AOAC INTERNATIONAL

Official Methods Board

Methods Reviewed by Official Methods Board (2014)

AOAC INTERNATIONAL
2275 Research Blvd., Suite 300
Rockville, MD, 20850
UNITED STATES
dboyd@aoac.org
301.924.7077 x126

2016 AOAC OFFICIAL METHODS BOARD AWARDS

CANDIDATES REVIEWED FOR

2014 METHOD OF THE YEAR

OFFICIAL METHODS OF ANALYSIS OF AOAC INTERNATIONAL

METHOD OF THE YEAR
OMB may select more than one method in this category each year.

Selection Criteria
The minimum criteria for selection are:

a. The method must have been approved for first or final action within the last three years.
b. Generally, some unique or particularly noteworthy aspect of the method is highlighted as making it worthy of the award, such as innovative technology or application, breadth of applicability, critical need, difficult analysis, and/or range of collaborators.
c. The method demonstrates significant merit in scope or is an innovative approach to an analytical problem.

Selection Process:

a. AOAC staff lists all eligible methods for consideration and forwards that list with supporting documentation (e.g. ERP chair recommendation(s)) to the Chair of the Official Methods Board (OMB).
b. The Chair forwards the list along with any supporting information to the members of the OMB.
c. The OMB selects the Method of the Year. The winner is selected by 2/3 vote. If necessary, the OMB chair may cast tie-breaking vote.

Award
An appropriate letter of appreciation and thanks will be sent to the author(s) of the winning method. The corresponding author will be announced at the appropriate session of the AOAC INTERNATIONAL annual meeting, with presentation of an award. All authors will be acknowledged at the annual meeting, will receive an award and a letter of appreciation. The name of the winner(s), with supporting story, will be carried in the announcement in the *ILM*.

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2014 OMB AWARDS – Descriptions of the awards from Board of Directors approved document in 2012.

Single Laboratory Validation of the Year

Selection Criteria

The minimum criteria for selection are:

a. The Single Laboratory Validation (SLV) study manuscript must have been submitted for publication within the last three years.

b. Generally, some unique or particularly noteworthy aspect of one or more single laboratory validation study and method is highlighted as making the study worthy of the award, such as innovative technology or application, breadth of applicability, critical need, difficult analysis, and/or range of collaborators.

c. The study demonstrates significant merit as to the scope of the method or an innovative approach to a difficult analytical problem.

Selection Process:

a. AOAC staff lists all eligible methods for consideration and forwards that list to the Chair of the Official Methods Board (OMB).

b. The Chair forwards the list along with any supporting information to the members of the OMB.

c. The OMB selects the SLV of the Year. The winner is selected by number of votes. If necessary, the OMB chair may cast tie-breaking vote.

Award

An appropriate letter of appreciation and thanks will be sent to the author of the winning SLV. The name of the winner, with supporting story, and names of all nominees, will be carried in the announcement in the *ILM*. The winner will be announced at the appropriate session of the AOAC INTERNATIONAL annual meeting, with presentation of an award.

Multi-Laboratory Study of the Year

Selection Criteria: The minimum criteria for selection are:

a. The multi-laboratory study must have been completed within the past 3 years.

b. Some unique or particularly noteworthy aspect of a multi-laboratory study and method should be highlighted as making the study worthy of the award, such as innovative technology or application, breadth of applicability, critical need, impact, difficult analysis, range of multi-laboratory collaborators, or special handling required for study materials.

Selection Process:

a. AOAC staff lists all eligible multi-laboratory studies for consideration and forwards that list to the Chair of the Official Methods Board (OMB). The OMB solicits the Method Committee Chairs, staff, communities and the Association for evidence in support of the nominees.

b. Written recommendations and supporting information will be submitted to the OMB chair. The information will be distributed to the members of the OMB.

c. The OMB selects the Multi-Laboratory Study of the Year. The winner is selected by number of votes. If necessary, the OMB chair may cast the tie-breaking vote.

Award

An appropriate letter of appreciation and thanks will be sent to the author Study Director of the winning multi-laboratory study. The name of the winner, with supporting story, and names of all nominees, will be carried in the announcement in the *ILM*. The winner will be announced at the appropriate session of the AOAC INTERNATIONAL annual meeting, with presentation of an award.

All multi-laboratory collaborators participating in the winning multi-laboratory study will receive an award and letter of appreciation.

MICROBIOLOGICAL METHODS

Evaluation of VIDAS® UP *Salmonella* (SPT) Assay for the Detection of *Salmonella* in a Variety of Foods and Environmental Samples: Collaborative Study

PATRICK BIRD, KIEL FISHER, MEGAN BOYLE, TRAVIS HUFFMAN, MARC JUENGER, M. JOSEPH BENZINGER, JR, PAIGE BEDINGHAUS, JONATHAN FLANNERY, ERIN CROWLEY, JAMES AGIN, and DAVID GOINS
Q Laboratories, Inc., 1400 Harrison Ave, Cincinnati, OH 45214
RONALD L. JOHNSON[1]
BioMérieux, Inc., 595 Anglum Rd, Hazelwood, MO 63042

Collaborators: M. Achen; J. Adams; M. Bandu; J. Clayborn; L. Drasher; H. Elgaali; W. Fedio; K. Goetz; Y. Huang; J. Jurgens; H. Kohler; D. Kondratko; S. Liles; T. Lonczynski; K. McCallum; J. Mills; S. Montez; J. Nogle; M. Oltman; J. Pickett; J. Rowe; L. Thompson; J. Walia; R. Zapata

The VIDAS® UP *Salmonella* (SPT) uses recombinant phage proteins to detect *Salmonella* species in human and animal food products and production environmental samples after 18–26 h of enrichment. The VIDAS SPT assay is performed with the automated VIDAS or mini-VIDAS instruments. The VIDAS SPT method was compared in a multilaboratory collaborative study to the U.S. Department of Agriculture/Food Safety and Inspection Service-*Microbiology Laboratory Guidebook* (USDA/FSIS-MLG) 4.05 (2011) *Isolation and Identification of Salmonella from Meat, Poultry, Pasteurized Egg and Catfish Products* reference method following the current AOAC guidelines. A total of 15 laboratories representing government, academia, and industry throughout the United States participated. One matrix, raw ground beef, was analyzed using two different test portion sizes, 25 and 375 g. Each test portion was artificially contaminated with *Salmonella* at three inoculation levels, an uninoculated control level (0 CFU/test portion), a low inoculum level (0.2–2 CFU/test portion), and a high inoculum level (2–5 CFU/test portion). In this study, 1656 unpaired replicate samples were analyzed. Of those unpaired replicates, 476 were presumptive positive by the VIDAS method, with 475 confirmed positive by the traditional confirmation procedures and 476 confirmed positive by an alternative confirmation procedure. There were 411 confirmed positive replicates by the USDA/FSIS-MLG reference method. Statistical analysis was conducted according to the probability of detection (POD). For the low-level 375 g test portions, the following dLPOD values, with 95% confidence intervals, were obtained: 0.01 (–0.12, +0.15) for samples confirmed following the traditional confirmation; 0.02 (–0.18, +0.2) for samples confirmed following traditional confirmation on IBISA and ASAP; and 0.03 (–0.18, +0.24) for samples confirmed following the alternative confirmation on IBISA and ASAP. For the low-level 25 g test portions, the following dLPOD values, with 95% confidence intervals, were obtained: 0.41, (0.32, +0.49) for samples confirmed following the traditional confirmation, the traditional confirmation on IBISA and ASAP, and the alternative confirmation on IBISA and ASAP. With 0.0 within the confidence intervals for the 375 g test portions, there was no statistically significant difference in the number of positive samples detected by the VIDAS SPT method and the USDA/FSIS-MLG method at the 0.05 level. For the 25 g test portions, a statistically significant difference was observed between the VIDAS SPT method and the reference method for the low inoculum level, where the VIDAS SPT method recovered a higher number of positive results than the reference method. It is recommended that the VIDAS SPT method with the optional ASAP and IBISA agar confirmation method be adopted for Official First Action status for the detection of *Salmonella* in a variety of foods and environmental samples.

Salmonellosis, the foodborne illness caused by the bacterium *Salmonella,* has been linked to numerous foodborne outbreaks associated with a wide range of products, such as meat, poultry, eggs, dairy products, fresh produce, spices, sauces, peanut butter, and chocolate (1). Taking up to 5 days to confirm, the detection of *Salmonella* species can be time-consuming and expensive for food manufacturers. With more than 2500 different serovars, *Salmonella* are antigenically complex due to variations in their lipopolysaccharide and flagellar protein antigens (1). The VIDAS UP *Salmonella* (SPT) assay, an automated enzyme phage-ligand-based assay for the detection of *Salmonella* in food and environmental samples, uses recombinant phage proteins to detect both motile and

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The recommendation was approved by the Methods Committee on Microbiology as First Action.
[1] Corresponding author's e-mail: ron.johnson@biomerieux.com
Appendixes are available on the *J. AOAC Int.* website, http://aoac.publisher.ingentaconnect.com/content/aoac/jaoac
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nonmotile *Salmonella.* The assay is performed in the automated VIDAS instrument.

The VIDAS SPT assay uses a primary enrichment (prewarmed to 42±1°C for 375 g samples), along with a proprietary supplement (SPT supplement). After 18–24 h of enrichment (22–26 h for 375 g samples), *Salmonella* detection is performed by the VIDAS SPT test. The new enrichment method eliminates the need for secondary enrichments [Tetrathionate Hanja (TTH), Rappaport-Vasilliadis (RV), and SX2 broths]. Negative results and presumptive positive results are available the day after enrichment.

Prior to the collaborative study, the VIDAS SPT method was validated according to AOAC guidelines for harmonized *Performance Tested Method*^{SM} (PTM) studies (2). The purpose of this study was to demonstrate that the VIDAS SPT method could detect *Salmonella* in a variety of foods and environmental surfaces as claimed by the manufacturer. For the VIDAS SPT PTM evaluation, 17 matrixes were tested using buffered peptone water (BPW) plus *Salmonella* supplement enrichment protocol: raw ground beef (25 and 375 g), processed American cheese (25 g), deli roast beef (25 g), liquid egg (25 g), peanut butter (25 g), vanilla ice cream (25 g), cooked shrimp (25 g), raw cod (25 g), bagged lettuce (25 and 375 g), dark chocolate (375 g), powdered eggs (25 g), instant nonfat dry milk (25 and 375 g), ground black pepper (25 g), dry dog food (375 g), and stainless steel, plastic, and ceramic environmental surfaces. In a matrix extension evaluation conducted in February 2012, three additional foods were evaluated using BPW plus *Salmonella* supplement enrichment protocol: raw ground turkey (375 g), almonds (375 g), and chicken carcass rinsates (30 mL). One matrix, raw ground beef (375 g), was evaluated using a different enrichment protocol, BPW plus vancomycin, to allow for a single enrichment when the VIDAS SPT and *E. coli* Phage Technology (bioMérieux) assays were used.

All other PTM parameters (inclusivity, exclusivity, ruggedness, stability, and lot-to-lot variability) tested in the PTM studies satisfied the performance requirements for PTM approval. The method was awarded PTM certification No. 071101 on July 15, 2011 with a matrix extension approval on March 23, 2012.

This collaborative study compared the VIDAS SPT method to the U.S. Department of Agriculture/Food Safety and Inspection Service-*Microbiology Laboratory Guidebook* (USDA/FSIS-MLG) 4.05 (2011) *Isolation and Identification of Salmonella from Meat, Poultry, Pasteurized Egg and Catfish Products* method (3) for raw ground beef at two test portion sizes, 25 and 375 g.

Collaborative Study

Study Design

For this collaborative study, one matrix, raw ground beef (80% lean), was analyzed using two different test portion sizes, 25 and 375 g. The raw ground beef was obtained from local retailers and screened for the absence of *Salmonella* by the USDA/FSIS-MLG reference method prior to analysis. The screening indicated an absence of indigenous *Salmonella*. For analysis of the 25 g test portions, the raw ground beef was artificially contaminated with *Salmonella* Enteritidis ATCC 13076 and with *Salmonella* Montevideo ATCC 8387 for the analysis of the 375 g test portions. There were two inoculation levels: a high inoculation level of approximately 2–5 CFU/test portion and a low inoculation level of approximately 0.2–2 CFU/test portion. A set of uninoculated control test portions were also included for each matrix at 0 CFU/test portion.

Twelve replicate samples from each of the three inoculation levels of product were analyzed. Two sets of samples (72 total) were sent to each laboratory for analysis by VIDAS SPT and the USDA/FSIS-MLG reference method due to different sample enrichments for each method. For both test portion sizes, collaborators were sent an additional 30 g test portion and instructed to conduct a total aerobic plate count on the day samples were received in order to determine the total aerobic microbial load in the matrix.

A detailed collaborative study packet outlining all necessary information related to the study, including media preparation, method-specific test portion preparation, and documentation of results, was sent to each collaborating laboratory before initiation of the study.

Preparation of Inocula and Test Portions

The *Salmonella* cultures used in this evaluation were propagated in 10 mL Brain Heart Infusion broth from a frozen stock culture held at –70°C at Q Laboratories, Inc. The broth was incubated for 18–24 h at 35±1°C. Appropriate dilutions were prepared based on previously established growth curves for both low and high inoculation levels, resulting in fractional positive outcomes for at least one level. For both test portion sizes, a bulk lot of the raw ground beef was inoculated with a liquid inoculum and mixed thoroughly by hand-kneading to ensure even distribution of microorganisms. The raw ground beef was inoculated on the day of shipment so that all test portions would have been held for 96 h by the day testing was initiated. For the analysis of the 25 g test portions, the bulk lot of test material was divided into 30 g portions for shipment to the collaborators. For the analysis of the 375 g test portions, 25 g of inoculated test product was mixed with 350 g of uninoculated test product for shipment to the collaborators for analysis by the VIDAS SPT method. Collaborators received 30 g portions for analysis by the USDA/FSIS-MLG method. To determine the level of *Salmonella* spp. in the raw ground beef, a five-tube MPN was conducted on the day of initiation of analysis. From both the high and low inoculated batches of raw ground beef, five 100 g test portions, five 25 g test portions, and five 10 g test portions were analyzed using a 1:10 dilution with BPW. The most probable number (MPN) and 95% confidence intervals were calculated from the high, medium, and low levels using the AOAC MPN Calculator (www.lcftld.com/customer/LCFMPNCalculator.exe; 4).

Confirmation of the samples was conducted according to the USDA/FSIS-MLG 4.05 reference method.

Test Portion Distribution

All samples were labeled with a randomized, blind-coded, three-digit number affixed to the sample container. Test portions were shipped on a Thursday via overnight delivery according to the Category B Dangerous Goods shipment regulations set forth by the International Air Transport Association. Upon receipt, samples were held by the collaborating laboratory at refrigeration

Table 2013.01A. Summary of results for the detection of *Salmonella* spp. in raw ground beef (25 g)

Method[a]	VIDAS SPT with traditional confirmation on BGSA and XLT4			VIDAS SPT with traditional confirmation on IBISA and ASAP[b]			VIDAS SPT with alternative confirmation on IBISA and ASAP[c]		
Inoculation level	Uninoculated	Low	High	Uninoculated	Low	High	Uninoculated	Low	High
Candidate presumptive positive/total samples analyzed	0/144	144/144	144/144	0/144	144/144	144/144	0/144	144/144	144/144
Candidate presumptive POD (CP)	0.00 (0.00, +0.03)	1.00 (+0.97, +1.00)	1.00 (+0.97, +1.00)	0.00 (0.00, +0.03)	1.00 (+0.97, +1.00)	1.00 (+0.97, +1.00)	0.00 (0.00, +0.03)	1.00 (+0.97, +1.00)	1.00 (+0.97, +1.00)
s_r[d]	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)
s_L[e]	0.00 (0.00 +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)
s_R[f]	0.00 (0.00, +0.22)	0.00 (0.00, +0.22)	0.00 (0.00, +0.22)	0.00 (0.00, +0.22)	0.00 (0.00, +0.22)	0.00 (0.00, +0.22)	0.00 (0.00, +0.22)	0.00 (0.00, +0.22)	0.00 (0.00, +0.22)
P-value	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
Candidate confirmed positive/total samples analyzed	0/144	143/144	144/144	0/144	143/144	144/144	0/144	143/144	144/144
Candidate confirmed POD (CC)	0.00 (0.00, +0.03)	0.99 (+0.96, +1.00)	1.00 (+0.97, +1.00)	0.00 (0.00, +0.03)	0.99 (+0.96, +1.00)	1.00 (+0.97, +1.00)	0.00 (0.00, +0.03)	0.99 (+0.96, +1.00)	1.00 (+0.97, +1.00)
s_r	0.00 (0.00, +0.16)	0.08 (+0.07, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.08 (+0.07, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.08 (+0.07, +0.16)	0.00 (0.00, +0.16)
s_L	0.00 (0.00, +0.16)	0.00 (0.00, +0.03)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.03)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.03)	0.00 (0.00, +0.16)
s_R	0.00 (0.00, +0.22)	0.08 (+0.08, +0.10)	0.00 (0.00, +0.22)	0.00 (0.00, +0.22)	0.08 (+0.08, +0.10)	0.00 (0.00, +0.22)	0.00 (0.00, +0.22)	0.08 (+0.08, +0.10)	0.00 (0.00, +0.22)
P-value	1.0000	0.4368	1.0000	1.0000	0.4368	1.0000	1.0000	0.4368	1.0000
Positive reference samples/total samples analyzed	0/144	84/144	138/144	0/144	84/144	138/144	0/144	84/144	138/144
Reference POD	0.00 (0.00, +0.03)	0.58 (+0.50, +0.67)	0.96 (+0.91, +0.98)	0.00 (0.00, +0.03)	0.58 (+0.50, +0.67)	0.96 (+0.91, +0.98)	0.00 (0.00, +0.03)	0.58 (+0.50, +0.67)	0.96 (+0.91, +0.98)
s_r	0.00 (0.00, +0.16)	0.50 (+0.45, +0.52)	0.19 (+0.17, +0.22)	0.00 (0.00, +0.16)	0.50 (+0.45, +0.52)	0.19 (+0.17, +0.22)	0.00 (0.00, +0.16)	0.50 (+0.45, +0.52)	0.19 (+0.17, +0.22)
s_L	0.00 (0.00, +0.16)	0.00 (0.00, +0.18)	0.06 (+0.02, +0.13)	0.00 (0.00, +0.16)	0.00 (0.00, +0.18)	0.06 (+0.02, +0.13)	0.00 (0.00, +0.16)	0.00 (0.00, +0.18)	0.06 (+0.02, +0.13)
s_R	0.00 (0.00, +0.22)	0.50 (+0.45, +0.52)	0.20 (+0.18, +0.24)	0.00 (0.00, +0.22)	0.50 (+0.45, +0.52)	0.20 (+0.18, +0.24)	0.00 (0.00, +0.22)	0.50 (+0.45, +0.52)	0.20 (+0.18, +0.24)
P-value	1.0000	0.6298	0.0179	1.0000	0.6298	0.0179	1.0000	0.6298	0.0179
dLPOD (candidate vs reference)	0.00 (–0.03, +0.03)	0.41 (+0.32, +0.49)	0.04 (0.01, +0.09)	0.00 (–0.03, +0.03)	0.41 (+0.32, +0.49)	0.04 (+0.01, +0.09)	0.00 (–0.03, +0.03)	0.41 (+0.32, +0.49)	0.04 (+0.01, +0.09)
dLPOD (candidate presumptive vs candidate confirmed)	0.00 (–0.03, +0.03)	0.01 (–0.02, +0.04)	0.00 (–0.03, +0.03)	0.00 (–0.03, +0.03)	0.01 (–0.02, +0.04)	0.00 (–0.03, +0.03)	0.00 (–0.03, +0.03)	0.01 (–0.02, +0.04)	0.00 (–0.03, +0.03)

[a] Results include 95% confidence intervals.

[b] Traditional confirmation on ASAP/IBISA = secondary enrichments streaked onto IBISA and ASAP.

[c] Alternative confirmation = direct streak of the primary enrichment onto IBISA and ASAP.

[d] Repeatability standard deviation.

[e] Among-laboratory standard deviation.

[f] Reproducibility standard deviation.

temperature (3–5°C) until the following Monday when analysis was initiated. All samples were packed with cold packs to target a temperature of <7°C during shipment. In addition to each of the test portions and the total plate count replicate, collaborators also received a test portion for each matrix labeled "temperature control." Participants were instructed to obtain the temperature of this portion upon receipt of the package, document results on the Sample Receipt Confirmation form provided, and fax to the Study Director.

Test Portion Analysis

Collaborators followed the appropriate preparation and analysis protocol according to the method for each test portion size. For both test portion sizes, each collaborator received 72 test portions of each food product (12 high, 12 low, and 12 controls for each evaluation). For the analysis of the 25 g test portions by the VIDAS SPT method, a 25 g portion was enriched with 225 mL BPW and homogenized for 2 min. *Salmonella* supplement (1 mL) was added to the enrichment, and the test portions were incubated for 18–24 h at 42 ± 1°C. For the 375 g test potions analyzed by the VIDAS SPT method, a 375 g portion was enriched with 1125 mL prewarmed (42 ± 1°C) bioMérieux BPW and homogenized for 2 min. *Salmonella* supplement (5 mL) was added to the enrichment, and test portions were incubated for 22–26 h at 42 ± 1°C.

After enrichment, samples were assayed by the VIDAS SPT method and confirmed using procedures outlined in the standard reference method by transferring an aliquot of the primary enrichment to secondary selective enrichment broths, TTH and RV. After incubation of the secondary selective enrichments, samples were struck to the selective agars specified in the USDA/FSIS-MLG and to two proprietary chromogenic agars, ASAP and IBISA. Presumptive positive samples from each agar were confirmed following the biochemical and serological procedures outlined in the USDA/FSIS-MLG.

An alternative confirmation for all VIDAS SPT samples was conducted by directly streaking an aliquot from the primary enrichment of each test portion to ASAP and IBISA chromogenic agar. Presumptive positive samples from each agar were confirmed following biochemical and serological procedures outlined in the USDA/FSIS-MLG method.

Both test portion sizes analyzed by the VIDAS SPT methods were compared to samples (25 g) analyzed using the USDA/FSIS-MLG reference method in an unpaired study design. Test portions of 25 g were enriched in BPW, homogenized for 2 min, and incubated at 35 ± 2°C for 24 ± 2 h. Samples were transferred to selective secondary enrichments and streaked to agars specified in the USDA/FSIS-MLG method. All positive test portions were biochemically confirmed by the API 20E biochemical test, AOAC *Official Method* **978.24** or the VITEK GN identification test, AOAC *Official Method* **2011.17**. Serological testing was also performed.

Statistical Analysis

Each collaborating laboratory recorded results for the reference method, VIDAS SPT results, and the results for both the traditional and alternative confirmation of the VIDAS SPT samples on the data sheets provided. The data sheets were submitted to the Study Director at the end of each week of testing for analysis. The results of each test portion for each sample were compiled by the Study Director, and the qualitative VIDAS SPT results were compared to the reference method for statistical analysis. Data for each test portion size were analyzed using the probability of detection (POD) statistical model (5). If the confidence interval of a dLPOD did not contain zero, that would indicate a statistically significant difference between the VIDAS SPT method and the USDA/FSIS-MLG reference method at the 5% probability level.

AOAC Official Method 2013.01
Salmonella in a Variety of Foods
VIDAS® UP *Salmonella* (SPT) Method
First Action 2013

[Applicable to detection of *Salmonella* in raw ground beef (25 and 375 g), processed American cheese (25 g), deli roast beef (25 g), liquid egg (25 g), peanut butter (25 g), vanilla ice cream (25 g), cooked shrimp (25 g), raw cod (25 g), bagged lettuce (25 and 375 g), dark chocolate (375 g), powdered eggs (25 g), instant nonfat dry milk (25 and 375 g), ground black pepper (25 g), dry dog food (375 g), raw ground turkey (375 g), almonds (375 g), chicken carcass rinsates (30 mL), and stainless steel, plastic, and ceramic environmental surfaces.]

See Tables **2013.01A** and **B** for a summary of results of the interlaboratory study. For detailed results of the interlaboratory study, *see* Tables A–F in Appendix 1 on *J. AOAC Int.* website, http://aoac.publisher.ingentaconnect.com/content/aoac/jaoac).

A. Principle

The VIDAS SPT method is for use on the automated VIDAS instrument for the detection of *Salmonella* receptors using the enzyme-linked fluorescent assay. The solid-phase receptacle (SPR) serves as the solid phase, as well as the pipetting device. The interior of the SPR is coated with proteins specific for *Salmonella* receptors. Reagents for the assay are ready-to-use and predispensed in the sealed reagent strips. The instrument performs all the assay steps automatically. The reaction medium is cycled in and out of the SPR several times. An aliquot of enrichment broth is dispensed into the reagent strip. The *Salmonella* receptors present will bind to the interior of the SPR. Unbound components are eliminated during the washing steps. The proteins conjugated to the alkaline phosphatase are cycled in and out of the SPR and will bind to any *Salmonella* receptors, which are themselves bound to the SPR wall. A final wash step removes unbound conjugate. During the final detection step, the substrate (4-methylumbelliferyl phosphate) is cycled in and out of the SPR. The conjugate enzyme catalyzes the hydrolysis of the substrate into a fluorescent product (4-methylumbelliferone), the fluorescence of which is measured at 450 nm. At the end of the assay, results are automatically analyzed by the instrument which calculates a test value for each sample. This value is then compared to internal references (thresholds) and each result is interpreted as positive or negative.

B. Apparatus and Reagents

Items (**a**)–(**h**) are available as the VIDAS SPT assay kit from bioMérieux Inc., Hazelwood, MO.

(**a**) *VIDAS or miniVIDAS automated immunoassay system.*

(**b**) *SPT reagent strips.*—60 polypropylene strips of 10 wells, each strip covered with a foil seal and label. The 10 wells contain the reagents in Table **2013.01**.

(**c**) *SPR.*—60 SPRs coated with proteins specific for *Salmonella* receptors.

(**d**) *Standard.*—One vial (6 mL). Contains purified and inactivated *Salmonella* receptors + preservative + protein stabilizer.

(**e**) *Positive control solution.*—One vial (6 mL). Contains purified and inactivated *Salmonella* receptors + preservative + protein stabilizer.

(**f**) *Negative control solution.*—One vial (6 mL). Contains Tris-buffered saline (150 mmol/L)–Tween pH 7.6 + preservative.

(**g**) *Master lot entry (MLE) card.*—One card providing specifications for the factory master data required to calibrate the test.

(**h**) *Package insert*.

(**i**) *Disposable pipet to dispense appropriate volumes.*

(**j**) *VIDAS Heat and Go.*—Available from bioMérieux, Inc.

(**k**) *Water bath (95–100°C) or equivalent system.*

(**l**) *Stomacher®-type bag with filter.*

(**m**) *Stomacher.*—Stomacher Lab Blender 400, available from Seward Medical (London, UK); Smasher, bioMérieux, Inc., or equivalent.

(**n**) *BPW.*—Available from bioMérieux, Inc.

(**o**) *Salmonella supplement.*—Available from bioMérieux, Inc.

(**p**) *Incubators.*—Capable of maintaining 42±1°C and 35±1°C.

(**q**) *Diagnostic reagents.*—Necessary for culture confirmation of assays. *See* AOAC *Official Method* **967.27**.

(**r**) *IBISA chromogenic agar.*—Necessary for cultural confirmation as an alternative to selective agar required by appropriate reference method. Available from bioMérieux, Inc.

(**s**) *ASAP chromogenic agar.*—Necessary for cultural confirmation as an alternative to selective agar required by appropriate reference method. Available from bioMérieux, Inc.

(**t**) *Vancomycin.*—Available from bioMérieux, Inc.

C. General Instructions

(**a**) Components of the kit are intended for use as integral unit. Do not mix reagents or disposables of different lot numbers.

(**b**) Store VIDAS SPT kits at 2–8°C.

(**c**) Do not freeze reagents.

(**d**) Bring reagents to room temperature before inserting them into the VIDAS instrument.

(**e**) Mix standard, controls, and heated test portions well before using.

(**f**) Include one positive and one negative control with each group of tests.

(**g**) Return unused components to 2–8°C immediately after use.

(**h**) *See* safety precautions in the VIDAS SPT package insert (refer to the following sections in the package insert: Warnings and Precautions and Waste Disposal).

D. Preparation of Test Suspension

(**a**) *Pre-enrichment.*—Pre-enrich test portion in BPW using filter Stomacher bags to initiate growth of *Salmonella.* For 25 g test portions, add 225 mL BPW to each test portion and homogenize thoroughly for 2 min. For 375 g test portions, pre-warm BPW to 42±1°C, add 1125 mL to each test portion, and homogenize thoroughly for 2 min.

(**b**) After homogenization add *Salmonella* supplement to each test portion. For 25 g test portions, add 1 mL of *Salmonella* supplement, mix samples manually, and incubate for 18–24 h at 42±1°C. For 375 g test portions, add 5 mL of *Salmonella* supplement, mix samples manually, and incubate for 22–26 h at 42±1°C.

(**c**) After incubation, homogenize samples manually. If a water bath is used, transfer 2–3 mL enrichment broth into a tube. Seal the tube. Heat for 5±1 min at 95–100°C. Cool the tube. Mix the boiled broth and transfer 0.5 mL into the sample well of the VIDAS SPT reagent strip. If the VIDAS Heat and Go is used, transfer 0.5 mL of the enrichment broth into the sample well of the VIDAS SPT reagent strip. Heat for 5±1 min (*see* VIDAS Heat and Go User's Manual). Remove the strip and allow to cool for 10 min prior to test initiation. Perform the VIDAS test.

E. Enzyme Immunoassay

(**a**) Enter factory master calibration curve data into the instrument using the MLE card.

(**b**) Remove the kit reagents and materials from refrigerated storage and allow them to come to room temperature.

(**c**) Use one VIDAS SPT reagent strip and one VIDAS SPT SPR for each sample, control, or standard to be tested. Reseal the storage pouch after removing the required number of SPRs.

(**d**) Enter the appropriate assay information to create a work list. Enter the test code by typing or selecting "SPT," and number of tests to be run. If the standard is to be tested, identify the standard by "S1" and test in duplicate. If the positive control is to be tested, identify it by "C1." If the negative control is to be tested, identify it by "C2."

Note: The standard must be tested upon receipt of a new lot of reagents and then every 14 days. The relative fluorescence value (RFV) of the standard must fall within the set range provided with the kit.

(**e**) Load the SPT reagents strips and SPRs into the positions that correspond to the VIDAS section indicated by the work list. Verify that the color labels with the assay code on the SPRs and reagent strips match.

(**f**) Initiate the assay processing as directed in the VIDAS operator's manual.

(**g**) After the assay is completed, remove the SPRs and reagent strips from the instrument and dispose of properly.

F. Results and Interpretation

The results are analyzed automatically by the VIDAS system. A report is printed which records the type of test performed, test sample identification, date and time, lot number, and expiration date of the reagent kit being used, each sample's RFV**,** test value, and interpreted result (positive or negative). Fluorescence is measured twice in the reagent strip's reading cuvette for each sample tested. The first reading is a background reading of the substrate cuvette before the SPR is introduced into the substrate. The second reading is taken after incubating the substrate with the enzyme remaining on the interior of

the SPR. The test value is calculated by the instrument and is equal to the difference between the background reading and the final reading. The calculation appears on the result sheet. A negative result has a test value less than the threshold (0.25) and indicates that the sample does not contain *Salmonella* spp. or contains *Salmonella* spp. at a concentration below the detection limit. A positive result has a test value equal to or greater than the threshold (≥0.25) and indicates that the sample may be contaminated with *Salmonella* spp. If the background reading is above a predetermined cutoff, then the result is reported as invalid (Table **2012.01D**).

G. Confirmation

All positive VIDAS SPT results must be culturally confirmed. Confirmation should be performed using the non-heated enrichment broth stored between 2 and 8°C, and should be initiated within 72 h after the end of incubation at 42 ± 1°C. Presumptive positive results may be confirmed by isolating on selective agar plates such as IBISA or ASAP, or on the appropriate reference method selective agar plates. Typical or suspect colonies from each plate are confirmed as described in AOAC *Official Method* **967.27**. As an alternative to the conventional tube system for *Salmonella*, any AOAC-approved commercial biochemical kits may be used for presumptive generic identification of foodborne *Salmonella* as described in AOAC *Official Methods* **978.24**, **989.12**, **991.13**, and **2011.17**.

Results of Collaborative Study

In this collaborative study, the VIDAS SPT method was compared to the to the USDA/FSIS-MLG reference method for one food product, raw ground beef, at two different test portion sizes, 25 and 375 g. A total of 15 laboratories throughout the United States participated in this study, with 14 submitting data for each matrix, as presented in Table 1. Each laboratory analyzed 36 test portions for each method: 12 inoculated with a high level of *Salmonella*, 12 inoculated with a low level, and 12 uninoculated controls. For each test portion size, the actual level of *Salmonella* was determined by MPN determination on the day of initiation of analysis. Individual laboratory and sample results are presented in Tables 2–5. Tables **2013.01A** and **B** summarize the interlaboratory results for all foods tested, including POD statistical analysis (6). Detailed results for each laboratory are presented in Tables A–F of the Appendix.

Raw Ground Beef (25 g Test Portions)

Raw ground beef test portions were inoculated at a low and high level, and analyzed (Tables 2 and 3) for the detection of *Salmonella* spp. Uninoculated controls were included in each analysis. Fourteen laboratories participated in the analysis of this matrix, and the results of 12 were included in the statistical analysis. Laboratory 8 reported that it was unable to confirm samples via serological testing and indicated that it did not conduct the alternative confirmation of the VIDAS SPT samples. Therefore, its results were not included in statistical analysis. Laboratory 12 produced a low-level presumptive positive result for one of its uninoculated control test portions, which could not be confirmed positive by the traditional reference method. Therefore, its results were not included in the statistical analysis. The MPNs obtained for this matrix, with 95% confidence intervals, were 1.10 CFU/test portion (0.49, 2.46) for the low inoculum level and 4.38 CFU/test portion (1.71, 11.20) for the high inoculum level.

Traditional Confirmation with Xylose-Lysine-Tergitol 4 (XLT4) and Brilliant Green Sulfa (BGS)

For the high inoculum level, all of the 144 test portions were reported positive by the VIDAS SPT method, with all portions confirming positive. For the low inoculum level, all 144 test portions were also reported as positive by the VIDAS SPT method, with 143 confirming positive, indicating one false unconfirmed positive result (Laboratory 6). For the uninoculated controls, none of the 144 samples produced a presumptive positive result by the VIDAS SPT method, and all samples confirmed negative. For test portions analyzed by the USDA/FSIS-MLG method, 138 out of 144 high and 84 out of 144 low inoculum test portions confirmed positive. For the uninoculated controls, none of the 144 test portions confirmed positive.

For the low-level inoculum, a $dLPOD_C$ value of 0.41 (+0.32, +0.49) was obtained between the USDA/FSIS-MLG method and the VIDAS SPT method. The confidence intervals obtained for $dLPOD_C$ indicated a statistically significant difference between the two methods. However, the VIDAS SPT method detected more positive samples than the USDA/FSIS-MLG reference method, indicating a higher level of sensitivity than the reference method. A $dLPOD_{CP}$ of 0.01 (–0.02, +0.04) was obtained between presumptive and confirmed VIDAS SPT results for both confirmation procedures. The confidence intervals obtained for $dLPOD_{CP}$ indicated no significant difference between the presumptive and confirmed results.

For the high-level inoculum, a $dLPOD_C$ value of 0.04 (+0.01, +0.09) was obtained between the USDA/FSIS-MLG method and the VIDAS SPT method. The confidence intervals obtained for $dLPOD_C$ indicated a statistically significant difference between the two methods. However, the VIDAS SPT method detected more positive samples than the USDA/FSIS-MLG reference method, indicating a higher level of sensitivity than the reference method. A $dLPOD_{CP}$ of 0.00 (–0.03, +0.03) was obtained between presumptive and confirmed VIDAS SPT results. The confidence intervals obtained for $dLPOD_{CP}$ indicated no significant difference between the presumptive and confirmed results. Results of the POD statistical analysis are presented in Table **2013.01A**, and in appended Table A and Figure 1A and B.

Traditional Confirmation with IBISA and ASAP

For the high inoculum level, all 144 test portions were reported as positive by the VIDAS SPT method, with all confirming positive. For the low inoculum level, all 144 test portions were also reported as positive by the VIDAS SPT method, with 143 confirming positive. For the uninoculated controls, none of the 144 samples produced a presumptive positive result by the VIDAS SPT method with all samples confirming negative. For test portions analyzed by the USDA/FSIS-MLG method, 138 of the 144 high inoculum test portions and 84 out of 144 low inoculum test portions confirmed positive. For the uninoculated controls, none of the 144 test portions confirmed positive.

For the low-level inoculum, a $dLPOD_C$ value of 0.41 (+0.32,

Table 2013.01B. Summary of results for the detection of *Salmonella* spp. in raw ground beef (375 g)

Method[a]	VIDAS SPT with traditional confirmation on BGSA and XLT4			VIDAS SPT with traditional confirmation on IBISA and ASAP[b]			VIDAS SPT with alternative confirmation on IBISA and ASAP[c]		
Inoculation level	Uninoculated	Low	High	Uninoculated	Low	High	Uninoculated	Low	High
Candidate presumptive positive/total samples analyzed	0/132	58/131	130/132	0/132	58/131	130/132	0/132	57/131	130/132
Candidate presumptive POD (CP)	0.00 (0.00, +0.03)	0.44 (+0.34, +0.55)	0.98 (+0.95, +1.00)	0.00 (0.00, +0.03)	0.44 (+0.34, +0.55)	0.98 (+0.95, +1.00)	0.00 (0.00, +0.03)	0.44 (+0.33, +0.54)	0.98 (+0.965, +1.00)
s_r[d]	0.00 (0.00, +0.16)	0.49 (+0.43, +0.52)	0.12 (+0.11, +0.16)	0.00 (0.00, +0.16)	0.49 (+0.43, +0.52)	0.12 (+0.11, +0.16)	0.00 (0.00, +0.16)	0.49 (+0.44. +0.52)	0.12 (+0.11, +0.16)
s_L[e]	0.00 (0.00, +0.16)	0.10 (0.00, +0.27)	0.00 (0.00, +0.05)	0.00 (0.00, +0.16)	0.10 (0.00, +0.27)	0.00 (0.00, +0.05)	0.00 (0.00, +0.16)	0.09 (0.00, +0.26)	0.00 (0.00, +0.05)
s_R[f]	0.00 (0.00, +0.23)	0.50 (+0.44, +0.52)	0.12 (+0.11, +0.14)	0.00 (0.00, +0.23)	0.50 (+0.44, +0.52)	0.12 (+0.11, +0.14)	0.00 (0.00, +0.23)	0.50 (+0.45, +0.52)	0.12 (+0.11, +0.14)
P-value	1.0000	0.1551	0.5190	1.0000	0.1551	0.5190	1.0000	0.1906	0.5190
Candidate confirmed positive/total samples analyzed	0/132	58/131	130/132	0/132	59/131	130/132	0/132	58/131	130/132
Candidate confirmed POD (CC)	0.00 (0.00, +0.03)	0.44 (+0.34, +0.55)	0.98 (+0.95, +1.00)	0.00 (0.00, +0.03)	0.45 (+0.35, +0.55)	0.98 (+0.95, +1.00)	0.00 (0.00, +0.03)	0.44 (+0.34, +0.55)	0.98 (+0.95, +1.00)
s_r	0.00 (0.00, +0.16)	0.49 (+0.43, +0.52)	0.12 (+0.11, +0.16)	0.00 (0.00, +0.16)	0.49 (+0.44, +0.52)	0.12 (+0.11, +0.16)	0.00 (0.00, +0.16)	0.49 (+0.43, +0.52)	0.12 (+0.11, +0.16)
s_L	0.00 (0.00, +0.16)	0.10 (0.00, +0.27)	0.00 (0.00, +0.05)	0.00 (0.00, +0.16)	0.09 (0.00, +0.25)	0.00 (0.00, +0.05)	0.00 (0.00, +0.16)	0.10 (0.00, +0.27)	0.00 (0.00, +0.05)
s_R	0.00 (0.00, +0.23)	0.50 (+0.45, +0.52)	0.12 (0.11, +0.14)	0.00 (0.00, +0.23)	0.50 (+0.45, +0.52)	0.12 (+0.11, +0.14)	0.00 (0.00, +0.23)	0.50 (+0.45, +0.52)	0.12 (+0.11, +0.14)
P-value	1.0000	0.1551	0.5190	1.0000	0.2060	0.5190	1.0000	0.1551	0.5190
Positive reference samples/total samples analyzed	0/132	57/132	132/132	0/132	57/132	132/132	0/132	54/132	131/132
Reference POD	0.00 (0.00, +0.03)	0.43 (+0.35, +0.52)	1.00 (+0.97, +1.00)	0.00 (0.00, +0.03)	0.43 (+0.35, +0.52)	1.00 (+0.97, +1.00)	0.00 (0.00, +0.03)	0.41 (+0.32, +0.50)	0.99 (+0.96, +1.00)
s_r	0.00 (0.00, +0.16)	0.50 (+0.45, +0.52)	0.00 (0.00, +0.17)	0.00 (0.00, +0.16)	0.50 (+0.45, +0.52)	0.00 (0.00, +0.17)	0.00 (0.00, +0.16)	0.49 (+0.44, +0.52)	0.09 (+0.08, +0.16)
s_L	0.00 (0.00, +0.16)	0.00 (0.00, +0.18)	0.00 (0.00, +0.17)	0.00 (0.00, +0.16)	0.00 (0.00, +0.18)	0.00 (0.00, +0.17)	0.00 (0.00, +0.16)	0.05 (0.00, +0.22)	0.00 (0.00, +0.04)
s_R	0.00 (0.00, +0.23)	0.50 (+0.45, +0.52)	0.00 (0.00, +0.23)	0.00 (0.00, +0.23)	0.50 (+0.45, +0.52)	0.00 (0.00, +0.23)	0.00 (0.00, +0.23)	0.49 (+0.44, +0.52)	0.09 (+0.08, +0.10)
P-value	1.0000	0.6261	1.0000	1.0000	0.6261	1.0000	1.0000	0.3313	0.4338
dLPOD (C vs R)	0.00 (–0.03, +0.03)	0.01 (–0.12, +0.15)	–0.02 (–0.05, +0.02)	0.00 (–0.03, +0.03)	0.02 (–0.18, +0.22)	–0.02 (–0.05, +0.02)	0.00 (–0.03, +0.03)	0.03 (–0.18, +0.24)	–0.01 (–0.05, +0.03)
dLPOD (CP vs CC)	0.00 (–0.03, +0.03)	0.00 (–0.15, +0.15)	0.00 (–0.04, +0.04)	0.00 (–0.03, +0.03)	–0.01 (–0.15, +0.14)	0.00 (–0.04, +0.04)	0.00 (–0.03, +0.03)	–0.01 (–0.21, +0.23)	0.00 (–0.04, +0.04)

[a] Results include 95% confidence intervals.

[b] Traditional confirmation on ASAP/IBISA = secondary enrichments streaked onto IBISA and ASAP.

[c] Alternative confirmation = direct streak of the primary enrichment onto IBISA and ASAP.

[d] Repeatability standard deviation.

[e] Among-laboratory standard deviation.

[f] Reproducibility standard deviation.

Table 2013.01C. Reagents included in 10-well reagent strip

Wells	Reagents (SPT)
1	Sample well: 0.5 mL of enrichment broth, standard or control
2	Prewash solution (400 µL): Buffer pH 7.8 + preservative
3–5, 7–9	Wash buffer (600 µL): TRIS-buffered saline (150 mmol/L) – Tween pH 7.6 + preservative
6	Conjugate (400 µL): alkaline phosphatase-labeled proteins specific for *Salmonella* receptors + preservative
10	Reading cuvette with substrate (300 µL): 4-methyl-umbelliferyl phosphate (0.6 mmol/L) + diethanolamine[a] (DEA; 0.62 mol/L or 6.6%, pH 9.2) + preservative

[a] Irritant reagent; *see* VIDAS SPT package insert for more information.

+0.49) was obtained between the USDA/FSIS-MLG method and the VIDAS SPT method. The confidence intervals obtained for $dLPOD_C$ indicated a statistically significant difference between the two methods. However, the VIDAS SPT method detected more positive samples than the USDA/FSIS-MLG reference method, indicating a higher level of sensitivity than the reference method. A $dLPOD_{CP}$ of 0.01 (–0.02, +0.04) was obtained between presumptive and confirmed VIDAS SPT results for both confirmation procedures. The confidence intervals obtained for $dLPOD_{CP}$ indicated no significant difference between the presumptive and confirmed results.

For the high-level inoculum, a $dLPOD_C$ value of 0.04 (+0.01, +0.09) was obtained between the USDA/FSIS-MLG method and the VIDAS SPT method. The confidence intervals obtained for $dLPOD_C$ indicated a statistically significant difference between the two methods. However, the VIDAS SPT method detected more positive samples than the USDA/FSIS-MLG reference method, indicating a higher level of sensitivity than the reference method. A $dLPOD_{CP}$ of 0.00 (–0.03, +0.03) was obtained between presumptive and confirmed VIDAS SPT results. The confidence intervals obtained for $dLPOD_{CP}$ indicated no significant difference between the presumptive and confirmed results. Detailed results of the POD statistical analysis are presented in Table **2013.01A** and in appended Table B and Figure 1C and D.

Alternative Confirmation with IBISA and ASAP

For the high inoculum level, all 144 test portions were reported as positive by the VIDAS SPT method, with all test portions confirming positive. For the low inoculum level, all 144 test portions were also reported as positive by the VIDAS SPT method, with 143 confirming positive. For the uninoculated controls, none of the 144 samples produced a presumptive positive result by the VIDAS SPT method, and all samples confirming negative. For test portions analyzed by the USDA/FSIS-MLG method, 138 of the 144 high inoculum test portions and 84 of the 144 low inoculum test portions confirmed

Table 2013.01D. Interpretation of test

Test value threshold	Interpretation
<0.25	Negative
≥0.25	Positive

Table 1. Participation of each collaborating laboratory[a]

Lab	Raw ground beef (25 g test portions)	Raw ground beef (25 g test portions)[b]	Raw ground beef (375 g test portions)	Raw ground beef (375 g test portions)[b]
1	Y	Y	Y	Y
2	Y	Y	Y[c]	Y
3	Y	Y	Y	Y
4	Y	Y	Y	Y
5	Y	Y	Y[c]	Y[c]
6	Y	Y	Y	Y
7	Y	Y	Y	Y
8	Y[c]	Y[c]	Y[c]	Y[c]
9	Y	Y	Y	Y
10	Y	Y	Y	Y
11	Y	Y	Y	Y
12	Y[c]	Y[c]	Y	Y[c]
13	Y	Y	Y	Y
14	Y	Y	N	N
15	N	N	Y	Y

[a] Y = Collaborator analyzed the food type; N = collaborator did not analyze the food type.

[b] Results were confirmed following the alternative confirmation procedure.

[c] Results were not used in statistical analysis due to laboratory error, or uninoculated control test portions were confirmed as *Salmonella*.

positive. For the uninoculated controls, none of the 144 test portions confirmed positive.

For the low level inoculum, a $dLPOD_C$ value of 0.41 (+0.32, +0.49) was obtained between the USDA/FSIS-MLG method and the VIDAS SPT method. The confidence intervals obtained for $dLPOD_C$ indicated a statistically significant difference between the two methods. However, the VIDAS SPT method detected more positive samples than the USDA/FSIS-MLG reference method, indicating a higher level of sensitivity than the reference method A $dLPOD_{CP}$ of 0.01 (–0.02, +0.04) was obtained between presumptive and confirmed VIDAS SPT results for both confirmation procedures. The confidence intervals obtained for $dLPOD_{CP}$ indicated no significant difference between the presumptive and confirmed results.

For the high-level inoculum, a $dLPOD_C$ value of 0.04 (+0.01, +0.09) was obtained between the USDA/FSIS-MLG method and the VIDAS SPT method. The confidence intervals obtained for $dLPOD_C$ indicated a statistically significant difference between the two methods. However, the VIDAS SPT method detected more positive samples than the USDA/FSIS-MLG reference method, indicating a higher level of sensitivity than the reference method A $dLPOD_{CP}$ of 0.00 (–0.03, +0.03) was obtained between presumptive and confirmed VIDAS SPT results. The confidence intervals obtained for $dLPOD_{CP}$ indicated no significant difference between the presumptive and confirmed results. Detailed results of the POD statistical analysis are presented in Table **2012.01A** and in appended Table C and Figure 1E and F.

Table 2. Individual collaborator results for raw ground beef (25 g test portions) using traditional confirmation[a]

	High-level test portions												Low-level test portions												Uninoculated test portions											
Lab	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12
VIDAS SPT (traditional confirmation)																																				
1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–[b]	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
8[c]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
11	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
12[c]	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–[c]	–	–	–	–	–	–	–	–	–
13	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
14	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
15	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
USDA/FSIS-MLG																																				
1	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	+	+	+	–	–	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–
2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
3	–	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
4	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+	–	+	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–
5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
6	+	+	+	+	+	+	–	+	+	+	+	–	–	–	–	–	+	+	+	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
7	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	–	–	–	+	–	–	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–
8[c]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
9	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	+	+	+	+	–	+	–	+	–	–	–	–	–[d]	–	–	–	–	–	–	–
10	–	+	+	+	+	+	+	+	+	+	–	–	+	–	–	–	–	+	+	+	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–
11	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	+	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
12[c]	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	+	+	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
13	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	–	+	+	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
14	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	+	–	+	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–
15	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

[a] + = Salmonella spp. were detected in samples; – = Salmonella spp. were not detected in sample; NA = laboratory did not participate in this matrix or results were not received.

[b] Sample was presumptive positive on VIDAS SPT, but confirmed negative indicating a false-positive result.

[c] Results were not used in statistical analysis due to laboratory error.

[d] Sample was confirmed negative on XLT4 and BGS, but confirmed positive on ASAP agar.

Raw Ground Beef (375 g Test Portions)

Raw ground beef test portions were inoculated at a low and a high level, and analyzed (Tables 4 and 5) for the detection of *Salmonella* spp. Uninoculated controls were included in each analysis. Fourteen laboratories participated in the analysis of this matrix; the results of 11 laboratories were included in the statistical analysis. Laboratory 8 reported that it was unable to confirm samples via serological testing and indicated that it did not conduct the alternative confirmation of the VIDAS SPT samples. Therefore, its results were not included in statistical analysis. Laboratory 5 detected the presence of *Salmonella* spp. in their reference method uninoculated control replicates; therefore, its results were not included for statistical analysis. Laboratory 2 detected the presence of *Salmonella* spp. in their VIDAS SPT replicates following the traditional confirmation procedure, and Laboratory 12 detected the presence of *Salmonella* spp. in the confirmation of VIDAS SPT replicates following the alternative confirmation procedure. Therefore, their results were not included in the statistical analysis for those categories. The MPN levels obtained for this test portion, with 95% confidence intervals, were 0.72 CFU/test portion

Table 3. Individual collaborator results for raw ground beef (25 g test portions) using alternative confirmation[a]

	High-level test portions												High-level test portions												High-level test portions											
Lab	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12
VIDAS SPT (alternative confirmation)[b]																																				
1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–[c]	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
8[d]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
11	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
12[b]	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–[c]	–	–	–	–	–	–	–	–	–
13	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
14	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
15	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
USDA/FSIS-MLG																																				
1	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	+	+	+	–	–	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–
2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
3	–	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
4	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+	–	+	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–
5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
6	+	+	+	+	+	+	–	+	+	+	+	–	–	–	–	–	+	+	+	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
7	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	–	–	–	+	–	–	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–
8[d]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
9	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	+	+	+	+	–	+	–	+	–	–	–	–	–[b]	–	–	–	–	–	–	–
10	–	+	+	+	+	+	+	+	+	+	–	–	+	–	–	–	–	+	+	+	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–
11	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	+	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
12[d]	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	+	+	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
13	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	–	+	+	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
14	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	+	–	+	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–
15	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

[a] += Salmonella spp. were detected in samples; – = Salmonella spp. were not detected in sample; NA = laboratory did not participate in this matrix or results were not received.

[b] Alternative confirmation = direct streak of the primary enrichment onto IBISA and ASAP.

[c] Sample was presumptive positive on VIDAS SPT but confirmed negative indicating a false-positive result.

[d] Results were not used in statistical analysis due to laboratory error.

[e] Sample was confirmed negative on XLT4 and BGS but confirmed positive on ASAP agar.

(0.31, 1.67) for the low level and 2.19 CFU/test portion (0.94, 5.12) for the high level.

Traditional Confirmation with XLT4 and BGS

For the high level, 130 of 132 test portions were reported as positive by the VIDAS SPT method, with all portions confirming positive. For the low level, 58 of 131 test portions were reported as positive by the VIDAS SPT method with 58 test portions confirming positive. For the uninoculated controls, none of the 132 samples produced a presumptive positive result by the VIDAS SPT method, and all confirmed negative. For test portions analyzed by the USDA/FSIS-MLG method, all of the 132 high and 57 of 132 low inoculum test portions confirmed positive. For the uninoculated controls, no test portions confirmed positive.

For the low-level inoculum, $dLPOD_C$ values of 0.01 (–0.12, +0.15) were obtained between the USDA/FSIS-MLG method and the VIDAS SPT method. The confidence intervals obtained for $dLPOD_C$ indicated no significant difference between the two methods. $dLPOD_{CP}$ values of 0.00 (–0.15, +0.15) were obtained

Table 4. Individual collaborator results for raw ground beef (375 g test portions) using traditional confirmation[a]

	High-level test portions												Low-level test portions												Uninoculated test portions											
Lab	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12
VIDAS SPT (traditional confirmation)																																				
1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
2[b]	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	–	–	–	+	+	–	+	–	–	–	–	–	–	–	–	–	–	+	–	–	–
3	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–[c]	–	–	–	–	–	–	NA	–	–	–	–	–	–	–	–	–	–	–	–
5[b]	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	–	–	–	–	+	–	+	+	–	–	–	–	–	–	–	–	–	+	–	+
6	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	–	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
8[b]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
11	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
12	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+	–	–	–	–	–	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
13	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
14	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
15	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–
USDA/FSIS-MLG																																				
1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
2[b]	+	+	+	+	+	+	+	+	+	+	+	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
3	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
5[b]	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–
6	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
7	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
8[b]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
9	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+	–	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
11	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
12	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	+	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
13	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	–	+	–	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
14	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
15	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

[a] + = Salmonella spp. were detected in samples; – =Salmonella spp. were not detected in sample; NA = laboratory did not participate in this matrix or results were not received.

[b] Results were not used in statistical analysis due to laboratory error.

[c] Sample was confirmed negative using the reference method agars XLT4 and BGS, but confirmed positive on ASAP agar.

between presumptive and confirmed VIDAS SPT results. The confidence intervals obtained for $dLPOD_{CP}$ indicated no significant difference between the presumptive and confirmed results using either confirmation process.

For the high-level inoculum, $dLPOD_C$ values of –0.02 (–0.05, +0.02) were obtained between the USDA/FSIS-MLG method and the VIDAS SPT method. The confidence intervals obtained for $dLPOD_C$ indicated no significant difference between the two methods. $dLPOD_{CP}$ values of 0.00 (–0.04, +0.04) were obtained between presumptive and confirmed VIDAS SPT results. The confidence intervals obtained for $dLPOD_{CP}$ indicated no significant difference between the presumptive and confirmed results. Detailed results of the POD statistical analysis are presented in Table **2013.01B** and in appended Table D and Figure 2A and B.

Traditional Confirmation with IBISA and ASAP

For the high level, 130 of the 132 test portions were reported as positive by the VIDAS SPT method, with all portions confirming positive. For the low level, 58 of 131 test portions were reported as positive by the VIDAS SPT method, with 59 confirming positive. For the uninoculated controls, none of the 132 samples produced a presumptive positive result by the VIDAS SPT method and all

Table 5. Individual collaborator results for raw ground beef (375 g test portions) using alternative confirmation[a]

	High-level test portions												Low-level test portions												Uninoculated test portions											
Lab	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12
VIDAS SPT (alternate confirmation)[b]																																				
1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
2	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	–	–	–	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
3	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–[c]	–	–	–	–	–	–	NA	–	–	–	–	–	–	–	–	–	–	–	–
5[d]	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	–	–	–	–	+	–	+	+	–	–	–	–	–	–	–	–	–	+	–	+
6	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	–	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
8[d]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
11	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
12[d]	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+	–	–	–	–	–	+	+	+	+	–	–	–	–	–	–	–	–	–	–[c]	–[c]	–[c]
13	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
14	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
15	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–
USDA/FSIS-MLG																																				
1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
2	+	+	+	+	+	+	+	+	+	+	+	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
3	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
5[d]	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–
6	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
7	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
8[d]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
9	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+	–	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
11	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
12	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	+	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
13	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	–	+	–	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
14	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
15	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

[a] + = *Salmonella* spp. were detected in samples; – = *Salmonella* spp. were not detected in sample; NA = laboratory did not participate in this matrix or results were not received.

[b] Alternative confirmation = direct streak of the primary enrichment onto IBISA and ASAP.

[c] Sample was confirmed negative using the reference method agars XLT4 and BGS, but confirmed positive on ASAP and IBISA agar.

[d] Results were not used in statistical analysis due to laboratory error.

samples confirmed negative. For test portions analyzed by the USDA/FSIS-MLG method, all of the 132 high and 57 of 132 low inoculum test portions confirmed positive. For the uninoculated controls, none of the 132 test portions confirmed positive.

For the low-level inoculum, $dLPOD_C$ values of 0.02 (–0.18, +0.22) were obtained between the USDA/FSIS-MLG method and the VIDAS SPT method. The confidence intervals obtained for $dLPOD_C$ indicated no significant difference between the two methods. $dLPOD_{CP}$ values of –0.01 (–0.15, +0.14) were obtained between presumptive and confirmed VIDAS SPT results. The confidence intervals obtained for $dLPOD_{CP}$ indicated no significant difference between the presumptive and confirmed results using either confirmation process.

For the high-level inoculum, $dLPOD_C$ values of –0.02 (–0.05, +0.02) were obtained between the USDA/FSIS-MLG method and the VIDAS SPT method. The confidence intervals obtained for $dLPOD_C$ indicated no significant difference between the two methods. $dLPOD_{CP}$ values of 0.00 (–0.04, +0.04) were obtained between presumptive and confirmed VIDAS SPT results. The confidence intervals obtained for $dLPOD_{CP}$ indicated no significant difference between the presumptive and confirmed results. Detailed results of the POD statistical analysis are

presented in Table **2013.01B** and in appended Table E and Figure 2C and D.

Alternative Confirmation with IBISA and ASAP

For the high level, 130 of 132 test portions were reported as positive by the VIDAS SPT method, with all portions confirming positive. For the low level, 57 of 131 test portions were reported as positive by the VIDAS SPT method, with 58 confirming positive. For the uninoculated controls, none of the 132 samples produced a presumptive positive result by the VIDAS SPT method, and all samples confirmed negative. For test portions analyzed by the USDA/FSIS-MLG method, 131 of 132 high and 54 of 132 low inoculum test portions confirmed positive. For the uninoculated controls, none of the 132 test portions confirmed positive.

For the low-level inoculum, $dLPOD_C$ values of 0.03 (–0.18, +0.24) were obtained between the USDA/FSIS-MLG method and the VIDAS SPT method. The confidence intervals obtained for $dLPOD_C$ indicated no significant difference between the two methods. $dLPOD_{CP}$ values of –0.01 (–0.21, +0.23) were obtained between presumptive and confirmed VIDAS SPT results. The confidence intervals obtained for $dLPOD_{CP}$ indicated no significant difference between the presumptive and confirmed results using either confirmation process.

For the high-level inoculum, $dLPOD_C$ values of –0.01 (–0.05, +0.03) were obtained between the USDA/FSIS-MLG method and the VIDAS SPT method. The confidence intervals obtained for $dLPOD_C$ indicated no significant difference between the two methods. $dLPOD_{CP}$ values of 0.00 (–0.04, +0.04) were obtained between presumptive and confirmed VIDAS SPT results. The confidence intervals obtained for $dLPOD_{CP}$ indicated no significant difference between the presumptive and confirmed results. Detailed results of the POD statistical analysis are presented in Table **2013.01B** and in appended Table F and Figure 2E and F.

IBISA and ASAP Chromogenic Agar

Results obtained from the IBISA and ASAP chromogenic agars were comparable to the results obtained from the XLT4 and BGS agars specified by the USDA/FSIS-MLG method. For the samples analyzed by the reference method, there were 412 positive results obtained from ASAP agar plates, compared to 411 positive results obtained from XLT4 and BGS agar plates. For samples analyzed by the VIDAS SPT method and confirmed following traditional procedures using IBISA and ASAP there were 476 positive results obtained from ASAP agar plates, compared to 475 positive results obtained from IBISA, XLT4 and BGS agar plates. For samples analyzed by the VIDAS SPT method and confirmed following the alternative procedure using IBISA and ASAP, there were 479 positive results obtained from IBISA and ASAP agar plates, compared to 475 positive results obtained from XLT4 and BGS agar plates.

Four uninoculated control samples produced positive results on the IBISA and ASAP chromogenic agar that were not detected on either the XLT4 or BGSA reference agars or during analysis with the VIDAS SPT assay. Because the *Salmonella* species was not detected on the two reference agar plates, the positive results produced by the chromogenic agar plates may be an artifact of cross-contamination or laboratory error.

Discussion

For this collaborative study, samples were analyzed at both 375 and 25 g test portions as required by the current AOAC guidelines, which require methods with more than one sample preparation or enrichment scheme to analyze one matrix per procedure.

For the analysis of 375 g test portions, no significant difference was observed using the POD statistical model in the number of positive results obtained between the two methods being compared using both the traditional and alternative confirmation procedures for the VIDAS SPT method. For the analysis of 25 g test portions, a significant difference was observed using the POD statistical model between the two methods for both the low and high levels of inoculation using both the traditional and alternative confirmation procedures, with more positive results obtained using the VIDAS SPT method, indicating a high level of sensitivity in the detection of the target analyte by the candidate method.

The results of the POD statistical analysis may indicate the high sensitivity of the VIDAS SPT assay. The VIDAS SPT showed a higher sensitivity than the reference method when test portions of the same size (25 g) were analyzed, and similar sensitivity to the reference method for test portions that were 15x larger (375 g VIDAS SPT test portions, compared to 25 g USDA/FSIS-MLG test portions).

No negative feedback was reported to the Study Directors from the collaborating laboratories with regard to the performance of the VIDAS SPT assay or the IBISA and ASAP chromogenic agar. Overall, the VIDAS SPT method recovered *Salmonella* in 475 test samples out of 826 samples analyzed, compared to 411 positive results out of 826 samples for the USDA/FSIS-MLG method. Only one unconfirmed positive result and no false-negative results were obtained using the VIDAS SPT method.

Recommendations

It is recommended that the VIDAS SPT method, with the optional ASAP and IBISA agar confirmation method, be adopted as Official First Action status for the detection of *Salmonella* in a variety of foods, including raw ground beef (25 and 375 g), processed American cheese (25 g), deli roast beef (25 g), liquid egg (25 g), peanut butter (25 g), vanilla ice cream (25 g), cooked shrimp (25 g), raw cod (25 g), bagged lettuce (25 and 375 g), dark chocolate (375 g), powdered eggs (25 g), instant nonfat dry milk (25 and 375 g), ground black pepper (25 g), dry dog food (375 g), raw ground turkey (375 g), almonds (375 g), chicken carcass rinsates, and stainless steel, plastic, and ceramic environmental surfaces.

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Willis Fedio and Ruben Zapata, New Mexico State University, Center for Animal Health, Food Safety and Biosecurity, Las Cruces, NM

Jas Walia, Certified Laboratories, Turlock, CA

Jack Rowe, Certified Laboratories, Buena Park, CA

Maya Achen and Heather Kohler, Ohio Department of Agriculture, Consumer Analytical Laboratory, Reynoldsburg, OH

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Hesham Elgaali, Indiana State Department of Health, Food and Dairy Microbiology Division, Indianapolis, IN

Kristi McCallum and Dominika Kondratko, Colorado Department of Agriculture, Inspection and Consumer Services, Biochemistry Laboratory, Denver, CO

Yanyan Huang, ConAgra Foods, Microbiology and Microphysical Laboratory, Omaha, NE

Katie Goetz, Q Laboratories Inc., Cincinnati, OH

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FOOD BIOLOGICAL CONTAMINANTS

Detection of *Salmonella* species in a Variety of Foods by the DuPont™ BAX® System Real-Time PCR Assay for *Salmonella*: First Action 2013.02

F. MORGAN WALLACE, BRIDGET ANDALORO, DAWN FALLON, NISHA CORRIGAN, STEPHEN VARKEY, DANIEL DEMARCO, ANDREW FARNUM, MONICA TADLER, STEVEN HOELZER, JULIE WELLER, EUGENE DAVIS, JEFFREY ROHRBECK, and GEORGE TICE
DuPont Nutrition & Health, ESL Building 400, Route 141 and Henry Clay Rd, Wilmington, DE 19880
PATRICK BIRD, ERIN CROWLEY, JONATHAN FLANNERY, KIEL FISHER, TRAVIS HUFFMAN, MEGAN BOYLE, M. JOSEPH BENZINGER, JR, PAIGE BEDINGHAUS, KATIE GOETZ, WILLIAM JUDD, JIM AGIN, and DAVID GOINS
Q Laboratories, Inc., 1400 Harrison Ave, Cincinnati, OH 45214

Collaborators: C. Churchill; D. Clark, Jr; B. Dieckelman; T. Donohue; H. Elgaali; W. Fedio; E. Galbraith; L. Hahn; D. Kondratko; B. Kupski; K. McCallum; G. McWhorter; J. Meyer; J. Putrow; R. Radcliff; D. Rodgers; S. Scott; D. Swift; L. Thompson

A multilaboratory study was conducted to evaluate the ability of the DuPont™ BAX® System Real-Time PCR Assay for *Salmonella* to detect the target species in a variety of foods and environmental surfaces. Internal validation studies were performed by DuPont Nutrition & Health on 24 different sample types to demonstrate the reliability of the test method among a wide variety of sample types. Two of these matrixes—pork and turkey frankfurters and pasteurized, not-from-concentrate orange juice without pulp—were each evaluated in 14 independent laboratories as part of the collaborative study to demonstrate repeatability and reproducibility of the internal laboratory results independent of the end user. Frankfurter samples were evaluated against the U.S. Department of Agriculture, Food Safety and Inspection Service reference method as a paired study, while orange juice samples were evaluated against the U.S. Food and Drug Administration reference method as an unpaired study, using a proprietary media for the test method. Samples tested in this study were artificially inoculated with a *Salmonella* strain at levels expected to produce low (0.2–2.0 CFU/test portion) or high (5 CFU/test portion) spike levels on the day of analysis. For each matrix, the collaborative study failed to show a statistically significant difference between the candidate method and the reference method using the probability of detection statistical model.

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The method was approved by the Expert Review Panel for Food Biological Contaminants as First Action.
The Expert Review Panel for Food Biological Contaminants invites method users to provide feedback on the First Action methods. Feedback from method users will help verify that the methods are fit for purpose and are critical to gaining global recognition and acceptance of the methods. Comments can be sent directly to the corresponding author or methodfeedback@aoac.org.
Corresponding author's e-mail: morgan.wallace@usa.dupont.com
Appendices are available on the *J. AOAC Int.* website, http://aoac.publisher.ingentaconnect.com/content/aoac/jaoac
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Salmonella is a leading cause of foodborne illness. The low infectious dose of the bacterium makes it critical to detect even low concentrations of the *Salmonella* in foods. Additionally, the presence of high concentrations of closely related nonpathogenic bacteria create the need for highly accurate methodologies. Traditionally, laboratories concerned with detection of *Salmonella* screened food samples with culture methods, such as those provided by the U.S. Department of Agriculture, Food Safety and Inspection Service (USDA-FSIS) and the U.S. Food and Drug Administration (FDA), which require several days to detect and confirm *Salmonella*. Rapid methods of screening for *Salmonella* have been developed, but these generally require 2 days of enrichment. By contrast, the DuPont™ BAX® System detects the pathogen less than 90 min after enrichment, and the DNA-based results are both reliable and reproducible, leading to quicker release of cleared product.

The BAX System Real-Time PCR Assay for *Salmonella* was certified by the AOAC Research Institute in August 2012 and designated *Performance Tested Method*℠ (PTM) No. 081201. No significant differences were reported for detection of *Salmonella* in the matrixes tested when comparing the BAX System method results to the standard reference culture procedures described in the USDA-FSIS *Microbiology Laboratory Guidebook* (MLG; 1), FDA *Bacteriological Analytical Manual* (BAM; 2), and Health Canada *Compendium of Analytical Methods* (HC CAM; 3). The matrixes validated in the PTM study included raw ground beef (85% lean, 25 and 375 g), chicken carcass rinse, cream cheese (34% fat), fresh bagged lettuce, dry pet food, and stainless steel. Inclusivity testing demonstrated that the BAX System method was reactive with 317 *Salmonella* isolates, representing over 100 different serotypes. The test method did not detect 37 different non-*Salmonella* strains tested (Appendix 1; *see* appendixes on *J. AOAC Int.* website, http://aoac.publisher.ingentaconnect.com/content/aoac/jaoac). After the PTM approval was achieved, a procedure change was applied to this validation to incorporate an eight-cycle increase in processing time in the BAX System Q7 instrument (Appendix 2).

Following the completion of the PTM study, a precollaborative study was conducted on an additional 18 matrixes, including

ground beef with soy (85% lean), beef trim, frankfurters (beef), shrimp, ground turkey, chicken wings, dried eggs (whole, powdered), shell eggs, frozen peas, orange juice, instant nonfat dry milk, ice cream (12% fat), peanut butter (52% fat), cocoa (unsweetened), white pepper, milk-based infant formula, ceramic tile, and plastic surfaces. The results obtained using the test method indicate no statistical difference with the reference method when compared to the corresponding reference method results (Appendix 3).

In addition, two of the precollaborative study matrixes—frankfurters (pork plus turkey) and orange juice (pasteurized not-from-concentrate)—were evaluated in a total of 15 independent laboratories as part of the collaborative study to demonstrate repeatability and reproducibility of the internal laboratory results independent of the end user. The results obtained using the BAX System method indicate no statistical difference when compared to the corresponding reference method results.

Collaborative Study

Study Design

Collaborators analyzed two representative matrixes (pork and turkey frankfurters and pasteurized, not-from-concentrate orange juice without pulp), 12 replicate test portions from each of three contamination levels (low, high, and uninoculated), comparing the performance of the BAX System Real-Time PCR Assay for *Salmonella* to appropriate reference culture methods. A total of 15 laboratories participated in the study, with 14 laboratories reporting data for each matrix. Each collaborator received instructions for performing the study and required materials prior to the start of the study. If necessary, training on the BAX System was provided to laboratory personnel by a DuPont representative.

The collaborative study was conducted in accordance with the *AOAC INTERNATIONAL Methods Committee Guidelines for Validation of Microbiological Methods for Food and Environmental Surfaces*, Appendix J (4). Frankfurter samples were evaluated against the USDA-FSIS MLG reference method as a paired study, as the test and reference method enrichment protocols are identical. Orange juice samples were evaluated against the FDA-BAM reference method as an unpaired study, as the BAX System method uses enrichment in proprietary media. Estimates of repeatability, reproducibility, and probability of detection (POD) were evaluated.

Preparation of Inocula and Test Portions

Sample product was obtained from a local retail outlet and screened by the organizing laboratory to identify any naturally contaminating *Salmonella* and determine a total aerobic plate count. For each sample type, five analytical size portions (25 g for orange juice and 325 g for frankfurters) were screened for *Salmonella* using the appropriate reference method. Although naturally contaminated samples would have been preferred, all samples tested returned negative results for *Salmonella*. Therefore, each sample matrix was artificially inoculated with a different serovar of *Salmonella* for use in this study.

Portions of each sample type were inoculated at levels that on the day of initiation of analysis produced a high spike level (POD approximately 1.0 or approximately 5 CFU/test portion) and a low spike level (POD 0.25–0.75 or 0.2–2.0 CFU/test portion). Additional matrix was left uninoculated to serve as negative controls.

To inoculate frankfurter samples, a pure colony of *Salmonella* Typhimurium was transferred from Trypticase Soy agar with 5% sheep's blood (SBA) into Brain Heart Infusion (BHI) broth and incubated at 35°C for 18–24 h. The inoculum was heat stressed in a 55°C water bath for 10 min to obtain a percent injury of approximately 70% as determined by plating onto selective Xylose Lysine Desoxycholate (XLD) agar and nonselective TSA. Four portions of equal size were inoculated drop-wise with an 18–22 h culture of the target organism, and then homogenized by hand. All four portions were combined one at a time into a single container, homogenizing the bulk material after each portion was added. The bulk lot was separated into two sampling containers and 40 samples (20 for each method) weighing 25 g each were removed from each container. Each 25 g sample was combined with 300 g uncontaminated matrix to create 325 g test portions. The remaining spiked matrix was rehomogenized by combining the material from both containers into one and mixing thoroughly for the purposes of maintaining an even distribution of the organism.

To inoculate orange juice, a pure colony of *Salmonella* Hadar was transferred from SBA into BHI broth containing 1% glucose and incubated at 35°C for 18–24 h. This stress protocol resulted in a percent injury of approximately 60% (as determined by plating onto selective XLD agar and nonselective TSA). The inoculum was added drop-wise to a bulk quantity of orange juice to reach the desired contamination level, and then mixed to achieve equal distribution of the inoculum throughout. This spiked bulk quantity was divided into 25 mL test portions for analysis.

Test Portion Distribution

All test portions were randomized and blind-coded by the organizing laboratory, then shipped overnight to each collaborating laboratory and maintained at 2–8°C until they were analyzed. The total hold time of samples was 48 h for frankfurters and 96 h for orange juice, including shipment time to each participating laboratory. On the first day of test sample analysis, a 5-tube, 3-level most probable number (MPN) estimation of contamination levels was conducted by the organizing laboratory using the appropriate reference method. The Least Cost Formulations, Ltd (Norfolk, VA) MPN Calculator-Version 1.6 (5) was used to determine the MPN values and 95% confidence intervals. The MPN is reported for each level of each matrix in Appendix 4, Tables 1–6 as MPN/test portion with 95% confidence intervals.

Test Portion Analysis

For testing frankfurters, each collaborator received 12 low-spike, 12 high-spike, and 12 uncontaminated 325 g test portions, blind-coded so that the contamination level was unknown to the collaborator. Approximately one-third to one-half of 2925 ± 58.5 mL of sterile buffered peptone water (BPW) was added to each portion, and each portion was homogenized approximately 2 min. The remainder of the

2925 mL BPW was added, and samples were incubated at 35°C for 18–24 h. For the test method, samples were tested directly from the BPW enrichment using the BAX System method. For the USDA-FSIS MLG reference method, 0.5 mL aliquots of each portion were transferred to 10 mL tetrathionate (TT) Hajna broth, and 0.1 mL sample was added to 10 mL modified Rappaport-Vassiliadis (mRV) broth. All secondary enrichments were incubated at 42 ± 0.5°C for 22–24 h (or in a water bath for 18–24 h). Secondary enrichments were streaked to brilliant green sulfa and either double modified lysine iron agar (LIA) or xylose lysine Tergitol^{TM} 4 agar plates and incubated 35 ± 2°C for 18–24 h. Isolated colonies were transferred to triple sugar iron (TSI) agar and LIA slants and incubated 35 ± 2°C for 22–26 h. *Salmonella* colonies were confirmed using serological (Somatic O and poly H agglutination) and biochemical procedures according to USDA-FSIS MLG.

For testing orange juice, each collaborator received 12 low-spike, 12 high-spike, and 12 uncontaminated 25 mL test portions blind-coded so that the contamination level was unknown to the collaborator. For the test method, samples were swirled with 225 mL BAX System MP media and incubated at 39–42°C for 22–26 h, then secondary enrichment was performed by transferring 10 µL primary enrichment to 500 µL prewarmed (37°C) BHI broth. Secondary enrichments were incubated at 37°C for 3 h, then tested with the BAX System method. For the FDA-BAM reference method, portions were swirled with 225 mL Universal Preenrichment Broth (UPB) and incubated at 35°C for 22–26 h. After primary enrichment, 1 mL of each enriched portion was transferred to 10 mL TT broth and 0.1 mL was transferred to 10 mL RV broth. RV tubes were incubated at 42 ± 0.2°C for 22–26 h using a circulating, thermostatically controlled water bath. TT tubes were incubated at 35 ± 2°C for 22–26 h. Secondary enrichments were streaked to bismuth sulfite, XLD, and Hektoen enteric agar plates and incubated at 35°C for 22–26 h. Isolated colonies were transferred to TSI and LIA slants and incubated 35 ± 2°C for 22–26 h. *Salmonella* colonies were confirmed using serological and biochemical procedures according to FDA-BAM.

Statistical Analysis

Data analysis was performed using each of the metrics below according to the format described in the *AOAC INTERNATIONAL Methods Committee Guidelines for Validation of Microbiological Methods for Food and Environmental Surfaces* and using the Least Cost Formulations, Ltd, AOAC Binary Data Interlaboratory Study Workbook (6).

For the purposes of this evaluation, POD was defined as the number of positive outcomes divided by the total number of trials. POD was estimated with a 95% confidence interval for each of the following levels: candidate presumptive results (POD_{CP}); candidate confirmatory results (POD_{CC}); candidate method results based on the presumptive and confirmatory results (POD_C); and reference method results (POD_R).

Candidate presumptive and confirmatory results were compared by determining the difference between the POD (dLOPD) values for each matrix and concentration ($dLPOD_{CP} = POD_{CP} - POD_{CC}$). If the confidence interval of a dLPOD does not contain zero, then the difference is statistically significant at the 5% level.

Candidate and reference method results were compared by determining the difference in POD values between the candidate and reference methods for each matrix and concentration ($dLPOD_C = POD_C - POD_R$). If the confidence interval of a dLPOD does not contain zero, then the difference is statistically significant at the 5% level.

AOAC Official Method 2013.02
Salmonella species in a Variety of Foods and Environmental Surfaces
BAX® System Real-Time PCR Assay for *Salmonella*
First Action 2013

[Applicable to the detection of *Salmonella* in a variety of foods, including raw ground beef (25 and 375 g), ground beef with soy (25 and 325 g), beef trim (25 and 325 g), frankfurters (325 g), shrimp (25 g), ground turkey (25 g), chicken wings (25 g), poultry rinse (30 mL), whole powdered (dried) eggs (25 g), shell eggs (1000 mL), fresh bagged lettuce (25 g), frozen peas (25 g), orange juice (pasteurized; 25 mL), cream cheese (25 g), nonfat dry milk (25 g), ice cream (25 g), peanut butter (25 g), cocoa (25 g), white pepper (25 g), milk-based infant formula (25 mL), and dry pet food (375 g), and on stainless steel, ceramic tile, and plastic surfaces.]

See Table **2013.02** for a summary of results of the collaborative study. *See* Appendix 4, Tables 1–6 for detailed results of the collaborative study.

Caution: Kits.—The reagents used in the BAX System should pose no hazards when used as directed. Dispose of lysate, PCR mixture, and other waste according to your site practices.

Cycler/detector.—Only qualified laboratory personnel should operate the cycler/detector. Do not attempt to repair the instrument. Live power may still be available inside the unit even when a fuse has blown or been removed. Refer to the BAX System User Guide for maintenance procedures when cleaning the unit or changing a fuse. The heating block can become hot enough during normal operation to cause burns or cause liquids to boil. Wear safety glasses or other eye protection at all times during operation.

Enrichment broths.—All enrichment broths may contain varying pathogens whether they contain *Salmonella* or not and thus should be sterilized and disposed of using proper procedures following any culture-based confirmatory steps.

Reference cultures.—When handling reference *Salmonella* cultures, always follow appropriate biosafety containment procedures as provided by your standard laboratory site practices, Centers for Disease Control and Prevention (CDC), or Canadian Pathogen Safety Data Sheets and Risk Assessment.

A. Principle

The DuPont™ BAX System uses the polymerase chain reaction (PCR) to amplify a specific fragment of bacterial

Table 2013.02. POD summary of results for the BAX System Real-Time PCR Assay for *Salmonella*

	Orange juice (25 g)			Frankfurters (325 g)		
Method[a]	Uninoculated	Low	High	Uninoculated	Low	High
Candidate presumptive positive/total No. of samples analyzed	0/168	94/168	168/168	0/168	87/168	168/168
Candidate presumptive POD (CP)	0.00 (0.00, +0.02)	0.56 (+0.48, +0.64)	1.00 (+0.98, +1.00)	0.00 (0.00, +0.02)	0.52 (+0.44, +0.60)	1.00 (+0.98, +1.00)
s_r[b]	0.00 (0.00, +0.15)	0.50 (+0.45, +0.52)	0.00 (0.00, +0.15)	0.00 (0.00, +0.15)	0.51 (+0.46, +0.52)	0.00 (0.00, +0.15)
s_L[c]	0.00 (0.00, +0.15)	0.00 (0.00, +0.18)	0.00 (0.00, +0.15)	0.00 (0.00, +0.15)	0.00 (0.00, +0.16)	0.00 (0.00, +0.15)
s_R[d]	0.00 (0.00, +0.21)	0.50 (+0.45, +0.52)	0.00 (0.00, +0.21)	0.00 (0.00, +0.21)	0.51 (+0.46, +0.52)	0.00 (0.00, +0.21)
P-value	1.0000	0.4901	1.0000	1.0000	0.6831	1.0000
Candidate confirmed positive/total No. of samples analyzed	0/168	92/168	168/168	0/168	85/168	168/168
Candidate confirmed POD (CC)	0.00 (0.00, +0.02)	0.55 (+0.47, +0.62)	1.00 (+0.98, +1.00)	0.00 (0.00, +0.02)	0.51 (+0.43, +0.58)	1.00 (+0.98, +1.00)
s_r	0.00 (0.00, +0.15)	0.50 (+0.45, +0.52)	0.00 (0.00, +0.15)	0.00 (0.00, +0.15)	0.50 (+0.45, +0.52)	0.00 (0.00, +0.15)
s_L	0.00 (0.00, +0.15)	0.00 (0.00, +0.17)	0.00 (0.00, +0.15)	0.00 (0.00, +0.15)	0.00 (0.00, +0.17)	0.00 (0.00, +0.15)
s_R	0.00 (0.00, +0.21)	0.50 (+0.45, +0.52)	0.00 (0.00, +0.21)	0.00 (0.00, +0.21)	0.50 (+0.46, +0.52)	0.00 (0.00, +0.21)
P-value	1.0000	0.5903	1.0000	1.0000	0.5570	1.0000
Positive reference samples/total No. of samples analyzed	0/168	88/168	168/168	0/168	85/168	168/168
Reference POD	0.00 (0.00, +0.02)	0.52 (+0.45, +0.60)	1.00 (+0.98, +1.00)	0.00 (0.00, +0.02)	0.51 (+0.43, +0.58)	1.00 (+0.98, +1.00)
s_r	0.00 (0.00, +0.15)	0.50 (+0.45, +0.52)	0.00 (0.00, +0.15)	0.00 (0.00, +0.15)	0.50 (+0.45, +0.52)	0.00 (0.00, +0.15)
s_L	0.00 (0.00, +0.15)	0.00 (0.00, +0.18)	0.00 (0.00, +0.15)	0.00 (0.00, +0.15)	0.00 (0.00, +0.17)	0.00 (0.00, +0.15)
s_R	0.00 (0.00, +0.21)	0.50 (+0.45, +0.52)	0.00 (0.00, +0.21)	0.00 (0.00, +0.21)	0.50 (+0.46, +0.52)	0.00 (0.00, +0.21)
P-value	1.0000	0.5021	1.0000	1.0000	0.5570	1.0000
dLPOD (C vs R)	0.00 (–0.02, +0.02)	0.02 (–0.08, +0.13)	0.00 (–0.02, +0.02)	0.00 (–0.02, +0.02)	0.00 (–0.11, +0.11)	0.00 (–0.02, +0.02)
dLPOD (CP vs CC)	0.00 (–0.02, +0.02)	0.01 (–0.10, +0.12)	0.00 (–0.02, +0.02)	0.00 (–0.02, +0.02)	0.01 (–0.10, +0.12)	0.00 (–0.02, +0.02)

[a] Results include 95% confidence intervals.

[b] Repeatability standard deviation.

[c] Among-laboratory standard deviation.

[d] Reproducibility standard deviation.

DNA, which is stable and unaffected by growth environment. The fragment is a genetic sequence that is unique to the genus *Salmonella*, thus providing a highly reliable indicator that the organism is present. The BAX System simplifies the PCR process by combining the requisite primers, polymerase, and nucleotides into a stable, dry, manufactured tablet already packaged inside the PCR tubes. After amplification, these tubes remain sealed for the detection phase, thus significantly reducing the potential for contamination with one or more molecules of amplified PCR product.

This automated BAX System method uses fluorescent detection to analyze PCR product. One PCR primer for each target (one *Salmonella*-specific target and an internal control) contains a fluorescent dye (two different dyes, one for each target) as a constituent of the primer as well as a quencher (the unimolecular combination of a primer, fluorescent dye, and quencher constitute a Scorpion™ Probe). When incorporated into a PCR product, the dye and quencher are spatially separated, which causes an increase in emission signal. The BAX System measures the magnitude and characteristics of fluorescent signal change. An analysis by the BAX System software algorithm then evaluates that data to determine a positive or negative result which is displayed as described below.

B. Apparatus and Reagents

Items (**a**)–(**h**) are part of the DuPont BAX System Start-Up Package available from DuPont Nutrition & Health (Wilmington, DE; www.fooddiagnostics.dupont.com).

Items (**i**)–(**l**) are part of the DuPont BAX System Real-Time PCR Assay for *Salmonella* available from DuPont Nutrition & Health (Cat. No. D14306040).

(**a**) *DuPont BAX System Q7 cycler/detector with computer workstation.*

(**b**) *DuPont BAX System application software.*

(**c**) *Cluster tubes with caps and racks.*—For lysis.

(**d**) *Capping/decapping tools.*—For removing and sealing cluster tube caps and PCR tube caps without jarring the contents.

(**e**) *Heating and cooling blocks with inserts.*—For maintaining lysis tubes at 37 ± 2, 95 ± 2, and 4°C. [*Note*: The DuPont Thermal Block (Cat. No. D14614252) may also be used to maintain appropriate temperatures for lysis tubes.]

(**f**) *Pipets.*—For transferring reagents; two adjustable mechanical pipets covering 20–200 and 5–50 µL; one repeating pipet; and one multichannel pipet covering eight channels and 550 µL. Pipets should be calibrated to deliver required volumes within 10%.

(**g**) *Pipet tips with barriers.*—0.5–250 µL, 0.5–100 µL extended barrier; 5 mL repeater pipet tips.

(**h**) *PCR tube holders.*—For transferring a rack of tubes from the cooling block to the cycler/detector.

(**i**) *PCR tubes with tablets.*

(**j**) *Flat optical caps for PCR tubes.*

(**k**) *Lysis buffer.*

(**l**) *Protease.*

(**m**) *Incubators.*—For maintaining media at 35 ± 1 and 39–42°C.

(**n**) *Stomacher.*—Seward model 400 or equivalent for mixing the sponge sample with enrichment media.

(**o**) *Appropriate confirmatory media for culture confirmation.*—Rappaport-Vassiliadis Soya Peptone (RVS), Selenite Cystine (SC), tetrathionate-Hajna (TT-Hajna) and tetrathionate (TT) broths, Xylose Lysine Desoxycholate (XLD), Xylose Lysine Tergitol 4 (XLT4), Hektoen Enteric (HE), Brilliant Green Sulfa (BGS), and Bismuth Sulfite (BS) agars.

C. Media

(**a**) *BAX System MP media.*—DuPont Cat. No. D12404925 (bulk powder) or D12745725 (StatMedia™ soluble packets).

(**b**) *Brain Heart Infusion (BHI) broth.*—Oxoid Cat. No. CM 1032 or equivalent.

(**c**) *Buffered Peptone Water (BPW).*—Oxoid Cat. No. CM 0509 or equivalent.

(**d**) *mTSB+n.*—Oxoid Cat. No. CM0989B or equivalent plus 2 mg/L novobiocin. Autoclave at 121°C for 15 min before addition of filter-sterilized novobiocin.

(**e**) *mTSB+caa+n.*—Oxoid Cat. No. CM0989B or equivalent plus 10 g/L casamino acids (casein acid hydrolysate) and 8 mg/L novobiocin. Autoclave at 121°C for 15 min before addition of filter-sterilized novobiocin.

(**f**) *Lactose broth (LB).*—Oxoid Cat. No. CM0137 or equivalent.

(**g**) *Brilliant green water.*—Prepare brilliant green water by adding 2 mL 1% brilliant green dye solution, **C**(**j**), per 1000 mL sterile distilled water. Let container stand undisturbed for 60 ± 5 min. Incubate loosely capped container, without mixing or pH adjustment, at 35°C for 24 ± 2 h.

(**h**) *Reconstituted nonfat dry milk.*—Suspend 100 g dehydrated nonfat dry milk in 1 L distilled water. Swirl until dissolved. Autoclave at 121°C for 15 min.

(**i**) *Universal preenrichment broth.*—Add 5 g tryptone, 5 g proteose peptone, 15 g potassium phosphate, 7 g sodium phosphate, 5 g sodium chloride, 0.5 g dextrose, 0.25 g magnesium sulfate, 0.1 g ferric ammonium citrate, and 0.2 g sodium pyruvate to 1 L distilled water. Heat ingredients with gentle agitation to dissolve, dispense, and autoclave at 121°C for 15 min. Final pH should be 6.3 ± 0.2.

(**j**) *1% Aqueous brilliant green dye solution.*—Dissolve 1 g dye in sterile water. Dilute to 100 mL.

(**k**) *Tryptic soy broth (TSB)*—Suspend 17 g tryptose, 3 g phytone, 5 g sodium chloride, 2.5 g potassium phosphate dibasic, and 2.5 g glucose in 1 L distilled water. Heat gently to dissolve, dispense into containers, and then autoclave 15 min at 121°C. Final pH is 7.3 ± 0.2.

D. Sample Enrichment

(**a**) *Ground beef, ground beef with soy, beef trim (25 g).*—Weigh 25 g test portion into sterile container. Use a stomacher, **B**(**n**), to homogenize sample for 2 min with 225 mL prewarmed (35°C) BPW, **C**(**c**). Incubate, **B**(**m**), at 35°C for 20–24 h.

(**b**) *Ground beef (375 g).*—Weigh 375 g test portion into sterile container. Use a stomacher, **B**(**n**), to homogenize sample for 2 min with 1500 mL prewarmed (45°C) mTSB+n, **C**(**d**). Incubate, **B**(**m**), at 39–42°C for 22–26 h.

(**c**) *Ground beef with soy (325 g).*—Weigh 325 g test portion into sterile container. Use a stomacher, **B**(**n**), to homogenize sample for 2 min with 975 mL prewarmed (35°C) mTSB+caa+n, **C**(**e**). Incubate, **B**(**m**), at 35°C for 20–24 h.

(**d**) *Beef trim (325 g).*—Weigh 325 g test portion into sterile container. Hand massage to homogenize sample for 2 min with

Figure 2013.02. Results are displayed on the computer screen after approximately 1 hr 10 min automated processing as a grid of icons representing the PCR outcome for each sample.

1500 mL prewarmed (41°C) BAX System MP media, **C**(**a**). Incubate, **B**(**m**), at 39–42°C for 16–24 h.

(**e**) *Frankfurters (325 g).*—Weigh 325 g test portion into sterile container. Use a stomacher, **B**(**n**), to homogenize sample for 2 min with 1400 mL prewarmed (35°C) BPW, **C**(**c**). Add additional BPW to reach a total media volume of 2925 mL. Incubate, **B**(**m**), at 35°C for 18–24 h.

(**f**) *Shrimp and peanut butter (25 g).*—Weigh 25 g test portion into sterile container. Use a stomacher, **B**(**n**), to homogenize sample for 2 min with 225 mL prewarmed (35°C) LB, **C**(**f**). Let stand at room temperature for 55–65 min. Adjust pH to 6.8 ± 0.2 using 1 N HCl or 1 N NaOH, if necessary. Incubate, **B**(**m**), at 35°C for 22–26 h.

Note: Regrowth is required for peanut butter.

(**g**) *Ground turkey and chicken wings (25 g).*—Weigh 25 g test portion into sterile container. Use a stomacher, **B**(**n**), to homogenize sample for 2 min with 225 mL prewarmed (35°C) BPW, **C**(**c**). Incubate, **B**(**m**), at 35°C for 16–24 h.

(**h**) *Poultry rinse (30 mL).*—Combine 30 mL BPW rinsate with 30 mL prewarmed (35°C) BPW, **C**(**c**), into sterile container. Incubate, **B**(**m**), at 35°C for 22–26 h.

(**i**) *Dried eggs (25 g).*—Weigh 25 g test portion into sterile container. Add approximately 15 mL prewarmed (35°C) LB, **C**(**f**), to sample and stir to smooth. Add three additional aliquots of LB of 10, 10, and 190 mL (total media volume 225 mL), stirring after each addition. Let stand at room temperature for 55–65 min. Adjust pH to 6.8 ± 0.2 using 1 N HCl or 1 N NaOH, if necessary. Incubate, **B**(**m**), at 35°C for 22–26 h.

(**j**) *Dried eggs, ice cream, and peanut butter (25 g).*—Weigh 25 g test portion into sterile container. Use a stomacher, **B**(**n**), to homogenize sample for 2 min with 225 mL prewarmed (35°C) BPW, **C**(**c**). Incubate, **B**(**m**), at 35°C for 22–26 h.

Note: Regrowth is required for peanut butter.

(**k**) *Shell eggs (approximately 1000 mL).*—Combine 20 eggs into sterile container with 2000 mL prewarmed (42°C) BAX System MP media, **C**(**a**). Incubate, **B**(**m**), at 42°C for 48 h.

(**l**) *Frozen peas, cream cheese, ice cream, and infant formula (25 g).*—Weigh 25 g test portion into sterile container. Use a stomacher, **B**(**n**), to homogenize sample for 2 min with 225 mL prewarmed (35°C) LB, **C**(**f**). Let stand at room temperature for 55–65 min. Adjust pH to 6.8 ± 0.2 using 1 N HCl or 1 N NaOH, if necessary. Incubate at 35°C for 22–26 h.

(**m**) *Frozen peas (25 g).*—Weigh 25 g test portion into sterile container. Use a stomacher, **B**(**n**), to homogenize sample for 2 min with 225 mL prewarmed (35°C) BAX System MP media, **C**(**a**). Incubate, **B**(**m**), at 35°C for 22–26 h.

(**n**) *Cream cheese (25 g).*—Weigh 25 g test portion into sterile container. Use a stomacher, **B**(**n**), to homogenize sample for 2 min with 225 mL prewarmed (35°C) BAX System MP media, **C**(**a**). Incubate, **B**(**m**), at 35°C for 12–24 h.

(**o**) *Fresh bagged lettuce (25 g).*—Weigh 25 g test portion into sterile container. Add 225 mL prewarmed (35°C) LB, **C**(**f**), and swirl 25 times clockwise and 25 times counterclockwise. Let stand at room temperature for 55–65 min. Adjust pH to 6.8 ± 0.2 using 1 N HCl or 1 N NaOH, if necessary. Incubate, **B**(**m**), at 35°C for 22–26 h.

(**p**) *Fresh bagged lettuce (25 g).*—Weigh 25 g test portion into sterile container. Add 225 mL prewarmed (35°C) BAX System MP media, **C**(**a**), and swirl 25 times clockwise and 25 times counterclockwise. Incubate, **B**(**m**), at 35°C for 10–24 h.

(**q**) *Ice cream (25 g).*—Weigh 25 g test portion into sterile container. Use a stomacher, **B**(**n**), to homogenize sample for 2 min with 225 mL prewarmed (35°C) brilliant green water, **C**(**g**). Incubate, **B**(**m**), at 35°C for 22–26 h.

(**r**) *Orange juice (25 mL).*—Weigh 25 g test portion into 225 mL prewarmed (35°C) universal preenrichment broth, **C**(**i**), and swirl thoroughly. Let stand at room temperature for 55–65 min. Do not mix or adjust pH. Incubate, **B**(**m**), at 35°C for 22–26 h.

Note: Regrowth is required for this sample type.

(**s**) *Orange juice (25 mL).*—Weigh 25 g test portion into 225 mL prewarmed (41°C) BAX System MP media, **C**(**a**), and swirl thoroughly. Incubate, **B**(**m**), at 39–42°C for 22–26 h.

Note: Regrowth is required for this sample type.

(**t**) *Nonfat dry milk (25 g).*—Pour 25 g sample slowly over the surface of 225 mL prewarmed (35°C) brilliant green water, **C**(**g**). Let stand at room temperature for 55–65 min. Do not mix or adjust pH. Incubate, **B**(**m**), at 35°C for 22–26 h.

Note: Regrowth is required for this sample type.

(**u**) *Stainless steel, ceramic tile, and plastic.*—Add 225 mL prewarmed (35°C) LB, **C**(**f**), to environmental sponge in sample bag and swirl thoroughly. Let stand at room temperature for 55–65 min. Adjust pH to 6.8 ± 0.2 using 1 N HCl or 1 N NaOH, if necessary. Incubate, **B**(**m**), at 35°C for 22–26 h.

(**v**) *Stainless steel, ceramic tile, and plastic.*—Add 225 mL prewarmed (35°C) BPW, **C**(**c**), to environmental sponge in sample bag and swirl thoroughly. Adjust pH to 6.8 ± 0.2 using 1 N HCl or 1 N NaOH, if necessary. Incubate, **B**(**m**), at 35°C for 18–24 h.

(**w**) *Cocoa (25 g).*—Weigh 25 g test portion into sterile container. Use a stomacher, **B**(**n**), to homogenize sample for 2 min with 225 mL reconstituted nonfat dry milk, **C**(**h**). Let stand at room temperature for 55–65 min, and then swirl thoroughly to mix. Adjust pH to 6.8 ± 0.2 using 1 N HCl or 1 N NaOH, if necessary. Add 0.45 mL 1% aqueous brilliant green dye solution, **C**(**j**), and mix well. Incubate, **B**(**m**), at 35°C for 22–26 h. Transfer 10 µL enrichment to 500 µL BHI broth, **C**(**b**), before processing. No additional incubation is required.

(**x**) *White pepper (25 g).*—Weigh 25 g test portion into sterile container. Use a stomacher, **B**(**n**), to homogenize sample for 2 min with 225 mL prewarmed (35°C) TSB, **C**(**k**). Let stand at room temperature for 55–65 min. Adjust pH to 6.8 ± 0.2 using 1 N HCl or 1 N NaOH, if necessary. Incubate, **B**(**m**), at 35°C for 22–26 h.

(**y**) *Dry pet food (375 g).*—Weigh 375 g test portion into sterile container. Use a stomacher, **B**(**n**), to homogenize sample for 2 min with approximately one-third to one-half of 3375 mL prewarmed (35°C) LB, **C**(**f**). Add the remainder of the prewarmed media. Let stand at room temperature for 55–65 min, and then swirl thoroughly to mix. Adjust pH to 6.8 ± 0.2 using 1 N HCl or 1 N NaOH, if necessary. Incubate, **B**(**m**), at 35°C for 22–26 h.

Note: Regrowth is required for this sample type.

(**z**) *Dry pet food (375 g).*—Weigh 375 g test portion into sterile container. Use a stomacher, **B**(**n**), to homogenize sample

for 2 min with approximately one-third to one-half of 3375 mL prewarmed (35°C) BPW, **C**(**c**). Add the remainder of the prewarmed media. Adjust pH to 6.8 ± 0.2 using 1 N HCl or 1 N NaOH, if necessary. Incubate, **B**(**m**), at 35°C for 22–26 h.

Note: Regrowth is required for this sample type.

E. Regrowth

(**a**) After incubation, transfer 10 µL of the enrichment to 500 µL prewarmed (37°C) BHI broth, **C**(**b**). Incubate, **B**(**m**), at 37°C for 3 h.

(**b**) Regrowth is required for orange juice, nonfat dry milk, peanut butter, and dry pet food samples. For cocoa, a dilution without additional incubation is required. For all other matrixes, regrowth is either optional or not required.

F. Assay

(**a**) After enriching the sample, turn on the heating blocks, **B**(**e**), and set temperatures to 37 and 95°C. Make sure that the cooling blocks have been refrigerated overnight or otherwise chilled at 2–8°C.

(**b**) Create a rack file by following prompts in the Rack Wizard, **B**(**b**), to enter identifying data on the entire rack and on the individual samples.

(**c**) Label and arrange cluster tubes, **B**(**c**), in the cluster tube rack, according to the rack file.

(**d**) Prepare the lysis reagent by adding 150 µL protease, **B**(**l**), to one 12 mL bottle lysis buffer, **B**(**k**). Transfer 200 µL prepared lysis reagent to each of the cluster tubes.

(**e**) Transfer 5 µL enriched sample to the corresponding cluster tubes. Secure caps with the capping/decapping tool, **B**(**d**).

(**f**) Heat cluster tubes at 37°C for 20 min.

(**g**) Heat cluster tubes at 95°C for 10 min.

(**h**) Cool cluster tubes at 2–8° for at least 5 min.

(**i**) Warm up the cycler/detector, **B**(**a**), by selecting RUN FULL PROCESS from the Operations menu of the application window, **B**(**b**).

(**j**) Place a PCR tube holder, **B**(**h**), on the PCR cooling block, **B**(**e**). Insert one PCR tube, **B**(**i**), per sample into the holder and remove caps with the capping/decapping tool, **B**(**d**).

(**k**) Using a multichannel pipet, **B**(**f**), transfer 30 µL of sample lysate to PCR tubes, **B**(**i**). Seal with flat optical caps, **B**(**j**), with the capping/decapping tool, **B**(**d**).

(**l**) Follow screen prompts, **B**(**b**), to load samples into the cycler/detector, **B**(**a**), and begin the program. At the completion of the PCR and detection process, follow the screen prompts to remove samples and display results.

G. Assay Results

The results are recorded on the rack display or from a spreadsheet printout of the results (called Detail View). Negative results are indicated by a green circle with (–) symbol, positive results are indicated by a red circle with (+) symbol, and indeterminate results are indicated with a yellow circle with (?) symbol. A yellow circle with a (?) symbol and a red slash indicate a low signal or signal error.

BAX System results are displayed as in Figure **2013.02**. figA

H. Confirmation

Presumptive positive results are confirmed by culture and the biochemical and serological protocols described in the appropriate reference method relevant to the matrix. For meat, poultry, and pasteurized egg products, follow the USDA-FSIS MLG Chapter 4 (http://www.fsis.usda.gov/wps/wcm/connect/700c05fe-06a2-492a-a6e1-3357f7701f52/MLG-4.pdf?MOD=AJPERES). For all other matrixes, follow the FDA-BAM Chapter 5 (http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm070149.htm). Alternatively, matrixes may be confirmed as described in the Health Canada Compendium, Vol. 3, Laboratory Procedures for the Microbiological Examination of Foods, Health Canada, Health Products and Food Branch, where appropriate (http://www.hc-sc.gc.ca/fn-an/res-rech/analy-meth/microbio/volume3-eng.php).

Results and Discussion

The results for orange juice are presented in Appendix 4, Tables 1–3. At each inoculation level, the BAX System method and the reference method demonstrated no significant statistical difference as indicated by POD analysis (the 95% confidence interval of the dLPOD included 0 in all cases). Two orange juice samples (one from each of two collaborator sites) returned a presumptive positive result with the test method but could not be culture confirmed. One sample indicated a very weak positive result, suggesting either a cross-contamination event (most likely during a sample transfer step) or a very low target cell density in the sample, which could be detected with the PCR method but was difficult to detect by culture. The second sample returned a strong positive result with the test method, so it is unclear what caused the discordant results between the test and reference methods. The remaining 502 orange juice samples tested from the alternative enrichment were in agreement with culture confirmation from the alternative enrichment broths.

The results for frankfurters are presented in Appendix 4, Tables 4–6. At each inoculation level, the BAX System method and the reference method demonstrated no significant statistical difference as indicated by POD analysis (the 95% confidence interval of the dLPOD included 0 in all cases). Two frankfurter samples, both from the same collaborator site, returned a presumptive positive result with the test method but could not be culture confirmed. Both samples indicated a very weak positive result, suggesting either a cross-contamination event or a very low target cell density in the sample, which could be detected with the PCR method but was difficult to detect by culture. The remaining 502 frankfurter samples analyzed with the alternative method were in agreement with culture confirmation results. One sample initially returned an indeterminate result with the test method and was retested according to the manufacturer's instructions. Upon retest, this sample returned a negative result, which was in agreement with culture confirmation results.

A POD summary of all test method results is shown in Table **2013.02**. Across all three inoculation levels for both matrixes, statistical analyses indicate that the test method presented demonstrates no significant differences from the reference methods. The within-laboratory component (S_r) of the reproducibility S_R value represents the sampling variability at very low spiking levels. It accounted for all of the S_R value

observed for each matrix collaboratively studied, the S_L value (between-laboratory effect components of S_R) being zero in both data sets at each partial response spike level. This acceptable interlaboratory reproducibility is supported by the insignificant homogeneity test P_T values (>0.1), which suggest that the laboratory POD values are not significantly different when allowance is made for the sampling variability. While interpretation of this latter test is subject to the study design, 10 or more laboratories with 12 replicate sample portions per level for each of three levels (high, low, and unspiked) per laboratory is deemed adequate for such studies.

The graphical representation of the data (Appendix 4, Figure 1) demonstrates that the dose-response curve for each matrix encompasses the partial response region required for qualitative detection method analysis. The 95% confidence interval of each dPOD value determined at each concentration contains zero, which is indicative of no significant difference between the candidate and reference methods and between the candidate presumptive result and candidate confirmed result.

Conclusions

Within the statistical constraints of these studies, no differences were found between the reference culture-based methods and the alternative BAX System method. These results indicate that the alternative method can be used to allow uncontaminated food to be released rapidly from a manufacturer's control and prevents *Salmonella*-contaminated foods from entering commerce. Furthermore, this test method can be a valuable tool for outbreak investigations when food contamination events occur.

Collaborator feedback indicated that the method was easy to use and that the clear yes/no results provided by the BAX System software were appreciated. Time and labor savings were cited as key advantages of the test method over the reference culture methods. No negative feedback regarding the method was provided by any of the collaborators.

The DuPont BAX System Real-Time PCR Assay for *Salmonella* was adopted as Official First Action status for the detection of *Salmonella* in a variety of foods, including raw ground beef, ground beef with soy, beef trim, frankfurters, shrimp, ground turkey, chicken wings, poultry rinse, dried eggs, shell eggs, fresh bagged lettuce, frozen peas, orange juice, cream cheese, nonfat dry milk, ice cream, peanut butter, cocoa, white pepper, infant formula, and dry pet food, and on stainless steel, ceramic tile, and plastic surfaces.

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FOOD COMPOSITION AND ADDITIVES

Analysis of Cocoa Flavanols and Procyanidins (DP 1–10) in Cocoa-Containing Ingredients and Products by Rapid Resolution Liquid Chromatography: Single-Laboratory Validation

PHILIP R. MACHONIS, MATTHEW A. JONES, and CATHERINE KWIK-URIBE
Mars Inc., 6885 Elm Street, McLean, VA 22101

Recently, a multilaboratory validation (MLV) of AOAC Official Method 2012.24 for the determination of cocoa flavanols and procyanidins (CF-CP) in cocoa-based ingredients and products determined that the method was robust, reliable, and transferrable. Due to the complexity of the CF-CP molecules, this method required a run time exceeding 1 h to achieve acceptable separations. To address this issue, a rapid resolution normal phase LC method was developed, and a single-laboratory validation (SLV) study conducted. Flavanols and procyanidins with a degree of polymerization (DP) up to 10 were eluted in 15 min using a binary gradient applied to a diol stationary phase, detected using fluorescence detection, and reported as a total sum of DP 1–10. Quantification was achieved using (-)-epicatechin-based relative response factors for DP 2–10. Spike recovery samples and seven different types of cocoa-based samples were analyzed to evaluate the accuracy, precision, LOD, LOQ, and linearity of the method. The within-day precision of the reported content for the samples was 1.15–5.08%, and overall precision was 3.97–13.61%. Spike-recovery experiments demonstrated recoveries of over 98%. The results of this SLV were compared to those previously obtained in the MLV and found to be consistent. The translation to rapid resolution LC allowed for an 80% reduction in analysis time and solvent usage, while retaining the accuracy and reliability of the original method. The savings in both cost and time of this rapid method make it well-suited for routine laboratory use.

Flavanols and procyanidins are subclasses of flavonoids naturally present in a variety of commonly consumed foods including cocoa, grapes, and apples. Over the past two decades, numerous studies have demonstrated that the specific consumption of cocoa products rich in flavanols and procyanidins can have a range of positive physiological effects in humans, most notably improvements in platelet function, vascular function, and blood pressure (1–3). Together, these data provide strong evidence that the regular dietary inclusion of these cocoa-based flavonoids may have important implications for cardiovascular health.

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Corresponding author's e-mail: philip.machonis@mss.effem.com
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Central to this research on the health-promoting properties of cocoa-based flavanols and procyanidins is the availability of robust, reliable, and efficient methods for their analysis. In cocoa, as in most plants and foods, flavanols and their oligomers, the procyanidins (Figure 1), are found together. Due to their general ease of use, methods such as Folin-Ciocalteu, oxygen radical absorbance capacity, and dimethylaminocinnemaldehyde have historically been used for the quantification of flavonoids like these; however, these methods are all nonspecific, providing no distinct molecular or structural information that is important to provide clear and accurate characterization of health-promoting components in plant-based foods. To begin to address this issue, several LC methods have been developed over the past decade to identify and quantify flavanols and procyanidins in cocoa, with the separation itself based on degree of polymerization (DP; 4–7). While these methods offer sufficient separation, they all have run times exceeding 1 h to achieve resolution of DP 1–10. Figure 2 compares the chromatography achieved for the conventional method and the rapid method on a representative cocoa matrix sample.

For the first time, one of these methods for the analysis of flavanols and procyanidins in cocoa was extensively evaluated through a multilaboratory validation (MLV); the results were recently published (7). This publication demonstrated that a method for the analysis of this complex mixture of flavonoids in cocoa could be reliable, robust, and readily transferable, opening the door to its broader implementation for the analysis of these specific flavonoids in cocoa. Demonstration of the method's potential suitability for broader use is evidenced by its recent advancement to status of First Action Official Method of Analysis by AOAC INTERNATIONAL (**2012.24**). Though this method represents a significant advancement in the field, one limitation of the method remains its extended run time. With a single injection requiring an 86 min run time and 86 mL of solvent, the existing method requires considerable resources that dramatically impact laboratory efficiency. In light of this limitation, we sought to improve this method's run time by transitioning from conventional LC to rapid resolution LC, while retaining the robust performance of the original validated method.

Building upon the recently published work, a rapid resolution LC method was developed, optimized, and evaluated through a single-laboratory validation (SLV) study. Utilizing a short, small bore diol-packed column, the normal phase separation of these cocoa components was accomplished in just 15 min,

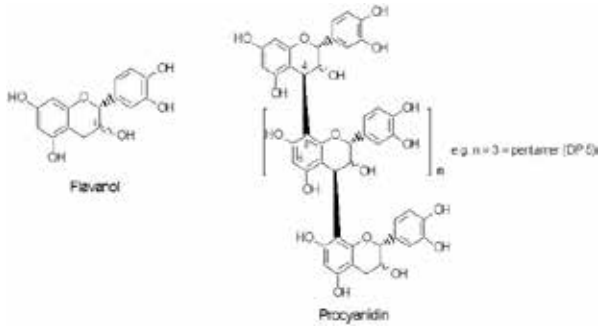

Figure 1. Chemical structure of flavanols and procyanidins. Basic structure is shown on the left, while an example of DP 5 is shown on the right.

achieving full resolution of the flavanols and procyanidins up to and including DP 1–10. Consistent with the previous method, epicatechin-based, method-specific relative response factors (RRFs) were used for the quantification of DP 2–10. This paper reports on the accuracy, linearity, repeatability, LOD, and LOQ in a single-laboratory setting. Samples ranging in concentration from 1 to 320 mg/g cocoa flavanols and procyanidins (CF-CP; DP 1–10) were evaluated so as to encompass what could be expected in commercially available ingredients and products.

Overall, the SLV reported herein demonstrates that by using this rapid resolution method, the analysis of CF-CP in a range of commercially representative samples can be done reliably, providing accurate and consistent results in a relatively short chromatographic run time. In addition, we also evaluated the performance of this rapid method against the previously validated conventional LC method (7), and the comparison of results provides initial demonstration that the methods have comparable accuracy.

Experimental

Samples

Seven cocoa-based ingredients and products were selected for this validation study. These samples were representative of the types of materials commercially available and therefore reflect the range of samples that a laboratory performing this type of analysis could likely encounter. The samples analyzed included a milk chocolate (Scharffen Berger, San Francisco, CA), natural and alkalized cocoa powders (Rushmore, Blommer Chocolate Company, Chicago, IL), dark chocolate and chocolate liquor (produced using the Cocoapro® process, Mars, Inc., McLean, VA), and cocoa nibs and cocoa extract (cocoa extract produced using the Cocoapro® process, Mars, Inc.).

Apparatus

(**a**) *HPLC system.*—An Agilent Technologies (Santa Clara, CA) 1200 HPLC system was used, consisting of a binary pump with a vacuum degasser, a thermostatted column compartment, an autosampler, and diode array and fluorescence detectors. Systems were controlled and data collected and analyzed by Agilent ChemStation software (version B.04.03).

(**b**) *Analytical column.*—Sepax Technologies (Newark, DE) HP-Diol (1.8 µm, 4.6 × 50 mm) diol-phase column.

(**c**) *Balance.*—Readability to 0.1 mg (Mettler-Toledo, Columbus, OH).

(**d**) *Ultrasonic bath.*—Model 150D (VWR, Radnor, PA).

(**e**) *Vortex mixer.*—VWR.

(**f**) *Centrifuge.*—Allegra X-22R (Beckmann Coulter, Brea, CA).

(**g**) *pH meter and probe.*—MultiSeven (Mettler-Toledo).

(**h**) *Syringe.*—Plastic, Luer lock, 1 mL (BD, Franklin Lakes, NJ).

(**i**) *SPE cartridges.*—Strata SCX (Phenomenex, Torrance, CA).

(**j**) *SPE manifold.*—VWR.

(**k**) *Freeze dryer.*—VirTus (SP Industries, Warminster, PA). Not needed for routine work.

(**l**) *PTFE syringe filter.*—0.45 µm (Whatman Inc., Florham Park, NJ).

(**m**) *Volumetric flask.*—Class A, various sizes (VWR).

(**n**) *Glass pipets.*—Class A, various sizes (VWR).

(**o**) *Graduate cylinders.*—Class A, various sizes (VWR).

(**p**) *Autosampler vials.*—12 × 32 mm, 2 mL amber vials (National Scientific, Rockwood, TN).

(**q**) *Vial caps.*—Standard crimp top (Agilent Technologies).

(**r**) *Disposable centrifuge tubes.*—15 and 50 mL polypropylene (VWR).

(**s**) *Bottle-top dispenser.*—(BRAND GMBH, Wertheim, Germany).

Reagents and Solvents

(**a**) *Water.*—Milli-Q (EMD Millipore, Billerica, MA).

(**b**) *Glacial acetic acid.*—HPLC grade (EMD Millipore).

(**c**) *Methanol.*—ACS/HPLC grade (Honeywell Burdick & Jackson, Muskegon, MI).

(**d**) *Acetone.*—ACS/HPLC grade (Honeywell Burdick & Jackson).

(**e**) *Acetonitrile.*—ACS/HPLC grade (Honeywell Burdick & Jackson).

(**f**) *Mobile phase A.*—Acetonitrile–glacial acetic acid (98 + 2, v/v).

(**g**) *Mobile phase B.*—Methanol–water–glacial acetic acid (95 + 3 + 2, v/v/v).

(**h**) *Enriched cocoa extract.*—Purity 65% for flavanols and procyanidins DP 1–10 (Mars Botanical, Germantown, MD).

(**i**) *Extraction solvent.*—In a suitable container, 700 mL acetone, 295 mL water, and 5 mL glacial acetic acid were combined, mixed, and allowed to equilibrate to room temperature.

(**j**) *Sodium hydroxide solution.*—50% (w/w; J.T. Baker, Phillipsburg, NJ).

(**k**) *Hydrochloric acid.*—OmniTrace Ultra, high purity (EMD Millipore).

(**l**) *(-)-Epicatechin.*—Purity 95.9% (Sigma-Aldrich, St. Louis, MO).

Standard Materials

The calibrant used for this method was exclusively (-)-epicatechin. Quantification of the oligomers (DP 2–10) was accomplished through the use of method-specific RRFs [RRF = signal of each DP relative to (-)-epicatechin]. Individually purified oligomeric fractions from cocoa were

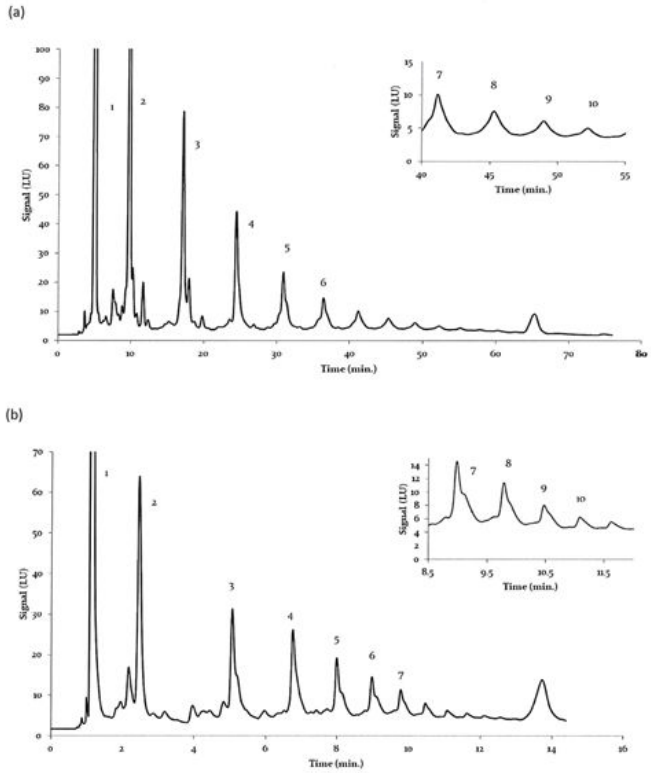

Figure 2. Sample chromatograms of natural cocoa powder showing (a) conventional LC diol trace of DP 1–10 on a 4.6 × 250 mm, 5 µm column, and (b) rapid resolution diol trace using a 4.6 × 50 mm, 1.8 µm column. Insets show expanded late elution range.

isolated as previously described (7) and subjected to extensive purity and identification assessments to support their use in the RRF determinations. Epicatechin, along with DP 2–7, had purities greater than 96%. The purities of DP 8, 9, and 10 were 93, 93, and 91%, respectively. As with the MLV, each RRF was determined by analyzing triplicate injections of a pure solution of epicatechin and a solution of each individual oligomeric fraction under the indicated detector conditions, and dividing the average response obtained of each oligomeric fraction by the response of epicatechin. This procedure was independently performed across three laboratories using the same standard materials. The method-specific RRFs for each oligomeric fraction was based on the average of all RRF determinations obtained from all three laboratories (n = 9), and are shown in Table 1.

Preparation of Test Solutions

(**a**) *Stock standard solution.*—25 mg (-)-epicatechin in a 25 mL volumetric flask and diluted to volume with extraction solvent. The concentration of the stock solution was 1 mg/mL. The solution was dispensed into an appropriate number of scintillation vials and sealed for storage at –20°C.

(**b**) *Working standard solutions.*—To generate a calibration curve that was used for quantification, seven serial dilutions of the stock standard solution of (-)-epicatechin were prepared in the extraction solvent. Stock and calibration solutions used for the study were prepared fresh each day and discarded at the end of the day.

(**c**) *Evaluation of solution stability.*—Additional standard solutions were prepared to evaluate stability of this standard over a 28-day period. Three dilutions of the stock standard were prepared, which represented a low, medium, and high concentration standard (corresponding to a low, medium, and high concentration calibration standard). These dilutions were dispensed into the appropriate number of vials needed for the study and stored at –20°C. In addition, aliquots of the stock standard solution were dispensed into scintillation vials and stored at –20°C. This was done to evaluate both working standard and stock standard stability. For this stability study, all samples were thawed only once, then discarded at the end of the day of use.

Sample Preparation

Cocoa and chocolate samples containing more than 10% total fat first required defatting using hexane, as outlined in Machonis et al. (8). The cocoa extract only contained 4% fat; therefore, fat extraction was not required for this specific sample. If the amount of fat in a sample is not known, quantitative defatting should be done so as to appropriately adjust the value to report total CF-CP content on a whole product basis. Following the defatting procedure, if applicable, samples were extracted following a procedure similar to the one outlined in Machonis et al. (8). In brief, the sample was weighed into a disposable centrifuge tube. A volumetric addition of extraction solvent was made, and the sample was vortexed. The sample was then sonicated at 50°C for 5 min, centrifuged at 1006 × g,

Table 1. Determined method-specific RRF for fractions DP 1–10

Oligomer	RRF[a]
Epicatechin	1.000
DP 2	0.377
DP 3	0.152
DP 4	0.146
DP 5	0.146
DP 6	0.119
DP 7	0.090
DP 8	0.071
DP 9	0.068
DP 10	0.067

[a] RRF determined for each oligomeric fraction. Values are based on the average RRF determined among three independent laboratories. Reported value is the average of the values obtained across three laboratories (three injections/oligomeric fraction, n = 9).

and the resulting supernatant was filtered with a 0.45 µm PTFE filter when dispensed into an autosampler vial for analysis. The only differences between this procedure and the one referenced were the use of a different extraction solvent and different sample weights and dilutions (listed in Table 2) to accommodate different CF-CP content in the samples.

Based on previous work (6), alkalized ingredients and finished product samples containing alkalized cocoa (cocoa powder or liquor) are known to contain compounds that can interfere with the analysis and thus require the use of a cation exchange SPE cartridge. This cleanup step has been shown to quickly and efficiently remove the interfering component(s) without affecting the CF-CP content in the sample (6). In this study, the alkalized liquor, alkalized cocoa powder, and the two chocolate samples containing alkalized components were first extracted with extraction solution, then the supernatant solution passed through a preconditioned Strata SCX cartridge. If it is unclear whether the sample contains any alkalized components, the SPE step can be applied without compromising CF-CP analysis. All samples were then filtered through a 0.45 µm PTFE syringe filter into amber HPLC vials for analysis.

LC Conditions

Separation was achieved on a Sepax Polar-Diol column (4.6 × 50 mm, 1.8 µm particle size) with a binary gradient consisting of solvent A: acetonitrile–glacial acetic acid (98 + 2, v/v) and solvent B: methanol–water–glacial acetic acid (95 + 3 + 2, v/v/v). The starting mobile phase condition was 0% B, which was held for 1 min. The mobile phase composition was subsequently increased to 30% solvent B over 10.5 min, then to 100% B over an additional 1.5 min. The 100% B condition was held for 1 min prior to a return to starting conditions over 1 min. Total run time was 15 min, followed by a 5 min post-run re-equilibration at the starting conditions (0% B).

For analysis, the column temperature was held at 35°C, and the autosampler temperature was set to 5°C. The flow rate was 1.0 mL/min with an injection volume of 1 µL. Detection was by fluorescence, with an excitation wavelength of 230 nm and an emission wavelength of 321 nm. The photomultiplier tube

Table 2. Sample weights (defatted, if required) and extraction solvent volumes used for sample preparation

Sample type	Weight, mg	Extraction solvent volume, mL
Cocoa extract	50	10
Cocoa nib	100	5
Cocoa liquor	250	5
Natural cocoa powder	500	5
Alkalized cocoa powder	1000	5
Dark chocolate	1000	5
Milk chocolate	1000	5

(PMT) gain was chosen based on the most sensitive setting at which the highest calibration standard was shown to achieve the greatest sensitivity without overloading the fluorescence detector. For our specific instrument, the PMT gain for the detector was set to 8 throughout the analysis. The appropriate PMT gain setting should be evaluated on a per-instrument basis.

Recovery Study

Given the additional sample handling required for samples with fat, two sample matrixes devoid of CF-CP, yet varying in fat content, were used to evaluate recovery: a highly alkalized reconstructed baking chocolate (>35% fat) and a cocoa extract negative control (<5% fat). These samples have been successfully applied in previous studies to evaluate recovery from cocoa-based samples (8).

Following preparation of the negative controls, the samples for the spike recovery experiments were prepared as follows. For the chocolate sample, 11.13 g reconstructed baking chocolate was melted and combined with 0.35 g cocoa extract containing DP 1–10. The mixture was stirred for 20 min to completely incorporate the spike and allowed to harden. For the spiked extract, 1.13 g cocoa extract negative control was combined with 0.39 g cocoa extract. This preparation was combined with 20 mL deionized water and stirred for 20 min to ensure homogenization of the components. Afterwards, the mixture was placed on the freeze dryer for drying. These spiked samples, i.e., 500 mg defatted baking chocolate (extracted with hexane) and 50 mg cocoa extract, were extracted with 5 mL extraction solvent and analyzed as described in the previous section.

Solution Stability

Standard stability, both stock solution and diluted preparation (working standard), was evaluated as part of this study. The study lasted 28 days, and samples were analyzed on Days 0, 1, 2, 3, 6, 9, 14, 21, and 28. On each study day, a single scintillation vial for stock standard or a set of three working standards was removed from the freezer and allowed to thaw and equilibrate to room temperature. The stock solution was diluted to prepare three standards at low, medium, and high standard concentrations within the calibrated range. All samples were then analyzed by LC according to the specified run conditions, and (-)-epicatechin concentration determined

Table 3. Repeatability results for cocoa-based samples

	Total CF-CP (DP 1–10)[a]			
	Mean content, mg/g[b]	Intraday RSD, %	Overall RSD, %	HorRat ratio
Cocoa extract[c]	318.88	1.15	3.97	1.68
Cocoa nib[c]	22.75	6.91	5.91	1.86
Cocoa liquor[c]	19.84	2.19	5.84	1.73
Natural cocoa powder[c]	13.57	2.63	6.48	1.71
Alkalized cocoa powder[d]	3.62	2.18	5.06	1.11
Dark chocolate[d]	3.56	2.54	8.06	1.92
Milk chocolate[d]	1.14	5.08	13.61	2.73

[a] All values are reported on a whole product basis.

[b] All sample concentrations fell within the specified calibration range.

[c] n = 19, analyzed on 4 different days; Q-test used for outlier identification with 95% confidence.

[d] n = 5, analyzed on 3 different days.

and each tested day compared to results obtained on Day 0 so as to evaluate the stability of the prepared standards.

Results and Discussion

Linearity/Calibration Curve

The linearity of (-)-epicatechin was determined using a seven-point calibration curve with effective concentrations of (-)-epicatechin at 0.02, 0.04, 0.08, 0.10, 0.16, 0.30, and 0.60 mg/mL. The seven standards were injected in triplicate, and the average areas of each were plotted against concentration. These concentrations of the calibration standards were suitable for the range of samples used for this study and reflect the range that could be expected in commercially available ingredients and finished products. The correlation coefficient was ≥0.998.

Calculation of CF-CP Results Using RRFs

Calculation of CF-CP (DP 1–10) was determined using RRFs. Monomer content was based on the external calibration standard, (-)-epicatechin. The concentration of DP 2–10 was calculated using the various RRFs in Table 1 by the following equation:

$$CF-CP_{DPn} \text{ in mg/g} = \frac{\left(\frac{Area_{DPn}}{m_{EC} \times RRF_{DPn}}\right)}{W_s} \times DF$$

where DPn is the degree of polymerization indicating the oligomer, m is the slope of the epicatechin calibration curve, W_s is sample weight in g, and DF is the appropriate dilution factor.

Since the concentration is based on the RRF to (-)-epicatechin, only a single calibration curve is needed to quantify DP 1–10. To construct the curve, (-)-epicatechin peak area was plotted versus concentration (mg/mL) as described above. The resulting slope of the calibration curve was used in the above calculation to quantify the various oligomers. The total CF-CP concentration was determined by summing the individual DP 1–10 concentrations, which can then be adjusted for the concentration of fat so as to express data on a whole product basis.

Table 4. Percent recoveries for total CF-CP in cocoa extract blank matrix and reconstructed baking chocolate

	Total CF-CP (DP 1–10)		
	Mean content, mg/g[a]	Avg. recovery, %	RSD, %[b]
High CF in cocoa extract negative control	221.93	99.79	1.55
Low CF in reconstructed baking chocolate	19.85	98.42	2.93

[a] All sample concentrations fell within the specified calibration range.

[b] n = 5.

LOQ/LOD

CF-CP is unique in that it is a group of various compounds. In previous work, LOD/LOQ were determined for each individual oligomer (6); however, this method, as well as the conventional LC method taken through the MLV (7), utilized a single calibrant, (-)-epicatechin, using epicatechin-based RRFs for quantification of DP 2–10. Therefore, the LOD and LOQ were determined using the S/N for a pure (-)-epicatechin solution. The LOD for (-)-epicatechin using this method was 0.4 µg/mL, and the LOQ was 1.2 µg/mL. It should be noted that within-matrix LOD/LOQ may vary slightly from what is reported due to the effects of individual cocoa-based sample matrixes.

Precision

Repeatability was evaluated by measuring five separate preparations of the seven sample types on three different days. This allowed evaluation of both interday and intraday repeatability. For cocoa extract, cocoa nib, and natural cocoa powder, the RSD for each of these sample types was higher than expected on one of the days; these results were further evaluated to determine the possibility of an outlier. In general, the RSD for all days was typically <5%. In the case of cocoa extract, cocoa nib, and natural cocoa powder (RSD of five sample results), the RSD was 8–10%. It was decided to analyze an extra set of five preparations, resulting in a fourth day. Using the Q-test and using a confidence interval of 95%, an outlier was determined in each of the "out of line" days and discounted for each of these three sample sets. Within-day variability ranged from 1.15 to 6.91% for all products, and the overall RSD spanned 3.97% for cocoa extract up to 13.61% for milk chocolate. For samples with lower total CF-CP concentrations (i.e., less than 3 mg/g whole product), such as milk chocolate, a higher RSD was expected, consistent with previous findings (7). The results of all the sample sets can be seen in Table 3, and are reported on a whole product (fat corrected) basis.

The Horwitz ratio (HorRat) can be a useful measure of overall method performance; however, as HorRat values are most generally applied to evaluate the precision of methods used in the analysis of individual compounds, the determination of HorRat values for methods that quantify mixtures of species (e.g., based on DP) may be less reliable. HorRat values for all seven sample types were calculated and are reported in

Table 5. Comparison of conventional diol methodology to rapid diol methodology[a]

Sample type	Rapid resolution LC result, mg/g	MLV results from our lab, mg/g	Collaborative range, mg/g[b]
Chocolate chips	7.64	9.28	7.62–12.58
Chocolate powder	65.07	61.61	51.81–67.71
Dark chocolate	6.94	7.44	6.54–9.65
Extract A	319.85	336.33	271.84–381.80
Extract B	434.13	471.06	394.30–566.07
Liquor	15.70	15.66	15.00–17.40
Milk chocolate	1.71	2.03	1.73–2.91

[a] Results are CF-CP DP 1–10 on a defatted basis (not corrected for fat content).

[b] Collaborative ranges are taken from reference 7.

Table 3. Despite the complex mixture of components being analyzed, the HorRat values for these samples ranged from 1.1 to 2.7. Historically, acceptable HorRat values in SLVs fall in the ranges of 0.5–2.0 and 0.3–1.3 for repeatability and intermediate reproducibility, respectively (9). With a value of 2.7, milk chocolate was the sample type to have a HorRat value outside this range. Given the low concentration of CF-CP in this sample, as well as the complex mixture of components (DP 1–10), a higher HorRat score is not unexpected and is consistent with what has been previously reported (7).

Accuracy

Because no certified reference material exists for the CF-CP (measured and reported as DP 1–10), our own matrix blanks were prepared and spiked with CF-CP. For this study, we evaluated a high fat (>35%) reconstructed baking chocolate matrix that was spiked with a lower amount of CF-CP, and a low fat (<10%) negative cocoa extract control spiked with a higher amount of CF-CP. Table 4 shows average recoveries for all samples. AOAC INTERNATIONAL establishes guidelines for acceptable recoveries (9), with the acceptable recovery at the 1% concentration level being 92–105%, and for the 10% concentration level 95–102%. Accuracy in both cases was excellent, with recoveries of 98.4% for the spiked baking chocolate and 99.8% for the spiked cocoa extract control. In addition to showing acceptable recovery, these results showed that sample preparation does not result in any significant loss of total CF-CP, consistent with previous findings (8).

Solution Stability

Standard samples were analyzed over a span of 28 days to evaluate stability. At each pre-selected time point, the concentration of (-)-epicatechin was determined and compared to the concentration on Day 0. For the stock standard at Day 28, the medium and high concentrations changed by 0.2 and 1.0%, respectively, from Day 0, while the low concentration had a 5.0% difference relative to Day 0. Differences between Day 0 and Day 28 for the working standard were 5.1% for the low level, 3.7% for the medium level, and 9.2% for the high level. These data support that the stock standard can be kept at –20°C for up to 28 days with no significant impact on performance. While the stock standard is stable, the working standards showed some degradation and should be prepared fresh at the time of analysis. While the (-)-epicatechin standards were not evaluated for solution stability in the method taken through the MLV, the same-day preparation of the working standards is consistent with what was used for that method (7).

Because the stock standard stability was not evaluated by drawing out of a single container, if the stock standard is to be used on multiple days, it should be proportioned into an adequate number of subsamples after initial preparation to avoid multiple freeze-thaw cycles that could negatively affect concentration and therefore stability.

Comparison to Conventional Methodology

In an effort to determine the equivalency between the newly developed rapid resolution method and the original LC method subjected to the MLV, the same samples used for the MLV (7) were also analyzed using the rapid resolution method. Sample types analyzed included chocolate chips, cocoa powder, dark and milk chocolates, cocoa extracts, and cocoa liquor. These samples were provided as blind duplicates and stored at –20°C. Samples for this comparative were prepared and analyzed as per the rapid resolution method described herein. Results reported herein for conventional method were taken from the results reported in the MLV and were not re-analyzed for this study. A comparison of the chromatography revealed that the resolution of CF-CP peaks was comparable between the conventional LC method and the rapid resolution method (Figure 3), indicating no loss in resolution despite the dramatically shortened run time. The average CF-CP values of each pair of samples tested determined using the rapid method are shown in Table 5, along with average concentrations for these samples obtained by our laboratory using the validated conventional LC method (which were previously reported to the Study Director as part of the MLV), as well as the ranges of the respective sample types from the MLV. In all but one case, the CF-CP value determined using the rapid resolution method was within the range of those same samples analyzed as part of the MLV. The only exception to this was the milk chocolate sample. With a determined concentration of 1.71 mg/g, this sample was just 0.02 mg/g below the range reported in the MLV (1.73–2.91 mg/g). In the repeatability section, the milk chocolate sample—with less than 3 mg/g CF-CP—had the highest RSD and a higher HorRat score relative to the other sample types evaluated. As previously noted, this may be due to the lower concentration of CF-CP in this sample type, the lowest concentration of all samples evaluated. Overall, these results demonstrate that the recently validated conventional LC method and newly developed rapid resolution method provide consistent results.

Conclusions

A validated method for determination of CF-CP (DP 1–10) was translated to rapid resolution chromatography, and a subsequent SLV was performed to evaluate method performance. The rapid resolution method not only demonstrated acceptable repeatability among seven distinct cocoa-based samples, but was also found to have good accuracy, as demonstrated by the results of the spike-recovery analyses with the high-fat,

low-cocoa flavanol matrix as well as in a low-fat, high-cocoa flavanol matrix. Importantly, the translation to rapid resolution chromatography maintained the required resolution and reliability of the recently published and validated method (7). The results of this SLV demonstrate that this rapid method is accurate and reliable, and with the 80% reduction in run time and solvent usage, the increased laboratory efficiency and quicker sample turnaround time offered by this method may make it more suitable in the routine analysis of CF-CP in a wide range of commercially available cocoa-based products. Furthermore, the same samples analyzed as part of the MLV of the conventional LC method on which this rapid method is based were also analyzed and showed that these two methods had comparable accuracy.

Acknowledgments

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FOOD COMPOSITION AND ADDITIVES

Method for the Determination of Catechin and Epicatechin Enantiomers in Cocoa-Based Ingredients and Products by High-Performance Liquid Chromatography: Single-Laboratory Validation

PHILIP R. MACHONIS, MATTHEW A. JONES, BRIAN T. SCHANEBERG, and CATHERINE L. KWIK-URIBE
Mars Incorporated, 6885 Elm St, McLean, VA 22101

A single-laboratory validation study was performed for an HPLC method to identify and quantify the flavanol enantiomers (+)- and (−)-epicatechin and (+)- and (−)-catechin in cocoa-based ingredients and products. These compounds were eluted isocratically with an ammonium acetate–methanol mobile phase applied to a modified β-cyclodextrin chiral stationary phase and detected using fluorescence. Spike recovery experiments using appropriate matrix blanks, along with cocoa extract, cocoa powder, and dark chocolate, were used to evaluate accuracy, repeatability, specificity, LOD, LOQ, and linearity of the method as performed by a single analyst on multiple days. In all samples analyzed, (−)-epicatechin was the predominant flavanol and represented 68–91% of the total monomeric flavanols detected. For the cocoa-based products, within-day (intraday) precision for (−)-epicatechin was between 1.46–3.22%, for (+)-catechin between 3.66–6.90%, and for (−)-catechin between 1.69–6.89%; (+)-epicatechin was not detected in these samples. Recoveries for the three sample types investigated ranged from 82.2 to 102.1% at the 50% spiking level, 83.7 to 102.0% at the 100% spiking level, and 80.4 to 101.1% at the 200% spiking level. Based on performance results, this method may be suitable for routine laboratory use in analysis of cocoa-based ingredients and products.

The flavanols, e.g., epicatechin and catechin, are a class of bioactive flavonoids that are widely found in many foods and beverages common in the human diet. Apples, grapes, red wine, and cocoa-based products have all been shown to be rich in flavanols. Epicatechin and catechin exist in distinct stereochemical forms (Figure 1), namely (+)- and (−)-epicatechin (structures **1**, **2**) and (+)- and (−)-catechin (structures **3**, **4**). Flavanols can undergo epimerization, specific stereochemical conversions at a single carbon center, leading to the conversion of (−)-epicatechin (structure **2**) to (−)-catechin (structure **4**) and (+)-catechin (structure **3**) to (+)-epicatechin (structure **1**) (1). The rates of these conversions are influenced by both pH and temperature, and have been shown to occur under conventional food processing conditions (2).

Specific to cocoa, the predominant flavanols that exist in the fresh cocoa bean (seed) are (−)-epicatechin and, to a much lesser extent, (+)-catechin. Although these specific flavanols are abundant in the fresh bean, as cocoa beans are processed (e.g., fermented, roasted, alkalized, etc.), the epimerization of (−)-epicatechin to (−)-catechin has been shown to readily occur, with the appearance of (−)-catechin suggested to be an indicator of the extent of handling and processing of cocoa itself (3). The epimerization of (−)-epicatechin is more than simply an interesting stereochemical conversion in the context of cocoa handling and processing. Over the past few years, research in humans has established that (−)-epicatechin is a key bioactive flavanol in cocoa, causally linked to the vascular improvements observed following the consumption of flavanol-rich cocoa (4). Furthermore, a recent study in humans demonstrated that the uptake and metabolism of flavanols is readily influenced by stereochemical configuration, with (−)-epicatechin demonstrated to have an oral absorbability of nearly six times that of (−)-catechin (5). Thus, in light of this emerging research, the identification of the distinct stereochemical forms of flavanols in food products may become of increasing scientific interest and nutritional relevance.

Although there are a number of published papers that present methods for the resolution of catechin from epicatechin, including a recent study in cocoa-based products (6), there are far fewer that specifically focus on the individual resolution of the distinct enantiomers of epicatechin or catechin. Additionally, the lack of clarity regarding the difference between methods that can resolve discrete epimers (e.g., catechin from epicatechin) versus those that can resolve discrete enantiomers [e.g., (−)-epicatechin from (+)-epicatechin] has led to errors in the reporting of identity within the recent published literature (7–9). In light of known processing-induced changes in stereochemistry (2, 3) along with recent data on the relative bioavailability and biopotency of flavanols (5), the resolution of epicatechin and catechin alone may be of limited value and, therefore, chiral methods may be of increased interest and need.

Although a wide array of analytical tools have been used for chiral separations in the field of chromatography, the use of HPLC has been reported in the bulk of the published literature within the past 2 years (10). Specific to enantiomers, Donovan et al. (11) reported a 75 min HPLC method for the four flavanol enantiomers; this method was then subsequently applied to the determination of flavanols present in a range of commercially available chocolates (3). Although separation of the four

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Corresponding author's e-mail: philip.machonis@mss.effem.com
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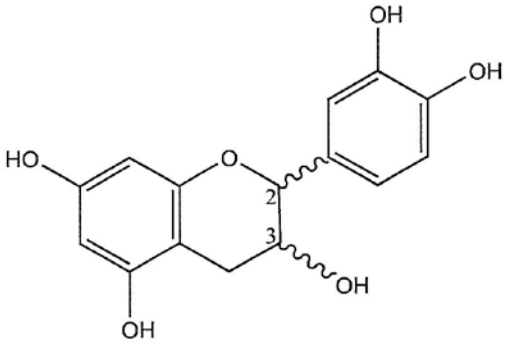

1. (+)-Epicatechin (2S,3S)
2. (-)-Epicatechin (2R,3R)
3. (+)-Catechin (2R,3S)
4. (-)-Catechin (2S,3R)

Figure 1. Enantiomers of epicatechin and catechin.

individual flavanols was reported in these papers, differentiation between (+)-epicatechin and (−)-epicatechin was not reproducible in our laboratory or by others (2). To address this issue, an isocratic method that uses fluorescence detection was developed to analyze the four individual enantiomers, (+/−)-epicatechin and (+/−)-catechin, in cocoa-based ingredients and products in a single 35 min run. This paper reports on the initial method optimization and the subsequent single-laboratory validation study—the first of its kind for this type of stereospecific analysis of flavanols—to evaluate the accuracy, selectivity, linearity, repeatability, LOQ, and LOD. As an enantiospecific method for catechin and epicatechin that is accurate and reliable, this method has the potential to be utilized more routinely in the analysis of cocoa-based ingredients and products.

Experimental

Samples

Three cocoa-based ingredients and products were selected for this validation. The samples are representative of the types of materials that a laboratory performing this analysis is likely to encounter. The samples analyzed were a dark chocolate (Mars, Inc., Hackettstown, NJ), a natural cocoa powder (Blommer Chocolate Co., Chicago, IL), and a cocoa extract (Mars Botanical, Gaithersburg, MD).

Apparatus

(**a**) *HPLC system*.—An Agilent Technologies 1100 HPLC system (Santa Clara, CA) was used, consisting of a quaternary pump with a vacuum degasser, thermostatted column compartment, autosampler, and diode array and fluorescence detectors. Systems were controlled and data collected and analyzed by Agilent ChemStation software (version B.04.03).

(**b**) *Analytical column*.—Astec Cyclobond I-2000 RSP (250 × 4.6 mm id, 5 µm particle size) chiral column (Supelco, Bellefonte, PA), preceded by a 4.0 × 2.0 mm id guard column with the same stationary phase.

(**c**) *Balance*.—Readability to 0.1 mg (Mettler-Toledo, Columbus, OH).

(**d**) *Ultrasonic bath*.—Model 150D (VWR, Radnor, PA).

(**e**) *Vortex mixer*.—VWR.

(**f**) *Centrifuge*.—Allegra X-22R (Beckmann Coulter, Brea, CA).

(**g**) *pH Meter and probe*.—MultiSeven (Mettler-Toledo).

(**h**) *Syringe*.—Plastic, Luer lock, 1 mL (BD, Franklin Lakes, NJ).

(**i**) *PTFE syringe filter*.—0.45 µm (Whatman Inc., Florham Park, NJ).

(**j**) *Volumetric flasks*.—Class A, various sizes (VWR).

(**k**) *Glass pipets*.—Class A, various sizes (VWR).

(**l**) *Graduated cylinders*.—Class A, various sizes (VWR).

(**m**) *Autosampler vials*.—12 × 32 mm, 2 mL amber vials (National Scientific, Rockwood, TN).

(**n**) *Vial caps*.—Standard crimp top (Agilent Technologies).

(**o**) *Disposable centrifuge tubes*.—15 and 50 mL polypropylene (VWR).

(**p**) *Bottle-top dispenser*.—Brand GmbH (Wertheim, Germany).

Reagents and Solvents

(**a**) *Water*.—Milli-Q (Millipore, Billerica, MA).

(**b**) *Glacial acetic acid*.—HPLC grade (EMD, Gibbstown, NJ).

(**c**) *Ammonium acetate, puriss*.—HPLC grade (Fluka, St. Louis, MO).

(**d**) *Methanol*.—ACS/HPLC grade (Honeywell Burdick & Jackson, Muskegon, MI).

(**e**) *Acetone*.—ACS/HPLC grade (Honeywell Burdick & Jackson).

(**f**) *20 mM Ammonium acetate buffer, pH 4.0*.—2.87 g ammonium acetate was dissolved in and brought to volume with water in a 1 L volumetric flask; pH was adjusted to 4.0 using glacial acetic acid.

(**g**) *HPLC mobile phase*.—20 mM ammonium acetate buffer, pH 4.0–methanol (70 + 30, v/v), allowed to equilibrate to room temperature.

(**h**) *Extraction solvent*.—In a suitable container, 700 mL

Table 1. Concentration of enantiomers spiked into cocoa extract negative control, alkalized cocoa powder, and reconstructed baking chocolate at the 100% concentration level

	Concentration, µg/mL			
Sample type	(+)-Epicatechin	(–)-Epicatechin	(+)-Catechin	(–)-Catechin
Cocoa extract	6.8	633.4	111.5	73.3
Cocoa powder	4.7	71.2	4.7	7.7
Reconstructed baking chocolate	4.7	71.2	4.7	7.7

Table 2. Parameters evaluated during method optimization; while one parameter was being evaluated, the other two were maintained at the initial conditions

Parameter	Condition		
	A	B	C
Column temperature, °C	25	35	45
Mobile phase composition, v/v	60 + 40	70 + 30	80 + 20
Aqueous buffer, pH	3.8	4.0	4.2

methanol, 295 mL water, and 5 mL glacial acetic acid were combined, mixed, and allowed to equilibrate to room temperature.

(**i**) *pH Calibration buffers*.—pH 4, 7, and 10 (VWR).

(**j**) *Sodium hydroxide*.—50% (w/w) solution (J.T. Baker, Phillipsburg, NJ).

(**k**) *Hydrochloric acid*.—OmniTrace Ultra, high purity (EMD).

Standard Materials

Standard reference materials were purchased or isolated in-house with identification and assigned purities based on a detailed characterization of the material. The materials' structural determination were supported by NMR spectrometry, MS, specific rotation, melting point, and microelemental data, while the purity was assessed by terms of UV spectrometry between 240 and 320 nm, water content, metal content, and residual solvent content.

(**a**) *(+)-Epicatechin*.—Purity 89.7% (Mars Botanical).

(**b**) *(−)-Epicatechin*.—Purity 97.3% (Sigma-Aldrich, St. Louis, MO).

(**c**) *(+)-Catechin hydrate*.—Purity 97.6% (Sigma-Aldrich).

(**d**) *(−)-Catechin*.—Purity 95.9% (Mars Botanical).

Preparation of Test Solutions

(**a**) *Stock standard solution*.—A stock standard solution was prepared by weighing 1.5 mg (+)-epicatechin, 17.6 mg (−)-epicatechin, 3.3 mg (+)-catechin, and 2.6 mg (−)-catechin into a 25 mL volumetric flask, dissolving in a portion of extraction solvent, then diluting to volume with extraction solvent. The resulting concentrations were approximately 59.8, 680.8, 130.7, and 104.0 µg/mL, respectively, for the four enantiomers. Concentrations of the stock standard solution were chosen based on the expected range of (−)-epicatechin, (+)-catechin, (−)-catechin, and (+)-epicatechin concentrations encountered in our own laboratory and reported in the literature (3). The stock solution was prepared fresh at the beginning of the day and discarded at the end of the day.

(**b**) *Calibration solutions*.—Serial dilutions of the stock solution prepared in extraction solvent, along with the stock solution, were used to generate a five-point calibration curve that

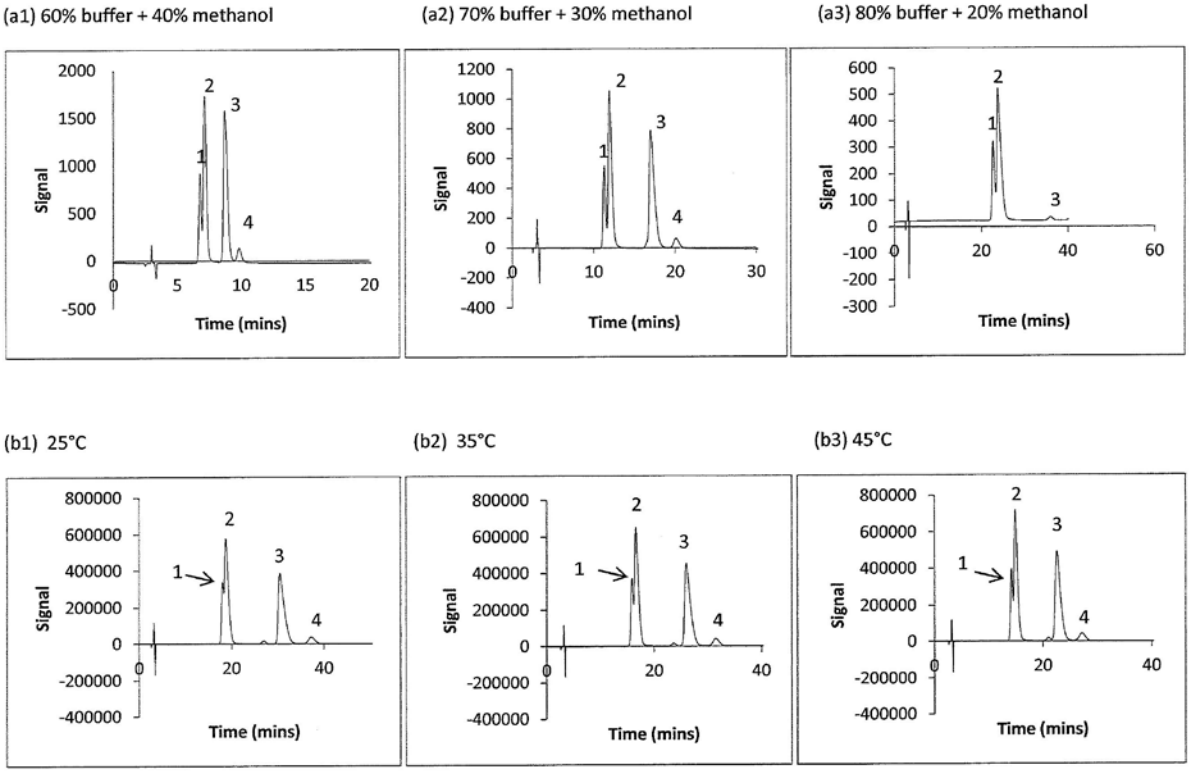

Figure 2. Effect of changes in (a1–a3) mobile phase composition and (b1–b3) column temperature on the separation of catechin and epicatechin enantiomers. (1) (+)-epicatechin, (2) (−)-epicatechin, (3) (+)-catechin, and (4) (−)-catechin.

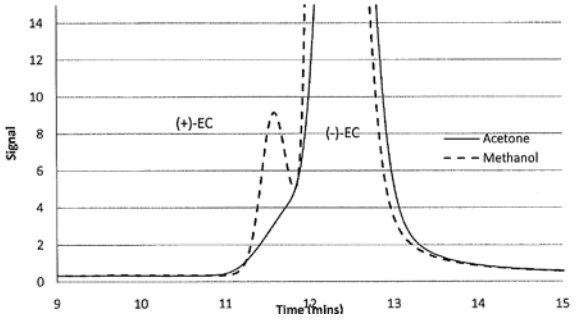

Figure 3. Effect of extraction solvent on the separation of (+)-epicatechin and (−)-epicatechin.

was used for sample quantification. Calibration solutions were also prepared fresh each day and discarded at the end of the day.

(**c**) *Linearity determination*.—Linearity for each of the individual flavanol enantiomers was determined by injecting the five standards in triplicate and plotting the average areas against concentration.

(**d**) *LOD/LOQ*.—LOD and LOQ were determined using standard solutions, or purified enantiomers dissolved in extraction solvent. The LOD and LOQ were calculated using S/N, with LOQ being 10:1 and LOD 3:1.

(**e**) *Spiking solution*.—The spiking solution was prepared by dissolving 5 mg (+)-epicatechin, 70 mg (−)-epicatechin, 5 mg (+)-catechin, and 20 mg (−)-catechin in methanol and bringing to volume in a 10 mL volumetric flask. A 1 mL amount of this solution was pipetted into a 100 mL volumetric flask and diluted to volume with methanol. Aliquots of this solution were used to spike the matrix blank samples at varying levels depending on the specific product.

Sample Preparation

Samples containing 10% or more total fat were first defatted using hexane. The natural cocoa powder and dark chocolate samples used in this study contained 10 and 45% total fat, respectively. The cocoa extract contained 4% fat; therefore, fat extraction was not required.

For defatting, approximately 10 g sample was weighed into a 50 mL disposable centrifuge tube and combined with approximately 45 mL hexane. The sample was vortexed for 1 min, sonicated at 50°C for 5 min, and centrifuged at 1006 × *g* for 5 min. Following centrifugation, the supernatant was decanted, and the remaining solids were carried forward to the next step. This fat extraction procedure was performed in triplicate. The sample was allowed to air-dry for at least 12 h to evaporate the residual hexane. Each sample (100 mg, defatted or nondefatted) was then weighed into a disposable 15 mL centrifuge tube. The flavanols were then extracted from the sample with extraction solvent. Using a bottle-top dispenser, 10 mL extraction solvent was used for the cocoa extract, while 5 mL extraction solvent was used for the cocoa powder and chocolate samples. These volumes of extraction solution were used based on the expected concentration of flavanols in the sample. The samples were subsequently vortexed for 1 min, sonicated at 50°C for 5 min, and centrifuged at 1006 × *g* for 5 min. The supernatant solution containing the extracted flavanols was collected and then passed through a 0.45 µm PTFE syringe filter into amber HPLC vials for analysis.

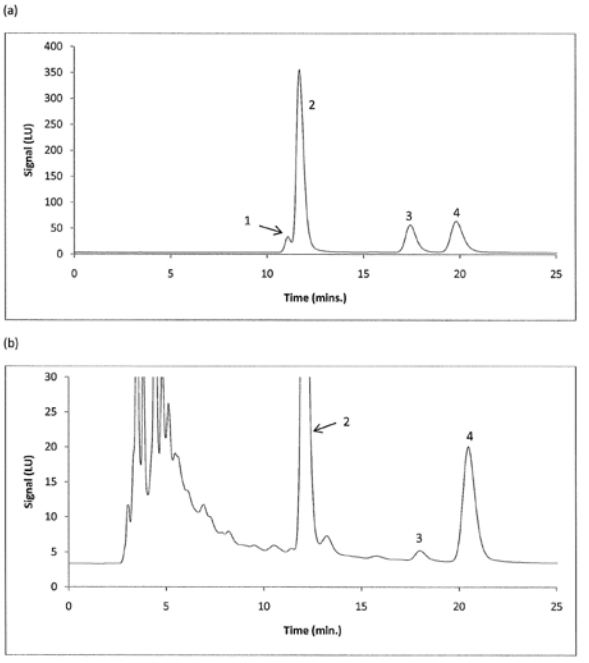

Figure 4. (a) Standard solution and (b) natural cocoa powder sample chromatograms showing separation of epicatechin and catechin enantiomers (1) (+)-epicatechin, (2) (−)-epicatechin, (3) (+)-catechin, and (4) (−)-catechin. The natural cocoa powder sample had no detectable (<LOD) (+)-epicatechin.

Determined HPLC Conditions

Separation was achieved using the isocratic mobile phase 20 mM ammonium acetate buffer, pH 4.0–methanol (70 + 30, v/v). Analysis time was 35 min, with a flow rate of 1.0 mL/min, and the injection volume was 10 µL. The stereoisomers were detected by fluorescence at an excitation wavelength of 276 nm and an emission wavelength of 316 nm. Column temperature was 35°C, and the autosampler was maintained at 5°C.

Recovery Study

Three distinct sample matrixes varying in fat content were obtained or created to evaluate recovery, as well as the potential for artifactual flavanol loss or epimerization during sample preparation. The samples used for this recovery study included a highly alkalized cocoa powder, a highly alkalized reconstructed baking chocolate, and a cocoa extract blank.

Jet Black cocoa powder (Blommer Chocolate Co., Chicago, IL) was used as a negative control for natural cocoa powder. Alkalization is known to destroy flavanols (7, 8), and previous work in our laboratory has demonstrated that this heavily alkalized Jet Black cocoa powder contained no detectable flavanols, thus making it a suitable blank matrix for the evaluation of recovery.

To evaluate recovery of the flavanol enantiomers from a higher fat matrix, a baking chocolate was reconstructed by combining cocoa butter and cocoa powder in the proper proportions (baking chocolate is simply unsweetened, ground cocoa beans). The reconstructed baking chocolate was made by melting 4 g cocoa butter in a water bath at 50°C and stirring in 4 g Jet Black cocoa powder until the mixture was well mixed and smooth. As the

Table 3. Range of standard concentrations for linear curves

Compound	Lower concn, µg/mL	Upper concn, µg/mL
(+)-Epicatechin	0.4	59.8
(−)-Epicatechin	6.8	680.8
(+)-Catechin	1.3	130.7
(−)-Catechin	1.0	104.0

Jet Black cocoa powder was used in the reconstructed baking chocolate, it too contained no detectable flavanols.

Because no suitable blank matrix exists for cocoa extract, a negative control was prepared by removing the flavanols from the cocoa extract. The cocoa extract was dissolved in acetone–water (80 + 20, v/v), resulting in a cocoa extract solution with a concentration of 1 mg/mL. To this solution Amberlite FPA90 CL resin (Rohm and Haas, Philadelphia, PA) was added in an excess of 20 times the amount of flavanols. The mixture was allowed to shake on a bench top shaker for 16 h. Afterwards, the spent resin was filtered out, and the solution was contacted with fresh resin for an additional 4 h, while shaking. This removed 95% of the flavanols from the cocoa extract. The remaining catechin and epicatechin were degraded by increasing the pH of the solution to 9.0 with concentrated sodium hydroxide and heating between 60 and 90°C for about 4 h. Afterwards, the solution pH was brought back down to 4.0 with concentrated hydrochloric acid, the solvent was removed using a rotary evaporator (Heidolph, Schwabach, Germany), and the mixture was freeze-dried to yield the cocoa extract negative control. Based on analysis, the level of flavanols in the treated extract was confirmed to be below the detectable limit.

To evaluate recovery, 100 mg of either Jet Black cocoa powder, reconstructed baking chocolate, or cocoa extract negative control were weighed into a centrifuge tube and spiked at varying levels with a spiking solution containing all four enantiomers in methanol. Based on previous analytical work in our laboratory, the content of flavanols in chocolate, cocoa powder, and extract can range from 0.2 to 100 mg/g; thus, the samples were individually spiked with varying amounts of flavanols to achieve final flavanol concentrations that were representative of what could be expected within the individual sample types (listed as the 100% level; Table 1). In addition, spiking was also performed at concentrations that were 50 and 200% of the representative concentration. After spiking the matrixes with the spiking solution, the spiked samples were vortexed for 5 min to ensure homogeneity and placed in a fume hood for 48 h to allow the solvent to fully evaporate. It should be noted that for baking chocolate, the samples were first liquefied by placing them in a water bath at 50°C for 5 min prior to adding in the spiking solution. The samples were defatted (if needed), and solvent was extracted as described previously. Samples were prepared and analyzed in duplicate.

Results and Discussion

Method Development

Although the Cyclobond I-2000 RSP stationary phase was ultimately selected, several columns were initially evaluated

Table 4. Repeatability results for cocoa extract, cocoa powder, and dark chocolate; all values are reported on a full-fat basis, *n* = 5, analyzed on 3 different days

	(−)-Epicatechin				(+)-Catechin				(−)-Catechin			
	Mean content, mg/g	Intraday RSD, %	Overall RSD, %	HorRat	Mean content, mg/g	Intraday RSD, %	Overall RSD, %	HorRat	Mean content, mg/g	Intraday RSD, %	Overall RSD, %	HorRat
Cocoa extract	59.0	0.5	1.5	0.5	1.5	5.1	3.7	0.7	4.5	1.2	1.7	0.4
Cocoa powder	2.5	0.2	1.8	0.4	0.1	6.0	6.9	0.8	1.1	1.1	5.9	1.1
Dark chocolate	1.85	1.5	3.2	0.6	0.1	5.4	6.6	0.8	0.5	1.2	6.9	1.2

Table 5. Recoveries for enantiomers in cocoa extract blank matrix, heavily alkalized cocoa powder, and reconstructed baking chocolate; samples were analyzed in duplicate, and results averaged

		Recovery, %		
	Compound	50% spiking level	100% spiking level	200% spiking level
Cocoa extract	(+)-Epicatechin	82.2	83.7	80.4
	(−)-Epicatechin	90.4	94.2	94.8
	(+)-Catechin	95.3	96.6	96.6
	(−)-Catechin	102.1	102.0	101.1
Cocoa powder	(+)-Epicatechin	85.0	94.5	89.8
	(−)-Epicatechin	91.4	95.9	94.4
	(+)-Catechin	100.2	98.1	92.6
	(−)-Catechin	95.9	94.7	92.8
Baking chocolate	(+)-Epicatechin	97.8	88.0	80.7
	(−)-Epicatechin	90.9	93.0	88.7
	(+)-Catechin	85.4	96.1	93.4
	(−)-Catechin	89.1	93.0	88.4

for their ability to separate the flavanol enantiomers. The four columns evaluated included three Astec Cyclobond columns with various chiral stationary phases (I-2000 DMP, I-2000 RSP, and I-2000 HP-RSP; Supelco) and a SCAS Sumichiral OA-7000 column (Sumika Chemical Analytical Services, Osaka, Japan). These columns were evaluated using a standard containing all four enantiomers and evaluating component separation using a variety of mobile phases. The Cyclobond I-2000 RSP column was selected due to its ability to adequately separate all four enantiomers using 20 mM ammonium acetate buffer, pH 4.0–methanol (70 + 30, v/v) mobile phase.

Following the initial selection of the Cyclobond I-2000 RSP column, selected method parameters were evaluated, specifically mobile phase buffer pH, mobile phase organic composition, and column temperature (Table 2). In the case of changes in mobile phase, an increase in the organic composition of the mobile phase to 80 + 20 (v/v) buffer–methanol decreased the resolution of the enantiomers and increased total run time. Figure 2a shows that a decrease in the organic composition to 60 + 40 (v/v) significantly decreased run time, but led to limited separation of (+)-epicatechin and (–)-epicatechin. Column temperature was evaluated while keeping a constant mobile phase of 20 mM ammonium acetate buffer–methanol (70 + 30, v/v). Decreasing the column temperature significantly reduced the resolution of epicatechin enantiomers with an accompanying increase in overall run time. There were no appreciable differences in peak resolution between 35 and 45°C and, as expected, the total run time was reduced about 5 min, with a column temperature of 45°C, as shown in Figure 2b. Modifying the pH of the ammonium acetate buffer solution from 3.8 to 4.2 had no significant effect on the chromatography (data not shown).

Initially, a sample extraction solvent of acetone–water–acetic acid (70 + 29.5 + 0.5, v/v/v) was to be used to keep the extraction solvent consistent with the sample preparation procedures commonly used in our laboratory for the analysis of cocoa flavanols and procyanidins (12), but it was observed that using acetone caused complete loss of resolution between (+)-epicatechin and (−)-epicatechin (Figure 3). By substituting methanol for acetone in the same proportions (70 + 29.5 + 0.5, v/v/v), this modified extraction solvent was found to improve resolution between (+)- and (–)-epicatechin and still retain a desirable level of recovery for these compounds from cocoa-containing samples (more details on recovery and accuracy are given).

Based on evaluation of column types and various method parameters, the Cyclobond I-2000 RSP column was selected, along with a 20 mM ammonium acetate buffer–methanol (70 + 30, v/v) mobile phase, a column temperature of 35°C, and a buffer pH of 4.0. Figure 4 shows chromatograms of both a standard (a) and a natural (nonalkalized) cocoa powder sample (b) using these conditions.

Linearity/Calibration Curve

Linearity for each flavanol enantiomer was determined. The range of concentrations evaluated for each enantiomer is listed in Table 3. These concentrations of the calibration standards were suitable for the range of samples used for this study and reflect the ranges that could be expected in commercially available ingredients and finished products. The correlation coefficients of all compounds were ≥0.9994.

LOQ/LOD

As the focus of this work was determination of the flavanol enantiomers in cocoa-based ingredients and products, the LOQ and LOD were established based on evaluation of the S/N of (+)-epicatechin, which is likely to be the least abundant flavanol enantiomer within cocoa-based ingredients and products. The LOQ of (+)-epicatechin was determined to be 0.6 µg/mL. Based on the mixed standard composition used in this work, the corresponding low-end concentrations of (−)-epicatechin, (+)-catechin, and (−)-catechin were 12.1, 2.3, and 2.2 µg/mL, respectively. LOD was determined in a similar manner and was 0.2 µg/mL for (+)-epicatechin, resulting in concentrations of 2.4 µg/mL for (−)-epicatechin, 0.4 µg/mL for (+)-catechin, and 0.4 µg/mL for (−)-catechin in the mixed standard solution. In looking at each enantiomer independently, LOQs were

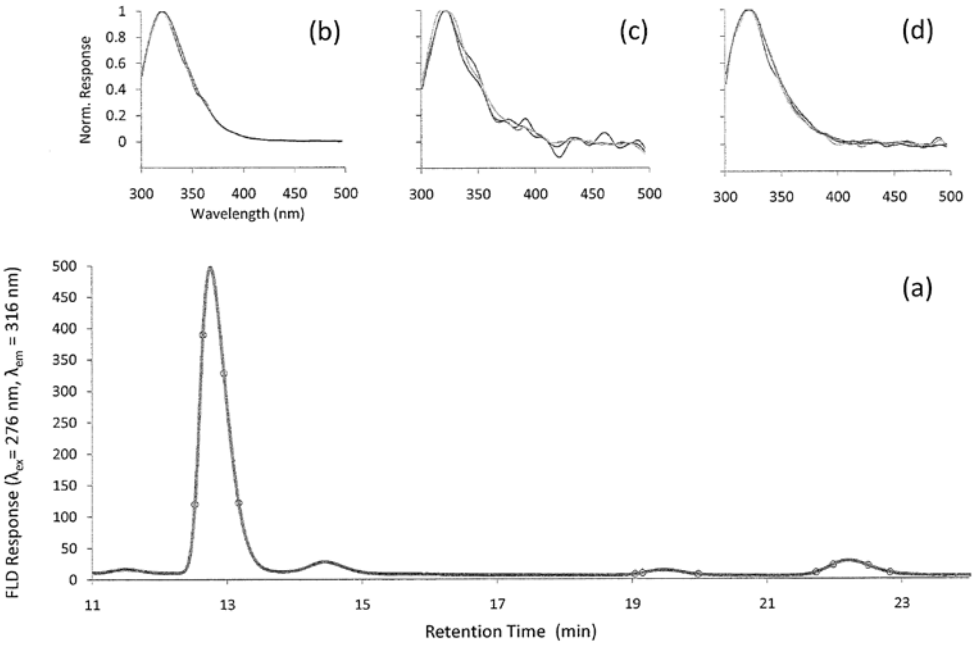

Figure 5. Peak purity analysis of the fluorescence emission signal for the separation of analytes in a representative cocoa extract sample. The chromatogram is presented in (a), while inserts (b)–(d) correspond to the normalized fluorescence emission spectra obtained for (−)-epicatechin, (+)-catechin, and (−)-catechin peaks, respectively. Visual comparison of the overlaid spectra indicates that the chromatographic peaks have high purity.

determined to be 0.6 µg/mL and LODs were 0.2 µg/mL for the other three enantiomers. As food formulations made with cocoa ingredients can vary considerably, not only in cocoa type and bean processing conditions, but also in the final product recipe, LOD and LOQ were determined using standard solutions rather than within the food matrix. Thus, as individual cocoa-based sample matrixes as well as instrumental dependencies may affect both LOQ and LOD, within-matrix LOQ/LOD may vary slightly from those reported herein.

Repeatability

Repeatability evaluation was performed by measuring five separate preparations of cocoa extract, natural cocoa powder, and dark chocolate on 3 different days. This allowed evaluation of both interday and intraday repeatability. The results can be seen in Table 3, reported on a whole product (fat corrected) basis. These results show that (−)-epicatechin, (+)-catechin, and (−)-catechin had strong repeatability. For (−)-epicatechin, the predominant flavanol monomer in cocoa-based products, intraday and interday precisions ranged from 0.2 to 1.5 and 1.5 to 3.2%, respectively, across the product types examined. The enantiomers (−)-epicatechin and (−)-catechin also had a strong indices of repeatability, as did (+)-catechin. In these samples, no (+)-epicatechin was detected.

The Horwitz ratio (HorRat) can be a useful measure of overall method performance (13). The HorRat values for (−)-epicatechin, (+)-catechin, and (−)-catechin in the three sample types across all 3 days (15 replicates/matrix) are displayed in Table 4. In the samples evaluated, calculated HorRat values were 0.4–0.6 for (−)-epicatechin, 0.7–0.8 for (+)-catechin, and 0.4–1.2 for (−)-catechin. Within the context of a single laboratory evaluation, a HorRat value between 0.3–1.3 indicates that the overall method variance is appropriate for a specific method.

Table 6. Fluorescence emission (λ_{ex} = 276 nm) peak purity factors

Component	Purity factor
(−)-Epicatechin	987.3
(+)-Catechin	988.4
(−)-Catechin	986.8

Accuracy

Accuracy was evaluated using duplicate preparations of the three spiking levels for each matrix. Table 5 shows average recoveries for all enantiomers of the three samples types. AOAC establishes guidelines for acceptable recoveries (14), with concentrations at the 100 and 200% levels expected to fall within the range of 80–115% recovery, and at the 50% relative concentration level to fall within an expanded range of 75–120% recovery. Recoveries for the three sample types investigated ranged from 82.2 to 102.1% at the 50% spiking level, 83.7 to 102.0% at the 100% spiking level, and 80.4 to 101.1% at the 200% spiking level. In addition to showing acceptable recovery, these results show that sample preparation does not result in appreciable loss or artifactual conversion of (−)-epicatechin to (−)-catechin or (+)-catechin to (+)-epicatechin.

Selectivity

Method selectivity was evaluated through a peak purity analysis of the fluorescence emission signal for a representative cocoa extract sample. Figure 5a shows the chromatographic response for the separation of the enantiomeric content present, while inserts b–d illustrate the normalized and overlaid spectral responses measured across the (−)-epicatechin, (+)-catechin, and (−)-catechin peaks, respectively. (+)-Epicatechin could not be evaluated since it was not present in the samples of interest. Visual consistency in the spectral responses, which are measured across each chromatographic peak at the points indicated by the symbols (**o**), provide an indication of the purity of the chromatographic peak and the method's selectivity for the analytes of interest in the cocoa extract sample. Table 6 presents the calculated purity factors obtained from a comparison of these spectral responses for the individual chromatographic peaks. A purity factor of 0 indicates no correspondence between spectra obtained across the chromatographic peak and, therefore, a highly impure peak. A value of 1000 indicates identical spectra and high peak purity. The purity factors found were ≥986, demonstrating that the spectral responses across the chromatographic peaks for (−)-epicatechin, (+)-catechin, and (−)-catechin were closely matched and the method has good selectivity for the analytes of interest in this matrix.

Solution Stability

An extensive evaluation of flavanol stability in solution was not conducted as part of this study (e.g., evaluation of stability over several days, under different temperatures or storage conditions, etc.); however, a comparison of area counts of standards injected at the beginning, middle, and end of runs found the change in peak areas to be <1% after 24 h. Therefore, in the absence of detailed information of the stability of flavanols in solution, a 24 h time period was adopted as a preliminary window of stability for the testing performed. As flavanols in solution remained stable over 24 h, this was established as a suitable period for sample usage. All analyses were thus conducted within 24 h of sample preparation and discarded after this time.

Conclusions

A method was developed for the identification and quantification of epicatechin and catechin enantiomers, namely, (+)- and (−)-epicatechin and (+)- and (−)-catechin, in cocoa-containing products; subsequently, a single-laboratory validation study was performed. The method demonstrated strong repeatability, as well as acceptable accuracy. In addition, the spike recovery experiments demonstrated that sample processing does not contribute to loss or epimerization of enantiomers, thus indicating that this method can likely be used for the analysis of a broad range of cocoa-containing sample and product matrixes.

While this method has thus far only been validated for cocoa-based ingredients and products, there may also be applicability to other non-cocoa-based food ingredients or food products that contain epicatechin and catechin. As environmental conditions (15) and food processing (3) have been shown to impact flavanol stereochemistry, methods that can resolve the individual flavanol enantiomers may be of increasing relevance to the food industry. Furthermore, in light of emerging data in support of the importance of the consideration of stereochemistry on the biological activity of flavanols (4), the ability to characterize not only the epicatechin and catechin composition of the food, but also the individual enantiomeric components, will likely become of increasing scientific interest and relevance to nutrition.

Acknowledgments

We would like to thank Charmonte Watkins (Mars Botanical, Gaithersburg, MD) for producing the (+)-epicatechin and (−)-catechin standards used in this work.

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FOOD CHEMICAL CONTAMINANTS

Determination of Aflatoxins B_1, B_2, G_1, and G_2 in Olive Oil, Peanut Oil, and Sesame Oil Using Immunoaffinity Column Cleanup, Postcolumn Derivatization, and Liquid Chromatography with Fluorescence Detection: First Action 2013.05

LEI BAO and CHENGZHU LIANG
Shandong Exit-Entry Inspection and Quarantine Bureau of China, Qing Dao, People's Republic of China 266002
MARY W. TRUCKSESS
U.S. Food and Drug Administration, 5100 Paint Branch Pkwy, College Park, MD 20740
YANLI XU, NING LV, ZHENXING WU, and PING JING
Shandong Exit-Entry Inspection and Quarantine Bureau of China, Qing Dao, People's Republic of China 266002
FRED S. FRY
U.S. Food and Drug Administration, 5100 Paint Branch Pkwy, College Park, MD 20740

A collaborative study of a method for determination of aflatoxins (AFs) B_1, B_2, G_1, and G_2 in olive oil, peanut oil, and sesame oil using immunoaffinity column cleanup, postcolumn derivatization, and LC with fluorescence detection, previously published in *J. AOAC Int.* 95, 1689–1700 (2012), was approved as First Action 2013.05 on March 29, 2013 by the Method-Centric Committee for Aflatoxins in Edible Oils. The method uses methanol for extraction followed by filtration. The extract is applied to an immunoaffinity column with antibodies specific for AFs, which are then eluted from the column with a methanol solution. Determination and quantification occur using RP-LC with fluorescence detection after postcolumn derivatization. The average recovery of AFs in olive, peanut, and sesame oils in spiked samples (levels between 2.0 and 20.0 µg/kg) ranged from 84 to 92%. The recoveries for AFs B_1, B_2, G_1, and G_2 were 86–93, 89–95, 85–97, and 76–85%, respectively. Within-laboratory RSD (RSD_r) values for AFs ranged from 3.4 to 10.2%. RSD_r values for AF B_1, B_2, G_1, and G_2 were 3.5–10.9, 3.2–9.5, 6.5–14.9, and 4.8–14.2%, respectively. Between-laboratory RSD (RSD_R) values for AFs were 6.1–14.5%. RSD_R values for AFs B_1, B_2, G_1, and G_2 were 7.5–15.4, 7.1–14.6, 10.8–18.1, and 7.6–23.7%, respectively. Horwitz ratio values were ≤2 for the analytes in the three matrixes.

Submitted for publication April 11, 2013.
The method was approved by the Method-Centric Committee for Aflatoxins in Edible Oils as First Action.
The AOAC Method-Centric Committee for Aflatoxins in Edible Oils invites method users to provide feedback on the First Action methods. Feedback from method users will help verify that the methods are fit for purpose and are critical to gaining global recognition and acceptance of the methods. Comments can be sent directly to the corresponding author or methodfeedback@aoac.org.
Corresponding author's e-mail: leibaoqd@yahoo.com
DOI: 10.5740/jaoacint.13-129

Aflatoxins (AFs) B_1, B_2, G_1, and G_2 represent the major AFs and are the most toxic of the mycotoxins (1). These AFs have also been determined to be mutagenic and teratogenic and can lead to health issues if consumed in food products. Because of the severity of health issues related to AFs, many countries have implemented regulatory limits for them in food commodities such as edible oils (2). With limits on AFs, a need exists for validated methods to generate reliable analytical results to allow a safe food supply and guarantee the health of the public. The following method has been found acceptable for the determination of AFs B_1, B_2, G_1, and G_2 in olive oil, peanut oil, and sesame oil.

AOAC Official Method 2013.05
Aflatoxins B_1, B_2, G_1, and G_2 in Olive Oil, Peanut Oil, and Sesame Oil
Immunoaffinity Column Cleanup, Postcolumn Derivatization, and LC with Fluorescence Detection
First Action 2013

[Applicable to the determination of total AFs (sum of AF B_1, AF B_2, AF G_1, and AF G_2) in olive oil, peanut oil, and sesame oil at 2–20 µg/kg and AF B_1 in the matrixes at 1–10 µg/kg.]

A. Principle

A test portion is extracted with methanol–water (55 + 45, v/v). After shaking and centrifuging, the extract is filtered, diluted with water, and applied to an immunoaffinity column containing antibodies specific for AFs. After washing with methanol–water (10 + 90, v/v), the AFs are eluted from the column with methanol and quantified by LC with fluorescence detection. For AF postcolumn derivatization, a photochemical derivatization device or Kobra cell is used. Refer to the published method for further details (2).

Results

The results from the collaborative study show the method is appropriate for the determination of AFs B_1, B_2, G_1, and G_2 in olive oil, peanut oil, and sesame oil.

The average recovery of AFs in olive, peanut, and sesame oil-spiked samples (levels between 2.0 and 20.0 µg/kg) ranged from 84 to 92%. The recoveries for AFs B_1, B_2, G_1, and G_2 were 86–93% at spiked levels of 1.0–10.0 µg/kg, 89–95% at spiked levels of 0.25–2.5 µg/kg, 85–97% at spiked levels of 0.5–5.0 µg/kg, and 76–85% at spiked levels of 0.25–2.5 µg/kg. Within-laboratory RSD (RSD_r) values for AFs ranged from 3.4 to 10.2%. RSD_r values for AFs B_1, B_2, G_1, and G_2 were 3.5–10.9, 3.2–9.5, 6.5–14.9, and 4.8–14.2%, respectively. Between-laboratory RSD (RSD_R) values for AFs were 6.1–14.5%. RSD_R values for AFs B_1, B_2, G_1, and G_2 were 7.5–15.4, 7.1–14.6, 10.8–18.1, and 7.6–23.7%, respectively.

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RESIDUES AND TRACE ELEMENTS

Determination of Arsenic, Cadmium, Mercury, and Lead in Foods by Pressure Digestion and Inductively Coupled Plasma/Mass Spectrometry: First Action 2013.06

KAARE JULSHAMN and AMUND MAAGE
National Institute of Nutrition and Seafood Research (NIFES), PO Box 2029 Nordnes, N-5817 Bergen, Norway
HILDE SKAAR NORLI
National Veterinary Institute, Department of Food and Feed Hygiene, Nordic Committee on Food Analysis, PO Box 8156, Dep., N-0033 Oslo, Norway
KARL H. GROBECKER
European Commission Joint Research Centre, Institute for Reference Materials and Measurements (IRMM), B-2440 Geel, Belgium
LARS JORHEM
National Food Administration, Box 622, SE-751 26 Uppsala, Sweden
PETER FECHER
Bayerisches Landesamt für Gesundheit und Lebensmittelsicherheit, Zentrallabor Chemie, Sachgebiet N6, Eggenreuther Weg 43 D-91058 Erlangen, Germany
DAWN DOWELL
AOAC INTERNATIONAL, 481 North Frederick Ave, Suite 500, Gaithersburg, MD 20877-2417

The method for the determination of As, Cd, Hg, and Pb in foods by pressure digestion and inductively coupled plasma (ICP)/MS, previously published in *J. AOAC Int.* 90, 844–856 (2007), was approved as First Action 2013.06 on April 9, 2013 by the Method-Centric Committee for Elemental Contaminants in Food. Digestion occurs using nitric acid in a closed vessel with elevated temperature and pressure by conventional or microwave-assisted heating. Determination occurs using ICP/MS. The elemental concentration ranges for As were 0.06–21.4, for Cd 0.03–28.3, for Hg 0.04–0.6, and for Pb 0.01–2.4 in mg/kg dry matter. The repeatability RSD (RSD_r) ranged from 3.8 to 24% for As, 2.6 to 6.9% for Cd, 4.8 to 8.3% for Hg, and 2.9 to 27% for Pb. Reproducibility RSD (RSD_R) ranged from 9.0 to 28% for As, 2.8 to 18% for Cd, 9.9 to 24% for Hg, and 8 to 50% for Pb.

Heavy metal poisoning from elements like As, Cd, Hg, and Pb has become a concern for most industrialized countries (1). These toxic metals have a negative effect on physiological processes. Because of the negative health effects, governments have begun to implement regulations on the levels of contaminants allowed in the food supply to protect the public. The implementation of these regulations raises a need to have validated analytical methods that produce reliable and accurate results to ensure compliance. The method has been reviewed and found acceptable for the determination of As, Cd, Hg, and Pb in a variety of foods.

Submitted for publication April 22, 2013.
The method was approved by the Method-Centric Committee for Elemental Contaminants in Food as First Action.
The AOAC Method-Centric Committee for Elemental Contaminants in Food invites method users to provide feedback on the First Action methods. Feedback from method users will help verify that the methods are fit for purpose and are critical to gaining global recognition and acceptance of the methods. Comments can be sent directly to the corresponding author or methodfeedback@aoac.org.
Corresponding author's e-mail: kju@nifes.no
DOI: 10.5740/jaoacint.13-143

AOAC Official Method 2013.06
Arsenic, Cadmium, Mercury, and Lead in Foods
Pressure Digestion and Inductively Coupled Plasma/Mass Spectrometry
First Action 2013

(Applicable to the determination of As, Cd, Hg, and Pb in a variety of foods by pressure digestion and ICP/MS. Method is capable of determining As, Cd, Pb, and Hg at or above 0.06, 0.03, 0.04, and 0.09 mg/kg dry matter, respectively.) For the complete method, *see* the publication in *J. AOAC Int.* (2).

Results

The results of the collaborative study (Table 1; 2) show this method to be suitable for the determination of As, Cd, Hg, and Pb in a variety of foods. The elemental concentration ranges for As were 0.06–21.4, for Cd 0.03–28.3, for Hg 0.04–0.6, and for Pb 0.01–2.4 in mg/kg dry matter. The repeatability RSD (RSD_r) ranged from 3.8 to 24% for As, 2.6 to 6.9% for Cd, 4.8 to 8.3% for Hg, and 2.9 to 27% for Pb. Reproducibility RSD (RSD_R) ranged from 9.0 to 28% for As, 2.8 to 18% for Cd, 9.9 to 24% for Hg, and 8 to 50% for Pb.

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Table 1. Interlaboratory study results for As, Cd, Hg, and Pb

Matrix	$\bar{x}$, mg/kg[a]	*n*[b]	s_r, mg/kg[c]	s_R, mg/kg[d]	RSD_r, %[e]	RSD_R, %[f]	r, mg/kg[g]	R, mg/kg[h]	HorRat[i]
				As					
Carrot	<LOD	4 [0/8]	—	—	—	—	—	—	—
Fish muscle	1.6	11 [0/1]	0.086	0.14	5.4	9	0.24	0.4	0.6
Mushroom	0.057	9 [1/2]	0.014	0.016	24	28	0.038	0.044	1.2
Wheat flour	<LOD	2 [0/10]	—	—	—	—	—	—	—
Simulated diet	<LOD	5 [1/6]	—	—	—	—	—	—	—
Scampi	19.1	12 [0/0]	0.73	2.3	3.8	12	3.8	6.4	1.2
Mussel	9.3	12 [0/0]	0.45	1.2	4.9	13	1.2	3.4	1.2
				Cd					
Carrot	0.3	13 [0/0]	0.008	0.027	2.7	9	0.023	0.076	0.47
Fish muscle	0.87	13 [0/0]	0.06	0.095	6.9	11	0.17	0.27	0.67
Mushroom	0.46	13 [0/0]	0.017	0.033	3.8	7.2	0.049	0.092	0.4
Wheat flour	0.03	12 [1/0]	0.002	0.006	6.4	18	0.084	0.24	0.8
Simulated diet	0.52	13 [0/0]	0.013	0.044	2.6	8.4	0.037	0.12	0.48
Scampi	0.078	11 [2/0]	0.0022	0.012	2.8	2.8	0.0062	0.032	0.63
Mussel	1.7	12 [1/0]	0.0043	0.17	2.5	9.9	0.12	0.47	0.67
				Hg					
Carrot	<LOD	4 [0/8]	—	—	—	—	—	—	—
Fish muscle	0.096	11 [1/0]	0.0079	0.016	8.2	17	0.022	0,045	0.74
Mushroom	0.23	10 [2/0]	0.011	0.023	5	9.9	0.032	0.063	0.5
Wheat flour	<LOD	3 [0/9]	—	—	—	—	—	—	—
Simulated diet	0.042	8 [2/2]	0.0035	0.01	8.3	24	0.0099	0.029	1.1
Scampi	0.56	12 [0/0]	0.027	0.093	4.8	17	0.075	0.26	0.96
Mussel	0.15	11 [1/0]	0.01	0.023	6.9	15	0.029	0.064	0.72
				Pb					
Carrot	0.086	13 [0/0]	0.0039	0.0091	4.5	11	0.011	0.025	0.45
Fish muscle	2.1	13 [0/0]	0.1	0.17	4.8	8	0.29	0.47	0.56
Mushroom	1.5	12 [1/0]	0.098	0.14	6.7	9.5	0.27	0.39	0.63
Wheat flour	0.013	7 [0/6]	0.0034	0.0063	27	50	0.0095	0.018	2.2
Simulated diet	0.26	13 [0/0]	0.023	0.029	8.7	11	0.063	0.082	0.57
Scampi	1.14	13 [0/0]	0.056	0.11	4.9	9.3	0.16	0.3	0.59
Mussel	2.4	13 [0/0]	0.068	0.19	2.9	8	0.19	0.53	0.57

[a] $\bar{x}$ = Mean.

[b] *n* = Number of laboratories remaining after elimination of outliers/reporting <LOD in brackets.

[c] s_r = Repeatability SD.

[d] s_R = Reproducibility SD.

[e] RSD_r = Repeatability RSD.

[f] RSD_R = Reproducibility RSD.

[g] r = Repeatability value.

[h] R = Reproducibility value.

[i] HorRat = Horwitz ratio.

VETERINARY DRUG RESIDUES

Determination and Confirmation of Nicarbazin, Measured as 4,4-Dinitrocarbanilide (DNC), in Chicken Tissues by Liquid Chromatography with Tandem Mass Spectrometry: First Action 2013.07

MARK R. COLEMAN
Elanco Animal Health, 2500 Innovation Way, Greenfield, IN 46140
JOHN M. RODEWALD
Covance Laboratories, 671 S. Meridian Rd, Greenfield, IN 46140
SHARON L. BRUNELLE
Brunelle Biotech Consulting, 14104 194th Ave NE, Woodinville, WA 98077
MARIA NELSON
Independent Consultant, 8637 Carriage Hill Ct, Savage, MN 55378
LAURYN BAILEY
Eurofins Central Analytical Laboratories, 2315 N. Causeway Blvd, Suite 150, Metairie, LA 7001
THOMAS J. BURNETT
Elanco Animal Health, 2500 Innovation Way, Greenfield, IN 46140

A single-laboratory validation (SLV) study was conducted on an LC/MS/MS method for the determination and confirmation of nicarbazin, expressed as 4,4-dinitrocarbanilide (DNC), in chicken tissues, including liver, kidney, muscle, skin with adhering fat, and eggs. Linearity was demonstrated with DNC standard curve solutions using a weighted (1/x) regression and confirmed with matrix-matched standards. Intertrial repeatability precision (relative standard deviation of repeatability; RSD_r) was from 2.5 to 11.3%, as determined in fortified tissues. The precision was verified with incurred tissue, and varied from 0.53 to 2.5%. Average recoveries ranged from 82% in egg to 98% in kidney. Although the average recoveries across all concentrations were within the acceptable range, the method was improved with the inclusion of an internal standard and the use of matrix-matched standards. Accuracy for the improved method in chicken liver varied from 93 to 99% across all concentrations (100–8000 ng/g) compared to recoveries below 80% at concentrations between 100–400 ng/g in chicken liver for the original method. The limit of detection was estimated to be less than 3.0 ng/g in all tissue types, and the limit of quantitation was validated at 20 ng/g. Based on confirmatory ion ratios and peak retention times, the false-negative rate was estimated as 0.00% (95% confidence limits 0.00, 0.74%) from 484 fortified samples and 12 incurred residue samples analyzed using the U.S. and EU confirmation criteria. Small variations to the method parameters, with the exception of injection volume, did not have a significant effect on recoveries. Stability was determined for fortified tissues, extracts, and standard curve solutions. The data collected in this study satisfy the requirements of SLV studies established by the AOAC Stakeholder Panel for Veterinary Drug Residue and the method was awarded First Action *Official Method*℠ status by the Expert Review Panel for Veterinary Drug Residues on May 7, 2013.

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The method was approved by the Expert Review Panel for Veterinary Drug Residues as First Action.
The AOAC Expert Review Panel for Veterinary Drug Residues invites method users to provide feedback on the First Action methods. Feedback from method users will help verify that the methods are fit for purpose and are critical to gaining global recognition and acceptance of the methods. Comments can be sent directly to the corresponding author or methodfeedback@aoac.org.
Corresponding author's e-mail: sbrunelle@aoac.org
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Nicarbazin is a broad-spectrum chemical anticoccidial agent used to prevent disease and treat infection in food-producing poultry. The drug residue is composed of equimolar quantities of 4,4-dinitrocarbanilide (DNC) and 2-hydroxy-4,6-dimethyl pyrimidine (HDP), with DNC serving as the primary component for tracking of the residue in edible tissues, including fat, muscle, liver, and kidney, and in eggs. Although nicarbazin is primarily used as a feed additive, most regulatory agencies require testing of edible tissues for residual DNC to ensure that significant levels of nicarbazin have not accumulated in the tissues of the treated animals.

Codex Alimentarius and EU Maximum Residue Limits (MRLs) and U.S. tolerances for nicarbazin, expressed as DNC concentrations, in various chicken tissues are presented in Table 1.

On December 7–8, 2010, an AOAC Expert Review Panel (ERP) for Veterinary Drug Residues met and reviewed 18 methods for the determination and/or confirmation of nicarbazin residues (1). The methods reviewed were chosen based on their applicability to poultry tissues and eggs, specificity for DNC, use of broadly available technology, and avoidance of

Table 1. Maximum residue limits (EU and Codex) and tolerances (U.S.) for nicarbazin in chicken tissues expressed as DNC concentration

	Nicarbazin (expressed as DNC)		
Tissue	Codex MRL, ng/g	EU MRL, ng/g	U.S. tolerance, ng/g
Liver	200	15000	4000
Kidney	200	6000	4000
Muscle	200	4000	4000
Fat	200	4000	4000
Eggs	NA[a]	NA	NA

[a] While there is no MRL in eggs, there is a "Maximum Limit" of 100 ng/g.

hazardous reagents. Each method was reviewed by two ERP members and the advantages and disadvantages of each method were discussed by the group. The ERP selected a multiresidue method using LC/MS/MS by Olejnik et al. (2) for further study. Recommendations to increase the suitability of the method included potential modifications to the sample size, run time, centrifugation, clean-up, detection, recovery, and applicability to fat. The use of solvent standards was recommended if no statistical difference to matrix-matched standards could be shown. The final candidate method was developed by Eurofins Central Analytical Laboratories, Metairie, LA, and is described in this paper. The candidate method provides the tissue concentration and confirmation of DNC as a marker for nicarbazin and for comparison to regulatory limits based on DNC concentration.

The AOAC Stakeholder Panel on Veterinary Drug Residues (SPVDR) met on October 2–3, 2008, and developed a general set of standard method performance requirements (SMPRs) for veterinary drug residue methods (3). The panel considered the most stringent requirements of guidelines from major developed countries during this process. The Veterinary International Conference on Harmonization (VICH) recently published a guidance document for analytical methods to be used in residue depletion studies (4), but the performance requirements followed for this single-laboratory validation (SLV) were in some cases more stringent than those prescribed by VICH. The parameters and criteria used for this validation are the same as those used previously for the validation of a method for determination of ractopamine in animal tissues (5). This paper describes the SLV of the candidate method in support of First Action Official Method status through an AOAC ERP (6).

SLV Study

The validation study was carried out by Covance Laboratories (Greenfield, IN) and included studies of linearity, matrix effect, precision, recovery/accuracy, bias, limits of detection and quantitation, and robustness. In addition to validation of method performance, data are presented for stability of the analyte in tissues, extracts, and standard solutions. Acceptance criteria are based on those developed by SPVDR (5).

Bulk tissues were initially processed by cryogenic grinding or blending of the tissues using a food grinder to produce finely powdered homogeneous samples, then subdividing the bulk into individual test portion sizes (1.0 g for kidney, 5.0 g for all other tissues and eggs). Individual test portions were then fortified with an appropriate volume of nicarbazin standard solution. After fortification, samples were briefly vortexed on a hand vortex unit and the test portions were allowed to sit undisturbed for approximately 10–30 min before the extraction procedure was initiated to more closely approximate incurred residues. Fortified matrixes were prepared at approximately ½ MRL/tolerance, at the MRL/tolerance, and at 2x MRL/tolerance. In some cases multiple MRL/tolerance values were accommodated by adding additional fortification levels.

Incurred tissues were provided to the validating laboratory by Elanco Animal Health.

Data were subjected to the Grubbs' test for removal of outliers.

AOAC Official Method 2013.07
Determination and Confirmation of Nicarbazin in Chicken Tissues
Liquid Chromatography with Tandem Mass Spectrometry
First Action 2013

[Applicable for the determination and confirmation of nicarbazin (expressed as 4,4-dinitrocarbanilide; DNC) in chicken liver, kidney, muscle, skin with adhering fat tissues, and in eggs.]

Caution: Solvents employed are common use solvents and reagents. Refer to adequate manuals or safety data sheets to ensure that the safety guidelines are applied before using chemicals. Store in a flammable liquid storage cabinet. Harmful if inhaled, swallowed, or absorbed through the skin. Use appropriate personal protective equipment such as a laboratory coat, safety glasses, rubber gloves, and a fume hood. Dispose of all materials according to federal, state, and local regulations.

A. Principle

Poultry tissue is cryogenically homogenized with solid sodium sulfate, and then extracted twice with acetonitrile. Extracts are combined, filtered, and diluted accordingly based on the regulatory limits being targeted and the working concentrations of the standards used for LC/MS/MS analysis. Confirmation of identity is accomplished by comparing the product ions measured in the samples to those present in the standard injections in mass and relative intensity, and comparison of chromatographic retention times between samples and standards. Nicarbazin determination and confirmation is based on the DNC portion of the molecule as are the regulatory limits and tolerances. Concentrations are determined by LC/MS/MS using a matrix-matched standard curve and DNC-d_8 internal standard.

B. Apparatus

(**a**) *Volumetric pipets*.—Class A, glass, assorted sizes.

(**b**) *Positive displacement pipets*.—Gilson, Inc. (Middleton, WI) Model No. M100 Part No. F148504 (10–100 µL), Model No. M250 Part No. F148505 (50–250 µL), and Model No. M1000 Part No. F148506 (100–1000 µL).

(**c**) *Volumetric flasks*.—Class A, glass, assorted sizes.

(**d**) *Analytical balances*.—Sensitive to at least 0.01 and 0.00001 g.

(**e**) *Actinic glassware*.—Or glassware covered with aluminum foil.

(**f**) *Spatulas*.—Stainless steel or Teflon-coated.

(**g**) *Glass bottles*.—Corning (Corning, NY), 1 or 2 L.

(**h**) *Graduated cylinders*.—Glass, assorted sizes.

(**i**) *Magnetic stirrer and Teflon-coated stir bars*.

(**j**) *Cryogenic grinding and homogenization equipment*.—Foss (Eden Prairie, MN) or Robot Coupe (Ridgeland, MS) grinder or a Waring blender or equivalent.

(**k**) *Multi-tube vortex mixer*.—VWR (Radnor, PA) Model No. DVX-2500.

(**l**) *Polypropylene centrifuge tubes*.—50 mL conical with closures.

(**m**) *Centrifuge*.—Refrigerated (temperature controlled), capable of 3000 rpm and 5°C.

(**n**) *Transfer pipets*.—Disposable.

(**o**) *Filters*.—Pall Gelman (Ann Arbor, MI) Acrodisc™, PTFE, 13 mm, 0.45 µm.

(**p**) *HPLC vials with caps*.

(**q**) *LC/MS/MS*.— AB Sciex (Framingham, MA) API4000, TurboIonSpray® probe, Analyst® software.

(**r**) *HPLC pump and autosampler*.

(**s**) *Chromatographic column*.—Restek (State College, PA) Aqueous C_{18}, 3 µm, 2.1 × 50 mm (Part No. 9178352 for 3 µm particle size or Part No. 9178552 for 5 µm particle size).

C. Materials and Reagents

(**a**) *Methanol (MeOH)*.—HPLC grade.

(**b**) *Water (H_2O)*.—HPLC grade or distilled, deionized.

(**c**) *Acetonitrile (ACN)*.—HPLC grade.

(**d**) *Sodium sulfate (Na_2SO_4)*.—Anhydrous granular, Certified ACS.

(**e**) *Ammonium acetate (NH_4OAc)*.—Certified ACS.

(**f**) *Formic acid (FA), concentrated*.—Certified ACS.

(**g**) *N,N'-Dimethyl formamide (DMF)*.—Certified ACS.

(**h**) *Nicarbazin reference standard*.—Eli Lilly and Co. (Indianapolis, IN). Composed of equimolar quantities of DNC and 2-hydroxy-4,6-dimethyl pyrimidine (HDP). When ordered from Eli Lilly and Co., the order will be accompanied by a certificate of analysis that gives details on the DNC purity. Store at 15 to 30°C. Consult the MSDS for safety and handling information.

(**i**) *DNC-d_8 internal standard*.—Sigma-Aldrich (St. Louis, MO), Part No. 34214.

D. Preparation of Reagents and Standards

(**a**) *Mobile phase solution A*.—To 1000 mL H_2O, add 1.0 mL FA and 0.38 ± 0.04 g NH_4OAc and mix thoroughly.

(**b**) *Mobile phase solution B*.—To 1000 mL MeOH, add 1.0 mL FA and 0.38 ± 0.04 g NH_4OAc and mix thoroughly.

(**c**) *Nicarbazin stock standard solution (1000 µg/mL DNC component)*.—Accurately weigh 141.4 mg nicarbazin reference standard, equivalent to about 100.0 mg DNC when compensated for purity, and transfer to a 100 mL volumetric flask. Dissolve with sonication (approximately 10 min) and dilute to volume with DMF. Mix thoroughly.

(**d**) *Nicarbazin intermediate standard solution (10 µg/mL DNC component)*.—Make a 100-fold dilution of the nicarbazin stock standard solution (1000 µg/mL DNC) with ACN.

(**e**) *Nicarbazin standard curve solutions*.—Make dilutions from the nicarbazin intermediate standard solution (10 µg/mL DNC) with ACN to prepare a standard curve of 25, 50, 125, 500, 1250, and 2500 ng/mL.

(**f**) *DNC-d_8 internal standard stock solution (1.0 mg/mL)*.—Using DMF, dissolve and transfer the 10 mg vial of DNC-d_8 standard into a 10 mL volumetric flask. Dilute to volume with DMF and mix thoroughly.

(**g**) *DNC-d_8 internal standard solution (1.0 µg/mL)*.—Make a 1000-fold dilution of the DNC-d_8 stock solution with ACN.

Note: Different volumes of equivalent concentrations may be substituted.

Note: Store all stock standards and standard solutions at room temperature protected from light. Stock standards are stable for 3 months and standard solutions for 14 days under these conditions.

E. Sample Preparation

(**a**) *Homogenization and storage of samples*.—Initial processing includes grinding or blending of the tissues using cryogenic grinding to produce homogeneous samples. Cryogenic grinding is carried out by freezing the tissue with liquid nitrogen and then grinding into a fine powder using a Foss or Robot Coupe grinder or a Waring blender. This process is used to produce a very fine homogeneous powder of the tissue for analysis. Grind a minimum 500 g sample of tissue when possible. Subsamples of 5.0 ± 0.5 g of tissue (1.0 g for kidney) may be weighed into 50 mL polypropylene tubes and frozen. This will minimize tissue exposure to multiple freeze/thaw cycles. Store all tissues at freezer temperatures (–20°C or below) when not processing or subsampling. It is advisable to store fortified samples of all tissues with experimental samples to verify storage stability.

(**b**) *Preparation of quality control (QC) and control tissues*.—On the day of analysis, prepare at least seven control matrix samples and a matrix sample fortified at MRL or tolerance [QC sample, *see* (**E**)(**c**)(*3*)]. Process QC and control samples as indicated in **E**(**c**).

(**c**) *Tissue extraction*.—Poultry muscle, liver, kidney, skin with adhering fat, and eggs:

(*1*) Accurately weigh 5.0 ± 0.05 g (1.0 ± 0.05 g for kidney) of a representative ground sample of frozen or partially thawed sample into a 50 mL conical polypropylene centrifuge tube.

(*2*) Fortify all samples with 200 µL (40 µL for kidney) of the 1.0 µg/mL DNC-d_8 internal standard solution.

(*3*) Fortify QC samples with nicarbazin (based on DNC content and purity) at MRL or tolerance.

(*4*) Add 10 ± 1 g anhydrous sodium sulfate to each tissue sample. If sample size is reduced to 1 g, reduce weight of sodium sulfate by a factor of 5.

(*5*) Thoroughly incorporate the sodium sulfate into the tissue sample using a stainless steel or disposable wooden spatula to generate a crumbly or pasty tissue homogenate.

(*6*) Add 20 mL ACN and mix using a multi-tube vortex mixer for 30 min.

(*7*) Centrifuge the sample at approximately 3000 rpm (RCF = approximately 2025 × *g*) for 10 min.

(*8*) Decant the supernatant into another graduated vessel (50 mL conical centrifuge tube or mixing cylinder).

(*9*) Re-extract the tissue pellet following steps (**E**)(**c**)(*6*)–(*8*) and combine the supernatants.

(*10*) Add 1.0 mL nicarbazin standard curve solutions to each of six control extracts to prepare the matrix-matched curve. Final concentrations are 0.5, 1.0, 2.5, 10, 25, and 50 ng/mL.

(*11*) Adjust all samples to 50 mL final volume with ACN and mix thoroughly.

(*12*) Filter the samples into LC vials for analysis.

F. Determination

(**a**) *LC operating conditions*.—*Note*: These guidelines may be modified to obtain the desired chromatography. Column temperature, 30°C; flow rate, 0.4 mL/min; autosampler temperature, ambient; injection volume, 10 µL; run time, 12 min; gradient, 0–2 min 0% mobile phase B, 2–3 min 0–80% mobile phase B, 3–6 min 80–100% mobile phase B, 6–8 min 100% mobile phase B, 8–8.2 min 100–0% mobile phase B.

(**b**) *MS/MS operating conditions*.—*Note*: Equivalent equipment can be substituted. The MS parameters provided are suggested values for the API 4000 instrument. For optimal analysis, MS parameters should be obtained by instrument tuning. Instrumentation, AB Sciex API 4000 Triple Quadrupole Mass Spectrometer; operating mode, negative ion, selected reaction monitoring [*Note*: Analyst software denotes this as multiple reaction monitoring (MRM)]; determinative transition, *m/z* 301.0→136.7; confirmatory transition, *m/z* 301.0→106.9; internal standard transition, *m/z* 308.7→140.6.

(**c**) *Mass spectrometer compound-specific parameters*.—DNC, Q1 mass 301.0 amu, Q3 mass 136.7 amu, collision energy –16 V, collision cell exit potential –11 V, entrance potential –6 V; DNC, Q1 mass 301.0 amu, Q3 mass 106.9 amu, collision energy –48 V, collision cell exit potential –7 V, entrance potential –4 V; DNC-d_8, Q1 mass 308.7 amu, Q3 mass 140.6 amu, collision energy –16 V, collision cell exit potential –7 V, entrance potential –6 V.

(**d**) *Mass spectrometer noncompound-specific parameters*.—ion source, turbospray; resolution (Q1 and Q3), unit; curtain gas (CUR), 20; ion spray (IS), –4500 V; collisional activated dissociation (CAD), 10; declustering potential (DP), –55 V; source temperature, 550°C.

(**e**) *System suitability*.—A sufficient number of injections should be made of the final control extract containing the internal standard such that the response of the internal standard has stabilized. It is left to the discretion of the analyst to determine when the *y*-axis response has stabilized. It may take anywhere from 5 to 10 injections for this to occur.

(**f**) *Quantitative determination*.—(*1*) Make single injections of the matrix-matched standard solutions, single injections of each sample extract solution, and then again single injections of the matrix-matched standard solutions. *Note*: Standard injections at the beginning and end of the run can be made out of the same HPLC vial. It is recommended to not exceed 12 sample injections between injections of a standard curve.

(*2*) Measure the peak areas for DNC and DNC-d_8 in the standard and sample solutions. Construct a 1/x weighted linear standard curve using determinative ion ratios of the standard responses (ratio of 301.0→136.7 to 308.7→140.6; DNC to DNC-d_8) vs concentration. From the standard curve, calculate the concentrations in ng/mL for each of the extracted samples.

(*3*) Using weight, volume, dilution from (**F**)(**f**)(*4*) if any, and concentrations from (**F**)(**f**)(*2*), calculate the DNC concentration in the samples.

$$\text{DNC tissue concentration} = \text{ng/g} = \frac{A \cdot B}{C} \cdot D$$

where A = sample concentration from standard curve (ng/mL); B = extract volume (mL); C = weight of tissue sample (g); D = dilution factor.

(*4*) If the determinative ion ratio exceeds the high end of the standard curve, the extracted sample should be diluted with control matrix extract and reinjected along with the standard curve.

For liver, if the tissue concentration is between 500 ng/g (equivalent of upper end of standard curve) and 8000 ng/g, the extracted sample should be diluted with control matrix extract and reinjected along with the standard curve.

For liver, if the tissue concentration of DNC exceeds 8000 ng/g, then the original tissue sample should be diluted in negative control tissue (for example, 1 g sample tissue + 4 g control tissue) and re-extracted.

(**g**) *Qualitative confirmation*.—Confirmation of identity is accomplished by comparing the product ions measured in the samples to those present in the standard injections in both mass and relative intensity.

(*1*) Obtain the individual ion chromatograms for the product ions and ensure that the chromatographic retention times for the analytes are ±5% relative to the mean retention time of the appropriate analyte in the standard. Extracts may be reinjected if there has been a sudden shift in retention time during the batch analysis exceeding the 5% tolerance.

(*2*) Integrate the area of the DNC peak for each SRM trace for the standards and samples. From the integrated area values for DNC, represent the determinative ion as 100% (*m/z* 301.0→136.7) and calculate the abundance of the confirmatory ion (*m/z* 301.0→106.9) as a relative percentage for each standard and sample. Using the mean ion abundance percentages (IAP) of the standard solutions within a chromatographic run, calculate the U.S. acceptance range (7) as mean ±20% arithmetic difference for the samples within that run. For example, at 20% relative abundance, the U.S. acceptance range would be 0–40% IAP. For the EU (8), the acceptance range depends on the IAP. For mean IAP >50%, the acceptance range is ±20% relative to mean IAP of standards. For mean ion abundance >20 to 50%, the acceptance range is ±25% relative to mean IAP of standards. For mean ion abundance >10 to 20%, the acceptance range is ±30% relative to mean IAP of standards. For mean ion abundance ≤10%, the acceptance range is ±50% relative to mean IAP of standards. For example, at 20% mean IAP, the EU acceptance range would be 14–26% for the samples within that run.

(**h**) *Standard curve acceptability criteria*.—The following criteria will be used for determining curve acceptability: (*1*) Back-calculated accuracy for any standard curve point must be within ±15% of the theoretical value (±20% of the theoretical value at the lower limit of quantitation).

(*2*) Individual data points may be excluded in a given batch provided the curve maintains a minimum of five different concentrations and the standards bracket the QC and unknown test portions.

(**i**) *QC acceptability criteria.*—The following criteria will be used for determining QC acceptability:

(*1*) Determine recovery of the QC test portions as recovery = (concentration/actual fortification level) × 100.

(*2*) QC test portions must meet the recovery requirements (e.g., 60–110% at <100 ng/g for U.S. Food and Drug Administration).

Results and Discussion

Matrix Effect

Standard solutions were compared to matrix-matched standards for muscle, liver, kidney, and skin/fat by constructing a standard curve in matrix extract or in solution and determining the standard curve slopes for each set of experiments. Skin/fat had the largest matrix effect, causing a decrease of 13.2% in the standard curve slope. Muscle had the smallest matrix effect with a decrease of 4.4%, and liver and kidney yielded matrix effects of 10.1 and 6.2%, respectively. Because these matrix effects are <20%, the method was made more efficient by utilizing solution standards, which allows for the analysis of multiple tissue types in a single chromatographic run.

Matrix Study—Fortified Tissues

Because the matrix effects were small, the method was initially developed with solution standards and did not include an internal standard. For determination of recovery and precision, matrixes were fortified at the concentrations indicated in Table 2, bracketing the Codex MRL and U.S. tolerance concentrations.

Repeatability precision was determined by testing six replicates at each fortification concentration in three independent trials. The independent trials included at least two operators and were performed on at least 2 different days. Intra- and intertrial means, standard deviations (s_r), and relative standard deviations (RSD_r) were calculated at each concentration for each matrix. Precision data are presented in Table 2. Acceptance criteria are based on the RSD_r values. At concentrations <100 ng/g DNC, the RSD_r must be ≤20% and at concentrations ≥100 ng/g DNC, the RSD_r must be ≤10%.

Eggs were the only matrix fortified with nicarbazin at <100 ng/g DNC. The intra-trial RSD_r values for eggs at 50 ng/g DNC ranged from 2.1 to 12.1%, and the intertrial RSD_r value was 8.6% and, thus, was within the acceptance criterion. At concentrations ≥100 ng/g DNC, the intra-trial RSD_r values ranged from 0.70 to 11.6% and intertrial RSD_r values ranged from 2.5 to 11.3% across all tissue types. There were six instances of intra-trial RSD_r values above 10%, including one each in liver, kidney, and eggs, and three in fat, ranging from 10.2 to 11.6%. Only one tissue, muscle, yielded an intertrial RSD_r value above the acceptance criterion, 11.3%, and only at one concentration (200 ng/g). Intra-trial RSD_r values for muscle were all below 10%, ranging from 0.81 to 7.0%.

HorRat values are a measure of the observed precision compared to the theoretical precision at a given concentration (9) and are calculated for repeatability as $HorRat_r = RSD_r, \%/PRSD_r, \%$, where $PRSD_r, \% = C^{-0.1505}$ and C = the estimated mean concentration. Intertrial RSD_r values were used for the observed precision. Values between 0.3–1.3 are generally considered acceptable (9). All tissues examined had $HorRat_r$ values within this range.

An average recovery for each matrix was determined from the slope of the regression line after plotting the candidate method results against the fortification concentrations. Recovery is defined as the ratio of the mean candidate result to the fortification concentration, expressed as a percentage (recovery % = $[mean_{cand}]/[fortification] \times 100$). Mean recoveries across concentration obtained from these plots for each tissue type are listed in Table 3. Average recoveries ranged from 82% in eggs to 98% in kidney tissue, and thus the method met the acceptance criteria for recovery in all tissue types tested.

The bias values for each fortification concentration are listed in Table 2. Bias is defined as the difference between the mean candidate result and the fortification concentration (bias = $[mean_{cand}] - [fortification]$). The average percent bias (bias/[fortification] × 100) across concentrations was determined for each matrix from the slope of the regression line after plotting the mean bias against the fortification concentrations. The average percent bias from these plots is related to the average percent recovery as recovery – bias = 100%. The average percent bias values are listed in Table 3. Kidney tissue had the smallest absolute average bias at –2.3% and eggs had the largest absolute average bias at –17.8%. There is no acceptance criterion for bias.

Twenty replicate test portions of control tissue were analyzed to determine the LOD for nicarbazin in each tissue type. The estimated LOD is defined as the mean result plus three standard deviations. Estimated LOD values for each matrix/tissue are presented in Table 3. Note that these data were generated using the original method developed without internal standard and with solution standards. An estimated LOD could not be determined for liver and muscle, as all results from the control tissue analyses were 0.00 ng/g. For those tissues where at least one replicate yielded a non-zero result, the estimated LOD values ranged from 0.37 to 2.90 ng/g.

LOQ was determined by fortifying control matrixes at 1/10 Codex MRL. Because there is no MRL for eggs, the LOQ was validated at the same level as the other tissues. Ten test portions of each fortified matrix were extracted and analyzed according to the candidate method. Note that these data were generated using the original method developed without internal standard and with solution standards. Two data points were determined as outliers per the Grubbs' test and removed from the analysis for muscle tissue. RSD_r values were ≤20% in all matrixes tested, which met the precision criterion (RSD_r ≤20% at concentrations <100 ng/g), validating the LOQ at 20 ng/g (Table 4).

Matrix Study—Incurred Tissues

The repeatability precision of the method was verified with incurred tissues from liver, kidney, muscle, and fat as listed in Table 5. Each animal was dosed through ingestion of feed containing the drug. Three replicate analyses were carried out for each available tissue using the original method developed without internal standard and with solution standards. The repeatability precision (RSD_r) varied from 0.33 to 2.5%. The

Table 2. Precision of initial method in fortified tissues

Fortification level, ng/g	Trial	Replicates	Mean, ng/g	s_r	RSD_r, %	Intertrial mean, ng/g	Intertrial s_r	Intertrial RSD_r, %	$HorRat_r$	Bias
					Liver					
100	1	6	77	8.0	10.4	77	6.3	8.2	0.73	–23.3
	2	6	81	1.8	2.2					
	3	6	72	4.4	6.1					
200	1	6	144	10.3	7.2	151	13.1	8.6	0.85	–48.9
	2	6	165	9.8	5.9					
	3	6	145	5.2	3.6					
400	1	6	312	6.9	2.2	314	15.0	4.8	0.52	–86
	2	6	329	7.1	2.2					
	3	6	301	14.4	4.8					
2000	1	6	1630	88	5.4	1660	80	4.8	0.67	–344
	2	6	1710	55	3.2					
	3	6	1630	73	4.5					
4000	1	6	3340	108	3.2	3270	180	5.5	0.85	–734
	2	6	3300	183	5.6					
	3	6	3170	214	6.8					
8000	1	6	7000	182	2.6	7100	180	2.5	0.43	–899
	2	6	7220	164	2.3					
	3	6	7080	145	2.1					
					Kidney					
100	1	6	85	1.1	1.3	85	5.6	6.6	0.58	–15.5
	2	6	85	9.2	10.8					
	3	6	84	4.1	5.0					
200	1	6	172	2.0	1.1	179	14.9	8.3	0.82	–21.1
	2	6	198	5.1	2.6					
	3	6	167	7.9	4.7					
400	1	6	369	4.3	1.2	392	27.1	6.9	0.75	–7.9
	2	5[a]	420	7.6	1.8					
	3	6	350	6.6	1.9					
2000	1	5[a]	1690	32.7	1.9	1770	95	5.4	0.75	–234
	2	6	1870	44.9	2.4					
	3	6	1720	68	4.0					
4000	1	6	3840	27.0	0.70	3890	169	4.4	0.67	–107
	2	6	4040	100	2.5					
	3	6	3800	217	5.7					
8000	1	5[a]	7260	84	1.2	7790	651	8.4	1.4	–209
	2	6	8480	461	5.4					
	3	6	7550	466	6.2					
					Muscle					
100	1	5[a]	95	0.77	0.81	85	8.5	10.0	0.89	–15.3
	2	6	76	4.0	5.3					
	3	5[a]	85	1.9	2.2					
200	1	6	190	3.0	1.6	169	19.1	11.3	1.1	–31.0
	2	6	151	10.5	7.0					
	3	5[a]	165	9.8	5.9					

Table 2. (*continued*)

Fortification level, ng/g	Trial	Replicates	Mean, ng/g	s_r	RSD_r, %	Intertrial mean, ng/g	Intertrial s_r	Intertrial RSD_r, %	$HorRat_r$	Bias
400	1	6	368	15.3	4.2	353	17.4	4.9	0.54	–46.9
	2	6	349	17.0	4.9					
	3	6	342	6.4	1.9					
2000	1	6	1820	92	5.0	1790	80	4.5	0.62	–207
	2	6	1840	33.9	1.8					
	3	6	1720	51	3.0					
4000	1	6	3650	73	2.0	3500	198	5.7	0.87	–505
	2	6	3570	49.8	1.4					
	3	6	3260	148	4.5					
8000	1	6	7370	125	1.7	7130	211	3.0	0.51	–868
	2	6	7020	109	1.6					
	3	6	7010	155	2.2					
Skin and fat										
100	1	6	90	1.9	2.1	97	6.6	6.8	0.60	–2.8
	2	6	101	5.2	5.2					
	3	6	101	5.6	5.6					
200	1	6	176	11.5	6.5	183	14.9	8.2	0.80	–17.5
	2	6	180	20.9	11.6					
	3	6	192	3.8	2.0					
400	1	6	350	5.9	1.7	365	28.6	7.8	0.85	–35.0
	2	6	356	40.8	11.5					
	3	6	386	7.8	2.0					
2000	1	6	1820	90	5.0	1880	129	6.9	0.95	–116
	2	6	1920	196	10.2					
	3	6	1920	38.3	2.0					
4000	1	6	3570	52	1.5	3610	211	5.8	0.90	–388
	2	6	3500	289	8.3					
	3	6	3760	123	3.3					
8000	1	6	7370	122	1.7	7450	456	6.1	1.1	–552
	2	6	7110	573	8.1					
	3	6	7860	128	1.6					
Eggs										
50	1	6	42.6	2.6	6.0	40.9	3.5	8.6	0.69	–9.1
	2	6	42.0	0.87	2.1					
	3	6	38.2	4.6	12.1					
100	1	6	84	3.2	3.8	85	5.3	6.2	0.55	–14.8
	2	6	84	2.9	3.5					
	3	6	87	8.4	9.6					
200	1	6	169	2.3	1.4	165	11.7	7.1	0.70	–35.1
	2	6	166	9.0	5.4					
	3	6	159	18	11.1					

[a] One data point removed as an outlier.

Table 3. Summary of mean recovery, mean bias, and LOD by matrix

Tissue	Mean recovery, %	Mean bias, %	LOD (mean + 3s_r, ng/g)
Liver	88	–11.7	ND[a]
Kidney	98	–2.3	2.9
Muscle	89	–11.0	ND[a]
Fat	93	–7.3	1.0
Eggs	82	–17.8	0.37

[a] ND = Not determined.

precision with incurred residue tissues is very tight and well within the limits established for the acceptance criteria.

Matrix Study—Modified Method

Initial method development data demonstrated no significant matrix effect and little difference in method performance with or without internal standard (ISTD). While the average recoveries for all matrixes were within the acceptable limits for the initial method, the individual recovery values for chicken liver at the lower concentrations were below 80% (76.7% at 100 ng/g; 75.5% at 200 ng/g; and 78.5% at 400 ng/g). To improve the accuracy of the method, a study was carried out in chicken liver using a modified method including matrix-matched standards and DNC-d_8 ISTD. Chicken liver was fortified at six concentrations and six replicates at each concentration were processed and analyzed on 2 different days. The data are presented in Table 6 for the modified method with and without ISTD. When ISTD is included, the data are referred to as accuracy. In the absence of ISTD, the data are referred to as recovery. When ISTD is included, all data points, as well as the means, meet the acceptance criteria for both precision and accuracy/recovery. Without ISTD, the recoveries are still acceptable, but the values at the lower concentrations are improved when ISTD is used. The authors recommend applying the modified method to all matrixes to ensure maximum accuracy. This revised method is reported herein and representative chromatograms are shown in Figure 1.

To evaluate the linearity of the improved method, DNC standard curve solutions in chicken liver extract with internal standard were analyzed in triplicate and a weighted (1/x) regression was performed. Residual analysis revealed small random residuals indicating linearity was achieved. The residuals ranged from –0.07 to 6.0%, with the highest residual percentages occurring at the two lowest concentrations. All residuals were within the acceptable range.

Confirmatory Ion Abundance Percentages

There are two criteria for confirmation of analyte in LC/MS/MS methods: (*1*) the retention time of the analyte in an unknown sample must be within 5% of the mean retention times observed in the standards; and (*2*) the IAP of the confirmatory product ion in an unknown sample must be within a specified range of the mean IAP observed in the standards (depending on whether U.S. or EU criteria are followed). Both criteria must be met in order for an unknown sample to be considered confirmed.

Table 4. LOQ by matrix

Matrix	Fortification, ng/g	Replicates	Mean concn, ng/g	s_r, ng/g	RSD$_r$, %
Liver	20	10	16.5	0.34	2.1
Kidney	20	10	12.5	1.9	15.1
Muscle	20	8[a]	17.7	0.45	2.5
Fat	20	10	17.9	0.35	2.0
Eggs	20	10	16.9	0.48	2.8

[a] Two data points removed as outliers.

One transition ion (*m/z* 300.95→106.88) was examined for confirmation of DNC. The IAP is determined from the relative abundance of the confirmatory ion compared to the determinative ion (*m/z* 300.95→136.88). IAP values from each of the standards within a chromatographic run were averaged and the IAP of each sample within that run was compared to the mean IAP value of the standards. There were two sets of standards analyzed in each run, one set at the beginning and one set at the end of each run. Both U.S. (7) and EU (8) confirmatory IAP criteria were used for comparison. The U.S. criterion for confirmation using two transition ions is an IAP value within 10% (arithmetic) of the mean IAP of the standards in that run. The EU criteria vary by the abundance as follows: at IAP >50%, tolerance is ±20% relative to the mean IAP of standards; at IAP >20 to 50%, tolerance is ±25% relative to the mean IAP of standards; at IAP >10 to 20%, tolerance is ±30% relative to the mean IAP of standards; and at IAP ≤10%, tolerance is ±50% relative to the mean IAP of standards. For example, a mean standard IAP of 50% would have acceptance ranges of 40–60% (±10% arithmetic) in the United States and 37.5–62.5% (±25%) in the EU for samples within that run.

A sample having an analyte retention time or an IAP value outside the acceptance range is considered a false-negative result. False negatives are known positive samples that do not meet the confirmation criteria. Data were collected from the fortified and incurred samples in the matrix studies from the method performed with solution standards and without ISTD.

All analyte retention times for the unknown samples were within 5% of the retention times of the standards for that run. The mean IAP of standards varied from 7.2 to 9.5% across all runs examined. There were no false-negative results observed out of 484 fortified samples and 12 incurred samples examined by the

Table 5. Precision in incurred samples

Tissue type	Inventory No.	Replicate 1	Replicate 2	Replicate 3	Mean, ng/g	s_r	RSD$_r$, %
Liver	12456004	8670	8500	8700	8630	108	1.3
Kidney	12456007	1870	1920	1820	1870	47	2.5
Muscle	12456005	1400	1420	1420	1410	11.0	0.78
Fat	12456006	1790	1800	1810	1800	9.5	0.53

Table 6. Nicarbazin accuracy in fortified chicken liver using matrix-matched standard curve with and without internal standard[a]

Fortification level, ng/g	Trial	Replicates	Mean, ng/g	s_r	RSD_r, %	Intertrial mean, ng/g	Intertrial s_r	Intertrial RSD_r, %	Accuracy, %
Method with matrix-matched curve and internal standard									
100	1	6	99	2.8	2.8	99	4.6	4.7	99
	2	6	98	6.2	6.4				
200	1	6	194	3.5	1.8	187	8.8	4.7	94
	2	6	181	7.2	4.0				
400	1	6	376	6.9	1.8	372	6.7	1.8	93
	2	6	369	4.9	1.3				
2000	1	6	1873	104	5.5	1955	121	6.2	98
	2	6	2037	71	3.5				
4000	1	6	3619	53	1.5	3758	161	4.3	94
	2	6	3896	89	2.3				
8000	1	6	7062	186	2.6	7470	465	6.2	93
	2	6	7878	205	2.6				
Method with matrix-matched curve									
100	1	6	87	12.6	14.5	88	20.8	23.8	88
	2	6	88	28.2	32.1				
200	1	6	179	5.8	3.3	174	11.3	6.5	87
	2	6	170	13.9	8.2				
400	1	6	348	63	18.2	366	47.4	13.0	92
	2	6	384	13.5	3.5				
2000	1	6	1858	136	7.3	1904	245	12.9	95
	2	6	1949	330	16.9				
4000	1	6	3671	190	5.2	3922	345	8.8	98
	2	6	4174	273	6.5				
8000	1	6	7159	308	4.3	7525	1042	13.8	94
	2	6	7892	1404	17.8				

[a] *Note*: Outliers, as confirmed by either the Dixon's or Grubbs' test, were not removed from the analysis.

U.S. and EU criteria for an overall estimated false-negative rate of 0.00% (95% confidence limits 0.00%, 0.74%).

Robustness

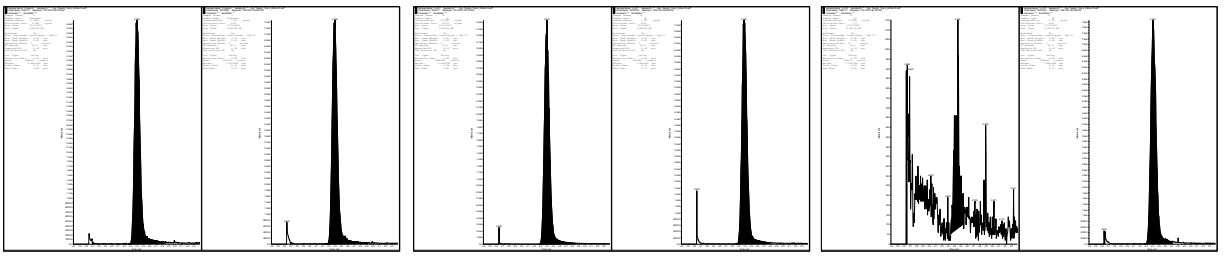
Chicken muscle was used as the matrix for the robustness study. Nine test portions of control tissue were fortified with 1000 ng DNC and the method was performed as initially developed, with solution standards and without ISTD. The method parameters used to test the robustness of the method included tissue weight, sodium sulfate weight, vortex time, shaking time, tissue temperature, fortification residence time, and injection volume.

Parameter conditions were tested according to the Plackett-Burman design (10) in which seven parameters are varied and tested in eight treatment combinations as shown in Table 7. The resulting recoveries for DNC were determined for each treatment combination. Not surprisingly, the recoveries appear to correlate with injection volume. After correction for injection volume, all treatment combinations were within the recovery criterion (80–110% at ≥100 ng/g), indicating that varying the remaining parameters had no significant effect on the method performance.

The data were also analyzed by multiple linear regression to determine whether any parameter variation is critical. Successive rounds of regression analysis were performed, each time eliminating the most nonsignificant variable to allow more degrees of freedom for estimation of error, until only one variable was left. As observed above, this variable was injection volume, with a *P*-value from the analysis of variance of 0.000115 (data not shown). When the recoveries were adjusted for injection volume, no significant parameters were found.

Stability Studies

Stability of DNC was examined in tissues, extracts, and



a b c

Figure 1. Method chromatograms: (a) DNC standard (2.5 ng/mL) in chicken liver extract with ISTD; (b) fortified chicken liver with ISTD; and (c) control chicken liver with ISTD.

standard curve solutions. Stability is defined as less than a 20% change in mean concentration or calibration slope. First, fortified tissue was frozen at –20°C, and then tested at 0, 14, and 28 days of storage. Mean DNC concentrations were compared to those of freshly fortified tissue, and stability was demonstrated in all tissues out to the 28 day time point (data not shown). The largest change was 9.8% in kidney tissue after 28 days, but all other tissues were less than 5%.

Next, fortified tissue extracts were stored at 2–8°C, and then analyzed at 0, 1, and 3 days. Mean concentrations were compared to those of freshly fortified tissue. All tissue extracts showed acceptable stability out to 3 days (data not shown).

Additionally, fortified tissues were subjected to freeze-thaw cycles. Tissues were prepared and examined after 0, 1, 2, or 3 repeated freeze-thaw cycles. Test portions were placed into a freezer set at –20°C for a minimum of 18 h to freeze, and then placed on the bench top at room temperature to thaw unassisted. The cycle was repeated as needed. Mean concentrations at each cycle were compared to those of freshly fortified tissue. DNC was shown to be stable for 3 freeze-thaw cycles in all tissue types (data not shown).

Finally, the standard curve solutions were subjected to stability testing at 2–8°C. The stored standard curve solutions were compared to freshly prepared standard curve solutions and tested at 0, 1, 3, 7, and 14 days. After analysis, the peak area ratios were plotted against concentration, and the concentration of each standard solution was calculated from the regression equation of the freshly prepared solutions. The standard solutions were deemed stable at each time point if (*1*) the back-calculated concentrations of the stored standard solutions were within 20% of those of the freshly prepared standard solutions, and (*2*) the slope resulting from regression analysis of the stored standard solutions did not differ from the slope of the fresh standard solutions by more than 20%. With the exception of the 2.5 ng/mL standard solution, the standard curve solutions met the acceptance criteria for stability in general (data not shown). There were two instances (1.0 ng/mL at day 3 and 0.5 ng/mL at day 14) where the standard curve solutions were outside the acceptable range for stability. The 2.5 ng/mL standard solution was significantly outside of the acceptability limit at 3 days and beyond. The % differences seen for the 2.5 ng/mL solution are somewhat out of line with the other data points and could warrant further investigation. The standard curve slopes were all within the acceptance criteria.

Conclusions

Data from fortified and incurred tissues generated by the initial candidate method with solution standards and without ISTD met the acceptance criteria for the determination of nicarbazin (DNC) in chicken tissues as established by the AOAC Stakeholder Panel for Veterinary Drug Residues for SLV studies. The method, however, had lower than desired recoveries at low DNC concentrations in chicken liver (76–79% at 100–400 ng/g) and, therefore, was revised to include matrix-matched standards and an ISTD to ensure accuracy in all tissue types at all concentrations. This revised method yielded accuracy values between 93 and 99% and repeatability

Table 7. Robustness study design and results

Treatment combination	Tissue weight, g	Sodium sulfate weight, g	Vortex time, min	Shaking time, min	Tissue temp., °C	Fortification residence time, min	Injection vol., µL	Recovery, %	Adjusted recovery[a], %
1	4.5	7.5	10	15	RT[b]	0	8	70.87	88.6
2	4.5	7.5	60	15	–70	30	12	107.6	89.7
3	4.5	12.5	10	60	RT	30	12	104.4	87.0
4	4.5	12.5	60	60	–70	0	8	77.03	96.3
5	5.5	7.5	10	60	–70	0	12	112.5	93.8
6	5.5	7.5	60	60	RT	30	8	74.84	93.6
7	5.5	12.5	10	15	–70	30	8	69.80	87.3
8	5.5	12.5	60	15	RT	0	12	97.13	80.9

[a] Recovery adjusted for injection volume.

[b] RT = Room temperature.

values (RSD_r) between 1.8 and 6.2% at fortification levels from 100–8000 ng/g in chicken liver and is presented here. It is expected that the revised method will perform at least as well in other chicken tissue types, including kidney, muscle, skin/fat, and eggs. Data for the revised method in these additional tissues will be available in the near future. Data from the initial candidate method demonstrated the ability of the method to confirm analyte, with no false negatives observed in 484 fortified samples and 12 incurred samples analyzed according to U.S. and EU confirmatory criteria. Robustness studies of the initial candidate method demonstrated no critical method parameters except for injection volume. Because injection volume is easily controlled with an autoinjector, the method is considered robust. Finally, DNC was shown to be stable in frozen tissue for up to 28 days and up to 3 freeze-thaw cycles; in chilled tissue extract for up to 3 days; and in chilled standard solutions for up to 14 days. The revised method presented here meets the stakeholder panel criteria in chicken liver at all concentrations tested and was granted First Action Official Method status on May 7, 2013.

Acknowledgments

James M. Turner, Elizabeth Fall, and Shawna Call, Covance Laboratories, Greenfield, IN

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[redacted] Lateral Flow Immunoassay for Ricin: Collaborative Study

Ted Hadfield and Valorie Ryan
MRIGlobal, 1470 Treeland Boulevard S.E., Palm Bay, FL 32909-2211

Sharon L. Brunelle
AOAC INTERNATIONAL, 481 N. Frederick Ave., Gaithersburg, MD 20877

Abstract

A collaborative study of the [redacted] Lateral Flow Immunoassay for Ricin was conducted. Eleven first responder collaborators participated at three test sites (4 collaborators at each of two sites and 3 collaborators at the third site). Each collaborator tested 24 randomized blind coded test portions consisting of 12 replicates of ricin at the acceptable minimum detection level (AMDL, 25 ng/mL) and 12 replicates of abrin, a structurally similar toxin, at 10 times the AMDL as required by SMPR 2010.005. One replicate of abrin resulted in an inconclusive result due to a clogged dropper bottle, but no retesting was performed so the test portion was excluded from the data analysis. Combined, the 132 ricin replicates resulted in a CPOD of 1.00 (95% confidence interval 0.97, 1.00) and the 131 replicates of abrin resulted in a CPOD of 0.00 (95% confidence interval 0.00, 0.03). These results meet the acceptance criteria outlined in SMPR 2010.005.

Introduction

Castor seeds contain ~50% castor oil and 1-5% ricin toxin by weight. The active toxin component of the castor bean is ricin (*Ricin communis* Agglutinin II, RCA 60) and is fully active. Exposure can occur from injection, inhalation or ingestion. The toxic dose is dependent on the route of exposure but in general a dose of 22 µg/kg of mass is toxic to 50% of those exposed by injection or inhalation (1.54 mg/154 lb person) while it takes a dose of 20-30 mg/kg of mass for 50% toxicity by ingestion (1.4-2.1 g/154 lb person)(1). At present there is no vaccine for protection against ricin poisoning nor are there antidotes for ricin toxicity. Symptomatic and supportive treatments are all that is available. Long term organ damage is likely in survivors. Ricin causes severe diarrhea and victims can die of shock. Death typically occurs within 3–5 days of the initial exposure. Abrin is a similar toxin, found in the highly ornamental rosary pea.

In 2007, the Department of Homeland Security contracted with AOAC INTERNATIONAL to develop consensus performance criteria for methods and equipment used by first responders and private-sector end-users (2). To fulfill this contract, AOAC created the Stakeholder Panel on Agent Detection Assays (SPADA), which included balanced representation from government, academia, and industry. SPADA,

through its Ricin Working Group, developed Standard Method Performance Requirement (SMPR) **2010.005** *Standard Method Performance Requirements for Immunological-Based Handheld Assays (HHAs) for Detection of Ricin in Visible Powders* (3). This document delineates the testing conditions and acceptance criteria for validation of ricin handheld assays in the method developer, independent laboratory and collaborative studies. The *AOAC INTERNATIONAL Methods Committee Guidelines for Validation of Biological Threat Agent Methods and/or Procedures* (BTAM Guidelines, 4) describes the study designs and statistical analyses used for validating biothreat detection methods. Together, these documents guide the Study Director through the *Performance Tested Methods*SM (PTM) and *Official Methods of Analysis*SM (OMA) pathways.

The PTM study preceded the collaborative study of the [redacted] Lateral Flow Immunoassay (LFI) for Ricin and included inclusivity and exclusivity studies, matrix studies by laboratory technicians and first responders, and environmental interference testing in both the method developer laboratory and the independent laboratory (5). The method developer laboratory additionally tested the robustness of the method. The data demonstrated equivalent performance between laboratory technicians and first responders and acceptable inclusivity/exclusivity and environmental interference results in both the method developer and independent laboratories. The method was awarded PTM certification #121201 on December 13, 2012.

It is important to note that although the method is intended for swab samples of visible powders, significant safety and sample variability concerns required the matrix studies to be carried out from the starting point of ricin in solution. In the PTM studies, the laboratory matrix studies were carried out using laboratory pipettes to dispense the sample into the LFI device while the first responder matrix studies were carried out using the dropper bottles as provided in the LFI kit to dispense the solution samples. The collaborative study went one step further and included the manipulations of opening the dropper bottle, inserting a swab, breaking the shaft, reclosing the dropper bottle and delivering sample dropwise to the LFI device to more closely simulate the method as performed in the field.

Collaborative Study

Study Design

The study was designed to include 4 collaborators at each of 3 study sites. At the last minute, one collaborator dropped out, leaving 11 collaborators total participating. The collaborators were first responders from local fire departments and the study sites were MRIGlobal laboratories in Palm Bay, FL, Kansas City, MO, and Rockville, MD. Each site had a designated Study Monitor to ensure that collaborators worked independently and to respond to questions or issues that arose during the study. The collaborators received training on the conduct of the study by an AOAC consultant, the conduct of the method by MRIGlobal staff, and safety procedures by MRIGlobal staff. The date of study was different at the three sites and depended on the availability of the collaborators. Training and conduct of the study were completed in one day.

Each collaborator was provided with 24 randomized and blind-coded liquid samples, consisting of 12 replicates of ricin (RCA 60) solution at the acceptable minimum detection level (AMDL, 25 ng/mL, 3) and

12 replicates of abrin solution at 10x the AMDL (250 ng/mL) packaged in the dropper bottles provided with the LFIs. To most closely simulate a field testing scenario, the first responders began the analysis by removing a swab from its sterile packaging, opening the first dropper bottle, and breaking off the swab shaft inside the dropper bottle. After replacing the cap and shaking the sample, 4-5 drops were added to the LFI device and the method instructions were followed for determining the results.

Safety

All manipulations of threat agents were performed in accordance with biosafety and bio-assurance practices stipulated by federal regulations. Equipment and facilities indicated by each institution were used for handling the test agent. Collaborators were referred to the CDC/NIH Biosafety in Microbiological and Biomedical Laboratories (BMBL) manual (7) for recommendations on safe handling. Collaborators received safety training at each MRIGlobal site and adhered to MRIGlobal safety requirements for personnel and facilities, which meet or exceed Federal guidance.

The concentration of ricin used in the validation study is well below the toxic dose described for toxicity in humans. First responders receive training from their unit on how to work with toxic substances including biological toxins. In addition, MRIGlobal provided training in how to work with ricin during this HHA study.

The CDC has interpreted 42 CFR Part 73 to mean the total ricin in aqueous castor seed extracts, regardless of concentration, counts toward the total quantity of ricin (100 mg) that a research laboratory can possess. At very low levels, ricin does not fall under select agent requirements. This study was conducted with an amount of ricin well below the quantity required for select agent tracking. During the course of this study, first responders were working with samples containing a total of 110 ng of ricin or 1100 ng of abrin provided as test solutions containing 25 ng/mL of ricin and 250 ng/mL of abrin. Consequently, the amount of ricin was well below the toxic dose for an adult. As part of this exercise, first responders were required to perform the testing in the personal protective equipment (PPE) they would wear when responding to a credible suspicious powder event. Part of the evaluation was to determine if performing this assay in PPE will yield reliable results.

Study Samples

Study samples were prepared by the coordinating laboratory (MRIGlobal, Palm Bay, FL). Ricin was purchased from Vector Laboratories (RCA60, Catalog No. L-1090). Abrin was purchased from Sigma (Catalog No. L9633). Certificates of Analysis are retained on file at MRIGlobal. Stock solutions of agent materials were prepared in the LFI sample buffer containing 0.8% sodium azide such that a spike volume of 100 µL of stock solution into the 4-mL dropper bottle resulted in a final concentration of 25 ng/mL ricin or 250 ng/mL abrin. Aliquots of 110 µL of agent were prepared in single cryo-compatible vials and labeled with a unique identifier. Aliquots of each agent were reserved for pre and post-shipment verification testing. All materials were stored at -80°C following preparation and during pre-shipment testing. A corresponding dropper bottle for each agent aliquot was labeled with a matching sample ID label and stored at room temperature until shipment.

Agent aliquots were shipped frozen on dry ice to the Kansas City and Rockville sites in approved toxin containment in accordance with IATA 6.2 regulations by an MRIGlobal IATA certified packaging and shipping specialist.

On the day of the study, the agent aliquots were thawed and a laboratory technician at the testing site transferred 100 µL from the blinded agent tube to the corresponding dropper bottle, replaced the screw cap on the dropper bottle, inverted to mix, and delivered a sample set of dropper bottles to each first responder.

Trial Run

A trial run consisting of 4 test samples per collaborator (2 ricin replicates and 2 abrin replicates) was conducted prior to the initiation of the validation study. The purpose of the trial run was to ensure that sample handling and data reporting processes were worked out and understood by all of the collaborators. The trial run was conducted under the same conditions as the validation study and a discussion was held with all of the collaborators to address issues and answer questions. The trial run data was not analyzed or included in this validation report, but was used as a demonstration of competency to qualify the collaborators to participate in the validation testing.

AOAC Official Method 2013.XX

[Applicable to the detection of residual ricin powder on non-porous surfaces in laboratories or by trained First Responders. The method was validated in a laboratory setting using first responders wearing personal protective equipment appropriate for a hazmat response and testing ricin in sample buffer at 25 ng/mL in compliance with the Standard Method Performance Requirements (SMPRs) developed by SPADA (SMPR 2010.005). The collaborators wore Tyvek® coveralls, safety glasses, sleeve covers and two pairs of latex gloves. This PPE is similar to standard PPE worn by the majority of the first responder collaborators.]

Safety Precautions

- When working in a laboratory, a Type II Biosafety cabinet must be used where there is a possibility of creating aerosols or splashes.
- When working with samples potentially contaminated with ricin, the appropriate personal protective equipment (PPE) must be worn. This would include a lab coat, latex or nitrile gloves, eye protection, and respiratory protection. Users should refer to the standard PPE required by their jurisdiction.
- When there is a potential for creating a ricin aerosol, such as powders, a full-face respirator should also be worn.
- For general biosafety guidelines, refer to *Biosafety in Microbiological and Biomedical Laboratories* (5th Edition), February 2009, Centers for Disease Control and Prevention and National Institutes of Health; available online at http://www.cdc.gov/biosafety/publications/bmbl5/index.htm.

- The customer is responsible for ensuring the necessary permits are issued before receipt and shipping of [redacted] material.
- Destroy negative/inconclusive assays by soaking the HHA in 5.0% commercial bleach solution for 30 minutes and dispose as medical waste. Positive assays will be processed according to Concept of Operations (CONOPS) for shipment to confirmatory laboratory.

See Table 2013.xx for the results of the collaborative study supporting acceptance of the method.

A. Principle

Lateral Flow Immunoassays (LFIs) identify biological warfare agents utilizing an antibody-based technology that provides a result in 15 minutes through a single- step operation. LFIs are based on the same simple technology utilized in home pregnancy test kits. This technology exploits the specific binding of antibodies to the appropriate antigen, resulting in a visible line for a positive result. LFIs are considered a presumptive test in the field and need to be confirmed by a more sensitive detection method.

B. Apparatus

Items (a)-(d) are available as part of the Biological Sampling Kit from the [redacted] (catalog # [redacted]) and items (e)-(f) are available as part of the Individual HHA Ricin Toxin Kit from the [redacted] (catalog # [redacted]). LFI products should be stored between 4°-10°C for up to three years of shelf-life and between 22°–25°C for up to one year of shelf-life.

a) Dacron Swabs (2). – Sterile package of 2 swabs
b) Phosphate Buffer Solution. – 4 mL in a dropper bottle with cap
c) Conical tube with screw cap. – for shipment of dropper bottle containing positive sample to confirmatory laboratory
d) Quick instruction guide.
e) Lateral flow immunoassay. – One device specific for Ricin toxin packaged in a foil pouch with desiccant
f) Package insert.

C. Additional Supplies

a) *Timing device*.

D. Preparation of Test Sample

a) Ensure HHAs are at room temperature (22-25°C) for at least one hour prior to use. Do not open HHA pouch until requirements in **Analysis** a) are met.

b) Take the dropper off the buffer bottle by using the cap to pop it off.

c) Open the Dacron® swabs from the opposite side of the tip. Insert one swab into buffer to saturate the swab.

d) Swipe the sample area (recommended 10 cm x 10 cm square). Rotate the swab between your fingers and swab the area going left to right and top to bottom. If the swab dries out, repeat steps 3 and 4 with a new swab.

e) Insert swab into buffer bottle.

i. Leave space between the swab and the bottom of the bottle.

ii. Break off swab tip(s).

iii. Replace cap and dropper top with swab tip(s) inside buffer bottle and shake for 30 seconds.

E. Analysis

a) Open the HHA barrier bag and remove the HHA. Check the desiccant inside the bag to test for HHA humidity exposure. If the desiccant is blue, the HHA has not been exposed to humidity. If the desiccant is pink, a new HHA should be used. Position the HHA on a flat surface in a manner that allows sample addition to the sample well.

b) Using the dropper bottle included in the Biological Sampling Kit (BSK), add 4-5 drops (approximately 100-120 µL) of sample to the sample well. Replace dropper lid onto the sample bottle to ensure sample integrity for confirmatory analysis.

c) Wait 15 ± 1 minutes before reading the results.

DO NOT READ PRIOR TO, OR AFTER THE READ TIME WINDOW.

Before 15 minutes, the assay has not fully developed and after 15 minutes, the sample can flow back across the membrane, which could produce a false result.

F. Interpretation and Test Results

a) Read the results. The results of the assay are presented as visual pink lines on the HHA strip.

i. The presence of both the control line and the sample line indicates a positive result.

ii. The presence of the control line without the presence of the sample line indicates a negative result.

iii. The presence of the sample line without the presence of the control line indicates an inconclusive result.

iv. The absence of both the control line and the sample line indicates an inconclusive result.

v. In the case of an inconclusive result, repeat the test with a new device.

NOTE: The lines should be a pink color. If the line is gray, the assay is inconclusive.

NOTE: It is generally recommended that all analyses be repeated to confirm results – follow your specific standard operating procedure for repeat testing.

b) Record the results. Ensure to include: result, time, date, location, operator, and lot number. If possible, a picture of the HHA result is recommended.

c) Following HHA analysis:

i. Destroy negative/inconclusive assays by soaking the HHA in 5.0% commercial bleach solution for 30 minutes and dispose as medical waste.

ii. Positive assays will be processed according to CONOPS for shipment to confirmatory laboratory.

NOTES:

- HHAs are one-time use only.
- There are possible effects that can cause aberrant results with the HHA:
    - The Matrix Effect: Something in the sample nonspecifically binds to the capture antibody.
        - May be encountered when testing soil, tap water, or sewage samples. Can result in false positive or false negative results.
    - The Hook Effect: Caused by too much antigen that overwhelms the assay and causes a false negative result. Dilution of sample is recommended.
    - Cross Reactivity: Two closely related organisms share a common antibody binding site and can cause false positive results.
    - Other factors that can cause false results:
        - Small particulate matter
        - pH extremes (<5 or >9)
        - High salt concentrations or lack of salt

Results and Discussion

The collaborative study data and statistics are presented in Table 2013.XX. In total, 132 replicates of ricin and 131 replicates of abrin were tested by 11 collaborators. Collaborator 7 had one dropper bottle of abrin solution clogged by the shaft of the swab, so no solution was delivered to the LFI device. Since

the collaborator did not alert the study monitor to obtain a replacement test sample, the replicate was dropped from the data analysis.

All 132 ricin replicates yielded positive results by all collaborators for a collaborative probability of detection (CPOD) of 1.00 (95% confidence interval 0.97 – 1.00). All 131 abrin replicates yielded negative results by all collaborators for a CPOD of 0.00 (95% confidence interval 0.00 – 0.03). These data meet the acceptance criteria of SMPR 2010.005, which requires an estimated 5% lower confidence limit on CPOD of 0.95 or higher for the ricin replicates and a 95% upper confidence limit on the CPOD of 0.05 or lower for the abrin replicates. The standard deviation of reproducibility (s_R) is 0.00, demonstrating that the method is reproducible when performed by first responders wearing standard PPE for a hazmat response. The collaborators wore Tyvek® coveralls, safety glasses, sleeve covers and two pairs of latex gloves. This PPE is similar to standard PPE worn by the majority of the first responder collaborators. One collaborator attempted testing using heavy rubber gloves which are standard issue for his organization but found they were too cumbersome to hold the swab or open the top of the dropper bottles. This collaborator requested substitution of two pairs of latex gloves to complete the testing and the request was granted.

Recommendation

The authors recommend the [redacted] Lateral Flow Immunoassay for Ricin for Official First Action status.

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Timothy Hall*, Firefighter/ Paramedic, Special Operations Team, Brevard County Fire/Rescue
Lieutenant Thomas Neidert, Special Operations Team, Brevard County Fire/Rescue
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Stephen Scott Clark, Kansas City Fire Department
Mike Jenkins, North Kansas City Fire Department
Carla Miranda, City of Omaha Fire and Rescue

At MRIGlobal Maryland
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4. Biothreat Agent Method Validation Guideline (2011) *J AOAC Int.* **94**, 1359-1382
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FOOD BIOLOGICAL CONTAMINANTS

Evaluation of 3M™ Molecular Detection Assay (MDA) *Salmonella* for the Detection of *Salmonella* in Selected Foods: Collaborative Study

PATRICK BIRD, KIEL FISHER, MEGAN BOYLE, TRAVIS HUFFMAN, M. JOSEPH BENZINGER, JR, PAIGE BEDINGHAUS, JONATHAN FLANNERY, ERIN CROWLEY, JAMES AGIN, and DAVID GOINS
Q Laboratories, Inc., 1400 Harrison Ave, Cincinnati, OH 45214
DEANN BENESH[1] and JOHN DAVID
3M Food Safety Department, 3M Center, Bldg 260-6B-01, St. Paul, MN 55144

Collaborators: D. Awad, M. Bandu, K. Blanchard, D. Bosco, R. Brooks, D. Clark Jr, H. Dammann, J. Dyszel, V. Gill, M. Greenwell, C. Gwinn, M. Horan, J. Jurgens, M. Kelly, D. Lewis, S. Luce, J. Marchent, W. McMahon, I. Mello, S. Montez, S. Moosekian, A. Morey, K. Newman, M. Oltman, M. Ontiberos, K. Rajkowski, J. Ruebl, B. Stawick, L. Thompson, M. Vross

The 3M™ Molecular Detection Assay (MDA) *Salmonella* is used with the 3M™ Molecular Detection System for the detection of *Salmonella* spp. in food, food-related, and environmental samples after enrichment. The assay utilizes loop-mediated isothermal amplification to rapidly amplify *Salmonella* target DNA with high specificity and sensitivity, combined with bioluminescence to detect the amplification. The 3M MDA *Salmonella* method was compared using an unpaired study design in a multilaboratory collaborative study to the U.S. Department of Agriculture/Food Safety and Inspection Service-*Microbiology Laboratory Guidebook* (USDA/FSIS-MLG 4.05), *Isolation and Identification of Salmonella from Meat, Poultry, Pasteurized Egg and Catfish Products* for raw ground beef and the U.S. Food and Drug Administration/*Bacteriological Analytical Manual* (FDA/BAM) Chapter 5 *Salmonella* reference method for wet dog food following the current AOAC guidelines. A total of 20 laboratories participated. For the 3M MDA *Salmonella* method, raw ground beef was analyzed using 25 g test portions, and wet dog food was analyzed using 375 g test portions. For the reference methods, 25 g test portions of each matrix were analyzed. Each matrix was artificially contaminated with *Salmonella* at three inoculation levels: an uninoculated control level (0 CFU/test portion), a low inoculum level (0.2–2 CFU/test portion), and a high inoculum level (2–5 CFU/test portion). In this study, 1512 unpaired replicate samples were analyzed. Statistical analysis was conducted according to the probability of detection (POD). For the low-level raw ground beef test portions, the following dLPOD (difference between the POD of the reference and candidate method) values with 95% confidence intervals were obtained: –0.01 (–0.14, +0.12). For the low-level wet dog food test portions, the following dLPOD with 95% confidence intervals were obtained: –0.04 (–0.16, +0.09). No significant differences were observed in the number of positive samples detected by the 3M MDA *Salmonella* method versus either the USDA/FSIS-MLG or FDA/BAM methods.

For over 100 years, *Salmonella*, one of the most frequently reported causes of foodborne outbreaks, has been known to cause foodborne illness in humans (1). The bacterium has been implicated in outbreaks from a variety of foods including raw animal products, such as meat, poultry, eggs, dairy products, seafood, and some fruits and vegetables (2). In order to reduce outbreaks of Salmonellosis, a comprehensive farm-to-fork approach is needed. The detection of *Salmonella* can often be very time-consuming and expensive, as the presence of the microorganism in food usually does not affect the taste, smell, or appearance (3). The 3M™ Molecular Detection Assay (MDA) *Salmonella* method, in conjunction with 3M Buffered Peptone Water ISO (BPW ISO; 4), uses a combination of loop-mediated isothermal DNA amplification and bioluminescence detection to detect *Salmonella* in enriched food, feed, and environmental samples.

The 3M MDA *Salmonella* method allows for next-day detection of *Salmonella* species. After 18–24 h of enrichment using prewarmed (37 ± 1°C) 3M BPW ISO medium, *Salmonella* detection is performed by the 3M MDA *Salmonella* method. Presumptive positive results are reported in real time; negative results are displayed after completion of the assay.

Prior to the collaborative study, the 3M MDA *Salmonella* method was certified as a *Performance Tested Method* (PTM) following the AOAC guidelines for harmonized PTM studies (5). The aim of the PTM study was to demonstrate that the 3M MDA *Salmonella* method could detect *Salmonella* in selected foods as claimed by the manufacturer. For the 3M MDA *Salmonella* evaluation, six matrices were analyzed: raw ground beef (25 g), processed breaded chicken (325 g), liquid egg (100 g), shrimp (25 g), fresh spinach (25 g), and wet dog food (375 g). All other

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[1] Corresponding author's e-mail: dbenesh1@mmm.com
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PTM parameters (inclusivity, exclusivity, ruggedness, stability, and lot-to-lot variability) tested in the PTM studies satisfied the performance requirements for PTM approval. The method was awarded PTM certification number 031208 on March 30, 2012.

The aim of this collaborative study was to compare the 3M MDA *Salmonella* method to the U.S. Department of Agriculture (USDA) Food Safety and Inspection Service (FSIS)-*Microbiology Laboratory Guidebook* (MLG) 4.05 (6) for raw ground beef and the U.S. Food and Drug Administration (FDA) *Bacteriological Analytical Manual* (BAM) Chapter 5 (7) method for wet dog food.

Collaborative Study

Study Design

For this collaborative study, two matrices, raw ground beef (80% lean) and wet dog food (canned beef chunks), were analyzed. The matrices were obtained from local retailers and screened for the absence of *Salmonella* by preparing one bulk sample and analyzing five sample replicates (25 g) by the appropriate reference method. The screening indicated an absence of the target organism. The raw ground beef was artificially contaminated with *Salmonella* Ohio Sequence Types (STS) 81 and the wet dog food with *Salmonella* Poona National Collection of Type Cultures (NCTC) 4840. There were two inoculation levels for each matrix: a high inoculation level of approximately 2–5 CFU/test portion and a low inoculation level of approximately 0.2–2 CFU/test portion. A set of uninoculated control test portions was also included for each matrix at 0 CFU/test portion.

Twelve replicate samples from each of the three contamination levels of product were analyzed. Two sets of samples (72 total) were sent to each laboratory for analysis by the 3M MDA *Salmonella* method and either the USDA/FSIS-MLG (raw ground beef) or FDA/BAM (wet pet food) reference method due to different sample enrichments for the candidate method and the reference methods. For both matrices, collaborators were sent an additional 30 g test portion and instructed to conduct a total aerobic plate count (APC) following the FDA/BAM Chapter 3 on the day samples were received to determine the total aerobic microbial load.

A detailed collaborative study packet outlining all necessary information related to the study including media preparation, method-specific test portion preparation, and documentation of results was sent to each collaborating laboratory prior to the initiation of the study.

Preparation of Inocula and Test Portions

The *Salmonella* cultures used in this evaluation were propagated in 10 mL of Brain Heart Infusion broth from a Q Laboratories frozen stock culture held at –70°C. The broth was incubated for 18–24 h at 35 ± 1°C. Appropriate dilutions were prepared based on previously established growth curves for both low and high inoculation levels, resulting in fractional positive outcomes for at least one level. For both test portion sizes, a bulk lot of each matrix was inoculated with a liquid inoculum and mixed thoroughly by hand-kneading to ensure an even distribution of microorganisms. The matrices were inoculated on the day of shipment so that all test portions would be held for 96 h before testing was initiated. For analysis of the raw ground beef, the bulk lot of test material was divided into 30 g portions for shipment to the collaborators. For analysis of the wet dog food, 25 g of inoculated test product was mixed with 350 g of uninoculated test product for shipment to the collaborators for analysis by the 3M MDA *Salmonella* method. For analysis by the reference method, collaborators received 30 g portions.

To determine the level of *Salmonella* spp. in the matrices, a five-tube most probable number (MPN) was conducted by the coordinating laboratory on the day of initiation of analysis using the FDA/BAM Chapter 5 reference method for wet pet food or the USDA/FSIS-MLG 4.05 reference method for raw ground beef. From both the high and low inoculated levels, five 100 g test portions, the reference method test portions, and five 10 g test portions were analyzed using the appropriate reference method enrichment broth. The MPN and 95% confidence intervals were calculated from the high, low, and uninoculated levels using the MPN Calculator (www.lcfltd.com/customer/LCFMPNCalculator.exe; 8). Confirmation of the samples was conducted according to either the USDA/FSIS-MLG 4.05 or FDA/BAM Chapter 5 reference method, dependent on the matrix.

Test Portion Distribution

All samples were labeled with a randomized, blind-coded three-digit number affixed to the sample container. Test portions were shipped on a Thursday via overnight delivery according to the Category B Dangerous Goods shipment regulations set forth by the International Air Transport Association. All samples were packed with cold packs to target a temperature of <7°C during shipment. Upon receipt, samples were held by the collaborating laboratory at refrigerated temperature (3–5°C) until the following Monday, when analysis was initiated. In addition to each of the test portions and the total plate count replicate, collaborators also received a test portion for each matrix labeled as "temperature control." Participants were instructed to record the temperature of this portion upon receipt of the shipment, document the results on the Sample Receipt Confirmation form provided, and fax to the Study Director.

Additional shipments of raw ground beef test portions were made by the sponsoring laboratory when aberrant results were observed. Further investigation of the results indicated that each participating collaborator detected the presence of the target analyte in the uninoculated control samples sent in the first shipment. In each case, the same species was reported for the control samples, which may have been due to cross-contamination. As a result, new test portions of raw ground beef were shipped and analyzed by each of the collaborating laboratories.

Test Portion Analysis

Collaborators followed the appropriate preparation and analysis protocol according to the method for each matrix. For both matrices, each collaborator received 72 test portions of each food product (12 high, 12 low, and 12 controls for each method). For the analysis of the raw ground beef test portions by the 3M MDA *Salmonella* method, a 25 g portion was enriched with 225 mL of prewarmed (37 ± 1°C) 3M BPW

ISO, homogenized for 2 min and incubated for 18 h at 37 ±1°C. For the wet dog food test portions analyzed by the 3M MDA *Salmonella* method, a 375 g portion was enriched with 3375 mL prewarmed (37 ± 1°C) 3M BPW ISO, homogenized for 2 min and incubated for 18 h at 37 ± 1°C.

Following enrichment, samples were assayed by the 3M MDA *Salmonella* method and confirmed following the standard reference method. Both test portion sizes analyzed by the 3M MDA *Salmonella* method were compared to samples (25 g) analyzed using either the USDA/FSIS-MLG or FDA/BAM reference method in an unpaired study design. All positive test portions were biochemically confirmed by the API 20E biochemical test, AOAC *Official Method* **978.24**, or by the VITEK 2 GN identification test, AOAC *Official Method* **2011.17**. Serological testing was also performed.

Statistical Analysis

Each collaborating laboratory recorded results for the reference method and the 3M MDA *Salmonella* method on the data sheets provided. The data sheets were submitted to the Study Director at the end of each week of testing for analysis. The results of each test portion for each sample were compiled by the Study Director and the qualitative 3M MDA *Salmonella* results were compared to the reference method for statistical analysis. Data for each test portion size were analyzed using the probability of detection (POD; 9). If the confidence interval of a dLPOD did not contain zero, then that would indicate a statistically significant difference between the candidate method and the reference method at the 5% confidence level (9).

AOAC Official Method 2013.09
Salmonella in Selected Foods
3M™ Molecular Detection Assay (MDA) *Salmonella* Method
First Action 2013

[Applicable to detection of *Salmonella* in raw ground beef (25 g), processed breaded chicken (325 g), liquid egg (100 g), shrimp (25 g), fresh spinach (25 g), and wet dog food (375 g)].

See Tables **2013.09A** and **B** for a summary of results of the inter-laboratory study.

See Appendix Tables A and B for detailed results of the inter-laboratory study.

A. Principle

The 3M Molecular Detection Assay (MDA) *Salmonella* method is intended for use with the 3M Molecular Detection System for the rapid and specific detection of *Salmonella* spp. in food, feed, and environmental samples after enrichment. After enrichment in prewarmed 3M Buffered Peptone Water ISO (3M BPW ISO) medium, the 3M MDA *Salmonella* test utilizes loop-mediated isothermal amplification to rapidly amplify *Salmonella* target DNA with high specificity and sensitivity, combined with bioluminescence to detect the amplification. Presumptive positive results are reported in real time; negative results are displayed after the assay is completed.

B. Apparatus and Reagents

Items (**b**)–(**g**) are available as the 3M MDA *Salmonella* kit from 3M Food Safety (St. Paul, MN).

(**a**) *3M Molecular Detection System*.—Available from 3M Food Safety.

(**b**) *3M MDA Salmonella reagent tubes*.—12 strips of eight tubes.

(**c**) *Lysis solution (LS) tubes*.—12 strips of eight tubes.

(**d**) *Extra caps*.—12 strips of eight caps.

(**e**) *Negative control (NC)*.—One vial (2 mL).

(**f**) *Reagent control (RC)*.—Eight reagent tubes.

(**g**) *Quick start guide.*

(**h**) *3M Molecular Detection Speed Loader Tray*.—Available from 3M Food Safety.

(**i**) *3M Molecular Detection Chill Block Tray and Chill Block Insert*.—Available from 3M Food Safety.

(**j**) *3M Molecular Detection Heat Block Insert*.—Available from 3M Food Safety.

(**k**) *3M Molecular Detection Cap/Decap Tool for reagent tubes*.—Available from 3M Food Safety.

(**l**) *3M Molecular Detection Cap/Decap Tool for lysis tubes*.—Available from 3M Food Safety.

(**m**) *Empty lysis tube rack*.—Available from 3M Food Safety.

(**n**) *Empty reagent tube rack*.—Available from 3M Food Safety.

(**o**) *3M BPW ISO*.—Available from 3M Food Safety. Formulation equivalent to ISO 6579:2002 Annex B (4).

(**p**) *Disposable pipet*.—Capable of 20 µL.

(**q**) *Multichannel (eight-channel) pipet.*—Capable of 20 µL.

(**r**) *Sterile filter tip pipet tips*.—Capable of 20 µL.

(**s**) *Filter stomacher bags*.—Seward Laboratory Systems Inc., Bohemia, NY, or equivalent.

(**t**) *Stomacher*.—Seward Laboratory Systems Inc. or equivalent.

(**u**) *Thermometer*.—Calibrated range to include 100 ± 1°C.

(**v**) *Dry double block heater unit or water bath*.—Capable of maintaining 100 ± 1°C.

(**w**) *Incubators*.—Capable of maintaining 37 ± 1°C.

(**x**) *Freezer*.—Capable of maintaining –10 to –20°C, for storing the 3M Molecular Detection Chill Block Tray.

(**y**) *Refrigerator*.—Capable of maintaining 2–8°C, for storing the 3M MDA.

(**z**) *Computer*.—Compatible with the 3M Molecular Detection Instrument.

C. General Instructions

(**a**) Store the 3M MDA *Salmonella* kit at 2–8°C. Do not freeze. Keep kit away from light during storage. After opening the kit, check that the foil pouch is undamaged. If the pouch is damaged, do not use. After opening, unused reagent tubes should always be stored in the resealable pouch with the desiccant inside to maintain stability of the lyophilized reagents. Store resealed pouches at 2–8°C for no longer than 60 days. Do not use 3M MDA *Salmonella* past the expiration date.

(**b**) The 3M Molecular Detection Instrument is intended for use with samples that have undergone heat treatment during the assay lysis step, which is designed to destroy organisms present in the sample. Samples that have not been properly heat-treated during the assay lysis step may be considered a potential

Table 2013.09A. POD summary of raw ground beef (25 g) results for the 3M MDA *Salmonella* method[a]

	Inoculation level		
	Uninoculated	Low	High
Candidate presumptive positive/total No. of samples analyzed	1/120	69/120	120/120
Candidate presumptive (CP) POD	0.01 (0.00, +0.05)	0.58 (+0.48, +0.67)	1.00 (+0.97, +1.00)
s_r[b]	0.09 (+0.08, +0.17)	0.51 (+0.45, +0.52)	0.00 (0.00, +0.18)
s_L[c]	0.00 (0.00, +0.04)	0.00 (0.00, +0.14)	0.00 (0.00, +0.18)
s_R[d]	0.09 (+0.08, +0.10)	0.51 (+0.45, +0.52)	0.00 (0.00, +0.24)
Candidate confirmed positive/total No. of samples analyzed	0/120	67/120	120/120
Candidate confirmed (CC) POD	0.00 (0.00, +0.03)	0.56 (+0.47, +0.65)	1.00 (+0.97, +1.00)
s_r[b]	0.00 (0.00, +0.17)	0.51 (+0.45, +0.52)	0.00 (0.00, +0.18)
s_L[c]	0.00 (0.00, +0.17)	0.00 (0.00, +0.11)	0.00 (0.00, +0.18)
s_R[d]	0.00 (0.00, +0.24)	0.51 (+0.46, +0.52)	0.00 (0.00, +0.24)
Positive reference samples/total No. of samples analyzed	0/120	68/120	119/120
Reference POD	0.00 (0.00, +0.03)	0.57 (+0.48, +0.66)	0.99 (+0.95, +1.00)
s_r[b]	0.00 (0.00, +0.17)	0.50 (+0.45, +0.52)	0.09 (+0.08, +0.17)
s_L[c]	0.00 (0.00, +0.17)	0.00 (0.00, +0.18)	0.00 (0.00, +0.04)
s_R[d]	0.00 (0.00, +0.24)	0.51 (+0.45, +0.52)	0.09 (+0.08, –0.11)
dLPOD (Candidate vs Reference)	0.00 (–0.03, +0.03)	–0.01 (–0.14, +0.12)	0.01 (–0.02, +0.05)
dLPOD (CP vs CC)	0.01 (–0.02, +0.05)	0.02 (–0.11, +0.15)	0.00 (–0.03, +0.03)

[a] Results include 95% confidence intervals.

[b] Repeatability SD.

[c] Among-laboratory SD.

[d] Reproducibility SD.

biohazard and should not be inserted into the 3M Molecular Detection Instrument.

(**c**) Follow all instructions carefully. Failure to do so may lead to inaccurate results.

(**d**) After use, the enrichment medium and the 3M MDA *Salmonella* tubes can potentially contain pathogenic materials. When testing is complete, follow current industry standards for the disposal of contaminated waste. Consult the Material Safety Data Sheet for additional information and local regulations for disposal.

Periodically decontaminate laboratory benches and equipment (pipets, cap/decap tools, etc.) with a 1–5% (v/v in water) household bleach solution or DNA removal solution.

D. Sample Enrichment

Prewarm 3M BPW ISO enrichment medium to 37 ± 1°C.

Aseptically combine the enrichment medium and sample following the outline in Table **2013.09C**. For all meat and highly particulate samples, the use of filter bags is recommended. Homogenize thoroughly for 2 min. Incubate at 37 ± 1°C.

E. Preparation of the 3M Molecular Detection Speed Loader Tray

Wet a cloth or paper towel with a 1–5% (v/v in water) household bleach solution and wipe the 3M Molecular Detection Speed Loader Tray. Rinse the tray with water. Use a disposable towel to wipe the tray dry. Ensure the 3M Molecular Detection Speed Loader Tray is dry before use.

F. Preparation of the 3M Molecular Detection Chill Block Insert

Before using the 3M Molecular Detection Chill Block Insert, ensure it has been stored on the 3M Molecular Detection Chill Block Tray in the freezer (–10 to –20°C) for a minimum of 2 h before use. When removing the 3M Molecular Detection Chill Block Insert from the freezer for use, remove it and the 3M Molecular Detection Chill Block Tray together. Use the insert and tray within 20 min.

G. Preparation of the 3M Molecular Detection Heat Block Insert

Place the 3M Molecular Detection Heat Block Insert in a dry double block heater unit. Turn on the dry block heater unit and set the temperature to allow the 3M Molecular Detection Heat Block Insert to reach and maintain a temperature of 100 ± 1°C.

Note: Depending on the heater unit, allow approximately 30–50 min for the 3M Molecular Detection Heat Block Insert to reach temperature. Using a calibrated thermometer, verify that the 3M Molecular Detection Heat Block Insert is at 100 ± 1°C.

Table 2013.09B. POD Summary of wet pet food (375 g) results for the 3M MDA *Salmonella* method[a]

	Inoculation level		
	Uninoculated	Low	High
Candidate presumptive positive/total No. of samples analyzed	1/132	65/132	131/132
Candidate presumptive (CP) POD	0.01 (0.00, +0.04)	0.49 (+0.40, +0.58)	0.99 (+0.96, +1.00)
s_r[b]	0.09 (+0.08, +0.16)	0.51 (+0.46, +0.52)	0.09 (+0.08, +0.16)
s_L[c]	0.00 (0.00, +0.04)	0.00 (0.00, +0.14)	0.00 (0.00, +0.04)
s_R[d]	0.09 (+0.08, +0.10)	0.51 (+0.46, +0.52)	0.09 (+0.08, +0.10)
Candidate confirmed positive/total No. of samples analyzed	0/132	65/132	131/132
Candidate confirmed (CC) POD	0.00 (0.00, +0.03)	0.49 (+0.40, +0.58)	0.99 (+0.96, +1.00)
s_r[b]	0.00 (0.00, +0.17)	0.51 (+0.46, +0.52)	0.09 (+0.08, +0.16)
s_L[c]	0.00 (0.00, +0.17)	0.00 (0.00, +0.14)	0.00 (0.00, +0.04)
s_R[d]	0.00 (0.00, +0.23)	0.51 (+0.46, +0.52)	0.09 (+0.08, +0.10)
Positive reference samples/total No. of samples analyzed	0/132	70/132	132/132
Reference POD	0.00 (0.00, +0.03)	0.53 (+0.44, +0.62)	1.00 (+0.97, +1.00)
s_r[b]	0.00 (0.00, +0.17)	0.52 (+0.46, +0.52)	0.00 (0.00, +0.17)
s_L[c]	0.00 (0.00, +0.17)	0.00 (0.00, +0.09)	0.00 (0.00, +0.17)
s_R[d]	0.00 (0.00, +0.23)	0.52 (+0.47, +0.52)	0.00 (0.00, +0.23)
dLPOD (Candidate vs Reference)	0.00 (–0.03, +0.03)	–0.04 (–0.16, +0.09)	–0.01 (–0.04, +0.02)
dLPOD (CP vs CC)	0.01 (–0.02, +0.05)	0.00 (–0.13, +0.13)	0.00 (–0.03, +0.03)

[a] Results include 95% confidence intervals.

[b] Repeatability SD.

[c] Among-laboratory SD.

[d] Reproducibility SD.

H. Preparation of the 3M Molecular Detection Instrument

Launch the 3M Molecular Detection Software and log in. Turn on the 3M Molecular Detection Instrument. Create or edit a run with data for each sample. Refer to the 3M Molecular Detection System User Manual for details.

Note: The 3M Molecular Detection Instrument must reach and maintain a temperature of 60°C before a run can be started. This heating step takes approximately 20 min and is indicated by an orange light on the instrument's status bar. When the instrument is ready to start a run, the status bar will turn green.

I. Lysis

Allow the LS tubes to warm up to room temperature by setting the rack on the laboratory bench for 2 h. Alternatives to equilibrate the LS tubes to room temperature are to incubate the LS tubes in a 37 ± 1°C incubator for 1 h or at room temperature overnight (16–18 h). Remove the enrichment broth from the incubator and gently agitate the contents. One LS tube is required for each sample and the NC sample. LS tube strips can be cut to the desired number. Select the number of individual LS tubes or eight-tube strips needed. Place the LS tubes in an empty rack. To avoid cross-contamination, decap strip at a time and use a new pipet tip for each transfer step. Transfer the enriched samples to LS tubes as described below:

Note: Transfer each enriched sample into individual LS tube first. Transfer the NC last.

Use the 3M Molecular Detection Cap/Decap Tool-Lysis to decap one LS tube strip—one strip at a time. Set the tool with cap attached aside on a clean surface. Transfer 20 µL of sample into an LS tube. Repeat transfer until each individual sample has been added to a corresponding LS tube in the strip. Use the 3M Molecular Detection Cap/Decap Tool-Lysis to recap the LS tube strip. Use the rounded side of the tool to apply pressure in a back-and-forth motion to ensure that the cap is tightly applied. Repeat as needed for the number of samples to be tested.

When all samples have been transferred, transfer 20 µL of NC into a LS tube. Use the 3M Molecular Detection Cap/Decap Tool-Lysis tool to recap the LS tube. Cover the rack of LS tubes with the rack lid and firmly invert three to five times

Table 2013.09C Sample enrichment protocols

Sample matrix	Sample size, g	Enrichment broth volume, mL	Enrichment time, h
Raw ground beef (27% fat)	25	225	18–24
Raw shrimp	25	225	18–24
Bagged spinach	25	225	18–24
Pasteurized liquid whole egg	100	900	18–24
Cooked breaded chicken	325	2925	18–24
Wet pet food (dog–beef cuts in gravy, canned)	375	3375	18–24

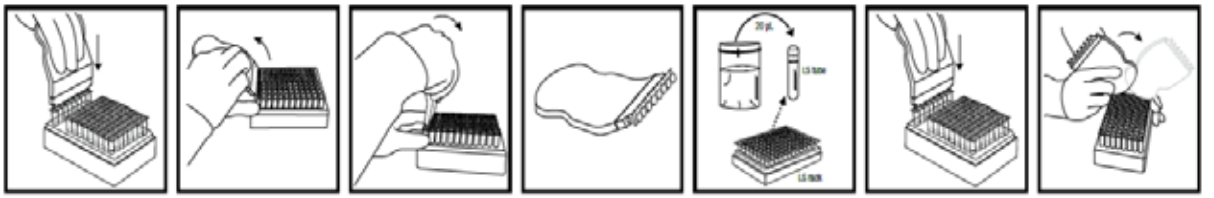

Figure 2013.09A. Transfer of enriched sample to Lysis Solution tube.

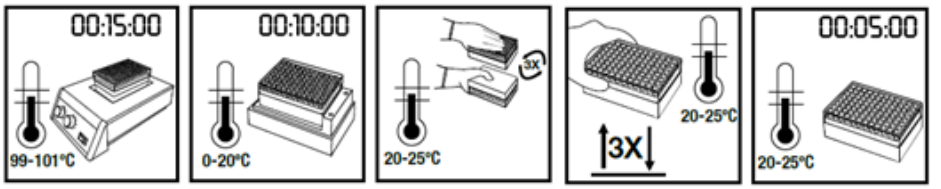

Figure 2013.09B. Sample Lysis.

to mix. Suspension has to flow freely inside the tube. *See* Figure **2013.09A**.

Verify that the temperature of the 3M Molecular Detection Heat Block Insert is at 100 ± 1°C. Place the rack of LS tubes in the 3M Molecular Detection Heat Block Insert and heat for 15 ± 1 min. An alternative to using dry heat for the lysis step is to use a water bath at 100 ±1°C. Ensure that sufficient water is used to cover up to the liquid level in the LS tubes. Place the rack of LS tubes in the water bath at 100 ± 1°C and heat for 15 ± 1 min. Samples that have not been properly heat-treated during the assay lysis step may be considered a potential biohazard and should not be inserted into the 3M Molecular Detection Instrument.

Remove the rack of LS tubes from the heating block and allow to cool in the 3M Molecular Detection Chill Block Insert for 10 ± 1 min. Remove the rack lid during incubation on the 3M Molecular Detection Chill Block Insert. The LS solution may freeze when processing less than 48 LS tubes. Freezing of the LS solution will not affect your test. If freezing is observed, allow the LS tubes to thaw for 5 min before mixing.

Remove the rack of LS tubes from the 3M Molecular Detection Chill Block Insert/3M Molecular Detection Chill Block Tray system. Replace the lid on the rack of LS tubes and firmly invert three to five times to mix. Suspension has to flow freely inside the tube. Firmly tap the lysis tubes rack on the laboratory bench three to five times. Place the rack on the laboratory bench. Let it sit undisturbed for at least 5 min to allow the resin to settle. Do not mix or disturb the resin at the bottom of the tube. *See* Figure **2013.09B**.

J. Amplification

One reagent tube is required for each sample and the NC. Reagent tube strips can be cut to desired tube number. Select the number of individual reagent tubes or eight-tube strips needed. Place reagent tubes in an empty rack. Avoid disturbing the reagent pellets from the bottom of the tubes.

Select one RC tube and place in rack. To avoid cross-contamination, decap one reagent tubes strip at a time and use a new pipet tip for each transfer step. Transfer lysate to reagent tubes and RC tube as follows:

Transfer each sample lysate into individual reagent tubes first followed by the NC. Hydrate the RC tube last.

Warning: Care must be taken when pipetting LS, as carry-over of the resin may interfere with amplification.

(*1*) Use the 3M Molecular Detection Cap/Decap Tool-Reagent to decap the reagent tubes–one strip at a time. Discard cap. (*2*) Transfer 20 µL of sample lysate from the upper portion of the fluid in the LS tube into corresponding reagent tube. Dispense at an angle to avoid disturbing the pellets. Mix by gently pipetting up and down five times. (*3*) Repeat until individual sample lysate has been added to a corresponding reagent tube in the strip. (*4*) Cover the reagent tubes with the provided extra cap and use the rounded side of the 3M Molecular Detection Cap/Decap Tool-Reagent to apply pressure in a back-and-forth motion, ensuring that the cap is tightly applied. Repeat steps (*1*) to (*4*) as needed for the number of samples to be tested. When all sample lysates have been transferred, repeat steps (*1*) to (*4*) to transfer 20 µL of NC lysate into a reagent tube. Transfer 20 µL of NC lysate into a RC tube. Dispense at an angle to avoid disturbing the pellets. Mix by gently pipetting up and down five times. Load capped tubes into a clean and decontaminated 3M Molecular Detection Speed Loader Tray. Close and latch the 3M Molecular Detection Speed Loader Tray lid. *See* Figure **2013.09C**.

Review and confirm the configured run in the 3M Molecular Detection Software. Click the start button in the software and select instrument for use. The selected instrument's lid automatically opens. Place the 3M Molecular Detection Speed Loader Tray into the 3M Molecular Detection Instrument and close the lid to start the assay. Results are provided within 75 min, although positives may be detected sooner.

After the assay is complete, remove the 3M Molecular Detection Speed Loader Tray from the 3M Molecular Detection

Figure 2013.09C. Transfer of lysate to reagent tube.

Instrument and dispose of the tubes by soaking in a 1–5% (v/v in water) household bleach solution for 1 h and away from the assay preparation area.

Notice: To minimize the risk of false positives due to cross-contamination, never open reagent tubes containing amplified DNA. This includes RC, reagent, and matrix control tubes. Always dispose of sealed reagent tubes by soaking in a 1–5% (v/v in water) household bleach solution for 1 h away from the assay preparation area.

K. Results and Interpretation

An algorithm interprets the light output curve resulting from the detection of the nucleic acid amplification. Results are analyzed automatically by the software and are color-coded based on the result. A positive or negative result is determined by analysis of a number of unique curve parameters. Presumptive positive results are reported in real time; negative and inspect results will be displayed after the run is completed. Presumptive positive results should be confirmed using your preferred method or as specified by the FDA/BAM (http://www.fda.gov/Food/ScienceResearch/LaboratoryMethods/BacteriologicalAnalyticalManualBAM/ucm070149.htm) or the USDA/FSIS-MLG (http://www.fsis.usda.gov/PDF/MLG_4_05.pdf; 6, 7), starting from the 3M BPW ISO, followed by secondary enrichment, plating, and confirmation of isolates using appropriate biochemical and serological methods.

Note: Even a negative sample will not give a zero reading as the system and 3M MDA *Salmonella* amplification reagents have a "background" relative light unit.

In the rare event of any unusual light output, the algorithm labels this as "inspect." 3M recommends the user to repeat the assay for any inspect samples. If the result continues to be inspect, proceed to confirmation test using your preferred method or as specified by local regulations.

Results

In this collaborative study, the 3M MDA *Salmonella* method was compared to the to the USDA/FSIS-MLG 4.05 reference method for raw ground beef and to the FDA/BAM, Chapter 5 reference method for wet dog food. A total of 20 laboratories throughout the United States participated in this study, with 14 laboratories submitting data for the raw ground beef and 16 laboratories submitting data for the wet dog food, as presented in Table 1. Each laboratory analyzed 36 test portions for each method: 12 inoculated with a high level of *Salmonella*, 12 inoculated with a low level of *Salmonella*, and 12 uninoculated controls. For each matrix, the actual level of *Salmonella* was determined by MPN determination on the day of initiation of analysis by the coordinating laboratory. The individual laboratory and sample results are presented in Tables 2 and 3. Tables **2013.09A** and **B** summarize the interlaboratory results for all foods tested, including POD statistical analysis (10). The results of the collaborating laboratories' APC analysis for each matrix are presented in Table C of the Appendix.

Table 1. Participation of each collaborating laboratory[a]

Lab	Raw ground beef[b] (25 g test portions)	Wet dog food (375 g test portions)
1	Y	Y
2	Y	Y
3	N	Y
4	N	Y[c]
5	N	Y[c]
6	N	Y
7	N	Y
8	N	Y
9	Y	Y
10	Y	Y[c]
11	Y	Y
12	Y[c]	Y[c]
13	Y	Y
14	Y	Y
15	Y	Y
16	Y[c]	Y[c]
17	Y	N
18	Y[c]	N
19	Y[c]	N
20	Y	N

[a] Y = Collaborator analyzed the food type; N = collaborator did not analyze the food type.

[b] Data obtained from additional shipment of raw ground beef. Initial shipment of raw ground beef was not used for evaluation purposes and therefore the data has not been presented.

[c] Results were not used in statistical analysis due to laboratory error, or uninoculated control test portions were confirmed as *Salmonella*.

Raw Ground Beef (25 g Test Portions)

Raw ground beef test portions were inoculated at a low and high level and were analyzed (Table 2) for the detection of *Salmonella* spp. Uninoculated controls were included in each analysis. The results presented for the raw ground beef were from a second shipment of test portions to the collaborating laboratories. The initial shipment of raw ground beef test portions sent to collaborators was discovered to contain contamination of the target analyte in the uninoculated control samples for each laboratory and therefore no data have been presented. Fourteen laboratories participated in the retest analysis of this matrix and the results of 10 laboratories were included in the statistical analysis. For the retest of the raw ground beef, laboratories 12, 16, 18, and 19 detected the presence of *Salmonella* spp. in either the candidate or reference method control replicates. Because of the potential for error, results from these laboratories were excluded from the statistical analysis. The MPN levels obtained for this test portion, with 95% confidence intervals, were 0.81 CFU/test portion (+0.62, +1.04) for the low level and 4.68 CFU/test portion (+3.22, +6.80) for the high level.

For the high level, 120 out of 120 test portions were reported as presumptive positive by the 3M MDA *Salmonella* method with all test portions confirming positive. For the low level, 67 out of 120 test portions were reported as presumptive positive by the 3M MDA *Salmonella* method with 65 test portions confirming positive. For the uninoculated controls, 1 out of 120 samples produced a presumptive positive result by the

Table 2. Individual collaborator results for raw ground beef (25 g test portions)[a]

	High-level test portions												Low-level test portions												Uninoculated test portions											
Lab	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12
	3M MDA *Salmonella*[b]																																			
1	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	–	–	+	+	+	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–
2	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	–	+	+	+	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–
3	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
4	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
5	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
6	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
7	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
8	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
9	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	–[c]	+	+	+	+	–	–	–[c]	–	–	–	–	–	–	–	–	–	–	–	–
10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
11	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
12[d]	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	–[c]	–[c]	–[c]	+	+	+	–[c]	–	–	–	–[c]	–[c]	–[c]	–[c]	–	–[c]	–	–[c]	–	–[c]
13	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	–	–	+	+	–	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
14	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	+	+	+	+	–	–	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–
15	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+	–	–	–	–	–	–	–	–	–[c]	–	–	–	–	–
16[d]	+	+	+	+	+	+	+	+	+	+	+	+	–[c]	–	+	+	+	–	+	+	+	+	+	+	–	–	–	–	–	–	–[c]	–[c]	–[c]	–[c]	–	–
17	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+	+	+	+	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
18[d]	+	+	+	+	+	+	+	+	+	+	+	+	–[c]	+	+	+	+	+	+	–[c]	+	+	–[c]	+	–[c]	+	–[c]	–[c]	–[c]	–[c]	–[c]	–[c]	–[c]	–[c]	–[c]	+
19[d]	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	+	+	–	+	–[c]	–	–	–	–[c]	–[c]	–[c]	–[c]	–	–[c]	–[c]	–[c]	–[c]	–[c]
20	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	+	–	+	+	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
	USDA/FSIS-MLG[b]																																			
1	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	+	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–
3	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
4	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
5	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
6	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
7	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
8	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
9	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	+	+	–	+	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
10	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+	–	+	–	–	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
11	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	–	+	–	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–
12[d]	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+	+	+	+	+	+	+	–	–	–	–	–	–	–	+	–	–	–
13	+	+	+	+	+	+	–	+	+	+	+	+	+	–	–	+	–	+	+	+	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–
14	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
15	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	+	–	+	+	+	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
16[d]	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	–	–	–	–	–	–	+	–	+	–	–	–	–	–	+	–	–	–	–	–	–
17	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	+	–	+	+	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–
18[d]	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+	+	+	+	+	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–
19[d]	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	–	–	+	–	+	+	–	+	+	–	–	–	–	–	–	–	–	–
20	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

[a] + = *Salmonella* spp. were detected in samples; – =*Salmonella* spp. were not detected in sample; NA = laboratory did not participate in this matrix, or results were not received.

[b] Sample results were obtained from the second shipment of raw ground beef test portions.

[c] Sample was presumptive positive on 3M MDA *Salmonella,* but confirmed negative, indicating a false-positive result.

[d] Results were not used in statistical analysis due to laboratory error.

Table 3. Individual collaborator results for wet dog food (375 g test portions)[a]

	High-level test portions												Low-level test portions												Uninoculated test portions											
Lab	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12
																	3M MDA *Salmonella*																				
1	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–
2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+	+	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–
3	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	+	–	–	+	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–
4[b]	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	–	+	+	–	–	+	–	–	–	–	–	+	–	–	–	–	–	–
5[b]	+	+	+	+	+	+	–	–	+	+	+	+	+	–	+	+	+	–	+	+	+	–	+	–	+	+	+	–	+	–	–	–	–	–	–	–
6	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+	–	–	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–
7	+	+	+	+	+	+	+	+	+	+	–	+	+	–	–	+	–	+	+	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–
8	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	–	–	–	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	–	–	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
10[b]	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–[c]	+	+	–[c]	–	+	+	+	–[c]	+	–	–	–[c]	–[c]	–	–	–	–[c]	–	–
11	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	–	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
12	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
13	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	+	–	+	+	–	–	+	+	–	–	–	–	–	–[c]	–	–	–	–	–	–	–
14	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	–	–	+	–	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
15	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	–	+	+	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
16[b]	+	+	+	+	–	–	+	+	+	+	+	+	+	+	–[c]	+	–	–	–	+	+	–	+	–	–	–[c]	–	+	–[c]	–[c]	+	+	+	–[c]	+	–
17	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
18	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
19	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
20	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
																	FDA/BAM																			
1	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	–	–	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–
2	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	–	+	–	+	–	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–
3	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	–	–	+	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–
4[b]	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	+	–	+	+	+	–	+	–	–	–	–	–	–	–	–	–	+	–	–
5[b]	+	+	+	+	–	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+
6	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	–	+	–	–	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–
7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	–	–	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–
8	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	–	–	+	+	+	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–
9	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	+	–	–	+	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–
10[b]	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	+	–	–	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–
11	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	–	–	–	+	–	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
12	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
13	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	+	–	–	–	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–
14	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+	+	+	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–
15	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	–	+	–	–	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
16[b]	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	–	+	+	+	–	–	+	+	+	–	–	+	–	+	–	–	–	–	–	–	–
17	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
18	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
19	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
20	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

[a] + = *Salmonella* spp. were detected in samples; – =*Salmonella* spp. were not detected in sample; NA = laboratory did not participate in this matrix or results were not received.

[b] Results were not used in statistical analysis due to laboratory error.

[c] Sample was presumptive positive on 3M MDA *Salmonella,* but confirmed negative, indicating a false-positive result.

3M MDA *Salmonella* method with all test portions confirming negative. For test portions analyzed by the USDA/FSIS-MLG Method, 119 out of 120 high inoculum and 68 out of 120 low inoculum test portions confirmed positive. For the uninoculated controls, 0 out of 120 test portions confirmed positive.

For the low-level inoculum, a $dLPOD_C$ value of –0.01 with 95% confidence intervals of (–0.14, +0.13) were obtained between the 3M MDA *Salmonella* method and the USDA/FSIS-MLG method. The confidence intervals obtained for $dLPOD_C$ indicated no significant difference between the two methods. A $dLPOD_{CP}$ value of 0.02 with 95% confidence intervals of (–0.11, +0.15) was obtained between presumptive and confirmed 3M MDA *Salmonella* results. The confidence intervals obtained for $dLPOD_{CP}$ indicated no significant difference between the presumptive and confirmed results using either confirmation process.

For the high-level inoculum, a $dLPOD_C$ value of 0.01 with 95% confidence intervals of (–0.02, +0.05) was obtained between the 3M MDA *Salmonella* method and the USDA/FSIS-MLG method. The confidence intervals obtained for $dLPOD_C$ indicated no significant difference between the two methods. A $dLPOD_{CP}$ value of 0.00 with 95% confidence intervals of (–0.03, +0.03) was obtained between presumptive and confirmed 3M MDA *Salmonella* results. The confidence intervals obtained for $dLPOD_{CP}$ indicated no significant difference between the presumptive and confirmed results. Detailed results of the POD statistical analysis are presented in Table **2013.09A** and Figures 1A and B of the Appendix.

Wet Dog Food (375 g Test Portions)

Wet dog food test portions were inoculated at a low and high level and were analyzed (Table 3) for the detection of *Salmonella* spp. Uninoculated controls were included in each analysis. Sixteen laboratories participated in the analysis of this matrix and the results of 11 laboratories were included in the statistical analysis. Laboratories 4, 5, 10, and 16 detected the presence of *Salmonella* spp. in either the candidate or reference method control replicates. Because of the potential for error, results from these laboratories were excluded from the statistical analysis. Laboratory 12 did not submit results due to cross-contamination of sample enrichments as reported by the analyst. The MPN levels obtained for this test portion, with 95% confidence intervals, were 0.72 CFU/test portion (+0.57, +0.90) for the low level and 5.34 CFU/test portion (+3.46, +8.24) for the high level.

For the high level, 131 out of 132 test portions were reported as presumptive positive by the 3M MDA *Salmonella* method with all test portions confirming positive. For the low level, 65 out of 132 test portions were reported as presumptive positive by the 3M MDA *Salmonella* method with all test portions confirming positive. For the uninoculated controls, 1 out of 132 samples produced a presumptive positive result by the 3M MDA *Salmonella* method with all test portions confirming negative. For test portions analyzed by the FDA/BAM method, 132 out of 132 high inoculum and 70 out of 132 low inoculum test portions confirmed positive. For the uninoculated controls, 0 out of 132 test portions confirmed positive.

For the low-level inoculum, a $dLPOD_C$ value of –0.04 with 95% confidence intervals of (–0.16, +0.09) was obtained between the 3M MDA *Salmonella* method and the FDA/BAM method. The confidence intervals obtained for $dLPOD_C$ indicated no significant difference between the two methods. A $dLPOD_{CP}$ value of 0.00 with 95% confidence intervals of (–0.13, +0.13) was obtained between presumptive and confirmed 3M MDA *Salmonella* results. The confidence intervals obtained for $dLPOD_{CP}$ indicated no significant difference between the presumptive and confirmed results using either confirmation process.

For the high-level inoculum, a $dLPOD_C$ value of –0.01 with 95% confidence intervals of (–0.04, +0.02) was obtained between the 3M MDA *Salmonella* method and the FDA/BAM method. The confidence intervals obtained for $dLPOD_C$ indicated no significant difference between the two methods. A $dLPOD_{CP}$ value of 0.00 with 95% confidence intervals of (–0.03, +0.03) was obtained between presumptive and confirmed 3M MDA *Salmonella* results. The confidence intervals obtained for $dLPOD_{CP}$ indicated no significant difference between the presumptive and confirmed results. Detailed results of the POD statistical analysis are presented in Table **2013.09B** and Figures 2A and B of the Appendix.

Discussion

For this collaborative study, samples were analyzed at both 25 and 375 g test portions as required by the current AOAC Guidelines (5), which require methods with more than one sample preparation or enrichment scheme to analyze one matrix per procedure. No negative feedback was provided by the collaborating laboratories in regard to the performance of the candidate method. Several collaborating laboratories expressed questions in regard to the AOAC study design of the collaborative study; others expressed concern with analyzing 375 g test portions. The concern with handling the larger test portions may have contributed to errors observed during testing that resulted in data not used in the statistical analysis.

During testing, four different laboratories detected the presence of *Salmonella* spp. in seven raw ground beef uninoculated control test portions. Additionally, four different laboratories detected the presence of *Salmonella* spp. in 15 wet pet food uninoculated control test portions. Due to detecting positive samples in the control test portions, the data provided by these laboratories were not included during the statistical analysis.

A root cause investigation to determine the source of contamination yielded the following possibilities: Due to the high number of samples analyzed, including test portions inoculated at a high inoculum level, contamination may have occurred during the transfer of enriched samples into the secondary selective enrichments or during the streaking of the reference agar plates. For the wet pet food, based on feedback from the collaborators, issues with storage during the incubation of the larger test portion sizes may have led to cross-contamination of the primary enrichments. Based on the fact that uninoculated control test portions were packaged 1 day prior to the inoculated test portions, contamination during test portion preparation at the coordinating laboratory is not believed to be the cause of the positive control samples.

During the analysis of both the raw ground beef and wet pet food, some laboratories produced false-positive results with the candidate method. The 3M Molecular Detection Assay is intended for use in a laboratory environment by professionals

trained in laboratory technique. Cross-contamination of samples resulting in false-positive results may occur if careful molecular techniques are not followed. To reduce the risk of cross-contamination, 3M recommends the use of sterile, aerosol barrier (filtered) molecular biology grade pipet tips. A new pipet tip should be used for each sample transfer, and the user may choose to add an intermediate transfer step in order to avoid pipet contamination, i.e., each enriched sample can be transferred into a sterile tube before proceeding to the lysis step. Discrepant results may be obtained if deviations from the method occur. Use of calibrated pipettors and thermometers is critical to ensure that correct volumes of samples, especially when hydrating the reagent tubes, and appropriate temperatures are utilized. It is recommended that users read and become familiar with the 3M MDA *Salmonella* product instructions and follow them carefully.

For either matrix, the collaborative study failed to show a statistically significant difference between the candidate method and the reference method using the POD model when the aforementioned four laboratories were removed from consideration.

Recommendations

It is recommended that the 3M MDA *Salmonella* method be adopted Official First Action for the detection of *Salmonella* in selected foods, including raw ground beef (25 g), processed breaded chicken (325 g), liquid egg (100 g), shrimp (25 g), fresh spinach (25 g), and wet dog food (375 g).

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Kyle Newman, Venture Laboratories, Inc., Lexington, KY

Mary Bandu and Matt Oltman, Chestnut Laboratory, Springfield, MO

Robert Brooks, ATC Microbiology, LLC, North Little Rock, AR

Christine Gwinn and Scott Moosekian, Covance Laboratories, Inc., Battle Creek, MI

Joey Marchent, Gulf Coast Seafood Laboratory, FDA, Dauphin Island, AL

Kathleen T. Rajkowski, USDA Agricultural Research Services, Eastern Regional Research Center, Food Safety and Technologies Initiative, Glenside, PA

Shaunti Luce, The National Food Lab, Livermore, CA

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FOOD BIOLOGICAL CONTAMINANTS

Evaluation of VIDAS® UP *Listeria* Assay (LPT) for the Detection of *Listeria* in a Variety of Foods and Environmental Surfaces: First Action 2013.10

ERIN CROWLEY, PATRICK BIRD, JONATHAN FLANNERY, M. JOSEPH BENZINGER, JR, KIEL FISHER, MEGAN BOYLE, TRAVIS HUFFMAN, BEN BASTIN, PAIGE BEDINGHAUS, WILLIAM JUDD, THAO HOANG, JAMES AGIN, and DAVID GOINS
Q Laboratories, Inc., 1400 Harrison Ave, Cincinnati, OH 45214
RONALD L. JOHNSON[1]
bioMérieux, Inc., 595 Anglum Rd, Hazelwood, MO 63042

Collaborators: A. Bollenbacher; B. Brahmanda; R. Burkhart; J. Cannon; A. Capps; L. Cesanas; D. Davis; D. Ebbing; H. Elgaali; B. Hand; R. Hiles; J. Hirsch; B. Howard; J. Jolly; S. Joseph; A. Kehres; K. Klemms; J. Li; B. May; M. Michels; J. Mills; S. Moore; N. Nagassar; S. Owusu; N. Palen; L. Parker; B. Paul; B. Perry; J. Pickett, N. Rogman; G. Rosario; P. Rule; C. Said; M. Sala-Rhatigan; A. Stegmann; T. Stubblefield; K. Wiggins; J. Zimmerman

The VIDAS® UP *Listeria* (LPT) is an automated rapid screening enzyme phage-ligand based assay for the detection of *Listeria* species in human food products and environmental samples. The VIDAS LPT method was compared in a multi-laboratory collaborative study to AOAC Official Method 993.12 *Listeria monocytogenes* in Milk and Dairy Products reference method following current AOAC guidelines. A total of 14 laboratories participated, representing government and industry, throughout the United States. One matrix, queso fresco (soft Mexican cheese), was analyzed using two different test portion sizes, 25 and 125 g. Samples representing each test portion size were artificially contaminated with *Listeria* species at three levels, an uninoculated control level [0 colony-forming units (CFU)/test portion], a low-inoculum level (0.2–2 CFU/test portion), and a high-inoculum level (2–5 CFU/test portion). For this evaluation, 1800 unpaired replicate test portions were analyzed by either the VIDAS LPT or AOAC 993.12. Each inoculation level was analyzed using the Probability of Detection (POD) statistical model. For the low-level inoculated test portions, difference in collaborator POD (dLPOD) values of 0.01, (–0.10, 0.13), with 95% confidence intervals, were obtained for both 25 and 125 g test portions. The range of the confidence intervals for dLPOD values for both the 25 and 125 g test portions contains the point 0.0 indicating no statistically significant difference in the number of positive samples detected between the VIDAS LPT and the AOAC methods. In addition to Oxford agar, VIDAS LPT test portions were confirmed using Agar Listeria Ottavani and Agosti (ALOA), a proprietary chromogenic agar for the identification and differentiation of *L. monocytogenes* and *Listeria* species. No differences were observed between the two selective agars. The VIDAS LPT method, with the optional ALOA agar confirmation method, was adopted as Official First Action status for the detection of *Listeria* species in a variety of foods and environmental samples.

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The method was approved by the Expert Review Panel for Microbiology Methods for Feed and Environmental Surfaces as First Action.
The Expert Review Panel for Microbiology Methods for Feed and Environmental Surfaces invites method users to provide feedback on the First Action methods. Feedback from method users will help verify that the methods are fit for purpose and are critical to gaining global recognition and acceptance of the methods. Comments can be sent directly to the corresponding author or methodfeedback@aoac.org.
[1] Corresponding author's e-mail: ron.johnson@biomerieux.com
Supplemental data is available on the *J. AOAC Int.* website, http://aoac.publisher.ingentaconnect.com/content/aoac/jaoac and follow link to supplemental data.
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The current classification of the genus *Listeria* includes six well-characterized species, with *L. monocytogenes* being the species of most concern in foodborne outbreaks (1). *Listeria* species are short, non-spore forming Gram-positive rods that are ubiquitous in the environment and can be found in soil, decaying vegetation, and most environments (2). While the number of people who become ill from listeriosis, the disease caused by *Listeria,* is relatively small, the high mortality rate from infection makes it one of the leading causes of death from foodborne illness (2). Of primary concern for illness from *Listeria* outbreaks are the elderly, pregnant women, infants, and people with compromised immune systems (3). Outbreaks from *Listeria* have been linked to such foods as ready-to-eat deli meats, hot dogs, pâtés, dairy products, soft cheeses, smoked seafood, raw sprouts, and most recently cantaloupes (4). The VIDAS UP *Listeria* (LPT) assay, an automated enzyme phage-ligand based assay for the screening of *Listeria* in food and environmental samples, provides the ability to detect *Listeria* after only 26 h of enrichment.

The VIDAS LPT assay uses a primary enrichment (prewarmed to 18–25°C) to detect *Listeria* species in 25 g test portions after 26–30 h of enrichment. For cantaloupe melons, whole melons are soaked in approximately 1 L LPT broth and incubated following conditions outlined for 25 g test portions. For larger

samples sizes, such as 125 g, following 24–30 h primary enrichment incubation, a transfer to a secondary enrichment in 10 mL LPT broth and an additional 22–26 h of incubation is required prior to detection. For smaller test portion sizes and cantaloupe melons, the new enrichment method eliminates the need for secondary enrichments and produces negative and presumptive positive results the following day.

Prior to the collaborative study, the VIDAS LPT method was validated by expert laboratories according to *AOAC INTERNATIONAL Methods Committee Guidelines for Validation of Microbiological Methods for Food and Environmental Surfaces*, Appendix J (5) in a precollaborative study. The objective of this study was to demonstrate that the VIDAS LPT method could detect *Listeria* spp. in a variety of foods and environmental surfaces as claimed by the manufacturer. For the VIDAS LPT evaluation, 19 matrixes were tested: deli ham (25 and 125 g), pepperoni (25 g), beef hot dogs (25 g), chicken nuggets (25 g), chicken liver pâté (25 g), ground beef (125 g), deli turkey (125 g), cooked shrimp (25 g), smoked salmon (25 g), whole cantaloupe melon, bagged mixed salad (25 g), regular peanut butter (25 g), black pepper (25 g), vanilla ice cream (25 g), queso fresco (25 and 125 g), and stainless steel, plastic, ceramic, and concrete environmental surfaces.

During the precollaborative method comparison evaluation, 525 unpaired samples were analyzed by the VIDAS LPT method. One false-positive result and 0 false-negative results were observed. Using the POD statistical model, no significant difference was observed between the reference method and the VIDAS LPT method for all matrixes analyzed except bagged mixed salad, beef hot dogs, and stainless steel environmental samples. For these three matrixes, the VIDAS LPT detected significantly more positive samples than the reference method, which resulted in the statistically significant difference. The inclusivity and exclusivity evaluation showed no unexpected results. The VIDAS LPT method detected all of the *Listeria* strains analyzed and none of the non-*Listeria* strains analyzed. The precollaborative data and report were reviewed by an expert review panel (ERP) prior to approval of the AOAC collaborative protocol. The precollaborative data are presented as supplemental data on the *J. AOAC Int.* website, http://aoac.publisher.ingentaconnect.com/content/aoac/jaoac.

This collaborative study compared the VIDAS LPT method to the AOAC **993.12** *Listeria monocytogenes* in Milk and Dairy Products (6) method for queso fresco at two test portion sizes, 25 and 125 g.

Collaborative Study

Study Design

For this collaborative study, one matrix, queso fresco, was analyzed using two test portion sizes: 25 and 125 g. The queso fresco was obtained from local retailers and screened for the absence of *Listeria* by AOAC **993.12** prior to analysis. The 25 and 125 g test portions of queso fresco were each inoculated with a different strain of *Listeria* at two inoculation levels: a high-inoculation level of approximately 2–5 colony-forming units (CFU)/test portion and a low-inoculation level of approximately 0.2–2 CFU/test portion. A set of uninoculated control test portions were also included for each matrix at 0 CFU/test portion. The 25 g test portions were artificially contaminated with *L. innocua* ATCC 33090 and the 125 g test portions with *L. monocytogenes* ATCC 19115.

Twelve replicate portions from each of the three inoculation levels of product were analyzed. Two sets of samples (72 total) were sent to each laboratory for analysis by VIDAS LPT and AOAC **993.12** due to different sample enrichments for each method.

A detailed collaborative study packet outlining all necessary information related to the study, including media preparation, method-specific test portion preparation, and documentation of results, was sent to each collaborating laboratory prior to the initiation of the study.

Preparation of Inocula and Test Portions

The *Listeria* cultures used in this evaluation were propagated in 10 mL brain heart infusion (BHI) broth from a frozen stock culture stored at –70°C at Q Laboratories, Inc. The broth was incubated for 18–24 h at 35 ±1°C. The inoculum was heat stressed in a 50 ± 1°C water bath for 10 min to obtain a percent injury of 50–80%, as determined by plating onto selective Oxford agar (OXA) and nonselective trypticase soy agar (TSA). The degree of injury was estimated as

$$(1-\frac{n_{select}}{n_{nonselect}})x100$$

where n_{select} = number of colonies on selective agar and $n_{nonselect}$ = number of colonies on nonselective agar. Appropriate dilutions of the heat-stressed cultures were prepared based on previously established growth curves for both low- and high-inoculation levels, resulting in fractional positive outcomes for at least one level. For both test portion sizes, a bulk lot of the queso fresco was inoculated with a liquid inoculum and mixed thoroughly by hand kneading to ensure an even distribution of microorganisms. The queso fresco was inoculated on the day of shipment so that all test portions would have been held for 96 h by the day testing was initiated. The shipment and hold times of the inoculated test material had been verified through 120 h as a quality control measure prior to study initiation. For the analysis of the 25 g test portions, the bulk lot of test material was divided into 30 g portions for shipment to the collaborators. For the analysis of the 125 g test portions, 25 g of inoculated test product was mixed with 100 g of uninoculated test product for shipment to the collaborators for the analysis by the VIDAS LPT method. Collaborators received 30 g portions for analysis by AOAC **993.12**. Validation criterion is satisfied when inoculated test portions produce fractional recovery of the spiked organism, defined as either the reference or candidate method yielding 25–75% positive results. To determine the level of *Listeria* spp. in the queso fresco, a 5-tube most probable number (MPN) was conducted on the day of initiation of analysis. From both the high- and low-inoculated batches of queso fresco, five 100 g test portions, the reference method test portions from the collaborating laboratories, and five 10 g test portions were analyzed following AOAC **993.12**. The MPN and 95% confidence intervals were calculated from the high, low, and uninoculated levels using the Least Cost Formulations (LCF; Norfolk, VA) MPN Calculator provided by AOAC (7).

Confirmation of the samples was conducted according to AOAC **993.12**.

Test Portion Distribution

All samples were labeled with a randomized, blind-coded 3-digit number affixed to the sample container. Test portions were shipped on a Thursday via overnight delivery according to the Category B Dangerous Goods shipment regulations set forth by the International Air Transportation Association. Upon receipt, samples were held by the collaborating laboratory at refrigeration temperature (3–5°C) until the following Monday when analysis was initiated. All samples were packed with cold packs to target a temperature of <7°C during shipment. In addition to each of the test portions and the total plate count replicate, collaborators also received a test portion for each matrix labeled as 'temperature control'. Participants were instructed to obtain the temperature of this portion upon receipt of the package, document results on the Sample Receipt Confirmation form provided, and fax to the study director.

Test Portion Analysis

Collaborators followed the appropriate preparation and analysis protocol according to the method for each test portion size. For both test portion sizes, each collaborator received 72 test portions of each food product (12 high, 12 low, and 12 controls for each evaluation). For the analysis of the 25 g test portions by VIDAS LPT, a 25 g sample replicate was enriched with 225 mL prewarmed (18–25°C) LPT broth and homogenized for 2 min. Test portions were incubated for 26–30 h at 30 ± 1°C. For the 125 g test portions analyzed by VIDAS LPT, a 125 g sample replicate was enriched with 375 mL prewarmed (18–25°C) LPT broth and homogenized for 2 min. Test portions were incubated for 24–30 h at 30 ± 1°C. For 125 g test portions, a 1.0 mL aliquot of the primary enrichment was transferred into 10 mL LPT broth and incubated for an additional 22–26 h at 30 ± 1°C.

Following enrichment, samples were assayed by VIDAS LPT and confirmed following procedures outlined in the standard reference method by streaking an aliquot of the primary enrichment onto OXA and a proprietary chromogenic agar, ALOA. Presumptive positive samples were streaked for isolation on TSA yeast extract (TSAYE) and biochemically confirmed by morphology verification via Gram stain, hemolysis test, and by AOAC **2012.02** VITEK 2 GP Biochemical Identification method (VITEK 2 GP) or API *Listeria* (1) biochemical test kits. Laboratories utilizing API *Listeria* kits were also required to conduct a catalase test and an oxidase test.

Both test portion sizes analyzed by the VIDAS LPT methods were compared to samples (25 g) analyzed using the AOAC **993.12** reference method in conjunction with VITEK 2 GP or API *Listeria* for the confirmation of *Listeria* in an unpaired study design. Twenty-five gram test portions were enriched in prewarmed (45°C) selective enrichment broth, homogenized for 2 min, and incubated at 30 ± 2°C for 48 h. Samples were streaked onto OXA and presumptive positive samples were streaked for isolation onto TSAYE. Colonies from TSAYE were confirmed by morphology verification via Gram stain, hemolysis test, and by VITEK 2 GP or API *Listeria* kits. Laboratories utilizing API *Listeria* kits were also required to conduct a catalase test and an oxidase test.

Statistical Analysis

Each collaborating laboratory recorded results for the reference method and VIDAS LPT results. The data sheets were submitted to the study director for analysis at the end of each week. The results of each test portion for each sample were compiled by the study director and the qualitative VIDAS LPT results were compared to the reference method for statistical analysis. Data for each test portion size was analyzed using the POD statistical model (5, 8). For each inoculation level, the probability of detection (POD) was calculated as the number of positive outcomes divided by the total number of trials. The POD was calculated for the candidate presumptive results, POD_{CP}, the candidate confirmatory results, POD_{CC}/POD_{C}, the reference method, POD_{R}, the difference in the candidate presumptive and confirmatory results, $dLPOD_{CP}$, and the difference in the candidate confirmed and reference methods, $dLPOD_{C}$. A confidence interval of a dLPOD not containing the point zero would indicate a statistically significant difference between VIDAS LPT and AOAC **993.12** at the 5% probability level (9).

AOAC Official Method 2013.10
Listeria species in a Variety of Foods and Environmental Surfaces
VIDAS® UP *Listeria* (LPT) Method
First Action 2013

[Applicable to detection of *Listeria* in deli ham (25 and 125 g), pepperoni (25 g), beef hot dogs (25 g), chicken nuggets (25 g), chicken liver pâté (25 g), ground beef (125 g), deli turkey (125 g), cooked shrimp (25 g), smoked salmon (25 g), whole cantaloupe melon, bagged mixed salad (25 g), peanut butter (25 g), black pepper (25 g), vanilla ice cream (25 g), queso fresco (25 and 125 g), stainless steel, plastic, ceramic and concrete environmental surfaces.]

See Tables **2013.10A** and **B** for a summary of results of the collaborative study. *See* supplemental data, Tables 2A–D, for detailed results of the collaborative study on *J. AOAC Int.* website, http://aoac.publisher.ingentaconnect.com/content/aoac/jaoac.

Caution: *Listeria monocytogenes* is of particular concern for pregnant women, the aged, and the infirmed. It is recommended that these concerned groups avoid handling this organism. Dispose of all reagents and other contaminated materials by acceptable procedures for potentially biohazardous materials. Some reagents in the kit contain 1 g/L concentrations of sodium azide. Check local regulations prior to disposal. Disposal of these reagents into sinks with copper or lead plumbing should be followed immediately with large quantities of water to prevent potential hazards. This kit contains products of animal origin. Certified knowledge of the origin and/or sanitary state of the animals does not totally guarantee the absence of transmissible pathogenic agents. It is, therefore, recommended that these products be treated as potentially infectious and

Table 2013.10A. Summary of results for the detection of *Listeria* spp. in queso fresco (25 g)

Method[a]	VIDAS LPT w/OXA			VIDAS LPT w/ALOA		
Inoculation level	Uninoculated	Low	High	Uninoculated	Low	High
Candidate presumptive positive/total No. samples analyzed	1/156	80/156	156/156	1/156	80/156	156/156
Candidate presumptive POD (CP)	0.01	0.51	1.00	0.01	0.51	1.00
	(0.01, 0.04)	(0.43, 0.59)	(0.98, 1.00)	(0.01, 0.04)	(0.43, 0.59)	(0.98, 1.00)
s_r[b]	0.08	0.51	0.00	0.08	0.51	0.00
	(0.07, 0.15)	(0.46, 0.52)	(0.00, 0.15)	(0.07, 0.15)	(0.46, 0.52)	(0.00, 0.15)
s_L[c]	0.00	0.00	0.00	0.00	0.00	0.00
	(0.00, 0.03)	(0.00, 0.13)	(0.00, 0.15)	(0.00, 0.03)	(0.00, 0.13)	(0.00, 0.15)
s_R[d]	0.08	0.51	0.00	0.08	0.51	0.00
	(0.07, 0.13)	(0.46, 0.52)	(0.00, 0.21)	(0.07, 0.13)	(0.46, 0.52)	(0.00, 0.21)
P value[e]	0.4395	0.9210	1.0000	0.4395	0.9210	1.0000
Candidate confirmed positive/total No. samples analyzed	0/156	78/156	156/156	0/156	78/156	156/156
Candidate confirmed POD (CC)	0.00	0.50	1.00	0.00	0.50	1.00
	(0.00, 0.02)	(0.42, 0.58)	(0.98, 1.00)	(0.00, 0.02)	(0.42, 0.58)	(0.98, 1.00)
s_r	0.00	0.51	0.00	0.00	0.51	0.00
	(0.00, 0.15)	(0.46, 0.52)	(0.00, 0.15)	(0.00, 0.15)	(0.46, 0.52)	(0.00, 0.15)
s_L	0.00	0.00	0.00	0.00	0.00	0.00
	(0.00, 0.15)	(0.00, 0.14)	(0.00, 0.15)	(0.00, 0.15)	(0.00, 0.14)	(0.00, 0.15)
s_R	0.00	0.51	0.00	0.00	0.51	0.00
	(0.00, 0.21)	(0.46, 0.52)	(0.00, 0.21)	(0.00, 0.21)	(0.46, 0.52)	(0.00, 0.21)
P value	1.0000	0.9161	1.0000	1.0000	0.9161	1.0000
Positive reference samples/total No. samples analyzed	0/156	76/156	156/156	0/156	76/156	156/156
Reference POD	0.00	0.49	1.00	0.00	0.49	1.00
	(0.00, 0.02)	(0.41, 0.57)	(0.98, 1.00)	(0.00, 0.02)	(0.41, 0.57)	(0.98, 1.00)
s_r	0.00	0.52	0.00	0.00	0.52	0.00
	(0.00, 0.15)	(0.46, 0.52)	(0.00, 0.15)	(0.00, 0.15)	(0.46, 0.52)	(0.00, 0.15)
s_L	0.00	0.00	0.00	0.00	0.00	0.00
	(0.00, 0.15)	(0.00, 0.10)	(0.00, 0.15)	(0.00, 0.15)	(0.00, 0.10)	(0.00, 0.15)
s_R	0.00	0.52	0.00	0.00	0.52	0.00
	(0.00, 0.21)	(0.47, 0.52)	(0.00, 0.21)	(0.00, 0.21)	(0.47, 0.52)	(0.00, 0.21)
P value	1.0000	0.9937	1.0000	1.0000	0.9937	1.0000
dLPOD (candidate vs reference)	0.00	0.01	0.00	0.00	0.01	0.00
	(–0.02, 0.02)	(–0.10, 0.13)	(–0.02, 0.02)	(–0.02, 0.02)	(–0.10, 0.13)	(–0.02, 0.02)
dLPOD (candidate presumptive vs candidate confirmed)	0.01	0.01	0.00	0.01	0.01	0.00
	(–0.02, 0.04)	(–0.10, 0.13)	(–0.02, 0.02)	(–0.02, 0.04)	(–0.10, 0.13)	(–0.02, 0.02)

[a] Results include 95% confidence intervals.

[b] Repeatability standard deviation.

[c] Among-laboratory standard deviation.

[d] Reproducibility standard deviation.

[e] *P* value = Homogeneity test of laboratory PODs.

handled observing the usual safety precautions (do not ingest or inhale).

A. Principle

VIDAS® UP *Listeria* (LPT) method is for use on the automated VIDAS instrument for the detection of *Listeria* antigens using the enzyme-linked fluorescent assay (ELFA) method. The assay also incorporates phage proteins allowing an increase in sensitivity and specificity compared to traditional immunoassay. The Solid Phase Receptacle (SPR®) serves as the solid phase as well as the pipetting device. The interior of the SPR is coated with proteins specific for *Listeria* receptors. Reagents for the assay are ready-to-use and predispensed in the sealed reagent

Table 2013.10B. Summary of results for the detection of *Listeria* spp. in queso fresco (125 g)

Method[a]	VIDAS LPT w/OXA			VIDAS LPT w/ALOA		
Inoculation level	Uninoculated	Low	High	Uninoculated	Low	High
Candidate presumptive positive/total No. of samples analyzed	0/144	70/144	144/144	0/144	70/144	144/144
Candidate presumptive POD (CP)	0.00	0.49	1.00	0.00	0.49	1.00
	(0.00, 0.03)	(0.40, 0.57)	(0.97, 1.00)	(0.00, 0.03)	(0.40, 0.57)	(0.97, 1.00)
s_r[b]	0.00	0.51	0.00	0.00	0.51	0.00
	(0.00, 0.16)	(0.46, 0.52)	(0.00, 0.16)	(0.00, 0.16)	(0.46, 0.52)	(0.00, 0.16)
s_L[c]	0.00	0.00	0.00	0.00	0.00	0.00
	(0.00, 0.16)	(0.00, 0.12)	(0.00, 0.16)	(0.00, 0.16)	(0.00, 0.12)	(0.00, 0.16)
s_R[d]	0.00	0.51	0.00	0.00	0.51	0.00
	(0.00, 0.22)	(0.46, 0.52)	(0.00, 0.22)	(0.00, 0.22)	(0.46, 0.52)	(0.00, 0.22)
P value[e]	1.0000	0.9730	1.0000	1.0000	0.9730	1.0000
Candidate confirmed positive/total No. of samples analyzed	0/144	70/144	144/144	0/144	70/144	144/144
Candidate confirmed POD (CC)	0.00	0.49	1.00	0.00	0.49	1.00
	(0.00, 0.03)	(0.40, 0.57)	(0.97, 1.00)	(0.00, 0.03)	(0.40, 0.57)	(0.97, 1.00)
s_r	0.00	0.51	0.00	0.00	0.51	0.00
	(0.00, 0.16)	(0.46, 0.52)	(0.00, 0.16)	(0.00, 0.16)	(0.46, 0.52)	(0.00, 0.16)
s_L	0.00	0.00	0.00	0.00	0.00	0.00
	(0.00, 0.16)	(0.00, 0.12)	(0.00, 0.16)	(0.00, 0.16)	(0.00, 0.12)	(0.00, 0.16)
s_R	0.00	0.51	0.00	0.00	0.51	0.00
	(0.00, 0.22)	(0.46, 0.52)	(0.00, 0.22)	(0.00, 0.22)	(0.46, 0.52)	(0.00, 0.22)
P value	1.0000	0.9730	1.0000	1.0000	0.9730	1.0000
Positive reference samples/total No. of samples analyzed	0/144	69/144	144/144	0/144	69/144	144/144
Reference POD	0.00	0.48	1.00	0.00	0.48	1.00
	(0.00, 0.03)	(0.39, 0.56)	(0.97, 1.00)	(0.00, 0.03)	(0.39, 0.56)	(0.97, 1.00)
s_r	0.00	0.51	0.00	0.00	0.51	0.00
	(0.00, 0.16)	(0.46, 0.52)	(0.00, 0.16)	(0.00, 0.16)	(0.46, 0.52)	(0.00, 0.16)
s_L	0.00	0.00	0.00	0.00	0.00	0.00
	(0.00, 0.16)	(0.00, 0.12)	(0.00, 0.16)	(0.00, 0.16)	(0.00, 0.12)	(0.00, 0.16)
s_R	0.00	0.51	0.00	0.00	0.51	0.00
	(0.00, 0.22)	(0.46, 0.52)	(0.00, 0.22)	(0.00, 0.22)	(0.46, 0.52)	(0.00, 0.22)
P value	1.0000	0.9672	1.0000	1.0000	0.9672	1.0000
dLPOD (C vs R)	0.00	0.01	0.00	0.00	0.01	0.00
	(–0.03, 0.03)	(–0.10, 0.13)	(–0.03, 0.03)	(–0.03, 0.03)	(–0.10, 0.13)	(–0.03, 0.03)
dLPOD (CP vs CC)	0.00	0.00	0.00	0.00	0.00	0.00
	(–0.03, 0.03)	(–0.12, 0.12)	(–0.03, 0.03)	(–0.03, 0.03)	(–0.12, 0.12)	(–0.03, 0.03)

[a] Results include 95% confidence intervals.

[b] Repeatability standard deviation.

[c] Among-laboratory standard deviation.

[d] Reproducibility standard deviation.

[e] *P* value = Homogeneity test of laboratory PODs.

strips. All of the assay steps are performed automatically by the instrument. The reaction medium is cycled in and out of the SPR several times. An aliquot of enrichment broth is dispensed into the reagent strip. The *Listeria* receptors present will bind to the interior of the SPR. Unbound components are eliminated during the washing steps. The proteins conjugated to the alkaline phosphatase are cycled in and out of the SPR and will bind to any *Listeria* receptors, which are themselves bound to the SPR wall. A final wash step removes unbound conjugate. During the final detection step, the substrate (4-methyl-umbelliferyl phosphate) is cycled in and out of the SPR. The conjugate enzyme catalyzes the hydrolysis of the

Table 2013.10C. Reagents included in 10-well reagent strip

Wells	Reagents (LPT)
1	Sample well: 0.5 mL of enrichment broth, standard or control
2	Prewash solution (400 µL): TRIS-NaCl (150 mmol/L) - Tween pH 7.6 + preservative
3–5, 7–9	Wash buffer (600 µL): TRIS-NaCl (150 mmol/L) - Tween pH 7.6 + preservative
6	Conjugate (400 µL): alkaline phosphatase-labeled proteins specific for *Listeria* receptors + preservative
10	Reading cuvette with substrate (300 µL): 4-Methyl-umbelliferyl phosphate (0.6 mmol/L) + diethanolamine[a] (DEA) (0.62 mol/L or 6.6%, pH 9.2) + preservative

[a] Irritant reagent: *See* VIDAS LPT package insert for more information.

substrate into a fluorescent product (4-methyl-umbelliferone), the fluorescence of which is measured at 450 nm. At the end of the assay, results are automatically analyzed by the instrument, which calculates a test value for each sample. This value is then compared to internal references (thresholds) and each result is interpreted as positive or negative.

B. Apparatus and Reagents

Items (**a**)–(**h**) are available as the VIDAS UP *Listeria* (LPT) assay kit from bioMérieux (595 Anglum Rd, Hazelwood, MO 63042-2330, USA).

(**a**) *VIDAS or miniVIDAS automated immunoassay system*.

(**b**) *LPT reagent strips.*—Sixty polypropylene strips of 10 wells, each strip covered with a foil seal and label. The 10 wells contain the reagents shown in Table **2013.10C**.

(**c**) *SPR.*—Sixty SPRs coated with proteins specific for *Listeria* receptors.

(**d**) *Standard.*—One vial (1 × 6 mL). Ready-to-use. Contains purified and inactivated *Listeria* receptors + preservative + protein stabilizer.

(**e**) *Positive control solution.*—1 × 6 mL. Contains purified and inactivated *Listeria monocytogenes* antigen + preservative + protein stabilizer.

(**f**) *Negative control solution.*—1 × 6 mL. Contains Tris-buffered saline (TBS; 150 mmol/l) – Tween pH 7.6 + preservative.

(**g**) *Master Lot Entry (MLE) card.*—One card providing specifications for the factory master data required to calibrate the test: To read the MLE data, please refer to the Operator's Manual.

(**h**) *Package insert*.

(**i**) *Disposable pipet.*—To dispense appropriate volumes.

(**j**) *VIDAS Heat and Go.*—Available from bioMérieux, Inc.

(**k**) *Water bath.*—95–100°C, or equivalent.

(**l**) *Bag with filter.*

(**m**) Smasher™ Blender/Homogenizer available from bioMérieux, Inc., or equivalent.

(**n**) *LPT broth.*—bioMérieux, Inc.

(**o**) *Incubators.*—Capable of maintaining 30±1°C and 35±1°C.

(**p**) *Diagnostic reagents.*—Necessary for culture confirmation of assays.

(**q**) *ALOA chromogenic agar.*—Necessary for cultural confirmation as an alternative to selective agar required by appropriate reference method. Available from bioMérieux, Inc.

(**r**) *Tryptic Soy Agar with yeast additive*.

C. General Instructions

(**a**) Components of the kit are intended for use as integral unit. Do not mix reagents or disposables of different lot numbers.

(**b**) Store VIDAS LPT kits at 2–8°C.

(**c**) Do not freeze reagents.

(**d**) Bring reagents to room temperature before inserting them into the VIDAS instrument.

(**e**) Standard, controls, and heated test portions are mixed well before using.

(**f**) Include one positive and one negative control with each group of tests.

(**g**) Return unused components to 2–8°C immediately after use.

(**h**) *See* safety precautions in the VIDAS LPT package insert (Warnings and Precautions and Waste Disposal).

(**i**) *See* Centers for Disease Control recommendations in handling pathogens. http:/www.cdc.gov/biosafety/publications/bmb15/index.htm/

D. Preparation of Test Suspension

(**a**) *Pre-enrichment.*—Pre-enrich test portion using filter Stomacher type bags to initiate growth of *Listeria.* For 25 g test portions, add 225 mL prewarmed (18–25°C) LPT broth to each test portion and homogenize thoroughly for 2 min. For cantaloupe melons, soak entire melon in approximately 1 L prewarmed (18–25°C) LPT broth. For 125 g test portions, add 375 mL prewarmed (18–25°C) LPT broth to each test portion and homogenize thoroughly for 2 min.

(**b**) *Test portions.—(1) 25 g test portions/cantaloupe melons rinses.*—After homogenization, incubate for 26–30 h at 30 ± 1°C.

(2) 125 g test portions.—After homogenization, incubate for 24–30 h at 30 ± 1°C.

From the primary enrichment broth, transfer a 1 mL aliquot into 10 mL prewarmed (18–25°C) LPT broth and incubate for 22–26 h at 30 ± 1°C.

(**c**) After incubation, homogenize samples manually. Follow appropriate instructions based on heating method.

(*1*) *Boiling.*—Transfer 2–3 mL of the enrichment broth into a tube. Seal the tube. Heat in a water bath for 5 ± 1 min at 95–100°C. Cool the tube. Mix the boiled broth and transfer 0.5 mL into the sample well of the VIDAS LPT reagent strip. Perform the VIDAS test.

(2) Heat and Go.—Transfer 0.5 mL of the enrichment broth into the sample well of the VIDAS LPT reagent strip. Heat for 5 ± 1 min (*See* VIDAS Heat and Go User's Manual). Remove the strip and allow to cool for 10 min prior to test initiation. Perform the VIDAS test.

Table 2013.10D. Interpretation of test

Test value threshold	Interpretation
<0.05	Negative
≥0.05	Positive

E. Enzyme Immunoassay

(**a**) Enter factory master calibration curve data into the instrument using the MLE card.

(**b**) Remove the kit reagents and materials from refrigerated storage and let them to come to room temperature for at least 30 min.

(**c**) Use one VIDAS LPT reagent strip and one VIDAS LPT SPR for each sample, control, or standard to be tested. Reseal the storage pouch after removing the required number of SPRs.

(**d**) Enter the appropriate assay information to create a work list. Enter the test code by typing or selecting "LPT," and number of tests to be run. If the standard is to be tested, identify the standard by "S1" and test in duplicate. If the positive control is to be tested, identify it by "C1." If the negative control is to be tested, identify it by "C2."

Note: The standard must be tested upon receipt of a new lot of reagents and then every 14 days. The relative fluorescence value (RFV) of the standard must fall within the set range provided with the kit.

(**e**) Load the LPT reagents strips and SPRs into the positions that correspond to the VIDAS section indicated by the work list. Verify that the color labels with the assay code on the SPRs and reagent strips match.

(**f**) Initiate the assay processing as directed in the VIDAS operator's manual.

(**g**) After the assay is completed, remove the SPRs and reagent strips from the instrument and dispose of properly.

F. Results and Interpretation

The results are analyzed automatically by the VIDAS system. A report is printed which records the type of test performed, the test sample identification, the date and time, the lot number and expiration date of the reagent kit being used, and each sample's RFV, test value, and interpreted result (positive or negative). Fluorescence is measured twice in the reagent strip's reading cuvette for each sample tested. The first reading is a background reading of the substrate cuvette before the SPR is introduced into the substrate. The second reading is taken after incubating the substrate with the enzyme remaining on the interior of the SPR. The test value is calculated by the instrument and is equal to the difference between the background reading and the final reading. The calculation appears on the result sheet. A "negative" result has a test value less than the threshold (0.05) and indicates that the sample does not contain *Listeria* spp. or contains *Listeria* spp. at a concentration below the detection limit. A "positive" result has a test value equal to or greater than the threshold (≥0.05) and indicates that the sample may be contaminated with *Listeria* spp. If the background reading is above a predetermined cutoff, then the result is reported as invalid (Table **2013.10D**).

G. Confirmation

All positive VIDAS LPT results must be culturally confirmed. Confirmation should be performed using the nonheated enrichment broth stored between 2–8°C, and should be initiated within 72 h following the end of incubation (AFNOR Certificate No. BIO 12/33-05/12). Presumptive positive results may be confirmed by isolating on selective agar plates such as ALOA or on the appropriate reference method selective agar plates. Typical or suspect colonies from each plate are confirmed as described in appropriate reference method. As an alternative to the conventional confirmation for *Listeria*, AOAC **2012.02** VITEK 2 GP Biochemical Identification or API *Listeria* biochemical kits may be used for presumptive generic identification of foodborne *Listeria*.

Results of Collaborative Study

In this collaborative study, the VIDAS UP *Listeria* (LPT) method was compared to AOAC **993.12** for one food product, queso fresco, at two test portion sizes: 25 and 125 g. A total of 14 laboratories throughout the United States participated in this study, with 14 laboratories submitting data for the 25 g test portions and 13 laboratories submitting data for the 125 g test portions as presented in Table 1. Each laboratory analyzed 36 test portions for each method—12 inoculated with a high level of *Listeria*, 12 inoculated with a low level of *Listeria*, and 12 uninoculated controls. A background screen of the matrix indicated an absence of indigenous *Listeria* species. As per criteria outlined in Appendix J of the AOAC guidelines, fractional positive results were obtained for both the 25 and 125 g test portions sizes. Cultures used to inoculate the matrix were heat stressed, and the results of the inoculum heat stress are presented in Table 2. For each test portion size, the actual level of *Listeria* was determined by MPN determination on the day of initiation of analysis. The individual laboratory and sample results are presented in Tables 3 and 4. Tables **2013.10A** and **2013.10B** summarize the collaborative study results for all foods tested, including POD statistical analysis (8). Detailed results for each

Table 1. Participation of each collaborating laboratory[a]

	Queso fresco	
Lab	25 g test portions	125 g test portions
1	Y	Y
2	Y	Y[b]
3	Y	Y
4	Y	Y
5	Y	Y
6	Y	Y
7	Y	Y
8	Y	Y
9	Y	Y
10	Y	Y
11	Y[c]	Y[c]
12	Y	Y
13	Y	Y
14	Y	Y

[a] Y = Collaborator analyzed the food type.

[b] Results were not submitted to the coordinating laboratory.

[c] Results were not used in statistical analysis due to laboratory error.

Table 2. Heat-stress injury

Matrix	Test organism	CFU/OXA (selective agar)	CFU/TSA (nonselective agar)	Degree injury, %
Queso fresco	*L. innocua*	5.3×10^8	1.3×10^9	59
LPT – 25 g	ATCC[a] 33091			
Queso fresco	*L. monocytogenes*	2.9×10^8	9.0×10^8	68
LPT – 125 g	ATCC 19115			

[a] ATCC = American Type Culture Collection.

laboratory are presented in Tables 2A–D, and Figures 1A–D and 2A–D as supplemental data on *J. AOAC Int.* website.

Queso Fresco (25 g Test Portions)

Queso fresco test portions, inoculated at a low and high levels, were analyzed for the detection of *Listeria* spp. (Table 3). Uninoculated controls were included in each sample set. Fourteen laboratories participated in the analysis of this matrix, and the results of 13 laboratories were included in the statistical analysis. Laboratory 11 reported data for eight reference method test portions (including seven uninoculated control test portions) that produced doubtful profiles of *L. grayi*. Colonies on these plates were also reported as beta-hemolytic, a characteristic not associated with *L. grayi.* The selective agar plates for these test portions were sent to the coordinating laboratory for further examination. Colonies present on the plates did not possess characteristics typical of *Listeria* spp. Colonies were identified as Gram-positive rods containing spores with morphology typical of *Bacillus* species. Based on the preliminary biochemical tests conducted, the test portions should not have been carried through for final biochemical identification on API *Listeria* strips, which resulted in the misidentification of the test portion as *Listeria* spp. The results for this laboratory were excluded from statistical analysis. The MPNs obtained for this matrix, with 95% confidence intervals, were 0.63 CFU/test portion (0.49, 0.79) for the low-inoculum level and 5.48 CFU/test portion (3.60, 8.36) for the high-inoculum level. For VIDAS LPT test portions, no differences were observed between confirmation of samples using the proprietary chromogenic ALOA and the reference method agar.

For the high-inoculum level, 156 out of 156 test portions were reported as positive by the VIDAS LPT method with all test portions confirming positive. For the low-inoculum level, 80 out of 156 test portions were reported as positive by the VIDAS LPT method with 78 test portions confirming positive, indicating two false-positive results. For the uninoculated controls, 1 out of 156 samples produced a presumptive positive result by the VIDAS LPT method with no samples confirming positive. All three false-positive samples were obtained from the same laboratory. For test portions analyzed by AOAC **993.12**, 156 out of 156 high-inoculum test portions and 76 out of 156 low-inoculum test portions confirmed positive. For the uninoculated controls, 0 out of 156 test portions confirmed positive.

For the low-level inoculum, a $dLPOD_C$ value of 0.01 (–0.10, 0.13) was obtained between AOAC **993.12** and VIDAS LPT. The confidence intervals obtained for $dLPOD_C$ indicated no significant difference between the two methods. A $dLPOD_{CP}$ of 0.01 (–0.10, 0.13) was obtained between presumptive and confirmed VIDAS LPT results for both confirmation procedures. The confidence intervals obtained for $dLPOD_{CP}$ indicated no significant difference between the presumptive and confirmed results.

For the high-level inoculum, a $dLPOD_C$ value of 0.00 (–0.02, 0.02) was obtained between AOAC **993.12** and VIDAS LPT. The confidence intervals obtained for $dLPOD_C$ indicated no significant difference between the two methods. A $dLPOD_{CP}$ of 0.00 (–0.02, 0.02) was obtained between presumptive and confirmed VIDAS LPT results. The confidence intervals obtained for $dLPOD_{CP}$ indicated no significant difference between the presumptive and confirmed results. Results of the POD statistical analysis are presented in Table **2013.10A**, Tables 2A–B, and Figures 1A–D, as supplemental data on the *J. AOAC Int.* website.

Queso Fresco (125 g Test Portions)

Queso fresco test portions were inoculated at a low and high level and analyzed for the detection of *Listeria* spp. (Table 4). Uninoculated controls were included in each sample set. Fourteen laboratories participated in the analysis of this matrix, and the results of 12 laboratories were included in the statistical analysis. Laboratory 2 did not report any data for this matrix. Laboratory 11 reported 10 reference method test portions (including five uninoculated control test portions) that produced non-*L. monocytogenes* profiles, with five of the test portions producing doubtful profiles of *L. grayi*. Colonies on these plates contained one or more of the following biochemical reactions not typically associated with *L. monocytogenes*: Gram-negative, non-beta-hemolytic, and catalase negative. Based on the preliminary biochemical tests conducted, the test portions should not have been carried through for final biochemical identification on API *Listeria* strips which resulted in the misidentification of the test portion as *Listeria* spp. The selective agar plates for these test portions were sent to the coordinating laboratory for further examination. The coordinating laboratory confirmed the supplementary results (Gram stain, hemolysis, and catalase reaction) reported by the participating laboratory and were not able to identify any *Listeria* species. The results from this laboratory were excluded from statistical analysis. The MPN levels obtained for this test portion, with 95% confidence intervals, were 0.59 CFU/test portion (0.46, 0.74) for the low level and 5.41 CFU/test portion (3.53, 8.30) for the high level. For VIDAS LPT test portions, no differences were observed between confirmation of samples using the proprietary chromogenic ALOA and the reference method agar.

For the high level, 144 out of 144 test portions were reported as positive by the VIDAS LPT method with all test portions confirming positive. For the low level, 70 out of 144 test portions were reported as positive by the VIDAS LPT method with all 70 test portions confirming positive. For the uninoculated controls, 0 out of 144 samples produced a presumptive positive result by the VIDAS LPT method and no samples confirming positive. For test portions analyzed by AOAC **993.12**, 144 out of 144 high inoculum and 69 out of 144 low inoculum test

Table 3. Individual collaborator results for queso fresco[a] (25 g test portions)

	VIDAS LPT[b]																																	
	High-level test portions												Low-level test portions												Uninoculated test portions											
Lab	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12
1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	+	–	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–
2	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	–	–	–	+	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–
3	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	+	–	+	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
4	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	–	+	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–
5	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	–	+	+	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–
6	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	+	–	+	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–
7	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	+	–	–	–	+	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	–	+	+	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–
9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	+	–	–	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–
10	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+	+	–	–	+	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
11[c]	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+	+	–	–	+	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
12	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+	–	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–
13	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+	+	–	+	+	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–
14	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	+	+	–	–	–	+	+	–[d]	–[d]	–	–	–	–	–	–	–	–	–	–[d]	–	–
	AOAC **993.12**																																			
1	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	+	+	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
3	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	–	–	–	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–
5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
6	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	+	+	+	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–
7	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	+	+	–	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
8	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
9	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	+	+	–	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
10	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	+	–	–	+	–	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–
11[c]	+	+	+	+	+	+	+	+	+	+	+	+	–	+[e]	+	+	+	+	–	–	+	–	+	+	+[e]	+[e]	+[e]	–	–	–	+[e]	+[e]	–	–	+[e]	+[c]
12	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	–	–	–	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–
13	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	+	+	–	–	–	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–
14	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	+	–	–	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–

[a] + = *Listeria* spp. were detected in samples; – = *Listeria* spp. were not detected in sample.

[b] Confirmed results from OXA and ALOA were identical for each test portion.

[c] Results were not used in statistical analysis.

[d] Sample was presumptive positive on VIDAS LPT but confirmed negative indicating a false-positive result.

[e] Result reported as *L. grayi* (doubtful API Profile).

portions confirmed positive. For the uninoculated controls, 0 out of 144 test portions confirmed positive.

For the low-level inoculum, $dLPOD_C$ values of 0.01 (–0.10, 0.13) were obtained between AOAC **993.12** and VIDAS LPT. The confidence intervals obtained for $dLPOD_C$ indicated no significant difference between the two methods. $dLPOD_{CP}$ values of 0.00 (–0.12, 0.12) were obtained between presumptive and confirmed VIDAS LPT results. The confidence intervals obtained for $dLPOD_{CP}$ indicated no significant difference between the presumptive and confirmed results using either confirmation process.

For the high-level inoculum, $dLPOD_C$ values of 0.00 (–0.03, 0.03) were obtained between AOAC **993.12** and VIDAS LPT. The confidence intervals obtained for $dLPOD_C$ indicated no significant difference between the two methods. $dLPOD_{CP}$ values of 0.00 (–0.03, 0.03) were obtained between presumptive and confirmed VIDAS LPT results. The confidence intervals obtained for $dLPOD_{CP}$ indicated no significant difference between the presumptive and confirmed results. Detailed results of the POD statistical analysis are presented in Table **2013.10B**, as supplemental data on the *J. AOAC Int.* website, Tables 2C–D, and Figures *2*A–D.

ALOA Chromogenic Agar

Confirmatory results obtained from the ALOA chromogenic

Table 4. Individual collaborator results for queso fresco[a] (125 g test portions)

	VIDAS LPT[b]																																	
	High-level test portions												Low-level test portions												Uninoculated test portions											
Lab	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12
1	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	–	–	–	–	–	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–
2[c]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
3	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	–	–	–	+	+	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–
4	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	+	–	+	–	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–
5	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	+	–	–	+	–	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–
6	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	+	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
7	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	–	–	+	+	+	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
8	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	–	–	+	–	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
10	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	–	+	+	–	–	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–
11[c]	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–
12	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	–	–	+	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
13	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	+	+	+	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–
14	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+	+	–	–	+	–	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–
	AOAC **993.12**																																			
1	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
2[c]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
3	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	+	–	+	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–
4	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	–	–	+	+	+	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–
5	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	–	+	–	+	+	–	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–
6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
7	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
8	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
9	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	+	–	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
11[c]	+	+	+	+	+	+	+	+	+	+	+	+	+[d]	+	+[e]	+	+	+[f]	+[f]	–	–	+[f]	+	+	–	–	+[f]	+[e]	–	–	–	–	–	+[g]	+[g]	+[f]
12	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+	+	–	–	–	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–
13	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
14	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	–	+	–	–	–	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–

[a] + = *Listeria* spp. were detected in samples; – = *Listeria* spp. were not detected in sample; NA laboratory did not participate in this matrix or results were not received.

[b] Confirmed results from OXA and ALOA were identical for each test portion.

[c] Results not used in statistical analysis.

[d] Result reported as *L. welshimeri.*

[e] Result reported as *L. innocua.*

[f] Result reported as *L. grayi.*

[g] Result reported as *L. ivanovii.*

agar were identical to the results obtained from the OXA agar specified by AOAC **993.12**. Out of a total of 451 positives detected by the VIDAS LPT, 448 were confirmed positive using OXA or ALOA selective agars.

Discussion

No negative feedback was reported to the study directors from the collaborating laboratories in regards to the performance of the VIDAS LPT assay or the ALOA chromogenic agar. Many laboratories indicated difficulty in identifying and isolating colonies from samples when using OXA plates, but not from test portions analyzed by the VIDAS LPT method. These results may be due to the higher selectivity of the ALOA agar to isolate and differentiate typical *Listeria* colonies from competing microflora, such as *Bacillus* colonies. The high selectivity of the proprietary LPT broth, the high background flora, and the low selectivity of the OXA agar most likely contributed to this observation, as well.

For the analysis of 25 g test portions by the VIDAS LPT method, three false positives were obtained. The test results produced by three false-positive test portions (average test value of 0.34) were much lower than the test values observed with true positives (average value >2.00). By the time the coordinating laboratory received the results, the primary enrichments for these samples had been discarded so no subsequent analysis on the VIDAS LPT was possible. However, the agar plates for these test portions were shipped to the coordinating laboratory for further analysis. Up to 20 different colonies were picked for morphological and biochemical analysis using VITEK 2 GP and no *Listeria* colonies were identified. Additionally, the entire lawn of growth from each agar plate was swabbed and enriched in separate LPT broth tubes and incubated for 26–30 h at 30 ± 1°C. An aliquot from each tube was analyzed by the VIDAS LPT assay and negative results for *Listeria* spp. were obtained. Results of this investigation lead the study directors to believe that the false positives were the result of contamination during the analysis of the samples.

For the analysis of both the 25 and 125 g test portions, Laboratory 11 detected the presence of multiple species of *Listeria*. An investigation into the results indicated that colonies picked for confirmation did not meet the characteristics of *Listeria* spp. (i.e., colonies produced Gram-negative stain reactions, non-motile, negative catalase, or produced hemolysis reactions not typically observed with *Listeria* spp.). The results of these tests should have precluded analysis using the API strips, which lead to an inaccurate identification. Due to the fact that final results reported were inconsistent with biochemical results, data produced by Laboratory 11 were removed from the statistical analysis of both the 25 and 125 g test portions.

Typical growth of *Listeria* spp. colonies from ALOA was easy to identify and the ALOA plates produced less background ground from the matrix than the OXA plates for both test portions sizes analyzed. Positive comments were received from collaborators about the ease of use associated with the ALOA plates.

Using the POD statistical model, no significant difference in the number of positive results obtained between the two methods being compared was observed at both the low- and high-inoculum levels for both the 25 and 125 g test portions. No significant difference was observed between presumptive and confirmed results for the candidate method.

Conclusions

The VIDAS UP *Listeria* (LPT) method with the optional ALOA agar confirmation method was adopted as Official First Action status for the detection of *Listeria* in a variety of foods and environmental surfaces including deli ham (25 and 125 g), pepperoni (25 g), beef hot dogs (25 g), chicken nuggets (25 g), chicken liver pâté (25 g), ground beef (125 g), deli turkey (125 g), cooked shrimp (25 g), smoked salmon (25 g), whole cantaloupe melon, bagged mixed salad (25 g), peanut butter (25 g), black pepper (25 g), vanilla ice cream (25 g), queso fresco (25 and 125 g), stainless steel, plastic, ceramic, and concrete environmental surfaces.

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FOOD BIOLOGICAL CONTAMINANTS

Evaluation of VIDAS® *Listeria monocytogenes* Xpress (LMX) for the Detection of *Listeria monocytogenes* in a Variety of Foods: First Action 2013.11

ERIN CROWLEY, PATRICK BIRD, JONATHAN FLANNERY, M. JOSEPH BENZINGER, JR, KIEL FISHER, MEGAN BOYLE, TRAVIS HUFFMAN, BEN BASTIN, PAIGE BEDINGHAUS, WILLIAM JUDD, THAO HOANG, JAMES AGIN, and DAVID GOINS
Q Laboratories, Inc., 1400 Harrison Ave, Cincinnati, OH 45214
RONALD L. JOHNSON[1]
bioMérieux, Inc., 595 Anglum Rd, Hazelwood, MO 63042

Collaborators: J. Adams; A. Bollenbacher; B. Brahmanda; R. Burkhart; J. Cannon; A. Capps; L. Cesanas-Tyson; D. Davis; D. Ebbing; H. Elgaali; B. Hand; R. Hiles; J. Hirsch; B. Howard; J. Jolly; S. Joseph; A. Kehres; K. Klemms; J. Li; B. May; M. Michels; J. Mills; S. Moore; N. Nagassar; S. Owusu; N. Palen; L. Parker; B. Paul; B. Perry; J. Pickett; N. Rogman; G. Rosario; P. Rule; C. Said; M. Sala-Rhatigan; A. Stegmann; T. Stubblefield; K. Wiggins; J. Zimmerman

The VIDAS® *Listeria monocytogenes* Xpress (LMX) is an automated rapid screening enzyme immunoassay for the detection of *Listeria monocytogenes* in food products. The VIDAS LMX method was compared in a multi-laboratory collaborative study to AOAC Official Method 993.12 *Listeria monocytogenes* in Milk and Dairy Products reference method following current AOAC guidelines. A total of 14 laboratories participated, representing government and industry, throughout the United States. One matrix, queso fresco (soft Mexican cheese), was analyzed using two different test portion sizes, 25 and 125 g. Samples representing each portion size were artificially contaminated with *L. monocytogenes* at three levels: an uninoculated control level [0 colony forming units (CFU)/test portion], a low inoculum level (0.2–2 CFU/test portion), and a high inoculum level (2–5 CFU/test portion). For this evaluation, 1800 unpaired replicate test portions were analyzed by either the VIDAS LMX or AOAC 993.12. Each level was analyzed using the Probability of Detection (POD) statistical model. For the low-level inoculated test portions, difference in collaborator POD (dLPOD) values of 0.04, (–0.08, 0.15) and 0.01, (–0.10, 0.13), with 95% confidence intervals, were obtained, respectively, for 25 and 125 g test portions. The range of the confidence intervals for dLPOD values for both the 25 and 125 g test portions contain the point 0.0 indicating no statistically significant difference in the number of positive samples detected between the VIDAS LMX and the AOAC method. In addition to Oxford Agar (OXA), VIDAS LMX test portions were confirmed using Agar Listeria Ottavani and Agosti (ALOA), a proprietary chromogenic agar for the identification and differentiation of *L. monocytogenes* and *Listeria* species. No differences were observed between the two selective agars. The VIDAS LMX method, with the optional ALOA agar confirmation method, was adopted as Official First Action status for the detection of *L. monocytogenes* in a variety of foods.

Submitted for publication October 31, 2013.
The method was approved by the Expert Review Panel for Microbiology Methods for Feed and Environmental Surfaces as First Action.
The Expert Review Panel for Microbiology Methods for Feed and Environmental Surfaces invites method users to provide feedback on the First Action methods. Feedback from method users will help verify that the methods are fit for purpose and are critical to gaining global recognition and acceptance of the methods. Comments can be sent directly to the corresponding author or methodfeedback@aoac.org.
[1] Corresponding author's e-mail: ron.johnson@biomerieux.com
Supplemental data is available on the *J. AOAC Int.* website, http://aoac.publisher.ingentaconnect.com/content/aoac/jaoac and follow link to supplemental data.
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Listeria monocytogenes is found widespread throughout the environment, having been isolated from soil, vegetation, marine sediments, and water as well as many different types of food products (1). While *L. monocytogenes* has long been known to cause illness in animals, it has only more recently been identified as the cause of listeriosis in humans (1). Listeriosis, while rare, can be of great concern for the elderly, pregnant women, infants, and the immunocompromised, as the disease can lead to septicemia, meningitis, encephalitis, or death (2, 3). Outbreaks from *L. monocytogenes* have been linked to such foods as ready-to-eat deli meats, hot dogs, pâtés, dairy products, soft cheese, smoked seafood, raw sprouts, and most recently cantaloupes (4). The VIDAS *Listeria monocytogenes* Xpress (LMX) assay, an automated enzyme-based assay for the screening of *L. monocytogenes* in food, provides the ability to rapidly detect the target analyte in only 1 to 2 days, depending on sample size.

The VIDAS LMX assay utilizes two proprietary enrichments to detect *L. monocytogenes* in food products, LMX broth with supplement for 25 g test portions and VIDAS UP *Listeria* (LPT) broth for 125 g test portions. The smaller test portions require 26–30 h of incubation, while larger test portions require a 24–30 h primary enrichment incubation followed by a secondary enrichment in 10 mL LPT broth for an additional 22–26 h of incubation. For smaller test portion sizes, the new enrichment

method produces negative and presumptive positive results the next day after enrichment.

Prior to the collaborative study, the VIDAS LMX method was validated according to *AOAC INTERNATIONAL Methods Committee Guidelines for Validation of Microbiological Methods for Food and Environmental Surfaces*, Appendix J (5) in a harmonized AOAC *Performance Tested Method*^{SM} (PTM) study. The objective of this study was to demonstrate that the VIDAS LMX method could detect *L. monocytogenes* in a variety of foods as claimed by the manufacturer. For the VIDAS LMX evaluation, 11 matrixes were originally evaluated: processed cheese (25 g), vanilla ice cream (25 g), cooked shrimp (25 g), smoked white fish (25 g), frozen spinach (25 g), peanut butter (25 g), and five "ready-to-eat" (RTE) 25 g meats (hot dogs, deli turkey, deli ham, fermented sausage, and pâtés). A matrix extension was conducted to evaluate four additional matrixes: deli ham (125 g), deli turkey (125 g), queso fresco (125 g), and ground beef (125 g).

All other PTM evaluation requirements (inclusivity, exclusivity, ruggedness, stability, and lot-to-lot variability) were satisfied. The method was awarded PTM certification No. 091103 on September 14, 2011 (6). The matrix extension was granted approval on January 15, 2013. This collaborative study compared the VIDAS LMX method to AOAC **993.12** *Listeria monocytogenes* in Milk and Dairy Products (7) method for queso fresco at two test portion sizes, 25 and 125 g.

Collaborative Study

Study Design

For this collaborative study, one matrix, queso fresco (soft Mexican cheese), was analyzed using two test portion sizes: 25 and 125 g. The queso fresco was obtained from local retailers and screened for the absence of *Listeria* by AOAC **993.12** prior to analysis. The 25 and 125 g test portions of queso fresco were inoculated with the same strain of *L. monocytogenes,* ATCC 19115, at two inoculation levels: a high inoculation level of approximately 2–5 colony-forming units (CFU)/test portion and a low inoculation level of approximately 0.2–2 CFU/test portion. A set of uninoculated control test portions were also included for each matrix at 0 CFU/test portion. Twelve replicate samples from each of the three inoculation levels of product were analyzed. Two sets of samples (72 total) were sent to each laboratory for analysis by VIDAS LMX and AOAC **993.12** due to different sample enrichments for each method.

A detailed collaborative study packet outlining all necessary information related to the study, including media preparation, method-specific test portion preparation, and documentation of results, was sent to each collaborating laboratory prior to the initiation of the study.

Preparation of Inocula and Test Portions

The *L. monocytogenes* culture used in this evaluation was propagated in 10 mL brain heart infusion (BHI) broth from a frozen stock culture stored at –70°C at Q Laboratories, Inc. (Cincinnati, OH). The broth was incubated for 18–24 h at 35 ± 1°C. The inoculum was heat stressed in a 50°C water bath for 10 min to obtain a percent injury of 50–80%, as determined by plating onto selective Oxford agar (OXA) and nonselective Tryptic Soy agar (TSA). The degree of injury was estimated as

$$(1 - \frac{n_{select}}{n_{nonselect}})x100$$

where n_{select} = number of colonies on selective agar and $n_{nonselect}$ = number of colonies on nonselective agar. Appropriate dilutions of the heat-stressed cultures were prepared based on previously established growth curves for both low and high inoculation levels, resulting in fractional positive outcomes for at least one level. For both test portion sizes, a bulk lot of the queso fresco was inoculated with a liquid inoculum and mixed thoroughly by hand kneading to ensure an even distribution of microorganisms. The queso fresco was inoculated on the day of shipment so that all test portions would have been held for 96 h by the day testing was initiated. The shipment and hold times of the inoculated test material had been verified through 120 h as a quality control measure prior to study initiation. For the analysis of the 25 g test portions by the VIDAS LMX and the AOAC **993.12** methods, the bulk lot of test material was divided into separate 30 g portions for shipment to the collaborators. For the analysis of the 125 g test portions by the VIDAS LMX method, 25 g of inoculated test product was mixed with 100 g of uninoculated test product for shipment to the collaborators. Validation criteria are satisfied when inoculated test portions produce fractional recovery of the spiked organism, defined as either the reference or candidate method yielding 25–75% positive results. To determine the level of *L. monocytogenes* in the queso fresco, a 5-tube most probable number (MPN) was conducted on the day of initiation of analysis. From both the high and low inoculated batches of queso fresco, five 100 g test portions, the reference method test portions from the collaborating laboratories, and five 10 g test portions were analyzed following AOAC **993.12**. The MPN and 95% confidence intervals were calculated from the high, medium, and low levels using the Least Cost Formulations (Norfolk, VA) MPN Calculator provided by AOAC (8). Confirmation of the samples was conducted according to AOAC **993.12**.

Test Portion Distribution

All samples were labeled with a randomized, blind-coded 3-digit number affixed to the sample container. Test portions were shipped on a Thursday via overnight delivery according to the Category B Dangerous Goods shipment regulations set forth by International Air Transportation Association regulations. Upon receipt, samples were held by the collaborating laboratory at refrigeration temperature (3–5°C) until the following Monday when analysis was initiated. All samples were packed with cold packs to target a temperature of <7°C during shipment. In addition to each of the test portions and the total plate count replicate, collaborators also received a test portion for each matrix labeled as 'temperature control'. Participants were instructed to obtain the temperature of this portion upon receipt of the package, document results on the Sample Receipt Confirmation form provided, and fax to the study director.

Test Portion Analysis

Collaborators followed the appropriate preparation and analysis protocol according to the method for each test portion size. For both test portion sizes, each collaborator received 72 test portions of each food product (12 high, 12 low, and 12 controls per method). For the analysis of the 25 g test portions by the

VIDAS LMX method, each sample was enriched with 225 mL prewarmed (18–25°C) LMX broth containing LMX supplement (500 µL supplement/225 mL LMX broth) and homogenized for 2 min. Test portions were incubated for 26–30 h at 37 ± 1°C. For the 125 g test portions analyzed by the VIDAS LMX method, each sample was enriched with 375 mL prewarmed (18–25°C) LPT broth and homogenized for 2 min. Test portions were incubated for 24–30 h at 30 ± 1°C. For 125 g test portions only, a 1.0 mL aliquot of the primary enrichment was transferred into 10 mL LPT broth and incubated for 22–26 h at 30 ± 1°C.

Following enrichment, samples were assayed by VIDAS LMX and confirmed following procedures outlined in the reference method by streaking an aliquot of the primary enrichment onto OXA and a proprietary chromogenic agar, ALOA. Presumptive positive samples were streaked for isolation on TSA yeast extract (TSAYE) and biochemically confirmed by morphology verification via Gram stain, hemolysis test, and by VITEK 2 GP Biochemical Identification method (AOAC **2012.02**) or API *Listeria* biochemical test kits (9). Laboratories utilizing API *Listeria* kits were also required to conduct a catalase test and an oxidase test.

Both test portion sizes analyzed by VIDAS LMX were compared to 25 g portions analyzed using AOAC **993.12** in conjunction with VITEK 2 GP Biochemical Identification (AOAC **2012.02**) or API *Listeria* for the confirmation of *Listeria* in an unpaired study design. Twenty-five gram test portions were enriched in prewarmed (45°C) selective enrichment broth, homogenized for 2 min and incubated at 30 ± 2°C for 48 h. Samples were streaked onto OXA and presumptive positive samples were streaked for isolation onto TSAYE. Colonies from TSAYE were confirmed by morphology verification via Gram stain, hemolysis test, and by VITEK 2 GP Biochemical Identification method or API *Listeria* biochemical test kits. Laboratories utilizing API *Listeria* kits were also required to conduct a catalase test and an oxidase test.

Statistical Analysis

Each collaborating laboratory recorded results for the reference method and VIDAS LMX results. The data sheets were submitted to the study director at the end of each week of testing for analysis. The results of each test portion for each sample were compiled by the study director, and the qualitative VIDAS LMX results were compared to the reference method for statistical analysis. Data for each test portion size was analyzed using the POD statistical model. For each inoculation level, the probability of detection (POD) was calculated as the number of positive outcomes divided by the total number of trials. The POD was calculated for the candidate presumptive results, POD_{CP}, the candidate confirmatory results, POD_{CC}/POD_{C}, the reference method, POD_{R}, the difference in the candidate presumptive and confirmatory results, $dLPOD_{CP}$, and the difference in the candidate confirmed and reference methods, $dLPOD_{C}$. A confidence interval of a dLPOD not containing the point zero would indicate a statistically significant difference between VIDAS LMX and AOAC **993.12** at the 5% probability level (10, 11).

AOAC Official Method 2013.11
Listeria monocytogenes in a Variety of Foods
VIDAS® *Listeria monocytogenes* Xpress (LMX) Method
First Action 2013

[Applicable to detection of *Listeria monocytogenes* in deli ham (25 and 125 g), fermented sausage (25 g), liver pâté (25 g), processed cheese (25 g), vanilla ice cream (25 g), cooked shrimp (25 g), smoked white fish (25 g), frozen spinach (25 g), peanut butter (25 g), deli turkey (25 and 125 g), queso fresco (125 g), and ground beef (125 g).]

See Tables **2013.11A** and **B** for a summary of results of the collaborative study. *See* supplemental data, Tables 2A–D, for detailed results of the collaborative study on *J. AOAC Int.* website, http://aoac.publisher.ingentaconnect.com/content/aoac/jaoac.

Caution: *Listeria monocytogenes* is of particular concern for pregnant women, the aged, and the infirmed. It is recommended that these concerned groups avoid handling this organism. Dispose of all reagents and other contaminated materials by acceptable procedures for potentially biohazardous materials. Some reagents in the kit contain 1 g/L concentrations of sodium azide. Check local regulations prior to disposal. Disposal of these reagents into sinks with copper or lead plumbing should be followed immediately with large quantities of water to prevent potential hazards. This kit contains products of animal origin. Certified knowledge of the origin and/or sanitary state of the animals does not totally guarantee the absence of transmissible pathogenic agents. It is therefore recommended that these products be treated as potentially infectious and handled observing the usual safety precautions (do not ingest or inhale).

A. Principle

The VIDAS® *Listeria monocytogenes* Xpress (LMX) method is for use on the automated VIDAS instrument for the detection of *L. monocytogenes* antigens using the enzyme-linked fluorescent assay (ELFA) method. The Solid Phase Receptacle (SPR®) serves as the solid phase as well as the pipetting device. The interior of the SPR is coated with proteins specific for *L. monocytogenes* receptors. Reagents for the assay are ready-to-use and predispensed in the sealed reagent strips. All of the assay steps are performed automatically by the instrument. The reaction medium is cycled in and out of the SPR several times. An aliquot of enrichment broth is dispensed into the reagent strip. The *L. monocytogenes* receptors present will bind to the interior of the SPR. Unbound components are eliminated during the washing steps. The proteins conjugated to the alkaline phosphatase are cycled in and out of the SPR and will bind to any *Listeria monocytogenes* receptors which are themselves bound to the SPR wall. A final wash step removes unbound conjugate. During the final detection step, the substrate (4-methyl-umbelliferyl phosphate) is cycled in and out of the SPR. The conjugate enzyme catalyzes the hydrolysis of the substrate into a fluorescent product (4-methyl-umbelliferone), the fluorescence of which is measured at 450 nm. At the end of the assay, results are automatically analyzed by the instrument which calculates a test value for each sample. This value is then compared to internal references (thresholds) and each result is interpreted as positive or negative.

B. Apparatus and Reagents

Items (**a**)–(**h**) are available as the 89 VIDAS *Listeria*

Table 2013.11A. Summary of results for the detection of *Listeria monocytogenes* in queso fresco (25 g)

Method[a]	VIDAS LMX w/OXA			VIDAS LMX w/ALOA		
Inoculation level	Uninoculated	Low	High	Uninoculated	Low	High
Candidate presumptive positive/total No. samples analyzed	0/156	77/156	156/156	0/156	77/156	156/156
Candidate presumptive POD (CP)	0.00	0.49	1.00	0.00	0.49	1.00
	(0.00, 0.02)	(0.41, 0.58)	(0.98, 1.00)	(0.00, 0.02)	(0.41, 0.58)	(0.98, 1.00)
s_r[b]	0.00	0.51	0.00	0.00	0.51	0.00
	(0.00, 0.15)	(0.46, 0.52)	(0.00, 0.15)	(0.00, 0.15)	(0.46, 0.52)	(0.00, 0.15)
s_L[c]	0.00	0.00	0.00	0.00	0.00	0.00
	(0.00, 0.15)	(0.00, 0.12)	(0.00, 0.15)	(0.00, 0.15)	(0.00, 0.12)	(0.00, 0.15)
s_R[d]	0.00	0.51	0.00	0.00	0.51	0.00
	(0.00, 0.21)	(0.47, 0.52)	(0.00, 0.21)	(0.00, 0.21)	(0.47, 0.52)	(0.00, 0.21)
P value[e]	1.0000	0.9772	1.0000	1.0000	0.9772	1.0000
Candidate confirmed positive/total No. samples analyzed	0/156	75/156	156/156	0/156	75/156	156/156
Candidate confirmed POD (CC)	0.00	0.48	1.00	0.00	0.48	1.00
	(0.00, 0.02)	(0.40, 0.56)	(0.98, 1.00)	(0.00, 0.02)	(0.40, 0.56)	(0.98, 1.00)
s_r	0.00	0.51	0.00	0.00	0.51	0.00
	(0.00, 0.15)	(0.46, 0.52)	(0.00, 0.15)	(0.00, 0.15)	(0.46, 0.52)	(0.00, 0.15)
s_L	0.00	0.00	0.00	0.00	0.00	0.00
	(0.00, 0.15)	(0.00, 0.14)	(0.00, 0.15)	(0.00, 0.15)	(0.00, 0.14)	(0.00, 0.15)
s_R	0.00	0.51	0.00	0.00	0.51	0.00
	(0.00, 0.21)	(0.46, 0.52)	(0.00, 0.21)	(0.00, 0.21)	(0.46, 0.52)	(0.00, 0.21)
P value	1.0000	0.8718	1.0000	1.0000	0.8718	1.0000
Positive reference samples/total No. samples analyzed	0/156	69/156	153/156	0/156	69/156	153/156
Reference POD	0.00	0.44	0.98	0.00	0.44	0.98
	(0.00, 0.02)	(0.36, 0.52)	(0.94, 0.99)	(0.00, 0.02)	(0.36, 0.52)	(0.94, 0.99)
s_r	0.00	0.51	0.13	0.00	0.51	0.13
	(0.00, 0.15)	(0.46, 0.52)	(0.12, 0.15)	(0.00, 0.15)	(0.46, 0.52)	(0.12, 0.15)
s_L	0.00	0.00	0.03	0.00	0.00	0.03
	(0.00, 0.15)	(0.00, 0.13)	(0.00, 0.07)	(0.00, 0.15)	(0.00, 0.13)	(0.00, 0.07)
s_R	0.00	0.51	0.14	0.00	0.51	0.14
	(0.00, 0.21)	(0.46, 0.52)	(0.12, 0.16)	(0.00, 0.21)	(0.46, 0.52)	(0.12, 0.16)
P value	1.0000	0.9320	0.0877	1.0000	0.9320	0.0877
dLPOD (candidate vs reference)	0.00	0.04	0.02	0.00	0.04	0.02
	(–0.02, 0.02)	(–0.08, 0.15)	(–0.01, 0.06)	(–0.02, 0.02)	(–0.08, 0.15)	(–0.01, 0.06)
dLPOD (candidate presumptive vs candidate confirmed)	0.00	0.01	0.00	0.00	0.01	0.00
	(–0.02, 0.02)	(–0.10, 0.13)	(–0.02, 0.02)	(–0.02, 0.02)	(–0.10, 0.13)	(–0.02, 0.02)

[a] Results include 95% confidence intervals.

[b] Repeatability standard deviation.

[c] Among-laboratory standard deviation.

[d] Reproducibility standard deviation.

[e] *P* value = Homogeneity test of laboratory PODs.

monocytogenes (LMX) assay kit from bioMérieux (595 Anglum Rd, Hazelwood, MO 63042-2330).

(**a**) *VIDAS or miniVIDAS automated immunoassay System.*

(**b**) *LMX reagent strips.*—Sixty polypropylene strips of 10 wells, each strip covered with a foil seal and label. The 10 wells contain the reagents in Table **2013.11C**.

(**c**) *SPR.*—Sixty SPRs coated with proteins specific for *Listeria* receptors.

(**d**) *Standard.*—One vial (1×6 mL). Ready-to-use. Contains

Table 2013.11B. Summary of results for the detection of *Listeria monocytogenes* in queso fresco (125 g)

Method[a]	VIDAS LMX w/OXA			VIDAS LMX w/ALOA		
Inoculation level	Uninoculated	Low	High	Uninoculated	Low	High
Candidate presumptive positive/total No. samples analyzed	0/144	70/144	144/144	0/144	70/144	144/144
Candidate presumptive POD (CP)	0.00	0.49	1.00	0.00	0.49	1.00
	(0.00, 0.03)	(0.40, 0.57)	(0.97, 1.00)	(0.00, 0.03)	(0.40, 0.57)	(0.97, 1.00)
s_r[b]	0.00	0.51	0.00	0.00	0.51	0.00
	(0.00, 0.16)	(0.46, 0.52)	(0.00, 0.16)	(0.00, 0.16)	(0.46, 0.52)	(0.00, 0.16)
s_L[c]	0.00	0.00	0.00	0.00	0.00	0.00
	(0.00, 0.16)	(0.00, 0.12)	(0.00, 0.16)	(0.00, 0.16)	(0.00, 0.12)	(0.00, 0.16)
s_R[d]	0.00	0.51	0.00	0.00	0.51	0.00
	(0.00, 0.22)	(0.46, 0.52)	(0.00, 0.22)	(0.00, 0.22)	(0.46, 0.52)	(0.00, 0.22)
P value[e]	1.0000	0.9730	1.0000	1.0000	0.9730	1.0000
Candidate confirmed positive/total No. samples analyzed	0/144	70/144	144/144	0/144	70/144	144/144
Candidate confirmed POD (CC)	0.00	0.49	1.00	0.00	0.49	1.00
	(0.00, 0.03)	(0.40, 0.57)	(0.97, 1.00)	(0.00, 0.03)	(0.40, 0.57)	(0.97, 1.00)
s_r	0.00	0.51	0.00	0.00	0.51	0.00
	(0.00, 0.16)	(0.46, 0.52)	(0.00, 0.16)	(0.00, 0.16)	(0.46, 0.52)	(0.00, 0.16)
s_L	0.00	0.00	0.00	0.00	0.00	0.00
	(0.00, 0.16)	(0.00, 0.12)	(0.00, 0.16)	(0.00, 0.16)	(0.00, 0.12)	(0.00, 0.16)
s_R	0.00	0.51	0.00	0.00	0.51	0.00
	(0.00, 0.22)	(0.46, 0.52)	(0.00, 0.22)	(0.00, 0.22)	(0.46, 0.52)	(0.00, 0.22)
P value	1.0000	0.9730	1.0000	1.0000	0.9730	1.0000
Positive reference samples/total No. samples analyzed	0/144	69/144	144/144	0/144	69/144	144/144
Reference POD	0.00	0.48	1.00	0.00	0.48	1.00
	(0.00, 0.03)	(0.39, 0.56)	(0.97, 1.00)	(0.00, 0.03)	(0.39, 0.56)	(0.97, 1.00)
s_r	0.00	0.51	0.00	0.00	0.51	0.00
	(0.00, 0.16)	(0.46, 0.52)	(0.00, 0.16)	(0.00, 0.16)	(0.46, 0.52)	(0.00, 0.16)
s_L	0.00	0.00	0.00	0.00	0.00	0.00
	(0.00, 0.16)	(0.00, 0.12)	(0.00, 0.16)	(0.00, 0.16)	(0.00, 0.12)	(0.00, 0.16)
s_R	0.00	0.51	0.00	0.00	0.51	0.00
	(0.00, 0.22)	(0.46, 0.52)	(0.00, 0.22)	(0.00, 0.22)	(0.46, 0.52)	(0.00, 0.22)
P value	1.0000	0.9672	1.0000	1.0000	0.9672	1.0000
dLPOD (C vs R)	0.00	0.01	0.00	0.00	0.01	0.00
	(–0.03, 0.03)	(–0.10, 0.13)	(–0.03, 0.03)	(–0.03, 0.03)	(–0.10, 0.13)	(–0.03, 0.03)
dLPOD (CP vs CC)	0.00	0.00	0.00	0.00	0.00	0.00
	(–0.03, 0.03)	(–0.12, 0.12)	(–0.03, 0.03)	(–0.03, 0.03)	(–0.12, 0.12)	(–0.03, 0.03)

[a] Results include 95% confidence intervals.

[b] Repeatability standard deviation.

[c] Among-laboratory standard deviation.

[d] Reproducibility standard deviation.

[e] *P* value = Homogeneity test of laboratory PODs.

purified and inactivated *L. monocytogenes* receptors + preservative + protein stabilizer.

(**e**) *Positive control solution.*—1 × 3 mL. Contains purified and inactivated *L. monocytogenes* antigen + preservative + protein stabilizer.

(**f**) *Negative control solution.*—1 × 6 mL. Contains Tris-buffered saline (TBS; 150 mmol/l) – Tween pH 7.6 + preservative.

(**g**) *Master lot entry (MLE) card.*—One card providing specifications for the factory master data required to calibrate the test: To read the MLE data, please refer to the Operator's Manual.

(**h**) *Package insert.*

Table 2013.11C. Reagents included in 10-well reagent strip

Wells	Reagents (LMX)
1	Sample well: 0.25 mL of enrichment broth, standard or control
2	Prewash solution (600 µL): TRIS-NaCl (150 mmol/L) – Triton X100 pH 7.6 + preservative
3, 4, 7–9	Wash buffer (600 µL): TRIS-NaCl (150 mmol/L) - Tween pH 7.6 + preservative
5	Conjugate (400 µL): biotin-labeled anti-*Listeria monocytogenes* antibodies + preservative
6	Streptavidin – ALP (400 µL)
10	Reading cuvette with substrate (300 µL): 4-Methyl-umbelliferyl phosphate (0.6 mmol/L) + diethanolamine[a] (DEA; 0.62 mol/L or 6.6%, pH 9.2) + preservative

[a] Irritant reagent: *See* VIDAS LPT package insert for more information.

(**i**) *Pipet*.—Disposable to dispense appropriate volumes.

(**j**) *VIDAS Heat and Go*.—Available from bioMérieux, Inc.

(**k**) *Water bath*.—95–100°C or equivalent system.

(**l**) *Bag with filter*.

(**m**) Smasher™ Blender/Homogenizer available from bioMérieux, Inc., or equivalent.

(**n**) *LMX broth*.—Available from bioMérieux, Inc.

(**o**) *Supplement for LMX broth*.

(**p**) *LPT broth*.—Available from bioMérieux, Inc.

(**q**) *Incubators*.—Capable of maintaining 37 ± 1°C, 35 ± 1°C, and 30 ±1°C.

(**r**) *Diagnostic reagents*.—Necessary for culture confirmation of assays.

(**s**) *ALOA Chromogenic Agar*.—Necessary for cultural confirmation as an alternative to selective agar required by appropriate reference method. Available from bioMérieux, Inc.

(**t**) *Tryptic Soy Agar with yeast additive*.

C. General Instructions

(**a**) Components of the kit are intended for use as integral unit. Do not mix reagents or disposables of different lot numbers.

(**b**) Store VIDAS LMX kits at 2–8°C.

(**c**) Do not freeze reagents.

(**d**) Bring reagents to room temperature before inserting them into the VIDAS instrument.

(**e**) Standard, controls and heated test portions are mixed well before using.

(**f**) Include one positive and one negative control with each group of tests.

(**g**) Return unused components to 2–8°C immediately after use.

(**h**) *See* Safety Precautions in the VIDAS LMX package insert (refer to the following sections in the package insert: Warnings and Precautions and Waste Disposal).

(**i**) Please review the policies recommended by the Centers for Disease Control and Protection (CDC) on dealing with pathogens. http://www.cdc.gov/biosafety/publications/bmb15/index.htm/.

D. Preparation of Test Suspension

(**a**) *Pre-enrichment*.—Pre-enrich test portion using filter Stomacher bags to initiate growth of *L. monocytogenes*. For 25 g test portions, add 225 mL pre-LMX broth brought to room temperature (18–25°C) and 500 µL LMX broth supplement to each test portion and homogenize thoroughly for 2 min. For 125 g test portions, add 375 mL LPT broth brought to room temperature (18–25°C) to each test portion and homogenize thoroughly for 2 min.

(**b**) *25 g Test portions*.—After homogenization, incubate for 26–30 h at 37 ± 1°C.

125 g Test portions.—After homogenization, incubate for 24–30 h at 30 ± 1°C.

After the primary enrichment, transfer a 1 mL aliquot into 10 mL LPT broth brought to room temperature (18–25°C) and incubate for 22–26 h at 30 ± 1°C.

(**c**) After incubation, homogenize samples manually and prepare samples for assay according the following procedures (based on sample size):

125 g Test portions.—No heating is necessary for method performance. Load 0.25 mL of enrichment into the VIDAS LMX reagent strip and perform the VIDAS test.

25 g Test portions.—Follow appropriate instructions based on heating method.

(*1*) *Boiling*.—Transfer 2–3 mL of the enrichment broth into a tube. Seal the tube. Heat in a water bath for 5 ± 1 min at 95–100°C. Cool the tube. Mix the boiled broth and transfer 0.25 mL into the sample well of the VIDAS LMX reagent strip. Perform the VIDAS test.

(*2*) *Heat and Go*.— Transfer 0.25 mL of the enrichment broth into the sample well of the VIDAS LMX reagent strip. Heat for 5 ± 1 min (*see* VIDAS Heat and Go User's Manual). Remove the strip and allow to cool for 10 min prior to test initiation. Perform the VIDAS test.

E. Enzyme Immunoassay

(**a**) Enter factory master calibration curve data into the instrument using the MLE card.

(**b**) Remove the kit reagents and materials from refrigerated storage and allow them to come to room temperature for at least 30 min.

(**c**) Use one VIDAS LMX reagent strip and one VIDAS LMX SPR for each sample, control or standard to be tested. Reseal the storage pouch after removing the required number of SPRs.

(**d**) Enter the appropriate assay information to create a work list. Enter the test code by typing or selecting "LMX", and number of tests to be run. If the standard is to be tested, identify the standard by "S1" and test in duplicate. If the positive control is to be tested, identify it by "C1". If the negative control is to be tested, identify it by "C2".

Note: The standard must be tested upon receipt of a new lot of reagents and then every 14 days. The relative fluorescence value (RFV) of the standard must fall within the set range provided with the kit.

(**e**) Load the LMX reagents strips and SPRs into the positions that correspond to the VIDAS section indicated by the work list. Verify that the color labels with the assay code on the SPRs and reagent strips match.

(**f**) Initiate the assay processing as directed in the VIDAS operator's manual.

(**g**) After the assay is completed, remove the SPRs and reagent strips from the instrument and dispose of properly.

Table 2013.11D. Interpretation of test

Test value threshold	Interpretation
<0.05	Negative
≥0.05	Positive

F. Results and Interpretation

The results are analyzed automatically by the VIDAS system. A report is printed which records the type of test performed, the test sample identification, the date and time, the lot number, and expiration date of the reagent kit being used, and each sample's RFV, test value, and interpreted result (positive or negative). Fluorescence is measured twice in the reagent strip's reading cuvette for each sample tested. The first reading is a background reading of the substrate cuvette before the SPR is introduced into the substrate. The second reading is taken after incubating the substrate with the enzyme remaining on the interior of the SPR. The test value is calculated by the instrument and is equal to the difference between the background reading and the final reading. The calculation appears on the result sheet. A "negative" result has a test value less than the threshold (0.05) and indicates that the sample does not contain *L. monocytogenes* or contains *L. monocytogenes* at a concentration below the detection limit. A "positive" result has a test value equal to or greater than the threshold (≥0.05) and indicates that the sample may be contaminated with *L. monocytogenes.* If the background reading is above a predetermined cutoff, then the result is reported as invalid (Table **2013.11D**).

G. Confirmation

All positive VIDAS LMX results must be culturally confirmed. Confirmation should be performed using the non-heated enrichment broth (the LMX primary enrichment broth for 25 g test portions and the LPT secondary enrichment broth for 125 g test portions) stored between 2–8°C, and should be initiated within 72 h following the end of incubation (AFNOR Certificate No. BIO 12/33-05/12). Presumptive positive results may be confirmed by isolating on selective agar plates such as ALOA or on the appropriate reference method selective agar plates. Typical or suspect colonies from each plate are confirmed as described in appropriate reference method. As an alternative to the conventional confirmation for *L. monocytogenes,* VITEK 2 GP Biochemical Identification (AOAC **2012.02**) or API *Listeria* biochemical kits may be used for presumptive generic identification of foodborne *L. monocytogenes*.

Results of Collaborative Study

In this collaborative study, the VIDAS *Listeria monocytogenes* (LMX) method was compared to the to AOAC **993.12** for one food product, queso fresco, at two test portion sizes, 25 and 125 g. A total of 14 laboratories throughout the United States participated in this study, with 14 laboratories submitting data for the 25 g test portions and 13 laboratories submitting data for the 125 g test portions as presented in Table 1. Each laboratory analyzed 36 test portions for each method 12 inoculated with a high level of *L. monocytogenes,* 12 inoculated with a low level of *L. monocytogenes,* and 12 uninoculated controls. A background screen of the matrix indicated an absence of indigenous *Listeria* species. As per criteria outlined in Appendix J, fractional positive results were obtained for both the 25 and 125 g test portions sizes. For each test portion size, the actual level of *L. monocytogenes* was determined by MPN determination on the day of initiation of analysis. The results of the inoculum heat-stress protocol are presented in Table 2. The individual laboratory and sample results are presented in Tables 3 and 4. Tables **2013.11A** and **B** summarize the collaborative study results for all foods tested, including POD statistical analysis (10). Detailed results for each laboratory are presented in Tables 2A–D, and Figures 1A–D and 2A–D as supplemental data on the *J. AOAC Int.* website.

Queso Fresco (25 g Test Portions)

Queso fresco test portions were inoculated at a low and high level and were analyzed (Table 3) for the detection of *L. monocytogenes*. Uninoculated controls were included in each analysis. Fourteen laboratories participated in the analysis of this matrix and the results of 13 laboratories were included in the statistical analysis. Laboratory 11 reported 11 test portions (including four uninoculated test portions) that produced non-*L. monocytogenes* profiles. Colonies on these plates contained one or more of the following biochemical reactions not typically associated with *L. monocytogenes*: Gram-negative, Gram-positive with spores, non-beta-hemolytic, and catalase negative. Based on the preliminary biochemical tests conducted, the test portions should not have been carried through for final biochemical identification on API *Listeria* strips which resulted in the misidentification of the test portion as *Listeria* spp. The selective agar plates for these test portions were sent to the coordinating laboratory for further examination. The coordinating laboratory confirmed the supplementary results (Gram stain, hemolysis, and catalase reaction) reported by

Table 1. Participation of each collaborating laboratory[a]

	Queso fresco	
Lab	25 g test portions	125 g test portions
1	Y	Y
2	Y[b]	Y
3	Y	Y
4	Y	Y
5	Y	Y
6	Y	Y
7	Y	Y
8	Y	Y
9	Y	Y
10	Y	Y
11	Y[c]	Y[c]
12	Y	Y
13	Y	Y
14	Y	Y

[a] Y= Collaborator analyzed the food type.

[b] Results were not submitted to the coordinating laboratory.

[c] Results were not used in statistical analysis due to laboratory error.

Table 2. Heat-stress injury results

Matrix (LMX test portion size)	Test organism[a]	CFU/OXA (selective agar)	CFU/TSA (nonselective agar)	Degree injury, %
Queso fresco LMX – 25 g	*L. monocytogenes* ATCC 19115	5.0×10^8	1.3×10^9	62
Queso fresco LMX – 125 g	*L. monocytogenes* ATCC 19115	2.9×10^8	9.0×10^8	68

[a] ATCC = American Type Culture Collection.

the participating laboratory and were not able to identify any *Listeria* species. Laboratory 11 also reported one uninoculated control portion positive for *L. monocytogenes*. Testing at the coordinating laboratory verified this result, which indicated cross contamination with that sample. The results from this laboratory were excluded from statistical analysis. The MPN obtained for this matrix, with 95% confidence intervals, were 0.55 CFU/test portion (0.43, 0.70) for the low inoculum level and 3.81 CFU/test portion (3.06, 5.48) for the high inoculum level. For VIDAS LMX test portions, no difference was observed between confirmation of samples using the proprietary chromogenic ALOA agar and OXA required by the reference method.

For the high inoculum level, 156 out of 156 test portions were reported as positive by the VIDAS LMX method with all test portions confirming positive. For the low inoculum level, 77 out of 156 test portions were reported as positive by the VIDAS LMX method with 75 test portions confirming positive, indicating two false-positive results. For the uninoculated controls, 0 out of 156 samples produced a presumptive positive result by the VIDAS LMX method with no samples confirming positive. For test portions analyzed by AOAC **993.12**, 153 out of 156 high inoculum test portions and 69 out of 156 low inoculum test portions confirmed positive. For the uninoculated controls, 0 out of 156 test portions confirmed positive.

For the low-level inoculum, a $dLPOD_C$ value of 0.04 (–0.08, 0.15) was obtained between AOAC **993.12** and VIDAS LMX. The confidence intervals obtained for $dLPOD_C$ indicated no significant difference between the two methods. A $dLPOD_{CP}$ of 0.01 (–0.10, 0.13) was obtained between presumptive and confirmed VIDAS LMX results for both confirmation procedures. The confidence intervals obtained for $dLPOD_{CP}$ indicated no significant difference between the presumptive and confirmed results.

For the high-level inoculum, a $dLPOD_C$ value of 0.02 (–0.01, 0.06) was obtained between AOAC **993.12** and VIDAS LMX. The confidence intervals obtained for $dLPOD_C$ indicated no significant difference between the two methods. A $dLPOD_{CP}$ of 0.00 (–0.02, 0.02) was obtained between presumptive and confirmed VIDAS LMX results. The confidence intervals obtained for $dLPOD_{CP}$ indicated no significant difference between the presumptive and confirmed results. Results of the POD statistical analysis are presented in Table **2013.11A**, Tables 2A–B, and Figures 1A–D as supplemental data on the *J. AOAC Int.* website.

Queso Fresco (125 g Test Portions)

Queso fresco test portions were inoculated at a low and high level and analyzed (Table 4) for the detection of *L. monocytogenes*. Uninoculated controls were included in each sample set. Fourteen laboratories participated in the analysis of this matrix and the results of 12 laboratories were included in the statistical analysis. Laboratory 2 did not report any data for this matrix. Laboratory 11 reported 10 reference method test portions (including five uninoculated control replicates) that produced non-*L. monocytogenes* profiles, with five of the test portions producing questionable API profiles of *L. grayi*. Colonies on these plates contained one or more of the following biochemical reactions not typically associated with *L. monocytogenes*: Gram-negative, non-beta-hemolytic, and catalase negative. Based on the preliminary biochemical tests conducted, the test portions should not have been carried through for final biochemical identification on API *Listeria* strips which resulted in the misidentification of the test portion as *Listeria* spp. The selective agar plates for these test portions were sent to the coordinating laboratory for further examination. The coordinating laboratory verified the supplementary results (Gram stain, hemolysis, and catalase reaction) reported by the participating laboratory and were not able to identify any *Listeria* species. The results from this laboratory were excluded from statistical analysis. The MPN levels obtained for this test portion, with 95% confidence intervals, were 0.59 CFU/test portion (0.46, 0.74) for the low level and 5.41 CFU/test portion (3.53, 8.30) for the high level. For VIDAS LMX test portions, no differences were observed between confirmation of samples using the proprietary chromogenic ALOA and the reference method agar.

For the high-level, 144 out of 144 test portions were reported as positive by VIDAS LMX with all test portions confirming positive. For the low level, 70 out of 144 test portions were reported as positive by VIDAS LMX with all 70 test portions confirming positive. For the uninoculated controls, 0 out of 144 samples produced a presumptive positive result by VIDAS LMX and no samples confirmed positive. For test portions analyzed by AOAC **993.12**, 144 out of 144 high inoculum and 69 out of 144 low inoculum test portions confirmed positive. For the uninoculated controls, 0 out of 144 test portions confirmed positive.

For the low-level inoculum, $dLPOD_C$ values of 0.01 (–0.10, 0.13) were obtained between AOAC **993.12** and VIDAS LMX. The confidence intervals obtained for $dLPOD_C$ indicated no significant difference between the two methods. $dLPOD_{CP}$ values of 0.00 (–0.12, 0.12) were obtained between presumptive and confirmed VIDAS LMX results. The confidence intervals obtained for $dLPOD_{CP}$ indicated no significant difference between the presumptive and confirmed results using either confirmation process.

For the high-level inoculum, $dLPOD_C$ values of 0.00 (–0.03, 0.03) were obtained between AOAC **993.12** and VIDAS LMX. The confidence intervals obtained for $dLPOD_C$ indicated no significant difference between the two methods. $dLPOD_{CP}$ values of 0.00 (–0.03, 0.03) were obtained between presumptive and confirmed VIDAS LMX results. The confidence intervals obtained for $dLPOD_{CP}$ indicated no significant difference between the presumptive and confirmed results. Detailed results of the POD statistical analysis are presented in Table **2013.11B**,

Table 3. Individual collaborator results for queso fresco[a] (25 g test portions)

	VIDAS LMX[b]																																	
	High-level test portions												Low-level test portions												Uninoculated test portions											
Lab	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12
1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	+	–	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
3	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	+	–	–[c]	–[c]		–	–	–	–	–	–	–	–	–	–	–	–
5	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	–	+	+	+	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
6	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	+	+	–	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–
7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	–	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
8	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	+	+	+	–	–	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–
9	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	–	–	–	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–
10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	–	–	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–
11[d]	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+[e]	+	–	–	–	+[f]	+	–	–	–	–	–	–	–	–	–	–	–	+[e]	–	–	+[e]
12	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	+	+	–	–	+	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–
13	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+	+	+	–	+	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–
14	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	+	–	–	+	–	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–
	AOAC **993.12**																																			
1	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	–	–	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
2	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	–	–	–	–	+	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–
3	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	–	–	+	+	+	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–
4	+	+	+	+	+	+	+	+	+	–	+	+	–	–	+	–	–	–	+	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
5	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+	+	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
6	+	+	+	+	+	+	+	+	+	+	–	–	–	+	–	+	+	–	–	+	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–
7	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	+	+	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–
8	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	+	+	+	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
9	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	+	–	+	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
10	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–
11[d]	+	+	+	+	+	+	+	+	+	+	+	+	+	+[g]	+[g]	+	+[h]	+	+	+[i]	+	+	–	+[h]	–	+[h]	–	–	–	+[h]	–	–	+[j]	–	–	–
12	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	–	+	–	–	–	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–
13	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	–	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–
14	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	+	–	+	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–

[a] + = *Listeria* spp. were detected in samples; – = *Listeria* spp. were not detected in sample.

[b] Confirmed results from OXA and ALOA were identical for each test portion.

[c] Sample was presumptive positive on VIDAS LMX and confirmed negative.

[d] Results were not used in statistical analysis.

[e] Sample presumptive negative on VIDAS LMX, result was reported positive as *L. seeligeri*.

[f] Sample was presumptive positive on VIDAS LMX but result was reported positive as *L. welshimeri*.

[g] Result was reported positive as *L. innocua*.

[h] Result was reported positive as *L. ivanovii*.

[i] Result was reported positive as *L. grayi*.

[j] Sample confirmed positive as *L. monocytogenes*.

Table 4. Individual collaborator results for queso fresco[a] (125 g test portions)

	VIDAS LMX[b]																																	
	High–level test portions												Low-level test portions												Uninoculated test portions											
Lab	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12
1	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	–	–	–	–	–	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–
2[c]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
3	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	–	–	–	+	+	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–
4	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	+	–	+	–	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–
5	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	+	–	–	+	–	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–
6	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	+	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
7	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	–	–	+	+	+	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
8	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	–	–	+	–	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
10	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	–	+	+	–	–	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–
11[c]	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–
12	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	–	–	+	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
13	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	+	+	+	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–
14	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+	+	–	–	+	–	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–
	AOAC **993.12**																																			
1	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
2[c]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
3	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	+	–	+	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–
4	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	–	–	+	+	+	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–
5	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	–	+	–	+	+	–	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–
6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
7	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
8	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
9	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	+	–	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
11[c]	+	+	+	+	+	+	+	+	+	+	+	+	+[d]	+	+[e]	+	+	+[f]	+[f]	–	–	+[f]	+	+	–	–	+[f]	+[e]	–	–	–	–	–	+[g]	+[g]	+[g]
12	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+	+	–	–	–	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–
13	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
14	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	–	+	–	–	–	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–

[a] + = *Listeria* spp. were detected in samples; – = *Listeria* spp. were not detected in sample.

[b] Results were not used in statistical analysis; NA = not applicable (data not submitted).

[c] Confirmed results from OXA and ALOA were identical for each test portion.

[d] Result reported as *L. welshimeri.*

[e] Result reported as *L. innocua.*

[f] Result reported as *L. grayi.*

[g] Results reported as *L. ivanovii.*

Tables 2C–D, and Figures 2A–D, as supplemental data on the *J. AOAC Int.* website.

ALOA Chromogenic Agar

Confirmatory results obtained from the ALOA chromogenic agar were identical to the results obtained from the OXA agar specified by AOAC **993.12**. Out of a total of 447 positives detected by the VIDAS LMX, 445 were confirmed positive using OXA or ALOA selective agars.

Discussion

No negative feedback was reported to the study directors from the collaborating laboratories in regards to the performance of the VIDAS LMX assay or the ALOA chromogenic agar. Many

laboratories indicated difficulty in identifying and isolating colonies from samples when using OXA plates, but not from test portions analyzed by the VIDAS LMX method. This may be due to the higher selectivity of the ALOA agar to isolate and differentiate typical *Listeria* colonies from competing microflora, such as *Bacillus* colonies. The high selectivity of the proprietary broth, the high background flora and the low selectivity of the OXA agar most likely contributed to this observation as well.

For the analysis of 25 g test portions by the VIDAS LMX method, two false positives were obtained. The test results produced by the false-positive test portions (average test value of 0.22) were much lower than the test values observed with true positives (average value >2.00). By the time the coordinating laboratory received the results, the primary enrichments for these samples had been discarded so no subsequent analysis on the VIDAS LMX was possible. However, the agar plates for these test portions were shipped to the coordinating laboratory for further analysis. Up to 20 different colonies were picked for morphological and biochemical analysis using VITEK 2 GP and no *Listeria* colonies were identified. Additionally, the entire lawn of growth from each agar plate was swabbed, enriched in LMX broth, and incubated for 26–30 h at 37 ± 1°C. An aliquot from each tube was analyzed by the VIDAS LMX assay and negative results for *L. monocytogenes* were obtained. Results of this investigation led the study directors to believe that the false positives were the result of possible cross-contamination during the analysis of the samples.

For the analysis of both the 25 and 125 g test portions, Laboratory 11 detected the presence of multiple types of *Listeria* spp. An investigation into the results indicated that colonies picked for confirmation did not meet the characteristics of *Listeria* spp., i.e. colonies produced Gram-negative stain reactions and were negative for motility and catalase. The results of these tests should have precluded analysis using the API strips which lead to an inaccurate identification. Due to the fact that final results reported were inconsistent with biochemical results, data produced by Laboratory 11 was removed from the statistical analysis of both the 25 and 125 g test portions.

Using the POD statistical model, no significant difference in the number of positive results obtained between the two methods being compared was observed at both the low and high inoculum levels for both the 25 and 125 g test portions. No significant difference was observed between presumptive and confirmed results for the candidate method.

Conclusions

The VIDAS *Listeria monocytogenes* Xpress (LMX) method with the optional ALOA agar confirmation method was adopted as Official First Action status for the detection of *L. monocytogenes* in a variety of foods, including deli ham (25 and 125 g), fermented sausage (25 g), liver pâté (25 g), processed cheese (25 g), vanilla ice cream (25 g), cooked shrimp (25 g), smoked white fish (25 g), frozen spinach (25 g), peanut butter (25 g), deli turkey (25 and 125 g), queso fresco (125 g), and ground beef (125 g).

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FOOD COMPOSITION AND ADDITIVES

Determination of Total Carbohydrates in Wine and Wine-Like Beverages by HPLC with a Refractive Index Detector: First Action 2013.12

STEVE KUPINA
Constellation Wines, U.S. Research and Development, 12667 Rd 24, Madera, CA 93637
MARK ROMAN
Tampa Bay Analytical Research, PO Box 931, Safety Harbor, FL 34695-0931

Collaborators: D. Anderson; S. Bhandari; M.S. Cardozo; A. Chandra; C. Hartless; M. Kelm; L.R. Masson; J. Neudahl; D. Roberts; J. Zhang

An international collaborative study was conducted of an HPLC-refractive index (RI) detector method for the determination of the combined amounts of sugars, glycerol, organic acids, and phenolic compounds in wines and wine-like beverages. Nine collaborating laboratories representing major winery, contract laboratories, and government laboratories tested eight different materials as blind duplicates using the proposed method. Sample materials included red and white wines, port, wine cooler, and nonalcoholic wine. One material was a negative control, and one material was a reference material. Samples were either treated with an ion-exchange resin to remove interfering organic acids prior to analysis or left untreated to include organic acids and phenolics. Red wine samples were treated with polyvinylpolypyrrolidone to remove potential interferences from phenolics prior to analysis. The HPLC analyses were performed on a Bio-Rad Fast Acid Analysis Column using RI detection. Reproducibility (RSD_R) for untreated samples (sugars + phenolics + organic acids) ranged from 6.6% for Titrivin AA4 reference material to 11.0% for dry red wine. RSD_R for treated samples (sugars only) ranged from 6.8% for white zinfandel to 18.9% for dry white wine. RSD_R for treated samples (sugars only) + glycerol ranged from 6.4% for white zinfandel to 19.8% for dry red wine. Based on these results, the method was adopted as Official First Action status for determination of total carbohydrates in wine and wine-like beverages.

Submitted for publication: September 25, 2013.
The method was approved by the Method-Centric Committee on Carbohydrates in Wine as First Action.
The Method-Centric Committee on Carbohydrates in Wine invites method users to provide feedback on the First Action methods. Feedback from method users will help verify that the methods are fit for purpose and are critical to gaining global recognition and acceptance of the methods. Comments can be sent directly to the corresponding author or methodfeedback@aoac.org.
Corresponding author's e-mail: steve.kupina@cbrands.com
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For years federal law has required the analysis and labeling of carbohydrate values for foodstuffs, and the U.S. Food and Drug Administration (FDA) AOAC-approved "By Difference" method (1) for carbohydrate determination has, in the past, been adequate. However, carbohydrate values for several categories of beverage alcohol, especially wines, would not be accurately reported if determined by this AOAC **985.10** Carbohydrate in Wine method. This method includes components not considered to be "carbohydrates," such as organic acids and tannins (polyphenolics). AOAC **985.10** is not, in fact, a stand-alone method, but a calculation obtained from the results of three separate AOAC methods: AOAC **920.56** "Specific Gravity of Wines," AOAC **945.09** "Extract of Beer," and AOAC **920.67** "Ash of Wines." The assumption in AOAC **985.10** is that all residue obtained after evaporation that is not ash is composed of carbohydrates.

In July 2007, the Alcohol, Tobacco Tax, and Trade Bureau (TTB) published Notice No. 73, "Labeling and Advertising of Wines, Distilled Spirits and Malt Beverages; Proposed Rule" (2). This Notice of Proposed Rulemaking introduced the Serving Facts Label, which included the total amounts of carbohydrates, expressed as grams/serving. Currently, the TTB is responsible for regulating wine labels, and TTB Procedure 2004-1 specifies that AOAC **985.10** must be used for the determination of carbohydrates in standard wines, ciders, and sake. The FDA, however, is responsible for defining what is a carbohydrate.

In 2004, the wine industry began looking into voluntarily labeling products and began testing wines. The key finding from these analytical results was that for some categories of wine, especially the widely popular dry red and white wine segment, carbohydrates are overstated by upward of 150% when determined by the currently approved AOAC method. This is primarily driven by the composition of wine, which in comparison to food has a much lower total solids value due to the high moisture content in wines. In beverage alcohol, to include wine, this results in a significant amount of noncarbohydrates such as organic acids (tartaric and malic acids), phenolic compounds (hydroxycinnamic acids and procyanidins), Maillard products, and glycerol being included in the calculation of the carbohydrate value, which in turn results in the overstatement of the value. Unfortunately, the FDA has not yet provided a definition for carbohydrates. The final definition may include just sugars, or compounds such as

Table 1. Reproducibility results from original multilaboratory study

Material	Avg, g/100 mL	s_r, g/100 mL	s_R, g/100 mL	RSD_r, %	RSD_R, %	HorRat	No. labs used	Outliers	TTB results, g/serving[a]
Untreated samples (sugars + glycerol + organic acids + phenolics)									
Nonalcoholic wine	5.27	0.0868	0.178	1.65	3.37	1.08	7	0	3.4
Port	13.1	0.252	0.48	1.92	3.66	1.35	6	0	10.8
White zinfandel	5.15	0.113	0.187	2.19	3.64	1.16	6	1	3.22
Wine cooler	12.8	0.0352	0.402	0.27	3.13	1.15	7	0	11.4
Dry white wine	2.26	0.0572	0.111	2.52	4.9	1.39	6	1	0.56
Dry red wine	2.4	0.0144	0.166	0.6	6.89	1.97	6	1	0.155
Ion-exchange resin treated samples (sugars only)									
Nonalcoholic wine	3.92	0.0303	0.218	0.77	5.56	1.71	6	2	3.4
Port	10.1	0.178	3.03	1.76	29.9	10.6	7	1	10.8
White zinfandel	3.89	0.189	0.341	4.85	8.77	2.69	7	1	3.22
Wine cooler	10.1	0.155	2.58	1.53	25.6	9.06	7	0	11.4
Dry white wine	0.876	0.0298	0.166	3.4	19.0	4.66	7	7	0.56
Dry red wine	0.589	0.0076	0.048	1.3	8.15	1.88	6	2	0.155
Ion-exchange resin treated samples (sugars + glycerol)									
Nonalcoholic wine	4.21	0.03	0.274	0.71	6.5	2.02	7	0	3.4
Port	11.7	0.188	1.40	1.61	12.0	4.35	7	0	10.8
White zinfandel	4.25	0.166	0.378	3.91	8.9	2.77	8	0	3.22
Wine cooler	10.8	0.174	1.6	1.6	14.8	5.29	7	0	11.4
Dry white wine	1.42	0.0357	0.207	2.52	14.6	3.85	8	0	0.56
Dry red wine	1.38	0.041	0.227	2.98	16.5	4.33	8	0	0.155

[a] Alcohol and Tobacco Tax and Trade Bureau results obtained from in-house method.

glycerol, organic acids, and/or phenolic compounds in addition to sugars.

As a result, the wine industry moved to develop a method that would produce results that the industry believed to be more accurate and, therefore, truthful for consumers. An HPLC-refractive index (RI) detector method was developed to remove potential interfering compounds such as organic acids, phenolics, and glycerol. The original multilaboratory study involving seven laboratories was conducted on the proposed method with good results.

Recently, the wine industry reconvened to reassess the need for an AOAC validated method that more accurately calculates carbohydrates for wines to maintain truth in labeling for consumer protection. This method will allow quantification of the combined amounts of sugars, glycerol, organic acids, and phenolic compounds, as well as just the sugars. The objective of this current study was to demonstrate that this method is suitable for adoption as an AOAC Official Method for the determination of total carbohydrates in wine and wine-like beverages containing total carbohydrates at a concentration of 0.2–14 g/100 mL.

Multilaboratory Study

The original multilaboratory study involving only seven laboratories was conducted on six materials submitted as blind duplicates: dry red wine, dry white wine, white zinfandel, wine cooler, port, and nonalcoholic wine. The results of this study supported this method for a collaborative study that meets AOAC requirements. Results of the original multilaboratory study are presented in Table 1.

Study Design

The HPLC-RI method was provided to nine laboratories participating in the collaborative study. The study was conducted on a total of seven materials submitted as blind duplicates. The test materials included red and white wines, port, wine cooler, and nonalcoholic wine. One material was a negative control, and one material was a reference material (Titrivin AA4 reference material, prepared by the Vine and Wine Department of the

Table 2. Study materials

Material	Approximate total carbohydrate concn, g/100 mL
Port	13
Dry red wine	2
Dry white wine	2
White zinfandel	5
Wine cooler (blush)	13
Titrivin AA4 reference material	10
Nonalcoholic red wine	5
Negative control[a]	0

[a] Mixture of alcohol and water containing food coloring and no carbohydrates.

Gironde Chamber of Agriculture, Blanquefort, France). The test samples were blinded in terms of identity and carbohydrate content. Random identification numbers were assigned to each of the blind duplicate materials. Table 2 provides a list of the test materials. All test samples were analyzed both as is (untreated) and after treatment with an ion-exchange resin (treated).

In addition, two practice samples were provided to each collaborating laboratory. The practice samples were first analyzed by the laboratories, and the results were submitted to the Study Director for approval. Upon successful completion of the practice samples, the laboratories were allowed to proceed with the collaborative study materials.

Collaborators

A total of nine laboratories participated in the collaborative study. The selected laboratories had experience with HPLC analysis and the analysis of wine and/or wine-like samples. The laboratories represented wine manufacturers, contract analytical laboratories, and government agencies.

Test Sample Preparation

Test materials were obtained from commercial sources, except for the negative control. The negative control was an alcohol–water (10 + 90, v/v) mixture containing food coloring (Red Dye No. 40). There was no additional processing by the originating laboratory. Finished wines were transferred from bottles or "bag in the box" containers to high-density polyethylene (HDPE) bottles for shipping.

Test Material Homogeneity

All samples were liquid samples. No homogeneity testing on the test materials was performed.

Preparation and Shipment of Samples

Sixteen test materials were shipped to each of the collaborating laboratories. A sufficient amount of each test material (approximately 100 mL) was packaged in suitable sized HDPE bottles by the Study Director, with the exception of the Titrivin AA4 reference material, which comes prepackaged in 10 mL ampules. The bottles were labeled with random identification codes. Test materials were shipped overnight at ambient temperature to the collaborating laboratories. Upon receipt, the laboratories were instructed to store the test materials at refrigeration temperature (4°C) and tightly sealed until use.

AOAC Official Method 2013.12
Determination of Total Carbohydrates in Wine and Wine-Like Beverages
HPLC with a Refractive Index Detector
First Action 2013

[Applicable to the determination of total carbohydrates in wine and wine-like beverages by using HPLC with a refractive index (RI) detector.]

A. Principle

This method is suitable for the determination of total sugars; total sugars plus glycerol; and total sugars plus glycerol, organic acids, and phenolics. Samples are analyzed either as is to quantify total sugars + glycerol, organic acids, and phenolics, or after treatment with an ion-exchange resin to remove interfering organic acids. Only red wine samples are treated with polyvinylpolypyrrolidone (PVPP) to remove potential interferences from phenolic compounds. The treated or untreated samples are subjected to ion-exchange chromatography with RI detection, and quantification is performed against glucose calibration standards.

B. Apparatus

Note: Equivalent apparatus may be substituted. All volumetric pipets and volumetric flasks are Class A.

(**a**) *HPLC system.*—Suitable system equipped with a pump, autosampler, thermostatted column oven, and RI detector. HPLC operating conditions: column temperature, 60°C; mobile phase flow rate, 0.8 mL/min; injection volume, 10 µL; and detection, RI (temperature 40°C).

(**b**) *LC column.*—Bio-Rad Fast Acid Analysis Column, 100 × 7.8 mm, No. 125-0100 (Bio-Rad Laboratories, Hercules, CA, www.biorad.com).

(**c**) *LC guard column (optional).*—Bio-Rad guard holder (No. 1250131) and Cation H refills, 30 × 4.6 mm (No. 1250129) or equivalent.

(**d**) *Analytical balance.*—Readability, ±1 mg.

(**e**) *Volumetric pipets.*—Various sizes.

(**f**) *Volumetric flasks*.—100 and 1000 mL.

(**g**) *Laboratory flatbed shaker.*

(**h**) *Tubes.*—15 mL graduated plastic centrifuge tubes, with caps.

(**i**) *Pipets.*—10 mL disposable, Pyrex.

(**j**) *Syringes.*—Disposable, 30 mL polypropylene, or equivalent.

(**k**) *Syringe filters.*—Millipore Millex-HV PVDF, 25 mm, 0.45 µm pore, No. SLHV025N (EMD Millipore Corp., Billerica, MA).

(**l**) *LC injection vials*.—2 mL, with Teflon-coated caps.

C. Reagents

Note: Chemicals from other suppliers meeting the specifications may also be used.

Table 2013.12A. Preparation of calibration solutions

Calibration solution	Vol. stock to pipet, mL	Flask vol., mL	Glucose final concn, g/100 mL
1	35	50	14
2	25	50	10
3	20	50	8
4	10	50	4
5	4	50	1.6
6	4	100	0.8
7	1	100	0.2

100

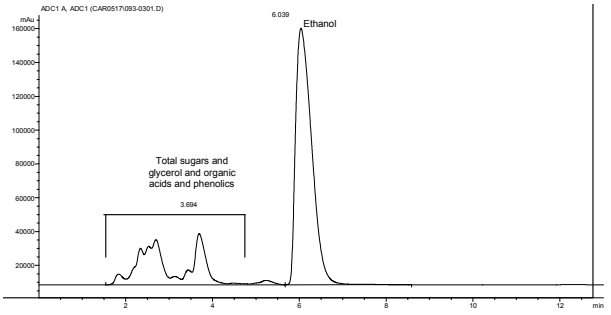

Figure 2013.12A. Untreated sample solution chromatogram. Total sugars + glycerol + organic acids + pheonolics elute at retention time (RT) 1.7 to 5.9 min.

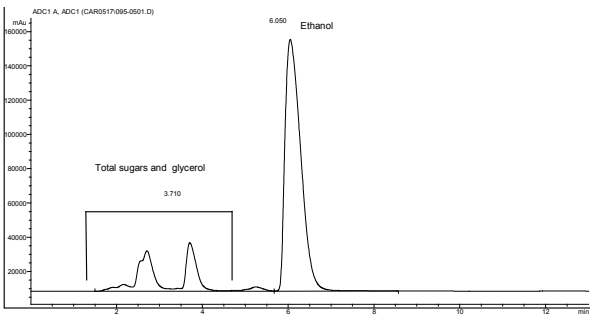

Figure 2013.12B. Ion-exchange resin treated sample solution chromatogram. Total carbohydrates treated (RT 1.7 to 3.4 min); total sugars + glycerol treated (RT 1.7 to 4.2 min).

(**a**) *Sulfuric acid.*—Concentrated, ACS grade. *Caution:* Sulfuric acid causes severe burns, reacts violently with water, and is harmful if swallowed or inhaled. Contact with other materials may cause fire. Use only in ventilated fume hood. Wear appropriate personal protective equipment.

(**b**) *Water.*—Deionized, >17 mΩ, 0.2 µm filtered.

(**c**) *PVPP.*—Sigma P6755 (Sigma-Aldrich, St. Louis, MO).

(**d**) *Ion-exchange resin.*—AG 501-X8, 20–50 mesh, Bio-Rad No. 142-6424 or equivalent. Prepare by slowly stirring approximately 50 g resin in 200 mL deionized water for 20 min. Filter (Whatman No. 2) and dry overnight at 40°C. Store in a sealed bottle.

(**e**) *Mobile phase.*—0.002 M sulfuric acid in water. Add 0.20 g concentrated sulfuric acid to 500 mL deionized water in a 1 L volumetric flask. Bring to volume with deionized water and mix well. Store at room temperature. Prepare fresh every 3 months. Alternately, 0.002 M sulfuric acid in water may be prepared by mixing 10 mL of commercially available 0.2 M sulfuric acid solution with 990 mL water.

(**f**) *Reference standards.*—D-(+) Glucose, anhydrous, ACS reagent grade, Sigma Part No. G-5767 or equivalent.

D. Preparation of Test Solutions

(**a**) *Preparation of stock standard solution.*—Accurately weigh to the nearest mg about 20.0 g anhydrous glucose reference standard and transfer into a 100 mL volumetric flask. Add deionized water and shake to dissolve. Dilute to volume with deionized water and mix thoroughly.

(**b**) *Preparation of calibration solutions.*—Prepare calibration solutions as presented in Table **2013.12A** by pipetting the indicated volume of stock standard solution into the indicated size volumetric flask and diluting to volume with deionized water. Filter an aliquot of each solution through a 0.45 µm PVDF syringe filter prior to analysis, discarding the first few mL filtrate. Store refrigerated. Prepare fresh every month.

(**c**) *Preparation of sample test solutions.*—(*1*) Untreated samples for analysis of total sugars plus glycerol, organic acids, and phenolics: filter a portion of the sample through a 0.45 µm PVDF syringe filter into an HPLC autosampler vial. Label: *Untreated Test Solution.*—(*2*) Treated samples for analysis of total sugars and total sugars plus glycerol: using a 10 mL disposable pipet, transfer 10 mL sample into a 15 mL tube (16 × 125 mm). Add 0.855 ± 0.005 g ion-exchange resin to the sample. If the wine is red or rose, add 1.0 g PVPP to the sample as well. Cap the tube and mix well to suspend all material. Shake for 20 min. Filter a portion of the sample through a 0.45 µm PVDF syringe filter into an HPLC autosampler vial. Label: *Treated Test Solution.*

E. Determination

(**a**) *Calibration.*—Make single 10 µL injections of each calibration standard solution and a deionized water blank. Calculate the slope, *y*-intercept, and r^2 value for the calibration curve. The r^2 value should be ≥0.99. Verify the calibration by analyzing a second source control sample. The recovery of the control sample must be within statistically determined control limits.

(**b**) *Sample analysis.*—Make single 10 µL injections of each test solution. After every 10 samples, inject 10 µL deionized water blank. Integrate all peaks from approximately 1.7 to approximately 3.4 min to calculate total sugars in the treated samples. Integrate all peaks from approximately 1.7 min to approximately 4.2 min to calculate total carbohydrates plus glycerol in the treated samples. Integrate all peaks from approximately 1.7 min to approximately 5.9 min in the untreated samples to calculate total sugars plus glycerol, organic acids, and phenolics. Example chromatograms are presented in Figures **2013.12A** and **B**.

F. Calculations

Concentrations are reported in g/100 mL total carbohydrates as glucose to two decimal places. Concentrations are calculated using an external seven-point calibration curve.

(**a**) The amount of total sugars in the treated test material in g/100 mL is calculated as follows:

$$\frac{A_s - B}{m}$$

where A_s = area of the peak from approximately 1.7 to approximately 3.4 min in the treated sample chromatogram, B = *y*-intercept of the calibration curve for glucose, and m = slope of calibration curve for glucose.

(**b**) The amount of total sugars + glycerol in the treated test material in g/100 mL is calculated as follows:

$$\frac{A_{sg} - B}{m}$$

where A_{sg} = total peak area (including glycerol) from approximately 1.7 to approximately 4.2 min in the treated

sample chromatogram, B = *y*-intercept of calibration curve for glucose, and m = slope of calibration curve for glucose.

(**c**) The amount of total sugars + glycerol, organic acid, and phenolics in the untreated test material in g/100 mL is calculated as follows:

$$\frac{A_p - B}{m}$$

where A_p = total peak area (including glycerol, organic acids, and phenolics) from approximately 1.7 to approximately 5.9 min in the untreated sample chromatogram, B = *y*-intercept of calibration curve for glucose, and m = slope of calibration curve for glucose.

Results and Discussion

Individual values for total sugars; total sugars plus glycerol; and total sugars plus glycerol, organic acids, and phenolics were reported for each material in units of g/100 mL to two decimal places and are presented in Tables 3–5. It should be noted that the results for the total sugars + glycerol were obtained from the same chromatograms as the total sugars results, with the glycerol peak results added to the total sugars results. The slope, *y*-intercept, and r^2 value for each of the calibration curves were also reported.

Cochran, Grubbs, and double Grubbs tests were used to remove outliers for total sugars; total sugars plus glycerol; and total sugars plus glycerol, organic acids, and phenolics. The average analyte concentration, repeatability SD (s_r), reproducibility SD (s_R), repeatability RSD (RSD_r), reproducibility RSD (RSD_R), predicted reproducibility RSD ($PRSD_R$), and HorRat values were calculated for all analytes in all materials after outlier removal.

Tables 6–8 present statistical summaries of the results. Statistical analysis to determine repeatability and reproducibility was performed using the AOAC INTERNATIONAL Interlaboratory Study Workbook for Blind (Unpaired) Replicates, v. 2.0 (3). S_r, S_R, RSD_r, RSD_R, and number of statistical outliers are presented. HorRat values are also presented in these tables, and are calculated as RSD_R (observed)/$PRSD_R$, where the $PRSD_R$ is calculated using the equation $RSD_R = 2C^{-0.1505}$, where C is the measured analyte concentration in decimal mass units (4). Cochran, Grubbs, and double Grubbs tests were used to remove statistical outliers where appropriate. It should be noted that HorRat values are not applicable to this method, as the analyte (total carbohydrates) is an indefinite analyte; HorRat values are provided, however, for informational purposes (5).

The initial interlaboratory study showed higher RSD and HorRat values than for this study. The number of laboratories in the original study did not meet the minimum requirements for a collaborative study, and therefore the statistical analysis has limited value and is not a reliable estimator of the reproducibility of the method.

Collaborator Comments

Laboratory 1 stated that it centrifuged the samples treated with PVPP for 10 min prior to analysis. Laboratory 2 noted that criteria used for peak identification in the method is not typical, and that sample chromatograms need to be provided with the final method. In addition, since peak retention time may differ depending upon the column age, a statement needs to be included in the method indicating that the peaks should be calculated based on elution as seen from the blank chromatogram. These additions will be included in the final method. Laboratory 1 also noted that the stock standard solution required vigorous stirring to dissolve the 20 g glucose, and that it had trouble getting the last 1 mL of calibrant using a 1 mL volumetric pipet. Instead it used 0.5 mL for the final dilution and adjusted the calibration curve appropriately. It also noted that there may have been a problem with one of the treated wine cooler samples based on the chromatography, but no attempt was made to rechromatograph the sample. Laboratory 4 used a four-point calibration curve instead of a seven-point curve.

Performance Characteristics

RSD_r was generally good for the untreated samples (measurement of sugars + organic acids + phenolics), and ion-exchange resin treated samples (measurement of sugars only and sugars + glycerol). RSD_R was significantly higher than the RSD_r, demonstrating that between-laboratory variability was much higher than within-laboratory variability. For the sugars-only result (ion-exchange resin treated), RSD_R ranged from 6.8 to 18.9%, with HorRat values ranging from 2.1 to 4.5. As noted previously, however, HorRat values are not applicable as this method quantifies an indefinite analyte. Comparison with other similar methods for indefinite analytes utilizing ion-exchange HPLC, such as AOAC **997.08** Fructans in Food Products (6) or AOAC **2000.11** Polydextrose in Foods (7), shows the RSD_R values obtained in this study are very consistent with those methods. It should also be noted that the TTB proposed labeling requirements for total carbohydrates in alcohol only requires reporting the value to two significant figures.

The Titrivin AA4 results are not directly comparable to the values assigned for this material on the certificate of analysis. Assigned values for the Titrivin AA4 by the Chambre d'Agriculture Gironde include glucose + fructose (0.826 g/100 mL), reducing sugars (0.926 g/100 mL), total acidity (0.06 g H_2SO_4/100 mL), acetic acid (0.068 g/100 mL), malic acid (0.325 g/100 mL), and lactic acid (0.019 g/100 mL). The current method does not distinguish among the individual sugars, reducing sugars, and organic acids.

RSD_r and RSD_R were noticeably better for the untreated samples (6.6 and 11.0%, respectively), which include sugars, phenolics, and organic acids in the results. This indicates that the addition of the ion-exchange resin in the treated samples has a slight negative effect on the performance of the method. It is not certain why the addition of the ion-exchange resin increases the variability. The ion-exchange resin must be prepared by the laboratory before use by stirring 50 g resin with 200 mL water for 20 min, filtering, and drying overnight at 40°C. It could be that variability in the manner in which laboratories prepared the resin prior to use could affect its sorption ability and thus increase the variability.

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We would like to acknowledge the following collaborators for participation in the study:

Denise Anderson, Diageo, Plainfield, IL

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Table 3. Results for blind replicates—untreated samples (sugars + glycerol = organic acids = phenolics)

	Results, g/100 mL, by laboratory number								
Material	1	2	3	4	5	6	7	8	9
Port	14.33	11.05	12.40	12.32	12.30	12.63	[a]	14.12	12.06
	14.37	11.37	12.40	12.34	12.43	12.48	10.97	14.03	12.06
Dry red wine	2.71	2.16	2.61	3.03	2.69	2.43	2.88	2.93	2.52
	2.73	2.12	2.64	2.91	2.71	2.12	2.62	2.96	2.52
White zinfandel	6.03	5.02	5.39	5.42	5.24	5.26	5.82	6.13	5.23
	6.02	5.01	5.39	5.41	5.15	5.23	5.62	6.14	5.24
Wine cooler	12.08	9.73	10.40	10.34	10.89	11.42	11.29	11.97	10.27
	12.03	9.59	10.30	10.34	11.02	10.91	11.19	11.98	10.25
Dry white wine	1.97	2.24	2.04	2.14	2.11	1.61	2.14	2.21	1.97
	1.96	2.28	2.01	2.12	2.09	8.06[b]	2.19	2.26	1.95
Titrivin AA4	2.58	2.18[b]	2.55	2.67	2.55	2.35	2.85	2.79	2.43
	2.57	2.29[b]	2.50	2.66	2.54	2.33	2.83	2.79	2.42
Nonalcoholic red wine	9.08	7.15	8.02	7.96	7.77	8.33	8.36	9.17	7.76
	9.06	7.37	8.01	8.01	7.64	8.31	8.55	9.16	7.77
Negative control	–0.32	0.11	0.09	0.20	0.01	0.09	0.00	0.00	0.13
	–0.32	0.09	0.08	0.16	0.01	0.09	0.05	3.95[b]	0.13

[a] Flask broken.

[b] Cochran outlier.

Table 4. Results for blind replicates—ion-exchange resin treated samples (sugars only)

	Results, g/100 mL, by laboratory number								
Material	1	2	3	4	5	6	7	8	9
Port	13.25	10.37	11.40	11.33	9.01	11.91	[a]	12.36	10.58
	13.27	10.31	11.60	11.37	9.11	11.79	11.29	8.62	10.30
Dry red wine	0.68	0.98	1.18	1.03	0.95	3.16[b]	0.92	0.88	0.91
	0.70	0.68	1.16	1.07	0.99	1.01	0.90	0.91	0.92
White zinfandel	4.63	3.83	4.35	4.16	4.04	4.49	4.33	4.61	3.94
	4.64	3.92	4.32	4.15	4.02	4.54	4.21	4.66	3.97
Wine cooler	12.11	6.02[b]	10.30	10.55	8.90	11.95	10.89	11.38	9.81
	12.35	0.14[b]	10.30	10.54	8.44	11.94	10.89	11.42	9.89
Dry white wine	0.42	0.80	0.89	0.85	0.84	0.74	0.70	0.71	0.71
	0.42	0.83	0.91	0.86	0.83	0.72	0.82	0.72	0.72
Titrivin AA4	0.84	1.31	1.36	1.19	1.20	1.64[b]	1.30	1.15	1.01
	0.91	1.19	1.34	1.17	1.20	0.53[b]	1.17	1.16	1.05
Nonalcoholic red wine	7.03	5.37	6.43	6.19	5.65	6.67	6.68	8.01[b]	5.89
	7.00	5.47	6.46	6.14	5.68	6.77	6.77	6.75	5.84
Negative control	–0.50	0.06	0.00	–0.01	0.01	0.09	0.00	0.00	0.00
	–0.50	0.06	0.00	–0.01	0.01	0.09	0.00	0.00	0.00

[a] Flask broken.

[b] Cochran outlier.

Table 5. Results for blind replicates—ion-exchange resin treated samples (sugars + glycerol)

	Results, g/100 mL, by laboratory number								
Material	1	2	3	4	5	6	7	8	9
Port	13.94	11.03	11.90	11.91	9.54	12.97	[a]	13.02	11.47
	13.96	11.02	12.10	11.95	9.65	12.35	11.98	9.29	11.16
Dry red wine	1.64	1.77	1.90	1.82	1.71	4.08[b]	1.94	0.88	1.68
	1.66	1.33	1.87	1.87	1.78	1.96	1.82	0.91	1.69
White zinfandel	5.16	4.29	4.75	4.59	4.48	5.00	4.79	5.15	4.51
	5.17	4.41	4.71	4.58	4.46	5.06	4.71	5.19	4.56
Wine cooler	12.11	6.32[b]	10.30	10.62	8.97	12.00	11.29	11.43	10.10
	12.35	0.06	10.30	10.61	8.50	11.99	11.19	11.47	10.17
Dry white wine	0.98	1.24	1.29	1.32	1.30	1.32	1.27	1.69[b]	1.18
	0.98	1.30	1.31	1.32	1.29	1.29	1.33	1.29	1.20
Titrivin AA4	1.46	1.83	1.85	1.71	1.74	1.78	1.69	1.80	1.57
	1.54	1.70	1.84	1.68	1.74	1.82	1.78	1.82	1.63
Nonalcoholic red wine	8.13	6.32	7.26	7.11	6.60	7.77	7.62	9.21[b]	6.91
	8.10	6.45	7.29	7.04	6.64	7.87	7.89	7.93	6.91
Negative control	–0.50	0.00	0.00	–0.01	0.01	0.09	0.00	0.00	0.00
	–0.50	0.00	0.00	–0.01	0.01	0.09	0.00	0.00	0.00

[a] Flask broken.

[b] Cochran outlier.

Table 6. Statistical analysis of blind replicates—untreated samples (sugars + glycerol + organic acids + phenolics)

Sample[a]	Average, g/100 mL	S_r	RSD_r, %	S_R	RSD_R, %	HorRat	Outlier labs	No. of labs used
A	12.6	0.10	0.78	1.09	8.76	3.2	0	9
B	2.63	0.10	3.83	0.29	10.95	3.2	0	9
C	5.49	0.05	0.96	0.39	7.12	2.3	0	9
D	10.9	0.13	1.22	0.81	7.48	2.7	0	9
E	2.07	0.02	1.14	0.17	8.14	2.3	0	9
F	2.59	0.02	0.59	0.17	6.61	1.9	1	8
G	8.19	0.08	0.93	0.63	7.71	2.6	0	9

[a] A = Port, B = dry red wine, C = white zinfandel, D = wine cooler, E = dry white wine, F = Titrivin AA4, and G = nonalcoholic red wine.

Table 7. Statistical analysis of blind replicates—ion-exchange resin treated samples (sugars only)

Sample[a]	Average, g/100 mL	S_r	RSD_r, %	S_R	RSD_R, %	HorRat	Outlier labs	No. of labs used
A	11.1	0.94	8.5	1.37	12.4	4.4	0	9
B	0.93	0.08	8.1	0.15	15.9	3.9	0	9
C	4.27	0.04	0.96	0.29	6.75	2.1	0	9
D	10.7	0.13	1.23	1.16	10.9	3.9	1	8
E	0.75	0.03	4.09	0.14	18.9	4.5	0	9
F	1.16	0.05	4.24	0.15	13	3.3	1	8
G	6.28	0.05	0.75	0.56	8.91	2.9	0	9

[a] A = Port, B = dry red wine, C = white zinfandel, D = wine cooler, E = dry white wine, F = Titrivin AA4, and G = nonalcoholic red wine.

Table 8. Statistical analysis of blind replicates—ion-exchange resin treated samples (sugars + glycerol)

Sample[a]	Average, g/100 mL	S_r	RSD_r, %	S_R	RSD_R, %	HorRat	Outlier labs	No. of labs used
A	11.7	0.95	8.1	1.4	11.9	4.3	0	9
B	1.66	0.12	6.95	0.33	19.8	5.4	0	9
C	4.75	0.04	0.87	0.31	6.42	2	0	9
D	10.8	0.14	1.25	1.14	10.5	3.8	1	8
E	1.24	0.02	1.92	0.11	8.96	2.3	0	9
F	1.72	0.05	2.71	0.11	6.65	1.8	0	9
G	7.28	0.08	1.12	0.62	8.42	2.8	0	9

[a] A = Port, B = dry red wine, C = white zinfandel, D = wine cooler, E = dry white wine, F = Titrivin AA4, and G = nonalcoholic red wine.

Sneh Bhandari, Silliker Laboratories, Chicago, IL
Maria Sarita Cardozo, Compliance Laboratory, TTB, Walnut Creek, CA
Amitabh Chandra, Access Business Group, Ada, MI
Chris Hartless, Diageo Wines, Rutherford, CA
Mark Kelm, Constellation Wines, Madera, CA
Larry Masson, Covance Laboratories, Madison, WI
James Neudahl, ETS Laboratories, St. Helena, CA
Dave Roberts, E&J Gallo Winery, Modesto, CA
Julia Zhang, Treasury Estates, Napa, CA

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INFANT FORMULA AND ADULT NUTRITIONALS

Determination of Folate in Infant Formula and Adult/Pediatric Nutritional Formula by Ultra-High Performance Liquid Chromatography-Tandem Mass Spectrometry: First Action 2013.13

KARINE MEISSER-REDEUIL, SYLVIE BÉNET, CATHERINE GIMENEZ, and ESTHER CAMPOS-GIMÉNEZ
Nestlé Research Center, Nestec Ltd, Vers-chez-les-Blanc, PO Box 44, CH-1000 Lausanne 26, Switzerland
MARIA NELSON
AOAC INTERNATIONAL, 481 N. Frederick Ave, Suite 500, Gaithersburg, MD 20877-2417

A UHPLC-MS/MS method for the determination of folate (vitamin B_9) in infant formula and adult/pediatric nutritional formula was assessed for compliance with standard method performance requirements set forth by the AOAC INTERNATIONAL Stakeholder Panel for Infant Formula and Adult Nutritionals (SPIFAN). A single-laboratory validation (SLV) study was conducted as the first step in the process to validate the method. In the study, 12 matrixes, representing the range of infant and adult nutritional products, were evaluated for folate [the sum of supplemental folic acid plus 5-methyl tetrahydrofolic acid (5-Me THF)]. Method response was linear in the range of 1.0–900 ng/mL, corresponding to 0.33–300 µg/100 g in reconstituted sample. LOD for folic acid and 5-Me THF, expressed in reconstituted product, were 0.10 µg/100 g and 0.05 µg/100 g, respectively, and LOQ were 0.33 µg/100 g and 0.10 µg/100 g, respectively. Repeatability was <5.3% and intermediate precision was <5.5%. Recovery rates of spiking at 50 and 100% of target values in nonfortified products were within 90–110%. Evaluation of trueness was performed on Certified Reference Material (SRM 1849 Infant/Adult Nutritional Formula) and gave 96.4% of theoretical value. Based on the results of the SLV, the method meets the SPIFAN requirements for AOAC First Action status for the determination of folates in infant formula and adult/pediatric nutritional formula.

A UHPLC-MS/MS method was developed to establish an international consensus method for the determination of folate in infant formula and adult/pediatric nutritional formula that will ensure results recognition and aid in dispute resolution. Standard method performance requirements (SMPRs) for total folates [supplemental folic acid plus 5-methyl tetrahydrofolic acid (5-Me THF)], approved by the AOAC INTERNATIONAL Stakeholder Panel for Infant Formula and Adult Nutritionals (SPIFAN), are described in AOAC SMPR **2011.006** (1). The single-laboratory validation (SLV) was conducted on 12 SPIFAN matrixes representing infant formulas and adult nutritionals made from any combination of milk, soy, rice, whey, hydrolyzed protein, starch, and amino acids, with and without intact protein, and a Certified Reference Material (SRM 1849 Infant/Adult Nutritional Formula). System suitability, linearity, LOD, LOQ, precision, and accuracy were assessed in the study.

In August 2013, an Expert Review Panel (ERP) for Infant Formula and Adult Nutritionals reviewed the SLV study on the UHPLC-MS/MS method for determination of folate and adopted the method First Action as AOAC *Official Method*^{SM} **2013.13**. The ERP will continue to monitor the method for approximately 2 years, after which the ERP may recommend the method to the AOAC Official Methods Board for Final Action status if the method is found to be suitable (2).

Received March 10, 2014.
The method was approved by the Expert Review Panel for Infant Formula and Adult Nutritionals as First Action.

The Expert Review Panel for Infant Formula and Adult Nutritionals invites method users to provide feedback on the First Action methods. Feedback from method users will help verify that the methods are fit for purpose and are critical to gaining global recognition and acceptance of the methods. Comments can be sent directly to the corresponding author or methodfeedback@aoac.org.

Corresponding author's e-mail: Karine.Meisser-Redeuil@rdls.nestle.com

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AOAC Official Method 2013.13
Folate in Infant Formula and Adult/Pediatric Nutritional Formula
UHPLC-MS/MS
First Action 2013

[Applicable to the determination of folate in ready-to-feed (RTF), liquid concentrate, and powder products from levels of 0.33 µg/100 g folic acid and 0.10 µg/100 g 5-Me THF and up to 300 µg/100 g folic acid and 5-Me THF in product as reconstituted.

See Figures **2013.13A** and **2013.13B** for the results of the SLV study supporting acceptance of the method. The method was evaluated against standard method performance requirement AOAC SMPR **2011.006** (1).

A. Principle

Powder samples were reconstituted by dissolving 25 g powder sample and 50 mg α-amylase in 200 g warm water (40°C). Samples were digested at 40°C for 15 min followed by dilution with 40 mL buffer [2% ascorbic acid, 0.1% dithiothreitol (DTT), pH 4.5] and heating at 90°C for 30 min

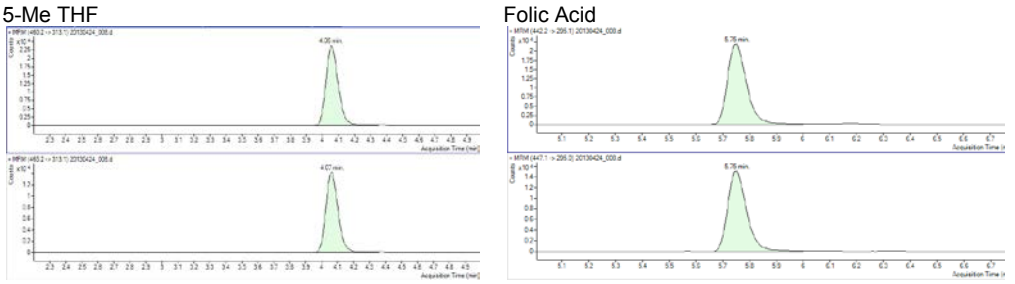

Figure 2013.13A. Representative chromatograms: Standard mix (concentration 100 ng/mL, corresponding to 33.33 µg/100 g in reconstituted product).

with stirring. Sample was then digested with protease solution (4 mg/mL) at 37°C for 30 min and transferred to a 100 mL volumetric flask with water. After filtration and addition of internal standard (IS), the filtrate was loaded on a strong anion exchange (SAX) cartridge, eluted, and evaporated at 50°C under nitrogen flow. Extracts were then reconstituted in 1.5 mL reconstitution solution (H_2O, 1% ascorbic acid, 0.5% DTT) and filtered through a 0.22 µm membrane into an amber LC vial for UHPLC-MS/MS analysis.

B. Apparatus

(**a**) *Column*.—UHPLC HSS T3, 1.8 µm; 2.1×150 mm (Waters Corp., Milford, MA) or equivalent.

(**b**) *Liquid chromatograph*.—Agilent 1290 Infinity (Agilent Technologies, Santa Clara, CA) or equivalent.

(**c**) *Detector*.—Agilent 6460 MS in positive electrospray ionization (ESI^+) mode operating at unit resolution, or equivalent.

(**d**) *Amber glassware*.—Standard laboratory Class A.

(**e**) *Micropipet*.—Adjustable (volumes from 2 to 20 µL) and disposable tips.

(**f**) *Micropipet*.—Adjustable (volumes from 10 to 100 µL) and disposable tips.

(**g**) *Micropipet*.—Adjustable (volumes from 100 to 1000 µL) and disposable tips.

(**h**) *Multipette® plus*.—Eppendorf (Hamburg, Germany), or equivalent.

(**i**) *Analytical balance*.—Precision 0.1 mg.

(**j**) *Homogenizer*.—Polytron 3100 (Kinematica, Lucerne, Switzerland), or equivalent.

(**k**) *pH meter*.—Mettler-Toledo (Columbus, OH), or equivalent.

(**l**) *Water bath (up to 90°C)*.—With magnetic stirrers (Labotech; DWB 16) or equivalent.

(**m**) *Folded filters*.—S&S 597½ (diameter 185 mm; Whatman, Piscataway, NJ), or equivalent.

(**n**) *Solid phase extraction (SPE) cartridges SAX*.—500 mg bed weight, 6 mL column volume, Supelco DSC-SAX (Supelco, St. Louis, MO) or Thermo HyperSep SAX (Thermo Scientific, Waltham, MA).

(**o**) *Disposable plastic syringe*.—10 mL (Becton Dickinson, Franklin Lakes, NJ), or equivalent.

(**p**) *Disposable plastic syringe*.—2 mL (Becton Dickinson), or equivalent.

(**q**) *Syringe-driven filter unit*.—0.22 µm, Millipore Millex GP (Bedford, MA), or equivalent.

(**r**) *HPLC amber vials*.—2 mL (Agilent Technologies), or equivalent.

C. Reagents

(**a**) *L-Ascorbic acid*.—Sigma (St. Louis, MO) A4544, or equivalent.

(**b**) *Ammonium acetate p.a.*—Merck (Darmstadt, Germany), or equivalent.

(**c**) *DTT*.—VWR (Radnor, PA), or equivalent.

(**d**) *Disodium hydrogen phosphate powder*.—VWR, or equivalent.

(**e**) *α-Amylase from porcine pancreas*.—Type VI, >10 units/mg (Sigma A3176), or equivalent.

(**f**) *α-Amylase from Bacillus subtilis*.—Approximately 50 units/mg (Fluka 10070; Buchs, Switzerland), or equivalent.

(**g**) *Protease from Streptomyces griseus*.—Type IV, >3.5 units/mg (Sigma P5147), or equivalent.

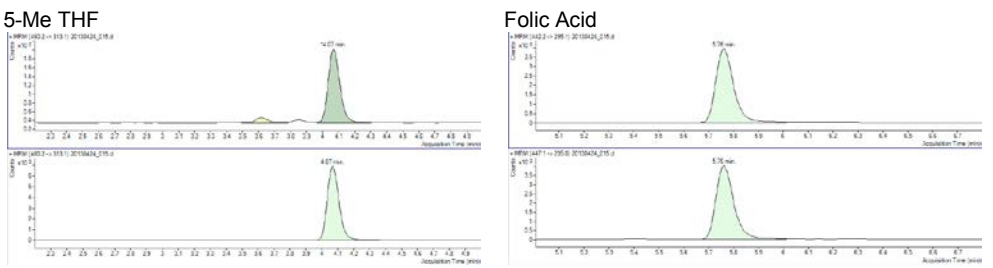

Figure 2013.13B. Representative chromatograms: USP SRM 1849 (Infant/Adult Nutritional Formula).

D. Solvents

(**a**) *Ethanol*.—HPLC grade (Merck), or equivalent.
(**b**) *Methanol*.—HPLC grade (Merck), or equivalent.
(**c**) *Isopropanol*.—LC-MS grade (Merck), or equivalent.
(**d**) *Acetonitrile*.—LC-MS grade (Merck), or equivalent.
(**e**) *Acetonitrile*.—HPLC grade (Sigma 34851).
(**f**) *Water*.—Milli Q (Millipore), or equivalent.

E. Solutions

(**a**) *Formic acid p.a*.—Merck, or equivalent.
(**b**) *Acetic acid glacial p.a.*—Merck, or equivalent.
(**c**) *Sodium hydroxide solution*.—1 M (Merck), or equivalent.
(**d**) *Hydrochloric acid*.—1 M (Merck), optional.
(**e**) *Hydrochloric acid*.—37% p.a. (Merck), or equivalent.
(**f**) *Ortho-phosphoric acid.*—85% (Merck), or equivalent.

F. Standards

(**a**) *Folic acid*.—Schirck Laboratories (Jona, Switzerland) 59-30-3, or equivalent.
(**b**) *(6R,S)-5-Me THF acid calcium salt*.—Schirck Laboratories 151533-22-1, or equivalent.
(**c**) *[$^{13}C_5$]-Folic acid*.—Merck, or equivalent.
(**d**) *[$^{13}C_5$]-(6S)-5-Me THF calcium salt.*—Merck, or equivalent.

G. Preparation of Solutions

(**a**) (*1*) *Mobile phase A*.—Acetic acid 0.5% (v/v) in water. Into a 1000 mL volumetric flask, add 5.00 mL acetic acid. Add about 800 mL water. Mix well. Make up to volume with water. This solution remains stable for 1 week at room temperature.

(*2*) *Mobile phase B*.—Acetonitrile.

(**b**) *Needle wash solvent.*—Water–acetonitrile–isopropanol (5+2+3) + 2% (v/v) formic acid. Into a 1000 mL bottle with cap, mix 500 mL water, 200 mL acetonitrile, and 300 mL isopropanol. Add 18 mL formic acid. Mix well. This solution remains stable for 1 month at room temperature. *Note*: Needle wash solvent is instrument-dependent. Solution to minimize carryover should be studied on each analytical system.

(**c**) *Extraction buffer.*—Sodium phosphate buffer 100 mmol/L, ascorbic acid 2% (w/v), DTT 0.1% (w/v), pH 4.5. Into a 1000 mL beaker, weigh 14.20 g disodium hydrogen phosphate (Na_2HPO_4), 20.0 g ascorbic acid, and 1.0 g DTT. Add about 800 mL water, dissolve, and adjust to pH 4.5 with *ortho*-phosphoric acid 85%. Transfer into a 1000 mL volumetric flask and make up to volume with water. This solution remains stable for 2 weeks at 4°C.

(**d**) *Protease solution*.—4 mg/mL in water. Into a 100 mL volumetric flask, weigh 400 mg protease. Dissolve and make up to volume with water. Prepare this solution fresh on the day of use.

(**e**) *SPE eluting solution*.—Acetonitrile–extraction buffer–acetic acid (6+3+1). Into a 250 mL bottle with cap, mix 150 mL acetonitrile, 75 mL extraction buffer, and 25 mL acetic acid using a measuring cylinder. This solution remains stable for 2 weeks at 4°C.

(**f**) (*1*) *Dissolution solution A*.—Sodium hydroxide 0.1 mol/L 5% (v/v)–ethanol 20% (v/v). Into a 100 mL volumetric flask containing about 50 mL water, mix 5.0 mL sodium hydroxide solution 1 mol/L and 20 mL ethanol. Make up to volume with water. This solution remains stable for 2 weeks at 4°C.

(*2*) *Dissolution solution B*.—Ammonium acetate 10 mmol/L, ascorbic acid 10% (w/v), DTT 2% (w/v)–methanol (1 + 3). Into a 50 mL beaker, weigh 38.5 mg ammonium acetate, 5.0 g ascorbic acid, and 1.0 g DTT. Add about 40 mL water, dissolve, and make up to volume with water.

Mix 50 mL of this solution with 150 mL methanol. This solution remains stable for 2 weeks at 4°C.

(*3*) *Dissolution solution C*.—Ascorbic acid 1% (w/v), DTT 0.5% (w/v). Into a 1000 mL volumetric flask, weigh 10.0 g ascorbic acid and 5.0 g DTT. Add about 800 mL water, dissolve, and make up to volume with water. This solution remains stable for 2 weeks at 4°C.

H. Other Standard Solutions

(**a**) *Folic acid stock standard solution*.—About 100 µg/mL. Into a 50 mL amber glass volumetric flask, weigh 5.00 ± 0.20 mg folic acid and record the mass to 0.01 mg. Dissolve and make up to volume with dissolution solution A. Store in aliquots flushed with N_2. This solution remains stable for 5 months at –20°C.

(**b**) *5-Me THF stock standard (approximately 100 µg/mL)*.—Into a 50 mL amber glass volumetric flask, weigh 5.00 ± 0.20 mg 5-Me THF acid calcium salt and record the mass to 0.01 mg. Dissolve and make up to volume with dissolution solution B. Store in aliquots flushed with N_2. This solution remains stable for 5 months at –20°C.

(**c**) *Standard Mix 1 (intermediate solution, 5000 ng/mL)*.—Into a 10 mL amber glass volumetric flask, transfer by pipetting the calculated amount of folic acid stock solution and the calculated amount of 5-Me THF (free form) stock solution to obtain an exact final concentration of folic acid and 5-Me THF in its free form of 500 ng/mL. Make up to volume with dissolution solution C. Store in aliquots flushed with N_2. This solution remains stable for 5 months at –20°C.

(**d**) *Standard Mix 2 (intermediate solution, 75 ng/mL)*.—Into a 10 mL amber glass volumetric flask, transfer by pipetting 150 µL of standard Mix 1. Make up to volume with dissolution solution C. Store in aliquots flushed with N_2. This solution remains stable for 3 months at –20°C.

(**e**) *[$^{13}C_5$]-Folic acid stock solution (approximately 200 µg/mL)*.—Into a 10 mL amber glass volumetric flask, weigh 2.00 ± 0.20 mg [$^{13}C_5$]-folic acid and record the mass to 0.01 mg. Dissolve and make up to volume with dissolution solution A. Store in aliquots flushed with N_2. This solution remains stable for 5 months at –20°C.

(**f**) *[$^{13}C_5$]-(6S)-5-Me THF IS stock solution (approximately 200 µg/mL)*.—Into a 10 mL amber glass volumetric flask, weigh 2.00 ± 0.20 mg [$^{13}C_5$]-(6S)-5-Me THF calcium salt and record the mass to 0.01 mg. Dissolve and make up to volume with dissolution solution B. Store in aliquots flushed with N_2. This solution remains stable for 5 months at –20°C.

(**g**) *IS mix working solution (5000 ng/mL)*.—Into a 10 mL amber glass volumetric flask, transfer by pipetting the calculated amount of folic acid IS stock solution and the calculated amount of 5-Me THF IS (free form) stock solution to obtain an exact final concentration of folic acid and 5-Me THF IS in its free form of 500 ng/mL. Make up to volume with dissolution solution C.

Table 2013.13. MS/MS transitions for folic acid and 5-Me THF on Agilent 6460

Time range, min	Analyte	Q1	Q3	Fragment or voltage, V	Collision energy, eV
2.0–5.0	5-Me THF (Q)	460.2	313.1	108	14
2.0–5.0	5-Me THF (q)	460.2	180.0	108	42
2.0–5.0	5-Me THF IS	465.2	313.1	120	15
5.0–8.0	Folic acid (Q)	442.2	295.1	90	10
5.0–8.0	Folic acid (q)	442.2	176.0	90	40
5.0–8.0	Folic acid IS	447.1	295.0	92	10

Store in aliquots flushed with N_2. This solution remains stable for 5 months at –20°C.

(**h**) *Working standards*.—Working standard solutions, 1 to 400 ng/mL. Into separate 5 mL amber glass volumetric flasks, transfer by pipetting the appropriate volume of standard Mix 1 or standard Mix 2 and IS mix working solution. Make up to volume with dissolution solution C. The final concentration of folic acid or 5-Me THF in the working standard solution ranges from 1 to 400 ng/mL with an IS concentration of 50 ng/mL.

I. Sample Preparation

Sample reconstitution.—Powder samples were reconstituted by dissolving 25 g powder sample and 50 mg α-amylase in 200 g warm water (40°C). The SRM was reconstituted by dissolving 10 g powder and 50 mg α-amylase in 90 g warm water (40°C). The samples were digested at 40°C for 15 min to let the enzyme work.

J. Extraction

(**a**) An aliquot of 15 g reconstituted sample or 15 g reconstituted RTF sample was weighed into a 100 mL amber glass volumetric flask.

(**b**) 40 mL extraction buffer (100 mmol/L phosphate buffer; 2% ascorbic acid; 0.1% DTT; pH 4.5) was added and the flask was then heated at 90°C for 30 min, while stirring.

(**c**) After cooling to room temperature, 2 mL protease solution (4 mg/mL) was added and incubation was carried out in a water bath at 37°C for 30 min.

(**d**) After cooling to room temperature, the volume was made up to the mark with water.

(**e**) After filtration through folded paper filter, 10 mL filtrate was transferred to a 10 mL amber glass volumetric flask and 50 µL of 5 µg/mL IS solution was added.

(**f**) From this solution, 3 mL was loaded on an SAX cartridge (previously conditioned with 4 mL acetonitrile and equilibrated with 10 mL extraction buffer).

(**g**) After loading, the cartridge was washed with 6 mL extraction buffer and analytes were then eluted with 4 mL SPE eluting solution into amber glass tubes.

(**h**) Eluate was then evaporated under controlled temperature at 55°C and nitrogen flow.

(**i**) Extracts were then reconstituted in 1.5 mL reconstitution solution (H_2O, 1% ascorbic acid, 0.5% DTT) and filtered through 0.22 µm membrane into an amber LC vial.

K. Instrument Operating Conditions

(**a**) *UHPLC conditions*.—5 µL of the reconstituted extract was injected onto an UHPLC system (Agilent 1290 Infinity) equipped with a Waters UHPLC HSS T3, 1.8 µm, 2.1 × 150 mm column. Mobile phase A consisted of H_2O, 0.5% acetic acid. Mobile phase B was acetonitrile. Following injection, isocratic conditions of 0% of solvent B were initially used for 0.5 min, then a step direct to 10% of solvent B was achieved in 0.1 min. Isocratic conditions of 10% solvent B were held for 1.4 min and followed by a linear gradient to 25% solvent B for 3.5 min. Then, a step directly at 99% B was achieved in 0.1 min and held for 1.9 min before going back to start conditions (0% of solvent B) in 0.1 min. Start conditions were kept for 2.4 min.

(**b**) *Mass spectrometer conditions*.—Mass spectrometry was performed on an Agilent 6460 MS in ESI + mode operating at unit resolution. ESI capillary voltage was set at 3.5 kV; nozzle voltage, 600 V; gas temperature, 300°C; sheath gas temperature, 350°C; gas flow, 10 L/min; sheath gas flow, 12 L/min; nebulizer pressure 30 psi.

Multiple-reaction monitoring mode was applied for quantification and compound identification confirmation. The transitions are shown in Table **2013.13**.

The dwell times were set up at 100, 200, and 75 msec for quantifier (Q), qualifier (q), and ISs, respectively.

L. Calculations

To accurately calculate the final folic acid (FA) concentration (expressed in µg/mL) of the stock solution, consider the following: purity and water content.

Calculate final concentration as follows: Purity: *x*%, water content: *y*%, and weight: *z* mg.

$$\text{FA concn} = [z \times 1000 \times (x/100) \times (1 - (y/100))]/50$$

To express the final 5-Me THF concentration (expressed in µg/mL) of the stock solution in its free form, consider the following: purity, water content, molecular weight (MW) of the salt form, and MW of the free form.

Calculate final concentration as follows: Purity: *x*%, water content: *y*%, weight: *z* mg, MW salt: 497.50 g/mol, and MW free form: 459.55 g/mol.

$$\text{5-Me THF concn} = [z \times (459.55/497.50) \times 1000 \times (x/100) \times (1 - (y/100))]/50$$

Calculate the FA and 5-Me THF final content (= w1) separately, in mg/100 g of product, using the following equation:

For powder samples:

$$w1 = C \times \frac{(m_1 + m_2) \times V_1 \times V_3 \times 100}{m_1 \times m_3 \times V_2 \times 1000}$$

where C = concentration in the test solution (ng/mL) of FA or 5-Me THF, calculated using the dedicated calibration curve; m_1 = mass of the sample weight for slurry, in g (= 25 g); m_2 = mass of water weight to prepare the slurry, in g

(= 200 g); m_3 = mass of the test portion, in g (= 15 g); V_1 = volume of the of sample extract, in mL (= 100 mL); V_2 = volume of sample loaded on SPE, in mL (= 3.0 mL); V_3 = volume of the reconstituted sample, in mL (= 1.5 mL); 100 = conversion to 100 g basis; 1000 = conversion from ng to µg.

For liquid samples:

$$w1 = C \times \frac{V_1 \times V_3 \times 100}{m_3 \times V_2 \times 1000}$$

where C = concentration in the test solution (ng/mL) of FA or 5-Me THF, calculated using the dedicated calibration curve; m_3 = mass of the test portion, in g (= 15 g); V_1 = volume of the of sample extract, in mL (= 100 mL); V_2 = volume of sample loaded on SPE, in mL (= 3.0 mL); V_3 = volume of the reconstituted sample, in mL (= 1.5 mL); 100 = conversion to 100 g basis; 1000 = conversion from ng to µg.

Verify that FA and 5-Me THF software calculated concentrations are >lower LOQ (LLOQ) and <upper LOQ (ULOQ).

If calculated concentration is <LLOQ, then this concentration cannot be taken into account for vitamin B_9 concentration.

Folate (vitamin B_9) concentration is the sum of folic acid plus 5-Me THF. Results in µg/100 g are expressed as folic acid in reconstituted product.

M. System Suitability

RSD of retention time and peak area should not be higher than 5% for FA and 5-Me THF.

N. Specificity

A tandem mass spectrometer was chosen as detection mode. Optimization consisted of selecting the precursor ion as well as the two main product ions for each analyte. The transition precursor ion/main product was defined as quantifier. The transition precursor ion/second main product ion was defined as qualifier. The ratio quantifier/qualifier was followed in all series with a defined limit to confirm peak identification.

Results

The validation study was conducted in accordance with the SPIFAN SLV Guidelines (3). System suitability was determined by checking retention times and peak shape for each analyte. RSD of retention time was below 0.5% for both folic acid and 5-Me THF in all analytical series. For specificity, native folate content was analyzed and calculated in three placebo samples supplied with the SPIFAN test material kit. A tandem mass spectrometer was chosen as detection mode. To confirm peak identification, the quantifier/qualifier ratio was followed in all series with a defined limit. Linearity was demonstrated by testing working solutions at 10 levels ranging from 0.3 to 9.0 ng/mL, each injected once, and resulting in $R^2 > 0.99$. A calibration range from 1 to 400 ng/mL was injected at the beginning of each analytical series. The SD of calibration point "STD 1" was <20%, while points "STD 2 to 8" were <15%. LOD and LOQ were estimated by performing 10 independent analysis of nonfortified sample (infant formula RTF, milk-based), spiked at low levels. LODs

Table 1. Precision results expressed in reconstituted product

Matrix[a]	Folate mean, µg/100 g	RSD_r, %[b]	RSD_{iR}, %[c]
SRM 1849	22.4	1.3	4.2
Adult nutritional powder milk protein-based	27.9	1.9	4.6
Infant formula powder part hyd milk-based	15.1	1.4	2.8
Infant formula powder part hyd soy-based	16.8	1.5	3.6
Adult nutritional powder low-fat	33.5	0.84	4.4
Child formula powder	18.6	4.0	5.1
Infant elemental powder	28.2	0.93	4.4
Infant formula powder milk-based	17.0	2.1	3.7
Infant formula powder soy-based	16.0	2.2	3.8
Infant formula RTF milk-based	16.1	2.0	5.3
Adult nutritional RTF high-protein	51.4	5.2	5.4
Adult nutritional RTF high-fat	57.9	2.4	5.1

[a] Fortified samples provided in the SPIFAN test material kit.

[b] RSD_r is the RSD of repeatability.

[c] RSD_{iR} is the RSD of intermediate reproducibility.

Table 2. Recovery results of spiking experiments for folic acid

		+50% of target		+100% of target	
	Target, µg/100 g	Avg., %	RSD, %	Avg., %	RSD, %
Nonfortified products					
Child formula powder	165	91	4.2	90	2.7
Infant elemental powder	240	94	0.36	94	3.1
Infant formula RTF, milk	20	90	10.4	95	3.8
Fortified products					
AN RTF high-fat	60	90	8.0	89	6.2
AN RTF high-protein	16.7	111	28	102	6.8

Table 3. Recovery results of spiking experiments for 5-Me THF

		+50% of target		+100% of target	
	Target (µg/100 g)	Avg., %	RSD, %	Avg., %	RSD, %
Nonfortified products					
Child formula powder	165	103	14.3	95	1.2
Infant elemental powder	240	98	1.5	99	1.7
Infant formula RTF, milk	20	96	7.4	101	2.1
Fortified products					
AN RTF high-fat	60	93	2.2	92	2.8
AN RTF high-protein	16.7	110	14	114	18

were 0.10 µg/100 g and 0.05 µg/100 g (expressed in product as reconstituted) for folic acid and 5-Me THF, respectively, and LOQs were 0.33 µg/100 g and 0.10 µg/100 g, respectively. Lower LODs and LOQs were achieved for 5-Me THF due to better ionization of this compound. Results of precision studies for folate expressed in reconstituted products are presented in Table 1. RSD_r ranged from 0.84 to 5.2%, and RSD_{iR} ranged from 2.8 to 5.4%, fulfilling the ≤7% and ≤16% performance requirements. Accuracy was proven by analyzing SRM 1849 in duplicate on 7 different days, and comparing the overall mean to the reference value. The overall mean was 22.4 µg/100 g for the reconstituted product with an SD of the mean duplicates of 0.3 µg/100 g, corresponding to 202 µg/100 g in powder with an SD of 2.69 µg/100 g, and matching the reference value of 211 ± 13 µg/100 g. Spike recovery was conducted on three nonfortified infant/child products and two fortified adult nutritional (AN) products. Fortified products were used because nonfortified products were not available. Samples were spiked with folic acid and 5-Me THF at 50 and 100% of target values. Spiked and nonspiked samples were analyzed in duplicate on three different days by two different analysts. Recovery results of the spiking experiments are shown in Tables 2 and 3. Recovery rates complied with the SMPR (90–110%) with the exception of AN RTF high-fat and AN RTF high-protein. The AN products were already fortified, and thus reached higher than intended levels; however, the levels were still within the calibration range.

Based on the results of this SLV study, the UHPLC-MS/MS method for the determination of folate was granted First Action status and adopted as AOAC *Official Method*^{SM} **2013.13**. The method will continue to be monitored for consideration as Final Action in the future.

References

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(3) *Official Methods of Analysis* (2012) 19th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, Appendix L: AOAC Recommended Guidelines for Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN) Single-Laboratory Validation. http://www.eoma.aoac.org

FOOD BIOLOGICAL CONTAMINANTS

Evaluation of the ANSR® for *Salmonella* Assay for Identification of *Salmonella* spp. from Colony Picks from Selective/Differential Agar Media: First Action 2013.14

MARK MOZOLA, MAXIMILIAN BOTIMER, CAROLYN JAGADICS, PAUL NORTON, OSCAR CABALLERO, NICOLE ENSLIN, PREETHA BISWAS, and JENNIFER RICE
Neogen Corp., 620 Lesher Pl, Lansing, MI 48912

Collaborators: E.S. Adams, H. Alnughaymishi, J.B. Barrett, J. Benzinger, M.E. Berrang, M. Boyle, J. Cannon, D. Clark, A. Copeland, M. Corebello, D.E. Cosby, N.A. Cox, N. Cuthbert, H. Dammann, J. Dyszel, E. Feldpausch, J. Flannery, C. Flores, J.G. Frye, R. Fuller, V. Gill, L.M. Hiott, B. Howard, M. Hudgens, C.R. Jackson, W. Jones, S.W. Knapp, L. Kuepfer, P. Kulkarni, B. Kupski, C. Pidgeon, A. Quenneville, L.L. Rigsby, N. Rogman, E. Sai, A. Scollon, M. Sisemore, J. Stepnitz, J. Van Brunt, M. Vross, D. Waltman, H. Wang, G. Whitbeck, S. York, L. Zhang

A collaborative study was conducted to evaluate performance of the ANSR® for *Salmonella* assay for identification of *Salmonella* spp. from colony picks taken from selective/differential agar media. The ANSR *Salmonella* assay is an isothermal nucleic acid amplification test based on the nicking enzyme amplification reaction chemistry. The test can be completed in less than 40 min including sample preparation. A total of 18 laboratories representing industry, government, academic, and commercial testing laboratories participated in the study. Each collaborator tested up to 84 samples, comprised of colony picks of six *Salmonella* spp. and six non-salmonellae taken from six selective/differential agar media as well as tryptic soy agar. A total of 1441 analyses were performed, 1416 of which gave the correct identification, for overall accuracy of 98.3%. For identification of *Salmonella* spp., 755 of 756 tests (99.9%) produced the correct result. For identification of non-salmonellae as such, 661 of 685 assays (96.5%) produced the correct result. Of the 18 laboratories, 15 produced data sets with 99–100% accuracy. The majority of false-positive results were clustered in three laboratories; analysis of raw data suggests procedural difficulties in at least two cases, which may explain the atypical data from these collaborators. The ANSR *Salmonella* assay can be used as a rapid, accurate adjunct or alternative to biochemical testing for identification of presumptive *Salmonella* spp. isolates.

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The method was approved by the Expert Review Panel for Food Biological Contaminants as First Action.
The Expert Review Panel for Food Biological Contaminants invites method users to provide feedback on the First Action methods. Feedback from method users will help verify that the methods are fit for purpose and are critical to gaining global recognition and acceptance of the methods. Comments can be sent directly to the corresponding author or methodfeedback@aoac.org.
Corresponding author's e-mail: mmozola@neogen.com
An appendix is available on the *J. AOAC Int.* website, http://aoac.publisher.ingentaconnect.com/content/aoac/jaoac
DOI: 10.5740/jaoacint.14-004

Identification of presumptive *Salmonella* colonies from selective/differential agar media as *Salmonella* spp. has historically been achieved using a variety of biochemical and serological procedures. In the case of food and environmental sample analysis, these procedures are specified in reference methods such as those in the U.S. Food and Drug Administration's *Bacteriological Analytical Manual* (BAM; 1) and the U.S. Department of Agriculture's *Microbiology Laboratory Guidebook* (MLG; 2). These methods include conventional biochemical tests, miniaturized biochemical test devices, automated biochemical identification platforms, and serological agglutination tests using *Salmonella*-specific antisera. The biochemical identification procedures, although accurate and reliable, generally require 6–24 h to obtain results. The serological procedures may be rapid, but often require subculture to enhance antigen expression, especially in the case of flagellar (H) antigen typing.

As an adjunct or alternative to biochemical and serological procedures, nucleic acid-based identification methods hold promise for providing timely and accurate results. This has been acknowledged, for example, by reference in both BAM and MLG to use of nucleic acid-based methods for identification of *Listeria monocytogenes* (3, 4).

The ANSR® *Salmonella* assay was originally developed for rapid screening of enriched food and environmental samples. The assay is an isothermal nucleic acid amplification procedure, based on the nicking enzyme amplification reaction (NEAR) technology (5). The ANSR method has been evaluated in three AOAC *Performance Tested Method*℠ (PTM) validation studies, leading to certification as PTM 061203, with claims for a variety of food and environmental sample types (6–7). In these studies, the ANSR method exhibited sensitivity comparable to that of the BAM and MLG reference culture methods by probability of detection statistical analysis, as well as >99% inclusivity and 100% exclusivity in testing of target and nontarget bacteria.

This method performance, coupled with the simplicity and rapidity of the assay (less than 40 min), suggested that the method could also serve as a useful tool for identification of presumptive *Salmonella* spp. isolates from selective/differential agar plating media. A precollaborative study has been completed in which colonies of 113 *Salmonella* spp. strains and 37 non-*Salmonella* strains were picked from tryptic soy agar (TSA) and six selective/differential agar media [Hektoen enteric agar (HE), xylose lysine

Table 1. Inclusive and exclusive isolates used in the ANSR *Salmonella* collaborative study

Organism	ID No.	Source	Origin (if known)
Salmonella enterica subsp. *arizonae*	700156	ATCC[a]	Poult
Salmonella enterica subsp. *enterica* Ser. Typhimurium	23566	ATCC	Unknown
Salmonella enterica subsp. *enterica* Ser. Cubana	12007	ATCC	Unknown
Salmonella bongori	43975	ATCC	Unknown
Salmonella enterica subsp. *enterica* Ser. Cerro	10723	ATCC	Unknown
Salmonella enterica subsp. *enterica* Ser. Enteritidis	4931	ATCC	Human GI tract
Enterobacter cloacae	13047	ATCC	Human CSF
Escherichia coli	25922	ATCC	Human
Proteus vulgaris	29905	ATCC	Unknown
Providencia alcalifaciens	27970	ATCC	Feces
Citrobacter freundii	8090	ATCC	Unknown
Klebsiella pneumoniae	13883	ATCC	Unknown

[a] American Type Culture Collection, Manassas, VA.

deoxycholate agar (XLD), bismuth sulfite agar (BS), brilliant green sulfa agar (BGS), xylose lysine tergitol agar (XLT-4), and double-modified lysine iron agar (DMLIA)] and tested in the ANSR assay. The former three media are specified for use in the BAM reference method, while the latter three are specified in the MLG method. One hundred and twelve *Salmonella* spp. strains produced positive results from all seven media, for inclusivity of 99.1%. One strain of *S.* Weslaco, previously identified as a non-inclusive strain lacking the genetic target for the ANSR assay, produced negative results from all seven media. In testing of exclusive strains, 248 of 251 assays produced negative results, for accuracy of 98.8%. The precollaborative study report is included as Appendix I on *J. AOAC Int.* website, http://aoac.publisher.ingentaconnect.com/content/aoac/jaoac.

Here we report results of an interlaboratory collaborative study conducted in 18 laboratories for further evaluation of the assay as a colony confirmation tool.

Collaborative Study

Study Design

This collaborative study was conducted in accordance with the *AOAC INTERNATIONAL Methods Committee Guidelines for Validation of Microbiological Methods for Food and Environmental Surfaces, Appendix J* (8). Eighteen laboratories participated in the collaborative study, representing industry, academic, government, and private testing laboratories. All collaborators were either established users of the ANSR test system or were expressly trained for the collaborative study prior to its commencement. A detailed set of instructions and data recording forms were sent to each collaborator in advance of the study. Collaborators were provided with all necessary agar plating media, test kits, ANSR system instrumentation, and a blind-coded set of 12 bacterial cultures for analysis.

Preparation of Isolates

All isolates were from the Neogen Corp. culture collection and consisted of six diverse strains of *S. enterica* and *S. bongori*, and six strains of Enterobacteriaceae belonging to other genera (Table 1). All strains were obtained directly from the American Type Culture Collection (ATCC; Manassas, VA). Identity of isolates was confirmed by API 20E testing. *Salmonella* isolates were also verified by O group serology. Isolates were cultured on TSA slants for 18–24 h at 36 ± 1°C. Slant cultures were labeled with a two-digit alphabetical code.

Distribution of Isolates

Cultures were shipped to collaborators via overnight delivery, at ambient temperature, using Category B Dangerous Goods packaging as set forth by International Air Transport Association regulations. Collaborators were instructed to store the cultures at 2–8°C until initiation of the analytical work (4–5 days). Collaborators were provided with a "Sample Receipt Form," to be completed and returned to the Study Director by email or fax, acknowledging that the samples were received in good condition.

Analysis of Isolates

To initiate the analysis, collaborators streaked each of the 12 bacterial isolates to each of the seven agar media, streaking for isolated colonies. Collaborators were provided with a sample randomization scheme by the Study Director and were instructed to blind-code each strain-agar medium combination with a unique number 1–84. This was performed by "Operator 1," who would have no involvement in the actual ANSR analyses. Plates were incubated for 24±2 h at 35±1°C and examined for the presence of isolated colonies. Plates without isolated colonies were reincubated for an additional 18–24 h. Plates containing distinct isolated colonies after 24 h were stored at 2–8°C. After a maximum of 48 h incubation, plates without growth or isolated colonies were noted as such on the Data Recording Form and analysis continued. Operator 1 then picked a single colony from each plate, including the refrigerated plates, using an inoculating loop or needle, and resuspended the colony in 0.5 mL phosphate-buffered saline (PBS). The coded tubes were transferred to "Operator 2," who then performed the ANSR analyses. ANSR testing was performed in blocks of up to 16 samples, starting with sample number 1 and continuing through sample number

84. Completed Data Recording Forms were returned to the Study Director by email or fax. ANSR assay raw data were provided to the Study Director by email as .json files. This raw data included the real-time fluorescence curves for each assay performed.

AOAC Official Method 2013.14
Identification of *Salmonella* spp. from Colony Picks
ANSR® *Salmonella* Confirmation Test
First Action 2013

(Applicable to the identification of *Salmonella* spp. from colony picks from selective/differential agar media: Bismuth sulfite agar, brilliant green sulfa agar, double-modified lysine iron agar, Hektoen enteric agar, tryptic soy agar, xylose lysine deoxycholate agar, and xylose lysine tergitol agar.)

See Tables **2013.14A** and **B** for a summary of results of the collaborative study.

Safety precautions.—Use of this test should be restricted to individuals with appropriate laboratory training in microbiology and molecular techniques. Reagents are for laboratory use only. Refer to the Material Safety Data Sheet from Neogen Corp. for more information. Enrichment cultures, used agar plates, and ANSR assay lysates and reaction tubes should be handled and disposed of as potentially infectious material and Biosafety Level 2 measures employed. The preferred method for disposal of contaminated materials, including cultures, pipet tips, tubes, etc., is autoclaving. Items that cannot be autoclaved should be decontaminated by treatment with disinfectant solution. ANSR reaction tubes should not be autoclaved in areas where they may open and possibly contaminate the laboratory environment with amplification products. Alternatively, they may be disposed of in a sealed container with a small amount of 10% household bleach added.

A. Principle

ANSR *Salmonella* is an isothermal nucleic acid amplification assay based on the nicking enzyme amplification reaction (NEAR) technology (5). The amplification mechanism involves binding of an oligonucleotide "template" to a specific sequence of target DNA. The template contains a recognition site for a specific endonuclease. The nicked strand is recognized as damaged and repaired by the action of a thermostable DNA polymerase, displacing the original strand with the newly-synthesized repaired portion. This displaced DNA "product" then binds to a second template and the same reactions lead to formation of a second product. Amplification products are detected using a specific molecular beacon probe. Fluorescent signal is generated in real time, with amplification and detection complete within 10 min. The entire assay is conducted at a constant temperature of 56°C using a temperature-controlled fluorescence detection instrument. Assay software analyzes the fluorescent signal over time; a data interpretation algorithm interprets results as negative, positive, or invalid based on baseline, rate-of-change, and other criteria. Each tube of ANSR reagents also contains an internal positive control, signaling in a second fluorescence channel irrespective of the presence of target DNA, and indicating proper functioning of the amplification reagents.

Table 2013.14A. Interlaboratory study results for the ANSR *Salmonella* test: Inclusive isolates

Organism	Correct	Misidentified	Total
Salmonella enterica subsp. *arizonae*	126	0	126
Salmonella enterica subsp. *enterica* Ser. Typhimurium	126	0	126
Salmonella enterica subsp. *enterica* Ser. Cubana	126	0	126
Salmonella bongori	126	0	126
Salmonella enterica subsp. *enterica* Ser. Cerro	126	0	126
Salmonella enterica subsp. *enterica* Ser. Enteritidis	125	1	126
Total isolates	755	1	756

B. Media and Reagents

(**a**) *ANSR® for Salmonella test kit.*—Available from Neogen Corp., Cat. No. 9843 (Lansing, MI, www.neogen.com). Contains: Lyophilized reagents in capped strip tubes, eight tubes per strip, 12 strips (96 tests) per kit, in two sealed foil pouches with desiccant packs; cluster tubes, eight tubes per strip, 12 strips per kit; permanent caps, eight caps per strip, 12 strips per kit; lysis buffer, one bottle, 60 mL; lysis reagent, three vials, lyophilized; kit insert. Store reagent tubes at 2–8°C, in sealed foil pouches with desiccant. Store lysis buffer at 2–8°C.

(**b**) *Phosphate-buffered saline (PBS).*—Per liter: 8.0 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4.

(**c**) *Hektoen enteric agar (HE).*—Available from Neogen Corp. and other suppliers. Follow manufacturer's instructions for preparation.

(**d**) *Xylose lysine deoxycholate agar (XLD).*—Available from Neogen Corp. and other suppliers. Follow manufacturer's instructions for preparation.

(**e**) *Bismuth sulfite agar (BS).*—Available from Neogen Corp. and other suppliers. Follow manufacturer's instructions for preparation.

(**f**) *Brilliant green sulfa agar (BGS).*—Available from Neogen Corp. and other suppliers. Follow manufacturer's instructions for preparation.

(**g**) *Xylose lysine tergitol agar (XLT-4).*—Available from Neogen Corp. and other suppliers. Follow manufacturer's instructions for preparation.

(**h**) *Double-modified lysine iron agar (DMLIA).*—Available

Table 2013.14B. Interlaboratory study results for the ANSR *Salmonella* test: Exclusive isolates

Organism	Correct	Misidentified	Total
Enterobacter cloacae	96	2	98
Escherichia coli	117	8	125
Proteus vulgaris	102	4	106
Providencia alcalifaciens	105	2	107
Citrobacter freundii	122	4	126
Klebsiella pneumoniae	119	4	123
Total isolates	661	24	685

from various suppliers. Follow manufacturer's instructions for preparation.

(**i**) *Tryptic soy agar (TSA).*—Available from Neogen Corp. and other suppliers. Follow manufacturer's instructions for preparation.

C. Apparatus

(**a**) *Incubator/reader.*—Available from Neogen Corp. Incubator/reader capable of operating at 56 ± 1°C and reading fluorescence in real time in two channels (485/535 nm and 540/590 nm).

(**b**) *Computer and ANSR software.*—Available from Neogen Corp. For connection to incubator/reader. Minimum requirements for computer: Intel® Core i3 processor, 1 GB RAM, Windows® 7, Ethernet, and USB connections.

(**c**) *Heater block.*—With insert for 1.2 mL cluster tubes, 80 ± 2°C.

(**d**) *Micropipettor.*—50 µL, fixed or adjustable volume.

(**e**) *Pipettor.*—100–1000 µL, adjustable volume.

(**f**) *8-Channel micropipettor.*—20–200 µL, adjustable volume.

(**g**) *Pipet tips.*—100 µL, with filter.

(**h**) *Pipet tips.*—1000 µL.

(**i**) *Tubes.*—Glass or plastic, 12 × 75 mm or similar, sterile, with caps.

(**j**) *Inoculating loops or needles.*—Sterile.

D. Preparation of Test Samples

Pick an isolated colony from nonselective or selective/differential agar medium (one of the media listed in section **B**) with an inoculating loop or needle and resuspend (vortex or otherwise thoroughly mix) in 0.5 mL PBS in a sterile, capped tube.

E. Test Procedure

(**a**) *General preparation.*—(*1*) This assay should be performed in a controlled laboratory environment.

(*2*) Do not use culture media or ANSR reagents beyond their expiration dates. Do not interchange reagents between ANSR kit lots.

(*3*) Remove ANSR reaction tubes from the foil pouch just before use. Avoid prolonged exposure to light. Tap reaction tubes on bench top to make sure that lyophilized reagents are at the bottom of the tube prior to adding the lysed sample.

(*4*) Complete all assay steps in sequence, avoiding delays between steps.

(*5*) Exercise care in pipetting steps to avoid cross-contamination of samples.

(*6*) Do not remove caps from reaction tubes at any point after the assay is started; this will prevent accidental contamination of the environment with amplification products.

(*7*) *Prior to starting the assay.*—(*i*) Preheat the lysis heater block to 80 ± 2°C. (*ii*) Start the ANSR software using the computer connected to the ANSR reader. Select "*Salmonella*" as the test type. Enter sample identifications and other experiment information. The reader will preheat to 56 ± 1°C.

(**b**) *Assay procedure.*—(*1*) Add 50 µL of colony resuspension to a 1.2 mL cluster tube. Use a new pipet tip for each sample.

(*2*) Add 450 µL lysis buffer to the cluster tube. *Note*: It is not necessary to use the lysis reagent provided with the test kit for this application.

(*3*) Transfer the cluster tubes to the 80°C heater block and incubate for 20 min. *Note:* The incubation time may be extended to a maximum of 60 min for the purpose of managing staggered assay start times.

(*4*) Approximately 3 min before the end of the lysis step, preheat the ANSR reaction tubes to 56°C by placing the tubes in the incubator/reader. *Note:* The strip of tubes may be cut to provide the number of tubes needed.

(*5*) At the end of the 20 min lysis incubation, remove and discard the caps from the reaction tubes.

Note: Steps (*6*)–(*8*) should be completed without delay (within 1 min).

(*6*) Using an 8-channel micropipettor and 100 µL tips with filters, carefully transfer 50 µL of the lysed samples to the reaction tubes. Mix by rapidly pipetting up and down at least 10 times until the sample appears homogenous in the pipet tip. Avoid excessive bubble formation by not depressing the pipettor plunger beyond the first stop.

(*7*) Place the permanent caps on the reaction tubes and close the lid of the incubator/reader.

(*8*) Click START in the ANSR software to begin the assay.

(*9*) The assay will complete in 10 min and results will be displayed.

F. Interpretation of Results

The ANSR software will indicate the test results as POSITIVE, NEGATIVE, or INVALID. A positive result indicates that the colony tested contains *Salmonella* spp. A negative result indicates that the colony tested does not contain *Salmonella* spp. Assays producing invalid results must be repeated. The real-time fluorescence curves for both the test and positive control channel can be viewed using the ANSR software.

G. Limitations

The assay detects serovars of both *S. enterica* and *S. bongori*, including all genetic subgroups. In testing of 113 strains of *Salmonella* spp., representing 108 serovars, only a single strain of *S.* Weslaco was not detected.

Results

A summary of results for inclusive and exclusive isolates is shown in Tables **2013.14A** and **B**, respectively. Detailed results, by collaborating laboratory, are shown in Tables 2 and 3. For inclusive strains, all collaborators reported that all six strains grew on all media and a total of 756 ANSR analyses were performed. There were 755 positive results, for accuracy of 99.9% in identification of presumptive *Salmonella* spp. colonies. Laboratory 2 reported a negative result for *S.* Enteritidis on HE agar. There is no obvious explanation for this result.

There were a maximum of 756 possible results on exclusive strains. There were 65 cases of reported no growth or lack of distinct isolated colonies. A detailed analysis of results showed that three collaborators (laboratories 3, 12, and 13) reported no growth for the *Enterobacter cloacae* culture on all seven media, accounting for 21 of the no-growth results. Most collaborators reported that neither *Providencia alcalifaciens* nor *Proteus*

Table 2. Inclusivity panel results[a]

Organism	Culture medium[b]	Collaborating laboratory																	
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
S. enterica subsp. *arizonae*	BGS	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	BS	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	DMLIA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	HE	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	TSA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	XLD	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	XLT-4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
S. enterica Ser. Typhimurium	BGS	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	BS	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	DMLIA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	HE	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	TSA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	XLD	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	XLT-4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
S. enterica Ser. Cubana	BGS	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	BS	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	DMLIA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	HE	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	TSA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	XLD	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	XLT-4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
S. bongori	BGS	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	BS	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	DMLIA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	HE	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	TSA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	XLD	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	XLT-4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
S. enterica Ser. Cerro	BGS	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	BS	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	DMLIA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	HE	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	TSA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	XLD	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	XLT-4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
S. enterica Ser. Enteritidis	BGS	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	BS	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	DMLIA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	HE	+	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	TSA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	XLD	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	XLT-4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

[a] + = Correctly identified as *Salmonella* spp.; – = incorrectly identified.

[b] BGS = brilliant green sulfa agar; BS = bismuth sulfite agar; DMLIA = double-modified lysine iron agar; HE = Hektoen enteric agar; TSA = tryptic soy agar; XLD = xylose lysine deoxycholate agar; XLT-4 = xylose lysine tergitol agar.

Table 3. Exclusivity panel results[a]

Organism	Culture medium[b]	Collaborating laboratory																	
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Enterobacter cloacae	BGS	–	–	NG[c]	–	–	–	–	–	–	–	+	NG	NG	–	–	+[d]	–	–
	BS	–	–	NG	–	–	–	–	–	–	–	–	NG	NG	–	–	+[d]	–	–
	DMLIA	–	–	NG	–	–	–	–	–	–	–	–	NG	NG	–	–	+[d]	–	–
	HE	–	–	NG	–	–	–	–	–	–	–	–	NG	NG	–	–	+[d]	–	–
	TSA	–	–	NG	–	–	–	–	–	–	–	–	NG	NG	–	–	+[d]	–	–
	XLD	–	+	NG	–	–	–	–	–	–	–	–	NG	NG	–	–	+[d]	–	–
	XLT-4	–	–	NG	–	–	–	–	–	–	–	–	NG	NG	–	–	NG	–	–
Escherichia coli	BGS	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	+	–	–
	BS	–	–	–	–	–	–	+	–	–	–	–	–	+	–	–	–	–	–
	DMLIA	–	+	–	–	–	–	–	–	–	–	–	–	–	NG	–	–	–	–
	HE	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–
	TSA	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	XLD	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	XLT-4	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Proteus vulgaris	BGS	–	–	–	–	–	–	–	–	–	–	–	–	–	NG	–	–	–	+
	BS	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–
	DMLIA	–	–	–	–	–	–	–	–	NG	–	–	–	–	NG	–	–	–	–
	HE	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–
	TSA	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–
	XLD	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	XLT-4	NG	NG	–	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG
Providencia alcalifaciens	BGS	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–
	BS	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–
	DMLIA	–	–	–	NG	–	–	–	–	NG	–	–	NG	–	NG	–	–	–	–
	HE	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	TSA	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	XLD	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	XLT-4	–	NG	–	NG	NG	NG	–	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG
Citrobacter freundii	BGS	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	BS	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	DMLIA	–	–	–	–	+	–	–	–	–	–	–	–	–	–	+	–	–	–
	HE	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–
	TSA	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	XLD	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	XLT-4	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Klebsiella pneumoniae	BGS	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	BS	–	–	–	–	–	–	–	–	–	–	–	–	–	NG	–	–	–	–
	DMLIA	–	–	–	–	–	–	–	–	–	–	–	–	–	NG	–	–	–	–
	HE	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	TSA	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–
	XLD	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–
	XLT-4	–	–	–	–	–	–	–	–	–	–	–	–	–	NG	–	+	–	–

[a] – = Correctly identified as not *Salmonella* spp.; + = incorrectly identified.

[b] BGS = brilliant green sulfa agar; BS = bismuth sulfite agar; DMLIA = double-modified lysine iron agar; HE = Hektoen enteric agar; TSA = tryptic soy agar; XLD = xylose lysine deoxycholate agar; XLT-4 = xylose lysine tergitol agar.

[c] No growth or no isolated colonies on plate.

[d] Suspected contaminated culture. Data removed from statistical analysis.

Table 4. Results by agar medium

Medium[a]		Correct	Misidentified	Total
BGS	Inclusive	108	0	108
	Exclusive	98	5	103
BS	Inclusive	108	0	108
	Exclusive	99	4	103
DMLIA	Inclusive	108	0	108
	Exclusive	93	3	96
HE	Inclusive	107	1	108
	Exclusive	100	4	104
TSA	Inclusive	108	0	108
	Exclusive	101	3	104
XLD	Inclusive	108	0	108
	Exclusive	101	3	104
XLT-4	Inclusive	108	0	108
	Exclusive	69	2	71
Total	Inclusive	755	1	756
	Exclusive	661	24	685

[a] BGS = brilliant green sulfa agar; BS = bismuth sulfite agar; DMLIA = double-modified lysine iron agar; HE = Hektoen enteric agar; TSA = tryptic soy agar; XLD = xylose lysine deoxycholate agar; XLT-4 = xylose lysine tergitol agar.

vulgaris produced colonies on XLT-4 agar. The remaining cases of no growth appeared to be random with respect to strain and medium. A total of 691 analyses were performed on exclusive strains. Collaborator 16 reported positive results on six of seven plates streaked with the *E. cloacae* culture. The remaining agar, TSA, was reported to have no growth. Collaborator 16 reported that the six plates all contained growth with colonies of a *Salmonella*-like appearance. It is concluded that this culture became contaminated at some point during preparation or analysis and therefore these data were eliminated from the statistical analysis. Of 685 remaining analyses, 661 produced negative results for accuracy with exclusive strains of 96.5%.

A summary of results by agar medium is shown in Table 4. The percentage of correct results was very similar for all seven media, ranging from 97.6 to 98.9%.

Discussion

In this multilaboratory evaluation of the ANSR *Salmonella* test for identification of presumptive *Salmonella* spp. isolates from agar media, the method exhibited exceptional accuracy with inclusive strains and a high degree of exclusivity with non-salmonellae. Of the 18 laboratories participating in the study, 15 reported results with overall accuracy of 99 to 100%. There was only a single false-negative result out of 756 *Salmonella* spp. colonies tested. Excluding data generated from a suspected contaminated slant culture, there were 24 false-positive results on non-*Salmonella* spp. colonies out of 685 colonies tested. All but seven of these aberrant results occurred in three laboratories. Laboratory 16 reported six false-positive results in addition to those linked to the contaminated slant culture. No further information is available for these samples, except that all six ANSR fluorescence curves were very strong, typical of true positive results. Laboratory 2 reported six false-positive results; four of these occurred in a single ANSR assay run of 15 samples. All but one of the false-positive results showed atypical, weak fluorescence curves, suggestive of cross-contamination during performance of the ANSR assay. Laboratory 13 reported five false-positive results. Again, all but one of these results showed atypical, weak fluorescence curves. Additionally, raw data received from this laboratory indicated that one assay run was repeated in total due to extreme aberrant results (i.e., invalid assays), suggesting that the technician was experiencing difficulty in performing the assay correctly.

Including data from all 18 laboratories (with the exclusion of the six suspected contaminated samples from laboratory 16), accuracy on inclusive and exclusive strains was 99.9 and 96.5%, respectively. Considering only data from the 15 laboratories without clusters of aberrant results, accuracy on exclusive strains was 98.8%.

Recommendations

The ANSR *Salmonella* test was adopted as Official First Action status for use as a rapid, accurate adjunct or alternative to biochemical testing for identification of presumptive *Salmonella* spp. isolates.

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