AOAC Standard Method Performance requirements 2020.XX, Version 6; July 02, 2020

Method Name: Detection of Shiga toxin-producing *Escherichia coli* in Cannabis and Cannabis Products

Purpose: AOAC SMPRs describe the minimum recommended performance characteristics and suggested inclusivity/exclusivity organisms 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 the 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 methods being considered for *Performance Tested Methods* or AOAC *Official Methods of Analysis*, 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:** Alternative methods used to detect certain Shiga toxin-producing *Escherichia coli* (STEC) serotypes in cannabis and cannabis products. Many regulatory bodies regulate cannabis products for seven STEC as adulterants. Either apply method to detect at least these seven *E. coli* serotypes (O157:H7, O26:H11, O45:H2, O103:H11, O111:H8, O121:H19, O145:NM) in cannabis and cannabis products, or to broadly detect STEC in cannabis and cannabis products, as declared by method developer. The United States Department of Agriculture, Food Safety and Inspection Service considers their regulated products (raw, non-intact beef products or the components of these products) found to have any of these seven specific STEC to be adulterated.[2]

3. **Analytical Technique**: Any analytical method that can meet the requirements to screen and confirm for the presence of Shiga toxin-producing *E. coli*.

4. **Definitions**:

***Candidate Method*.**— The method submitted for validation[3]
***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.

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

Exclusivity.—Study involving pure nontarget bacterial strains, some of which are potentially cross-reactive, that shall be not detected or enumerated by the candidate method. *See* Table 10 for a list of recommended nontarget strains.[3]

Fractional positive.—Validation criterion that is satisfied when an unknown sample yields both positive and negative responses within a set of replicate analyses. The proportion of positive responses should fall within 25% and 75% and should ideally approximate 50% of the total number of replicates in the set. A set of replicate analyses are those replicates analyzed by one method. Only one set of replicates per matrix is required to satisfy this criterion.

Inclusivity.—Study involving pure target bacterial strains that shall be detected or enumerated by the candidate method. *See* Tables 8 and 9 for a list of recommended target strains.[3]

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.[4]

LCL.—Lower confidence limit.

Shiga toxin-producing *Escherichia coli*

Shiga toxin-producing *E.coli* are Gram negative, facultative anaerobes that are characterized by the production of *Stx*1 and/or *Stx*2 and the potential to cause hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) in humans, which can be fatal. STEC O157:H7, the prototypic serotype, exhibits slow or no fermentation of sorbitol and does not have glucuronidase activity.[5] STEC are hosted in the intestines of cattle and have corresponding prevalence in the environment and animals that can be transmitted through the production and distribution of food and agricultural products.

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.[6,7] See Table 2 for minimum test portion requirements.

UCL. —Upper confidence limit.

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

Positive and negative 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 controls are not appropriate to an assay.

6. Reference Material(s):

The use of live cultures (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[3,8]:

At the time of the publication, no national reference method exists for the confirmation of STEC from cannabis products. Until a suitable reference method is established the following is recommended for method developers:

To screen samples for the presence or absence of the target analyte, two methods that employ different technologies (agar plate, PCR, ELISA) must be used, followed by cultural confirmation onto selective agars.

To ensure the viability of the inoculating organism (both for confirming presumptive results or determining false negative results) a minimum 18 h primary enrichment is required prior to beginning confirmation. For matrices with known inhibitory properties, a secondary enrichment is recommended. Confirmation of all samples, regardless of presumptive results, must include plating of the sample to a minimum of two types of agar plates, one of which is recommended to be chromogenic agar (Table 6). Final confirmation should include biochemical confirmation and verification of *stx* virulence genes from STEC isolates. Optional confirmation steps can include tests for glucuronidase (MUG), galactopyranosidase (X-gal), indole and latex agglutination tests.

When performing the validation, bulk inoculation of test material is required. In certain instances (e.g., therapeutic patches) individual item inoculation may be required.

For the Single Laboratory Validation with artificial contamination, matrix naturally contaminated with non-target organisms (when available) shall be used. For at least one matrix evaluated during the single laboratory validation, competing non-target microflora must be at least 10x the level of the target microorganism. If the concentration of competing microflora does not exceed 10x, the target organism for any matrix, artificial contamination of one matrix with non-target organism(s) is required.

A minimum three level most probable number (MPN) study should be performed to determine the concentration of the target organism used in the validation. If possible, the use of test portions included in the matrix study should be included as a level in the MPN study. See AOAC Appendix J guidelines for details on performing the MPN study. [3]

8. Maximum Time-To-Determination: None

9. Method Performance Requirements

See Table 1 for acceptance criteria for validation
See Table 2 for category test portion requirement
See Table 3 for matrix claims acceptance criteria
See Table 4 for descriptions of MPN analysis
See Table 5 condition of inoculating culture and stabilization of matrix for inoculation
See Table 6 for selective broth and agar recommendations
See Table 7 for inclusivity and exclusivity performance requirements
See Tables 8 & 9 for inclusivity organisms
See Table 10 for exclusivity organisms

Table 1. Validation Acceptance Criteria (Plants/Flowers, Concentrates, Infused Edibles, Infused Non-Edibles)

Parameter	Parameter Requirements	Target Test Concentration[a]	Minimum Acceptable Results
Single Laboratory Validation with artificial contamination			
Fractional Concentration (low level)	Replicates per matrix: 20 Inoculation procedure: AOAC Appendix J	Low level to produce fractional positive results Ex. 0.2-2 CFU/Test Portion	Fractional positive results, 25-75% (5-15 positive test replicates) $dPOD_{CP}$ 95% CI: LCL < 0 < UCL[b]
High Concentration	Replicates: 5 Inoculation procedure: AOAC Appendix J	High level to produce consistently positive results Ex. 2-10 CFU/Test Portion	POD of 1.00[c]
Non-Inoculated (Zero) concentration	Replicates: 5	0 CFU/Test Portion	POD of 0.00[c]
Single Laboratory Validation with natural contamination			
Acceptable minimum detection level (low level)	2 separate lots of 20 replicates	N/A	Fractional positive results, 25-75% (5-15 positive test replicates) for minimum 1 lot $dPOD_{CP}$ 95% CI: LCL < 0 < UCL[b]
Multi Laboratory Validation			
LPOD	12	1-10 CFU/Test Portion	0.15 ≥ LPOD ≥ 0.85 $dPOD_{CP}$ 95% CI: LCL < 0 < UCL[b]
	12	10-50 CFU/ Test Portion	LPOD ≥ 0.95
$LPOD_{(0)}$	12	0 CFU/Test Portion	LPOD ≤ 0.05
[a]Determined through MPN Procedures (see Table 4)			
[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			

Table 2. Category Test Portion Requirements

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

Table 3. 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 4. Minimum Most Probable (MPN) Number Recommendation

Category	Inoculation Level	Large Test Portions	Medium Test Portions	Small Test Portions
Plants & Flowers Concentrates	Low	20 x 10 g*	3 x 5 g	3 x 1 g
	High	5 x 10 g*	3 x 5 g	3 x 1 g
Concentrates	Low	20 x 5 g	3 x 2.5 g	3 x 1 g
	High	5 x 5 g*	3 x 2.5 g	3 x 1 g
Infused Edibles	Low	20 x 25 g*	3 x 10 g	3 x 5 g
	High	5 x 25 g*	3 x 10 g	3 x 5 g
Infused Non-Edibles	Low	20 x 10 g*	3 x 5 g	3 x 1 g
	High	5 x 10 g*	3 x 5 g	3 x 1 g

*Test portions from matrix study

Table 5. Condition of Inoculating Culture and Stabilization of Matrix

Matrix	Inoculating Cells	Stabilization Conditions
Perishable product	Liquid non-stressed culture	4°C, 48-72 h
Heat processed perishable product	Liquid heat stressed	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: Recommended Selective Broths and Agars for STEC

Media Name	Media Type
Chromogenic agar	Agar
Sorbitol Macconkey	Agar
Rainbow agar	Agar
TBX agar	Agar
SHIBAM agar	Agar
Tryptic soy broth + novobiocin	Broth
Modified BPW with pyruvate + ACV	Broth
mEC + novobiocin	Broth

Table 7. Inclusivity/Exclusivity Performance Requirements

Parameter	Parameter Requirements	Final Test Concentration (CFU/mL)	Minimum Acceptable Results
Inclusivity	Single-laboratory validation (SLV) study: For the seven STEC claim, at least 5 strains per required STEC serotype (Table 8) cultured by the candidate method enrichment procedure. For the broad STEC claim, at least 5 strains per required STEC serotype (Table 8) and 3 strains of 5 additional STEC serogroups (Table 9) cultured by the candidate method enrichment procedure. A minimum of 50 total strains is required.	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 (e.g., ISO 16140[8], AOAC OMA Appendix J[3]). Some unexpected results may be acceptable if the unexpected results are investigated, and acceptable explanations can be determined and communicated to method users. It is possible that the incorporation of *Shigella dysentery* in the exclusivity portion of the study may result in a positive determination by the screening method.

b In instances where an exclusivity culture produces a positive 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.

Tables 8 & 9: Inclusivity Panel

The following are lists of required serotypes (Table 8) and suggested serogroups (Table 9) that method developers can use to validate their methods.

Table 8: Required STEC serotypes for Inclusivity for Seven STEC Claim:

Serotype	**Minimum Strains Required**
O157:H7	5
O45:H2	5
O121:H19	5
O26:H11	5
O103:H11	5
O111:H8	5
O145:NM	5

Table 9: Recommended STEC Serogroup for Inclusivity for Broad STEC Claim[a]:

Serogroup	**Minimum Strains Recommended**
O118	3
O186	3
O71	3
O80	3
O91	3
O113	3
O5	3
O3	3

a At least five serogroups in addition to the seven serotypes listed in Table 8 are required to be included. It is recommended that serotypes with varying virulence and attachment gene expression are included.

Table 10: Exclusivity Panel (Recommended)

Organism	
Aeromonas hydrophila	*Hafnia* species
Additional *Aeromonas species*	*Klebsiella oxytoca*
Burkholderia species	*Klebsiella pneumonia*
Bacillus subtilis	*Listeria monocytogenes*
Campylobacter jejuni	*Morganella morganii*
Candida tropicalis	*Pantoea species*
Citrobacter braakii	*Proteus hauseri*
Citrobacter farmerii	*Proteus mirabilis*
Citrobacter freundii	*Proteus vulgaris*
Citrobacter murliniae	*Pseudomonas aeruginosa*
Citrobacter youngae	*Pseudomonas fluorescens*
Citrobacter species	*Pseudomonas species*
Edwardsiella tarda	*Ralstonia species*
Enterobacter aerogenes	*Rhanella species*
Enterobacter amnigenus	*Salmonella spp.*
Enterobacter cancerogenus	*Serratia marcesens*
Enterobacter cloacae	*Shigella dysenteriae*
Enterobacter gergoviae	*Shigella flexneri*
Enterobacter sakazakii	*Shigella sonnei*
Erwinia species	*Trichoderma harzianum*
Escherichia coli (non-STEC)	*Yersinia* species
Escherichia fergusonii	*Vibrio vulnificus*
Escherichia hermanii	
Escherichia vulneris	

References

(1) Appendix F: *Guidelines for Standard Method Performance Requirements, Official Methods of Analysis of AOAC INTERNATIONAL,* (2016). AOAC INTERNATIONAL, Rockville, MD, USA.

(2) *Microbiology Laboratory Guidebook, 5C.00.* (2019). United States Department of Agriculture, Food Safety and Inspection Service, Athens, GA, USA.

(3) 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.

(4) 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.

(5) Feng, P., Weagant, S.D., Grant, M.A., Burkhardt, W. 2017. *Bacteriological Analytical Manual: Chapter 4 Enumeration of Escherichia coli and the Coliform Bacteria.* US Food and Drug Administration, Washington, DC, USA.

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

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

(8) 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.

(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.