Refrigerated Foods Association (RFA) Standardized Protocol for Determining the Shelf Life of Refrigerated Ready-To-Eat (RTE) Foods (revised January 2009)

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EXECUTIVE SUMMARY:

The Refrigerated Foods Association (RFA) Standardized Protocol for Determining Shelf Life of Refrigerated Foods was developed in 2002 (http://www.refrigeratedfoods.org/ShelfLifeProtocol.doc). Since that time, industry's experience and a number of important regulatory and technological changes have been adopted within the food industry, necessitating the need for updating this protocol. The purpose of the original standardized protocol was to enable individual manufacturers, and or their customers if they so desired, to determine their product shelf life and compare their stated shelf life with the shelf life of similar products produced by other manufacturers through the utilization of this standardized method. The standardized protocol has been updated, specifically to include with it issues raised by the proposed FDA Compliance Guideline (http://www.fda.gov/ora/compliance_ref/cpg/cpgfod/draft_cpg555-320.html) allowing a tolerance for the psychrotrophic pathogen *Listeria monocytogenes* in foods that do not support its growth. In addition, the protocol has been revised to employ mathematical modeling to address growth and/or control of nonproteolytic *C. botulinum* type E strains in seafood products where modified atmosphere packaging is utilized.

1. Background

The original RFA standardized protocol was developed for determining the shelf life of refrigerated RTE foods with a focus on the following products: potato salad, tuna salad, and refrigerated pizza. It was not the intent of the project to determine the shelf life of these products. Instead, the project goal was to establish a standardized protocol by which individual manufacturers, and/or their customers if they so desired, could determine their product shelf life, and compare their stated shelf life with the shelf life of these and/or similar products produced by other manufacturers through utilization of this standardized method.

Since 2002, a number of important regulatory and technological changes have been adopted within the food industry, necessitating a need for revision of the RFA standardized protocol. Chief among these were addressing concerns regarding issues raised by the proposed FDA Compliance Guideline (U.S. FDA, 2008) allowing a tolerance for the psychrotrophic pathogen *Listeria monocytogenes* in foods that do not support its growth, as well as issues of nonproteolytic *C. botulinum* type E in refrigerated foods. Europe and Canada permit a regulatory tolerance of 100 cfu *L. monocytogenes*/g of food for RTE foods that do not support *Listeria* growth. The FDA is expected to revise policy regulations for establishment of a regulatory limit of 100 colony forming units per gram (CFU/g) for *Listeria monocytogenes* in foods that do not support the growth of the organism (http://www.fda.gov/ora/compliance_ref/cpg/cpgfod/draft_cpg555-320.html). In anticipation of adoption of this regulatory standard, which has been shown in countries outside of the U.S. to be protective to public health, the RFA Standardized Protocol has been revised to provide validation for this standard.

In order to develop a standardized protocol, a comprehensive literature review was conducted in order to overview current best practices in shelf life determination for refrigerated RTE foods. Consideration in this review was given to product handling during shelf life by consumers; holding temperatures which are appropriate for shelf life determination based upon product handling; and nature of product composition- whether the refrigerated RTE foods contain preservatives; the nature of the packaging material used and whether products were packaged under modified atmospheres or had pH adjustment to extend shelf life. A comprehensive search of the literature from 1990-2008 was performed using the Agricola, Medline and CAB abstracts databases and searching the Keywords “shelf life”, “determination” “foods”; “*Salmonella*” “*Campylobacter* infections”, “*Listeria* infections”, “disease outbreaks”, “food preservation” “tuna salad” “potato salad”, “cold salads”, and “shelf life”. Enforcement Reports on the FDA website were searched using the keywords “pizza”, “tuna salad” and “potato salad” and a search of the CFSAN database using the keywords “potato” or “salad”. Citations most appropriate for this study were selected for review.

A risk assessment was conducted for these products in order to determine how criteria for refrigerated shelf life based on safety could be accomplished. The literature cited above was

reviewed to determine risks posed by bacterial pathogens in the study foods and in refrigerated RTE foods in general. A number of bacterial pathogens have been shown to be capable of growth at refrigeration temperatures. These include: *Aeromonas hydrophila,* nonproteolytic strains of non-proteolytic *Clostridium botulinum* type E, *Listeria* spp., *Yersinia enterocolitica*, certain strains of *Bacillus cereus*, enteropathogenic *E. coli*, and *Vibrio parahaemolyticus* (Marth, 1998; NACMCF, 2005). A review of the literature indicated that certain of these pathogens have been associated with the study foods. In assessing the risks posed by foodborne pathogens in these products, consideration was given to the food characteristics, including potential microbiological hazards and the history of causing food-borne illness. Due to the ubiquitous nature of *L. monocytogenes*, its potential for growth at refrigeration temperatures and its high mortality rate, it was considered a primary pathogen of concern in these products. The risk of *Listeria* outgrowth in these products is a function of pH, temperature, salt concentration and A_w. If products are packaged under reduced oxygen conditions, consideration was given to performance of challenge studies that consider risk of non-proteolytic *Clostridium botulinum* type E outgrowth using either predictive mathematical modeling or conducting challenge studies, where appropriate. Microbiological parameters were established to dictate shelf life based upon a log growth tolerance.

Based upon the results of review above, best practices were proposed. These practices are the basis of a standardized protocol that facilitates experimentation, evaluation and validation of shelf life for refrigerated RTE foods, based upon quality and safety parameters.

2. Risk Assessment for Refrigerated Foods

The shelf life of refrigerated food products is dependent upon the interactive effects of several factors including the raw material quality, formulation of product, sanitary conditions during production, processes applied, storage temperature, the numbers and types of cold tolerant spoilage and pathogenic bacteria, the packaging materials used and the gaseous environment of the product (Mead, 1990; Walker, 2000). Winger (2000) defines shelf life as "the period of time at the end of which the 'quality' of a given food product is perceived as significantly, unacceptably different from the expected 'fresh' quality". Shelf life may be assessed through microbiological parameters, chemical or physical changes of foods, or sensory tests. The leading causes of food deterioration are attributable to physical, chemical and microbiological changes during storage (Singh, 2000). Physical changes may occur as the result of mishandling of foods during processing, while chemical changes occur as a result of enzymatic action, which is highly temperature-dependent and proceeds rapidly with increases in temperature. Oxygen, water and pH are also variables that dictate enzymatic as well as microbiological activities. The presence of bacteria, yeasts and molds can have an adverse impact upon refrigerated product shelf life.

To prevent microbial growth, a number of interventions are available to food processors including alteration of temperature, removal of water, reduction of oxygen, increase in CO_2, use of traditional antimicrobials such as sorbic acid and benzoic acid, and use of novel antimicrobials (such as sodium lactate/potassium diacetate, cultured dextrose and/or

maltodextrin, etc.). Trends in the food industry to extend the shelf life of products increasingly employ the use of modified atmosphere packaging (MAP). While MAP is effective in controlling spoilage organisms that limit shelf life, it is not effective in controlling pathogens. Fortunately, for mayonnaise-based products such as potato and tuna salads, these products are likely to exhibit decomposition, as evidenced by watery or oily separation, prior to outgrowth of pathogens such as *L. monocytogenes* during shelf life. Most of these salads, when commercially prepared, are formulated using chemical barriers, such as acetic acid, sodium benzoate and potassium sorbate, which are inhibitory to the growth of pathogens. New lactic acid and sodium/potassium lactate based products are commercially available to control outgrowth of *L. monocytogenes*, as well as to control the growth of a broad range of other organisms including yeasts and lactic acid bacteria. The 2005 Food Code exempts deli salads (ham, chicken, egg, seafood, pasta, potato and macaroni) prepared and packaged in a food processing plant from product dating since scientific data support the exemption because of sufficient acidity and preservatives to prevent growth of *L. monocytogenes* (Eblen, et al, 2002).

Organisms of concern from a spoilage standpoint in mayonnaise-based products include *Lactobacillus fructivorans*, *Lactobacillus plantarum, Lactobacillus fermentum, Lactobacillus brevis, Bacillus megaterium,* and fungi in the genus *Saccharomyces, Geotrichum* and *Zygosaccharomyces* (Smittle and Cirigliano, 2001). Since most lactobacilli and yeasts die quickly in mayonnaise-based products, their presence is usually indicative of potential spoilage problems and poor sanitation. Most products are processed to contain < 10 per g of these organisms where possible. Any increase above this level indicates a potential sanitation and/or spoilage problem (Smittle and Cirigliano, 2001). Lactic acid bacteria isolated from spoiled delicatessen salads included *Lactobacillus acetolerans, L. brevis, L. plantarum* and *L. buchneri. L. acetolerans* has been shown to have a high tolerance to acetic acid (Entani et al. 1986).

With respect to products such as fresh, refrigerated pizza, maintaining the quality of cheese during storage requires protection from dehydration and reduction of undesirable microorganisms including pathogens (Eliot et al. 1998). These undesirable organisms can cause odor and flavor changes and may also modify cheese texture and appearance. Yeasts and lactic acid bacteria were found to be the main microorganisms of concern in tomato paste and mozzarella cheese as components of fresh pizza. Elliot et al. (1998) examined the stability of shredded Mozzarella cheese under modified atmosphere storage. These authors found that modified atmospheres containing carbon dioxide were effective in stabilizing lactic and mesophilic flora and were inhibitory to staphylococci, molds and yeasts. Psychrotrophic organisms were found to be less numerous when stored in high CO_2 atmospheres. Optimal levels were determined to be 75% CO_2, where suppression of undesirable flora and reduced gas formation were observed. Cabo et al. (2001) determined that acidification and release of CO_2 as a result of the fermentative metabolism of lactic acid bacteria and yeasts were the main reasons for development of off-odors and off-flavors in ham pizza stored in MAP containing 20% CO_2. Fasano and Gallo (2001) conducted research to determine the optimal hygienic conditions for production of fresh pizza packaged by modified atmosphere with a shelf life of 15 days at 6-8^{o}C. Target organisms for analysis were APC, lactobacilli, yeasts and molds, *Listeria*, and *Salmonella.* Limits for acceptability during the 15-day shelf life were established as follows:

Table 1. Proposed limits of acceptability during the 15 day shelf life of fresh pizza packaged by modified atmosphere and stored at 6-8°C (Fasano and Gallo, 2001)

APC	$<10^3$ CFU/g
Lactobacilli	$<10^2$ CFU/g
Yeasts and Molds	<10 CFU/g
Listeria monocytogenes	Absent in 25 g
Salmonella	Absent in 25 g

Between 1990 and 2005, contaminated multi-ingredient foods such as salads, pizza and sandwiches were the causative agents of 367 outbreaks involving 23,142 cases of food poisoning (CSPI, 2005; viewed at http://www.cspinet.org/foodsafety/outbreak_alert.pdf). Of those foods, 93 outbreaks with 12,036 cases were linked to salads such as tuna, egg and potato. Of the three food products, presence of pathogens appeared to be most problematic in potato salad. Prepared potato salads contain boiled, diced potato with mayonnaise dressing, flavoring, herbs and other vegetables, and are marketed to foodservice outlets and through supermarkets and delicatessens to consumers (Wills and Taabe, 1998). These products have been shown to be vulnerable to contamination during handling by viral agents and group A streptococci which enter these products as a result of poor hygiene. Additionally, organisms which have caused outbreaks/recalls of potato salad include: *Salmonella typhi, Salmonella typhimurium, Salmonella enteritidis, Salmonella muenchen, Salmonella infantis, Salmonella Heidelberg, Shigella sonnei, Staphylococcus aureus, Listeria monocytogenes, Clostridium botulinum* (restaurant associated botulism), enterotoxigenic *E. coli*, *E. coli* 0157:H7, *E. coli* 06:H16, norwalk-like virus, and *Shigella flexneri*. Organisms that have caused outbreaks/recalls of tuna salad include: *Vibrio parahaemolyticus*, *Listeria monocytogenes, Clostridium perfringens* (suspected), *Salmonella enteritidis,* Hepatitis A, *Campylobacter jejuni,* and Scrombrotoxin. An outbreak of *Campylobacter jejuni* (O:33) infection at a summer camp was reported, with tuna salad implicated as the infective vehicle (Roels et al. 1998). It is believed that cross contamination with another food product, the food preparation surface, or hand contact by a food service worker were the most likely sources of contamination leading to this outbreak. This remains, however, an outbreak involving a rare strain in an unusual food vehicle. Outbreaks linked to pizza, while infrequent, show Hepatitis A, *S. aureus* and Norwalk-like virus as primary agents of infection.

PATHOGENS

1. Listeria

Storage temperature has a profound impact upon not only refrigerated product shelf life, but also upon the microbiological safety of the study products. The HHS *Listeria* Risk Assessment (USFDA/CFSAN-USDA/FSIS-CDC, 2003) utilized results of a survey conducted by Audits International (1999) in which home refrigerator temperature was analyzed for 939 refrigerators in the U.S. The survey results revealed that 27.4% of the refrigerators had storage temperatures exceeding 5^{o}C (41^{o}F), with 1.4% of refrigerators exceeding 51^{o}F (10.5^{o}C). Subsequent data from an Audits International (1999) U.S. Food Temperature Evaluation Survey revealed that 41% of retail food product temperatures were in the range of 41-50^{o}F, while 6.7% of products were at temperatures between 51-65^{o}F (Table 3). Ecosure, a division of Ecolab, updated the Audits International data in 2007 and revealed similar findings (http://foodrisk.org/exclusives/EcoSure). Thus, in determining the shelf life of refrigerated RTE foods, the potential for product to be held under less than optimal conditions must be considered and the potential for growth of spoilage flora and pathogens carefully evaluated. It is essential to insure that refrigerated RTE foods such do not support growth of pathogens during periods of temperature abuse by consumers and retailers.

Table 2. Frequency Distribution of Home Refrigerator Temperature from a Survey of 939 U.S. Refrigerators (Adapted from Table 111-8 FDA *Listeria* Risk Assessment)

Refrigerator Temperature (^{0}F)	Refrigerator Temperature (^{0}C)	Frequency (%)
<32	0	9
33-35	0.5-1.6	10
36-38	2.2-3.3	25
39-41	3.8-5.0	29
42-44	5.5-6.6	18
45-47	7.2-8.3	5
48-50	8.8-10	3
51-53	10.5-11.6	0.4
54-56	12.2-13.3	0.5
57-59	13.8-15	0.4
60-63	15.5-17.2	0.1

Table 3. Frequency Distribution of Retail Refrigerator Product Temperatures

Product Temperature (^{o}F)	Frequency (%) Audits International 1999	Frequency (%) Ecosure 2007
≤ 26F	-	0.7
27-29	-	0.7
30-32	-	6
<32	6	-
33-35	5	8
36-38	15	22
39-41	27	32
42-44	21	15
45-47	10	7
48-50	10	6
51-53	3	1
54-56	2	0.8
57-59	0.8	0.3
60-65	0.9	0.5
≥ 66F	-	0.05

Table 3. Home Refrigerator Temperature (based on temperature of 1793 food samples following placement in home refrigerator for 24 hours)

	F
Mean Product Temperature	38.2
Standard Deviation	4.40
Minimum Temperature	23
Maximum Temperature	63
Percent above 0F	-
Percent above 32F	-
Percent above 41F	17
Percent above 45F	5
Percent above 50F	0.7

Table Product Temperature Frequency Distribution EcoSure 2007

Product Temperature	Frequency (%)
<26 F	0.5
27-29	3
30-32	7
33-35	13
36-38	28
39-41	32
42-44	10
45-47	4
48-50	2
51-53	0.2
54-56	0.2
57-59	0.2
60-62	0.1
63-65	0.1
>66F	0

The HHS Listeria Risk Assessment (USFDA/CFSAN-USDA/FSIS-CDC, 2003) highlighted the risks posed to consumers by consumption of foods contaminated by *L. monocytogenes*. The Risk Assessment identified the consumption of non-reheated RTE deli meat and poultry products as presenting the highest risk to public health. The FDA/FSIS *L. monocytogenes* action plan sought advice on the scientific basis for establishing safety-based "use by" date labeling for refrigerated, ready-to-eat foods. The FDA expressed concern that consumers interpret the labeling terms "consume by" and "best if used by" as synonymous with "safe to consume until". The National Advisory Committee on the Microbiological Criteria for Food (NACMCF) was asked to provide assistance to the FDA in developing a scientific framework for the establishment of safety-based "use by" date labels (SBDL's). Specifically, the Committee considered 5 questions:

1. What are the scientific parameters for establishing safety-based "use by" date labels

for refrigerated RTE foods?

2. Should safety-based "use-by" dates for refrigerated RTE foods be established using mathematical modeling techniques? If so, what modeling approaches are best suited to the development of safety-based "use-by" date labels for refrigerated RTE foods?
3. What data needs to be acquired to scientifically validate and verify the adequacy of a proposed safety-based "use by" date label for a refrigerated RTE food?
4. What effects do the multiple factors that influence the growth and survival of *L. monocytogenes*, ie strain differences, food matrices, production and distribution systems, consumer susceptibility, etc., have on the establishment of safety-based "use by" date labels for refrigerated foods?
5. What impact would safety-based "use by" date labels likely have on the control of other foodborne pathogens in RTE foods?

The NACMCF (2005) concluded that *L. monocytogenes* was the appropriate target for a SBDL, and if established correctly, a SBDL could have a beneficial impact on public health. In response to question 1, the NACMCF advised that the pathogen of concern (*L. monocytogenes*) must be able to grow under refrigeration to levels which can cause illness in the food product under consideration. Scientific evidence documenting that a SBDL will reduce the risk of illness from that food must be available, and safety-based end points are necessary for establishing a SBDL. Other considerations included strain differences, the effect of various food matrices on these strains, the impact of competing microflora and packaging conditions, the impact of product distribution and handling practices, the susceptibility of consumers to the pathogens of concern, initial pathogen levels, and the kinetics of growth of the pathogen of concern (NACMCF, 2005). The FDA risk assessment concluded that limitation of storage time coupled with temperature control would have a major impact on reduction of the incidence of listeriosis.

Because presence of *Listeria* in refrigerated RTE foods continues to be of concern to the FDA, the fate of this pathogen in the study foods was included as a focus in developing a standardized protocol. Stresses to which bacteria in foods are subjected may result in increased expression of virulence in stressed pathogens and may promote adaptive mutations resulting in more virulent pathogens. The employment of processing procedures that tend to promote stress adaptation of organisms (extended refrigeration, cook/chill procedures, sous-vide) may generate organisms that become not only more adaptive to hostile environments, but also more virulent. A host of genes control stress adaptation in *Listeria* through production of heat shock proteins, cold acclimating proteins, and acid tolerance response proteins. As acidulants are utilized to extend refrigerated product shelf life, the impact upon this acidic environment upon *Listeria* growth and recovery must be considered. Hill and Gahan (2000) showed that exposure of *L. monocytogenes* to pH 5.5 for a short period (30 min) can result in the subsequent survival of these organisms to a normally lethal pH of 3.5. Repair enrichment procedures that incorporate a nonselective repair step provide a more accurate assessment of the true presence or absence of *Listeria* and also provide for a margin of safety. Research on stress adaptation of bacterial pathogens has revealed a host of genetic mechanisms that allow a both Gram positive and Gram negative bacteria to adapt to hostile environments. These mechanisms play a role in predicting the fate of pathogens in acidic foods. Acid adaptation increases the general resistance, including acid tolerance, of *L. monocytogenes, S. typhimurium,* and *E. coli*, so that acid-adapted pathogens survive better in

both acidic and fermented foods than unadapted cultures. Roth and Donnelly (1996) assessed survival of acid-injured *Listeria* in acidic food products such as apple cider, yogurt, fresh cole slaw and salsa. Temperature was found to greatly dictate survival of *Listeria* in salsa. When stored at 30°C, populations declined rapidly within 3 days. At 4°C, populations persisted for up to 17 days of storage (Donnelly, 2002). Johnson (1993), in a study conducted for the Refrigerated Foods Association, examined the fate of *L. monocytogenes* in refrigerated salads and determined that the presence of organic acids, and particularly acetic acid, coupled with the low pH of these products, were effective at controlling the growth of *Listeria*. Differential survival of *Listeria* was found in the various salads. Eblen et al. (2002) inoculated a 3-strain cocktail of *L. monocytogenes* at levels of 3 log 10 CFU/g into deli salads which were either manufactured by a retail store or by large-scale food manufacturers. Salads consisted of chicken, tuna, ham, egg, shrimp, crab, imitation crab, potato or cole slaw. Control and inoculated salads were stored up to 30 days at 5°C, 7°C, or 12° C and analyzed for *Listeria*, titratable acidity, water activity and pH. In salads produced by large scale food manufacturers, these products were found to contain chemical barriers to growth, and *Listeria* populations decreased or remained static during shelf life testing. In contrast, salads prepared by retail establishments lacked chemical preservatives, however they exhibited decomposition prior to outgrowth by *L. monocytogenes*. PURAC® Fresh, a lactic acid based product, is an example of technological improvement shown to be effective in controlling growth of *L. monocytogenes* in deli salads.

Almost without exception, foods involved in outbreaks or sporadic cases of listeriosis are those foods that can support the growth of *L. monocytogenes* and are ready to eat (examples being coleslaw fresh soft cheese, frankfurters, deli meats and butter, FDA 2008). Extensive industry data confirms the absence of *L. monocytogenes* in most RTE foods (Gombas, et al.2003; Frye and Donnelly, 2005). Europe and Canada permit a regulatory tolerance of 100 cfu *L. monocytogenes*/g of food for RTE foods that do not support *Listeria* growth. The FDA is expected to revise policy regulations for establishment of a regulatory limit of 100 colony forming units per gram (CFU/g) for *Listeria monocytogenes* in foods that do not support the growth of the organism. In anticipation of adoption of this regulatory standard, which has been shown in countries outside of the U.S. to be protective to public health, the RFA Standardized Protocol has been revised to provide validation for this standard. Considerations include validation of detection capabilities and the ability to determine with confidence that a food contains <100 cfu/g. Considerations of refrigerated and/or RTE foods that do not support the growth of *Listeria* will be made as follows:

1. Has a low pH (4.4), (*some fermented products, pickles)* or:
2. Has a low moisture content (water activity <0.92), (*Parmesan, other hard cheeses, bacon, and dried sausages)*; and
3. Is normally held or consumed in the frozen state
4. Processing involves an effective listeristatic control measure

The use of chromagenic media has greatly facilitated direct plating and enumeration of colonies for assessment of compliance with the <100 cfu/g *L. monocytogenes* regulatory standard. There are two classes of chromagenic media, the first of which utilizes cleavage by PI-PLC of L-α-phosphatidyl-inositol, forming a zone of precipitation around the colony, combined with the chromogenic substrate 5-bromo-4-chloro-3-indoxyl-β- glucopyranoside for detection of β- -

glucosidase, which occurs in all *Listeria* spp. All *Listeria* spp. produce turquoise colonies on these media. Further differentiation is facilitated by use of phosphatidylinositol or lecithin, hydrolysis of which indicates presence of phospholipase production by *L. monocytogenes* and *L. ivanovii.* Media containing both substrates in a nutrient-rich selective agar base is now known as "Agar Listeria according to Ottaviani and Agosti (ALOA™)". Media based on similar detection systems have been introduced by other manufacturers and include CHROMagar™ *Listeria*, BBL™ CHROMagar™ *Listeria*, and OCLA. For detection of *L. monocytogenes* in foods, the FDA recommends procedures outlined in the Bacteriological Analytical Manual (U.S. Food and Drug Administration 2003. Chapter 10, in Bacteriological Analytical Manual Online.) FDA recommends use of ALOA or BCM media. BCM™ *Listeria monocytogenes* plating medium belongs to a second group of chromagenic media which utilizes 5-bromo-4-chloro-3-indoxyl-myoinositol-1-phosphate. Pathogenic *Listeria* spp. form blue-turquoise colonies, while non-pathogenic *Listeria* spp. form white colonies. D'Amico et al (2008) used CHROMagar *Listeria* Plates (CHROMagar, Paris, France) to enumerate *Listeria monocytogenes* in soft ripened cheeses. A detection limit of $\geq$ 5 cfu/g was reached by use of 150 x 15 mm Petri dishes containing 30 ml of CHROMagar to which 1 ml of homogenate (1:10 dilution with food) was inoculated in duplicate. Turquoise colonies surrounded by a white halo were counted and sugar tubes further discriminated *L. monocytogenes* from *L. ivanovii*.

Food Safety legislation implemented in Europe establishes criteria for *L. monocytogenes* which either specifies absence in a 25 g food sample, or presence at a level below 100 cfu/g at any point in the shelf life of an RTE food. A national standard method has been established by the National Health Service Standards Unit (Health Protection Agency, 2007) for direct enumeration of *L. monocytogenes* based on BS EN ISO 11290 parts 1 and 2 (1997, 1998; see http://www.hpa-standardmethods.org.uk/pdf sops.asp. The method involves primary enrichment of 25 g or 25 ml of food product in 225 ml half Fraser broth at 30C for 24 hours, followed by secondary enrichment of 0.1 ml of half Fraser enrichment into 10 ml of Fraser broth, incubation at 37C for 48 hrs, and subculture to *Listeria* Chromagenic Agar. Samples negative by enrichment are reported as *Listeria* species not detected in 25 g. If *Listeria* are not isolated by enumeration but are isolated by enrichment, results are reported as "*Listeria* species detected in 25 g." For enumeration, food samples are diluted 1:10 in appropriate diluent, stomached or homogenized, then plated on *Listeria* Chromagenic Agar. If *Listeria* species are detected by enumeration, counts are calculated per gram or ml of food.

Scott et al (2005) published guidelines for conducting *L. monocytogenes* challenge testing of foods. These guidelines can be used to assess growth potential of *Listeria* in foods, for validation of the efficacy of inhibitors, or for validating process lethality. The guidelines address factors which should be considered in conducting studies of this nature, including choice and number of strains to be included in testing, initial inoculum levels, preparation and methods for inoculation, conditions of sample incubation, the length of incubation and sampling frequency. The method offers a standardized approach to challenge testing, including validating process lethality. The guidelines recommend use of the challenge testing in conjunction with a predictive model. A

number of mathematical models have been developed to predict the growth of *Listeria* and other foodborne pathogens in foods. These include the USDA Pathogen Modeling Program (http://ars.usda.gov/services/docs.htm?docid=6786); the U.K. Food MicroModel; ComBase (USDAUKFSA 2003) a joint USDA-ARS/U.K. Institute of Food Research and the U.K. Food Standards Agency program (http://ars.usda.gov/services/docs.htm?docid=8392); and Microfit software, developed by the U.K. Institute of Food Research (http://www.ifr.ac.uk/MicroFit), which is based on the Baranyi model. PURAC® has recently developed the PURAC Fresh *Listeria* Control Model, which is available upon request from the manufacturer.
If the predictive model indicates that growth is not likely during product shelf life, challenge studies become unnecessary. The guidelines recommend conducting challenge studies with 3-5 strains of *L. monocytogenes*, either individually or in combination. The guidelines recommend use of surrogates (*L. innocua*) only when there are no other options (such as challenge testing in a pilot plant or food processing facility). The level of inoculation for growth potential should reflect the expected level of contamination for the product. If process lethality is being assessed, high inoculation levels may be appropriate. Guidance on preparation of inocula, the need for pre-adaptation, and the need to minimize culture transfer, along with recommendations on the appropriate use of surface inoculation versus homogeneous incorporation into foods, is provided. It is recommended that challenge studies be conducted at more than one temperature (40-45^0 F and 50-55^{0}F) particularly when function of antimicrobials or other inhibitors may be temperature dependent. The recommended duration of shelf life testing is desired shelf life plus an additional margin to assess fate of *Listeria* if the product were used past recommended shelf life. The guidelines recommended 5-7 sample intervals to provide accurate information of behavior of inoculated strains. The guidelines recommend use of selective-differential plating media but caution the need to consider sublethal injury when utilizing selective media. Analysis of changes in product parameters such as moisture, salt, pH, titratable acidity, gaseous composition, preservative concentrations, and other parameters is necessary to understand behavior of *L. monocytogenes* in the tested product. The guidelines indicate that a <1 log increase in *L. monocytogenes* throughout the shelf life and across replicate trails would be indicative of a product which does not support *Listeria* growth and meets acceptance criteria.

In February 2008, the U.S. Food and Drug Administration published draft guidance for industry on the control of *L. monocytogenes* in refrigerated or frozen RTE foods (http://www.cfsan.fda.gov/~dms/lmrtegui.html). It is noted that with rare exceptions, foods involved in outbreaks of listeriosis have been those that support growth of the pathogen and are ready-to-eat. Published studies confirm that most RTE foods surveyed do not contain detectable levels of *L. monocytogenes* (Gombas et al. 2003, Frye and Donnelly, 2005). The FDA joint risk assessment concluded that *L. monocytogenes* does not grow in foods having a pH of less than or equal to 4.4; a Aw of less than or equal to 0.92 or in frozen foods. In deli type salads, *Listeria* growth can be controlled when acid is added to bring the pH to less than or equal to 4.4, or through use of sorbate and benzoate. Sorbate is used to control *L. monocytogenes* growth in cheeses. Control of *Listeria* has been achieved when shelf life studies demonstrate a less than 1 log increase of *L. monocytogenes* in replicate trials using test foods or formulations.

More recently (Beaufort et al. 2008), the EU Community Reference Laboratory (CRL) for *Listeria monocytogenes*, in collaboration with a working group of 10 laboratories, nine of which are National

Reference Laboratories for *Listeria monocytogenes*, prepared a technical guidance document providing detailed and practical information on how to conduct shelf life studies for *L. monocytogenes* in RTE foods
(see
http://ec.europa.eu/food/food/biosafety/salmonella/docs/shelflife_listeria_monocytogenes_en.pdf).
The report delineates challenge tests as those which are conducted with *L. monocytogenes* which has been artificially inoculated into foods to assess growth potential or to estimate growth parameters (maximum growth rate). Growth potential (δ) is defined as the difference between the $\log_{10}$ cfu/g at the beginning versus the end of the test. When $\delta > 0.5$ $\log_{10}$ cfu/g, the food is classified as RTE food able to support the growth of *L. monocytogenes* (other than those intended for infants and special medical purposes). When $\delta \leq 0.5$ $\log_{10}$cfu/g, the food is classified as a RTE food unable to support the growth of *L. monocytogenes* (other than those intended for infants or special medical purposes). The maximum allowable concentration of *L. monocytogenes* in the food must comply with the 100 cfu/g at any point during product shelf life. Combining challenge testing with the use of predictive models overcomes some of the limitations afforded by the use of challenge studies alone and are useful to predict the maximum growth rate of *L. monocytogenes* in the tested food. These tests are delineated from durability studies which examine *L. monocytogenes* in a naturally contaminated food. Durability tests are most useful when the prevalence of *L. monocytogenes* in a test food is high. The EU protocol recommends testing at least 3 batches of the same product. It also recommends use of 3 different strains of *L. monocytogenes*, at least one of which is a reference strain. Guidance is offered on preparation of the inoculum to obtain late exponential/early stationary phase cultures which are suitably adapted to the test food conditions. The recommended target level of contamination is 50 cfu/g, not to exceed 100 cfu/g. Detection is accomplished using *Listeria* chromagenic media specified in EN ISO 11290-1, amended.

2. *Clostridium botulinum* type E

The NACMCF (2005), in its consideration of safety based date labeling for refrigerated ready to eat foods, considered the threat of *C. botulinum* toxigenic type E strains involved in outbreaks in the United States. Between 1973 and 2000, CDC collected data from 150 patients with botulism from botulinum toxin type E in Alaska. None of these illnesses were from foods produced by a commercial source. Between 1990 and 2000, data from 24 outbreaks of botulism from botulinum toxin type E collected by CDC revealed that all outbreaks except two occurred in Alaska and only 1 was from a commercial source. As noted by the NACMCF, a 1989 CDC analysis of U.S. outbreaks indicated that no case of botulism due to non-proteolytic strains were obviously due solely to growth at refrigeration temperatures, and the committee therefore concluded that a SBDL would have little impact on preventing outbreaks associated with psychrotrophic *C. botulinum*. Outbreaks of botulism linked to seafood products in modified atmosphere packaging have almost exclusively involved uncooked/minimally processed seafood products (Gram, 2001). In a comprehensive report which examined *C. botulinum* in vacuum packed and MAP chilled foods, Peck et al (2006) note that there has not been a single outbreak of botulism associated with short shelf life chilled foods produced in the U.K. and concluded that unknown hurdles may have prevented growth and toxin formation by non-proteolytic *C. botulinum*. The unknown hurdles include: low spore contamination; a heat process that damages or reduces spore numbers; inhibitory background flora; a reduced pH, low water activity, presence of salt of

preservatives, inhibitory modified atmosphere, or an effect of food structure; storage at less than 8C through all or part of the food chain; product consumption prior to the end of shelf life; or heating of food prior to consumption to inactivate toxin. Peck et al (2006) noted that some foods (raw fish) have a much higher risk than others. The Peck study did note a dramatic effect of storage temperature on toxin formation by non-proteolytic *C. botulinum* type E. The model in ComBase Predictor (http://wyndmoor.arserrc.gov/combase) predicted time to toxin formation as 16 days at 5C and 24 days at 4C. Peck et al. (2006) therefore encouraged the maintenance of chilled food at 4C/5C to extend the safety margin. Groups in the US have advocated use of TTI's to assure the safety of MAP seafood products (Welt, et al., 2003; Mendoza, et al, 2004).

The Botulinum Working Party of the The European Chilled Foods Federation (ECFF) in 1996 specified that controls for long shelf life hermetically sealed products should be either:

- subjected to a 6D process (non-proteolytic *C. botulinum* spores): 90^0C/10 min and $<10^0$C
- <6D process (non-proteolytic *C. botulinum* spores) and $<3^0$C, or
- Demonstrably effective hurdles versus non-proteolytic *C. botulinum* spores

Guidelines issued in 1996 by the European Chilled Food Federation specify the following: "if a chilled product is to be packed in a reduced oxygen atmosphere and has a shelf life of >10 days, one or more of the following hurdles should also be used to control non-proteolytic (psychrotrophic) *C. botulinum*:

- heat to a temperature/time combination equivalent to 90C for 10 min (or equivalent); or
- Adjust Aw to < 0.97; or
- Increase acidity to <pH 5.0; or
- Use combinations of Aw, pH, atmosphere, temperature etc. that demonstrably will inhibit the growth of non-proteolytic (psychrotrophic) *C. botulinum* within the shelf life and storage conditions"

Safe harbors, as defined by the Advisory Committee on the Microbiological Safety of Food (ACMSF), Food Standards Agency, U.K. in 1992 include: storage at <3C; a heat treatment of 90^0 C for 10 min (or a process of equivalent lethality-80^0C for 129 min, 85^0C for 36 min) coupled with chilled storage temperature (designed to give a 6D process for nonproteolytic *C. botulinum*).; a pH of $\leq$ 5 throughout the food, combined with refrigerated storage; Aw of $\leq$ 0.97 throughout the food, combined with refrigerated storage; or combinations of heat treatment and other preservative factors which have been shown to prevent growth and toxin production by non-proteolytic *C. botulinum* type E, combined with refrigerated storage. In 1995, the ACMSF recommended "storage at $\leq$ 5C and a shelf life of $\leq$ 10 days, or storage at 5-10C and a shelf life of $\leq$ 5 days." Peck et al. (2006) noted the absence of reports of foodborne botulism that involved toxin formation by non-proteolytic *C. botulinum* in commercial chilled foods for which the shelf life and storage temperature were maintained under manufacturer's specifications. Outbreaks of non-proteolytic *C. botulinum* type E in commercial chilled foods have been confined to products where the shelf life or storage temperature has been abused.

Year	Country	Product	Toxin type	Cases (deaths)	Factors contributing to botulism outbreak	Reference
1981	USA	Uneviscerated salted, air-dried fish ("kapchunka")	B	1	Poorly controlled salting, lack of refrigeration	California State Health Department, 1981
1982	Madagascar	Commercial pork sausage	E	60 (30)	Inadequate preservation	Viscens et al., 1985
1985	USA	Uneviscerated salted, air-dried fish ("kapchunka")	E	2 (2)	Poorly controlled salting, lack of refrigeration	CDC, 1985
1987	USA and Israel	Commercial uneviscerated salted, air-dried fish ("kapchunka")	E	8(1)	Poorly controlled salting, lack of refrigeration	Slater *et al.*, 1989
1991	Sweden	Vacuum-packed hot-smoked rainbow trout	E	?	?	Korkeala *et al.*, 1998
1991	Egypt	Commercial uneviscerated salted fish ("faseikh")	E	>91(18)	Putrefaction of fish before salting	Weber *et al.*, 1993
1992	USA	Commercial uneviscerated salted fish ("moloha")	E	8	Insufficient salt	CDC, 1992
1994	Sweden	Vacuum-packed hot-smoked rainbow trout	E	?	?	Korkeala *et al.*, 1998
1995	Canada	"Fermented" seal or walrus (4 outbreaks)	E	9	Unsafe process	Proulx *et al.*, 1997
1997	France	fish	E	1	?	Boyer *et al.*, 2001
1997	Germany	Commercial hot-smoked vacuum-packed fish ("Raucherfisch")	E	2	Suspected temperature abuse	Jahkola and Korkeala, 1997; Korkeala *et al.*, 1998
1997	Argentina	Home cured ham	E	6	?	Rosetti *et al.*, 1999
1997	Germany	Home smoked vacuum-packed fish ("Lachsforellen")	E	4	Temperature abuse	Anon, 1998
1998	Germany	Commercial smoked vacuum-packed fish	E	4	?	Therre, 1999
1998	France	Commercial frozen vacuum packed scallops	E	1	Temperature abuse (?)	Boyer *et al.*, 2001
1998	France	Commercial frozen vacuum packed prawns	E	1	Temperature abuse (?)	Boyer *et al.*, 2001
1999	Finland	Whitefish eggs	E	1	Temperature abuse	Lindström *et al.*, 2004
1999	France	Salmon or fish soup	E	1	?	Boyer *et al.*, 2001
1999	France	Grey mullet	E	1	Temperature abuse (?)	Boyer *et al.*, 2001
2001	Australia	Reheated chicken	E	1	Poor temperature control	Mackle *et al.*, 2001
2001	USA	Home-made fermented beaver tail and paw	E	3	Temperature abuse	CDC, 2001
2001	Canada	Home-made fermented salmon roe (2 outbreaks)	E	4	Unsafe process	Anon, 2002
2002	USA	Home-made "mukluk" (from Beluga whale)	E	12	Unsafe process	McLaughlin *et al.*, 2004
2003	Germany	Home salted air-dried fish	E	3	Temperature abuse (?)	Eriksen *et al.*, 2004
2004	Germany	Commercial vacuum-packed smoked salmon	E	1	Consumed after "use-by date"	Dressler, 2005

Food MicroModel (FMM, version 2.5; Leatherhead Food Research Association, Leatherhead, Surrey, U.K.) and Pathogen Modeling Program (PMP, version 5.0, USDA ERRC, Wyndmoore, PA) have been used to generate predictive values for safe storage times for sous vide products potentially contaminated with non-proteolytic *C. botulinum* type E spores (Hyytia-Trees et al. 2000). Peck et al (2006) reported on four predictive models for determining growth of non-proteolytic *C. botulinum*:

1. Combase Predictor Model (www.combase.com) developed by Graham et al. (1996b). This model is based upon growth curves performed in microbiological broth media.
2. Pathogen Modeling Program (PMP). This probability model was developed by Whiting and Oriente (1997) and is available at www.arserrc.gov/mfs/PMP6 CurMod.htm).
3. The Baker/Genigeorgis Model was developed by Baker and Genigeorgis (1992) and is based on experiments conducted in 17,000 raw fish homogenates. This model is available in the PMP site (above).
4. Skinner/Larkin Model (Skinner and Larkin, 1998).

All four models predicted toxin formation to occur in microbiological broth media in less than 10 days at 8C. Peck et al (2006) report that, if present, non-proteolytic *C. botulinum* type E can form toxin in 10 days and less at 8C. ComBase Predictor estimates toxin formation in 6 days at 8C.

Table 1.1 Characteristics of the two physiologically and genetically distinct clostridia most frequently associated with foodborne botulism

	Proteolytic *C. botulinum* (mesophilic)	Non-proteolytic *C. botulinum* (psychrotrophic)
neurotoxins formed	A, B, F	B, E, F
minimum growth pH	4.6	5.0
minimum growth temperature	10-12°C	3.0°C
maximum growth NaCl	10%	5%
spore heat resistance ($D_{100°C}$)	>15 min	<0.1 min

As it is impractical to examine the test foods on a routine basis for all potential pathogens and spoilage flora of concern, it is recommended that a standardized protocol target analysis for the following: APC, coliforms, *E. coli*, *Staphylococcus aureus*, *L. monocytogenes,* lactobacilli, and yeasts and molds.

Revised Standardized Protocol:

The following is a recommendation for a standardized protocol for shelf-life determination of refrigerated RTE foods. It is applicable to many refrigerated RTE foods provided that a risk assessment is conducted to assure that this protocol sufficiently covers the hazards of concern. In order to establish the shelf life of perishable refrigerated RTE foods, it is necessary to perform careful experimentation, evaluation and adequate validation through establishment of microbiological and chemical testing and appropriate criteria of specified sensitivity and specificity. Validation of shelf life differs substantially from verification and routine monitoring. Validation is conducted prior to the initiation of a new food safety system to document that it is capable of achieving the desired food safety goal. In this standardized protocol, challenge studies will be considered as validation of shelf life safety parameters, while shelf life studies which measure indigenous flora and sensory parameters will be considered verification.

Purpose:

To provide the framework for a standardized protocol for determining the shelf life of refrigerated RTE foods so that microbiological criteria, chemical analysis and sensory profiles

are analyzed over time to substantiate the maintenance of quality and safety throughout the shelf life of these products.

Scope:

This standardized protocol is intended to provide a framework such that individual manufacturers or their customers can compare their stated shelf life against like product produced by other manufacturers. The standardized protocol establishes a shelf life program that meets basic requirements set out in this document and will include:

1. Shelf Life Analysis- Verification: complete testing to assure that consistent microbiological, chemical and sensory standards are measured, and parameters that dictate end of shelf life are identified. From a microbiological perspective, the standardized protocol will analyze potential for growth of indigenous organisms that may comprise both spoilage flora and bacterial pathogens.

a. Microbiological: For shelf life verification, products will be subjected to analysis for the following: Aerobic Plate Count (APC), coliforms, *E. coli, Staphylococcus aureus,* lactobacilli and yeasts and molds.

b. Chemical: pH, TA. A_w and temperature

c. Sensory Analysis: Sensory specification that identifies important organoleptic attributes and acceptable tolerances around them.

2. Challenge Studies: performed in order to assess the fate of two specific pathogens (*Listeria monocytogenes* and in the case of reduced oxygen packaging, non-proteolytic *Clostridium botulinum* type E) Mathematical modeling is recommended to determine the potential for growth and toxin production by non-proteolytic *C. botulinum* type E in the product.

General Shelf Life Analysis Requirements:

1. Selection of Samples for Shelf Life Analysis:

For measurement of shelf life from a specific production run, sufficient samples should be set aside to complete 2 full schedules of tests at storage temperatures of 4^oC and 10^oC. For microbiological and chemical analysis, at each time point during the analysis, samples should be prepared from a minimum of two (duplicate) unopened containers for each storage temperature (N=4x 7 time points=28 total containers) to account for variations in test product composition, testing each package separately. Once samples have been aseptically removed for microbiological and chemical analysis, containers can be used for sensory analysis.

2. Sample Storage:

Samples will be stored at two specified temperatures: 4 ± 1^oC (optimal refrigeration) and 10 ± 1^oC (abuse temperature). Storage temperature will be monitored by use of an appropriate recording device. Samples will be properly labeled to clearly identify the shelf life protocol for which they are intended. Records will be kept as to the location and number of shelf life samples and when samples are removed for shelf life testing.

3. Testing Protocol for Refrigerated RTE Food Shelf Life:

The purpose of conducting shelf life analysis is to validate that the shelf life that has been established for a specific product is appropriate. All products will be tested at seven different ages regardless of length of expected shelf life. Shelf life analysis is dependent upon examination of batches of samples until the shelf life becomes unacceptable. Sampling intervals should be determined at 20% of product shelf life, which comprise seven different ages from fresh to full shelf life, plus a sampling to allow an interval 20% beyond shelf life for a total time to encompass 120% of shelf life. Therefore, if shelf life of refrigerated salads is expected to be 30 days, sampling intervals should consist of day 0, 6, 12, 18, 24 and 30 and 36. A 35 day expected shelf life would call for analysis at 0, 7, 14, 21, 28, 35 and 42 days. Refrigerated pizza with an expected shelf life of 90 days would be analyzed at 0, 18, 36, 54, 72 and 90, and 108 days.

The reliability of the analytical testing is directly related to the precision of the employed analytical methodology as well as the sampling plan employed. The use of appropriate and validated analytical methods and sampling plans are essential when performing shelf life analysis using this standardized protocol. Testing methods will be performed in accordance with either (1) methods specified in the FDA Bacteriological Analytical Manual, USDA Microbiology Laboratory Guidebook Number 8, Compendium of Methods for the Microbiological Examination of Foods, or AOAC; or (2) validated methods with an approved technical report that addresses specificity and accuracy.

4. Microbiological Analysis:

Aerobic Plate Count: The aerobic plate count should be performed in accordance with methods prescribed in Chapter 7 of the *Compendium of Methods for the Microbiological Examination of Foods, 4th Edition*; Chapter 3 of the FDA Bacteriological Manual, 8th Edition, Revision A. 1998; or the AOAC *Official Methods of Analysis,* sec. 986.33 (AOAC International, Gaithersburg, Md.).

Coliforms and *E. coli*: Coliforms should be enumerated using procedures described in Chapter 8.7 of the *Compendium of Methods for the Microbiological Examination of Foods, 4th Edition,* FDA Bacteriological Analytical Manual, using the MPN, conventional solid medium method or the Dry Rehydratable Film Method (AOAC Official Method 991.14 as described in *Official Methods of Analysis of AOAC International*).The Dry Rehydratable Film Method for the enumeration of *Escherichia coli* can be performed using the Petrifilm™ *E. coli* Count plate methods are described in the APHA Standard Methods for the Examination of Dairy Products and in the Official Methods of Analysts of AOAC International. The MPN method or the Dry Rehydratable Film Method can be used to confirm fecal coliforms and *E. coli* can proceed using methods outlined in the FDA BAM Manual, including use of the or the *Compendium of Methods for the Microbiological Examination of Foods*, Chapter 8.8.

Yeasts and Molds: Enumeration of yeasts and molds using Dichloran Rose Bengal Chloramphenicol Agar (DRBC) as specified in Chapter 20.51 of the *Compendium of Methods*

for the Microbiological Examination of Foods, 4th Edition, is recommended.

Lactobacilli: Perform enumeration based upon methods described in the *Compendium of Methods for the Microbiological Examination of Foods,* Chapter 53.85 (Smittle and Cirigliano, 2001), Chapter 19.529 (Hall et al. 2001) using *Lactobacillus*-selective MRS agar or MRS-S agar, or Chapter 19.521 using MRS agar with an APT agar overlay.

Staphylococcus aureus: Staphylococcus aureus should be enumerated according to procedures described in the FDA Bacteriological Analytical Manual, 8th Edition, Revision A 1998, Chapter 12 or in the Compendium of Methods for the Microbiological Examination of Foods, 4th Edition. Quantitative pathogen detection can be facilitated through employment of CHROMagar *Staph aureus* (CHROMagar Microbiology, Paris, France).

Interpretation of Results:

Microbiological results will be interpreted through use of pass/fail criteria.
Pass Criteria:
APC counts: Will not exceed 1x 10^4 CFU/gm upon initial sampling(except in cases were there are cultured ingredients or other ingredients were "high" APC is acceptable)
Total increase of $\leq$3 log APC over product shelf life.

Coliforms: Will not exceed 1 x 10^2 CFU/gm upon initial sampling
Total increase of $\leq$2 log over product shelf life.

Yeasts and Molds: Not to exceed 10^2 within 24 h. Total increase should be limited to $\leq$3 log increase at end of shelf life.

Lactobacilli: $<10^2$ initially; Total increase should be limited to $\leq$3 log increase at end of shelf life.

S. aureus: Will not exceed 1 x 10^2 CFU/gm upon initial sampling. No more than a 1 log CFU/gm increase for any two consecutive time points. Total increase of $\leq$ 3 log throughout product shelf life

Criteria	Class 1[a]	Class 2[b]	Class 3[c]	Fail
$T_0 < 10^4$ APC $T_{end} \leq 3$ log increase	met	met with 1 borderline (≤ 0.5 log increase within 1 category	met with 2 borderlines within 2 categories	exceeds 2 borderline tests across all categories
Initial coliform counts $<10^2$ <2 log increase	met			
Y&M $<10^2$ initially <3 log increase	met			
Lactobacilli $<10^2$ initially <3 log increase	met			
T_0 *S. aureus* $<10^2$ < 1 log increase between time points T_{end} <3 log increase	met			
TOTAL	5/5	4/5	3/5	2/5

[a] product meets all acceptance criteria
[b] 1 borderline test result
[c] no more than 2 borderline results

5. Chemical Analysis:

a. pH Analysis**:** Should be conducted according to protocol specified in the Compendium of Methods for the Microbiological Examination of Foods, 4th Edition, Chapter 64.6, or 21 CFR Volume 2 (revised April 1, 2001).

b. Titratable Acidity: Should be conducted according to protocol specified in the Compendium of Methods for the Microbiological Examination of Foods, 4th Edition, Chapter 64.8.

c. $A_{w:}$ Should be conducted according to protocol specified in the Compendium of Methods for the Microbiological Examination of Foods, 4th Edition, Chapter 64.1.

6. Sensory Evaluation:

a. All determinations should be made based upon comparison to a reference sample.

b. For each test temperature, 2 containers each of representative product should be removed from storage for analysis.

c. Each container or package should be inspected for gas formation by spoilage flora as evidenced by doming of lids or blowing in the package.

d. Containers/packages should be opened and held at room temperature for at least 10 minutes to allow samples to equilibrate (Mead, 1990).

e. For refrigerated salads, sensory properties associated with deterioration during refrigerated shelf life (odor, color, syneresis and taste) should be evaluated by at least three individuals, and results expressed as a mean score using a four point scale (see sample score sheet) where:

Odor:

1=Normal odor
2=Slight off-odor
3=Moderate off-odor
4=Strong off odor

Color:
1=Normal Color
2=Slight browning
3=moderate browning
4=pronounced browning

Syneresis:
1=normal appearance-no evidence of watery/oily separation
2=Slight watery/oily separation
3=moderate watery/oily separation
4=pronounced watery/oily separation

Taste:
1=Normal/fresh taste
2=Slight rancidity/spoilage
3=Moderate rancidity/spoilage
4=Pronounced rancidity/spoilage

A mean score of 2.5 or below indicates an acceptable product. A mean score above 2.5 marks the end of product shelf life.

f. Sensory Evaluation of Pizza: Will proceed based upon a modification of the methods described by Cabo (2001). Briefly, sensory evaluation should be conducted by a three-member panel. A quality evaluation is carried out by monitoring inflation of packages, examination for presence of yeasts and molds, and changes in the color of pizza components. Subsequently, pizzas are oven cooked for 10 min at 250^{o}C and sensory properties are scored on a four point

scale where:

1=extremely like
2=acceptable
3=moderately dislike
4=extremely dislike

A mean score of 2.5 or below indicates an acceptable product. A mean score above 2.5 marks the end of refrigerated pizza shelf life.

7. Challenge Studies for assessing *Listeria monocytogenes* growth potential during shelf life:

The challenge tests proposed herein are designed to provide a laboratory simulation of what may happen to *L. monocytogenes* in a test food or formulation during its refrigerated self-life. Challenge studies can be costly and time consuming, and results will apply only to those specific conditions tested. Therefore, these studies should proceed with these considerations in mind. Any changes in product formulation or use of storage temperatures different than those used in challenge studies may invalidate the results of the challenge study. All challenge studies assessing potential for *L. monocytogenes* growth should proceed with utilization of known, well-characterized strains in a referenced collection, such as the ILSI Strain Collection (Fugett et al 2006). Cultural conditions prior to inoculation are important and may affect subsequent growth, thus they are defined herein. Inoculation conditions should not alter test medium (i.e. increase the water activity of test foods). The goal of the challenge studies is to determine whether the test product is able to support to growth of *L. monocytogenes*, and if so, to what level under different storage conditions. The FDA has stated that *Listeria* control measures are deemed effective if challenge studies show a less than 1 log increase in *L. monocytogenes* levels during replicate trials of the specific food being tested (USFDA, 2008).

Challenge testing for *L. monocytogenes* is often conducted with simultaneous use of predictive modeling. Predictive models recommended include the USDA Pathogen Modeling Program (http://www.arserrc.gov/cemmi); Microfit software, developed by the U.K. Institute of Food Research (http://www.ifr.ac.uk/MicroFit; and ComBase (USDAUKFSA 2003) a joint USDA-ARS/U.K. Institute of Food Research and the U.K. Food Standards Agency program ((http://wyndmoor.arserrc.gov/combase). If predictive modeling indicates that growth during product shelf life is unlikely to occur within a reasonable safety margin, challenge studies are unnecessary (Scott et al, 2005).

Listeria Challenge Studies:

Recommended Strains: *Listeria monocytogenes* strains FSL N1-227 (serotype 4b/ribotype DUP-1044A; outbreak strain originally isolated from frankfurters), FSL R2-764 (serotype 4b/ribotype DUP-1044A; outbreak strain originally isolated from turkey deli meat, and

FSL R2-499 (serotype 1/2a/ribotype DUP-1053A; outbreak strain originally isolated from turkey deli meat (CDC, 2000; Hise et al., 2004) were shown by CDC PulseNet data to be the most commonly distributed outbreak serotypes between 1998 and 2002.These strains are recommended for use in challenge studies and can be obtained from the ILSI *Listeria monocytogenes* Strain Collection (Fugett et al., 2006), through Dr. Martin Wiedmann (Cornell University, Ithaca, NY). Cultures should be stored as frozen stock cultures (-78C). Prior to use, inoculate cultures to 9 ml of trypticase soy broth with 0.6% yeast extract and incubate at 30 $\pm$ 1°C for 18 h for two transfers prior to use. Determine viable counts of *L. monocytogenes* in suspensions from aerobic plate counts on Petrifilm AC films (3M Microbiology, St. Paul, Minn) incubated at 32 $\pm$ 1°C for 48 $\pm$ 2 hrs.

Insure that test product is free of *L. monocytogenes* contamination through dual primary enrichment of a 25 g test portion in 225 ml University of Vermont-Modified *Listeria* Enrichment Broth (UVM) incubated at 30C for 24 h, along with a 25 g portion in Listeria Repair Broth (see Figure 1). The mUSDA method is carried out as described by Pritchard et al. (1999). Samples should undergo dual primary enrichment in both UVM and *Listeria* repair broth (LRB) incubated at 30°C for 24 h. Filter sterilized solutions (0.45µm nitrocellulose filter; General Electric Water and Process Technologies) resulting in 40mg/L of nalidixic acid-sodium salt (Sigma-Aldrich. St. Louis, MO), 50mg/L of cyclohexamide (Sigma-Aldrich) and 15 mg/L of acriflavine-HCL (Sigma-Aldrich) are added to LRB after 4-5h incubation at 30°C. For secondary enrichment, 0.1ml each of UVM and LRB are inoculated into 10 ± 0.5 ml FB incubated at 35 ± 2°C for 26 ± 2h. A 0.1 ml aliquot from Fraser Broth enrichment should be plated on CHROMagar *Listeria* plates incubated at 37C for 24 h as recommended by D'Amico et al. (2008). A detection limit of $\geq$ 5 cfu/g was reached by use of 150 x 15 mm Petri dishes containing 30 ml of CHROMagar to which 1 ml of homogenate (1:10 dilution with food) was inoculated in duplicate. A product free of *Listeria* contamination will show an absence of turquoise colonies surrounded by white halos.

Assessment of Growth Potential:

***L. monocytogenes* Strains**: A three strain cocktail comprised of equal portions of *L. monocytogenes* FSL N1-227, FSL R2-764, and FSL R2-499 should be inoculated to test product at initial levels of 50 cfu/g of product (not to exceed 100 cfu/g product). Use 1 ml of the appropriate serial dilution from stock culture in Butterfield's Phosphate Buffer (BPB) to obtain initial target levels. The FDA Draft Compliance Guideline specifies the use of ISO 11290-2:1998/Amd.1:2004(E) for enumeration of *L. monocytogenes* (http://www.iso.org/iso/en/ISOOnline.frontpage). Enumerate *L. monocytogenes* by colony count on Listeria Chromagenic Media (CHROMagar *Listeria* plates incubated at 37C for 24 h. in conjunction with MPN enumeration using selective enrichment in MOPS-BLEB with subsequent plating on CHROMagar *Listeria* plates incubated at 37C for 24 h is recommended).

Inoculation and sampling for shelf life studies: Select 14 representative containers of product from the same production lot; inoculate containers with 1 ml of a 3 strain cocktail of *L. monocytogenes*, initial inoculation target 50 cfu/g, not to exceed 100 cfu/g and place 7 inoculated containers at 4°C and 7 inoculated containers at 10°C. Select 4 additional containers; inoculate 1

with 1 ml of Butterfield's phosphate buffer to serve as inoculation control and a second uninoculated container which will serve as an uninoculated control. Place one set of controls at 4C and the second set of controls at 10C. Sample at intervals specified in section 3 (Testing Protocol for Refrigerated Salads). Aseptically remove 25 gram samples of product and prepare serial dilutions. Directly plate product samples at each sampling interval. A detection limit of $\geq$ 5 cfu/g can be reached by use of 150 x 15 mm Petri dishes containing 30 ml of CHROMagar to which 1 ml of homogenate (1:10 dilution with food) is inoculated in duplicate. Turquoise colonies surrounded by a white halo are counted. Biochemical tests can be used to further discriminate *L. monocytogenes* from *L. ivanovii* if desired.

Acceptance criteria: Compare mean counts at time zero with mean counts at sampling intervals. An increase of $\leq$ 1 log over shelf life of product is indicative of a product which will not support *Listeria* growth. Counts in product should not exceed 100/g above initial inoculum levels to meet compliance with regulatory criteria of <100 cfu/g product during product shelf life.

Clostridium botulinum:
Assess potential for growth/toxin production in product through use of one of the following predictive models:

1. Combase Predictor Model (www.combase.com) developed by Graham et al. (1996b). This model is based upon growth curves performed in microbiological broth media.
2. Pathogen Modeling Program (PMP). This probability model was developed by Whiting and Oriente (1997) and is available at www.arserrc.gov/mfs/PMP6 CurMod.htm).
3. The Baker/Genigeorgis Model was developed by Baker and Genigeorgis (1992) and is based on experiments conducted in 17,000 raw fish homogenates. This model is available in the PMP site (above).

If predictive models indicate potential for growth, this is indicative of the need for strict temperature control, and shelf life limitations as recommended in the FDA/CFSAN FDA 2001 Food Code. Inoculated pack studies may be warranted.

Methods and Considerations: Sublethal Injury

Numerous studies have evaluated the performance of different isolation methods for their ability to detect low levels of *L. monocytogenes* and sublethally injured cells in foods and food processing environment and established that highly selective media may not recover sublethally injured cells (Pritchard and Donnelly, 1999; Donnelly, 2002; Bruhn et al., 2005). Accordingly, procedures that allow for repair of injured cells have been recommended when attempting to recover injured *Listeria*. Failure to account for injured *L. monocytogenes* in food and environmental samples presents a significant public health risk. Processing stresses such as the addition of sodium nitrite ($NaNO_2$), NaCl, lactic acid, heating, freezing, and contact with sanitizers reduce levels of *Listeria* and may cause sublethal injury. There is a need for improved isolation methods with increased

ability to detect both low level and injured *Listeria* populations. Mead (1999) documented that low numbers of *Listeria* (0.3 CFU/g) were recovered from frankfurters involved in the 1998/1999 outbreak. Sodium nitrite ($NaNO_2$) is used as a curing agent in meat products including frankfurters, smoked ham and luncheon meats. Currently, the USDA/FSIS permits between 100-200ppm of $NaNO_2$ in cured meats. Pelroy et al. (1994) demonstrated the delay of growth in *L. monocytogenes* when 190-200ppm $NaNO_2$ in conjunction with an oxygen permeable film, 5% salt and low temperature (5^0C) was used. Ngutter and Donnelly (2003) determined that $NaNO_2$ has the ability to injure *L. monocytogenes*; nitrite-injury is reversible; and recovery of *L. monocytogenes* from frankfurters containing nitrite is improved using dual-enrichment in *Listeria* Repair Broth (LRB) and University of Vermont-Modified *Listeria* Enrichment Broth (UVM). $NaNO_2$, when used at concentrations of 100 and 200ppm, was found to injure *L. monocytogenes.* The injury was completely reversible or growth of uninjured *Listeria* occurred in LRB when injury was between 98.5 and 98.7%. However, total recovery was not observed in LRB when injury exceeded 99%. UVM was unable to reverse the effects of nitrite-injured *L. monocytogenes*. With respect to time, inoculum, and meat type, LRB was found to consistently be superior to UVM at recovering *L. monocytogenes* from frankfurters (Table 1).

Table 1. Recovery of *L. monocytogenes* in chicken frankfurters using two primary enrichment broths.

Time (days)	*% Recovery (LRB)*	*% Recovery (UVM)*
0	100	100
4	100	100
7	100	75
14	100	58.3
21	66.6	25
28	50	0

LRB= *Listeria* Repair Broth and UVM= University of Vermont *Listeria* Enrichment Broth

Table 1 indicates that both LRB and UVM recovered 100% of *Listeria*, until day 7 when UVM only recovered 75%, while, LRB continued to recover *Listeria* (100%). By the end of the study (day 28), *Listeria* was undetectable in all frankfurters using UVM, but LRB was able to recover *Listeria* 50% of the time. Given that the shelf life of commercial frankfurters is 70 days, these results are significant.

The USDA-HHS Risk Assessment (USFDA/CFSAN-USDA/FSIS-CDC, 2003) identified frankfurters, deli meats, smoked seafood and preserved fish as foods at high risk for transmission of listeriosis. Nyachuba and Donnelly (2007) determined the impact of nitrite ($NaNO_2$) in

smoked salmon, smoked ham and bologna on viability and detection/recovery of *L. monocytogenes.* Nitrite-containing (NC-200 ppm $NaNO_2$) or nitrite-free (NF) foods were inoculated with a 5-strain cocktail of *L. monocytogenes* at 2.5 x 10^2 CFU/g, vacuum-packed, and stored at 5^{o}C. Samples were analyzed weekly for presence of *L. monocytogenes* using modified University of Vermont (UVM) broth, *Listeria* enrichment broth (LEB), Listeria repair broth (LRB), a combination of UVM and LRB (dual enrichment), and the PCR-based BAX system during 4-8 weeks of storage. Residual $NaNO_2$, total aerobic count, and percent injury were also determined at each sampling. *L. monocytogenes* were directly enumerated on Modified Oxford Agar (MOX) and CHROMagar. By the end of week 1, plating on CHROMagar and MOX resulted in 1 x 10^2 CFU and <1 x 10^2 *Listeria*/g, respectively, for NC foods. This decrease in *L. monocytogenes* is attributed to $NaNO_2$-indiced injury, which ranged from 83-99%. $NaNO_2$ decreased from 200 ppm at week 0 to <20 ppm at week 4 and thereafter. The BAX system and the dual enrichment detected 100 and 92.4% of positive samples, respectively, in all products throughout the storage period. While LRB recovered 43/48 *Listeria*-positive samples and UVM recovered 39/48 in NC smoked salmon, both media recovered *Listeria* in NF salmon-positive samples 100% of the time. As for smoked ham and bologna, more positive samples escaped detection at the earlier sampling times, indicating that high $NaNO_2$ masked detection of positive samples. These results provide further evidence that $NaNO_2$-injury masks detection/recovery of *L. monocytogenes* in NC ready-to-eat meat and seafood products, which can repair and grow to high levels over extended refrigerated storage periods

A further complication posed during isolation of *L. monocytogenes* from foods is the presence of other *Listeria* spp. Silk, Roth and Donnelly (2002) found the levels of selective agents used in Fraser broth to be sufficient enough to cause an extended lag period and an increased repair time for injured *L. monocytogenes* cells. In further studies, the efficacy of two different enrichment media for recovery of acid-injured *Listeria* from acidic foods was assessed using *Listeria* Repair Broth (LRB) and UVM. At time points where differences were detected, LRB detected the organism in 22 of 54 samples, compared to UVM, which detected only 3 of 54 contaminated samples. These findings have important implications for the safety of refrigerated foods that are dependent upon chemical barriers to acidify foods and extend shelf life.

Incidence data has shown that nonpathogenic *Listeria* are as common as *L. monocytogenes* in food samples, and that *L. innocua* can exceed the numbers of *L. monocytogenes* in selective enrichment, masking its detection. Curiale and Lewis (1994) found *L. innocua* to exceed numbers of *L. monocytogenes* by 100 fold in both primary and secondary enrichments. Therefore, the expected rate of isolation of *L. monocytogenes* is low, with only one chance in 100 of a colony being *L. monocytogenes* on an isolation plate. Therefore, incidence of *L. monocytogenes* may be underreported, with enrichment favoring nonpathogenic *L. innocua. L. monocytogenes* consists of three genetic lineages and 13 serotypes. Strains belonging to three serotypes (1/2a, 1/2b and 4b) are associated with the majority of human cases of listeriosis. Serotype 1/2b and 4b belong to lineage 1, with serotype 1/2a and most other food isolates belonging to lineage 2. Bruhn et al. (2005) reported enrichment bias when using UVM media to enrich samples co-inoculated with *L. innocua, L. monocytogenes* lineage 1 strains and *L. monocytogenes* lineage 2 strains. *L. innocua* outcompeted *L. monocytogenes* lineage 1 but not lineage 2 strains. Further, *L. monocytogenes* lineage 2 strains consistently outcompeted lineage 1 strains. This significant study confirms that existing enrichment procedures have shortcomings

associated with identification of those *L. monocytogenes* serotypes of most significance to human health.

The use of chromagenic media has greatly facilitated direct plating and enumeration of colonies for assessment of compliance with the <100 cfu/g *L. monocytogenes* regulatory standard. Method BS EN ISO 11290 parts 1 and 2 (British Standards Institution) incorporate use of chromagenic media that takes advantage of B-glucosidase activity of *Listeria* species. Further differentiation is facilitated by use of phosphatidylinositol or lecithin, hydrolysis of which indicates presence of phospholipase production by *L. monocytogenes* and *L ivanovii*. D'Amico et al (2008) used CHROMagar Listeria Plates (CHROMagar, Paris, France) to enumerate *Listeria monocytogenes* in soft ripened cheeses. A detection limit of $\geq$ 5 cfu/g was reached by use of 150 x 15 mm Petri dishes containing 30 ml of CHROMagar to which 1 ml of homogenate (1:10 dilution with food) was inoculated in duplicate. Turquoise colonies surrounded by a white halo were counted and sugar tubes further discriminated *L. monocytogenes* from *L. ivanovii*.

Food Safety legislation implemented in Europe establishes criteria for *L. monocytogenes* which either specifies absence in a 25 g food sample, or presence at a level below 100 cfu/g at any point in the shelf life of an RTE food. A national standard method has been established by the National Health Service Standards Unit (Health Protection Agency, 2007) for direct enumeration of *L. monocytogenes* based on BS EN ISO 11290 parts 1 and 2 (1997, 1998; see http://www.hpa-standardm,ethods.org.uk/pdf sops.asp. The method involves primary enrichment of 25 g or 25 ml of food product in 225 ml half Fraser broth at 30C for 24 hours, followed by secondary enrichment of 0.1 ml of half Fraser enrichment into 10 ml of Fraser broth, incubation at 37C for 48 hrs, and subculture to *Listeria* Chromagenic Agar. Samples negative by enrichment are reported as *Listeria* species not detected in 25 g. If *Listeria* are not isolated by enumeration but are isolated by enrichment, results are reported as "*Listeria* species detected in 25 g." For enumeration, food samples are diluted 1:10 in appropriate diluent, stomached or homogenized, then plated on *Listeria* Chromagenic Agar. If *Listeria* species are detected by enumeration, counts are calculated per gram or ml of food.

Figure 1. Assessment of presence of *Listeria* in test products

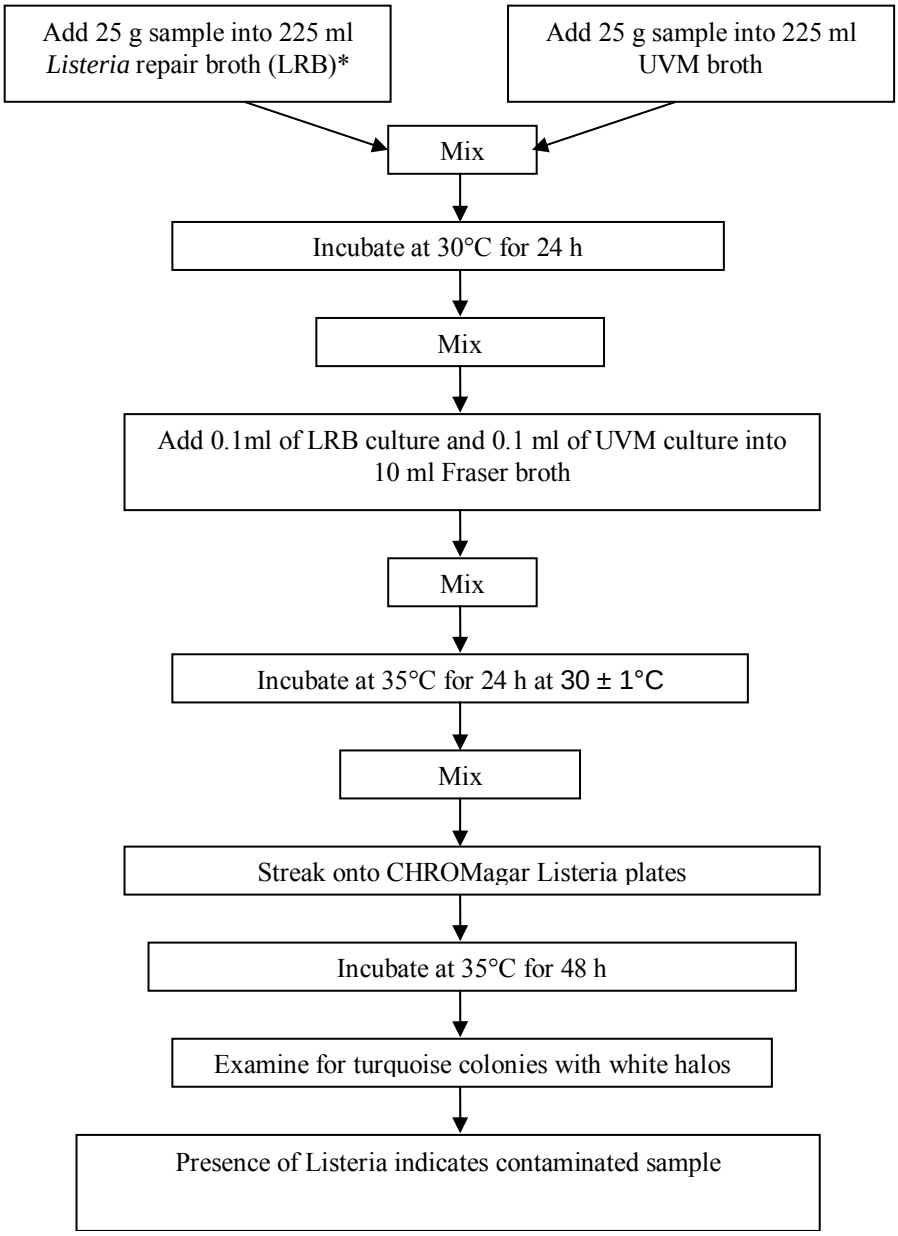
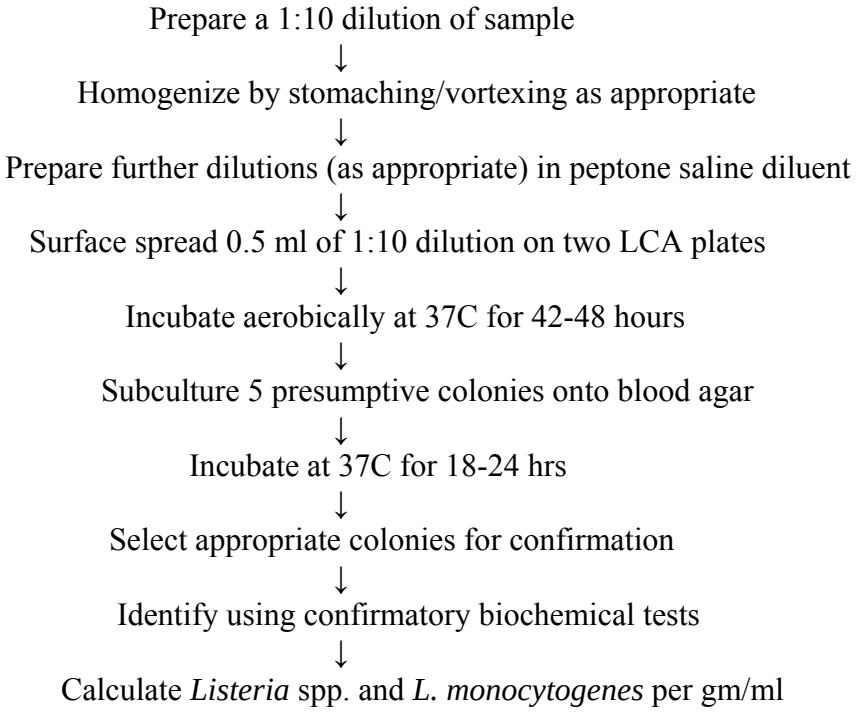


Figure 2. Overview of ISO 11290-2:1998/Amd. 1:2004(E)
Microbiology of food and animal feeding stuffs-Horizontal method for the detection and enumeration of *Listeria monocytogenes*-Part 2:Enumeration method



Conclusions:

The development of a standardized protocol for shelf life determination of refrigerated RTE foods is an important step in assuring the quality and safety of these products. It is essential that this protocol be reviewed on a periodic basis to insure that it incorporates the best possible procedures for analysis of microbiological, chemical and sensory parameters important in establishing product shelf life. It is also essential that the protocol considers the

threat of new and emerging microbial pathogens, and incorporates procedures for addressing new pathogens of concern should the need arise.

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The Refrigerated Foods Association (RFA) has announced the release of an updated Shelf Life Protocol, available now to both members and non-members. The RFA Standardized Protocol for Determining Shelf Life of Refrigerated Foods was originally developed in 2002 and subsequently revised in 2009. The purpose of the standardized protocol was to enable individual manufacturers, and/or their customers if they so desired, to determine their product shelf life and compare their stated shelf life with the shelf life of similar products produced by other manufacturers. Read More. Recap: RFAâ€™s 40th Annua Developing a Messaging Graphic for Storage Times of Refrigerated Ready to Eat (RTE) Foods for a Consumer Food Safety Health Campaign. by. Delores Chambers.Â Determining the best way to educate consumers on food safety can be challenging but data show that many methods can work, including both in person and on-line educational programs, if the educational materials are appropriately developed [37]. However, some racial and ethnic differences have been noted for food safety messaging, which suggests that some people prefer more informational messaging, while others may prefer more guilt- or fear-inducing information [38]. A recent study showed that results can vary depending on the demographics of the participants in a major food safety campaign Refrigerating certain fruits and vegetables can rob them of flavor and nutrients, ruin their texture, and quicken their spoiling. The following is a list of 6 foods that should not (or need not) be refrigerated to keep them fresh. , lively and delicious for the longest period: 1. Avocado. Avocados can be frustrating. Unique and nutritious, there. Continue Reading. Since the advent of agriculture and food storage, itâ€™s been natural to chill or refrigerate many food items.