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**

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**Dated: 7th May, 2024**

## Order

**Subject: Insertion of Method for Determination of Ochratoxin A in Wine and other Fermented Alcoholic Beverages under FSSAI Manual of Methods of Analysis of Foods- Alcoholic Beverages - reg.**

The Method for Determination of Ochratoxin A in Wine and other Fermented Alcoholic Beverages (FSSAI 13.051:2024) has been approved by Food Authority in its 43rd meeting.

2. The Method is enclosed herewith and the same has also been inserted in the Manual of Methods of Analysis of Foods- Alcoholic Beverages.

2. This method shall be used by the laboratories with immediate effect.

3. Since the process of updation of test methods is dynamic, any changes happening from time to time will be notified separately. Queries/concerns, if any, may be forwarded to email: sp-sampling@fssai.gov.in, dinesh.k@fssai.gov.in.

*Encl: as above*

Dr. SATYEN KUMAR PANDA

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(Dr. Satyen Kumar Panda)
Advisor (QA)

To:

1. All FSSAI Notified Laboratories
2. All State Food Testing Laboratories
3. CEO, National Accreditation Board for Testing and Calibration Laboratories (NABL)

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| 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 | **Determination of Ochratoxin A in Wine and other Fermented Alcoholic Beverages** | | |
| **Method No.** | FSSAI 13.051:2024 | **Revision No. & Date** | 0.0 |
| **Scope** | Applicable to the determination of ochratoxin A in Wine and Other Fermented alcoholic Beverages at >0.06 ng/L. | | |
| **Caution** | OTA is toxic as well as carcinogenic in nature, use nitrile gloves while handling these substances. Prior to sample extract disposal, the solutions must be treated with 5–6% sodium hypochlorite. All glass ware exposed to the residues of these toxins must be rinsed with methanol and 1% sodium hypochlorite solution and then washed. | | |
| **Principle** | Wine and beer are diluted with a solution containing polyethylene glycol and NaHCO3, and the diluted solutions are filtered and cleaned up on an immunoaffinity column. OTA is eluted with methanol and quantified by reversed-phase liquid chromatography (HPLC) with fluorometric detection. | | |
| **Apparatus/Instruments** | 1. Microbalance (Measuring to within ± 0.01 mg).<br>2. Glass vials - 4 mL. (Note: Certain types of vials might lead to losses of OTA during evaporation. To avoid this, silanization can be used. Prepare vials by filling them with silanizing reagent and leave this reagent in vials for 1 min. Rinse vials twice with a solvent [toluene, acetone, or hexane] followed by water [twice], and dry vials).<br>3. Volumetric flasks - 5 mL, with accuracy of at least ± 0.5%.<br>4. Vacuum manifold - To accommodate immunoaffinity columns.<br>5. Reservoirs and attachments -To fit immunoaffinity columns.<br>6. Glass microfiber filters - Whatman GF/A, or equivalent.<br>7. Immunoaffinity columns - Containing antibodies against OTA with a total binding capacity of ≥100 ng OTA and a recovery of ≥85% when a diluted wine solution containing 100 ng OTA is applied.<br>8. Solvent evaporator.<br>9. Syringe and microliter pipet(s).—250 µL.<br>10. HPLC system equipped with pump (Isocratic; delivering constant flow rate of 1.0 mL/min.), Injection system (Syringe-loading injection valve with 100 µL injection loop, or equivalent.<br>11. HPLC analytical column - Stainless steel (150×4.6 mm id) packed with 5 µm C18 reversed-phase material.<br>12. Reversed-phase guard column (i.e., 20×4.6 mm id, 0.5 µm particle size) or guard filter (i.e., 0.5 µm, Rheodyne); Columns of different dimensions may be used, if they adequately resolve the OTA peak from all other peaks.<br>13. Fluorescence detector - Fitted with a flow cell and set at 333 nm (excitation) and 460 nm (emission) indicating a peak from ≥0.02 ng of OTA.<br>14. Data collection system.<br>15. UV spectrophotometer. | | |
| **Materials and Reagents** | 1. Polyethylene glycol (PEG) - PEG 8000.<br>2. Methanol - HPLC grade.<br>3. Acetonitrile - HPLC grade.<br>4. Water - HPLC grade.<br>5. Glacial acetic acid - 99% purity.<br>6. Toluene - Analytical grade.<br>7. Ochratoxin A (OTA).—Crystalline form, film, or solution (stored in | | |

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| | the dark at 4°C). |
| **Preparation of Reagents** | 1. Diluting solution (1% PEG + 5% $NaHCO_3$, pH 8.3) - Dissolve PEG (10 g) and $NaHCO_3$ (50 g) in water (950 mL) and dilute to 1 L with water.<br>2. Washing solution (2.5% NaCl + 0.5% $NaHCO_3$, pH 8.1) - Dissolve NaCl (25 g), and $NaHCO_3$ (5 g) in 950 mL water and dilute to 1 L with water.<br>3. HPLC mobile phase [Water–acetonitrile–glacial acetic acid (99 + 99 + 2, v/v/v; pH 3.2)] - Mix 990 mL water, 990 mL and 20 mL acetic acid, filter through 0.45 μm filter and degas (e.g., with He).<br>4. Solvent mixture [Toluene–acetic acid (99 + 1, v/v)] - Mix 99 parts, by volume of toluene with 1 part by volume of acetic acid.<br>5. OTA stock solution - Dissolve OTA (1 mg) or the contents of 1 ampule (if OTA has been obtained as a film) in the solvent mixture, (4), to prepare a solution containing OTA at approximately 20–30 μg/mL. To determine the exact concentration, record the absorption curve between 300 and 370 nm in a 1 cm quartz cell with the solvent mixture (4) as the reference. Identify the maximum absorption, and calculate the mass concentration of OTA, $C_{OTA}$, in μg/mL, using the following equation:<br>$$C_{OTA} = A_{max} \times M \times 100/\varepsilon \times \delta$$<br>Where $A_{max}$ is the absorption determined at the maximum of the absorption curve (at 333 nm); M is the relative molecular mass of OTA (M = 403.8 g/mol); ε is the relative molar absorption coefficient of OTA in the solvent mixture (4), (ε = 544 $m^2$/mol); and δ is the path length of the quartz cell in cm. This solution is stable at –18°C for ≥4 years.<br>6. OTA standard solution [2 μg/mL in toluene–acetic acid (99 + 1, v/v)]. Dilute stock solution (5), with solvent mixture (4) to obtain a standard solution with a mass concentration of OTA of 2 μg/mL. Store standard solution at +4°C.<br>**7.** Calibration solutions - Pipet 0.5 mL standard solution containing OTA at 2 μg/mL, into a glass vial, and evaporate the solvent under a stream of N. Re-dissolve contents of vial in 10 mL HPLC mobile phase, which has been filtered through a 0.45 μm filter. This gives a solution containing OTA at 100 ng/mL. Take 6 different volumes (30, 100, 300, 1000, 2000, 3000 μL) of this solution in separate 5mL volumetric flasks and Dilute each standard solution to volume (5 mL) with filtered HPLC mobile phase (3) to obtain solutions with following concentrations (0.6, 2.0, 6.0, 20, 40 and 60 ng/mL). Inject 100 μL of each calibration solution (containing 0.06, 0.20, 0.60, 2.00, 4.00 and 6.00 ng respectively) into the HPLC system. |
| **Sample Preparation** | 1. Cool beer at +4°C for 30 min to prevent fast foam formation.<br>2. Degas by sonicating for 1 h.<br>3. Pour 10 mL of alcoholic beverage into a 100 mL conical flask.<br>4. Add 10 mL diluting solution. Mix vigorously. Filter through glass microfiber filter, if solution is cloudy solutions or if solid residue is formed after dilution.<br>5. Connect the immunoaffinity column to the vacuum manifold and attach the reservoir to the immunoaffinity column. Add 10 mL (equivalent to 5 mL alcoholic beverage) diluted solution to the reservoir, and pass solution through the immunoaffinity column at a flow rate of about 1 drop/s. Do not permit the immunoaffinity column to run dry.<br>6. Wash the immunoaffinity column with 5 mL washing solution and |

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| | then with 5 mL water at a flow rate of 1–2 drops/s. Dry the column by passing air through it.<br>7. Elute OTA into the vial by passing 2 mL methanol at a flow rate of 1 drop/s.<br>8. Evaporate the eluate to dryness at 50°C under N. Re-dissolve eluate immediately in 250 μL HPLC mobile phase and store at +4°C until HPLC analysis. |
| **Method of analysis** | 1. Set flow rate of the mobile phase at 1.0 mL/min.<br>2. Inject 100 μL reconstituted extract (equivalent to 2 mL alcoholic beverage) into the HPLC system.<br>3. Quantify OTA by comparing OTA peak area with the relevant calibration curve. If the content of OTA in the test solutions fall outside the calibration range, dilute test extracts.<br>4. Prepare a calibration curve at the beginning of every day of analysis and whenever chromatographic conditions change. |
| **Calculation with units of expression** | Determine from the calibration curve the amount of OTA (in ng) in the aliquot of test solution injected into the HPLC system.<br><br>Calculate the concentration of OTA ($C_{OTA}$; in ng/mL) from the following equation:<br>$C_{OTA} = M_A \times (2/V_1) \times (V_3/V_2)$<br><br>Where, $M_A$ is the mass of OTA (in ng) in the aliquot injected on column, determined from the calibration graph;<br>$2$ is the dilution factor;<br>$V_1$ is the volume of solution taken for analysis (10 mL);<br>$V_2$ is the volume of test solution injected on column (100 μL);<br>$V_3$ is the volume of solution used to dissolve the dried eluate (250 mL). |
| **Reference** | Angelo Visconti, Michelangelo Pascale, And Gianluca Centonze; Determination of Ochratoxin A in Wine and Beer by Immunoaffinity Column Cleanup and Liquid Chromatographic Analysis with Fluorometric Detection: Collaborative Study; Journal of AOAC International. 84, (6), 2001; 1818-1827. |
| **Approved by** | Scientific Panel on Methods of Sampling and Analysis |