FOOD COMPOSITION AND ADDITIVES

Determination of Vitamins A (Retinol) and E (alpha-Tocopherol) in Foods by Liquid Chromatography: Collaborative Study

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A collaborative study was conducted for the determination of vitamins A and E. Existing AOAC liquid chromatographic (LC) methods are suited for specific vitamins A and E analytical applications. This method differs from existing methods in that it can be used to assay samples in all 9 sectors of the food matrix. Standards and test samples are saponified in basic ethanol–water solution, neutralized, and diluted, converting fats to fatty acids and retinol esters and tocopherol esters to retinol and tocopherol, respectively. Retinol and alpha-tocopherol are quantitated on separate LC systems, using UV detection at 313 or 328 nm for retinol, and fluorescence detection (excitation 290 nm, emission 330 nm) for alpha-tocopherol. Vitamin concentrations are calculated by comparison of the peak heights or peak areas of vitamins in test samples with those of standards.

Vitamin A is extremely important for health: eye function, antioxidant activity, the immune system, cell growth, fetal development/reproduction, hormone function, maintenance of skin condition, bone and teeth formation, and neurotransmitter function. Vitamin E is also important for health: muscular function, reproductive function, and antioxidant activity. A difficult but important task of analytical laboratories is the accurate determination of vitamins A and E in foods. These determinations have traditionally been made using AOAC Methods **960.45**, **974.29**, **971.30**, **975.43** and/or **948.26** (1). In recent years, liquid chromatographic (LC) technology has been applied and successfully adopted for a number of specific vitamins A and E analytical applications. Broad application of LC to the analysis of vitamins A and E in foods leading to adoption of the technology as part of an AOAC *Official Method*SM or as an approved method of the American Association of Cereal Chemists has not been achieved. No modern methods exist for the simultaneous determination of vitamins A and E by LC for all food categories. With the need for simplicity and desire for a single flask work-up for more than one analyte, a method that has been successfully used routinely for nearly 25 years in the authors' laboratories was submitted to collaborative study. Ruggedness testing of the method was performed as part of its initial development and improvement (2–4).

The concentration of vitamins A and E in foods and supplements is usually given in international units (IU). The scientific literature often uses the unit, retinol equivalents (RE) to account for the fact that different vitamers of vitamin A differ in activity. Analysts determine the quantities of the vitamers themselves in mass units such as μg/g or mg/g, units which are readily converted to the desired bioactive units.

Collaborative Study

Twelve food samples were selected on the basis of their fat, protein, and carbohydrate content. The test samples were chosen to match the ratios expressed in the AOAC food analysis triangle (5) to cover the breadth of macro food properties. One test sample representative of each of the 9 food sectors in the triangle was selected. In addition, an extra test sample was selected from each of the sectors 5–7 because most foods consumed lie in these sectors. Judicious selection provided test samples with a wide range of vitamins A and E content. Eight of the foods were spiked with known levels of retinol palmitate and alpha-tocopherol acetate so that typical recoveries to be expected from this method could be determined. The test samples and their respective protein, fat, and carbohydrate content are listed in Table 1.

All test samples were thoroughly homogenized in a blender. Spikes were added as appropriate, and the spiked samples were homogenized a second time. Subsamples of about 20 g were packaged in glass containers, sealed, coded, and held at or below –20°C until shipment. Test samples were removed from frozen storage just before shipment, shipped to collaborators at ambient temperature overnight, with instructions to place in frozen storage until the time of analysis. All

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Vitamin A was approved for Official First Action. Vitamin E was not accepted for Official First Action.
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Table 1. Foods selected for collaborative study

Food matrix sector	Test samples	Protein, %	Fat, %	Carbohydrate, %
1	Margarine/butter (50/50 mix)	0	100	0
2	Chicken gravy (canned)	6	42	52
3	Cheese sauce	19	60	21
4	Whole egg powder	42	48	10
5	Multigrain cereal	17	6	77
5	Corn cereal	9	0	91
6	Infant formula (powdered)	24	17	59
6	NIST SRM 1846	24	17	59
6	Baked beans with franks (60/40, w/w)	23	26	51
7	Dried milk	41	1	58
7	Full fat soy flour	42	23	35
8	Cottage cheese	66	20	14
9	Canned tuna in oil	86	14	0

collaborators were provided with one "check sample" to assess their performance before beginning the assays.

AOAC Official Method 2001.13
Determination of Vitamins A and E in Foods
Liquid Chromatography
First Action 2001
(Vitamin E was not accepted for Official First Action)

(Applicable for the determination of retinol from 0.15 μg/g to 1 g/g and for the determination of alpha-tocopherol from 0.001 mg/g to 1 g/g in foods.)

Caution: Potassium hydroxide is extremely caustic. This chemical can cause severe burns. Protect skin and eyes while performing this method. This method involves the use of flammable liquids. Perform behind a barrier when using hot water, steam, or an electric heating mantle. Use an effective fume removal device to remove flammable vapors as produced. Leave ample headroom in flask and add boiling chips before heating is begun. Place all controls, unless vapor sealed, outside of vapor area. This method utilizes toxic chemicals. Use an effective fume removal device to remove vapors as produced.

See Tables **2001.13A–D** for the results of the inter- and intralaboratory studies supporting acceptance of the method.

A. Principle

Standards and test samples are saponified in basic ethanol–water solution, neutralized, and diluted, converting fats to fatty acids and retinol esters and tocopherol esters to retinol and tocopherol, respectively. Retinol and alpha-tocopherol are quantitated on separate LC systems, using UV detection at 313 or 328 nm for retinol, and fluorescent detection (excitation 290 nm, emission 330 nm) for alpha-tocopherol. Vitamin concentrations are calculated by comparison of peak heights or peak areas of vitamins in test samples with those of standards.

B. Apparatus and Materials

Note: Two separate simple isocratic LC systems can be used to perform the determination steps of this method for vitamins A and E simultaneously. Alternatively, the retinol and alpha-tocopherol can be analyzed sequentially on a single LC system since the alpha-tocopherol is sufficiently stable in the diluted saponification solution to be stored while vitamin A is being run.

(**a**) *HPLC system*.—(*1*) *Pump*.—A high pressure pump operating continuously at 1.0 to 2.0 mL/min with a flow precision of ±1% or better. (*2*) *Injector*.—A manual injector or autosampling injector with a 20 μL fixed loop having a typical sampling precision of ±0.25% or better. (*3*) *Chromatography columns*.—(*a*) *For retinol (vitamin A)*.—Reversed-phase C18, 10 μ (4.6 × 250 mm) capable of separating *cis* and *trans* isomers of retinol with a resolution of 1.0 or greater. *Cis* retinol typically elutes prior to *trans* retinol on columns providing effective separation. (*b*) *For alpha-tocopherol (vitamin E)*.—Reversed-phase C8, 10 μ (4.6 × 250 mm) capable of separating alpha- and beta- or alpha- and gamma-tocopherol with resolution of 1.5 or better. The tocopherols will typically elute in the order of delta, gamma, and beta (beta and gamma are rarely well separated on reversed-phase columns), then alpha. It is important to achieve adequate resolution between alpha and the other tocopherols; otherwise low levels of alpha-tocopherol will be masked by high levels of gamma- and beta-tocopherol in products with high levels of gamma- and beta-tocopherol (i.e., oleomargarines produced from soy oil). (*4*) *Detectors*.—(*a*) *Vitamin A*.—Photometric detector mon-

Table 2001.13A. Interlaboratory study results for the determination of vitamin A in foods by LC

Food matrix sector No.	Matrix	Labs[a(b)]	Mean, µg/100 g	Rec., %	s_R	RSD_R, %	R	HORRAT
1	Margarine/butter (50/50 mix)	12(0)	857.08		68.36	7.98	191.41	0.69
1	Margarine/butter (50/50 mix; spiked)	10(2)	1457.20	106.6	89.90	6.17	251.72	0.58
2	Chicken gravy (canned; spiked)	9(1)	131.13	97.1	19.17	14.62	53.68	0.95
3	Cheese sauce (spiked)	11(0)	271.45	103.7	34.96	12.88	97.89	0.94
4	Whole egg powder	10(0)	161.98		62.48	38.57	174.94	2.59
4	Whole egg powder (spiked)	12(0)	426.12	91.7	205.71	48.28	575.99	3.75
5	Multigrain cereal	12(0)	634.38		114.85	18.10	321.58	1.49
5	Corn cereal	12(1)	945.52		86.78	9.18	242.98	0.80
5	Corn cereal (spiked)	12(1)	1395.91	108.5	76.11	5.45	213.11	0.51
6	Infant formula (powdered)	12(0)	584.15		95.98	16.43	268.74	1.34
6	NIST SRM 1846	12(0)	464.28	79.5	49.06	10.57	137.37	0.83
7	Dried nonfat milk	12(0)	817.27		102.94	12.60	288.23	1.08
7	Dried nonfat milk (spiked)	12(0)	1708.25	125.5	195.68	11.46	547.90	1.10
8	Cottage cheese	8(1)	46.02		8.19	17.80	22.93	0.99
8	Cottage cheese (spiked)	10(2)	411.03	98.9	69.29	16.86	194.01	1.30
9	Canned tuna in oil (spiked)	11(1)	262.36	89.5	56.11	21.39	157.11	1.55
Avg.				100.1 ± 13.2				

[a(b)] Number of laboratories where a = number of laboratories retained after outliers removed and b = number of outlier laboratories.

itoring absorbance at 328 nm. (Alternatively a wavelength of 313 nm can be used.) (*b*) *Vitamin E*.—Fluorescence detector, excitation 290 nm, emission 330 nm. (*5*) *Recorder, intergrator, or data collection system*.—Compatible with detectors used.

(**b**) *Erlenmeyer flasks*.—Low actinic 125 mL with neck adapted for connecting reflux condenser.

(**c**) *Hot plate*.—With sufficient heating surface area to handle multiple reflux apparatus setups preferred.

(**d**) *Reflux condensers*.—With adapters (if necessary) to attach 125 mL low actinic Erlenmeyer flasks and nitrogen lines.

(**e**) *Volumetric flasks*.—Low actinic 100 and 10 mL.

(**f**) *Nitrogen blanket apparatus*.—A supply of nitrogen gas with appropriate tubing and connectors to provide a constant nitrogen atmosphere blanket in the reflux apparatus during saponification.

Table 2001.13B. Interlaboratory study results for the determination of vitamin A in foods by LC (Youden pair statistical treatment)

Youden pairs	Labs[a(b)]	Mean, µg/100 g	s_r	RSD_r, %	s_R	RSD_R, %	r	R	HORRAT
Dried nonfat milk (spiked) and margarine/butter (50/50 mix; spiked)	10(2)	1595.1	107.29	6.73	112.22	7.04	300.41	314.22	0.67
Corn cereal and corn cereal (spiked)	10(2)	1173.3	48.33	4.12	85.49	7.29	135.32	239.37	0.66
Margarine/butter (50/50 mix) and dried nonfat milk	12(0)	837.18	66.23	7.91	87.38	10.44	185.44	244.66	0.90
Multigrain cereal and infant formula (powdered)	12(0)	609.27	87.42	14.35	105.83	17.37	244.78	296.32	1.42
Cottage cheese (spiked) and NIST SRM 1846	10(2)	436.18	60.4	13.85	61.23	14.04	169.12	171.44	1.10
Canned tuna in oil (spiked) and cheese sauce (spiked)	11(1)	269.25	39.14	14.54	45.07	16.74	109.59	126.20	1.21
Chicken gravy (canned; spiked) and whole egg powder	8(1)	137.94	22.19	16.09	36.58	26.52	62.13	102.42	1.74

[a(b)] Number of laboratories where a = number of laboratories retained after outliers removed and b = number of outlier laboratories.

Table 2001.13C. Interlaboratory study results for determination of vitamin E in foods by liquid chromatography

Food matrix sector	Matrix	Labs[a]	Mean, mg/100 g	s_R	RSD_R, %	R	HORRAT	Rec., %
1	Margarine/butter (50/50 mix)	7(0)	3.75	0.68	18.21	1.90	1.96	
1	Margarine/butter (50/50 mix; spiked)	8(0)	13.77	2.07	15.05	5.80	1.97	103.03
2	Chicken gravy (canned; spiked)	8(0)	4.31	0.99	23.03	2.77	2.54	108.05
3	Cheese sauce	6(0)	1.44	0.19	13.39	0.53	1.25	
3	Cheese sauce (spiked)	7(1)	3.77	0.45	11.96	1.26	1.29	114.37
4	Whole egg powder	8(2)	3.89	0.44	11.40	1.23	1.24	
4	Whole egg powder (spiked)	8(2)	5.16	0.46	8.96	1.29	1.01	88.91
5	Multigrain cereal	10(0)	125.53	11.97	9.54	33.52	1.74	
5	Corn cereal	10(0)	145.32	16.19	11.14	45.33	2.08	
5	Corn cereal (spiked)	10(0)	155.65	26.34	16.92	73.75	3.20	63.10
6	Infant formula (powdered)	10(0)	15.84	2.20	13.91	6.16	1.86	
6	NIST SRM 1846	10(0)	32.13	3.74	11.65	10.47	1.74	118.56
6	Beans with franks (60/40, w/w)	8(0)	0.87	1.02	117.59	2.86	11.56	
7	Full fat soy flour	5(0)	0.69	0.10	14.85	0.28	1.24	
7	Nonfat dry milk (spiked)	8(1)	2.08	0.28	13.35	0.78	1.32	106.06
8	Cottage cheese (spiked)	10(0)	7.49	1.48	19.76	4.14	2.37	103.22
9	Canned tuna in oil	7(1)	1.70	0.20	11.60	0.56	1.11	
9	Canned tuna in oil (spiked)	9(1)	3.68	0.33	9.01	0.92	0.97	97.84
Avg.								100.35 ± 16.43

[a] Number of laboratories retained after outliers removed. Number of outlier laboratories shown in parentheses.

C. Reagents

(**a**) (*1*) *Certified vitamin A acetate concentrate (USP).*—Equivalent to ca 30 mg of retinol/g of oil (content certified by United States Pharmacopeia, Rockville, MD 20852 USA; www.usp.org); or (*2*) *Retinyl palmitate, all-trans*.—Fluka Chemical Co., Ronkonkoma, NY, +1-800-358-5287. Request Certificate of Lot Analysis when ordering. If manufacturer's certification is unavailable, or purity of standards needs to be verified, test vitamin A palmitate purity as follows: Dissolve 50 mg (record to the nearest 0.1 mg) of retinol palmitate standard in 2-propanol (UV-spectroscopy grade) in a 500 mL flask and dilute to volume. Dilute 10 mL of this solution to 100 mL with 2-propanol (final concentration is ca 10 mg/L). Measure maximum absorbance obtained at 325–328 nm using 1 cm pathlength cell and 2-propanol as a blank. Calculate the purity of the retinol palmitate as follows (6):

$$\text{Percent purity} = (\text{ABS} \times 5 \times 10^6) / (960 \times \text{W})$$

where ABS = absorbance maximum; 960 = absorbance of pure retinol palmitate (1% solution in 1 cm cell); W = weight of test portion in mg; and 5×10^6 = combined dilution factors, conversion to 1% equivalent solution, and conversion to %.

Store retinol palmitate standard at 0–4°C to allow for easier handling while weighing.

(**b**) *Vitamin E acetate*.—Aldrich Chemical Co. (Milwaukee, WI).

(**c**) *Mixed tocopherol standard*.—Prepare 50 + 50 chromatographic resolution test mixture of alpha- and beta- or alpha- and gamma-tocopherol in the mobile phase, at concentration level of highest alpha-tocopherol standard.

(**d**) *Acetic acid*.—Glacial.

(**e**) *Methanol*.—HPLC grade.

(**f**) *Ethanol*.—95%.

(**g**) *Tetrahydrofuran*.

(**h**) *Hexane*.

(**i**) *Pyrogallic acid*.—Crystals.

(**j**) *Vitamin A mobile phase*.—Combine 860 mL methanol (HPLC grade) and 140 mL water. Mix well. Stir overnight to degas or mechanically degas prior to use.

(**k**) *Vitamin E mobile phase*.—Combine 940 mL methanol (HPLC grade) and 60 mL water. Mix well. Stir overnight to degas or mechanically degas before use.

(**l**) *THF–ethanol (50 + 50)*.—Combine 500 mL tetrahydrofuran and 500 mL 95% ethanol. Mix well.

(**m**) *Potassium hydroxide solution, 50%*.—Slowly add 500 g of KOH pellets to 500 mL water contained in a 2 L thick wall Erlenmeyer flask. (*Caution*: The solution gives off substantial heat while KOH is dissolving; add the KOH in 100 g portions while the flask is being cooled with cold water. Swirl the flask gently to aid in dissolution of the KOH. Store in glass container with cork stopper.)

Table 2001.13D. Intralaboratory study results for determination of vitamin E in foods by liquid chromatography

Youden pairs	Labs[a]	Mean, mg/100 g	s_r	RSD_r, %	s_R	RSD_R, %	r	R	HORRAT
Corn cereal (spiked) and corn cereal	10(0)	150.45	12.03	8.00	21.86	14.53	33.68	61.21	2.73
Multigrain cereal and NIST SRM 1846	10(0)	79.24	8.97	11.32	8.97	11.32	25.12	25.12	1.93
Infant formula (powdered) and margarine/butter (50/50 mix; spiked)	7(1)	14.76	0.22	1.50	2.24	15.16	0.62	6.27	2.01
Cottage cheese (spiked) and whole egg powder (spiked)	10(0)	6.40	0.51	7.94	0.65	10.17	1.43	1.82	1.19
Margarine/butter (50/50 mix) and canned tuna in oil (spiked)	10(0)	3.67	0.44	12.12	0.53	14.35	1.23	1.48	1.54
Whole egg powder and chicken gravy (canned; spiked)	8(0)	4.10	0.71	17.33	0.77	18.74	1.99	2.16	2.05
Cheese sauce (spiked) and nonfat dry milk (spiked)	7(0)	2.90	0.21	7.36	.36	12.23	0.59	1.01	1.27
Canned tuna in oil and cheese sauce	6(0)	1.571	0.033	2.10	0.201	13.04	0.09	0.56	1.23

[a] Number of laboratories retained after outliers removed. Number of outlier laboratories shown in parentheses.

(**n**) *Vitamin A working standard (ca 15 μg/mL).*—(*1*) *Using USP standard.*—Weigh 50 mg vitamin A acetate concentrate into a 100 mL low actinic volumetric flask. Record weight to nearest 0.1 mg. Record concentration in mg/g per USP certification. Add small amount of acetone (<3 mL) to aid dissolution. Dilute to volume with 95% ethanol. Store at 4°C in dark. Solution is stable for 2 weeks. (*2*) *Using retinyl palmitate.*—Weigh 55 mg of retinyl palmitate into a 100 mL low actinic volumetric flask. Record weight to nearest 0.1 mg. Record purity per supplier certification or purity test. Add pea-sized piece of pyrogallic acid, ca 50 mg. Dissolve and dilute to volume with hexane. Pipet 5 mL of solution to second 100 mL low actinic flask and dilute to volume with 95% alcohol. Store at 4°C in dark. Solution is stable for 2 weeks.

(**o**) *Vitamin E stock solution.*—ca 500 μg/mL. Weigh 50 mg vitamin E acetate into 100 mL low actinic volumetric flask. Record weight to nearest 0.1 mg. Add small amount of acetone (<3 mL) to aid dissolution. Dilute to volume with 95% ethanol. Store at 4°C in dark. Solution is stable for 1 month.

(**p**) *Vitamin E working standard.*—ca 50 μg/mL. Pipet 10 mL vitamin E stock solution, (**o**), into 100 mL low actinic volumetric flask. Dilute to volume with 95% ethanol. Store at 4°C in dark. Solution is stable for 1 month.

D. Extraction and Saponification

Turn on hot plate to preheat. Start and adjust cooling water flow to precool reflux condensers.

Prepare high standard by pipeting 5 mL vitamin A working standard, **C**(**n**), and 10 mL vitamin E working standard, **C**(**p**), into a 125 mL low actinic Erlenmeyer flask. Add 25 mL of 95% ethanol. Proceed to addition of pyrogallic acid.

Prepare intermediate standard by pipeting 2 mL vitamin A working solution and 5 mL vitamin E working solution into a second 125 mL low actinic Erlenmeyer flask. Add 33 mL of 95% ethanol. Proceed to addition of pyrogallic acid.

Prepare low standard by pipeting 0.5 mL vitamin A working standard and 2 mL vitamin E working standard into a third 125 mL low actinic Erlenmeyer flask. Add 37.5 mL of 95% ethanol. Proceed to addition of pyrogallic acid.

Grind solids to pass a 40 mesh sieve. Blend liquid or wet materials to homogeneity and store ≤4°C in the dark.

To prepare low fat (<40% fat) test samples, weigh enough test sample (≤5 g) to give ca 50 μg of vitamin A and/or 1.0 mg vitamin E into a 125 mL low actinic Erlenmeyer flask. For test samples high in sugar, add 3 mL water and disperse the test portion as a slurry. Add 40 mL of 95% ethanol.

To prepare high fat test samples, weigh test sample (≤2 g) to give ca 50 μg of vitamin A and/or 1.0 mg vitamin E into a 125 mL low actinic Erlenmeyer flask. Add 40 mL of 95% ethanol.

Add a pea-sized piece (ca 50 mg) of pyrogallic acid (antioxidant) to each standard and test flask. Add a glass bead or boiling stone to promote even boiling.

Swirl all flasks to ensure that all materials are thoroughly dispersed in the solution.

Turn on N flow and ensure N atmosphere for all flasks before and while refluxing.

Pipet 10 mL of 50% KOH solution into each flask and immediately place flask on hot plate under reflux condenser. Swirl.

Reflux 45 min. Swirl flasks every 10 min.

Remove reflux flasks from hot plate, stopper with corks, and quickly cool flasks to room temperature using cold water or ice water.

Pipet 10 mL of glacial acetic acid into each flask to neutralize the KOH. Mix well and let flasks cool again to room temperature.

Quantitatively transfer the solution in each flask to a 100 mL low actinic volumetric flask using THF–95% ethanol (50 + 50). Dilute to volume with the same solvent mixture.

Stopper and invert volumetric flask 10 times.

Allow flasks to set for at least 1 h at room temperature and preferably overnight in refrigerator to precipitate fatty acid salts formed during saponification. In some cases, centrifugation may reduce settling time.

E. Determination

Start HPLC system(s) and allow to warm up and equilibrate for a minimum of 30 min with mobile phase flowing at flow rate of 1.0 mL/min.

Inject vitamin A standards that have been taken through saponification onto HPLC system. Adjust mobile phase to achieve a resolution of 1.5 or better for *cis* and *trans* forms. All *trans* retinol should elute in ca 9 min. Inject chomatographic resolution test mixture onto vitamin E LC system. Adjust mobile phase to achieve resolution of 1.5 or better for the 2 forms of tocopherol present in the resolution test mix. alpha-Tocopherol should elute in ca 10 min.

Inject high, medium, and low standards. Adjust detector sensitivity to give peak heights of 50–90% of full scale for the vitamin of interest at the high standard. Repeat injection of standard until peak height(s) are reproducible.

Inject test solutions. Intersperse with standard solution injections after every 9 tests. [If retinol or alpha-tocopherol in test exceeds the peak height of their respective high standard by more than 25%, dilute test solutions using a solution of 10 mL 50% KOH, 40 mL of 95% ethanol, 10 mL glacial acetic acid, and 40 mL THF–95% ethanol (50 + 50).]

F. Calculations

(**a**) *Vitamin A*.—Calculate μg/g of vitamin A (as retinol) as follows: Measure the peak heights or areas of the standards.

(*1*) *Using USP standard*.—Determine the response factor for vitamin A (RF_A) using the following calculation:

$$RF_A = \frac{mg_{std} \times mL_{std} \times conc_{std}}{PkHT_{std} \times 10000}$$

where $PkHT_{std}$ = peak height or area of standard from chromatogram; mL_{std} = mL of working standard used in procedure; $conc_{std}$ = concentration of USP vitamin A (as retinol) per USP certification (mg/g); mg_{std} = mg of USP standard weighed in reagents section; 10000 = combined dilution factors for vitamin A standard.

(*2*) *Using retinyl palmitate*.—Determine the response factor for vitamin A (RF_A) using the following calculation:

$$RF_A = \frac{mg_{std} \times mL_{std} \times purity_{std} \times 0.5458}{PkHT_{std} \times 200}$$

where $purity_{std}$ = percent purity certified by supplier or determined, divided by 100; mg_{std} = mg of retinyl palmitate weighed; $PkHT_{std}$ = peak height or area of standard from chromatogram; mL_{std} = mL of working standard used in procedure; 0.5458 = ratio of retinol to retinyl palmitate molecular weights; and 200 = combined dilution factors/ conversion from mg to μg.

The RF_A values of the low, medium, and high standards should agree with each other within 3% relative since the detector response should be linear across this concentration range. Use an average of RF_A values calculated from high, medium, and low standards for test sample quantitation.

Measure the peak heights or areas corresponding to retinol (vitamin A) in the test sample extracts. The 13-*cis* isomer of retinol (eluting immediately proceeding the all *trans* isomer) might be present in some test samples. Measure the 13-*cis* peak also.

Multiply the height or area of the 13-*cis* retinol peak by 1.08 (to compensate for difference in absorbance compared to the *trans* isomer).

Add the corrected peak height or area for the 13-*cis* isomer to that of the all-*trans* isomer to give total test sample peak height or area. Calculate the concentration of vitamin A (in μg/g as retinol) using the following equation:

$$\text{Vitamin A, } \mu g/g \text{ (as retinol)} = \frac{RF_A \times PkHT_{SPLE} \times 100}{W}$$

where RF_A = response factor for vitamin A; $PkHT_{SPLE}$ = total test sample peak height or area of all *trans* and 13-*cis* retinol; 100 = dilution volume of test portion, mL; and W = weight of test portion, g.

(**b**) *Vitamin E*.—Calculate mg/g vitamin E (as alpha-tocopherol acetate) as follows: Measure peak height or area of alpha-tocopherol in the standards. Determine response factor for vitamin E (RF_E) using the following equation:

$$RF_E = \frac{mg_{std} \times mL_{std}}{PkHT_{std} \times 100000}$$

where $PkHT_{std}$ = peak height or area of standard from chromatogram; mL_{std} = mL of working standard used in procedure; mg_{std} = mg alpha-tocopherol acetate standard weighed; and 100 000 = combined dilution factors in vitamin E standard.

The RF_E values of the low, medium, and high standards should agree with each other within 3% relative since the detector response should be linear across this concentration range. An average of the low, medium, and high RF_E standards should be used for test sample quantitation.

Measure peak height or area of peak corresponding to alpha-tocopherol on chromatogram of test sample extract.

Use the following equation to calculate the concentration of vitamin E (as alpha-tocopherol) in mg/g:

$$\text{Vitamin E, mg/g (as alpha-tocopherol acetate)} = \frac{RF_E \times PkHT_{SPLE} \times 100}{W}$$

Table 2. Collaborative study data for vitamin A

Food matrix sector	Vitamin A samples	Spike level, µg/100 g	Laboratory											
			1	2	3	4	5	6	7	8	9	10	11	12
1	Margarine/butter (50/50 mix)		874	806	732	871	860	960	820	934	832	948	781	867
1	Margarine/butter (50/50 mix; spiked)	563	1470	1283	969[a]	1480	1480	1570	1390	1492	1347	1530	4278[a]	1530
2	Chicken gravy (canned)						10							
2	Chicken gravy (canned; spiked)	135	140	92	127	158		140	140.2		113	141	342[a]	129
3	Cheese sauce		39	64	80				29.5			35		
3	Cheese sauce (spiked)	214	270	258	304	296	300	250	222	227	263	338		258
4	Whole egg powder		176	67	144	188	160		186.8		118	191	293	96
4	Whole egg powder (spiked)	288	467	155	680	433	430	180	337.4	479	881	506	282	283
5	Corn cereal		968	1008	766	970	950	100[a]	847.7	894	1043	1063	910	981
5	Corn cereal (spiked)	415	1430	1369	1316	1340	1390	1380	1279	1455	1465	1551	359[a]	1380
5	Multigrain cereal		599	571	500	717	620	630	627.6	841	727	778	561	441
6	Baked beans with franks (60/40 w/w)		2	395			40							
6	Infant formula (powdered)		620	575	604	608	350	660	593.8	635	563	737	475	589
6	NIST SRM 1846	584	499	453	362	457	450	450	468.3	400	480	547	508	497
7	Nonfat dry milk		896	822	658	725	750	890	780.2	691	914	1005	794	882
7	Nonfat dry milk (spiked)	710	1720	1738	1924	1750	1520	1800	1625	1748	1825	1994	1245	1610
7	Full fat soy flour			15				40	363.8				208	
8	Cottage cheese		36	39	84[a]	57.7	50	50	38.19			55		42
8	Cottage cheese (spiked)	369	407	404	459	354	270	1430[a]	392.3	491	391	512	838[a]	430
9	Canned tuna in oil													
9	Canned tuna in oil (spiked)	293	287	163	227	263	530[a]	260	284	266	338	351	187	260

[a] Dixon outlier.

Table 3. Collaborative study data for vitamin E

Food matrix sector		Vitamin E samples	Laboratory									
			Spike level, mg/100 g	1	2	3	4	5	6	7	8	9	10
1	213	Margarine/butter (50/50 mix)		4.04	2.5	3.7		4.8	—[a]	—[a]	3.71	3.6	3.9
1	241	Margarine/butter (50/50 mix; spiked)	9.25	12.5	11.8	11.7	17.2	15	—[a]	—[a]	12.36	16	13.6
2	215	Chicken gravy (canned)				0.1	3.9				0.26		
2	243	Chicken gravy (canned; spiked)	3.99	3.47	3.6	3.5		5.5	3.71		3.91	4.8	6
3	217	Cheese sauce		1.33	1.3	1.3			—[a]	—[a]	1.49	1.8	1.4
3	245	Cheese sauce (spiked)	1.96	3.42	3.5	3.3	6[b]	4.3	—[a]	—[a]	3.48	4.4	3.97
4	219	Whole egg powder		3.51	3.9	3.4	9[b]	4.4	4.62	6.1[b]	3.62	4.1	3.6
4	247	Whole egg powder (spiked)	1.42	5.32	5.2	4.9	13.7[b]	4.5	4.89	8.65[b]	5.23	6.1	5.11
5	221	Multigrain cereal		122	127	125.9	121.2	144	120.4	113.84	106	144	131
5	223	Corn cereal		149	154.4	147.1	146.9	134	148.3	119.54	123	174	157
5	249	Corn cereal (spiked)	16.36	163	158.5	170.4	156.2	110	152.6	153.77	116	198	178
6	225	Infant formula (powdered)		15.2	14	14.1	20	13.4	15.3	18.21	14.4	18.2	15.6
6	227	NIST SRM 1846	27	33.7	27.3	31.6	30.2	26.4	32.48	32.91	31.9	39.3	35.5
6	229	Baked beans with franks (60/40 w/w)		0.2	0.147	2.9		0.65	2.01	0.33	0.4	0.3	
7	231	Full fat soy flour		0.7	0.6	0.7			—[a]	—[a]	0.85	0.6	
7	235	Nonfat dry milk				0.009				0.62			
7	251	Nonfat dry milk (spiked)	1.96	2.01	2.3	2	1.7		2.17	5.86[b]	1.86	2.6	1.99
8	237	Cottage cheese				0.1					0.23	2.2	
8	253	Cottage cheese (spiked)	7.26	7.25	7.2	7	4.2	8.2	6.55	9.56	7.67	8.9	8.41
9	239	Canned tuna in oil		1.61	1.6	1.5	5.6[b]	1.7			1.8	2.1	1.62
9	257	Canned tuna in oil (spiked)	1.92	3.55	3.1	3.4	7.4[b]	3.5	3.88	4.19	3.78	4	3.75

[a] Laboratory failed to obtain required chromatographic resolution.

[b] Dixon outlier.

where RF_E = response factor for vitamin E; $PkHT_{SPLE}$ = test sample peak height or area of alpha-tocopherol; 100 = dilution volume of test portion, mL; and W = weight of test portion, g.

Alternatively, a 3 level calibration using a zero order polynomial fit (linear) can be used to calculate vitamins A and E.

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Results and Discussion

Vitamin A

Collaborative study data for vitamin A were received from 13 laboratories. The data from one of the laboratories showed a systematically high bias compared to the other laboratories. A detailed investigation of the procedure used by that laboratory showed that the method specified was not followed with regard to standard preparation. The results of that laboratory are not included in this report. Table 2 provides a tabulation of the results obtained from the remaining 12 laboratories. For 4 of the test samples, chicken gravy, cheese sauce, baked beans with franks, and canned tuna, insufficient data were received from the reporting laboratories to conduct an adequate statistical evaluation. Full fat soy flour, as a plant-based material, will not have a measurable quantity of retinol (test samples were selected based on published theoretical data for both vitamins A and E content). All of the remaining food samples were expected to have nutritionally significant quantities of vitamin A based on published food table data. The typical LC operating limit of detection/quantitation for retinol is 15 μg/100 g in most laboratories. It is possible that the actual values for the 4 samples listed are <15 μg/100 g, or that the detectors used by some of the laboratories were not adequately tuned to a sufficiently low signal-to-noise ratio to detect the low levels of retinol actually present. In the case of each of these 5 test samples, chicken gravy, cheese sauce, baked beans with franks, canned tuna, and soy flour, at least one additional test sample from that food sector was present for which valid data were obtained.

The AOAC guidebook for Study Directors does not provide guidance for statistical processing of individual test samples. It provides guidance only on statistical evaluation applicable to duplicates and/or Youden pairs. Therefore, we applied the Dixon test to each of the individual test samples having a sufficient number of data points to obtain the between-laboratory statistical data for the test samples. The results are shown in Table **2001.13A**. Dixon outliers are noted in Table 2. The results obtained were good across all sectors except sector 4 (33–67% fat, 33–67% protein, and 0–33% carbohydrates) represented by whole egg samples. The variability of results [expressed as relative standard deviations (RSDs) at various concentrations] is in agreement with what one would expect as indicated by the Horwitz ratio (HORRAT column) for expected collaborative results relative to level of analyte. (Normally one expects a HORRAT of <2.) For whole egg samples, it is uncertain whether the particular fat, carbohydrate, or protein ratio present is the source of the analytical variability, if egg as a matrix is difficult to digest and extract, or if homogeneity of the whole egg powder sample was not achieved. It does not appear to be an issue with the protein content of the test sample, since test samples in adjacent sectors 7 and 8, which have similar protein content, were analyzed with satisfactory results.

The Youden pair statistics approach should be applicable for all pairs of test samples where the variance of the pair is expected to be similar. Since the method being studied is applicable to all foods, similar variance would be expected for test samples of similar analyte level, regardless of their matrix. Therefore, the Youden pair statistical approach was applied to the data to determine the within-laboratory variability for the method. Youden pairs were set up for those test samples having the closest analyte levels. The results are shown in Table **2001.13B**. The 14 test samples can be combined into 7 pairs. The between-laboratory variability calculated using the Youden pair approach is very similar to the between-laboratory variability arrived at by treating each test sample singularly. As expected, the within-laboratory variability for each pair of test samples is less than the between-laboratory variability.

To assess the recovery by the method, 8 test samples were spiked with a range of vitamin concentrations. In addition, NIST SRM 1846 was included in the test sample set. As a result, a recovery sample was included for each of the sectors of the food analysis triangle. The results of the recovery calculations are shown in Table **2001.13A**. The overall recovery for the method was 100 ± 13%, which is very much in line with the expected variability of recovery for a method with an expected RSD_R of approximately 13% (*see* the Horwitz ratio values in Tables **2001.13A** and **B**).

SRM 1846, a powdered, milk-based infant formula available from NIST, was included as a test sample with the unknowns sent to participating laboratories as a check on accuracy of the method. The NIST Certificate of Analysis lists the noncertified vitamin A content of the NIST sample as 5.84 ± 0.68 mg/kg (584 ± 68 μg/100 g), 95% uncertainty range. The collaborative study results were 464 ± 31 μg/100 g, 95% uncertainty range. This represents a significantly lower result, i.e., recovery of 79.5%. Packets of SRM 1846 had been procured at least 6 months in advance of the study and stored, unopened, in a dark cabinet in an office where the temperature typically ranged from 20 to 25°C (68 to 77°F) per storage instructions supplied with the SRM. Because the level of *cis*-retinol was somewhat elevated beyond what might be expected in the SRM, the authors decided to investigate the possible loss of retinol on storage of this material. New packets of SRM 1846 were purchased and analyzed side by side with retained packets of SRM that had now been stored for an additional 8 months (at least 14 months total). The NIST samples were analyzed at ca 2 weeks, 5 weeks, and 6 months after receipt of the new packets. The results for the stored NIST sample were 80.7, 78.6, and 81.1% compared to the newly received materials, respectively (average 80.1%). Average concentration for stored NIST samples was 465 μg/100 g. The average concentration was 580 μg/100 g for the newly purchased NIST samples. The actual cause of the reduced level of vitamin A in the SRM used in the collaborative study is un-

known, but the level found is consistent with the comparative result in the subsequent study. Although the office where the SRM was stored prior to the collaborative study was climate-controlled, the area of the building immediately adjacent to the office is a food production pilot plant facility. Investigation with the sanitation officer revealed that this food production area was periodically heated well over 100°F on weekends for pest control purposes. It may be that the cabinet in the office, against the wall adjacent to the food production facility, experienced periods of high temperature during the time the NIST sample was stored there, thus exposing the NIST sample used in the collaborative study to degradation temperatures.

Vitamin E

Collaborative study data for vitamin E was received from 10 laboratories. Table 3 provides a tabulation of results obtained from all 10 laboratories. For 5 of the test samples, margarine/butter mixture, spiked margarine/butter mixture, cheese sauce, spiked cheese sauce, and full fat soy flour, results from several laboratories were at odds with results from the remainder of the laboratories. Review of the chromatograms and communication with participating laboratories indicated that, although specified in the method, the recommended adequate resolution of the various tocopherols was not achieved before analyses were conducted, i.e., several laboratories failed to ascertain adequate chromatographic resolution. Excellent agreement was achieved between laboratories that routinely analyze for mixed tocopherols. Although the method submitted to the collaborators specified a minimum resolution for separation of tocopherol isomers, some collaborators misinterpreted the instructions; therefore, the explanation of the means of establishing adequate resolution was expanded by adding more detail to the method. The data from these particular laboratory/sample combinations, therefore, had to be eliminated (*see* Table 3, footnote a). As a result, for 2 of the samples, cheese sauce and full fat soy flour, data for the statistical evaluation are limited. The number of samples is noted in Table **2001.13C**. For the nonfat dry milk sample and the cottage cheese sample, only 2 and 3 laboratories reported detecting alpha-tocopherol, respectively. The typical LC operating limit of detection/quantitation for alpha-tocopherol is 0.1–0.2 mg/100 g in most laboratories. It is most likely that the actual values for these test samples are <0.2 mg/100 g. For these 2 test samples, at least one additional test sample from that food sector was present for which valid data were obtained. All of the remaining food samples were expected to have measurable quantities of vitamin E based on published food table data.

As with vitamin A, we applied the Dixon test to each test sample having a sufficient number of data points to obtain the between-laboratory statistical data for the samples. The results are shown in Table **2001.13C**. Dixon outliers are noted in Table 3. The variability of results (expressed as RSDs at various concentrations) are generally in agreement with what one would expect as indicated by the Horwitz ratio (HORRAT column) for expected collaborative results relative to level of analyte. (Normally one expects a HORRAT of <2.) Baked beans with franks had an exceptionally high variability. An intuitive review of the data for baked beans and franks would cause one to reject the data from laboratories 4 and 7; however, the results of the laboratories were not removed by the statistical outlier test.

Again, as with vitamin A, the Youden pair statistical approach was applied to the data to determine the within-laboratory variability for the method. Youden pairs were set up for those test samples having the closest analyte levels. The results are shown in Table **2001.13D**. The 16 samples can be combined into 8 pairs. The between-laboratory variability calculated using the Youden pair approach is very similar to the between-laboratory variability arrived at by treating each sample singularly. As expected, the within-laboratory variability for each pair of test samples is less than the between laboratory variability.

To assess the recovery by the method, 8 test samples were spiked with a range of vitamin concentrations. In addition, NIST SRM 1846 was included in the test sample set. As a result, a recovery sample was included for each of the sectors of the food analytical triangle. The results of the recovery calculations are shown in Table **2001.13C**. The overall recovery for the method was 100 ± 16%. This is very much in line with the expected variability of the recovery for a method that has an expected RSD_R of ca 9% (*see* the Horwitz ratio values in Tables **2001.13C** and **D**).

Collaborators' Comments

Vitamin A.—Collaborating laboratories reported no difficulties regarding the vitamin A assay. In general, collaborators found that the method performed well, and they appreciated the easy preparation procedure. One collaborator disagreed with some of the terminology used in the calculations, but did not offer alternative suggestions.

Vitamin E.—Conditions proposed for vitamin E collaborative study are the same as routinely used in the laboratory of one of the collaborators. The detector is linear well beyond calibrated range. The procedure was easy to follow and does not involve lengthy preparation times.

Recommendations

Collaborators who followed the instructions and method provided were successful in performing the procedure, and there is a need for an LC method for vitamins A and E across all food categories. As a result, the Study Coordinators recommend that this method for the determination of vitamins A and E in foods by liquid chromatography be adopted Official First Action by AOAC INTERNATIONAL.

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