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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

Dated, the 10th June 2022

ORDER

Subject: Method for Determination of Niacin in Foodstuffs

The Food Authority has approved the "Method for determination of niacin in food stuff" **(Annexure-1)** in its 38th meeting held on 02.03.2022.

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

3. Any issue related to these methods may be forwarded to the Scientific Panel On Methods of Sampling and Analysis for its consideration at email: sp-sampling@fssai.gov.in .

(Dr. Harinder Singh Oberoi)
Advisor (QA)

Enclosed: Method

To:

i. All FSSAI notified Laboratories
ii. All State Food Testing Laboratories
iii. IT Division for uploading on the FSSAI website

Annexure-I

FOOD SAFETY AND STANDARDS AUTHORITY OF INDIA Inspiring Trust, Assuring Safe & Nutritious Food Ministry of Health and Family Welfare, Government of India	**Method for Determination of Niacin in foodstuffs**		
Method No.	**01**	**Revision No. & Date**	10th June 2022
Scope	• This is a method for the determination of the mass fraction of niacin in foodstuffs by high performance liquid chromatography (HPLC) by two different ways of hydrolysis, (A) acid hydrolysis (bioaccessible / bioavailable), (B) acid/alkaline hydrolysis (Total) based on References 1 and 2 (The method has been validated in inter laboratory tests on fortified and non-fortified samples such as breakfast cereal powder, chocolate cereals, cooked ham, green peas, lyophilized green peas with ham, lyophilized soup, nutritive orange juice, milk powder and wheat flour, at levels from 0.5 mg/100 g to 24 mg/100 g). • In options A niacin is calculated as the sum of nicotinamide and nicotinic acid, and expressed as nicotinic acid. • Option B gives higher results than A for niacin with non-supplemented cereals, but similar results for other products. In option B, niacin is calculated and expressed as nicotinic acid after transformation of nicotinamide into nicotinic acid. • Option A is faster and cheaper than B. • Option B quantifies total niacin. The alkaline hydrolysis is able to liberate other forms giving higher results for niacin, which in some foods such as maize and cereals are not normally biologically available. • The niacin content (mg/100 g dry weight) obtained with acid hydrolysis ranged from a low level in corn flour (0.26), white wheat flour (0.45) and oat flakes (0.48), to a higher level in wholegrain flours (rye: 0.79, barley: 0.99, wheat: 0.88), wheat bran (2.7) and wheat germ (2.7). The niacin content with the acid–alkaline hydrolysis, however, was 1.9 – 11-fold the value measured after extraction with acid hydrolysis. In general, the niacin content found in the databases is closer to the results obtained after the acid–alkaline extraction, suggesting that the niacin values reported in the databases may not reflect actual bioaccessible niacin but total niacin.		
Caution	1. **Hydrochloric acid:** It is a hazardous liquid which must be used with care. The acid itself is corrosive, and concentrated forms release acidic mists that are also dangerous. If the acid or mist, come into contact with the skin, eyes, or internal organs, the damage can be irreversible or even fatal in severe cases. 2. **Acetic acid:** Acetic acid can be a hazardous chemical if not used in a safe and appropriate manner. This liquid is highly corrosive to the skin and eyes and, because of this, must be handled with extreme care.		

	Acetic acid can also be damaging to the internal organs if ingested or in the case of vapor inhalation. 3. **Sodium hydroxide:** Sodium hydroxide is strongly irritating and corrosive. It can cause severe burns and permanent damage to any tissue that it comes in contact with. Sodium hydroxide can cause hydrolysis of proteins, and hence can cause burns in the eyes which may lead to permanent eye damage. 4. **Hydrogen peroxide:** Hydrogen peroxide is a strong oxidizer (moderate oxidizer in lower concentrations), and can be corrosive to the eyes, skin, and respiratory system. This chemical can cause burns to the skin and tissue damage to the eyes. Take special caution to avoid contact with hydrogen peroxide. 5. **Copper sulphate:** Copper sulfate can cause severe eye irritation. Eating large amounts of copper sulfate can lead to nausea, vomiting, and damage to body tissues, blood cells, the liver, and kidneys. 6. **Sodium acetate:** May cause irritation to skin, eyes, and respiratory tract. 7. **Potassium dihydrogen phosphate:** Acute Inhalation - irritation; Skin Contact - irritation, chronic exposure may also cause dermatitis. Eye Contact - mild irritation; Ingestion - nausea, vomiting, diarrhea, and stomach pain and bone disorders (including bone and joint pain).
Principle	Niacin vitamers are extracted from food by an acid (option A), or an acid/alkaline (option B) treatment and quantified by HPLC with a fluorometric detection after a post-column derivatization with UV irradiation. For option A, niacin is determined as the sum of nicotinamide and nicotinic acid. Niacin is expressed as nicotinic acid after correction of the molecular weights. For option B, niacin is determined and expressed as nicotinic acid. The alkaline treatment transforms all nicotinamide into nicotinic acid.
Apparatus/Instruments	1. General-Usual laboratory apparatus and glassware. 2. UV vis Spectrophotometer is capable of measurement of absorbance at defined wavelength. 3. Oven, capable of maintaining a temperature of 37 °C. 4. Autoclave, capable of maintaining a temperature of 120 °C. 5. A HPLC system with fluorometric detector (322 nm excitation and 380 nm emission wavelengths) and integrator. 6. A normal phase silica (HSS) T3 column (2.1 × 150 mm, 1.8 µm) **1.** Note: Equivalent products may also be used if they can be shown to lead to the same results; i.e., Other phases, particle sizes, or column dimensions. **2.** [e.g., Reference 2: HPLC system fluorescence detector with excitation and emission wavelengths set at 322 nm and 380 nm, and an integrator. Analytical reverse phase separating column - LiChrospher® 60 RP-18

	Select B endcapped and VL-120 BLB column (a) a length of 25 cm; (b) an inner diameter of 4,0 mm; (c) a particle size of 5 µm. **3.** Reference 3. A reversed-phase column (HSS T3 C18; 1.8 µm, 2.1mmx 150 mm;). Separation parameters shall be adapted to such other materials to guarantee equivalent results]. 7. Filter device - Membrane filter with a pore size of for example 0.45 µm. / syringe-filter of 0.2 µm. 8. Post-column derivatization tube and UV lamp - A polytetrafluoroethylene (PTFE) tube (length of 5 m, inner diameter of 0.17 mm, external diameter of 1.59 mm) surrounding a UV light (366 nm, 8 W). WARNING 1 — Harmful UV light could come out of the metal box containing the lamp. WARNING 2 — If bubble formation occurs in the tube due to overheating, the tube should be efficiently cooled by air circulation, for example by lifting the box.
Materials and Reagents	All reagents are analytical grade. 1. Sodium acetate, CH_3COONa > 99% 2. Potassium dihydrogen phosphate, KH_2PO_4 > 99.5% 3. Non stabilized hydrogen peroxide solution, H_2O_2 = 30% 4. Copper sulfate, Cu(II)$SO_4 \cdot 5H_2O$ > 99% 5. Acetic acid, CH_3COOH > 99.8% 6. Concentrated hydrochloric acid solution (option A and B), HCl = 37.0% 7. Sodium hydroxide (option B), (NaOH) ≥ 99% 1. Standard substances 8. Nicotinic acid, (C_5H_4NCOOH) ≥ 99,5% 9. Nicotinamide, ($C_5H_4NCONH_2$) ≥ 99.5% Note: The purity may vary from different suppliers and it is therefore necessary to determine the concentration of the calibration solution by a spectrometric determination
Preparation of Reagents	1. Acetic acid solution, substance concentration (CH_3COOH) = 5 mol/L 2. Sodium acetate solution, (CH_3COONa) = 2.5 mol/L 3. Sodium acetate solution (option B), (CH_3COONa) = 0.05 mol/L, pH = 4.5 Dissolve 4.10 g of sodium acetate in 900 ml of water. Adjust the solution to pH = 4.5 with acetic acid and then dilute to 1000 mL with water. 4. Hydrochloric acid solution (options A and B), (HCl) = 0.1 mol/L. 5. Copper sulfate solution, [Cu(II)$SO_4.5H_2O$] (It should be suitable for dilution to prepare mobile phase). 6. Potassium dihydrogen phosphate (KH_2PO_4) solution (It should be suitable for dilution to prepare mobile phase).

	7. Hydrogen peroxide solution (H_2O_2) (It should be suitable for dilution to prepare mobile phase). 8. Sodium hydroxide solution (option B), (NaOH) = 5 mol/L. Dissolve 20 g of sodium hydroxide in 80 mL of water. After cooling dilute to 100 mL. Standard substances - Stock solutions 9. Nicotinic acid stock solution, mass concentration = 1 mg/mL Dissolve an amount of the nicotinic acid standard substance e.g. approximately 100 mg (to the nearest 1 mg) in 100 mL of water. This solution is stable for 1 week at -18 °C ± 1. 10. Nicotinamide stock solution, (options A and B) = 1 mg/mL Dissolve an amount of the nicotinamide standard substance e.g. approximately 100 mg (to the nearest 1 mg) in 100 mL of water. This solution is stable for 1 week at -18 °C ± 1.
Method of analysis	Concentration tests Nicotinic acid solution = 1 mg/mL 1. Dilute 1 mL of the nicotinic acid stock solution in 100 mL of hydrochloric acid (0.1 mol/L) solution and measure the absorbance at 260 nm in a 1 cm cell using a UV vis Spectrophotometer spectrometer against hydrochloric acid solution as reference. 2. Calculate the mass concentration, ρ, in milligram per millilitre of the stock solution, using the following Equation: $$\rho = \frac{A_{260} \times 1000}{420}$$ where A_{260} is the absorbance value of the solution at 260 nm; 420 is the $E_{1cm}^{1\%}$ value for nicotinic acid in 0.1 mol/L HCl, Nicotinamide solution = 1 mg/mL 3. Dilute 1 mL of the nicotinamide stock solution in 100 mL of hydrochloric acid (0.1 mol/L) solution and measure the absorbance at 260 nm in a 1 cm cell using a UV vis Spectrophotometer spectrometer against hydrochloric acid (0.1 mol/L) solution as reference. 4. Calculate the mass concentration, ρ, in milligram per millilitre of the stock solution using the following Equation: $$\rho = \frac{A_{260} \times 1000}{410}$$ where A_{260} is the absorbance value of the solution at 260 nm. 410 is the $E_{1cm}^{1\%}$ value for nicotinamide in 0.1 mol/L HCl. Nicotinic acid and nicotinamide standard solutions = 0.05 - 5 µg/mL

	5. Prepare e.g. a first solution with 1 mL of each stock solution in 100 mL of water. 6. From this solution prepare four standard solutions (0.5 mL, 2.5 mL, 10 mL and 50 mL) in 100 mL of water. These solutions are stable for one day at room temperature. Acid extraction 7. Analytical samples (0.5 – 1.0 g) were vortexed with 25 mL of 0.1 M hydrochloric acid. 8. The tubes were placed in a boiling water bath for 1 h with an occasional shaking of the tubes (2 – 3 times). 9. After cooling on an ice bath, the pH of the extracts was adjusted to 4.5 with sodium acetate solution (2.5 M). 10. The extracts were then transferred into 50 mL volumetric flasks and filled up to the mark with d-H_2O. 11. The sample extracts were syringe-filtered (0.2 μm) into 2 mL HPLC vials prior to the HPLC analysis. Acid–alkaline extraction 12. The samples (0.5 – 2.0 g) were vortexed with 25 mL of 0.1 M hydrochloric acid. 13. The tubes were heated for 1 h in a boiling water bath. 14. The extracts were then transferred into Erlenmeyer flasks (250 mL). 20 mL of d-H_2O and 4 mL of 5M sodium hydroxide were added and the flasks were autoclaved (121 °C ± 1; 1 h). 15. After cooling, the pH of the extracts was adjusted to 4.5 first with concentrated and then with dilute (0.1 M) hydrochloric acid. 16. The extracts were diluted to achieve 100 mL with d-H_2O and filtered into HPLC vials. HPLC analysis: (Reference 2.) 17. The niacin vitamers (NA and NAM) were separated with a normal phase silica (HSS) T3 column (2.1 × 150 mm, 1.8 μm). 18. The chromatographic separation was performed at 30 °C using an isocratic flow of the mobile phase (MP) (0.3 mL/min) consisting of an optimized concentration of copper sulphate ($CuSO_4$, 5 μM) and hydrogen peroxide (H_2O_2, 150 mM) in a potassium phosphate buffer (70 mM of potassium dihydrogen phosphate; pH 4.5). 19. The run time as 15 min. 20. The eluent flow from the column was exposed to a long-wavelength UV light (366 nm, 8 W) in a knitted PTEE reaction coil (1.59 mm o.d., 0.17 mm i.d. and 5 m length). 21. NA and NAM were detected fluorometrically (322 nm excitation and 380 nm emission wavelengths). 22. The sample extracts were injected (10 μL) in duplicate.

	[Note1. Retention times - NA, 3.6 min; NAM, 10.6 min; These may vary depending on the column phase and dimensions as well as mobile phase flow rate. Note 2. The LOD for NA and NAM was found to be 0.02 ng and 0.01 ng respectively. The LOQ (3-fold the LOD) for NA and NAM was thus 0.06 ng and 0.03 ng respectively] 23. The actual concentration of the NA and NAM standards was confirmed spectrophotometrically (Reference 2 EN 15,652 method 2009) at 420 nm and 410 nm for NA and NAM, respectively, using equations described earlier.
Calculation with units of expression	1. The NA and NAM concentrations were calculated using external calibration curves (calibration range: 0.2 – 20 ng). 2. In terms of the cereal sample (for a 5 g sample), the LOQ for NA was 0.3 μg/g and it was 0.03 μg/g for NAM. The linear response for both NA and NAM were in the range of 0.2–1000 ng, with consistently excellent linearity (average $R^2 = 0.9985$; RSD of < 3.5%). Niacin is expressed as mg/100 g foodstuff.
Reference	1. Niacin contents of cereal-milling products in food-composition databases need to be updated; Journal of Food Composition and Analysis (2020), 91, 103518. 2. EN 15652:2009- Foodstuffs - Determination of niacin by HPLC. 3. Riboflavin, niacin, folate and vitamin B12 in commercial microalgae powders; Journal of Food Composition and Analysis (2019) 82, 103226. 4. Determination of niacin profiles in some animal and plant based foods by high performance liquid chromatography: association with healthy nutrition; Journal of Animal Science Technology 2019; 61(3), 138-146.
Approved by	Scientific Panel on Methods of Sampling and Analysis

Note: The test methods given in the manual are standardised/ validated/ taken from national or international methods or recognised specifications, however it would be the responsibility of the respective testing laboratory to verify the performance of these methods onsite and ensure that it gives proper results before putting these methods in to use".