NISIN

Prepared at the 12th JECFA (1968), published in NMRS 45A (1969) and in FNP 52 (1992). Metals and arsenic specifications revised at the 63rd JECFA (2004). An ADI 0-33,000 units /kg bw was established at the 12th JECFA (1968)

SYNONYMS INS No. 234

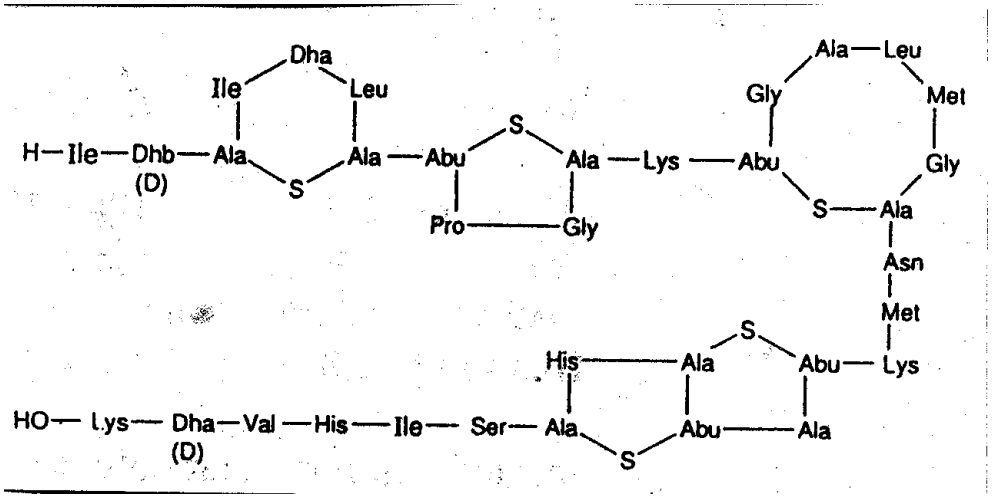
DEFINITION Consists of several closely related polypeptide antibiotics produced by strains of *Streptococcus lactis*, Lancefield group N of which major component is shown below.

Nisin concentrate contains not less than 900 units per mg. In a mixture of non-fat milk solids and a minimum sodium chloride content of 50%. The most potent preparation of nisin yet obtained is 40,000 units/mg. The Unit has been redefined by Tramer and Fowler, *J.Sci.Fd.Agric.*, 15, 522 (1964) in terms of a standard preparation. This approximates to the activity unit described by Berridge (*Biochem.J.* 45, 436, 1949).

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

Chemical formula $C_{143}H_{230}N_{42}O_{37}S_7$

Structural formula Abu=alpha-aminobutylic acid, Dha=dehydroalanine, Dhb=dehydrobutyrine



Formula weight 3354.12

DESCRIPTION Nisin concentrate is a white, micronized, spray-dried powder. Nisin concentrate is stable at ambient temperatures. Both purified nisin and nisin concentrate are stable to heating under acid conditions, and will withstand 121° for 30 min at pH 2.0 and 15 min at pH 3.0. Nisin is less stable at higher pH values; heating at 121° in buffer for 15 min. results in the following percentage decreases in activity: pH 4.0-29%, pH 5.0-69%, pH 6-86%, pH 7.0-99.7%.

FUNCTIONAL USES Antimicrobial preservative

CHARACTERISTICS

IDENTIFICATION

Solubility	Passes test See description under TESTS
Melting point (Vol. 4)	None. Chars on heating
Differentiation from other antibiotics	Passes test See description under TESTS

PURITY

Loss on drying (Vol. 4)	Not more than 3.0% (102-103°, to constant weight)
Lead (Vol. 4)	Not more than 1 mg/kg Determine using an atomic absorption technique appropriate to the specified level. The selection of sample size and method of sample preparation may be based on the principles of the method described in Volume 4, “Instrumental Methods.”

TESTS

IDENTIFICATION TESTS

Solubility	Purified preparation of nisin containing 40,000 units/mg: Solvent / Water, pH 2.5 : 4.8 x 10^6 Units/ml Solvent / Water, pH 5.0 : 1.6 x 10^6 Units/ml Solvent / Methanol, acidified with HCl to pH 2.8 : 3.2 x 10^6 Units/ml Insoluble in non-polar solvents Nisin concentrate: Forms a cloudy suspension in water due to the presence of denatured protein, but the nisin component behaves similarly to the purified material.
Differentiation from other antibiotics	The microbiological assay of nisin in foods is not specific and other antibiotics present in food could interfere. In preparation for nisin assay, samples of food are acidified and boiled in order to bring nisin into the aqueous phase. As a second stage the nisin-containing extracts are made alkaline and heated, causing rapid inactivation of nisin, in order to provide a suitable nisin-free diluent for the nisin standard. The fate of other antibiotics when subjected to heat under acid and alkaline conditions is an important factor when considering the possibility of interference during nisin assay. a. Suspension of processed cheese to which various antibiotics had been added were adjusted to pH 2.0 by the addition of conc. HCl and boiled for 5 min. Antibiotic activity in the acidified suspensions was measured by diffusion assay against M. flavus before and after boiling. Table I gives the loss in activity, expressed as a percentage, attributable to boiling at pH 2.0. Table I "Name of antibiotic" and "% loss of activity" are as follows: Nisin 0 Tylosin 0 Polymyxin B 0 Tetracycline 68

Cloxacillin 32
Gramicidin 61
Bacitracin 50
Ampicillin 72
Phenoxyethylpenicillin 94
Benzylpenicillin 98
Neomycin 87
Novobiocin 93
Streptomycin 96
Erythromycin 99
Chloramphenicol 99
Phenoxybenzylpenicillin 97
Phenylmetylpenicillin 97
Phenoxypropylpenicillin 74

b. Cheese suspensions as in (a) were adjusted to pH 11.0 by the addition of 5 N NaOH and heated at 63^{o} for 30 min. The suspensions were cooled and the pH adjusted back to 2.0 with conc. HCl. Antibiotic activity was measured before and after treatment. Percentage losses are listed in Table II.

Table II
"Name of antibiotic" and "% loss of activity" are as follows:
Nisin 100
Tylosin 100
Polymyxin B 100
Tetracycline 64
Cloxacillin 58
Gramicidin 100
Bacitracin 100
Ampicillin 87
Benzylpenicillin 64
Neomycin 78
Novobiocin 100
Streptomycin 99
Erythromycin 100
Chloramphenicol 99
Phenoxybenzylpenicillin 24
Phenoxymethylpenicillin 93
Phenoxyethylpenicillin 66
Phenoxypropylpenicillin 75

Tylosin, polymyxin, gramicidin, bacitracin, novobiocin and erythromycin behaved in the same way as nisin. From the results obtained in a. and b. above it appears that two antibiotics in particular, namely tylosin and polymyxin B, might be confused with nisin when measuring antibiotic activity in foods.
It is a known that the *Streptococcus lactis* strains which produce nisin will grow in reasonably high concentrations of the antibiotic. The same strains may, however, be sensitive to a wide range of other antibiotics, thus providing a fairly simple means of distinction.

Sensitivity tests were set up in which sterile litmus milk containing aerial dilution of antibiotics was inoculated with 0.1% of an overnight milk culture

of *Streptococcus lactis* NCIB 8586. After 18 h incubation at 30° the sensitivity of the organism to a particular antibiotic was taken as the lowest concentration in which no growth occurred (MIC). The results are given in Table III.

Table III. Sensitivity of Streptococcus lactis to antibiotics
"Name of antibiotic" and "MIC" are as follows:
Nisin > 5000 units/ml
Polymyxin B > 100 µg/ml
Gramicidin > 100 µg/ml
Benzylpenicillin 1.5 µg/ml
Bacitracin 12 µg/ml
Tylosin 1.0 µg/ml
Chloramphenicol 3 µg/ml
Tetracycline 1.5 µg/ml
Streptomycin 0.75 µg/ml
Neomycin 2.5 µg/ml
Novobiocin 1.5 µg/ml
Erythromycin 0.3 µg/ml

METHOD OF ASSAY

Determination of sodium chloride content of nisin concentrate
Weigh ca. 5 g material, transfer with 80% alcohol to 100 ml volumetric flask and add enough 80 percent alcohol to give volume of ca 50 ml. Shake well to suspend all insoluble material. Add 1 ml HNO_3 and with pipet add excess of 0.1N $AgNO_3$ soln. Dilute to 100 ml with alcohol. Transfer mixture to centrifuge bottle and centrifuge 5 min. at ca 1800 rpm. Pipet 50 ml supernatant into 300 ml Erlenmeyer flask, add 2 ml saturated $FeNH_4(SO_4)$ solution and 2 ml HNO_3 and titrate to permanent light brown with 0.1N NH_4CNS. Divide ml 0.1N $AgNO_3$ used by 2 and subtract ml NH_4CNS solution used. Multiply difference by 0.005844 to obtain g NaCl present.

Preparation of test organism
Streptococcus cremoris. 1P5 (NCDO 495) (available from National collection of Dairy Organism, National Institute for Research in Dairying, Shinfield, Berkshire, England.) is subcultured daily in sterile separated milk by transferring (Based on the method of Friedman and Epstein, J. Gen. Microbiol. 5: 830, 1951) one loopful to a McCartney bottle of litmus milk and incubating at 30°. Inoculated milk for the assay is prepared by inoculating a suitable quantity of sterile separated milk with 2 percent of a 24 h culture, and placing it in a water-bath at 30° for 1-½ h. It is then used immediately.

Preparation of standard solution
The Standard Stock Solution is prepared by dissolving an accurately weighed quantity of standard nisin in 0.02N hydrochloric acid to give a solution containing 5 000 units/ml. The stock solution is diluted further with 0.02N hydrochloric acid immediately before use to give 50 units/ml.
Preparation of sample solution
The weight of sample taken for preparing the stock solution of the sample is such that corresponding tubes of the sample and standard series match, i.e. within close limits the sample and standard are of the same concentration with respect to nisin content. The sample stock solution is diluted in 0.02N hydrochloric acid to give an estimated concentration of 50 units of nisin per ml.

Preparation of resazurin solution

A 0.0125% solution of resazurin in distilled water is prepared immediately before use.

Assay procedure

Graded volume (0.60, 0.55, 0.50, 0.45, 0.41, 0.38, 0.34, 0.31, 0.28, 0.26 ml) of the 50 unit per ml sample and standard solutions are pipetted into rows of 10 dry 6-inches x 5/8-inch bacteriological test-tubes, and 4.6 ml of the inoculated milk is added to each by means of an automatic pipetting device. The addition of inoculated milk is made in turn across each row of tubes containing the same nominal concentration not along each row of ten tubes. The tubes are placed in a water-bath at 30^{o} for 15 min, then cooled in an ice water bath while 1 ml resazurin solution is added to each. The addition is made with an automatic pipetting device, in the same order used for the addition of inoculated milk. The contents of the tubes are thoroughly mixed by shaking, and incubation at 30^{o} is continued in a water-bath for a further 3-5 min.

The tubes are examined under 2 x 20 watt Osram "Natural" fluorescent light in a black matt-finish cabinet. The sample tube of the highest concentration which shows the first clear difference in colour (i.e. has changed from blue to mauve) is compared with tubes of the standard row to find the nearest in colour. Further matches are made at the next two lower concentrations of the sample and standard. Interpolation of matches may be made at half dilution steps. As the standard tubes contain known amounts of nisin, the concentration of nisin in the sample solution may be calculated. The three results are averaged.