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Method Name: **Standard Method Performance Requirements for Viable Yeast and Mold Count Enumeration in Cannabis and Cannabis Products**

Purpose: AOAC *Standard Method Performance Requirements*[SM] (SMPRs) describe the minimum recommended performance characteristics to be used during the evaluation of a method. The evaluation may be an on-site verification, a single-laboratory validation, or a multi-site collaborative study. SMPRs are written and adopted by AOAC stakeholder panels composed of representatives from industry, regulatory organizations, contract laboratories, test kit manufacturers, and academic institutions. AOAC SMPRs are used by AOAC expert review panels in their evaluation of validation study data for method being considered for *Performance Tested Methods*[SM] or AOAC *Official Methods of Analysis*[SM] and can be used as acceptance criteria for verification at user laboratories.[1]

Approval Body: AOAC Cannabis Analytical Science Program
Approval Date:
Final version date:

1. **Intended Use**: Consensus-based Reference method.

2. **Applicability**: Candidate methods used to detect and quantify viable yeast and mold in cannabis and/or cannabis products. Candidate methods may be validated for specific matrices, categories or broader claims.

   See Table 4 for acceptable matrix/category claims.

3. **Analytical Technique**: Any analytical technique that meets the method performance requirements is acceptable.

4. **Definitions**:

   Candidate Method.— The method submitted for validation[2]

   Candidate Method Presumptive Result.—Preliminary result for a test portion produced by following a candidate method's instructions for use.

   Candidate Method Confirmed Result.—Final result obtained for a test portion after cultural confirmation of a candidate method.

   Cannabis.—genus of flowering plants within the *Cannabinaceae* family that commonly contain 9-tetrahydrocannabinol (THC), cannabidiol (CBD), and other cannabinoids and terpenes. Cannabis includes, but is not limited to, high-THC and high-CBD cultivars.

   Cannabis Concentrates.—Extracts (primarily composed of cannabinoids and/or terpenes) manufactured through the extraction and concentration of compounds derived from the cannabis plant or flower. Final products can be many forms including oils, wax, or hash (Category II).

   Cannabis Infused Edibles.—Food and drinks containing extracts of cannabis and/or cannabis materials (Category III).

Cannabis Infused Non-Edibles.—Products containing extracts of cannabis and/or cannabis materials intended to be applied to the human body or any part thereof. Final products can be many forms including creams, ointments, cosmetics and therapeutic pads (Category IV).

***Cannabis Plant and Flower*.**—General terms for the structural and flowering unadulterated parts of the cannabis plant (Category I).

Cannabis Products.—Products (Edible, and non-edible) extracted or infused with compounds derived from the cannabis plant including but not limited to CBD and THC.

Confidence Interval. —The estimated range in which an obtained result should enclose the actual concentration. For the purpose of this SMPR, a 90% and/or 95% confidence interval is used, depending on the type of method validated.

Probability of detection (POD).—The portion of positive analytical outcomes for a semi-quantitative method for a given matrix at a given analyte level or concentration. This difference in POD values between presumptive and confirmed results is termed $dPOD_{CP}$.

Enumeration. – The determination of the number of viable microorganisms in a given test portion.

Exclusivity.—Study involving nontarget strains, some of which are potentially cross-reactive, that are not detected or enumerated by the candidate method. *See* Table 8 for a list of recommended nontarget strains.[2]

Inclusivity.—Study involving pure target strains that can be detected or enumerated by the candidate method. *See* Table 7 for a list of recommended target strains.[2]

Laboratory probability of detection (LPOD).—The POD value obtained from combining all valid collaborator data sets for a method for a given matrix at a given analyte level or concentration.[3]

Lower Confidence Limit (LCL).—Lower confidence limit.

Limit of Detection. - The analyte concentration at which the probability of detection (POD) is equal to 50%.

Limit of Quantitation. - lowest analyte concentration that can be quantified with an acceptable level of precision and trueness under the conditions of the test.

Quantitative Method. – Method of analysis whose response is the amount (count or mass) of the analyte measured either directly (e.g., enumeration in a mass or a volume), or indirectly (e.g., color absorbance, impedance, etc.) in a specified test portion.[2]

***Test portion*.**—The test portion is the sample size used in most validation studies. For cannabis flower/plant and cannabis infused non-edible products a 10 g test portion is used. For cannabis concentrates, a 5 g test portion is used. For cannabis infused edibles, a 25 g test portion is used. A larger test portion can be used in validation studies when appropriate.[4,5] *See* Table 3 for minimum test portion requirements.

Upper Confidence Limit (UCL). —Upper confidence limit.

5. System suitability tests and/or analytical quality control:

The use of controls shall be embedded in assays as appropriate. Inhibition controls should be used for method verification for each new matrix. Manufacturer must provide written justification if the use of controls is not appropriate to an assay.

6. Reference Material(s):

The use of live cultures and/or fungal spores (liquid stressed/non-stressed, lyophilized) is required for inclusivity and exclusivity testing and for inoculation of test matrices during the matrix studies. Extracted DNA is not suitable for use in validating methods against this SMPR but may be used to develop supplemental information.

7. Validation Guidance[2,6]:

At the time of the publication, no national reference method exists for viable yeast and mold count determination from cannabis products. Until a suitable reference method is established the following is recommended for method developers:

Natural contamination, artificial contamination, or a combination of both are acceptable for the validation study. Method developers must specify which mode of contamination is used for which sample subset upon method submission. If artificial contamination is used, a non-inoculated contamination level must be included, and matrix naturally contaminated with non-target organisms (when available) shall be used. Additionally, if artificial contamination is used for the matrix study, an inoculating culture containing at least five different organisms (two fungal organisms, two yeast organisms, and another target organism of choice) that are co-inoculated must be included.

When performing the validation, use of bulk contaminated test material or bulk inoculation of test material is required. In certain instances (ex. therapeutic patches) individual item inoculation may be required (reference Table 5).

It is recommended that in addition to the required confirmatory agar, Dichloran Rose-Bengal Chloramphenicol agar (DRBC), a secondary, non-selective agar is included in the cultural confirmation portion of the study and that suitability testing of media be performed.[4,7,8]

For quantitative methods, at least three contamination levels (low, medium, and high) shall be included. The concentration of target organisms in each contamination level may be determined by the method developer. Each contamination level should be prepared to have a concentration of target organisms that is approximately one logarithmic different from adjacent contamination levels. It is expected that when applicable, contamination levels align with local regulatory requirements and thresholds.

Data Analysis (reference Table 2):

For the data analysis portion of the method, the following guidance should be followed per method type:

Quantitative Methods:

a. Each lot of matrix must be analyzed separately for each candidate and confirmation method.
b. Perform a logarithmic transformation on the reported CFU/g: Log_{10}[CFU/g + 0.1]
    i. Where 0.1 provides an offset to allow inclusion of "0 CFU/g" data points.
c. Perform outlier tests (Cochran and Grubbs). Remove outliers from data analysis only if there is a justifiable cause.
d. Plot the candidate method result (y-axis) vs. the culture result (x-axis). Usually major discrepancies will appear.
e. Calculate repeatability as the standard deviation of replicates at each concentration of each matrix for each method.

$$s_r = \sqrt{\frac{\sum_{i=1}^{n}(X_i - \bar{X})^2}{n-1}}$$

f. Calculate the relative standard deviation:

$$RSD_r = [s_r/mean_{cand}] \times 100$$

g. Calculate the mean difference between the candidate and reference method log_{10} transformed results with 90% and 95% confidence intervals for matrix at each contamination level using the Least Cost Formulations, Ltd., Paired Method Analysis for Micro Testing Excel worksheet v1.2.
h. Prepare a summary table of mean, s_r, RSD_r and difference of means with 90% and 95% CI data.

Qualitative Methods:

a. Analyze the data by Probability of Detection (POD) statistics according to the AOAC OMA Appendix J Guidelines.[2]
b. Analyze each contamination level separately.
c. Calculate the probability of detection (POD) as the number of positive outcomes divided by the total number of trials. Estimate the POD with a 95% confidence interval for:
    i. the candidate method presumptive results (POD_{CP}), and
    ii. the candidate method confirmed results (POD_{CC}).
d. Compare the presumptive and confirmed results by determining the $dPOD_{CP}$ at each contamination level and report results:

$$dPOD_{CP} = POD_{CP} - POD_{CC}$$

where $dPOD_{CP}$ is the difference in POD values between the candidate and reference methods. If the confidence interval of a dPOD does not contain zero, then the difference is statistically significant at the 5% level. For this validation, method comparisons are paired.

8. Method Performance Requirements:

See Table 1 for contamination levels and replicates required for quantitative and qualitative candidate methods.

See Table 2 for validation data analysis and acceptance criteria.

See Table 3 category test portion requirements.

See Table 4 for acceptable matrix claims.

See Table 5 condition of inoculating culture and stabilization of matrix for inoculation.

See Table 6 for inclusivity and exclusivity performance requirements.

See Table 7 for the inclusivity panel.

See Table 8 for the recommended exclusivity panel.

Table 1. Contamination Levels and Replicates Required for Quantitative and Qualitative Candidate Methods

Type of Method	Contamination Type	Contamination Level	Replicates/ Method	Cultural Confirmation
Quantitative	Natural Contamination/ Artificial Contamination	Low	5	DRBC[d] 25°C 5-7 days
		Medium	5	
		High	5	
Qualitative	Natural Contamination/ Artificial Contamination	Below Lowest Threshold[a]	5	
		Low Action Limit[b]	20	
		High Action Limit[b]	20	
		Above Highest Threshold[c]	5	

a. A lower contamination level targeted to be approximately one logarithmic difference from the lowest threshold determined in b.
b. Target threshold to determine presence or absence at a set threshold, or a detected or non-detected, result
c. A higher contamination level targeted to be approximately one logarithmic difference from the highest threshold determined in b.
d. Dichloran Rose-Bengal Chloramphenicol

Table 2. Validation Data Analysis and Acceptance Criteria (Plants/Flowers, Concentrates, Infused Edibles, Infused Non-Edibles)

Type of Method	Contamination Level	Required Performance Characteristics	Acceptance Criteria/Confidence Interval
Quantitative	Low	Mean Repeatability (s_r) Relative Standard Deviation (RSD_r) Difference of Means	Report LCL and UCL Statistics at 90% & 95% Confidence Intervals[d]
	Medium		
	High		
Qualitative	Below Lowest Threshold	Probability of Detection Statistics[a] $dPOD_{CP} = POD_{CP} – POD_{CC}$	POD of 0.00[c]
	Low Action Limit		$dPOD_{cp}$ 95% CI: LCL < 0 < UCL[b]
	High Action Limit		
	Above Highest Threshold		POD of 1.00[c]

a. Per AOAC OMA Appendix J Guidelines[2] (http://www.eoma.aoac.org/app_j.pdf). $dPOD_{CP}$ = difference in probability of detection between candidate presumptive and candidate confirmation methods
b. The range between the lower and upper confidence interval should encompass 0, if not, the results must be investigated, and an explanation provided.
c. If acceptance criteria is not observed, results must be investigated, and an explanation provided.
d. LCL and UCL for 90% and 95% confidence intervals for quantitative methods must fall within -0.5, 0.5

Table 3. Category Test Portion Requirements

Category Number	Category Name	Minimum Test Portion Size[a]
I	Plants & Flowers	10 g
II	Concentrates	5 g
III	Infused Edibles	25 g
IV	Infused Non-Edibles	10 g
[a]Minimum test portion size required for validation. Alternatively, larger test portions may be validated.		

Table 4. Acceptable Matrix Claims[9]

Matrix Claim	Criteria	
	Number of Matrices	Minimum Number of Categories
Broad Range of Cannabis & Cannabis Products	15 (minimum 3 matrices/category)	4 categories
Variety of Cannabis & Cannabis Products	≥ 10 (minimum 2 matrices/category)	4 categories
Select Cannabis Products	≥ 5	2 categories
Specific Category	≥ 5	1 category
Specific Matrix (s)	≥ 1	1 category

Table 5. Condition of Inoculating Culture and Stabilization of Matrix for Inoculation[2]

Matrix	Inoculating Cells	Stabilization Conditions
Perishable product	Liquid non-stressed culture	4°C, 48-72 h
Heat processed perishable product	Liquid heat stressed culture	4°C, 48-72 h
Frozen Product	Liquid non-stressed culture (If frozen food is processed, cells must be heat stressed)	-20°C, 2 weeks
Shelf stable dry product	Dried culture	Ambient Temperature (20-25°C), 2 weeks
Shelf stable liquid product (heat processed)	Liquid non-stressed culture (If shelf stable product is processed, cells must be heat stressed)	Ambient Temperature (20-25°C), 2 weeks

Table 6. Inclusivity/Exclusivity Performance Requirements

Parameter	Parameter Requirements	Final Test Concentration (CFU/mL)	Minimum Acceptable Results
Inclusivity	Single-laboratory validation (SLV) study: target strains cultured by the candidate method procedure. A minimum of 50 strains is required (reference Table 7).	10-100 x limit of detection of the candidate method	100% positive results[a]
Exclusivity	SLV study: At least 30 non-target organisms, cultured under optimal conditions for growth[b]	Overnight growth undiluted	100% negative results[a]

a 100% correct analyses are expected. All unexpected results are to be retested following internationally recognized guidelines.[2,6] Some unexpected results may be acceptable if the unexpected results are investigated, and acceptable explanations can be determined and communicated to method users.

b In instances where an exclusivity culture produces a positive or detected result by the candidate method, the culture may be reanalyzed after culture following the candidate method enrichment procedure. Both results (optimal growth conditions and candidate method enrichment) must be reported.

Table 7. Inclusivity Panel[a]

No.	Organism
1	*Alternaria alternata*
2	*Arthrinium* species
3	*Aspergillus aculeatus*
4	*Aspergillus brasiliensis*
5	*Aspergillus caesiellus*
6	*Aspergillus flavus*
7	*Aspergillus fumigatus*
8	*Aspergillus niger*
9	*Aspergillus oryzae*
10	*Aspergillus terreus*
11	*Aureobasidium* species
12	*Botrytis cinerea*
13	*Candida albicans*
14	*Candida tropicalis*
15	*Cladosporium* species
16	*Cryptococcus laurentii*
17	*Cryptococcus neoformans*
18	*Erysiphe* species[b, c]
19	*Fusarium proliferatum*
20	*Fusarium oxysporum*
21	*Fusarium solani*
22	*Golovinomyces cichoracearum*[b, c]
23	*Hyphodontia* species[c]
24	*Microsphaera* species[b, c]
25	*Mucor circinelloides*
26	*Mucor hiemalis*
27	*Paecilomyces* species
28	Penicillium chrysogenum
29	*Penicillium rubens*
30	*Penicillium venetum*
31	*Phytophthora infestans*
32	*Podosphaera* species[b, c]
33	*Purpureocillium* species
34	*Rhizopus oryzae*
35	*Rhizopus stolonifera*
36	*Scopulariopsis acremonium*
37	*Sphaerotheca* species[b, c]
38	*Yarrowia lipolytica*
39	*Talaromyces pinophilus*

[a]Method developers must test 50 total species to meet inclusivity requirements. The list in this table shall be included unless organisms cannot be obtained or cultured, per footnotes b and c.

[b]Organisms associated with powdery mildew that may potentially have difficulty growing in culture media

[c]Organism availability may be limited. Every effort to obtain these strains should be made. Non-traditional culture collections should be contacted for availability. In cases where strains are not available, different species may be supplemented. Expert reviewers will make the final determination if species analyzed are sufficient for certification.

Table 8. Exclusivity Panel[a]

No.	Organism
1	*Acinetobacter baumanii*
2	*Aeromonas hydrophila*
3	*Burkholderia cepacia*
4	*Bacillus subtilis*
5	*Citrobacter braakii*
6	*Citrobacter farmeri*
7	*Edwardsiella tarda*
8	*Enterobacter aerogenes*
9	*Enterobacter cloacae*
10	*Erwinia amylovora*
11	*Escherichia coli*
12	*Escherichia coli* O157:H7
13	*Escherichia hermanii*
14	*Escherichia vulneris*
15	*Hafnia alvei*
16	*Klebsiella oxytoca*
17	*Klebsiella pneumonia*
18	*Listeria monocytogenes*
19	*Morganella morganii*
20	*Pantoea agglomerans*
21	*Proteus mirabilis*
22	*Pseudomonas aeruginosa*
23	*Pseudomonas fluorescens*
24	*Pseudomonas putida*
25	*Ralstonia insidiosa*
26	*Rahnella aquatilis*
27	*Salmonella* Agona
28	*Stenotrophomonas maltophilia*
29	*Staphylococcus aureus*
30	*Serratia marcescens*

[a]List of suggested organisms method developers can use to validate their methods. A minimum of 30 non-target organisms are required for AOAC adoption. Organisms utilized should be well characterized and information provided must include source, strain numbers and origin (if available)

References

(1) Appendix F: *Guidelines for Standard Method Performance Requirements, Official Methods of Analysis of AOAC INTERNATIONAL, 21st Ed.* (2019). AOAC INTERNATIONAL, Rockville, MD, USA.

(2) Appendix J: *AOAC INTERNATIONAL Methods Committee Guidelines for Validation of Microbiological Methods for Food and Environmental Surfaces, Official Methods of Analysis of AOAC INTERNATIONAL, 21st Ed.* (2019). AOAC INTERNATIONAL, Rockville, MD, USA.

(3) Appendix H: *Probability of Detection (POD) as a Statistical Model for the Validation of Qualitative Methods, Official Methods of Analysis of AOAC INTERNATIONAL*, *21st Ed.* (2019). AOAC INTERNATIONAL, Rockville, MD, USA.

(4) General Chapter <61> *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests, USP 40*. (2016). United States Pharmacopeia, Rockville, MD, USA.

(5) General Chapter <62> *Microbiological Examination of Nonsterile Products: Tests for Specified Microorganisms, USP 40.* (2016). United States Pharmacopeia, Rockville, MD, USA.

(6) ISO 16140-2:2016, *Microbiology of the Food Chain — Method Validation Part 2: Protocol for the validation of alternative (proprietary) methods against a reference*

method, (2016). International Organization for Standardization, Geneva, Switzerland.

(7) ISO 16212:2017, *Cosmetics — Microbiology — Enumeration of yeast and mould,* (2017). International Organization for Standardization, Geneva, Switzerland.

(8) European Pharmacopeia 01/2005:20612, *Microbiological examination of non-sterile products (total viable aerobic count)*, *European Pharmacopeia 5.6.* (2013). European Pharmacopeia, Strasbourg, France.

(9) McKenzie, D. 2016. *Technical Bulletin: TB02MAY2016: Acceptable Validation Claims for Proprietary/Commercial Microbiology Methods for Foods and Environmental Surfaces.* AOAC INTERNATIONAL, Rockville, MD, USA.