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Homogeneity study of candidate reference material in fish matrix

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Abstract. A material is perfectly homogeneous with respect to a given characteristic, or composition, if there is no difference between the values obtained from one part to another. Homogeneity is usually evaluated using analysis of variance (ANOVA). However, the requirement that populations of data to be processed must have a normal distribution and equal variances greatly limits the use of this statistical tool. A more suitable test for assessing the homogeneity of RMs, known as "sufficient homogeneity", was proposed by Fearn and Thompson. In this work, we evaluate the performance of the two statistical treatments for assessing homogeneity of methylmercury (MeHg) in candidate reference material of fish tissue.

1. Introduction

The homogeneity study is an integral part of planning the production of certified reference material (CRM), allowing the evaluation of one of the key uncertainty components in the certification model. Clearly, a high degree of homogeneity is anticipated during the preparation of reference materials. However, despite all efforts to ensure this, materials can still exhibit a degree of heterogeneity. The producer of the material thus has to ensure and demonstrate the level of homogeneity through the use of appropriate procedures to detect any impurities or interferences, caused by problems not detected during preparation.

According to ISO guide 35 [1], a material is perfectly homogeneous with respect to a particular characteristic or composition if there is no difference between the obtained values from one party to another. As a consequence, the amount of material necessary for homogeneity studies and the choice of appropriate measurement methods for determining the characteristics or chemical composition must be integrated from the beginning of experimental protocols.

A basic model for the evaluation of a homogeneity study comprises bottles (or parts) of the material and measured values that can be expressed by the relationship between the overall mean value of the measurements, error for homogeneity between bottles and random measurement error. The variances of these terms are represented by the variance between bottles and repeatability variance. Such evaluations are typically performed using one-way analysis of variance (ANOVA). However, this strategy can lead to erroneous conclusions in two cases: if the analytical method used is inaccurate, possible heterogeneities may not be detected and the test can incorrectly indicate that the material is homogeneous or that the variability in the results is significant; conversely, if the analytical



method is highly sensitive, small differences the composition among bottles can be inappropriately interpreted as heterogeneity. Moreover, populations of data to be processed by ANOVA must have a normal distribution and equal variances, which limits the use of this statistical tool.

Given the limitations of ANOVAs, a new methodology to evaluate the homogeneity of reference materials was proposed by Fearn and Thompson [2]. Known as "sufficient homogeneity", this method (unlike ANOVA) imposes a limit to the analytical variance, designated s_{an} , in relation the "target standard deviation (or expected)", designated σ_p . This limitation requires that the ratio s_{an}/σ_p should be less than 0.5, if possible. Another difference from ANOVA is the calculation of the critical value for comparison, calculated from obtaining the values of allowable variance between-samples (s_{all}^2), the analytical variance (s_{an}^2) and factors (F1 and F2) extracted from a specific table to test "sufficient homogeneity" [2].

Due to the superior performance of the sufficient homogeneity test, it was incorporated into an international protocol [3] and has been widely cited in the literature [4,5].

The objective of the present study is to compare analysis by ANOVA and "sufficient homogeneity" in the assessment of homogeneity study for methylmercury (MeHg) in candidate reference material in a fish matrix.

2. Experimental

2.1 Planning the homogeneity study

Between-bottle homogeneity of MeHg was determined for the contents of 10 bottles. For evaluation of the data by ANOVA, three portions of approximately 0.5 g of sample material from each bottle were tested. Each portion was analysed three times, obtaining three average values for each bottle. Subsequently, "sufficient homogeneity" was evaluated using two more portions of 0.5 g each and measured in triplicate, producing two average values for each bottle.

2.2 Sample preparation and determination of MeHg

Determination of MeHg is based on the acid leaching with hydrochloric acid solution (HCl) 6 mol L⁻¹ (by volume) and mercury separation of the organic and inorganic ion exchange resin (Dowex 1×8 100–200 mesh). The methodology was based on Horvat and May [6,7].

Once separated, MeHg was decomposed into inorganic Hg²⁺ by ultraviolet (UV) irradiation and the final solution was diluted to 30 g with demineralised water and inserted into the sample introduction system of an atomic absorption spectrophotometer (FS-SpectrAA220 Varian Australia Pty Ltd.). Methylmercury (such as mercury) is determined by atomic absorption spectrophotometry with cold vapor generation and flow injection (FIA-CV-AAS).

3. Results and discussion

3.1 Determination of methylmercury (MeHg) in candidate CRMs.

The portions from 10 bottles were analyzed and outliers were tested using the Grubbs test [8]. The analysis did not indicate the presence of any outliers.

3.1.1 One-way Analysis of Variance. Table 1 shows the results of ANOVA for MeHg.

Table 1. Data ANOVA of the homogeneity study for MeHg

Source of variation	SS	Degrees of freedom	MS	F _{calculated}	F _{critic}
Between groups	0.0950	9	0.0106	2.72	2.39
Within groups	0.0774	20	0.0039		
Total	0.1724	29			

As can be seen from table 1, F_{calculated} is higher than the critical value F_{critic}, indicating that the sample is not homogeneous (i.e. the material has not passed the test for homogeneity). Comparing the calculated variances with the measured values (table 2) reveals that one of the assumptions of the ANOVA was not supported because the variances are different. This is further substantiated by dividing the highest value obtained at the lower variance giving a value of 223, as compared to recommended values of 3 or 4 [9]. The F test used is a hypothesis test based on sample variances. Thus, the fact that the variances are different is further evidence that the test does not support or reject the hypothesis of equality of MeHg values.

Table 2. Results of mean values, standard deviation and variance of the results of replicates analyzed for MeHg.

Bottle N ^o .	Mean (µg g ⁻¹)	Standard deviation (µg g ⁻¹)	Variance	Replicate (n _j)
2	0.277	0.032	0.00104	3
9	0.205	0.033	0.00107	3
14	0.388	0.134	0.01789	3
26	0.241	0.017	0.00030	3
31	0.265	0.100	0.01001	3
36	0.317	0.074	0.00545	3
50	0.334	0.009	0.00008	3
55	0.216	0.022	0.00047	3
63	0.237	0.044	0.00195	3
78	0.223	0.021	0.00044	3

The difference in variance demonstrated by the ANOVA can be derived from the analytical method used in the measurements (FIA-CV-AAS), as performed on different days. This showed variability in the response signals of both the calibration curve and in the signals of the samples.

3.1.2 "Sufficient homogeneity" test. As recommended in the protocol [2], samples in duplicate were analyzed from 10 bottles under replicable conditions (results in Table 3). The items following table 3 are the equations used for the calculations. In item (a), the analytical variance (s_{an}^2) is obtained by dividing the sum of the squares of the differences (D^2) by $2m$, where m is the number of bottles. Item (b) gives the analytical variance as the standard deviation. Item (c) is the value obtained for the estimation of variance between bottles (S_{sam}^2). The value of the allowable variance between the sample (s_{all}^2) calculated in Item (d), obtained from the value of the target standard deviation (σ_p) calculated by the Horwitz function [3,10]. The critical value for the homogeneity study, called "c" is calculated in

Item (e), being obtained by summing the results of multiplying specific factors [2] with the values of (s_{all}^2) and (s_{an}^2). The significance of this test is determined by the following criteria: if " $S_{sam}^2 > c$ " homogeneity test failed and this cannot be proven and " $S_{sam}^2 < c$ " homogeneity test was accepted and this is proven.

Table 3. Test of homogeneity between bottles, applied to the data of MeHg

Bottle N.	Result 1 (a)	Result 2 (b)	D = a - b	S = a + b	D ² = (a - b) ²
2	0.296	0.295	0.001	0.591	0.000001
9	0.195	0.241	-0.046	0.436	0.002116
14	0.291	0.333	-0.042	0.624	0.001764
26	0.245	0.255	-0.010	0.500	0.000100
31	0.379	0.226	0.153	0.605	0.023409
36	0.382	0.332	0.050	0.714	0.002500
50	0.328	0.329	-0.001	0.657	0.000001
55	0.217	0.237	-0.020	0.454	0.000400
63	0.208	0.215	-0.007	0.423	0.000049
78	0.243	0.224	0.019	0.467	0.000361
Sum =					0.030701

Item (a) $s_{an}^2 = (\Sigma D^2)/2m = 0.030701/(2 \cdot 10) = 0.001535 \mu g g^{-1}$ (analytical variance)

Item (b) $s_{an} = \sqrt{s_{an}^2} = \sqrt{0.001535} = 0.039179 \mu g g^{-1}$ (analytical variance expressed how standard deviation)

Item (c) $S_{sam}^2 = ((\text{variance of the sums } S=a+b)/2 - S_{an}^2)/2 = (0.010686/2 - 0.001535)/2 = 0.001904$

Item (d) $\sigma_{all}^2 = (0.3\sigma_p)^2 = (0.3 \cdot 0.05326)^2 = 0.000255 \mu g kg^{-1}$.

σ_p (obtained by Horwitz function) = $0.02c^{0.8495} = 0.02(0.274 \cdot 10^{-6})^{0.8495}/10^{-6} = 0.05326 \mu g g^{-1}$ ("c" is average concentration of MeHg obtained, expressed as mass fraction).

Item (e) $c = F_1 s_{all}^2 + F_2 s_{an}^2 = 1.88 \cdot 0.0002553 + 1.01 \cdot 0.001535 = 0.002030$.

Using the criteria, $S_{sam}^2 = 0.001904 < c = 0.002030$ the homogeneity is proved.

4. Conclusions

Tests for homogeneity of MeHg for candidate reference material in a fish matrix were carried out using two statistical tools.

The ANOVA test indicated that the material was not homogeneous. In contrast, the "sufficient homogeneity" test proposed by Fearn and Thompson indicated an acceptable level of homogeneity of the material.

The homogeneity study provides one of the components of uncertainty required to evaluate combined standard uncertainty in certified reference material, ensuring minimum levels of quality and acceptability.

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