U.S. FOOD & DRUG ADMINISTRATION

Memorandum

To: Foods Program Governance Board
From: FDA Foods Program Regulatory Science Steering Committee (RSSC)
Date: October 17, 2019
Subject: Guidelines for the Validation of Chemical Methods for the FDA Foods Program, 3rd Edition

The FDA Foods Program Regulatory Science Steering Committee (RSSC), made up of representatives from the Center for Food Safety and Applied Nutrition (CFSAN), the Center for Veterinary Medicine (CVM), the Office of Regulatory Affairs (ORA), the National Center for Toxicological Research (NCTR), and the Office of the Chief Scientist of the FDA, is charged with the task of prioritizing, coordinating and integrating human food- and animal food-related science and research activities across the operating units of FDA's Foods Program.

As a regulatory agency tasked with ensuring the safety of the nation's food supply, it is imperative that the laboratory methods needed to support regulatory compliance, investigations and enforcement actions meet the highest analytical performance standards appropriate for their intended purposes. Development of standardized validation requirements for all regulatory methods used in our laboratories to detect chemical and radiological contaminants, as well as microbial pathogens, is a critical step in ensuring that we continue to meet the highest standards possible.

The attached document, now formally adopted by the RSSC, updates and renews the requirements that must be fulfilled in the evaluation of chemical methods to be used in our testing laboratories and supersedes the prior guidelines. These updated guidelines are posted on FDA's Foods Program Methods website. Please share these chemical methods validation guidelines with anyone who may be conducting or supervising chemical methods validation projects or otherwise needs to be aware of these updated requirements.

As one of the hierarchical committees under the RSSC, the Chemical Methods Validation Subcommittee (CMVS) is charged with providing guidance and oversight to all validation studies and is principally responsible for the content of these Guidelines, with input from the Chemistry Research Coordination Group (CRCG) and associated Technical Advisory Groups. Additional questions and comments about the Guidelines may be directed to the CMVS or CRCG.

Thank you,

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Guidelines for the Validation of Chemical Methods in Food, Feed, Cosmetics, and Veterinary Products

3rd Edition

U.S. Food and Drug Administration

Foods Program

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Center for Food Safety and Applied Nutrition
Office of Regulatory Science
Office of Food Safety
Office of Applied Research and Safety Assessment

Center for Veterinary Medicine
Office of Research
Office of New Animal Drug Evaluation

Office of Regulatory Affairs
Office of Regulatory Science

APPROVAL PAGE

This document is approved by the FDA Foods and Veterinary Medicine (FVM) Regulatory Science Steering Committee (RSSC). RSSC is the new committee that replaces the Science and Research Steering Committee. The **FVM RSSC Project Manager is responsible for updating the document as change requirements are met, and disseminating updates to the RSSC and other stakeholders, as required.**

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Guidelines for the Validation of Chemical Methods for the FDA FVM Program, 3rd *Edition*

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1.0 INTRODUCTION

1.1 Purpose

The U.S. Food and Drug Administration (FDA) is responsible for ensuring the safety of approximately 80% of the nation's food supply. FDA laboratories contribute to this mission through routine surveillance programs, targeted regulatory analyses, and emergency response when contaminated food or feed is detected or suspected in a public health incident. The effectiveness of these activities is highly dependent on the quality and performance of the laboratory methods needed to support regulatory compliance, investigations and enforcement actions. To ensure that the chemical methods employed for the analysis of foods and feeds meet the highest analytical performance standards appropriate for their intended purposes the Regulatory Science Steering Committee (SRSC) has established criteria by which all Foods and Veterinary Medicine (FVM) Program chemical methods shall be evaluated and validated. This document defines four standard levels of performance for use in the validation of analytical regulatory methods for chemical analytes in foods and feeds.

1.2 Scope

These criteria apply to FDA laboratories as they develop and participate in the validation of analytical regulatory methods for chemical analytes in food, feed, and cosmetics in anticipation of Agency-wide FVM Program implementation. These criteria do not apply to methods developed by or submitted to FDA under a codified process or official guidance (e.g., in the Code of Federal Regulations, CPGs, etc.), such as for veterinary drug approval. For such studies, the appropriate Center for Veterinary Medicine (CVM) or other Program guidance documents should be followed. This guidance is a forward-looking document; the requirements described here will only apply to *newly*-developed methods and significant modifications to existing methods (see Requirements). Once a method has been validated at the appropriate level, it can be implemented according to document, FDA-OFVM-3, "Methods Development, Validation, and Implementation Program," which establishes a standard operating procedure for the methods development, validation and implementation process [1]. For example, a multi-laboratory validated method to be used in a widespread regulatory application can be implemented by other FDA laboratories following the method verification process.

1.3 Administrative Authority and Responsibilities

All criteria established in this document for analytical method validation have been adopted and approved by the RSSC. The document, FDA-OFVM-3, establishes the standard operating procedure for the approval and tracking of method development and validation activities within the FVM Program [1]. Single laboratory validation (SLV) studies (including both Level 1 and Level 2 validations) can be managed wholly by the respective Center and Office line management structure. Oversight and coordination of multi-laboratory validation (MLV) studies (including both Level 3 and Level 4 validations) are the responsibility of the Methods Validation Subcommittees (MVS).

1.4 The Method Validation Subcommittee

Under the charge of the RSSC, the Chemistry Methods Validation Subcommittee (CMVS) will have oversight responsibility for MLV studies involving chemical methods associated

with the FVM Program which are intended for use in a regulatory context. The CMVS is a subcommittee of the Chemistry Research Coordinating Group (CRCG), which reports directly to the RSSC. The CMVS is governed by the organizational structure, roles and responsibilities as detailed in its charter [2]. Briefly, the CMVS will oversee and coordinate, in collaboration with the originating laboratory, all MLV studies for chemical methods developed within the FDA FVM Program to support regulatory analytical needs. This includes the evaluation and prioritization of proposed MLV studies as well as evaluation of completed MLV studies and reports. Submissions of chemical validation proposals, reports, questions, *etc.* can be directed to the CMVS through a central email account:

Chemistry.mvs@fda.hhs.gov

However, where possible, MLVs should be discussed in appropriate Technical Advisory Groups or with the CRCG to ensure the broadest possible consideration of factors before committing resources to an MLV.

1.5 General Responsibility of the Originating Laboratory

It is the responsibility of the originating laboratory to ensure proper adherence to all criteria described in this document. The originating laboratory should work in consultation with the CMVS and/or its designated Technical Advisory Group (TAG) throughout the multi-laboratory validation process. It will be the responsibility of the originating laboratory to include their respective QA/QC manager in all aspects of the validation process.

1.6 Overview of Method Validation

Method validation is the process of demonstrating or confirming that a method is suitable for its intended purpose. The purpose of these methods may include but is not limited to qualitative analysis, quantitative analysis, screening analysis, confirmatory analysis, limit tests, matrix extensions, platform extensions, and emergency/contingency operations. Validation includes demonstrating performance characteristics such as accuracy, precision, sensitivity, selectivity, limit of detection, limit of quantitation, linearity, range, and ruggedness, to ensure that results are meaningful.

Method validation is a distinct phase from method development/optimization and should be performed *subsequent* to method development. Methods may be validated for one or more analytes, one or more matrices, and one or more instruments or platforms. The method is validated by conducting experiments to determine the specific performance characteristics that serve to define and quantify method performance.

1.7 Applicability

This document establishes validation criteria for regulatory methods that are to be widely used to detect and quantitate chemical analytes in food, feed and other FDA regulated products covered by the FVM Program including, but not limited to, the following:

Chemotherapeutic Residues
Color Additives
Decomposition Products
Dietary Supplement Ingredients/Adulterants
Elemental and Metals
Food and Feed Additives and Preservatives
Food Allergens
Gluten

Intentional Adulterants/Poisons
Mycotoxins
Nutrients
Persistent Organic Pollutants
Pesticides
Seafood and plant toxins
Toxic Elements
Veterinary Drug Residues

1.8 Requirements

Method validation is required for:

- Submission of a new or original method.
- Expansion of the scope of an existing method to include additional analytes.
- Expansion of the scope of an existing method to include additional matrices.
- Changes in the intended use of an existing method (*e.g.,* screening vs. confirmatory).
- Modifications to a method that may alter its performance specifications (*e.g.*, modifications that could significantly affect the precision and accuracy, changes to the fundamental science of an existing method, significant changes to reagents, apparatus, instrumental parameters, sample preparation and/or extraction, or modification of a method's range beyond validated levels). Allowable modifications that would not require further validation are provided in Appendix 6 for Mass Spectrometry (GC and LC) methods and in the document ORA-LAB.5.4.5 Attachment A-Modification Criteria [3] for HPLC and GC (non-MS) methods.

2.0 CRITERIA AND GUIDANCE FOR THE VALIDATION OF CHEMICAL METHODS

2.1 General Validation Tools and Protocol Guidance

There are a number of excellent references and guides available providing further information on method validation for chemical methods [3-20]. The following provides some general guidelines/tools that should be used to assess method performance:

General Protocol: Prepare and analyze method blanks, matrix blanks, reference materials (if available) and matrix spikes (using matrix blanks if available) of known concentration as generally described under the Methods Validation Levels section and Table 1 below. Accuracy or bias and precision are calculated from these results. Data will also be used to evaluate matrix effects and ruggedness/robustness of the method resulting from changes in the sample matrix.

The following general validation tools should be used to generate method performance characteristics as described in the Performance Characteristics section below.

Blanks: Use of various types of blanks enables assessment of how much of the result is attributable to the analyte in relation to other sources. Blanks are useful in the determination of limit of detection.

Reference materials and certified reference materials: The use of known reference materials (when available and applicable) should be incorporated to assess the accuracy or bias of the method, as well as for obtaining information on interferences.

Matrix Blank: This type of blank is a substance that closely matches the samples being analyzed with regard to matrix components. Matrix blanks are used to establish background level (presence or absence) of analyte(s) and to verify that sample matrix and equipment used does not interfere with or affect the analytical signal.

Matrix Spikes (Laboratory Fortified Matrix): Recovery determinations can be estimated from fortification or spiking with a known amount of analyte and calculation of spike recoveries. (Note: spike recovery may not be accurately representative of recovery from naturally incurred analytes.) Matrix effects can also be assessed with these samples. Accuracy or bias and precision are calculated from these results. The data can also be used to evaluate robustness of the method resulting from changes in the sample matrix.

Incurred Samples: This type of sample contains (not laboratory fortified) the analyte(s) of interest (if available) and can be used to evaluate precision and bias (if analyte concentration(s) are reliably known). Analyte recovery can also be evaluated through successive extractions of the sample and/or comparison to another analytical procedure with known bias.

Reagent Blank: This type of blank incorporates all reagents used in the method and is subjected to all sample processing operations. It serves to verify that reagents are analyte free and the equipment used does not interfere with or affect the analytical signal.

Replicate Analyses: The precision of the analytical process can be evaluated using replicate analyses. The originating laboratory should assure that adequate sample replicates are

performed and that results from replicate measurements of each analyte are compared. Minimally, the method repeatability should be evaluated.

Interferences: Spectral, physical, and chemical interferences can be evaluated by analyzing samples containing various suspected interferences. Carryover should be evaluated using the incorporation of blanks immediately following standards and samples.

Statistics: Statistical techniques are employed to evaluate accuracy, trueness (or bias) precision, linear range, limits of detection and quantitation, and measurement uncertainty.

2.2 Reference Method

A reference method is a method by which the performance of an alternate or new method may be measured or evaluated. For chemical analytes, an appropriate reference method is not always identifiable or available. However, there are some instances in which the use of a reference method is appropriate such as when replacing a method specified for use in a compliance program. Consultation between the originating laboratory and the CMVS and the Program Office is suggested when deciding if the use of a reference method will be necessary.

2.3 Performance Characteristics

Performance characteristics that should be evaluated in order to validate a method will vary depending on the intended use of the method, the type of method (*e.g.*, quantitative vs. qualitative), and the degree to which it has been previously validated (e.g., matrix extension, analyte extension, platform extension). Although definitions of these characteristics are included in Appendix 1, this document is not meant to address the various ways of calculating characteristics such as method detection level, limit of detection or limit of quantitation.

Performance Characteristics for Validation of New Quantitative Methods: Validation of new quantitative methods should include at a minimum evaluation of the following performance characteristics: accuracy, precision, selectivity, limit of detection, limit of quantitation, linearity (or other calibration model), range, measurement uncertainty, ruggedness, confirmation of identity and spike recovery.

Performance Characteristics for Validation of New Qualitative Methods: Validation of new qualitative methods should include at a minimum evaluation of the following performance characteristics: sensitivity, selectivity, false positive rate, false negative rate (guidance for determining false positive/negative rates is in Appendix 2B), minimum detectable concentration, ruggedness, and confirmation of identity.

Performance Characteristics for Validation of Method Extensions: Validating the extension of methods that have previously been validated requires a careful evaluation of the intended purpose of the extension. In cases where the sample preparation and/or the extraction procedure/analytical method is modified from the existing test procedure, it should be demonstrated that the modifications do not adversely affect the precision and accuracy of the data obtained. In order to implement the modified method, generally the standard or existing method is first performed. The modified method performance then is verified by comparison with that of the original method.

2.4 Confirmation of Identity

Confirmation of identity for each analyte must be performed as part of the method validation for regulatory enforcement for both qualitative and quantitative methods. Unambiguous confirmation of identity usually requires analytically identifying key features of each analyte in the scope of the new method being validated such as with mass spectral fragmentation patterns or by demonstration of results in agreement with those obtained using an independent analysis.

FDA has issued guidance documents on the development, evaluation, and application of mass spectrometric methods for confirming the identity of target analytes including: CVM Guidance for Industry 118: Mass Spectrometry for Confirmation of the Identity of Animal Drug Residues [4] and Acceptance Criteria for Confirmation of Identity of Chemical Residues using Exact Mass Data within the Office of Foods and Veterinary Medicine [5] Following the CVM guidance is required for veterinary drug residue methods. For other types of chemical contaminants in food (e.g. food additives, mycotoxins, etc.), the CVM document should be followed because it was written as a Guidance for Industry and therefore has been more widely internally and externally reviewed and distributed.

2.5 Method Validation Levels

The following describes the four standard levels of performance defined for method validation of analytical regulatory methods for chemical analytes in foods. This approach is based on the Food Emergency Response Network (FERN), SOP No: FERN-ADM.0008.00, FERN Validation Guidelines for FERN Chemical, Microbiological, and Radiological Methods [6], as well as AOAC guidelines for single laboratory validation [7] and collaborative studies [8]. Key validation parameters for each level are summarized in Table 1. It is the responsibility of the originating (developing) laboratory to determine the appropriate level of validation required up to and through single laboratory validations. It is highly recommended that originating laboratories work with the appropriate Technical Advisory Group when determining the appropriate level of validation.

NOTE: *Not all methods will or should be validated to the highest level.*

Level One

This is a single laboratory validation level with the lowest level of validation requirements and is appropriate for emergency/limited use. Performance of the method at this initial level of scrutiny will determine, in part, whether further validation is useful or warranted.

Intended Use: emergency/limited use/matrix extension/analyte extension/platform extension. Examples of where Level One validation would be acceptable include isolated consumer complaints, single-occurrence samples, and application of a method developed for a specific analyte(s) to a matrix not previously validated, in response to a real or perceived threat to food safety or public health. Validation of method performance with a new matrix is intended to assure that the new matrix will produce accurate and reliable results for all the analytes in the scope of the method. Generally, all targeted analytes still must be included in matrix spikes at this level, if widespread use in this matrix is anticipated for regulatory purposes. As the first level of validation of methods for matrix, analyte or platform extension/emergency use, it would be expected that a more rigorous single laboratory validation at least equivalent to Level Two below would be performed before more widespread non-emergency regulatory use. For further guidance on extensions, see Appendix 5.

Level Two

This is a single laboratory validation level. The originating lab has conducted a comprehensive validation study, with performance criteria similar to an AOAC Single

Laboratory Validation study. If appropriate, a comparison with an existing reference method has been performed. Some of the criteria of the study may be at a lower level than the AOAC Single Laboratory Validation study,but are appropriate for the developing method at this stage.

Intended Use: Routine regulatory testing, emergency needs, minor method modifications, analyte and matrix extensions of screening methods. If a method validated at this level is expected to have use that is widespread, long term, of high public visibility or potentially involved in international trade conflicts, its validation should be extended to at least Level Three below.

Level Three

This is a multi-laboratory validation level. Level Three validation employs a minimum of one collaborating laboratory in addition to the originating laboratory. Most of the criteria followed by the originating lab are at a level similar to the AOAC full collaborative study level with comparison to an existing reference method when available and appropriate. The additional collaborating laboratories follow many of the criteria found in an AOAC collaborative study. The main differences are that Level Three validation employs at least one additional collaborating laboratory instead of the eight to ten used by AOAC and requires fewer replicates for each food matrix/spike level. MLV's are studies of the method, not the laboratory. The method must be followed as closely as practicable, and any deviations by participants from the method described, no matter how trivial they may seem, must be noted on the report form [8].

Intended Use: Methods validated to this level of scrutiny are acceptable for use in all regulatory circumstances including screening analyses, confirmatory analyses, regulatory surveys, and compliance support. If the method is expected to have use that is widespread, long term, of high public visibility or involved in international trade conflicts, it may be appropriate to have its validation extended to Level Four.

Level Four

This validation level has criteria equivalent to a full AOAC or ISO Collaborative Study. Any method reaching this level of validation should be able to be submitted for adoption by the AOAC as a fully collaborated method. MLV's are studies of the method, not the laboratory. The method must be followed as closely as practicable, and any deviations by participants from the method described, no matter how trivial they may seem, must be noted on the report form [8].

2.6 Acceptability Criteria

There are various acceptability ranges for method validation performance criteria that may be appropriate depending on the application or intended use of the methodology and especially the levels of concern, action levels or tolerance for the chemical analyte. Some examples of acceptability ranges used by various national and international organizations and their sources are provided in Appendix 2. Acceptable spike recoveries vary with analyte concentration as indicated in Appendix 2 (e.g., recoveries may fall in approximately the 80- 120% range for quantitative methods at the 1 µg/g (ppm) concentration). Repeatability and reproducibility also vary with analyte concentration. The acceptability ranges in Appendix 2 provide approximate target ranges for method developers and the MVS and are not rigid binding guidelines. It is recognized that for some situations such as with difficult matrices, extremely low analyte concentrations (e.g., chlorinated dioxins, persistent organic pollutants), multi-residue methods and with emergency situations these general acceptability ranges may not be achievable or required.

Table 1. Key Validation Parameter Requirements for Chemical Methods

	Level One: Emergency/ Limited Use	Level Two: Single Laboratory Validation	Level Three: Multi-Laboratory Validation	Level Four: Full Collaborative Study
Number participating labs	1	1	≥ 2	8 (quantitative) 10 (qualitative)
Number of matrices*	≥1	≥3 recommended where available	≥3 recommended where available	≥3 recommended where available
Number of analyte(s) spike levels for at least one matrix source**	≥2 spike levels + 1 matrix blank	≥3 spike levels + 1 matrix blank	≥3 spike levels + 1 matrix blank	≥3 spike levels + 1 matrix blank
Replicates required per matrix source at each level tested per laboratory	≥2 (quantitative) ≥2 (qualitative)	≥2 (quantitative) ≥3 (qualitative)	≥2 (quantitative) ≥3 (qualitative)	≥2 (quantitative) ≥3 (qualitative)
Replicates required at each level tested per laboratory if only one matrix source used	≥4 (quantitative) ≥6 (qualitative)	≥6 (quantitative) ≥9 (qualitative)	≥3 (quantitative) ≥6 (qualitative)	≥2 (quantitative) ≥6 (qualitative)

*If a variety of food matrices with differing physical and chemical properties are selected, the number of sources for each food sample matrix may be one or more, but if only one food matrix is studied then ≥3 sources are recommended, where available. The number of matrix sources may be reduced, particularly if it is difficult to obtain blank matrix sources, as long as the total number of spike levels and matrix combinations are adequate (e.g., 6 replicates or greater at each spike level for quantitative methods and 9 replicates or greater for qualitative methods). Certified reference materials/ incurred tissues should be used, when available, and can replace one of your spiking levels.

** Number of spike levels is recommended for at least one source of matrix. Other similar sources of matrix (*e.g.,* within the same category; see Appendix 4) may be studied at one or two spike levels (*e.g.*, at an action/guidance or tolerance level or close to the lower limit of quantitation/detection). Certified reference materials/ incurred tissues should be used, when available, and can replace one of your spiking levels. For some analytes, spiking with pure standard alone does not sufficiently demonstrate method performance (i.e. BPA in can coatings contain oligomeric interferences, gluten in fermented/hydrolyzed products, protein-bound veterinary drug metabolites, sulfites binding irreversibly after spiking). In these cases, reference materials and/or real samples must be used to demonstrate method performance.

3.0 ADDITIONAL PROCEDURAL GUIDANCE

In addition to the criteria described above in Table 1 for standard quantitative and qualitative methods, additional guidance is provided in this section for specific types of methods or validation situations.

3.1 Platform/Instrumentation Extension

Expanding the use of a validated method to include another significantly different instrument or platform requires further validation. Such instances include the use of an instrument or platform similar in scope and function to that currently validated and approved for use; however, it may have major differences in configuration, or detection scheme. Detailed guidance for platform extensions are in Appendix 5.

3.2 Analyte Extension

Multi-residue, multi-class methods are becoming more common. Many of these methods are semi-quantitative (limits tests) or qualitative broad band screens. Performance requirements for these types of procedures are described below. However, if a multi-residue method is meant to be used for quantitation, the same performance characteristics as required for single analyte methods should be evaluated for each analyte (accuracy, precision, selectivity, limit of detection, limit of quantitation, linearity range, uncertainty, and ruggedness). It is understood that with a large multi-residue method, not all analytes will meet the recommended acceptability ranges listed in Appendix 2, but the performance for each compound should be tested and reported so that the accuracy and precision are known for any given analyte and are sufficient for the intended purpose of the method. Detailed guidance for validation required for analyte extensions is described in Appendix 5.

3.3 Food Matrix Extension

The validation of method performance with a new matrix is intended to assure that the method will continue to produce accurate and reliable results. It is generally assumed that the more closely related a new food matrix is to a previously validated matrix for a defined analyte, the greater the probability that the new matrix will behave similarly. It is also usually the case that the regulatory chemical methods employed by FDA are used to analyze a diversity of products representing a large spectrum of matrices. Detailed guidance for matrix extensions is in Appendix 5.

3.4 Limit Tests (common semi-quantitative screening method)

One specific category of qualitative methods includes limit tests (binary or pass/fail tests) for analytes that have a defined level of concern. The purpose of these screening methods is to determine if analyte is present with a concentration near or above the level of concern. This is in contrast to screening methods whose intended purpose is to determine the presence or absence of an analyte at any level. Limit test method validations must include determination of the precision of the method for an analyte(s) at the level(s) of concern.

Limit test screening methods, in general, should avoid false negatives with false negative rates representing less than 5% of the analytical results (see Appendix 2B for determining false positive/negative rates). The occurrence of false positives is less critical since presumptive positives are further analyzed by quantitative or confirmatory

methods. However, false positive rates should typically be less than 10-15% to avoid unnecessary confirmatory testing. Ideally, limit tests are capable of rapidly screening a large number of samples to minimize the need for additional analysis. A common approach used in limit test screening methods is to use a confidence interval to set a laboratory threshold or cut-off value whereby only responses above that value require further testing. For a limit test based on an instrument response, a threshold or cut-off value can be determined by a confidence limit, based on an estimate of the standard deviation of the response or concentration of an analyte in samples fortified with the analyte at the level of concern.

Example:
Milk samples (n=21) were fortified with sulfamethazine at the level of concern (10 ng/mL). A LC-MS/MS limit test screening method was used to measure this drug in the extracted milk samples. The mean concentration found was to be 10.99 ng/mL with a standard deviation of 2.19. A threshold or cut-off value was calculated so that 95% of samples containing sulfamethazine at or above 10 ng/mL would have a response above the threshold value:

Threshold value = [mean concentration – (t * standard deviation)]
= [10.99 – (1.725 * 2.19)] = 7.21 ng/mL

Where t = one-tailed Student's t value for n-1 degrees of freedom at the 95% confidence level

This approach can also be used for immunosorbent assays such as enzyme linked immunosorbent assay (ELISA) or optical biosensor assays. These tests may be non-competitive (direct measurement of analyte response) or competitive (indirect measurement). Analysis of data from a competitive immunosorbent test should account for the fact that the observed response decreases with increasing analyte concentration; therefore, a response lower than the threshold or cut-off would be considered a presumptive positive response. For immunosorbent assays, it is also important to measure the response observed for blank matrix samples and to verify that the blank response is distinguishably (statistically) different from that of the threshold.

Performance characteristics of limit tests:
Validation of new limit tests should include, at a minimum, evaluation of the following performance characteristics: sensitivity, specificity, precision, threshold or cut-off value, false positive rate, false negative rate, minimum detectable concentration (should be lower than the threshold/cut-off value), and ruggedness/robustness.

3.5 Qualitative Broad-band Analyte Screening

Broad-band methods that can detect many compounds are being utilized more frequently as an initial screening step as part of chemical contaminant testing in FDA laboratories. These methods usually involve mass spectrometric analyses and provide qualitative information. For example, the data obtained may be compared to an established reference such as a database of compounds with exact mass and molecular formula information or spectra in a compiled library.

Typically, initial validation of these methods is performed using a limited set of representative analytes and representative matrices. For example, sets of analytes that contain compounds from a variety of chemical classes from the area of interest (e.g. pesticides,

veterinary drug residues, or common chemical toxins) are tested with the method using representative matrices. The performance characteristics that may be evaluated include: sensitivity, selectivity, false positive rate, false negative rate (see Appendix 2B for calculation of false positive/negative rates), minimum detectable concentration, ruggedness, and confirmation of identity. It is understood that the method performance may vary with the different classes of compounds, but it is important to have an initial evaluation of the method's capabilities.

Laboratories continuously expand the scope of these broad-band methods by adding new analytes that come to their attention through various sources of intelligence. In addition, a new compound might be found in a sample after acquired data are compared to the reference databases. In these cases, some verification that the analyte can be detected reliably by the screening method is required. When a new compound is added to the scope of a qualitative method, it should first be determined whether this compound belongs to a class of compounds that has already been validated for the broad-band method. If the new compound shares chemical characteristics with an existing class of compounds in the scope of the method, then it may suffice to select a few representative matrices, perform a single level spike in these representative matrices in duplicate and determine that reproducible recovery is obtained in order to assess whether the analyte can be detected effectively by the method. Scenarios that may require a full validation would include a new analyte being added to the scope of the broad-band method that was not represented by any of the compound classes already in the scope. Also, if the new analyte requires modifications in the extraction protocol due to its chemical characteristics, then its inclusion in the scope should be fully validated as recommended by this guidance.

Although positive findings by the broad-band method are subjected to confirmatory testing using a targeted method, it is still important to determine, through proper validation and verification protocols, that the broad-band method does not give rise to a high number of false negative findings. False negative in this context means the method fails to detect a residue in its scope when the residue is present in the matrix at or above the level of concern or minimum detectable concentration. While the positive finding by the broad-band method is subjected to further analysis and scrutiny, negative findings are upheld as such and a regulatory decision is made based on these results, *e.g.*, to release the products into commerce.

4.0 REFERENCES AND SUPPORTING DOCUMENTS

[1] Food and Drug Administration, Office of Foods and Veterinary Medicine, "Methods Development, Validation and Implementation Program", Document #: FDA-OFVM-3. Effective Date 10/16/2014.

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APPENDIX 1 - Glossary of Terms

Generally, references 13-17 were utilized in preparation of this glossary.

Accuracy: The closeness of agreement between a test result and an accepted reference value. When applied to test results, accuracy includes a combination of random and systematic error. When applied to test method, accuracy refers to a combination of trueness and precision.

Action level: Level of concern or target level for an analyte that must be reliably identified or quantified in a sample.

Analyte: The chemical substance measured and/or identified in a test sample by the method of analysis.

Analytical batch: An analytical batch consists of samples, standards, and blanks which are analyzed together with the same method sequence and same lots of reagents and with the manipulations common to each sample within the same time period (usually within one day) or in continuous sequential time periods.

Bias: The difference between the expectation of the test result and the true value or accepted reference value. Bias is the total systematic error, and there may be one or more systematic error components contributing to the bias.

Blank: A substance that does not contain the analytes of interest and is subjected to the usual measurement process. Blanks can be further classified as method blanks, matrix blanks, reagent blanks, instrument blanks, and field blanks.

Calibration: Determination of the relationship between the observed analyte signal generated by the measuring/detection system and the quantity of analyte present in the sample measured. Typically, this is accomplished through the use of calibration standards containing known amounts of analyte.

Calibration Standard: A known amount or concentration of analyte used to calibrate the measuring/detection system. May be matrix matched for specific sample matrices.

Carryover: Residual analyte from a previous sample or standard which is retained in the analytical system and measured in subsequent samples. Also called *memory*.

Certified Reference Material (CRM): Reference material accompanied by documentation (certificate) issued by an authoritative body and providing one or more specified property values with associated uncertainties and traceability, using valid procedures. Note: Standard Reference Material (SRM) is the trademark name of CRMs produced and distributed by the National Institute of Standards and Technology (NIST).

Check Analysis: Result from a second independent analysis which is compared with the result from the initial analysis. Typically, check analyses are performed by a different analyst using the same method.

Confirmation of Identity: Unambiguous identification of an analyte(s) by a highly specific technique such as mass spectrometry or by demonstration of results from two or more independent analyses in agreement.

Confirmatory Analysis/Method: Independent analysis/method used to confirm the result from an initial or screening analysis. A different method is often used in confirmation of screening results.

Cut-off Concentration: In qualitative analysis, the concentration of the analyte that is either statistically lower than the level of concern (for limit tests) or at which positive identification ceases (for confirmation of identity methods). See also *Threshold Value*.

False Negative Rate: In qualitative analysis, a measure of how often a test result indicates that an analyte is not present, when, in fact, it is present or, is present in an amount greater than a threshold or designated cut-off concentration. See Appendix 2B.

False Positive Rate: In qualitative analysis, a measure of how often a test result indicates that an analyte is present, when, in fact, it is not present or, is present in an amount less than a threshold or designated cut-off concentration. See Appendix 2B.

Fitness for Purpose: Degree to which data produced by a measurement process enables a user to make technically and administratively correct decisions for a stated purpose.

Guidance Level: Level of concern or action level issued under good guidance practices that must be reliably identified or quantified in a sample.

Incurred Samples: Samples that contain the analyte(s) of interest, which were not derived from laboratory fortification but from sources such as exogenous exposure or endogenous origin. Exogenous exposure includes, for example, pesticide use, consumption by an animal, or environmental exposure.

Interference: A positive or negative response or effect on response produced by a substance other than the analyte. Includes spectral, physical, and chemical interferences which result in a less certain or accurate measurement of the analyte.

Intermediate Precision: Within-laboratory precision obtained under variable conditions, e.g., different days, different analysts, and/or different instrumentation.

Internal Standard: A chemical added to the sample, in known quantity, at a specified stage in the analysis to facilitate quantitation of the analyte. Internal standards are used to correct for matrix effects, incomplete spike recoveries, etc. Analyte concentration is deduced from its response relative to that produced by the internal standard. The internal standard should have similar physico-chemical properties to those of the analyte.

Laboratory Fortified Matrix: See *Matrix Spike*.

Level of Concern: Level of concern is the concentration of an analyte in a sample that has to be exceeded before the sample can be considered violative. This concentration can be a regulatory tolerance, safe level, action level, guidance level or a laboratory performance level.

Limit of Detection (LOD): The minimum amount or concentration of analyte that can be reliably distinguished from zero. The term is usually restricted to the response of the detection system and is often referred to as the *Detection Limit*. When applied to the complete analytical method it is often referred to as the *Method Detection Limit* (MDL). Sample calculations are in references [21] and [22].

Limit of Quantitation (LOQ): The minimum amount or concentration of analyte in the test sample that can be quantified with acceptable precision. Limit of quantitation (or quantification) is variously defined but must be a value greater than the MDL and should apply to the complete analytical method. Sample calculations are in references [21] and [22].

Limit Test: A type of semi-quantitative screening method in which analyte(s) has a defined level of concern. Also referred to as binary or pass/fail tests.

Linearity: The ability of a method, within a certain range, to provide an instrumental response or test results proportional to the quantity of analyte to be determined in the test sample.

Matrix: All the constituents of the test sample with the exception of the analyte.

Matrix Blank: A substance that closely matches the samples being analyzed with regard to matrix components. Ideally, the matrix blank does not contain the analyte(s) of interest but is subjected to all sample processing operations including all reagents used to analyze the test samples. The matrix blank is used to determine the absence of significant interference due to matrix, reagents and equipment used in the analysis.

Matrix Effect: An influence of one or more components from the sample matrix on the measurement of the analyte concentration or mass. Matrix effects may be observed as increased or decreased detector responses, compared with those produced by simple solvent solutions of the analyte.

Matrix Source: The origin of a test matrix used in method validation. A sample matrix may have variability due to its source. Different food matrix sources can be defined as different commercial brands, matrices from different suppliers, or in some cases different matrices altogether. For example, if a variety of food matrices with differing physical and chemical properties are selected, the number of sources for each food sample matrix may be one or more.

Matrix spike: An aliquot of a sample prepared by adding a known amount of analyte(s) to a specified amount of matrix. A matrix spike is subjected to the entire analytical procedure to establish if the method is appropriate for the analysis of a specific analyte(s) in a particular matrix. Also referred to as a *Laboratory Fortified Matrix*.

Method blank: A substance that does not contain the analyte(s) of interest but is subjected to all sample processing operations including all reagents used to analyze the test samples. An aliquot of reagent water is often used as a method blank in the absence of a suitable analyte-free matrix blank.

Method Detection Limit (MDL): The minimum amount or concentration of analyte in the test sample that can be reliably distinguished from zero. MDL is dependent on sensitivity, instrumental noise, blank variability, sample matrix variability, and dilution factor.

Method Development: The process of design, optimization and preliminary assessment of the performance characteristics of a method.

Method Validation: The process of demonstrating or confirming that a method is suitable for its intended purpose. Validation criteria include demonstrating performance characteristics such as accuracy, precision, specificity, limit of detection, limit of quantitation, linearity, range, ruggedness and robustness.

Method Verification: The process of demonstrating that a laboratory is capable of replicating a validated method with an acceptable level of performance.

Minimum Detectable Concentration (MDC): In qualitative analysis, an estimate of the minimum concentration of analyte that must be present in a sample to ensure at a specified high probability (typically 95% or greater) that the measured response will exceed the detection threshold, leading one to correctly conclude that an analyte is present in the sample.

Precision: The closeness of agreement between independent test results obtained under specified conditions. The precision is described by statistical methods such as a standard deviation or confidence limit of test results. See also *Random Error*. Precision can be further classified as *Repeatability, Intermediate Precision*, and *Reproducibility*.

Qualitative Analysis/Method: Analysis/method in which substances are identified or classified on the basis of their chemical, biological or physical properties. The test result is either the presence or absence of the analyte(s) in question.

Quantitative Analysis/Method: Analysis/method in which the amount or concentration of an analyte may be determined (or estimated) and expressed as a numerical value in appropriate units with acceptable accuracy and precision.

Random error: Component of measurement error that in replicate measurements varies in an unpredictable manner. See also *Precision*.

Range: The interval of concentration over which the method provides suitable accuracy and precision.

Reagent Blank: Reagents used in the procedure taken through the entire method. Reagent Blanks are used to determine the absence of significant interference due to reagents or equipment used in the analysis.

Recovery: The proportion of analyte (incurred or added) remaining at the point of the final determination from the analytical portion of the sample measured. Usually recovery is expressed as a percentage.

Reference material: A material, sufficiently homogenous and stable with respect to one or more specified properties, which has been established to be fit for its intended use in a measurement process or in examination of nominal properties.

Reference standard: A standard, generally having the highest metrological quality available at a given location in a given organization, from which measurements are made or derived. Note: Generally, this refers to recognized national or international traceable

standards provided by a standards producing body such as the National Institute of Standards and Technology (NIST).

Repeatability (RSD_r): Precision obtained under observation conditions at a specific concentration/spike level where independent test results are obtained with the same method on identical test items in the same test facility by the same operator using the same equipment within short intervals of time. Should be included in all quantitative MLV reports.

Representative Analyte: An analyte used to assess probable analytical performance with respect to other analytes having similar physical and/or chemical characteristics. Acceptable data for a representative analyte are assumed to show that performance is satisfactory for the represented analytes. Representative analytes should include those for which the worst performance is expected. Representative analytes are used mostly for non-targeted analysis and unknown screening procedures.

Representative Matrix: Matrix used to assess probable analytical performance with respect to other matrices, or for matrix-matched calibration, in the analysis of broadly similar commodities. For food matrices, similarity is usually based on the amount of water, fats, protein, and carbohydrates. Sample pH and salt content can also have a significant effect on some analytes.

Reproducibility (RSD_R): Precision obtained at a specific concentration/spike level under observation conditions where independent test results are obtained with the same method on identical test items in different test facilities with different operators using different equipment. Should be included in all quantitative MLV reports.

Ruggedness/Robustness: A measure of the capacity of an analytical procedure to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

Screening Analysis/Method: An analysis/method intended to detect the presence of analyte in a sample at or above some specified concentration (action or target level). Screening methods typically attempt to use simplified methodology for decreased analysis time and increased sample throughput.

Selectivity: The extent to which a method can determine particular analyte(s) in a mixture(s) or matrix(ces) without interferences from other components of similar behavior. Selectivity is generally preferred in analytical chemistry over the term *Specificity*.

Sensitivity: The change in instrument response which corresponds to a change in the measured quantity (*e.g.*, analyte concentration). Sensitivity is commonly defined as the gradient of the response curve or slope of the calibration curve at a level near the LOQ.

Specificity: In quantitative analysis, specificity is the ability of a method to measure analyte in the presence of components which may be expected to be present. The term *Selectivity* is generally preferred over Specificity.

Spike Recovery: The fraction of analyte remaining at the point of final determination after it is added to a specified amount of matrix and subjected to the entire analytical procedure. Spike Recovery is typically expressed as a percentage. Spike recovery should be calculated for the method as written. For example, if the method prescribes using deuterated internal standards or matrix-matched calibration standards, then the reported analyte recoveries should be calculated according to those procedures.

Standard: A substance of known identity and purity and/or concentration.

Standard Reference Material (SRM): A certified reference material issued by the National Institutes of Standards and Technology (NIST) in the United States. (www.nist.gov/SRM).

Systematic error: Component of measurement error that in replicate measurements remains constant or varies in a predictable manner. This may also be referred to as *Bias*.

Threshold Value: In qualitative analysis, the concentration of the analyte that is either statistically lower than the level of concern (for limit tests) or at which positive identification ceases (for confirmation of identity methods). See also *Cut-off Concentration*.

Trueness: The degree of agreement of the mean value from a series of measurements with the true value or accepted reference value. This is related to systematic error (bias).

Uncertainty: Non-negative parameter characterizing the dispersion of the values being attributed to the measured value.

APPENDIX 2 – Examples of Acceptability Criteria for Certain Performance Characteristics

Examples of acceptability criteria are found in references 7,9,10,14 and 18, Table A below summarizes what is included in references 7 (AOAC) and 10 (CODEX). No single set of acceptability is going to be truly applicable to all methodology covered in the FVM program. For example, a single analyte method, particularly an isotope dilution method, is expected to have better recoveries than a multi-analyte method. However, a good starting point for many methods is found in Table A below and in the Codex Alimentarius Commission, Procedural Manual, Twenty-second ed., 2014 [10]

A. Quantitative Method Acceptability Criteria

Table A2.1. Method Criteria for Method Levels at Increasing Orders of Magnitude
(reproduced in part from reference 10, Table 4, p. 72 and reference 7)

ML* unit	0.001 mg/kg	0.01 mg/kg	0.1 mg/kg	1 mg/kg	10 mg/kg	100 mg/kg	1 g/kg	10 g/kg
Alternative ML* unit	1 ppb	10 ppb	100 ppb	1 ppm	10 ppm	100 ppm	0.1%	1 %
Concentration ratio of ML (C_{ML})	10^{-9}	10^{-8}	10^{-7}	10^{-6}	10^{-5}	10^{-4}	10^{-3}	10^{-2}
Minimum applicable range	From 0.0006 to 0.0014 mg/kg	From 0.006 to 0.014 mg/kg	From 0.03 to 0.17 mg/kg	From 0.52 to 1.48 mg/kg	From 6.6 to 13.3 mg/kg	From 76 to 124 mg/kg	From 0.83 to 1.2 g/kg	From 8.8 to 11 g/kg
LOD (≤ mg/kg)	0.0002	0.002	0.01	0.1	1	10	100	1000
LOQ (≤ mg/kg)	0.0004	0.004	0.02	0.2	2	20	200	2000
RSD_r**	22%	22%	11%	8%	6%	4%	3%	2%
$PRSD_R$#	22%	22%	22%	16%	11%	8%	6%	4%
RSD_R##	≤ 44%	≤ 44%	≤ 44%	≤ 32%	≤ 22%	≤ 16%	≤ 12%	≤ 8%
Recovery	40%-120%	60%-115%	80%-110%	80%-110%	80% - 110%	90% - 107%	95% – 105%	97%-103%

* ML is a method level and can be defined for the analyte(s)/sample matrix(ces) combination as a maximum level, minimum level, normative level or concentration range depending on the intended use of the method.

[**]The RSD_r or Repeatability Precision refers to the degree of agreement of results when conditions are maintained as constant as possible within a short period of time (e.g., relative standard deviation of replicates or best precision exhibited by a single laboratory). Typically, acceptable values for RSD_r are between ½ and 2 times the value shown (Horwitz Ratio ($HorRat_r$) = RSD_r (found, %)/ RSD_r (calculated, %)). For concentration ratios ≥ 10^{-7} Horwitz theory is applied [10]. For concentration ratios < 10^{-7}, Thompson theory is applied [10].
[#]The $PRSD_R$ or Predicted Relative Reproducibility Standard Deviation is based on the Horwitz/Thompson equation. For concentration ratios < 10^{-7}, Thompson theory is applied [10].
[##] The RSD_R or Reproducibility Precision refers to the degree of agreement of results when operating conditions are as different as possible (*e.g.*, same test samples in different laboratories) and should be calculated from the Horwitz/Thompson equation. When the Horwitz/Thompson equation is not applicable (for an analytical purpose or according to a regulation) or when "converting" methods into criteria then it should be based on the RSD_R from an appropriate method performance study. The ratio between the found and predicted value should be ≤ 2. ($HorRat_R$ = RSD_R / $PRSD_R$ ≤ 2)

B. Qualitative Method Acceptability Criteria

Example statistical approach to confirm false negative (FN) and false positive (FP) rates as <5%

Zero acceptance number sampling is a statistical approach commonly used to test a hypothesis (or criteria) for the frequency of defective items in a population (e.g., such as FN or FP rates with repeated testing). For this approach, all tested samples must have the correct response in order to accept the hypothesis (i.e., accept only when zero "defective" responses observed). The minimum number of samples that must be tested depends on the criteria for the defect rate and the level of statistical confidence:

$$n = \log(\alpha) \Big/ \log(1 - p)$$

where 1-α is the confidence level and *p* is the maximum acceptable defect rate per sample (e.g., FN or FP rate). Sample sizes to assess selected criteria for FN or FP rates with varying levels of confidence are provided in the following table.

Table A2.3. Samples required to determine false positive/negative rates

	Confidence Level			
FN or FP rate	80%	90%	95%	99%
<1%	161	230	299	459
<2%	80	114	149	228
<5%	***32***	***45***	***59***	***90***
<10%	16	22	29	44

For example, if the goal is to have 95% confidence that the FN rate is <5% then test 59 samples with the analyte present at the concentration of interest, typically the LOD or a relevant level of concern, in a range

of matrices. The criteria are satisfied if all 59 test results are positive for the target.

This sample size formula is related to the Clopper-Pearson confidence interval for Binomial proportions and frequently used for zero defect acceptance sampling plans for commodity lots. The rationale for the sample size is that when the probability of a defective (incorrect) test response is p for each sample then $(1 - p)^n$ is the probability that n samples will have the correct response. The minimum sample size required for a specified level of confidence follows from setting the probability of that outcome equal to the type I error rate α and solving for n.

APPENDIX 3 - Examples of Validation Plans

A. Extension to other matrices with the same analyte(s) at Level One Validation

This scheme represents an emergency use method extension plan for Matrix Y and Analyte Z. This plan utilizes two different sources of matrix. *In cases where a representative matrix is being used to characterize a whole family of commodities, it is recommended that additional, different commodities from that family are used as "sources".* Note that this plan is for emergency use only – the new matrix (or matrices) cannot be officially included in the scope of the method until at the minimum a Level Two Validation is performed.

Table A3.1. Plan for Matrix Extension (Level One Validation, Example)

	Matrix	Samples 1 & 2	Analyte Z Fortified Samples 3 & 4	Analyte Z Fortified Samples 5 & 6	Analyte Z Fortified Samples 7 & 8
Day 1	Matrix Y (Source 1)	Blank	½X Spike Level	X Spike Level	2X Spike Level
Day 1	Matrix Y (Source 2)	Blank	½X Spike Level	X Spike Level	2X Spike Level

Notes:
i. Test portion matrices listed as Matrix Y represent 2 different commercial brands.
ii. Fortification levels: fortification will be at the level of concern or action level (X) as stated in the method and at levels corresponding to 1/2X and 2X.
iii. Fortification of each matrix can be done on the same day.
iv. Other fortification plans meeting requirements specified in Table 1 may be used.

B. Extension to similar analytes in the same matrix at Level Two Validation

A validated method can be extended to other potential analyte(s) belonging to the same chemical group. For example, a toxin method can be extended to other toxins. An example of the composition of a set of validation studies for method extension is shown in the following table for new analytes Y and Z in canned corn from 3 different sources where the method is validated originally for analyte A in corn.

Table A3.2. Plan for Extension to Similar Analytes (Level Two Validation, Example)

	Matrix	Analyte Y fortification levels	Analyte Z fortification levels
Day 1	Corn 1,2,3	0, 1/2X, X, 2X	0, 1/2X, X, 2X
Day 2	Corn 1,2,3	0, 1/2X, X, 2X	0, 1/2X, X, 2X
Day 3	Corn 1,2,3	0, 1/2X, X, 2X	0, 1/2X, X, 2X

Notes:
i. Three different commercial brands of same product will be analyzed.
ii. Fortification levels: fortification will be at the level of concern or action level (X) as stated in the method and at levels corresponding to 1/2X and 2X.
iii. Each analyte will be analyzed in blank matrix and in duplicate at 1/2X, X and 2X fortification levels.
iv. Simultaneous analysis of the analytes can be undertaken if warranted.
v. Other fortification plans meeting requirements specified in Table 1 may be used.

C. Validation at Level Two for single matrix and single analyte

This plan utilizes 3 different commercial brands of one matrix. The single matrix is being validated for a single analyte.

Table A3.3. Plan for Single Matrix and Single Analyte Level Two Validation (Example)

	Matrix 1 Source 1	Matrix 1 Source 2	Matrix 1 Source 3
Day 1	Blank Fortified (X)	Fortified (X) Fortified (2X)	Blank Fortified (1/2X)
Day 2	Fortified (2X) Fortified (1/2X)	Blank Fortified (1/2X)	Blank Fortified (2X)
Day 3	Fortified (1/2X) Fortified (X)	Fortified (2X) Blank	Fortified (X) Fortified (X)
Day 4	Fortified (2X) Blank	Fortified (X) Fortified (1/2X)	Fortified (2X) Fortified (1/2X)

Notes:
i Sample matrix, represents one matrix from 3 different sources of matrix.
ii Fortification levels: fortification will be at the level of concern or action level (X) as stated in the method and at levels corresponding to 1/2X and 2X.
iii Each of 3 different sources of matrix will be analyzed 8 times (replicate analyses) over the course of experiment, two times unfortified, two times fortified at each level.
iv. The validation in this example will take place over a period of 4 days. It is acceptable to complete the validation in a single day.
v. Other fortification plans meeting requirements specified in Table 1 may be used.

APPENDIX 4 – Selection of Representative Matrices

Two tools that can aid in selection of representative matrices and CRMs when designing a validation protocol for a method intended to have applicability to a broad scope of products are shown below. Food composition varies greatly, and the performance of some methods is more impacted than others by differences in matrix composition, making the validation of methods intended for a wide variety of foods a difficult balance between available resources and sufficient validation with a variety of food types.

A. Commodity groups and representative commodities

Table A4.1. Vegetable and Fruits, Cereals and Food of Animal Origin (reproduced in part from reference 14)

Commodity groups	Typical commodity categories	Typical representative commodities
1. High water content	Pome fruit	Apples, pears
	Stone fruit	Apricots, cherries, peaches
	Other fruit	Bananas
	Alliums	Onions, leeks
	Fruiting vegetables/cucurbits	Tomatoes, peppers, cucumber, melon
	Brassica vegetables	Cauliflower, Brussels sprouts, cabbage, broccoli
	Leafy vegetables and fresh herbs	Lettuce, spinach, basil
	Stem and stalk vegetables	Celery, asparagus
	Fresh legume vegetables	Fresh peas with pods, peas, mange tout, broad beans, runner beans, French beans
	Fresh Fungi	Champignons, canterelles
	Root and tuber vegetables or feed	Sugar beet and fodder beet roots, carrots, potatoes, sweet potatoes
2. High acid content and high water content	Citrus fruit	Lemons, mandarins, tangerines, oranges
	Small fruit and berries	Strawberry, blueberry, raspberry, black currant, red currant, white currant, grapes

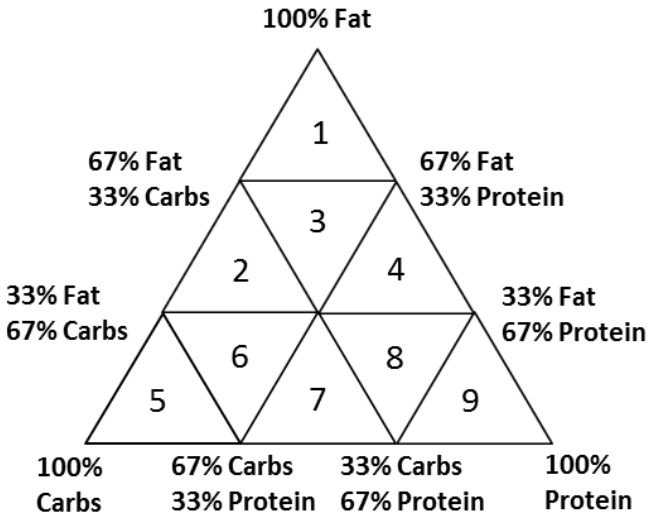
Table A4.1. Vegetable and Fruits, Cereals and Food of Animal Origin (continued)

Commodity groups	Typical commodity categories	Typical representative commodities
3. High sugar and low water content	Honey, dried fruit	Honey, raisins, dried apricots, dried plums, fruit jams
4a. High oil content and very low water content	Tree nuts	Walnuts, hazelnuts, chestnuts
	Oil seeds	Oilseed rape, sunflower, cotton-seed, soybeans, peanuts, sesame, etc.
	Pastes of tree nuts and oil seeds	Peanut butter, tahini, hazelnut paste
4b. High oil content and intermediate water content	Oily fruits and products	Olives, avocados and pastes thereof
5. High starch and/or protein content and low water and fat content	Dry legume vegetables/pulses	Field beans, dried broad beans, dried haricot beans (yellow, white/navy, brown, speckled), lentils
	Cereal grain and products thereof	Wheat, rye, barley and oat grain; maize, rice, whole meal bread, white bread, crackers, breakfast cereals, pasta, flour.
6. “Difficult or unique commodities”		Hops, cocoa beans and products thereof, Coffee, tea, spices
7. Meat (muscle) and Seafood	Red muscle	Beef, pork, lamb, game, horse
	White muscle	Chicken, duck, turkey
	Offal	Liver, kidney
	Fish	Cod, haddock, salmon, trout
8. Milk and milk products	Milk	Cow, goat and buffalo milk
	Cheese	Cow and goat cheese
	Dairy products	Yogurt, cream
9. Eggs	Eggs	Chicken, duck, quail, and goose eggs
10. Fat from food of animal origin	Fat from meat	Kidney fat, lard
	Milk fat	Butter

B. AOAC Food Matrix Triangle

The AOAC Food Matrix Triangle (Figure A4.1) can be used to categorize foods and food matrix reference materials into nine sectors based on relative fat, protein and carbohydrate content [9, 19, 20]. This tool can be useful in the validation of methods intended for a wide variety of food matrices and to help in categorizing similar food matrices for methods intended for more limited applicability.

Figure A4.1. Foods Partitioned into Sectors Based on Their Protein, Fat, and Carbohydrate Content



APPENDIX 5 – Verifications and Extensions of Existing Methods

Method Verification [24]: Method verification is a demonstration that a laboratory can properly perform a standard method that has been previously validated elsewhere. Verification of a quantitative or qualitative method that has undergone Multi-Laboratory Validation (Level III or Level IV) through the established food and feed program process, as well as compendial methods that have undergone MLVs that meet or exceed the requirements set in the Chemical Methods Validation Guidelines, requires analysis of spikes at two concentration levels, each extracted and run in triplicate, along with a matrix blank (when available) and a method blank. A single matrix can be selected even if the original method is applicable to multiple matrices. The selected spiking concentrations (reference materials should be used, if available) should ensure that the method meets the requirements of the particular Program Area and consider any relevant regulatory limits/action levels (e.g. spiking at 0.5x any applicable limit). These spikes should be run prior to the analysis of regulatory samples. For some analytes, spiking with pure standard alone does not sufficiently demonstrate method performance (e.g., BPA in can coatings contain oligomeric interferences; gluten in fermented/hydrolyzed products; protein-bound veterinary drug metabolites do not perform the same as unbound analytes, elemental analysis of matrices resistant to digestion). In these cases, reference materials and/or real samples should be analyzed, in place of spikes, to demonstrate method performance. Method performance results should be

approved by the supervisor, local QA manager (if applicable), and Laboratory Director, and shared with the ORA/ORS Program Coordinator. Results should also be shared with the MLV organizer/ORA/Center Subject Matter Expert (SME) (when possible), and the appropriate TAG for review if/when additional evaluation is needed.

Verification of a quantitative or qualitative method that has NOT undergone Multi-Laboratory Validation (Level III or Level IV) through the established food and feed program process requires Level II SLV unless the method is intended for one time or emergency use, in which case analyzing two matrix spike levels (each in triplicate) along with a matrix blank and a method blank is acceptable. Verifications should be performed prior to the analysis of regulatory samples. Spiking requirements are summarized in Table 1.

Validation Level of the Original Method (Quantitative or Qualitative)	Minimum Requirements for Verification	Notes
Level II SLV: to be used routinely/long term by adopting laboratory	Level II SLV	Must be run prior to the analysis of regulatory samples
Level II SLV: for one time/short term/emergency use by adopting laboratory	Two matrix spike levels, run in triplicate, along with a matrix blank and a method blank	Must be run prior to the analysis of regulatory samples
Level III MLV or Level IV Collaborative Study or equivalent compendial method	Two matrix spike levels, run in triplicate, along with a matrix blank and a method blank	Must be run prior to the analysis of regulatory samples

Table 1: Guidance for Method Verifications

Matrix Extensions: It is critical l to note that it is impossible to provide exhaustive guidance on when a matrix extension is required. For example, Elemental Analysis Manual Method 4.10 was validated for grape, pear, and apple juice, but required modification to perform acceptably for pomegranate, cherry, and prune juice. The perspective of analysts with subject matter expertise and close monitoring of QA/QC data are necessary to ensure differences in method performance in different matrices are identified, and that the method is fit for use.

The identification and classification of a new matrix is dependent on the programmatic area. Pesticides should refer to Appendix 4, Table 1 of the Guidelines for the Validation of Chemical Methods for the FDA FVM Program. For DNA identification methods, consult the Guidelines for the Validation of Analytical Methods for Nucleic Acid Sequence-Based Analysis of Food, Feed, and Cosmetics. Other program areas should contact the ORA/ORS program

contact, the ORA/Center SME, and the ORA/ORS Office of Research Coordination and Evaluation (ORCE) for guidance. All spiking concentrations used should ensure that the method meets the requirements of the particular Program Area and consider any relevant regulatory limits/action levels. The results should be shared with the local QA manager and the ORA/ORS program contact.

If the original method uses isotopically labeled internal standards for each analyte of interest or matrix matched calibration curves, a spike (in duplicate) along with a matrix blank analyzed concurrently with a regulatory sample is sufficient to demonstrate suitable performance (see Table 2). For other methods, spikes at two concentrations, each analyzed in duplicate, along with a matrix blank should be performed. This can be performed concurrently with or prior to the analysis of regulatory samples. All recoveries should be within the range of those reported for the matrices in the original validation, and consistent with Appendix 2A of the Guidelines for the Validation of Chemical Methods for the FDA FVM Program. The results should be shared with the lab supervisor, the local QA manager, and the ORA/ORS program contact. Once completed, the matrix can now be analyzed by other regulatory labs using the same harmonized method without further validation.

Technique used in the Original Method	Minimum Requirements for Matrix Extensions	Notes
Methods using isotopically labeled internal standards or matrix matched calibration curves	Spike run in duplicate, along with a matrix blank (if available).	Can be run prior to or concurrent with regulatory samples
All other methods	Two matrix spike levels, run in duplicate, along with a matrix blank (if available)	Can be run prior to or concurrent with regulatory samples

Table 2: Guidance for Matrix Extensions

Analyte Extensions: For the addition of a new analyte (quantitative or qualitative) to an existing validated method, a Level II SLV should be undertaken for that analyte, as well as determinations of LOD, LOQ, and linearity. In cases where the method performance for existing analytes may be impacted (e.g. optical methods, analytes with similar chromatographic retention, isobaric target analytes in mass spectrometry, duty cycle issues with mass spectrometry, multiplexed antibody-based methods), the validation must ensure standards continue to perform acceptably for those existing analytes. This must include confirming the absence of interferences and maintenance of linearity and sensitivity for all existing analytes. In the case of mass spectrometric methods, a sufficient number of data points (>10) must be maintained for quantitation of all analytes. All recoveries should be

consistent with Appendix 2A of the Guidelines for the Validation of Chemical Methods for the FDA FVM Program. The results should be shared with the lab supervisor, the local QA manager, and the ORA/ORS program contact. Assuming methods are harmonized between multiple laboratories, this will only need to be performed in one laboratory. Once completed, the analyte can now be analyzed by other regulatory labs using the same harmonized method following a verification.

Platform Extensions: When switching to a new platform that applies the same technique as used in the initially validated quantitative or qualitative method (e.g. an Agilent LC-QQQ to an AB SCIEX LC-QQQ), provided the remainder of the original method is unchanged, a full validation is unnecessary. However, analysts should evaluate the LOD/LOQ to ensure they are still suitable and determine that the original calibration curve fit and linear dynamic range have not changed (e.g., the curve has not become a quadratic/reached saturation). The results should be shared with the lab supervisor, the local QA manager, and the ORA/ORS program contact.

When switching a method to a new instrument (e.g., LC-MS/MS to LC-Q Exactive, ICP-Q to ICP-QQQ, GC-MS to GC-MS/MS), a Level II SLV should be performed for all target analytes (performed by one lab, verified by additional labs). This can be performed via the determination of spike recoveries, or by standard addition to extracted matrices, provided the number of samples meets or exceeds the requirements of a Level II SLV. Analysts should also evaluate the LOD/LOQ and linear dynamic range for all analytes to ensure they still meet the needs of the particular Program. The results should be shared with the lab supervisor, the local QA manager, and the ORA/ORS program contact. Once completed, the new platform can now be used by other regulatory labs using the same harmonized method following a verification.

APPENDIX 6 – Acceptable Modifications to Mass Spectrometry Methods

Modification Guidelines for Chromatography - Mass Spectrometry Methods

1.0: Scope

This document establishes guidance on the acceptable instrumental modifications to liquid chromatography- and gas chromatography-mass spectrometry methods for determination of chemical analytes in food, feed and cosmetics.

2.0 Introduction/Applicability

Multi-laboratory validation (MLV) studies are performed to ensure that the methodology will accurately measure, within an acceptable precision, the target analyte(s) in matrices defined in the method scope. Subsequent use of a validated method for regulatory testing, requires that the analyst perform the analysis, (sample preparation and instrumental analysis) as described in the validated method. Therefore, when utilizing a validated method, modification to the method procedure should not be made except in cases of critical necessity. The guidelines presented herein address changes to the analytical conditions (e.g., chromatographic separation and/or mass spectrometric detection) and method parameters that are acceptable within the boundaries

of a validated method. In situations where a change to the validated method falls outside the scope of the original method or the ranges described herein, additional testing is required. Depending on the change, these additional tests may either extend the original scope of the method or require the identification of an entirely new method.

These guidelines are applicable to methods being performed on the same analytes, matrices/matrix type and the same mass spectrometer (make and model) as described in the MLV. These guidelines **do not** address extension of methods to new instrument platforms (i.e., different make or model), extension to new analytes, extension to new matrices/matrix types or changes to the sample preparation procedures.

If a validated method specifically addresses any of the modifications listed in these guidelines, then the statements in the method supersede these general guidelines.

2.1 Applicability to Collaborative Studies

Participation in a multi-laboratory collaborative study involves strict adherence to the developed method procedure. Modifications from the collaborative study protocol should be avoided except in cases of critical necessity. When a modification is required, even if within the limits listed in these guidelines, it should be reviewed and agreed to by the study director and must be documented in the final validation report for review by the CMVS. If a modification falls outside the limits listed in these guidelines, additional testing (e.g., additional lab) or the removal of the data from the collaborative study may be required based on the review and recommendations made by the CMVS and/or CRCG.

During the running of an MLV, deviations (i.e., unplanned modifications) can occur. These guidelines can be used by the study director and the CMVS to determine the impact of each deviation. If the deviation falls within the limits listed in these guidelines, additional testing should not be required. However, the deviation must still be captured in the final validation report. If the deviation falls outside of the limits listed in these guidelines, additional testing (e.g., additional lab) or the removal of the data from the collaborative study may be required based on the review and recommendations made by the CMVS and/or the CRCG.

3.0 Liquid Chromatography-Mass Spectrometry Modifications

3.1 Acceptable Modifications

The following modifications represent minor changes to a method that may be made if critically required. All modifications should be shared with the local QA manager and reported through the appropriate TAG. The modifications listed below do not need to be captured as part of the scope of the compendial method.

A. **Liquid Chromatography Column**: The specific column(s) (manufacturer, bonded phase, particle size, particle type, pore size) identified in the multi-laboratory validated method should be used when running the method. The column dimensions (either length or diameter, not both) can be altered if subsequent changes are made to the flow rate to achieve the same separation reported in the validated method (relative retention time and chromatographic resolution ±20%). If comparable chromatography cannot be achieved

with the new column/flow rate, further testing is required before the column can be used (Section 3.2A).

B. Mobile Phase Modifiers: Small changes in the concentration of mobile phase modifiers (e.g., salts) (±10 %) and pH (± 0.2 units), except ion pair reagents, are acceptable and should be within the robustness of a well-developed method. This applies to both gradient and isocratic separations. Chromatographic separation should be maintained (relative retention time and chromatographic resolution ±20 %).

C. Injection Volume: Given possible differences in the performance characteristics between instruments or in response to changes made to the column dimensions and flow rates (Section 3.1A), it may be necessary to change the sample injection volume. The change should not impact peak symmetry, resolution, and method sensitivity (no statistical difference at 95% CL). The analyst must verify that the calibration range is maintained (no statistically significant variation) or improved.

D. Reproduction of HPLC methods UHPLC instrumentation: Methods developed and validated with HPLC hardware may be reproduced using modern UHPLC equipment, as long as the original column is utilized in the UHPLC system.

E. Source Conditions: Instruments of the same series from the same vendor (i.e., Sciex 6500 QTRAP) may have performance differences which would require different source conditions (e.g., temperature, gas flow rate) to be used to meet the same performance specifications. Generally, these differences are small and therefore any changes to the source conditions should be minor adjustments to temperature, voltages and/or gas flows. Additionally, analysts should avoid making multiple changes that could have detrimental additive effects, such as reducing both temperature and gas flow. The new source conditions should maintain (or improve) the calibration range. If any loss (statistically significant) in the calibration range is detected then matrix extracts (n ≥ 2 replicates per validated matrix) at critical concentrations (e.g., Level of Concern, LOQ) should be evaluated to establish that under the new conditions the analytes can be accurately quantitated at these critical concentrations. Any changes to the conditions should be recorded for use by other analysts on the system.

F. Collision Cell: A change to the collision gas and/or the collision energy is/are allowable and may be necessary. The impact of the new collision cell conditions should be evaluated on calibration standards across the entire calibration range. The new collision cell conditions should maintain (or improve) the calibration range. If any loss (statistically significant) in the calibration range is detected then matrix extracts (n ≥ 2 replicates per validated matrix) at critical concentrations (e.g., Level of Concern, LOQ) should be evaluated to establish that under the new conditions the analytes can be accurately quantitated at these critical concentrations. Any changes to the conditions should be recorded for use by other analysts on the system.

G. Mass Spectra Ion Monitoring Window: The time and width of ion monitoring window may be adjusted to account for changes in the chromatographic elution profile. The number of concurrent transitions should not be changed.

H. Number of Analytes Monitored: The number of analytes monitored may be reduced during a confirmatory run, check analysis, or to perform the analysis on a smaller number of target analytes. To increase the number of analytes an Analyte Extension Study as described in *Appendix 5 Verifications and Extensions of Existing Methods* of the *Guidelines for the Validation of Chemical Methods for the FDA FVM Program, 3rd Ed.* should be performed.

3.2 Liquid Chromatography-Mass Spectrometry Modifications that Require Specific Extension Study or Verification Study

A. Liquid Chromatography Column: The column used in the original MLV should be used when performing the method. If a critical need arises, such as discontinuation of the analytical column used in the validation, or unavailability due to supply issues, it may be necessary to use a different column. The new column must use the same bonded phase chemistry (e.g., C18), particle type (e.g., solid-core or porous), similar particle and pore size (± 30%) and provide the same elution order. The separation performance of the potential column should be compared to the reference separation reported in the validated method, choosing the column that best reproduces the reference separation. The method modification and associated verification or validation data, as described below, must be collected, reviewed by the CRCG and added as an addendum to the MLV or documented in the method compendium as an extension to the scope of the method. If a new column produces a different separation (i.e., elution order) or does not meet these requirements, then it is deemed a different method and the column cannot be added as an addendum to the original method. The studies below also apply to columns of the same characteristics but different dimensions, where the separation of the original method cannot be duplicated (Section 3.1A).

1. Single Analyte Method or Multi-analyte method without stable isotope or non-coeluting* stable isotope internal standard: A method verification study as described in *Appendix 5 Verifications and Extensions of Existing Methods* of the *Guidelines for the Validation of Chemical Methods for the FDA FVM Program, 3rd Ed.* should be performed on the new column.

2. Single Analyte Method or Multi-analyte method Multi-analyte with coeluting stable isotope internal standard: A platform extension (new platform) study as described *Appendix 5 Verifications and Extensions of Existing Methods* of the *Guidelines for the Validation of Chemical Methods for the FDA FVM Program, 3rd Ed.* should be performed on the new column.

* This is not intended to be a comparison to the original method, therefore even if the stable isotope internal standard does not coelute in the original method the studies described should be performed.

3.3 Liquid Chromatography-Mass Spectrometry Modifications that Require Further Validation (i.e., create a new method)

The following characteristics of the method cannot be changed. Any change would represent a new method, which should undergo the appropriate validation according to Section 2.5 of the Guidelines for the Validation of Chemical Methods for the FDA FVM Program, 2nd Ed. prior to

use.

a. Column Characteristics (e.g., separation mode [reverse phase to normal phase], particle type)
b. Column Setpoint Temperature
c. Data Collection Mode (e.g., Full Scan, MRM, DDA): There may be changes to data collection modes that would not constitute a new method. Requests to allow a change should be submitted for review by the CRCG.
d. Ion Pair Reagent Composition
e. Ionization Polarity
f. Ion Selection of precursor and product ions: For confirmatory analysis the use of additional structurally significant products ions is allowable, provided they are compared to a standard analyzed at the time of use and do not reduce the dwell times of the quantifying and qualifying ions listed in the method.
g. Ionization Source (e.g., ESI, APCI)
h. Mass Resolution
i. Mobile Phase (composition and gradient, except for changes related to Section 3.1A)

4.0 Gas Chromatography-Mass Spectrometry Modifications

4.1 Acceptable Modifications

The following modifications represent minor changes to a method that may be made if critically required. All modifications should be shared with the local QA manager and reported through the appropriate TAG. The modifications listed below do not need to be captured as part of the scope of the compendial method.

A. Gas Chromatography Column: The specific column used in the initial method development and validation is the preferred column option. Columns of the same dimensions and stationary phase (e.g., chemistry, thickness) but from different vendors can be used in place of the original column. The separation characteristics should be evaluated to ensure they remain consistent with the original method (relative retention time and chromatographic resolution ±20%). When necessary, it is acceptable to shorten the column during routine maintenance to maintain chromatographic performance. Such changes and the means for assessing chromatographic performance should be captured as part of routine laboratory QA.

B. Injection Volume: Given possible differences in performance characteristics between instruments, it may be necessary to change the injection volume. Any increase should not exceed 2x the validated injection volume and any decrease should not exceed 0.5x the validated injection volume. For split injections, a change to split ratio is permitted but should not exceed 30%. The change should not impact peak symmetry, resolution, and method sensitivity (no statistical difference 95% CL). The analyst must verify that the calibration range is maintained (no statistically significant variation) or improved.

C. Collision Cell: A change to the collision gas and/or the collision energy is/are allowable and may be necessary. The impact of the new collision cell conditions should be evaluated on calibration standards across the entire calibration range. The new collision cell conditions should maintain (or improve) the calibration range. If any loss (statistically significant) in the calibration range is detected then matrix extracts (n≥2 per validated

matrix) at critical concentrations (e.g., Level of Concern, LOQ) should be evaluated to establish that the new conditions can accurately determine the analytes at the relevant concentrations.

D. **Inlet Pressure or Flow Rate or Linear Velocity:** When using a new column, the inlet pressure, flow rate and linear velocity should be set to the values defined in the collaboratively studied method. However, with use, and to maintain method performance, many GC methods may require shortening of the column during routine maintenance. After column maintenance, changes in inlet pressure or flow rate or linear velocity may be required to maintain chromatographic performance. Such changes and the means for assessing chromatographic performance should be captured as part of routine laboratory QA.

4.3 Gas Chromatography-Mass Spectrometry Modifications that Require Further Validation (i.e., create a new method)

The following characteristics of the method cannot be changed. Any change would represent a new method, which should undergo appropriate validation according to Section 2.5 of the Guidelines for the Validation of Chemical Methods for the FDA FVM Program, 2nd Ed. prior to use.

a. Carrier Gas
b. Column Stationary Phase Chemistry (e.g., cyanopropyl to phenyl)
c. Data Collection Mode (e.g., Full Scan, MRM, DDA): There may be changes to data collection modes that would not constitute a new method. Requests to allow a change should be submitted for review by the CRCG.
d. Injection Type
e. Inlet Pressure or Flow Rate or Linear Velocity with new column (See Section 4.2D)
f. Ionization Mode (e.g., EI, CI) CI reagent and purity
g. Ionization Polarity
h. Ion Selection of precursor and product ions (including isolation width): For confirmatory analysis the use of additional structurally significant products ions is allowable, provided they are compared to a standard analyzed at the time of use and do not reduce the dwell times of the quantifying and qualifying ions listed in the method.
i. Mass Spectrometer Resolution
j. Mass Spectrometer Source Conditions
k. Temperature Program

5.0 Further Guidance

It is critical to note that it is impossible to provide comprehensive guidance across all methods, which will ensure that a modification to a method will yield comparable results. Therefore, if the modifications allowed in this document lead to changes in method performance, they should not be implemented as an addendum to the original method and should be communicated to the TAG, and the ORA/ORS Program Coordinator. The perspective of analysts with subject matter expertise, and close monitoring of QA/QC data, is necessary to ensure differences in method performance are adequately assessed, and that the method is fit for use.

All verification or validation results should be shared with the local QA manager and the

ORA/ORS program coordinator. Any SLV, that may constitute a method extension must be submitted to the CRCG for review and consideration as an addendum to the MLV. Any changes initiated due to critical need (e.g., discontinuation of a column) should be reported to QA manager, ORA/ORS program coordinator and the CRCG.

6.0 Acronyms

APCI	Atmospheric Pressure Chemical Ionization
CI	Chemical Ionization
CL	Confidence Level
CMVS	Chemical Methods Validation Subcommittee
CRCG	Chemical Research Coordination Group
DDA	Data Dependent Acquisition
EI	Electron Ionization
ESI	Electrospray Ionization
GC	Gas Chromatography
HPLC	High Performance Liquid Chromatography
LC	Liquid Chromatography
LOQ	Limit of Quantitation
MLV	Multi-Laboratory Validation
MRM	Multiple Reaction Monitoring
ORA/ORS	Office of Regulatory Affairs/Office of Regulatory Science
QA	Quality Assurance
SLV	Single Laboratory Validation
TAG	Technical Advisory Group
UHPLC	Ultra-High Performance Liquid Chromatography