Determination of Vitamin A as Retinyl Palmitate in Processed-Grain Commodities

WORKING INSTRUCTIONS & METHOD VALIDATION

United States Department of Agriculture
Grain Inspection, Packers and Stockyards Administration
Technical Services Division
Analytical, Reference and Testing Services Branch

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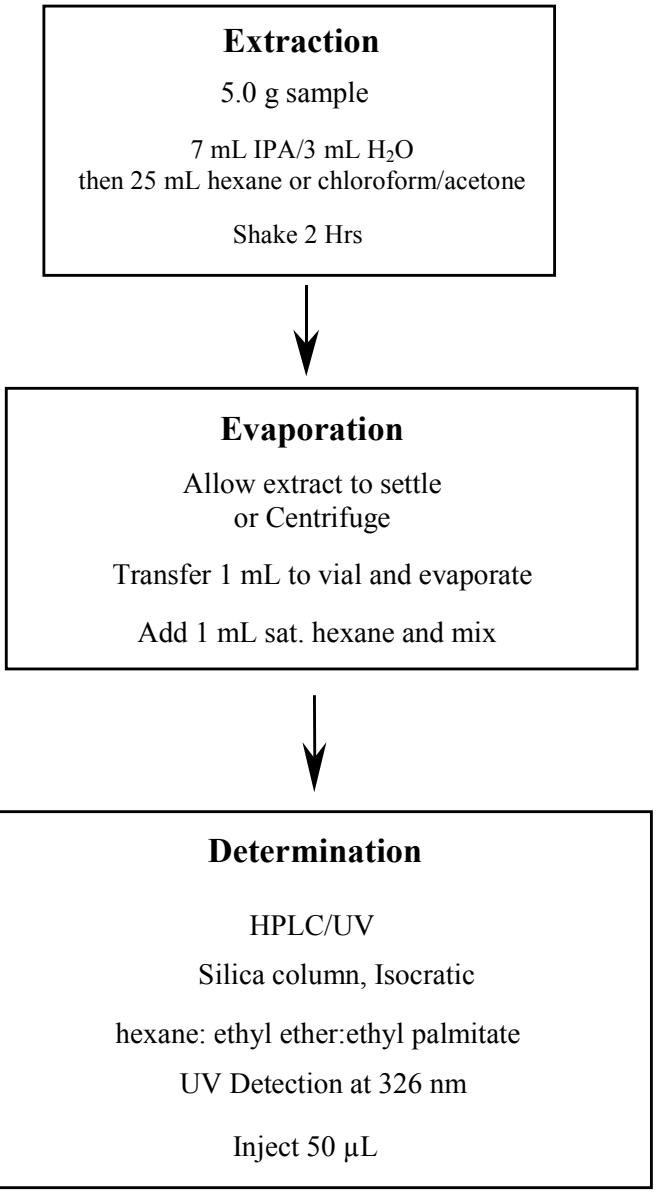
Method Flowchart



Summary Procedure for the Validation of Vitamin A Samples

1. Scan each chromatogram to verify that the integration has been applied properly and consistently and that the peaks have been assigned to the right compound.

2. Run the "VitaQC" macro on each calibration curve and check that each calibration parameter is within the specification (CV values for the 13-cis and the all-trans compounds, the 13-cis/all-trans ratio, and the target areas).

3. Run the "VitaResults" macro to generate the summary file. In this file look at each "check calibration" (CC) sample and confirm that the value is within 5% of the target value of 24,200 IU/Kg.

4. Compare each check sample result with the results found in the "Trace_Lab" database. Any result that is over 3 standard deviations from the average result in the database shows that the method was not in statistical control when the batch was analyzed and the samples must be rerun. If a result is between 2 and 3 standard deviations from the mean, the batch QC results should be carefully inspected. Only one out of twenty results (5%) should be in this range.

5. Create a copy of the results batch file at this point. The name should be "Copy of batch_name.xls." Remove lines from the actual results file that are not real sample results, but leave QC samples in the file. Run the "VitaCert" macro to generate the certificate. Double-check to see that the internal ID numbers have been properly assigned and that the quality control naming conventions have been adhered to. QC samples will have the following designations in the internal ID field and will have a null customer ID.

   QC naming specifications:

   RB for reagent blank
   CC for calibration check
   CS for check sample
   Full name for check sample includes a data label designating the check sample and the commodity type for that check sample. An example would be CS012000APF. The date is in month/day/year format with no spaces between.

6. Finally run the "Vita_Access" macro to generate an input file to the Trace_lab database. Then go into the database and run the "samplehistory" and "results" macros to pull the data in. Check the tables to be sure the data was pulled in properly.

If any of the specifications outlined in 1-4 are out of the target range, the lab supervisor must be notified. Most of these situations will require rerunning that batch of samples.

Method Revision History

Changes in Revision 1

The method was changed so that all processed-grain commodities except all-purpose and bread flours are analyzed using the acetone:chloroform solvent system. Comparison analyses showed that acetone:chloroform often gave higher results than hexane on duplicate samples run in the same batch. In sets of four or more bulgur samples, the results were also less variable with acetone:chloroform than with hexane.

The column was changed from the Zorbax Sil column to the Zorbax RX-Sil column. Due to an apparent change in manufacturing, it is now necessary to use the RX-Sil column. This column contains a silica which is purer than that in the Sil column. The standard Sil column caused irreversible binding of the retinyl palmitate and this was observed as a slowly increasing peak size when replicate injections were made of the retinyl palmitate standard. This occurred even after careful conditioning of the column with isopropyl alcohol and water.

The peak width in the integrator events table was changed from 0.10 to 0.01 minutes to allow for a more accurate integration of the 13-cis retinyl palmitate peak.

All references to “solid commodities” were changed to “processed-grain commodities.”

Table of Contents

1. Purpose and Scope of Application

The purposes of these Working Instructions (WI) are to establish the operational parameters, methodology, and requirements for the quality assurance and acceptability of data in the determination of vitamin A as retinyl palmitate in processed-grain commodities. These commodities include all-purpose and bread flour (APF and BF), bulgur (BUL), soy-fortified bulgur (SFB), soy-fortified sorghum grits (SFSG), corn meal (CM), soy-fortified corn meal (SFCM), corn-soy blend (CSB), and wheat-soy blend (WSB). A mixture of 30% acetone and 70% chloroform is used as the extraction solvent for all commodities except APF and BF. For these samples hexane is used as the extraction solvent.

2. Analyst Qualifications and Responsibilities

The analyst(s) will receive proper training in the conduct of these WI and will follow the WI as written. The Supervisory Chemist (or Project Leader) is responsible for ensuring that the WI are followed and modified as necessary or appropriate. All revisions to these WI must be approved by the Chief of the Analytical, Reference and Testing Services Branch prior to implementation.

3. References

- Thompson, J. N.; Hatina, G.; Maxwell, W. B. "A High Performance Liquid Chromatographic Determination of Vitamin A in Margarine, Milk," Partially Skimmed Milk, and Skimmed Milk, *J. AOAC Int.* **1980**, *63*, 894-898. (HPLC method is based on this reference).

- Woollard, D. C.; Indyk, H. "The HPLC Analysis of Vitamin A Isomers in Dairy Products and their significance in Biopotency Estimations," *J. Micronutrient Analysis* **1986**, *2*, 125-146. (Basis for the inclusion of the 13-cis isomer in the Vitamin A concentration).

- Ross, A. C. "Separation of Long-Chain Fatty Acid Esters of Retinol by High-Performance Liquid Chromatography," *Anal. Biochem*. **1981**, *115*, 324. (Extinction coefficients for retinol and retinyl esters are identical).

- Hubbard, R. "Geometric Isomerization of Vitamin A, Retinene and Retinene Oxime," *J. Am. Chem. Soc.* **1956**, *78*, 4662-4667. (Reference for the extinction coefficients for all-trans and 13-cis retinyl palmitate in hexane).

- Baldingh, J.; Cama, H. R.; Collins, F. D.; Morton, R. A.; Gridgeman, N. T.; Isler, O.; Kofler, M.; Taylor, R. J.; Welland, A. S.; Bradbury, T. "Pure All-trans Vitamin A Acetate and the Assessment of Vitamin A Potency by Spectrophotometry," *Nature (London)* **1951**, *168*, 598. (Reference for the extinction coefficients for all-trans and 13-cis retinyl palmitate in hexane).

- Qian, H.; Sheng, M. "Simultaneous determination of fat-soluble vitamins A, D and E and pro-vitamin D2 in animal feeds by one-step extraction and high-performance liquid chromatography analysis," *J. Chrom.* **1998**, *825*, 127-133. (Basis for the use of acetone:chloroform as an extraction solvent).

- Analytical Methods Committee "Determination of Vitamin A in Animal Feedingstuffs by High-performance Liquid Chromatography," *Analyst (London)* **1985**, *110*, 1019-1026. (Traditional saponification method used in the method validation studies).

4. Safety and Hazardous Waste

Hexane, chloroform, and ethyl ether are extremely flammable and are health hazards. They can be absorbed through the skin or the lungs. Ethyl ether is a peroxide former and should not be stored for more than one year. Please date each can of ether when it is received. Use latex/nitrile gloves, eye protection, and perform operations with these solvents in the hood.

For additional information refer to the Technical Services Division Chemical Hygiene Plan.

5. Equipment

This section is meant to specify the equipment being used in the laboratory during the method validation process. Other equipment can be substituted for these WI as long as the supervising chemist/technician approves. In some cases, side-by-side testing will be necessary to verify equivalency.

a) High-performance liquid chromatograph, Model 1100 with auto sampler, quaternary pumping system, vacuum degasser, variable-wavelength UV detector, and Chemstation software version 7. (Agilent Technologies, Wilmington, DE).

b) Analytical balance, capable of weighing to 0.0001 g.

c) Ultraviolet spectrophotometer, HP 8452 (Agilent Technologies, Wilmington, DE).

d) Centrifuge, Model GS-6 (Beckman Instruments, Palo Alto, CA).

e) Orbital shaker, Model 3520 (Lab-line Instruments Inc., Melrose Park, IL).

f) Nitrogen evaporator, Turbovap LV (Zymark Corporation, Hopkinton, MA).

6. Materials

This section is meant to specify the materials being used in the laboratory during the method validation process. Other materials can be substituted for these WI as long as the supervising chemist/technician approves. In some cases, side-by-side testing will be necessary to verify equivalency.

a) Hexane, Burdick and Jackson, UV grade (#BJ216-4, VWR Scientific, Chicago, IL).

b) Ethyl Ether, anhydrous, BHT stabilized, ACS Grade (#E138-4, Fisher Scientific, Pittsburgh, PA).

c) Ethyl Palmitate, 99% (#28,691-5, Aldrich Chemical, Milwaukee, WI).

d) Water, high purity, through Milli-Q RO Plus/Milli-Q UV water purification system (Millipore Corp., Marlborough, MA).

e) Acetone, Burdick and Jackson, HPLC grade (#BJ015-4, VWR Scientific, Chicago, IL).

f) Chloroform, HPLC grade (#C607SK-4, Fisher Scientific, Pittsburgh, PA).

g) 2-Propanol, HPLC grade (#A451-4, Fisher Scientific, Pittsburgh, PA).

h) Retinyl Palmitate, all trans, type IV, synthetic (#R3375, Sigma, St. Louis, MO).

i) Cottonseed Oil (#17,991-4, Aldrich Chemical, Milwaukee, WI).

j) Syringe Filters, 25 mm, PTFE, 0.45 µ, Whatman (09-927-30D, Fisher Scientific, Pittsburgh, PA).

k) Hamilton Gastight Syringes, 5 mL; #1005, 1.0 mL; #1001, 500 µL; #1750, 250 µL; #1725, 100 µL; #1710, 50 µL; #1705, 25 µL; #1702, 10 µL; #701 (Hamilton Company, Reno, NV).

l) Centrifuge Tubes, Falcon, 50 mL, polypropylene (#14-959-49A, Fisher Scientific, Pittsburgh, PA).

m) Autosampler vials and 11 mm snap caps, Agilent #5182-0545 and #5182-0550, respectively (Agilent Technologies, Wilmington, DE).

n) Adjustable 1-5 mL FinnPipet with disposable tips (#21-377-196 and #21-377-51, Fisher Scientific, Pittsburgh, PA).

o) Pipettor, 100 to 1000 µL, Eppendorf (#53511-582, #53508-819 (tips), VWR Scientific, Chicago, IL)

p) Analytical column, Zorbax RX-SIL (normal phase silica), 250 x 4.6 mm, 5µ (#880975-901, Agilent Technologies, Wilmington, DE).

q) Guard Column, Zorbax SIL, 4.6 x 12.5 mm, 5µ (#820950-901, Agilent Technologies, Wilmington, DE).

r) Guard Column Holder (#820777-901, Agilent Technologies, Wilmington, DE).

s) Precolumn filter with 0.5 µ frit (#A-101x, Upchurch Scientific, Oak Harbor, WA).

t) Vials, 20 mL, pre-cleaned (Quorpak Company, Bridgeville, PA).

u) Bottle-top Dispensers, Dispensette III, 1-10 mL (#4701 341, BrandTech Scientific, Inc).

v) Bottle-top Dispensers, Dispensette III, 2.5-25 mL (#4731 351, BrandTech Scientific, Inc).

w) Culture Tubes, 16 x 100 mm (#60825-618, VWR Scientific, Chicago, IL).

x) Volumetric Flasks, class A, 10 mL (VWR Scientific, Chicago, IL).

7. Quality Control Procedures

7.1 Definitions

Reagent Blank (RB). Sample where the method is run with only reagents. Its purpose is to check for impurities that may interfere with the analysis.

Check sample (CS). A portion of each commercially-fortified commodity containing approximately the target level (24,200 IU/Kg) of retinyl palmitate. The check sample is analyzed with each batch of samples as a check on the method performance. It indicates the performance of the method on a "real" sample.

Neat Standard. Pure all-trans retinyl palmitate standard purchased from a commercial vendor.

Concentrated Standard. Standard solution prepared from the neat standard and UV-grade hexane (*ca* 1000 µg/mL).

Calibration Standard (STD1, STD2, or STD3). A solution containing a known quantity of retinyl palmitate prepared from the concentrated standard and UV-grade hexane.

Calibration Check (CC). A calibration standard that is run during sample analysis to check for any drift in the instrumental response.

Internal ID (VIAYCONT). Sample ID that is assigned when the sample enters the Trace Analysis Laboratory. "VIA" stands for vitamin A analysis, "Y" is a single digit refering to the fiscal year, and "CONT" is a counter that starts with 0001 and continues for each sample assigned until the end of that fiscal year.

Batch (VIAYRCNT). A batch of samples consists of one reagent blank, one check sample for each commodity being analyzed, and up to ninety samples. A batch of samples is processed, as much as possible, as a single unit. This means that all the samples are processed on the same day, in the same lab environment, and in exactly the same way. When the batch is analyzed on the instrument it includes a calibration standard analyzed at 3 levels, one reagent blank, at least one check sample, one to ninety samples, and a check calibration every 10 injections. In naming the batch "VIA" refers to vitamin A analysis, "YR" is the fiscal year the batch was analyzed in , and "CNT" is a batch counter starting at 1 that begins day one of that fiscal year.

7.2 Preparation of Standards

7.2.a Preparation of the Concentrated Standard

1. Bring the neat all-trans retinyl palmitate standard to 30 °C.

2. Weigh 10-12 mg of retinyl palmitate into a 20 mL, amber, precleaned vial using a 500 µL Hamilton syringe (about 1 drop) or a disposable Pasteur pipet.

3. Add 10 mL of hexane using the adjustable pipet and disposable 5 mL tip. Cap the vial.

4. Mix by sonicating for 15 minutes.

5. Label the vial with the chemical name, concentration (1 mg/mL), date, solvent, lot number, and your initials.

7.2.b Determination of the Concentrated Standard

This determination must be performed each time a batch of samples is analyzed, due to the rapid evaporation of hexane from the standard.

1. Make sure that the standard has come fully to room temperature if it has been refrigerated.

2. Add 50.0 µL of the 1 mg/mL concentrated standard to a 10 mL volumetric flask and dilute to the mark with hexane.

3. Mix the solution by vortexing or inverting. The concentration will be about 5 µg/mL.

4. Turn on the HP 8452 diode-array UV spectrophotometer and the computer. The lamp must be on for at least one hour before performing this test. Set the system for "General Scanning" from 200 nm to 400 nm.

5. Fill a clean quartz UV cell with hexane, put it into the instrument, and run "Scan Blank." Remove the cell, discard the hexane, and dry the cell with a stream of nitrogen. Transfer about 3 mL of the *ca* 5 µg/mL solution prepared above into the UV cell. Put the lid on the UV cell. Place the cell back into the spectrophotometer as soon as possible and activate "Scan."

6. Place the cursor at the highest point on the curve near 325 nm and record the displayed absorbance value. The maximum must occur at 326 nm +/- 2 nm. If the maximum is not in the range of 324-328 nm, inform the supervising

chemist/technician.

7.2.c Preparation of the Calibration Standard

1. Make sure that the concentrated standard has come fully to room temperature if it has been refrigerated.

2. Calculate the volume of concentrated standard to add to generate a standard containing 121.0 IU (24,200 IU/Kg sample) of total retinyl palmitate:

   Volume (µL) = 33.25 / ABS Where ABS is the absorbance for the 5 µg/mL solution measured in step 7.2.b.

   IMPORTANT: This equation is correct only for cis/trans peak area ratios of 0.035 to 0.055. If analysis of the standard is out of this range, a new standard should be prepared or refer to section 12, equation (11) for the calculation that includes the actual area ratio.

3. Label a centrifuge tube "STD" and add 7 mL of 2-propanol, 3 mL of Millipore water and 25 mL of a 30% acetone:70% chloroform mixture using the bottle-top dispensers. To prepare a standard for APF and BF use 25 mL of hexane instead of the acetone:chloroform solution. Then add 100 µL of cottonseed oil to the tube with an Eppendorf pipet.

4. Add the calculated volume of the concentrated standard to the centrifuge tube using a 50 µL or 100 µL (closest match) Hamilton syringe. Do not push the plunger abruptly to 0, but push it slowly and steadily to deliver the standard. Do not immerse the needle into the hexane. When the entire standard is expelled from the syringe, touch the needle to the inside of the centrifuge tube to remove the last drop. Put the cap onto the tube snugly and shake.

5. Let the solution settle for a few minutes.

6. For hexane standards, transfer portions of the top layer to several 2 mL autosampler vials and label the vials, STD. Prepare enough vials to run a check calibration every 10 injections and at the end of the sequence. The concentration is 24,200 IU/Kg total retinyl palmitate. For acetone:chloroform standards, the portions should be taken from the lower layer. Be sure to push air out while inserting the pipet through the top aqueous layer to avoid drawing in any of this layer.

7.3 Preparation of Quality Control Samples

7.3.a Reagent Blank

1. Label a centrifuge tube RB.

2. Add 7 mL of 2-propanol, 3 mL of Millipore water and either 25 mL of 30% acetone:70% chloroform solution or 25 mL of hexane to a 50 mL centrifuge tube using the bottle-top dispensers.

7.3.b Check Sample

1. Label a collection vial "CSDATECOM" where DATE is the date label on the check sample in "MODYYR" format and "COM" refers to the abbreviation for the commodity (*ie,* APF, BF, BUL, SFB, SFSG, CM, SFCM, WSB, CSB).

2. Weigh 4.95 to 5.04 g of the ground check sample into the centrifuge tube.

3. Add 7 mL of 2-propanol, replace the cap, and tap firmly on the edge of the benchtop to fully wet the commodity. Add 3 mL of water to the tube and again tap on the benchtop to mix. Wait at least 10 minutes and then add 25 mL of hexane or acetone:chloroform solution.

8. Instrument Parameters

8.1 High-Performance Liquid Chromatograph (HPLC)

8.1.a HPLC Conditions

Pump (1100)	Flow	2.0 mL/min
	Mobile Phase (isocratic)	96% water sat. hexane: 4% ethyl ether: 0.1% ethyl palmitate
	Stop Time	10 min
Column	Type	Zorbax RX-SIL
	Particle Size	5 micron
	Dimensions	4.6 mm x 250 mm
	Temperature	40 °C
Injection	Volume	50.0 µL
	Draw Speed	200 µL/min

UV Detector	Stop Time	as Pump: 10 min
	Wavelength	326, 4 nm
	Reference Wavelength	450, 80 nm
	Slit Width	4 nm
	Flow Cell Path Length	10 mm
	Peak width	0.05 min
	Sampling Interval	0.320 sec
Calibration	Replace	All Levels
Integration Events:	Area Reject	0.50
(“Event_VWD1A”)	Slope Sensitivity	1.75
	Peak Width	0.01
	Height Reject	0.50
	Shoulders	OFF
	Baseline at Valleys On	2.20
	Integration On	2.20
	Integration Off	5.0
Report Parameters	Destination	Printer, File
	File Types	.DIF, CSV, TXT, XLS
	Report Style	Short
	Quant. Results Sorted By	Signal
	Chrom. Orientation	Portrait
	Chrom. Time Size	0.00 to 10.00 min
	Chrom. Response Range	Full
Calibration Table Parameters	Type	External Standard
	Basis	Peak Areas
	Rel. Reference Window	0.5 min

	Curve Type	Linear
	Origin	included
Calibration Table	Cis/trans=0.045 (0.035-0.055)	
13-cis retinyl palmitate	Level 1	433.5
	Level 2	867
	Level 3	1,734
All-trans retinyl palmitate	Level 1	11,666.5
	Level 2	23,333
	Level 3	46,666

8.1.b HPLC Performance Evaluation/Troubleshooting

1. Check the instrument pressure reading on the Chemstation after the system has been on for one hour with the correct mobile phase. The pressure should not be more than 10 bar higher than the normal reading for the HPLC using the same mobile phase and flow rate. The normal pressure reading will be different for each HPLC/column combination. To determine the correct reading, check the logbook for the last pressure reading entry for a vitamin A batch. The reading will probably be between 70-80 bar.

2. If the reading is too high, install a new precolumn filter. If this does not bring down the pressure, install a new guard column. If the pressure is still high, report the problem to the supervising chemist/technician.

3. If the reading is too low, look for leaks. The most common place for a leak is at the precolumn filter, guard cartridge holder, or at the column. Tighten fittings carefully, only applying just enough pressure to stop the leak. “Cranking” on fittings will ruin the fitting and cause a larger leak.

4. Adjust the UV signal attenuation on-screen until the “noise” fills up 10-20% of the chromatogram. Pump for at least 60 minutes to fully equilibrate the system. Check to see that the baseline (imaginary line through the center of the noise) has been flat for at least 5 minutes.

5. Inject the standard (using the Vita_com method) and wait 10 minutes until the report emerges at the printer.

6. The retention time for retinyl palmitate must be between 2.5 and 3.5 minutes. If it is not within this range, carefully prepare a new batch of mobile phase

according to these WI and equilibrate the HPLC for at least 30 minutes before reinjecting the standard. If the peak is still not within this range, report this to the supervising chemist/technician.

7. The retinyl palmitate peak must be gaussian-shaped with no "leading edge" or "trailing edge." The main thing to look for is a symmetrical peak. That is, if you draw an imaginary line down through the center of the peak, the resulting halves are mirror images. If you see a fronting peak (leading edge) or a tailing peak (trailing edge) report this to the supervising chemist/technician. If you must troubleshoot this problem, first try mixing up a new batch of mobile phase. If that doesn't work, try a new guard and/or analytical column.

8. Measure the noise on the lowest-level standard report chromatogram, just after the retinyl palmitate peak. Measure the peak height from the center of the noise (imaginary line) to the top of the peak. Calculate the signal-to-noise by dividing the peak-height measurement by the noise-height measurement. This result must be at least 10.0 to proceed. If the signal-to-noise ratio is <10.0, report this to the supervising chemist/technician.

9. The last performance parameter to check is the target area. The middle-level standard should give an area of 299 +/-5% for the hexane standard and 273 +/-5% for the acetone:chloroform standard. If the areas are not within the specified range, report this to the supervising chemist/technician. (NOTE: These numbers are instrument dependent. Determine these numbers before running samples).

10. If all of these performance parameters are within range, the sequence for the determination of this batch of samples can be started. Refer to section 9.4 to start the HPLC determination. Also see section 10 for additional performance checks to be made after the sequence has begun.

9. Sample Analysis

9.1 Solvent Preparation

9.1.a Acetone:choloroform Solvent: 30% acetone:70% chloroform

1. Rinse a clean 4L jug and a 2000 mL graduated cylinder with HPLC grade acetone.

2. Measure 1200 mL of HPLC grade acetone using the 2000 mL graduated cylinder and add it to the 4L jug.

3. Measure 1400 mL of chloroform using the 2000 mL graduated cylinder and add it to the 4L jug. Repeat this process for a total of 2800 mL chloroform.

4. Invert the jug several times to mix (careful to release pressure, if heating occurs) and allow the solvent to come to room temperature. It will help to place the jug in a sink of room temperature water to speed up the process.

9.1.b HPLC Mobile Phase: 96% wet hexane/4% ethyl ether/0.1% ethyl palmitate

1. To prepare the "wet hexane" solution, add at least 200 mL of Millipore water to a 4L jug of hexane and shake well. Let this jug stand overnight.

2. Rinse a 1L HPLC solvent reservoir and a 1000 mL graduated cylinder with methanol and then with hexane.

3. Add 960 mL of wet hexane to the 1L reservoir using a 1000 mL graduated cylinder.

4. Add 40 mL of ethyl ether to the 1L reservoir using a 50 mL graduated cylinder or a syringe.

5. Add 1 mL of ethyl palmitate to the reservoir using the FinnPipet. It may be necessary to heat the ethyl palmitate in the GC oven at 30 °C to liquify.

6. Swirl the reservoir for 15 seconds to mix. After installing the reservoir on the HPLC, open the prime/purge valve and pump at 10.0 mL/min for a few minutes to purge the air bubbles from the system. Reset the flow and close the valve.

9.2 Extraction

1. Weigh 4.95 to 5.04 g of the commodity sample into a 50 mL centrifuge tube.

2. Continue to weigh out all samples including the check samples (one for each commodity being analyzed).

3. Add 7 mL of 2-propanol to the first sample tube, replace the cap and tap firmly on the edge of the benchtop to fully wet the commodity. Repeat this procedure for all the samples.

4. Add 3 mL of water to each tube, replace each cap and again tap on the benchtop to mix. Wait at least 10 minutes and then add 25 mL of acetone:chloroform solution to each tube or hexane if running APF or BF samples.

5. Shake the tubes for 2 hours at 250 rpm on the orbital shaker.

9.3 Evaporation

1. Place the tube racks upright and let sample extracts settle out. For acetone:chloroform extracts, centrifuge at 2500 rpm for 5 minutes. Label one set of autosampler vials with the appropriate sample IDs.

2. For acetone:chloroform extracts, filter about 3 mL of the lower layer through the syringe filter (0.45 micron, PTFE) into a culture tube. Be aware that there may be only a single layer. In this case just take the aliquot from the middle of the tube. Filtering can be performed with the FinnPipet by cutting off about 5 mm from the end of a pipet tip so that it will fit snugly into the syringe filter. Measure 1 mL of the filtered extract into an autosampler vial. For hexane extracts, transfer 1 mL of the top hexane layer from each sample tube to an autosampler vial using the FinnPipet.

3. Place the vials in the custom vial rack and then into the Turbovap LV nitrogen evaporator. Evaporate the samples at 45 °C with a very low flow of nitrogen. The nitrogen flow should be adjusted by starting with the pressure at 0 and increasing carefully until the liquid surface in each vial is moving, but without any splashing. Evaporate just to dryness (about 15 minutes for hexane and about 30 minutes for acetone:chloroform). Remove the vials promptly.

4. Add 1 mL of water-saturated hexane to the first autosampler vial and cap immediately. Repeat until all vials are capped. Shake each vial briefly to mix.

9.4 Determination

1. Load the method, "Vita_com" and turn on the pump and lamp if they are not already on.

2. The instrument should equilibrate at 2.0 mL/min and 40 °C for at least 60 minutes before sample analysis begins. The HPLC pump should be started before the extraction and evaporation steps to allow enough time for equilibration and analysis of the calibration standards.

3. Enter the analysis sequence so that the calibration standards are analyzed first, from low to high, the QC samples, and finally the samples to be determined. The calibration levels consist of 12,100, 24,200 and 48,400 IU/Kg of total retinyl palmitate in the solid sample. The levels are obtained using a single calibration standard with injection sizes of 25 µL, 50 µL, and 100 µL. A check calibration using a separate calibration vial must be run after every 10 samples to check for instrument drift. The continuing calibration is run as "sample," not as "calibration" in the sequence table. Save the sequence and print out a copy. Start the sequence by choosing "Run Sequence."

4. Load the standard, QC samples, and samples into the autoinjector and double-check the sequence printout to be sure the samples are in the correct order. Start the sequence.

5. Check the first report to be sure the retinyl palmitate peak is found and integrated. If the

retention times are slightly out of range, abort the sequence, enter the new retention time of the retinyl palmitate peaks into the calibration table, save the method, and restart the sequence. If the peaks are not integrated properly, check the integrator parameters in the method against those listed in section 8.1.a. After adjusting the parameters, the method can be saved and the sequence restarted.

6. When the three calibration levels have been analyzed, run the Excel macro, VitQC, to determine if the CV, %CIS, and Target area values are within specification. If the curve is not within the specifications listed on the report, prepare a new calibration curve and restart the sequence.

10. Data Validation

10.1 Peak Shape and Retention Time

Refer to section 8.1.b for the acceptable retention time range and peak shape. If the peak shape has deteriorated or the retention time has drifted out of range, the whole sequence must be reinjected. If more than 24 hours have passed since the initial sequence was started, the samples must be reanalyzed from the beginning.

10.2 Integration of Peaks

Correct integration of the retinyl palmitate peaks can be determined by observing the "integration line" drawn at the base of the peak in the "chromatogram" section of the report. Ideally, the line should begin at the point where the response just increases above the baseline and stop at the point where the response just meets the baseline. Unfortunately, it is not always easy to determine where the baseline really is. For these WI we will define two cases.

In the first case, the baseline has "shifted" while the peak eluted and either the front edge of the peak is higher or lower than the back edge. When this occurs, the peak integration line should stay along the baseline and another vertical line should be drawn up to the start or end of the peak. This shifting may have occurred because of a "drifting" of the instrument response, or because of an interfering peak. Under no circumstances should the peak be integrated using this method if the baseline has shifted more than 25% of the total peak height. If this is the case, the sample must be reinjected or reanalyzed to eliminate the interference.

In the second case, a broad interference peak exists under the peak and both the front and the back edges of the peak are elevated off of the baseline. Be careful that you know where the baseline really is. You can determine this by looking at the whole chromatogram and drawing a line from the response at 0 time to that at 10 minutes. This is usually the true baseline. If it is determined that the retinyl palmitate peaks occur on a broad interference, then the peak must be "skimmed" off of the interference peak. That is, the integration line should be drawn from the point where the response just increases above the "interference baseline" and stop at the point where the response just meets the "interference baseline." The "interference baseline" is an imaginary line drawn that

outlines the expected shape of the broad interference peak.

10.3 Signal-to-Noise Ratio

Refer to section 8.1.b for the acceptable signal-to-noise ratio for the 12,100 IU/Kg standard. If the signal-to-noise ratio is <10.0, the quantitative result is not reliable and must not be reported. This signal-to-noise ratio does not include separated background interferences. It refers only to the random noise created by the instrumentation. If this noise cannot be determined due to matrix interferences, and standard peaks have acceptable signal-to-noise ratios, it can be concluded that the results for the sample are within the signal-to-noise ratio specification.

10.4 Interferences

When another peak is merged with the retinyl palmitate peak such that either the start or end of the peak is elevated more that 25% of the total peak height and the interfering peak's width at half height is within 3 times that of the retinyl palmitate peak, the sample must be reinjected. After reinjection, if the interference peak still appears, the sample must be reanalyzed. When the interfering peak is >3 times the width of the analyte peak, it is classified as a broad interference and can be integrated as described in section 10.2.

10.5 Calibration Table

When the coefficients of variation of the response factors in the calibration table are out of range (>3% for the 13-cis and >1% for the all-trans) and the sequence was analyzed anyway, results that are "not detected" can still be reported. Other sample results will have increased variability and should not be reported. In this case new standards must be prepared and all samples reinjected.

10.6 Sample Dilutions

Samples with results that are >75,000 IU/Kg must be diluted and reinjected so that they are within the calibrated range. A standard dilution factor of 5 should be used. For example, if a sample result was 80,000 IU/Kg, the sample must be diluted by adding 100 µL of the original sample and 400 µL of water-saturated hexane to a new autosampler vial. The sample result must be multiplied by 5 to get the final result.

10.7 Check Calibration

Check calibration injections must fall within the range of 0.95 to 1.05 times that of the 24,200 IU/Kg responses. If they do not, the ten samples after this check calibration standard must be reinjected along with a new calibration curve. Reprocessing is not an option here since the out-of-

range result could have been caused by an injection problem.

10.8 Control Charting

One check sample is analyzed for each commodity in each batch of samples. The result should be plotted on a control chart with previous results for that commodity. For most commodities, a difference of >15% from the mean should be a warning sign. If the result is greater than 2 standard deviations from the mean, the supervising chemist/technician should be notified. If the result does not lie within 3 standard deviations of the mean the whole batch of samples for that commodity must be reanalyzed.

11. Data Reporting

11.1 Vitamin A Results Macro

Running this macro will produce a summary of all sample results for this batch. It is important to check each injection for proper integration before running the macro. The macro adds results for the 13-cis and all-trans isomer to give a total retinyl palmitate result. It also rounds the numbers so that 3 significant figures are reported in IU/Kg. The Hamilton syringes used to make the calibration standard are good only to 3 significant figures. For this reason, the final result must be rounded to 3 significant figures. A result of 24,350 IU/Kg will be rounded and reported as 24,400 IU/Kg.

A control chart should be generated showing the historical results of each check sample analysis. The chart should show the mean, two, and three standard deviations. The charts allow for a quick check that the analyses were in statistical control (within 3 standard deviations of the mean).

11.2 Reporting Procedure

The Vitamin A Certificate macro should be run to generate the lab certificate. The certificate will list the customer ID (or LAB ID), the internal ID, the sample type, and the result in both IU/Kg and IU/lb. The supervising chemist/technician must sign the certificate before the results can be released.

The Vitamin A Access macro should be run to generate an input file into the Trace_Lab database.

12. Isomer Calculation Equations

It has not proven possible to obtain a 100% pure all-trans retinyl palmitate standard. The Sigma standard used in the GIPSA lab usually contains about 4.5% of the 13-cis isomer. For this reason the 13-cis

compound must be taken into account when the standard is prepared. In addition, the 13-cis isomer also has biological activity at about 75% the level of the all-trans isomer. The 13-cis isomer should be quantified in samples containing this isomer and the amount added to the all-trans result. The following derivation was used to generate the equation used in section 7 and to generate the equations used to calculate the concentrations of the 13-cis and all-trans isomers in the HPLC calibration table given in section 8.

The extinction coefficients for all-trans and 13-cis retinyl palmitate were taken from the literature (see section 3). The λ_{max} values for the all-trans and 13-cis isomers in hexane are 326 and 328 nm, respectively. Since our measurements are being taken 326 nm instead of the λ_{max} of the 13-cis isomer, the extinction coefficient for this isomer has been adjusted downward by 2% from the literature value.

ε_{cis} = 47,300 cm^{-1} l mol^{-1}
ε_{trans} = 52,100 cm^{-1} l mol^{-1}
Molecular weight = 524.9 g/mol (all-trans or 13-cis retinyl palmitate)
Molecular weight = 286.5 g/mol (retinol)
1 IU = 0.3 µg of retinol

In this method, the UV spectrophotometer is used to standardize the concentration of the all-trans retinyl palmitate. Since it is actually a mix of the two isomers, the following equation follows:

(1) $$A_{326nm} = A_{cis} + A_{trans}$$

where A_{326nm} is the total UV absorbance at 326 nm
A_{cis} is the UV absorbance contributed by 13-cis retinyl palmitate
A_{trans} is the UV absorbance contributed by all-trans retinyl palmitate

From Beer's Law we know that

(2) $$A_{cis} = \varepsilon_{cis} b c_{cis}$$

(3) $$A_{trans} = \varepsilon_{trans} b c_{trans}$$

where ε is the extinction coefficient
b is the path length
c is the concentration

Analysis of the standard by HPLC allows the determination of the area ratios of the two isomers by UV detection at 326 nm and allows us to write equation (4).

(4) $$\frac{Peak\_Area_{cis}}{Peak\_Area_{trans}} = \frac{\varepsilon_{cis} b' c_{cis}}{\varepsilon_{trans} b' c_{trans}} = \sigma$$

where Peak_Area is the area of the HPLC peaks for the two isomers detected at 326 nm.
Rearranging equation (4) gives (4a):

(4a) $$c_{cis} = \frac{c_{trans}\varepsilon_{trans}\sigma}{\varepsilon_{cis}}$$

Combining equations (1)-(3) and plugging (4a) back in gives equations (5) and (6).

(5) $$c_{cis} = \frac{A_{326}(524,900)}{\varepsilon_{cis}(1+\frac{1}{\sigma})}$$

And

(6) $$c_{trans} = \frac{A_{326}(524,900)}{\varepsilon_{trans}(1+\sigma)}$$

where c in both cases is in units of µg/mL

These equations allow the calculation of the concentration of either isomer from the total absorbance of the solution and the isomer ratio σ of that same solution determined by HPLC.

The target concentration of total retinyl palmitate for processed-grain commodities is 24,200 IU/Kg. This leads to equation (7):

(7) $$c_{cis} + c_{trans} = 24,200$$

Adding a factor of 0.75 to equation (4a) to account for the lower biological activity of 13-cis retinyl palmitate and then plugging (4a) into equation (7) gives equation (8):

(8) $$c_{cis} = \frac{(0.75)(24,200)\sigma\varepsilon_{trans}}{\varepsilon_{cis} + (0.75)\varepsilon_{trans}\sigma}$$

Equation (8) allows the calculation of the concentration of the cis isomer from the total retinyl palmitate in IU/Kg. For a σ of 0.045 and a total retinyl palmitate concentration of 24,200, the concentration of the 13-cis isomer is 867 IU/Kg. This makes the all-trans isomer 23,333 IU/Kg.

Dividing the all-trans target concentration by 1000 and multiplying by 5 gives 116.7 IU, which is the amount in a single sample tube required to give a concentration of 23,333 IU/Kg in the sample. This amount in International Units of Vitamin A can be converted to µg of all-trans retinyl palmitate using the ratio of the molecular weights of retinol and retinyl palmitate along with the definition that says 1 IU = 0.3 µg of retinol.

$$(9) \qquad \left(116.67 IU_{atrp}\right)\left(\frac{524.9 g/m}{286.5 g/m}\right)\left(\frac{0.3 ug}{1 IU}\right) = 64.12 ug_{atrp}$$

where atrp = all-trans retinyl palmitate

Equation (9) shows that a 5g sample with a concentration of 24,200 IU/Kg contains 64.12 µg of all-trans retinyl palmitate.

The equation for the volume of standard solution required in µL to prepare a 24,200 IU/Kg standard (or to add 64.12 µg of all-trans retinyl palmitate) is given in equation (10) where c_{trans} is in µg/µL.

$$(10) \qquad (Vol_{\mu L})(c_{trans}) = 64.12 \mu g$$

We can substitute equation (6) in for c_{trans} in equation (10) along with a factor of 200 to account for the dilution of the concentrated standard (1000 µg/mL) to that used to measure the UV (5 µg/mL) to give equation (11).

$$(11) \qquad Vol_{\mu L} = \frac{31.82(1+\sigma)}{A_{326}}$$

For $\sigma = 0.045$ equation (12) results.

$$(12) \qquad Vol_{\mu L} = \frac{33.25}{A_{326}}$$

13. Excel Macros

13.1 Vitamin A Results

This macro produces a spreadsheet summarizing the results for the batch result information. No template is stored in Sheet1 or in Sheet2.

```
'
' Vitamin A Res Macro
' Macro written 3/16/99 by Tim Norden
'
'
Sub VitaminA_Res()
'
'Prompt the user for the subdirectory and datafile name.
'The MainDir and TemplateName statements can be changed to reflect
'where the datafiles are located.
```

```
'
   Instrument = InputBox("Please enter the instrument number:", "Instrument")
   If Instrument = "1" Then
   MainDir = "Q:\1\DATA\"
   Else: MainDir = "Q:\2\DATA\"
   End If
   BatchName = InputBox("Enter the Batch Name:", "Batchname")
   StoreFile = MainDir & BatchName & "\" & BatchName
   Row = 4
'
'Test each datafile to determine if the directory holds the valid calibration data.
'This is done by looking in Report00.dif see if it is a "Level 4" calibration sample.
'
   NextFile = Dir(MainDir & BatchName & "\*.d", vbDirectory)
   Workbooks.Add
   Cells(1, 1) = BatchName
   Range("A1").Select
   With Selection.Font
      .Name = "Arial"
      .FontStyle = "Regular"
      .Size = 12
      .Strikethrough = False
      .Superscript = False
      .Subscript = False
      .OutlineFont = False
      .Shadow = False
      .Underline = xlNone
      .ColorIndex = xlAutomatic
   End With
   Selection.Font.Bold = True
   ActiveWorkbook.SaveAs Filename:="New.xls"
10  Do While NextFile <> ""
   FirstFile = MainDir & BatchName & "\" & NextFile & "\" & "Report00.dif"
   Workbooks.Open Filename:=FirstFile
   Test = Cells(1, "A")
   ActiveWorkbook.Close
   If Test = "Report Title" Then
   InfoFile = MainDir & BatchName & "\" & NextFile & "\" & "Report02.dif"
   DataFile = MainDir & BatchName & "\" & NextFile & "\" & "Report03.dif"
   Else
   InfoFile = MainDir & BatchName & "\" & NextFile & "\" & "Report00.dif"
   DataFile = MainDir & BatchName & "\" & NextFile & "\" & "Report01.dif"
   End If
'
'First, paste in the BatchName and get the Sample Name from the datafile.
```

```
'
    Row = Row + 1
    If Row <> 5 Then GoTo 20
    Workbooks.Open Filename:=DataFile
    Range("H1:H4").Select
    Selection.Copy
    Windows("New.xls").Activate
    Range(Cells(Row - 2, 2), Cells(Row - 2, 2)).Select
    Selection.PasteSpecial Paste:=xlAll, Operation:=xlNone, SkipBlanks _
       :=False, Transpose:=True
    Windows(2).Activate
    ActiveWorkbook.Close


20  Workbooks.Open Filename:=DataFile
    Range("E1:E4").Select
    Application.CutCopyMode = False
    Selection.Copy
    Windows("New.xls").Activate
    Range(Cells(Row, 2), Cells(Row, 2)).Select
    Selection.PasteSpecial Paste:=xlAll, Operation:=xlNone, SkipBlanks _
       :=False, Transpose:=True
    Windows(2).Activate
    ActiveWorkbook.Close

    Workbooks.Open Filename:=InfoFile
    Range("B1").Select
    Application.CutCopyMode = False
    Selection.Copy
    Windows("New.xls").Activate
    Range(Cells(Row, 1), Cells(Row, 1)).Select
    ActiveSheet.Paste
    Windows(2).Activate
    ActiveWorkbook.Close
    NextFile = Dir()
    Loop
'
'Check for dashes and replace them with zeros
'
    Windows("New.xls").Activate
    For i = 5 To Row
    check = ActiveSheet.Cells(i, 2)
    If check = "-" Then Cells(i, 2) = "0"
    check = ActiveSheet.Cells(i, 3)
    If check = "-" Then Cells(i, 3) = "0"
```

```
    Next i
'
'We just need to saveas, print and do some file cleanup.
'The intent is not to change any files except the newly created one.
'
    ActiveSheet.Cells(3, 4) = "total retinyl palmitate"
    ActiveSheet.Cells(3, 5) = "total retinyl palmitate"
    ActiveSheet.Cells(4, 4) = "(IU/Kg)"
    ActiveSheet.Cells(4, 5) = "(IU/LB)"
    Range("D5").Select
    ActiveCell.FormulaR1C1 = "=IF((RC[-2]+RC[-1])-10000>0, ROUND(RC[-2]+RC[-1], -2),
ROUND(RC[-2]+RC[-1], -1))"
    Range("D5").Select
    Selection.AutoFill Destination:=Range(Cells(5, 4), Cells(Row, 4)), Type:=xlFillDefault
    Range("E5").Select
    ActiveCell.FormulaR1C1 = "=IF(((RC[-3]+RC[-2])*0.454)-10000>0, ROUND(((RC[-3]+RC[-
2])*0.454), -2), ROUND(((RC[-3]+RC[-2])*0.454), -1))"
    Range("E5").Select
    Selection.AutoFill Destination:=Range(Cells(5, 5), Cells(Row, 5)), Type:=xlFillDefault
    Columns("A:E").AutoFit
      Columns("A:A").Select
    Selection.NumberFormat = "@"
      Columns("B:E").Select
    Selection.NumberFormat = "#,##0"
    With Selection
      .HorizontalAlignment = xlCenter
      .VerticalAlignment = xlBottom
      .WrapText = False
      .Orientation = xlHorizontal
    End With
      Columns("B:E").Select
    Selection.Columns.AutoFit
    With ActiveSheet.PageSetup
      .PrintTitleRows = ""
      .PrintTitleColumns = ""
    End With
    ActiveSheet.PageSetup.PrintArea = ""
    With ActiveSheet.PageSetup
      .LeftHeader = ""
      .CenterHeader = ""
      .RightHeader = ""
      .LeftFooter = ""
      .CenterFooter = ""
      .RightFooter = ""
      .LeftMargin = Application.InchesToPoints(0.75)
```

```
        .RightMargin = Application.InchesToPoints(0.75)
        .TopMargin = Application.InchesToPoints(1)
        .BottomMargin = Application.InchesToPoints(1)
        .HeaderMargin = Application.InchesToPoints(0.5)
        .FooterMargin = Application.InchesToPoints(0.5)
        .PrintHeadings = False
        .PrintGridlines = True
        .PrintComments = xlPrintNoComments
        .PrintQuality = 600
        .CenterHorizontally = False
        .CenterVertically = False
        .Orientation = xlPortrait
        .Draft = False
        .PaperSize = xlPaperLetter
        .FirstPageNumber = xlAutomatic
        .Order = xlDownThenOver
        .BlackAndWhite = False
        .Zoom = 100
    End With
    Sheets("Sheet1").Name = BatchName
    Workbooks("New.xls").SaveAs Filename:=StoreFile
    Kill "New.xls"
    Windows("VitaRes.XLS").Activate
    ActiveWorkbook.Close saveChanges:=False
End Sub
```

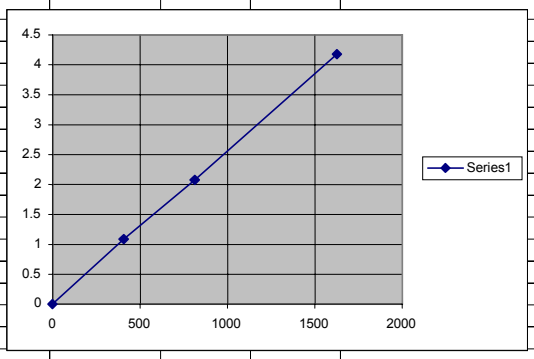
13.2 Vitamin A QC

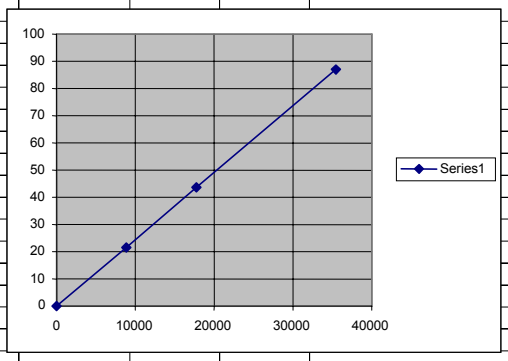
This macro generates means and coefficients of variation (CV) for each set of response factors. To be acceptable, the CV must be <1.0% (or 3.0% for the cis isomer). The correlation coefficient must be at least 1.0000 to continue analyzing the batch. Percentage cis isomer must be 0.035 to 5.5%.

The spreadsheet contains the following template stored in Sheet1 and saved with the macro:

Template

0	0	0	0	13-cis retinyl palmitate		0	0	0	0	all trans retinyl palmitate
1	406.5	1.083593	375.1408	13-cis retinyl palmitate		1	8869.5	21.60995	410.436	all-trans retinyl palmitate
2	813	2.0748	391.8451	13-cis retinyl palmitate		2	17739	43.60413	406.8192	all-trans retinyl palmitate
3	1626	4.176197	389.3495	13-cis retinyl palmitate		3	35478	87.06573	407.4852	all-trans retinyl palmitate
	mean		385.4451				**mean**		408.2468	
	std. dev.		9.0106				**std. dev.**		1.9249	
	CV		2.34%	<3.00			**CV**		0.47%	<1.00
	Corr. Coeff.	0.9999	=1.0000				**Corr. Coeff.**	1.0000	=1.0000	
	CIS	24.14783								
	%CIS	27.7%	3.5-5.5%				**Target Area**	43.60	299h (273ac)	






```
'
' Vitamin A QC Macro
' Macro written 3/19/98 by Tim Norden
'
'
Sub VitA_QC()
'
'Prompt the user for the subdirectory and datafile name.
'The MainDir and TemplateName statements can be changed to reflect
'where the datafiles are located.
'
    Instrument = InputBox("Please enter the instrument number:", "Instrument")
    If Instrument = "1" Then
    MainDir = "Q:\1\DATA\"
    Else: MainDir = "Q:\2\DATA\"
    End If
    BatchName = InputBox("Enter the Batch Name:", "Batchname")
    filename = InputBox("Enter the File Name containing the Calibration Data without the
Extension or Press Enter to Automatically Find the Files:", _
```

```
                    "Calibration Data File Directory")
    If filename <> "" Then
    CalFile = MainDir & BatchName & "\" & filename & ".d\" & "Report01.dif"
    InfoFile = MainDir & BatchName & "\" & filename & ".d\" & "Report02.dif"
    ExcelFile = MainDir & BatchName & "\" & filename & ".d\" & "Report01.xls"
    StoreFile = MainDir & BatchName & "\" & filename & ".d\" & filename
    GoTo 20
    End If
'
'Test each datafile to determine if the directory holds the valid calibration data.
'This is done by looking in Report00.dif see if it is a "Level 3" calibration sample.
'
    NextFile = Dir(MainDir & BatchName & "\*.d", vbDirectory)
10  Do While NextFile <> ""
    Pos = InStr(NextFile, ".D")
    filename = Left(NextFile, Pos - 1)
    LevelFile = MainDir & BatchName & "\" & filename & ".d\" & "Report00.dif"
    CalFile = MainDir & BatchName & "\" & filename & ".d\" & "Report01.dif"
    InfoFile = MainDir & BatchName & "\" & filename & ".d\" & "Report02.dif"
    ExcelFile = MainDir & BatchName & "\" & filename & ".d\" & "Report01.xls"
    StoreFile = MainDir & BatchName & "\" & filename & ".d\" & filename
    Workbooks.Open filename:=LevelFile
    level = Cells(17, "B")
    ActiveWorkbook.Close
    NextFile = Dir()
    If level <> "3" Then GoTo 10
'
'First, get the calibration data from the datafile, Report01.dif.
'
20  Workbooks.Open filename:=CalFile
    Columns("G:I").Select
    Selection.Delete Shift:=xlToLeft
    Range("C1:G3").Select
    Application.CutCopyMode = False
    Selection.Copy
    Windows("vitaQC.XLS").Activate
    Sheets("Template").Select
    Range("A4").Select
    ActiveSheet.Paste

    Windows("report01.dif").Activate
    Range("C4:G6").Select
    Application.CutCopyMode = False
    Selection.Copy
    Windows("vitaQC.XLS").Activate
    Sheets("Template").Select
```

```
    Range("G4").Select
    ActiveSheet.Paste
'
'Next, get the areas of the cis and trans isomers from the datafile, Report01.xls.
'
    Workbooks.Open filename:=ExcelFile
    Sheets("Peak").Select
    check = "0"
    Row = 2
    Do While check <> ""
    check = Cells(Row, 18)
    If check = 1 Then GoTo 30
    Row = Row + 1
    Loop
30  ActiveSheet.Cells(Row, 14).Select
    Selection.Copy
    Windows("vitaQC.XLS").Activate
    Sheets("Template").Select
    Range("C15").Select
    ActiveSheet.Paste
'
'Now get the pathname and sampleinfo data from the datafile, Report02.dif.
'
    Workbooks.Open filename:=InfoFile
    Range("B1").Select
    Selection.Copy
    Windows("vitaQC.XLS").Activate
    Sheets("Template").Select
    Range("A1").Select
    ActiveSheet.Paste
    Application.CutCopyMode = False
    With Selection.Font
       .Name = "Arial"
       .FontStyle = "Regular"
       .Size = 14
       .Strikethrough = False
       .Superscript = False
       .Subscript = False
       .OutlineFont = False
       .Shadow = False
       .Underline = xlNone
       .ColorIndex = xlAutomatic
    End With
    Selection.Font.Bold = True
    Windows("REPORT02.DIF").Activate
    Range("B3").Select
```

```
    Selection.Copy
    Windows("vitaQC.XLS").Activate
    Sheets("Template").Select
    Range("A2").Select
    ActiveSheet.Paste
    Windows("REPORT02.DIF").Activate
    Range("C3").Select
    Application.CutCopyMode = False
    Selection.Copy
    Windows("vitaQC.XLS").Activate
    Sheets("Template").Select
    Range("E2").Select
    ActiveSheet.Paste
    Application.CutCopyMode = False
'
'We just need to saveas, print and do some file cleanup.
'The intent is not to change any files except the newly created one.
'
    Windows("vitaQC.XLS").Activate
    Sheets("Template").Select
    Sheets("Template").Copy
    Sheets("Template").Name = BatchName
    ActiveWorkbook.SaveAs filename:=StoreFile, FileFormat:=xlNormal _
       , Password:="", WriteResPassword:="", ReadOnlyRecommended:= _
       False, CreateBackup:=False
    ActiveWindow.SelectedSheets.PrintOut Copies:=1
    Windows("Report01.xls").Activate
    ActiveWorkbook.Close
    Windows("Report02.dif").Activate
    ActiveWorkbook.Close
    Windows("Report01.dif").Activate
    ActiveWorkbook.Close saveChanges:=False
    Loop
    Windows("vitaQC.XLS").Activate
    ActiveWorkbook.Close saveChanges:=False
 End Sub
```

13.3 Vitamin A Certificate

The spreadsheet contains the following template stored in Sheet1 and saved with the macro:

United States Department of Agriculture				
Grain Inspection, Packers and Stockyards Administration				
Technical Services Division				
Trace Analysis Laboratory				
Analysis Type:	Vitamin A in Commodities			
Trace Lab Batch:				
Analyst:				
Date Analyzed:				
LAB_ID	**Internal_ID**	**Sample Type**	**Result (IU/Kg)**	**Result (IU/LB)**
Approval:	Joe Barney 816-891-0465			

```
Sub Vitamin_Certificate()
'
' Vitamin_Certificate Macro
' Macro recorded 12/15/1999 by Tim Norden
'
'
'Prompt the user for the subdirectory and datafile name.
'The MainDir and TemplateName statements can be changed to reflect
'where the datafiles are located.
'
    Instrument = InputBox("Please enter the instrument number:", "Instrument")
    If Instrument = "1" Then
    MainDir = "Q:\1\DATA\"
    Else: MainDir = "Q:\2\DATA\"
    End If
    BatchName = UCase(InputBox("Enter the Batch Name:", "Batchname"))
    InternalID = UCase(InputBox("Enter the Starting InternalID", "InternalID"))
    SheetName = BatchName & "_Cert.xls"
    StoreFile = MainDir & BatchName & "\" & SheetName
    InfoFile = MainDir & BatchName & "\" & "vita0003.d" & "\" & "Report02.dif"
    Workbooks.Open FileName:=InfoFile
    Analyst = Cells(9, 2)
    InjDate = Left$(Cells(11, 2), 9)
    ActiveWorkbook.Close
    Cells(7, 2) = BatchName
```

```
    Cells(8, 2) = Analyst
    Cells(9, 2) = InjDate
    Range("B7:B9").Select
    With Selection.Font
        .Name = "Arial"
        .FontStyle = "Regular"
        .Size = 12
        .Strikethrough = False
        .Superscript = False
        .Subscript = False
        .OutlineFont = False
        .Shadow = False
        .Underline = xlNone
        .ColorIndex = xlAutomatic
    End With
    Range("B9:C9").Select
    Selection.Merge
    Selection.NumberFormat = "mmmm d, yyyy"
    Selection.HorizontalAlignment = xlLeft
    ActiveWorkbook.SaveAs FileName:=StoreFile
    ResultFile = MainDir & BatchName & "\" & BatchName & ".xls"
    Workbooks.Open FileName:=ResultFile
'
'Insert a column and parse the first column into two columns with the
'"-" as a delimiter.
'
    Columns("B:B").Select
    Selection.Insert Shift:=xlToRight
    Columns("A:A").Select
    Selection.TextToColumns Destination:=Range("A1"), DataType:=xlDelimited, _
        TextQualifier:=xlDoubleQuote, ConsecutiveDelimiter:=False, Tab:=False, _
        Semicolon:=False, Comma:=False, Space:=False, Other:=True, OtherChar _
        :="-", FieldInfo:=Array(Array(1, 2), Array(2, 1))
    Columns("A:A").Select
    Selection.Insert Shift:=xlToRight
'
'Check each cell in column one for the string, "std" and then delete
'the rows containing this string.
'
    Test = "not_null"
        chkcell = 5
        CellCount = 0
        IDCount = Right(InternalID, 4)
        IDString = Left(InternalID, 4)
```

```
10  Do While Test <> ""
    Test = UCase(Cells(chkcell, "B"))
    If Test = "" Then GoTo 10
    CellCount = CellCount + 1
    If Left(Test, 3) = "STD" Then
    Rows(chkcell).Select
    Selection.Delete Shift:=xlUp
    CellCount = CellCount - 1
    GoTo 10
    Else
    If Left(Test, 2) = "CC" Then
    ccCount = ccCount + 1
    ccID = Test & "_" & ccCount
    Cells(chkcell, 1) = ccID
    Cells(chkcell, 2) = ""
    chkcell = chkcell + 1
    GoTo 10
    Else
    If Left(Test, 2) = "RB" Or Left(Test, 2) = "MB" _
    Or Left(Test, 2) = "CS" Or Left(Test, 2) = "SK" Then
    Cells(chkcell, 1) = Test
    Cells(chkcell, 2) = ""
    chkcell = chkcell + 1
    GoTo 10
    Else
    Cells(chkcell, 1) = IDString & IDCount
    IDCount = IDCount + 1
    chkcell = chkcell + 1
    End If
    End If
    End If
    Loop
'
'Now insert new rows into temp.xls and copy the appropriate columns from
'the results file to the certificate file, temp.xls.
'
    Windows(SheetName).Activate
    Range(Rows("13"), Rows(CellCount + 11)).Select
    Selection.Insert Shift:=xlDown
    Selection.Font.Bold = False
    Selection.RowHeight = 18

    Windows(2).Activate
    Range(Cells(5, 2), Cells(CellCount + 4, 2)).Select
```

```
    Application.CutCopyMode = False
    Selection.Copy
    Windows(SheetName).Activate
    Range(Cells(13, 1), Cells(CellCount + 12, 1)).Select
    Selection.PasteSpecial Paste:=xlValues, Operation:=xlNone, SkipBlanks:= _
    False, Transpose:=False

    Windows(2).Activate
    Range(Cells(5, 1), Cells(CellCount + 4, 1)).Select
    Application.CutCopyMode = False
    Selection.Copy
    Windows(SheetName).Activate
    Range(Cells(13, 2), Cells(CellCount + 12, 2)).Select
    Selection.PasteSpecial Paste:=xlValues, Operation:=xlNone, SkipBlanks:= _
    False, Transpose:=False

    Windows(2).Activate
    Range(Cells(5, 3), Cells(CellCount + 4, 3)).Select
    Application.CutCopyMode = False
    Selection.Copy
    Windows(SheetName).Activate
    Range(Cells(13, 3), Cells(CellCount + 12, 3)).Select
    Selection.PasteSpecial Paste:=xlValues, Operation:=xlNone, SkipBlanks:= _
    False, Transpose:=False

    Windows(2).Activate
    Range(Cells(5, 6), Cells(CellCount + 4, 7)).Select
    Application.CutCopyMode = False
    Selection.Copy
    Windows(SheetName).Activate
    Range(Cells(13, 4), Cells(CellCount + 12, 5)).Select
    Selection.PasteSpecial Paste:=xlValues, Operation:=xlNone, SkipBlanks:= _
    False, Transpose:=False
    Selection.NumberFormat = "#,##0"

    Windows(2).Activate
    ActiveWorkbook.Close saveChanges:=False
    ActiveWorkbook.Save
End Sub
```

13.4 Vitamin A Access

The spreadsheet contains the following template stored in Sheet1 and saved with the macro:

Customer ID	Internal ID	Sample Type	Batch ID	Date Completed	Customer	External ID

The spreadsheet contains the following template stored in Sheet2 and saved with the macro:

Internal ID	Batch ID	Analyte	Test	Result

```
 Sub Macro1()
'
' Macro1 Macro
' Macro recorded 1/5/00 by Pesticide Lab
'

'
    Instrument = InputBox("Please enter the instrument number:", "Instrument")
    If Instrument = "1" Then
    MainDir = "Q:\1\DATA\"
    Else: MainDir = "Q:\2\DATA\"
    End If
    BatchName = UCase(InputBox("Enter the Batch Name:", "BatchName"))
    Infofile = MainDir & BatchName & "\" & BatchName & "_cert.xls"
    Workbooks.Open FileName:=Infofile


    Test = Range("B13").Select


    Counter = 0
    Do Until Test = ""
    Counter = Counter + 1
```

```
ActiveCell.Offset(1, 0).Select
Test = ActiveCell
Loop


Range(Cells(13, 1), Cells(Counter + 12, 1)).Select
Application.CutCopyMode = False
Selection.Copy
Windows("VitA_Acc_samphist.xls").Activate
Cells(2, 1).Select
Selection.PasteSpecial Paste:=xlValues, Operation:=xlNone, SkipBlanks:= _
False, Transpose:=False

Windows(BatchName & "_cert.xls").Activate

Range(Cells(13, 2), Cells(Counter + 13, 2)).Select
Application.CutCopyMode = False
Selection.Copy
Windows("VitA_Acc_samphist.xls").Activate
Cells(2, 2).Select
Selection.PasteSpecial Paste:=xlValues, Operation:=xlNone, SkipBlanks:= _
False, Transpose:=False

Windows(BatchName & "_cert.xls").Activate

Range(Cells(13, 3), Cells(Counter + 13, 3)).Select
Application.CutCopyMode = False
Selection.Copy
Windows("VitA_Acc_samphist.xls").Activate
Cells(2, 3).Select
Selection.PasteSpecial Paste:=xlValues, Operation:=xlNone, SkipBlanks:= _
False, Transpose:=False

Windows(BatchName & "_cert.xls").Activate

Let BatchID = Range("B7")
Let DateAnalyzed = Range("B9")
Let Analyte = "Vitamin A"
Let Com = "CTL"

Windows("VitA_Acc_samphist.xls").Activate

Counter2 = 0
Do While Counter2 < Counter
```

```
Cells(2 + Counter2, 4) = BatchID
Cells(2 + Counter2, 5) = DateAnalyzed
Cells(2 + Counter2, 6) = Com
Counter2 = Counter2 + 1
Loop



Windows(BatchName & "_cert.xls").Activate

Range(Cells(13, 2), Cells(Counter + 13, 2)).Select
Application.CutCopyMode = False
Selection.Copy
Windows("VitA_Acc_samphist.xls").Activate
Sheets("Sheet2").Select
Cells(2, 1).Select
Selection.PasteSpecial Paste:=xlValues, Operation:=xlNone, SkipBlanks:= _
False, Transpose:=False

Windows(BatchName & "_cert.xls").Activate

Range(Cells(13, 4), Cells(Counter + 13, 4)).Select
Application.CutCopyMode = False
Selection.Copy
Windows("VitA_Acc_samphist.xls").Activate
Sheets("Sheet2").Select
Cells(2, 5).Select
Selection.PasteSpecial Paste:=xlValues, Operation:=xlNone, SkipBlanks:= _
False, Transpose:=False

Windows(BatchName & "_cert.xls").Activate

Let Com = Cells(13, 3)
If Com = "OIL" Then Let Test = "VIAOIL" Else Let Test = "VIACOM"

Windows("VitA_Acc_Samphist.xls").Activate
Sheets("Sheet2").Select

Counter3 = 0
Do While Counter3 < Counter
Cells(2 + Counter3, 4) = Test
```

```
Cells(2 + Counter3, 3) = Analyte
Cells(2 + Counter3, 2) = BatchID
Counter3 = Counter3 + 1
Loop


Windows("VitA_Acc_Samphist.xls").Activate

SheetName = BatchName & "_ACC.xls"
StoreFile = MainDir & BatchName & "\" & SheetName

ActiveWorkbook.SaveAs FileName:=StoreFile
ActiveWorkbook.SaveAs FileName:="C:\Docs\Lims\Temp\VitA_Temp.xls"

Windows(BatchName & "_cert.xls").Activate
ActiveWorkbook.Close

End Sub
```

14. Method Validation

14.1 Overview

The determination of vitamin A as retinyl palmitate in processed-grain commodities is a new method for GIPSA. The Farm Services Administration has determined that the target fortification level for these samples will be 11,000 IU/LB or 24,200 IU/Kg. Samples will include all-purpose flour, bread flour, bulgur, soy-fortified bulgur, corn meal, soy-fortified corn meal, soy-fortified sorghum grits, corn-soy blend, and wheat-soy blend samples. Historical analysis has revealed that it may be necessary to run an average of 200 samples per week with a maximum of 400 during a heavy week. The method must allow for the analysis of at least 75 samples per day to provide the necessary two-day reporting time.

A search of the chemical literature in this area reveals that most of the published methods for grains or feeds utilize saponification during the extraction step. This involves refluxing the sample in a basic aqueous alcohol solution. The resulting extract must then go through extensive cleanup by extracting with large volumes of hexane. The hexane is then washed with water and evaporated. This whole process is time and resource intensive. Running 75 samples per day would not be cost effective using such a method. Work in our lab suggested that it might be possible to extract retinyl palmitate using a two-phase solvent system without saponification. An aqueous alcohol solution is used to dissolve the hydrophilic coating that encapsulates the vitamins and the retinyl palmitate is partitioned into an organic layer. After evaporation, the organic solvent is analyzed directly using liquid chromatography.

The liquid chromatographic determinative part of the method is based on a paper published in 1980 by Thompson, Hatina and Maxwell entitled, “High Performance Liquid Chromatographic Determination of Vitamin A in Margarine, Milk, Partially Skimmed Milk, and Skimmed Milk.” This part of the method is essentially identical to the GIPSA method for vitamin A in vegetable oils. This LC method is highly efficient, resolving the 13-cis isomer from the all-trans isomer in 3 minutes with a total run time of 10 minutes. The new GIPSA method for processed-grain commodities allows for the extraction and determination of as many as 90 samples in less than 24 hours.

This method was validated using the GIPSA-ARTS method validation protocol for fortified samples. All-purpose flour, bulgur and corn-soy blend samples were chosen for the fortification experiments. These experiments involve fortifying blank commodities with commercial vitamin premixes at three levels and then analyzing them over a period of four days with three different analysts. The resulting data is used to determine the accuracy and precision of this method in the GIPSA laboratory. The method was also compared to a traditional saponification method (see reference list). One randomly chosen sample of each of the nine commodities was run by the new method and by the saponification method.

14.2 Results and Discussion

Comparison with a Traditional Method

One sample was chosen from each commodity and run by several different extraction methods, including a traditional saponification method. The results of these experiments are given in Table I. The first three columns represent modifications of the new solvent extraction method and the last is the traditional saponification method. E/H stands for ethanol:water:hexane, E/CA for ethanol:water:chloroform:acetone, and I/H for isopropyl alcohol:water:hexane. According to the results, the I/H method gives comparable results to the E/H method, but in 2 hours rather than 5 hours. For all of the commodities except the APF and BF samples, the E/CA method gives higher results than the I/H method. For the flour samples (APF and BF), the E/CA method gives much lower results than the I/H or E/H methods. In nearly all comparisons, the optimum solvent extraction method gives higher results than the tradtional saponification method. The average percentage difference between these two methods for this set of samples is 16.8%.

To confirm these results, additional samples were run using the I/H and the E/CA method. A third method, I/H-Ex, was run to determine the maximum amount of retinyl palmitate that could be recovered using hexane. This method involved shaking the samples with two additional portions of hexane with the same sample and combining them. The results for these samples are given in Table II. For the flour and bulgur samples there is very little difference between the I/H and I/H-Ex methods. For the corn-soy blend samples the difference is much larger suggesting an incomplete extraction when using the I/H method. The E/CA results confirm the previous conclusion that this method works well for the CSB samples, but poorly for the flour samples. Additional experiments showed that substituting isopropyl alcohol for ethyl alcohol when using the choroform:acetone solvent system had no significant effect on the results.

On the basis of these experiments the isopropyl alcohol/water/acetone/chloroform method was chosen for all commodities except APF and BF samples. These samples were validated using the isopropyl alcohol/water/hexane solvent system.

Validation using Fortified Samples

Three different commercial vitamin premixes were used to fortify the three commodities: all-purpose flour, bulgur, and corn-soy blend. The flour premix was obtained from Richard Thornhill of ADM Arkady and the bulgur and corn-soy blend premixes were obtained from Bill Nienkamp of Crete Mills. The exact concentrations of each premix were determined by weighing *ca* 3,000 IU (30 – 130 mg) into a 250 mL flask, dissolving in 75 mL of water or 1% acetic acid, and diluting to the mark with isopropyl alcohol. All premix solutions were clear at this point showing that all of the retinyl palmitate is dissolved. Ten mL of this solution was combined with 25 mL of hexane, shaken, and analyzed according to the working instructions. The premixes were each run using both 1% acetic acid and water and the highest result was chosen to calculate the recoveries.

The method recovery and precision phase of the validation is meant to determine the labs best on any given day. A total of 18 samples are analyzed by a single analyst and consisting of:

1. Three (3) procedural blanks: Reagent blanks; no matrix and no retinyl palmitate.
2. Three (3) blank samples: Samples that do not contain retinyl palmitate.
3. Three (3) blank samples fortified at 6,050 IU/Kg.
4. Six (6) blank samples fortified at 24,200 IU/Kg
5. Three (3) blank samples fortified at 72,600 IU/Kg.

Tables III –V give the results from this phase of the study. Results for flour, bulgur and corn-soy blend samples all show mean recoveries close to 100%. Coefficients of variation (CV) are from 2 to 7% with the highest numbers reported for the bulgur samples. Results from this phase of the study are quite acceptable and show little if any bias.

The purpose of the method ruggedness testing is to determine the accuracy and precision of the results under "real" lab conditions. Two additional analysts analyzed the following set of randomly labeled samples over a period of three days.

1. One (1) procedural blanks: Reagent blanks; no matrix and no retinyl palmitate.
2. One (1) blank samples: Samples that do not contain retinyl palmitate.
3. Three (3) blank samples fortified at 6,050 IU/Kg.
4. Three (3) blank samples fortified at 72,600 IU/Kg
5. Three (3) commercially fortified samples at 24,200 IU/Kg.

Tables VI-VIII give the results of all four days of analyses with all three analysts. All mean recoveries were consistent with those obtained in the method recovery and precision phase. The CV values range from 2-9%, with the largest CV values generally resulting from the analysis of the lowest (*ca* 6000 IU/Kg) level.

14.3 Summary and Tables

This method, based on a two-phase solvent extraction of retinyl palmitate followed by LC analysis, provides for an efficient, accurate, and precise method for determining vitamin A in processed-grain commodity samples. Traditional methods for the analysis of vitamin A require a labor-intensive method that results in a low sample throughput. The new method allows for the analysis of 90 samples in a one workday by a single technician and uses $1/20^{th}$ of the solvent compared to the traditional method.

Table I. Comparison of Extraction Methods for Vitamin A in Ground Commodities

Commodity	E/H (5hr)*	E/CA (2hr)*	I/H (2hr)*	TM*	*delta*
BF	8,936	2,015	9,250	8,178	13.1%
CM	14,242	15,386	13,513	13,607	13.1%
SFB	10,402	11,448	8,607	10,322	10.9%
APF	16,964	6,594	18,580	14,612	27.2%
SFSG	10,821	9,891	9,330	8,982	10.1%
CSB	23,736	25,369	18,178	20,310	24.9%
BULG	18,989	20,881	17,615	15,484	34.9%
SFCM	21,851	29,673	24,693	24,867	19.3%
WSB	14,381	17,662	10,698	18,031	-2.0%
* Units are IU/Kg				Average *delta*	16.8%

E/H: ethanol/hexane; **E/CA:** ethanol/chloroform acetone;

I/H: isopropyl alcohol/hexane; **TM:** traditional saponification method

Table II. Comparison of Extraction Methods for Vitamin A in Ground Commodities

Sample ID	Type	I/H*	I/H-Ex*	I/CA*
3328635	APF	14,900	15,075	8,728
3328686	APF	18,700	18,559	10,973
3328708	APF	14,800	15,256	6,711
3328945	APF	13,900	14,314	-
3328988	APF	17,700	18,090	-
3329046	BF	11,000	11,328	-
3328791	BUL	6,150	6,103	5,323
3329070	BUL	4,710	5,152	-
99027021	CSB	20,800	-	20,858
99028247	CSB	14,000	19,160	21,493
99028249	CSB	14,800	18,910	17,865
99028254	CSB	14,900	20,930	22,400
99028259	CSB	14,700	19,407	23,306
99028325	CSB	25,200	26,403	-
99028341	CSB	24,600	28,862	-

*Units are IU/Kg

I/H: isopropyl alcohol/hexane; **I/H-Ex:** isopropy alcohol/hexane exhaustive extraction;

I/CA: isopropyl alcohol/choloroform acetone

Table III. Recovery and Precision for All-Purpose Flour

Sample	Procedure Blank	Flour Blank	1LOQ 6,450 IU/kg	5LOQ 24,600 IU/Kg	10LOQ 73,200 IU/Kg
1	ND	ND	95.2%	102.0%	97.8%
2	ND	ND	97.5%	97.2%	100.5%
3	ND	ND	95.5%	98.8%	98.4%
4				99.2%	
5				104.9%	
6				106.5%	
		Mean	96.1%	101.4%	98.9%
		Std. Dev.	1.3%	3.7%	1.4%
		CV	1.3%	3.6%	1.4%

Premix concentration was 54,398 IU/g

Table IV. Recovery and Precision for Bulgur

Sample	Procedure	Matrix	1LOQ	5LOQ	10LOQ
	Blank	Blank	6,050	24,600	73,200
			IU/kg	IU/Kg	IU/Kg
1	ND	ND	101.4%	94.4%	99.7%
2	ND	ND	97.2%	98.5%	103.4%
3	ND	ND	110.3%	94.8%	97.6%
4				100.5%	
5				87.5%	
6				96.1%	
		Mean	103.0%	95.3%	100.2%
		Std. Dev.	6.7%	4.5%	2.9%
		CV	6.5%	4.7%	2.9%

Premix concentration was 106,428 IU/g

Table V. Recovery and Precision for Corn-soy Blend

Sample	Procedure Blank	CSB Blank	1LOQ 6,110 IU/Kg	5LOQ 24,300 IU/Kg	10LOQ 72,700 IU/Kg
1	ND	ND	102.2%	104.1%	98.2%
2	ND	ND	100.4%	106.5%	103.5%
3	ND	ND	102.2%	111.0%	99.8%
4				103.2%	
5				101.2%	
6				106.1%	
		Mean	101.6%	105.4%	100.5%
		Std. Dev.	1.0%	3.4%	2.7%
		CV	1.0%	3.2%	2.7%

Premix concentration was 24,341 IU/g

Table VI. Method Ruggedness for All-Purpose Flour

	Analyst 1					Analyst 2					Analyst 3				
Sample	**Procedure**	**Flour**	**1LOQ**	**10LOQ**	**Comm.**	**Procedure**	**Flour**	**1LOQ**	**10LOQ**	**Comm.**	**Procedure**	**Flour**	**1LOQ**	**5LOQ**	**10LOQ**
	Blank	**Blank**	**6,170**	**72,600**	**Fortified**	**Blank**	**Blank**	**6,170**	**72,600**	**Fortified**	**Blank**	**Blank**	**6,170**	**24,500**	**72,600**
			IU/Kg	**IU/Kg**	**IU/Kg**			**IU/Kg**	**IU/Kg**	**IU/Kg**			**IU/Kg**	**IU/Kg**	**IU/Kg**
Day1-1	ND	ND	107.3%	97.4%	20,000	ND	ND	105.0%	94.8%	20,400	ND	ND	106.5%	98.8%	99.0%
Day1-2	ND	ND	110.4%	99.7%	20,100	ND	ND	104.5%	98.2%	21,400	ND	ND	110.0%	101.2%	100.5%
Day1-3	ND	ND	91.6%	94.6%	21,700	ND	ND	103.4%	96.4%	20,400	ND	ND	88.4%	86.6%	98.5%
Day1-4														101.6%	
Day1-5														98.4%	
Day1-6														101.6%	
Day2-1	ND	ND	93.7%	96.3%	19,200	ND	ND	93.8%	98.2%	19,600	ND	ND	95.2%	102.0%	97.8%
Day2-2	ND	ND	92.7%	95.0%	19,600	ND	ND	100.8%	95.6%	19,300	ND	ND	97.5%	97.2%	100.5%
Day2-3	ND	ND	89.5%	95.3%	20,000	ND	ND	98.2%	101.9%	19,800	ND	ND	95.5%	98.8%	98.4%
Day2-4														99.2%	
Day2-5														104.9%	
Day2-6														106.5%	
Day3-1	ND	ND	93.5%	92.1%	20,000	ND	ND	102.4%	93.1%	20,900	ND	ND	102.1%	96.7%	98.2%
Day3-2	ND	ND	99.8%	93.7%	19,700	ND	ND	99.0%	96.0%	19,800	ND	ND	89.6%	99.6%	98.2%
Day3-3	ND	ND	91.2%	92.4%	20,100	ND	ND	87.4%	94.2%	18,800	ND	ND	99.7%	98.8%	98.8%
Day3-4														96.3%	
Day3-5														95.9%	
Day3-6														90.6%	
Mean			96.6%	95.2%	20,044			99.4%	96.5%	20,044			98.3%	98.6%	98.9%
Std. Dev.			7.5%	2.4%	688			5.7%	2.6%	809			7.2%	4.6%	1.0%
CV			7.8%	2.5%	3.4%			5.8%	2.7%	4.0%			7.3%	4.7%	1.0%

*** % recoveries based on premix = 54,398 IU/g**

Table VII. Method Ruggedness for Bulgur

	Analyst 1					Analyst 2					Analyst 3				
Sample	**Procedure**	**Matrix**	**1LOQ**	**10LOQ**	**Comm.**	**Procedure**	**Matrix**	**1LOQ**	**10LOQ**	**Comm.**	**Procedure**	**Matrix**	**1LOQ**	**5LOQ**	**10LOQ**
	Blank	**Blank**	**6,050**	**72,800**	**Fortified**	**Blank**	**Blank**	**6,050**	**72,800**	**Fortified**	**Blank**	**Blank**	**6,050**	**24,200**	**72,800**
			IU/Kg	**IU/Kg**	**IU/Kg**			**IU/Kg**	**IU/Kg**	**IU/Kg**			**IU/Kg**	**IU/Kg**	**IU/Kg**
Day1-1	ND	ND	96.2%	91.7%	23,500	ND	ND	97.1%	95.5%	25,200	ND	ND	101.4%	95.9%	100.2%
Day1-2			98.7%	96.8%	23,800			103.3%	100.7%	23,200	ND	ND	97.2%	100.0%	103.9%
Day1-3			110.8%	94.8%	23,600			103.3%	96.9%	24,400	ND	ND	110.3%	96.3%	98.1%
Day1-4														102.1%	
Day1-5														88.9%	
Day1-6														97.6%	
Day2-1	ND	ND	97.1%	101.2%	24,800	ND	ND	100.2%	92.0%	22,000					
Day2-2			98.7%	101.4%	25,100			84.2%	96.6%	23,300					
Day2-3			102.5%	95.5%	24,000			86.7%	91.4%	21,600					
Day3-1	ND	ND	89.3%	100.9%	23,400	ND	ND	84.0%	90.3%	22,600					
Day3-2			97.2%	95.7%	23,100			87.8%	94.3%	21,500					
Day3-3			100.7%	98.5%	23,900			84.5%	94.4%	22,600					
Mean			99.0%	97.4%	23,911			92.3%	94.7%	22,933			103.0%	96.8%	100.7%
Std. Dev.			5.7%	3.4%	653			8.5%	3.2%	1246			6.7%	4.5%	2.9%
CV			5.8%	3.4%	2.7%			9.2%	3.4%	5.4%			6.5%	4.7%	2.9%

*** % recoveries based on premix = 106,428 IU/g**

Table VIII. Method Ruggedness for Corn-Soy Blend

	Analyst 1					Analyst 2					Analyst 3				
Sample	**Procedure Blank**	**CSB Blank**	**1LOQ 6,110 IU/Kg**	**10LOQ 72,700 IU/Kg**	**Comm. Fortified IU/Kg**	**Procedure Blank**	**CSB Blank**	**1LOQ 6,110 IU/Kg**	**10LOQ 72,700 IU/Kg**	**Comm. Fortified IU/Kg**	**Procedure Blank**	**CSB Blank**	**1LOQ 6,110 IU/Kg**	**5LOQ 24,300 IU/Kg**	**10LOQ 72,700 IU/Kg**
Day1-1	ND	ND	105.8%	103.9%	20,800	ND	ND	107.3%	108.9%	22,800	ND	ND	102.2%	104.1%	98.2%
Day1-2			107.6%	105.6%	21,800			109.6%	109.6%	21,600	ND	ND	100.4%	106.5%	103.5%
Day1-3			97.9%	102.4%	21,200			104.3%	109.9%	21,500	ND	ND	102.2%	111.0%	99.8%
Day1-4														103.2%	
Day1-5														101.2%	
Day1-6														106.1%	
Day2-1	ND	ND	101.7%	111.1%	25,200	ND	ND	108.3%	109.4%	22,300					
Day2-2			96.1%	123.2%	20,100			90.1%	107.5%	20,900					
Day2-3			104.2%	101.5%	23,400			100.1%	110.8%	20,500					
Day3-1	ND	ND	96.8%	94.3%	19,300	ND	ND	97.9%	97.9%	21,900					
Day3-2			111.4%	96.2%	21,300			98.8%	102.6%	19,900					
Day3-3			95.5%	99.7%	20,800			99.3%	98.4%	21,500					
Day4-1	ND	ND	100.9%	93.5%	20,600	ND	ND	90.3%	94.9%	22,000	ND	ND	99.9%	96.6%	100.1%
Day4-2			100.1%	97.5%	20,500			91.6%	94.7%	21,400	ND	ND	95.2%	107.3%	98.4%
Day4-3			106.8%	97.5%	21,700			108.9%	100.2%	22,000	ND	ND	112.3%	102.0%	98.6%
Day4-4														99.9%	
Day4-5														106.9%	
Day4-6														101.2%	
Day5-1	ND	ND	101.9%	99.5%	21,100	ND	ND	93.9%	82.5%	21,300					
Day5-2			119.5%	104.4%	21,900			117.1%	102.4%	19,900					
Day5-3			103.2%	94.3%	21,800			106.5%	85.4%	20,200					
Mean			103.3%	101.6%	21,433			101.6%	101.0%	21,313			102.0%	103.8%	99.8%
Std. Dev.			6.4%	7.7%	1401			8.1%	8.9%	874			5.7%	3.9%	2.0%
CV			6.2%	7.6%	6.5%			8.0%	8.8%	4.1%			5.6%	3.8%	2.0%

*** % recoveries based on premix = 24,341 IU/g**