AOAC *OFFICIAL METHODS*℠ NEWS

First Action *Official Method*℠ 2011.20 (5′-Mononucleotides in Infant and Nutritional Formula) Successfully Completes SLV; Method to Proceed to Multilaboratory Study

In support of the Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN) initiative, on March 12, 2013 at the AOAC Mid-Year Meeting in Rockville, MD, USA, an expert review panel (ERP) focused on a group of high-priority analytes for which at least two AOAC First Action *Official Methods*℠ were available. After comparison of standard method performance requirements (SMPRs; 1) with data from single-laboratory validation (SLV) studies and information resulting from a detailed questionnaire and scoring system regarding method performance and suitability, the ERP selected one method each for vitamins A, B_{12}, D, E, inositol, and nucleotides to undergo multilaboratory studies. The goal is to provide internationally approved consensus methods to be available as Codex Type II dispute resolution methods.

The following is part of a series of articles on the methods selected for multilaboratory studies. These articles report on the SLV studies that followed a common protocol and study design and used an appropriate test materials kit organized by SPIFAN and the International Formula Council (IFC). This article describes the SLV of a method for the analysis of 5′-mononucleotides for which AOAC First Action *Official Method*℠ status was granted in 2011.

An ERP reviewed two AOAC *Official Methods*℠ for nucleotides, **2011.20** (2) and **2012.21** (3). While both methods were considered acceptable, an evaluation of SLV data for Method **2011.20** received a higher score in the questionnaire completed by reviewers and was therefore selected as the candidate method for reproducibility assessment by a multilaboratory study. Panel members expressed concern, however, over the applicability of the method to starch-based products; therefore an additional sample preparation step was incorporated to accommodate the testing of these matrices.

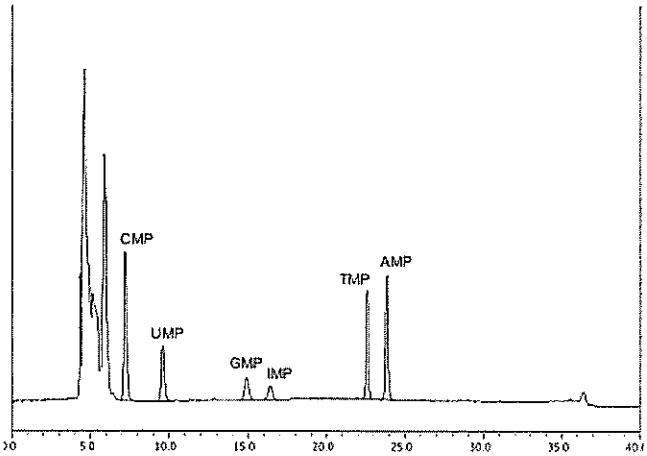

Figure1. Chromatogram of milk-based infant formula sample.

Principle of the Assay

Method **2011.20** is used for the determination of CMP (cytidine 5′-monophosphate), UMP (uridine 5′-monophosphate), GMP (guanosine 5′-monophosphate), IMP (inosine 5′-monophosphate), and AMP (adenosine 5′-monophosphate) in infant formula and adult nutritional products. These are quantified utilizing TMP (thymidine 5′-monophosphate) as internal standard.

Infant formula samples are dissolved in high-salt solution to inhibit protein and fat interactions. Nucleotides are extracted by strong anion exchange solid-phase extraction, and then analyzed by reversed-phase liquid chromatography using photo-diode array detection. Quantitation is by internal standardization using TMP. Figure 1 is an example of chromatogram obtained using Method **2011.20.**

Reference Material

The performance of the method was assessed using a test materials kit, organized by SPIFAN and IFC. These matrices included NIST SRM 1849a (standard reference material), milk protein-based adult nutritional powder, partially hydrolyzed milk-based infant formula powder, partially hydrolyzed soy-based infant formula powder, low-fat adult nutritional powder, child formula powder, infant elemental powder, milk-based infant formula powder, milk-based infant formula ready-to-feed (RTF) liquid, soy-based infant formula

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Table 1. Method performance requirements[a]

Parameter	SMPR 2011.008	Method 2011.20				
		CMP	UMP	GMP	IMP	AMP
Analytical range	0.02-3.5[b]	0.017-5.5	0.017-5.5	0.017-5.5	0.017-5.5	0.017-5.5
	0.31-22.3[c]	0.085-27.5	0.085-27.5	0.085-27.5	0.085-27.5	0.085-27.5
Limit of detection	<0.006[b]	0.005	0.005	0.005	0.005	0.005
Limit of quantitation	<0.02	0.017	0.017	0.017	0.017	0.017
Repeatability (RSD_r)	0.02 ≤ 10%	5.2	3.2	13.1	5.9	13.1
	0.1 ≤ 8%	1.6	2.5	0.3	1.7	2.3
	1 ≤ 6%	1.6	1.3	2.1	1.5	1.8
	5	1.3	2.8	1.1	1.6	2.7
Recovery	90–100% Mean spiked recovery over range of assay	99.4	97.5	101.1	99.8	98.7
Reproducibility (RSD_R)[d]	0.02 ≤ 20%					
	0.1 ≤ 16%					
	1 ≤ 11%					
	5					

[a] Concentrations apply to (*1*) ready-to-feed liquids, "as is"; (*2*) reconstituted powders (25 g in 200 g water); and (*3*) liquid concentrates diluted 1:1 by weight.
[b] mg/100 mL individual nucleotide results reported.
[c] mg/100 mL aggregate of all five nucleotide results reported.
[d] Reproducibility to be established during upcoming multilaboratory study.

powder, high-protein adult nutritional RTF liquid, and high-fat adult nutritional RTF liquid. Only the NIST SRM 1849a, partially hydrolyzed milk-based infant formula powder, and milk-based infant formula powder were fortified with nucleotides during manufacture.

The reference materials were used to determine analytical range, limit of quantification (LOQ), limit of detection (LOD), repeatability, and recovery as outlined in AOAC SMPR 2011.008 (Table 1). Reproducibility will be determined in the upcoming multilaboratory study.

Linearity

Eight standards were prepared over the range specified in the nucleotides SMPR. Three linearity experiments were performed with standards analyzed in random order. The peak area was plotted against concentration and regression analysis performed. Linear regression plots with correlation coefficients of >0.9995 confirm the linearity of the method. Residual plots showed a random distribution of values above and below the line of best fit.

Precision

Four samples were selected for precision studies: an in-house QC milk-based infant formula powder, a milk-based infant formula powder, a milk-based infant formula RTF liquid, and SRM 1849a from the SPIFAN kit. All samples selected for precision studies were analyzed in duplicate on each of 6 days using three different analysts and two different HPLC instruments. Fresh reagents and working standards were made each day.

Repeatability for the method in typical samples ranged between 1.2–4.1%. Repeatability was poorest in the milk-based infant formula RTF liquid, due to the low unfortified concentrations in this sample close to the LOQ.

Repeatability was also assessed in a sample (low-fat adult nutritional powder) spiked with nucleotides at the concentrations specified in the SMPR. For the higher concentrations (1 and 5 mg/100 mL), repeatability ranged from 1.1–2.8%, well below the limit of 6% set in the SMPR. The repeatability for the 0.1 mg/100 mL concentration was 0.3–2.5%, well below the limit of 8% set in the SMPR. The lowest concentration (0.02 mg/100 mL) is near the LOQ for this method, and the poorer repeatability for GMP and AMP (13.1%) reflects this with values slightly above the limit of 10% set in the SMPR (*see* Table 1).

Recovery

Recovery was evaluated in all of the SPIFAN matrices and an in-house QC infant formula powder. Each matrix was spiked with two nucleotide concentrations, at 50 and 150% of typical infant formula concentrations. Duplicate spiked samples were analyzed at each spike level on a single day. Recoveries were between 91.6 and 106.4% within the 90–110% limit set in the SMPR (Table 2).

Recovery was also evaluated in a single SPIFAN matrix (low-fat adult nutritional powder) at each of the four concentrations as defined in the SPMR. The recoveries measured for

Table 2. Mean recovery of duplicate spiked samples at 50 and 150% of typical concentrations					
Sample	Percent mean recovery (standard deviation)				
	CMP	UMP	GMP	IMP	AMP
In-house QC milk-based infant formula powder	102.9 (5.3)	102.2 (8.0)	96.5 (5.1)	103.2 (4.7)	94.2 (2.8)
Milk protein-based adult nutritional powder	98.3 (3.4)	92.8 (3.1)	104.3 (3.9)	99.3 (2.5)	97.7 (2.6)
Partially hydrolyzed milk-based infant formula powder	100.9 (3.1)	94.1 (8.8)	106.1 (2.2)	96.5 (0.6)	105.8 (1.1)
Partially hydrolyzed soy-based infant formula powder	99.3 (0.9)	103.1 (4.4)	96.7 (6.6)	95.7 (1.0)	97.0 (1.7)
Low-fat adult nutritional powder	100.5 (2.6)	98.0 (4.6)	102.0 (3.9)	102.1 (1.7)	95.3 (4.1)
Child formula powder	100.2 (2.8)	94.6 (1.2)	100.5 (1.9)	101.7 (0.7)	91.6 (0.4)
Infant elemental powder	97.8 (1.2)	100.0 (1.7)	103.8 (2.8)	97.9 (2.2)	98.0 (1.2)
Milk-based infant formula powder	99.9 (5.0)	97.3 (5.9)	101.6 (7.7)	102.6 (1.5)	97.7 (5.6)
Milk-based RTF powder	100.6 (3.2)	96.9 (3.5)	102.1 (2.5)	101.1 (3.2)	100.5 (2.9)
Soy-based infant formula powder	96.5 (2.3)	97.6 (4.2)	99.2 (6.5)	98.8 (5.7)	106.4 (2.4)
High-protein adult nutritional RTF powder	996.3 (4.6)	98.6 (3.5)	100.5 (1.5)	100.8 (3.5)	102 (1.3)
High-fat adult nutritional RTF liquid	97.2 (3.9)	95.2 (3.9)	100.0 (6.4)	97.3 (3.6)	98.2 (6.2)

three concentration levels (0.1, 1, 5 mg/100 mL) were 92.5–103.4% and within the 90–110% limit specified in the SMPR. The lowest concentration (0.02 mg/100 mL) had recoveries outside the limits set in the SMPR of 115.1 and 124.7% for CMP and AMP, respectively. Since this concentration is near the LOQ, higher uncertainty in results is expected and a wider range of recoveries was observed (Table 3).

LOD and LOQ

The LOD for the method was evaluated by dilution of nucleotide standards of known concentration to obtain a signal 3 times that of the noise with the LOQ calculated as the concentration equivalent to 10 times signal to noise. These standard concentrations were converted to sample concentrations on a dry-weight (powder) and RTF (liquid) basis. The measured LOQ of 0.017 mg/100 mL is less than that specified in the SMPR of 0.02 mg/100 mL.

Summary

An SLV of AOAC Method **2011.20** was performed with the assessment of method recovery, linearity, precision, and LOD and LOQ to demonstrate compliance with the SPIFAN SMPR utilizing a SPIFAN test materials kit. LOQ was estimated to be 0.017 mg/100 mL, lower than the limit set in the SMPR. Linearity was demonstrated over the range defined in the SMPR of 0.02–5 mg/100 mL.

Repeatability in accordance with the SMPR was demonstrated at the 0.1, 1, and 5 mg/100 mL concentration levels. Repeatability at the 0.02 mg/100 mL level was outside the 10% limit set in the SMPR for GMP and AMP, although CMP, UMP, and IMP were within the limits.

Recovery experiments demonstrated the method was applicable to all the SPIFAN matrices with recoveries measured between 90–110% at typical sample concentrations. Recoveries determined at the concentrations specified in the SMPR were acceptable at the 0.1, 1, and 5 mg/100 mL concentration levels. Recovery at the 0.02 mg/100 mL concentration level was outside the 90–110% limit set in the SMPR for CMP and AMP, but acceptable for UMP, IMP, and GMP.

The method met all the criteria specified in the SMPR at the 0.1, 1, and 5 mg/100 mL concentration levels. Although the method did not meet all of the criteria for precision and accuracy at the lowest concentration (0.02 mg/100 mL) in the SMPR, this concentration is well below levels measured in nucleotide-supplemented infant formulas.

Further evaluation of the method, including bias, intermediate precision, and selectivity, was also performed. The method was found to be suitable for its intended purpose of measuring 5′-mononucleotides in infant formula and adult/pediatric nutritional formula.

As of date, 20 laboratories around the world have expressed interest in participating in this study which would generate valuable method performance information. ■

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Table 3. Mean recovery in an adult nutritional product spiked at SMPR concentrations					
Concentration of spike to low-fat adult nutritional powder(mg/100 mL)	Percent mean recovery[a]				
	CMP	UMP	GMP	IMP	AMP
0.02	115.1	91.3	102.1	91.8	124.7
0.1	96.5	94.8	101.5	94	103.4
1	99.8	92.5	101.2	98.4	99.6
5	101.8	91.9	102.3	99.9	102.3

[a] Triplicate measurements.

References

(1) AOAC SMPR 2011.008 (2012) *J. AOAC Int.* 95, 296

(2) Gill, B.D., Indyk, H.E., Kumar, C.M., Sievwright, N.K., Manley-Harris, M., & Dowell, D. (2012) *J. AOAC Int.* 95, 599–602

(3) Inoue, K., & Dowell, D. (2012) *J. AOAC Int.* 95, 603–605