

**AS 475:2014**

**Afghanistan National Standard**

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**FORTIFIED EDIBLE OILS AND GHEE- SPECIFICATION**

**ANNEX 1: FORTIFICATION, SAMPLING AND TESTING**

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**Afghanistan National Standards Authority**

## 0. FOREWORD

0.1 Fortification of food is a public health measure aimed at improving and maintaining the health of individuals in the population through provision of an adequate level of nutrient intake by the addition of nutrients to processed foods or food products

0.2 Fats and oils are considered to be one of the most cost effective, technically feasible and widely used vehicle for vitamins A, and D fortification because the two vitamins are fat soluble and production of fats and oils to a greater extent involve a centralized process. Vegetable fats and oils are also consumed by most sectors of the population.

0.3 This Afghanistan Standard is drafted and prepared in order to assist in alleviating of problem of vitamin A and D deficiency and to ensure that the safety and quality of fortified products are complied with, and the respective vitamins are supplied in the right amount and form.

0.4 When added to oils of high quality and stored at temperatures below 25°C, vitamin A is relatively stable to changes during storage. Losses may reach 5-15% during storage, 5% during boiling to 20% when food is fried. Losses of higher magnitude will occur during high temperature/repeated frying.

If the oil is of low quality in terms of high peroxide levels (5.8 mEq/kg) losses of vitamin A can reach 26% in the semi dark in 4 weeks compared with 0.7% at a PV of 0.4 mEq/kg (Laillou et.al, Food Nutr. Bull. 2012, Sep33 (3) 186-93. Oil for fortification must be of the highest quality, and wherever possible stored under cool, dark conditions.

## 1. SCOPE AND FIELD OF APPLICATION

This Afghanistan standard prescribes requirements, methods of sampling and tests for fortified edible fats and oils intended for human consumption. The standard is applicable only where there exists no other specific prescribed Afghanistan standard for a particular edible fat or oil fortified with any of the vitamin A and D

## 2. Terminologies

**2.1** For the purpose of this Afghanistan standard the following definitions shall apply:

**2.1.1 Ghee and oils** – Foodstuffs which are composed of glycerides of fatty acids of vegetable origin. They may contain small amounts of other lipids such as phosphatides, unsaponifiable constituents and free fatty acids naturally present in the ghee or oil.

**2.1.2 Fortificant** – A micronutrient compound to be added to food vehicle.

**2.1.3 Fortification** - The addition of one or more micronutrients to food for purpose of preventing or correcting a demonstrated deficiency of one or more micronutrients in the population or specific population group.

**2.1.4 Food vehicle** - A food stuff identified to be fortified with the prescribed micronutrient (s) as prescribed in this standard.

**2.1.5 Micronutrient** - A natural or synthesized vitamin, mineral or trace element that is essential for normal growth, development and maintenance of life; and of which a deficit will cause characteristic biochemical or physiological changes.

**2.1.6 Edible ghee and Oils** - Ghee and oils obtained by mechanical procedure of expelling or pressing and/or solvent extraction and subjected to refining to make it suitable for human consumption as prescribed in this standard. Ghee is prepared by the partial hydrogenation of refined vegetable oil.

### **3. REQUIREMENTS**

#### **3.1 Description**

Fortified edible ghee and oils shall be a food stuff composed of glycerides of fatty acids of vegetable origin to which vitamin A and D have been added. Such fats and oils may contain traces or small amounts of other lipid such as phosphatides, unsaponifiable constituent's free fatty acid and other breakdown by products of the ghee or oil.

#### **3.2 Physical and chemical requirements**

**3.2.1** The quality of edible ghee and oils shall conform to chemical requirements given in this Afghanistan Standard (Ref No) based on Codex Standard 19-1981 Edible oils not covered by individual standards (Rev 2 -1999).

*Exception:* At the point of fortification the maximum peroxide value for oil should be 2meq/kg.

#### **3.3 Antioxidant**

**3.3.1** Permitted additives:

No.	Antioxidant	Max individual level
1	Tertiary butyl hydroquinone (TBHQ)	120 mg/kg
2	Butylated hydroxyanisole (BHA)	175 mg/kg
3	Butylated hydroxytoluene (BHT)	75 mg/kg
4	Any combination of BHA, BHT, and/or TBHQ	200 mg/kg but limits above not to be exceeded

#### **4.4 FORTIFICATION**

**4.4.1** The level of micronutrients added in edible fats and oils shall be given in

Table 3. Levels are based on an adult consumption of 40g of oil containing 40% of Recommended Daily Intake (RDI) of Vitamin A (See Appendix 1)

**Table 3: Micronutrient content in fortified edible fats and oils.**

S/No. شماره مستند	Micronutrient	Minimum Content	Maximum content	Method of reference test
1	Vitamin A, (retinyl palmitate)  Target 30,000 IU/kg	24,000 IU /kg	36,000 IU/kg	EN12823-1:2000 Foodstuffs: determination of vitamin A by high performance Liquid Chromatography. Measurement of all-trans-retinol and 13-cis-retinol. (See Appendix 2) EN 12821: 2009 Foodstuffs: determination of vitamin D by high performance liquid chromatography. Measurement of cholecalciferol (D3) and ergocalciferol (D20). (See Appendix 4)
2	Vitamin D <sub>3</sub> , (cholecalciferol)  Target 3,000 IU/kg	2,400 IU/kg (60 microgram/kg)	3,600 IU/kg (75 microgram/kg)	

\*Note: Shall also serve as an antioxidant

#### 4.4.2 Permitted fortificants

For the purpose of vitamin fortification of edible fats and oils, the fortificants which shall be used are the vitamin concentrated or premixes of oily nature in form of retinyl palmitate and vitamin D<sub>3</sub>, (cholecalciferol). The fortificants shall conform to the United State Pharmacopoeia, British Pharmacopoeia, Food Chemical Codex, the FAO/WHO General Principles for the use of Food Additives of the Codex Alimentarius, Volume 1 or the European Pharmacopoeia.

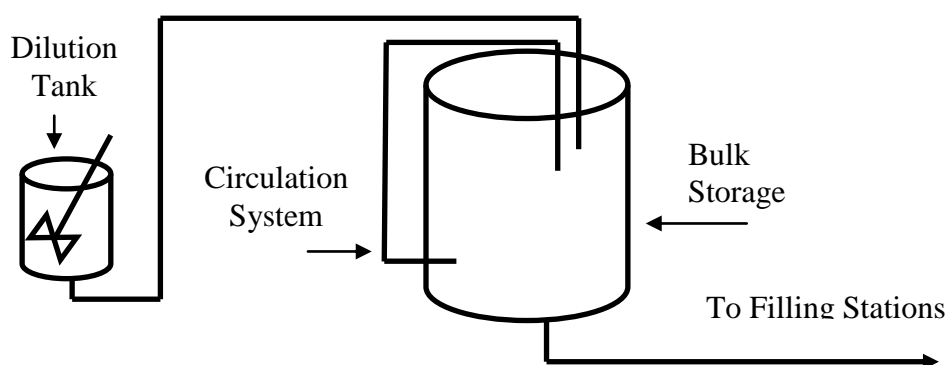
## 5. EXAMPLE METHOD OF FORTIFICATION

The method of fortification must ensure uniform distribution of the added fortificants within the oil or ghee such that the all samples provide levels for vitamin A and vitamin D within the minimum and maximum limits set in Table 3. Batch or continuous fortification processes may be used. A batch process is illustrated in Fig1. The refined oil should be less than 55°C in temperature before the addition of the vitamin blend.

*Stage 1.* Dilute the required quantity of combined Vitamin A and D fortificant in 50 times its weight of refined oil or ghee (see Box 1). Preferably add the fortificant to about 20% of this refined oil, mix well then continue to dilute to full volume. Mix gently without air inclusion until uniformity is obtained. (The time required will require testing by sample analysis).

*Stage 2.* Place 20% of the bulk oil in the Bulk storage tank. Transfer the Diluted fortificant from Stage 1 into this Bulk tank and commence circulation to uniformity. Then continue to fill bulk tank to the final volume while circulation occurs. Maintain circulation while the Bulk tank is filled to the desired final weight. Continue circulation until the required uniformity of vitamin distribution is obtained. Each process must determine the appropriate mixing time to obtain uniformity.

Fig 1 Example Batch Fortification Method



An equivalent protocol would be applied to a continuous fortification process whereby the diluted fortificant is dosed into a flow line fitted with an in-line mixer.

An example of the derivation of quantities of fortificants used in a fortification process is given in Box 1. This example is based on the inclusion of a standard commercial blend of Vitamin A and Vitamin D comprising 1million IU/g Vitamin A and 100,000 IU/g Vitamin D<sub>3</sub>. Permitted antioxidants should be included in the diluted premix in accordance with the manufacturer's guidelines.

Box 1:

If the Vitamin A is 1 million units and a concentration in the final product is 30,000 IU/litre (30IU/g) (see Table 3) or 10 micrograms retinol equivalent /g then the fortification addition rate is 33.3g/MT.

To fortify 38 MT of vegetable oil or ghee you should add 1265g (33.3g x 38) of fortificant to at least 63.25 kg of oil and mix uniformly.

Include the antioxidants at the manufacturer's recommended level.

Add all of the diluted premix to the bulk batch.

For continuous systems the doser should be set to deliver the diluted premix at the desired level.

## **6. RECORDS OF PERFORMANCE**

It is a requirement within this Standard that all vegetable oil / ghee processors retain for inspection records relating to the performance of fortification. These are:

1. Certificate of license to prepare fortified vegetable oil /ghee
2. Certificate of analysis and inclusion level of fortificant from the supplier
3. Date of fortificant delivery/batch number and date of usage.
4. Daily record of flow rate of vegetable oil / ghee through the process
5. Daily record of quantity of fortificant used
6. Daily record of calibration of fortificant feeders
7. Daily record of confirmatory tests that vegetable oil / ghee has been fortified using the rapid vitamin A assay procedure (Appendix A1)
8. Written Standard Operating Procedures(SOP) related to the calibration of all components of the vegetable oil / ghee fortification system.

## **7. HYGIENE AND QUALITY MANAGEMENT**

Edible fats and oils fortified with Vitamins A and D shall be produced in accordance with Recommended General Principles of Food Hygiene CAC/RCP 1-1969-Rev 4 -2003 including the adoption of the principles of Hazard Analysis Critical Control Point (HACCP). Appropriate standards of health and safety must be applied due to the nature of Vitamins A and D in high concentration

## **8. SAMPLING AND TEST**

**8.1** The method of drawing representative samples shall be in accordance with Codex Sampling Plans For prepackaged Foods (XOT13) (or the new Afghanistan protocol under review Ref No....)

**8.2** Testing shall be done in accordance with the respective prescribed methods provided in Table 3 and Appendix 2, 3 and 4. Methods in Table 3 shall be used as reference methods for the determination of Vitamins A and D. The rapid spectrophotometric method in Appendix 3 shall be used as the routine method for Vitamin A. There is no rapid spectrophotometric method for the measurement of Vitamin D.

**NOTE:** “Pure chemicals” shall mean chemicals that do not contain impurities which affect the results of analysis (chemicals of analytical grade).

**8.3** In reporting the results of a test or analysis made in accordance with this standard, if the final value, observed or calculated is to be rounded off, it shall be done in accordance with Afghanistan Standard XYZ

## **9. PACKAGING, MARKING AND LABELING**

**9.1** Packing – Fortified edible fats and oils shall be packed in food grade, non absorbent material which has no adverse influence upon the composition of the product, its properties and appearance. The container shall be sunlight proof and sealed to safeguard the product in terms of the following.

- a) Safety and hygiene, that is, prevent contamination of the product
- b) Nutrition value; that is minimizes loss of nutrients.
- c) Technological qualities of the products; and
- d) Organoleptic qualities of the product and the food to which is to be Applied

### **9.2 Marking and labeling**

Containers of fortified edible fats and oils shall be legibly and indelibly marked with the following information

- a) Name of the product  
The name of the product shall be fortified “X fat or oil, where X is the name of the specific vegetable or animal; including a statement “Refined fat or oil”. Where the vegetable fat has been subjected to any process of esterification or to processing which alters the fatty acid composition or its consistency, the name of

the product or any synonym shall not be used unless qualified to indicate the nature of the product.

- b) Date marking.  
The date of minimum durability (preceded by the words “Date of manufacture and expiry date”) shall be declared by the day, month and year in uncoded numerical sequence except that the products with a shelf life of more than three months; the month and year shall suffice.
- c) List of ingredients
- d) The statement “store in cool and dry place away from sunlight” shall be included in close proximity to the date marking.
- e) The fortified vitamin nutrient and the average quantity in mg per 100 g of the product.
- f) The name, postal and physical address of the manufacturer of the product.
- g) Country of origin
- h) Net content by mass in SI units
- i) Lot/Batch identification number in code or in clear
- j) Manufacturers registered trade mark; if any.

### **9.3 Certification mark**

- In the case of voluntary implementation, each container of fortified oil/ghee may also be marked with the recognized mark of competent certification body.
- After the implementation has become compulsory, Each container shall be marked with the logo of supervising body.

## **10. CAVEAT**

**Separation:** If any part, section or provision of this Act shall be declared invalid or unconstitutional, other provisions or parts thereof which are not affected thereby shall remain in full force and effect.



## APPENDICES

Based on Afghanistan Statistical yearbook's data (2011/12) and decision of ANSA/TC3/SC1 members it is to be agreed that the daily adult intake of edible oil/ghee in Afghanistan is around 40gr/day.

40g oil containing 30,000 IU/kg will provide 1200IU/day

If the Recommended Daily Intake (RDI) for an adult is 3000 IU/ day then 40g oil will provide 40% of this RDI

### Appendix 2

#### RECOMMENDED ROUTINE METHOD FOR THE MEASUREMENT OF VITAMIN A IN FORTIFIED OIL OR GHEE

Principle of method:

Retinol is saponified using methanolic or ethanolic potassium hydroxide solution and extracted by an appropriate solvent. The determination is carried out by a high performance liquid chromatography (HPLC) with either a fluorometric or ultra violet detection. The substances are identified on the basis of the retention times and determined by the external standards procedure using peak areas or heights.

#### RECOMMENDED ROUTINE METHOD FOR THE MEASUREMENT OF VITAMIN A IN FORTIFIED OIL OR GHEE

A rapid spectrophotometric assay based on the Carr Price reaction between antimony trichloride and retinol which gives quantitative results with validated comparability to high pressure liquid chromatography, for example Rohner, F et al (2011) Int.J Vitam.Nutr.Res. 81 (5) 335-342 .

The method is a modern rapid kit assay based on the reaction of vitamin A in oil with a colour complexing reagent.

The kit is available from [www.bioanalyt.com](http://www.bioanalyt.com) using extraction reagent vials iEx ELAN and photometer iCheck CHROMA

#### Method outline:

1. Draw 0.5ml fortified oil into a syringe.
2. Inject the oil sample into a reaction vial iExElan.
3. Invert the vial twice to develop the colour reaction.
4. Immediately measure the colour absorbance in the iCheck Chroma photometer with readout as mgRE/kg oil.

**45.1.01**  
**AOAC Official Method 960.45**  
**Vita min A in Margarine**  
**Spectrophotometric Method**  
**First Action 1960**

**A. Principle**

Unsaponifiable portion of margarine is chromatographed on adsorption column consisting of 2 segments of activated and standardized alumina separated by middle segment of alkaline alumina. (If eluent fraction containing vitamin A is colored, it must be further chromatographed on column of MgO.) Top segment of alumina column prevents caking and initiates separation of vitamin A from carotene and other interfering substances. Middle section of alk. alumina separates persistent interference that cannot be separated by other adsorbents. Final portion of column is non fluorescent and provides suitable background for observing vitamin A fluorescence on column, thus facilitating control of the chromatography. Carotene elutes from column first, second fraction of eluent is discarded, and third fraction contains vitamin A. Better control of chromatography than is possible by observing colored and fluorescent bands on column is achieved by observing fluorescence in 1 mL portions from final parts of fluorescent and colored eluents. This technic achieves separation of vitamin A from impurities that cause erroneously high values. Adequacy of separation is determined from ratio of A of chromatographed vitamin A solution at 310 and 325 nm. A of sample solution at 325 nm multiplied by factor 18.3 gives concentration of vitamin A in units/mL.

**B. Apparatus**

(a) *Spectrophotometer and cells.*—UV spectrophotometer with suitable source of UV light is required. (Incandescent lamp is not suitable source.) Spectrophotometer (such as Beckman DU, or equivalent) with continuous spectrum source and reading to 200 nm is recommended. Matched quartz cells with 1.0 cm internal light path are preferable. If cells are not matched, suitable corrections must be made. Periodically check wavelength and A scales of spectrophotometer. (See Definitions of Terms and Explanatory Notes, item (23).)

(b) *Chromatographic tubes.*—(1) 12 mm od × 90 mm with sealed-in disk of medium porosity and with funnel on upper end and stem on lower end, 8 mm od × 40 mm. (Available from SGA Scientific, Inc.) (2) 8 mm od × 250 mm long with lower 50 mm pulled out to form tapered constricted exit 2 mm id. Plug ca 10 mm of upper part of constricted section with glass wool. Fuse flared tube, 18 mm od × 140 mm to top of 6 mm section.

(c) *Vacuum gage with bleeder valve or pinchcock regulator.*—Use vacuum micro bell jar large enough to hold 100 mL beaker or flask for applying vacuum and collecting eluents. Control vacuum from line or for applying vacuum and collecting eluents. Control vacuum from line or H<sub>2</sub>O aspirator by gage and stopcock or screw-clamp bleeder on T-tube.

(d) *Long wavelength ultraviolet lamp.*—Use lamp source of weak UV for observing fluorescent bands on chromatographic columns. Lamp should provide radiation in long (300 nm) wavelength region. (Suitable lamp is available from Ultra-Violet Products, Inc.; No. UVSL-55: LW 240.) With com. lamps, narrow aperture or screen may be necessary to reduce amount of destructive radiation. (Vitamin A is readily destroyed by too intense UV light.)

**C. Reagents**

(a) *Potassium hydroxide solution.*—50%, w/w (780 g/L).

(b) *Alcohol.*—Absolute and 95%. Shall not show A >0.05 when measured at 300 nm in suitable spectrophotometer in 1.0 cm quartz cell against H<sub>2</sub>O. Isopropanol USP reagent of same spectral purity may be substituted for absolute alcohol in A measurements.

(c) *Ethyl ether*.—Peroxide-free. Use USP, freshly distilled, discarding first and last 10% of distillate; or use USP anesthesia grade in 0.5 lb cans. Must meet same A requirement as for alcohol, (b).

(d) *Petroleum ether*.—Bp 30–60°, free from fluorescence and with  $T$  at 300 nm >85% when measured against air in quartz spectrophotometer fitted with 1 cm cell. This solvent, available in 5 lb cans, should be suitable for chromatic purposes. Also, in adsorbent activity test, eluent effect of 10 mL pet ether by itself must cause movement of visible color  $\leq 1$  cm below surface of column. To meet these requirements, purification by adsorption and/or distn may be necessary.

(e) *Eluting solutions*.—(1) 16% redistilled ether in petroleum ether; (2) 25% redistilled ether in petroleum ether; (3) 10% absolute alcohol in petroleum ether. Dry (1) and (2) with anhydrous Na<sub>2</sub>SO<sub>4</sub> and store over bright Cu strip or turnings to inhibit peroxide formation.

(f) *Sodium sulfate*.—Anhydrous, granular; 10% solution must not be acid (red) to Me red. Must not adsorb vitamin A.

(g) *Alumina*.—Alcoa grade F-20 or Fisher “Alumina, Adsorption,” Cat. No. A-540. Before working with alumina it is essential to determine that the following specifications for particle size have been met: Not >50% of alumina should pass No. 160 sieve; ca 50% should pass No. 100 sieve, but not No. 160 sieve. Remainder ( $\leq 20\%$ ) which does not pass through No. 100 sieve should pass No. 60 sieve. Blend thoroughly before use.

(h) *Standardized alumina*.—Heat portion of alumina 3 h in furnace at 600°, and after partial cooling, place in tightly closed screw-cap glass jar. Cool to room temperature, pass through No. 80 sieve, weigh, and place in weighed screw-cap glass jar of such size that only 2/3 of volume is used. Add H<sub>2</sub>O dropwise, with frequent shaking of capped bottle, until alumina contains 3% by weight of added H<sub>2</sub>O. (Proportion of H<sub>2</sub>O required may vary from 2 to 4%; 3% is usually sufficient for new alumina and 2% for rejuvenated material.) Continue shaking  $\geq 15$  min until no lumps remain and material is uniform. Transfer batch to several small tightly capped jars. Determine adsorption index as in A, after alumina has remained in tightly capped jar overnight. (Since change in moisture content will affect absorptivity of reagent, container must be kept tightly closed, except while removing portion of contents for use.) Adsorption index of stored material decreases with time and should be checked periodically. Alumina suitable for chromatography has adsorption index of 30–40; extremely retentive alumina with index >50 will not permit clean-cut separations. When index is <10, adsorbent has lost most of its retentiveness. Decreased retentiveness may be due to excess H<sub>2</sub>O content or to changed physical state caused by overheating.

(i) *Standardized alkaline alumina*.—Mix portion of alumina, C(g), with equal weight of 10% (w/w) aqueous KOH solution in evaporating dish. Decant excess liquid, and dry moist alumina overnight at 100°. Pass dry material through No. 60 sieve and place in capped bottle filled  $\leq 2/3$  full. Add H<sub>2</sub>O dropwise with frequent shaking until alumina contains 3% by weight of added H<sub>2</sub>O.

Determine adsorption index as in C. To be suitable for use, alkaline alumina should have index of 7–12. If desired adsorption index is not attained with 3% H<sub>2</sub>O, add additional H<sub>2</sub>O in 2% increments to index of 7–12. Store in tightly capped jars.

(j) *Standardized adsorptive magnesia*.—(Westvaco Sea Sorb 43, Fisher Scientific Co., No. S-120.) Heat portion of magnesia 4 h at 600°. After cooling, mix with equal portion of Hyflo Super-Cel (Celite), in 1/2 full, tightly closed jar. Determine adsorption index as in E. To be suitable for use, the magnesia–Celite mixture should have index of 20–35.

(k) *Extract D&C Yellow No. 10 solution*.—Dissolve 20 mg dye (Yellow OB; formerly FD&C Yellow No. 4; Color Index No. 11390) in 1 L petroleum ether.

#### **D. Determination of Adsorption Index of Alumina**

Place adsorbent to be tested in chromatographic tube 8 mm od × 220 mm containing glass wool plug at bottom. Tap material into settled position, making column 10 cm high, and attach to vacuum controlled bell jar. Add 1.0 mL dye solution, **C(k)**, to top of column.

From accurately filled 50 mL graduate, add small portions of eluting solution, 16% ether in petroleum ether. Apply slight vacuum [5" (16.9 kPa); or 635 mm Hg pressure]. Accurately determine volume of eluent required to elute dye completely from column. This volume in mL is adsorption index. For easier recognition of end point, collect eluent until all apparent color on column has been removed, and then collect 2 mL fractions in small beakers until colorless fraction is obtained.

#### **E. Determination of Adsorption Index of Magnesia**

Place magnesia–Celite mixture to be tested in chromatographic tube 12 mm od × 90 mm, and fitted with sealed-in fritted glass disk.

Apply 25" (635 mm; 84.7 kPa) vacuum (125 mm, 16.7 kPa pressure) and, with aid of tamper of suitable diameter, tightly pack column to height of 1.5 cm. Release vacuum, and add 1.0 mL dye solution, **C(k)**. From accurately filled 50 mL graduate add small (ca 2 mL) portions eluting solution, 10% absolute alcohol in petroleum ether.

Apply 20" (510 mm; 68.0 kPa) of vacuum (250 mm, 33.3 kPa pressure) and continue to add portions of eluent until most of color is eluted. Collect final eluents in 1.0 mL portions under 5" (130 mm; 17.3 kPa) of vacuum (630 mm, 84.0 kPa pressure). Volume in mL of eluent required to produce first colorless 1 mL fraction is adsorption index of MgO–Celite mixture. (Removal of individual fractions is easily accomplished at 5" (130 mm; 17.3 kPa) of vacuum by slipping edge of micro bell jar over edge of its base plate.)

#### **F. Sampling**

Store sample in refrigerator. Remove outer layers from 1 lb prints and take sample from interior. Remove end slices from 1/4 lb prints and take sample from remainder.

#### **G. Determination**

(*Caution: See Appendix B, Distillation, Extraction, and Evaporations; Hazardous Radiations; Flammable Solvents; Diethyl Ether and Petroleum Ether.*)

(a) *General precautions.*—Protect vitamin A from strong illumination by working in subdued light or by using no actinic glassware. Avoid undue exposure of vitamin A solutions to air.

Perform chromatographic steps in completely darkened room to make possible adequate monitoring of chromatographic columns with UV light. Complete all steps as promptly as possible.

(b) *Saponification.*—Weigh  $10 \pm 0.1$  g sample into wide-mouth 500 mL Erlenmeyer equipped with cold finger condenser, and add 75 mL 95% alcohol and 25 mL 50% KOH solution. Heat on electric hot plate and stir to break up lumps and completely disperse sample.

Maintain solution at vigorous boil 5 min. Remove heat and let stand at room temperature 20 min with occasional stirring. Avoid rapid cooling.

(c) *Extraction.*—Transfer solution to 500 mL separator. Rinse saponification flask with 100 mL H<sub>2</sub>O in several portions and add rinsings to separator. Add 100 mL ether, shake vigorously, and let stand ca 2 min. Transfer aqueous portion to another 500 mL separator and extract with four 50 mL portions ether. (In case of slow separation, add 2–5 mL 95% alcohol and swirl gently.) Combine ether extracts, pour two 100 mL portions H<sub>2</sub>O into combined ether extracts, swirl gently, and separate. Extract these 2 combined rinses with 2 consecutive 50 mL portions ether, adding ether to original ether extracts. Pour two 100 mL portions H<sub>2</sub>O through combined ether

extracts and discard each washing without shaking. Add ca 10 mL 0.02 N KOH, shake vigorously, and discard after separation.

Rinse with successive 50 mL portions H<sub>2</sub>O, with gentle agitation, until rinse H<sub>2</sub>O is alkali-free to phthln. Let ether solution stand 5 min, discard separated H<sub>2</sub>O, transfer with rinsing to 400–500 mL tall beaker, add 3–5 g anhydrous Na<sub>2</sub>SO<sub>4</sub>, and stir gently to remove traces of H<sub>2</sub>O. Decant ether extract into another clean 400–500 mL beaker, and rinse Na<sub>2</sub>SO<sub>4</sub> thoroughly (ca 6 times) with small portions ether. Combine rinses with extract.

(d) *Preparation of solution for chromatography.*—Evaporate extract on steam bath to volume of ca 25 mL. Transfer to 50 mL beaker and continue evaporation on steam bath until viscous oily residue forms which, when stirred with small rod, shows no indication of volatilizing liquid. Heat ca 20 s, but  $\leq 2$  min, until droplets of oil form. Remove from steam bath and immediately apply stream of nitrogen 1 min. Add 5 mL petroleum ether, transfer to 10 mL volumetric flask, and dilute to volume with petroleum ether. This is sample solution.

(e) *Alumina chromatography.*—Pack each adsorbent in chromatographic tube 12 mm od  $\times$  90 mm by gravity and slight tapping. Add standardized alumina to height of 1 cm, then segment of alkaline alumina 2 cm high, and another segment of standardized n alumina 4 cm high. Apply 5" (130 mm; 17.3 kPa) of vacuum (630 mm, 84.0 kPa pressure), and add 5 mL petroleum ether, followed by 5 mL sample solution, then another 5 mL petroleum ether. As last of solution disappears into column, add 5 mL portions 16% ether eluent until all carotene elutes from column. Determine completeness of elution by collecting final part of eluent in 1 mL beakers and observing color against white background. Elution is complete when carotene color cannot be seen in last 1 mL fraction observed. Combine all carotene fractions and reserve for concentration, below. Continue elution with 5 mL portions 16% ether. Examine column regularly with UV lamp and observe progress of fluorescent vitamin A band. (Total time required to elute vitamin A should be  $\leq 20$  min. If it is desirable to accelerate movement of vitamin A down column, use 25% ether eluting solvent in 3 mL portions.) Discard eluent that collects after carotene fraction has been collected and before vitamin A band begins to elute. Collect all of vitamin A eluent in separate beaker. Elution of vitamin A is complete when 1 mL portion of eluent collected in 1 mL beaker shows no vitamin A fluorescence when examined with UV lamp. Determination of cut-off point for collection of vitamin A fraction is very important. If it goes too far, extraneous material absorbing at 325 nm will be present, giving erroneously high results; if chosen too early, vitamin A values will be low. Combine all vitamin A-containing fractions. (*Note:* Some food dyes may not be separated from vitamin A by alumina chromatography. Whenever the fluorescent vitamin A eluent is colored, rechromatograph on magnesia column as in (f).) Treat carotene eluent and vitamin A eluent separately, maintaining identity of each solution.

Reduce volume of carotene eluent and of vitamin A eluent to ca 2 mL by evaporation on steam bath. Completely remove remaining solvent by evaporation at temperature  $\leq 40^\circ$  under vacuum or with stream of N. Dissolve carotene in 5 mL petroleum ether, transfer to 10 mL volumetric flask, and dilute to volume with petroleum ether.

This is carotene solution for spectrophotometric measurement.

If vitamin A eluent shows no indication of color, dissolve residue in 5 mL absolute alcohol, transfer to 10 mL volumetric flask, and dilute to volume with absolute alcohol. If vitamin A eluent is colored, dissolve residue in ca 2 mL petroleum ether and proceed with chromatography on magnesia–Celite.

(f) *Magnesia oxide chromatography.*—Pack magnesia–Celite mixture in chromatographic tube 12 mm od  $\times$  s 65 mm, using full vacuum and light tapping. Column should be 4 cm high. Add 5 mL petroleum ether and apply 15" (380 mm; 50.8 kPa) of vacuum (380 mm, 50.8 kPa pressure). When pet ether disappears into column, add petroleum ether sample solution. Rinse container with three 2 mL portions petroleum ether and add each rinse to column.

Elute vitamin A from column with 0.5% absolute alcohol in petroleum ether. Use technic of adding eluent, observing movement of vitamin A fluorescence, and collecting vitamin A eluent similar to that described for alumina chromatography. This separation should take  $\leq 10$  min. Loss of vitamin A may result if this chromatographic step is too slow. Evaporate solvent as before, dissolve residue in 5 mL absolute alcohol, transfer to 10 mL volumetric flask, and dilute to volume with absolute alcohol.

(g) *Spectrophotometric measurements and calculations.*—(1) *Carotene.*—Determine  $A$  of petroleum ether solution of carotene at 450 nm in 1 cm cell.

$\mu\text{g Carotene/lb} = A \times 4.17 \times 454/W$  Carotene as units vitamin A/lb  $= A \times 6.95 \times 454/W$  where  $W$  = g sample/mL solution.

(2) *Vitamin A.*—Determine  $A$  of absolute alcohol solution of vitamin A at 310 and 325 nm in 1 cm cell.

$\mu\text{g Vitamin A/lb} = A_{325} \times 5.5 \times 454/W$  units Vitamin A/lb  $= A_{325} \times 18.3 \times 454/W$  where  $A_{325}$  =  $A$  at 325 nm and  $W$  = g sample/mL solution.

Determine ratio of  $A$  at 310 and 325 nm; this ratio is usually  $\leq 1$ .

References: *JAOC* **43**, 6(1960); **45**, 442(1962). CAS-36-88-4 (carotene)  
CAS-68-26-8 (vitamin A)

## **Appendix 4**

### **RECOMMENDED REFERENCE METHOD FOR THE MEASUREMENT OF VITAMIN D IN FORTIFIED OIL OR GHEE**

Principle of method:

Vitamin D3 and vitamin D2 are saponified in the foodstuffs using alcoholic potassium hydroxide solution and extracted by an appropriate solvent. The determination of vitamin D3 or vitamin D2 in an appropriate sample extract solution is carried out by semi-preparative normal phase HPLC followed by reverse-phase analytical HPLC.

If vitamin D3 is to be determined, then vitamin D2 is used as an internal standard. If vitamin D2 is to be determined, then vitamin D3 is used as an internal standard.

Vitamin D is detected by ultraviolet (UV) spectrometry and peaks are identified on the basis of retention times and additionally by UV spectral profile if diode-array detection is used. The determination is carried out by the internal standard procedure using peak areas or peak heights, see [1] to [8].