NIST Special Publication 260-218

Value Assignment of Reference Material 8404
Almond Flour for Allergen Detection

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Almond Flour for Allergen Detection

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National Institute of Standards and Technology
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Abstract

The National Institute of Standards and Technology (NIST) recently released RM 8404 Almond Flour for Allergen Detection, which is intended for harmonizing measurements of allergenic proteins in foods. The material was purchased from a commercial vendor and data was obtained from an interlaboratory comparison exercise and collaborating laboratories. A description of the material, results, and data analysis are discussed in the following report.

Keywords:

Allergen; Almond; Protein; Proximates; Reference Material.

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1 Introduction

In 2017 and 2019, NIST held workshops to identify needs of the food industry and federal regulators. Among other things, NIST was asked to continue production of food-matrix SRMs for use by laboratories making measurements in support of food safety [1]. One described need was for additional commodity materials for method comparison and harmonization of food allergen testing, as laboratories need a means for demonstrating method validity and accuracy when analyzing food products. RM 8404 Almond Flour for Allergen Detection was also requested by various stakeholders through AOAC INTERNATIONAL to assist in the evaluation of allergen determination in food matrices. NIST currently offers commodity reference materials for many important allergenic foods (e.g., milk, egg, wheat, soy, peanuts, fish, shellfish), but RMs for tree nut allergens are currently unavailable from NIST or any other reference material producer. Availability of reference materials for tree nuts, such as almond, will facilitate the development and harmonization of methods for detecting trace levels of these allergenic foods in finished products.

2 Material

2.1 Acquisition & Packaging

Based on the intended use of this RM, selection of a material that contains proteins from a single nut source was critical. Numerous manufacturers were evaluated via websites and product claims and several contacted to determine whether the nut products that they produce are likely to be pure or may have come in contact with other tree nuts or allergenic foods. In August 2019, 2.2 kg (1 pound) of blanched almond flour was purchased from Mandelin (San Luis Obispo, CA). The candidate material was aliquoted into 100-g packets and sent in blind triplicate to Romer Labs (Union, MO) to be tested for the presence of protein allergens from cashew, wheat, hazelnut, macadamia, peanut, pecan, pine nut, pistachio, and walnut. Results of the allergen testing conducted by Romer Labs in August 2019 are described in Table 1, which identified possible presence of macadamia protein in the sample. After consulting with experts, the presence of macadamia protein in the almond flour was deemed questionable given the low prevalence of macadamia nut production in California and the potential for cross-reactivity of the ELISA assay utilized by Romer Labs.

Table 1. Results of August 2019 allergen screening by Romer Labs for candidate almond flour.
LOD = limit of detection; LOQ = limit of quantification

	Result mg/kg (ppm)			**Method**		
Parameter	**A**	**B**	**C**	**Name**	**LOD mg/kg (ppm)**	**LOQ mg/kg (ppm)**
Cashew	<LOD	<LOD	<LOD	US-AgraQuant ELISA Allergen Test Kit	0.2	2.0
Gluten	<LOD	<LOD	<LOD	US-AgraQuant ELISA Allergen Test Kit	2.0	4.0
Gluten	<LOD	<LOD	<LOD	US-RIDASCREEN Gliadin Test Kit	3.0	5.0
Hazelnut	<LOD	<LOD	<LOD	US-AgraQuant ELISA Allergen Test Kit	0.3	1.0
Macadamia	11.7	12.7	10.5	US-AgraQuant ELISA Allergen Test Kit	1.0	2.0
Peanut	<LOD	<LOD	<LOD	US-AgraQuant ELISA Allergen Test Kit	0.1	1.0
Pecan	<LOD	<LOD	<LOD	US-Pecan ELISA	1.0	2.0
Pine Nut	<LOD	<LOD	<LOD	US-Pine Nut ELISA	0.7	1.5
Pistachio	<LOD	<LOD	<LOD	US-AgraQuant ELISA Allergen Test Kit	0.1	1.0
Walnut	<LOD	<LOD	<LOD	US-AgraQuant ELISA Allergen Test Kit	0.3	2.0

In September 2019, a 30-g aliquot of the candidate almond material was sent to BioFront Technologies (Tallahassee, FL) to be evaluated using different ELISAs than those used at Romer Labs. The material was tested for the presence of protein allergens from almond, brazil nut, cashew, hazelnut, macadamia nut, peanut, pecan, pine nut, pistachio, and walnut. Results of the allergen testing conducted by BioFront Technologies in September 2019 are described in Table 2. The candidate material was found to contain less than 1 mg/kg (ppm) macadamia protein, indicating that the positive result found by Romer Labs may have been a result of assay cross-reactivity.

Table 2. Results of September 2019 allergen screening by BioFront Laboratories for candidate almond flour. *ROQ = range of quantification; LOD = limit of detection; LLOQ = lower limit of quantification; ULOQ = upper limit of quantification.*

		MonoTrace ELISA		
Parameter	**Result mg/kg (ppm)**	**ROQ mg/kg (ppm)**	**LOD mg/kg (ppm)**	**LLOQ mg/kg (ppm)**
Almond	>ULOQ	1-40	0.23	1
Brazil Nut	<LLOQ	1-40	0.14	1
Cashew	<LLOQ	1-40	0.12	1
Hazelnut	<LLOQ	1-40	0.04	1
Macadamia	<LLOQ	2-80	0.13	2
Peanut	<LLOQ	1-40	0.24	1
Pecan	<LLOQ	1-40	0.17	1
Pine Nut	<LLOQ	1-40	0.24	1
Pistachio	<LLOQ	1-40	0.12	1
Walnut	<LLOQ	1-40	0.22	1

Final packaging of the RM was completed by High-Purity Standards (North Charleston, SC). Prior to receiving materials, Neogen Reveal kits for Multi-Treenut were shipped by NIST to High-Purity Standards. High-Purity Standards was asked to use these kits to test for cross-contact with other allergen-containing materials during packaging. In December 2019, the room and equipment at High-Purity Standards were thoroughly cleaned and the Reveal kit used to test swabs of the tabletop and packaging components. No tree nuts were detected prior to receipt of the almond flour, as shown in the report from High-Purity Standards (Figure 1).

high-purity standards

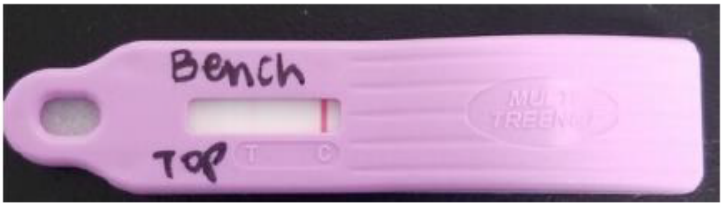
December 30, 2019

National Institute of Standards and Technology
100 Bureau Drive
Bldg 301, Room B130
Gaithersburg, MD 20899-1410

Reference: Allergen test report for contract 1333ND19PNB640946P20001 sales Order 17598

Dear Dr. Melissa Phillips,

High-Purity Standards received a test kit from NIST for the Neogen Reveal® for Multi-Treenut test. It was stored in a refrigerator maintained at 2°C – 8°C. Prior to the test, the room and equipment were thoroughly cleaned. The test was then performed according to manufacturer's instructions. We tested the tabletop and inner surfaces of the polybag, drum, air vent, balance, weigh-boat and gas nozzle. Tree nut was not detected on all equipment confirming that they are all clean and free of tree nut. Shown below is a representative image of the results for the tabletop.



HPS is now ready to receive the almond powder.

Participants:

Cleaning: Carley Freeland – Inorganic Lab Technician
Analysis: Tommy Breen – Inorganic Manufacturing Manager

Sincerely,

Tommy Breen, Inorganic Lab Manager

7221 Investment Drive • North Charleston, SC 29418
843.767.7900 • info@highpuritystandards.com • www.highpuritystandards.com

Figure 1. Report of facility cleaning quality from High-Purity Standards prior to receipt of RM 8404 Almond Flour for Allergen Detection.

In January 2020, 34 kg (75 pounds) of blanched almond flour was purchased from Mandelin and shipped directly to High-Purity Standards. Prior to packaging, the material was transferred to a 56.8 L (15 gallon) polyethylene mixing vessel and blended for 18 h by a rocking and rolling technique. After blending, the material was transferred to 0.10 mm (4 mil or 0.004 in) plastic polyethylene bags with a 4 kg capacity each. To verify the homogeneity of the blended flour, six samples were analyzed by High-Purity Standards using inductively coupled plasma optical emission spectrometry (ICP-OES) following microwave digestion. The results for Ca, K, Mg, Mn, S, and Zn (Table 3) indicate that sufficient blending was achieved, and that the material could be packaged as requested. Aliquots (5.0 g ± 0.1 g) of the almond flour were weighed using a static free container on a platform balance with the accuracy of ± 0.0001 g and immediately transferred to 0.10 mm (4 mil or 0.004 in) plastic polyethylene bags through a solid funnel. The polyethylene bags were flushed with dry nitrogen and immediately heat sealed and over-packed aluminized plastic packets with two 0.5 g packets of SORB-IT while being purged with dry nitrogen before double sealing. The aluminized packets were placed in rows in 35.56 cm x 35.56 cm x 35.56 cm (14 in x 14 in x 14 in) cardboard boxes. The packets were arranged into sections (1 through 4), placed from back to front of the box. The front and back of each box were marked, and the boxes were numbered sequentially and sealed. A total of 10 boxes were produced containing 500 packets each, giving a grand total of 5000 packets. Fourteen packets were removed after packaging for additional homogeneity testing (Table 3). The remaining material was repackaged and included with the packaged material on a pallet and sealed in a plastic film for shipment to NIST. 10 boxes of packaged RM 8404 Almond Flour for Allergen Detection, as well as remaining unpackaged material, were received at NIST on February 25, 2020.

Table 3. Pre- and post-packaging homogeneity testing report from High-Purity Standards for RM 8404 Almond Flour for Allergen Detection.

	% RSD			
Analyte	**Pre-Packaging[a]**	**Post-Packaging[b]**	**Criteria (%)**	**Result**
Ca	1.96	2.23	≤ 3.0	Pass
K	1.94	2.89	≤ 3.0	Pass
Mg	1.54	1.91	≤ 3.0	Pass
Mn	1.91	2.80	≤ 3.0	Pass
S	2.64	2.58	≤ 3.0	Pass
Zn	1.69	2.75	≤ 3.0	Pass

[a] RSD for 6 randomly selected samples.
[b] RSD for 14 randomly selected samples.

2.2 Storage

The packets of RM 8404 have been stored at −20 °C at NIST since their receipt.

3 Experimental Procedures

3.1 Interlaboratory Studies for Value Assignment

RM 8404 was distributed in Exercise 5 of the NIST Health Assessment Measurements Quality Assurance Program (HAMQAP). Laboratories participating in the HAMQAP Exercise were provided with 3 packets of RM 8404 and were asked to use their in-house analytical methods to determine the mass fraction (percent) of proximates (fat, protein, carbohydrates, solids, and ash) as well as calories (kcal/100 g) in each packet. The quantitative results from this study are reported here in full, and the full report from Exercise 5 is published elsewhere [2]. Results were reported by the participants listed in Table 4, using the methods described in Section 4.1. The reported results from each participating organization have been assigned an arbitrary numeric code.

Table 4. Participants in the proximates study of HAMQAP Exercise 5.

Company	Location	Country
Advanced Botanical Consulting & Testing, Inc.	Tustin, CA	USA
Analytical Resource Labs	Lehi, UT	USA
Anonymous*	--	USA
Exact Scientific Services, Inc.	Ferndale, WA	USA
Intertek Champaign Laboratories	Champaign, IL	USA
SORA Labs	Forsyth, MO	USA

*This laboratory did not give consent to be named as a HAMQAP participant.

3.2 Collaborating Laboratories for Value Assignment

Eurofins Food Chemistry Testing US (Madison, WI) was provided with 14 samples of RM 8404 for determination of total protein. To establish the repeatability of the laboratory's method, 5 samples were provided from the beginning of the production lot. Nine additional samples were provided from across the production lot to evaluate material homogeneity.

Total protein was determined in 0.2 g to 0.3 g samples of RM 8404 using Dumas combustion based on AOAC Official Method 968.06 Protein (Crude) in Animal Feed [3]. In summary, the samples were combusted at ≥ 850 °C and the nitrogen generated was carried by CO_2 for quantitation by thermal conductivity. The nitrogen content determined in the samples was converted to crude protein using a generic conversion factor of 6.25, common for legumes, corn, and many animal proteins [4]. Jones factors for nuts and seeds, however, are lower and should be used in the case of almond testing. The data provided by the collaborating laboratory has been adjusted using a more appropriate Jones factor for almond measurement (5.18).

$$crude\ protein\ (\%) = nitrogen\ (\%)\ x\ conversion\ factor$$

3.3 Statistical Approaches for Value Assignment

Statistical analysis was provided by the NIST Statistical Engineering Division (SED). Where more than one method is available for a measured analyte, the estimated value is a weighted mean of the method estimates available for this analyte. The weighted mean used is the Dersimonian-Laird estimate [5], the uncertainty of which is estimated using a bootstrap procedure based on a Gaussian random effects model for the between-method effects [6-9]. If only one method is available for an analyte, then that method estimate is the analyte estimate.

Significant differences are often observed between the results from the different laboratories participating in an interlaboratory study. For the interlaboratory study, the estimate for each analyte is the weighted median of the individual laboratory means for that analyte, where the weights are based on a Laplace random effects model [10]. For this RM, the weighted median is equal to the unweighted median of laboratory means for all analytes. The uncertainty of the weighted median is estimated using a bootstrap procedure based on a Laplace random effects model for the between-laboratory and within-laboratory effects [6-10].

Some measurements from the interlaboratory studies were flagged by the analysts and omitted from the calculations. The deviance of these measurements from the others exceeded the usual variation, often differing by an order of magnitude or more. Other measurements may be questionable but could not be determined to be unrepresentative extreme outliers because of the sparseness and variation of the rest of the data. Some measurements were revised for incorrect reporting units or incorrect Jones factors (for protein) and are noted in the sections below.

Some of the estimates and uncertainties in this report are purposely listed with more significant digits than is scientifically warranted. The relevant technical experts trim any estimates and uncertainties to the number of significant digits that are scientifically warranted prior to inclusion on the Reference Material Information Sheet as non-certified values [11].

3.4 Screening for Trace Allergen Contaminants

3.4.1 Eurofins GeneScan

Eurofins GeneScan (New Orleans, LA) was provided with 3 samples of RM 8404 for testing by R-Biopharm R6802 Hazelnut Allergen (ELISA). The RIDASCREEN FAST Hazelnut (Product R6802) is a sandwich enzyme immunoassay for the quantitative analysis of hazelnut (or hazelnut proteins) in food with a limit of quantification (LOQ) of 2.5 mg/kg (ppm) hazelnut [12]. The test principle is described below in an excerpt from reference [12].

> The basis of the test is the antigen-antibody reaction. The wells of the microtiter strips are coated with specific antibodies against hazelnut proteins. By adding the standard or sample solution to the wells, present hazelnut protein will bind to the specific capture antibodies. The result is an antibody-antigen-complex. Components not bound by the antibodies are then removed in a washing step. Then, antibody conjugated to peroxidase is added. This conjugate is bound to the Ab-Ag-complex. An antibody-antigen-antibody (sandwich) complex is formed. Any unbound conjugate is then removed in a washing step. Substrate/Chromogen are added to the wells and incubated. Bound conjugate converts the colorless chromogen into a blue product. The addition of the stop solution leads to a color change from blue to yellow. The measurement is made photometrically at 450 nm. The absorbance is proportional to the hazelnut content of the sample. The result is expressed in mg/kg hazelnut.

3.4.2 Food Allergen Research and Resource Program (FARRP) at the University of Nebraska Lincoln

The Food Allergen Research and Resource Program (FARRP) at the University of Nebraska Lincoln (Lincoln, NE) was provided with samples of RM 8404 for testing by numerous commercial approaches as described in the sections below. FARRP was provided with 3 samples of RM 8404 for each test.

3.4.2.1 Neogen Veratox Hazelnut ELISA

The Neogen Veratox for Hazelnut Allergen Quantitative Test (Product 8420) is a sandwich enzyme-linked immunosorbent assay intended for the full quantitative analysis or simple screening of hazelnut

protein residues in food products with an LOQ of 2.5 mg/kg (ppm) total hazelnut [13]. The test principle is described below in an excerpt from reference [13].

> The Veratox for Hazelnut Allergen test is a sandwich enzyme-linked immunosorbent assay (S-ELISA). Hazelnut protein is extracted from samples with a phosphate buffered salt solution (PBS) by shaking in a heated water bath, followed by centrifugation or filtration. Extracted hazelnut protein is sampled and added to antibody-coated wells (capture antibody) where it binds to the antibody during an incubation. Any unbound hazelnut protein is washed away and a second antibody (detector antibody), which is enzyme labeled, is added. The detector antibody binds to the already bound hazelnut protein. After a second wash, substrate is added. Color develops as a result of the presence of bound detector antibody. Red Stop reagent is added and the color of the resulting solution is observed. The test is read in a microwell reader to yield optical densities. The optical densities of the controls form a standard curve, and the sample optical densities are plotted against the curve to calculate the exact concentration of hazelnut expressed as parts per million of hazelnut.

3.4.2.2 Neogen Veratox Peanut ELISA

The Neogen Veratox for Peanut Allergen Quantitative Test (Product 8420) is a sandwich enzyme-linked immunosorbent assay intended for the quantitative analysis of peanut protein in food products with an LOQ of 2.5 mg/kg (ppm) total peanut [14]. The test principle is described below in an excerpt from reference [14].

> The Veratox for Peanut Allergen test is a sandwich enzyme-linked immunosorbent assay (S-ELISA). Peanut protein residue is extracted from samples with a phosphate buffered salt solution (PBS) by shaking in a heated water bath, followed by centrifugation or filtration. Extracted peanut residue is sampled and added to antibody-coated wells (capture antibody) where it binds to the antibody during an incubation. Any unbound peanut residue is washed away and a second antibody (detector antibody), which is enzyme labeled, is added. The detector antibody binds to the already bound peanut residue. After a second wash, substrate is added. Color develops as a result of the presence of bound detector antibody. Red Stop reagent is added and the color of the resulting solution is observed. The test is read in a microwell reader to yield optical densities. The optical densities of the controls form a standard curve, and the sample optical densities are plotted against the curve to calculate the exact concentration of peanut protein.

3.4.2.3 BioFront Technologies MonoTrace for Pecan ELISA and Walnut ELISA

The BioFront Technologies MonoTrace for Pecan ELISA (Product PC4-EK-48) is monoclonal antibody-based assay intended for the qualitative or quantitative detection of pecan protein in food products with an LOQ of 1.0 mg/kg (ppm) total pecan [15]. BioFront Technologies MonoTrace for Walnut ELISA (Product WJ4-EK-48) is monoclonal antibody-based assay intended for the qualitative or quantitative detection of walnut protein in food products with an LOQ of 1.0 mg/kg (ppm) total walnut [15]. As summarized from the graphical representation in [15], the target protein residue is extracted from samples with a buffer solution. Extracted residue is sampled and added to monoclonal antibody-coated wells (capture antibody) where it binds to the antibody during an incubation. Any unbound residue is washed away in a series of wash steps and a second horseradish peroxidase (HRP)-conjugated monoclonal antibody (detector antibody) is added. The detector antibody binds to the already bound residue. After a second series of washes, substrate is added. Color develops as a result of the presence of bound detector antibody. HRP-quench solution is added to stop the reaction and the color of the resulting solution is observed. The test is read in a microwell reader to yield optical

densities. The optical densities of the controls form a standard curve, and the sample optical densities are plotted against the curve to calculate the exact concentration of trace protein.

3.4.2.4 3M Macadamia Protein ELISA

The 3M Macadamia Protein ELISA (Product E96MAC) is a sandwich enzyme-linked immunosorbent assay intended for the detection of macadamia proteins in clean-in-place water (CIP) final rinse water, environmental swab samples, food ingredients, and processed food products with an LOQ of 0.33 mg/kg (ppm) macadamia protein [16]. The test principle is described below in an excerpt from reference [16].

> The 3M Macadamia Protein ELISA Kit utilizes a sandwich ELISA. The macadamia proteins present in the sample react with the anti-macadamia antibody, which have been adsorbed to the surface of polystyrene microtiter wells. After the removal of unbound proteins by washing, anti-macadamia antibodies conjugated with horseradish peroxidase (HRP) are added. These enzyme-labeled antibodies form complexes with the previously bound macadamia protein. Following a second washing step, the enzyme bound to the immunosorbent is detected by the addition of a chromogenic substrate, 3,3',5,5'-tetramethylbenzidine (TMB). The color development from this enzymatic reaction varies directly with the concentration of macadamia protein in the sample tested; thus, the absorbance, at 450 nm, is a measure of the concentration of macadamia protein in the test sample. The quantity of macadamia protein in the test sample can be extrapolated from the standard curve, constructed from standards of known concentration, and adjusted to consider the sample dilution.

3.4.3 Microbac Laboratories

Microbac Laboratories (Oak Ridge, TN) was provided with 6 sets each of 3 samples of RM 8404 for testing by R-Biopharm SureFood Allergen PCR for peanut (S3603), hazelnut (S3602), cashew (S3615), pecan (S3618), pistachio (S3614), and walnut (S3607). SureFood Allergen PCR is a polymerase chain reaction technology that utilizes an internal amplification control for qualitative and quantitative detection of allergenic foods with a limit of detection of 0.4 mg/kg (ppm) of DNA for peanut and all tree nuts of interest [17]. As summarized in reference [17], the substance to be tested is lysed in buffer and proteinase K at 65 °C for one hour. After centrifugation and filtration via a spin filter, the DNA is bound to a spin filter, washed several times with wash buffer and eluted with of elution buffer. In a two-step thermal profile, the DNA is amplified for 45 cycles. A positive result for a qualitative test shows an exponential curve and a cycle threshold (Ct) value.

3.5 Customer Feedback

Samples of RM 8404 Almond Flour for Allergen Detection (2 packets of 5 g of material, for a total of 10 g of material) were provided to interested stakeholders for evaluation of fitness-for-purpose. Several laboratories provided feedback to NIST regarding their use of RM 8404, as summarized below.

3.5.1 Bia Diagnostics, LLC

Bia Diagnostics, LLC (Colchester, VT) utilized 3M Protein ELISA Kits for coconut, almond, hazelnut, pecan, macadamia, walnut, pistachio, cashew, and Brazil nut testing, which are quantitative kits utilizing polyclonal antibodies.

3.5.2 Eurofins Analytik GmbH

Eurofins Analytik GmbH (Hamburg, Germany) utilized internal methods for allergen testing, including ELISA testing for β-lactoglobulin and casein (milk proteins), cashew, peanut, coconut,

hazelnut, lupin, macadamia, Brazil nut, pecan, sesame, soy, egg, walnut, and mustard, and polymerase chain reaction (PCR) testing for cashew, peanut, fish, oat, hazelnut, lupin, pistachio, celery, mustard, sesame, soy, walnut, and wheat. All tests were conducted in duplicate, and the results of the single determinations did not vary significantly. Additionally, paprika powder and chocolate were spiked with RM 8404 leading to a final concentration of 1 % (10000 mg/kg, 10000 ppm) almond and tested with the almond ELISA.

3.5.3 Eurofins Immunolab

Eurofins Immunolab (Kassel, Germany) utilized internal ELISA methods (three kit lots) for allergen testing. Proteins from RM 8404 were extracted according to the kit instructions for use and diluted in extraction buffer.

3.5.4 Hygenia

Hygenia (Camas, Sevilla, Spain) used RM 8404 in quality control and cross reactivity studies. No additional information was provided.

3.5.5 Neogen

Neogen (Lansing, MI) used Veratox Almond (8440) kits to evaluate the samples via ELISA. 1000 µg/ml samples were prepared by adding 1 mg of the reference material to 1 mL of phosphate-buffered saline (PBS). The sample was vortexed until dissolved and then diluted 1:10 to prepare a 100 µg/mL spike stock. Five grams of material (rice or PBS) was spiked at (0, 5, 10, and 20) mg/kg (ppm) commodity using the 100 µg/mL stock. The samples were extracted and tested in duplicate following the kit insert.

Neogen also used their lateral flow assay Reveal 3D Almond (902086G) to evaluate RM 8404. The 100 µg/mL spike stock described above was used to spike 0.25 g of commodity (rice flour and PBS) at (0, 5, and 10) µg/mL. These samples, in addition to the 1000 µg/mL spike stock described above, were extracted and tested in duplicate via kit insert.

4 Results and Discussion

4.1 Proximates

Results for proximates provided by Exercise 5 of HAMQAP and by Eurofins Food Chemistry Testing are summarized in the sections below. All results were provided on an as-received basis.

4.1.1 Fat

The fat values reported by laboratories participating in HAMQAP Exercise 5 are summarized in Table 5. The table also provides several summary values: *N* = number of values, Mean = mean of values, SD = standard deviation of values, and Pooled SD = square-root of the sum of the squared within-participant standard deviations.

Table 5. Summary of Results for Fat, %

Lab	A	B	C	Mean	SD	Method
E002	58.64	58.26	59.01	58.6	0.4	Sum of fatty acids as triglycerides
E030	51.5	50	49.7	50.4	1.0	Sum of fatty acids as triglycerides
E033	62.34	59.6	59.2	60.4	1.7	Sum of fatty acids as triglycerides
E047	53.71	53.09		53.4	0.4	Roese-Gottlieb/Mojonnier
			***N*:**	4		
			Mean, Pooled SD:	55.9	2.0	
			SD:	4.4		

4.1.2 Protein

The protein values reported by Eurofins Food Chemistry Testing are summarized in Table 6. In the first column of data, as reported by Eurofins, the results for total nitrogen were converted to protein using a Jones factor of 6.25. In the second column of data, the results have been adjusted by NIST to reflect a proper Jones factor of 5.18. The table also provides several summary values: *N* = number of values, Mean = mean of values, and SD = standard deviation of values.

Table 6. Summary of Eurofins Results for Protein, %

	Jones Factor	
Box	**6.25**	**5.18**
1-1	28.2	23.4
1-1A	28.1	23.3
1-1B	27.8	23.0
1-1C	27.8	23.0
1-1D	28.1	23.3
1-3	28.3	23.5
2-4	28.0	23.2
3-4	28.2	23.4
4-3	28.5	23.6
6-1	28.4	23.5
7-1	28.3	23.5
8-1	27.9	23.1
8-2	28.1	23.3
10-4	27.6	22.9
***N*:**	10	10
Mean:	28.1	23.3
SD:	0.3	0.2

Figure 2 displays the protein results reported by Eurofins Food Chemistry Testing as a function of the sample box number using the Jones factor of 5.18. The blue circles in the figure represent the individual test results for each sample. The mean mass fraction is indicated by the solid line, and the dashed lines bound the interval Mean ± 2×SD.

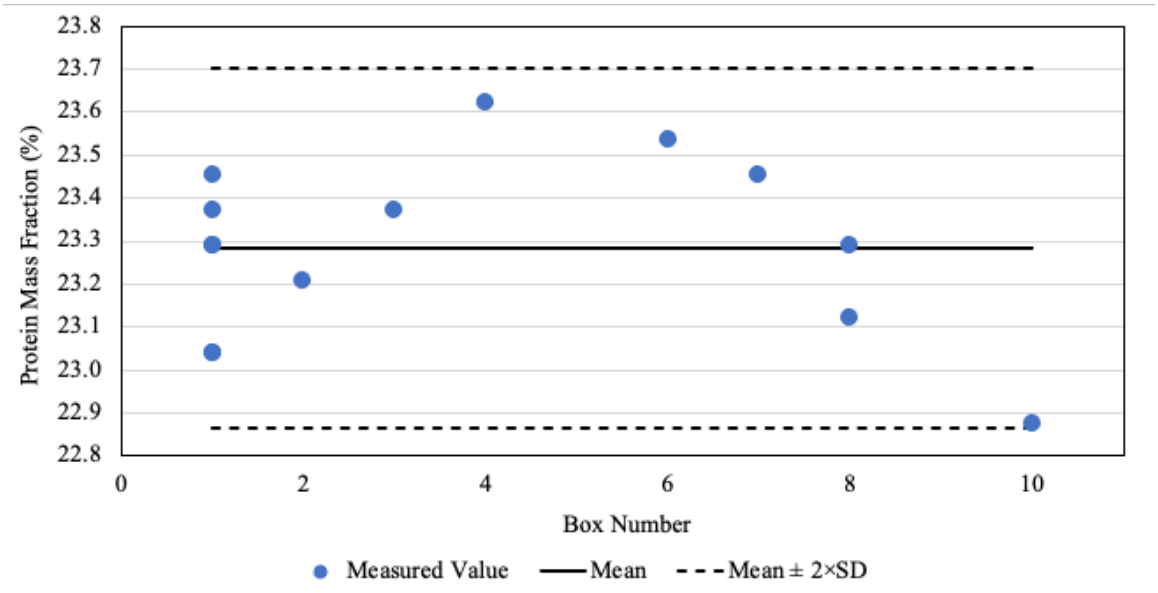


Figure 2. Protein Mass Fraction Results Reported by Eurofins as a Function of Box Number

The protein values reported by laboratories participating in HAMQAP Exercise 5 are summarized in Table 7. The upper portion of the table summarizes the results as reported by the participants in the study. The lower portion of the table repeats this information, replacing the results for two laboratories who reported using a Jones factor of 6.25. The table also provides several summary values: *N* = number of values, Mean = mean of values, SD = standard deviation of values, and Pooled SD = square-root of the sum of the squared within-participant standard deviations.

Table 7. Summary of HAMQAP Exercise 5 Results for Protein, %

HAMQAP Exercise 5 (as reported by participants)

Lab	A	B	C	Mean	SD	Method (Jones Factor)
E002	24.9	25.3	24.8	25	0.3	Kjeldahl (5.18)
E030	22.4	22.7	22.7	22.6	0.2	Kjeldahl (5.18)
E033	28.8	28.3	28.8	28.6	0.3	Combustion (6.25)
E047	28.0	28.1		28.1	0.1	Combustion (6.25)
			***N*:**	4		
			Mean, Pooled SD:	25.9	0.4	
			SD:	2.6		

HAMQAP Exercise 5 (corrected for Jones factor)

Lab	A	B	C	Mean	SD	Method (Jones Factor)
E002	24.9	25.3	24.8	25	0.3	Kjeldahl (5.18)
E030	22.4	22.7	22.7	22.6	0.2	Kjeldahl (5.18)
E033	23.9	23.5	23.9	23.7	0.2	Combustion (5.18)
E047	23.2	23.3		23.2	0.1	Combustion (5.18)
			***N*:**	4		
			Mean, Pooled SD:	23.7	0.4	
			SD:	1.0		

4.1.3 Carbohydrates

The carbohydrate values reported by laboratories participating in HAMQAP Exercise 5 are summarized in Table 8. The table also provides several summary values: *N* = number of values, Mean = mean of values, SD = standard deviation of values, and Pooled SD = square-root of the sum of the squared within-participant standard deviations.

Table 8. Summary of Results for Carbohydrates, %

Lab	A	B	C	Mean	SD	Method
E002	7.02	6.95	7.16	7	0.1	*Not specified*
E030	18.9	19.8	20.2	19.6	0.7	Calculation [100-(solids+protein+fat+ash)]
E033	1.5	5.2	4.8	3.8	2	Calculation [100-(solids+protein+fat+ash)]
E047	11.7	12.21		12	0.4	Calculation [100-(solids+protein+fat+ash)]
			***N*:**	4		
			Mean, Pooled SD:	10.5	2.2	
			SD:	6.6		

4.1.4 Ash

The ash values reported by laboratories participating in HAMQAP Exercise 5 are summarized in Table 9. The values from Lab E035 were implausibly large and therefore were omitted from the statistical analysis. The table also provides several summary values: *N* = number of values, Mean = mean of values, SD = standard deviation of values, and Pooled SD = square-root of the sum of the squared within-participant standard deviations.

Table 9. Summary of Results for Ash, %

Lab	A	B	C	Mean	SD	Method
E002	3.21	3.17	3.10	3.16	0.06	Thermogravimetry
E009	2.61	2.81	2.37	2.6	0.22	*Not specified*
E030	2.69	2.67	2.67	2.68	0.01	Muffle furnace
E033	2.83	2.85	2.87	2.85	0.02	Muffle furnace
E035	84.6	78.4	69.9	77.63	7.38	Muffle furnace
E047	3.19	3.1		3.15	0.06	Muffle furnace
			***N*:**	6		
			Mean, Pooled SD:	16.1	7.4	
			SD:	29.5		
			***N*:**	5		**removing outlying data from E035*
			Mean, Pooled SD:	2.87	0.24	
			SD:	0.26		

4.1.5 Solids

The solids values reported by laboratories participating in HAMQAP Exercise 5 are summarized in Table 10. The table also provides several summary values: *N* = number of values, Mean = mean of values, SD = standard deviation of values, and Pooled SD = square-root of the sum of the squared within-participant standard deviations.

Table 10. Summary of Results for Solids, %

Lab	A	B	C	Mean	SD	Method
E002	97.67	97.7	97.73	97.7	0.03	Thermogravimetry
E030	95.5	95.22	95.3	95.34	0.14	Drying in Forced Air Oven
E033	95.5	95.9	95.7	95.7	0.2	Drying in Vacuum Oven
E047	96.3	96.5		96.4	0.14	Drying in Forced Air Oven
			***N*:**	4		
			Mean, Pooled SD:	96.3	0.3	
			SD:	1.0		

4.1.6 Calories

The calories values reported by laboratories participating in HAMQAP Exercise 5 are summarized in Table 11. The upper portion of the table summarizes the results as reported by the participants in the study. The lower portion of the table repeats this information, replacing the results for two laboratories who reported results in incorrect units. The table also provides several summary values: *N* = number of values, Mean = mean of values, SD = standard deviation of values, and Pooled SD = square-root of the sum of the squared within-participant standard deviations.

Table 11. Summary of Results for Calories, kcal/100 g

HAMQAP Exercise 5 (as reported by participants)

Lab	A	B	C	Mean	SD	Method
E002	655.44	653.26	658.93	656	3	Calculation [9(fat)+4(protein)+4(carbohydrate)]
E030	0.629	0.62	0.619	0.623	0.006	Calculation [9(fat)+4(protein)+4(carbohydrate)]
E033	682.4	670.2	667.3	673	8	Calculation [9(fat)+4(protein)+4(carbohydrate)]
E047	0.642	0.639		0.641	0.002	Calculation [9(fat)+4(protein)+4(carbohydrate)]
			***N*:**	4		
			Mean, Pooled SD:	362.8	8.5	
			SD:	346.8		

HAMQAP Exercise 5 (corrected for units)

Lab	A	B	C	Mean	SD	Method
E002	655.44	653.26	658.93	656	3	Calculation [9(fat)+4(protein)+4(carbohydrate)]
E030	629	620	619	623	6	Calculation [9(fat)+4(protein)+4(carbohydrate)] - corrected
E033	682.4	670.2	667.3	673	8	Calculation [9(fat)+4(protein)+4(carbohydrate)]
E047	642	639		641	2	Calculation [9(fat)+4(protein)+4(carbohydrate)] - corrected
			***N*:**	4		
			Mean, Pooled SD:	648.8	10.4	
			SD:	20.9		

4.2 Trace Allergen Contaminants

4.2.1 Eurofins GeneScan

Data provided by Eurofins GeneScan for hazelnut protein in RM 8404 Almond Flour for Allergen Detection is reported in Table 12. No hazelnut protein was detected by the R-Biopharm ELISA kit above the lower limit of quantitation for all three samples.

Table 12. Results provided by Eurofins for hazelnut allergen in RM 8404 using R-Biopharm Hazelnut Allergen ELISA

Sample Number	Result mg/kg (ppm)	LOQ mg/kg (ppm)
1	< 2.5	2.5
2	< 2.5	2.5
3	< 2.5	2.5

4.2.2 Food Allergen Research and Resource Program (FARRP) at the University of Nebraska Lincoln

Data provided by FARRP for hazelnut, peanut, pecan, walnut, and macadamia proteins in RM 8404 Almond Flour for Allergen Detection are reported in Table 13. No hazelnut, peanut, pecan, or walnut protein was detected by the Neogen and BioFront ELISA kits above the lower limits of quantitation for the assays in all three samples. A level of macadamia protein just above the LOQ was detected using the 3M kit. As described in Section 2.1, the likelihood of macadamia contamination of this almond flour is low based on low prevalence of macadamia nut production in the area where this material was sourced and is likely a result of potential cross-reactivity of the ELISA kit in the presence of the high level of almond protein.

Table 13. Results provided by FARRP for various allergens in RM 8404

Allergen	Test	LOQ mg/kg (ppm)	Sample Result mg/kg (ppm)		
			1	2	3
Hazelnut	Neogen Veratox ELISA	2.5	< 2.5	< 2.5	< 2.5
Peanut	Neogen Veratox ELISA	2.5	< 2.5	< 2.5	< 2.5
Pecan	BioFront Technologies MonoTrace ELISA	1.0	< 1	< 1	< 1
Walnut	BioFront Technologies MonoTrace ELISA	1.0	< 1	< 1	< 1
Macadamia	3M ELISA	0.33	0.37	0.37	0.76

4.2.3 Microbac Laboratories

Data provided by Microbac Laboratories for hazelnut, peanut, pecan, walnut, pistachio, and cashew DNA in RM 8404 Almond Flour for Allergen Detection are reported in Table 14. No DNA from any tested source were detected above the method LOQ by the R-Biopharm PCR kits.

Table 14. Results provided by Microbac Laboratories for various allergens in RM 8404 using R-Biopharm SureFood PCR Kits

Allergen	LOQ mg/kg (ppm)	Qualitative Sample Result		
		1	2	3
Hazelnut	0.4	Negative	Negative	Negative
Peanut	0.4	Negative	Negative	Negative
Pecan	0.4	Negative	Negative	Negative
Walnut	0.4	Negative	Negative	Negative
Pistachio	0.4	Negative	Negative	Negative
Cashew	0.4	Negative	Negative	Negative

4.3 Customer Feedback

4.3.1 Bia Diagnostics, LLC

As shown in Table 15, no cross-reactivity to or contamination with Brazil nut, cashew, coconut, hazelnut, macadamia, pecan, pistachio, or walnut was observed for RM 8404. The almond response for this kit was extremely high, with results above LOQ of 27 mg/kg (ppm) at a 1/62500 dilution, which equates to a result of over 1687500 mg/kg (ppm) almond protein. Because the almond antibody used in this kit was raised against roasted almonds, the blanched almonds used to prepare this RM may react more strongly than anticipated due to the abundance of intact, folded protein compared to

those in roasted almonds as roasting may be more destructive to the protein 3D structure than blanching.

Table 15. Summary of cross reactivity and contamination results provided by Bia Diagnostics ELISA testing of RM 8404. *ND = not detected*.

Allergen	Test LOQ mg/kg (ppm)	Test Result mg/kg (ppm)
Almond	27	> 1 687 500
Brazil Nut	1	ND
Cashew	0.9	ND
Coconut	2	ND
Hazelnut	1	ND
Macadamia	0.3	ND
Pecan	0.66	ND
Pistachio	1	ND
Walnut	2	ND

4.3.2 Eurofins Analytik GmbH

Eurofins Analytik GmbH reported that RM 8404 was homogenous and weighing and dissolution was straightforward, permitting spiking experiments without issue. As shown in Table 16, no cross-reactivity to or contamination with bovine beta-lactoglobulin (a milk protein), casein (a milk protein), cashew, peanut, coconut, hazelnut, lupin, macadamia, Brazil nut, pecan, sesame, soy, egg, walnut, mustard, fish, oat, pistachio, celery, or wheat was observed for RM 8404 based on the ELISA and PCR tests conducted.

Table 16. Results provided by Eurofins Analytik GmbH for RM 8404. *ND = not detected, NT = not tested.*

	ELISA		PCR	
Allergen	LOQ (mg/kg)	Result (mg/kg)	LOQ (mg/kg)	Result (mg/kg)
beta-lactoglobulin	0.031	< 0.031	NT	NT
Brazil Nut	4	< 4	NT	NT
Casein	0.25	< 0.25	NT	NT
Cashew	2	< 2	Undefined	ND
Celery	NT	NT	Undefined	ND
Coconut	2	< 2	NT	NT
Egg	0.31	< 0.31	NT	NT
Fish	NT	NT	Undefined	ND
Hazelnut	0.16	< 0.16	Undefined	ND
Lupin	2	< 2	Undefined	ND
Macadamia Nut	1	< 1	NT	NT
Mustard	2	< 2	Undefined	ND
Oat	NT	NT	Undefined	ND
Peanut	0.2	< 0.2	Undefined	ND
Pecan Nut	2	< 2	NT	NT
Pistachio	NT	NT	Undefined	ND
Sesame	2	< 2	Undefined	ND
Soy	0.31	< 0.31	Undefined	ND
Walnut	2	< 2	Undefined	ND
Wheat	NT	NT	Undefined	ND

Additionally, Eurofins Analytik GmbH reported spiking RM 8404 into paprika and chocolate at 10 000 mg/kg (ppm) to evaluate recovery of their commodity assays. The paprika spiked with RM 8404 was tested with the almond ELISA and 8 400 mg/kg (ppm) of almond was recovered (84 %). The chocolate spiked with RM 8404 was tested with the almond ELISA and 7 700 mg/kg (ppm) of almond was recovered (77 %). Eurofins Analytik GmbH did not provide an acceptance range but indicated that recovery for all samples was in the range of normal acceptance criteria.

4.3.3 Eurofins Immunolab

Eurofins Immunolab tested two dilutions of RM 8404 and reported a mean activity of 95 % compared to the raw almond material applied for the calibration of the ELISA (Table 17).

Table 17. Results for almond ELISA kit reactivity provided by Eurofins Immunolab for dilutions of RM 8404.

		Result mg/kg [ppm]		Reactivity [%]
Kit-Lot	**Sample**	**1:100000**	**1:1000000**	**Mean**
MDL-148	Sample 1	9.60	0.80	88
	Sample 2	9.81	0.96	97
MDL-149	Sample 1	9.93	0.92	96
	Sample 2	9.75	0.98	98
MDL-150	Sample 1	9.87	0.99	99
	Sample 2	9.80	0.89	94
			Mean	**95**

4.3.4 Hygenia

Hygenia reported use of RM 8404 in quality control and cross reactivity studies and that the materials worked well, but that not enough material was provided for full characterization. No additional data or information was provided by Hygenia.

4.3.5 Neogen

Results for Neogen Veratox assay and Reveal 3D lateral flow kit testing of PBS and rice spiked with RM 8404 are provided in Table 18. Results for both assays are consistent with expectations.

Table 18. Results provided by Neogen for ELISA and lateral flow kit testing of materials spiked with RM 8404.

		Veratox Almond ELISA			Reveal 3D Almond Lateral Flow	
Matrix	**Sample**	**Spike mg/kg (ppm)**	**Result mg/kg (ppm)**	**Recovery**	**Spike mg/kg (ppm)**	**Result**
PBS	**Neg**	0	0.07	-	0	Negative
	RM 8404	5	5.01	100 %	5	Positive
		10	10.63	106 %	10	Positive
		20	18.88	94 %	1000	Positive
Rice Flour	**Neg**	0	0.18	-	0	Negative
	RM 8404	5	5.32	106 %	5	Positive
		10	10.42	104 %	10	Positive
		20	23.16	116 %	1000	Positive

5 Conclusions

5.1 Value Assignment for Proximates

As described in Section 3.3, available data for each measurand was used to provide an estimate of the mass fraction present in RM 8404 where x is the mean and $U_{95}(x)$ is the 95 % confidence interval. The summary of these estimates is provided in Table 19, along with a summary of the datasets used to arrive at these estimates.

Table 19. Summary of Estimates for Proximates in RM 8404

Analyte	x	$U_{95}(x)$	Units	Based on
Fat	56.02	8.03	%	HAMQAP
Protein	23.29	0.46	%	HAMQAP, Eurofins
Carbohydrates	9.50	10.77	%	HAMQAP[a]
Ash	2.85	0.35	%	HAMQAP[a]
Solids	96.05	1.61	%	HAMQAP
Calories	648.19	35.23	kcal/100 g	HAMQAP

[a] Not all laboratories reported methods used.

5.2 Trace Allergen Contaminants

All testing results and customer feedback regarding the potential contamination of RM 8404 with other trace allergens is summarized in Table 20. Observed presence of macadamia nut is most likely explained as cross-reactivity of the 3M and Romer assays to the high levels of almond protein and therefore not of concern with respect to the proteins present in RM 8404.

Table 20. Summary of all results for trace allergen contamination in RM 8404. *ND = Not detected.*

Allergen	Presence mg/kg (ppm)	Based on
β-Lactoglobulin	< 0.031	Eurofins Analytik GmbH ELISA
Brazil Nut	< 1	3M ELISA
	< 1	BioFront MonoTrace ELISA
	< 4	Eurofins Analytik GmbH ELISA
Casein	< 0.25	Eurofins Analytik GmbH ELISA
	< 0.4	R-Biopharm SureFood PCR
Cashew	< 0.2	Romer US-AgraQuant ELISA
	< 0.9	3M ELISA
	< 1	BioFront MonoTrace ELISA
	< 2	Eurofins Analytik GmbH ELISA
	ND	Eurofins Analytik GmbH PCR
Celery	ND	Eurofins Analytik GmbH PCR
Coconut	< 2	3M ELISA
	< 2	Eurofins Analytik GmbH ELISA
Egg	< 0.31	Eurofins Analytik GmbH ELISA
Fish	ND	Eurofins Analytik GmbH PCR
Hazelnut	< 0.16	Eurofins Analytik GmbH ELISA
	< 0.3	Romer US-AgraQuant ELISA
	< 0.4	R-Biopharm SureFood PCR
	< 1	BioFront MonoTrace ELISA
	< 1	3M ELISA
	< 2.5	R-Biopharm ELISA
	< 2.5	Neogen Veratox ELISA
	ND	Eurofins Analytik GmbH PCR
Lupin	< 2	Eurofins Analytik GmbH ELISA
	ND	Eurofins Analytik GmbH PCR
Macadamia	< 0.3	3M ELISA
	0.5	**3M ELISA**
	35	**Romer US-AgraQuant ELISA**
	< 1	Eurofins Analytik GmbH ELISA
	< 2	BioFront MonoTrace ELISA
Mustard	< 2	Eurofins Analytik GmbH ELISA
	ND	Eurofins Analytik GmbH PCR

Allergen	Presence mg/kg (ppm)	Based on
Oat	ND	Eurofins Analytik GmbH PCR
Peanut	< 0.1	Romer US-AgraQuant ELISA
	< 0.2	Eurofins Analytik GmbH ELISA
	< 0.4	R-Biopharm SureFood PCR
	< 1	BioFront MonoTrace ELISA
	< 2.5	Neogen Veratox ELISA
	ND	Eurofins Analytik GmbH PCR
Pecan	< 0.4	R-Biopharm SureFood PCR
	< 0.66	3M ELISA
	< 1	Romer US ELISA
	< 1	BioFront MonoTrace ELISA
	< 2	Eurofins Analytik GmbH ELISA
Pine Nut	< 0.7	Romer US ELISA
	< 1	BioFront MonoTrace ELISA
Pistachio	< 0.1	Romer US-AgraQuant ELISA
	< 0.4	R-Biopharm SureFood PCR
	< 1	BioFront MonoTrace ELISA
	< 1	3M ELISA
	ND	Eurofins Analytik GmbH PCR
Sesame	< 2	Eurofins Analytik GmbH ELISA
	ND	Eurofins Analytik GmbH PCR
Soy	< 0.31	Eurofins Analytik GmbH ELISA
	ND	Eurofins Analytik GmbH PCR
Walnut	< 0.3	Romer US-AgraQuant ELISA
	< 0.4	R-Biopharm SureFood PCR
	< 1	BioFront MonoTrace ELISA
	< 2	3M ELISA
	< 2	Eurofins Analytik GmbH ELISA
	ND	Eurofins Analytik GmbH PCR
Wheat	< 2	Romer US-AgraQuant ELISA
	< 3	Romer US-RIDASCREEN
	ND	Eurofins Analytik GmbH PCR

5.3 Customer Feedback

Various laboratories spiked RM 8404 into various solutions or other food products to evaluate the recovery of their assays and fitness-for-purpose of the material. All assays responded acceptably and as expected.

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