Published, May 24, 2021
TFDAA0087.00

Method of Test for Nisins in Foods

1. Scope

This method is applicable to the determination of nisin including nisin A and nisin Z in cheese and liquid egg.

2. Method

After extraction, analytes are determined by liquid chromatography/tandem mass spectrometry (LC-MS/MS).

2.1. Equipment

2.1.1. Liquid chromatograph/tandem mass spectrometer.

2.1.1.1. Ion source: electrospray ionization, ESI.

2.1.1.2. Column: Poroshell 120 EC-C18, 2.7 μm, 4.6 mm i.d. × 5 cm, or an equivalent product.

2.1.2. Homogenizer.

2.1.3. Ultrasonicator.

2.1.4. Centrifuge: centrifugal force ≧ 5000 ×g.

2.1.5. Vortex mixer.

2.2. Chemicals

Methanol, HPLC grade;
Acetonitrile, HPLC grade;
Formic acid, reagent grade;
Sodium chloride, reagent grade;
Acetic acid, reagent grade;
Deionized water, resistivity ≧ 18 MΩ • cm (at 25°C);
Nisin A (purity 2.5%) and nisin Z, reference standard.

2.3. Apparatus

2.3.1. Volumetric flask: 10 mL, 50 mL and 100 mL.

2.3.2. Centrifuge tube: 50 mL, PP.

2.3.3. Membrane filter: 0.22 μm, PVDF.

2.4. Reagents preparation

2.4.1. 0.5% formic acid in 20% acetonitrile: mix 5 mL of formic acid and 200 mL of acetonitrile, and dilute with deionized water to 1000 mL.

2.4.2. Extraction solution: dissolve 5.7 g of acetic acid and 58.5 g of sodium chloride in 500 mL deionized water, and dilute with methanol to 1000 mL.

2.5. Mobile phase

2.5.1. Solvent A: dilute 1 mL of formic acid with deionized water to 1000 mL, and

filter with a membrane filter.

2.5.2. Solvent B: acetonitrile.

2.6. Standard solution preparation

Accurately weigh about 100 mg of nisin Z reference standard into a 100 mL volumetric flask, dissolve and dilute with 0.5% formic acid in 20% acetonitrile to volume as the standard stock solution. And accurately weight equivalent 10 mg of nisin A reference standard into a 10 mL volumetric flask, dissolve and dilute with 0.5% formic acid in 20% acetonitrile to volume as the standard stock solution. Store in the dark in the freezer. When to use, mix appropriate volume of the stock solutions and dilute with 0.5% formic acid in 20% acetonitrile to 1000 ng/mL as the standard solution.

2.7. Sample solution preparation

Take about 2 g of the homogenized sample accurately weighed, add 25 mL of extraction solution. Homogenize for 1 min, and transfer into a 50 mL volumetric flask. Wash the blender container with 15 mL of extraction solution. Combine the extraction solution into the volumetric flask, and dilute with extraction solution to volume. Transfer the extraction solution into a centrifuge tube, centrifuge at 5000 ×g for 10 min, filter the supernatant with a membrane filter. Take 200 µL (a) of filtrate, dilute with 0.5% formic acid in 20% acetonitrile to 1000 µL (b) and mix well as the sample solution.

2.8. Matrix-matched calibration curve preparation

Take a blank sample and follow the procedure described in section 2.7 to obtain the eluent. Take 200 µL of the eluent, add 40 - 200 µL of the standard solution, and dilute with 0.5% formic acid in 20% acetonitrile to 1000 µL. Mix well as the matrix-matched standard solutions. Operate LC-MS/MS according to the following conditions. Establish the matrix-matched calibration curve of each nisin by the peak area of each nisin vs. the added concentrations (40 - 200 ng/mL).

LC-MS/MS operating conditions(note)

Column: Poroshell 120 EC-C18, 2.7 µm, 4.6 mm i.d. × 5 cm.

Column temperature: 25°C.

Mobile phase: a gradient program of solvent A and solvent B is as follows:

Time (min)	A (%)	B (%)
0.0 → 0.5	98 → 98	2 → 2

0.5 → 6.0	98 → 2	2 → 98
6.0 → 10.0	2 → 2	98 → 98
10.0 → 12.0	2 → 98	98 → 2
12.0 → 14.0	98 → 98	2 → 2

Flow rate: 0.40 mL/min.

Injection volume: 10 μL.

Ion spray voltage: 5.5 kV.

Ionization mode: ESI^+.

Ion source temperature: 550°C.

Nebulizer gas, GS1: 50 psi.

Heated gas, GS2: 60 psi.

Curtain gas: 25 psi.

Collision gas: 8 psi.

Detection mode: multiple reaction monitoring (MRM). Detection ion pair, declustering potential and collision energy are as follows:

Analyte	Ion pair*	Declustering potential (V)	Collision energy (eV)
	Precursor ion (*m/z*) > product ion (*m/z*)		
Nisin A	671.6 > 810.9**	50	25
	671.6 > 744.5		21
Nisin Z	667.1 > 804.9**	60	22
	667.1 > 801.0		23

* The charge numbers of the precursor ion and the product ion are different.

** The quantitative ion pair.

Note: All the parameters can be adjusted depending on the instruments used if the above conditions are not applicable.

2.9. Identification and quantification

Accurately inject 10 μL of the sample solution and the matrix-matched standard solutions into LC-MS/MS separately, and operate according to the conditions in section 2.8. Identify each nisin based on the retention time and the relative ion intensities(note). Calculate the amount of each nisin (g/kg) in the

sample by the following formula:

$$\text{The amount of nisin in the sample (g/kg)} = \frac{\sum C \times V \times F}{M} \times 10^{-6}$$

Where,

C: the concentration of each nisin in the sample solution calculated by the matrix-matched calibration curve (ng/mL)

V: the final volume of the sample (50 mL)

M: the weight of the sample (g)

F: the dilution factor, b/a

Note: Relative ion intensities are calculated by peak areas of qualitative ions divided by peak areas of quantitative ions ($\leqq$ 100%). Maximum permitted tolerances for relative ion intensities by LC-MS/MS are as follows:

Relative ion intensity (%)	Tolerance (%)
> 50	± 20
> 20-50	± 25
> 10-20	± 30
$\leqq$ 10	± 50

Remark

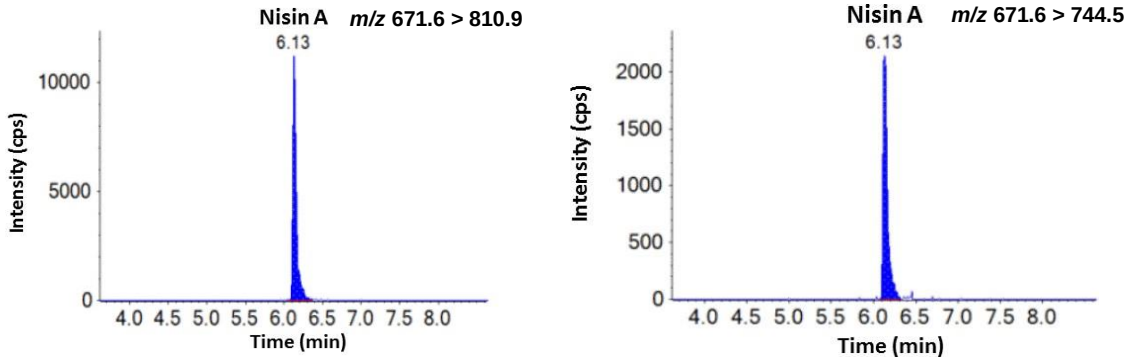
1. Limits of quantitation (LOQs) for nisin A and nisin Z are both 0.005 g/kg.
2. Further validation should be performed when interference compounds appear in samples.

References

1. Ko, K. Y., Park, S. R., Lee, C. A. and Kim, M. 2015. Analysis method for determination of nisin A and nisin Z in cow milk by using liquid chromatography-tandem mass spectrometry. J. Dairy Sci. 98: 1435- 1442.
2. Schneider, N., Werkmeister, K. and Pischetsrieder, M. 2011. Analysis of nisin A, nisin Z and their degradation products by LCMS/MS. Food Chem. 127: 847-854.
3. Lu, S. F., Chang, H. S., Yeh, L. Y. and Fu, W. G. 2020. Expanded evaluation of test methods in food additives and adulteration. Commissioned Research Report of Taiwan Food and Drug Administration.

Reference chromatogram

(a)



(b)

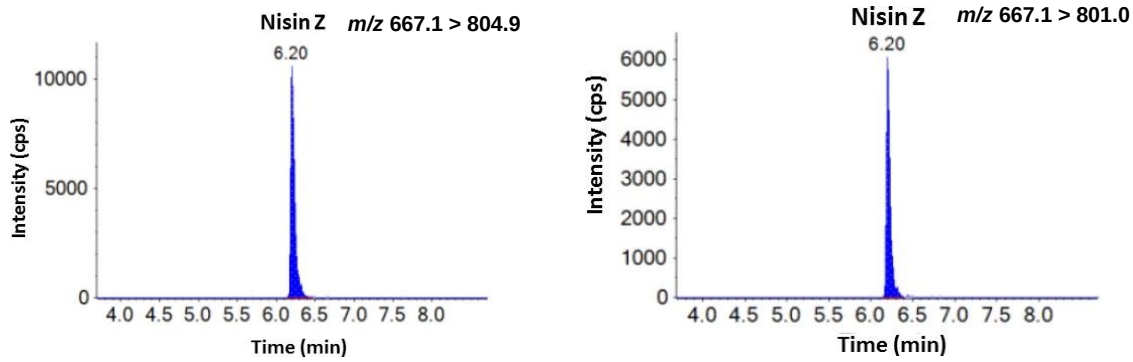


Figure. MRM chromatograms of nisin A (a) and nisin Z (b) standards analyzed by LC-MS/MS.