AOAC SMPR® 2020.012

Standard Method Performance Requirements (SMPRs®) for Detection of Shiga Toxin-Producing *Escherichia coli* in Cannabis and Cannabis Products

Intended Use: Consensus-Based Reference Method

1 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 (SLV), or a multi-site collaborative study. SMPRs are written and adopted by AOAC stakeholders 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 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).

2 Applicability

Alternative methods used to detect certain Shiga toxin-producing *Escherichia coli* (STEC) serogroups in cannabis and cannabis products. Many regulatory bodies regulate cannabis products for seven STECs as adulterants. Either apply method to detect at least these seven *E. coli* serogroups (O157:H7, O26, O45, O103, O111, O121, and O145) in cannabis and cannabis products, or to broadly detect STEC in cannabis and cannabis products, as declared by method developer. The U.S. Department of Agriculture-Food Safety and Inspection Service considers its regulated products (raw, nonintact beef products, or components of these products) found to have any of these seven specific STECs to be adulterated (2).

3 Analytical Technique

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

4 Definitions

[*See* Section 9 Method Performance Requirements for a comprehensive list of tables outlining validation criteria and corresponding method performance requirements.]

Candidate method.—Method submitted for validation (3).

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

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

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 extraction and concentration of compounds derived from 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 nonedibles.—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 nonedible) extracted or infused with compounds derived from cannabis plant, including but not limited to, CBD and THC.

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. 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* Table 8 for required target serogroups/serotype and Table 9 for recommended target serogroups (3).

Laboratory probability of detection (LPOD).—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.

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

Shiga toxin-producing Escherichia coli.—Gram-negative, facultative anaerobes that are characterized by production of *Stx*1 and/or *Stx*2 and 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). STECs are hosted in ruminant animals, including cattle, goats, sheep, deer, and elk, with cattle as the major source for human illnesses (6). STEC have corresponding prevalence in the environment and animals that can be transmitted through production and distribution of food and agricultural products.

Test portion.—Sample size used in most validation studies. For cannabis flower/plant and cannabis-infused nonedible 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 (7, 8). *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)

Use of live cultures (liquid-stressed/no-stressed, lyophilized) is required for inclusivity and exclusivity testing and for inoculation of test matrices during 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

See refs 3 and 9.

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

To screen samples for presence or absence of target analyte, two methods that employ different technologies (agar plate, PCR, ELISA) must be used.

To ensure viability of 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 validation, bulk inoculation of test material is required. In certain instances (e.g., therapeutic patches), individual item inoculation may be required. *See* Table 5 for further information regarding the condition of inoculating culture and stabilization of matrix for inoculation.

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

A minimum 3-level most probable number (MPN) study should be performed to determine concentration of the target organism used in the validation. If possible, use of test portions included in the matrix study should be included as a level in the MPN study. *See* Table 4 and 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 for 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 (required serogroups/serotype) and 9 (recommended serogroups) for inclusivity organisms that method developers can use to validate their methods.

See Table 10 for exclusivity organisms.

References

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

(2) *Microbiology Laboratory Guidebook, 5C.00* (2019) U.S. 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* (2019) *Official Methods of Analysis of AOAC INTERNATIONAL,* 21st Ed., AOAC INTERNATIONAL, Rockville, MD, USA

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

(5) Feng, P., Weagant, S.D., Grant, M.A., & Burkhardt, W. (2020) *Bacteriological Analytical Manual: Chapter 4 Enumeration of Escherichia coli and the Coliform Bacteria,* U.S. Food and Drug Administration, Washington, DC, USA

(6) *E. coli* Index: Questions and Answers (2014) Centers for Disease Control and Prevention, U.S. Department of Health and Human Services, https://www.cdc.gov/ecoli/general/index.html

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

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

(9) 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

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

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Table 1. Validation acceptance criteria (plants/flowers, concentrates, infused edibles, and infused nonedibles)

Parameter	Requirements	Target test concentration[a]	Minimum acceptable results
Single-laboratory validation (SLV) 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]
Noninoculated (zero) concentration	Replicates: 5	0 CFU/test portion	POD of 0.00[c]
SLV with natural contamination			
Acceptable minimum detection level (low level)	2 separate lots of 20 replicates	NA	Fractional positive results, 25–75% (5–15 positive test replicates) for minimum 1 lot $dPOD_{CP}$ 95% CI: LCL < 0 < UCL[b]
Multilaboratory 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] Range between lower and upper confidence interval should encompass 0; if not, 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, g[a]
Plants and flowers	10
Concentrates	5
Infused edibles	25
Infused nonedibles	10

[a] Minimum test portion size required for validation. Alternatively, larger test portions may be validated.

Table 3. Acceptable matrix claims (ref. 10)

	Criteria	
Matrix claim	No. matrices	Minimum No. categories
Broad range of cannabis and cannabis products	15 (Minimum 3 matrices/category)	4
Variety of cannabis and cannabis products	≥10 (Minimum 2 matrices/category)	4
Select cannabis products	≥5	2
Specific category	≥5	1
Specific matrix(es)	≥1	1

Table 4. Minimum most probable number (MPN) recommendation

		Test portions, g		
Category	Inoculation level	Large	Medium	Small
Plants and flowers	Low	20 × 10[a]	3 × 5	3 × 1
	High	5 × 10[a]	3 × 5	3 × 1
Concentrates	Low	20 × 5	3 × 2.5	3 × 1
	High	5 × 5[a]	3 × 2.5	3 × 1
Infused edibles	Low	20 × 25[a]	3 × 10	3 × 5
	High	5 × 25[a]	3 × 10	3 × 5
Infused nonedibles	Low	20 × 10[a]	3 × 5	3 × 1
	High	5 × 10[a]	3 × 5	3 × 1

[a] Test portions from matrix study.

Table 5. Condition of inoculating culture and stabilization of matrix

Matrix	Inoculating cells	Stabilization conditions
Perishable product	Liquid nonstressed culture	4°C, 48–72 h
Heat-processed perishable product	Liquid heat-stressed	4°C, 48–72 h
Frozen product	Liquid nonstressed culture (if frozen food is processed, cells must be heat-stressed)	–20°C, 2 weeks
Shelf-stable dry product	Dried culture	Ambient temp. (20–25°C), 2 weeks
Shelf-stable liquid product (heat-processed)	Liquid nonstressed culture (if shelf-stable product is processed, cells must be heat-stressed)	Ambient temp. (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	Requirements	Final test concn, CFU/mL	Minimum acceptable results
Inclusivity	Single-laboratory validation (SLV) study: For the 7 STEC claim, at least 5 strains per required STEC serogroup/serotype (Table 8) cultured by candidate method enrichment procedure. For the broad STEC claim, at least 5 strains per required STEC serogroup/serotype (Table 8) and 3 strains of 5 additional STEC serogroups (Table 9) cultured by candidate method enrichment procedure. A minimum of 50 total strains is required.	10–100 × limit of detection of the candidate method	100% positive results[a]
Exclusivity	SLV study: At least 30 nontarget 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 (ref 9), AOAC OMA Appendix J (ref 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 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 candidate method enrichment procedure. Both results (optimal growth conditions and candidate method enrichment) must be reported.

Table 8. Required STEC serogroup/serotypes for inclusivity for seven STEC claim

Serogroup/serotype	Minimum strains required
O157:H7	5
O45	5
O121	5
O26	5
O103	5
O111	5
O145	5

Table 9. Recommended STEC serogroups 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 5 serogroups in addition to the seven serogroups 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	