AOAC INTERNATIONAL

Expert Review Panel

Guidelines for Validation of Botanical Identification Methods

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1. Scope

The purpose of this document is to provide comprehensive technical guidance for conducting AOAC INTERNATIONAL (AOAC) validation studies for botanical identification methods submitted for *Performance Tested Methods* (PTM) status and/or for AOAC *Official Methods of Analysis* (OMA) status. The requirements for single laboratory validation studies, independent validation studies, and collaborative validation studies for those methods are described.

2. Applicability

These guidelines are intended to be applicable to the validation of all candidate botanical identification methods (Appendix A) are submitted to AOAC for (1) a Collaborative Study or an Alternative Pathway (AP) Study to achieve OMA status or for (2) a PTM certification.

3. Terms and Definitions

3.1 Botanical: Of, or relating to, plants or botany. May also include algae and fungi. May refer to the whole plant, a part of the plant (e.g. bark, woods, leaves, stems, roots, rhizomes, flowers, fruits, seeds, extracts, etc.), or an extract of the plant.

3.2 Botanical identification method (BIM): A method that establishes identity specifications for a botanical material and allows determination, within a specified statistical limit, that a test material is a true example of the target botanical material and meets the identity specifications. Thus, a method answers the question, "*Is the test material the same as the target material?*" not "*What is this material?*". In most cases, the method will achieve this goal by comparison of the test material with botanical material(s) from the inclusivity panel and will return a yes/no (or, in some cases, a consistent/non-consistent) answer.

3.3 Candidate method: The method to be validated or submitted for validation (Appendix A).

3.4 Exclusivity: Ability of a BIM to correctly reject non-target botanical materials.

3.5 Exclusivity sampling frame: A list of practically obtainable non-target botanical materials that have similar taxonomic, physical, or chemical composition characteristics to the target botanical that must give a negative result when tested by the BIM.

3.6 Exclusivity panel: A subset of the exclusivity sample frame that is selected for the validation study. These materials should be authenticated by an appropriate method.

3.7 Identity specification (IS): The morphological, genetic, chemical or other characteristics that define a target botanical material. Specifications may include, but are not limited to, data from macroscopic, microscopic, genetic (e.g., DNA sequencing), chromatographic fingerprinting (e.g., CE, GC, LC, TLC), and spectral fingerprinting (e.g., IR, NIR, NMR, MS, UV/Vis) methods.

3.8 Inclusivity: Ability of a BIM to correctly identify variants of the target material that meet the identity specification.

3.9 Inclusivity sampling frame: A list of practically obtainable botanical materials that are expected to give a positive result when tested by the BIM. The inclusivity frame should be sufficiently large that the botanical variation is adequately represented. Sources of variation may include, but are not limited to, species, sub-species, cultivar, growing location, growing conditions, growing season, and post-harvest processing.

3.10 Inclusivity panel: A subset of the inclusivity sample frame that is selected for the validation study. These materials should be authenticated by an appropriate method.

3.11 Laboratory sample: Sample as prepared for sending to the laboratory intended for inspection or testing.

3.12 Non-target botanical material: Any botanical material that does not meet the identity specification.

3.13 Physical form: Botanical materials exist in a number of physical forms. The form(s) will be specified by the SMPRs.

3.14 Probability of identification (POI): The expected or observed fraction of test portions at a given concentration that give a positive result when tested by the BIM. A general description is provided in Appendix B)

3.15 Sample: A small portion or quantity, taken from a population or lot that is ideally a representative selection of the whole. Sample homogeneity is usually determined with multiple samples.

3.16 Specified inferior test material (SITM): A botanical material mixture that has the maximum concentration of target material that is considered unacceptable, as specified by the SMPRs. The BIM must reject this material with a specified minimum level of (1 – POI) with 95% confidence. The ideal BIM would reject the SITM 100% of the time (i.e., identify 0% of the time). The SITM will typically be high quality target material mixed with the worst-case (for identification) non-target material.

3.17 Specified superior test material (SSTM): A botanical material mixture that has the minimum acceptable concentration of the target material, as specified by the SMPR. The

BIM must identify this material with a specified minimum level of POI with 95% confidence. The ideal BIM would identify the SSTM 100% of the time. The SSTM will typically be high quality target material mixed with a small amount of worst-case (for identification) non-target material.

3.18 Standard method performance requirements (SMPRs): Performance requirements based on the fitness for purpose statement for each method. For BIMs, the SMPRs should include the physical form of the sample, the ISF, the ESF, the SSTM, the SITM, the number of samples for the inclusivity/exclusivity panels, and the desired probability and confidence limits for the method.

3.19 Target botanical material: The botanical material of interest as described in the identity specification.

3.20 Test portion: The portion of the laboratory sample that is subjected to analysis by the method.

4 Validation Study Guidelines

A validated BIM requires a method validation study that demonstrates its acceptability according to the SMPRs. The guidelines presented here are intended to be applied to any qualitative BIM that returns a single, binary result (Appendix A). The guidelines provide technical guidance in validating the method based on the POI model (Appendix B).

4.1 Standard Method Performance Requirements

The SMPRs will be prepared by the appropriate AOAC body as per AOAC policy. The SMPRs will specify (a) the target botanical material, (b) the physical form of the material, (c) a list of botanical materials for the inclusivity/exclusivity frames,(d) composition of the SSTM and SITM, (e) maximum POI for the SITM and minimum POI for the SSTM, and (f) the desired probability and confidence limits for the inclusivity/exclusivity and SSTM/SITM measurements.

The SMPRs will consider the nature of the material being tested and determine the necessary breadth and depth of the inclusivity and exclusivity panels. In some cases, a few, very similar exclusivity panel materials may require in depth testing (more test portions of a smaller group of materials). Conversely, the nature of the material may require greater breadth (fewer test portions of a greater number of materials).

The number of test portions needed should be determined on sound statistical grounds and subject matter expertise.

4.2 Single Laboratory Validation Study

4.2.1 Scope

An SLV study is intended to determine the performance of a candidate method (Appendix A). For validation purposes, the candidate botanical identification method may be regarded as a black box providing a single, yes/no test result. The study is designed to evaluate performance parameters for the candidate method including (1) inclusivity/exclusivity, (2) POI for the specific superior test material (SSTM) and the specific inferior test material (SITM), and (3) POI as a function of the concentration of the target material (analytical response curve). This last parameter may be optional.

4.2.2 Inclusivity/Exclusivity Study

The purpose of this study is to confirm the ability of the candidate method to provide positive results (YES answers) for botanical materials on the inclusivity panel and negative results (NO answers) for materials on the exclusivity panel.

4.2.2.1 Inclusivity/Exclusivity Panel Selection

Botanical materials selected from the inclusivity/exclusivity sampling frames will comprise the inclusivity/exclusivity panels. If the Inclusivity/exclusivity sampling frames are sufficiently large, a representative sub-group will be selected for the panels. Primary requirements for the panel materials are their availability and their authentication by an appropriate method. All test portions should be as uniform and homogeneous as possible. The level of replication of the inclusivity/exclusivity panels will be specified in the SMPRs.

4.2.2.2 Study Design

Prepare the test samples in a form appropriate for the candidate method. All test samples will be blinded and randomized so that the analyst(s) cannot know the identity of the samples. Analyze the test samples following the instructions of the candidate method.

4.2.2.3 Data Analysis and Reporting

The data will be analyzed for positive and negative responses. Unexpected results will be investigated, evaluated, and resolved prior to continuing the validation. The data is reported for individual inclusivity/exclusivity material as the number correctly identified. For example, “Of the 30 specific inclusivity botanical materials tested, 28 were correctly identified and 2 were not identified. Those materials not identified correctly were the following:…” or “Of the 30 specific exclusivity botanical materials tested, 27 were identified correctly and 3 were not identified correctly. Those not identified correctly were the following:…” The study report should include a table titled “Inclusivity/Exclusivity Panel Results,” which lists all materials tested, their source, origin, and essential characteristics and testing outcome. The implications of each unexpected result should be discussed and evaluated.

4.2.3 SSTM/SITM Study

The purpose of this study is to demonstrate method performance at two concentrations, the SSTM and the SITM.

4.2.3.1 Test Samples

The appropriate amount of a target material is selected from the inclusivity panel and is mixed with an appropriate amount of a non-target material from the exclusivity panel to produce the SSTM and SITM as specified by the SMPRs. The test materials may be prepared using a mixture of botanical materials from the inclusivity list as the target material and a mixture of materials from the exclusivity list as the non-target material as specified by the SMPRs.

All test portions should be as uniform and homogeneous as possible. The level of replication of the SSTM and SITM will be specified in the SMPR.

4.2.3.2 Study Design

Prepare the test samples in a form appropriate for the candidate method. All test samples will be blinded and randomized so that the analyst(s) cannot know the identity of the samples. Analyze the test samples following the instructions of the candidate method.

4.2.3.3 Data Analysis and Reporting

The data will be analyzed for positive and negative responses. For the SSTM and the SITM, report the POI results with 95% confidence intervals and the total number tested and the total number correctly identified. Comparison to SMPRs should be made and discussed.

4.2.4 Analytical Response Curve

This study will characterize the POI curve for mixtures of SSTM and SITM.

4.2.4.1 Test Samples

The appropriate amount of a target material is selected from the inclusivity panel and is mixed with an appropriate amount of a non-target material from the exclusivity panel to produce the mixtures with concentrations intermediate between the SSTM and SITM. The test materials shall be prepared using the same target and non-target botanical material samples used in the SSTM and SITM study.

4.2.4.1 Study Design

Prepare the test samples in a form appropriate for the candidate method. All test samples will be blinded and randomized so that the analyst(s) cannot know the identity of the samples. Analyze the test samples following the instructions of the candidate method.

4.2.4.2 Data Analysis and Reporting

The data will be analyzed for positive and negative responses. For each mixture, report the POI results with 95% confidence intervals, the total number of samples tested, and the total number of positive responses. Plot the POI curve and confidence intervals.

4.3 Independent Validation Study

This study is identical to the Single Laboratory Validation Study in **Section 4.2**.

4.4 Collaborative Study

The collaborative study is a route to an OMA method. The purpose of the Collaborative Study is to estimate the reproducibility and determine the performance of the candidate method among collaborators.

4.4.1 Number of Collaborators

A minimum of 10 independent laboratories reporting valid data is required. The study director should plan on including additional laboratories in the case of invalid data sets.

4.4.2 Number of Tests

Each collaborator receives 12 replicates of each material to be studied. At a minimum these materials will include the SSTM and SITM. Prepare the test samples in a form appropriate for the candidate method. All test samples will be blinded and randomized so that the analyst(s) cannot know the identity of the samples. Analyze the test samples following the instructions of the candidate method.

4.4.3 Data Analysis and Reporting

The data will be analyzed by laboratory for positive and negative responses. For the SSTM and the SITM, report the POI results with confidence intervals for each laboratory, and for the combined results. Estimate reproducibility as in Appendix XX and evaluate compared to the SMPR.

5 References – To be added

Appendix A - Pre-Validation Study

A.1 Scope

The candidate method must measure appropriate characteristics that are suitable to the question being asked and that will meet pre-determined SMPRs. The method may be based on new principles or modifications of an existing method. The identity specifications will be based on morphological, genetic, and/or chemical characteristics, or any other defining feature of the botanical material. The candidate method may use visual inspection, DNA sequencing, instrumental analysis, or any other appropriate measurement. The measured characteristics will collectively provide a single analytical parameter that will be used to determine the final yes or no result. The analytical parameter may be based on the degree of similarity or the degree of difference of the test sample and the reference material. Thus the AP may either increase of decrease with similarity.

A.2 Inclusivity/Exclusivity Panel Selection

The method developer will select representative botanical materials from the inclusivity and exclusivity sample frames for use as target and non-target botanical materials, respectively, in development of the method. These materials must be authenticated by an appropriate method.

A.3 Analytical Parameter

The method developer will prepare all the botanical samples in a form appropriate for the candidate method. The developer will analyze the target and non-target botanical materials using the candidate method and develop an analytical parameter that is suitable for distinguishing between the two sets of materials.

A.4 Probability of Identification

Target materials will be mixed with systematically increasing amounts of non-target materials to produce a series of target materials whose concentrations range from 100% to a concentration below the minimum acceptable concentration specified by the SMPRs. The developer will analyze the target and diluted target materials using the candidate method and determine the analytical parameter for each concentration.

A.5 Specific Superior/Inferior Test Materials

Based on the analytical parameters measured for the diluted target materials, a threshold value will be established that will permit positive identification of the minimum acceptable concentration of the target material with the specified confidence (e.g. 95%). The developer will use the threshold to determine a probability of identification (POI) for each concentration (Appendix B). The POIs measured for each concentration will be used to construct the POI curve.

A.6 Data Analysis and Reporting

The method developer will document the candidate method and the POI results.

Appendix B: Understanding the POI Model

(Double click on Abstract to open the PDF file)

Probability of Identification (POI): a Statistical Model for the Validation of Qualitative Botanical Identification Methods

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Abstract

A qualitative botanical identification method (BIM) is an analytical procedure which returns a binary result (1 = Identified, 0 = Not Identified). A BIM may be used by a buyer, manufacturer or regulator to determine whether a botanical material being tested is the same as the target (desired) material or whether it contains excessive non-target (undesirable) material. We describe the development and validation of studies for a BIM based on the idea of a proportion of replicates identified, or probability of identification (POI), as the basic observed statistic. The statistical procedures proposed for data analysis follow closely those of the probability of detection (POD), and harmonize the statistical concepts and parameters between quantitative and qualitative method validation. Use of POI statistics also harmonizes statistical concepts for botanical, microbiological, toxin and other analyte identification methods that produce binary results. The POI statistical model provides a tool for graphical representation of response curves for qualitative methods, reporting of descriptive statistics, and application of performance requirements. Single collaborator and multi-collaborative study examples are given.

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Appendix C: Number of Test Portions

Sample Size Required for Proportion **LOW** ***Version:*** ***1.1***

ASSUME: 1. Binary outcome (occur / not occur).
2. Constant probability rho of event occurring.
3. Independent trials (e.g., simple random sample).
4. Fixed number of trials N.

INFERENCE: 95% confidence interval lies entirely at or BELOW specified maximum rho.
DESIRED: Sample size N needed.
NOTES: 1. Based on modified Wilson score 1-sided confidence interval.
2. AOQL = Average Outgoing Quality Level

Maximum Probability rho	Sample Size N	**Maximum** Number Events x	**Minimum** Number Non-events y	1-sided **Upper** Confidence Limit on rho	Expected Lower Confidence Limit on rho	Expected Upper Confidence Limit on rho	Effective AOQL rho
50%	3	0	3	47.4%	0.0%	56.1%	28.1%
50%	10	2	8	45.9%	5.7%	51.0%	28.3%
50%	20	6	14	48.4%	14.5%	51.9%	33.2%
50%	40	14	26	48.0%	22.1%	50.5%	36.3%
50%	80	32	48	49.2%	30.0%	51.0%	40.5%
45%	2	0	2	57.5%	0.0%	65.8%	32.9%
45%	10	1	9	34.8%	0.0%	40.4%	20.2%
45%	20	5	15	43.2%	11.2%	46.9%	29.0%
45%	40	12	28	42.9%	18.1%	45.4%	31.8%
45%	80	28	52	44.1%	25.5%	45.9%	35.7%
40%	5	0	5	35.1%	0.0%	43.4%	21.7%
40%	10	1	9	34.8%	0.0%	40.4%	20.2%
40%	20	4	16	37.8%	8.1%	41.6%	24.8%
40%	40	10	30	37.6%	14.2%	40.2%	27.2%
40%	80	24	56	39.0%	21.1%	40.8%	30.9%
35%	6	0	6	31.1%	0.0%	39.0%	19.5%
35%	10	1	9	34.8%	0.0%	40.4%	20.2%
35%	20	3	17	32.2%	5.2%	36.0%	20.6%
35%	40	9	31	34.9%	12.3%	37.5%	24.9%
35%	80	21	59	35.0%	17.9%	36.8%	27.3%
30%	7	0	7	27.9%	0.0%	35.4%	17.7%
30%	10	0	10	21.3%	0.0%	27.8%	13.9%
30%	20	2	18	26.2%	2.8%	30.1%	16.4%
30%	40	7	33	29.3%	8.7%	31.9%	20.3%
30%	80	17	63	29.6%	13.7%	31.4%	22.6%
25%	9	0	9	23.1%	0.0%	29.9%	15.0%
25%	10	0	10	21.3%	0.0%	27.8%	13.9%
25%	20	1	19	19.6%	0.0%	23.6%	11.8%
25%	40	5	35	23.5%	5.5%	26.1%	15.8%
25%	80	13	67	24.1%	9.7%	25.8%	17.8%
20%	11	0	11	19.7%	0.0%	25.9%	12.9%
20%	20	1	19	19.6%	0.0%	23.6%	11.8%
20%	24	1	23	16.7%	0.0%	20.2%	10.1%
20%	36	3	33	19.1%	2.9%	21.8%	12.4%
20%	40	3	37	17.3%	2.6%	19.9%	11.2%
20%	48	5	43	19.9%	4.5%	22.2%	13.3%
20%	60	6	54	18.2%	4.7%	20.1%	12.4%
20%	72	8	64	18.7%	5.7%	20.4%	13.1%
20%	80	10	70	19.8%	6.9%	21.5%	14.2%
15%	20	0	20	11.9%	0.0%	16.1%	8.1%
15%	24	0	24	10.1%	0.0%	13.8%	6.9%
15%	36	1	35	11.5%	0.0%	14.2%	7.1%
15%	40	2	38	14.0%	1.4%	16.5%	8.9%
15%	48	3	45	14.6%	2.1%	16.8%	9.5%
15%	60	4	56	14.0%	2.6%	15.9%	9.3%
15%	72	5	67	13.6%	3.0%	15.2%	9.1%
15%	80	6	74	13.9%	3.5%	15.4%	9.4%
10%	40	0	40	6.3%	0.0%	8.8%	4.4%
10%	48	1	47	8.8%	0.0%	10.9%	5.4%
10%	60	2	58	9.6%	0.9%	11.4%	6.1%
10%	72	3	69	10.0%	1.4%	11.5%	6.5%
10%	80	3	77	9.0%	1.3%	10.5%	5.9%
5%	60	0	60	4.3%	0.0%	6.0%	3.0%
5%	72	0	72	3.6%	0.0%	5.1%	2.5%
5%	80	0	80	3.3%	0.0%	4.6%	2.3%
5%	90	1	89	4.8%	0.0%	6.0%	3.0%

Notes:

1. Enter the first column with the maximum error fraction tolerated by the SMPR, e.g., 10%.

2. Select the sample size required by the number of misclassifications to be allowed, e.g., 1 erroneous result gives a sample size of n = 48 for a maximum error probability of 10%.
3. Allowing more erroneous results increases the sample size required.
4. The last (AOQL) column indicates the maximum error probability of a method which passes the SMPR for the test. For the example sampling plan indicated, this is 5.4%, approximately ½ of the maximum error probability in the SMPR. Typically the AOQL must be only 50-60% of the SMPR value to reliably pass the validation test. Method developers should take this into account.