NSF International Standard for Dietary Supplements — Dietary supplements

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7.3 Test methods for microbiological contaminants

7.3.1 Reference methods

Testing shall be performed based on the currently promulgated version of the USP. With the exception of Pseudomonas, the methods described in USP Chapter 2021 “Microbial Enumeration Tests – Nutritional and Dietary Supplements” and Chapter 2022 “Microbiological Procedures for Absence of Specified Microorganisms – Nutritional and Dietary Supplements” shall be adhered to. For Pseudomonas, the methods described in USP Chapter 61 “Microbial Test Limits” shall be adhered to.

Alternatively, methods that have been validated according to the guidelines set forth in Appendix D and have been demonstrated to yield equivalent or better results compared to the aforementioned USP methodologies may be substituted.

7.3.2 Preparatory Testing

Preparatory testing, as specified in the currently promulgated version of the USP, shall be performed on all products. Certain products may themselves inhibit the multiplication of microorganisms that might be present, thus interfering with quantitative and qualitative microbiological assays detailed in section 7.3. Products shall be inoculated with the challenge microorganisms specified in USP Chapter 2021 and 2022. For the quantitative assays, at least a 70% bioburden recovery compared to a control medium must be demonstrated. For the qualitative assays, the challenge organism must be recovered on the applicable selective media. If a product fails to meet the recovery limit, a suitable neutralizer (e.g. soy lecithin, 0.5%; or polysorbate 20, 4.0%) shall be added to the culture medium to neutralize inhibitory substances.

Alternatively, *in lieu* of performing preparatory testing, a suitable neutralizer may be automatically added to the product and testing for the individual indicator organisms and pathogens may proceed as described in the following sections.

7.3.3 Total Aerobic Microbial Counts

Per the USP, the Membrane Filtration Method or Plate method shall be used for products that are freely soluble. Moderately soluble and translucent products shall be processed via the Plate method. The Multiple-Tube Method shall be used for all other products. The media, diluent and incubation conditions specified by the USP shall be used.

7.3.4 Total Combined Molds and Yeasts Count

Per the USP, the Membrane Filtration Method or Plate method shall be used for products that are freely soluble. Moderately soluble and translucent products shall be processed via the Plate method. The Multiple-Tube Method shall be used for all other products. The media, reagents and incubation conditions specified by the USP shall be used.

7.3.5 Enterobacteriaceae

Per the USP, the sample shall be dissolved or suspended in Phosphate buffer or Fluid soybean casein digest medium and diluted to 100 mL with Fluid soybean casein digest medium. The suspension shall be pre-incubated and subsequently processed using the media, reagents and incubation conditions stated by USP.

7.3.6 *Salmonella spp*
Testing shall be performed based on the USP Test for the Absence of *Salmonella sp.* (Chapter 2022).

7.3.7 *Escherichia coli*
7.3.7.1 Generic *Escherichia coli*
For finished products, testing shall be performed based on the qualitative USP Test for the Absence of *Escherichia coli* (Chapter 2022).

7.3.7.2 Pathogenic *Escherichia coli*
If the presence of *E. coli* is confirmed, then testing shall be performed based the USFDA BAM in Chapter 4A to determine whether the product contains pathogenic *Escherichia coli*, including but not limited to 0157:H7.

7.3.8 *Staphylococcus aureus*
Testing shall be performed based on the USP Test for Absence of *S. aureus* (Chapter 2022).

7.3.9 *Clostridium species*
Testing shall be performed based on the USP Test for Absence of *Clostridium* species (Chapter 2022).

7.3.10 *Pseudomonas aeruginosa*
For semisolid or liquid products containing less than 25% alcohol v/v, testing shall be performed based on the USP Chapter 62 “Microbiological Examination of Nonsterile Products: Tests for Specified Microorganisms”.

~~**7.3.1 Aflatoxins**~~

~~Testing shall be performed based on the methods described in Chapter 49, Natural Toxins, pp 49-1 to 49-49 of the AOAC *Official Methods of Analysis*.~~

~~**7.3.2 Yeast and mold**~~

~~Testing shall be performed based on the USP Plate Count Method under Total Aerobic Microbial Count substituting Potato Dextrose Agar and altering the incubation time/temperature to 5-7 d at 25 °C (77 °F) or appropriately validated rapid testing procedure such as the Soleris system[4] “dilute to spec” protocol for yeasts and molds.~~

~~[4] The user’s attention is called to the possibility that compliance with this standard may require use of an invention covered by patent rights. By publication of this standard, no position is taken with respect to the validity of this claim or of any patent rights in connection therewith. The patent holder has, however, filed a statement of willingness to grant a license under these rights on reasonable and nondiscriminatory terms and conditions to applicants desiring to obtain such license. Patent holder for the Soleris system is Neogen, 620 Lesher Place Lansing, MI 48912, www.neogen.com.~~

~~**7.3.3 Bacteria – total aerobic count**~~

~~Testing shall be performed based on the USP Total Aerobic Microbial Count or appropriately validated rapid testing procedure such as the Soleris[29] system protocol for Total Viable Count.~~

~~**7.3.4 Enterobacteriaceae**~~

~~Testing shall be performed based on the USP Total Aerobic Microbial Count substituting m-ENDO agar as the agar medium or appropriately validated rapid testing procedure such as the Soleris system "dilute to spec" protocol for Enterobacteriaceae.~~

~~**7.3.5 *Salmonella sp***~~

~~Testing shall be performed based on the USP Test for *Salmonella sp*.~~

~~**7.3.6 *Escherichia coli***~~

~~**7.3.6.1 Generic *Escherichia coli***~~

~~For finished products, testing shall be performed based on the qualitative USP Test for *Escherichia coli*. For raw materials, testing shall be performed based on a quantitative method adapted from the Enterobacteriaceae method (7.3.4). One to five colonies exhibiting a green metallic sheen shall be confirmed as generic *Escherichia coli* using the Conventional Method for coliforms, fecal coliforms, and *E. coli* presented in the USFDA Bacteriological Analytical Manual, Chapter 4.~~

~~**7.3.6.2 Pathogenic *Escherichia coli***~~

~~If the presence of *Escherichia coli* is confirmed, then testing shall be performed based on the USFDA Bacteriological Analytical Manual in Chapter 4A to determine whether the colonies are pathogenic *Escherichia coli*, including but not limited to 0157:H7.~~

~~**7.3.7 *Staphylococcus aureus***~~

~~Testing shall be performed based on the USP Test for *S. aureus*.~~

~~**7.3.8 *Pseudomonas aeruginosa***~~

~~Testing shall be performed based on the USP Test for *P. aeruginosa*.~~

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Annex D
(informative)

This annex contains guidance for validating alternative microbiological methodology to the accepted USP procedures.

D.1 Method Validation

Method validation is required to demonstrate that a non-reference method can deliver equivalent results to a standard reference method (i.e. USP). Prior to incorporating a non-reference method into the every-day testing of a supplement product, a full scale method validation should be carried out and documented. The method validation may consist of the following steps:

- Feasibility plan
- Validation plan
- Performance qualification
- Installation qualification
- Operation qualification
- SOP creation

D.2 Feasibility plan – Prior to initiating a method validation, a feasibility plan should be drafted. The feasibility plan should include: reason / justification for the adoption of the new non-standard method or process change, a description of required equipment, a description of safety considerations.

D.3 Validation plan - A validation plan is required for new non-standard reference methods and analytical process changes to non-standard reference methods. It is recommended that all validation studies shall be repeated 30 times in duplicate on at least 5 separate days. The performance characteristics required for qualitative methods quantitative methods are outlined below in the performance qualification section. All supporting data generated during the verification study shall be retained.

D.4 Performance qualification (PQ) - Prior to accepting a method as equivalent to the reference (i.e. USP) methods, a performance qualification should be performed. PQ provides documented evidence the system performs as expected when testing is performed using the media and test substrates that will be used for testing using this system. A PQ should include the following:

D.4.1 For a qualitative method, a side-by-side comparison of the non-reference method to the reference method must be performed. The Chi Square analysis is typically used to determine if the method mean is not statistically different from the reference method mean. The following performance indicators may be included in the validation:

D.4.1.2 Matrix Effect – the effect of different matrices should have been properly examined for the method in question. This would involve examining different matrices for potential interferences. Internal controls (matrix spikes) are typically used to analyze the matrix effect.

D.4.1.3 Sensitivity rate (p+) – the probability that the method will classify a test sample a positive, given that a test sample is a known positive. This is further defined as the total number of confirmed positive test portions by the method divided by the total number of confirmed positive test portions by both the new and reference method.

D.4.1.4 Specificity rate (p-) – the probability that the method will classify a test sample a negative, given that a test sample is a known negative. This is further defined as the total number of analyzed negative test portions by the method divided by the total number of confirmed negative test portions by both the new and reference method.

D.4.1.5 False negative - A false negative is a test in which samples that are inoculated or naturally contaminated generate a positive result in the reference method but are negative in the alternative method.

D.4.1.5.1 False negative rate (pf-) – the probability that a test sample is a known positive, given that the test sample has been classified as a negative by the method. This is further

defined as the number of misclassified known positives divided by the total number of positive test samples (misclassified positives plus the number of correctly classified known positives) obtained with the method.

D.4.1.5.2 The incidence of false negatives equals 100 minus the sensitivity rate.

D.4.1.6 False positive - A false positive is a test in which a sample that is not inoculated with the target organism generates a positive result in the alternative method but not in the reference method.

D.4.1.6.1 False positive rate (pf+) - the probability that a test sample is a known negative, given that the test sample has been classified as a positive by the method. This is further defined as the number of misclassified known negatives divided by the total number of negative test samples (misclassified negatives plus the number of correctly classified known negatives) obtained with the method.

D.4.1.6.2 The incidence of false negatives equals 100 minus the specificity rate.

D.4.1.7 Inclusivity and exclusivity: For each of the different assays the specificity needs to be determined. Inclusivity is the ability of the method to detect a target analyte from a wide range of strains. Exclusivity is the lack of interference in the method from a relevant range of non-target strains, which are potentially cross-reactive

D.4.2 For a quantitative method, a side-by-side comparison of the non-reference method to the reference method must be performed. A one-way analysis of variance (ANOVA) or a paired t-test by matrix type is typically used to determine if the method mean is not statistically different from the reference method mean. The following performance indicators may be included in the validation:

D.4.2.1 Detection limit - The limit of detection for the alternative method must be determined and compared to the reference method.

D.4.2.2 Repeatability (sr) – the closeness of agreement between successive and independent results obtained by the same method on identical test material, under the same conditions (i.e. apparatus, operator, laboratory and incubation time). Repeatability is a measure of precision within a laboratory.

D.4.2.3 Repeatability value (r) – the value below which the absolute difference between 2 single test results obtained under repeatability conditions may be expected to lie within 95% probability.

D.4.2.4 Reproducibility (sR) – the closeness of agreement between single test results on identical test material using the same method and obtained by operators in different laboratories using different equipment. Reproducibility is a measure of precision among laboratories. This may be obtained via help of a system vendor or independent laboratory.
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D.4.2.4 Reproducibility value (R) – the value below which the absolute difference between single test results under the reproducibility conditions may be expected to lie within 95% probability.

D.4.2.5 Relative Standard Deviation (RSD) – a measure of precision in quantitative studies which compares the variability of sets with different means. RSD is computed by dividing sR by sr by the mean. RSD values are independent of the amount of analyte over a reasonable range and facilitate comparison of variabilities at different concentrations. The result of a collaborative test may be summarized by giving the RSD for repeatability (RSDr) and RSD for reproducibility (RSDR).

D.4.2.6 Uncertainty – this a parameter associated with the result of a measurement that characterizes the dispersion of the values that could reasonably attributed to the measure and (i.e. what is being measured). Uncertainty encompasses the variance or standard deviation, precision and degree of confidence of a given result. Uncertainty is typically calculated from 30 measurements performed in duplicate.

D.4.2.6.1 The laboratory should document all possible components that would contribute to uncertainty. Examples of components of uncertainty which are associated with quantitative analysis are as follows.

D.4.2.6.2 Sampling – source of sample, sample container, methods of sampling, transportation time and temperature of sample, storage time and temperature of sample after receipt until analysis

D.4.2.6.3 Method of analysis – source (SMEWW, AOAC, ASTM, In-house), degree of validation, level of performance verification, performance characteristics (specificity, sensitivity, recovery, ruggedness)

D.4.2.6.4 Culture media and reagents – formulation specifications, preparation protocols, glassware/plastic ware, water quality, performance acceptability criteria, storage conditions, shelf-life

D.4.2.6.5 Analytical procedure – sample homogenization/mixing, sub sampling, preparing and dispensing dilutions, inoculation procedure (i.e. spread plate, membrane filtration), incubation conditions, reading/interpreting and recording results

D.4.2.6.6 Equipment – acquisition, maintenance, calibration, repair

D.4.2.6.7 Personnel – qualifications, validating performance, maintaining competency

D.4.2.7 Inclusivity and exclusivity: For each of the different assays the specificity needs to be determined. Inclusivity is the ability of the method to detect a target analyte from a wide range of strains. Exclusivity is the lack of interference in the method from a relevant range of non-target strains, which are potentially cross-reactive

D.4.3 Two additional performance characteristics should be considered for both qualitative and quantitative methods: ruggedness and robustness.

D.4.3.1 Ruggedness - Precision of a sample analyzed by different conditions (such as different analyst, instrument, lot of media, etc.)

D.4.3.2 Robustness - Capacity of method to remain unaffected by small deliberate variations in method parameters

D.4.4 In addition to the performance characteristics for methods involving novel systems of equipment, the laboratory should verify the correct performance of all software functions and review the QC data of all system components generated by vendor

D.5 Installation qualification (IQ) - For methods that involve novel equipment, documented evidence should be provided indicating that the system is installed as specified by the manufacturer in the location where it will be operated. IQ includes: Verification that all system components were installed and validation of these components; validation of environmental conditions; electrical requirements, computer qualification; installation checklist; and verification that the instrument was properly calibrated.

D.6 Operation qualification (OQ) - For methods that involve novel equipment, documented evidence should be provided indicating that the system operates as specified by the manufacturer when installed in its intended location. OQ includes: a unique standard operating procedure (SOP) for all product assay combinations that need to be performed; training documents; software characteristics; certification and verification of 21 CFR part 11 compliance; and an operation checklist.

D.7 Creation/converting method to SOP format – the proposed new method or alteration shall be written in SOP format. Include the following: equipment, reagents, methodology, calculations, on-going method quality control, positive and negative controls. All methods which the new methods are based or are used as a reference shall be obtained and retained by the laboratory.

D.8 Example Checklist for Method Validation:

A. Feasibility Plan

B. Validation Plan

C. Performance Qualification

1. Side-by side comparison of method to the USP methodology (all assays should be tested) to verify equivalency
2. Verification of correct performance of all software functions
3. QC data of all system components generated by vendor
4. Specificity data (inclusivity and exclusivity) for each of the assays
5. Limit of detection (lowest number of organism in the sample that can be detected)
6. Repeatability (ability to get consistent results)
7. Robustness (measure capacity of method to remain unaffected by small deliberate variations in method parameters)
8. Ruggedness (measures the degree of precision of a sample analyzed by different conditions such as different analyst, instrument, lot of media, etc)
9. False negative rate of true positive samples
10. False positive rate of true negative samples.

D. Installation Qualification

1. Reference Documents
2. Major Component Identification
3. Verification of Environmental Conditions
4. Verification of Electrical Requirements
5. Verification of Computer Requirements
6. Installation Checklist
7. Calibration Documentation

E. Operation Qualification

1. Standard Operating Procedures on System Operation
2. Software Identification
3. 21 Part 11 software compliance
4. Operation Checklist (verification that all function operate as expected)

5. Temperature Verification (instrument incubator)
6. Alarm Checklist
7. Radio Frequency Interference/Electro-Mechanical Interference

F. SOP Creation

References

P19 - CAEAL Policy on the Estimation of Uncertainty of Measurement in Environmental Testing. Rev 1.7. October 24, 2003. http://www.caeal.ca/uncertaintypolicy.html
Feldsine etal. 2002. AOAC International Methods Committee Guidelines for Validation of Qualitative and Quantitative Food Microbiological Official Methods of Analysis. Journal of AOAC International. Vol. 85, No. 5.
Zar, J.H. 1996. Biostatistical Analysis. Prentice Hall. New Jersey
Code of Federal Regulations, Parts 11, 210 and 211
USP <2021> Microbial Enumeration Tests - Nutritional and Dietary Supplements
USP <2022>
USP <2023>
USP <1223> Validation of Alternative Microbiological Methods
PDA Technical Report Number 33, Evaluation, Validation and Implementation of New Microbiological Methods

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