



Fortification  
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## STANDARD OPERATING PROCEDURE Fortified Milk Testing

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### Scope and Objective

The objective is to ensure that the fortified milk carry required level of vitamins upon fortification. Due to cost implications, vitamin analysis in fortified food is not applied routinely; however, random samples may be analyzed periodically. Moreover, these methods are important for completing Quality assurance and ensuring the activities of fortification.

### Estimation of Vitamin A and Vitamin D

Some of methods (HPLC based) for quantification of vitamin A & D in fortified milk are expensive and needs analytical skills since the test procedures are lengthy, require sophisticated equipment including analytical grade (AR) reagents and skilled and trained personnel. The method based on HPLC can be used by external well equipped/ sophisticated analytical laboratory. However there are the methods (Colorimetric methods) for quantifications of vitamin A & D in fortified milk which can be performed at Dairy Federation/ Milk Union's Centralized laboratory. Nowadays Kit/ ELISA methods are also available for analysis of Vitamin A & D in milk at dairy QC laboratory.

#### 1. Colorimetric methods of analysis- Vitamin A

##### A. Estimation of vitamin A (Neeld and Pearson, 1963)

###### Principle

The sample is saponified with ethanolic potassium hydroxide solution and vitamin A is extracted into petroleum ether. The unsaponified fraction which is obtained after boiling contains vitamin A. This is extracted thrice with petroleum ether. The pooled petroleum ether is washed with aqueous KOH and then with water to remove excess alkali. The ether extract is dried and the residue obtained is dissolved in chloroform and trifluoroacetic acid (TFA) and optical density is measured at 620 nm.

**Reagents:** 60% Potassium hydroxide solution, Ethanol, Petroleum ether (boiling range 40°C to 60°C), 0.5 N potassium hydroxide, Chloroform, Anhydrous sodium sulphate, All-*trans*-retinol, Pyrogallol, Trifluoroacetic acid (TFA), TFA reagent: Mix 1 part TFA and 2 parts chloroform (v/v).

###### Laboratory equipment:

1. Spectrophotometer – suitable to measure absorbance at 620 nm
2. Nitrogen Water Bath/ vacuum rotary evaporator

###### Preparation of Standards:



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Prepare stock solution of vitamin A (Retinyl palmitate (20 µg/ml) in ethanol. From this stock solution, make solutions of 0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 µg/ml in chloroform (required IU or µg to be prepared based on the label claim).

### Vitamin conversion

1 IU retinyl palmitate = 0.55 µg (mcg)

1 IU retinyl acetate = 0.344 µg (mcg)

### Extraction of samples

- Take 10-20 ml milk in a 50 ml stoppered test tube.
- Add 5 ml of absolute ethanol containing 0.1% (w/v) ascorbic acid or 1% pyrogallol (w/v) and 2ml of 60% KOH.
- Agitate the tubes carefully and place in a water bath at 80°C for 20 min.
- After saponification, cool the tubes with running water and place in an ice water bath.
- Add 10 ml petroleum ether (40-60°C) and shake for 15 minutes.
- Transfer the upper ether layer in another tube. Repeat the extraction thrice and collect the ether portion.
- Transfer the combined ether extract to a separating funnel, wash with 10 ml of 0.5 N KOH and subsequently with distilled water (2-3 times) to remove excess alkali.
- Pass the ether extract through phase separator filter paper to remove water, if any.
- Evaporate the ether extract under nitrogen in a water bath maintained at 37°C.  
(Note: Perform all the extractions under subdued incandescent light using amber colored glassware.)
- Dissolve the residue in known volume of chloroform.
- Take 2 ml of sample/standard solution in a test tube and add 2 ml of TFA reagent. Blue color is developed which is not very stable. Immediately measure the optical density at 620 nm in a spectrophotometer.

Plot the standard curve and find out the concentration of vitamin A in the sample.

### B. Estimation of vitamin A

- (i) Carr-Price method; Section 89.1, pages 101 to 102 - Handbook of food analysis Part XI, Dairy Products-BIS
- (ii) Spectrophotometric method; Section 89.2, pages 102 to 103 - Handbook of food analysis Part IX, Dairy Products - BIS



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### Carr-Price Method

#### Principle:

Estimation of vitamin A is based on the reaction of preformed vitamin A with antimony trichloride (Carr-Price reaction). When a solution of antimony trichloride in chloroform is added to a dilute solution of a vitamin A containing sample, a blue colour appears which soon reaches a maximum intensity and then rapidly fades or changes to reddish brown or other colours, depending on the nature of sample. Under carefully controlled conditions, the blue colour persists long enough to make accurate readings possible. A comparison of the blue colour of the unknown, with colour formed by a standard solution of vitamin A, is used to determine vitamin A in unknown sample.

#### Laboratory equipment:

1. Spectrophotometer – suitable to measure absorbance at 620 nm

**Reagents:** Chloroform, 95% ethyl alcohol, antimony trichloride - 25% solution in dry chloroform (discard if solution is turbid), KOH solution - 50% in distilled water, vitamin A standard - 0.1 g of vitamin A acetate, anhydrous sodium sulphate, diethyl ether, 0.5 N KOH in distilled water.

#### Procedure:

In case of dairy products containing fat, saponification and extraction is necessary, for vitamin A estimation.

- Weigh 0.1g of standard vitamin A acetate and 1g of milk fat/milk powder. Transfer these separately to two saponification flasks.
- To each, add 40 ml ethyl alcohol and 7 ml of 50% KOH. Reflux in boiling water bath for 30 minutes.
- Cool and add 30 ml of distilled water. Mix thoroughly and transfer into a separating funnel.
- Extract thrice, with 50 ml portions of ether and discard aqueous phase.
- Combine the ether extracts (these contain vitamin A) in another separating flask; wash with 100 ml water followed by 50 ml of 0.5 N KOH.
- Again wash with 100 ml portions of water till the washing give no color with phenolphthalein.
- Remove moisture from the ether extract by adding 5-10 g of anhydrous sodium sulphate and allowing it to settle. Thereafter, decant the ether extract carefully into another flask. Rinse the first flask with ether to remove any traces and add this wash to ether extract.
- Evaporate the extract to dryness. Dissolve the residue obtained in 100 ml of chloroform.



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- Dilute 1ml of this solution to 10 ml with chloroform to get final concentration of 0.1 mg/ml for standard. For sample, dilution may be carried out depending upon the concentration.
- To 1ml of the sample/standard chloroform solution, add 9 ml of antimony trichloride solution.
- Read the blue colour obtained at 620 nm within 4 seconds. This can be done by adding antimony trichloride just when taking colorimetric reading.
- From the readings of standard, equate the values mathematically to determine concentration of vitamin A in the sample.

### 2. Kits/ ELISA Methods

#### **ELISA Kit by Crystal Chem**

Vitamin A Food ELISA Kit

Vitamin D Food ELISA Kit

### 3. HPLC Methods:-

**Vitamin A-** AOAC 2011.07 & AOAC 2012.10

**Vitamin D-** AOAC 995.05, AOAC 2011.11 & AOAC 2002.05 (20<sup>th</sup> Edn., 2016)

The above methods of estimating Vitamin A & D in milk can be applicable at analytical laboratory, since needs skilled manpower and sophisticated analytical instruments.

Dairy plants shall sent the samples of Vitamin premix (A and D) and Fortified milk to the external laboratory as per the specified frequency mentioned in this document to verify right premix at appropriate rate being fortified in milk at specified concentration.

## Frequency of Testing

### A. Vitamin Pre-mix Testing

- Vitamin premix for fortification – Ensure that premix supplied manufactured of single lot/Batch.
- Fix the premix batch size with the supplier and get the Certificate of Analysis (COA) for each lot of consignment supplied.
- Each lot of premix shall be sent to external laboratory for analysis.
- Assay of premix/vitamin concentrates shall be ensured periodically (every quarterly for a batch/lot which is stored for longer time).



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### **B. Finished product testing (Fortified Milk)**

- The unit starts fortification of milk on first time, the unit shall send the samples of fortified milk for quantification of Vitamin A & D in the finished product at NABL accredited and FSSAI approved laboratory.
- Once standardized the process for fortification of milk, the dairy unit should send the samples of finished product (fortified milk) for analysis at NABL accredited and FSSAI approved laboratory.

### **Record keeping**

- The quantity of vitamin concentrates used must be recorded and cross-verified with the quantity of product fortified.
- The test reports (internal and external) for finished product testing shall be available with the dairy units.