

## SPECIAL GUEST EDITOR SECTION: FOOD ALLERGENS NEW METHODS

# Development and Validation of a Quantitative ELISA for the Detection of Almond Residues in Foods

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**Neogen Corp. has developed Veratox for Almond Allergen for use in the quantitative analysis and screening of almond protein residues in food products, such as cookies, crackers, chocolate bars, cereals, beverages, and clean-in-place rinses. Quantitation with Veratox for Almond Allergen ranges from 2.5 to 25 ppm and, with dilution, it can be extended for highly positive samples. This paper describes the findings of internal testing and validation studies designed to establish product claims for the assay of Veratox for Almond Allergen.**

According to the culinary definition, tree nuts comprise true tree nuts (e.g., hazelnuts and Brazil nuts) as well as some fruits and seeds (e.g., coconuts and almonds). Tree nuts are used worldwide as a food ingredient because of their high oil and protein content. Almonds are a fruit that is classified under the tree nut category of foods. Production of almonds has increased by 25% in the last 10 years to almost 1.2 million metric tons in 2016–2017, making them the most popular of all the tree nuts (1).

Although almond popularity and production continues to increase, so, too, does the prevalence of food allergies. The top eight food types—peanut, milk, egg, tree nuts, fish, wheat, fish, and crustacean shellfish—are responsible for the majority of food allergies (2, 3). All food allergens can cause an allergic reaction from the ingestion of minute amounts of protein from the allergenic food. Reactions to food allergens range from mild (itchiness and hives) to severe (anaphylaxis; 4–6).

Due to the increasing popularity of almonds, it is important to have a rapid, quantitative test method that is capable of detection in a variety of ingredients and processed foods. Veratox for Almond Allergen is intended for the full quantitative analysis or the simple screening of almond protein residues on surfaces and in clean-in-place rinses and food products, such as beverages, cookies, crackers, chocolate bars, ice cream, and cereals. The almond allergen ELISA provides food processors with a method to easily determine whether an ingredient or product has been subjected to cross-contact with almond residue.

## Materials and Methods

### *Reference Material Used in Standard Preparation and Spiking*

The almond positive control was prepared for the sample spike solution using almond paste. The almond paste was prepared by mixing multiple sources of almonds, both raw and roasted, and blending into a uniform paste. One hundred milligrams of extract from almond paste was combined with 100 mL of 10 mM phosphate buffered saline (PBS; composed of 10 mM sodium phosphate, 138 mM NaCl, and 2.7 mM KCl; pH 7.4) in a shaker water bath at 60°C for 15 min. This constituted the 1 mg/mL almond extract solution. This solution was subsequently diluted 1:10 (10 mL stock plus 90 mL of 0.25% porcine gelatin in PBS), resulting in a stock solution of 100 µg/mL.

### *Sample Preparation and Extraction*

All samples were numbered, randomized, and blind-coded. The 100 µg/mL stock solution described above was used to spike almond-negative samples. Spiked samples were prepared fresh each day for the 5 and 15 ppm levels. Negative samples (0 ppm) were also prepared fresh for each day of testing. Extractions were performed following the manufacturer's instructions. Three technicians were selected for the study on 2 consecutive days. Each technician used all three levels of prepared spiked material on each of three lots of Veratox for Almond Allergen kits. On both days, all three manufactured lots were tested by each operator, starting with lot 1, followed by lot 2, and finishing with lot 3.

### *ELISA Method*

All assays were performed per the manufacturer's method, except those described in the *Ruggedness Testing* section, which describes the intentional deviations that were performed.

### *Interpretation of Results*

This test detects almond protein, and the results are expressed as parts per million of total almond.

The LOQ is 2.5 ppm almond, as defined by the lowest concentration point on the calibration curve in which this test could reliably detect almond. The range of quantitation is 2.5–25 ppm almond. Depending on the negative sample tested, it is not uncommon for low-level optical densities (ODs) to occur due to matrix effects. Samples that reported

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ODs above the 0 ppm standard and lower than 2.5 ppm standard are considered negative or below the LOQ. Quantitating samples above 25 ppm can be performed by diluting the extracted samples.

The percent recovery is calculated as

$$[\text{ppm almond}]_{\text{predicted}} / [\text{ppm almond}]_{\text{actual}} \times 100$$

The RSD is defined as

$$(\text{SD}/\text{mean}) \times 100$$

## Results

### LOD

The LOD was determined by testing 10 extracts of a known negative sample (ground coffee). The LOD is expressed as the mean value (in parts per million of the negative samples plus three SDs). As shown in Table 1, the LOD was determined to be 0.3 ppm almond. This value was confirmed by testing lowly positive almond extracts between 0.1 and 2.5 ppm almond (not shown).

### Cross-Reactivity

A panel of 39 samples, including nuts, seeds, grain, and protein samples, were tested for cross-reactivity. These foods were purchased at local stores and tested per the kit instructions. No cross-reactivity was found in these samples (Table 2). Veratox for Almond Allergen cross-reacted with other *Prunus* genus seeds, including apricot, cherry, nectarine, peach, and plum. This cross-reactivity was isolated to the seed of these fruits and not the flesh.

### Spike and Recovery

Blind-coded samples were prepared from four locally purchased almond-free foods: vanilla ice cream, cookies, pasta

**Table 1. LOD<sup>a</sup>**

| Replicate             | OD    | ppm  |
|-----------------------|-------|------|
| 1                     | 0.071 | 0.28 |
| 2                     | 0.071 | 0.28 |
| 3                     | 0.069 | 0.27 |
| 4                     | 0.064 | 0.25 |
| 5                     | 0.068 | 0.27 |
| 6                     | 0.066 | 0.26 |
| 7                     | 0.067 | 0.29 |
| 8                     | 0.072 | 0.27 |
| 9                     | 0.068 | 0.28 |
| 10                    | 0.07  | 0.26 |
| Mean                  |       | 0.27 |
| SD                    |       | 0.01 |
| LOD = mean + (3 × SD) |       | 0.30 |

<sup>a</sup> Replicate extracts of ground coffee were used as the negative sample. All calculated parts-per-million values were below the LOQ (2.5 ppm). The LOD was verified by testing lowly positive controls at 0.1–2.5 ppm almond.

**Table 2. Cross-reactivity<sup>a</sup>**

| No. | Commodity           |
|-----|---------------------|
| 1   | Adzuki bean         |
| 2   | Anasazi bean        |
| 3   | Beef                |
| 4   | Black-eyed pea      |
| 5   | Black turtle bean   |
| 6   | Brazil nut          |
| 7   | Black walnut        |
| 8   | Cashew              |
| 9   | Chick pea           |
| 10  | Cocoa               |
| 11  | Coconut             |
| 12  | Corn meal           |
| 13  | Great northern bean |
| 14  | Hazelnut            |
| 15  | Kidney bean         |
| 16  | Lentil              |
| 17  | Lima bean           |
| 18  | Lecithin            |
| 19  | Macadamia nut       |
| 20  | Navy bean           |
| 21  | Peanut              |
| 22  | Pecan               |
| 23  | Pepper seed         |
| 24  | Pine nut            |
| 25  | Pinto bean          |
| 26  | Pistachio           |
| 27  | Poppy seed          |
| 28  | Pork                |
| 29  | Rice                |
| 30  | Rice (brown)        |
| 31  | Rye                 |
| 32  | Sesame seed         |
| 33  | Skim milk           |
| 34  | Split pea           |
| 35  | Turkey              |
| 36  | Walnut              |
| 37  | Wheat               |
| 38  | Wheat gluten        |
| 39  | Wheat flour         |

<sup>a</sup> Locally-sourced almond-free foods tested and not found to cross-react with the almond ELISA.

sauce, and milk chocolate. Foods were spiked with almond paste extract at two levels (5 and 15 ppm), and one nonspiked negative control was prepared as described in the *Reference Material Used in Standard Preparation and Spiking* section. Each sample was independently analyzed by three technicians in the same laboratory using the same microwell strip reader.

The above samples were tested using the procedures described in the *Sample Preparation and Extraction* sections.

The spike recovery experiments showed an average recovery of 94.3% for 5.0 ppm samples and 90.1% for 10 ppm samples. Across all levels, the recovery was 92.2%. RSD<sub>r</sub> was 6.6% and 4.9% for 5 and 10 ppm, respectively. Overall, the RSD<sub>r</sub> was 5.8% (Table 3).

**Table 3. Spike recovery testing<sup>a</sup>**

| Results             | Spike level, ppm |      |       | Overall Performance |
|---------------------|------------------|------|-------|---------------------|
|                     | 0                | 5    | 10    |                     |
| Vanilla ice cream   |                  |      |       |                     |
| Mean, ppm           | 0.1              | 5.1  | 9.1   | 90.6                |
|                     | 0.1              | 5.0  | 9.7   |                     |
|                     | 0.1              | 4.9  | 9.5   |                     |
|                     | 0.1              | 5.0  | 9.4   |                     |
| Mean recovery, %    |                  | 100  | 94.3  |                     |
| SD                  |                  | 0.1  | 0.3   |                     |
| RSD, %              |                  | 2.0  | 3.2   |                     |
| Cookies             |                  |      |       |                     |
| Mean, ppm           | 0.3              | 4.3  | 7.9   | 73.3                |
|                     | 0.1              | 3.3  | 6.9   |                     |
|                     | 0.2              | 3.8  | 7.2   |                     |
|                     | 0.2              | 3.8  | 7.3   |                     |
| Mean recovery, %    |                  | 76   | 73.3  |                     |
| SD                  |                  | 0.5  | 0.5   |                     |
| RSD, %              |                  | 13.2 | 7.0   |                     |
| Pasta sauce         |                  |      |       |                     |
| Mean, ppm           | 0.1              | 5.4  | 9.8   | 103.7               |
|                     | 0.0              | 5.4  | 10.9  |                     |
|                     | 0.1              | 5.7  | 10.4  |                     |
|                     | 0.1              | 5.5  | 10.4  |                     |
| Mean recovery, %    |                  | 110  | 103.7 |                     |
| SD                  |                  | 0.2  | 0.6   |                     |
| RSD, %              |                  | 3.1  | 5.3   |                     |
| Milk chocolate      |                  |      |       |                     |
| Mean, ppm           | 0.0              | 5.0  | 9.3   | 89                  |
|                     | 0.0              | 4.3  | 8.8   |                     |
|                     | 0.0              | 4.4  | 8.6   |                     |
|                     | 0.0              | 4.6  | 8.9   |                     |
| Mean recovery, %    |                  | 91.3 | 89    |                     |
| SD                  |                  | 0.4  | 0.4   |                     |
| RSD, %              |                  | 8.3  | 4.1   |                     |
| Overall recovery, % |                  | 94.3 | 90.1  | 90.6                |
| Overall RSD, %      |                  | 6.6  | 4.9   | 5.8                 |

<sup>a</sup> Sample resulting in ODs greater than that of the 0 ppm control, but less than that of the LOQ (2.5 ppm) are considered to be negative.

### Incurred Almond Detection

In an effort to characterize the detection of almond in a variety of food matrixes and processing methods, several foods were obtained from local markets. Eight foods with almond listed as an ingredient were obtained as positive samples. Two foods containing no almond as a listed ingredient were obtained as negative controls. Each sample was tested with neat extraction per the procedures described in the *Sample Preparation and Extraction* sections. The extracts were diluted further for all samples to obtain a concentration

that fell within the range of quantitation. The calculated parts per million almond for each food were determined by multiplying the assay result by the dilution factor. Quantitation was expressed in parts per million for only those dilutions that produced a result within the range of quantitation (Table 4).

### Inter- and Intra-Assay Variability

To determine the within-lot variability of the Veratox for Almond Allergen assay, 16 replicates of the assay controls were tested by one operator using one lot of the almond ELISA. The RSDs for each level were 10.0, 6.1, 5.9, 8.7, and 7.4% for 0, 2.5, 5.0, 10, and 25 ppm, respectively (Table 5). The overall RSD was 7.6%.

To determine the between-lot variability of the Veratox for Almond Allergen assay, three kit lots were tested with two users on the same day of experimentation. Soy milk was prepared as a sample at 0, 5, and 15 ppm almond. For each user and kit lot, four replicates were measured for 0 and

**Table 4. Incurred foods screening<sup>a</sup>**

| Commodity                   | Dilution | ppm  | Calculated ppm |
|-----------------------------|----------|------|----------------|
| Vanilla coffee              | Neat     | 0    | 0              |
|                             | 1 + 100  | 0    | 0              |
|                             | 1 + 500  | 0    | 0              |
| Sharp cheddar cheese spread | Neat     | 0    | 0              |
|                             | 1 + 100  | 0    | 0              |
|                             | 1 + 500  | 0    | 0              |
| Rice pilaf with almond      | Neat     | >25  | >25            |
|                             | 1 + 1000 | >25  | >25            |
|                             | 1 + 4000 | 22.5 | 90,000         |
| Almond ice cream            | Neat     | >25  | >25            |
|                             | 1 + 1000 | >25  | >25            |
|                             | 1 + 3000 | 13.4 | 40,200         |
| Almond biscotti             | Neat     | >25  | >25            |
|                             | 1 + 100  | >25  | >25            |
|                             | 1 + 500  | 19.7 | 9861           |
| Belgian almond thin         | Neat     | >25  | >25            |
|                             | 1 + 100  | >25  | >25            |
|                             | 1 + 500  | 16.3 | 81,500         |
| Vanilla almond protein bar  | Neat     | >25  | >25            |
|                             | 1 + 1000 | >25  | >25            |
|                             | 1 + 2000 | 19.5 | 38,960         |
| Almond milk, organic        | Neat     | >25  | >25            |
|                             | 1 + 1000 | >25  | >25            |
|                             | 1 + 3000 | 16.1 | 48,216         |
| Almond milk, vanilla        | Neat     | >25  | >25            |
|                             | 1 + 1000 | >25  | >25            |
|                             | 1 + 3000 | 11.0 | 33,054         |
| Almond milk, chocolate      | Neat     | >25  | >25            |
|                             | 1 + 1000 | >25  | >25            |
|                             | 1 + 3000 | 12.0 | 35,967         |

<sup>a</sup> All samples were neat-extracted per the kit instructions. Positive samples were diluted in PBS until the result was within the LOQ. The concentration of almond was calculated by multiplying the dilution factor by the assay output in parts per million.

**Table 5. Within-run precision<sup>a</sup>**

| Results    | 0 ppm |       | 2.5 ppm |       | 5 ppm |       | 10 ppm |       | 25 ppm |       |
|------------|-------|-------|---------|-------|-------|-------|--------|-------|--------|-------|
| OD650nm    | 0.267 | 0.210 | 0.587   | 0.622 | 0.863 | 0.929 | 1.402  | 1.388 | 2.154  | 2.219 |
|            | 0.272 | 0.284 | 0.592   | 0.572 | 0.887 | 0.880 | 1.452  | 1.375 | 2.213  | 2.160 |
|            | 0.295 | 0.344 | 0.566   | 0.587 | 0.801 | 0.848 | 1.205  | 1.331 | 1.944  | 2.081 |
|            | 0.256 | 0.264 | 0.551   | 0.572 | 0.787 | 0.813 | 1.216  | 1.092 | 1.919  | 1.995 |
|            | 0.261 | 0.287 | 0.580   | 0.571 | 0.838 | 0.860 | 1.364  | 1.298 | 2.036  | 1.900 |
|            | 0.287 | 0.285 | 0.609   | 0.576 | 0.840 | 0.841 | 1.102  | 1.284 | 2.042  | 1.780 |
|            | 0.302 | 0.270 | 0.535   | 0.538 | 0.726 | 0.791 | 1.135  | 1.201 | 1.831  | 1.820 |
|            | 0.286 | 0.265 | 0.586   | 0.472 | 0.795 | 0.789 | 1.233  | 1.239 | 1.880  | 1.815 |
| Mean OD    | 0.277 |       | 0.570   |       | 0.831 |       | 1.270  |       | 1.987  |       |
| SD         | 0.028 |       | 0.035   |       | 0.049 |       | 0.110  |       | 0.148  |       |
| CV, %      | 10.0  |       | 6.1     |       | 5.9   |       | 8.7    |       | 7.4    |       |
| Mean CV, % |       |       |         |       | 7.6   |       |        |       |        |       |

<sup>a</sup> Sixteen replicates for each kit control were tested on one assay lot to determine within-run precision. The resulting ODs are shown in the table above.

15 ppm and six replicates for the 5 ppm samples. Mean recoveries of 124 and 130% were observed for the 5 and 15 ppm samples, respectively (Table 6). Lot-to-lot reproducibility was found to have an RSD of 10.4% for the 5 ppm sample and an RSD of 14.2% based on the calculated parts per million of the replicates described above.

### Ruggedness Testing

The ruggedness of the assay was evaluated by varying multiple test parameters, including reagent incubation time, volume, and shaking time. Each condition was tested by one operator over 2 days with triplicate measurements for each variable. Soy milk was spiked with amounts of paste extract according to the description in the *Sample Preparation and Extraction* sections.

**Incubation timing.**—The recommended incubation time was 10 + 10 + 10 min. Studies were performed with different incubation times to determine the effect of timing: less (8 + 8 + 8), more (12 + 12 + 12), and variable (8 + 10 + 12). These alterations to the incubation time appear to have had no

effect when compared with the optimum timing condition of 10 + 10 + 10 (Table 7).

**Reagent volume.**—The recommended reagent volume at each step is 100 µL. This study was performed using 95, 100, and 105 µL reagent volumes to determine the effect of reagent volume on assay performance. The data demonstrated that there was no effect on assay results when altering the reagent volume to 95 or 105 µL relative to the optimum volume of 100 µL (Table 8).

**Shaking time.**—The recommended shaking time at the beginning of each incubation step is 20 s. This study compared no shaking and 30 s shaking with the standard of 20 s shaking to determine the effect of shaking time on the assay results. Elimination (0 s) or increasing (30 s) shaking did not appear to have an effect on the assay when compared with the optimum condition of 20 s (Table 9).

### Discussion

This study shows the Veratox for Almond Allergen quantitative ELISA is capable of the quantitative measurement of processed almond in multiple food matrixes. The almond ELISA showed no significant cross-reactivity to other foods except the seeds of other *Prunus* species (e.g., apricot and cherry). Lastly, the assay demonstrated excellent ruggedness when the method parameters were intentionally modified from the recommended protocol.

### Acknowledgments

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### References

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**Table 6. Lot-to-lot variability<sup>a</sup>**

| Parts per million | Parameter   | Value           |
|-------------------|-------------|-----------------|
| 0                 | Mean        | 0.02            |
|                   | SD          | NA <sup>b</sup> |
|                   | CV, %       | NA              |
| 5                 | Mean        | 6.22            |
|                   | Recovery, % | 124             |
|                   | SD          | 0.65            |
|                   | CV, %       | 10.4            |
| 15                | Mean        | 19.5            |
|                   | Recovery, % | 130             |
|                   | SD          | 2.77            |
|                   | CV, %       | 14.2            |

<sup>a</sup> Soy milk spiked with almond extract. Three lots were tested and the calculated parts per million were recorded for four replicates of each sample.

<sup>b</sup> NA = Not Applicable.

**Table 7. Incubation time<sup>a</sup>**

| Minutes                   | Recovery after the incubation time challenge, % |     |      |     |            |     |      |     |             |            |      |      |      |             |
|---------------------------|-------------------------------------------------|-----|------|-----|------------|-----|------|-----|-------------|------------|------|------|------|-------------|
|                           | 0 ppm                                           |     |      |     | 5 ppm      |     |      |     |             | 15 ppm     |      |      |      |             |
|                           | Replicates                                      |     | Avg. |     | Replicates |     | Avg. |     | Recovery, % | Replicates |      | Avg. |      | Recovery, % |
| Day 1                     |                                                 |     |      |     |            |     |      |     |             |            |      |      |      |             |
| 8 + 8 + 8                 | 0.0                                             | 0.0 | 0.1  | 0.0 | 6.2        | 5.4 | 5.7  | 5.8 | 115.3       | 18.1       | 15.3 | 18.1 | 17.2 | 114.4       |
| 10 + 10 + 10 <sup>b</sup> | 0.3                                             | 0.3 | 0.1  | 0.2 | 5.6        | 5.7 | 4.7  | 5.3 | 106.7       | 16.3       | 16.7 | 14.7 | 15.9 | 106.0       |
| 12 + 12 + 12              | 0.4                                             | 0.4 | 0.3  | 0.4 | 5.2        | 5.2 | 5.4  | 5.3 | 105.3       | 15.4       | 16.5 | 16.1 | 16.0 | 106.7       |
| 8 + 10 + 12               | 0.0                                             | 0.0 | 0.1  | 0.0 | 5.5        | 5.4 | 5.7  | 5.5 | 110.7       | 15.6       | 15.6 | 16.7 | 16.0 | 106.4       |
| Day 2                     |                                                 |     |      |     |            |     |      |     |             |            |      |      |      |             |
| 8 + 8 + 8                 | 0.0                                             | 0.0 | 0.0  | 0.0 | 4.5        | 4.1 | 5.1  | 4.6 | 91.3        | 12.8       | 13.2 | 14.6 | 13.5 | 90.2        |
| 10 + 10 + 10 <sup>b</sup> | 0.2                                             | 0.0 | 0.2  | 0.1 | 4.8        | 4.5 | 4.8  | 4.7 | 94.0        | 14.5       | 13.3 | 14.6 | 14.1 | 94.2        |
| 12 + 12 + 12              | 0.1                                             | 0.4 | 0.3  | 0.3 | 5.0        | 4.6 | 5.0  | 4.9 | 97.3        | 15.2       | 13.8 | 15.5 | 14.8 | 98.9        |
| 8 + 10 + 12               | 0.1                                             | 0.3 | 0.0  | 0.1 | 4.9        | 4.6 | 4.8  | 4.8 | 95.3        | 14.4       | 13.4 | 14.0 | 13.9 | 92.9        |

<sup>a</sup> Recovery of almond in spiked soy milk samples tested over 2 days. There were three replicates for each timing scheme.<sup>b</sup> Kit insert-recommended timing.**Table 8. Reagent volume challenge<sup>a</sup>**

| Reagent volume, $\mu$ L | Recovery after the reagent volume challenge, % |     |      |     |            |     |      |     |             |            |      |      |      |       |
|-------------------------|------------------------------------------------|-----|------|-----|------------|-----|------|-----|-------------|------------|------|------|------|-------|
|                         | 0 ppm                                          |     |      |     | 5 ppm      |     |      |     | Recovery, % | 15 ppm     |      |      |      |       |
|                         | Replicates                                     |     | Avg. |     | Replicates |     | Avg. |     |             | Replicates |      | Avg. |      |       |
|                         |                                                |     |      |     |            |     |      |     |             |            |      |      |      |       |
| Day 1                   |                                                |     |      |     |            |     |      |     |             |            |      |      |      |       |
| 95                      | 0.2                                            | 0.0 | 0.2  | 0.1 | 5.5        | 5.8 | 6.2  | 5.8 | 116.7       | 15.8       | 17.5 | 18.0 | 17.1 | 114.0 |
| 100 <sup>b</sup>        | 0.0                                            | 0.0 | 0.0  | 0.0 | 5.9        | 5.4 | 5.6  | 5.6 | 112.7       | 16.9       | 16.7 | 18.4 | 17.3 | 115.6 |
| 105                     | 0.4                                            | 0.0 | 0.2  | 0.2 | 5.8        | 5.4 | 4.9  | 5.4 | 107.3       | 15.7       | 15.6 | 14.3 | 15.2 | 101.3 |
| Day 2                   |                                                |     |      |     |            |     |      |     |             |            |      |      |      |       |
| 95                      | 0.0                                            | 0.1 | 0.1  | 0.1 | 3.4        | 3.3 | 3.3  | 3.3 | 66.7        | 9.8        | 9.7  | 9.3  | 9.6  | 64.0  |
| 100 <sup>b</sup>        | 0.1                                            | 0.0 | 0.1  | 0.1 | 3.5        | 3.1 | 3.6  | 3.4 | 68.0        | 9.1        | 8.6  | 9.1  | 8.9  | 59.6  |
| 105                     | 0.1                                            | 0.1 | 0.1  | 0.1 | 3.5        | 3.2 | 3.5  | 3.4 | 68.0        | 9.0        | 8.8  | 9.3  | 9.0  | 60.2  |

<sup>a</sup> Reagent volume testing for spiked soy milk samples.<sup>b</sup> Kit insert-recommended volume (100  $\mu$ L) for each reagent.**Table 9. Shaking time<sup>a</sup>**

| Seconds         | Recovery after the shaking time challenge, % |     |      |     |            |     |      |     |             |            |      |      |      |             |
|-----------------|----------------------------------------------|-----|------|-----|------------|-----|------|-----|-------------|------------|------|------|------|-------------|
|                 | 0 ppm                                        |     |      |     | 5 ppm      |     |      |     | Recovery, % | 15 ppm     |      |      |      |             |
|                 | Replicates                                   |     | Avg. |     | Replicates |     | Avg. |     |             | Replicates |      | Avg. |      | Recovery, % |
|                 |                                              |     |      |     |            |     |      |     |             |            |      |      |      |             |
| Day 1           |                                              |     |      |     |            |     |      |     |             |            |      |      |      |             |
| 0               | 0.2                                          | 0.1 | 0.0  | 0.1 | 4.0        | 4.0 | 3.3  | 3.8 | 75.3        | 10.9       | 11.8 | 10.3 | 11.0 | 73.3        |
| 20 <sup>b</sup> | 0.4                                          | 0.3 | 0.3  | 0.3 | 3.5        | 3.6 | 3.2  | 3.4 | 68.7        | 9.1        | 9.9  | 9.2  | 9.4  | 62.7        |
| 30              | 0.0                                          | 0.0 | 0.0  | 0.0 | 3.9        | 3.7 | 3.2  | 3.6 | 72.0        | 9.0        | 9.0  | 9.2  | 9.1  | 60.4        |
| Day 2           |                                              |     |      |     |            |     |      |     |             |            |      |      |      |             |
| 0               | 0.0                                          | 0.0 | 0.2  | 0.1 | 4.7        | 4.5 | 5.0  | 4.7 | 94.7        | 13.6       | 12.9 | 14.1 | 13.5 | 90.2        |
| 20 <sup>b</sup> | 0.0                                          | 0.0 | 0.4  | 0.1 | 4.5        | 4.5 | 4.9  | 4.6 | 92.7        | 13.1       | 12.8 | 12.0 | 12.6 | 84.2        |
| 30              | 0.2                                          | 0.0 | 0.0  | 0.1 | 4.6        | 4.4 | 4.5  | 4.5 | 90.0        | 12.5       | 12.4 | 14.4 | 13.1 | 87.3        |

<sup>a</sup> Shaking time was tested with spiked soy milk as the sample.<sup>b</sup> Kit insert-recommended shaking time (20 s).

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