NIST Special Publication 260-219

Value Assignment of Reference Material 8405

Hazelnut Flour for Allergen Detection

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Abstract

The National Institute of Standards and Technology (NIST) recently released RM 8405 Hazelnut 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; Hazelnut; 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 8405 Hazelnut 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 hazelnut, 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 July 2019, 2.2 kg (1 pound) of hazelnut flour was purchased from the American Hazelnut Company (Gays Mills, WI). A representative from the American Hazelnut Company indicated that their hazelnuts are grown on a single-crop farm and processing of hazelnuts into flour was performed in a shared facility where other allergens might be present, such as gluten. 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 egg, wheat, milk, peanut, pecan, soy, and walnut protein allergens. Results of the allergen testing conducted by Romer Labs in July 2019 are described in Table 1, which identified possible presence of peanut protein in the sample.

Table 1. Results of July 2019 allergen screening by Romer Labs for candidate hazelnut flour. *LOD = limit of detection; LOQ = limit of quantification; *Result found to be > LOD but < LOQ.*

	Result mg/kg (ppm)			Method		
Parameter	**A**	**B**	**C**	**Name**	**LOD mg/kg (ppm)**	**LOQ mg/kg (ppm)**
Egg	<LOD	<LOD	<LOD	US-AgraQuant ELISA Allergen Test Kit	0.5	1.0
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	2.0
Milk Protein	<LOD	<LOD	<LOD	US-AgraQuant ELISA Allergen Test Kit	0.05	0.4
Peanut	--*	<LOD	<LOD	US-AgraQuant ELISA Allergen Test Kit	0.1	1.0
Pecan Protein	<LOD	<LOD	<LOD	US-Pecan ELISA	1.0	2.0
Soy Protein	<LOD	<LOD	<LOD	US-AgraQuant ELISA Allergen Test Kit	16.0	40.0
Walnut	<LOD	<LOD	<LOD	US-AgraQuant ELISA Allergen Test Kit	0.3	2.0

In September 2019, a 30-g aliquot of the candidate hazelnut 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 almond, brazil nut, cashew, hazelnut, macadamia nut, peanut, pecan, pine nut, pistachio, and walnut protein allergens. 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) peanut protein, indicating that the positive result found by Romer Labs may have been a result of assay cross-reactivity or contamination of the single sample during testing.

Table 2. Results of September 2019 allergen screening by BioFront Laboratories for candidate hazelnut 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	<LLOQ	1-40	0.23	1
Brazil Nut	<LLOQ	1-40	0.14	1
Cashew	<LLOQ	1-40	0.12	1
Hazelnut	>ULOQ	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 October 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 hazelnut flour, as shown in the report from High-Purity Standards (Figure 1).

October 29, 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 two test kits from NIST for the Neogen Reveal® for Multi-Treenut test. They were 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 tabletop and inner surfaces of the polybag, drum, 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 one of the tree nut powders. We would prefer to start with hazelnut powder.

Participants:

Cleaning: Kevin Dowen – Inorganic Chemist
Analysis: Tommy Breen – Inorganic Manufacturing Manager

Sincerely,

Moven Mututuvari, VP Manufacturing

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 8405 Hazelnut Flour for Allergen Detection.

In November 2019, 30 kg (66 pounds) of hazelnut flour was purchased from American Hazelnut Company 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 8 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 hazelnut 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 8405 Hazelnut Flour for Allergen Detection, as well as remaining unpackaged material, were received at NIST on January 3, 2020.

Table 3. Pre- and post-packaging homogeneity testing report from High-Purity Standards for RM 8405 Hazelnut Flour for Allergen Detection.

	% RSD			
Analyte	**Pre-Packaging[a]**	**Post-Packaging[b]**	**Criteria (%)**	**Result**
Ca	1.05	0.91	≤ 3.0	Pass
K	1.08	0.97	≤ 3.0	Pass
Mg	1.68	0.97	≤ 3.0	Pass
Mn	0.69	0.85	≤ 3.0	Pass
S	1.37	1.01	≤ 3.0	Pass
Zn	1.33	0.76	≤ 3.0	Pass

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

2.2 Storage

The packets of RM 8405 have been stored at −20 °C at NIST since their receipt.

3 Experimental Procedures

3.1 Interlaboratory Studies for Value Assignment

RM 8405 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 8405 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 8405 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 8405 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 hazelnut testing. The data provided by the collaborating laboratory has been adjusted using a more appropriate Jones factor for hazelnut measurement (5.30).

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

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 8405 for testing by R-Biopharm R6901 Almond Allergen (ELISA). The RIDASCREEN FAST Hazelnut (Product R6901) is a sandwich enzyme immunoassay for the quantitative analysis of almond or parts of almond in food with a limit of quantification (LOQ) of 2.5 mg/kg (ppm) almond [12]. The test principle is described below in an excerpt from reference [12].

> The wells of the microtiter strips are coated with specific antibodies to almond proteins. By adding standards and samples to the wells, almond protein present will bind to the specific antibodies. In a washing step, components not bound are removed. Then antibody conjugated to peroxidase is added. This antibody 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. The detection of almond protein takes place by adding substrate/chromogen. The enzyme conjugate converts the 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 almond protein concentration of the sample. The result is expressed in mg/kg almond.

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 8405 for testing by numerous commercial approaches as described in the sections below. FARRP was provided with 3 samples of RM 8405 for each test.

3.4.2.1 Neogen Veratox Almond ELISA

The Neogen Veratox for Almond Allergen Quantitative Test (Product 8440) is a sandwich enzyme-linked immunosorbent assay intended for the full quantitative analysis or simple screening of almond protein residues in food products with an LOQ of 2.5 mg/kg (ppm) total almond [13]. The test principle is described below in an excerpt from reference [13].

The Veratox for Almond is a sandwich enzyme-linked immunosorbent assay (S-ELISA). Almond residues are extracted from samples with a buffered salt solution, Phosphate Buffered Saline (PBS), by shaking in a heated water bath, followed by centrifugation or filtration. Extracted almond residue is sampled and added to capture antibody-coated wells where it binds to the antibody during an incubation. Any unbound almond residue is washed away and a second detector antibody, which is enzyme labeled, is added. The detector antibody binds to the already bound almond residue. After a second wash, the substrate is added. Color develops as a result of the presence of bound detector antibody. Red Stop solution 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 almond residue.

3.4.2.2 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 [14]. 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 [14]. As summarized from the graphical representation in [14], 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.

3.4.3 Microbac Laboratories

Microbac Laboratories (Oak Ridge, TN) was provided with 3 samples of RM 8405 for testing by R-Biopharm SureFood Allergen PCR for almond (S3604). 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 almond [15]. As summarized in reference [15], 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 8405 Hazelnut 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 8405, 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, lupin, macadamia, Brazil nut, pecan, sesame, soy, egg, walnut, mustard, and almond, and polymerase chain reaction (PCR) testing for cashew, peanut, fish, oat, 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, chocolate was spiked with RM 8405 leading to a final concentration of 0.1 % (1000 mg/kg, 1000 ppm) hazelnut and tested with the hazelnut ELISA.

3.5.3 Eurofins Immunolab

Eurofins Immunolab (Kassel, Germany) utilized internal ELISA methods (three kit lots) for allergen testing. Proteins from RM 8405 were extracted according to the kit instructions for use and diluted in extraction buffer.

3.5.4 Hygenia

Hygenia (Camas, Sevilla, Spain) used RM 8405 in quality control and cross reactivity studies. No additional information was provided.

3.5.5 Neogen

Neogen (Lansing, MI) used Veratox Hazelnut (8420) 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 Hazelnut (902087E) to evaluate RM 8405. 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	12.41	12.78	12.58	12.6	0.2	Sum of fatty acids as triglycerides
E030	10.8	10.8	10.5	10.7	0.2	Sum of fatty acids as triglycerides
E033	9.5	9.4	9.4	9.4	0.1	Sum of fatty acids as triglycerides
E047	13.06	14.73		13.9	1.2	Roese-Gottlieb/Mojonnier
			***N*:**	4		
			Mean, Pooled SD:	11.5	1.2	
			SD:	1.8		

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 5.30. 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**	**5.30**
1-1	40.0	33.9
1-1A	40.0	33.9
1-1B	40.1	34.0
1-1C	39.8	33.8
1-1D	39.9	33.8
1-3	39.7	33.7
2-4	39.9	33.8
3-4	39.9	33.8
4-3	39.7	33.7
6-1	39.9	33.8
7-1	39.5	33.5
8-1	39.8	33.8
8-2	39.6	33.6
10-4	39.7	33.7
***N*:**	10	10
Mean:	39.82	33.77
SD:	0.17	0.14

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 5.30. 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.

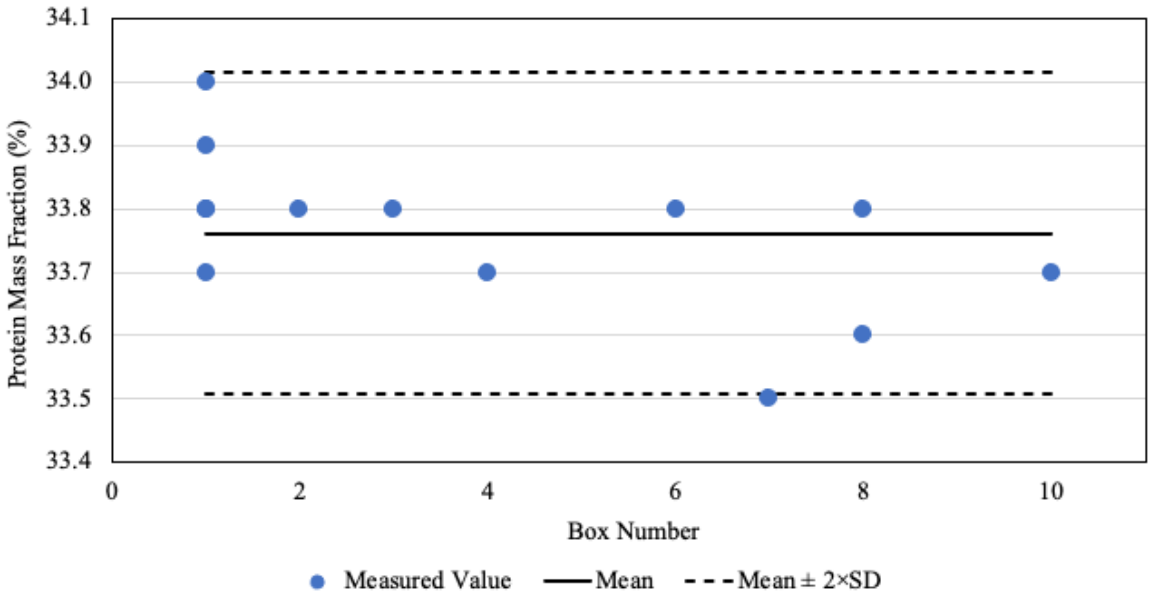

Figure 2. Protein Mass Fraction Results Reported by Eurofins as a Function of Box Number

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 one laboratory 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	35.38	35.58	35.86	35.6	0.2	Kjeldahl (5.30)
E030	33.7	33	31.9	32.9	0.9	Kjeldahl (5.30)
E033	32.8	30.9	31.4	31.7	1	Combustion (5.30)
E047	40.6	40.4		40.5	0.1	Combustion (6.25)
			***N*:**	4		
			Mean, Pooled SD:	34.7	1.4	
			SD:	3.3		

HAMQAP Exercise 5 (corrected for Jones factor)

Lab	A	B	C	Mean	SD	Method (Jones Factor)
E002	35.38	35.58	35.86	35.6	0.2	Kjeldahl (5.30)
E030	33.7	33	31.9	32.9	0.9	Kjeldahl (5.30)
E033	32.8	30.9	31.4	31.7	1	Combustion (5.30)
E047	34.4	34.3		34.3	0.1	Combustion (5.30)
			***N*:**	4		
			Mean, Pooled SD:	33.6	1.4	
			SD:	1.7		

The measured protein content of RM 8405 (34 %) is significantly greater than expected based on label claims of many commercial hazelnut flour products (7 % to 21 %). Further communication with American Hazelnut Company, the source of RM 8405, indicated that much of the hazelnut oil in their product is extracted prior to milling, resulting in a higher protein fraction of the finished product.

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	29.43	29.4	29.46	29.4	0	*Not specified*
E030	43.2	43.5	45.6	44.1	1.3	Calculation [100-(solids+protein+fat+ash)]
E033	45.2	47.8	31.4	41.5	8.8	Calculation [100-(solids+protein+fat+ash)]
E047	34.41	33.17		33.8	0.9	Calculation [100-(solids+protein+fat+ash)]
			***N*:**	4		
			Mean, Pooled SD:	37.5	9.0	
			SD:	7.5		

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	6.2	6.18	6.25	6.21	0.04	Thermogravimetry
E009	3.57	3.43	3.3	3.43	0.14	*Not specified*
E030	5.68	6.13	5.73	5.85	0.25	Muffle furnace
E033	5.91	5.83	5.45	5.73	0.25	Muffle furnace
E035	93.01	92.66	92.35	92.67	0.33	Muffle furnace
E047	6.41	6.24		6.33	0.12	Muffle furnace
			***N*:**	6		
			Mean, Pooled SD:	20.8	0.5	
			SD:	34.3		
			***N*:**	5		**removing outlying data from E035*
			Mean, Pooled SD:	5.47	0.39	
			SD:	1.11		

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	96.65	96.96	96.43	96.68	0.27	Thermogravimetry
E030	93.38	93.48	93.7	93.52	0.16	Drying in Forced Air Oven
E033	93.4	93.9	93.9	93.73	0.29	Drying in Vacuum Oven
E047	94.48	94.54		94.51	0.04	Drying in Forced Air Oven
			***N*:**	4		
			Mean, Pooled SD:	94.6	0.4	
			SD:	1.4		

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	370.93	374.94	374.5	373	2	Calculation [9(fat)+4(protein)+4(carbohydrate)]
E030	0.405	0.403	0.404	0.404	0.001	Calculation [9(fat)+4(protein)+4(carbohydrate)]
E033	397.5	399.3	400.8	399	2	Calculation [9(fat)+4(protein)+4(carbohydrate)]
E047	0.418	0.454		0.436	0.025	Calculation [9(fat)+4(protein)+4(carbohydrate)]
			***N*:**	4		
			Mean, Pooled SD:	210.9	2.8	
			SD:	201.8		

HAMQAP Exercise 5 (corrected for units)

Lab	A	B	C	Mean	SD	Method
E002	370.93	374.94	374.5	373	2	Calculation [9(fat)+4(protein)+4(carbohydrate)]
E030	405	403	404	404	1	Calculation [9(fat)+4(protein)+4(carbohydrate)] - corrected
E033	397.5	399.3	400.8	399	2	Calculation [9(fat)+4(protein)+4(carbohydrate)]
E047	418	454		436	25	Calculation [9(fat)+4(protein)+4(carbohydrate)] - corrected
			***N*:**	4		
			Mean, Pooled SD:	400.2	25.6	
			SD:	23.3		

4.2 Trace Allergen Contaminants

4.2.1 Eurofins GeneScan

Data provided by Eurofins GeneScan for almond protein in RM 8405 Hazelnut Flour for Allergen Detection is reported in Table 12. No almond 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 almond allergen in RM 8405 using R-Biopharm Almond 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 almond, pecan, and walnut proteins in RM 8405 Hazelnut Flour for Allergen Detection are reported in Table 13. No almond, 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.

Table 13. Results provided by FARRP for various allergens in RM 8405

Allergen	Test	LOQ mg/kg (ppm)	Sample Result mg/kg (ppm)		
			1	2	3
Almond	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

4.2.3 Microbac Laboratories

Data provided by Microbac Laboratories for almond DNA in RM 8405 Hazelnut Flour for Allergen Detection are reported in Table 14. No almond DNA was detected above the method LOQ by the R-Biopharm PCR kit.

Table 14. Results provided by Microbac Laboratories for almond allergen in RM 8405 using the R-Biopharm SureFood PCR Kit

Allergen	LOQ mg/kg (ppm)	Qualitative Sample Result		
		1	2	3
Almond	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 coconut, almond, pecan, macadamia, walnut, pistachio, cashew, or Brazil nut was observed for RM 8405. The hazelnut response for this kit was high, giving a result of 5.2 mg/kg (ppm) at a 1/50000 dilution. This equates to a result of 260000 mg/kg (ppm) hazelnut protein, which is quite high for hazelnuts. However, as described in Section 4.1.2, the protein content for RM 8405 is higher than expected in comparison to other commercial products based on the manufacturing process of this specific material.

Table 15. Summary of cross reactivity and contamination results provided by Bia Diagnostics ELISA testing of RM 8405. *ND = not detected*.

Allergen	Test LOQ mg/kg (ppm)	Test Result mg/kg (ppm)
Almond	27	ND
Brazil Nut	1	ND
Cashew	0.9	ND
Coconut	2	ND
Hazelnut	1	> 260 000
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 8405 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 almond, bovine beta-lactoglobulin (a milk protein), casein (a milk protein), cashew, peanut, coconut, sesame, soy, egg, walnut, mustard, fish, oat, pistachio, celery, or wheat was observed for RM 8405 based on the ELISA and PCR tests conducted. Traces of lupin, macadamia, and pecan were identified in the ELISA kits for these proteins, but these are known to cross-react and cause false positives with hazelnut products. Additionally, the level of Brazil nut measured was close to the LOQ and thus a second test was conducted. The result of the second test was a positive result near the LOQ with a mean of 4.7 mg/kg Brazil nut in two test portions tested. In parallel, the lab tested another hazelnut sample (washed in order to avoid cross contact) and this sample came out clearly negative for Brazil nut. Therefore, a false-positive or cross-reactivity is not assumed, but instead a lab-generated contamination of RM 8405 with Brazil nut or other foods leading to known false positive results in this test, like pecan or paprika.

Table 16. Results provided by Eurofins Analytik GmbH for RM 8405. *ND = not detected, NT = not tested*.

	ELISA		PCR	
Allergen	**LOQ (mg/kg)**	**Result (mg/kg)**	**LOQ (mg/kg)**	**Result (mg/kg)**
Almond	0.4	< 0.4	NT	NT
beta-lactoglobulin	0.031	< 0.031	NT	NT
Brazil Nut[a]	4	< 4	NT	NT
Brazil Nut[a]	4	4.7	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
Lupin[b]	2	3.6	Undefined	ND
Macadamia Nut[b]	1	1.7	NT	NT
Mustard	2	< 2	Undefined	ND
Oat	NT	NT	Undefined	ND
Peanut	0.2	< 0.2	Undefined	ND
Pecan Nut[b]	2	3.8	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

[a] Assay for Brazil nut was run twice because first run was close to LOQ.
[b] Known cross reactivity with hazelnut, so results are assumed to be false positive.

Additionally, Eurofins Analytik GmbH reported spiking RM 8405 into chocolate at 1 000 mg/kg (ppm) to evaluate recovery of their commodity assays. The chocolate spiked with RM 8405 was tested with the hazelnut ELISA and 800 mg/kg (ppm) of hazelnut was recovered (80 %). Eurofins Analytik GmbH did not provide an acceptance range but indicated that recovery was in the range of normal acceptance criteria.

4.3.3 Eurofins Immunolab

Eurofins Immunolab tested two dilutions of RM 8405 and reported a mean activity of 176 % compared to the raw hazelnut material applied for the calibration of the ELISA (

Table 17). The increased hazelnut activity is likely a result of the higher than anticipated protein content of RM 8405.

Table 17. Results for hazelnut ELISA kit reactivity provided by Eurofins Immunolab for dilutions of RM 8405.

		Result mg/kg [ppm]		Reactivity [%]
Kit-Lot	**Sample**	**1:100 000**	**1:1 000 000**	**Mean**
HSN-151	Sample 1	18.71	1.64	175
	Sample 2	16.39	1.60	162
HSN-152	Sample 1	19.24	2.05	199
	Sample 2	18.87	1.75	182
HSN-153	Sample 1	17.82	1.69	174
	Sample 2	17.70	1.56	166
			Mean	**176**

4.3.4 Hygenia

Hygenia reported use of RM 8405 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 8405 are provided in Table 18. The Veratox Hazelnut recoveries were higher than expected but may be due to calibration issues resulting from the higher than anticipated protein content of RM 8405.

Table 18. Results provided by Neogen for ELISA and lateral flow kit testing of materials spiked with RM 8405.

		Veratox Hazelnut ELISA			Reveal 3D Hazelnut Lateral Flow	
Matrix	**Sample**	**Spike mg/kg (ppm)**	**Result mg/kg (ppm)**	**Recovery**	**Spike mg/kg (ppm)**	**Result**
PBS	**Neg**	0	0.00	-	0	Negative
	RM 8405 1	5	9.79	196 %	5	Positive
		10	19.70	197 %	10	Positive
		20	35.81	179 %	1000	Positive
	RM 8405 2	5	8.02	160 %	5	Positive
		10	14.17	142 %	10	Positive
		20	31.65	158 %	1000	Positive
Rice Flour	**Neg**	0	0.07	-	0	Negative
	RM 8405 1	5	39.29	786 %	5	Positive
		10	98.20	982 %	10	Positive
		20	13.22	66 %	1000	Positive
	RM 8405 2	5	9.95	199 %	5	Positive
		10	24.11	241 %	10	Positive
		20	41.81	209 %	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 8405 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 8405

Analyte	x	$U_{95}(x)$	Units	Based on
Fat	11.64	3.27	%	HAMQAP
Protein	33.77	0.31	%	HAMQAP, Eurofins
Carbohydrates	37.63	11.85	%	HAMQAP[a]
Ash	5.85	1.22	%	HAMQAP[a]
Solids	93.73	1.67	%	HAMQAP
Calories	401.6	36.4	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 8405 with other trace allergens is summarized in Table 20. As described in Section 4.3.2, all positive tests (highlighted in bold below) are explained as known assay cross reactivity (lupin, macadamia, pecan) or lab-generated contamination (Brazil nut), and do not reflect contamination of RM 8405 with these proteins.

Table 20. Summary of all results for trace allergen contamination in RM 8405

Allergen	Presence mg/kg (ppm)	Based on
Almond	< 0.4	Eurofins Analytik GmbH ELISA
	< 0.4	R-Biopharm SureFood PCR
	< 1	BioFront MonoTrace ELISA
	< 2.5	R-Biopharm ELISA
	< 2.5	Neogen Veratox ELISA
	< 27	3M ELISA
β-Lactoglobulin	< 0.031	Eurofins Analytik GmbH ELISA
Brazil Nut	< 1	3M ELISA
	< 1	BioFront MonoTrace ELISA
	4.7	**Eurofins Analytik GmbH ELISA**
Casein	< 0.25	Eurofins Analytik GmbH ELISA
Cashew	< 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	< 0.5	Romer US-AgraQuant ELISA
	< 0.31	Eurofins Analytik GmbH ELISA
Fish	ND	Eurofins Analytik GmbH PCR
Lupin	**3.6**	**Eurofins Analytik GmbH ELISA**
	ND	Eurofins Analytik GmbH PCR
Macadamia	< 0.3	3M ELISA
	1.7	**Eurofins Analytik GmbH ELISA**
	< 2	BioFront MonoTrace ELISA
Milk	< 0.05	Romer US-AgraQuant 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
	< 1	BioFront MonoTrace ELISA
	ND	Eurofins Analytik GmbH PCR
Pecan	< 0.66	3M ELISA
	< 1	Romer US ELISA
	< 1	BioFront MonoTrace ELISA
	3.8	**Eurofins Analytik GmbH ELISA**
Pine Nut	< 1	BioFront MonoTrace ELISA
Pistachio	< 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
	< 16	Romer US-AgraQuant ELISA
	ND	Eurofins Analytik GmbH PCR
Walnut	< 0.3	Romer US-AgraQuant ELISA
	< 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 8405 into various solutions or other food products to evaluate the recovery of their assays and fitness-for-purpose of the material. Several users reported assays giving higher than expected response or reactivity. This elevated response is likely a result of the higher-than-expected protein level of RM 8405 compared to commercial hazelnut flours based on the material preparation described in Section 4.1.2. Customers of RM 8405 should be aware of this difference and instructed to use care in design and conduct of spiking studies using this material.

6 References

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