File No: 11014/07/2021-QA
Food Safety and Standards Authority of India
(A Statutory Authority established under the Food Safety and Standards Act, 2006)
(Quality Assurance Division)
FDA Bhawan, Kotla Road, New Delhi – 110002

दिनांक: 07 मई, 2024

आदेश

Subject: Methods for testing of Fortificants in Salt, Milk and Oil- reg.

The Scientific Panel on methods of Sampling and Analysis has approved the following methods -

(i) Method for determination of **Iodine** in Double Fortified Salt: **FSSAI.FS.16.011.2024 (Annexure-I)**
(ii) Method for determination of **Iron** in Double Fortified Salt: **FSSAI.FS.16.012.2024 (Annexure-II)**
(iii) Method for determination of **Vitamin A** in Milk: **FSSAI.FM.16.013.2024 (Annexure-III)**
(iv) Method for determination of **Vitamin A** in Oils & Fats: **FSSAI.FO.16.015.2024 (Annexure-IV)**

2. The food testing laboratories are hereby requested to use the aforesaid methods with immediate effect.

3. This issues with the approval of competent authority.

Enclosure: As above

Dr. SATYEN KUMAR PANDA
Digitally signed by Dr. SATYEN KUMAR PANDA
Date: 2024.05.07 18:11:47 +05'30'

(डॉ. सत्येन कुमार पंडा)
सलाहकार (गुणवत्ता आश्वासन)

To:
1. All FSSAI Notified Laboratories
2. All State Food Testing Laboratories
3. CEO, NABL

Annexure I

एफएसएसएआई fssai भारतीय खाद्य सुरक्षा और मानक प्राधिकरण Food Safety and Standards Authority of India स्वास्थ्य और परिवार कल्याण मंत्रालय Ministry of Health and Family Welfare	**Method for determination of Iodine in Double Fortified Salt (Quantitative)**		
Method No.	FSSAI.FS.16.011.2024	**Revision No. & Date**	0.0
Scope	The iodine content can be measured by conventional iodometric titration using sulphuric acid, but H_2SO_4 interferes with the estimation of iodine leading to erroneous results. Hence a modified method with orthophosphoric acid has been validated for the estimation of iodine in Double Fortified Salt (DFS).		
Caution	Caution should be taken while preparing the solutions and also while analyzing the samples.		
Principle	Iodine estimation by Titration Method. The Iodine content in DFS is measured by a modified iodometric titration.		
Apparatus/Instruments	Weighing balance		
Materials and Reagents	***Materials*** 1. Burette 2. Erlenmeyer flask with stopper, 250 mL 3. Beakers, 250mL and 500 mL 4. Pipettes ***Reagents*** 1. Potassium Iodide 2. Orthophosphoric acid 3. Sodium thiosulphate 4. Starch 5. Sodium chloride 6. Potassium iodate 7. Double distilled water		
Preparation of Reagents	**1. Potassium Iodide, KI (1% solution):** Dissolve 1 g of KI (LR grade) in 100 mL water. Store in a cool, dark place. The solution is stable for at least 3 months if stored properly. **2. Orthophosphoric acid(H_3P0_4), 4 N:** Slowly add 75.4 mL of AR grade orthophosphoric acid to 900 mL of ice-cold distilled water. Dilute and make to 1000 mL with water. The volume is sufficient for 200 samples. The solution is stable. ***Note:*** Always add acid to water dropwise, not water to acid. Stir the solution while adding acid. **3. Sodium thiosulphate ($Na_2S_2O_3$), 0.0005M:** Dissolve 1.24 g $Na_2S_2O_3.5H_2O$ (AR grade) in 1000 mL water. Store in a cool place. This volume is sufficient for nearly 200 samples. The solution is stable at least for 1 month, if stored properly. **4. Starch indicator solution:** **4a. Preparation of saturated NaCl solution** Make 100 mL of a saturated NaCl solution, by adding NaCl in small quantities at a time, to approximately 80 mL water in a beaker, with stirring and heating, until no further Nacl dissolves. This solution is stable for one		

	year. **5. Preparation of Starch:** Weigh one gram soluble starch (potato, extra pure/LR grade) into a 100 mL beaker, add 10 mL water and make a paste, heat to dissolve. Add saturated NaCl solution to the hot starch solution to make to 100 mL. Store in a cool, dark place. This volume is sufficient for 200 samples. The solution is stable for up to one month, and should be heated (not boiled) each day before use to resuspend any solids. **6. Standard KIO_3 :** Weigh accurately 0.167 g of KIO_3 (AR grade) and dissolve in water in a standard measuring flask (100 mL) and make up the volume to 100 mL. This will give a concentration of 1 mg of iodine/mL.
Sample Preparation	***A. DFS Sample Preparation:*** a. Weigh accurately 10 g DFS into a 250 mL Erlenmeyer flask with stopper b. Add 0.5 mL of 1% KI (CAUTION: Do not pipette by mouth) c. Add 46 mL of water. Swirl the flask to dissolve the salt. d. Add 5 mL of 4 N H_3PO_4. The solution will turn yellow if iodine is present. e. Stopper the flask and put it in the dark (cup board) for 10 min. (Caution: The reaction mixture should be kept in the dark before titration because a side reaction can occur when exposed to light that causes iodide ions to be oxidized to iodine) ***B. Standard KIO_3:*** Run standard KIO_3 (1 mg iodine/mL) with 10 g of non-iodized salt as part of quality control. Take 46 mL of water into a 250 mL Erlenmeyer flask with stopper. Add 1 mL of standard KIO_3 (1mg of iodine/mL) and 10 g of non-iodized salt. Add 0.5 mL 1% KI. Add 5 mL 4 N H_3PO_4. Stopper the flask and put in the dark for 10min. **<u>Precautions:</u>** Inaccurate results may occur if starch solution is used while still warm. If starch indicator is added too early, a strong iodine-starch complex is formed which reacts slowly and gives falsely elevated results. The reaction should be performed at room temperature (< 30 ° C), as iodine is volatile and the indicator solution will lose sensitivity when exposed to high temperature.
Method of analysis	***Sample Analysis*** a. Rinse and fill the burette with 0.005 M Sodium thiosulphate and adjust the level to zero. b. Remove the flask from the dark and titrate against $Na_2S_2O_3$ from the burette until the solution turns pale yellow (straw yellow) c. Add 0.5 mL of starch indicator solution and continue titration until the solution becomes colorless. d. Record the volume of thiosulfate in the burette and convert to ppm using the "conversion table". Refer to conversion table for iodine content. ***Standard KIO_3 Analysis*** i. Rinse and fill the burette with 0.005 M Sodium thiosulphate and adjust the level to zero. ii. Then titrate the standard KIO3 solution against 0.005 M $Na_2S_2O_3$ (repeat steps b to d as indicated above) to calculate the iodine content. This will give an iodine value of 100 ppm (100 µg/g).
Calculation with units of expression	The unit of expression is µg/g (ppm)

Inference (Qualitative Analysis)	NA, Quantitative Analysis
Reference	S. Ranganathan & M. G. Karmarkar, Indian Journal of Medical Research 123, April 2006, p,531-540; Estimation of Iodine in salt fortified with Iodine & Iron
Approved by	Scientific Panel on Methods of Sampling and Analysis

Annexure II

<table>
<tr><td>एफएसएसएआई
fssai
भारतीय खाद्य सुरक्षा और मानक प्राधिकरण
Food Safety and Standards Authority of India
स्वास्थ्य और परिवार कल्याण मंत्रालय
Ministry of Health and Family Welfare</td><td colspan="3">Method for determination of Iron in Double Fortified Salt
(Quantitative)</td></tr>
<tr><td>Method No.</td><td>FSSAI.FS.16.012.2024</td><td>Revision No. & Date</td><td>0.0</td></tr>
<tr><td>Scope</td><td colspan="3">This method is used for the estimation of Iron calorimetrically in Double Fortified Salt (DFS).</td></tr>
<tr><td>Caution</td><td colspan="3">Caution should be taken while preparing the solutions and also while analyzing the samples.</td></tr>
<tr><td>Principle</td><td colspan="3">Iron is determined calorimetrically by the principle that ferric ion (Fe^{3+}) gives a blood red color with potassium thiocyanate (KCNS).</td></tr>
<tr><td>Apparatus/Instruments</td><td colspan="3">1. Weighing Balance
2. Colorimeter</td></tr>
<tr><td>Materials and Reagents</td><td colspan="3">1. Sulphuric Acid
2. Potassium Persulphate
3. Potassium thiocyanate
4. Standard Iron solution
5. Working standard solution</td></tr>
<tr><td>Preparation of Reagents</td><td colspan="3">1. Sulphuric Acid, H_2SO_4 (30%)
Take 60 mL distilled water in a beaker. Keep in an ice bath and add slowly drop-wise 30 mL of concentrated H_2SO_4 with constant stirring. Make the volume to 100 mL with distilled water.
2. Potassium persulphate, $K_2S_2O_8$ (7%)
Dissolve seven grams of $K_2S_2O_8$ in distilled water and make up the volume to 100 mL with distilled water.
3. Potassium thiocyanate, KCNS (40%)
Dissolve 40 g of KCNS in 90 mL distilled water. Add four mL acetone and make up the volume to 100 mL.
4. Standard Iron Solution
Dissolve 702.2 mg ferrous ammonium sulphate in 100 mL distilled water. Add five mL of 1:1 hydrochloric acid (HCL) and make up the volume to 100 mL (0.1 mg/mL). The standard solution is prepared fresh and can be kept for 6 months. From this prepare the working standard.
4a. Working Standard (10 µg iron/mL)
Dilute 10 mL of the standard iron solution (0.1 mg/mL) to 100 mL with distilled water. This will give 1010 µg iron/mL concentration.</td></tr>
<tr><td>Sample Preparation</td><td colspan="3">Take one gram of DFS in a 100 mL standard measuring flask using a glass funnel. Add 2.5 mL of concentrated HCL and make up the volume to 100 mL with distilled water. Mix and use 1 mL – 2 mL aliquots for the estimation of iron as given below.

<table>
<tr><th>Reagent</th><th>Test Tube 1</th><th>Test Tube 2</th><th>Test Tube 3</th><th>Test Tube 4</th><th>Test Tube 5</th><th>Test Tube 6</th><th>Test Tube 7</th><th>Test Tube 8</th></tr>
<tr><td>Distilled water (mL)</td><td>6.5</td><td>5.5</td><td>4.5</td><td>6.0</td><td>5.5</td><td>4.5</td><td>3.5</td><td>2.5</td></tr>
<tr><td>Iron working standard(mL)</td><td>0</td><td>0</td><td>0</td><td>0.5</td><td>1.0</td><td>2.0</td><td>3.0</td><td>4.0</td></tr>
<tr><td>DFS Solution(mL)</td><td>0</td><td>1.0</td><td>2.0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td></tr>
</table></td></tr>
</table>

	30% H_2SO_4(mL)	1	1	1	1	1	1	1	1
	7% $K_2S_2O_8$(mL)	1	1	1	1	1	1	1	1
	40% KCNS(mL)	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Method of analysis	Prepare the test tubes as above adding all other solutions except 40% KCNS solution. Add 40% KCNS solution just before taking the readings. Measure the red color developed within 20 min of addition of 40% KCNS at 540 nm.								
Calculation with units of expression	Draw a standard graph of the iron standards by taking iron concentration (µg) on the X-axis and the OD on the Y-axis and calculate the iron content from the standard graph.								
Inference (Qualitative Analysis)	NA, Quantitative Analysis								
Reference	Wong, SY, Hawk' s. Physiological Chemistry,14th Edition, New York: McGraw Hill, 1965, page 1094								
Approved by	Scientific Panel on Methods of Sampling and Analysis								

Annexure III

एफएसएसएआई fssai भारतीय खाद्य सुरक्षा और मानक प्राधिकरण Food Safety and Standards Authority of India स्वास्थ्य और परिवार कल्याण मंत्रालय Ministry of Health and Family Welfare	**Method for determination of Vitamin A in Milk**		
Method No.	FSSAI.FM.16.013.2024	**Revision No. & Date**	0.0
Scope	Determination of Vitamin A in Milk. The limit of Quantification is 90 µg/kg (300 IU/L).		
Caution	1. Refer to the Material Safety Data sheets for all chemicals prior to use. 2. Wear eye protection, gloves, and lab coat. Use only with adequate ventilation. Keep away from heat, sparks, and open flames. 3. Vitamin A is sensitive to light; perform all steps under UV- shielded lighting or in dark.		
Principle	1. Samples are subjected to saponification, which converts retinol esters to retinol. 2. These are extracted in petroleum ether, which is made free from alkali, evaporated, dissolved and analyzed on HPLC system with UV detection at 325 nm. 3. Vitamin concentration is calculated by comparing peak heights of vitamins in test samples with those of standards.		
Apparatus/Instruments	1. Heating Water Bath with concentric ring arrangement 2. HPLC system with Photodiode Detector, pumps, Oven and auto- sampler 3. Sonicator 4. Vortex-mixer 5. Weighing balance (Range: 0.1 mg -200 g) 6. Rotatory vacuum evaporator 7. Spectrophotometer 8. Nitrogen distribution system during saponification 9. Beakers - 50 ml 10. Glass bottle – 1000 ml 11. Clear screw vial with patch, 9 mm 2ml 12. Eppendorf tube 2 ml 13. Erlenmeyer flask with ground-glass neck – 500 ml 14. HPLC Column : 150×4.6 mm,5 µm 15. Measuring cylinder – 100 ml, 500 ml 16. Micropipettes - 2-200 µl, 100-1000 µl 17. Reflux condenser – 1 m 18. Round bottom flask – 500 ml 19. Separator funnel - 250 ml , 500 ml 20. Extraction Cylinder - 1000 ml 21. Volumetric flask – 5 ml, 10 ml, 25 ml, 500 ml, 1000 ml		
Materials and Reagents/Standard	1. Alcohol (95%) 2. Butylated hydroxyl toluene (BHT) 3. Pyrogallic acid 4. Distilled Water 5. Methanol: HPLC grade 6. Petroleum Benzene (40 ° C – 60 °) 7.Potassium hydroxide (KOH) 8. Sodium Sulphate 9. Retinol standard- Sigma		

Preparation of Standards/Reagents

- **Reagent Preparation**

1. Potassium hydroxide solution 50 %(w/v):

Weigh 50 g KOH & add 50 ml water, mix well and allow it to cool to ambient temperature.

2. 95% Alcohol:

Takes 95 ml ethanol and make up or add the volume with 5 ml water.

- **Standard preparation**

1. Vitamin A (Retinol) standard stock

Weigh 20 ± 1 mg Retinol in 50 ml Volumetric flask and make up the volume with 2- propanol.

- **Standardization**:

a) Take 1 ml from standard stock & make it 50 ml in 2-propanol and follow below steps:
b) Set the spectrophotometer in spectrum (scan) mode between 300 to 400 nm.
c) Set the instrument zero with 2-propanol.
d) Read the absorption of prepared solution against the 2-propanol.
e) Read the absorption of the solution at 310,325 and 334nm. The absorption will be between 0.7-0.8, if necessary an intermediate dilution may be used.
f) Calculate the corrected absorption of retinol by following equation- A325corr = 6.15 * A325 – 2.555*A310-4.26*A334
g) A325corr/A325 is less than 0.97 use the value A325cor for standardization; Otherwise use A325
h) Vitamin A concentration IU/mL = A325 X 18.3 Or
i) Vitamin A concentration IU/mL = A325corr X 18.3

Standard dilution	Volume taken (mL)	Volume made(ml) in Methanol	Final Concentration (IU/mL)
Dilution 1 (From stock)	0.1	2	Concentration depend on standardization of standard
Dilution 2 (From D1)	1	2	
Dilution 3 (From D2)	1	2	
Dilution 4 (From D3)	1	2	
Dilution 5 (From D4)	1	2	
Dilution 6 (From D5)	1	2	
Dilution 7 (From D6)	1	2	
Dilution 8 (From D7)	1	2	
Dilution 9 (From D8)	0.1	1	

Sample Preparation and Method of analysis	• **Saponification** a) Turn the water bath/hotplate on for preheat. b) Preparation of sample: Milk sample should be homogenized at 37 ° C. c) Accurately weigh 10 g Milk in 500 ml flat bottomed flask. d) Add 50 mg pyrogallic acid and 100 ml 95% ethanol into it. e) After the addition to all reagents swirl all the flasks to ensure uniform dispersal of all ingredients in the solution. f) Turn on Nitrogen supply and ensure N_2 atmosphere for the all the flasks before and during refluxing. g) Pipette 10 ml of 50% KOH solution into each flask and immediately place flask on boiling water bath/hot plate under reflux condenser and swirl. h) Reflux for 45 min after the boiling starts. Swirl flasks every 10 min. i) Remove flasks from water bath/hotplate and quickly cool to room temperature using cold water or ice water bath. • **Extraction** a) Transfer the solution of each flask to a 1000 ml glass cylinder. b) Rinse the flasks 1 time with 50 ml ethanol, 100 ml water and 100 ml petroleum ether and transfer each extraction to glass cylinder containing saponified sample extracts. c) Shake the cylinders well and allow to stand till phases separates. d) Collect organic phase in separating funnel with application of positive pressure. e) Again re-extract the aqueous phase three times with 100 ml Petroleum ether and add to separating funnel. • **Washing /Removal of impurities** a) Collect the organic phase and wash it with water 4 times or till free from alkalinity. b) Discard the aqueous phase. c) Transfer the content (organic phase) to the round bottomed flask through a fliter containing sodium sulphate in a glass funnel d) Rinse sodium sulphate with 20-50 ml Petroleum Benzene. e) Add BHT granules in extracted solvent. f) Evaporate the content at 40 ° C and dissolve in 10 ml methanol. • **HPLC Condition:** Column: C18, 150×4.6mm, 5 µm Oven temperature: 28° C PDA Detector wavelength: 325 nm Flow rate: 1.2 ml/min Injection volume: 20 µl • **Mobile Phase:** Methanol: Water [950 ml + 50 ml (v + v)]. Shake well and degas, if equipment not equipped with degasser.
Calculation with units of expression	$Vitamin\ A(Retinol)$ µg/100 g = $(Hp * C * Vo * P * 0.3 / Hs * m) * 100$ Where, • Hp- Height/Area of Vitamin A peak in the sample solution. • Hs- Height/Area of Vitamin A peak in the standard solution. • C-Concentration of Vitamin A in the standard solution used for quantitation, in IU/ml.

	• M-Mass of test sample in grams • Vo- Final Volume made(ml) • P- Purity of Standard
References	1. IS 15120-2002 2. AOAC Official method 2001.13- Chapter 45.1.34,21st Edition, 2019
Approved by	Scientific Panel on Methods of Sampling and Analysis

Annexure IV

एफएसएसएआई fssai भारतीय खाद्य सुरक्षा और मानक प्राधिकरण Food Safety and Standards Authority of India स्वास्थ्य और परिवार कल्याण मंत्रालय Ministry of Health and Family Welfare	**Method for determination of Vitamin A in Edible Oils and Fats**		
Method No.	FSSAI.FO.16.015.2024	**Revision No. & Date**	0.0
Scope	Determination of Vitamin A in Edible oils and Fats. The Limit of Quantification is 0.1 mg/kg.		
Caution	1. Always wear gloves and mask while doing sample Analysis. 2. Dark room is required for sample Analysis and Standard Preparation.		
Principle	Vitamin A is extracted and separated by liquid chromatography (LC) on C_{18} column, detected by ultraviolet (UV) detector at 326 nm.		
Apparatus/Instruments	1. High performance liquid chromatography: with Ultraviolet (UV) detector. 2. Concentrator- with N_2 flow 3. Water bath 4. Reflux apparatus: Flat bottom flask (Amber colour) 150ml 5. Pipettes: graduated 100 - 1000 µl and 20 - 200µl		
Materials and Reagents/Standard	1. Retinyl acetate standards 2. Methanol: HPLC grade 3. Water: Millipore Milli-Q system to>18 M-ohm resistivity, or equivalent. 4. Petroleum Ether: AR Grade 5. KOH: AR Grade 6. Pyrogallol: AR Grade 7. Volumetric flask: 10ml and 100ml 8. Beaker: 100ml 9. Separating funnel:250ml 10. Measuring Cylinder: 50 ml		
Preparation of Standards/Reagents	1. Transfer 10mg of Vitamin A Standard into 10ml Volumetric Flask and add 7mL Methanol and sonicate for 10 minutes. Maintain the volume as 10mL in Methanol and mix thoroughly. Label with name of Standard, Concentration, date of preparation, date of expiry. The stock standard solution is stable up to 15 days Standard. 2. **Saponification solution –** 10.5M potassium hydroxide (KOH). Dissolve 673g KOH in 1 L H2O. 3. **Antioxidant solution** -1% pyrogallol. Dissolve 5.0 g pyrogallol in 500 ml ethanol.		

Sample Preparation and Method of analysis	1. Take approximately 2 g of sample into 150 ml flat bottom flask (Amber Colour). 2. Add 5 ml saponification solution (10.5 M KOH). 3. Add 20ml antioxidant solution (1% pyrogallol), reflux for 45min at 70°C in Water bath. 4. Remove sample and place in ice 5 min, or until contents Cool to room temperature sample transfer to separate 250mL separatory funnels. 5. Add 30 ml extraction solvent (Petroleum Ether) into funnel and shake well 5 min and separate upper layer. 6. Repeat the above step 3 times. 7. Wash it with distilled water up to alkali free. 8. Evaporate aliquot up to dryness under nitrogen gas. 9. Dissolve the residue in 1ml methanol, as per requirement of the sample. 10. Filter the solution with the 0.45µm (PVDF) syringe filter. 11. Now inject 20µl of the filtered solution on HPLC system.
Instruments Conditions (Details Required)	1. LC Column: C18 (250mm×4.6mm) , 5µm 2. Detector- Ultraviolet (UV) 3. Wavelength-326 nm 4. Mobile phase- A) Methanol (98%) B) Milli-Q (2%) Filter through a membrane (porosity 0.45 µm). 5. Flow rate-1mL/min. 6. Flow Type- Isocratic 7. Column Temperature- Ambient 8. Run Time- 10 min. 9. Injection Volume- 20 µL
Calculation with units of expression	$Vitamin\ A\ (Retinol), ppm\ or \frac{mg}{Kg} = \frac{\text{Peak area of Unknown X Std. conc. (ppm) X Dilution (ml)}}{\text{Peak area of Std. area X Sample weight (gm)}}$
References	1. IS-15120:2002, Animal Feeding Stuffs Determination of Vitamin A 2. Kienen et.al talanta 75 (2008) 141-146 3. AOAC 992.06,21th Ed. 2019
Approved by	Scientific Panel on Methods of Sampling and Analysis