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# Nisin A

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# Nisin A

*Revised specifications prepared at the 99th JECFA (2024) and published in FAO JECFA Monographs 34 (2025), superseding specifications for Nisin prepared at the 77th JECFA (2013). The Committee reaffirmed the ADI of 0-2 mg/kg bw established at the 77th JECFA.*

**SYNONYMS** INS No. 234

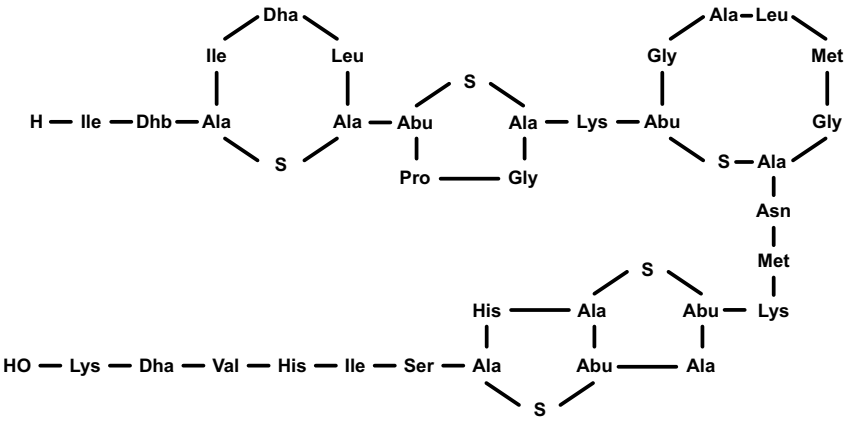
**DEFINITION** Nisin A, produced under appropriate fermentation conditions by *Lactococcus lactis* subsp. *lactis* strains, is an antimicrobial polypeptide. Nisin A is produced in a sterilized medium of non-fat milk solids or non-milk-based fermentation source, such as yeast extract and carbohydrate solids. The fermentation process is controlled for time and pH, until optimum nisin A production has been achieved. The nisin is then concentrated, recovered and purified from the fermentation medium by various methods, such as sterile injection, membrane filtration, acidification, salting out, ultrafiltration or spray-drying. The purified nisin A is then standardized with sodium chloride to achieve desired activity levels of nisin preparation. Nisin A is stable, at ambient temperatures and when heated, under acidic conditions (up to pH 3). Nisin A is commercially available as nisin preparation, which contains 2.5% w/w nisin A, >50% sodium chloride; the remaining components of the preparation are products of fermentation that include proteins and carbohydrates related to the starting material used for fermentation.

The activity of nisin A is measured in International Units (IU). One IU is defined as the amount in micrograms of nisin A required to inhibit the growth of 1 bacterial cell/1 ml of broth of Lactococcus lactis subsp. Cremoris. 1 IU of nisin A is equivalent to 0.025 µg.

**C.A.S. number** 1414-45-5

**Chemical formula** $C_{143}H_{230}O_{37}N_{42}S_7$ (Nisin A)

**Structural formula**




Abu =alpha-aminobutyric acid, Dha=dehydroalanine, Dhb=dehydrobutyrine (Nisin A)

**Formula weight** 3354.12 (Nisin A)

**Assay** Not less than 900 IU of nisin per mg (or 22.5 µg/mg)

| | |
|---|---|
| **DESCRIPTION** | White to light brown micronized powder |
| **FUNCTIONAL USES** | Antimicrobial preservative |

## CHARACTERISTICS

### IDENTIFICATION

| | |
|---|---|
| **Solubility (Vol. 4)** | Soluble in water at pH 2.5, sparingly soluble at pH 5, insoluble at pH>7 and in non-polar solvents |
| **Differentiation from other antimicrobial substances** | Passes test<br>See description under TESTS |
| **Nisin A Activity** | The sample shows nisin A activity<br>See description under METHOD OF ASSAY |

### PURITY

| | |
|---|---|
| **Loss on drying (Vol. 4)** | Not more than 3.0% (105 °C, 2 h) |
| **Sodium Chloride (Vol. 4)** | Not less than 50% |
| **LEAD (Vol. 4)** | Not more than 1 mg/kg<br>Determine using a method appropriate to the specified level. The selection of sample size and method of sample preparation may be based on the principles of the methods described in Vol. 4 (under "General methods, metallic impurities"). |
| **Microbiological criteria (Vol. 4)** | *Salmonella* species: Absent in 25 g of sample<br>Total coliforms: Less than 30 cfu per gram<br>*Escherichia coli*: Absent in 25 g of sample |

## TESTS

### IDENTIFICATION TESTS

**Differentiation from other antimicrobial substances**

**STABILITY IN ACIDIC CONDITIONS**

Sample stock solution: Suspend 1 g of sample in 1 liter of 0.02 N hydrochloric acid to give a solution containing 1 000 IU/ml.

**Sample Preparation:** Make a dilution of the Sample stock solution with 0.02 N hydrochloric acid to arrive at a concentration of 50 IU/ml. Boil this solution for 5 min and measure the nisin A activity as directed under 'Determination of Nisin A Activity', in METHOD OF ASSAY.

The calculated nisin A concentration of the boiled sample should be 100% (+/- 5%) of the assay value indicating no significant loss of activity following this heat treatment.

**STABILITY IN ALKALINE CONDITIONS**

Adjust the pH of the unused portion of the boiled nisin A solution from 'Stability in acidic conditions' to 11.0 by adding 5 N sodium hydroxide. Heat the solution at 65 °C for 30 min, and then cool.

Adjust the pH to 2.0 by adding hydrochloric acid dropwise. Measure the nisin A activity as directed under 'Determination of Nisin A Activity' in METHOD OF ASSAY. Record loss of the antimicrobial activity of nisin A following this treatment. Total loss of the antimicrobial activity should be observed following the treatment described.

**TOLERANCE OF *LACTOCOCCUS LACTIS* TO HIGH CONCENTRATIONS OF NISIN A**

Prepare cultures of *L. lactis* (ATCC 11454, NCIMB 8586) in sterile skim (<1% fat) milk by incubating for 18 h at 30 °C. Prepare one or more flasks containing 100 ml of litmus milk and sterilize at 121 °C for 15 min. Suspend 0.1 g of sample in the sterilized litmus milk, and allow to stand at room temperature for 2 h. Add 0.1 ml of the *L. lactis* culture, and incubate at 30 °C for 24 h. Record *L. lactis* growth. *L. lactis* will grow at this concentration of sample (about 1 000 IU/ml); however, it will not grow in similar concentrations of other antimicrobial substances. (NOTE: This test will not differentiate nisin A from subtilin.)

## METHOD OF ASSAY

**Transglucosidase activity**

**DETERMINATION OF NISIN A ACTIVITY**

**Principle**

Nisin A activity, expressed in International Units (IU), refers to the amount of nisin A required to inhibit growth of 1 bacterial cell in 1 ml of broth. 1 IU of nisin A is equivalent to 0.025 µg. Commercial nisin A preparations consist typically of 2.5% w/w of nisin A, sodium chloride and other solids from the fermentation medium.

**Solution A (*L. lactis* subsp. *cremoris* (ATCC 14365, NCDO 495)):** Prepare a subculture daily by transferring one loopful of the test culture to a McCartney bottle of litmus milk and incubating at 30 °C for 24 h (subculture A).

Inoculate a suitable quantity of sterile skim milk with 2 percent of subculture A and place it in a water bath at 30 °C for 90 min. Use immediately.

**Solution B (Standard solution of nisin A):** Dissolve 1 g of Nisin A preparation in 1 L of 0.02 N hydrochloric acid and dilute to 1 000 IU/ml. Dilute a portion of the standard stock in 0.02 N hydrochloric acid to a final concentration of 50 IU/ml. Prepare a nisin A solution containing 5,000 IU/ml (125 micrograms/ml). Dissolve an accurately weighed quantity of standard nisin A in 0.02 N hydrochloric acid. Immediately before use, dilute the solution further with 0.02 N hydrochloric acid to give 50 units/ml (dilution factor 100).

**Solution C (Unknown sample):** Weigh an amount of sample sufficient to ensure that corresponding tubes of the sample and standard series match, i.e., within close limits, so that the nisin A content in the sample and standard are similar. Dilute the sample solution in 0.02 N hydrochloric acid to obtain an approximate concentration of 50 IU per ml (IU/ml).

**Solution D (Resazurin):** Prepare a 0.0125% w/v solution of resazurin in water immediately prior to use.

**PROCEDURE**

- Pipet volumes of the 50 IU/ml nùisin standard (Solution B) and unknown sample (Solution C) into two rows of ten 12-ml bacteriological test tubes as depicted in the table below.

**Pipet volumes of nisin standard and unknown sample**

| Dilution number | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| Solution B (ml) | 0.60 | 0.55 | 0.50 | 0.45 | 0.41 | 0.38 | 0.34 | 0.31 | 0.28 | 0.26 |
| Solution C (ml) | 0.60 | 0.55 | 0.50 | 0.45 | 0.41 | 0.38 | 0.34 | 0.31 | 0.28 | 0.26 |

- Add 4.6 ml of the inoculated milk (Solution A) to each by means of an automatic pipetting device. The addition of inoculated milk should be made in turn across each duplicate row of tubes containing the same nominal concentration, and not along each row of ten tubes (e.g., dilution 1 (C and B), dilution 2 (C and B), etc.).
- Place the tubes in a water-bath at 30 °C for 15 min, then cool in an ice-water bath while adding 1 ml resazurin solution to each.
- Add the resazurin solution in the same order as the addition of inoculated milk, using an automatic pipetting device. Thoroughly mix the contents of the tubes by shaking. Continue incubation at 30 °C in a water bath for a further 3 – 5 min.
- Examine the standard and sample tubes under fluorescent light in a black matte-finish cabinet. Compare the sample tube of the highest concentration that shows the first clear difference in colour (i.e., has changed from blue to mauve) with tubes of the standard to find the nearest match in colour. Make further matches at the next two lower concentrations of the sample with the standard. Interpolation of matches may be made at half dilution steps. Obtain three readings of the sample solution and average them. As the standard tubes contain known amounts of nisin A, calculate the concentration of nisin in the sample solution.
- If necessary, convert nisin A activity from IU to micrograms of nisin A, using the conversion factor 1 IU = 0.025 micrograms of nisin A.

**Alternative method**

Quantification of Nisin A using HPLC

- Apparatus and reagents: HPLC equipped with an UV-Vis or Diode Array Detector (DAD); solvents HPLC purity grade.
- **External HPLC calibration curve and retention time of nisin:** Use dilutions of solution B. Filtrate through a syringe filter (0.45µm) and inject into the HPLC. Establish the retention time of nisin and build a calibration curve. Express nisin in mg/kg.
- **Sample injection:** Inject the same aliquot of the unknown samp into the HPLC, measure the area of the corresponding nisin peak. Substitute it in the calibration curve to obtain the corresponding concentration of natamycin in the unknown sample.

Adjust the injection volumes and dilutions of the samples to ensure that the response remains within the linear response range of the detector.

Column: reversed-phase Jupiter, 5µm, C18, 300A,
250 mm × 4.6 mm or equivalent
Flow rate: 1.0 ml/min
Detector: 210-260 nm, quantitative at 214 nm
Solvents: A (H2O (0.1% Trifluoracetic acid); B (Acetonitrile/H2O / Trifluoracetic acid (90/10/0.1 V/V)
Gradient:

| Time (min) | A (%) | B (%) |
|---|---|---|
| 0 | 78 | 22 |
| 30 | 43 | 57 |
| 35 | 0 | 100 |
| 45 | 78 | 22 |

Expected Retention Time of nisin A: 25 minutes (35-37% of acetonitrile)