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PRACTITIONER'S REPORT

Preparation and characterization of a new reference material for the inorganic analysis of corn flour

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Abstract Corn is one of the most consumed cereals in world and the second most cultivated in Brazil. Cereal flour has a flavor well accepted by the Brazilian population. This work describes the preparation and characterization of a new corn flour reference material which can be used as a quality assurance tool to demonstrate the reliability of the analytical results obtained from inorganic analysis of cereals and their derivatives. The studied elements were Ca, K, Mg, P, Cu, Fe, Mn and Zn. Homogeneity and stability tests, material characterization and attribution of uncertainty value were performed in this work by following recommendations of ISO Guides 30–35. The material was shown to be homogeneous and stable for 15 months at temperatures of −10 °C, +25 °C and +45 °C, with small uncertainty. Alternatively, the data were analyzed by hierarchical clustering technique and the stability was confirmed. The confidence ellipse technique enabled a simple and fast evaluation of data supplied by collaborating laboratories allowing the exclusion of outlying values and indicating possible causes of the deviations.

Keywords Reference material · Corn flour · HCA · Confidence ellipse

Introduction

Celiac disease is worldwide considered a public health problem, and because of the severity of this disease in Brazil, there is a permanent demand for dietary foods.

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According to the Codex Alimentarius [International Food Standards established by the Food and Agriculture Organization (FAO) of the United Nations and the World Health Organization (WHO)] [1], dietetic foods are composed by one or more ingredients which do not contain wheat, rye, barley, oat or their derivatives so that the level of gluten in food sold or distributed to consumers does not exceed 20 mg kg^{-1} [1]. The most common ingredients in gluten-free foods are starch, corn flours, potato, tapioca (a kind of starch extracted from cassava), rice, etc. [2]. The daily consumption of products derived from cereal is recommended due to their content of fiber, minerals and vitamins, which are supposed to prevent various diseases [3].

Due to the importance of cereal flours in human nutrition, the development of analytical methods for assessing the levels of essential elements contained in these foods is relevant not only for healthcare professionals, but also for food industry, in order to propose nutritional improvements and contributions in the food composition table, where data of nutrient content of gluten-free food do not exist [4].

Having knowledge about the need of monitoring levels of nutrients in cereal flours, the choice of a suitable sample is important to check the accuracy of analytical measurements, in order to ensure reliability [5]. Some certified reference materials (CRM) of cereals are available, but they do not comprise all kinds of cereals and concentration ranges, especially in the case of foods of vegetable origin such as gluten-free foods [6]. However, a reference material (RM) which is homogeneous, stable and well characterized may serve as an alternative to a CRM in method development and quality control. According to ISO Guides 30–35, the development of an RM involves three steps [7]: the property values (e.g., content of analytes) measured in several containers for assessing the homogeneity of the material, the short- and long-term stability study to ensure conditions of transportation and storage and, finally, the material characterization that is to conduct a series of measurements of fractions of the material with the participating laboratories.

Corn was chosen as the food matrix, because it is one of the most consumed cereals in the world and the second most cultivated in Brazil. Nowadays, more than corn, its derived products are especially relevant, from which corn flour is one worth mentioning. Corn flour has several applications such as cakes, breads, biscuits, among others, not only because its flavor is well accepted by consumers, but also because of its low cost and commercial availability [8]. In this sense, the aim of this work was to develop an RM to be used to demonstrate the reliability of analytical results for the content of inorganic elements in cereal flours.

Experimental

Preparation of the candidate RM

The samples of corn flour used in this work were obtained from the same manufacturer with different batches and acquired in a supermarket from Salvador City, Bahia, Brazil. For the production of the candidate RM, 8 kg of corn flour was used. After irradiation with 15 kGy gamma radiation to prevent the development of fungi and bacteria, the material was placed in a polyethylene bucket for homogenization. Six subsamples were taken from the material bulk for the preliminary study of homogeneity. After a satisfactory level of homogeneity was achieved, the corn flour was then transferred to 100 polyethylene bottle, each of them with approximately 80 g of the material. The bottles were labeled with the name of the material, numbered from 1 to 100 and airtight stored. These bottles were randomly chosen for the stability and homogeneity studies as well as for the characterization of the material.

Sample digestion and measurement of element mass fractions

The material contained in each bottle was analyzed in triplicate to evaluate the content of elements of interest. About 2.0 g was placed in each digestion tube, followed by the addition of 2.0 mL of 65% (by mass) nitric acid and 1 mL of 30% (by mass) hydrogen peroxide. The mixture was heated on a digester block until obtaining a limpid solution and then quantitatively transferred into polyethylene tubes and diluted with ultrapure water up to a final volume of 12.0 mL. The digestion of a blank sample was carried out in the same way as the samples. The multi-element measurement of Ca, K, Mg, P, Cu, Fe, Mn and Zn mass fraction was performed by the technique of inductively coupled plasma optical emission spectrometry (ICP OES). This digestion method was used to study the homogeneity and stability.

Homogeneity study

The homogeneity study was performed with samples representative of the whole lot. After remixing a sample inside the bottle, three subsamples from each one were taken for analysis and twenty bottles of corn flour candidate RM were randomly selected for this study. The steps of this test were described by Santos [8].

Stability study

The short- and long-term stability study was performed to evaluate conditions of transportation and storage of the candidate RM. Adopting the isochronous design, all analyses were performed under repeatability conditions to minimize the variability of the measurement [19]. A total of 18 bottles were randomly selected, and groups of six of them were kept at −10 °C, +25 °C and +45 °C, respectively, one each for 0, 3, 6, 9,12 and 15 months. Each material was treated and analyzed in triplicate for quantification of the analytes by the same procedure employed in the homogeneity study previously described [8].

On a basis of the ISO Guide 35 [7] recommendations, stability was estimated by the ratio

$$R_t(T) = w_t(T)/w_t(T_{\text{ref}}) \quad (1)$$

where $w_t(T)$ is the measured mean mass fraction of a given element when the material was stored at the temperature T for a length of time t, and $w_t(T_{\text{ref}})$ is the corresponding value of material stored at the chosen reference temperature $T_{\text{ref}} = +25$ °C. The mass fractions were measured in triplicate to obtain their mean and relative standard deviation $s_{\text{rel},t}(T)$, from which the measurement uncertainty of $R_t(T)$ was derived by

$$u_t = \left(s^2_{\text{rel},t}(T) + s^2_{\text{rel},t}(T_{\text{ref}})\right)^{1/2} R_t(T) \quad (2)$$

For ideal stability, the ratio R_t should be 1. In practice, however, there are random variations because of measurement uncertainty [18]. As long as the interval $R_t \pm u_t$ includes the value 1, the material is considered stable [2].

The hierarchical cluster analysis (HCA) is an exploratory multivariate technique that seeks to group the samples based on the similarity of the participants' results and the differences between members of different groups. There are a number of methods available to establish groups, such as the Ward's method using the analysis of variance to evaluate the distances between groups [20].

In this work, this technique was also used to assess the stability. The similarities of results are shown in two-dimensional diagrams known as dendrograms. HCA can be used as an alternative statistical technique to evaluate the stability of the candidate RM, once it allows the evident formation of homogeneous groups [10, 11]. The formation of homogeneous groups of samples evaluated in heterogeneous temperature is an indication of stability. To assess the stability grouping formation by samples evaluated at the same temperature would provide a hint on instability.

Material characterization

The material characterization was performed with an interlaboratory study with 13 laboratories, each of which received two bottles of the candidate RM, and the analytes were quantified by routine procedures.

The set of results presented by the participant was analyzed statistically by Grubbs and Cochran tests [2]. The Grubbs test was applied to detect possible outliers in the population of individual results and in the population of laboratory measured, whereas the Cochran test was employed to identify outliers in variance of laboratories. For the set of accepted data, the confidence ellipse technique was applied.

Confidence ellipse

The confidence ellipse graph is used to demonstrate the compatibility among the laboratories, and it follows Youden's method [12]. The construction of this graph predicts the distribution of one pair of samples for each element, and each laboratory is represented by a score. The robust statistic [22] was employed to calculate robust z-scores (Eq. 3) and robust mean for ellipses, as recommended by ISO 13528 [13]. The version 4.3.01 Labwin-PEP software [14] was used to draw the ellipses.

$$z = (x - X)/IQR \quad (3)$$

where x is the measurement result reported by a participant, X is the reference value (robust mean), and IQR is the normalized interquartile range being the difference between the highest quartile ($Q3$) and the lowest quartile ($Q1$): $IQR = Q3 - Q1$. To obtain a value which can be compared to a standard deviation, IQR is multiplied by a factor of 0.7413 which is based on the standard normal distribution.

This way, all scores within the 95% confidence ellipse refer to the laboratories which have obtained satisfactory results. Scores between the 95% and 99% ellipses denote questionable results. Scores out of the confidence ellipse of 99% refer to unsatisfactory results [15].

The standard uncertainty associated with the characterization (u_{char}) was calculated from parameters obtained from ANOVA [7], as shown in the following Eq. 4.

$$u^2_{\text{char}} = \left[\left(MS_{\text{among}} - MS_{\text{within}}\right) + MS_{\text{within}}\right]/np = MS_{\text{among}}/np \quad (4)$$

where MS_{among} and MS_{within} are mean squares among and within the groups, provided by ANOVA, n is the number of measurements performed by each participant, and p is the number of laboratories participating in the study.

Combined and expanded uncertainty

The expanded uncertainty U_{RM} of the measured values of element mass fractions in the RM of corn flour is obtained combining the uncertainties of homogeneity (u_{bb}), stability (u_t) and material characterization (u_{char}), as shown in Eq. 5. The interval of $\pm U_{\text{RM}}$ around the attributed value of element mass fraction encompasses measured values that may represent the measurand at a given level of confidence [2, 3]. The choice of the coverage factor k is based on the confidence level intended, where for about 95%, the value of k is 2 [17].

$$U_{\mathrm{RM}} = k\left(u_{\mathrm{char}}^2 + u_{\mathrm{bb}}^2 + u_t^2\right)^{1/2} \tag{5}$$

Results and discussion

Stability study

The stability of the corn flour reference material candidate was evaluated for storage conditions. Examples of the ratios R_t (Eq. 1) and their standard uncertainties u_t (Eq. 2) are shown as regression graphs in Fig. 1a, b: The data of all elements are presented as Electronic Supplementary Material.

The results show that the corn flour RM is stable at the considered storage temperatures for 15 months. Alternatively, the stability was also evaluated by applying the results to HCA analysis (Fig. 2). As shown in the dendrogram obtained using Ward's clustering algorithm [6], the results are separated into two groups at linkage distances between 80 and 100. Each group has samples corresponding to all temperature values as such these multivariate results do not provide any strong evidence to question the stability of the corn flour samples. It was possible to identify the formation of groups composed by heterogeneous temperatures revealing that the RM

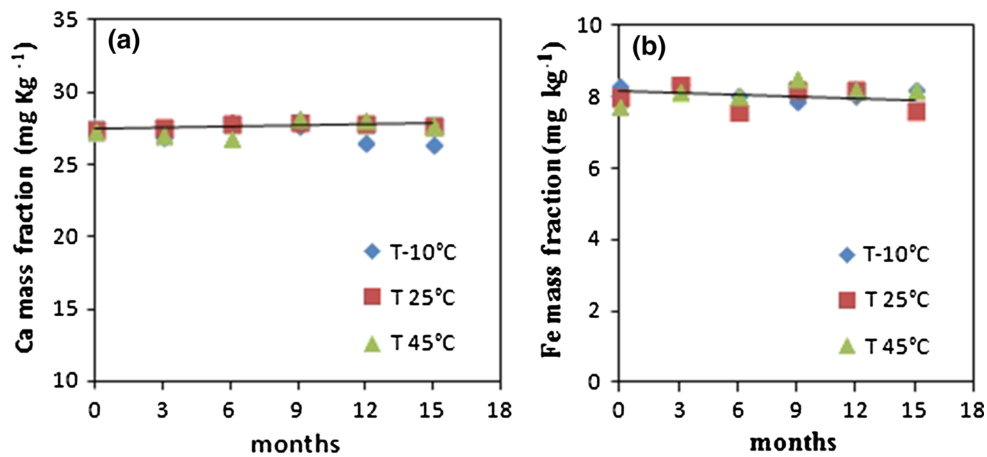

Fig. 1 Stability study applying the linear regression model of the candidate RM, of the elements **a** Ca, **b** Fe

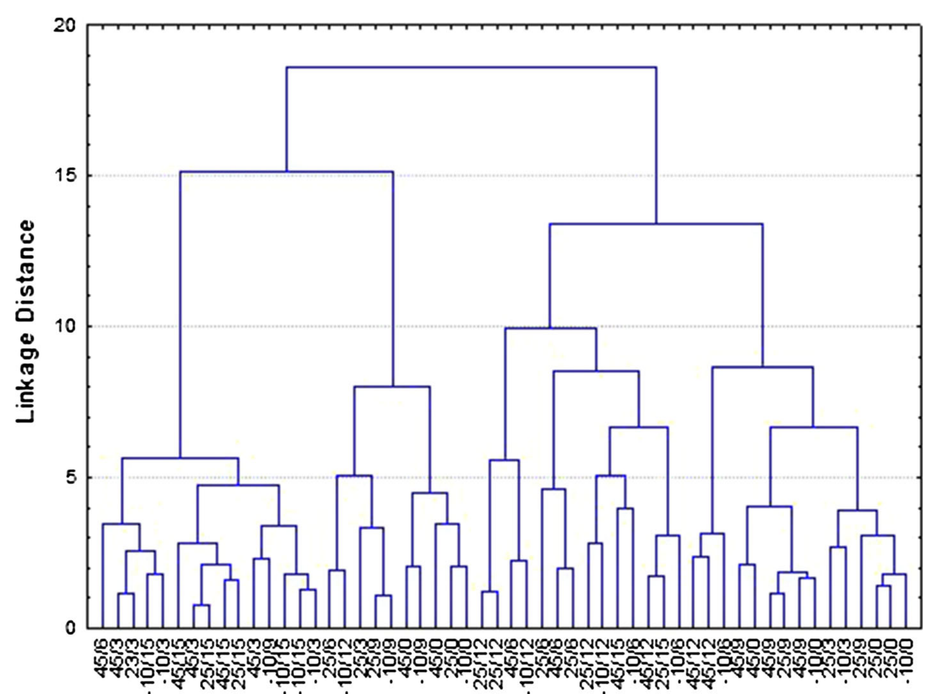

Fig. 2 Dendrogram of the stability study by Ward's method obtained from hierarchical cluster analysis (HCA) of the candidate RM

candidate is stable at the temperatures and period evaluated, demonstrating that regardless of the period and the storage time the mass fraction of the analytes did not change.

Material characterization

After having received the results from the participants of the interlaboratory comparison, an identification number was attributed to each laboratory. Reference mass fraction values were given for elements that are easily measurable according to the results furnished by the participating laboratories.

The compatibility among the laboratories was checked by confidence ellipses on the basis of the *z*-scores (Eq. 3) which had been obtained from results provided by the laboratories participating in this study. Figure 3 shows straight dotted lines that pass through the robust mean of laboratory results in *x* and *y* and divide the diagram into quadrants, where their intersection point is accepted as the most probable value [21]. Scores in the right upper and left lower quadrants represent laboratories which tend to obtain high or low values in both samples possibly due to systematic errors.

Scores outside the ellipse, far from the major axis of the ellipse, indicate significant random measurement error and may occur due to unstable conditions inside the laboratory, related to an operator incorrectly trained, random measurement error (signal, calculus, conversion of data, transcription of data, etc.). So, for Mg (Fig. 3b), the results from Laboratory 5 were in the range between the 99% and 95% ellipses and because of this it was considered questionable, probably due to random measurement error.

Results near the major axis of the ellipse indicate significant systematic errors, which may occur due to adverse conditions in the laboratory and can have their origin in unauthorized changes in methodology or in equipment that was not calibrated or checked. So, for Mg (Fig. 3 b), K, Fe and Mn, Laboratory 5 presented questionable results and for Zn unsatisfactory results, indicating a systematic error. Laboratories 2 and 8 presented questionable results for Mn, denoting also systematic error. The Ca (Fig. 3a) results from Laboratory 4 indicate random and systematic measurement errors out of the range of the 99% ellipse and so the performance was considered unsatisfactory.

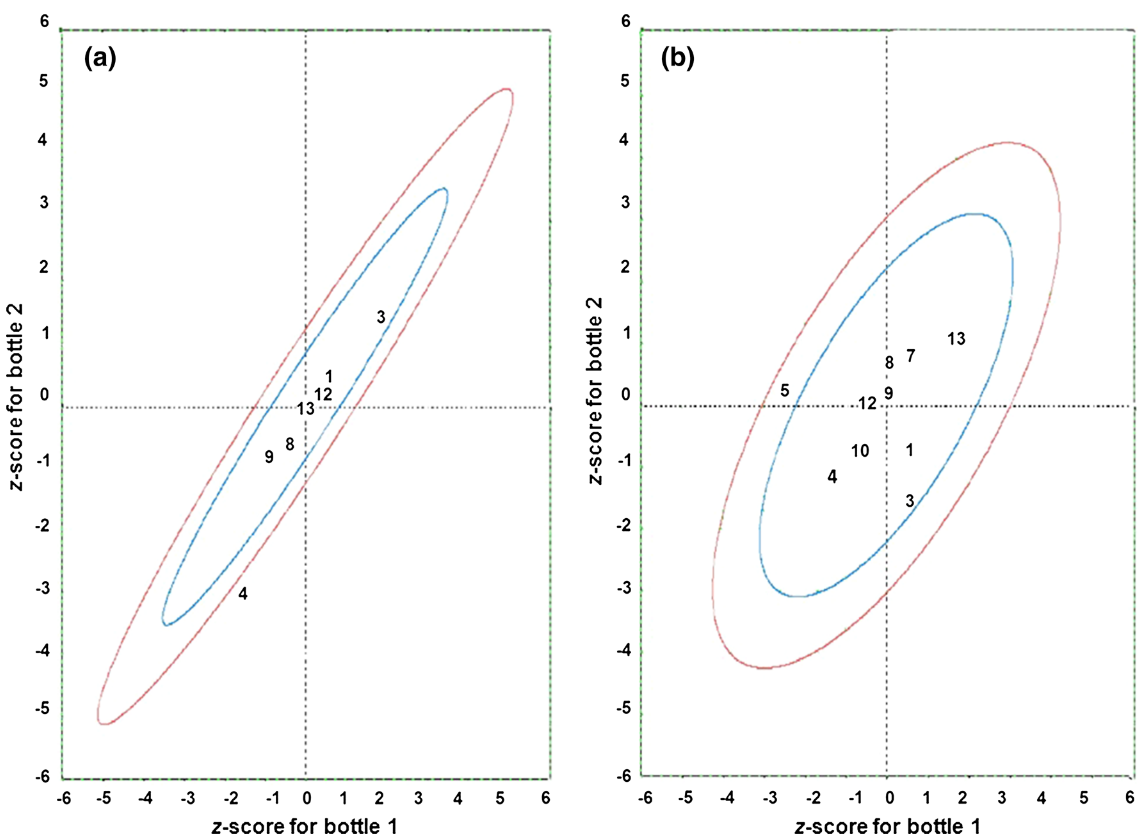

Fig. 3 Youden plots based on *z*-scores for mass fractions of the elements **a** Ca, **b** Mg. The *ellipses* enclose the confidence ranges of 95% and 99%; numbers identify the participants

Table 1 Reference values of element mass fractions present in the new RM of corn flour and their expanded uncertainty values

Element	Mass fraction (mg kg^{-1})	U_{RM} (mg kg^{-1})
Ca	24.6	1.5
K	1307	10
Mg	184.2	1.7
P	527.1	11.1
Cu	0.61	0.24
Fe	6.31	0.49
Mn	0.91	0.10
Zn	4.93	0.17

Expanded uncertainty

The expanded uncertainty of the mass fractions attributed to the new RM was calculated from the data obtained using Eq. 5, as recommended by ISO Guide 35 [7]. Table 1 shows the mass fraction values obtained as well as the respective expanded measurement uncertainties.

Conclusion

The performed study confirmed that the corn flour material is stable for 15 months at the temperatures of −10 °C as well as of +45 °C. The hierarchical cluster analysis (HCA) showed to be applicable as alternative method in studies of stability for RM candidates. The multivariate evaluation of data proved to be an interesting tool because of its simplicity and practicality.

The confidence ellipse technique enables improved discrimination of results and can be considered a powerful tool, because it allows identifying outliers, and gives information about the kind of error (random or systematic). The developed RM can be used to demonstrate the reliability of analytical results for the mass fractions of Ca, K, Mg, P, Cu, Fe, Mn and Zn, in samples of flour cereals and can be also used for routine checks, quality control and validation studies of analytical methods.

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