

Statement of measurement



4005

Allergen kit – Milk, Egg, Almond, Hazelnut and Walnut Reference Material LGC746-KT

Assessed Values

Material number	Description	Property	Content g/100 g	Uncertainty ³ g/100 g	k ⁴
LGC7421	Skimmed milk powder	Nitrogen ¹	5.40	0.17	2
		Water ²	4.22	0.40	2
LGC7422	Egg white powder	Nitrogen ¹	13.49	0.41	2
		Water ²	6.01	0.53	2
LGC7424	Almond powder	Nitrogen ¹	4.19	0.13	2
		Water ²	4.22	0.45	2
LGC7425	Hazelnut powder – partially defatted	Nitrogen ¹	4.99	0.16	2
		Water ²	8.6	1.1	2.03
LGC7426	Walnut powder – partially defatted	Nitrogen ¹	6.15	0.19	2
		Water ²	6.11	0.65	2

Material number	Description	Property	Content mg/kg allergen protein	Uncertainty mg/kg allergen protein ³	k ⁴
LGC7461	Chocolate paste – no added allergenic ingredients	Milk protein ⁵	<0.05	-	-
		Egg white protein ⁵	<0.05	-	-
		Hazelnut protein ⁵	<0.04	-	-
LGC7462	Chocolate paste with added allergenic ingredients	Milk protein ⁶	10.0	1.8	2.06
		Egg white protein ⁶	10.0	1.5	2.04

Notes:

1. Determined by an automated Dumas procedure. See later section for metrological traceability.
2. Determined by oven Karl Fischer titration. See later section for metrological traceability.
3. The quoted uncertainty is the half-width of the expanded uncertainty interval calculated using a coverage factor ⁴, which gives a level of confidence of approximately 95 %.
4. Coverage factor.
5. Based on analysis using a single ELISA platform. See later section for metrological traceability.
6. Based on the gravimetric preparation and the calculated values for protein content. See later section for metrological traceability.

Date of Issue: December 2019

Updated: September 2020

Signed: _____

Gill Holcombe (Mrs)
for the Government Chemist

Material number: LGC746-KT

Batch number: 1

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Indicative Values

Material number	Description	Property	Content mg/kg allergen protein ¹	Uncertainty mg/kg allergen protein ²	k ³
LGC7462	Chocolate paste with added allergenic ingredients	Almond protein	9.7	1.9	2.11
		Hazelnut protein	9.8	+10.5/-5.1	2.97
		Walnut protein	10.0	2.3	2.12

Notes:

- Based on the gravimetric preparation data.
- The quoted uncertainty is the half-width of the expanded uncertainty interval calculated using a coverage factor ³, which gives a level of confidence of approximately 95 %.
- Coverage factor

Additional information

The results obtained by analysis using various commercial ELISA kits are given below, for information. In most cases the mean result measured is lower than the prepared concentration, which is not unexpected. For example, for ELISAs calibrated using 'whole' hazelnuts (not defatted), low recovery from partially defatted nut powders is well known, and may be related to the impact of lipid removal on proteins. The table shows the range of individual results obtained; however this should be used with caution, as the range is likely to be less where a small number of units are examined, and a user may obtain results outside this range in their laboratory.

LGC7462 Chocolate paste with added allergenic ingredients						
Allergen	Number of units examined	Replication	Number of runs	Study design		Range of individual results mg/kg ⁶
Milk protein ¹	15	triplicate	3	Randomised block design	Kit A	3.9 – 7.5
Milk protein	6	duplicate	1	Single run	Kit B	11.0 – 16.8
Egg white protein	15	triplicate	3	Randomised block design	Kit A	4.2 - 8
Egg white protein ²	6	duplicate	1	Single run	Kit B	7.8 – 13.1
Hazelnut protein ⁴	15	triplicate	3	Randomised block design	Kit A	0.9 - 5.0
Hazelnut protein ⁴	4	triplicate	1	Single run	Kit B	1.2 – 3.5
Almond protein ³	16	duplicate	2	Nested design	Kit A	7.4 – 13.9
Walnut protein ⁵	16	duplicate	2	Nested design	Kit A	0.5 – 1.0

Notes:

- Where applicable, milk protein was calculated from the measured casein content using a conversion factor of 1.25
- Where applicable, egg white protein was calculated from whole egg powder content using a conversion factor of 0.263
- Calculated from the measured almond content using a conversion factor of 0.211
- Calculated from the measured hazelnut content using a conversion factor of 0.141
- Calculated from the measured walnut content using a conversion factor of 0.1523
- Not within LGC's scope of accreditation to ISO 17034

Calculated values

Material number	Description	Protein Content ¹ g/100 g	Uncertainty ² g/100 g	Nitrogen factor ³
LGC7421	Skimmed milk powder	34.4	1.1	6.38
LGC7422	Egg white powder	84.3	2.6	6.25
LGC7424	Almond powder	21.24	0.68	5.18
LGC7425	Hazelnut powder – partially defatted	25.86	0.85	5.30
LGC7426	Walnut powder – partially defatted	32.6	1.0	5.30

1. Calculated using the nitrogen content from Page 1 and the nitrogen factor³
2. Calculated using the uncertainty value from Page 1 and the nitrogen factor³
3. Food Standards Agency (2002) McCance and Widdowson's The Composition of Foods, Sixth, summary edition. Cambridge: Royal Society of Chemistry

The calculated values table has been included for information. Whilst it is known that the nitrogen factor will vary according to the source of the ingredient, no allowance associated with the uncertainty of the factor (which is known to be non-zero) has been included in the calculation of the protein content and its uncertainty, nor has this source of uncertainty been included in the uncertainty for the assessed and indicative values for allergen proteins. Users may wish to include their own estimates of this source of uncertainty.

Kit contents

Each kit contains:

- One vial of each:
LGC7421 Skimmed milk powder
LGC7422 Egg white powder
LGC7424 Almond powder
LGC7425 Hazelnut powder – partially defatted
LGC7426 Walnut powder – partially defatted
- Five bottles of:
LGC7461 Chocolate paste – no added allergenic ingredients
LGC7462 Chocolate paste with added allergenic ingredients

Material sourcing and preparation

LGC7421, LGC7422, LGC7424, LGC7425 and LGC7426

The raw materials were sourced by the University of Manchester from reputable suppliers to minimise the risk of contamination, and were described as follows:

Material number	Description
LGC7421	Organic skimmed milk powder. Produced in Austria from Austrian or EU pasteurised, skimmed milk.
LGC7422	Origin: Austria
LGC7424	Origin: California, USA. Blanched ground almonds.
LGC7425	Origin: South Island New Zealand <i>Corylus avellana</i> . Fine ground flour produced from the "cake" after the oil (fats) have been cold pressed out of raw hazelnut.
LGC7426	Origin: Italy. <i>Juglans regia</i> cultivar Lara. Lipid content 27 g/100g

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LGC7421, LGC7422, LGC7424, LGC7425 and LGC7426

LGC7426 was prepared by grinding, using a centrifugal mill, to pass a 0.5 mm sieve. All other materials were packaged as received.

For all five products, the material was combined and mixed before weighing in (1.1 ± 0.1) g portions into amber glass vials. The vials were closed under argon with a rubber stopper and a crimp cap. Each unit was sealed inside a metallised sachet to prevent changes in water content. 550 units were packaged for each material and stored at (5 ± 4) °C.

LGC7461

A chocolate dessert paste was prepared, based on the recipe described by Cochrane *et al.* [1].

The ingredients used were cold swelling starch, cocoa powder, icing sugar, maize oil and emulsifier (Polysorbate 60). The paste was mixed well and weighed into amber glass bottles in 5 g (minimum) portions. Each bottle was closed using a tamper-evident screw-cap with integral plug. 3000 units were prepared and stored at (5 ± 4) °C.

LGC7462

A chocolate dessert paste was prepared, based on the recipe described by Cochrane *et al.* [1]. The ingredients used were as for LGC7461. The paste was mixed well and used as the base for the material with added allergen ingredients. LGC7462 was prepared in a three stage process by serial dilution of a high concentration paste with 'blank paste'. LGC7424 and LGC7425 were sieved before use and the portion passing a 0.5 mm sieve was used to prepare LGC7462. Each level of material was mixed thoroughly before the next level was prepared. The final paste was mixed well and weighed into amber glass bottles in 5 g (minimum) portions. Each bottle was closed using a tamper-evident screw-cap with integral plug. 3000 units were prepared and stored at (5 ± 4) °C.

Homogeneity Assessment

LGC7421, LGC7422, LGC7424, LGC7425 and LGC7426

Homogeneity was assessed for nitrogen (which is assumed to arise mainly from the protein present) and water content. For the procedure used, please see the section entitled 'Analytical Methods Used'. In each case, fifteen units were analysed in triplicate over three runs using a randomised block design.

LGC7461

Homogeneity was assessed at LGC using a single ELISA platform. Thirty units were analysed singly in two runs using a randomised run order.

LGC7462

Homogeneity was assessed using a single ELISA platform under ISO/IEC 17025 testing accreditation.

Stability Assessment

A short-term stability study was carried out on LGC7462. Material was stored at nominal temperatures of 5 °C, 18 °C, 37 °C and 60 °C for periods of 1 day, 7 days and 14 days and then analysed for casein, egg white protein and hazelnut protein content using a single ELISA platform. There was no evidence of instability for casein, egg white protein and hazelnut protein when stored at 5 °C or 18 °C for up to 14 days.

A long-term stability study was carried out on LGC7462. Material was stored at nominal temperatures of 5 °C, 18 °C, and -20 °C for periods of 1 day, 1 month, 2 months and 3 months and then analysed for casein, egg white protein and hazelnut protein content using a single ELISA platform. There was no evidence of instability for casein, egg white protein and hazelnut protein when stored at 5 °C for 3 months. However, for egg white protein, there was marginal statistical evidence of instability at 18 °C (p -value=0.0225).

Deterioration is not expected over the lifetime of the kit when stored under the recommended conditions; however LGC746-KT will be subjected to testing under LGC's stability monitoring programme and purchasers will be informed of any changes to the assessed values.

Characterisation

This reference material kit has been prepared by LGC and characterised as described below.

Analytical Methods Used

LGC7421, LGC7422, LGC7424, LGC7425 and LGC7426

Nitrogen was determined at LGC using an automated Dumas procedure using a rapid N cube nitrogen analyser (Elementar Analysensysteme GmbH) which is within LGC's scope of accreditation to ISO/IEC 17025 for testing. The instrument was calibrated using an EDTA Organic Analytical Standard which has a certified value for nitrogen traceable to NIST SRM 143d.

Water was determined by oven Karl Fischer (KF) coulometry using a Metrohm 774 oven sample processor and 831 KF coulometer. This method is within LGC's scope of accreditation to ISO/IEC 17025 for calibration.

DNA sequencing

Purified DNA that had been extracted from the almond, hazelnut and walnut candidate reference material samples prior to inclusion in the nut allergens reference material, was subject to testing for species identity using a Sanger DNA sequencing-based approach.

Pairs of species-specific oligonucleotides were used to direct the PCR amplification of polymorphic regions for the plastid *matK* and *rbcL* loci for each of the samples. Sanger DNA sequencing was performed on each of the amplified products in both forward and reverse directions.

The resulting DNA sequence information, consisting of sequence read lengths between 328bp – 979bp, were evaluated for sequence identity by comparison to annotated DNA sequence data using the BLASTn (v. 2.9.0) sequence alignment software accessible at the National Centre For Biotechnology Information, Washington, USA (NCBI).

All of the analysed sequences exhibited at least 99.04 % sequence identity with DNA sequences for the respective nut species (*Corylus avellana*, *Prunus dulcis* and *Juglans regia*) accessible at the NCBI GenBank database (accessed 06/09/2019). Corresponding E-values (probability that a score as high as the ones observed between two DNA sequences arising by chance alone) were equal to or less than 10^{-155} .

Proteomics

These materials were also characterised at the University of Manchester, UK, by a combination of advanced proteomic techniques including gel electrophoresis, immune-based analysis and mass spectrometry, confirming the presence of the relevant allergen protein molecules.

LGC7461

The homogeneity assessment described above was also used for the purpose of characterisation of the material.

LGC7462

The assessed value for each allergen protein was determined using the gravimetric preparation details and the nitrogen value determined for the allergen ingredient, converted to protein. The uncertainty of the assessed value was calculated by combining the gravimetric uncertainty with a contribution for the nitrogen content uncertainty and homogeneity uncertainty.

Conversion factors

Conversion from	Conversion to	Factor
Casein	milk protein	1.25
Hazelnut	hazelnut protein	0.141
Almond	almond protein	0.211
Walnut	walnut protein	0.1523

The information above has been derived from Pearson's Composition and Analysis of Foods, Ninth edition. Longman Group UK Ltd. 1991, Food Standards Agency (2002), McCance and Widdowson's The Composition of Foods, Sixth summary edition. Cambridge: Royal Society of Chemistry, or from ELISA kit inserts.

Intended Use

The allergen food ingredients (LGC7421, LGC7422, LGC7424, LGC7425 and LGC7426) are intended for use in method development: e.g. allergen kit calibrator extract solutions, method validation: e.g. external check calibrator extract solutions, and recovery estimates e.g. by spiking food matrices for which no RMs are available. They can also be used in the quality control of methods for the determination of nitrogen and water in food ingredients and processed food products.

The blank matrix (LGC7461) is intended for use (a) as a 'no-template' control to provide assurance of absence of in-lab allergen cross contamination (either environmentally, from personnel, or in reagents) and (b) a material to assist method limit of detection calculation (as 3.3 times the standard deviation of a 'blank' dataset).

The incurred matrix (LGC7462) is intended for use (a) to optimise analytical recovery from a chocolate-type matrix, (b) inform risk assessors of the possible 'true' estimate of allergen in a questioned product, and (c) in checking in-house quality control materials.

Metrological Traceability

For LGC7421, LGC7422, LGC7424, LGC7425 and LGC7426, the values for nitrogen and water are traceable to the SI.

For nitrogen, the instrument was calibrated using an EDTA Organic Analytical Standard (Elemental Microanalysis Ltd., Devon, UK) which has a certified value for nitrogen traceable to NIST SRM 143d (Gaithersburg, USA), which in turn is traceable to the SI.

The value for water content by coulometric Karl Fischer titration is traceable to the SI via (a) the use of coulometric titration equipment certified for performance using electrical test equipment calibrated and traceable to national and/or international standards (Certificate of Performance no. 1831001014106), (b) the use of certified reference materials for performance verification and quality control, and (c) the use of calibrated balances for mass determination.

The assessed values for LGC7462 are traceable to the SI unit of the kg through the gravimetric preparation of the material.

Accreditation

All values are within LGC's scope of accreditation to ISO 17034, unless otherwise marked.

Instructions for Use

For LGC7421, LGC7422, LGC7424, LGC7425 and LGC7426, the sample should be brought to room temperature before opening. The contents of the vial should be mixed using a clean spatula, being careful to avoid contamination. The sample portion should be removed and used rapidly to prevent changes in the water content. Opened vials should not be stored for reuse.

For LGC7461 and LGC7462, the sample should be brought to room temperature before opening. The contents of the bottle should be mixed using a clean spatula or similar tool, being careful to avoid contamination. Opened bottles may be stored in a fridge at (5 ± 4) °C and used within one week.

The minimum recommended sample size is based on the amount of material taken for the homogeneity assessment and is shown below:

- 0.25 g for nitrogen in LGC7421, LGC7422, LGC7424, LGC7425 and LGC7426
- 0.01 g for water in LGC7421, LGC7422, LGC7424, LGC7425 and LGC7426
- 1 g for milk protein, egg white protein and hazelnut protein in LGC7461 and LGC7462

Use in allergen analysis should follow published guidance.

Storage Conditions

The kit should be stored unopened at (5 ± 4) °C.

Shelf Life

This statement is valid for 6 months from the date of shipment provided the kit is stored unopened under the recommended conditions.

Acknowledgements

The University of Manchester School of Biological Sciences, Division of Infection, Immunity and Respiratory Medicine, together with Romer Laboratories, are thanked for their advice and technical contribution to the production of this material.

References

[1] Cochrane *et al.*, *Allergy* **67** (2012) 107-113

Document Revision

In September 2020, this document was updated to confirm the reference material kit is now within LGC's scope of accreditation to ISO 17034 as a reference material producer.

Kit Number

Date of Shipment

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