NATIONAL INSTITUTE OF STANDARDS AND TECHNOLOGY
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Reference Material 8258
Wild-caught Shrimp
REFERENCE MATERIAL INFORMATION SHEET

Purpose: This Reference Material (RM) is a fresh frozen shrimp homogenate prepared from wild-caught shrimp collected off the coast of South Carolina, USA. RM 8258 is intended to support investigations of seafood safety and seafood authenticity using genetics, crude fat, fatty acids and total protein. All constituents for which non-certified values are provided are naturally present in the homogenate.

Description: A unit of RM 8258 includes two glass jars, each containing approximately 6 g to 8 g (wet basis) of frozen tissue homogenate.

Non-Certified Values: Non-certified values are suitable for use in method development, method harmonization, and process control but do not provide metrological traceability to the International System of Units (SI) or other higher order reference systems [1]. Non-certified values were calculated where the estimated value is the mean of the measurements for that analyte, with the standard uncertainty being evaluated by the conventional Type A method [2] and the expanded uncertainty being a multiple of the standard uncertainty to achieve 95 % coverage. Non-certified mass fraction values and expanded uncertainties for crude fat and fatty acid measurements are provided in Table 1 and the non-certified mass fraction value and expanded uncertainty for crude protein is provided in Table 2.

A set of heuristic, experience-based rules were used to establish confidence estimates for the species identification of RM 8258 based on genetic sequencing methods and phylogenetic analysis (Table 3) [3].

Additional Information: Additional information is provided in Appendix A.

Period of Validity: The non-certified values are valid within the measurement uncertainty specified until **31 August 2028**. The value assignments are nullified if the material is stored improperly, damaged, contaminated, or otherwise modified.

Maintenance of Non-Certified Values: NIST will monitor this material to the end of its period of validity. If substantive technical changes occur that affect the non-certified values during this period, NIST will update this Reference Material Information Sheet. Before making use of any of the values delivered by this material, users should obtain the most recent version of this documentation, available through the NIST SRM website (https://www.nist.gov/srm).

Marcela Najarro, Acting Chief
Chemical Sciences Division

Steven J. Choquette, Director
Office of Reference Materials

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Safety: RM 8258 IS INTENDED FOR RESEARCH USE; NOT FOR HUMAN CONSUMPTION. Standard biohazard safety practices and precautions for the handling of biological tissues should be exercised.

Storage: This material has been stored at NIST at −80 °C or lower since preparation and should be stored by the user at this temperature. The material should not be allowed to thaw prior to subsampling for analysis for the non-certified values to be valid within the stated uncertainties.

Use: This material is a frozen tissue homogenate and will lose its powder-like form if allowed to warm or with extended storage at temperatures of −25 °C or higher. The following procedures and precautions are recommended when handling this material for sample preparation. If weighing relatively large quantities, pre-weigh the bottle, remove a portion of homogenate and reweigh the bottle to determine the mass of the subsample, taking care to avoid heavy frost buildup by handling the bottles quickly and wiping them prior to weighing. Subsamples of this RM for analysis should be withdrawn from the jar immediately after opening and used without delay for the non-certified values listed to be valid within the stated uncertainties. Additionally, the use of pre-chilled subsampling implements at −25 °C or lower is recommended to avoid thawing. Non-certified values provided herein may not apply to previously thawed material.

Source and Preparation: RM 8258 was prepared from wild-caught shrimp that arrived at NIST fresh and headed and were stored at −40 °C until processing. The shrimp were thawed for approximately 16 hr before being rinsed with fresh water, peeled, chopped into small pieces, refrozen and stored in liquid nitrogen (LN_2) vapor phase freezers (at or below −150 °C) until cryomilling. The material was cryomilled three times prior to bottling to generate a fresh, frozen powder homogenate and to ensure complete blending [4]. Subsamples (approximately 6 g to 8 g) were aliquoted into pre-cooled glass jars and stored at or below −80 °C.

Crude Fat Analysis: Total crude fat analysis was conducted according to AOAC Official Method 948.15 [5]. Approximately 8 g test portions taken from each of 10 jars of RM 8258 were hydrolyzed with hydrochloric acid and extracted with a combination of ethyl and petroleum ethers. The ethers containing the fat were collected and dried and the resulting extracted fat was used to calculate crude fat as a percent of the total (Table 1).

Fatty Acid Analysis: Fatty acid analysis was conducted according to AOAC Official Method 996.06 [6]. Approximately 6 g test portions taken from each of 6 jars of RM 8258 underwent hydrolytic extraction and methylation. Value assignment of mass fractions of individual fatty acids in RM 8258 was conducted using gas chromatography with flame ionization detection (GC-FID) of the resulting fatty acid methyl esters (FAMEs). Each measured fatty acid mass fraction was then converted to its free fatty acid equivalent (Table 1).

Crude Protein Analysis: Crude protein analysis was conducted according to AOAC Official Method 968.06 [7]. Approximately 0.2 g to 0.3 g test portions taken from each of 3 jars of RM 8258 underwent combustion at or greater than 850 °C, in oxygen with quantitation by thermal conductivity of nitrogen. Nitrogen content was converted to crude protein using a conversion factor of 6.25 (Table 2).

Genetic Analysis: A Next Generation DNA Sequencer was used to sequence the genome of RM 8258 and a reference DNA sequence library, sourced from the National Center for Biotechnology Information (NCBI) and other public access domains, was used for species authentication. This analysis indicated the presence of two different species in the material, *Farfantepenaeus aztecus* (brown shrimp) and *Litopenaeus setiferus* (Atlantic white shrimp). NIST was provided with the FASTQ file and the reference sequences to develop a novel method for calculating confidence estimates around species identification. Briefly, reference sequences of nucleotides from the cytochrome c oxidase subunit 1 mitochondrial gene (CO1) were used as genetic barcodes that serve as digital proxies for the holotypes of each of the two species represented in RM 8258. Short-reads described in the FASTQ files were matched against the reference sequences using MUSCLE (multiple sequence alignment with high accuracy and high throughput) [8]. For each short-read (33,277 total) the longest block of contiguous loci that matched to the U.S. Food and Drug Administration (FDA) Reference Standard Sequence Library (RSSL) National Seafood Inspection Laboratory (NSIL) sequence NSILBS-16 (for *F. aztecus*) [9] and the University of Louisiana at Lafayette (ULL) sequence ULLZ-11629 (for *L. setiferus*) [10] was determined. For each species, a single short-read was chosen from those that achieved at least 50 % coverage (proportion of loci of the reference that were matched) to serve as identifier that a.) achieved the highest coverage, b.) had a relative matching error (evaluated using the Damerau-Levenshtein distance) of less than 5 %, and c.) had a confidence of at least 95 %. NIST is Most Confident (0) that the biological species in RM 8258 are *Farfantepenaeus aztecus* (brown shrimp) and *Litopenaeus setiferus* (Atlantic white shrimp).

Additionally, phylogenetic analysis was performed by extracting DNA from RM 8258 and amplifying the mtDNA 16S region for sequencing. This DNA sequence is considered the source of “comparability of identity” for shrimp species. 16S mtDNA sequences from authenticated brown shrimp samples are used to establish inclusivity;

16S mtDNA sequences from close relatives are used to establish exclusivity. The 16S sequences from RM 8258 were aligned with a proprietary reference database and identifications were made based on sequence similarity and phylogeny reconstruction. The evolutionary history was inferred using the Neighbor-Joining method [11] and the optimal tree is shown in Figure 1. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches [12]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method [13] and are in the units of the number of base differences per site. This analysis involved 103 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Non-coding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There was a total of 581 positions in the final dataset. Evolutionary analyses were conducted in MEGA X [14]. The sequences for RM 8258 clustered with *Farfantepenaeus aztecus* (brown shrimp) clade in 100 % of the bootstrap replicates. The confidence estimate for the species identification of RM 8258 as *Farfantepenaeus aztecus* (brown shrimp) is therefore Most Confident (0).

Table 1. Non-Certified Values for Fatty Acids (as Free Fatty Acids) and Crude Fat in RM 8258

Analyte	Common Name	Mass Fraction[(a)] (g/100 g)
Hexadecanoic acid (C16:0)	Palmitic acid	0.074 ± 0.007
Hexadecenoic acid (C16:1)	Palmitoleic acid	0.028 ± 0.002
Octadecanoic acid (C18:0)	Stearic acid	0.063 ± 0.004
Octadecenoic acid (C18:1)	Oleic acid	0.041 ± 0.011
(Z,Z,Z,Z)-5,8,11,14-Eicosatetraenoic acid (C20:4n-6)	Arachidonic acid	0.039 ± 0.002
(Z,Z,Z,Z,Z)-5,8,11,14,17-Eicosapentaenoic acid (C20:5n-3)	EPA	0.083 ± 0.004
(Z,Z,Z,Z,Z,Z)-4,7,10,13,16,19-Docosahexaenoic acid (C22:6n-3)	DHA	0.063 ± 0.005
Monounsaturated Fat		0.069 ± 0.012
Omega-3 fatty acids		0.146 ± 0.009
Omega-6 fatty acids		0.039 ± 0.002
Polyunsaturated Fat		0.185 ± 0.011
Saturated Fat		0.137 ± 0.010
Total Fat		0.390 ± 0.030
		%
Crude Fat		0.721 ± 0.050

[(a)] The assigned value is the average of the values measured for the analyte, and the corresponding expanded uncertainty listed here is a suitable multiple of the standard uncertainty to achieve 95 % coverage.

Table 2. Non-Certified Value for Crude Protein in RM 8258

Analyte	Percent[(a)] (%)
Total Protein	21.63 ± 0.14

[(a)] The assigned value is the average of the values measured for the analyte, and the corresponding expanded uncertainty listed here is a suitable multiple of the standard uncertainty to achieve 95 % coverage.

Table 3. Definitions of Heuristic Rules for Confidence Estimates of Species Identity

Confidence Level	***Species Identity***
Most Confident (0)	Have very well-supported and well-resolved phylogeny and/or multiple diagnostic nucleotides differentiating species from closest relatives; have data from multiple samples of both an inclusivity and exclusivity panel; data from multiple independent gene regions agree; ≥95 % confidence based on FASTQ file analysis.
Very Confident (1)	Have reasonably well-supported and well-resolved phylogeny and/or a few diagnostic nucleotides differentiating species from close relatives; have data from multiple samples of both an inclusivity and exclusivity panel; data from one gene, or data from multiple independent gene regions agree, <95 % but ≥90 % confidence based on FASTQ file analysis.
Confident (2)	Have a reasonably well-supported and well-resolved phylogeny and/or one or a few diagnostic nucleotides differentiating species from close relatives; have data from a few samples of both an inclusivity and exclusivity panel; data from one gene or data from multiple independent gene regions generally agree, <90 % but ≥80 % confidence based on FASTQ file analysis.
Ambiguous (3)	Have a poorly supported and poorly resolved phylogeny and/or no diagnostic nucleotides differentiating species from close relatives; have data from a few or multiple samples of both an inclusivity and exclusivity panel; data from one gene, or data from multiple independent gene regions generally disagree, <80 % confidence based on FASTQ file analysis.

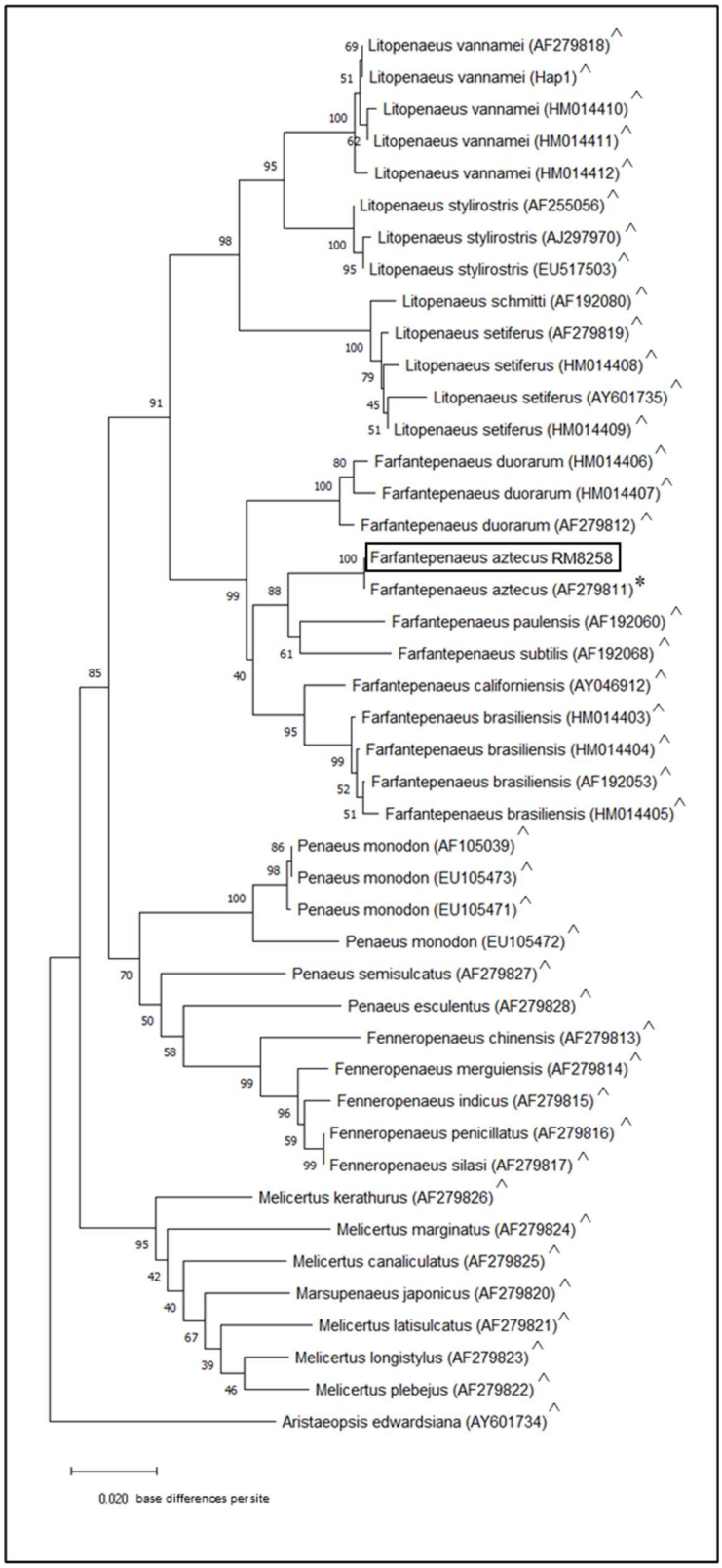


Figure 1. Phylogeny reconstruction results for RM 8258. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The scale (0.020) represents the number of base differences per site, which is the proportion of nucleotide sites at which the two sequences compared are different (i.e., number of nucleotide differences/total number of nucleotides compared). The taxon names followed by an "^" indicate the Exclusivity Panel samples while those followed by an "*" indicate the Inclusivity Panel samples. The confidence estimate for the species identification of RM 8258 as *Farfantepenaeus aztecus* is Most Confident (0) and is indicated by the black box labeled "Farfantepenaeus aztecus RM8258".

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If you use this RM in published work, please reference:

Ellisor DL, Place B, Phillips M, Yen J (2021) Analysis of Seafood Reference Materials: RM 8256, RM 8257, RM 8258, and RM 8259, Wild-Caught Coho Salmon (RM 8256), Aquacultured Coho Salmon (RM 8257), Wild-Caught Shrimp (RM 8258), Aquacultured Shrimp (RM 8259). (National Institute of Standards and Technology, Gaithersburg, MD), NIST Special Publication (SP) 260-214. https://doi.org/10.6028/NIST.SP.260-214.

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Certain commercial equipment, instruments, or materials may be identified in this Reference Material Information Sheet to adequately specify the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

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APPENDIX A

Responsibilities for RM 8258:

Coordination of technical measurements was provided by D. Ellisor of the NIST Chemical Sciences Division.

Material acquisition was coordinated by D. Ellisor of the NIST Chemical Sciences Division and B.T. Knott of the NOAA Northwest Fisheries Science Center Forensics Laboratory.

Material production was performed by D. Ellisor, J. Hoguet, A. Moors, J. Ness, R. Pugh, and J. Ragland of the NIST Chemical Sciences Division.

Fat measurements were coordinated by D. Ellisor and B. Place of the NIST Chemical Sciences Division.

Total protein measurements were coordinated by D. Ellisor and M. Phillips of the NIST Chemical Sciences Division.

Genetic measurements were coordinated by C. Rimmer and B. Place of the NIST Chemical Sciences Division.

Genetic measurements were completed by B.T. Knott of the NOAA Northwest Fisheries Science Center Forensics Laboratory.

Statistical consultation and analyses were performed by J. Yen and A. Possolo of the NIST Statistical Engineering Division.

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