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Preparation and evaluation of a new reference material for macro- and micronutrients in fish feed

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ABSTRACT

A new reference material for inorganic constituents analysis in fish feed was produced and characterized according to the ISO Guides 30-35. The evaluation of the estimated minimum mass, homogeneity, and short- and long-term stability of the material was performed using microwave-assisted digestion and inductively coupled plasma optical emission spectroscopy as an in-house validated technique for Ca, Cu, Fe, K, Mg, Mn, Na, P, and Zn analysis. The accuracy of the measurements results was confirmed by analyzing the certified reference materials Oyster Tissue and Tomato Leaves, NIST SRM 1566b and SRM 1573a. The chemical characterization concerning elemental composition was performed through a collaborative trial with the participation of 35 laboratories by using spectroscopic and instrumental neutron activation analysis techniques. One-way analysis of variance (ANOVA) and regression analysis were the statistical methods applied to achieve the consensus values and their combined uncertainty, and the homogeneity and stability of the material presented averages within the 95% confidence interval. The expanded uncertainty considered all the instability observed during the stability, homogeneity, and characterization tests. The certified values and expanded uncertainties (k = 2) for the candidate reference were (28.65 ± 1.84) g kg^{-1} Ca, (10.51 ± 0.87) mg kg^{-1} Cu, (231.97 ± 20.17) mg kg^{-1} Fe, (5.86 ± 0.31) mg kg^{-1} K, (1.55 ± 0.16) g kg^{-1} Mg, (19.46 ± 2.12) mg kg^{-1} Mn, (2.16 ± 0.19) g kg^{-1} Na, (16.06 ± 0.97) g kg^{-1} P, and (129.56 ± 6.79) mg kg^{-1} Zn. The produced material proved its suitability for internal instrumental quality control or for the quality assurance of Ca, Cu, Fe, Mg, Mn, Na, P, and Zn analysis in fish feed for evaluation the laboratories quality control.

1. Introduction

One of the main technical difficulties in the development and expansion of aquaculture in Brazil is the feeding of cultivated organisms. A straight influence on the productive chain, on the healthy growth and development of the species, is observed. Furthermore, there is a need to ensure the availability of adequate feed formulations that meet the nutritional requirements of each species at the various stages of aquaculture production [1,2]. Fish feed is also the item with the highest cost in the aquaculture and can represent up to 70 to 80% of the total costs.

Brazil is one of the largest fisheries producers [3], and there is a growing demand for food and agricultural products, in quantity and quality. During the last decade, domestic consumption of fish and fish products has steadily increased by imports and the construction of reservoirs to growth fisheries production [4]. The outlook for aquaculture projects a production growth to 52% above the average level for 2012–14 by 2024, propelled by the national demand and policies to support the development of the sector [5]. For the development and growth of Brazilian aquaculture production in a sustainable way, the scientific and technological actions are necessary, addressing the current demands in the various links in the production chain. The quality of the chemical analytical results is fundamental. In this context, reference materials (RMs), certified reference materials (CRM), and the use of accredited analytical procedures are routinely used to ensure reliability and accuracy of measurement results [6–9].

Fish feed choice should be guided by the quality criteria, which should consider the nutritional composition and the physical and chemical evaluations of feed pellets [1]. Despite the relevance of chemical measurements for the fisheries and aquaculture sector, Brazil still does not produce RMs in enough quantity to supply the demand. The accessibility of RMs with designated concentrations of nutrients is required to assist fisheries producers in quality control. Furthermore, it is

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of essential to prepare a RM with similar matrix and composition of the samples to be analyzed [10]. Matrix-matched RMs can provide a better verification of a measurement method, resulting in an improved agreement with regulation organisms. A survey in COMAR, the international RM database, showed no specific RM of fish feed or even one material with a similar composition for macro- and micronutrients purposes [11].

Considering the previous discussion, we present the preparation and characterization of a new RM of fish feed. The development was according to ISO Guides 30-35 [12,13], including results obtained in a collaborative trial regarding the mass fraction of macro- and micronutrients. Sample preparation, homogeneity and stability studies were performed at Embrapa Pecuaria Sudeste, a governmental Brazilian agriculture research institution. Statistical techniques were applied to the evaluation of homogeneity and stability studies to calculate the uncertainty contribution of each component. Results from the preparation and interlaboratory comparison allowed the estimation of consensus values and its expanded measurement uncertainties for Ca, Cu, Fe, K, Mg, Mn, Na, P and Zn.

2. Experimental

2.1. Preparation of the reference material

About 40 kg of fish feed samples of the same batch were obtained from a commercial manufacturer, to produce the RM candidate. It was used a fish feed with a typical constitution to those used by the producers, which has about 32% crude protein. Feed samples were dried for 48 h at 60 °C ± 5 °C in an air convection oven (Fanem, Sao Paulo, Brazil), ground using an ultra-centrifugal mill (ZM200, Retsch, Germany) fitted with a 750 μm sieve, three times homogenized in a "Y" shaped polytetrafluorethylene coated bowl (MA 201/5MO Marconi, Piracicaba SP, Brazil), and randomly bottled after division in a PT-100 rotary divider (Retsch Technology, Haan, Germany). Aliquots of about 85 g were distributed into 300 demineralized amber glass bottles with screw caps and packaged in zippered metalized polyethylene terephthalate/low-density polyethylene stand-up pouches (metalized PET/LDPE). For longer shelf life, after bottling the material was sterilized using gamma irradiation (5–10 kGy) at the Nuclear and Energetic Research Institute (IPEN, São Paulo, Brazil). After sterilized, the bottles were stored at room temperature (25 °C ± 5 °C).

Taking into consideration that homogeneity can be affected by particle size, one randomly selected bottle was analyzed, in three subsamples, for particle size distribution in the equipment Analysette 22 (MicroTec Plus, Fritsch, Germany), using wet dispersion.

Residual moisture content was carried out by measuring mass loss after oven drying. Three bottles were randomly selected, and three separate portions (approximately 1 g each) were taken, from each bottle, and placed 24 h into an air convection oven at 105 °C ± 4 °C. Mass fractions of macro- and micronutrients were converted based on dry mass using the weight difference for individual test portions.

2.2. Reagents and solutions

All reagents used were of analytical grade and prepared using high-purity water (18 mΩ cm resistivity), obtained from a Milli-Q® water system (Millipore, Billerica, USA). All glassware, including amber glass bottles to package samples and polypropylene flasks, was washed with soap and soaked for 24 h in 10% (v v^{-1}) HNO_3, rinsed with water, and dried in a laminar flow hood. High-purity nitric acid (Synth, São Paulo, Brazil) was prepared by sub-boiling distillation system (BSB-939-IR, Berghof, Germany) and 30% (m v^{-1}) H_2O_2 (Sigma Aldrich, Germany) were used for sample preparation.

The measurement calibration solutions (Ca, Cu, Fe, K, Mg, Mn, Na, P, and Zn) were prepared from mon-element containing 1000 mg L^{-1} (TEcLab, São Paulo, Brazil, traceable to NIST reference solutions). Liquid argon was used as purge and plasma gas (99.999% purity, White Martins, São Paulo, Brazil). Oyster Tissue, SRM 1566b, and Tomato Leaves, SRM 1573a, from the National Institute of Standards and Technology (NIST, Gaithersburg, USA), were the CRMs used to accuracy evaluation.

2.3. Sample preparation and mass fraction determination

Macro- and micronutrients (Ca, Cu, Fe, K, Mg, Mn, Na, P, and Zn) mass fractions were determined in solution by ICP OES with an iCAP 6000-series dual-view emission spectrometer (Thermo Fisher Scientific, Waltham, MA, USA), operating in axial and radial view mode, equipped with CID detector and Echelle grating.

Sample pretreatment procedure was performed using a closed vessel microwave digestion system (Ethos 1, Milestone, Sorisole, BG, Italy) fitted with perfluoroalkoxy alkane (PFA) vessels and equipped with pressure and temperature sensors. A volume of 6 mL of 7 mol L^{-1} HNO_3 and 2 mL of 30% (m v^{-1}) H_2O_2 were added to a blank sample, and 250 mg accurately weighed of each evaluated sample, RM candidate and CRMs (SRM 1566b and SRM 1573a) in the microwave vessels. The experiment was performed in triplicates. Samples digestions were performed according to the following microwave program: 1300 W, 120 °C, 15 min, 1300 W, 200 °C, 40 min, and 0 W, 10 min respectively considering power, temperature and time. After the vessels were cooled to room temperature, the digests were transferred to volumetric flasks, and the volume was set to 50 mL. Each sample was analyzed (in triplicates), followed ICP OES quantification to achieve the total mass fraction of elements for all studies carried out with the RM.

To determine the minimum sample able to be determined, four sample sizes, ranging from 100 mg to 300 mg with the precision of ± 0.001 mg were tested taking three replicates and were evaluated based on the relative standard deviation (RSD) of the obtained results.

2.4. Homogeneity study

Among reference material preparation, homogenization is one of the most critical steps, and good homogeneity is a crucial characteristic. Two types of homogeneity should be considered: within-bottle and between-bottle. Within-bottle validates the homogenization step and determines the minimum sample intake for which the reference value is valid. Between-bottle homogeneity endorses that there is no bottle-to-bottle variability, which ensures that the batch has only identical samples [14,15]. The homogeneity of the RM candidate was evaluated according to the statistical procedures recommended by the ISO Guide 35 [13]. Homogeneity within-bottles was accessed with analysis of ten sub-samples obtained from one randomly selected bottle of RM. A Grubbs test was applied in the analyte results, to identify and excluded anomalous results and significant outliers. The value of the ratio between the difference of each result (x_i) and the mean of all results (x) concerning the standard deviation (s) is compared to calculate the Grubbs (G) test (Eq. (1)). If the value of "G" is higher than the critical value corresponding to tabulated values, the suspicious value is considered outlier at a significance level of 95% [19,20].

$$G = \frac{|(x_i - \overline{x})|}{s} \quad (1)$$

Between-bottles homogeneity study provides homogeneity uncertainty and is calculated according to the equations presented in the scope of ISO Guide 35 [13,16], and it was evaluated by analysis of 250 mg, in three replicates in ten randomly selected bottles from the

prepared batch and mass fraction quantification of Ca, Cu, Fe, K, Mg, Mn, Na, P, and Zn. The obtained results from both tests were analyzed using a one-way analysis of variance (ANOVA), and the evaluation of the homogeneity was performed employing the Fisher test (*F* test).

According to the ISO 35 [13], Eq. (2) was used to determine the contribution of uncertainty from inhomogeneity between-bottles.

$$s_{bb}^2 = \frac{MS_{among} - MS_{within}}{n_0} \quad (2)$$

where: S_{bb} – between-bottle variance; MS_{among} is the mean square for the between-bottle from a one-way ANOVA, MS_{within} is the mean square for the within-bottle from a one-way ANOVA, and n_o is the number of replicates. In these cases, the between-bottle variance S_{bb}^2 is equal to ${u_{bb}}^2$ In the case of the insufficient repeatability of the measurement method [13,21,22], the Eq. (3) was used to calculate the uncertainty component relating to inhomogeneity:

$$u^*bb = \frac{\sqrt{MSwithin}}{n}\sqrt[4]{\frac{2}{\nu MSwithin}} \quad (3)$$

where: u^*_{bb} – uncertainty between-bottles; n – number of replicates; MS_{whitin} – mean square within bottles; ν_{MSwithin} – degrees of freedom of mean square within bottles.

2.5. Short- and long-term stability study

Combining with homogeneity study, stability has a crucial role in the RM production process. The assigned value of a property should be ensured for all the expected lifetime of a RM [17]. There are two relevant types of stability in the production of RMs: short-term stability, which simulates material stability during transport conditions, and long-term stability, which simulates shelf life [13,14]. Assessment of both types of stability was made through a classical study design, where a prepared batch is measured as time progresses and the measurements are carried out under intermediate conditions of measurement [13]. In this context, the first test was performed in a short-term and high temperature and humidity using three bottled units randomly selected. Chosen bottles were set into a glass apparatus filled with water in the bottom and left in a conventional oven for four weeks, with the controlled temperature at 37 °C ± 3 °C and 100% ± 4% relative humidity. Three sub-samples of each bottle were analyzed in the beginning and at the end of the experiment for the macro- and micronutrients analysis. For a long-term stability assessment, ten bottles were randomly chosen and monitored periodically (every three months) for about 1 year by using the bottles stored at room temperature (25 °C ± 5 °C). Stability was evaluated based on the ANOVA results of a linear regression of respective residual macro- and micronutrients in the stability data according to ISO 35 guidelines [13]. According ISO Guide 35, an analyte is considered stable if the absolute value of the slope, |b|, is smaller than the product of Student's *t* factor ($t_{0.95,\ n-2}$) by the uncertainty associated with the slope, $s(b)$, as presented in Eqs. (4)–(7):

$$b_0 = \overline{Y} - b_1\overline{X} \quad (4)$$

$$b_1 = \frac{\sum_{i=1}^{n}(X_i - \overline{X})(Y_i - \overline{Y})}{\sum_{i=1}^{n}(X_i - \overline{X})2} \quad (5)$$

$$s^2 = \frac{\sum_{i=1}^{n}(Y_i - b_0 - b_1X_1)2}{n-2} \quad (6)$$

$$s(b_1) = \frac{s}{\sqrt{\sum_{i=1}^{n}(X_i - \overline{X})2}} \quad (7)$$

where: X – time of the experiment; Y – analyte mass fraction; b_0 – intercept; s^2 – standard deviation; $s(b_1)$ – uncertainty associated with slope.

2.6. Material characterization

Characterization of the material was performed using a collaborative study. One bottle of fish feed RM candidate was distributed for each one of 35 laboratories that agree to participate in the collaborative trial, during the Annual Workshop of Proficiency Test for Animal Nutrition Laboratories at Embrapa Pecuaria Sudeste. It was requested the quantification of six replicates for each analyte, and the results should be provided individually. The *z*-score was adopted to evaluate the performance of each laboratory for each analyte test. Results in the limit $|z| \leq 2$ were considered satisfactory; *z*-score results between the range $2 < |z| < 3$ were considered questionable, and *z*-score results above the limit $|z| \geq 3$ were considered unsatisfactory. The $|z| \geq 2$ were not considered for the final calculation.

2.7. Estimation of measurement uncertainties

The RM combined uncertainty takes into consideration the individual uncertainties related to homogeneity, stability and characterization studies. Therefore, uncertainty related to all measurands of the RM of fish feed was calculated according to ISO Guide 35 [13]. A coverage factor (k) was determined from the distribution function assumed for the property values (normal distribution) and the level of confidence of 95%. Therefore, as described by the ISO 35, based on the normal distribution, 95% level of confidence, a coverage factor k = 2 was assigned.

3. Results and discussion

3.1. Residual moisture and particle size

Average mass fraction moisture of the RM of fish feed candidate was evaluated using three bottles and three sub-samples from each bottle (n = 18). Residual moisture was assessed using approximately 1 g of dried sample. For the three analyzed bottles, the residual moisture was 5.43% ± 0.20%. This result was used for mass fraction correction to a dry mass basis.

Fig. 1 presents the particle size distribution profile for the fish feed RM, indicating that about 90% of the particles presented a diameter under 625 μm (n = 3). It is important to emphasize that we intend to produce a material similar to the normal feed fish samples that are sent to the laboratories.

3.2. Method validation

Selectivity, linearity, working range, quantification and detection limits, trueness and method repeatability were the parameters evaluated for the validation of the used homogeneity and stability method. Each day of the validation, a blank (no matrix) was prepared and analyzed to check for any possible reagent or material cross-contamination.

The accuracy of the evaluated nutrients was accessed based on the certified reference values of two CRMs: NIST SRM 1566b – Oyster tissue and NIST SRM 1573a – Tomato Leaves, prepared and measured using the same procedure as applied to the samples. The results, presented in Table 1, showed good agreement with the certified values, with recovery percentages ranging from 91 to 107%. Elemental quantification was performed by ICP OES, and the obtained results were statistically satisfactory for all the nutrients under a paired *t*-test at 95% confidence level.

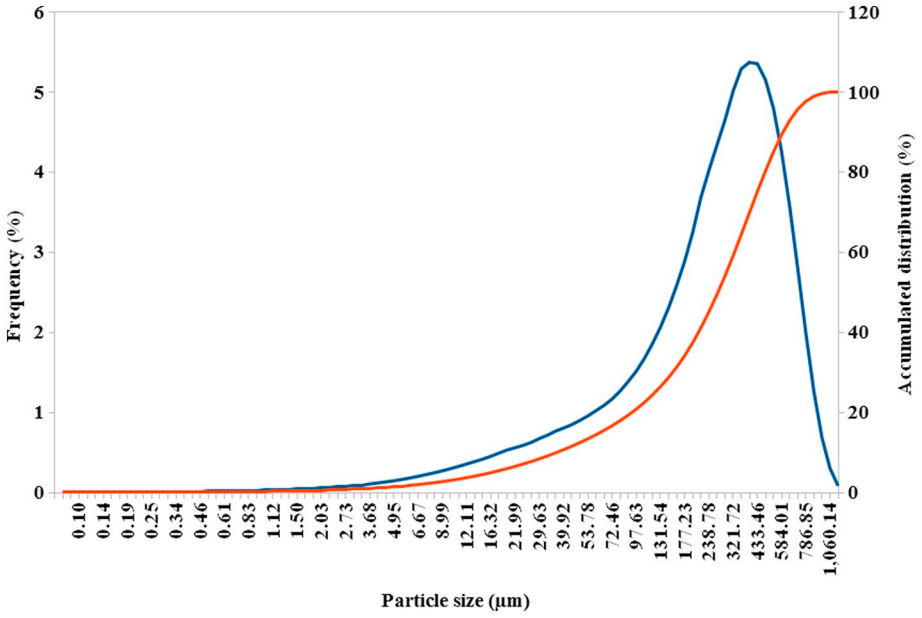

Fig. 1. Particle size distribution for the reference material of fish feed. Laser diffraction with wet dispersion was applied.

Table 1
Certified and determined mass fraction (mean ± SD, n = 3) and calculated accuracy (%) for Ca, Cu, Fe, K, Mg, Mn, Na, P and Zn in oyster tissue (NIST SRM 1566b) and tomato leaves (NIST SRM 1573a), obtained by ICP OES.

Element	CRM	Certified value	Determined value	Recovery (%)
Ca	Oyster tissue	0.838 ± 0.020[b]	0.83 ± 0.04[b]	99
	Tomato leaves	0.505 ± 0.09[b]	0.54 ± 0.04[b]	107
Cu	Oyster tissue	71.6 ± 1.6[a]	76.6 ± 1.1[a]	107
	Tomato leaves	4.70 ± 0.14[a]	4.81 ± 0.23[a]	102
Fe	Oyster tissue	205.8 ± 6.8[a]	201.1 ± 1.1[a]	98
	Tomato leaves	368 ± 7[a]	368.1 ± 1.1[a]	100
K	Oyster tissue	6.52 ± 0.09[b]	6.22 ± 0.01[b]	95
	Tomato leaves	27 ± 0.5[b]	28.9 ± 0.1[b]	107
Mg	Oyster tissue	1.085 ± 0.023[b]	1.07 ± 0.01[b]	99
	Tomato leaves	12[b]	11.44 ± 0.01[b]	95
Mn	Oyster tissue	18.5 ± 0.2[a]	20.05 ± 0.22[a]	108
	Tomato leaves	246 ± 8[a]	250.47 ± 1.15[a]	102
Na	Oyster tissue	3.297 ± 0.053[b]	3.15 ± 0.01[b]	96
	Tomato leaves	136 ± 4[b]	150 ± 1[b]	110
P	Tomato leaves	2.16 ± 0.04[b]	1.96 ± 0.01[b]	91
Zn	Oyster tissue	1424 ± 46[a]	1474 ± 1[a]	104
	Tomato leaves	30.9 ± 0.7[a]	30.82 ± 0.24[a]	100

[a] mg kg^{-1}.
[b] g kg^{-1}.

3.3. Homogeneity study

According to ISO Guide 33 [18], the RM user should consider that the homogeneity is directly associated to a defined sample size and the uncertainty associated with the reference value, that is guaranteed only with the use of a minimum sample mass specified by the RM producer.

Four different masses, 100, 200, 250, and 300 mg were used to evaluate the minimum sample size for the mass fraction quantification of Ca, Cu, Fe, K, Mg, Mn, Na, P, and Zn. The average results (n = 3) of the evaluated analytes in the RM for the four masses analyzed by ICP OES after sample digestion are presented in Table 2. Following ISO Guide 35 [13], ANOVA at 5% significance was applied to determine the significance of differences between the results.

For Ca, Fe, P, and Zn, ANOVA showed no significant differences between the results obtained from the four sample sizes, while the other elements showed significant differences (p-value < 0.05 and $F > F_{crit} = 4.10$). It was possible to observe a decrease in RSD value for the mass of 250 mg. Thus, the minimum sample size was set to homogenize the acquired results, considering the lowest RSDs. Consequently, the mass of 250 mg which presents the lowest RSD, was established for the subsequent studies. The within bottle homogeneity results indicated that at a level of 95% confidence, no outlier was found, suggesting that the data are homogeneous.

The ANOVA was used to statistically evaluate the between-bottles

Table 2
Mass fractions of macro- and micronutrients in the fish feed reference material obtained for different sample masses (n = 3).

	100 mg		200 mg		250 mg		300 mg	
	Mean ± SD	RSD (%)	Mean ± SD	RSD (%)	Mean ± SD	RSD (%)	Mean ± SD	RSD (%)
Ca[b]	29.93 ± 1.52	5.07	30.00 ± 0.68	2.25	29.57 ± 0.51	1.73	31.09 ± 2.18	6.99
Cu[a]	11.51 ± 0.38	3.34	10.44 ± 0.43	4.15	10.37 ± 0.17	1.66	10.55 ± 0.26	2.42
Fe[a]	267.8 ± 57.9	21.64	247.6 ± 3.8	1.53	247.6 ± 1.6	0.65	241.1 ± 15.3	6.34
K[b]	5.70 ± 0.05	0.85	6.15 ± 0.06	0.97	6.23 ± 0.01	0.18	6.28 ± 0.05	0.83
Mg[b]	1.36 ± 0.02	1.37	1.47 ± 0.01	0.52	1.50 ± 0.01	0.06	1.55 ± 0.01	0.57
Mn[a]	17.11 ± 0.58	3.37	16.95 ± 0.65	3.82	19.92 ± 0.14	0.70	18.32 ± 0.13	0.72
Na[b]	2.15 ± 0.01	0.51	2.20 ± 0.02	1.01	2.25 ± 0.01	0.16	2.30 ± 0.04	1.68
P[b]	15.68 ± 0.36	2.32	15.65 ± 0.30	1.94	15.59 ± 0.14	0.88	16.07 ± 1.05	6.52
Zn[a]	128.3 ± 1.0	0.79	126.8 ± 0.4	1.15	126.1 ± 0.4	0.33	127.0 ± 0.5	0.42

[a] mg kg^{-1}.
[b] mg kg^{-1}.

Table 3
Statistical evaluation of the between-bottle homogeneity study.

Analyte	$F_{calculated}$	u_{bb}	p-Value
Ca	1.77	0.44[b]	0.14
Cu	0.72	0.17[a]	0.68
Fe	1.83	6.40[a]	0.12
K	3.44	0.07[b]	0.01
Mg	1.37	0.01[b]	0.26
Mn	1.97	0.52[a]	0.10
Na	1.96	0.03[b]	0.10
P	2.81	0.28[b]	0.03
Zn	4.02	1.44[a]	0.01

[a] mg kg^{-1}.
[b] g kg^{-1}. $F_{critical} = 2.39$.

homogeneity, taking into consideration a critical value of $F = 2.39$, at a level of significance of 5% ($\alpha = 0.05$). Variation in the obtained concentration as a function of the sub-sample mass used for several elements was verified, and the ANOVA test was repeated after evaluation with Grubbs test and removal of anomalous results and significant outliers observed for some results of Fe and Zn. These anomalous results were removed to obtain average values. The uncertainty due to homogeneity and p-value arising from the between-bottle test were calculated and estimated according to ISO Guide 35 [13].

The statistical data of ANOVA for the between-bottles homogeneity study can be observed in Table 3.

The F calculated values smaller than critical F value (2.39 at 0.05 significance) was obtained for Ca, Cu, Fe, Mg, Mn, and Na since no significant differences were verified in the considered confidence level. The RM had significant differences between-bottles when $F > F_{crit}$ and $p < 0.05$ for K, P, and Zn (Table 3). The contribution of uncertainty inherent to the degree of heterogeneity was calculated using Eqs. (2) and (3) as described in the Experimental, and the highest value was chosen, as a worst-case estimation for the uncertainty contribution from possible inhomogeneity.

3.4. Short- and long-term stability

Stability study aimed to evaluate the level of instability of the candidate RM of fish feed or establish the stability of the material under various conditions. To evaluate if the targeted macro- and micronutrients are stable or not under transport conditions, three bottles randomly selected were placed into an apparatus with controlled temperature and humidity for 4 weeks. After this time, the samples were prepared as described before and analyzed by ICP OES. One-way ANOVA and simple linear regression analysis were used to assess the short-term stability of the RM and calculated F value was then compared to the critical F value. The ANOVA statistical data are in the supplementary material. It was possible to observe that for Ca, Cu, Fe, K, Mn, P and Zn, $F_{calculated} < F_{critical}$, while for Mg and Na was the opposite, probably by the instability of the measurement system or reproducibility aspects, such as equipment or calibration, considering the classical design [13].

Simple linear regression analysis assumes a normally distributed data [13]. Primarily, the data were checked for outliers using the Grubbs test, and linear regression analysis as a function of time was performed. Slopes were tested for significance using t-test. After the verification, this statistic tool was used to estimate the uncertainty associated with the short-term stability of the RM. The obtained standard error is multiplied by the time of analysis, in this case, 4 weeks, to get the short-term uncertainty component.

Long-term stability must be performed to verify stability during storage conditions. It was performed considering the samples stored at the reference temperature of 25 °C ± 5 °C, and the results were evaluated through ANOVA and regression analysis. Table 4 presents the average concentrations obtained for the time of storage, while Fig. 2 shows regression analysis graphs for some of the evaluated elements. Regression analysis graphs for selected elements are presented in the supplementary material. The same procedure employed for short-term uncertainty assessment was used for long-term stability. The uncertainty values were calculated as the product of the standard deviation and the time of the experiment (12 months).

As previously discussed, ISO Guide 35 [13] establishes that an analyte is considered stable if the absolute value of the slope, $|b|$, is smaller than the product of Student's t factor ($t_{0.95,\ n-2}$) by the uncertainty associated with the slope, $s(b)$ (Eqs. (4)–(7) of Experimental item). If this condition is confirmed, no instability is observed. Table 5 shows the statistical results obtained from this evaluation.

By analyzing the data from Table 5, stability can be observed for Fe, K, Mg, Mn and Na in a confidence level of 95%. Considering the linear regression analysis according to ISO Guide 35, Ca, Cu, P and Zn could be considered unstable for long-term room temperature storage since a possible degradation or concentration increment were observed. However, estimating regression parameters and evaluating significance without a previous verification of regression assumptions could generate ambiguous inferences about the RM stability [13,19]. The experiment was performed by using the classic layout. In this case, the work is carried out under (within-laboratory) reproducibility conditions, which leads to relatively high uncertainty, as the instability of the measurement system.

Observed variability can be due to the precision of ICP OES method used, rather than to any significant instability of the RM. ISO Guide 35 points out that the main disadvantage of the classical design is that the results can be unfavorably affected by variations in the measurement process. Fish feed RM has been monitored every 6 months, and recoveries between 93 and 113% were found for all evaluated analytes.

3.5. Interlaboratory comparison

The RM characterization was performed through a collaborative trial, and the consensus value for macro- and micronutrients were obtained from the results reported by the participants. After homogeneity and stability studies, a bottle of candidate RM of fish feed was sent to the laboratories that accepted to take part in the collaborative trial, as previously described. The number of laboratories that reported results varied according to parameters, ranging from 22 (Cu) to 35 (Ca) participants, as well as analytical techniques utilized for Ca, Cu, Fe, K, Mg, Mn, Na, P, and Zn quantification. A variety of techniques ranging from ICP OES, flame atomic absorption spectrometry (FAAS) and flame photometry (FP), to instrumental neutron activation analysis (INAA).

According to ISO Guide 35, one of the ways for characterization assessment is through a collaborative study utilizing different methods or various laboratories. The interlaboratory comparison is the most applied method for establishing assigned values of properties of a RM candidate. The use of different sample preparation procedures and analytical techniques can lead to a difference between the results. Otherwise, as can be observed in Fig. 3, the majority of results were within z-score previously defined as satisfactory values. The statistical study was carried out based on ISO/IEC 17043:2010 [23], using the z-score for trueness assessment, according to ISO Guide 35 [13]. For each analyte, it was primarily applied Grubbs test at a 95% confidence level to identify anomalous data and remove potential outliers [13,20,24]. After removing outliers, it was utilized Cochran's test to verify the homogeneity of variances. Horwitz [25] and Thompson [26] models were used for estimation of the standard deviation for the proficiency

Table 4
Average concentration (n = 3) for the long-term stability study.

Analytes	0 month	3 months	6 months	9 motnhs	12 months
Ca[b]	31.78 ± 1.32	35.15 ± 1.66	33.51 ± 1.29	31.34 ± 1.67	28.44 ± 0.89
Cu[a]	11.40 ± 0.39	10.76 ± 0.39	10.49 ± 0.16	10.70 ± 0.18	11.97 ± 0.48
Fe[a]	246.0 ± 15.9	268.7 ± 16.1	236.5 ± 10.5	253.7 ± 11.6	245.4 ± 20.0
K[b]	6.08 ± 0.10	6.92 ± 0.17	6.93 ± 0.12	6.76 ± 0.03	6.26 ± 0.05
Mg[b]	1.58 ± 0.04	1.76 ± 0.05	1.68 ± 0.03	1.72 ± 0.02	1.72 ± 0.02
Mn[a]	17.11 ± 1.03	22.42 ± 1.77	16.65 ± 1.14	19.38 ± 2.60	17.9 ± 4.29
Na[b]	2.18 ± 0.05	3.16 ± 0.36	2.89 ± 0.07	2.70 ± 0.05	2.45 ± 0.03
P[b]	14.16 ± 0.48	14.93 ± 0.33	14.91 ± 0.41	14.66 ± 0.50	15.4 ± 0.35
Zn[a]	124.0 ± 2.1	119.0 ± 4.3	120.2 ± 1.8	123.5 ± 1.6	130.9 ± 3.9

[a] mg kg^{-1}.
[b] g kg^{-1}.

assessment (σ_p). As described in the Experimental item, results in the limit $|z| \leq 2$ were considered satisfactory and both z-score results, the $2 < |z| < 3$, considered questionable, and above the limit $|z| \geq 3$, unsatisfactory, were not considered in the final calculations of the characterization uncertainty.

Considering this classification, the following results were discarded, as can be observed in Fig. 2: for Ca the results provided by laboratories 35 and 54; for K the laboratory 29; for Fe the laboratory 15; for Mg the laboratories 3, 16, and 62; for Mn the laboratories 6 and 21; for Na the laboratory 61; for P the laboratories 4 and 44, and for Zn the laboratory 62.

3.6. Estimated mass fractions with associated uncertainties

Assignment of associated uncertainty implies greater confidence in the trueness of the measurement result, and the reference value

Table 5
Statistical results obtained by linear regression for macro- and micronutrients during the long-term study.

Analytes	$\lvert b \rvert$	$t_{(0.95,n-2)} \times s(b)$	u_{lts}
Ca[b]	0.349	0.275	0.51
Cu[a]	0.052	0.036	0.15
Fe[a]	0.543	1.714	4.25
K[b]	0.007	0.057	0.09
Mg[b]	0.0080	0.0081	0.01
Mn[a]	0.049	0.336	0.72
Na[b]	0.003	0.055	0.09
P[b]	0.073	0.042	0.11
Zn[a]	0.608	0.527	1.02

[a] mg kg^{-1}.
[b] g mg^{-1}.

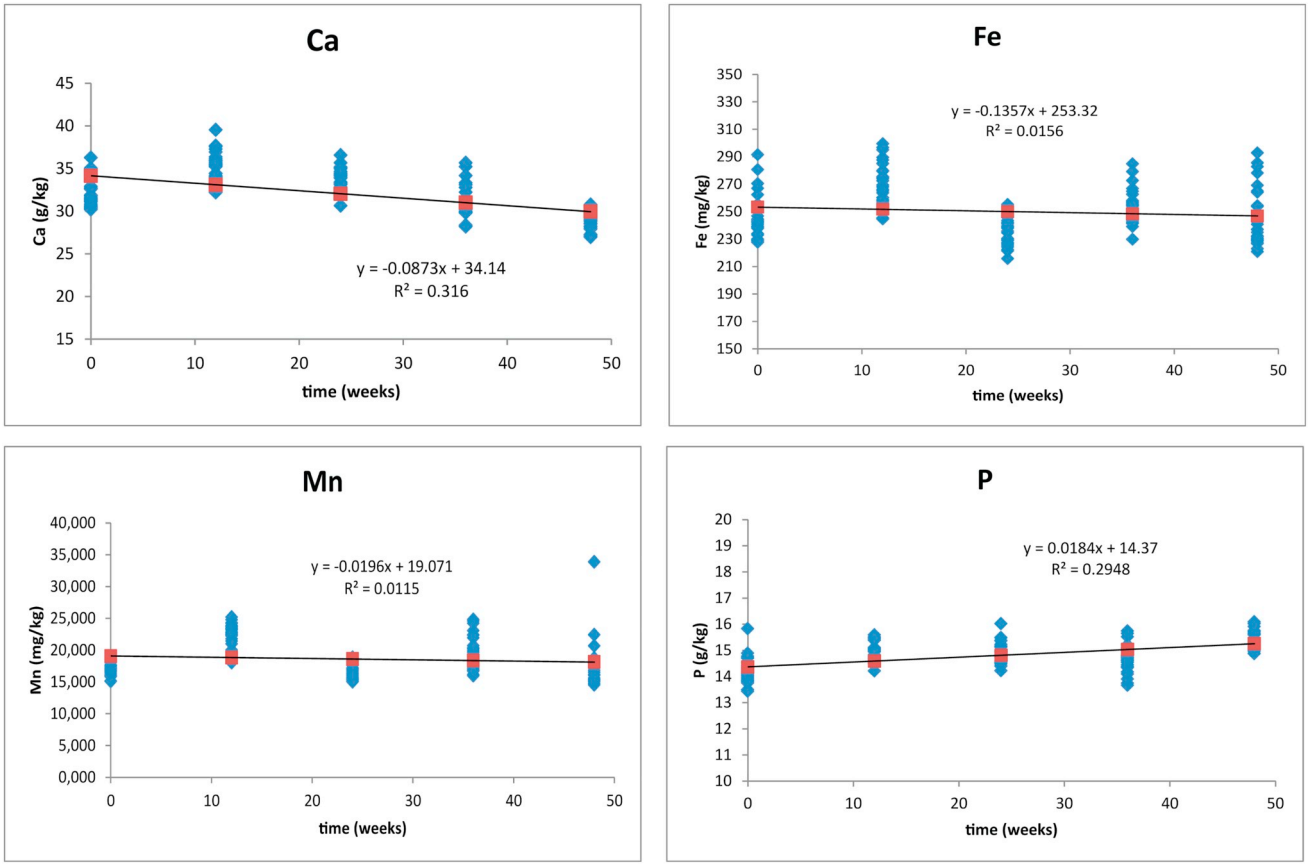

Fig. 2. Typical linear regression plots obtained for long-term stability study during 12 months of storage at room temperature for the RM of fish feed for Ca, Fe, Mn, and P.

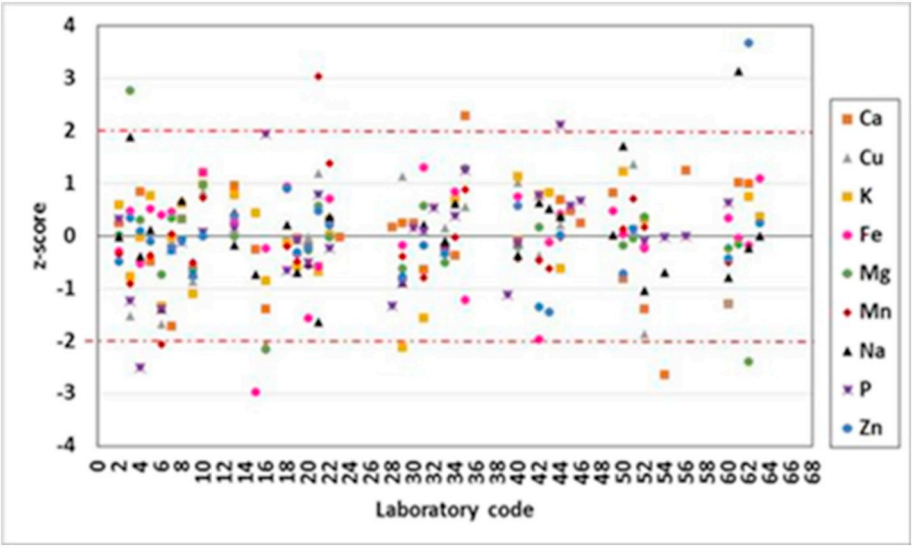

Fig. 3. Obtained *z*-scores by the laboratories in the collaborative study for mass fractions of the macro- and micronutrients.

Table 6
Estimates of standard uncertainties of characterization (u_{char}), between-bottle homogeneity (u_{bb}), short-term stability (u_{sts}) and long-term stability (u_{lts}) along with the combined uncertainty (u_{RM}).

	u_{char}	u_{bb}	u_{sts}	u_{lts}	u_{RM}
g kg^{-1} (m m^{-1})					
Ca	0.57	0.44	0.43	0.51	0.98
K	0.12	0.07	0.04	0.09	0.17
Mg	0.08	0.01	0.01	0.01	0.08
Na	0.06	0.03	0.03	0.09	0.11
P	0.32	0.28	0.22	0.11	0.49
mg kg^{-1} (m m^{-1})					
Cu	0.32	0.17	0.21	0.15	0.45
Fe	5.12	6.40	4.94	4.25	10.47
Mn	0.65	0.52	0.38	0.72	1.16
Zn	2.93	1.44	0.56	1.02	3.46

Table 7
Fish feed mass fraction robust means with expanded uncertainties. $k = 2$, at dry mass basis, in a confidence interval of 95%. Values for macro- and micronutrients.

Analyte	Mass fraction	U_{RM} (%)
Ca[b]	28.65 ± 1.84	6.43
Cu[a]	10.51 ± 0.87	8.28
Fe[a]	231.97 ± 20.17	8.70
K[b]	5.86 ± 0.31	5.30
Mg[b]	1.55 ± 0.16	10.23
Mn[a]	19.46 ± 2.12	10.91
Na[b]	2.16 ± 0.19	8.76
P[b]	16.06 ± 0.97	6.06
Zn[a]	129.56 ± 6.79	5.24

[a] mg kg^{-1}.
[b] g kg^{-1}.

presented with its uncertainty makes it possible to use the RM material in the assessment of the accuracy of an analytical method and an establishment of quality control of procedures.

Table 6 presents the standard uncertainties associated with characterization (u_{char}), between-bottle homogeneity (u_{bb}), short-term stability (u_{sts}) and long-term stability (u_{lts}) along with the combined uncertainty. All uncertainties were calculated as described by ISO Guide 35 [13]. Expanded uncertainty, U_{RM} was calculated, as described in Eq. (8) taking into consideration contributions from all the components at a coverage factor, $k = 2$ (for 95% confidence interval) [13].

$$U_{RM} = k\sqrt{u_{char}^2 + u_{bb}^2 + u_{sts}^2 + u_{lts}^2} \quad (8)$$

Reference values for mass fractions of targeted macro- and micronutrients in the final fish feed RM were calculated from the results provided by the collaborative study. Table 7 shows the reference values associated with its expanded uncertainties.

Homogeneity, stability and characterization uncertainty components are equally important and emphasizing not all of them could result in a decrease of the material quality. When assessing a RM uncertainty, all the components must be included, and contributions from homogeneity and stability are often more important than characterization uncertainty of the batch [14], but it is essential to consider that the characterization component is sensitive to deviation due to variation from the laboratories of the collaborative trial. Fig. 4 shows the individual contribution of each component and the combined uncertainty.

According to Table 7 and Fig. 4, most analytes (Ca, Cu, K, Mg, P, and Zn) had significant influence from the characterization uncertainty component, while homogeneity uncertainty component was the major contributor for Fe uncertainty and long-term stability was the main contribution for Mn and Na.

4. Conclusions

Production and characterization of a new fish feed RM were accomplished. Long-term stability study of the MR of fish feed was performed by the residual analysis in regression and ANOVA, and the short-term stability study was performed by simple linear regression. The RM was considered sufficiently stable to be stored at room temperature. The data obtained along with the production and through the collaborative trial allowed the characterization of the fish feed RM and the assignment of mass fractions of elements. The contribution of homogeneity, stability, and characterization was taken into consideration for the estimation of uncertainties components. For most of the elements, the characterization component was the major uncertainty contribution, due to the variation of the results provided by the participants in the collaborative trial. Moreover, participation of the laboratories was fundamental to obtain the reference values for Ca, Cu, Fe, K, Mg, Mn, Na, Pb, and Zn in the produced RM fish feed. The new material can be used as a quality control tool to support the development of aquaculture production sustainable, ensuring the quality of products.

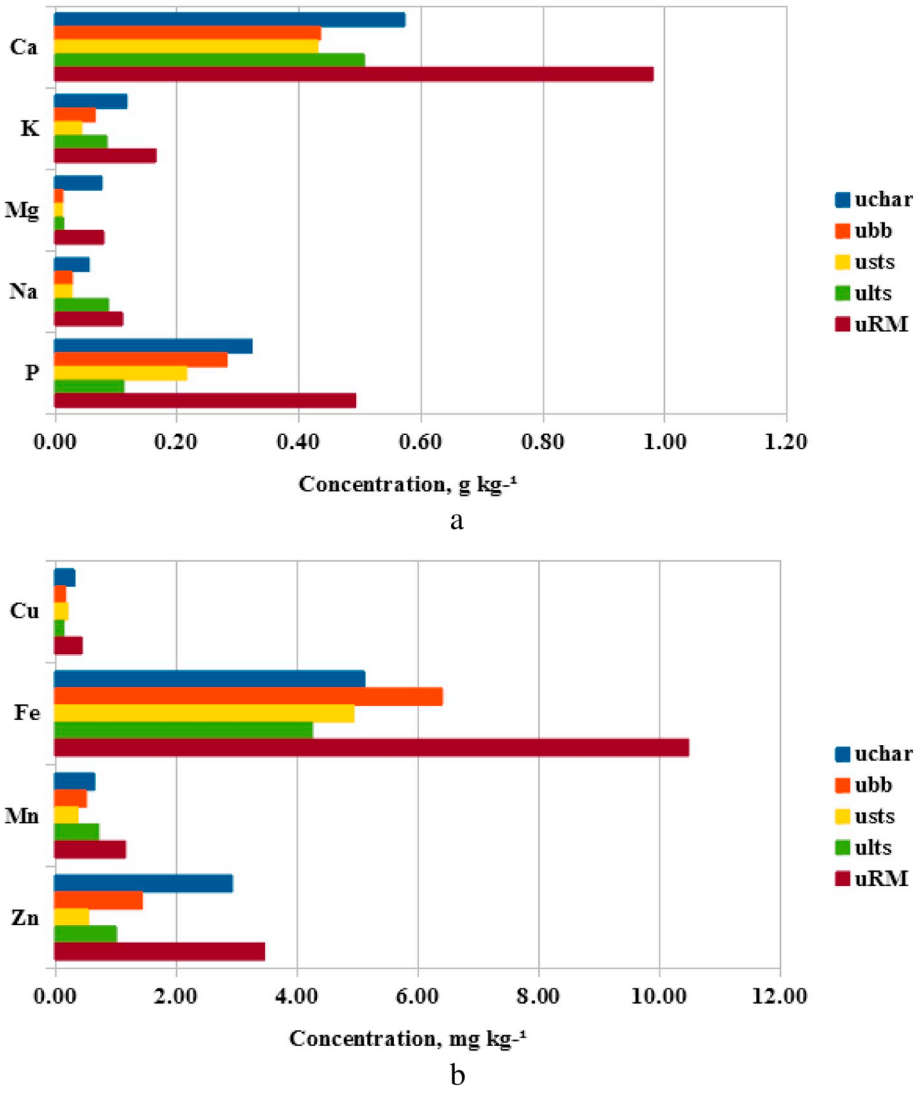

Fig. 4. Contribution of individual uncertainty components to fish feed RM uncertainty for (a) macronutrients and (b) micronutrients. Presented are individual uncertainty components and combined uncertainty.

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