A | 2.82 | 0.101 | 0.42 | 0.520 | 0.14 | 0.705 |
| B | 0.11 | 0.739 | 0.49 | 0.485 | 0.04 | 0.838 |
| C | 0.60 | 0.444 | 0.00 | 0.959 | 3.48 | 0.066 |
| D | – | – | 1.14 | 0.287 | 0.02 | 0.875 |
| AB | 0.30 | 0.584 | 0.02 | 0.880 | 0.02 | 0.896 |
| AC | 1.08 | 0.305 | 2.42 | 0.122 | 0.44 | 0.507 |
| AD | – | – | 0.43 | 0.513 | 0.01 | 0.940 |
| BD | – | – | 0.07 | 0.796 | 1.96 | 0.166 |
| BC | 0.05 | 0.824 | 0.42 | 0.521 | 1.51 | 0.223 |
| CD | – | – | 0.27 | 0.604 | 0.49 | 0.486 |
| ABC | 0.62 | 0.437 | 0.00 | 0.985 | 1.37 | 0.245 |
| ABD | – | – | 0.43 | 0.514 | 0.08 | 0.772 |
| ACD | – | – | 0.32 | 0.570 | 0.00 | 0.960 |
| BCD | – | – | 0.07 | 0.793 | 0.00 | 0.961 |
| ABCD | – | – | 0.00 | 0.990 | 1.72 | 0.193 |

**Table 7** Optimized irradiation and measurement parameters, defined at the $2^3$ and $2^4$ factorial designs experiments

| Parameter | Condition | |
|---|---|---|
| | Mg and V | Br, Cl, K, Mn and Na |
| Irradiation time, s | 60 | 60 |
| Sample–detector distance, mm[a] | 60 | 60 |
| Counting time, s | 300 | 2700 |
| Decay time, s | – | 1800 |

[a]Shelf 2

experimental designs. Analysis of variance (ANOVA) was used to verify the influence of factors and their interactions on the observed total variability in the factorial designs [13, 14]. In this case, the null hypothesis ($H_0$) is that all obtained results in the response are equal, while the alternative hypothesis ($H_1$) states that there are significant differences between the results and that these differences are caused by the influence of a given factor. $p$ values greater than 0.05 indicate that the influence of factors is not significant [13].

Table 6 summarizes the ANOVA results for the $2^3$ and $2^4$ factorial designs for all elements, treated in the Mg–V; K–Mn–Na and Br–Cl subsets. No factor or factor interaction was significant at the 95% confidence level even though significant influences were observed when elements were evaluated separately, such as factor B (sample–detector distance) for V and Br ($p$ value $< 0.05$). As a general trend, there was no influence of a specific factor or factor interactions on the variability in the obtained results for the analyzed elements under the experimental conditions used.

Concluding the factorial design output analyses, the coefficients of the equations for the linear regression model that takes into account the factors and their interactions obtained with the Minitab 18 program were obtained. Once again, it was observed the difficulty of the model to explain the variability of the results as a function of the factors and level employed as observed for Mg and K, where adjusted determination coefficients ($R^2$) were less than 35%. Still, adjusted $R^2$ greater than 90% was obtained for Br and V and adjusted $R^2$ between 60 and 90% was obtained for Cl, Mn and Na.

Table 7 presents the optimized irradiation and measurement conditions obtained by using $z$-scores and $2^3$ and $2^4$ factorial designs. These conditions will be used in the following formal validation process of the method.

## Conclusions

In the study, the mass fractions of Br, Cl, K, Mg, Mn, Na and V were determined in a mussel tissue reference material by INAA as part of a factorial experimental design, with the intention to choose the best conditions for irradiation and measurement and to investigate the most comprehensive effects that may occur. As INAA is a robust technique, employed factor levels did not affect obtained mass fraction results as for choosing the best conditions. Using a $z$-score analysis however, it was observed that the best irradiation and measurement conditions for the simultaneous optimization of the INAA method were similar. It was concluded that with the two factorial designs ($2^3$ and $2^4$) the same analysis protocol could be adopted to optimize the measurement results for simultaneous element determination in biological materials via INAA. Also the crucial role that proper selection of factor levels play in factorial designs became evident and the use of the tool is dependent of the careful selection of the response parameter for the successful use of the statistical tool.

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