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Research Article

MICROBIOLOGICAL METHODS

Validation of the Soleris® *Enterobacteriaceae* Method for Detection of *Enterobacteriaceae* in Dried Cannabis Flower: AOAC *Performance Tested Method*SM 121901

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Abstract

Background: The Soleris® *Enterobacteriaceae* vial is a growth-based, automated method for detection of bacteria of the family *Enterobacteriaceae* in foods and other sample types including nutraceuticals and cosmetics. The Soleris method is used in a "dilute-to-specification" or threshold manner, in which a result is scored as positive or negative around a predetermined cutoff (in CFU/g) established by the dilution and volume of sample homogenate tested. The Soleris method was granted AOAC *Performance Tested Method*SM (PTM) status for select foods after successful completion of a validation study (PTM 121901).

Objective: The objective of this study was to validate the method for the detection of *Enterobacteriaceae* in dried cannabis flower [>0.3% delta-9-tetrahydrocannabinol (THC)].

Methods: The matrix study included comparison of Soleris method presumptive results to confirmation from the Soleris vials, and comparison of the Soleris confirmed results to those of the ISO 21528-2:2017 colony count method. Test materials at four different levels of contamination ranging from 7.8 to 3500 CFU/g were tested at three dilutions, corresponding to test thresholds.

Results: Probability of detection analysis at $P < 0.05$ showed there were no significant differences between Soleris presumptive and confirmed results, and no significant differences between Soleris confirmed and ISO 21528-2:2017 results.

Conclusion: The results provided evidence that the Soleris *Enterobacteriaceae* test is an accurate method for detection of *Enterobacteriaceae* in dried cannabis flower.

Highlights: The Soleris *Enterobacteriaceae* method provides cannabis industry QC personnel with an effective method for analysis of dried cannabis flower and produces results in 20–24 h.

General Information

Cannabis products, including dried cannabis flower, present a variety of potential microbial hazards including *Salmonella* spp., pathogenic *Escherichia coli*, and pathogenic yeasts and molds. State regulatory authorities have responded by prescribing limits for microorganisms of particular concern (1). To ensure product safety and compliance with regulatory limits, cannabis

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products are routinely tested for total aerobic microorganisms, yeasts and molds, indicator organisms such as coliform bacteria and *Enterobacteriaceae* (EBAC), and, in some cases, specific pathogens (2).

EBAC are Gram-negative, rod-shaped facultative anaerobes that are non-spore forming and can be motile or nonmotile. This family of bacteria can cause a wide range of illnesses. Not all types of bacteria in this family are truly pathogenic but can instead be regarded as opportunistic. EBAC are well dispersed in the environment and have been detected in soil, water, plants, and the gastrointestinal tracts of animals and humans. In the food industry, EBAC are used as indicator organisms for poor hygiene practices or problems in the manufacturing process (3).

The Soleris® *Enterobacteriaceae* test was granted *Performance Tested Method*SM (PTM) status (Certificate No. 121901) in 2019 following a successful validation study. Validated matrixes include pasteurized milk, yogurt, mozzarella cheese, ice cream, dried milk, pasteurized liquid egg, frozen cooked chicken, deli ham, lettuce, and dry dog food (4). A method modification study was performed in 2020 to validate the change of the agar plug to comprise agarose, which is a more consistent product and improves material stability and robustness during manufacture and transport. Inclusivity/exclusivity testing, matrix testing, and a reagent stability/lot-to-lot consistency trial were included in that study (5).

Principle

The Soleris *Enterobacteriaceae* test is a growth-based, automated method for detection of bacteria of the family *Enterobacteriaceae*. The method consists of a Soleris test vial and instrument. The test vial is comprised of a growth chamber with a selective medium and a detection window for optical determination of changes in color resulting from metabolism-induced pH changes. The detection window contains an agar plug that equilibrates with the medium in the growth chamber while excluding particulates that could potentially interfere with the optical readings. For the EBAC test, the color of the growth medium and agar plug changes from purple to yellow due to the growth of EBAC, resulting in a reduction in pH from the fermentation of glucose. The Soleris instrument consists of a temperature-controlled incubation chamber with positions for 32 vials per chamber. At each position, a light-emitting diode (LED) light source takes periodic readings through the detection window of the vials. For the EBAC test, presumptive results are typically available in 18–24 h or less.

Scope of Method

(a) *Analytes*.—*Enterobacteriaceae*.
(b) *Matrixes*.—Pasteurized whole milk, vanilla yogurt (1.5% fat), mozzarella cheese, vanilla ice cream, dried milk, pasteurized liquid egg, frozen cooked chicken, deli ham, Romaine lettuce (chopped, bagged), and dry dog food (main ingredients: beef, corn, barley, rice, and wheat); 10 g portions of dried cannabis flower [>0.3% delta 9-tetrahydrocannabinol (THC)].
(c) *Summary of validated performance claims*.—No statistical difference compared to the ISO 21528–2:2017 colony count method [*Microbiology of food and animal feeding stuffs—Horizontal methods for the detection and enumeration of Enterobacteriaceae—Part 2: Colony-count method* (6)] as determined by probability of detection (POD) analysis (7) at $P < 0.05$.

Definitions

(a) *Probability of detection (POD)*.—The proportion of positive analytical outcomes for a qualitative method for a given matrix at a given analyte level or concentration. POD is concentration dependent.
(b) *Difference of probabilities of detection (dPOD)*.—Difference of probabilities of detection is the difference between any two POD values. If the confidence interval of a dPOD does not contain zero, then the difference is statistically significant at the 5% level.

Materials and Methods

Test Kit Information

(a) *Kit name*.—Soleris *Enterobacteriaceae* vial.
(b) *Cat. No*.—S2-EBAC9.
(c) *Ordering Information*.—*In the United States*.—NEOGEN Corp., 620 Lesher Pl., Lansing, MI 48912, Tel: 800-234-5333 or 517-372-9200, Fax: 517-372-2006, Website: www.neogen.com. *Outside the United States*.—Contact U.S. office for ordering or distributor information.

Test Kit Components

(a) *Soleris Enterobacteriaceae vial, 9 mL*.—Sterile medium in plastic vial devices, box of 100, one test per vial, pH 6.7 ± 0.2, sample capacity 1 mL. Requires Soleris instrument or equivalent.

Additional Supplies and Reagents

(a) *Stomacher-type bags with mesh filter (Product No. 6827 or equivalent)*.
(b) *Hydrochloric acid solution*.—1 N, sterile., for adjusting pH of sample.
(c) *Sodium hydroxide solution*.—1 N, sterile, for adjusting pH of sample.
(d) *pH paper*.
(e) *Buffered peptone water (Product No. NCM0015 or equivalent)*.
(f) *Tryptic soy broth (TSB; Product No. BLX-TSB90 or equivalent)*.
(g) *Violet red bile glucose agar (VRBG; Product No. NCM0041A or equivalent)*.—500 g (other sizes available).
(h) *Inoculating loops*.—10 μL.

Apparatus

(a) *Neogen Rapid Microbiology instrument (Product Nos. BSX-32, BSX-128, BLX-INS32, SNG-INS32)*.—Containing one or four temperature-controlled (18–60 ± 0.5°C) incubator drawers with 32 test locations per drawer. Each test location contains an LED-based optical sensor for measurement of changes in absorbance over time.
(b) *Soleris computer system (Product No. BSC01)*.—Includes vial rack.
(c) *Soleris computer only (Product No. SCT-01 or equivalent)*.
(d) *Soleris vial rack (Product Nos. VR-200, VR-300 or equivalent)*.—Holds 32 vials.

(e) *Soleris vial rack transfer mechanism (Product No. VRTM-200)*.
(f) *Soleris operator's manual (Product No. OM-710)*.
(g) *Stomacher® or equivalent*.
(h) *Balance*.—For weighing samples, minimum 100 g ± 0.1 g capacity.
(i) *Micropipettor and tips*.—20–200 μL.
(j) *Micropipettor and tips*.—100–1000 μL.

Safety Precautions

Use of this test should be restricted to individuals with appropriate laboratory training in microbiology as some *Enterobacteriaceae* are potentially infectious. Reagents are for laboratory use only. Test samples, used Soleris vials, and agar media may contain potentially infectious microorganisms; follow appropriate laboratory procedures for handling of microbial pathogens (U.S. Department of Health and Human Services, *Biosafety in Microbiological and Biomedical Laboratories (BMBL)*, 6th Edition, HHS Publication No. (CDC) 300859, Revised June 2020 [(8); or most current version found at cdc.gov]. All pipetting transfers must be made using either a disposable pipet and pipetting aid or micropipettor with disposable tips. Refer to the safety data sheet available from NEOGEN Corp. for more information. The preferred method for decontamination of contaminated material is autoclaving. Items that cannot be autoclaved may be decontaminated by using a disinfectant solution, e.g., 10% household bleach, followed by rinsing with water. Consult with your facility safety director for specific instructions.

General Preparation

This test should be performed under normal laboratory conditions with respect to humidity, temperature, lighting, etc. Do not use Soleris vials beyond their expiration date.

Sample Preparation—Dried Cannabis Flower

(a) Prepare a 1:10 sample homogenate by adding 90 mL TSB to 10 g cannabis flower in a Stomacher-type bag. Massage/homogenize thoroughly by hand.
(b) Decant TSB for testing. Squeeze remaining sample to remove as much TSB as possible.
(c) Prepare further decimal dilutions from the 1:10 sample homogenate in TSB to establish the test threshold as appropriate depending on the expected contamination level of the matrix. For example, for >10 CFU/g, use sample homogenate, for >100 CFU/g, prepare further 1:10 dilution, for >1,000 CFU/g, prepare further 1:100 dilution, etc.
(d) Add 1.0 mL sample homogenate or further dilution to the Soleris vial. Cap the vial tightly and invert three times to mix. Keep the cap tight.
(e) Proceed to Soleris analysis.

Sample Preparation—Foods

(a) Combine 10 g sample and 90 mL sterile buffered peptone water in a Stomacher-type bag, homogenize thoroughly.
(b) Check pH and adjust if necessary to pH 7.0 ± 1.0.
(c) For testing at a threshold level of >10 CFU/g, use the sample homogenate without further dilution. For testing at higher threshold levels, prepare the appropriate dilution in buffered peptone water.

Analysis

Note: The Soleris system requires installation and operator training. Both are provided by NEOGEN Corp.

(a) In the Soleris software, select the test type and enter sample identification information into the sample position grid.
(b) Insert the vial into the Soleris instrument programmed with the following settings:
  (1) Test: S2-EBAC9.
  (2) Threshold: 10.
  (3) Skip: 1.
  (4) Shuteye: 25.
  (5) Duration: foods—18 h, dried cannabis flower—20–24 h.
  (6) Temperature: 35 ± 0.5°C.
(c) Click "Start Run".

Conclusions, Interpretation, and Test Result Report

(a) A detection curve will be generated in real time. The Soleris software will indicate a positive or negative test result. Positive results will generally be reported in less than the designated test duration.
(b) *Negative result*.—Tests producing no detection within the designated test duration are considered negative at the test threshold selected.
(c) *Positive result*.—Tests with detection times within the designated test duration indicate a positive result at the test threshold selected.

Confirmation

(a) At the conclusion of the test, remove the vials from the instrument, invert to mix, and streak a 10 μL aliquot of the vial contents to a VRBG plate.
(b) Incubate plates for 24 ± 2 h at 37 ± 1°C.
(c) Examine plates for presumptive *Enterobacteriaceae* colonies and continue with confirmation following ISO 21528-2:2017 procedures.

Validation Study

This validation study was conducted under the AOAC Research Institute PTM program and in accordance with the Appendix J: *AOAC INTERNATIONAL Methods Committee Guidelines for Validation of Microbiological Methods for Food and Environmental Surfaces* (9). Matrix testing was conducted as an independent laboratory study at TEQ Analytical Labs (Aurora, CO, USA) and included the analysis of one matrix, dried cannabis flower (>0.3% THC).

Independent Laboratory Study—Matrix Testing

(a) *Methodology*.—Dried cannabis flower (>0.3% THC) was obtained by the independent laboratory and screened for the presence of EBAC using the ISO 21528-2:2017 method. Based on the screening results, artificial contamination was required to achieve the required EBAC levels for the validation study. A lyophilized cell pellet of *Escherichia coli*, American Type Culture Collection (ATCC, Manassas, VA, USA) 8739 was crushed and mixed with nonfat dried milk powder to create the inoculum. The dried cannabis flower was broken up by hand and placed into a large, sterile Whirl-Pak® bag. The material was further hand massaged to create small, uniform particles. The *E. coli* inoculum was added to the bag of dried

cannabis flower and further hand mixed and shaken to create a uniform material. After 2 weeks at 15–20°C, the inoculated matrix was screened for EBAC using the ISO 21528–2:2017 method to check the contamination level.

Four contamination levels were then created from this material by adding non-inoculated material to produce test materials targeting <10 CFU/g, approximately 10 CFU/g (intermediate 1), approximately 100 CFU/g (intermediate 2), and >100 CFU/g (high) contamination levels. Mixing was performed in sterile Whirl-Pak bags using extensive manual mixing to ensure maximum homogeneity. One of the two intermediate levels was required to produce fractional positive results (25–75% of test portions testing positive) for at least one of the test thresholds chosen. The low level is intended to produce all or mostly negative results and the high level all or mostly positive results for at least one of the test thresholds. Test portion numbers were blind coded so the analyst running the method was unaware of the contamination level.

For the low and high levels, five 10 g test portions were tested. For the two intermediate levels, twenty 10 g test portions were tested. Test portions were analyzed using the ISO 21528–2:2017 method and the Soleris method at three test threshold levels (dilutions). Samples were prepared by adding 90 mL TSB to each 10 g cannabis portion and then massaged or homogenized by hand. The homogenized cannabis and TSB mixture was poured off and used for testing. Further dilutions were made using TSB to achieve 1:100 and 1:1,000 dilutions. The sample homogenates and the dilutions correspond to the test thresholds of >10, >100, and >1000 CFU/g. From each sample homogenate and dilution prepared, 1 mL was transferred to the corresponding Soleris vial. Following Soleris incubation for 24 h using Soleris SNG-INS32 instruments, the vial contents were confirmed for the presence of EBAC by streaking a 10 μL loopful to VRBG agar and continuing with confirmation procedures of the ISO 21528-2:2017 method.

For the ISO 21528-2:2017 colony count method, the same set of sample homogenates and dilutions were used to transfer 1 mL to VRBG agar using the pour plate method. An overlay of VRBG was added to the solidified pour plate, and plates were incubated at 37 ± 1°C for 24 ± 2 h. Following incubation, plates were examined for growth and confirmation of the presumptive EBAC colonies was performed following the ISO method. Briefly, presumptive EBAC colonies (pink to red to purple) were subcultured to nutrient agar and oxidase and glucose fermentation tests performed. Oxidase-negative, glucose-positive colonies are confirmed as EBAC. Plates with one or more EBAC colonies were reported as positive and plates with no EBAC colonies were reported as negative.

POD statistical analysis at $P < 0.05$ was performed for each contamination level and test threshold using the Least Cost Formulations POD calculator, v. 5.1 (Virginia Beach, VA, USA). The Soleris presumptive and confirmed results were compared using paired POD analysis. The Soleris confirmed results and modified ISO method positive results were compared using unpaired POD analysis.

(b) *Results.*—Results and POD analysis are shown in Tables 1 and 2. Results of the MPN analysis, conducted on the day of testing, were interpreted using the Least Cost Formulations MPN calculator (Virginia Beach, VA, USA). EBAC levels for the low, intermediate 1, intermediate 2, and high test materials were 7.8, 70, 1200, and 3500 CFU/g, respectively.

There were no statistically significant differences between the Soleris presumptive and confirmed results, and no

Table 1. Soleris *Enterobacteriaceae* presumptive and confirmed results for testing of dried cannabis flower

Matrix	Strain	Level, MPN/g[a]	Test threshold, CFU/g[b]	N[c]	Soleris presumptive			Soleris confirmed				
					x[d]	POD_{CP}[e]	95% CI	x	POD_{CC}[f]	95% CI	$dPOD_{CP}$[g]	95% CI[h]
Dried cannabis flower	*E. coli* ATCC 8739	Low 7.8 (2.0, 23)	>10	5	3	0.60	0.23, 0.88	3	0.60	0.23, 0.88	0	−0.47, 0.47
			>100	5	0	0	0, 0.43	0	0	0, 0.43	0	−0.47, 0.47
			>1000	5	0	0	0, 0.43	0	0	0, 0.43	0	−0.47, 0.47
		Intermediate 1 70 (44, 120)	>10	20	20	1	0.84, 1	20	1	0.84, 1	0	−0.13, 0.13
			>100	20	14	0.70	0.48, 0.85	14	0.70	0.48, 0.85	0	−0.13, 0.13
			>1000	20	2	0.10	0.03, 0.30	2	0.10	0.03, 0.30	0	−0.13, 0.13
		Intermediate 2 1 200 (700, 2300)	>10	20	20	1	0.84, 1	20	1	0.84, 1	0	−0.13, 0.13
			>100	20	20	1	0.84, 1	20	1	0.84, 1	0	−0.13, 0.13
			>1000	20	15	0.75	0.53, 0.89	15	0.75	0.53, 0.89	0	−0.13, 0.13
		High 3 500 (1100, 11000)	>10	5	5	1	0.57, 1	5	1	0.57, 1	0	−0.47, 0.47
			>100	5	5	1	0.57, 1	5	1	0.57, 1	0	−0.47, 0.47
			>1000	5	5	1	0.57, 1	5	1	0.57, 1	0	−0.47, 0.47

[a] From most probable number determination, with 95% confidence interval.
[b] Based on dilution and volume of sample tested. A positive result indicates contamination above the test threshold level.
[c] N = Number of test portions.
[d] x = Number of positive test portions.
[e] POD_{CP} = Candidate method presumptive positive outcomes divided by the total number of trials.
[f] POD_{CC} = Candidate method confirmed positive outcomes divided by the total number of trials.
[g] $dPOD_{CP}$ = Difference between the candidate method presumptive result and candidate method confirmed result POD values.
[h] 95% CI = If the confidence interval of a dPOD does not contain zero, then the difference is statistically significant at the 5% level.

Table 2. Soleris *Enterobacteriaceae* confirmed and ISO 21528-2:2017 reference method results for testing of dried cannabis flower

				Soleris confirmed				ISO 21528-2:2017				
Matrix	Strain	Level, MPN/g[a]	Test threshold, CFU/g[b]	N[c]	x[d]	POD_C[e]	95% CI	x	POD_R[f]	95% CI	$dPOD_C$[g]	95% CI[h]
Dried cannabis flower	*E. coli* ATCC 8739	Low 7.8 (2.0, 23)	>10	5	3	0.60	0.23, 0.88	3	0.60	0.23, 0.88	0	−0.46, 0.46
			>100	5	0	0	0, 0.43	0	0	0, 0.43	0	−0.43, 0.43
			>1000	5	0	0	0, 0.43	0	0	0, 0.43	0	−0.43, 0.43
		Intermediate 1 70 (44, 120)	>10	20	20	1	0.84, 1	20	1	0.84, 1	0	−0.16, 0.16
			>100	20	14	0.70	0.48, 0.85	11	0.55	0.34, 0.74	0.15	−0.14, 0.41
			>1000	20	2	0.10	0.03, 0.30	0	0	0, 0.16	0.10	−0.08, 0.30
		Intermediate 2 1 200 (700, 2300)	>10	20	20	1	0.84, 1	20	1	0.84, 1	0	−0.16, 0.16
			>100	20	20	1	0.84, 1	20	1	0.84, 1	0	−0.16, 0.16
			>1000	20	15	0.75	0.53, 0.89	14	0.70	0.48, 0.85	0.05	−0.22, 0.31
		High 3 500 (1100, 11000)	>10	5	5	1	0.57, 1	5	1	0.57, 1	0	−0.43, 0.43
			>100	5	5	1	0.57, 1	5	1	0.57, 1	0	−0.43, 0.43
			>,000	5	5	1	0.57, 1	5	1	0.57, 1	0	−0.43, 0.43

[a]From most probable number determination, with 95% confidence interval.
[b]Based on dilution and volume of sample tested. A positive result indicates contamination above the test threshold level.
[c]N = Number of test portions.
[d]x = Number of positive test portions.
[e]POD_C = Candidate method confirmed positive outcomes divided by the total number of trials.
[f]POD_R = Reference method confirmed positive outcomes divided by the total number of trials.
[g]$dPOD_C$ = Difference between the candidate method and reference method POD values.
[h]95% CI = If the confidence interval of a dPOD does not contain zero, then the difference is statistically significant at the 5% level.

significant differences between the Soleris confirmed and ISO 21528-2:2017 method results as determined by POD analysis. The requirement for fractional positives was achieved for both intermediate levels: intermediate 1 had fractional positive results at the >100 CFU/g testing threshold and intermediate 2 had fractional positive results at the >1000 CFU/g testing threshold. All negative results were seen for the low level at the >100 CFU/g and >1000 CFU/g testing thresholds, and all positive results were seen for the high level at all three testing thresholds.

No discrepancies were seen between Soleris presumptive and confirmed results. Soleris detection times ranged from 7.7 to 19.8 h. For the 150 samples tested in total, there were 109 positive results with the Soleris method and 103 positive results with the ISO 21528-2:2017 colony count procedure.

Discussion

This independent laboratory study for the Soleris *Enterobacteriaceae* method provided data to support the effectiveness of screening for EBAC in dried cannabis flower (>0.3% THC) at a 10 g portion size. The Soleris EBAC method can provide results within 20–24 h, and the use of pre-prepared vials instead of agar and agar overlays dramatically reduces sample processing time. The use of the "dilute-to-specification" methodology would be useful in the cannabis industry, allowing ease in the adjustment of testing thresholds to adhere to failure limits for specific product types in progressively changing state-mandated testing.

Conclusions

It is recommended that a matrix extension for dried cannabis flower (>0.3% THC) be approved for PTM 121901 for the detection of *Enterobacteriaceae*.

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Conflict of Interest

All authors declare no conflict of interest.

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