

New Analytical Methods

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Running title: Validation of xMAP FADA

**Single Laboratory Validation of the Multiplex xMAP Food Allergen Detection Assay
(xMAP FADA) with Incurred Food Samples**

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ABSTRACT

An xMAP Food Allergen Detection Assay (xMAP FADA) was developed to meet analytical needs when responding to complaints by individuals with multiple food allergies and to address potential ambiguities associated with cross-reactive proteins. A Single Laboratory Validation (SLV) was conducted to examine the reliability of the xMAP FADA to detect 15 analytes individually, or as part of a mixture, at ≥ 6 concentrations, in four foods. The xMAP FADA reliably detected the analytes despite the incurred dark chocolate and incurred baked muffins displaying recoveries of 10-20% and $< 60\%$, respectively. The high reliability for recoveries less than 60% in part reflects the statistical strength of the design of the xMAP FADA. Only crustacean, egg, and milk incurred in dark chocolate were not reliably detected using the PBST buffered-detergent protocol. Following the reduced-denatured protocol no problems were encountered detecting milk, though egg did not display a dynamic response in dark chocolate.

The ruggedness of the xMAP FADA was ascertained by the ability of novice analysts to detect food allergens in baked rice cookies. Despite one analyst losing $> 80\%$ of the beads and the count for one bead set dropping to 7, the assay displayed only a decrease in precision (increased standard deviations) and a change in the ratios between complementary antibody pairs.

INTRODUCTION

The increased prevalence of individuals with multiple food allergies and the expanding global market^{1–5} make the availability of analytical methods that can simultaneously detect multiple allergens and distinguish between cross-reactive antigens necessary for efficient support of the Food Allergen Consumer Protection Act of 2004 (FALCPA) and the Gluten-Free Regulation issued by the FDA on August 5, 2013^{6,7}. The methods routinely employed by the FDA involve the use of single-analyte specific ELISA technology. These antibody-based methods display limits of detection (LoD) between 0.2 µg/mL and 3 µg/mL, with dynamic ranges typically spanning one order of magnitude and recoveries varying with the type of processing used in the production of the food and extraction protocol employed to mobilize the target analyte. Major problems associated with classical ELISA methods include must first know what food allergen to test for, ELISAs for the target analyte must be available, cross-reactivity, and a complete kit must be discarded after opening. Thus, when testing for the presence of one of a multitude of possible food allergens, it becomes necessary to conduct many ELISAs. Further, due to the possibility of cross-reactivity to homologous proteins, the ELISA protocol employed by the FDA requires that all results be confirmed using a second ELISA, preferably employing different extraction conditions and antibodies displaying different specificities. Despite the increased reliability provided by using multiple ELISA test kits, the conclusions are still not definitive, especially when dealing with related, homologous foods or novel products.

An alternative approach involves the use of dipsticks, a.k.a. lateral flow devices. The advantages associated with dipsticks are primarily two-fold. One, being able to conduct a single analysis rather than consuming either a whole ELISA plate or test strip (typically 8 wells). The second advantage is turn-around time, typically 10 – 15 minutes per dipstick versus one to three hours for a 48- or 96-well ELISA plate. Thus, a single sample can be analyzed for numerous target analytes with minimal effort at a fraction of the cost required for ELISA test kits. However, when

high-throughput, quantitative analyses which include demonstration of reliability (measure error), are necessary, the use of dipsticks is no longer suitable. Primary concerns include increased variance, lower sensitivity, and the propensity of dipsticks to generate false negative results upon saturation with the target analyte, referred to as the Hook Effect.

An alternative antibody-based technology that has shown a high level of reliability with sensitivities comparable to or better than ELISAs is multi-analyte profiling (xMAP®) technology ^{8, 9}. This bead-based technology has been demonstrated to be reliable in the detection of various proteinaceous toxins in foods, with a throughput exceeding ELISA test kits ¹⁰. In an xMAP® assay, each analytical sample can be simultaneously screened for multiple analytes and subjected to simultaneous confirmatory testing. In addition, the antibody capture and detection reagents are supplied in individual vials giving the analyst freedom to perform one or as many analyses at a time as needed (typically a maximum of either 48 or 96), thereby mimicking one of the advantages associated with dipsticks. Furthermore, the analysts may determine the composition of the multiplex cocktail and 'mix and match' the assay to interrogate the extracted samples for allergens of choice.

The use of xMAP® technology for regulatory purposes is not new. xMAP®-based assays have been validated and adopted by multiple governmental agencies and industries, including the FDA and the Laboratory Response Network (LRN) to support the CDC's detection of biothreat agents.

Recently, the FDA worked with Radix BioSolutions, Ltd. (Georgetown, Texas) to develop a commercial multiplex xMAP® Food Allergen Detection Assay (xMAP FADA) using established antibodies already employed in commercial ELISA test kits marketed by Morinaga Institute of Biological Science, Inc. (MioBS, Yokohama-Shi, Japan), IEH Laboratory and Consulting Group (Lake Forest Park, WA), and Elution Technologies - 3M Food Safety (Colchester, VT) ^{11 - 13}. The xMAP FADA includes 29 bead sets for the detection of food allergens extracted using buffered-

detergent (PBST or UD Buffer) and an additional five bead sets for use with a reduced-denatured (SDS/ β -mercaptoethanol) extraction protocol. Additionally, four AssayChex Process Control Panel[®] bead sets are used to confirm assay - instrumental performance in both the buffered-detergent and reduced-denatured phases of the assay. In total, the xMAP FADA includes one bead set for crustacean; two bead sets for each of soy and nine tree nuts (almond, Brazil nut, cashew, coconut, hazelnut, macadamia, pine nut, pistachio, and walnut); three bead sets for egg, gluten, and peanut; and four bead sets for milk detection.

To ascertain the performance reliability of the xMAP FADA it was subjected to an extensive single laboratory validation (SLV). Included was an examination of the detection of analyte (individually or as a mixture) spiked into food extracts and incurred into food prior to processing and subsequently subjected to either the PBST buffered-detergent or reduced-denatured extraction process. The foods included in the validation were orange juice, dark chocolate, pancake batter, and baked muffin. Additionally, analytes in assay buffer were included. Overall, the reliability and sensitivity of the xMAP FADA exceeded expectations and showed unique robustness that more than met current and anticipated analytical needs for regulatory allergen testing, particularly when testing for multiple food allergens or when the allergen present is unknown.

MATERIALS AND METHODS

Reagents Phosphate buffered saline (PBS, cat# P5368), Tween-20 (cat# P9416), and wheat gluten (cat# G5004) were purchased from Sigma-Aldrich Inc. (St. Louis, MO). BD Difco skim milk powder and Sodium Dodecyl Sulfate (SDS, cat# 28312) were purchased from Fisher Scientific (Waltham, MA). Whole, raw nuts and legumes were acquired from nuts.com as previously described ¹¹. All other reagents were of the highest technical grade available. Ingredients to prepare the orange juice, chocolate, pancake batter, baked muffin, and baked rice cookie samples were acquired locally being selective that none of the ingredients contained any food allergens or gluten except for the pancake mix which contained soy flour.

Protein extracts of the food allergens and gluten, as described in the xMAP product insert, were used to prepare the orange juice, chocolate, pancake batter, and baked muffin samples. Ground almond, coconut, gluten, soy and NIST SRM 2387 peanut butter were used to prepare the rice cookies.

Validation Before an assay can be adopted for routine use with regulatory samples it must first be validated. The xMAP FADA single laboratory validation exceeded the requirements established by the Office of Foods and Veterinary Medicine, FDA ¹⁴. Specifically, as itemized in Table 1, the xMAP FADA was validated for all 15 target allergens individually and as a mixture, spiked into both food extracts and as incurred food samples. The matrices included in this study were chosen to represent extremely different, but common matrix groups for allergen testing.

Preparation of Food Samples Overview The concentrations of each analyte examined are listed in Table 2 and encompass the dynamic ranges and the calibration standards for each allergen. The analyses of analyte spiked into food extract were performed at each of the seven concentrations as listed. The incurred samples were designed to yield analytical samples at five concentrations (S1, S2, S4, S6, S7). To achieve these concentrations with the incurred samples, the amount of analyte added to the food prior to processing was designed to compensate for dilutions associated with sample extraction and preparation. Further, to reduce variability in analyte content and improve the assessment of the accuracy of recovery measurements, these samples were prepared by incurring the analyte(s) into 1 gram aliquots of the food. For example, to generate gluten at 69 ng/mL in the analytical sample, 1.38 µg gluten was added to one gram of muffin batter prior to baking and the subsequent 20-fold dilution associated with PBST buffered-detergent extraction. For the reduced-denatured protocol, the dilution associated with extraction and sample preparation is 2,000-fold; as such, 138 µg gluten was incurred into 1 gram muffin batter. No correction was made for the loss of mass (water) during baking since the focus is the amount of analyte present (0.69 ng) in the original sample as may apply to potential oral dose

exposure. Disadvantages of this approach include omission of potential sources of error associated with weighing 1 gram samples and that the processing (e.g., baking) of 1 gram samples may not identically reproduce the effects of commercial food production. However, considering the variability in commercial production, the preparation of baked muffins is primarily to observe the effects associated with heating in the presence of batter.

Orange Juice The orange juice (OJ) samples were prepared using a commercial product that contained medium levels of pulp and was enriched with calcium and vitamin D. The incurred samples entailed adding analyte to one gram of juice at amounts that upon extraction generated the desired concentration in the analytical sample. As such, the OJ samples mimicked inadvertent cross-contact that may occur post pasteurization.

Chocolate Incurred chocolate samples were prepared by mixing the appropriate amount of (2000X) analyte into 0.663 grams (0.5 mL) melted dark chocolate. The samples were allowed to solidify and stored at 4 °C prior to analysis. Extraction entailed melting the chocolate at 58 °C for 15 minutes prior to addition of 9.5 mL of extraction buffer.

Pancake Batter Soy flour-based pancake mix was used as prescribed by the manufacturer. Incurred samples entailed adding (2000X) analyte to the batter, which was subsequently stored at 4 °C until analyzed.

Baked Muffins Gluten-free (and allergen-free) commercial cake mix was used as prescribed by the manufacturer with vegetable oil spread and water added to create a batter with a density of 1.2 g/mL. One gram samples of batter were incurred with (2,000X) analyte to yield samples containing the desired amounts of analyte. The 1 g 'mini-muffins' were baked for 9 min at 350 °F and stored at – 20 °C prior to analysis.

Baked Rice Cookies Rice cookies were prepared either incurred or spiked with whole ground almond, coconut, gluten, soy, and NIST SRM 2387 peanut butter. Rice cookie batter was

prepared from scratch by mixing 770 g rice flour with 175 mL canola oil and 525 mL water. To two gram portions of batter either 10 µg or 100 µg of each analyte was incurred and the 'mini cookies baked at 350 °F for 24 minutes (final baked mass 1.2 – 1.3 g). Whole mini rice cookies were each ground and extracted with 20 mL PBST. The extracted sample is akin to what would be expected when analyzing a 1 g portion of a baked cookie containing 10 µg or 100 µg analyte (designated A-10 and A-100) with only a slight (0.2-0.3 g) increase in cookie matrix in the 20 mL of extract. Spiked samples were prepared by adding 10 µg or 100 µg of the five analytes to ground analyte-free mini cookies (designated B-10 and B-100). The extracts from the samples containing 100 µg of the analytes (A-100 and B-100) were analyzed as-is or diluted 10-fold with PBS plus 0.1% Tween-20 (designated A100/10 and B100/10), prior to mixing with the bead cocktail.

xMAP Food Allergen Detection Assay Overview xMAP FADA analyses were performed according to the manufacturer's recommended instructions as delineated in the product insert (see electronic supporting information file) and in prior publications which also describe the antibodies ¹¹⁻¹³. A feature of the xMAP FADA that increases its effectiveness is the use of two separate analytical phases; analysis of an extract using buffered-detergent and analysis of an extract using reduced-denatured conditions. The antibodies used with the reduced-denatured extracts were developed exclusively for such and have been recognized for their performance ¹⁵⁻¹⁷. The buffered-detergent extraction is performed either using a PBST protocol or a more stringent UD buffer protocol which has been effective in reducing non-specific interactions ^{13,18,19}. The UD buffer protocol is often used with pure plant products and problematic samples where increased stringency is needed. For this validation, only the PBST buffered-detergent protocol was employed, not the UD buffered-detergent protocol. Further, unique to this validation and not included in prior publications are the addition of two egg (-25 and -26) and two milk (-35 and -36) bead sets to the buffered detergent protocol; thereby increasing the number of unique bead sets in the buffered-detergent protocol to 29 (plus the 4 AssayChex[®] bead sets, Radix BioSolutions,

192 Georgetown, TX). Irrespective of the extraction protocol employed, between all incubation steps,
193 the bead sets were washed using either a BioTek ELX50/8MF or a Bio-Rad Bio-PlexPro micro-
194 plate washer.

195 *Sample Analysis and Instrumentation* All incurred samples were prepared in triplicate. Once
196 prepared, the samples were placed into conical tubes for storage and subsequently extracted and
197 analyzed twice.

198 *Comparison Between Luminex and MagPix* All results were read sequentially in Luminex 200 #1,
199 Luminex 200 #2, and a MagPix. This order was necessary because the Luminex 200 deposits
200 the analyte (after counting) back into the original well with sheath fluid, while the MagPix disposes
201 the captured beads used to measure the MFI. The concentration of the food allergens and gluten
202 in the analytical samples (mixed with the bead cocktail) were the same as the calibration
203 standards typically analyzed using the assay and listed in Table 2.

204 Though the Luminex 200 data sets were usually indistinguishable, quite often the MagPix derived
205 responses slightly exceeded those measured using the Luminex 200 instruments despite claims
206 by the manufacturer and an end-user of minimal differences ²⁰. This may be associated with the
207 differences between laser-based reading and camera-based. This observation is not unique to
208 the xMAP FADA and has been observed with other commercial kits (data not shown). Further,
209 the magnitude of these differences is inconsequential relative to the variances routinely accepted
210 when analyzing food samples for allergens and gluten (typically 5%).

211 *Ruggedness Evaluation* Besides the extensive single laboratory validation described above, a
212 two-laboratory validation involving novice analysts was conducted. The novice analysts had
213 extensive experience performing ELISA analyses. However, they were only provided with two
214 days of training on the xMAP FADA and a single practice sample prior to being asked to analyze
215 incurred and spiked baked rice cookies containing either 10 µg or 100 µg of each of five analytes

per cookie. Each cookie was ground and completely mixed with 20 mL PBST according to the protocol; resulting in analytical samples of 0.5 µg/mL and 5 µg/mL. The rice cookie samples were designed to exceed the dynamic range of the assay and focus on qualitative reproducibility, while the calibration standards examined performance within the dynamic range. The xMAP FADA reagents used in this evaluation were approximately 2 years old and thereby did not contain egg-25, egg-26, milk-35, or milk-36 bead sets and the peanut calibration standards were at ten-times the concentration in the calibration standards used in the single lab validation ¹³.

RESULTS AND DISCUSSION

Overview The detection and accurate quantification of food allergens in oral portions using antibody-based methods is often limited by multiple variables. These include artifacts that may arise from components in the food released during the extraction process, changes in antigenicity associated with food processing or other factors, and antigen extraction mobilization which, if insufficient results in recoveries less than 100%, while by increasing availability – accessibility, relative to the calibration standards, may appear as exceeding 100%. To ascertain the effects of these potential sources of analytical ambiguity, the validation was conducted in two parts. First, the detection of the analytes spiked into extract prepared from various foods and second, the detection of analyte incurred into foods prior to processing (e.g., baking) and subjected to the extraction process prior to analysis. Further, the four foods were chosen to represent common problematic foods due to either extensive processing or components that might be problematic in the extraction or antibody binding aspects of the assay (e.g., incurred into melted dark chocolate that is solidified prior to analysis, acidic conditions, reducing agents, and complex ‘sticky’ batter that might interfere with bead interaction and assay performance).

Analytes Spiked into Food Extracts *Overview* To ascertain the effects of food extracts on the reliability of the xMAP FADA, measurements were made of analyte spiked into extracts prepared from analyte-free samples and compared to the responses observed using either calibration

standards or analyte spiked into PBS with 0.05% Tween-20 (PBST). Though not perfect, due to other potential sources of analytical error, this approach provides a starting point to better appreciate variances that might be observed when analyzing food samples. Since, results discussed in this section have already been partially observed using the calibration standards in PBST, the detailed data is presented as electronic Supplementary Information delineated in Tables SM1 - SM3 and Figures SM1 – SM3 with the hope that by comparing these results to those generated using incurred food samples (below) analyzed using the same lot(s) of assay reagents will provide more detailed information not otherwise possible but beyond the immediate scope of the validation.

Analyte Detection Presented in the supplementary information electronic file as Table SM-1 and graphed in Figure SM-1 are the responses curves observed for the 29 analytes individually spiked into PBST buffered-detergent and averaged with the MFI responses for comparable amounts of analyte spiked into extracts prepared from analyte-free foods (OJ, dark chocolate, pancake batter, and baked muffins). The titration curves are characteristic of the calibration curves routinely generated with the error bars representing one standard deviation between the five matrices being averaged. Almost all analytes could be described using third order polynomial trendlines with R^2 coefficients ≥ 0.99 to describe the relationship between the seven concentration points and their respective MFI. In addition, many analytes displayed x-axis intercepts of zero, consistent with the subtraction of background. The exception to this pattern was macadamia-33 which agreed best with a logarithmic trendline ($R^2 = 0.992$ versus 0.9727 for a third order polynomial). A few of the analytes displayed a slight inconsistency when fitting the trendlines at the highest concentrations, possibly due to the onset of saturation. The largest differences between extracts were usually observed between the background responses generated by pancake batter and PBST, with 10-out-of-29 bead set analyses being >10-fold. Chocolate extract displayed the second highest background. In contrast, variation in the MFI generated in the presence of the extracts for the

different analytes was minor with usually the MFI in the presence of PBST greater than in the presence of one of the food extracts. An interesting conclusion from this data is that there is a high likelihood that interpolation using buffer-based calibration standards (PBST) will underestimate the amount of free analyte; estimated at 25% or less for the effect of carry-over OJ, chocolate, pancake batter, and baked muffin in the extract.

A similar analysis for the performance of the reduced-denatured detection of egg, milk (casein and β -lactoglobulin), peanut, and gluten are presented in the electronic supplementary information as Figure SM-2 which plots each analyte in the reduced denatured extracts derived from PBST and the four foods separately or averaged. The reduced-denatured extracts displayed slightly different traits from the buffered-detergent extracts. Greater differences between the responses generated by the analytes in the presence of the different extracts were observed than for the buffered -detergent samples. Despite this increase, these variances were comparable to variations often observed when analyzing food samples.

Limits of Detection The LoD values for the various analytes spiked into PBST and buffered-detergent extracts are presented in electronic supplementary information Table SM-2. Tabulated are the manufacturer's ascribed LoQ based on the lowest non-zero calibration standard (green font), interpolated LoD values based on a signal-to-noise ratio of 3 (S/N=3, blue font), and for the overall average MFI an additional LoD calculated based on 3-times the standard deviation (3D, purple font). The use of S/N=3 may over- or under-estimate the LoD depending on the magnitude of the background and its precision; variables that may be bead set specific and affect the reliability of S/N include those that depend on the degree of conjugation and the antibodies employed. In the xMAP FADA the %CV values were typically < 10%, thus the use of S/N=3 is expected to overestimate LoD values. The inclination of S/N=3 to overestimate LoD values was especially observed with milk bead sets-35 and -36 due to exceptionally high backgrounds. Using S/N=3, bead sets -35 and -36 displayed LoD values ranging from 5 to 8 ng/mL, while the LoD

291 calculated using the 3D method was 3 ng/mL and closer to the manufacturer's LoQ of 1.5 ng/mL
292 (see incurred sample LoD for a more detailed analysis). Similarly, pancake batter samples
293 displayed higher backgrounds and LoD values than the other matrices. This was observed with
294 all bead sets, especially the walnut bead sets. The ubiquitously higher background due to
295 pancake batter may represent the mediation of non-specific interactions by a component in the
296 pancake batter extract; thus, the more stringent UD Buffer protocol may be more appropriate than
297 the PBST protocol for analyses in this matrix.

298 *Complementary Antibody Ratios* Complementary antibody ratios provide a powerful confirmatory
299 test in the identification of food allergens and differentiation from cross-reactive foods. This
300 secondary, confirmatory end-point is only valid provided the standards and samples have
301 undergone the same processing and likelihood to undergo modification. The complementary
302 antibody ratios of the various analytes in PBST, OJ, chocolate, pancake batter, and muffin
303 buffered-detergent extracts and the overall average between the matrices are listed in electronic
304 supplementary information Table SM-3 and the overall average ratios for all five matrices graphed
305 in Figure SM-3, with the error bars representing one standard deviation to provide a better
306 depiction of the variance between the matrices. As previously observed and theoretically
307 expected, the complementary antibody ratios varied with analyte concentration ¹¹. Further, as
308 expected by the minimal effect of buffered-detergent food extracts on the measured responses,
309 for 10 of the 14 complementary antibody pairs, the %CV values indicated no obvious selective
310 interactions with an extract; almond (CV = 7%), Brazil nut (7%), cashew (7%), egg (4%), gluten
311 (8%), milk (6%), peanut (3%), pine nut (10%), soy (11%) and walnut (8%). Several tree nuts were
312 the only exceptions: coconut (S1 displayed a CV of 31%, average S2-S7 11%), hazelnut (12%),
313 and macadamia (20%) displayed ratios and concentration dependencies in the presence of
314 pancake batter considerably different from the other four extracts. Pistachio (13%) was unique in
315 displaying ratios and concentration dependencies comparable between pancake batter and

1.

muffins, while PBST, OJ, and chocolate were similar. To compensate for the possibility of specific interactions, the ratios should be compared to appropriate standards and, when possible, also compared to direct comparison controls (DCCs). DCCs entail spiking or incurring analyte at the target (threshold) level in a comparable sample of food (matrix), which is extracted and diluted alongside the sample so the amount of carry over matrix and composition of the solvent milieu are identical.

Analytes Incurred into Food Samples Overview The analysis of undeclared food allergens in prepared or ready-to-eat food products is substantially more challenging than in ingredients. The virtually unlimited number of different forms of processing employed and preparation conditions make it likely that the antigenic elements in a food may be uniquely altered along with the ability to be mobilized (extracted) and subsequently detected and quantified. The incurred samples included in this study were chosen to represent extremely different common food occurrences and a commonly used buffer (PBS with 0.05% Tween-20, PBST).

Detection of Incurred Analytes Figure SM-4 depicts the detection of each of the 15 analytes incurred in PBST, OJ, dark chocolate, pancake batter, and baked muffins and extracted using the PBST buffered-detergent protocol. The allergens were added individually at concentrations that following the 20-fold dilution associated with extraction, generate their respective S1, S2, S4, S6, and S7 concentrations (see Table 2). Plotted in Figure SM-4 are the average of triplicates after subtraction of the background responses; error bars represent one standard deviation. The three curves for each bead set depict the measurements made using two Luminex 200 and one MagPix instrument. For example, the top row depicts for almond-12 and almond-13 the MFI generated by 58, 104, 340, 1100, 1998 ng almond protein in the five matrices (2.9, 5.2, 17, 55, and 99 ng almond protein / mL in the analytical samples following buffered-detergent extraction). The analyte is scaled identically for each matrix to better visualize changes in detectability. Further, each plot depicts the responses for both bead sets targeting the same analyte to better compare

341 differences in affinity constants and capacity within the dynamic range of the assay (S1 – S7).
342 Included in the plots are inserts designed to help visualize bead sets with low affinity constants or
343 binding capacity. For example, coconut-21 plotted on the same scale as coconut-20 appears non-
344 responsive to the presence of analyte. However, upon expanding the vertical scale, see insert, it
345 is obvious that it also displays a dynamic response with excellent precision. Similarly, when the
346 vertical scale for Brazil nut detection in for OJ, chocolate, and baked muffins appears non-
347 responsive relative to PBST, but upon expansion (see inserts) displays a dynamic response with
348 excellent signal-to-noise to support Brazil nut detection. Only rarely was it impossible to reliably
349 detect any of the analytes. These unique cases include the detection of crustacean, egg, and milk
350 incurred into dark chocolate and milk incurred into OJ. The drop in MFI observed with egg-25 and
351 egg-26 for egg incurred at 198 ng/mL (S7) in buffer was not observed with OJ, pancake batter, or
352 baked muffins nor when spiked at 198 ng/mL into extracts prepared from analyte-free matrices.
353 Inasmuch as the analytical protocol includes wash steps between incubation of the bead sets with
354 the analytical sample and the addition of detector antibody, the decrease is unlikely to represent
355 a Hook Effect but more likely operator error in the preparation of the samples. No issues were
356 encountered recovering milk and egg in dark chocolate or milk in OJ using the reduced-denatured
357 protocol.

358 Figure SM-5 depicts the detection of the 15 analytes simultaneously incurred into each of the five
359 matrices and extracted using the PBST buffered-detergent protocol. As observed with the
360 incurred individual analytes, changing the matrix affected detection.

361 Figure SM-6 depicts the MFI responses of the five bead sets associated with the reduced-
362 denatured (SDS/ β -mercaptoethanol) extraction protocol for each of the five matrices incurred with
363 the four individual analytes or a mixture. The five bead sets displayed excellent specificity. Egg-
364 65 only displayed a positive response for egg incurred in the matrix. Similarly, milk-66 and milk-
365 67 were specific for milk while peanut-72 was specific for peanut and gluten-73 for gluten. Of the

four analytes, milk was the only analyte that generated definitive positive responses characteristic of a titration curve with the incurred chocolate samples. The other three analytes displayed irregularities in the titration curves when incurred in chocolate. These irregularities likely represent an increase in the LoD values as evidenced by the onset of a dynamic response for gluten and peanut at the higher concentration(s). Similarly, peanut was not detectable above background at the lowest concentrations incurred into baked muffins, indicating an increase in the LoD value. The loss of a dynamic response throughout the concentration range examined for egg in dark chocolate may indicate either a unique problem or massive shifting of the dynamic range.

Limits of Detection The LoD values for the various incurred analytes in the five matrices were determined using three popular approaches. These included a signal-to-noise ratio (S/N) of 3, three-times the standard deviation of the background ($3D$ of S_0), and three-times the sum of the standard deviations of the background and the lowest non-zero sample ($3 \times \Sigma D$ of S_0 and S_1). These three approaches have different advantages and disadvantages. Table 3 compares the interpolated LoD values (blue font) to the LoQ listed by the test kit manufacturer (S_1 , green font) for individually incurred samples extracted using the PBST buffered-detergent protocol. Table 4 presents the same information for the analytes individually incurred and as a 15-analyte mixture using the reduced-denatured extraction protocol. Interpolated LoD values exceeding the LoQ are in red font (interpolated LoD > S_1). To further help visualize the interpolated LoD data, the cells were highlighted in different colors; blue - LoD < $S_1/10$, grey - $S_1/10$ < LoD < $0.9 \times S_1$, no highlight - $0.9 \times S_1$ < LoD < S_1 , yellow - S_1 < LoD < $1.1 \times S_1$, pink - $1.1 \times S_1$ < LoD < $10 \times S_1$, and red - LoD > $10 \times S_1$. Not surprisingly, the majority of LoD values that exceeded the LoQ (S_1) as defined by the manufacturer, were derived using S/N=3; specifically, 50-of-61 of the buffered-detergent analyses and 22-of-26 reduced denatured analyses. Further, approximately 80% of the buffered detergent interpolated LoD values that exceeded S_1 were either chocolate or pancake batter samples, consistent with the increased backgrounds observed in the studies of analytes spiked

391 into food extracts. Not listed in Table 3 are the interpolated LoD values for crustacean, egg, and
392 milk in dark chocolate and milk in OJ due to the lack of a sufficient dynamic response increasing
393 over multiple concentrations of analyte (see Figure SM-5). Similarly, the interpolated LoD values
394 for egg, gluten, and peanut in chocolate are questionable due what appears to be a dramatic shift
395 in the dynamic response depicted in Figure SM-6.

396 The preponderance of interpolated LoD values derived from $3D$ or $3xSD$ that are less than one-
397 tenth the manufacturer's LoQ makes the xMAP FADA ideal for the analysis of problematic
398 samples. Exploiting this feature, it is possible to employ dilutions that minimize (or eliminate)
399 potential artifacts caused by matrix carry-over or analyze samples displaying less than ideal
400 recoveries (see below).

401 *Complementary Antibody Ratios* The complementary antibody ratios for each of the 15 analytes
402 (14 food allergens plus gluten) incurred individually or as mixture into PBS, OJ, dark chocolate,
403 pancake batter, and baked muffins are tabulated in Table 5. The ratios are divided by analyte with
404 the ratios generated by the mixtures highlighted in grey. The analytes displayed an acceptable
405 consistency between the different matrices except for soy and several of the tree nuts (coconut,
406 cashew, macadamia, pine nut, pistachio, and walnut) which displayed significant differences
407 between different matrices.

408 Compared to the ratios generated by analyte in the presence of PBST or analyte-free extract, the
409 ratios observed with the incurred samples were typically similar except the variances across the
410 different matrices were greater. In addition, there were several cases when the incurred analytes
411 displayed considerable differences from the patterns observed for analyte in the presence of
412 extract. These included cashew, coconut, gluten, and macadamia in chocolate, and egg,
413 pistachio, and walnut (individually) in baked muffin. Overall, there appeared to be a re-occurring
414 pattern whereby the incurred muffin samples had a greater likelihood of deviating from the other
415 samples while analytes spiked into the muffin extract did not display such differences. While this

may be due to the additional processing (baking) of the baked muffins, to apply generalizations requires more statistical information. It is recommended that complementary antibody ratios be only applied to distinguish between large differences or for analyzing samples identically processed and compared to controls analyzed alongside the sample.

Recovery The efficiency of analyte detection (recovery) in the presence of extracts prepared from analyte-free matrices and incurred in the same matrices are presented in Tables 6 and 7. The analytes when added to PBST at the concentrations in Table 2 are identical to the calibration standards for the buffered-detergent analyses. Thus, by comparing the intensities (average MFI) between the analytes in the different foods, incurred and subjected to the extraction process (designate OJ_{incurred} , $Choc_{\text{incurred}}$, $Panc_{\text{incurred}}$, or $Muff_{\text{incurred}}$) or spiked into extract (e.g., OJ_{extract} , $Choc_{\text{extract}}$, $Panc_{\text{extract}}$, or $Muff_{\text{extract}}$), at the same concentrations provides a measure of recovery and the influence of the various food matrices, processing, and extraction. To help visualize the data, the cells were highlighted depending on the level of recovery; dark blue > 150%; 120% < light blue < 150%; 80% < no highlight < 120%; 50% < yellow < 80%; red < 50%. Not surprisingly, the recovery of analyte spiked into an extract was greater than when incurred with a majority between 80% - 120% (no highlight). Interestingly, the recovery of analyte incurred into PBST, OJ, and pancake batter at the two lowest concentrations (S1, S2) and subjected to the buffered-detergent extraction process (Table 7) was greater than analyte spiked into extracts with average recoveries of $252 \pm 77\%$, $189 \pm 101\%$, and $131 \pm 52\%$, across all 29 bead sets. In contrast, the three higher concentrations (S4, S6, S7) averaged $109 \pm 42\%$, $83 \pm 43\%$, and $89 \pm 21\%$ for PBST, OJ, and pancake, respectively. This pattern is consistent with the extraction process increasing the availability of analyte that at low concentrations may not otherwise be as readily detectable. Analytes added to pancake batter extract displayed an average recovery of $80 \pm 18\%$ relative to detection from PBST. The recovery of analyte incurred in pancake batter versus incurred in PBST ($63 \pm 32\%$) and analyte incurred in pancake batter versus in the presence of pancake batter extract

441 (106 $\pm$ 42%) also indicate that the detection of analyte was not significantly affected by pancake
442 batter. This is despite a dramatic increase in background associated with pancake batter, and
443 associated increases in LoD values calculated based on S/N=3 or standard deviations (3D or
444 $3 \times \Sigma D$). This supports earlier indications that the effects of pancake batter on the MFI background
445 was mostly independent of analyte detection and probably due to a component in the extract
446 derived from pancake batter mediating binding between bead sets and either detector antibodies
447 or the streptavidin-phycoerythrin conjugate. As such, the use of more stringent binding conditions
448 (e.g., UD buffer) should reduce the background and by improving the signal / noise ratio, improve
449 sensitivity while not changing the dynamic properties of the complexation (antibody – antigen
450 binding) being measured.

451 Chocolate displayed very different properties than pancake batter. Analyte incurred in chocolate
452 displayed extremely poor levels of recovery, while spiked into chocolate extracts displayed
453 excellent levels of recovery (86 $\pm$ 16% relative to in PBST). However, once incurred, the average
454 recovery of analyte relative to analyte incurred in PBST or in the presence of chocolate extract
455 were 12 $\pm$ 11% and 18 $\pm$ 12%, respectively. This pattern may correlate with the previously
456 mentioned inability to detect milk and egg in chocolate using the buffered detergent protocol. The
457 milk bead sets -35 and -36 displayed the highest backgrounds at 2406 $\pm$ 799 and 1859 $\pm$ 567,
458 respectively (across all matrices) and egg-26 displayed the third highest background relative to
459 the milk bead sets (913 $\pm$ 203). Interestingly, the background for the milk and egg bead sets used
460 in the buffered-detergent protocol appeared (in some cases) to increase with time. Such
461 backgrounds are expected not to affect detection provided the causes of the background and the
462 effects of the matrix function independently. Since chocolate greatly affects recovery, it is possible
463 that these two variables are not independent. There can be numerous causes for the poor
464 recovery of analyte incurred in dark chocolate using the buffered-detergent extraction protocol.
465 One potential solution to both the effects of chocolate on milk and egg detection and the poor

recovery values may be to employ different extraction conditions that improve mobilization of the antigens or increase stringency to prevent non-specific interactions between components in the chocolate (e.g., polyphenols) and the antigens that may alter antigen recognition.

The detection of analyte incurred in baked muffins also displayed reduced recoveries. Analyte in the presence of muffin extract versus PBST displayed an average recovery of $76 \pm 25\%$. However, when incurred in baked muffins the recoveries relative to incurred in PBST and in the presence of muffin extract were $33 \pm 21\%$ and $56 \pm 34\%$, respectively. This is likely due to the baking process affecting the antigenicity of the proteins.

The detection of analyte in the presence of OJ was similar to PBST as evidenced by the average recovery of analyte in the presence of OJ extract relative to the presence of PBST being $87 \pm 15\%$. Further, when incurred in OJ the recovery of analyte relative to analyte incurred in PBST or relative to analyte in the presence of OJ extract were $72 \pm 38\%$ and $125 \pm 89\%$, respectively. Despite this high level of similarity, it is not understood why bead sets -35 and -36 were ineffective in detecting milk in the presence of OJ. Several possible explanations can explain these results ranging from sample preparation error to the high MFI associated with S1 ($>S_0$) representing saturation of the beads.

The low levels of recovery associated with some of the samples were not an analytical problem due to the high level of sensitivity (LoD values). Only, crustacean, egg, and milk incurred in chocolate, and milk in OJ, at the concentrations used in this study were impossible to reliably detect due to the loss of a dynamic response. Otherwise, as depicted, there were no problems detecting the various analytes. A concern associated with low recoveries is whether the loss of detectable analyte skews the population of detectable antigens sufficiently to mislead when quantifying to ascertain potential health risks. However, these problems are not new and there are no available analytical methods capable of fully resolving this problem.

Of the extraction protocols available, the use of reduced-denatured conditions, as employed by MloBS and related ELISA test kits, has shown the most promise in reliably detecting incurred analytes. It is for this reason that the xMAP FADA includes a second phase whereby SDS/ β -mercaptoethanol is used to generate comparable reduced-denatured extraction conditions. Table 7 lists the recoveries calculated for incurred samples extracted using SDS/ β -mercaptoethanol or in the presence of SDS/ β -mercaptoethanol extracts of PBST, OJ, dark chocolate, pancake batter, or baked muffins. Compared to the buffered-detergent recoveries, the incidence of recoveries exceeding 100% were greater. However, in general the patterns were similar with the lowest recoveries associated with analytes incurred in chocolate ($21 \pm 15\%$ and $39 \pm 39\%$ relative to analyte incurred in PBST or relative to analyte in the presence of matrix extract, respectively) and then incurred in baked muffins ($32 \pm 21\%$ and $60 \pm 42\%$). Interestingly, the percent recoveries for analytes incurred in chocolate averaged about twice the level observed using the buffered-detergent analyses while the percent recoveries for the incurred muffin samples were similar. The improved recovery with chocolate is consistent with analyses conducted using the MloBS ELISA versus buffered-detergent extraction protocols (data not shown). The pancake batter samples displayed no significant reduction in recovery ($156 \pm 55\%$, $96 \pm 39\%$, $90 \pm 23\%$) as observed with the buffered-detergent analyses.

Ruggedness Overview To ascertain the ruggedness of the xMAP FADA, two novice analysts with backgrounds performing ELISA analyses but only two days training on the use of the MagPix and BioPlex instruments, and a single practice sample ran using the xMAP FADA were asked to analyze incurred and baked rice cookies containing either 0, 10, or 100 $\mu\text{g/g}$ of each of five analytes (almond, coconut, gluten, peanut, and soy). The incurred samples were designated A-10 or A-100 and the spiked samples were designated B-10 or B-100. The centrifuged extracts prepared from the samples containing 100 $\mu\text{g/g}$ of each analyte were also analyzed following a 10-fold dilution with PBST (designated A-100/10 and B-100/10).

Bead Count Bead count, an indicator of the loss of beads during the analysis of a sample, is often used to assess analyst performance since variance in MFI is often inversely proportional to bead count. Analyst A generated the data depicted in Figure 1 with an average bead count per bead set in each well of 218 ± 97 , comparable to the 100 – 200 beads typically observed using the xMAP FADA. In contrast, the second analyst generated the results depicted in Figure 2 with an average bead count per bead set in each well of 28 ± 36 ; many wells displaying bead counts formally classified as 'FAIL' (less than 10) with a high probability of analytical error (Figure 2). Below each column in Figures 1 and 2 are listed the average bead count for the specific bead set across all micro-wells.

Qualitative Detection Figures 3A and 4A tabulate the interpolated amount of analyte detected in the rice cookie samples and associated direct comparison controls (DCC) for the two novice analysts. The DCCs were prepared by the novice analysts by adding either 20 μg gluten, 50 μg milk, or 10 μg peanut to one ground baked rice cookie and performing the buffered-detergent extraction protocol and analysis alongside the samples. The concentrations were interpolated assuming linearity between the two adjacent calibration standards. If an MFI response was less than the LoQ (S1) it was designated as 'under' and highlighted in grey. Samples exceeding the LoQ (S1) are highlighted in green with those exceeding the dynamic range ($> S7$) designated as 'over'; Before a sample was concluded to include an analyte, both complementary antibodies had to have MFI responses above S1.

Despite, the significant differences in bead count observed between the two analysts, the data were qualitatively identical, successfully detecting the qualitative presence in rice cookies of incurred and spiked almond, coconut, gluten, peanut, and soy in the rice cookie samples (Figures 3A and 4A, areas highlighted in green and boxed using a red line). Further, the DCC generated responses as expected (areas boxed in red) for both analysts with only the second analyst generating what may be false positives probably due to contamination of the M-50.

Unexpected was a slight positive response with Brazil nut-14 by both analysts and a weak positive response with Brazil nut-15 by the second novice analyst. The difference in behavior by the two Brazil nut bead sets make it unlikely that cross-contact occurred. The possibility of cross-reactivity with one of the target analytes (almond, coconut, gluten, peanut, and soy) as the cause is inconclusive. In a recent extensive study of cross-reactivity, soy was the only one of the five analytes that displayed cross-reactivity with the Brazil nut bead sets ^{12, Table 2}. Further, responses with bead sets pine nut-39, -42, and walnut-47 were not observed despite an expected greater cross-reactivity with these bead sets than the Brazil nut bead sets. In contrast, in an earlier study using a different lot of reagents, the cross-reactivity with pine nut-39, -42, and walnut-47 were not observed despite presence of a very weak cross-reactivity with Brazil nut-14 being detectable ¹¹. The other positive responses for the rice cookie samples (e.g., incurred, spiked, and DCC) were common to both analysts and displayed patterns characteristic of cross-reactivity patterns previously observed. Specifically, cross-reactivities were repeatedly observed with cashew-18, -19, hazelnut-29, -30 and walnut-48. Consistent with the responses representing cross-reactivities were the dependence on analyte concentration, the analytical samples were deliberately prepared omitting the optional dilution steps routinely performed (typically > 10-fold for 10 µg/g analyte) and correlated with expected excessive concentrations, spiked at 100 µg/g analyte (B-100) being the greatest and incurred at 10 µg/g analyte (A-10) the least.

Calibration Curves Further, illustrating the ruggedness of the xMAP FADA are the performance of the calibration standards used to interpolate analyte concentrations. Figures 3B and 4B depict the titration curves generated by the calibration standards in PBST, with the error bars representing one standard deviation between the triplicate analyses. Of the possible calibration plots, depicted are those for three of the analytes present; almond, gluten, and peanut (designated I, ii, and iii, respectively). Despite the differences in bead count and the analyses being performed by two novice analysts, the calibration curves were comparable, with most re-occurring

differences being the size of the error bars. A unique advantage of binding assays that measure the binding of a ligand, is the effect of multiple binding sites that enhance the signal/noise and sensitivity, as typified by the gluten-28 assay as previously discussed ¹¹. Thus, the gluten-28 calibration standard curves (Figures 3Bii and 4Bii, gluten plots, orange curves) were comparable for the two analysts despite the average bead counts for the gluten-28 bead sets being 230 ± 29 (ideal) and 6 ± 4 ('FAIL'), respectively. This similarity also applied throughout the samples despite the overall gluten-28 bead count for the second analyst being 7 ± 9 (n=48) across all samples.

The increase in variance and loss of precision is potentially problematic as illustrated for the peanut calibration curves whereby the ratios between the two bead sets are different for the two analysts (Figure 3Biii and 4Biii). What appears to be a lower response by peanut-37 and increase in response by peanut-38 alters the ratio profile (peanut-37/peanut-38) that may be used as a secondary end-point. It is therefore recommended that data from bead counts classified as 'FAIL' (<10 beads) be carefully reviewed prior to use in secondary end-point analysis.

Another utility of bead count is in troubleshooting poor assay technique. This was observed with a third novice analyst in which 'borderline/ and 'FAIL' bead counts for cells A11 – H11 in an experiment in which alternating columns with acceptable bead counts in columns 1, 3, 5, 7, and 9. One explanation was that the pattern was due to improper holding of the microtiter plate against the magnetic plate during a manual wash step (data not shown). Despite this error, the qualitative conclusions were unaffected with only a minor effect on the precision of the data.

Application The xMAP Food Allergen Detection Assay (xMAP FADA) displayed excellent performance in a single laboratory validation and when used by novice analysts demonstrating suitability as a potential regulatory method. Replicate samples displayed good reproducibility (%CV typically < 5%) and the results read using a Luminex 200 were comparable to those using a MagPix. Though the recovery of analyte that was incurred in chocolate and baked muffins averaged < 60%, the high sensitivity of the xMAP FADA meant that only crustacean, egg, and

590 milk incurred in dark chocolate had problems being detected using the buffered-detergent
591 extraction protocol; the reduced-denatured protocol reliably detected milk incurred in dark
592 chocolate. Unlike chocolate and baked muffins, pancake batter caused the greatest increase in
593 background (and LoD values) while displaying no differential effect on whether the analyte was
594 added to pancake batter extract or incurred into pancake batter and extracted. Orange juice had
595 minimal effect on analyte detection except for the lack of a dynamic response by milk-35 and milk-
596 36, presumably related to the atypically high background (>1,000 MFI versus < 100 MFI) that
597 appeared to increase with time after preparation of the bead cocktail.

598 The ruggedness of the xMAP FADA was illustrated by the ability of two independent novice
599 analysts to generate qualitatively comparable results despite one analyst losing most of the beads
600 (estimated at > 85%) during analysis.

601

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612

613 **CONFLICT OF INTEREST**

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ELECTRONIC SUPPORTING INFORMATION

Table SM-1. MFI and Ratios Between Complementary Antibodies for Allergens and Gluten in the Presence of PBST Buffered-Detergent Derived Extracts.

Table SM-2. Limits of Detection (LOD values) of Food Allergens in PBST Buffered-Detergent Extracts using xMAP FADA.

Table SM-3. Ratios Between Complementary Antibodies in the Presence of PBST and PBST Buffered-Detergent Extracts.

Figure SM-1. Detection of 14 Food Allergens plus Gluten in the Presence of PBST and Buffered-Detergent Extracts.

Figure SM-2. Detection of Food Allergens plus Gluten in the Presence of Reduced-Denatured Food Extracts.

Figure SM-3. Variation in Antibody Ratio of Complementary Antibodies in the Presence of Food Extract.

Figure SM-4. Comparison between Luminex and MagPix Detection of 14 Food Allergens plus Gluten Individually Incurred into Matrices and Analyzed Using the PBST Buffered Detergent Extraction Protocol.

Figure SM-5. Detection of 14 Food Allergens plus Gluten Incurred Simultaneously.

Figure SM-6. Detection of Food Allergens plus Gluten Incurred in PBST and Food Using the Reduced-Denatured Extraction Protocol.

xMAP FADA product insert ver G

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700 **FIGURE LEGENDS**701 **Figure 1. Bead Count Recovery of xMAP FADA by a Novice Analyst with Good Technique.**

702 Rice cookies incurred or spiked with a mixture of either 10 µg/g or 100 µg/g of each of five analytes
703 (ground-whole-raw almond, ground-whole-raw coconut, wheat gluten, NIST SRM 2387 peanut
704 butter, and ground-whole-dried soy beans) or prepared analyte-free (0 µg/g) were analyzed by a
705 novice analyst (extensive background with ELISAs but only two days of training and one practice
706 exercise with the xMAP FADA). Listed are the bead counts per bead set per sample / well. Green
707 indicates bead count > 25, yellow between 10 and 25, red < 10. The average content across all
708 sample wells is listed at the bottom of the table.

709 **Figure 2. Bead Count Recovery of xMAP FADA by a Novice Analyst with Poor Technique.**

710 Details the same as delineated for Figure 1. Green indicates bead count > 25, yellow between 10
711 and 25, red < 10. The average content across all sample wells is listed at the bottom of the table.

712 **Figure 3. Detection of Food Allergens in Baked Rice Cookies Using xMAP FADA by a**
713 **Novice Analyst with Good Technique.**

Rice cookies incurred or spiked with a mixture of either
714 10 µg/g or 100 µg/g of each of five analytes (ground-whole-raw almond, ground-whole-raw
715 coconut, wheat gluten, NIST SRM 2387 peanut butter, and ground-whole-dried soy beans) or
716 prepared analyte-free (0 µg/g) were analyzed by a novice analyst (extensive background with
717 ELISAs but only two days of training and one practice exercise with the xMAP FADA). **A-**
718 interpolated concentration (ng protein per gram sample) for responses within the dynamic range
719 as defined by calibration standards S1 and S7, assuming linearity between the two most adjacent
720 calibration standards. Responses less than S1 are listed as 'under' and highlight in grey. Positive
721 responses (>S1) are highlighted in green with 'over' used to indicate > S7 (greater than the
722 quantitative dynamic range of the assay). Red boundaries define the areas where positive
723 responses are expected. A blue box defines unexpected cross-reactivity, possibly due to cross

724 contamination. **B-** calibration standard curves for almond, gluten, and peanut. Plotted are the
725 average MFI of triplicate analyses; error bars represent one standard deviation.

726 **Figure 4. Detection of Food Allergens in Baked Rice Cookies Using xMAP FADA by a**
727 **Novice Analyst with Poor Technique.** Details the same as delineated for Figure 3. Besides
728 differences in performance, a possible cross-contamination resulting in false positives in 4A,
729 marked with blue boundaries.

730

TOC GRAPHIC

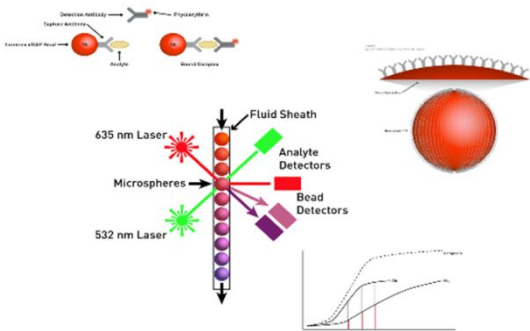


Table 1. Requirements and Approach Taken for Single Laboratory Validation (Level 2+)

Validation Parameter	Minimum Requirement ^a	Approach
Number participating labs	1	1
Number of matrices	≥ 3 recommended	4 foods representing different properties & processing + buffer
Number of analyte(s) spike levels per matrix	≥ 3 levels + a blank	All 15 target analytes spiked and incurred separately and as a mixture. 5 levels emphasizing lower and upper (onset of saturation) limits of the dynamic range + a blank. Samples incurred prior to processing for 3-out-of-4 of the foods; OJ & buffer (matrices) spiked.
Replicates required per matrix at each level tested	≥ 2 (quantitative) ≥ 3 (qualitative)	3 (quantitative, from scratch), with results read using 3 different instruments (two Luminex 200 & a MagPix). All xMAP bead set analyses of extracts repeated on separate microtiter plates.
Replicates required at each level tested per laboratory if only one matrix source used	≥ 6 (quantitative) ≥ 9 (qualitative)	N/A

^a Adapted from Food & Drug Administration, Office of Foods and Veterinary Medicine. Guidelines for the Validation of Chemical Methods for the FDA FVM Program, 2nd Edition. 2015. <https://www.fda.gov/downloads/ScienceResearch/FieldScience/UCM273418.pdf>; These requirements are comparable to those stipulated by Food Emergency Response Network (FERN), SOP No: FERNADM.0008.00, FERN Validation Guidelines for FERN Chemical, Microbiological, and Radiological Methods; and the draft AOAC International, "Standard Method Performance Requirement (SMPR) Documents" (Version 12.1; 31-Jan-11).

Table 2. Concentration of Food Allergen Protein in Analytical Samples ^a

<i>b</i>	S1 (LoQ) (ng/mL)	S2 (ng/mL)	S3 (ng/mL)	S4 (ng/mL)	S5 (ng/mL)	S6 (ng/mL)	S7 (ng/mL)
Almond	2.9	5.2	9.4	17	30	55	99
Brazil Nut	2.9	5.2	9.4	17	30	55	99
Cashew	0.5	0.8	1.5	2.7	4.8	8.7	16
Coconut	0.6	1.1	2.1	3.7	6.7	12	22
Crustacean	15	28	50	90	162	292	525
Egg	5.8	10	19	34	61	110	198
Gluten	3.7	6.6	12	21	39	69	125
Hazelnut	1.2	2.1	3.8	7	12	22	40
Macadamia Nut	4.8	8.7	16	28	51	91	165
Milk	1.5	2.6	4.7	8	15	27	49
Peanut	1.5	2.8	5	9	16.2	29.2	52.5
Pine Nut	8.1	15	26	47	85	152	274
Pistachio	2.9	5.2	9.4	17	30	55	99
Soy	9.2	17	30	54	96	174	313
Walnut	9.2	17	30	54	96	174	313

^a Concentration (ng allergen-derived protein per mL) in the analytical sample immediately prior to addition of bead cocktail.

^b Calibration standards prepared at 0 ng / mL (aka S0 or background), S1, S2, S3, S4, S5, S6, S7 in buffer. Spiked foods samples prepared by mixing analyte (or mixture) with analyte-free food extract to yield concentrations S1 -S7. Incurred samples prepared by mixing allergen-derived protein that upon completion of the extraction / sample preparation process yielded concentrations of S1, S2, S4, S6, and S7. For example, peanut at 1.5 ng/mL in the samples derived from incurred muffins extracted according to the buffered-detergent protocol, which entails 20-fold dilution (1 g + 20 mL PBST), the 1 g muffin batter was incurred with 30 ng peanut protein prior to baking and the complete muffin (irrespective of mass change during baking) extracted for analysis.

Table 3. Limits of Detection of Incurred Food Allergens Using the PBST Buffered-Detergent Extraction Protocol ^a

LoD ^c	PBST Buffer ^b	Orange Juice	Choc	Pancake Batter	Baked Muffin	PBST Buffer ^b	Orange Juice	Choc	Pancake Batter	Baked Muffin	LoD	PBST Buffer ^b	Orange Juice	Choc	Pancake Batter	Baked Muffin	PBST Buffer ^b	Orange Juice	Choc	Pancake Batter	Baked Muffin
2.9 ng/mL^c																					
ALMOND - 12					ALMOND - 13					BRAZIL NUT - 14					BRAZIL NUT - 15						
S/N=3	0.05	0.06	3.1	3.3	0.61	0.14	0.18	4.1	3.5	0.73	S/N=3	0.10	2.4	3.5	5.1	2.6	0.15	1.4	3.6	4.4	3.9
3xD	0.001	0.001	0.14	0.05	0.11	0.006	0.008	0.000	0.07	0.02	3xD	0.006	0.07	0.12	0.03	0.10	0.003	0.02	0.09	0.04	0.08
3xSD	0.17	0.04	0.26	0.08	0.19	0.09	0.07	0.20	0.19	0.11	3xSD	0.22	0.26	0.54	0.10	0.40	0.16	0.17	0.45	0.25	0.91
0.50 ng/mL																					
CASHEW - 18					CASHEW - 19					COCONUT - 20					COCONUT - 21						
S/N=3	0.05	0.07	3.1	2.0	0.21	0.08	0.30	2.2	3.5	0.35	S/N=3	0.02	0.04	11	0.33	0.12	0.49	0.38	14	4.2	1.1
3xD	0.001	0.003	0.19	2.0	0.005	0.002	0.009	0.02	0.05	0.03	3xD	0.000	0.002	0.42	0.002	0.005	0.009	0.010	0.22	0.12	0.05
3xSD	0.07	0.07	0.42	2.2	0.10	0.06	0.06	0.16	0.11	0.09	3xSD	0.06	0.04	0.49	0.03	0.03	0.08	0.03	1.0	0.20	0.27
15 ng/mL																					
CRUSTACEAN - 22					EGG - 25					EGG - 26											
S/N=3	1.5	1.1	e	22	2593	S/N=3	0.31	0.33	e	7.6	1.9	2.4	2.8	e	8.9	12.53	0.03	0.06	e	0.06	0.16
3xD	0.02	0.02	e	0.90	40	3xD	0.003	0.004	e	0.09	0.06	0.03	0.06	e	0.06	0.16	0.37	0.82	e	0.50	0.66
3xSD	2.2	1.8	e	4.0	110	3xSD	0.37	0.82	e	0.35	0.75	0.41	0.74	e	0.50	0.66					
3.7 ng/mL																					
GLUTEN - 27					GLUTEN - 28					HAZELNUT - 29					HAZELNUT - 30						
S/N=3	0.98	0.93	32	13	1.9	0.82	2.19	62	7.7	2.9	S/N=3	0.07	0.10	9.1	5.4	0.61	0.24	0.46	13	5.5	0.88
3xD	0.03	0.03	0.34	0.23	0.11	0.02	0.07	1.7	0.07	0.03	3xD	0.001	0.003	0.32	0.04	0.05	0.000	0.02	0.45	0.17	0.04
3xSD	0.17	0.19	1.6	0.5	0.14	0.26	0.19	3.4	0.19	0.55	3xSD	0.05	0.32	1.1	0.52	0.31	0.07	0.32	1.8	0.55	0.41
4.8 ng/mL																					
MACADAMIA - 33					MACADAMIA - 34					MILK - 35					MILK - 36						
S/N=3	0.11	0.13	3.2	0.86	0.52	0.68	1.0	8.6	5.8	2.1	S/N=3	3.1	e	e	11	5.9	3.8	e	e	15	7.2
3xD	0.001	0.003	0.05	0.008	0.02	0.01	0.02	0.09	0.04	0.09	3xD	0.06	e	e	0.2	0.2	0.07	e	e	0.11	0.15
3xSD	0.10	0.57	0.23	0.10	0.22	0.21	1.1	0.26	0.29	0.56	3xSD	0.6	e	e	0.4	0.7	0.7	e	e	0.8	0.7
1.5 ng/mL																					
PEANUT - 37					PEANUT - 38					PINE NUT - 39					PINE NUT - 42						
S/N=3	0.02	0.42	0.47	0.91	0.10	0.04	0.73	1.3	1.6	0.28	S/N=3	4.7	8.9	132	17	7.9	5.7	8.4	48	45	10.4
3xD	0.001	0.02	0.013	0.03	0.005	0.001	0.006	0.04	0.03	0.02	3xD	0.03	0.4	0.9	0.2	0.2	0.05	0.2	0.7	0.5	0.000
3xSD	0.05	0.09	0.16	0.06	0.06	0.08	0.14	0.19	0.11	0.18	3xSD	0.4	1.1	4.5	0.6	1.3	0.5	0.9	3.9	2.8	0.2
2.9 ng/mL																					
PISTACHIO - 43					PISTACHIO - 44					SOY - 45					SOY - 46						
S/N=3	0.05	0.06	0.70	2.1	0.26	0.52	1.1	8.5	8.4	1.3	S/N=3	1.6	0.17	34	f	5.4	0.24	5.1	5.5	f	1.3
3xD	0.001	0.001	0.03	0.05	0.003	0.01	0.04	0.19	0.24	0.10	3xD	0.06	0.01	0.31	f	0.80	0.01	0.13	0.06	f	0.02
3xSD	0.09	0.31	0.40	0.37	0.29	0.23	0.32	1.3	0.44	0.33	3xSD	0.60	0.03	1.8	f	1.1	0.60	1.14	0.57	f	0.35
9.2 ng/mL																					
WALNUT - 47					WALNUT - 48																
S/N=3	1.7	2.1	124	44	6.2	3.6	8.8	60	42	12	S/N=3	1.6	0.17	34	f	5.4	0.24	5.1	5.5	f	1.3
3xD	0.01	0.06	2.6	1.2	0.2	0.11	0.2	2.4	0.8	0.7	3xD	0.06	0.01	0.31	f	0.80	0.01	0.13	0.06	f	0.02
3xSD	1.0	0.7	9.7	2.9	1.8	1.1	0.6	8.3	2.0	2.6	3xSD	0.60	0.03	1.8	f	1.1	0.60	1.14	0.57	f	0.35

^a LoD values (ng protein/mL) interpolated assuming linearity between analyte-free matrix (background) and the first non-zero incurred sample (after subtraction of background) prepared to yield upon preparation concentrations of allergen in the analytical portion identical to the first non-zero calibration standard (manufacturer's LoD), assuming 100% recovery.

^b Matrix- allergens incurred into PBST Buffer (0.05% Tween-20), Orange Juice, Dark Chocolate, Pancake Batter, and Baked Muffins. Samples extracted using the PBST buffered-detergent protocol

^c S1, the first non-zero calibration standard is defined by the product insert as the limit of quantification (LoQ) in ng protein/mL and listed in the table using a green font. LoD values (blue font) were interpolated based on three commonly used approaches: a signal/noise ratio of 3 (S/N=3), three-times the standard deviation of the background (3xD), and three-times the sum of the standard deviations of the background and the first non-zero sample (3xSD). A Red font indicates that the interpolated LoD exceeded S1 (LoQ). Cells highlighted in Blue LoD < 0.1xS1; grey LoD > 0.1xS1; no highlight LoD > 0.9xS1; yellow LoD >S1; pink LoD > 1.1xS1; red LoD > 10xS1.

^d Samples were prepared in triplicate and the average responses (av) and associated standards of deviation (stdev) calculated.

^e lack of demonstrated dynamic response made interpolation of LoD values not possible. ^f Pancake batter made with soy flour, thus the MFI of soy-45 and soy-46 not representative of the incurring concentrations. Interestingly, 3xD and 3xSD yielded borderline LoD values due to the high level of precision between replicates despite a greatly inflated background.

Table 4. Limit of Detection of Food Allergens Incurred into Matrices using the Reduced-Denatured Extraction Protocol of the xMAP FADA

		Egg - 65 detection of 5.8 ng Egg Protein/mL ^b					Gluten - 73 detection of 3.7 ng Gluten Protein/mL					Peanut-72 detection of 1.5 ng Peanut Protein/mL				
		PBST Buffer ^c	Orange Juice	Dark Choco	Pancake Batter	Baked Muffin	PBST Buffer	Orange Juice	Dark Choco	Pancake Batter	Baked Muffin	PBST Buffer	Orange Juice	Dark Choco	Pancake Batter	Baked Muffin
LoD ^d	S/N=3	0.6	0.5	1.6	1.2	1.2	0.09	0.38	1.3	0.59	0.15	1.54	0.31	3.6	0.36	3.1
	S/N=3*	2.9	3.0	2.2	1.5	4.4	0.21	2.7	1.2	0.67	0.17	3.4	4.1	5.1	0.91	4.0
	3D	0.04	0.03	0.09	0.03	0.03	0.005	0.011	0.11	0.010	0.006	0.05	0.01	0.19	0.013	0.17
	3D*	1.3	0.3	0.6	0.3	0.2	0.07	0.69	0.45	0.11	0.14	0.000	0.34	0.35	0.04	0.28
	3SD	0.6	0.2	0.3	0.4	0.4	0.04	0.25	0.67	0.09	0.22	1.1	0.31	0.37	0.17	0.98
	3SD*	1.5	0.5	0.7	0.3	0.3	0.08	0.77	0.55	0.12	0.14	0.12	0.47	0.62	0.08	0.50
Backgd	av ^e	16	19	30	24	24	27	20	24	124	23	16	19	29	27	22
	stdev	1	1	2	1	1	2	1	2	2	1	1	1	2	1	1
LLoQ - bkgd	av ^e	507	640	327	345	345	3435	577	201	2324	1724	48	275	37	340	31
	stdev	16	8	4	7	7	11	13	10	17	33	11	18	2	12	6
Mix LLoQ-bkgd	av ^e	98	109	237	278	94	1393	80	219	2058	1543	22	20	26	133	24
	stdev	7	2	8	4	1	8	5	9	21	19	0	2	2	1	2

		Milk - 66 detection of 1.5 ng Milk Protein/mL					Milk - 67 detection of 1.5 ng Milk Protein/mL				
		PBST Buffer	Orange Juice	Dark Choco	Pancake Batter	Baked Muffin	PBST Buffer	Orange Juice	Dark Choco	Pancake Batter	Baked Muffin
LoD	S/N=3	0.57	0.58	8.4	4.5	5.8	0.33	0.55	6.7	1.6	1.7
	S/N=3*	3.0	3.5	12	6.6	18	2.2	4.0	12	2.4	6.5
	3D	0.015	0.02	0.15	0.08	0.10	0.01	0.20	0.27	0.05	0.06
	3D*	0.25	0.28	0.53	0.25	0.35	0.17	2.0	6.5	0.12	0.22
	3SD	0.30	0.30	0.27	0.55	0.42	0.73	0.53	0.73	0.30	0.28
	3SD*	0.32	0.43	0.75	0.37	0.66	0.24	3.4	6.9	0.20	0.44
Bkgd	av			304	438	955			151	203	92
	stdev			8	18	17			6	7	3
LLoQ - bkgd	av			2382	3369	513			102	559	239
	stdev			149	204	14			10	30	12
Mix LLoQ-bkgd	av			451	556	348			57	385	64
	stdev			25	35	41			81	11	3

^a LoD interpolated assuming linearity between analyte-free matrix (background) and the first non-zero incurred sample (minus background).

^b The incurred samples were designed to yield in the analytical portions concentrations of allergen (egg-5.8, gluten-3.7, peanut-1.5, and milk-1.5 ng protein/mL) comparable to the first calibration standard, assuming 100% recovery (<100% would decrease the interpolated LoD, accordingly).

^c Matrix- PBST Buffer (0.05% Tween-20), Orange Juice, Dark Chocolate, Pancake Batter, and Baked Muffins.

^d LoD is defined by the xMAP FADA insert as the first non-zero calibration standard (S1) in ng protein/mL (green font); LoD values (blue font) were interpolated based on a signal/noise ratio of 3 (S/N=3), three-times the standard deviation of the background (3xD), and three-times the sum of the standard deviations of the background and the sample. LoD analyses were done using pure analyte and a mixture of all 15 analytes indicated by an '*'. Red font indicates elevated LoD values, which may arise from many causes, including high background responses.

^e Bkgd indicates analyte-free matrix, LLoQ represents samples incurred with analyte at a concentration that upon extraction and 100% recovery would be comparable to the concentration in the analytical sample equivalent to the lowest calibration standard . The use of an '*' indicates that the analyte was present as a mixture of all 15 analytes, each at comparable concentrations (e.g., all a their LLoQ). The samples were prepared in triplicate, subjected to two independent xMAP assays, and read using two Luminex 200 and one MagPix instrument. The results in this table were generated by one experimental analysis of the three replicates, all using the same Luminex 200.

ng protein/ mL ^b	PBST Buffer		Orange Juice		Dark Chocolate		Pancake Batter		Baked Muffins		ng protein/ mL	PBST Buffer		Orange Juice		Dark Chocolate		Pancake Batter		Baked Muffins		ng protein/ mL	PBST Buffer		Orange Juice		Dark Chocolate		Pancake Batter		Baked Muffins																																
	indiv ^c	mix	indiv	mix	indiv	mix	indiv	mix	indiv	mix		indiv	mix	indiv	mix	indiv	mix	indiv	mix	indiv	mix		indiv	mix	indiv	mix	indiv	mix	indiv	mix	indiv	mix																															
ALMOND^c - 12 / ALMOND - 13																																BRAZIL NUT - 14 / BRAZIL NUT - 15																CASHEW - 18 / CASHEW - 19															
2.9	2.4	3.1	2.8	3.3	2.0	2.1	2.9	2.8	2.9	3.0	2.9	2.3	2.5	1.0	2.0	2.5	3.0	2.5	3.3	2.1	3.5	0.47	2.7	3.4	6.9	7.2	1.9	3.6	2.5	2.5	2.2	2.9																															
5.2	2.1	3.0	2.5	3.2	2.1	2.1	2.7	2.6	2.6	3.0	5.2	2.4	2.6	1.0	2.0	2.4	3.2	4.0	3.8	2.5	3.2	0.85	2.5	3.3	6.7	6.7	2.1	3.6	2.3	2.7	2.4	3.1																															
17	1.9	2.5	2.3	2.9	2.1	2.1	2.3	2.1	2.3	2.5	17	2.4	2.8	1.0	2.2	2.6	3.2	2.6	2.6	2.5	3.5	2.7	2.3	2.8	6.4	5.2	2.5	3.4	2.7	2.2	2.5	3.0																															
55	1.7	1.9	2.2	2.2	2.1	2.0	1.8	1.7	1.9	2.3	55	2.3	2.7	1.0	2.6	2.8	3.2	2.5	2.4	2.8	3.6	8.9	1.8	2.0	5.4	3.1	2.6	3.0	1.9	1.8	3.0	3.0																															
99	1.6	1.7	2.0	1.9	2.2	1.8	1.6	1.6	1.6	2.0	99	2.2	2.4	1.1	2.6	2.9	3.2	2.9	2.3	2.8	3.6	16	1.5	1.7	4.0	2.3	2.3	2.4	1.8	1.6	2.9	2.6																															
overall average ± standard deviation										2.3	±	0.5	overall average ± standard deviation										2.6	±	0.7	overall average ± standard deviation										3.1	±	1.5																									
COCONUT - 20 / COCONUT - 21																																EGG - 25 / EGG - 26																GLUTEN - 27 / GLUTEN - 28															
0.65	45	23	40	24	14	7.3	19	12	19	18	5.8	0.56	0.59	0.57	0.65	0.45	0.46	0.56	0.55	0.48	0.48	3.7	0.32	0.41	0.35	0.40	0.20	0.29	0.50	0.55	0.42	0.47																															
1.2	46	25	42	27	31	7.5	28	16	32	19	10	0.55	0.57	0.56	0.64	0.46	0.44	0.54	0.55	0.45	0.47	6.6	0.29	0.41	0.33	0.42	0.22	0.28	0.42	0.47	0.43	0.47																															
3.8	43	29	41	31	27	7.3	36	23	34	31	34	0.58	0.55	0.57	0.57	0.46	0.43	0.53	0.52	0.43	0.45	21	0.25	0.37	0.31	0.39	e	0.30	0.33	0.38	0.43	0.50																															
12	40	27	38	30	36	9.6	36	25	44	34	110	0.60	0.59	0.58	0.59	0.49	0.49	0.57	0.57	0.43	0.44	69	0.24	0.32	0.28	0.33	0.36	0.34	0.27	0.30	0.54	0.58																															
22	34	24	33	26	41	11	32	23	39	32	198	0.56	0.63	0.62	0.62	0.54	0.53	0.60	0.59	0.43	0.43	125	0.22	0.29	0.27	0.30	0.38	0.34	0.24	0.26	0.38	0.54																															
overall average ± standard deviation										28.3	±	10.6	overall average ± standard deviation										0.5	±	0.1	overall average ± standard deviation										0.4	±	0.1																									
HAZELNUT - 29 / HAZELNUT - 30																																MACADAMIA - 33 / MACADAMIA - 34																MILK - 35 / MILK - 36															
1.2	6.5	3.3	9.1	5.6	3.4	2.5	5.1	4.0	4.9	3.6	4.9	14.2	27	23	32	13	7.2	10.1	10	12	17	1.4	1.7	1.5	1.5	1.2	1.4	1.6	1.5	1.3	1.5	0.83																															
2.1	6.3	3.5	9.2	5.6	3.9	2.6	4.6	3.5	4.4	3.7	8.7	10.4	23	16	30	17	8.0	10.9</																																													

^d Overall average and standard deviation across all five matrices and five concentrations of analyte. High variances are expected since differences across analyte concentration are expected..

Table 6. Percent Recovery of Incurred and Spiked Individual Analytes Analyzed According to the PBST Buffered - Detergent xMAP FADA Protocol ^a

[] ^b		Almond ^c		Brazil Nut		Cashew		Coconut		Crust		Egg		Gluten		Hazelnut		Macadamia		Milk		Peanut		Pine Nut		Pistachio		Soy		Walnut	
designation		12	13	14	15	18	19	20	21	22	25	26	27	28	29	30	33	34	35	36	37	38	39	42	43	44	45	46	47	48	
PBST incur / PBST	s1	258	286	322	319	114	128	338	188	310	299	330	194	227	281	145	208	300	252	231	444	444	203	204	291	257	335	263	306	124	
	s2	258	278	296	293	130	146	303	178	255	292	302	175	224	259	143	154	274	278	284	380	363	179	179	228	219	318	251	263	109	
	s4	258	149	159	160	80	79	146	102	155	145	145	109	145	144	83	131	158	171	165	162	158	111	90	125	120	172	144	150	67	
	s6	258	107	107	103	72	72	106	67	114	104	107	83	101	92	56	107	90	106	101	107	106	88	51	103	70	106	97	94	47	
	s7	258	105	103	104	79	79	94	60	116	114	109	84	106	98	66	98	89	105	99	107	107	85	48	106	80	97	93	99	56	
OJ extract / PBST	s1	79	74	66	57	67	63	79	83	68	61	61	68	68	72	73	81	79	45	42	103	108	114	101	97	96	77	96	101	96	
	s2	80	75	64	56	70	64	78	79	73	72	65	70	70	80	84	93	91	101	120	104	105	117	100	101	96	80	99	105	97	
	s3	80	76	68	57	71	66	77	87	72	72	69	81	78	83	81	102	89	114	133	101	113	109	99	99	78	99	100	92		
	s4	82	79	69	61	74	67	82	85	79	78	78	77	74	84	84	103	90	107	113	101	98	108	97	99	94	79	97	101	87	
	s5	85	82	74	64	77	70	85	88	79	85	85	80	79	91	91	104	96	113	120	96	96	106	93	102	94	77	93	94	90	
Choc extract / PBST	s1	88	85	79	66	79	73	88	90	84	87	88	80	77	92	90	103	87	115	115	100	98	101	88	102	92	78	94	97	88	
	s2	89	87	81	73	83	74	92	89	81	96	98	86	89	98	96	107	94	123	120	103	100	102	91	102	95	85	98	102	91	
	s3	49	56	71	57	69	65	57	67	80	89	91	82	81	83	86	97	61	61	100	101	103	108	94	90	100	118	92	109		
	s4	52	58	68	54	69	66	55	52	69	83	94	100	89	82	88	90	97	93	103	101	98	109	105	95	85	99	115	96	107	
	s5	54	58	69	56	68	65	56	55	70	84	89	99	89	85	81	96	90	99	107	100	97	90	105	93	91	99	118	92	104	
Panc extract / PBST	s1	62	64	70	60	76	66	62	57	76	93	97	100	88	85	82	100	91	103	98	104	102	100	110	93	97	106	123	98	109	
	s2	68	68	75	62	70	69	67	69	59	83	96	100	97	91	90	87	102	100	102	96	94	89	106	95	90	97	109	87	109	
	s3	75	72	78	66	80	73	76	65	77	94	97	92	75	88	81	95	81	98	95	97	95	92	101	95	84	95	105	88	98	
	s4	81	81	83	74	87	77	83	71	76	102	101	93	77	93	87	93	86	108	105	96	92	83	99	94	79	92	101	90	92	
	s7	70	67	62	51	50	50	86	47	73	67	73	77	71	77	103	91	149	54	59	96	87	93	122	83	95	#	#	75	87	
Muff extract / PBST	s1	68	68	58	50	53	47	81	72	78	68	69	68	74	75	105	84	140	94	115	89	82	100	102	83	110	44	3	85	90	
	s2	68	67	59	50	53	45	77	65	75	66	69	78	75	75	94	87	134	97	108	86	77	96	91	80	110	30	0	80	84	
	s3	74	69	62	52	57	46	79	59	82	71	75	74	75	73	95	90	125	92	97	87	83	93	89	83	110	#	#	88	84	
	s4	76	72	66	54	59	48	80	60	79	74	78	71	74	76	98	91	129	90	97	82	78	92	85	85	106	#	#	81	84	
	s5	79	75	71	59	63	55	83	65	81	71	75	69	73	77	91	89	110	86	88	86	82	91	85	89	101	-3	0	80	84	
OJ incur / PBST incur	s1	79	80	74	66	66	57	86	66	82	79	81	74	80	82	98	93	115	90	87	88	85	91	86	91	99	17	-2	86	86	
	s2	58	51	3	7	46	18	73	82	81	65	63	77	66	59	42	86	57	#	#	#	7	7	105	91	95	57	64	87	115	54
	s4	62	51	3	7	41	15	71	78	97	64	63	84	69	68	48	95	67	#	#	#	98	99	109	92	102	58	62	80	106	51
	s6	67	55	3	7	48	17	81	83	106	75	75	75	56	67	45	92	70	#	#	#	99	98	106	95	103	56	69	84	105	52
	s7	71	56	4	8	46	15	88	91	86	82	85	77	59	79	51	95	75	#	#	#	99	99	110	105	106	57	71	86	112	57
OJ incur / OJ extract	s1	82	67	5	9	53	20	91	92	96	305	272	83	63	79	49	90	63	#	#	#	102	102	115	110	102	53	74	91	0	0
	s2	190	197	16	40	79	38	313	187	369	318	340	221	221	230	83	219	213	#	#	#	29	29	287	184	285	152	278	238	349	69
	s4	172	189	14	37	75	35	275	175	342	260	291	213	222	220	81	158	203	#	#	#	358	344	166	165	230	134	246	205	264	58
	s5	107	105	6	18	51	20	143	100	206	139	139	106	110	116	45	117	123	#	#	#	158	159	109	88	130	72	150	125	156	40
	s7	86	70	5	12	42	15	106	68	117	98	103	80	78	79	31	99	77	#	#	#	105	107	97	61	106	44	96	90	108	31
Choc incur / PBST incur	s1	98	81	6	13	50	22	94	63	118	105	107	81	75	78	34	83	60	#	#	#	107	109	96	58	105	45	85	86	0	0
	s3	3	4	6	5	3	4	1	4	#	#	#	9	13	2	3	13	15	#	#	#	7	7	16	14	9	8	6	17	3	8
	s4	4	4	3	3	3	4	2	3	#	#	#	9	11	2	4	12	8	#	#	#	7	7	22	13	14	9	5	15	1	3
	s5	8	7	7	7	5	4	2	4	#	#	#	3	1	3	4	15	7	#	#	#	5	5	5	20	16	14	6	4	12	4
	s6	14	11	12	10	8	6	7	8	#	#	#	35	22	5	5	43	17	#	#	#	10	10	40	24	37	11	12	30	10	6
Choc incur / Choc extract	s1	18	13	16	12	14	9	13	10	#	#	#	36	20	9	8	39	11	#	#	#	43	40	39	16	61	18	15	34	9	5
	s2	17	20	26	29	5	8	7	13	#	#	#	19	37	5	5	32	46	#	#	#	29	29	33	27	28	22	21	37	11	10
	s3	16	17	14	17	6	8	13	12	#	#	#	15	27	7	6	20	22	#	#	#	25	25	36	22	33	25	16	33	3	3
	s4	17	17	17	18	5	5	6	7	#	#	#	3	2	5	4	20	12	#	#	#	7	7	22	13	19	8	7	15	6	2
	s7	20	17	17	16	8	6	10	8	#	#	#	32	29	5	4	49	19	#	#	#	11	11	39	12	40	9	14	27	11	3
Panc incur / PBST	s1	23	17	19	16	13	9	14	9	#	#	#	32	27	9	6	41	11	#	#	#	48	47	40	8	68	18	15	31	10	3
	s3	36	31	36	33	40	45	37	86	35	26	25	68	41	32	41	67	100	23	27	46	45	62	102	34	59	#	#	51	105	
	s2	39	30	22	13	27	29	35	59	31	21	21	53	33	38	54	58	59	16	17	41	40	69	84	37	29	#	#	43	71	
	s4	52	42	42	40	44	37	58	68	55	47	50	61	43	51	73	76	103	35	38	69	65	83	101	68	85	#	#	54	66	
	s6	76	72	70	66	62	57	82	91	64	63	66	78	62	74	105	87	133	644												

[] ^b						
designation						
	Egg	Milk	Peanut	Gluten		
	65	66	67	72	73	
PBST _{incur} /	s1	216	410	320	249	201
	s2	242	356	282	228	167
	s4	160	170	137	102	110
	s6	84	100	86	80	93
	s7	104	91	72	100	96
PBST						
OJ _{extract} /	s1	123	137	178	130	112
	s2	135	128	176	117	117
	s3	155	119	168	118	120
	s4	152	106	154	127	116
	s5	149	104	143	118	
PBST	s6	149	106	157	156	118
	s7	138	107	152	168	113
OJ _{incur} /	s1	126	141	70	102	17
	s2	127	119	114	84	21
	s4	105	111	99	83	25
	s6	137	110	86	84	35
	s7	116	130	97	89	45
PBST _{incur}						
OJ _{incur} /	s1	222	424	126	196	30
	s2	227	331	182	164	30
	s4	111	178	88	67	24
	s6	77	105	47	43	27
	s7	88	111	46	53	38
OJ _{extract}						

Table 7. Percent Recovery of Incurred and Spiked Individual Analytes Analyzed According to the Reduced - Denatured xMAP FADA Protocol ^a																		
[] ^b						[] ^b						[] ^b						
designation						designation						designation						
Egg						Egg						Egg						
Milk						Milk						Milk						
Peanut						Peanut						Peanut						
Gluten						Gluten						Gluten						
65						65						65						
66						66						66						
67						67						67						
72						72						72						
73						73						73						
Choc _{extract} /	s1	90	86	86	71	88	s1	133	151	364	194	129	s1	63	108	146	39	84
	s2	109	93	97	84	105	s2	138	124	289	166	130	s2	77	100	51	41	96
	s3	137	80	122	113	105	s3	153	105	246	173	127	s3	84	86	48	41	95
	s4	149	70	137	128	98	s4	157	91	202	165	124	s4	88	78	48	48	94
	s5	148	66	140	160	96	s5	149	86	173	189	124	s5	89	77	54	61	92
PBST	s6	147	75	146	163	97	s6	149	92	165	180	123	s6	100	84	78	82	93
	s7	145	87	135	165	99	s7	150	97	147	172	120	s7	107	88	94	102	92
Panc _{extract} /	s1	133	151	364	194	129	s1	68	48	88	126	68	s1	48	19	38	12	50
	s2	138	124	289	166	130	s2	43	35	60	84	67	s2	29	11	29	3	50
	s3	153	105	246	173	127	s3	81	54	86	146	85	s3	28	8	20	8	68
	s4	157	91	202	165	124	s4	136	80	122	176	104	s4	39	11	19	32	70
	s5	149	86	173	189	124	s5	126	101	163	148	110	s5	32	8	41	54	76
PBST	s6	149	92	165	180	123	s6						s6					
	s7	150	97	147	172	120	s7						s7					
Muff _{extract} /	s1	63	108	146	39	84	s1	110	130	78	162	106	s1	163	73	82	75	120
	s2	77	100	51	41	96	s2	76	102	58	116	86	s2	91	39	158	19	88
	s3	84	86	48	41	95	s3	82	100	58	90	76	s3	51	18	57	17	80
	s4	88	78	48	48	94	s4	76	88	64	78	79	s4	33	14	21	31	70
	s5	89	77	54	61	92	s5	88	95	79	86	88	s5	31	9	31	52	80
PBST	s6	100	84	78	82	93	s6						s6					
	s7	107	88	94	102	92	s7						s7					
Muff _{incur} /	s1	48	19	38	12	50	s1	110	130	78	162	106	s1	163	73	82	75	120
	s2	29	11	29	3	50	s2	76	102	58	116	86	s2	91	39	158	19	88
	s3	28	8	20	8	68	s3	82	100	58	90	76	s3	51	18	57	17	80
	s4	39	11	19	32	70	s4	76	88	64	78	79	s4	33	14	21	31	70
	s5	32	8	41	54	76	s5	88	95	79	86	88	s5	31	9	31	52	80
PBST _{incur}	s6						s6						s6					
	s7						s7						s7					
Muf _{extract}	s1	163	73	82	75	120	s1	110	130	78	162	106	s1	163	73	82	75	120
	s2	91	39	158	19	88	s2	76	102	58	116	86	s2	91	39	158	19	88
	s3	51	18	57	17	80	s3	82	100	58	90	76	s3	51	18	57	17	80
	s4	33	14	21	31	70	s4	76	88	64	78	79	s4	33	14	21	31	70
	s5	31	9	31	52	80	s5	88	95	79	86	88	s5	31	9	31	52	80
Muff _{incur} /	s6						s6						s6					
	s7						s7						s7					

^a % Recovery based on MFI (after subtracting background) using a Lumines 200. The incurred and spiked samples were prepared using soluble protein extracts of each individual analyte prepared as described for the 200X calibration standards. Incurred samples were extracted following the Reduced-- Denatured Protocol. Spiked samples were prepared by spiking the analyte into either PBST or extracts prepared from analyte-free samples to the specified concentrations without further extraction or dilution. Red highlight indicates recovery < 50%, Yellow < 80%, no highlight if between 80% and 120%, Light Blue if <150%, Dark Blue if > 150%.

^b Samples prepared so the concentrations of protein in the analytical portion (mixed 1:1 with bead cocktail) the same as designated for S1, S2, S3, S4, S5, S6, S7 in product insert. Incurred samples were added to PBST and food matrices prior to processing (e.g., prior to baking of the muffin). Spiked samples were added to either PBST or extracts prepared from analyte-free samples to the final (designated) concentrations.

BEAD COUNTS (Fail (<10, red highlight), Borderline (between 10 and 25) OK (≥25 ok (green))																												
cell	designation	average count of a bead set in the well	Almond		Brazil Nut		Cashew		Coconut		Crust	Gluten		Hazelnut		Macadamia		Peanut		Pine Nut		Pistachio		Soy		Walnut		
			12	13	14	15	18	19	20	21	22	27	28	29	30	33	34	37	38	39	42	43	44	45	46	47	48	
A1	S	289	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	
A3	S	244	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	
A5	S	293	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	
B1	S1	268	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	
B3	S1	280	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	
B5	S1	279	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	
C1	S2	256	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	
C3	S2	239	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	
C5	S2	268	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	
D1	S5	236	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	
D3	S5	238	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	
D5	S5	230	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	
E11	S7	248	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	
E3	S7	250	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	
E5	S7	243	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	
F1	G20	237	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	
F3	G20	238	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	
F5	G20	227	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	
G1	M50	281	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	
G3	M50	273	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	
G5	M50	275	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	
H1	P10	198	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	
H3	P10	217	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	
H5	P10	220	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	
A7	A-10	208	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	
A9	A-10	232	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	
A11	A-10	89	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	
B7	A-100	171	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	
B9	A-100	159	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	
B11	A-100	110	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	
C7	B-10	156	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	
C9	B-10	179	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	
C11	B-10	154	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	
D7	B-100	131	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	
D9	B-100	151	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	
D11	B-100	109	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	
E7	0	284	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	
E9	0	224	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	
E11	0	126	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	
F7	0	197	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	
F9	0	270	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	
F11	0	222	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	
G7	A-100/10	232	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	
G9	A-100/10	245	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	
G11	A-100/10	232	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	
H7	B-100/10	194	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	
H9	B-100/10	190	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	
H11	B-100/10	194	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	
av ct / set			218±97	166	206	182	167	141	277	130	211	295	246	229	159	163	142	246	335	224	143	193	163	303	282	355	288	249

FIGURE 1

BEAD COUNTS (Fail (<10, red highlight), Borderline (between 10 and 25) OK (≥25 ok (green))																																															
average count																																															
cell	designation	of a bead set in the well	Almond		Brazil Nut		Cashew		Coconut		Crust		Gluten		Hazelnut		Macadamia		Peanut		Pine Nut		Pistachio		Soy		Walnut																				
			12	13	14	15	18	19	20	21	22	27	28	29	30	33	34	37	38	39	42	43	44	45	46	47	48																				
A1	S	59	ok	ok	borderline	ok	ok	borderline	ok	ok	ok	ok	borderline	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok																			
A3	S	39	ok	ok	borderline	ok	borderline	borderline	ok	ok	ok	ok	FAIL	ok	ok	ok	ok	borderline	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok																			
A5	S	24	ok	ok	borderline	ok	borderline	borderline	borderline	borderline	ok	ok	FAIL	borderline	borderline	ok	borderline	borderline	borderline	ok	borderline	borderline	FAIL	ok	borderline	borderline	borderline	borderline																			
B1	S1	34	ok	ok	borderline	ok	ok	borderline	ok	ok	ok	ok	borderline	borderline	borderline	ok	ok	ok	borderline	ok	borderline	ok	borderline	borderline	ok	ok	ok	borderline																			
B3	S1	19	ok	borderline	FAIL	ok	borderline	FAIL	ok	borderline	ok	borderline	FAIL	FAIL	borderline	ok	borderline	borderline	ok	ok	FAIL	borderline	borderline	ok	borderline	borderline	borderline	borderline																			
B5	S1	14	ok	borderline	FAIL	ok	FAIL	FAIL	borderline	FAIL	borderline	borderline	FAIL	FAIL	FAIL	ok	borderline	borderline	borderline	borderline	borderline	FAIL	borderline	FAIL	borderline	borderline	borderline	borderline																			
C1	S2	40	ok	ok	ok	ok	ok	borderline	ok	ok	ok	ok	FAIL	ok	borderline	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok																			
C3	S2	13	borderline	borderline	FAIL	ok	FAIL	FAIL	borderline	borderline	borderline	borderline	FAIL	borderline	borderline	borderline	borderline	borderline	borderline	borderline	borderline	borderline	FAIL	borderline	borderline	borderline	FAIL	FAIL																			
C5	S2	13	borderline	ok	FAIL	borderline	borderline	FAIL	borderline	borderline	borderline	borderline	FAIL	borderline	FAIL	FAIL	borderline	borderline	borderline	borderline	ok	FAIL	borderline	FAIL	borderline	FAIL	FAIL	borderline																			
D1	S5	37	ok	ok	borderline	ok	borderline	borderline	ok	ok	ok	ok	FAIL	ok	borderline	ok	ok	ok	ok	ok	ok	ok	borderline	ok	borderline	ok	ok	ok																			
D3	S5	17	ok	ok	FAIL	ok	borderline	borderline	borderline	borderline	ok	borderline	FAIL	borderline	FAIL	borderline	borderline	borderline	borderline	borderline	ok	FAIL	borderline	FAIL	borderline	borderline	borderline	FAIL																			
D5	S5	9	borderline	borderline	FAIL	ok	borderline	FAIL	FAIL	FAIL	borderline	borderline	FAIL	borderline	FAIL	borderline	FAIL	FAIL	FAIL	borderline	FAIL	FAIL	FAIL	FAIL	FAIL	FAIL	borderline	FAIL																			
E11	S7	31	ok	ok	borderline	ok	borderline	borderline	ok	ok	ok	ok	borderline	ok	borderline	ok	ok	borderline	borderline	ok	borderline	borderline	borderline	borderline	borderline	borderline	ok	ok																			
E3	S7	8	borderline	borderline	FAIL	borderline	borderline	FAIL	FAIL	FAIL	borderline	FAIL	FAIL	borderline	FAIL	FAIL	FAIL	FAIL	FAIL	borderline	FAIL	FAIL	FAIL	FAIL	FAIL	FAIL	FAIL	FAIL																			
E5	S7	11	borderline	borderline	FAIL	ok	FAIL	FAIL	borderline	borderline	borderline	borderline	FAIL	borderline	borderline	borderline	borderline	FAIL	borderline	borderline	borderline	borderline	FAIL	borderline	FAIL	FAIL	FAIL	FAIL																			
F1	G20	22	ok	ok	borderline	ok	borderline	FAIL	ok	borderline	ok	ok	FAIL	borderline	borderline	ok	borderline	borderline	borderline	borderline	ok	borderline	borderline	borderline	borderline	borderline	borderline	borderline																			
F3	G20	26	ok	ok	FAIL	ok	ok	FAIL	borderline	ok	ok	ok	FAIL	borderline	borderline	ok	ok	borderline	borderline	borderline	ok	borderline	ok	borderline	ok	borderline	borderline	borderline																			
F5	G20	23	ok	ok	FAIL	ok	borderline	borderline	ok	ok	ok	ok	FAIL	borderline	FAIL	ok	borderline	borderline	borderline	borderline	ok	borderline	borderline	borderline	borderline	borderline	borderline	borderline																			
G1	M50	32	ok	ok	borderline	ok	ok	borderline	ok	borderline	ok	ok	FAIL	borderline	ok	ok	ok	ok	ok	ok	ok	ok	ok	borderline	ok	borderline	ok	borderline																			
G3	M50	25	ok	ok	borderline	ok	borderline	FAIL	borderline	borderline	ok	ok	FAIL	borderline	borderline	ok	ok	borderline	borderline	borderline	ok	borderline	ok	borderline	borderline	borderline	borderline	borderline																			
G5	M50	24	ok	ok	borderline	ok	ok	borderline	borderline	borderline	ok	ok	FAIL	FAIL	borderline	ok	borderline	borderline	borderline	borderline	ok	borderline	borderline	borderline	borderline	borderline	borderline	ok	ok																		
H1	P10	138	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok																			
H3	P10	161	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok																			
H5	P10	98	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	borderline	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok																			
A7	A-10	10	borderline	borderline	FAIL	ok	borderline	borderline	FAIL	borderline	borderline	FAIL	FAIL	FAIL	FAIL	borderline	borderline	FAIL	borderline	borderline	FAIL	borderline	borderline	FAIL	borderline	borderline	FAIL	FAIL																			
A9	A-10	40	ok	ok	borderline	ok	ok	borderline	ok	ok	ok	ok	FAIL	ok	ok	ok	ok	ok	ok	ok	ok	ok	borderline	ok	ok	ok	ok	ok																			
A11	A-10	24	ok	ok	FAIL	ok	ok	FAIL	ok	borderline	ok	ok	FAIL	borderline	FAIL	ok	borderline	borderline	borderline	ok	borderline	borderline	borderline	borderline	ok	ok	borderline	borderline																			
B7	A-100	7	FAIL	borderline	FAIL	ok	FAIL	FAIL	FAIL	FAIL	borderline	FAIL	FAIL	FAIL	FAIL	FAIL	FAIL	FAIL	FAIL	borderline	FAIL	FAIL	FAIL	FAIL	FAIL	FAIL	FAIL	FAIL																			
B9	A-100	19	ok	ok	FAIL	ok	borderline	FAIL	borderline	borderline	ok	ok	FAIL	borderline	FAIL	ok	borderline	borderline	borderline	borderline	borderline	borderline	borderline	borderline	borderline	borderline	borderline	borderline																			
B11	A-100	25	ok	ok	FAIL	ok	ok	borderline	ok	ok	ok	ok	FAIL	borderline	borderline	ok	ok	borderline	borderline	ok	borderline	borderline	borderline	ok	borderline	borderline	borderline	borderline																			
C7	B-10	19	ok	ok	borderline	ok	FAIL	FAIL	ok	borderline	ok	ok	FAIL	FAIL	borderline	ok	borderline	borderline	borderline	ok	borderline	borderline	FAIL	borderline	FAIL	borderline	borderline	borderline																			
C9	B-10	17	ok	ok	FAIL	ok	borderline	FAIL	borderline	borderline	borderline	borderline	FAIL	borderline	borderline	ok	borderline	borderline	borderline	borderline	borderline	borderline	FAIL	borderline	borderline	borderline	borderline	borderline																			
C11	B-10	18	ok	ok	FAIL	ok	borderline	borderline	ok	borderline	ok	borderline	FAIL	borderline	FAIL	ok	borderline	borderline	borderline	ok	borderline	borderline	borderline	borderline	borderline	borderline	borderline	borderline																			
D7	B-100	10	borderline	borderline	FAIL	borderline	borderline	FAIL	borderline	borderline	borderline	borderline	FAIL	FAIL	FAIL	borderline	FAIL	FAIL	FAIL	borderline	FAIL	FAIL	FAIL	FAIL	FAIL	FAIL	borderline	FAIL																			
D9	B-100	6	borderline	FAIL	FAIL	borderline	borderline	FAIL	FAIL	FAIL	FAIL	FAIL	FAIL	FAIL	FAIL	FAIL	FAIL	FAIL	FAIL	borderline	FAIL	FAIL	FAIL	FAIL	FAIL	FAIL	FAIL	FAIL																			
D11	B-100	6	FAIL	FAIL	FAIL	borderline	borderline	borderline	FAIL	FAIL	FAIL	borderline	FAIL	FAIL	FAIL	borderline	FAIL	FAIL	FAIL	borderline	FAIL	FAIL	FAIL	FAIL	FAIL	FAIL	FAIL	FAIL																			
E7	0	12	borderline	ok	FAIL	borderline	borderline	FAIL	borderline	borderline	borderline	borderline	FAIL	FAIL	FAIL	borderline	borderline	borderline	borderline	borderline	borderline	FAIL	borderline	FAIL	FAIL	FAIL	FAIL	borderline																			
E9	0	13	borderline	borderline	FAIL	ok	borderline	borderline	borderline	FAIL	borderline	borderline	FAIL	borderline	borderline	borderline	borderline	borderline	FAIL	borderline	borderline	FAIL	borderline	FAIL	borderline	borderline	FAIL	FAIL																			
E11	0	20	ok	ok	borderline	ok	borderline	FAIL	ok	borderline	borderline	ok	FAIL	borderline	FAIL	ok	borderline	borderline	borderline	borderline	ok	FAIL	borderline	borderline	borderline	borderline	borderline	borderline																			
F7	0	15	borderline	ok	FAIL	ok	borderline	borderline	borderline	borderline	borderline	ok	borderline	FAIL	borderline	FAIL	borderline	borderline	borderline	borderline	ok	borderline	borderline	borderline	borderline	FAIL	borderline	borderline																			
F9	0	11	borderline	borderline	FAIL	ok	borderline	FAIL	borderline	FAIL	borderline	borderline	FAIL	borderline	FAIL	borderline	FAIL	borderline	FAIL	borderline	borderline	FAIL	borderline	FAIL	borderline	FAIL	borderline	borderline																			
F11	0	13	borderline	borderline	FAIL	ok	borderline	FAIL	borderline	borderline	borderline	borderline	FAIL	borderline	FAIL	borderline	borderline	borderline	FAIL	FAIL	borderline	FAIL	FAIL	borderline	FAIL	borderline	borderline	borderline																			
G7	A-100/10	10	borderline	borderline	FAIL	borderline	borderline	FAIL	borderline	borderline	FAIL	FAIL	FAIL	FAIL	FAIL	borderline	borderline	FAIL	FAIL	borderline	borderline	borderline	borderline	borderline	borderline	borderline	borderline	FAIL																			
G9	A-100/10	8	borderline	FAIL	FAIL	borderline	FAIL	FAIL	borderline	FAIL	FAIL	borderline	FAIL	FAIL	FAIL	borderline	FAIL	FAIL	FAIL	borderline	FAIL	FAIL	FAIL	FAIL	FAIL	FAIL	FAIL	FAIL																			
G11	A-100/10	7	borderline	borderline	FAIL	borderline	FAIL	FAIL	FAIL	FAIL	FAIL	FAIL	FAIL	FAIL	FAIL	borderline	FAIL	FAIL	FAIL	borderline	FAIL	FAIL	FAIL	FAIL	FAIL	FAIL	FAIL	FAIL																			
H7	B-100/10	43	ok	ok	borderline	ok	ok	borderline	ok	ok	ok	ok	borderline	ok	borderline	ok	ok	ok	ok	ok	ok	ok	borderline	ok	ok	ok	ok	ok																			
H9	B-100/10	29	ok	ok	borderline	ok	ok	borderline	ok	ok	ok	ok	borderline	borderline	borderline	ok	ok	ok	borderline	ok	ok	borderline	ok	borderline	borderline	borderline	ok	borderline																			
H11	B-100/10	32	ok	ok	borderline	ok	borderline	borderline	ok	ok	ok	ok	ok	borderline	borderline	ok	ok	ok	ok	ok	ok	borderline	borderline	borderline	borderline	ok	ok	ok																			
av ct / set		28 ±36	48	48	12	64	23	12	29	25	41	33	7	20	16	42	28	24	24	44	17	24	16	24	23	26	20																				

FIGURE 2

A. STEPWISE LINEAR INTERPOLATION (≤ S1 defined as 'under', >S7 defined as over)

0

Location

Sample

Almond

12

13

Brazil Nut

14

15

Cashew

18

19

Coconut

20

21

Crust

22

Gluten

27

28

Hazelnut

29

30

Macadamia

33

34

Peanut

37

38

Pine Nut

39

42

Pistachio

43

44

Soy

45

46

Walnut

47

48

confirm
specificity
at target
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G-20

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Analysis of samples,
red blocks indicate
conclusions

A-10

0.34

0.24

under

under

under

under

0.06

0.05

under

OVER

OVER

under

under

under

under

0.37

under

under

under

under

under

under

under

under

under

under

under

under

A-100

OVER

1.16

under

under

under

under

OVER

0.30

under

OVER

OVER

under

0.05

under

under

1.75

1.35

under

under

under

under

under

under

0.53

0.26

under

0.64

B-10

1.41

1.58

under

under

under

under

0.11

0.13

under

OVER

OVER

under

0.08

under

under

2.01

2.01

under

under

under

under

0.88

1.19

under

0.96

B-100

OVER

OVER

0.12

under

0.02

0.04

OVER

OVER

under

OVER

OVER

under

0.05

0.26

under

under

OVER

OVER

under

under

under

0.08

5.69

OVER

3.69

A100/10

0.36

0.11

under

under

under

under

0.08

0.05

under

1.90

0.64

under

under

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under

0.48

under

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under

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under

B100/10

1.42

1.51

under

under

0.01

0.01

0.11

0.20

under

OVER

OVER

under

0.09

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under

0.93

1.19

under

1.02

Almond

Coconut

Gluten 27

Peanut

Soy

Green highlight > 0.03. Grey highlights indicate < LoQ (S1). Blue boxed data suspected of cross-contamination

B. CALIBRATION STANDARD PLOTS

almond

gluten

peanut

FIGURE 3B and 3C

ACS Paragon Plus Environment

A. STEPWISE LINEAR INTERPOLATION of MFI Responses minus Background ($\leq S1$ defined as 'under', $>S7$ defined as over)																											
Location		Almond		Brazil Nut		Cashew		Coconut		Crust	Gluten		Hazelnut	Macadamia		Peanut		Pine Nut		Pistachio		Soy		Walnut			
	Sample	12	13	14	15	18	19	20	21	22	27	28	29	30	33	34	37	38	39	42	43	44	45	46	47	48	
confirm specificity at target conc.	G-20	under	under	under	under	under	under	under	under	under	OVER	OVER	under	under	under	under	under	under	under	under	under	under	under	under	under	under	
	M-50	under	under	under	under	under	under	under	under	under	OVER	2.445	under	under	under	under	under	under	under	under	under	under	under	under	under	under	
	P-10	under	under	under	under	under	under	under	under	under	under	under	under	under	under	under	0.287	0.219	under	under	under	under	under	under	under	under	
Stepwise Linear Analysis of samples, red blocks indicate conclusions	A-10	0.375	0.122	under	under	under	under	0.066	0.040	under	1.417	0.723	under	under	under	under	0.352	under	under	under	under	under	under	under	under	under	
	A-100	OVER	1.772	under	under	under	under	0.413	0.433	under	OVER	OVER	under	0.062	under	under	4.353	2.606	under	under	under	under	under	0.768	0.412	under	
	B-10	1.427	1.265	under	under	under	under	0.111	0.149	under	OVER	OVER	under	0.057	under	under	2.435	2.133	under	under	under	under	under	0.921	1.140	under	
	B-100	OVER	OVER	0.105	0.132	0.016	0.032	OVER	OVER	under	OVER	OVER	0.040	0.219	under	under	10.417	OVER	under	under	under	under	under	OVER	OVER	#VALUE!	
	A100/10	0.482	0.206	under	under	under	#VALUE!	0.076	0.054	under	2.047	1.529	under	under	under	under	0.582	under	under	under	under	under	under	under	under	under	
	B100/10	1.555	1.199	under	under	under	under	0.111	0.183	under	OVER	OVER	under	0.064	under	under	2.613	2.056	under	under	under	under	under	0.801	1.097	under	
		Almond						Coconut				Gluten 27					Peanut						Soy				
Green highlight > 0.03. Grey highlights indicate < LoQ (S1). Blue boxed data suspected of cross-contamination																											

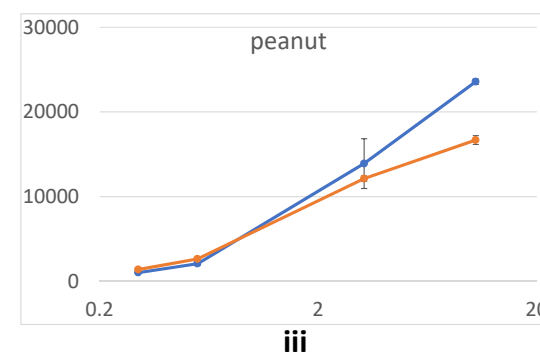
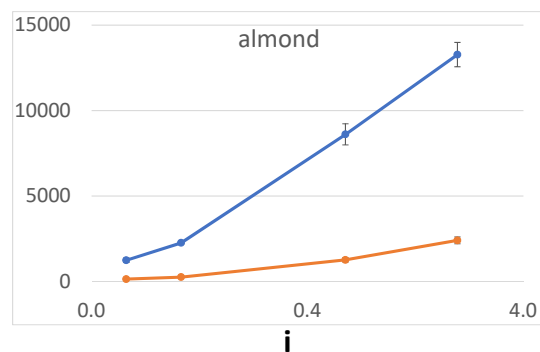
B. CALIBRATION STANDARD PLOTS

FIGURE 4A and 4B