U.S. Department of Health & Human Services

FDA U.S. Food and Drug Administration

Elemental Analysis Manual

for Food and Related Products

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Elemental Analysis Manual

for Food and Related Products

3.5 Reference Materials

Version 3.0 (December 2021)

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GLOSSARY and ACRONYMS

Reference materials (RMs) are used for method validation, verification of correct use of a method, calibration, and quality control. The sections below provide information on some of FDA's elemental analysis thinking on the use of RMs but additional information is available [1] [2] [3].

- Reference Material (RM)—Material or substance one or more of whose property values are sufficiently homogeneous, stable, and well established to be used for the calibration of an apparatus, the assessment of a measurement method, or for assigning values to materials [4].
- Certified Reference Material (CRM)—Reference material, accompanied by a certificate, one or more of whose property values are certified by a procedure which establishes its traceability to an accurate realization of the unit in which the property values are expressed, and for which each certified value is accompanied by an uncertainty at a stated level of confidence [4].
- Matrix Reference Material-Reference material that has a composition similar to that of the material being investigated (food, leaves, water, soil, etc.).
- In-house Reference Material (in-house RM)—Reference material developed by a laboratory for its own internal use.

A CRM is a RM with an associated certificate that satisfies traceability requirements. When a certificate expires, the material in the unit (or container) continues to be an RM but is, by definition, no longer traceable and therefore no longer a CRM. The name "standard reference material" (SRM) is sometimes used generically when discussing any CRM but is actually a trade name that the U. S. National Institute of Standards and Technology (NIST) uses for a CRM. In-house RMs can be tailored to provide suitable analyte and matrix characteristics. Although they do not carry a certifying body traceability, they can be extremely useful to show consistent performance over time (i.e., “repeatability”).

3.5.1. MATRIX REFERENCE MATERIAL USE FOR QUALITY CONTROL

Quality control instructions and specifications are given in each method. This discussion should not be taken to supersede those instructions but it provides FDA’s elemental analysis thinking on the function of RMs in the laboratory.

Matrix RMs are included with a sample analysis batch to verify the accuracy and overall performance of the analysis. Unlike CRMs used for instrument calibration, matrix RMs serve primarily to confirm (not establish) that the analysis is in control and that it is unlikely for there to be unexpected anomalies. Matrix CRMs provide traceability. Other non-certified RMs, including expired CRMs and RMs with established consensus values (*e.g.*, Roelandts and Gladney [5]), can be used to demonstrate repeatability. Matrix CRMs may be used in any RM application but must be included, alone or in combination with non-certified RMs, in regulatory analyses. CRMs are typically not required for investigational and surveillance analyses.

RM certificates provide instructions for the recommended minimum analytical portion mass, the procedure for determining mass at a reference moisture condition, storage requirements, etc. The use of z-scores (see §3.5.3) is an accepted procedure for demonstrating the quality of RM analysis results.

> *For RM analysis results to be properly evaluated, analysis uncertainty MUST BE COMBINED with the RM’s analyte uncertainty. Use of the RM’s uncertainties alone is inappropriate.*

RMs are chosen to closely match the matrix and analyte levels of interest. However, this is often

difficult because of the relatively small variety of RMs available. For FDA labs, the difficulty in choosing an appropriate RM is compounded by the large variety of food matrices and numerous analytes.

3.5.2. REFERENCE MATERIAL FOR INSTRUMENT CALIBRATION

The standardization of analyte response in a measurement system (*i.e.*, instrument calibration) is normally carried out using "standard" analyte solutions prepared from RMs. Commercially available single and multi-element stock standards, when produced by an ISO Guide 34 accredited RM provider meet the definition of CRMs. Calibration solutions, and corresponding verification solutions, prepared from CRMs contribute to the traceability of the measurement system.

3.5.3. IN-HOUSE REFERENCE MATERIAL DEVELOPMENT

An in-house RM is usually developed when the matrices or reference levels of commercially available RMs do not closely match the samples to be analyzed or when an ample supply is desired that will be available for many years. The suggested steps for development of an in-house RM for elemental analysis are outlined below.

3.5.3.1. Selection

Ideally, the material will be available in an ample supply, with minimal cost, and needing little or no preparation. An ideal choice, for example, is a food that is in powdered form, packaged in desired quantity, in sealed containers, and has a long shelf life. Materials requiring freezing, pulverizing, sieving, blending, sterilization, packaging, etc., should be selected only if necessary.

Logically, the material would be of a form or consistency that is expected to be homogeneous and stable with a long shelf life. If refrigerated storage is required, cryogenic temperatures (-80 C or colder) is assumed. Analyte levels, interference issues, and analysis difficulty need to be compatible with the analysis goals. Challenging materials having interferences or digestion complications are generally undesirable but could be useful to demonstrate ruggedness.

3.5.3.2. Analytical

Analysis data are used to characterize the material's behavior, set analyte levels, determine minimum analytical portion mass, and set uncertainties.

A procedure for bringing the material to a reproducible moisture condition must be defined so that during use the correct analytical portion mass can be known. Typical moisture conditions include as received, freeze-dried, oven-dried, desiccator-dried, equilibrium mass state, and reconstituted.

Analyte levels are best established using a variety of analytical techniques and methods in different laboratories and by different analysts. Use of multiple sets of data in this way compensates for the small and unknown amounts of method bias that are always assumed to exist. [6] [7] Limits can be deduced on how large the bias may be and the certificate uncertainties can be adjusted accordingly.

For each analyte, the reference value should be established using a combination of at least 2 different analytical techniques or laboratories. The use of a consensus mean approach such as by DerSimonian and Laird [8] is recommended. This, which FDA has used, and other approaches are commonly used in CRM production at NIST [9].

When analysis data are available for only one source (method/laboratory/analyst), then there is no experimental confirmation for the reference values and it is much more difficult to arrive at a reasonable uncertainty component for bias.

3.5.3.3. Random Error and Homogeneity

A study specifically to address heterogeneity would not, necessarily, be necessary to set reference values for the analytes of interest because heterogeneity will be reflected in the observed random error. However, an understanding of heterogeneity is quite useful when evaluating reported data, setting the minimum analytical portion mass specification, and assigning reference values and uncertainties.

Analyte heterogeneity can be defined as the RSD from analyte variations within the material ($RSD_{heterog}$) and expressed as relative percent. It is a characteristic that, as shown in equation 1, combines with random error from the measurement process (RSD_{meas}) to give the total, or observed, RSD (RSD_{obs}). [10]

$$(RSD_{meas})^2 + \left(RSD_{heterog}\right)^2 = (RSD_{obs})^2 \qquad \text{3.5 Equation 1}$$

Ideally, heterogeneity will be negligible for all analytes and RSD_{obs} will be due entirely to analytical factors. It should be expected, though, that heterogeneity will be significant for at least some analytes and be an increasing issue as the analytical portion mass decreases. When developing an in-house RM, the objective is therefore to find the smallest mass that will result in a trivial amount of heterogeneity for as few analytes as is practical. The minimum mass specified for commercial RMs is often ~300 mg.

Use of equation 1 is straightforward when RSD_{meas} (the random uncertainty component) is known. It enables several pieces of information to be discovered about the RM material when measurement results are available from different sources (different methods, laboratories, analysts).

If RSD_{obs} is greater than RSD_{meas}, then heterogeneity can be calculated by re-arranging Equation 1. $RSD_{heterog}$ is a characteristic of the material whereas RSD_{meas} is a characteristic of a laboratory/method. This means that $RSD_{heterog}$ should be the same for all laboratories and is logically no larger than the smallest RSD_{obs}. Larger RSD_{obs} values therefore indicate variations in RSD_{meas} at the different laboratories.

The following procedure was used for CFSAN in-house reference materials to decide how much larger RSD_{obs} must be relative to RSD_{meas} to conclude $RSD_{heterog}$ has been detected:

The adequacy of random error alone to account for an observed data distribution can be evaluated on the basis of the integral of the distribution function $P_x(\chi^2,v)$ from $x^2 = \chi^2_{(observed)}$ to $x^2 = \infty$, where χ^2 is chi-square distribution and v is the number of degrees of freedom [11]. *Here, the integral of the distribution function is referred to simply as probability. When the probability is ≤10%, a heterogeneity component can be calculated.*

In general, heterogeneities that are equal to the associated random measurement uncertainties would be expected to have probabilities <10%. Therefore, when a probability is >10%, the heterogeneity is known to be less than the random measurement uncertainty and the latter can be taken as an upper limit for heterogeneity. However, when RSD_{obs} is lower than the random measurement uncertainty, RSD_{obs} is taken instead as an upper limit for heterogeneity.

3.5.3.4. Uncertainties

Uncertainties need to be included with the analyte reference values. Very commonly, uncertainties are reported at a 95% confidence level, which is sometimes referred to as a "two-sigma" uncertainty. Methods for determining uncertainties vary and depend on the data sets and associated analytical information.

Caution is necessary when setting uncertainties and especially when choosing which generic ("canned") routine to use. Several are available in the software noted above (section 3.5.2.2). [9] Since the uncertainties given on the certificate will be used to judge individual analysis results, the RM uncertainties must be appropriate for individual test portions and not just for the bulk material. The concern here is associated with heterogeneity. If a large amount of data are available from many sources, analyte levels in the bulk RM material can be known extremely well (i.e., known with very small uncertainty). It is assumed, however, that RM uncertainties are used for routine quality assurance to confirm individual analysis results. Therefore, the certificate uncertainties should instead predict the errors expected for individual analyses.

The significance of heterogeneity in this regard stems from Equation 1. It affects analysis results and is a characteristic of the RM - not of the analysis. The in-house RM producer is therefore responsible for propagating heterogeneity in the certificate's uncertainty or providing it separately. If provided separately, an analyst would need to combine heterogeneity with the in-house RM uncertainty (in addition to combining with their own measurement uncertainty) before evaluating their analysis results. FDA propagates heterogeneity in the elemental analysis in-house RM uncertainties.

In a practical sense, a useful rule when assigning an RM uncertainty is that it should probably not be any smaller than the smallest standard deviation associated with the data from which a reference value is derived. This follows from the likelihood that accurate data are used to generate the reference values. This, of course, would not always be true, with an obvious exception being for RM solutions.

> *RM uncertainty example for element "X" - In the case where many data are available (say, >20 analyses by each lab), the RSDs for labs A, B, and C are 4.8%, 7.2%, and 5.5%, and there is excellent agreement (lab averages very close together), the RM bulk uncertainty RSD could end up being 1.5%. This would mean the level in the bulk material is extremely well known. It does NOT mean, however, that the RSD for individual portions should be 1.5%. By considering the results from Lab A, the "useful rule" noted above suggests to not set the RM uncertainty for element X any lower than 4.8%.*

3.5.3.5. Instructions

Although an in-house RM would not be certified, it needs to have documentation (i.e., a non-certified certificate) with the same types of information provided on certificates from certifying bodies [12]. In addition to recommended analyte values and uncertainties, it needs instructions. At a minimum, it will explain how to use the in-house RM and give storage requirements, a procedure for determining the reference moisture condition, minimum analytical portion mass, and reference values for the analytes.

3.5.4. REFERENCE MATERIAL RE-VERIFICATION

Continued use of RMs is subject to re-verification. This is the process of showing that an RM is still fit for purpose and is based on observations and analytical results. The analytical results may be obtained

specifically for re-verifying the RM or generated during its routine analysis. The re-verification may also be issued as a general update to the bulk material or may be limited to one or more analytes and/or be applicable only within one laboratory. As such, re-verification follows the data. CFSAN has not assigned expiration dates for in-house RMs.

Whereas CRMs are used to demonstrate traceability, in-house RMs demonstrate repeatability. As such, when measurement results agree with established levels, not only does this show the analytical process is correct but also that the analyte level(s) in the in-house RM are unchanged. Thus, the levels for analytes of interest are effectively re-verified with each use of the RM.

Re-verification is accomplished by showing that the measured results agree with the reference values, relative to the measurement uncertainties. Measurement uncertainties are ideally determined along with analyses but they could also be generically assigned for well-defined methods (e.g., 10% for some elements in EAM methods). It is not uncommon for analysis uncertainty to be ignored which, in essence, sets measurement uncertainty at zero. This is problematic and unnecessarily restrictive.

Continuous monitoring of RM results is useful because changes in element levels in an RM are observed in a timely fashion. Plotting element levels over time will show trends. Visual inspection can be used to verify the absence of obvious evidence that would cause one to question a RM unit's physical integrity. For example, change in color, presence of mold or seeing liquid when the material should be dry would disqualify an RM unit.

For a designated re-verification exercise, at least 2 analytical portions of the RM are analyzed concurrently with at least one analytical portion of a CRM. The CRM and RM results are compared with the certified and reference values, respectively, by using z-scores [13]. A z-score is equal to the difference between the result and certified value divided by the square root of the sum of the squares of the uncertainties from both the reference and analysis results (see Explanatory Note below). Absolute values are used for z-scores and interpreted as follows:

|z-score| of 2 or less is acceptable (agreement with reference value)

|z-score| between 2 and 3 is questionable (questionable agreement with reference value)

|z-score| of 3 or more is unacceptable (disagreement with reference value)

Re-verification is successful for an element if at least two-thirds of the z-scores are in the acceptable range and none are in the unacceptable range. Thus, when only 1 analytical portion of a CRM and 2 analytical portions of an RM are analyzed, every z-score must be in the acceptable range. When 3, 4, or 5 analytical portions are analyzed, one may be in the questionable range. When 6, 7, or 8 are analyzed, two can be in the questionable range, etc.

If re-verification is unsuccessful, all analysis information is examined. Mistakes such as data entry or calculation errors may only need correcting. Analytical problems may require repairing equipment or obtaining new reagents. Unexplained findings may require reanalysis. Repeated failure to re-verify may indicate a faulty RM or CRM unit.

Note about z-scores:

A z-score [13] *indicates how many standard deviations a result is from the reference value. Use of a z-score to examine data quality is a standardized way to evaluate results and provides an additional perspective besides recoveries, which do not account for the reference or measurement uncertainties, and precision, which accounts for only random error. For this application, the z-score is defined as:*

$$z = \frac{x_m - x_c}{\sigma}$$ 3.5 Equation 2

where: $\sigma = \sqrt{\sigma_m^2 + \sigma_c^2}$

x_m = *measured analyte level*

x_c = *is the certificate (reference) level*

σ_m = *total combined measurement uncertainty (one sigma, corresponding to a confidence level of approximately 67%)*

σ_c = *certificate uncertainty (one sigma).*

Example:

The CRM reference value for element "X" is 45.7 ± 8.3 mg/kg and the CRM's certificate states the uncertainty is at a 95% confidence level. The results of analysis of three analytical portions are 41.6, 33.4, and 51.1 mg/kg. The method is well-defined, LOQ for this element is 5.0 mg/kg, and the decision has been made to assign 10% uncertainty (at the one sigma, or 67%, confidence) for values above LOQ. The following uses 3.5 Equation 2, carries extra significant digits within the equation, and rounds the z-score to a tenths digit:

$$\sigma_c = 8.3/2 = 4.15\ mg/kg$$

$$\sigma_m = 41.6 \times 10\% = 4.16\ mg/kg$$

$$\sigma = \sqrt{(4.15)^2 + (4.16)^2} = \sqrt{34.53} = 5.876\ mg/kg$$

$$z = \frac{x_m - x_c}{\sigma} = \frac{41.6 - 45.7}{5.876} = \frac{-4.1}{5.876} = -0.7$$

This calculation is performed for all three results to give the following (absolute value) z-scores:

For 41.6 mg/kg = 0.7 (acceptable)

For 33.4 mg/kg = 2.3 (questionable)

For 51.1 mg/kg = 0.8 (acceptable)

Two out of three are acceptable and none are unacceptable so the RM is re-verified.

3.5.5. REFERENCE MATERIAL SOURCES

Below are several sources for obtaining RMs. This listing is not meant to be complete and some of the websites are interconnected (can access one from others). All are current as of September, 2021.

- National Institute of Standards and Technology
- National Research Council of Canada
- LGC Group (formerly Laboratory of the Government Chemist)
- European Reference Materials (ERM)
- Joint Research Centre - Institute for Reference Materials and Measurements (IRMM)
- Bundesanstalt für Materialforschung und -prüfung (BAM)
- National Institute for Environmental Studies
- International Atomic Energy Agency
- COMAR Internet location with a searchable database of CRMs (not a producer)

3.5.6. IN-HOUSE REFERENCE MATERIAL CERTIFICATES

By their very nature, in-house RMs are developed for internal use and are not certified. However, FDA has used "certificate" and "certificate of analysis" to summarize the material's documentation because these terms clearly convey the types of information contained therein.

3.5.6.1. FDA Cocoa Powder (CP)

Current certificate of analysis: FDA Cocoa Powder Certificate (2012, amended 2013) Excel 2010 (.xlsx) file

FDA CP Certificate (2012) was issued May 16, 2012. With this issuance, FDA CP was revalidated for continued use as an in-house RM for quality control/quality assurance for determination of element mass fractions in food and other biological materials.

Development work for FDA CP started in 1994 when approximately 10 kg of commercially-produced cocoa powder from a single production lot was obtained from a local grocery store. The material was re-packaged into pre-washed amber glass bottles and has been stored at FDA CFSAN. Bottles are sent to FDA labs on request. Each bottle contains about 300 grams of cocoa powder.

Reference values are derived from analytical results that have been reported. Since data have been received on an on-going basis, the reference values have evolved over time. The first FDA CP certificate was issued August 9, 1996. Minor updates occurred in 1998, 1999, and 2000 and a major update occurred May 4, 2006. As expected, changes in numerical values have been quite small.

Several enhancements were realized with the 2012 certificate:

- The certificate is presented electronically in the form of an Excel 2010 workbook named "FDA Cocoa Powder Certificate (2012).xlsx". It replaces the traditional hard-copy certificate but one may be obtained by printing the first three worksheets. The workbook is password-protected with changes permitted only in specific cells designed for user input. Calculations are therefore protected from being altered inadvertently.

- Basis mass is relative to the material exposed to air at 30% relative humidity (RH), which is expected to be the median condition for typical FDA laboratories. An analytical portion is exposed to laboratory air for at least 2 hours before measuring the mass. If a mass uncertainty of ±1% is acceptable, then no further calculation is needed. If ±0.5% is desired, an adjustment is performed using the actual laboratory RH. This calculation can be done automatically using a tool within the certificate.
- The data set used to derive the reference values has expanded considerably. With the 2010 update, data were available for 43 elements from 17 sources (methods, laboratories, techniques, etc.). Detection limits are provided for 6 additional elements.
- Reference values were set using state-of-the-art "concensification" software developed at NIST for generating SRM certificate values. Values are provided for Confirmed Values (those confirmed via multiple sources) and Unconfirmed Values (those from a single source).
- Reference values are provided in duplicate listings. One listing applies for the bulk material and is useful for evaluating means obtained by averaging results from replicate analyses. The second listing applies for individual analytical portions and is useful for evaluating individual analysis results. The former is typical for commercial CRM certificates while the latter includes sample-to-sample non-homogeneity effects.
- Users select the coverage factor for displaying uncertainties. For example, by entering the number one (1), the reference values will have standard uncertainties (1-sigma or about 67% confidence); or, by entering the number two (2), the reference values will have uncertainties at about 95% confidence, which is typical for commercial RM certificates.
- The certificate has a z-score evaluation capability. When a user enters analysis uncertainty (e.g., 10%), ranges are automatically generated to show the acceptable and questionable ranges for results. And, a hard-copy z-score evaluation may be generated to include with a report of analysis. A user needs only to enter the individual results for an analysis.

3.5.7. HISTORY

EAM 3.5 Table 1. History

Version	Revisions Made	Effective Date
1.0	*Reference Materials*	June 2008
1.1	Added subsection 3.5.6 (*In-House Reference Material Certificates*)	December 2012
2.0	Merged *RM Organizations* (3.5.5) with *RM Sources* (3.5.4); the numbering for Subsection RM Certificates changed to 3.5.5 (formerly 3.5.6); converted to PDF for web posting.	September 2014
3.0	Updated; added *History* section.	December 2021

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