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CONFERENCE CONTRIBUTION

Wayne R. Wolf · Denis E. Lacroix

Sampling constants for niacin content in standard reference material 1846 Infant Formula

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Abstract Modern measurement systems for food components often require use of ever smaller sample sizes, down to mg for some new microtechniques, which puts a stronger demand on development of reference materials with defined homogeneity for subsampling. One approach to evaluate the homogeneity of materials is the characterization of sampling constants, defined as that amount of material that gives a 1% error for subsampling. This approach was developed for geological sampling and has been applied in a limited way for inorganic components in food/biological materials. We have extended this approach to the determination of the sampling constants for an organic component, niacin, in the SRM 1846 Infant Formula material. This material was produced by blending of a dry vitamin mix (5% weight) into the bulk spray dried powder, for long term stability purposes. By analyzing similar aliquots of a reconstituted homogeneous fluid solution of a large sample size, in comparison to smaller portions of dry powder, an estimate of the variation due to sampling can be separated from estimates of variation due to analysis. Using either the AOAC microbiological method or a newly developed HPLC method, sampling constants for the niacin content of SRM 1846 are in the range of 1–3 g; use of smaller sub-samples can introduce significant variation into determinations using this SRM.

Introduction

Modern measurement systems for food components often require a low level of uncertainty, in the range of 2–5% or less. Development of new analytical techniques is moving towards use of ever smaller sample sizes, i.e. 0.5 g or much less for some microtechniques. Present reference materials for food components often have uncertainties of ± 5–10% or higher for the characterized values. These uncertainties take into account random measurement errors and/or systematic bias between different characterization methods. In addition, the uncertainties account for sample inhomogeneities, and the certificates of analysis usually have an applicability statement regarding recommended minimal sample size, often 0.5 to 1.0 g or more. Thus the ability for most effective use of many of the available reference materials to assess and improve measurement accuracy and validity is somewhat limited. While there have been several studies on methods to obtain highly homogeneous biological reference materials [1, 2] these have generally produced highly specific matrices of single component materials.

One approach to study and evaluate the degree of homogeneity of materials for sampling purposes is the determination of sampling constants, as introduced for geological samples. Use of small geological samples for chemical analysis of materials that may be inhomogeneous for specific elements can give rise to large uncertainties [3]. This is particularly of concern when a large concentration of one element is contained in one ore of a mixture, for example a small fraction of chromite (high in chromium) in a reference granite G-1 [4]. In this case the number of grains of the minor component varied in subsamples, leading to an increased variance of chromium measurements on those subsamples. The variance due to the number of grains is a function of the subsample size, and can be predicted. To estimate the mass of an adequate geological sample for determining the component of interest in relation to accuracy of the analytical method, Wilson [5] calculated the subsampling error (S_s), which is expressed as a relative standard deviation of repeated subsamplings. This uncertainty depends upon the mass (m, in g) of sample, particle size distribution, density, number of components, and distribution of the analyte among the components [6]. For a binary mixture with a minor component

W. R. Wolf (✉) · D. E. Lacroix
Food Composition Laboratory,
Beltsville Human Nutrition Research Center,
Agricultural Research Service,
United States Department of Agriculture, Beltsville,
MD 20705, USA

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present in phase A and absent in phase B, (S_s) can be expressed as:

$$S^2_s = 100 \times [4\pi r^3 \rho_A / 3B_A m]^{1/2} \quad (1)$$

In absence of accurate information on all of these factors, it has been found that a well mixed material could be characterized by its sampling constant, K_s, which is defined as that amount of material which gives a 1% error for subsampling [3]. The value of K_s is related to the subsampling error (S_s) by:

$$S_s = [K_s/m]^{1/2} \quad (2)$$

or

$$m \times (S_s)^2 = K_s = 100 \times [4\pi r^3 \rho_A / 3B_A]^{1/2} \quad (3)$$

wherein K_s is a constant for the analyte under consideration in a given mass fraction (B_A) of a particular particle size distribution ($4\pi r^3/3$) and density (ρ_A).

If the measurement error (S_m) can be precisely defined, the subsampling error (S_s) can be determined from observed overall variance (S_o = RSD) of a series of measurements at various sample sizes

$$S_o{}^2 = S_m{}^2 + S_s{}^2 \quad (4)$$

Using neutron activation analysis, Chatt applied this approach to determine sampling constants for one element, selenium, in a variety of reference materials [6]. He states that: "The sampling constant concept is not restricted only to sampling of geological materials, but can be applied to biological samples as well as to any other uniform material under investigation."

Homogeneity studies in a variety of reference materials have been studied for trace element contents using slurry sample introduction furnace atomic spectroscopy [7–9]. Additional sampling constant characterization of reference materials would allow much greater use of these materials to evaluate and validate development and implementation of analytical methodology in making food component measurements. Up to the present, for foods or biological materials, this sampling constant approach has been applied solely to inorganic components. It has not been applied to organic compounds such as vitamins in food related materials.

The Standard Reference Material (SRM-1846) Infant Formula-Milk Based, available from the National Institute of Standards and Technology (NIST), was developed primarily as a standard for organic nutrient content, including water-soluble vitamins, for which very few food matrix reference materials are presently available [10]. This commercially prepared material was produced by mixing various components such as mineral and encapsulated fat soluble vitamin premixes into the bulk fluid milk base. For purposes of longer term stability, the water soluble vitamins are dry mixed into the bulk material following spray drying. Since the entire vitamin content is located in a small fraction (5%) of the total mass of the mixed material, there is concern with homogeneity of small analytical subsamples. These small subsamples may not be representative of the whole amount due to variation of number of vitamin particles (analogous to grains in geological sampling) in each subsample. A detailed study of the homogeneity of this SRM material as a function of analytical subsample size offers an excellent opportunity to address the applicability of the sampling constant approach to assess sample homogeneity for organic components. Larger amounts of material (20–30 g) can readily be reconstituted to give a homogeneous fluid mixture. Smaller amounts of material have sufficient levels of vitamins to be well above detection limits for methods of analysis including microbiological methods [11]. Thus, a series of determinations giving precision of analysis of aliquots of the reconstituted homogeneous fluid will lead to an estimate of measurement error (S_m), since the sampling error is minimal for this large amount of subsample. Determination of precision of analysis of small subsamples of the dry material will give an estimate which includes both measurement and sampling error. The difference of these two estimates will enable determination of sampling error (S_s) and thus sampling constants for this material. It will also allow better evaluation of measurement and sampling components of the uncertainty statements for the Certificates of Analysis of this material.

Methods and materials

Microbiological. Methods 960.46 Vitamin Assays, Microbiological Methods, and 985.34 Niacin and Nicotinamide in Ready to Feed Milk Based Infant Formula: Microbiological-Turbimetric Method were used [11]. The latter assay measures growth of the organism *Lactobacillus Plantarum* in a vitamin free culture media in response to added amounts of an assay solution. The infant formula assay solution is prepared by digestion of the analytical sample with H_2SO_4, followed by a pH adjustment to 6.5–6.8 to precipitate proteins, filtration and final adjustment to pH 4.5. This method has been well established and is being used routinely by the Food and Drug Administration (FDA) laboratory to determine niacin content in a variety of food materials.

HPLC. A simple rapid solid-phase column procedure for the extraction of niacin from a complex food matrix has been developed. This procedure uses a vacuum manifold solid phase extraction column system to quickly and efficiently process sample digests for liquid chromatographic analysis. Samples of SRM-1846 are digested with H_2SO_4 using the procedure specified in AOAC 985.34 [11]. The digested solution is filtered and passed through an aromatic sulfonic acid solid phase extraction column. Niacin is eluted with 0.25 mol/L sodium acetate/acetic acid buffer at pH 5.6. Aliquots of the resulting filtrate are analyzed by anion exchange liquid chromatography using 0.1 mol/L sodium acetate/acetic acid buffer at pH 4.0 as mobile phase. This method is an adaptation of that in [12], described elsewhere [13], and is presently undergoing evaluation for acceptance as an AOAC Peer Validated Method.

Results and discussion

Results from an initial study conducted at the Food and Drug Administration, Nutrient Surveillance Branch, Washington D.C., to gain preliminary information on sampling constants for niacin in SRM 1846 by the AOAC microbiological method are reported in this paper. Subsequently methodology was developed at the Food Composition Laboratory (FCL), USDA to determine niacin in Infant Formula by an HPLC method [13]. More detailed studies of the sampling constants for niacin in SRM 1846 were carried out using this HPLC method, and are reported herein.

Microbiological method. This initial study consisted of multiple determinations of niacin content on two sample sizes, 0.1 and 0.5 g, of SRM 1846. The first set of determinations (0.1 g) were carried out by an experienced FDA scientist with observation of the visiting FCL scientist. The second set of samples (0.5 g) were analyzed by the FCL scientist with the oversight of the experienced FDA scientist.

For the first set of determinations, the experimental design entailed taking a sufficiently large sample (20 g) of SRM-1846 from one packet (approximate weight of 30 g) of the material and reconstituting it to 200 mL of fluid (0.1 mg/mL). Multiple subsamples (6) of 1 mL (0.1 g) aliquots of the homogeneous fluid mixture were taken for individual determinations of niacin. Duplicate subsamples of an equivalent amount of dry material (0.1 g) were taken from each of three additional packets of the SRM. Niacin content of these six subsamples was determined in conjunction with the six aliquots from the fluidized sample. These data are shown in Table 1 (Supplementary material). The variance of the values from these determinations on the small size subsamples of dry material gives an observable variance S_o^2 which reflects both sampling and measurement error. From Eq. (4), the difference between these two variances is that variance due to sampling

$$S_o^2 - S_{meas}^2 = S_{samp}^2 \tag{5}$$

From Table 1, the data for the 0.1 g samples show that: $S_{samp}^2 = (6.46)^2 - (3.03)^2 = 32.55$. From Eq. (3)

$$m \times S_{samp}^2 = 0.1 \times 32.55 = 3.26 \text{ g} = K_{s(0.1\text{ g})} \tag{6}$$

This experiment was then repeated, by the second analyst on a different day, using 5 mL of the fluid solution and 0.5 g of dry powder. Results of these determinations are also shown in Table 1. Similar to the 0.1 g sample size, the data for the 0.5 g samples show: $(S_{samp})^2 = (2.13)^2 - (1.43)^2 = 2.49$.
and

$$m \times (S_{samp})^2 = 0.5 \times 2.49 = 1.24 \text{ g} = K_{s(0.5\text{ g})} \tag{7}$$

The observed variances of the second experiment were consistently lower than that of the first as would be expected in moving further away from the detection limitations of the system. In summary, these preliminary experiments showed a sampling constant in the range of 1.2–3.3 g for SRM 1846.

HPLC Method. In the course of development and validation of the HPLC method [13], a number of experiments were run to determine the repeatability at various sample weights as shown in Tables 2–5 (SM). These experiments were carried out by accurately weighing out multiple (6) subsamples of the SRM. Each subsample was carried through the digestion/extraction/cleanup steps of the procedure to give a final liquid extract for analysis.

Variations in the niacin content of these six extracts would reflect both sampling and preparation variances. The final step of the analytical procedure was the injection, using an autosampler, onto the HPLC column, separation, detection, and quantitation. Variance due to this portion of the measurement process can be determined from the values obtained from multiple injections of each extract. Thus, three injections of each of the six subsample extracts were carried out for each of the four sample weights. Due to the length of time for an individual HPLC run, the samples were run in two batches of 36 determinations each. The 0.25 g and 0.50 g samples were run in one batch and the 1.0 g and 2.0 g samples in a second.

Using the data in Table 2 for the 2.0 g samples as an example, the following calculations were carried out. *Mean values* for each subsample extract were calculated from the individual injections, along with an *overall mean* of the six subsamples. These results for all four sample weights were comparable to the certified niacin value and uncertainty of 63.3 ± 7.6 mg/kg given in the SRM 1846 Certificate of Analysis [14]. An *overall standard deviation* was calculated from the 18 values (15 for the 0.5 g sample, one extract was lost). This standard deviation (reflecting errors resulting from sampling, sample preparation and injections) is subsequently used as the value for the overall variance (S_o) as discussed above, giving $S_{o2.0g} = 2.89\%$.

Next a one way analysis of variance (ANOVA) statistical analysis was carried out. Following the design given in [15], a combined estimate of the sample variance due to injection/chromatography within a subsample, $(S_{inj})^2$ can be obtained by computing: $(a)_{2.0g} = \Sigma x_i^2 = 66652.67$ and $(b)_{2.0g} = \Sigma(\text{Extract Sum})^2/5 = 66641.62$.

Subtracting (b) from (a) and dividing by the total number of within extract degrees of freedom 6 × 2 = 12, we obtained a "mean square", which is an estimate of the within injection analytical error variance: $S_{inj\ 2.0g} = (66652.67 - 66641.62)/12 = 0.921$.

The standard deviation (SD) for the injection error is $(S_{inj})^{1/2}$ and the relative standard deviation RSD is 100 × SD/Mean: $RSD_{inj}(\%) = 100 \times (0.921)^{1/2}/60.83 = 1.58 = S_{inj\ 2.0g}$.

The variability among the extract means is obtained by computing: $(c)_{2.0g} = (\text{Grand Sum})^2/18 = 66600.33$. Subtracting (c) from (b) and dividing by the "among-extracts" degrees of freedom, (6 – 1 = 5) gives: $S_{among\ 2.0g} = (66641.62 - 66600.33)/5 = 8.258$. The mean square obtained is 9.0 (8.26/0.92) times the variance estimated from the within injection means. This value is the F_{samp} ratio

which can be used to test the null hypothesis that the mean results from each sample extract are means of samples of three from the same population, which have a standard deviation of $SD_{inj}/(3)^{1/2}$. This F_{samp} value is included in each of the Tables 2–5. From tables of the F distribution [15] the critical values for 5 and 12 degrees of freedom at the upper 95% point of the distribution curve is 3.11 (F_{null}). As summarized in Table 6: For the 2.0 and 1.0 g samples, the sample-to-sample variation is greater than can be attributed to the analytical error within multiple injections. ($F_{samp} > F_{null}$). Conversely for the 0.25 and 0.5 g sample weights, the analytical variance is greater than that between samples ($F_{samp} < F_{null}$).

Calculation of sampling constants from HPLC data. As shown in Eq. (4) above, the observed variance is comprised of the sampling variance and that due to measurement. In order to calculate the sampling constant (K_s) an estimate must be made of the observed variation and an independent estimate of the measurement variation.

Through use of an Instrumental Neutron Activation Analysis technique [6], the portion of the overall variation (S_o) due to measurement error could be assigned predominately to counting statistics and thus a value for S_m could be obtained. In our procedure for the determination of niacin by HPLC the measurement process has two major parts. Once the analytical subsample is weighed out, it goes through a preparation procedure involving digestion, extraction and a simple column clean-up resulting in a final solution, which is assumed to be homogeneous. Multiple aliquots of this solution are then injected into the HPLC for separation and instrumental measurement. Thus, the measurement variability in our process can be represented in two parts, preparation and instrumental measurement:

$$S_m^2 = S_{prep}^2 + S_{inst}^2 \quad (8)$$

and thus from Eq. (1);

$$S_o^2 = S_s^2 + S_{prep}^2 + S_{inst}^2 \quad (9)$$

Overall variability is obtained by determination of the content of multiple samples. The variability due to instrumental measurement, S_{inst}^2, can be determined by injection of multiple aliquots of the prepared digest of each sample. Thus:

$$S_o^2 - S_{inst}^2 = S_s^2 + S_{prep}^2. \quad (10)$$

This experiment was run at various sample sizes and estimates for S_o^2 and S_{inst}^2 are shown in Table 2–5 (SM). Figure 1 shows that both the overall observed standard deviation S_o and the analytical standard deviation S_{inst} increase with decreasing sample weight. Chatt [6] found very similar results in the study of sampling constants for selenium in several biological reference materials. From Eq. (10) the residual variations due to sampling-preparation are calculated and shown in Table 6. In order to determine sampling constants as outlined above, an estimate must be made of the S_{prep} in order to separate it from S_s. If one makes the assumption that the variation due to preparation is negligible ($S_{prep} = 0$) [an obviously poor assumption] then the data in Table 6 can be utilized to determine an upper limit for the sampling constant $K_{s,limit}$. As shown in Table 6, if all the unaccounted variances were due to sampling, the resulting sampling constant would be between 2–15 g. As expected, these results are similar to but larger than the upper end of those estimated by the microbiological assay approach.

Experimental data on the variability due to the sample preparation is difficult to obtain, since each analytical subsample is carried through the entire preparation process and

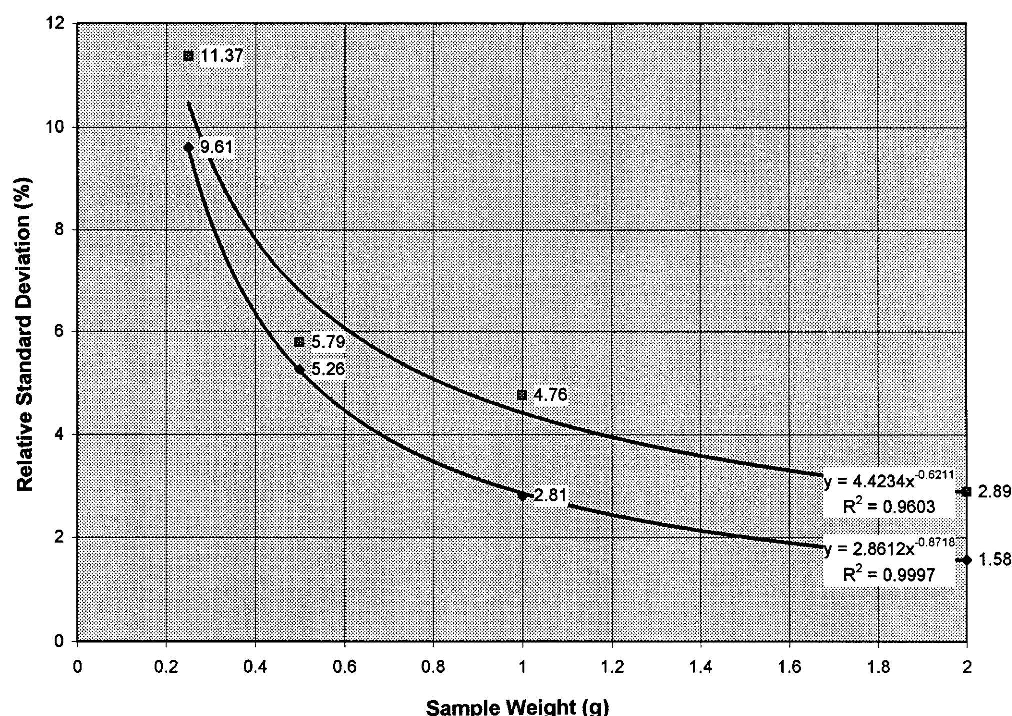

Fig. 1 Variation of RSD with sample weight, where ◆ = S_o and ■ = S_{inst} and the lines are power law fits as shown

Table 6 Sampling constants and limiting value K(samp + prep) for niacin in SRM 1846 Infant Formula using the HPLC Method

Sample mass (g)	0.25	0.5	1.0	2.0
S_o	11.37	5.76	4.76	2.88
S_o^2	129.28	33.18	22.66	8.29
S_{inst}	9.61	5.26	2.81	1.58
S_{inst}^2	92.35	27.67	7.90	2.50
$S_s^2 + S_{prep}^2$ [a]	36.92	5.51	14.76	5.80
K_s(lim)	9.23	2.75	14.76	11.60
F	2.36	1.79	7.38	8.97
F(null)	3.11	3.11	3.11	3.11

[a] $= S_o^2 - S_{inst}^2$

Table 7 Sampling constants for niacin in SRM 1846 Infant Formula using the HPLC method

Sample weight (g)	1.0 Niacin (mg/g)		0.5 Niacin (mg/g)
Dry powder sample			
1	61.2		60.2
2	59.4		58.3
3	57.1		59.7
4	54.0		73.9
5	59.7		65.2
6	59.5		53.1
	58.48	Mean	61.73
	2.56	SD	7.11
	4.38	RSD (%) (S_o)	11.52
	19.15	(S_o^2)	132.70
Reconstituted sample			
C1	62.4		57.1
C2	60.1		62.4
C3	59.5		58.6
C4	61.2		59.5
C5	55.8		64.8
C6	57.3		75.8
	59.88	Mean	63.03
	2.45	SD	6.84
	4.13	RSD (%) (S_m)	10.85
	17.08	(S_m^2)	117.72
	2.07	(S_s^2)	14.98
	2.07	K_s	7.5

thus sampling variability is part of the preparation process. Our basic assumption is that the sampling variability decreases at higher sample mass until it becomes non-significant. The sampling constant has been defined as that mass for which the variability due to sampling has been reduced to 1%. If the above data show an upper limit for K_s of 15 g, then our use of a 20 g sample for the microbioloigcal studies was justified. Figure 1 shows that above approximately 1 g, the decrease in overall variance level begins to reach a plateau at a relatively constant value of about 3%. We assume that this is due primarily to the preparation steps in the procedure. This qualitative estimate of about 1–2 g for sampling variability significance is in line with the sampling constant determined by the microbiological procedure.

Fortunately, the Infant Formula SRM-1846 of interest in this paper is readily soluble in water; it was manufactured to be reconstituted before use. Thus, the experiment done for the microbiological assay can be repeated for the HPLC assay. Reconstituting a large sample of the dry powder Infant Formula gives a solution that can be considered to be homogeneous. Determinations on multiple aliquots of this reconstituted material then minimizes sampling variability to a negligible amount and gives a measure of the variability due to preparation. These data for aliquots representing various weights of the dry powder material are shown in Table 7. These data then allow estimation of the sampling constant for the dry powder material based upon the various sample weights similar to that done for the microbiological method. Resultant values of 2.0 and 7.5 g for K_s for the 1 g and 0.5 g samples thus confirm our earlier measurements. The high value for the 0.5 g sample reflects the extreme sensitivity of determining K_s close to the levels of detection of the analytical measurement. At this level, the variation due to the measurement (S_m) is large (about 10% CV). The determination of K_s involves determination of a small difference between two large numbers, therefore at low levels this determination is not as reliable. Qualitative agreement for the 0.5 g sample is taken as confirmation of the more precise estimate at a larger sample mass.

Conclusion

Estimations of the sampling constant for niacin in SRM 1846 Infant Formula give a value of about 2–3 g. Use of subsample sizes much lower than this amount of material can introduce significant variation into determinations for organic nutrients using this SRM to validate analytical methodology.

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