Final Report

CCQM–K62: Nutrients in Infant/Adult Formula – Vitamins

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ABSTRACT

Key Comparison CCQM-K62 was designed to enable demonstration of the equivalence in capabilities for measurement of vitamins in a food matrix. A milk-based fortified human infant/adult formula was selected as the matrix based upon material availability and relevance. Because vitamins were added to the CCQM-K62 study material in a single form and at levels significantly higher than those that would be naturally occurring in the milk base, the ability of a laboratory to measure the study vitamins is only indicative of a laboratory's ability to measure vitamins in fortified foods. Target analytes were selected for study because of the ready availability of suitable standard materials and the range of their chemical properties: folic acid (vitamin B9) is a single water-soluble molecular entity that typically occurs at low levels and can be unstable, niacin (vitamin B3) is a single stable molecular entity and is typically present at higher concentrations than the other water-soluble vitamins, vitamin A has multiple molecular forms (including retinol and retinyl palmitate), is fat-soluble and typically occurs at relatively high levels. Results for participants measuring only folic acid or niacin are only indicative of their ability to make that measurement; results for participants measuring both folic acid and niacin are indicative of a laboratory's ability to measure folic acid, thiamine, niacin, and riboflavin in fortified foods but not vitamin C or other water-soluble vitamins. The ability to measure vitamin A (reported as retinol equivalents) in this material is also indicative of the participant's ability to measure vitamin E (as alpha-tocopherol and alpha-tocopheryl acetate) but is not indicative of the ability to measure vitamins D and K, which typically occur at much lower concentrations. The relative degrees of equivalence of the reported measurements for all three analytes in CCQM-K62 were within 10%; however, since only two results were submitted for niacin and vitamin A and one of the three results for folic acid was withdrawn, no strong conclusions as to the expected comparability of higher-order vitamin measurements can be established.

INTRODUCTION

At the April 2005 CCQM Organic Analysis Working Group (OAWG) Meeting in Sévres, France, measurement of vitamins in food was identified as a pilot study; NIST's candidate Standard Reference Material (SRM) 1849 Infant/Adult Nutritional Formula was chosen as the food matrix to be tested. At the CCQM OAWG meeting in September 2005 (Geel, Belgium), a B vitamin, vitamin A or E, and folic acid were discussed as analytes to be measured. Niacin, vitamin A, and folic acid were ultimately selected for determination, based on the ready availability of pure standards and stable isotope-labeled standards (for use as internal standards in mass spectrometric methods).

The OAWG conducted CCQM-P78 in July 2006, in which six laboratories received samples and five laboratories returned data. Results were reported in September 2006. The OAWG deemed the results of the pilot acceptable, and proceeded to a key comparison. The study sample was a material related to SRM 1849. Samples for CCQM-K62 were distributed in September 2007, with results due February 15, 2008. Three laboratories participated in the key comparison. Only NIM and NIST reported results for all three vitamins.

MATERIALS AND CONDUCT OF STUDY

The CCQM-K62 study material was a spray-dried milk-based hybrid of infant and adult nutritional formulas, packaged in nitrogen-flushed foil pouches with a paper over-wrap in 10 g quantities. Along with many of the ingredients currently found in a typical milk-based infant formula in the U.S. (e.g., vitamins, long-chain polyunsaturated fatty acids, nucleotides, elements), the material also contains carotenoids, fructooligosaccharides, and elements such as Se that would not be added to infant formula but might be commonly found in adult nutrition products. Vitamin A was added in the form of retinyl palmitate, niacin was added as niacinamide, and folic acid was added as folic acid. This material is similar in composition to the pilot material (SRM 1849 Infant/Adult Nutritional Formula), but is not the same material. SRM 1849 was provided for participants to use as a quality control material if they chose to do so. Both materials were stored by NIST at -80 °C to enhance long-term stability, but study samples were shipped at room temperature to facilitate shipment and expedite receipt. Participants were advised to store material at 0 °C or colder upon receipt.

Prior to distribution, NIST analyzed the material for vitamin A and folic acid. No significant variability among packets was observed.

Each participant received twenty packets of the study material – five packets for practice, five packets for determination of vitamin A, five packets for determination of niacin, and five packets for determination of folic acid. Participants were asked to measure each vitamin individually in a single test portion from each of the five packets and to report five individual results for each of the three vitamins. Results for vitamin A were requested as retinol equivalents without the inclusion of any vitamin A contribution from beta-carotene. Participants were expected to use their usual methods of sample

preparation and analysis, which potentially included liquid chromatography with absorbance or mass spectrometric detection. NIST has developed methods using stable isotopally-labeled analogues as internal standards in the liquid chromatography/mass spectrometry measurement of retinyl palmitate (vitamin A palmitate), niacin, and folic acid. Sources of these stable isotopically labeled vitamins were identified in the original study proposal and in the cover letter that accompanied study samples.

RESULTS

Results were discussed at the OAWG meeting in April 2008 in Sévres. Results for retinol and niacin were considered to be in reasonable agreement, but the NIST results for folic acid did not adequately agree with those reported by KRISS and NIM. *After investigating potential sources of bias, NIST found that the purity correction for their folate standard did not include a moisture correction. The original reported purity was 99.7 %; the moisture-corrected purity was 91.8 %.* NIST agreed to the exclusion of its folic acid results from the Key Comparison Reference Value (KCRV) estimation. The reported values for all three CCQM-K62 analytes are summarized in Table 1.

KCRV and U_{95}(KCRV)

Given only two eligible results for each of the analytes, the OAWG agreed to estimate each of the KCRVs as the arithmetic mean, and the standard uncertainty of the KCRV, u(KCRV), by combining the standard deviation, SD, of the results and the pooled value of their reported uncertainties, $\overline{u} = \sqrt{\sum_{i=1}^{n} u^2(\text{Value}_i)} \Big/ n$: $u(\text{KCRV}) = \sqrt{\text{SD}^2 + u_c^2} \Big/ \sqrt{n}$

where n is the number of results. Using the conventional coverage factor of k=2, the approximate 95 % expanded uncertainty of the KCRV is U_{95}(KCRV) = 2×u(KCRV). These estimates are listed in Table 1.

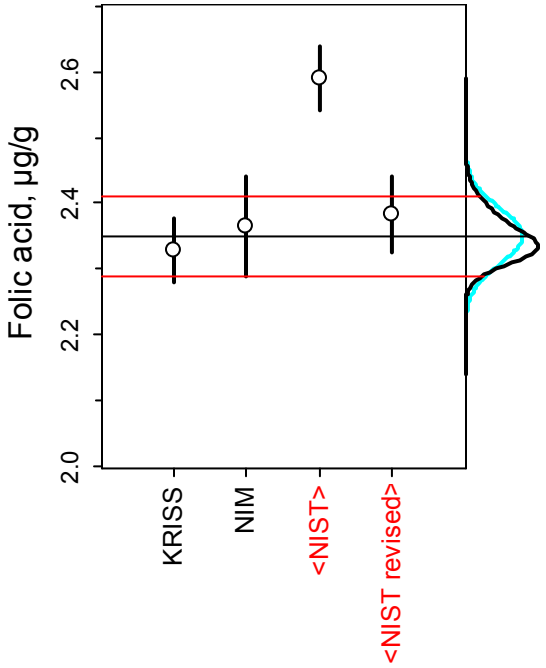
Table 1. Reported Results and Summary Statistics for CCQM-K62.

	Folic Acid				Niacin			Vitamin A as Retinol		
Participant	Value	u	U_{95}	Purity	Value	u	U_{95}	Value	u	U_{95}
KRISS	**2.329**	**0.024**	**0.049**	90.6						
NIM	**2.364**	**0.038**	**0.076**	91.8	**107.0**	**1.1**	**2.1**	**16.66**	**0.50**	**1.01**
NIST	2.590	0.024	0.048	99.7	**106.4**	**3.2**	**6.5**	**15.52**	**0.29**	**0.57**
NIST revised	2.380	0.030	0.060	91.8						
n	2				2			2		
Mean	2.347				106.70			16.09		
SD	0.025				0.42			0.81		
$\overline{u}$		0.032				2.39			0.41	
KCRV	2.35	0.03	0.06		106.7	1.7	3.4	16.1	0.6	1.3

Results are presented as the reported mean value (Value), standard uncertainty (u), and expanded uncertainty using k=2 (U_{95}); all results are in units of μg/g. The purity for the folic acid standard (Purity) is in %. Only results shown in bold are included in the summary statistics.

Figure 1 displays the reported results and the KCRV ±U_{95}(KCRV) for folic acid. Figure 2 displays the reported results and the KCRV ±U_{95}(KCRV) for niacin and vitamin reported as retinol.

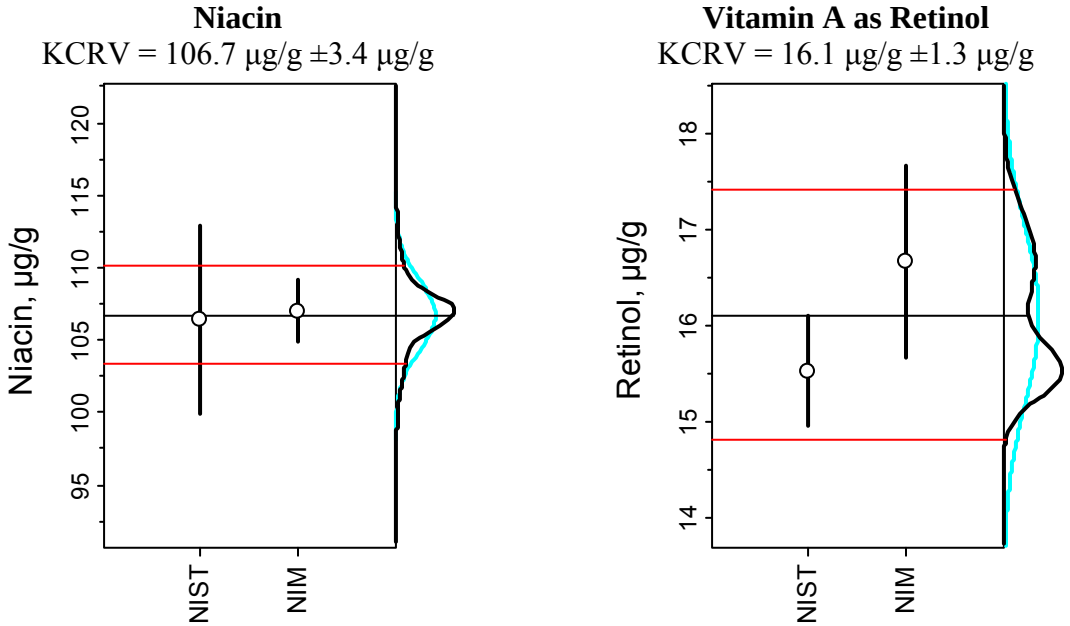
Figure 1. CCQM-K62 Nutrients in Infant/Adult Formula:
Reported Results for Folic Acid. KCRV = 2.35 µg/g ±0.06 µg/g



Error bars represent U_{95} expanded uncertainties as reported by participants. Results labeled in <red> are not used in the assignment of the KCRV. The black horizontal line represents the proposed KCRV; the red lines bound the proposed KCRV ±U_{95}(KCRV) interval. The black curve to the right edge is the empirical probability density function for the values used to assign the KCRV. The blue curve to the right is the Gaussian distribution having mean = KCRV and SD = U_{95}(KCRV)/2.

The mass-fraction axis in this and both subplots of Figure 2 spans ±15 % of the KCRV.

Figure 2. CCQM-K62 Nutrients in Infant/Adult Formula:
Reported Results for Niacin and Vitamin A as Retinol.



Degrees of Equivalence

The Degree of Equivalence, D_i, between the i^{th} reported value and the KCRV is the difference between the reported value and the KCRV: $D_i = \text{Value}_i - \text{KCRV}$. Following current OAWG procedure, the uncertainty of D_i, $u(D_i)$, is estimated as $u(D_i) = \sqrt{u^2(\text{KCRV}) + u^2(\text{Value}_i)}$. By convention, the expanded uncertainty of the D_i at the 95 % level of confidence is twice $u(D_i)$: $U_{95}(D_i) = 2 \times u(D_i)$. Figures 3 to 5 provide these values for the three analytes in graphical format.

Figure 3. CCQM-K62 Nutrients in Infant/Adult Formula:
Degrees of Equivalence for Folic Acid.

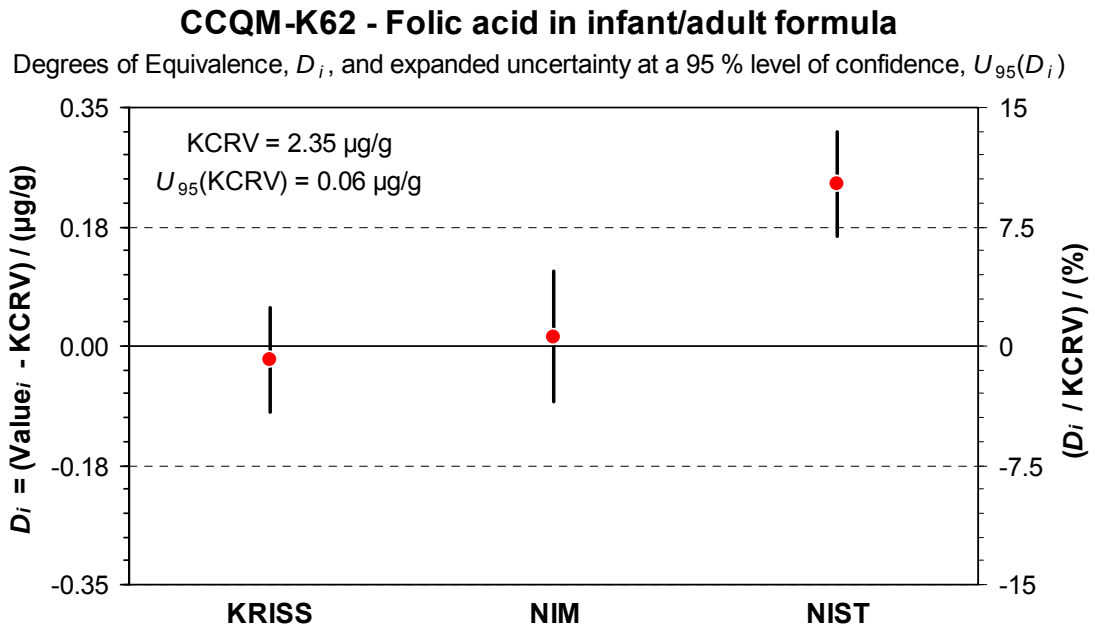


Figure 4. CCQM-K62 Nutrients in Infant/Adult Formula:
Degrees of Equivalence for Niacin.

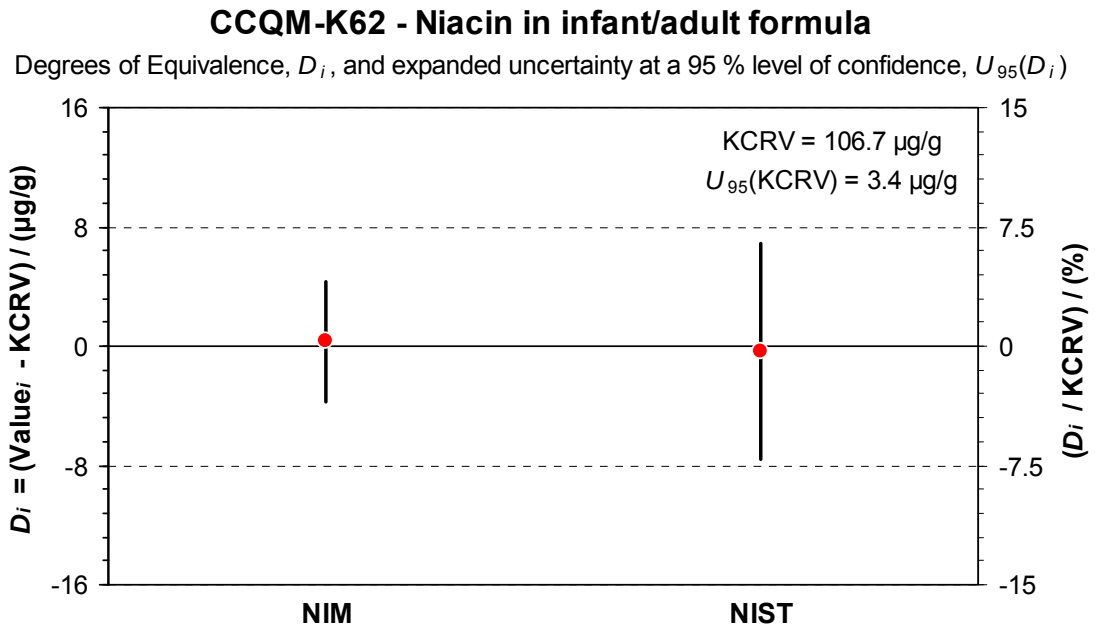
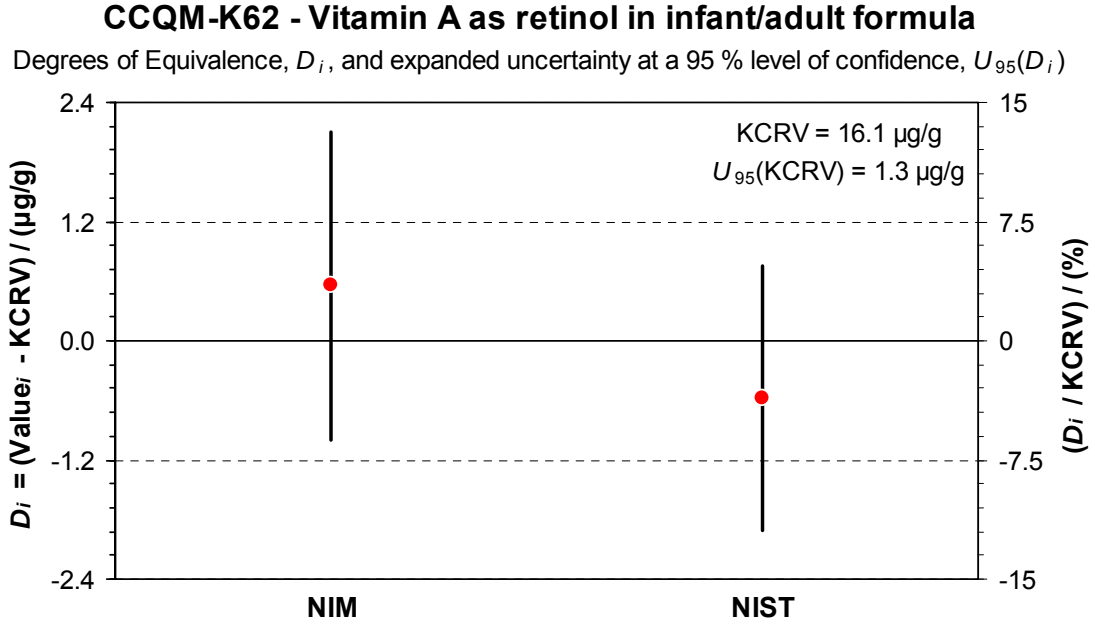


Figure 5. CCQM-K62 Nutrients in Infant/Adult Formula:
Degrees of Equivalence for Vitamin A as Retinol.



ANALYTICAL METHODOLOGY

A summary of methods employed is provided in Table 2.

Table 2. Summary of analytical methodology.

Participant	Folic Acid	Niacin	Vitamin A as Retinol
KRISS	1 g, extraction, IS, LC/MS/MS		
NIM	2 g, extraction, IS, LC/MS/MS	2 g, extraction, IS, LC/MS	2 g, saponification, IS, LC/MS
NIST	1 g, extraction, IS, LC/MS/MS	2 g, extraction, IS, LC/MS	2 g, extraction, IS, LC/MS

ES = external standard, IS = internal standards, LC = liquid chromatography, IEC = ion exchange chromatography, fluor = fluorescence detection, UV = ultraviolet absorbance detection, MS = mass spectrometric detection, MS/MS = tandem mass spectrometric detection.

UNCERTAINTY COMPONENTS

Laboratories calculated their own uncertainties. The components included in the uncertainty calculations are as follows:

<u>Uncertainties for Folic Acid:</u>

KRISS
- Purity of standard solution
- Repeatability of gravimetric preparation of standard solution
- Repeatability of gravimetric preparation of isotope ratio standard
- Repeatability of analysis (includes measurement of the isotope ratio of the sample, weighing the sample taken for analysis, weighing the internal standard solution added to the sample, and measurement of the isotope ratio of the {standard plus internal standard mixture})

NIM
- Reference material
- Balance
- RSD of measurement

NIST
- Purity of calibration material
- Calibration response factor, including gravimetric uncertainty in the preparation of calibrants and repeatability of the LC system
- Chromatographic component, including gravimetric uncertainty in the preparation of the samples and the repeatability of the LC system

Uncertainties for Niacin:

NIM
- Reference material
- Balance
- RSD of measurement

NIST
- Purity of calibration material
- Calibration response factor, including gravimetric uncertainty in the preparation of calibrants and repeatability of the LC system
- Chromatographic component, including gravimetric uncertainty in the preparation of the samples and the repeatability of the LC system

Uncertainties for Vitamin A as Retinol:

NIM
- Reference material
- Balance
- RSD of measurement

NIST
- Purity of calibration material
- Calibration response factor, including gravimetric uncertainty in the preparation of calibrants and repeatability of the LC system
- Chromatographic component, including gravimetric uncertainty in the preparation of the samples and the repeatability of the LC system

HOW FAR DOES THE LIGHT SHINE?

Because vitamins were added to the CCQM-K62 study material in a single form and at levels significantly higher than those that would be naturally occurring in the milk base, the consensus opinion of the OAWG is that the ability to measure the study vitamins is indicative of a laboratory's ability to measure vitamins in fortified foods.

- The ability to measure vitamin A (as retinol and retinyl palmitate) in this material is indicative of a laboratory's ability to measure vitamin E (as alpha-tocopherol and alpha-tocopheryl acetate) as well as vitamin A; vitamin E occurs at a higher concentration and is usually easier to measure than vitamin A. Measurement of vitamin A is not indicative of the ability to measure vitamins D and K, which typically occur at lower concentrations.

- Folic acid occurs at low levels and can be unstable; if a laboratory measures only folic acid, it is only indicative of the laboratory's ability to make that measurement in fortified foods.

- Niacin is stable and present at higher concentrations than the other water-soluble vitamins. If a laboratory measures only niacin, it is only indicative of the laboratory's ability to make that measurement in fortified foods. Successful measurement of both niacin and folic acid can be considered indicative of a laboratory's ability to measure folic acid, thiamine, niacin, and riboflavin in fortified foods. These measurements are not indicative of a laboratory's ability to measure vitamin C, which is unstable and typically measured separately, nor are they indicative of a laboratory's ability to measure the other water-soluble vitamins.

APPENDIX A
Participant Reports

KRISS

Folic Acid

FOLIC ACID IN STUDY SAMPLE

CCQM-K62
Nutrients in Infant/Adult Formula: Vitamins

Reporting Date (m/d/y):	02/12/2008
Laboratory:	KRISS
Submitted by:	Byungjoo Kim

BRIEF DESCRIPTION OF PROCEDURES USED:

Approximate amount of sample used for analysis: 1 g

Source of material used for calibration	LGCPromochem
Purity of material used for calibration	90.60%

Brief description of sample preparation (e.g., saponification, extraction, etc.)
Extraction by 0.1 M dibasic potassium phosphate solution (pH 6)
C18 SPE

Analytical method used (e.g., LC/MS, LC/fluorescence):

Analyt. Method	Column Phase	Solvents	Isocratic/Gradient	Column Temperature	Detection Parameters
LC/MS/MS	Luna C18(2)	64% Water 23.4% acetonitrile 12.6% Methanol 0.1% formic acid	Isocratic	25 oC	MS

Method of quantitation (IS = internal standard, ES = external standard):
IS

If an internal standard was used, what was it?
Folic acid -$^{13}C_5$

If internal standard method was used, when was the internal standard added?
Before reconstitution

Type of calibration done - single point, bracketing, or calibration curve?
Single Point

Calibration Curve (if used)

	Points	Conc. Range	Was the analyte outside of calibration curve calibration range?
What control material did you use?	SRM 1849		
Did you obtain the expected value?	Yes		

RESULTS:

Folic Acid	CCQM-K62 Packet 1	CCQM-K62 Packet 2	CCQM-K62 Packet 3	CCQM-K62 Packet 4	CCQM-K62 Packet 5
Mass fraction (micrograms per gram)	2.300	2.342	2.341	2.346	2.319

	CCQM-K62 mean	CCQM-K62 combined std unc	CCQM-K62 expanded unc
Mass fraction (micrograms per gram)	2.329	0.024	0.049

Discuss uncertainty calculations below along with any additional information.

Source of Uncertainty	Relative Uncertainty (rel%)
Standard solution	**0.83**
purity	0.71
repeatabilality of gravimetric preparation	0.43
Isotope Ratio Standard(repeatabilality of gravimetric preparation)	**0.25**
Repeatabilality of Analysis	**0.83/sqrt(5)=0.38**
Measurement uncertainty	**0.43**
measurementof isotope ratio of sample	included in the repeatability of analysis
weighing sample taken for analysis	included in the repeatability of analysis
weighing IS sol addede to the sample	included in the repeatability of analysis
measurement of isotope ratio of (standard+IS Mix)	0.43

NIM

Folic Acid

FOLIC ACID IN STUDY SAMPLE

CCQM-K62

Nutrients in Infant/Adult Formula: Vitamins

Please fill in all blanks, and use requested units of concentration
DO NOT INSERT ROWS OR COLUMNS WITHIN THIS TABLE. DO NOT MOVE CELLS.
- If necessary, add additional data/information at the end of the table.

Reporting Date (m/d/y): 01/18/2008
Laboratory: NIM
Submitted by: LIU Jun, HUANG Ting, ZHANG Wei

BRIEF DESCRIPTION OF PROCEDURES USED:

Approximate amount of sample used for analysis: 2 g

Source of material used for calibration: Folic acid (Sigma)
Purity of material used for calibration: 91.8%

Brief description of sample preparation (e.g., saponification, extraction, etc.)

A test portion of 2 g was weighed. 0.1 g of ascorbic acid, 10 mL of HCl (0.01 mol/L) and 5 mL of trichloroacetic acid (5%) was added. The sample was placed in a water bath of 95 C for 30 min. After cooling to room temperature, 0.1 mL of NaOH (5 mol/L) was added. It was diluted to 25 mL with water, and then it was filtered. (Exposure to bright light was avoided throughout the entire process.)

Analytical method used (e.g., LC/MS, LC/fluorescence):

Analyt. Method	Column Phase	Solvents	Isocratic/Gradient	Column Temperature	Detection Parameters
LC/MS(ESI)	ODS	Methanol:formic acid	Isocratic	Room temperature	MRM: m/z=442 -> 295 and m/z=447 -> 295

Method of quantitation (IS = internal standard, ES = external standard):
IS

If an internal standard was used, what was it?
Folic acid-$^{13}C_5$

If internal standard method was used, when was the internal standard added?
Before dissolvation of test portions

Type of calibration done - single point, bracketing, or calibration curve?
Bracketing

Calibration Curve (if used)

Points	Conc. Range	Was the analyte outside of calibration curve calibration range?
-	-	-

What control material did you use? SRM 1849
Did you obtain the expected value? Yes

RESULTS:

Folic Acid	CCQM-K62 Packet 1	CCQM-K62 Packet 2	CCQM-K62 Packet 3	CCQM-K62 Packet 4	CCQM-K62 Packet 5
Mass fraction (micrograms per gram)	2.365	2.368	2.369	2.353	-

	CCQM-K62 mean	CCQM-K62 combined std unc	CCQM-K62 expanded unc
Mass fraction (micrograms per gram)	2.364	0.038	0.076

Discuss uncertainty calculations below along with any additional information.

Source	Standard uncertainty	
Reference material	1.5%	
Balance	0.07%	
RSD of measurement	0.31%	
Combined std unc as %	1.6%	
Expanded unc as %	3.2%	
Combined std unc	0.038	
Expaned unc	0.076	(coverage factor k=2)

NIACIN IN STUDY SAMPLE

CCQM-K62
Nutrients in Infant/Adult Formula: Vitamins

Please fill in all blanks, and use requested units of concentration
DO NOT INSERT ROWS OR COLUMNS WITHIN THIS TABLE. DO NOT MOVE CELLS.
- If necessary, add additional data/information at the end of the table.

Reporting Date (m/d/y): 01/18/2008
Laboratory: NIM
Submitted by: LIU Jun, HUANG Ting, ZHANG Wei

BRIEF DESCRIPTION OF PROCEDURES USED:

Approximate amount of sample used for analysis: 2 g

Source of material used for calibration: Nicotinamide (Fluka)
Purity of material used for calibration: 99.7%

Brief description of sample preparation (e.g., saponification, extraction, etc.)

A test portion of 2 g was dissolved in 5 mL of warm water and ultrasonicated for 10 min.
The pH of sample was adjusted to 1.7 by 5 mol/L HCl. After 2 min, it was adjusted to 4.5 by 5 mol/L NaOH.
It was diluted to 50 mL with water, and then it was filtered.(Exposure to bright light was avoided throughout the entire process.)

Analytical method used (e.g., LC/MS, LC/fluorescence):

Analyt. Method	Column Phase	Solvents	Isocratic/Gradient	Column Temperature	Detection Parameters
LC/MS(ESI)	ODS	Methanol:ammonium acetate	Isocratic	Room temperature	m/z=123 and m/z=127

Method of quantitation (IS = internal standard, ES = external standard):
IS

If an internal standard was used, what was it?
Nicotinamide-D_4

If internal standard method was used, when was the internal standard added?
Before dissolvation of test portions

Type of calibration done - single point, bracketing, or calibration curve?
Bracketing

Calibration Curve (if used)

Points	Conc. Range	Was the analyte outside of calibration curve calibration range?
-	-	-

What control material did you use? SRM 1849
Did you obtain the expected value? Yes

RESULTS:

Niacin	CCQM-K62 Packet 1	CCQM-K62 Packet 2	CCQM-K62 Packet 3	CCQM-K62 Packet 4	CCQM-K62 Packet 5
Mass fraction (micrograms per gram)	107.0	106.3	106.5	108.1	-

	CCQM-K62 mean	CCQM-K62 combined std unc	CCQM-K62 expanded unc
Mass fraction (micrograms per gram)	107.0	1.1	2.1

Discuss uncertainty calculations below along with any additional information.

Source	Standard uncertainty	
Reference material	0.5%	
Balance	0.07%	
RSD of measurement	0.75%	
Combined std unc as %	1.0%	
Expanded unc as %	2.0%	
Combined std unc	1.1	
Expaned unc	2.1	(coverage factor k=2)

Vitamin A as Retinol

VITAMIN A IN STUDY SAMPLE

CCQM-K62
Nutrients in Infant/Adult Formula: Vitamins

Please fill in all blanks, and use requested units of concentration
DO NOT INSERT ROWS OR COLUMNS WITHIN THIS TABLE. DO NOT MOVE CELLS.
- If necessary, add additional data/information at the end of the table.

Reporting Date (m/d/y): 01/18/2008
Laboratory: NIM
Submitted by: LIU Jun, HUANG Ting, ZHANG Wei

BRIEF DESCRIPTION OF PROCEDURES USED:

Approximate amount of sample used for analysis: 2 g

Source of material used for calibration: Vitamin A palmitate (Dr. Ehrenstorfer)
Purity of material used for calibration: 87.9%

Brief description of sample preparation (e.g., saponification, extraction, etc.)

A test portion (2 g) was weighed into a flask and dissolved by 6 mL of warm water.
20 mL of pyrogallol(15g/L) and 10 mL potassium hydroxide (50%) was added. The samples were saponified under nitrogen at 27°C for 16 h.
The sample was extracted by petroleum ether (30~60°C) in separator funnels for 3 times . The organic phase was filtered through anhydrous sodium sulphate.
The filtrate was evaporated and the residue was dissolved into methanol. (Exposure to bright light was avoided throughout the entire process.)

Analytical method used (e.g., LC/MS, LC/fluorescence):

Analyt. Method	Column Phase	Solvents	Isocratic/Gradient	Column Temperature	Detection Parameters
LC/MS(APCI)	ODS	Methanol:acetonitrile:acetic acid	Isocratic	Room temperature	m/z=269 and m/z=273

Method of quantitation (IS = internal standard, ES = external standard):
IS

If an internal standard was used, what was it?
Retinol-D_4 solution obtained by retinyl palmitate-D_4 through saponification procedures described above

If internal standard method was used, when was the internal standard added?
Before dissolvation of test portions

Type of calibration done - single point, bracketing, or calibration curve?
Bracketing

Calibration Curve (if used)

Points	Conc. Range	Was the analyte outside of calibration curve calibration range?
-	-	-

What control material did you use? SRM 1849
Did you obtain the expected value? Yes

RESULTS:

Vitamin A - as retinol equivalents	CCQM-K62 Packet 1	CCQM-K62 Packet 2	CCQM-K62 Packet 3	CCQM-K62 Packet 4	CCQM-K62 Packet 5
Mass fraction (micrograms per gram)	17.49	16.33	16.36	16.59	16.53

	CCQM-K62 mean	CCQM-K62 combined std unc	CCQM-K62 expanded unc
Mass fraction (micrograms per gram)	16.66	0.50	1.01

Discuss uncertainty calculations below along with any additional information.

Source	Standard uncertainty	
Reference material	1.0%	
Balance	0.07%	
RSD of measurement	2.85%	
Combined std unc as %	3.0%	
Expanded unc as %	6.1%	
Combined std unc	0.50	
Expaned unc	1.01	(coverage factor k=2)

Folic Acid

FOLIC ACID

CCQM-K62
Nutrients in Infant/Adult Formula: Vitamins

Please fill in all blanks, and use requested units of concentration
DO NOT INSERT ROWS OR COLUMNS WITHIN THIS TABLE. DO NOT MOVE CELLS.
- If necessary, add additional data/information at the end of the table.

Reporting Date (m/d/y): 8/1/2007
Laboratory: NIST
Submitted by: Bryant Nelson

BRIEF DESCRIPTION OF PROCEDURES USED:

Approximate amount of sample used for analysis: 1.0 g

Source of material used for calibration: Sigma
Purity of material used for calibration: 99.7%

Brief description of sample preparation (e.g., saponification, extraction, etc.)

Dilute with K2HPO4 containing trifluoroacetic acid and ascorbic acid. Vortex. Immerse in boiling water bath 30 min.
Cool on ice for 20 min. Centrifuge for 10 min.
Transfer 1 mL supernatant to centrifuge tube; dilute 1+2 with aqueous ascorbic acid.
Extract folic acid and 13C5-folic acid (internal standard) on solid-phase extraction cartridge.

Analytical method used (e.g., LC/MS, LC/fluorescence): LC/MS/MS

Analyt. Method	Column Phase	Solvents	Isocratic/Gradient	Column Temperature	Detection Parameters
LC/MS/MS	Supelco Discovery HS-F5 (pentafluorophenyl)	1% formic acid in water, 1% formic acid in methanol	Gradient	30 degrees C	folic acid, m/z 442 to m/z 295; labeled folic acid, m/z 447 to m/z 295.

Method of quantitation (IS = internal standard, ES = external standard):
internal standard

If an internal standard was used, what was it?
13C5-folic acid
If internal standard method was used, when was the internal standard added?
Prior to dilution with K2HPO4
Type of calibration done - single point, bracketing, or calibration curve?
five calibrants bracketing expected value
Calibration Curve (if used)

Points	Conc. Range	Was the analyte outside of calibration curve calibration range?
five	approx 200 ng/uL	no

What control material did you use? SRM 1849 Infant/Adult Nutritional Formula
Did you obtain the expected value? yes

RESULTS:

Folic Acid	CCQM-K62 Packet 1, #1	CCQM-K62 Packet 2, #1	CCQM-K62 Packet 3, #1	CCQM-K62 Packet 4, #1	CCQM-K62 Packet 5, #1	CCQM-K62 Packet 6, #1
Mass fraction (micrograms per gram)	2.48	2.68	2.67	2.6	2.54	2.56
	CCQM-K62 Packet 1, #2	CCQM-K62 Packet 2, #2	CCQM-K62 Packet 3, #2	CCQM-K62 Packet 4, #2	CCQM-K62 Packet 5, #2	CCQM-K62 Packet 6, #2
	2.54	2.63	2.64	2.53	2.5	2.63

	CCQM-K62 mean	CCQM-K62 combined std unc	CCQM-K62 expanded unc
Mass fraction (micrograms per gram)	2.59	0.024	0.048

Discuss uncertainty calculations below along with any additional information.

NIACIN IN STUDY SAMPLE

CCQM-K62
Nutrients in Infant/Adult Formula: Vitamins

Please fill in all blanks, and use requested units of concentration
DO NOT INSERT ROWS OR COLUMNS WITHIN THIS TABLE. DO NOT MOVE CELLS.
- If necessary, add additional data/information at the end of the table.

Reporting Date (m/d/y): 2/20/2008
Laboratory: NIST
Submitted by: Karen Phinney

BRIEF DESCRIPTION OF PROCEDURES USED:

Approximate amount of sample used for analysis: 2.0 g

Source of material used for calibration: USP
Purity of material used for calibration: 99.3%

Brief description of sample preparation (e.g., saponification, extraction, etc.)

Extraction with 1% acetic acid in water (sonication)
Centrifugation
Filtration

Analytical method used (e.g., LC/MS, LC/fluorescence):

Analyt. Method	Column Phase	Solvents	Isocratic/Gradient	Column Temperature	Detection Parameters
LC/MS	Cadenza CD-C18	Ammonium formate buffer	Gradient	22° C	SIM m/z 123

Method of quantitation (IS = internal standard, ES = external standard):
IS

If an internal standard was used, what was it?
niacinamide-d4
If internal standard method was used, when was the internal standard added?
prior to extraction
Type of calibration done - single point, bracketing, or calibration curve?
bracketing
Calibration Curve (if used)

Points	Conc. Range	Was the analyte outside of calibration curve calibration range?

What control material did you use? none
Did you obtain the expected value?

RESULTS:

Niacin	CCQM-K62 Packet 1	CCQM-K62 Packet 2	CCQM-K62 Packet 3	CCQM-K62 Packet 4	CCQM-K62 Packet 5
Mass fraction (micrograms per gram)	102.24	116.25	99.85	107.72	105.98

	CCQM-K62 mean	CCQM-K62 combined std unc	CCQM-K62 expanded unc
Mass fraction (micrograms per gram)	106.4		106.4 ± 6.5 (k = 2)

Discuss uncertainty calculations below along with any additional information.

Vitamin A as Retinol

VITAMIN A

CCQM-K62
Nutrients in Infant/Adult Formula: Vitamins

Please fill in all blanks, and use requested units of concentration
DO NOT INSERT ROWS OR COLUMNS WITHIN THIS TABLE. DO NOT MOVE CELLS.
- If necessary, add additional data/information at the end of the table.

Reporting Date (m/d/y): 4/2/2007
Laboratory: NIST
Submitted by: C.A. Rimmer

BRIEF DESCRIPTION OF PROCEDURES USED:

Approximate amount of sample used for analysis: 2 g

Source of material used for calibration: Fluka-lot 1179930
Purity of material used for calibration: 95.959% by ELSD and UV at 325 nm

Brief description of sample preparation (e.g., saponification, extraction, etc.)

five successive 60 min extractions with 40 mL ethyl acetate in an ultrasonic bath. Samples were centrifuged and solvent was decanted between extractions. The five portions of ethyl acetate (~200 mL combined) were blown down to ~10 mL with nitrogen.

Analytical method used (e.g., LC/MS, LC/fluorescence):

Analyt. Method	Column Phase	Solvents	Isocratic/Gradient	Column Temperature	Detection Parameters
LC/MS	ACE C18	50/50 Acetonitrile/Methano	isocratic	25 C	The MS detector was used with atmospheric pressure chemical ionization (APCI): capillary voltage – 3000 V; fragmentor voltage 70 V; corona current 4 mA; gas temp 350 °C; vaporizer temperature °C; drying gas flow rate 6 l/min; and a nebulizer pressure of 35 psig. Two fragment ions were monitored 269 and 273, representing retinyl palmitate and retinyl palmitate-d4 respectively.

Method of quantitation (IS = internal standard, ES = external standard):
IS

If an internal standard was used, what was it?
retinyl palmitate d4
If internal standard method was used, when was the internal standard added?
added by mass directly to the samples prior to extraction
Type of calibration done - single point, bracketing, or calibration curve?
5 calibrants were used, they bracketed the samples and an average response factor was used in the calculation.
Calibration Curve (if used)

Points	Conc. Range	Was the analyte outside of calibration curve calibration range?

What control material did you use? SRM 1849
Did you obtain the expected value? yes

RESULTS:

Vitamin A

	CCQM-K62 Packet 1, #1 (Box 1)	CCQM-K62 Packet 2, #1 (Box 2)	CCQM-K62 Packet 3, #1 (Box 4)	CCQM-K62 Packet 4, #1 (Box 5)	CCQM-K62 Packet 5, #1 (Box 7)	CCQM-K62 Packet 6, #1 (Box 8)
Mass fraction (micrograms per gram)	14.089	14.873	15.917	15.439	15.848	16.201

	CCQM-K62 Packet 1, #2 (Box 1)	CCQM-K62 Packet 2, #2 (Box 2)	CCQM-K62 Packet 3, #2 (Box 4)	CCQM-K62 Packet 4, #2 (Box 5)	CCQM-K62 Packet 5, #2 (Box 7)	CCQM-K62 Packet 6, #2 (Box 8)
Mass fraction (micrograms per gram)	14.947	16.205	15.710	15.239	15.352	16.528

	CCQM-K62 mean	CCQM-K62 combined std unc	CCQM-K62 expanded unc		
Mass fraction (micrograms per gram)	15.52		0.63		95 % expanded uncertainty
	15.52		0.57	Neff=12.4	k=2 expanded uncertainty

Discuss uncertainty calculations below along with any additional information.

Results reported as retinol equivalents
Uncertainty worksheet is attached to this e-mail.

The uncertainty is based upon three areas, the standard purity, the response factor (which includes gravimetric uncertainty in the preparation of calibrants and repeatability of the LC system), and the LC (which includes gravimetric uncertainty in the preparation of the samples and the repeatability of the LC system).