

Variation of Analytical Results for Peanuts in Energy Bars and Milk Chocolate

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Peanuts contain proteins that can cause severe allergic reactions in some sensitized individuals. Studies were conducted to determine the percentage of recovery by an enzyme-linked immunosorbent assay (ELISA) method in the analysis for peanuts in energy bars and milk chocolate and to determine the sampling, subsampling, and analytical variances associated with testing energy bars and milk chocolate for peanuts. Food products containing chocolate were selected because their composition makes sample preparation for subsampling difficult. Peanut-contaminated energy bars, noncontaminated energy bars, incurred milk chocolate containing known levels of peanuts, and peanut-free milk chocolate were used. A commercially available ELISA kit was used for analysis. The sampling, sample preparation, and analytical variances associated with each step of the test procedure to measure peanut protein were determined for energy bars. The sample preparation and analytical variances were determined for milk chocolate. Variances were found to be functions of peanut concentration. Sampling and subsampling variability associated with energy bars accounted for 96.6% of the total testing variability. Subsampling variability associated with powdered milk chocolate accounted for >60% of the total testing variability. The variability among peanut test results can be reduced by increasing sample size, subsample size, and number of analyses. For energy bars the effect of increasing sample size from 1 to 4 bars,

subsample size from 5 to 20 g, and number of aliquots quantified from 1 to 2 on reducing the sampling, sample preparation, and analytical variance was demonstrated. For powdered milk chocolate, the effects of increasing subsample size from 5 to 20 g and number of aliquots quantified from 1 to 2 on reducing sample preparation and analytical variances were demonstrated. This study serves as a template for application to other foods, and for extrapolation to different sizes of samples and subsamples as well as numbers of analyses.

Allergy to peanuts is a significant health problem because of the high prevalence, potential severity, and chronicity of the reaction. To avoid accidental ingestion of peanut-contaminated food, methods of analysis for the determination of the allergenic proteins in foods are needed tools. The need to determine the validation performance of qualitative screening methods as well as quantitative methods remains. The variability of test results depends on variability generated by sampling, sample preparation, and analytical steps of the test procedure used to measure protein in foods. According to recent statistical studies, the sampling variability can be reduced by increasing sample size; the sample preparation variability can be reduced by grinding the food matrix into smaller particle sizes and/or increasing subsample size; and the analytical variability can be reduced by increasing the number of aliquots quantified (T.B. Whitaker, U.S. Department of Agriculture, unpublished data, 2004).

Two scientific approaches for quantifying peanuts in a food matrix can be applied. They are the protein- and DNA-based methods; the former tests for peanut proteins, the latter detects DNA sequences of peanuts. Enzyme-linked

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immunosorbent assay (ELISA) techniques have been used for protein, whereas the polymerase chain reaction (PCR) has been used for DNA.

ELISA is rapid, simple, specific, sensitive, and portable, and it is similar to other analytical methods because it consists of 4 components: sample preparation, extraction, cleanup or isolation, and determination. ELISA is based on interactions between antibodies and antigens. Enzymes and colorimetric substrates are used to produce observable responses. Microplate ELISA is most commonly used. The microplate ELISA can be used qualitatively (yes/no) or quantitatively.

Guidelines for validation and use of immunoassays have been discussed (1). These include standardized reference materials, documentation, method description, accuracy, precision, sensitivity, specificity, ruggedness, and limitations. Currently no procedures have been established for determining variability associated with sampling, sample preparation, and analysis for peanut cross-contacted foods. The major problem has been the preparation of a peanut spiking solution for recovery studies. To solve this problem, a reference peanut butter, a standard reference material (SRM 2387), was obtained from the National Institute of Standards and Technology (NIST; Gaithersburg, MD). A peanut butter suspension was prepared from this SRM. The suspension was evaluated and found to be stable and homogeneous and was used for our study (2).

Three commercially available kits, Biokits for Peanuts (Tepnel BioSystems Ltd., Flintshire, UK), the Veratox for Peanut Allergens (Neogen Corp., Lansing, MI), and the RIDASCREEN®FAST Peanut (R-Biopharm AG, Darmstadt, Germany), were evaluated by using the peanut suspension for spiking. They have been certified as *Performance-Tested Methods*SM by the AOAC Research Institute and as effective methods for the detection of peanuts at levels of 5 µg/g in breakfast cereal, cookies, ice cream, and milk chocolate (S. Coates, AOAC INTERNATIONAL, Gaithersburg, MD, personal communication, 2003).

Chocolate brownie energy bars and milk chocolate were selected for this study because they are both ready-to-eat products. The former contains particles, whereas the latter is smooth and homogeneous. The energy bars are widely sold in health clubs (3). In a telephone survey, 45 health clubs in the Boston area were contacted. Almost half of the clubs sold some type of nutritional products. Of those who did, 75% sold various energy bars. Milk chocolate is widely consumed by children and adults. Contamination with peanuts in these commodities could cause adverse effects in individuals who are allergic to peanuts.

The objectives of this study were to (a) measure the percent recovery of added peanuts in food using an ELISA test kit; (b) determine the total variability of test procedures used to detect peanuts in food matrixes; (c) partition the total variability of the test procedure into sampling, sample preparation, and analytical variance components; and (d) demonstrate how to reduce the sampling, sample preparation, and analytical variability associated with the test

procedure to measure peanut protein in energy bars and milk chocolate.

Experimental

Test Materials

(a) *Control material*.—The major ingredients of the energy bars were soy protein isolate, whey protein, glycerin, natural flavor, water, canola oil, almond butter, alkalized cocoa powder, almonds, and vitamin and mineral blend. Four energy bars, uncontaminated with peanuts, were each ground and analyzed. They were tested to ensure peanuts at <1 µg/g.

Four milk chocolate bars (247 g/bar) were ground and analyzed. They were tested to ensure peanuts at <1 µg/g.

(b) *Naturally contaminated material*.—From a box of 12 energy bars naturally contaminated with peanuts (energy bars made on different dates by the manufacturer of the control bars), 4 samples, 1 bar each, were removed from the shipment and ground separately for testing.

(c) *Incurred material*.—The process of adding known levels of peanut to a food matrix during processing produces incurred milk chocolate. Defatted peanut flour was added to melted milk chocolate. After cooling, each batch of incurred milk chocolate was ground to a fine powder. This material was provided by the National Food Processors Association (NFPA). Four batches of powdered milk chocolate containing known levels of 0, 5, 10, and 50 µg defatted peanut flour/g chocolate (equivalent to 0.0, 9.3, 18.7, and 93.5 µg peanuts/g, respectively) were analyzed for protein.

(d) *Spiked material*.—Control energy bars were spiked at 10 µg peanuts/g by adding 400 µL reagent [see Reagents (i)] to a 5 g test sample.

Subsamples from the control milk chocolate were spiked at 5 µg peanuts/g and 10 µg peanuts/g by adding 200 and 400 µL reagent [see Reagents (i)], respectively, to the 5 g subsample.

Spiked energy bars and spiked milk chocolate were used to measure the recovery of the ELISA test kit. The variability associated with the sampling, sample preparation, and analytical steps was measured by using the naturally contaminated energy bars and the incurred milk chocolate.

Design

Recovery.—A single energy bar was cut into small pieces and kept frozen at −40°C. Powdered dry ice and frozen sample were added to a blender jar and ground to a fine powder. After the evaporation of the dry ice, four 5 g subsamples were removed from the comminuted energy bar and spiked as described above. Each spiked subsample was solvent-extracted, and the peanut level was quantified in each of 4 aliquots from each subsample. The 4 measured values per subsample were averaged and compared with the spiking level of 10 µg peanuts/g.

Four 5 g subsamples were removed from the comminuted milk chocolate at each of the 2 spiking levels (5 and 10 µg/g). Each subsample was extracted, and the peanut level was quantified in each of 4 aliquots from each subsample. A total of 4 peanut determinations were made per subsample per

spiking level. The 4 peanut values per milk chocolate subsample were averaged and compared with the appropriate spiking level of either 10 or 5 µg/g.

Precision.—By using a nested design, 4 samples of 1 bar each were removed from a single shipment of naturally contaminated energy bars. Each sample of energy bar was ground. Four 5 g subsamples were taken from each comminuted sample (1 energy bar) and analyzed in 4 replicates with the Neogen Veratox for Peanut Allergen kit.

Four batches of incurred milk chocolate, each batch at a different peanut concentration (0.0, 9.3, 18.7, and 93.5 µg/g), were used to measure the subsampling and analytical variability associated with measuring protein in milk chocolate. The experimental design used to measure variability at each incurred level of milk chocolate was similar to that used for the energy bars. Because the entire batch of incurred milk chocolate was ground, there was no measure of sampling variability associated with measuring protein at each incurred level. Four 5 g subsamples were taken from each batch or incurred level of protein. The protein in each subsample was solvent-extracted, and the peanut level was quantified in each of 4 aliquots from each subsample.

Apparatus

(a) *Polytron homogenizer.*—Model PT10/35 (Cat. No. 27 11 2005, Brinkmann Instruments, Inc., Westbury, NY).

(b) *Generator.*—Model PTA 20TS (Cat. No. 27 11 3959, Brinkmann).

(c) *Centrifuge.*—Operating at $>23\,500 \times g$.

(d) *Shaker.*—IKA-Schuttler MTS 4 shaker (Fisher Scientific, orbital shaker), or equivalent.

(e) *Pipet and pipet tips.*—20, 100, and 1000 µL; 5 and 10 mL adjustable air-displacement pipets with disposable tips.

(f) *Multichannel pipet.*—Labsystems 8- or 12-channel, 200 µL (Fisher Scientific), or equivalent.

(g) *ELISA strip reader.*—Stat Fax 302 plus (Awareness Technology Inc., Palm City, FL).

(h) *Reagent boat.*—Plastic, 30 mL, wedge-shaped troughs for use with multichannel pipet.

(i) *Software.*—Log/logit (Awareness Technology Inc.).

Reagents

(a) *Veratox for Peanut Allergen test.*—Neogen Corp.

(b) *SRM 2387 peanut butter.*—NIST (Gaithersburg, MD). The SRM 2387 peanut butter was prepared by a peanut butter manufacturer using its usual recipe for creamy peanut butter: roasted peanuts, sugar, partially hydrogenated vegetable oils, and salt. Most of the peanuts were Florunner. The final product contained about 90% peanuts and included no other sources of protein. The reference value for total protein in SRM 2387 is $22.2 \pm 0.5\%$. Protein was calculated from nitrogen by using a factor of 5.46 (4).

(c) *Carboxymethylcellulose (CMC), sodium salt.*—Cat. No. C9481 (Sigma-Aldrich Chemicals, St. Louis, MO).

(d) *Bovine serum albumin.*—Cat. No. B4287 (Sigma-Aldrich Chemicals).

(e) *Thimerosal.*—Cat. No. T8784 (Sigma-Aldrich Chemicals).

(f) *Phosphate-buffered saline (PBS).*—Cat. No. P-3813 (Sigma-Aldrich Chemicals).

(g) *2% CMC, sodium salt, solution.*—Prepare by using a polytron to homogenize 12 g CMC, sodium salt, in 600 mL distilled water for 5 min, and then add 300 mg thimerosal and 1.5 g bovine serum albumin. Homogenize the mixture for an additional 1 min. If the appearance of the solution is milky, let stand at room temperature for 1 h or until solution is clear.

(h) *Peanut butter suspension.*—Weigh 50 mg peanut butter into a 100 mL beaker, and add CMC solution to the same beaker to a total weight of 70 g. Use a small, flat, stainless steel spatula to disperse the peanut butter into the solution until there is no visible peanut butter adhering to the spatula. Homogenize the mixture with the polytron for 5 min. The concentration (w/w) of peanuts in this suspension is 643 µg/g, calculated on the basis of the peanut concentration of SRM 2387 peanut butter of 90% (concentration of peanut = $50\text{ mg}/70\text{ g} \times 90\%$, or $50\,000\text{ µg}/70\text{ g} \times 0.9 = 643\text{ µg/g}$).

(i) *Peanut butter spiking solution.*—Weigh 4.9 g peanut butter suspension in a 25 mL tared volumetric flask. Add 10 mL PBS (10mM, pH 7.4), and mix well. Dilute solution to 25 mL with PBS. The peanut concentration of this solution is 126 µg/mL. Prepare this solution daily.

Method of Analysis

The Veratox for Peanut Allergen kit (Neogen Corp.) was used to quantify peanut in the energy bars and milk chocolate. Reagents and the analytical procedure are included in the kit. The test sample is extracted with PBS. The extract is added to test wells coated with polyclonal antibodies raised against peanut proteins. The peanut proteins in the extract are sandwiched between 2 antibodies, one to capture the analyte and the other conjugated to the enzyme horseradish peroxidase. Tetramethylbenzidine/peroxide substrate is added to produce a blue color. The reaction is stopped with a stopping solution, and the color is stabilized. The intensity of the color is proportional to the peanut protein concentration and is measured with an ELISA reader. Standards included in the test kit have been corrected for dilution with the extraction buffer. The unit of expression of the standards is µg peanut/g food. Peanut concentrations of the test samples are determined from the standard curve.

Mathematical Analysis of Data

Recovery.—Recovery is expressed as a ratio of the measured to the known peanut concentration and is usually expressed as a percent. If multiple measurements are made per sample or per subsample, the measurements are averaged and compared to the known peanut concentration.

Precision (variability).—The total variability associated with the test procedure to measure peanuts in foods can be the sum of sampling, sample preparation, and analytical variances. Differences among the 4 aliquot peanut values per subsample are a measure of analytical variability; differences among the 4 subsample peanut values per sample are a

Table 1. Recovery of peanuts added to an energy bar and powdered milk chocolate

Food type	Peanuts, µg/g	Test sample	Recovery, % ^a	SD, %	Variance	CV, %
Energy bar	10	Portion 1	85.5	4.0	16.3	4.7
		Portion 2	77.5	1.3	1.7	1.7
		Portion 3	73.0	9.1	83.3	12.5
		Portion 4	89.0	6.4	40.7	7.2
		All portions	81.3	8.4	71.1	10.4
Milk chocolate	5	Portion 1	91.0	5.8	33.3	6.3
		Portion 2	78.0	4.9	24.0	6.3
		Portion 3	88.5	6.0	3.0	6.8
		Portion 4	99.5	5.3	27.7	5.3
		All portions	89.3	9.3	86.9	10.4
Milk chocolate	10	Portion 1	104.3	3.9	15.6	3.8
		Portion 2	92.8	6.1	37.6	6.6
		Portion 3	95.0	6.7	44.7	7.0
		Portion 4	94.5	2.6	7.0	2.8
		All portions	96.6	6.5	42.4	6.7

^a Recovery is based on the average of 4 replicates per portion.

measure of subsampling variability; and differences among the 4 test sample peanut values per lot are a measure of sampling variance. The sampling and/or subsampling variances cannot be measured directly because there is always an analytical measurement associated with determining peanuts in either the test sample or comminuted subsample. The estimated sampling and subsampling variances can be measured by subtraction of the analytical variance from the total variance. The total, sampling, subsampling, and analytical variances were estimated by using a nested procedure in statistical analysis system (Proc Nested) for each concentration level and each food matrix.

Results and Discussion

Recovery

The recovery of the Veratox analytical method was evaluated by spiking the control matrixes with the diluted peanut butter suspension. Table 1 shows average recovery,

standard deviation (SD), and coefficient of variation (CV) for 4 replicate analyses of each of 4 portions of energy bars spiked at 10 µg/g and milk chocolate spiked at 5 and 10 µg/g. Recovery averaged about 81% for the energy bar and 89 and 97% for the milk chocolate at 5 and 10 µg/g, respectively. The overall analytical CVs of the 2 matrixes were about 10% at the spiked levels and were within the limits of the Horwitz ratio (5).

Variability

The mean, range, and SD of the peanut measurements for a single lot of energy bars and each batch or level of incurred milk chocolate are shown in Table 2. For energy bars, the mean peanut level for the 64 peanut measurements was 4800 µg/g, and peanut measurements ranged from 3100 to 7700 mg/g. The overall CV for the 64 measurements was about 26% and reflected variability in sampling, sample preparation, and analysis.

Table 2. Peanut levels found in a naturally contaminated energy bar and in incurred milk chocolate

Food	Concentration, µg/g	No. of analyses	Mean, µg/g	Minimum, µg/g	Maximum, µg/g	SD, µg/g	CV, %
Energy bar	4800 ^a	64	4800	3100	7700	1243.6	25.8
Milk chocolate	9.3 ^b	16	8.8	7.7	10	0.7	7.9
	18.7 ^b	16	20	18	22.4	1.5	7.5
	93.5 ^b	16	113.8	91	130	13.3	11.7

^a Natural concentration.

^b Incurred concentration.

Table 3. Sampling, subsampling, and analytical variances associated with 2 test procedures to measure peanuts in naturally contaminated energy bars

Test procedure	Test procedure steps	Test specification	Variance	CV	Variance ratio to total, %
1	Sampling	1 bar	821666	18.9	53.1
	Subsampling	5 g	672083	17.1	43.5
	Analysis	1 aliquot	52934	4.8	3.4
	Total		1546683	26.0	100.0
2	Sampling	2 bars	410833	13.4	67.9
	Subsampling	20 g	168021	8.6	27.8
	Analysis	2 aliquot	26467	3.4	4.4
	Total		605321	16.3	100.0

Table 3 shows the total, sampling, subsampling, and analytical variances and CVs for 2 test procedures (1 and 2) to measure peanuts in energy bars. Test procedure 1 was used in this study (1 bar sample, 5 g subsample, quantifying peanut in 1 aliquot by Veratox). For test procedure 1, the sampling step was the major source of variability and accounted for 53.1% of the total variability because of the large difference in the peanut contamination level from bar to bar. The variability associated with the subsampling step was also large and accounted for 43.5% of the total variability. Collectively, the variability associated with the sampling and subsampling steps to measure peanuts in energy bars contributed >97% of the total error. Analytical error accounted for only 3% of the total error. Both the sampling and subsampling variances were much larger than the analytical variability because the high sugar content affected the particle size.

Increasing sample size, subsample size, and number of aliquots quantified can reduce the variability associated with test procedure 1 to measure peanuts in energy bars. Variances from test procedure 1 (sample size of 1 energy bar, subsample of 5 g ground bar, and single analysis) were used to calculate variances associated with test procedure 2 (sample size of 2 energy bars, subsample of 20 g ground bar, and duplicate

analysis. For example, if sample size is doubled, the sampling variance is cut in half. This can be expressed mathematically as follows:

Sampling variance (vs) becomes

$$vs = (1/n) \times 821\,666, \quad (1)$$

subsampling variance (vss) becomes

$$vs = (5/nss) \times 672\,083, \quad (2)$$

analytical variance (va) becomes

$$va = (1/na) \times 52\,934, \quad (3)$$

and total variance (vt) becomes

$$vt = vs + vss + va \quad (4)$$

where n = number of bars, nss = subsample size, and na = number of aliquots.

The total variability for test procedure 1 is reduced by 61% [= {(vt of test procedure 1 – vt of test procedure 2)/vt of procedure 1} × 100] by using sampling plan 2 (Table 3).

Table 4. Subsampling and analytical variances associated with measuring peanuts in known (incurred) levels in milk chocolate

Test procedure (5 g, 1 aliquot)	Level, µg/g		Variance	CV, %	Variance ratio to total, %
	Incurred	Found			
Subsampling	9.3	8.8	0.327	6.48	61
Analysis	9.3	8.8	0.209	5.23	39
Subsampling	18.7	20.0	1.479	6.10	66.4
Analysis	18.7	20.0	0.749	4.35	33.6
Subsampling	93.5	113.8	162.708	11.21	91.6
Analysis	93.5	113.8	14.833	3.4	8.4

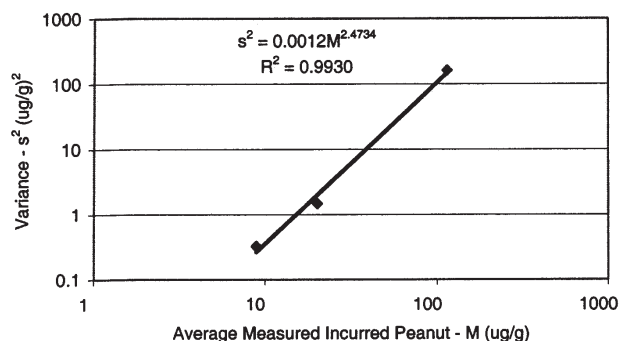


Figure 1. Subsampling variance for a 5 g subsample of milk chocolate using the Veratox analytical method to measure peanut concentration.

The variance can be used to predict the range among replicated peanut test results for a given lot when a given test procedure is used. The range (R) among 95% of the test results can be calculated from the SD or the square root of the variance (vt).

Normal distribution 95% all peanut test results will fall between $M \pm 2*SD$ (M is the mean peanut concentration) or more precisely $M \pm 1.96*SD$. The expected low value is $M - 1.96*SD$ and the expected high value is $M + 1.96*SD$. The range R is the high value minus the low value. As the variance becomes smaller, the range becomes narrower. The ranges of test results for a peanut level of 4800 $\mu\text{g/g}$ for test procedures 1 and 2 are 2298–7272 and 3229–6341 $\mu\text{g/g}$, respectively. The range calculations assume the distribution among peanut test results is Normal, which may not be the case. The assumption is better for powdered milk chocolate than for energy bars.

Peanut levels in the incurred milk chocolate are much lower than those found in the energy bars. Variability among peanut measurements in the milk chocolate reflected subsampling and analytical variability, but not sampling

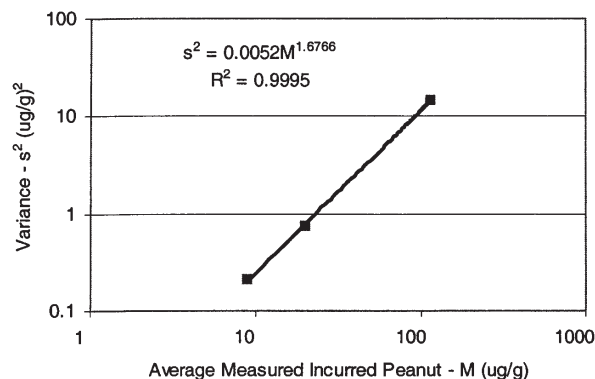


Figure 2. Analytical variance for a 5 g subsample of milk chocolate using the Veratox analytical method to measure peanut concentration.

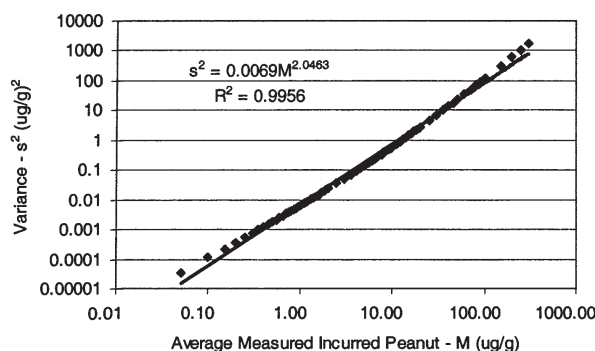


Figure 3. Total variance (subsampling plus analytical variances) associated with measuring peanut concentration in a 5 g subsample of milk chocolate using the Veratox analytical method.

variability. The CV of the 16 peanut measurements for each batch varied from about 7 to 12% (Table 2). Four 5 g subsamples were removed from each of the 3 incurred batches of powdered milk chocolate. Therefore, the variability associated with the sampling step could not be measured. Table 4 shows subsampling and analytical variances and CVs for a test procedure that used a 5 g subsample and a single analysis by the Veratox method. Subsampling contributes 60–90% of the total testing error. The total variability can be reduced by >50% by increasing the subsampling size to 20 g and running duplicate analyses with the same mathematical strategy used for energy bars.

The variances appear to increase with an increase in the incurred peanut level (Table 4). The subsampling, analytical, and total variances shown in Figures 1–3 demonstrate the effect of peanut concentration on variances. Regression equations were developed to describe the relationship between peanut concentration and the subsampling, analytical, and total variances. Figures 1–3 show that the predicted relationship between variance and peanut concentration (solid line) and the experimental data (individual points) correlated well. By using the regression equation, the total variance of other levels of peanuts in similar cross-contacted foods can be extrapolated.

Conclusions

The total variability associated with testing energy bars for peanut contamination was related to sampling, subsampling, and analytical components. The sampling and subsampling steps were the major source of variability, contributing >97% of the total variance. Because sampling and subsampling variabilities are much larger than analytical variability, the best use of resources to increase the precision (reduce variability) of the peanut test procedure is to increase sample and subsample sizes. For energy bars or similar snack foods such as candy bars and breakfast bars, the preferred approach is to use a 4 bar sample, 20 g subsample, and duplicate analyses of 1 extract. The subsampling and analytical variabilities associated with peanuts in powdered milk

chocolate were also determined. The subsampling variability tended to be larger than the analytical variability. Both variance components increased with the protein concentration. For milk chocolate the preferred test procedure would use a minimum of a 20 g subsample and duplicate analyses of 1 extract.

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