

Determination of Vitamin A in Animal Feed by HPLC/UV

Friday August 11th, 2017

2017 AAFCO Annual Meeting

Bellevue, Washington

Overview

- Added to scope of accreditation: May 2017
- Dec 2016-May 2017
- Based on principles utilized nationally: saponification, cleanup and LC/UV measurement
- Different lab methods report Vitamin A into the same AAFCO LC method code.
- Considered a “consensus” method.

Method Proficiency Testing Report

Method Code	Analyte Name and Method (Units)	Lab Code	Lab Data		Method Values				AAFCO CS Z Score	Threshold %RSD	Flag
			Value	Range	Rob Mean	Robust SD	R-bar	# Labs			
106.00	Vitamin A, Color (KU / kg)	0019	41.665	1.7100			1.7100	1			0
106.01	Vitamin A, UV (KU / kg)	0098	36.000	0.80000			0.80000	1			0
106.02	Vitamin A, LC (KU / kg)	0861	23.200	10.800	42.849	13.503	5.3265	22	-1.46	23%	0
106.02	Vitamin A, LC (KU / kg)	2053	28.600	8.2000	42.849	13.503	5.3265	22	-1.06	17%	0
106.02	Vitamin A, LC (KU / kg)	0227	28.900	1.4000	42.849	13.503	5.3265	22	-1.03	16%	0
106.02	Vitamin A, LC (KU / kg)	0027	29.901	6.6404	42.849	13.503	5.3265	22	-0.96	15%	0
106.02	Vitamin A, LC (KU / kg)	0904	30.670	0.00000	42.849	13.503	5.3265	22	-0.90	14%	0
106.02	Vitamin A, LC (KU / kg)	2004	32.450	0.70000	42.849	13.503	5.3265	22	-0.77	12%	0
106.02	Vitamin A, LC (KU / kg)	0941	34.000	6.6200	42.849	13.503	5.3265	22	-0.66	10%	0
106.02	Vitamin A, LC (KU / kg)	0610	36.550	15.300	42.849	13.503	5.3265	22	-0.47	7%	0
106.02	Vitamin A, LC (KU / kg)	0003	38.500	1.0000	42.849	13.503	5.3265	22	-0.32	5%	0
106.02	Vitamin A, LC (KU / kg)	2103	40.980	3.8400	42.849	13.503	5.3265	22	-0.14	2%	0
106.02	Vitamin A, LC (KU / kg)	0208	41.116	5.5110	42.849	13.503	5.3265	22	-0.13	2%	0
106.02	Vitamin A, LC (KU / kg)	0004	42.515	1.6500	42.849	13.503	5.3265	22	-0.02	0%	0
106.02	Vitamin A, LC (KU / kg)	0563	44.569	0.46570	42.849	13.503	5.3265	22	0.13	2%	0
106.02	Vitamin A, LC (KU / kg)	2068	46.420	0.23900	42.849	13.503	5.3265	22	0.26	4%	0
106.02	Vitamin A, LC (KU / kg)	0148	46.680	2.6600	42.849	13.503	5.3265	22	0.28	4%	0
106.02	Vitamin A, LC (KU / kg)	0905	49.060	6.9660	42.849	13.503	5.3265	22	0.46	7%	0
106.02	Vitamin A, LC (KU / kg)	0676	51.450	8.5000	42.849	13.503	5.3265	22	0.64	10%	0
106.02	Vitamin A, LC (KU / kg)	0010	51.915	3.7500	42.849	13.503	5.3265	22	0.67	11%	0
106.02	Vitamin A, LC (KU / kg)	0014	55.900	13.000	42.849	13.503	5.3265	22	0.97	15%	0
106.02	Vitamin A, LC (KU / kg)	0675	67.700	9.5400	42.849	13.503	5.3265	22	1.84	29%	0
106.02	Vitamin A, LC (KU / kg)	0169	90.500	7.0000	42.849	13.503	5.3265	22	3.53	56%	0
106.02	Vitamin A, LC (KU / kg)	0555	202.80	3.4000	42.849	13.503	5.3265	22	11.85	187%	0

Procedure

Weigh samples;
aliquot standards

→ Add pyrene

→ Add 80 mL of
pyragoll/ethanol
solution

→ Add 20 mL KOH

→ Shake on shaker
for 16-18 hours

Procedure Cont.

Add 50 mL of
neutralizing solution



Decant 20 mL into
centrifuge tube



Centrifuge



Dilutions in ACN



HPLC/UV

Pyrene Surrogate Standard

- Pyrene added to samples and standards as surrogate standard
- If recovery is outside the expected range (90-110%) professional judgement is used to determine if additional analysis is needed

New Method Performance

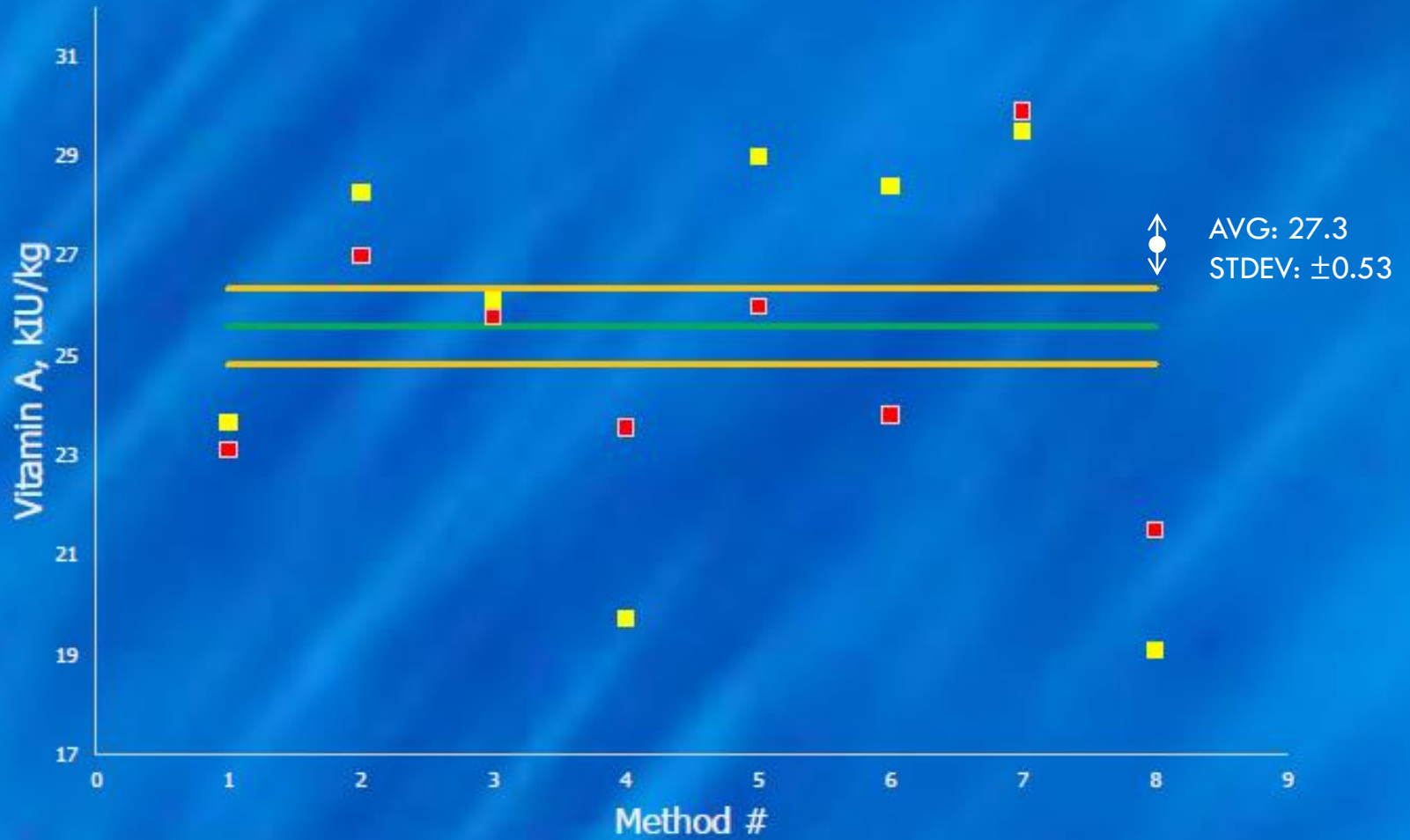
QC Summary	
	LAB
NIST SRM 1849 α N=16	1.91% RSD
AAFCO 1621 N=12	26.26% RSD
Split Study N=7	17.6% RSD
Duplicates N=12	23.8% RPD

STD Rerun	
DATE	0.374 IU/mL
2.17.17	25251
3.2.17	26343
3.9.17	26076
3.14.17	26170
3.23.17	25572
3.30.17	25764
AVG	25863
STDEV	410
%RSD	1.58

New Method Performance

DATE	AAFCO	Description	Result	Z-score	Range	%RPD
2.28.17	201721	Dry dog food	23.68 KU/KG	0.841	0.563	2.4%
3.31.17	201722	Poultry layer feed-medicated	29.26 KU/KG	-0.144	3.48	11.9%
4.30.17	201723	Milk replacer-medicated	44.72 KU/KG	0.465	3.92	8.8%

Vitamin A



New Method Summary

- Strong method performance
 - NIST
 - Pyrene
 - Chromatography
- Improvements:
 - Efficiency-more samples
 - Prefer shake vs boil
 - Pyrene surrogate QC
 - Measurement precision on the bench
- Large MU: Dups, splits LCS
- Not as good a cleanup as SPE

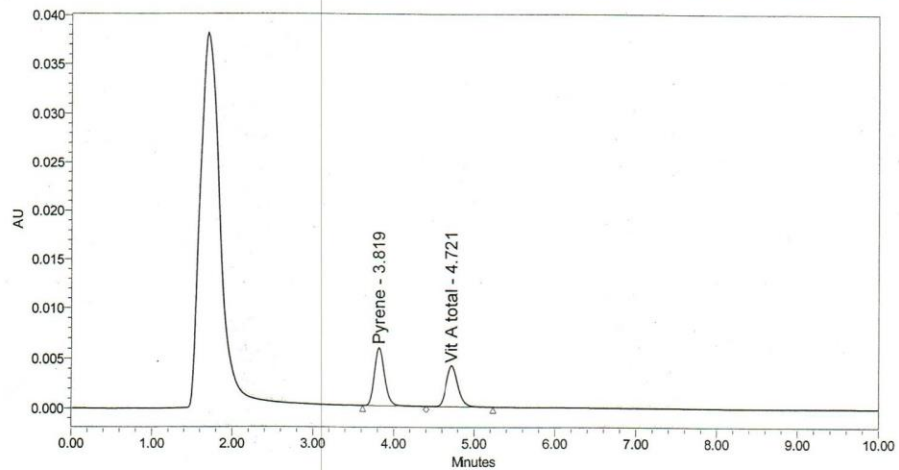
New Method Chromatogram



Vitamin A component summary

SAMPLE INFORMATION

Sample Name:	0.145	Acquired By:	System
Sample Type:	Unknown	Sample Set Name:	122016 Vit A new meth
Vial:	26	Acq. Method Set:	Vitamin A
Injection #:	1	Processing Method:	Vitamin A_PM IS
Injection Volume:	30.00 ul	Channel Name:	W2489 ChA
Run Time:	10.0 Minutes	Proc. Chnl. Descr.:	W2489 ChA 325nm
Date Acquired:	12/20/2016 3:18:42 PM MST		
Date Processed:	12/22/2016 8:57:51 AM MST		



	Peak Name	RT	Area	Amount	Units
1	Pyrene	3.819	51231		
2	Vit A total	4.721	41758	0.141	ppm

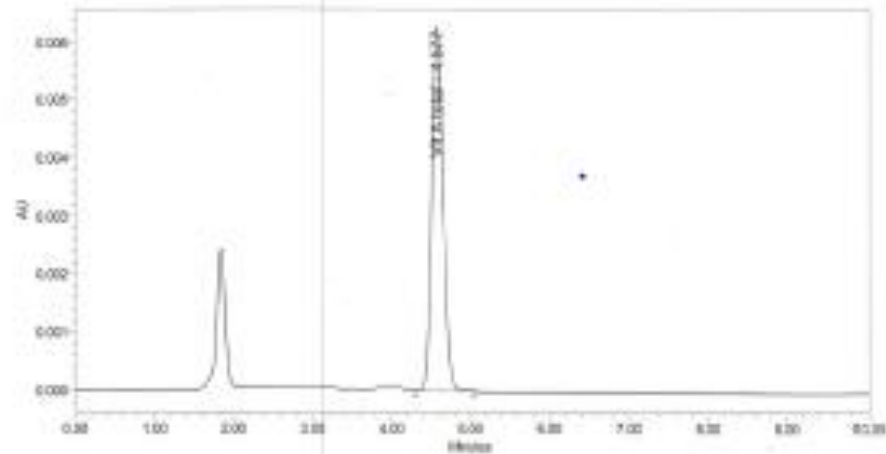
Old Method Chromatogram

Empower²
DATAWORKS

Vitamin A component summary

SAMPLE INFORMATION

Sample Name:	5.260 ppm	Acquired By:	System
Sample Type:	Standard	Sample Set Name:	Vit A 20161125 (1)
Vial:	4	Acq. Method Set:	Vitamin A_M
Injection #:	1	Processing Method:	Vitamin A_PM
Injection Volume:	30.00 ul	Channel Name:	W0466 C0A
Run Time:	19.0 Minutes	Proc. Chnl. Descr.:	W0466 C0A S25nm
Date Acquired:	11/25/2016 12:35:30 PM MST		
Date Processed:	11/25/2016 9:44:06 AM MST		



Peak Name	RT	Area	Amount	Units
1 vit A total	4.577	62633	0.260	ppm ✓

Overview

1992-2016

- OLD: The Extraction of Vitamin A From Feeds, Premixes and Animal Remedies Using Solid Phase Extraction Disk Cartridges, pp. 1-6, Olson Biochemistry Laboratories, Department of Chemistry and Biochemistry, South Dakota State University, Box 1270, Brookings, SD 57007-1217.

2016-current

- NEW: Analysis for Vitamin A in Feeds by HPLC Pyrene Internal Standard Overnight Shake Assay, Method 118-40, Revised on April 21, 1999, Feed Chromatography Laboratory, Office of Indiana State Chemist

Method Comparison

	NEW METHOD	OLD METHOD
Grind	0.75 mm	2.0 mm
Weigh	5-10 g	10-40 g
Saponification	ETOH/KOH, Shake 16-18 hrs	ETOH/KOH, Boil 45 mins
Cleanup	Neutralization, ACN dilutions	HCB Oasis SPE cleanup
Measure	LC/UV	LC/UV
Surrogate	pyrene	NA

Method Performance

	NEW METHOD	OLD METHOD
Dup RPD	23.8 ± 21.8 N=12	16.7 ± 20.8 N=17
Split RSD	17.6 ± 8.5 N=5	NA

	AAFCO/NIST	NEW METHOD	OLD METHOD
AAFCO 1621	42.85 ± 13.5 N=21	36.7 ± 9.6 N=12	33.5 ± 4.81 N=3
AAFCO 1591	51.73 ± 16.0 N=21	NA	47.82 ± 9.65 N=12
NIST SRM 1849 _a	7.68 ± 0.23	8.2 ± 0.16 N=16	8.7 ± 0.025 N=2

New vs OLD

- No data trends indicating one better than another
- Both have large MU issues
- New: strong method performance---
- SPE gives better cleanup
- Not able to overcome large MU
- How to handle large MU reporting results

Measurement Uncertainty

- Category III Method
 - Requires a measurement uncertainty estimate

Symbol	Source of Uncertainty	Value (%)	Distrb	Div	Uncert (1 σ)	Degree Freedom
U	Sample Preparation & Testing process	17.64	N	1	17.64	14
U	Expanded Uncertainty (K=2.14)					37.75 %
MU estimated to be 38% (95% CL, k=2)						

Measurement Uncertainty Cont.

- To reduce MU for deficient samples:
 - Measure multiple times
 - Determine the mean
 - Apply the following equation

$$\text{Adjusted MU} = \frac{MU}{\sqrt{N}}$$

N= number of measurements used to determine the mean

Examples of Adjusted MU

	AB70328	AB70187	AB70140
Result 1	64,315	10,990	148,000
Result 2	64,405	11,987	132,000
Result 3	70,218	14,922	149,000
Result 4	61,931		
AVG	65,217	12,633	143,000
MU	38%	38%	38%
\sqrt{N}	2	1.73	1.73
Adjusted MU (MU/ \sqrt{N})	19%	22%	22%
Adjusted MU*AVG	12,391	2,772	31,374
Non-Adjusted 95% Confidence Interval	40,435 - 89,999	7,832 - 17,434	88,660 - 197,340
Adjusted 95% Confidence Interval	52,826 - 77,609	9,861 - 15,405	111,626 - 174,374
Bottom of AV Range	280,000	28,000	168,000

Conclusions

- Benefits of ISO method
- Help regulatory group with MU
- MU: spectrum from protein to Vitamin A
- Could not overcome struggle with MU
- Future improvements: incorporate SPE cleanup?

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