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

1 General

(**a**) All methods for (a) given analyte(s) will be subjected to single-laboratory validation (SLV) using approved SLV protocols following this guideline utilizing available AOAC Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN) matrices. When SPIFAN matrices are not available, sample types to be considered can be found in the associated AOAC *Standard Method Performance Requirements* (SMPRs®).

(**b**) SLV protocols may vary somewhat between analytes, depending on the specific demands associated with each. The scope of the SLV should be compliant with the SMPR applicability statement.

(**c**) Study directors (SDs) for each analyte will agree on final details of the required SLV protocol.

(**d**) System suitability criteria indicating method/system performance is required and will be generated during the SLV (1).

(e) Ruggedness of an analytical method is evaluated to measure the capacity to remain unaffected by small but deliberate variations in method parameters (2–4). It provides an indication of the method's robustness during normal use. 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 SMPRs.

2 Definitions

(a) *Reference material*.—Sufficiently stable, homogeneous sample matrix containing a specified analyte or group of analytes with a content that is reliable and reproducible (5). The sample has been established to be fit for its intended use in a measurement process between two or more laboratories (6). The stability and homogeneity may be determined as described elsewhere (7).

(b) *Certified Reference Material (CRM)*.—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 (8).

(c) *Reference standard.*—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).*—Lowest concentration or mass of analyte in a test sample that can be distinguished from a true blank sample at a specified probability level.

(e) *Limit of quantification (LOQ).*—Lower LOQ below which the measured concentration of the analyte does not meet the SMPR for bias (recovery) or repeatability.

(f) *Selectivity*.—Accuracy of measurement in the presence of interferences, such as competing nontarget impurities, degradants, and matrix components.

(g) *Accuracy.*—Closeness of agreement between the test result and the accepted reference value (9).

(h) *Reagent blank.*—Reagents used during the analytical process (including solvents used for extraction or dissolution) analyzed in order to determine whether they contribute to the measurement signal.

(i) *Sample blank.*—Essentially sample matrices with concentrations of analyte of interest close to or below the expected LOD.

(j) *Precision.*—Closeness of agreement between independent test results obtained under stipulated conditions. Precision is typically subdivided into three types (9):

- *Repeatability*.—Precision under repeatability conditions, where independent test results are obtained with the same method on identical test items in the same laboratory by the same operator using the same equipment within short intervals of time.
- *Intermediate precision*.—Precision under conditions in which independent tests results are obtained in the same laboratory at different times using different operators or different equipment or in which a calibration has been carried out between measurements (10).
- *Reproducibility*.—Precision under reproducibility conditions, where independent test results are obtained with the same method on identical test items in different laboratories with different operators using different equipment.

3 Materials

(a) The use of a CRM is recommended to assess method trueness 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* section **3(c)**.

(c) Where a CRM is not available, the concentration of the analyte(s) being studied in a reference material is assessed preferably using two appropriate but different analytical procedures. Statistically equivalent results from these analytical methods are requested with a minimum of two independent analyses in replicate, preferably determined by different laboratories. Follow relevant WG or SMPR guidelines or consult a statistician. 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 (including moisture levels if relevant) should be established, understood, and fit for purpose.

The SPIFAN SLV guidelines were approved by the AOAC Expert Review Panel on Infant Formula and Adult Nutritionals in September 2011. Original publication date: September 2011.

The guidelines were revised in May 2022 and approved by the AOAC Official Methods Board on June 9, 2022. Publication date: July 2022.

If a noncommercial reference standard is used, its origin needs to be clearly identified along with all pertinent information demonstrating purity and/or analyte concentration and how 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) A minimum of six levels (three replicates per each level) that span the desired working range as described in the SMPR. In case the number of calibration levels used is <6, the report must substantiate reasons to be fit for purpose, based on the response of the instrumentation and methodology used.

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

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

5 LOD/LOQ

Ten independent analyses of blank or blank spiked at low level (toApproaches using reagent blanks and/or matrix blanks are possible as suggested in literature (11); these approaches need to be described in the SLV report.

The LOD could also be estimated by a statistical analysis of the calibration data according to ISO standards, ISO 11843-2 (12) for linear data or ISO 11843-5 (13) for linear and nonlinear data. When doing this estimation, include as many sources of variation as possible within a single laboratory. Calibration data from at least three analysts over a minimum of three different runs should be included, preferably using different instruments, if possible (14).

SMPRs are met at or above LOQ.

6 Selectivity

(a) The 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 relevant matrices. The recovery of the analyte(s) of interest should be determined and the influences of any suspected interferences should be stated in the SLV report (1). Examples of selectivity tests for chromatographic methods are described elsewhere (11, 15). Methods with a known interference should be modified to be selective prior to SLV study. 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, SD(s), in consultation with the appropriate expert review panel (ERP) and working group chair(s), will decide on an acceptable practice for each analyte.

7 Precision

All test materials selected for precision studies will be analyzed in duplicate on each of 6 days using multiple analysts and instruments as practical on the different days. Fresh reagents and working standards should be prepared and used each day. Reports will include information on the 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) Determine repeatability, intermediate precision, and measurement uncertainty for each sample type (9, 10, 16).

8 Accuracy

(a) *Analysis of CRM or reference material.—(1)* Where a CRM is not available, a suitable reference material may be substituted and compared to the established levels [*see* section **3(c)**].

(2) A minimum of nine independent replicates of CRM or reference material should be tested across 3 days (e.g., triplicate over 3 days) and compared to certified or reference values.

(b) *Spike recovery.—(1)* Recovery will be determined from a range of infant formula and adult nutritional matrices (use of the recognized SPIFAN kit is recommended where appropriate, otherwise SDs may agree on the sample types to be used for recovery studies).

(2) Each selected matrix will be spiked at three levels: low, middle, and high end of calibration range. Use spike levels covering the analytical range specified in the SMPR.

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

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

9 References

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(2) ISO 17034:2016: *General requirements for the competence and consistent operation of reference material producers*, International Organization for Standardization, Geneva, Switzerland

(3) ISO 5725-1:1994: *Accuracy (trueness and precision) of measurement methods and results–Part 1: General principles and definitions*, International Organization for Standardization, Geneva, Switzerland

(4) ISO 5725-3: *Accuracy (trueness and precision) of measurement methods and results–Part 3: Intermediate measures of the precision of a standard measurement method*, International Organization for Standardization, Geneva, Switzerland

(5) Eurachem/CITAC Guide: *Guide to Quality in Analytical Chemistry: An Aid to Accreditation* (2016) 3rd Ed., V. Barwick (Ed), Appendix B-Instrument calibration and performance checks, ISBN 978-0-948926-32-7 (available from www.eurachem.org)

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(9) ISO Guide 30:2015: *Reference materials–selected terms and definitions*, International Organization for Standardization, Geneva, Switzerland

(10) ISO 13528: *Statistical methods for use in proficiency testing by interlaboratory comparison*, Annex B, International Organization for Standardization, Geneva, Switzerland
(11) Eurachem Guide: *The Fitness for Purpose of Analytical Methods–A Laboratory Guide to Method Validation and Related Topics* (2014) 2nd Ed., Magnusson and U. Örnemark (Eds), ISBN 978-91-87461-59-0 (available from www.eurachem.org)
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