Proposed Revisions 2-2021:

Appendix L: AOAC Recommended Guidelines for Stakeholder Program on Infant Formula and Adult Nutritionals (SPIFAN) Single-Laboratory Validation

1 Definitions

(a) Reference Material: A sufficiently stable, homogeneous sample matrix containing a specified analyte or group of analytes with a content that is reliable and reproducible [1]. The sample has been established to be fit for its intended use in a measurement process between two or more laboratories [2]. The measurable quantity is the mean of a specified population of measurements [11]. The stability and homogeneity may be determined as described elsewhere [3].

(b) Certified Reference Material (CRM): A reference material characterized by a recognized procedure for determining analyte concentration accompanied by a certificate issued by an authoritative body that provides the value of the concentration, its associated uncertainty, and a statement of metrological traceability [4].

(c) Reference Standard: A substance of known identity and purity with accompanying certificate of analysis from an authoritative body and used to prepare calibration standards and/or for the calibration of other measurement standards. Moisture content should be monitored to ensure stability and purity.

(d) Limit of Detection (LoD): the lowest concentration of analyte that can be confidently detected.

(e) Limit of Quantification (LoQ): the lowest concentration of analyte that can be determined with acceptable precision and accuracy.

(f) Matrix Blank: A product matrix that does not contain the analyte of interest (it may contain endogenous levels below LOD/LOQ) but does contain all the same components as the sample solution.

(g) Method Reagent Blank: Blank (e.g. water, buffer, solvent, or any other diluent which is free from the analyte) analyzed by the method in place of a sample. It identifies the amount of the signal that is due to the reagents used in the preparation of the samples.

2 General

(a) All methods for (a) given analyte(s) will be subjected to a common SLV protocol utilizing available SPIFAN matrices. When SPIFAN matrices are not available, sample types to be considered can be found in the associated SMPR.

(b) Assessment of the various parameters for single laboratory validation are described elsewhere [5].

(c) SLV protocols may vary somewhat between analytes, depending on the specific demands associated with each.

Study directors (SDs) for each analyte willagree on final details of the required SLV protocol.

(d) System Suitability criteria indicating method/system performance is acceptable and will be generated during SLV [6, Annex B].

(e) Ruggedness of an analytical method could be evaluated to measure the capacity to remain

unaffected by small but deliberate variations in method parameters [7, 8, 9]. By evaluating ruggedness, one provides an indication of the method's robustness during normal usage. If robustness/ruggedness was part of the method development phase, results of this can be documented in the SLV report.

(f) Units of measure for reported values and figures of merit must be consistent with those stated in the associated Standard Method Performance Requirement (SMPR).

3 Materials

(a) Use of a CRM where available is recommended to assess method accuracy as bias. The CRM should be accompanied by documentation (certificate) issued by an authoritative body.

(b) If a variety of matrices with different physical and chemical properties are defined in the SMPR, then a CRM of each type of matrix shall be included if available, otherwise see 3C.

(c) Where a CRM is not available, the concentration of the analyte(s) being studied in a reference material are assessed using preferably two appropriate orthogonal techniques. Statistically equivalent results from these analyses are requested with a minimum of two independent analyses, preferably determined by different laboratories. The completed SLV report should be accompanied by assessment protocols and results.

(d) Any reference standard used needs to be accompanied with a certificate of analysis, stating supplier, identity, batch number, purity and basis for the purity statement in the SLV report. The purity of the reference standards used should be established, understood and fit for purpose. If a non-commercial reference standard is used, its origin needs to be clearly identified along with all pertinent information demonstrating purity and/or analyte concentration and the means with which these were determined.

(e) If a variety of matrices with different physical and chemical properties are defined in the SMPR, the number of matrices needs to be at least one for each matrix type. The matrix sources should cover the range of expected concentrations of the analyte(s) of interest. If only a single matrix is studied, then ≥3 sources are recommended, preferably with different attributes (e.g. maturity, varieties, age).

4 Linearity/Calibration Fit

(a) Minimum of six (6) levels that span the desired working range as described in the SMPR.

(b) Assessment of heteroscedasticity should be performed; for example, calculation of relative error of back-calculated concentrations[1]

(c) Minimum of three (3) independent experiments to confirm the linearity/calibration fit.

5 LOD/LOQ

To determine the LOD and LOQ, the Standard Deviation and Blank Mean of sample measurements is obtained from repeated measurements of samples with a relevant low concentration (e.g. a homogeneous Matrix Blank or a Method Reagent Blank spiked at low level)[3] [12, 13]. It is recommended that a minimum of twenty (20) sample measurements be used and that these measurements, where possible, come from a variety of samples and over several days.

LOD = Blank Mean + 3 Standard Deviations

[1] No specific criterion in SMPR; recommend calibration errors to be <15%. Along the whole range. Higher calibration error at LLOQ can be accepted (*see* FDA 2018, Bioanalytical Method Validation, guidance for industry).

LOQ = Blank Mean + 10 Standard Deviations

The LOD/LOQ could also be determined via the signal/noise ratio procedure. Relevant low concentration samples (close to expected LOD/LOQ in at least 3 concentration levels) are analyzed three times. The signal/noise ratio can be calculated for each replicate of the spiked samples. A graph can be plotted of the signal/noise ratio versus the concentration. After a linear regression analysis, the intercept and slope determine the LOD/LOQ:

$$LOD = \frac{3 - \text{Intercept}}{\text{Slope}}$$

$$LOQ = \frac{10 - \text{Intercept}}{\text{Slope}}$$

Alternative approaches are possible as suggested in literature elsewhere [13].

6 Selectivity

(a) Selectivity is the degree to which the method can quantify the target analyte in the presence of other analytes, matrices, or other potentially interfering materials. No explicit proposals for evaluating selectivity are suggested, however methods must be tested in the presence of accompanying analytes or matrices most likely to interfere. The freedom from effects of interfering materials can be studied using various samples, ranging from pure measurement standards to mixtures with complex matrices. The recovery of the analyte(s) of interest should be determined and the influences of suspected interferences stated [6]. Examples of selectivity tests for chromatographic methods are described elsewhere [10, 13]. Methods with a known interference should be modified prior to SLV. If method selectivity was part of the method development phase, results of this can be documented in the SLV report.

(b) Useful strategies for completing selectivity vary from analyte to analyte. Therefore, the Study Directors for each analyte will decide on an acceptable practice.

7 Precision

All samples selected for precision studies will be analyzed in duplicate on each of six (6) days using multiple analysts and instruments as practical for the different days. Fresh reagents and working standards should be used each day. Reports will include information of number of analysts, instruments, etc. The number of matrices may vary between analytes.

(a) Precision data using a CRM or reference material should be included for all candidate methods.

(b) Estimate within-day repeatability, between-day repeatability (intermediate precision) and an, estimation of measurement uncertainty for each sample type. Estimates pooled across the sample types may also be useful, if appropriate.

8 Accuracy (Trueness)

(a) *Analysis of CRM or Reference Material*.

- A minimum of nine (9) independent replicates of CRM should be tested across three (3) days (e.g. 3 x 3) and compared to certified or reference values provided.
- Where a CRM is not available, a suitable reference material may be substituted and compared to

the established levels (see paragraph 3C). A *t*-test or other statistical test should be used to evaluate method bias.

(b) *Spike recovery.*

(*1*) Recovery will be determined from an appropriate sampling of a range of infant formula and adult nutritional matrices (use of the recognized SPIFAN kit is recommended where appropriate, otherwise the Study Directors may agree on the samples to be included).

(2) Each selected matrix will be spiked at three levels. Use spike levels covering the analytical range specified in the SMPR.

(3) Spiked and unspiked samples will be analyzed in triplicates on each of three (3) days.

(4) The daily mean of unspiked samples will be used for calculating individual recoveries.

References

1. ISO Guide 30:2015, Reference materials – selected terms and definitions. International Organization for Standardization, Geneva, Switzerland (2015)
2. ISO 13528: Statistical methods for use in proficiency testing by interlaboratory comparison. Annex B
3. NACRW Reference Material Use in Trace Analysis, NACRW Reference Materials Working Group, Edition 1; 1-20-2021
4. ISO17034:2016: General requirements for the competence and consistent operation of reference material producers.
5. Gill, B.D., Indyk, H.E., Blake, C.J., Konings, E.J., Jacobs, W.A., & Sullivan, D.M. (2015). Evaluation Protocol for Review of Method Validation Data by the AOAC Stakeholder Panel on Infant Formula and Adult Nutritionals Expert Review Panel. *Journal of AOAC International*, 98, 112–115.
6. V. Barwick (Ed), Eurachem/CITAC Guide: Guide to Quality in Analytical Chemistry: An Aid to Accreditation (3rd ed. 2016). ISBN 978-0-948926-32-7. Available from www.eurachem.org
7. Youden, W.J., & Steiner, E.H. (1975) *Statistical Manual of the AOAC*, AOAC INTERNATIONAL, Gaithersburg, MD
8. Vander Heyden, Y., Nijhuis, A., Smeyers-Verbeke, J., Vandeginste, B.G.M., & Massart, D.L. (2001). Guidance for robustness/ruggedness tests in method validation. *Journal of pharmaceutical and biomedical analysis*. 24, 723–753
9. Dong, F. (1993). On the identification of active contrasts in unreplicated fractional factorials. *Statistica Sinica* 3, 209–217.
10. FDA Center for Drug Evaluation and Research (1994) Reviewer Guidance/Validation of Chromatographic Methods.
11. ISO 3534-1: 1993: Statistics – vocabulary and symbols – Part 1: probability and general statistic terms.
12. Armbruster, D. A., & Pry, T. (2008). Limit of blank, limit of detection and limit of quantitation. The Clinical biochemist. Reviews, 29 Suppl 1(Suppl 1), S49–S52.
13. Magnusson and U. Örnemark (eds.) Eurachem Guide: The Fitness for Purpose of Analytical Methods – A Laboratory Guide to Method Validation and Related Topics, (2nd ed. 2014). ISBN 978-91-87461-59-0. Available from www.eurachem.org

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