OMA | Detecting Salmonella in a Variety of Foods and Environmental Surfaces

Introduction

As part of the validation process required by AOAC International for acceptance as an Official Method of Analysis (OMA), the following internal method comparison study was performed to compare the BAX® System Real-Time PCR Assay for *Salmonella* to the reference methods described in the U.S. Department of Agriculture-Food Safety and Inspection Service Microbiological Laboratory Guidebook (USDA-FSIS MLG), U.S. Food and Drug Administration Bacteriological Analytical Manual (FDA BAM), and Health Canada Compendium of Analytical Methods (HC CAM). A total of 24 different sample types were evaluated to demonstrate the reliability of the BAX System method. Two of these matrices – frankfurters and orange juice – were each evaluated in 14 external laboratories as part of the collaborative study to demonstrate repeatability of the internal laboratory results independent of the end user.

These studies, within the statistical constraints, indicate that the BAX System method is sensitive, specific, and rapid in the detection of *Salmonella* in a variety of food and environmental matrices.

As a result, AOAC International has adopted this method as ***Official Method***℠ **2013.02.**

Methodology

Equipment, reagents and supplies

- BAX System Real-Time PCR assay for *Salmonella* (KIT2006)
- BAX System standard equipment and supplies

Enrichment media – BAX System method

- BAX System MP media
- Modified tryptic soy broth with 2 mg/L novobiocin (mTSB+n)
- Modified tryptic soy broth with 10 g/L casamino acids and 8 mg/L novobiocin (TSB+caa+n)
- Buffered peptone water (BPW)
- Brain-heart infusion (BHI) broth

Enrichment media – reference methods

- Buffered peptone water (BPW)
- Lactose broth (LB)
- Tryptic soy broth (TSB)
- Universal pre-enrichment broth (UPB)
- Brain-heart infusion (BHI) broth
- Brilliant green water (BGW)
- Nonfat dry milk

Sample Preparation and Inoculation

Most sample types were artificially inoculated with a different *Salmonella* strain from the Hygiena Culture Collection or the American Type Culture Collection (ATCC); chicken wings and poultry rinses were naturally contaminated (see Table 1). Stainless steel samples were also co-spiked with a *Citrobacter* strain to evaluate the ability of the BAX System method to detect *Salmonella* in the presence of high levels of a competing organism. To artificially contaminate samples, a pure culture of each strain was grown overnight in BHI broth at 35-37°C, then diluted in additional BHI broth to levels expected to produce low (0.2–2.0 CFU/test portion) or high (5 CFU/test portion) spike levels. Some portions of each sample type were left unspiked to serve as negative controls.

Table 1. Inoculation Strain Selection

Sample Type	Strain Name	Strain ID	Strain Source
Meat and Seafood			
Raw ground beef	*Salmonella* Stanley	DD1333	Chicken
Raw ground beef with soy	*Salmonella* Typhimurium	DD1467	Unknown
Beef trim	*Salmonella* Berta	DD1331	Sausage
Frankfurters	*Salmonella* Thompson	DD1336	Chicken
Shrimp	*Salmonella* Anatum	DD1332	Shrimp
Poultry and Eggs			
Ground turkey	*Salmonella* Heidelberg	DD 12913	Turkey
Chicken wings	Natural Flora	n/a	n/a
Poultry rinses	Natural Flora	n/a	n/a
Dried eggs	*Salmonella* Braendrup	DD1329	Sausage
Shell eggs	*Salmonella* Enteritidis	ATCC13076	Unknown
Produce			
Lettuce	*Salmonella* Newport	DD1261	Duck
Frozen peas	*Salmonella* California	DD1668	Unknown
Orange juice	*Salmonella* Worthington	DD4043	Unknown
Dairy Products			
Nonfat dry milk	*Salmonella* Haardt	DD1343	Environmental
Ice cream	*Salmonella* Agona	DD1333	Chicken
Cream cheese	*Salmonella* Typhimurium	DD586	Animal tissue
Environmentals			
Stainless steel	*Salmonella* Senftenberg *Citrobacter brakii*	DD13056 DD13477	Food processing Environmental strain
Ceramic tile	*Salmonella* Lexington	DD13068	Environmental strain
Plastic	*Salmonella* Mbandaka	DD13240	Environmental strain
Miscellaneous			
Peanut butter	*Salmonella* St Paul	DD4102	Nuts
Cocoa	*Salmonella* Sya	DD 2380	Unknown
White pepper	*Salmonella* Newport	DD13079	Basil
Infant formula	*Salmonella* Ealing	DD1469	Infant formula
Dry pet food	*Salmonella* Kentucky	DD2826	Turtle

Sample Enrichment

Many sample types were evaluated using multiple primary enrichment media to accommodate the validation requirements of multiple countries, which require comparisons to different reference methods. Furthermore, in order to best meet the needs of a wide variety of end users, many sample types were evaluated both before and after a re-growth step. Table 2 describes the enrichment protocols approved for each sample type.

Note: Samples for which the secondary enrichment is listed as optional were validated with and without a re-growth step to best meet the needs of a wide variety of end users. Specific varieties of these sample types should be evaluated to determine if re- growth is necessary before using the BAX System method.

Table 2. Sample Enrichments

Sample Type	Size	Primary Enrichment	Secondary Enrichment
Ground beef Ground beef w/soy Beef trim	25 g	Homogenize sample with 225 mL pre-warmed (35°C) BPW. Incubate at 35°C for 20-24 hours.	None
Ground beef	375 g	Homogenize sample with 1,500 mL pre-warmed (35°C) mTSB+n. Incubate at 35°C for 22-26 hours.	None
Ground beef with soy	325 g	Homogenize sample with 975 mL pre-warmed (35°C) mTSB+caa+n. Incubate at 35°C for 20-24 hours.	None
Beef trim	325 g	Homogenize sample with 1,500 mL pre-warmed (41°C) BAX System MP media. Incubate at 39-42°C for 16-24 hours.	None
Frankfurters	325 g	Homogenize sample with 1,400 mL pre-warmed (35°C) BPW. Add additional BPW to reach a total media volume of 2,925 mL. Incubate at 35°C for 18-24 hours.	None
Shrimp	25 g	Homogenize sample with 225 mL pre-warmed (35°C) LB. Let stand at room temperature for 55-65 minutes. Adjust pH to 6.8 ± 0.2, if necessary. Incubate at 35°C for 22-26 hours.	None
Peanut butter	25 g	**LB Enrichment** - Homogenize sample with 225 mL pre-warmed (35°C) LB. Let stand at room temperature for 55-65 minutes. Adjust pH to 6.8 ± 0.2, if necessary. Incubate at 35°C for 22-26 hours. **BPW Enrichment** - Homogenize sample with 225 mL pre- warmed (35°C) BPW. Incubate at 35°C for 22-26 hours.	Transfer 10 µL primary enrichment to 500 µL BHI broth. Incubate at 37°C for 3 hours.
Ground turkey Chicken wings	25 g	Homogenize sample with 225 mL pre-warmed (35°C) BPW. Incubate at 35°C for 16-24 hours.	None
Poultry rinse	30 mL	Combine 30 mL BPW rinsate with 30 mL pre-warmed (35°C) BPW. Incubate at 35°C for 22-26 hours.	None
Dried eggs	25 g	**LB Enrichment** - Add approximately 15 mL pre-warmed (35°C) LB to sample and stir to smooth. Add 3 additional aliquots of LB of 10 mL, 10 mL, and 190 mL (total media volume 225 mL), stirring after each addition. Let stand at room temperature for 55-65 minutes. Adjust pH to 6.8 ± 0.2, if necessary. Incubate at 35°C for 22-26 hours. **BPW Enrichment** - Homogenize sample with 225 mL pre- warmed (35°C) BPW. Incubate at 35°C for 22-26 hours.	*Optional* - Transfer 10 µL primary enrichment to 500 µL BHI broth. Incubate at 37°C for 3 hours.
Ice cream	25 g	**LB Enrichment** - Homogenize sample with 225 mL pre-warmed (35°C) LB. Let stand at room temperature for 55-65 minutes. Adjust pH to 6.8 ± 0.2, if necessary. Incubate at 35°C for 22-26 hours. **BPW Enrichment** - Homogenize sample with 225 mL pre- warmed (35°C) BPW. Incubate at 35°C for 22-26 hours. **BGW Enrichment** - Homogenize sample with 225 mL pre- warmed (35°C) Brilliant green water. Incubate at 35°C for 22-26 hours	*Optional* - Transfer 10 µL primary enrichment to 500 µL BHI broth. Incubate at 37°C for 3 hours.
Shell eggs	1,000 mL	Combine 20 eggs into sterile container with 2,000 mL pre-warmed (42°C) BAX System MP media. Incubate at 42°C for 48 hours.	*Optional* - Transfer 10 µL primary enrichment to 500 µL BHI broth. Incubate at 37°C for 3 hours.

Table 2. Sample Enrichments (continued)

Sample Type	Size	Primary Enrichment	Secondary Enrichment
Infant formula	25 g	Homogenize sample with 225 mL pre-warmed (35°C) LB. Let stand at room temperature for 55-65 minutes. Adjust pH to 6.8 ± 0.2, if necessary. Incubate at 35°C for 22-26 hours.	*Optional* - Transfer 10 µL primary enrichment to 500 µL BHI broth. Incubate at 37°C for 3 hours.
Frozen peas	25 g	**MP Media Enrichment** - Homogenize sample with 225 mL pre-warmed (35°C) BAX System MP media. Incubate at 35°C for 22- 26 hours. **LB Enrichment** - Homogenize sample with 225 mL pre-warmed (35°C) LB. Let stand at room temperature for 55-65 minutes. Adjust pH to 6.8 ± 0.2, if necessary. Incubate at 35°C for 22-26 hours.	*Optional* - Transfer 10 µL primary enrichment to 500 µL BHI broth. Incubate at 37°C for 3 hours.
Cream cheese	25 g	**MP Media Enrichment** - Homogenize sample with 225 mL pre-warmed (35°C) BAX System MP media. Incubate at 35°C for 12- 24 hours. **LB Enrichment** - Homogenize sample with 225 mL pre-warmed (35°C) LB. Let stand at room temperature for 55-65 minutes. Adjust pH to 6.8 ± 0.2, if necessary. Incubate at 35°C for 22-26 hours.	None
Fresh bagged lettuce	25 g	**MP Media Enrichment** - Homogenize sample with 225 mL pre-warmed (35°C) BAX System MP media. Incubate at 35°C for 10- 24 hours. **LB Enrichment** - Add 225 mL pre-warmed (35°C) LB to sample and swirl 25 times clockwise and 25 times counterclockwise. Let stand at room temperature for 55-65 minutes. Adjust pH to 6.8 ± 0.2, if necessary. Incubate at 35°C for 22-26 hours.	None
Orange juice	25 mL	**MP Media Enrichment** - Swirl sample thoroughly with 225 mL pre-warmed (41°C) BAX System MP media. Incubate at 39-42°C for 22-26 hours. **UPB Enrichment** - Swirl sample thoroughly with 225 mL pre-warmed (35°C) UPB. Let stand at room temperature for 55-65 minutes. Do not mix or adjust pH. Incubate at 35°C for 22-26 hours.	Transfer 10 µL primary enrichment to 500 µL BHI broth. Incubate at 37°C for 3 hours.
Nonfat dry milk	25 g	Pour sample slowly over the surface of 225 mL pre-warmed (35°C) Brilliant green water. Let stand at room temperature for 55-65 minutes. Do not mix or adjust pH. Incubate at 35°C for 22-26 hours.	Transfer 10 µL primary enrichment to 500 µL BHI broth.Incubate at 37°C for 3 hours.
Stainless steel, ceramic tile, plastic	–	**LB Enrichment** - Add 225 mL pre-warmed (35°C) LB to environmental sponge in sample bag and swirl thoroughly. Let stand at room temperature for 55-65 minutes. Adjust pH to 6.8 ± 0.2, if necessary. Incubate at 35°C for 22-26 hours. **BPW Enrichment** - Add 225 mL pre-warmed (35°C) BPW to environmental sponge in sample bag and swirl thoroughly. Adjust pH to 6.8 ± 0.2, if necessary. Incubate at 35°C for 18-24 hours.	None
Cocoa	25 g	Homogenize sample with 225 mL reconstituted nonfat dry milk. Let stand at room temperature for 55-65 minutes, then swirl thoroughly to mix. Adjust pH to 6.8 ± 0.2, if necessary. Add 0.45 mL 1% aqueous brilliant green dye solution and mix well. Incubate at 35°C for 22-26 hours.	Transfer 10 µL enrichment to 500 µL BHI broth before processing. Optional - Incubate BHI broth at 37°C for 3 hours.
White pepper	25 g	Homogenize sample with 225 mL pre-warmed (35°C) TSB. Let stand at room temperature for 55-65 minutes. Adjust pH to 6.8 ± 0.2, if necessary. Incubate at 35°C for 22-26 hours.	*Optional* - Transfer 10 µL primary enrichment to 500 µL BHI broth. Incubate at 37°C for 3 hours.
Dry pet food	375 g	**LB Enrichment** - Homogenize sample with approximately one-third to one-half of 3,375 mL pre-warmed (35°C) LB. Add the remainder of the pre-warmed media. Incubate at 35°C for 22-26 hours. **BPW Enrichment** - Homogenize sample with approximately one-third to one-half of 3,375 mL pre-warmed (35°C) BPW. Add the remainder of the pre-warmed media. Incubate at 35°C for 22-26 hours.	Transfer 10 µL primary enrichment to 500 µL BHI broth. Incubate at 37°C for 3 hours.

Method

BAX System method – BAX System lysis reagent was prepared by adding 150 µL protease to 12 mL lysis buffer. For each sample, 5 µL enrichment was added to 200 µL prepared lysis reagent in cluster tubes. Tubes were heated for 20 minutes at 37°C and 10 minutes at 95°C, then cooled for at least 5 minutes at 4°C. PCR tablets were hydrated with 30 µL lysate and a full process was run in the BAX System Q7 instrument. For the purposes of validation, all test method samples were confirmed by culture and biochemical and serological protocols, regardless of presumptive results, as described in U.S. FDA Bacteriological Analytical Manual (FDA-BAM) Chapter 5, USDA-FSIS Microbiology Laboratory Guidebook (MLG) Chapter 4.05, and/or Health Canada Compendium of Analytical Methods MFHPB-20, using the appropriate confirmation media.

USDA-FSIS method – Ground beef, ground beef with soy, beef trim, frankfurters, ground turkey, chicken wings, poultry rinses and environmental sponges were evaluated using the USDA-FSIS reference culture method as described in MLG Chapter 4C.03: Use of a PCR Assay for Screening *Salmonella*. For each sample, 5 µL enrichment was added to 200 µL prepared BAX System lysis reagent, then lysed and processed with the BAX System PCR Assay for *Salmonella* according to the instructions in the BAX System User Guide. Secondary enrichments were performed in TT and mRV broths, then streaked onto BGS and XLT-4 agars to confirm typical *Salmonella* colonies with the appropriate biochemical and serological methods as described in MLG Chapter 4.05: Isolation and Identification of *Salmonella* from Meat, Poultry, Pasteurized Egg and Catfish Products.

FDA BAM method – Shrimp, dried eggs, shell eggs, lettuce, frozen peas, orange juice, nonfat dry milk, ice cream, cream cheese, peanut butter, cocoa, white pepper, infant formula and environmental sponges were evaluated using the FDA-BAM reference culture method as described in BAM Chapter 5: *Salmonella*. Secondary enrichments were performed in TT and RV broths, then streaked onto HE, BS and XLD agars to confirm typical *Salmonella* colonies with the appropriate biochemical and serological methods.

Health Canada method – Dried eggs, ice cream, peanut butter and dry pet food were evaluated using the Health Canada reference method as described in MFHPB-20. Secondary enrichments were performed in TT and RVS broths, then streaked onto BGS, BS and XLT-4 agars to confirm typical *Salmonella* colonies with the appropriate biochemical and serological methods.

Results and Discussion

The results for all sample types tested are summarized in Tables 3 - 8 below. For each sample type tested, the BAX System method and the reference method demonstrated no significant statistical difference as indicated by POD analysis (the dPOD 95% confidence interval included 0) and either McNemar (for paired samples) or Mantel-Haenszel (for unpaired samples) Chi-square analysis (the X^2 value was less than 3.84).

One finding from this study is that *Salmonella* will reliably grow from cocoa enrichments to relatively high cell densities, which allow for a dilution to remove PCR inhibitors without a three-hour regrowth time.Though this may not be applicable to all chocolate matrix variants, this option allows for a reduced time to result for this matrix.

Conclusion

These studies, within the statistical constraints, indicate that the BAX System Real-Time PCR Assay for *Salmonella* is a sensitive, specific, and rapid method for the detection of *Salmonella* in a variety of food and environmental matrices. As a result, AOAC International has adopted this method as ***Official Method***^{SM} **2013.02**.

Table 3. BAX System vs. Reference Method Results – Meat and Seafood Samples

Sample Type	Media	Spike Level	Test Portions	BAX Positive	BAX Confirmed	Reference Positive	dPOD 95% CI	X^2
Ground beef (25 g)	BPW	Neg Control	5	0	0	0	(-0.45, 0.45)	0.0
		Low	20	4	4	4	(-0.25, 0.25)	0.0
Ground beef (375 g)	mTSB+n	Neg Control	5	0	0	0	(-0.45, 0.45)	0.0
		Low	20	5	5	4**	(-0.21, 0.30)	0.0
Raw ground beef with soy (25 g)	BPW	Neg Control	5	0	0	0	(-0.45, 0.45)	–
		Low	60	36	36	36	(-0.049, 0.049)	–
		High	5	5	5	5	(-0.45, 0.45)	–
Raw ground beef with soy (325 g)	mTSB+caa+n	Neg Control	5	0	0	0	(-0.43, 0.43)	–
		Low	60	39	39	36**	(-0.12, 0.22)	0.32
		High	5	5	5	5**	(-0.43, 0.43)	–
Beef trim (25 g)	BPW	Neg Control	5	0	0	0	(-0.45, 0.45)	–
		Low	20	7	7	7	(-0.14, 0.14)	–
		High	5	5	5	5	(-0.45, 0.45)	–
Beef trim (325 g)	MP media	Neg Control	5	0	0	0	(-0.43, 0.43)	–
		Low	20	10	10	7**	(-0.15, 0.41)	0.90
		High	5	5	5	5**	(-0.43, 0.43)	–
Frankfurters (325 g)	BPW	Neg Control	5	0	0	0	(-0.45, 0.45)	–
		Low	20	10	10	10	(-0.14, 0.14)	–
		High	5	5	5	5	(-0.45, 0.45)	–
Shrimp (25 g)	LB	Neg Control	5	0	0	0	(-0.45, 0.45)	–
		Low	20	11	11	11	(-0.14, 0.14)	–
		High	5	4	4	4	(-0.45, 0.45)	–

*** unpaired samples (the reference method enrichment differs from the test method enrichment)*

Table 4. BAX System vs. Reference Method Results – Poultry and Egg Samples

Sample Type	Media	Spike Level	Test Portions	BAX Positive	BAX Confirmed	Reference Positive	dPOD 95% CI	X^2
Ground turkey (25 g)	BPW	Neg Control	5	0	5	0	(-0.45, 0.45)	–
		Low	20	8	8	8	(-0.14, 0.14)	–
		High	5	5	5	5	(-0.45, 0.45)	–
Dried eggs (25 g)	LB	Neg Control	5	0	0	0	(-0.45, 0.45)	–
		Low	20	15	15	15	(-0.14, 0.14)	–
		High	5	5	5	5	(-0.45, 0.45)	–
Dried eggs (25 g)	BPW	Neg Control	5	0	0	0	(-0.43, 0.43)	–
		Low	20	16	16	16	(-0.14, 0.14)	–
		High	5	5	5	5	(-0.43, 0.43)	–
Shell eggs (1,000 mL)	MP media	Neg Control	5	0	0	0	(-0.43, 0.43)	–
		High	20	17	17	14**	(-0.11, 0.39)	1.3
Chicken wings (25 g)	BPW	n/a	20	5	5	5	(-0.14, 0.14)	–
Poultry rinse	BPW	n/a	20	11	11	11	(-0.28, 0.28)	0.00

*** unpaired samples (the reference method enrichment differs from the test method enrichment)*

Table 5. BAX System vs. Reference Method Results – Produce Samples

Sample Type	Media	Spike Level	Test Portions	BAX Positive	BAX Confirmed	Reference Positive	dPOD 95% CI	X^2
Lettuce (25 g)	BPW	Neg Control	5	0	0	0	(-0.45, 0.45)	–
		High	20	10	10	10	(-0.28, 0.28)	0.0
Lettuce (25 g)	MP media	Neg Control	5	0	0	0	(-0.45, 0.45)	–
		High	20	10	10	10**	(-0.28, 0.28)	–
Peas (25 g)	LB	Neg Control	5	0	0	0	(-0.45, 0.45)	–
		Low	20	7	7	7	(-0.14, 0.14)	–
		High	5	4	4	4	(-0.45, 0.45)	–
Peas (25 g)	MP media	Neg Control	5	0	0	0	(-0.43, 0.43)	–
		Low	20	8	8	7**	(-0.23, 0.32)	1.0
		High	5	5	5	4**	(-0.27, 0.62)	–
Orange juice (25 mL)	UPB / BHI	Neg Control	5	0	0	0	(-0.45, 0.45)	–
		High	20	3	3	3	(-0.14, 0.14)	–
Orange juice (25 mL)	MP media / BHI	Neg Control	5	0	0	0	(-0.43, 0.43)	–
		High	20	7	7	3**	(-0.070, 0.44)	2.1

*** unpaired samples (the reference method enrichment differs from the test method enrichment)*

Table 6. BAX System vs. Reference Method Results – Dairy Samples

Sample Type	Media	Spike Level	Test Portions	BAX Positive	BAX Confirmed	Reference Positive	dPOD 95% CI	X^2
Cream cheese (25 g)	MP media	Neg Control	5	0	0	0	(-0.45, 0.45)	–
		High	20	2	2	5	(-0.38, 0.022)	1.5
Cream cheese (25 g)	LB	Neg Control	5	0	0	0	(-0.45, 0.45)	–
		High	20	5	5	5**	(-0.38, 0.022)	–
Nonfat dry milk (25 g)	Brilliant green water / BHI	Neg Control	5	0	0	0	(-0.45, 0.45)	–
		Low	20	11	11	11	(-0.14, 0.14)	–
		High	5	5	5	5	(-0.45, 0.45)	–
Ice cream (25 g)	LB	Neg Control	5	0	0	0	(-0.45, 0.45)	–
		Low	20	9	9	6**	(-0.14, 0.14)	0.94
		High	5	3	3	4**	(-0.45, 0.45)	–
Ice cream (25 g)	BPW	Neg Control	5	0	0	0	(-0.45, 0.45)	–
		Low	20	6	6	6**	(-0.14, 0.14)	0.0
		High	5	4	4	4**	(-0.45, 0.45)	–
Ice cream (25 g)	Brilliant green water	Neg Control	5	0	0	0	(-0.45, 0.45)	–
		Low	20	6	6	6	(-0.27, 0.27)	–
		High	5	5	5	4	(-0.27, 0.62)	–

*** unpaired samples (the reference method enrichment differs from the test method enrichment)*

Table 7. BAX System vs. Reference Method Results – Environmental Samples

Sample Type	Media	Spike Level	Test Portions	BAX Positive	BAX Confirmed	Reference Positive	dPOD 95% CI	X^2
Stainless steel	LB	Neg Control	5	0	0	0	(-0.45, 0.45)	–
		High	20	13	13	13	(-0.28, 0.28)	–
Stainless steel	BPW	Neg Control	5	0	0	0	(-0.45, 0.45)	–
		High	20	14	13	13	(-0.28, 0.28)	–
Ceramic tile	LB	Neg Control	5	0	0	0	(-0.45, 0.45)	–
		Low	20	9	9	9	(-0.14, 0.14)	–
		High	5	5	5	5	(-0.14, 0.14)	–
Ceramic tile	BPW	Neg Control	5	0	0	0	(-0.45, 0.45)	–
		Low	20	6	6	6	(-0.14, 0.14)	–
		High	5	5	5	5	(-0.45, 0.45)	–
Plastic	LB	Neg Control	5	0	0	0	(-0.45, 0.45)	–
		Low	20	13	13	13	(-0.14, 0.14)	–
		High	5	5	5	5	(-0.45, 0.45)	–
Plastic	BPW	Neg Control	5	0	0	0	(-0.45, 0.45)	–
		Low	20	11	11	11	(-0.14, 0.14)	–
		High	5	5	5	5	(-0.45, 0.45)	–

Table 8. BAX System vs. Reference Method Results – All Other Samples

Sample Type	Media	Spike Level	Test Portions	BAX Positive	BAX Confirmed	Reference Positive	dPOD 95% CI	X^2
Dry pet food	LB	Neg Control	5	0	0	0	(-0.45, 0.45)	0.0
		High	20	5	5	5	(-0.26, 0.26)	0.0
Dry pet food	BPW	Neg Control	5	0	0	0	(-0.45, 0.45)	0.0
		High	20	5	5	5	(-0.26, 0.26)	0.0
Peanut butter (25 g)	LB / BHI	Neg Control	5	0	0	0	(-0.45, 0.45)	–
		Low	20	9	9	9	(-0.14, 0.14)	–
		High	5	3	3	3	(-0.14, 0.14)	–
Peanut butter (25 g)	BPW / BHI	Neg Control	5	0	0	0	(-0.45, 0.45)	–
		Low	20	10	10	9**	(-0.28, 0.33)	0.10
		High	5	5	5	3**	(-0.11, 0.77)	–
Cocoa (25 g)	Nonfat dry milk	Neg Control	5	0	0	0	(-0.45, 0.45)	–
		Low	20	13	13	13	(-0.14, 0.14)	–
		High	5	5	5	5	(-0.45, 0.45)	–
White pepper (25 g)	TSB	Neg Control	5	0	0	0	(-0.45, 0.45)	–
		Low	20	14	14	14	(-0.14, 0.14)	–
		High	5	5	5	5	(-0.45, 0.45)	–
Infant formula (25 g)	LB	Neg Control	5	0	0	0	(-0.45, 0.45)	–
		Low	20	16	16	16	(-0.25, 0.25)	–
		High	5	5	5	5	(-0.45, 0.45)	–

*** unpaired samples (the reference method enrichment differs from the test method enrichment)*