



Microbiology Methods Task Force presents:

# *Salmonella* Detection Methods and Laboratory Best Practices for Seasonings, Herbs, and Spice Matrices





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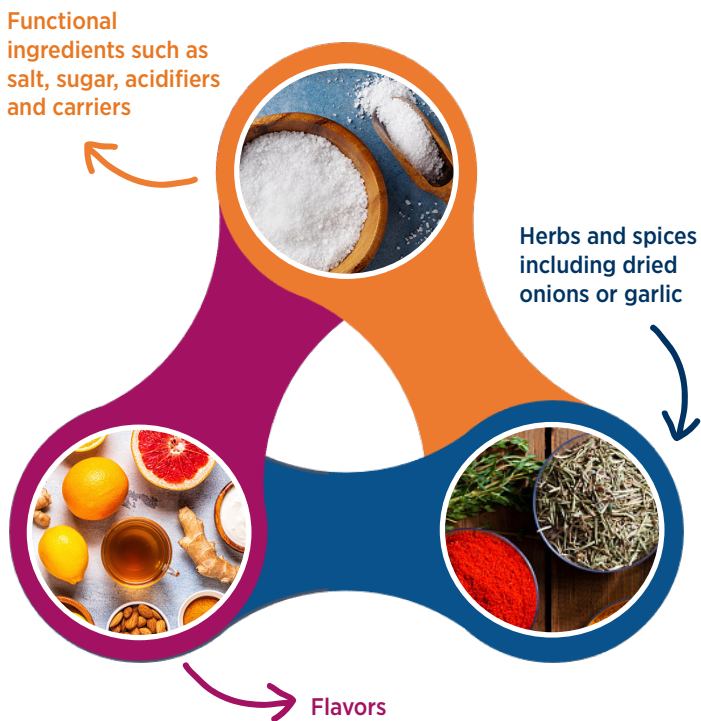
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## PREFACE

Seasonings are functional dry ingredients and blends used in a wide range of foods and snack products to impart or enhance various sensory characteristics such as saltiness, sourness, sweetness, bitterness or umami. Seasonings are also used to impart a distinct taste profile and can enhance the texture, “mouth feel”, and the appearance of foods. The composition of seasonings can be grouped in three main classes:



Seasonings are often considered as microbiologically sensitive ingredients with regard to the food safety of snack foods because they are often added to the surface of ready to eat snacks foods at a point in the process without a subsequent microbial lethality process (e.g., thermal process steps such as baking, frying, extrusion, etc.). The major contributor to the overall microbiological risk of a seasoning is the group of herbs and spices while carriers and flavors are generally

considered very low risk, because of their chemical nature, low aW values and high degree of processing. Therefore, seasoning manufacturers focus on proper risk assessment and thorough controls of the spices, dried vegetables and herbs used in the recipes followed by strict process controls and bacterial pathogen environmental monitoring in production zones to avoid contamination.

However, spices have been linked to foodborne disease outbreaks (FDA 2017), with non-Typhoidal *Salmonella* as the primary cause of these outbreaks. Of the twenty-one recalls of spices between 1973 and 2003, twenty were recalled because of *Salmonella* (Vij et al., 2006). The U.S. FDA collected 1,406 samples of imported spices at the port of entry between 2013 and 2017 and found that the prevalence of *Salmonella* ranged from 3.5% to 18% (Zhang et al., 2017). The same study. FDA collected 7,250 samples of various spices at retail and found that the incidence of *Salmonella* ranged from undetectable to as high as 0.64%. The difference in the prevalence between port of entry and retail is attributable to the microbial interventions which are routinely applied to imported spices. Because of this, the control of non-Typhoidal *Salmonella* is an important part of quality assurance and verification testing.

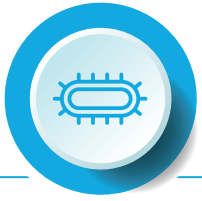
In addition to microbial interventions, seasoning manufacturers rely on good manufacturing practices and have implemented quality and food safety monitoring steps for spices and seasonings. *Salmonella* spp. testing of spices and seasonings is an important part of the verification programs. Snack producers are committed to the highest levels of food safety, and thus they require validated methods for *Salmonella* testing to avoid erroneous results in general, but most importantly to minimize the chance for false negative results. Therefore, the co-authors listed on page 2 took the initiative to create this global guidance document to recommend and cascade best practices for the detection of *Salmonella* spp. in herbs, spices, seasonings and their subcomponents.



## PURPOSE

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To address the need for industry-wide expert guidance, PepsiCo formed a Supplier Task Force and collaborated with the American Spice Trade Association to develop a guidance document (i.e., “white paper”) that can be used to benchmark best practices when testing for the presence of *Salmonella* spp. in seasoning blends and spices. This white paper will allow best practices and current technical expertise to be shared with key stakeholders, including secondary suppliers (regional and global) to foster a more consistent approach to *Salmonella* verification testing of individual herbs and spices, as well as complex seasoning/spice blends. The intent is for this guidance document to be revisited and updated in the future, when significant new technical advances are made.



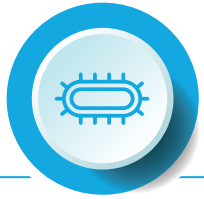
# SECTION 1: ANTIMICROBIAL SUBCOMPONENTS OF CONCERN

## Background and Objectives

Low water activity (aw) seasoning blends are often formulated with a variety of ingredients including items such as salt and acids that may have antimicrobial properties. Seasoning blends may also contain spices, herbs, cocoa and other ingredients with natural bacteriostatic or bactericidal constituents. Vegetative pathogens like non-typhoidal *Salmonella* are most susceptible to antimicrobial activity when samples are diluted for pre-enrichment. For this reason, it is important to address the individual and synergistic antimicrobial effects of the ingredients when testing seasoning blends for non-typhoidal *Salmonella* so that accurate results can be obtained.











## Best Practices

- Identify antimicrobial ingredients in a formula including, but not limited to, acids, salt (NaCl), onion, garlic, cinnamon, allspice, oregano, cloves and cocoa. The ingredients may be present in various forms such as powders, granules, extracts, concentrates, distillates, flavors and oils. While concentrated forms of inhibitory ingredients may be present in minute amounts, their antimicrobial impact may be more pronounced and potentially in much higher percentages than when in their natural forms. For this reason, it is important to consider not only the type and percentage of ingredients in a blend but also the form of the ingredients. As a general rule of thumb sample may need to be enriched at a sample: broth ratio >1:9 if there are more than 10% antimicrobial components in the formula. However the user should always consider pre-enrichment verification activities as per section 4 to address any combination unanticipated effects from different components.
- Become familiar with protocols to counteract known antimicrobials to allow for growth of the *Salmonella* target. Sample pre-enrichment protocols include neutralization, dilution, and partitioning, and can be used alone or in combination.
- For example, the FDA (2018), ISO (2017) and Canadian (Government of Canada, 2009) methods for *Salmonella* pre-enrichment of onion and garlic include the addition of K<sub>2</sub>SO<sub>3</sub> to the pre-enrichment broth to achieve a final concentration of 0.5% (w/v). This addition would also be appropriate for seasoning blends containing onion and garlic.
- Another option is to dilute the antimicrobials beyond their toxicity as recommended in the FDA BAM (2018), ISO (2017) and Canadian (Government of Canada, 2009) methods for pure cinnamon/cassia, oregano, allspice and cloves. This concept can be applied to other antimicrobial ingredients as well as noted by the USP (2014) for phenolics, aldehydes and sorbate. For seasoning blends, take into consideration the dilution aspect of the ingredient in the seasoning coupled with a standard 1:9 sample to broth pre-enrichment ratio. As an example, the ISO (2017) method advises dilution of salt when it exceeds 10% (w/v) in a sample so that the final concentration in the pre-enrichment medium is no higher than 1% (w/v). Unpublished research indicates that the percentage of salt may be as high as 40% in a blend with no impact on *Salmonella* recovery at the standard 1:9 sample to pre-enrichment broth ratio as long as there are no other inhibitory ingredients present. As with any analytical method, the final salt concentration in the pre-enrichment medium should be validated to have no effect on recovery, if it exceeds 1%.
- Partitioning methods move the antimicrobial compounds away from microorganisms. One partitioning method evaluated by Beaubrun et al. (2016) utilized 2% corn oil in the pre-enrichment medium for oregano followed by shaking incubation.

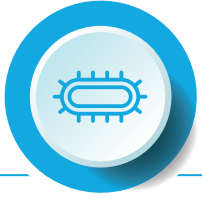


## SECTION 1: ANTIMICROBIAL SUBCOMPONENTS OF CONCERN

- The following table summarizes established pre-enrichment protocols for inhibitory foods in their pure form from the FDA BAM (2018), ISO (2017) and Canadian (Government of Canada, 2009) methods. Refer to the reference documents for detailed instructions. These protocols or a modification of the protocols may be applicable to blends containing these ingredients. A combination of techniques may be required based on the type of ingredients. An example is a blend containing garlic, oregano and other spices. The addition of K<sub>2</sub>SO<sub>3</sub> to the pre-enrichment medium along with a dilution higher than the standard 1:9 sample:broth ratio may be required to address all of the inhibitory properties.

Individual Antimicrobial Food Component	<i>Salmonella</i> Pre-Enrichment Steps
 <p><i>Acids and acid ingredients</i></p>	<p>FDA BAM: Measure pH and adjust the pH of the sample pre-enrichment mixture to 6.8 + 0.2            ISO: Measure pH and adjust the pH of the sample pre-enrichment to 7.0 + 0.5; For pH adjustment of moderately acidic samples (pH &gt; 3.5 to &lt; 4.5) using double-strength Buffered Peptone Water            Government of Canada: If the pH of the pre-enrichment mixture lies outside the range of 6.0 to 7.0, adjust with 1 N NaOH</p>
  <p><i>Onion and garlic</i></p>	<p>FDA BAM, ISO, Government of Canada: Add K<sub>2</sub>SO<sub>3</sub> to pre-enrichment medium to achieve a final concentration of 0.5% (w/v). Alternatively, the task force indicates onion and garlic may be diluted in the pre-enrichment medium beyond their toxicity</p>
  <p><i>Cinnamon, allspice, oregano ground</i></p>	<p>FDA BAM: Dilute cinnamon, oregano and allspice 1:100 in pre-enrichment broth. Sample size 37.5 g for every 375 g composite.            ISO: Dilute cinnamon and oregano 1:100 in pre-enrichment broth            Government of Canada: Cinnamon sticks and allspice 1:100 spice to pre-enrichment broth ratio (w/v); Oregano and ground cassia 1:50 spice to pre-enrichment broth ratio (w/v)</p>
 <p><i>Cloves</i></p>	<p>FDA BAM, ISO, Government of Canada: Dilute sample 1:1000 in pre-enrichment broth. Sample size 10 aliquots of 3.75 g for every 375 g composite</p>
 <p><i>Cocoa and chocolate</i></p>	<p>FDA BAM: Prepare a 1:9 sample to broth ratio in sterilized reconstituted non-fat dry milk. Add 0.45ml 1% aqueous brilliant green dye solution per 25 g sample and mix well.            ISO: Prepare 1:9 sample to broth ratio in UHT milk or sterilized reconstituted non-fat dry milk. For cocoa powder and other samples that may be highly contaminated with Gram-positive bacteria, add 0.45 ml 1% (w/v) aqueous brilliant green solution per 25 g sample and mix well.            Government of Canada: Suspend 25 g sample in 225 mL skim milk medium and blend</p>
  <p><i>Leafy spices (such as basil and parsley)</i></p>	<p>FDA BAM: Prepare a 1:19 sample to broth ratio.</p>
 <p><i>Salt</i></p>	<p>ISO: Dilute samples containing more than 10% (w/w) sodium chloride to ensure the total concentration in the initial suspension does not exceed 1% (w/v)</p>



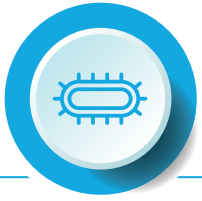


## SECTION 1: ANTIMICROBIAL SUBCOMPONENTS OF CONCERN

- The FDA BAM (2018) method instructs to leave sample pre-enrichments at room temperature for 60 + 5 minutes prior to pH determination and adjustment. The purpose is to allow time for pH equilibration. A resuscitation period at laboratory ambient temperature of up to one hour for dry products and 20-30 minutes for flour is advised by ISO (2017). For samples containing acids and acidic ingredients, these waiting periods are not recommended prior to pH adjustment due to the potential for the acid to inactivate or injure the target bacterial cells immediately following the addition of the sample to the medium. Therefore, immediately after the sample is added to the pre-enrichment broth and mixed, check the pH with pH paper and adjust as necessary with 1N NaOH.
- Following the same thought process, if it has been verified that a pre-enrichment dilution higher than the standard 1:9 sample to broth ratio is required for a seasoning, it is important to fully dilute the sample in the shortest period of time so that the antimicrobial ingredients do not have time to negatively impact the target bacteria in a concentrated suspension.
- Verify the effectiveness of the pre-enrichment protocol for seasoning blends with inoculated samples as directed in Section 2. Historical verification data will provide direction on the pre-enrichment protocols for new formulas.
- If substantial historical verification data and historical insights for seasoning formulas are available, it may be possible to group formulas based on the type and percentage of ingredients such that the number of new formula verifications may be reduced.

### Conclusion

Spices and seasoning blends are among the most difficult foods to test for pathogens due to their inherent and natural antimicrobial constituents. When those antimicrobial properties are combined with other antimicrobial ingredients such as acids and salt, the complexity of accurately analyzing seasoning blends becomes apparent. Knowledge of and experience with ways to mitigate the antimicrobial properties of these kinds of matrices is important for the laboratory analyzing seasoning blends for *Salmonella* and other pathogens.



## SECTION 2: *SALMONELLA* PRE-ENRICHMENT VERIFICATION PROTOCOL

### Background and Objectives

Because of the complexity of seasonings, verification of the *Salmonella* pre-enrichment protocol is required if an assessment is not possible based on experience. To achieve a high-quality and comparable verification, the relevant testing criteria must be defined. The most relevant parameters are listed in this section.

### Best Practices

Verification testing should be conducted by experienced laboratory analysts who have been trained and have demonstrated competence in assessing and executing microbiological test methods. The testing is preferably conducted by a Corporate laboratory or a well-respected 3rd-party commercial laboratory.

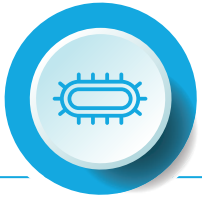
- Verify the pre-enrichment protocol initially with 375 g samples. Based on the outcome of this testing, multiple smaller samples at higher dilutions may be needed. Consider the method's limit of detection when sample dilutions are high and determine the appropriate pre-enrichment incubation time as needed.
- Verify the pre-enrichment protocol with 3 samples of each blend from different commercially manufactured lots. Do not use lab prepared samples.
- A minimum of 1 *Salmonella* strain from the original method validation should be used as the inoculum. A lyophilized culture that is labeled with a marker such as Green

Fluorescing Protein is preferred as a Good Laboratory Practice.

- Measure the pH of the pre-enrichment mixture and adjust, if necessary. Quickly hydrate the culture and inoculate the pre-enrichment mixture so that the amount of time that passes between hydration and inoculation is minimized.
- Target inoculum: Maximum of 25 CFU/375 g
- Quantify the inoculum using a direct plating method on a non-selective agar to confirm the target inoculum level has been reached.
- Commercially released lots should not be used as negative controls for pre-enrichment verification studies.
- It is recommended run a positive control each day verification testing is conducted.
- The acceptance criteria are 3 inoculated samples test positive and the positive control is positive, for any given pre-enrichment test protocol.

### Conclusion

To obtain accurate results, it is necessary to verify the effectiveness of the pre-enrichment protocol for samples that demonstrate or have the known potential for *Salmonella* growth inhibition. For correct verification, the test protocol must meet the above-mentioned best practice requirements.



## SECTION 3: *SALMONELLA* DETECTION METHODS AND CONFIRMATION FOR SEASONING AND SPICES

### Background and Objectives

Successful enrichment resulted in a sufficient enrichment of all *Salmonella* present. Even sublethally damaged *Salmonella* cells should be resuscitated as far as possible. The detection methods selected should demonstrate the lowest possible rates of false-positive and false-negative results. Among the commonly used *Salmonella* detection methods in use at the time of this publication are: conventional bacteriology, ELFA/ELISA immunoassays, a wide array of PCR-based methods targeting specific DNA or RNA sequences, methods that utilize recombinant phage proteins that are specific for *Salmonella* spp. and labeled with alkaline phosphatase, and methods that employ immunomagnetic beads and specialized magnets to enhance the speed or efficiency of the detection process. They are based on the application of re-combinant phage proteins, which are specific for *Salmonella* spp. and labeled with alkaline phosphatase

These have all different advantages and disadvantages, so each laboratory need to assess and make a science-based decision as to which detection technique is to be used for each type of herb, spice and seasoning matrix.

#### *Genetics-based methods:*

- Appropriate commercial test kits may be used to identify *Salmonella*. These test kits shall be used according to the manufacturer's specifications and should be validated for the appropriate food matrix.
- The development of sensitive and rapid gene amplification methods (Polymerase chain reaction, PCR) provides a rapid alternative to conventional methods. Various manufacturers offer so-called open (more manually based) and closed (mostly automated) PCR systems, each with its own sets of advantages and potential disadvantages.
- As a general rule, qualitative PCR-based results are generally available after 1 day (24-30 hours) with this method of determination detection. (ISO 20837, ISO 20838, ISO 22118, ISO 22119)

### Best Practices

The following aspects are key to generating consistent and reliable results:

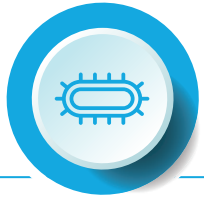
- Food matrix interference can occur when using PCR-based methods. In these scenarios, the polymerase chain reaction or certain key enzymes used within the test system may be disrupted so that there is inhibited or insufficient propagation of the target sequence, resulting in an invalid result.
- Matrix ingredients can disrupt or interfere with the photometric detection step after amplification, so that no correct measurement is made.
- Potential sources of error must be excluded by

verification/validation interpretation of photometric measurement curves from PCR based systems requires proper training and experience.

- The use of positive controls and maintaining close communication with the manufacturer's technical support team are both important.

#### *Conventional Culture-based Methods*

Conventional methods for cultural detection of *Salmonella* spp. generally assess several biochemical properties of the bacteria isolated to obtain reactions that can be evaluated on selective semi-solid agar media. In order to increase the



## SECTION 3: *SALMONELLA* DETECTION METHODS AND CONFIRMATION FOR SEASONING AND SPICES

reliability of detection, various properties are evaluated and made visible by, for example, color reactions of the colonies on various differential agar media, and by colony morphology.

There are many well-suited nutrient media from different manufacturers on the market. (ISO 6579), (Mikrothek, G Klein), (FDA BAM) and each lab must evaluate and select those most suitable to their needs.

ISO 6579 defines a standard for testing foodstuffs and animal feeding stuffs: after two-stage enrichment (non-selective, selective) cultivation is carried out on two different selective culture media. One suspected colony must then be confirmed biochemically or serologically. If negative four additional colonies must be tested (see below).

The FDA BAM (2019) requires that confirmation be carried out on three different selective agars. After incubation, two or more typical colonies from each selective agar must be picked to continue for biochemical and/or serological confirmation. In the absence of typical colonies atypical colonies must be picked.

It is essential for successful identification of typical and atypical colonies to familiarize oneself with the selective media used in order to drive consistency and avoid misinterpretation of the results.

### Conclusion

Various technologies are available to provide assurance that the presence of *Salmonella* is genuine. Biochemical, serological, genetics-based PCR test kits and ELISA tests are commercially available from numerous manufacturers. Here, the user has to weigh the pros and cons, such as approval by authoritative expert associations such as ISO, AOAC, FDA-BAM, AFNOR, etc., time-to-results, ease of use, pricing and local availability of kits/reagents, and the quality of customer service from the respective kit or diagnostic equipment vendors.

In order to generate meaningful and accurate pathogen test results, these assays shall always be carried out according to the manufacturer's specifications. Regardless of the standard method chosen by the laboratory, the method must be verified and validated that it is appropriate for the range of food and ingredient matrices being tested.

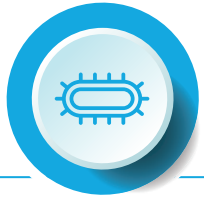
### *Biochemical and Serological Confirmation Methods*

There are a variety of commercial test kits may be used to perform biochemical and serological confirmation of *Salmonella*. These test kits shall be used according to the manufacturer's specifications. Laboratories that have few samples for confirmation may opt to use an accredited third-party laboratory to avoid the expense of maintaining kits and to avoid misinterpretation by laboratory personnel.

### *ELISA/ELFA Methods*

Appropriate immunoassay-based commercial test kits may be used to identify *Salmonella*. These test kits shall be used according to the manufacturer's specifications. As with the biochemical test kits, laboratories may find that some test kits are more useful than others for the serovars of *Salmonella* that they routinely encounter, or may have less cross-reactivity (false positive results) with the microbiome that they encounter in their samples.

The ELISA assay relies on the ability to quickly and effectively wash away unbound molecules. This allows ELISA assays to capture specific *Salmonella* antigens or reagent-based antibodies from the test samples. Optimizing the parameters of the wash steps is critical to obtaining the best results from an ELISA assay.



## SECTION 4: VERIFYING A RAPID METHOD FOR SEASONINGS AND SPICES

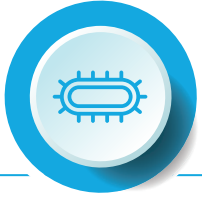
### Background and Objectives

Incorporating more rapid methodology into a *Salmonella* testing program for seasonings and spices is an attractive option to reduce testing turnaround time and allow for faster production lot release. However, as pointed out in sections 1 and 2, seasoning and spices may present unique matrix/microbial inhibition challenges. The performance of any rapid method must be verified to demonstrate its equivalence to an appropriate reference method before it can be confidently used in routine testing. According to the FDA's Food Safety Modernization Act regulation 21 CFR Part 117.165, all routine food safety program verification methods must be "scientifically valid" (US FDA, 2019). Therefore, proper rapid method selection and verification activities are critical steps in ensuring all parties can be confident in *Salmonella* testing results for the matrices being analyzed by the laboratory.

### Best Practices

Always select methods that has been validated by internationally recognized validation schemes such as AOAC Official Method of Analysis (OMA), Health Canada, AFNOR, ISO, or the equivalent. Those methods designated as "Official Methods of Analysis" have a more robust set of multi-laboratory verification and reproducibility data than do those methods that are designated as AOAC "R.I." (Research Institute) or as "Performance Tested Methods".

- Elements of evaluating a validated or alternate method are: sensitivity, specificity, limit of detection (LOD), Inclusivity, and Exclusivity, and Probability of Detection (POD) (AOAC International, 2012).
- Evaluate if the method has been validated for larger sample size or if compositing is permitted.
- When selecting a method consider ease of use in the laboratory, space, turn-around time, practicality, capacity and/or throughput, cost, and laboratory schedules.
- Consider availability of detection kit and media before approving a method for use globally or in each geographical area of interest.
- Determine if the existing documented validation studies included a seasoning or spice matrix or matrices. If the validation does not include the matrix in consideration, additional verification studies should be strongly considered to confirm the method is fit for intended purpose.
- If additional verification studies are needed, all steps of the candidate method shall be verified. Pre-enrichment, secondary enrichment, and detection (diagnostic) are key steps to include as part of the verification study. Best practices when conducting a verification study are:
  - Use low levels of *Salmonella* (<30 cfu of analyte per 25 g) to evaluate the suitability dilution ratio, growth media, and incubation condition of the pre-enrichment step (US FDA, 2019).
  - Method verification should include at least 3-5 replicate samples per sample matrix.
  - For samples matrix selection, select samples that represent the entire range of known variability such as highly acidic, dark-colored, high percentage of gums or thickening agents, known inhibitory subcomponents, high salt, high sugar, and so on.
  - It is recommended to select at least 10 of the most complex matrices and method verifications should be performed in triplicate e.g., 10x3 = 30 samples per verification study. This will provide a robust method evaluation.
  - In a spiked study, all samples should yield positive results.
  - All samples should be culturally confirmed follow FDA BAM or ISO protocols.

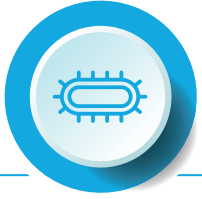


## SECTION 4: VERIFYING A RAPID METHOD FOR SEASONINGS AND SPICES

- False negatives should be investigated. Identifying where the failure occurred could allow the user to make adjustments that may correct the issue. For instance, the cause for false negative could be non-recovery during pre-enrichment and adjusting dilutions may be an appropriate corrective action.
- It is recommended to perform spiked method verification studies at an ISO 17025 accredited 3rd party laboratory or at a well-controlled internal corporate microbiology laboratory.
- Typically, method verification is undertaken for the purpose of identifying a more efficient or cost-effective method. It is recommended to concurrently run the current approved method with the candidate rapid method. This type of approach will allow for side by side comparison of method.
- If a negative control is required for the verification study; irradiating the sample prior to the study may be an alternate option.

### Conclusion

Rapid microbiological methods offer many benefits over the traditional/reference method. However, it is important to understand not all rapid methods are created equal. It is critical to select rapid methods that are scientifically validated and fit for purpose. Most rapid methods are designed to perform at the edge of method sensitivity therefore any level of matrices inhibition could have an impact on their performance. Given the complexity of seasonings, herbs, and spice matrices, even a validated method should be verified before use by seasoning suppliers or their customers within the food industry.



## SECTION 5: PRE-ENRICHMENT AND SELECTIVE ENRICHMENT MEDIA HANDLING

### Background and Objectives

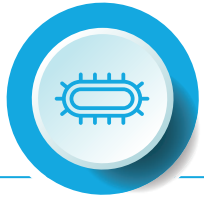
When selecting a pre-enrichment broth for *Salmonella* testing it is important that the selected broth(s) can resuscitate injured bacterial cells, provide adequate buffering capacity, and support the growth of *Salmonella* spp. in spices, herbs and seasoning blends. If present in a given sample, *Salmonella* may occur at very low populations and among a population of many other non-pathogenic microorganisms and bacterial spores. *Salmonella* cells may also be potentially be “injured”, “stressed” or otherwise difficult to resuscitate due to environmental stresses imposed by pre- or post-harvest food processing, and/or by the intrinsic property of the seasonings or spices themselves. Therefore, it is critical that the pre-enrichment broths selected provide the appropriate conditions necessary to resuscitate the target *Salmonella* cells, thereby allowing them to multiply to a level (i.e., CFU/mL of enrichment media) that they can be detected (D’aoust and Jean-Yves, 1981; Bedu-Amoako et al., 1992).

For selection and combination of enrichment steps there are numerous preconditions prescribed in the standard methods (e.g., ISO 6887-1,4,5, ISO 6579-1, FDA-BAM). These pre- and selective-enrichment conditions have been developed based upon the known inhibitory characteristics of certain ingredients and sub-components of the samples being tested. To define and select a suitable pre-enrichment, the food microbiologist must have appropriate training and experience, and must verify or validate the method, as needed based upon a technical risk assessment (see section 2).

There are many commercially available kits and alternate methods that utilize:

- a. A single non-selective enrichment; or
- b. Two non-selective enrichments (i.e. BPW and BHI = Brain Heart Infusion Broth); or
- c. A single non-selective enrichment followed by a selective enrichment (ISO 6579-1)





Any of these kinds of approaches may be considered acceptable, provided internal laboratory studies are carried out and documented to validate the method is suitable for the matrix been tested.



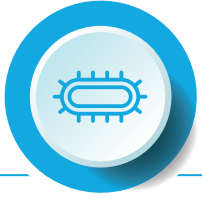
## SECTION 5: PRE-ENRICHMENT AND SELECTIVE ENRICHMENT MEDIA HANDLING

### Best Practices

Most methods involve selection of pre-enrichment broth, incubation, detection and decontamination. Here are some best practices to consider in each step.

 <p><i>Prewarming the pre-enrichment</i></p>	<ul style="list-style-type: none"><li>• Liquid media within sealed flasks or bottles which has been prewarmed in incubator prior to use (minimum 12 hours). (Compendium 2015 Chapter 52)</li><li>• A pre-warmed filter-sterilized deionized water system</li><li>• A media preparation system – enables to be pumped directly from media preparation into the sample; e.g., a bioMérieux Masterclave 60™ (with 60 Liters capacity); Systec Mediaprep 120™ (with 120 Liters capacity)</li></ul>
 <p><i>Media</i></p>	<ul style="list-style-type: none"><li>• Media sterility must be considered when sterilizing large volumes of media.</li><li>• Guar gums, similar thickening agents, and other additives that absorb a high volume of liquid when pre-enriching seasoning blends. Therefore, the microbiologist may need to dilute samples beyond the typical 1:10 sample: broth ratio; otherwise samples will become too viscous to pipette during subsequent steps of the test protocol (ISO 6887-4:2017; Hayman et al., 2015).</li><li>• The enzymatic treatment of botanical gum containing matrices may be useful in certain circumstances. The enrichment of Guar gum at a 1:9 sample to lactose broth ratio provides a good illustration of this principle. In 1996, Amaguana et al. of the U.S. FDA reported that the addition of 0.01% cellulase enzyme to the lactose broth reduced the viscosity of the final enrichment, making it readily pipettable and much easier to work with during subsequent <i>Salmonella</i> testing steps. The step-by-step details on how to apply the cellulase enzyme during pre-enrichment are explained in the FDA-BAM (Dec. 2019), Chap. 5: <i>Salmonella</i>.</li><li>• The use of double-strength buffered peptone water for neutralization of pre-enrichment is admissible, but the pH of such products should be always checked when these are tested for the first time, to ensure the required pH range is achieved (ISO 6887-1:2017, Chapter 5.2.3.)</li></ul>
 <p><i>Neutralization of the non-selective pre-enrichment</i></p>	<ul style="list-style-type: none"><li>• Using pH meter or pH paper to adjust the final pH to 6.5 to 7.5. FDA BAM, ISO 6887-1:2017(Chapter 8.6.) aseptically to prevent contamination</li><li>• Usage of 0.1 N, 1 N or 2 N NaOH strength are typically used. It is important to ensure that the characteristics of the enrichment broth not be significantly altered during the pH titration and adjustment process.</li></ul>
 <p><i>Incubation Conditions</i></p>	<ul style="list-style-type: none"><li>• The temperature and time of incubation must be strictly followed as prescribed by the Standard method laboratory is using. Failure to do so may result in erroneous results (e.g., false negatives).</li><li>• The approved test laboratory must have a procedure to document and track the incubation times for each sample being analyzed.</li></ul>



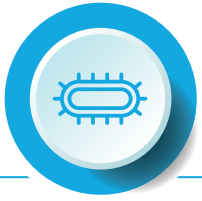


## SECTION 5: PRE-ENRICHMENT AND SELECTIVE ENRICHMENT MEDIA HANDLING

### Conclusion

When testing seasoning and spices for *Salmonella* it is important to select the correct enrichment broth if using a one-step enrichment method or pre-enrichment/selective enrichment broth if using a two-step method. Once selected, there are other aspects of testing such as pH, pre-warming the broths, addition of larger volume due to matrix property or to reduce the toxicity effect of matrix and incubation temperature and time that need to be considered for recovery of *Salmonella* in seasoning and spices.

If a given company does not have the expertise to validate or verify the methods internally, then the advice and guidance of 3rd-party experts in food microbiology diagnostics must be sought out and followed. The importance of this statement cannot be over-emphasized. Once all testing is completed, it is important that appropriate measures are taken to ensure that all biological waste generated is properly decontaminated prior to disposing.



## SECTION 6: SELECTION CRITERIA FOR 3RD-PARTY LABS AND INTERNAL LAB QUALITY SYSTEMS

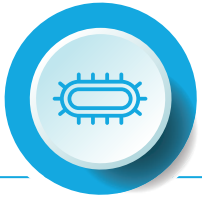
### Background and Objectives

There are many external or 3rd-party laboratories that offer *Salmonella* testing and other microbiological testing services around the world. However, to enable detection of pathogens in microbiologically sensitive ingredients with a high degree of confidence, it is important to only use laboratories that have demonstrated the required competency and proficiency with the kinds of matrices being analyzed. Competency is defined in understanding and implementation of laboratory quality management programs, the use of suitable methods, and properly trained personnel to perform the test on a consistent basis. This section provides guidance on key considerations and core requirements for the selection of laboratories for the pathogen testing of seasonings, herbs, and spices, and the kinds of Good Laboratory Practices (GLP) that one should assess when selecting a lab to carry out such testing. The USDA – FSIS (2013) published a guidance document on the selection of a commercial laboratory for testing, and while the document is oriented towards meat and poultry, the basic laboratory principles are relevant to all food testing laboratories.

### Best Practices

#### *3rd-Party Laboratory best practices*

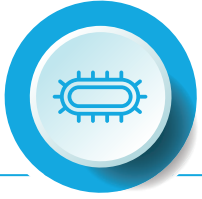
- Select 3rd-party labs that is ISO 17025 Accredited (ISO 2005), or equivalent or local government certification.
- Confirm that each desired *Salmonella* test method is covered under the scope of accreditation for the desired product category.
- Larger 3rd-party food microbiology lab testing “networks” with a good reputation tend to have robust, centrally managed lab quality systems and will often be more familiar with testing complex matrices such as seasoning and spices.
- Verify that the 3rd-party lab has systems in place to assure that the client’s instructions regarding sample management and *Salmonella* pre-enrichment procedures are followed every time.
- Selected 3rd-party labs must participate in a proficiency testing program (PTP) that meets or exceeds the ISO/IEC 17025 accreditation standard.
- Review of the laboratory’s proficiency testing program (PTP) results on a consistent basis is critical. As part of this review, confirm that all technicians performing testing are participating in the PTP. Verify if PTP covers the kinds of matrices being requested by you, the client, and includes the target pathogen of interest.
- Laboratory technicians conducting *Salmonella* assays must adequately trained and their training records must be maintained and accessible.
- Whenever possible, select a laboratory that is located within same country/region and in close proximity of the sites submitting samples for analysis. This will improve the odds of faster test turnaround times and may avoid delays incurred if samples must be inspected by customs officials.
- The testing lab should be well designed with separate rooms (walled-off areas) for testing, media preparation, and the handling of waste materials and biohazardous materials (EPA 2017).
- Performance and calibration of equipment critical to the method (e.g., autoclaves, incubators, balances, thermometers, etc.) should be properly documented. Calibration and maintenance records must be accessible and provided for review upon request.
- A corrective and preventive action (CAPA) should be in place to document laboratory action plans to address any gaps identified during ISO 17025 audits (ISO 2005), proficiency testing failures, failure or improper calibration of critical pieces of equipment, false positives, and customer complaints.



## SECTION 6: SELECTION CRITERIA FOR 3RD-PARTY LABS AND INTERNAL LAB QUALITY SYSTEMS

### *Internal or Supplier Microbiology laboratories conducting pathogen testing*

- Microbiology laboratories testing for pathogen should preferably be ISO 17025 approved strive to be compliant to ISO 17025 or equivalent accreditation.
- The laboratory shall be supervised by a competent microbiologist.
- Laboratories conducting pathogen testing must implement the Biological Safety Level-2 (BSL-2) or equivalent standard. BSL-2 standard is an important containment protocols to prevent cross-contamination within microbiology lab (EPA 2017).
- Procedure should be in place to minimize cross-contamination risk. This procedure should include having a dedicated technician for handling high risk activities such as confirmation of suspect or presumptive samples handling within the laboratory.
- Both internal or supplier microbiology laboratories should participate in proficiency testing program as on-going basis. Proficiency testing program (PTP) should meets or exceeds the ISO/IEC 17025 accreditation standard. A review of historical perform of PTP should be incorporated as a key criterion during laboratory approval process. Incorrect pathogen results on a proficiency testing program is a serious deviation and requires immediate attention. It is recommended to perform proficiency test semi-annually or quarterly per sample regime at a minimum.
- Each *Salmonella* test method must have a written procedure or SOP that is customized to that the particular laboratory. Only methods that are validated/verified for specific matrices (ISO 2016) shall be used (see Section 5 of this guidance document).
- Incorporate microbiological laboratory Quality Systems audits as part of the holistic supplier qualification for those suppliers that manage microbiology laboratory. This may require developing a special checklist and training standards for the auditing team.
  - Review of the most updated lab accreditation documents if applicable.
  - Proficiency test date and results
- Training records for each technician
- Test method and the SOP's for pathogen testing
- Any validation or verification studies on the test method, especially the pre-enrichment media selection
- Aseptic sampling procedures
- A review of any test failures and the corrective actions taken in response
- Management of Change protocols and training programs
- Lab internal and external audit records and findings
- In case of emergencies (e.g., electrical power outages, natural disasters), external test lab options and emergency preparedness plans.
- Technicians shall be trained on all aspects of pathogen testing that may be assigned to them, and training must be documented.
- If a culture positive control is used, laboratories should always use positive control strains of *Salmonella* that are rare and with a well-characterized strain (e.g., based on the serovar and/or the genetic profile of the strain). This will enable a more efficient and accurate investigation of a possible laboratory cross-contamination event. After the test, the positive pathogen strain should be autoclaved and handled correctly, according to established biosafety protocols.
- Laboratories located on the same location to the manufacturing plant should limit and carefully control any use of pathogen culture positive controls. These labs should have a separate air flow system or be outside of the food production floor. Proper BSL-2 controls must be in place to protect against cross-contamination (WHO 2004).
- The Micro testing lab must have key or ID badge-controlled access to prevent access by any non-authorized personnel.

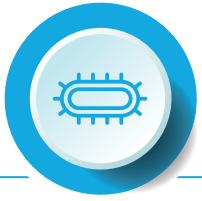


## SECTION 6: SELECTION CRITERIA FOR 3RD-PARTY LABS AND INTERNAL LAB QUALITY SYSTEMS

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### **Conclusion**

Laboratories selected, audited, and used for pathogen testing of seasonings, herbs and spices should be able to meet the basic requirements that are listed above. The laboratory used should meet ISO 17025 or equivalent quality standards and be audited on a regular basis. The test method used should be validated or verified for testing of the specific matrices of concern. All laboratory technicians performing the tests shall be well trained and competent, as documented through training records and periodic inoculated sample lab proficiency test results.



## SECTION 7: POSITIVE RELEASE AND INVENTORY TRACEABILITY

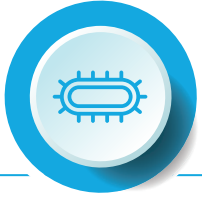
### Background and Objectives

It is possible for companies that create customized flavor blends to receive multiple shipments of the same vendor batch of certain high-volume usage ingredients, especially spices, herbs and dehydrated vegetables. Given the criticality of these materials, many of which are *Salmonella*-sensitive ingredients, it is important to establish and deploy well-designed Food Safety programs that minimize the potential for accidental re-testing of materials that have already undergone the appropriate microbiological analyses to verify conformance to specifications.

The primary objective is to prevent sites from retesting vendor lots (the same batch) that were already shipped to a flavor site multiple times thus stopping redundant testing and eliminating the risk of a supply chain recalls or other means of disruption. The risks of repeat analysis of seasoning batches that already have been shipped will also be addressed. Best practices regarding inventory traceability will also be outlined; the authors of this document suggest that the current GFSI Standards be followed as the best practice.

### Best Practices

- As a general practice, both ingredient and seasoning full or partial batches that have been shipped to commerce must never be tested again for *Salmonella*.
- Whenever ingredients are tested, it is important to place on hold the entire batch/lot at all sites that may have received the same unique batch/lot until ALL relevant testing is completed.
- When an ingredient is tested and the result is presumptive positive, it must be confirmed from the same enrichment broths following the ISO or FDA BAM confirmation protocols. A separate sample must never be started in response to the presumptive positive.
- Under extraordinary circumstances such as investigating a potential food safety incident or a request from a regulatory agency, an ingredient or finished product may be retested for *Salmonella*. As best practice, if an ingredient lot is re-tested, all products which contain that ingredient should be identified in case the ingredient tests positive upon re-testing.
- In these kinds of scenarios, it is important to understand all potential outcomes and the consequences of retesting, and concrete contingency plans must be in place before initiating such retesting.
- All impacted parties such as suppliers and customers must be informed of the decision to retest. Moreover, this activity requires approval from the appropriate senior level food safety and quality management leadership team.
- In case of retesting: If all material of a retested seasoning batch or produced with an affected ingredient batch is still under control of the producer, it should be placed “ON HOLD”.
- In case of retesting: If not all of the material is still under the control of the producer, the retesting strategy needs to be carefully designed. In addition, a risk assessment is needed to evaluate the extent of possible customer impact and communication.
- The use of Enterprise Resource Planning (ERP) software systems such as SAP™ to prevent re-testing is strongly encouraged. In some cases, existing systems will need to be modified for this purpose to assure the required levels of inventory control and communications are in place.
- Understanding ingredient supplier’s lot/batch assignment criteria can help to avoid unintended redundant testing.



## SECTION 7: POSITIVE RELEASE AND INVENTORY TRACEABILITY

### Conclusion

It is critically important that the proper systems are in place to maintain and assure traceability of all incoming raw materials and production lots of seasoning blends, spices and herbs throughout the end-to-end supply chain. Unique vendor lots / batches previously tested must not be retested, and this can be accomplished by eliminating the potential for human error (mistakes) or errors in judgement by using well designed, automated ERP systems as much as possible. In certain extraordinary circumstances retesting may be required. In such cases it is important to understand the consequences of retesting and concrete contingency plans to address all possible outcomes must be in place. In some cases, working with the ingredient supplier to better understand each scenario may reduce the need for such retesting. But this risk mitigation work must always be carried out using an individualized and risk-based approach to assure consumer food safety and public health.



## DEFINITIONS

**Alternative method** – New or different method of analysis for detection of the same analyte that is measured using the corresponding reference method, for a given category of products (ISO, 16140:2003)

**Antimicrobial** – an agent that kills microorganisms or stops their growth

**Bactericidal** – an agent that kills or inactivates bacteria

**Bacteriostatic** – an agent that prevents or significantly inhibits the growth rates of certain bacteria

**Batch** – A definite quantity of some commodity manufactured or received under conditions, which are presumed uniform for the purpose of these guidelines. To manage logistics, it may be necessary to create multiple smaller batches of a seasoning blend to fulfill customer needs for large lot sizes. At a minimum, the certificate of analysis should be generated using samples that represent the composition of the entire campaign used to create the production lot.

**GFSI** – Global Food Safety Initiative, a private organization that maintains various schemes used to benchmark food safety standards, some examples being BRC, FSSC22000 and SQF.

**Good Microbiological Practice (GMP)** – consists of aseptic techniques and other good microbiological practices that are not uniformly defined but are necessary to prevent contamination of the laboratory with the agents being handled and contamination of the work with agents from the environment.

**Inhibitory** – hinders or prevents growth

**Limit of detection (LOD)** – Lowest amount or concentration of analyte that a specific method can statistically differentiate from analyte-free sample matrix. This is dependent on sensitivity, instrumental noise, blank variability, sample matrix variability, and dilution factor.

**Lot** – Sometimes used interchangeably to mean a batch. According to ISO, Lot defined as “a collection of unit of product from which a sample shall be drawn and inspected to determine conformance with acceptability criteria, and which may differ from a collection of units designated as a lot for other purposes (for example production, shipment, etc.)”

**Matrix Extension** – a process of verifying a method to add additional matrices beyond those covered under the method validation scope.

**Non-Selective Enrichment broth** – Liquid media that is non-inhibitory, nutritionally complex with few or no selective agents and promotes the growth of other micro flora including *Salmonella*. Some examples of non-selective enrichment broth are: Buffered Peptone Water, Lactose Broth, Tryptic Soy Broth, Brilliant Green Water, Ringer’s with Brilliant Green, and Brain Heart Infusion Broth (BHI)

**Positive Release** – Results must be obtained prior to release of product.

**Proficiency Testing (PT) Program** – A structured and centrally managed Lab QA program that allows participating labs to demonstrate that they can consistently meet relevant internal or external standards for accuracy, reliability, and compliance, as it pertains to the detection of bacterial pathogens (or other targets) in inoculated food matrix samples. Each lab participating in a given PT program is sent multiple inoculated food samples for analysis using the laboratory’s established methods for testing actual “field” or commercial/QA samples. The test results are submitted to the PT program administrator for analysis, and the lab’s individual performance is assessed using specified statistical criteria with either a peer group of similar testing labs, or an assigned target determined by the organization that administers the PT program.

**Qualitative method** – Method of analysis whose result is the presence or absence of the target analyte, detected directly or indirectly, in a specified quantity of sample - for example, *Salmonella* detection, commercial sterility evaluation.



## DEFINITIONS

**Quality Management System (QMS)** – QMS is plans, controls, and improves the elements that impact on the achievement of the desired results by the laboratory and on the satisfaction of the users.

**Reference method** – Pre-existing recognized (international, regional, or national) standard method against which the candidate method will be compared.

**Salmonella Sensitive Ingredients** – Ingredients that have a history and increased probability of being contaminated with *Salmonella*.

**SAP™** – A widely used type of Enterprise Resource Planning (ERP) software. SAP stands for Systems, Applications, and Products, and is the trademark name for an ERP software system marketed by SAP AG (Walldorf, Baden-Württemberg, Germany).

**Selective enrichment broth** – Designed to inhibit the growth of other microorganisms while promoting the growth of *Salmonella*. Some examples of selective enrichment broth are: Selenite Cysteine broth; Tetrathionate Broth, and Rappaport-Vassiliadis Broth

**Sensitivity** – Sensitivity: The lowest concentration that can be distinguished from background noise or the smallest amount of a substance or organism that can accurately be measured by a method or test system is the analytical sensitivity.

**Specificity** – The capability of a method to discriminate between the analyte of interest and other components of the sample including matrix components.

**Traceability** – An activity of collecting and managing information regarding what has been done in manufacturing processes from acceptance of raw materials and parts to shipment of products.

**Validation** – A process by which a laboratory confirms by examination, and provides objective evidence, that the requirements for specific uses are fulfilled (US FDA, 2015). Validation studies typically involve the use of inoculated matrix or microbial challenge studies.

**Verification** – The confirmation by examination, and the provision of objective evidence, that specified requirements have been fulfilled (US FDA, 2015). Verification studies are required when a laboratory implements a fully validated, published, or reference standard method. A verification study is typically a much-abbreviated version of a validation study and is often focused on examining sample matrix effects to assure method accuracy or precision.





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