
AOAC Official Method 2001.13 Determination of Vitamin A (Retinol) in Foods

Liquid Chromatography
First Action 2001

(Applicable for the determination of retinol from 0.15 µg/g to 1 g/g.)

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** and **2001.13B** for the results of the interlaboratory 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 to retinol. Retinol is quantitated using a high pressure liquid chromatography (HPLC) system with UV detection at 313 or 328 nm. Vitamin concentration is calculated by comparison of peak heights or peak areas of vitamins in test samples with those of standards.

B. Apparatus and Materials

(**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 column*.—Reverse 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. (*4*) *Detector*.—Photometric detector monitoring absorbance at 328 nm. (Alternatively a wavelength of 313 nm can be used.) (*5*) *Recorder, intergrator, or data collection system*.—Compatible with detector 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.

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:

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

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**) *Acetic acid*.—Glacial.

(**c**) *Methanol*.—HPLC grade.

(**d**) *Ethanol*.—95%.

(**e**) *Tetrahydrofuran*.

(**f**) *Hexane*.

(**g**) *Pyrogallic acid*.—Crystals.

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

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

(**j**) *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.)

(**k**) *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.

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**(**k**), 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 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 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 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 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 hotplate, 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. *Cis* retinol will elute as a small peak just prior to the all *trans* form.

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 in test exceeds the peak height of the 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:

$$RF_A =$$

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:

$$RF_A =$$

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 workings tandard 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_Avalues 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:

$$\text{Vitamin A, µg/g (as retinol)} =$$

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.

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

Reference:
J. AOAC Int. (future issue).

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

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