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**Short Communication**

# HPLC-pharmaceutical Analysis of Lantibiotic Nisin in the Industrial Samples Including Expired Sample

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*Nisin is the most well-known representative of subtype A1 lantibiotics. This small (<5 kDa) peptide ribosomally produced by Gram-positive bacteria belonging to Lactococcus, Streptococcus, Staphylococcus and Blautia species. Nisin exhibits antibacterial activity against a broad range of Gram-positive bacteria and may be effective against Gram-negative pathogens. For analytical semi-preparative purposes, gradient HPLC is often used in the acetonitrile concentration range from 20 to 30% (solvent B) and a retention time of 20 to 50 min. In this study the optimal conditions for the analysis of nisin by RP-HPLC were determined: a gradient from 23 to 28% acetonitrile (buffer B) when used as solvents: buffer A: [4M $LiClO_4$ – 0.1M $HClO_4$] : $H_2O$=1:19; and buffer B: 100% $CH_3CN$ with a retention time up to 12 min. Differences between the chromatographic profiles of expired and non-expired nisin samples have been identified. The expired nisin sample differs from the non-expired samples by the presence of asymmetric nisin A/Z peaks with significant degradation of the nisin A peak. The results of the study indicate the possibility using RP-HPLC for checking the quality and shelf life of commercial nisin samples without the need for additional purification.*

**Keywords:** reverse-phase high performance liquid chromatography (RP-HPLC), bacteriocins, nisin, expired and non-expired nisin samples

Nisin is the most well-known representative of subtype A1 lantibiotics. This small peptide is ribosomally produced by Gram-positive bacteria belonging to *Lactococcus, Streptococcus, Staphylococcus,* and *Blautia* species [1-4]. Nisin exhibits antibacterial activity against a broad range of Gram-positive bacteria, such as *Staphylococci*, *Streptococci*, *Listeria*, *Bacilli*, *Enterococci,* etc. As a rule, it is inactive against Gram-negative species. However, some studies indicate that purified nisin and certain bioengineered variants of nisin may be effective against Gram-negative pathogens [1, 5]. Nisin is granted generally regarded as safe (GRAS) status by the Food and Drug Administration (FDA). Over the past few decades, nisin is widely used as a natural biopreservative in the food industry due to its ability to effectively inhibit numerous food-borne pathogens and spoilage bacteria. However, recently the use of nisin has advanced beyond its role as a biopreservative for food products. The application of nisin has been extended to biomedical fields. Unique bacteriotropic and immunotropic properties of natural and genetically modified variants of nisin open wide prospects for their clinical use. Studies have reported that nisin can prevent the growth of multidrug-resistant bacterial strains, such as *Streptococcus pneumoniae*, *Clostridium difficile,* methicillin-resistant *Staphylococcus aureus* (MRSA), and vancomycin-resistant *Enterococci* [1, 3, 5, 6]. Nisin can work synergistically in combination with conventional therapeutic drugs. It is also considered promising in the fight against bacterial biofilms due to its increased diffusion properties [5, 7]. In addition, like host-defense peptides, nisin may activate the adaptive immune response and have an immunomodulatory role. Increasing evidence indicates that nisin can influence the growth of tumors and exhibit selective cytotoxicity toward cancer cells [1]. Nisin has demonstrated anticancer properties in various types of cancers such as head and neck squamous cell carcinoma, colorectal cancer, breast adenocarcinoma, astrocytoma, neuroblastoma, hepatocellular cancer carcinoma, and melanoma [8].

Peptide analysis using reverse-phase HPLC (RP-HPLC) is widely used in the analysis of oligo-peptide drugs and in the development of new genetically engineered peptide drugs, such as insulins [13]. For analytical semi-preparative purposes, gradient HPLC is often used in the concentration range of acetonitrile (solvent B) from 20 to 30% and a retention time of 20 to 50 min. Nisin peptides are well separated in the gradient region of 23-28% solvent B [14].

The aim of the present study was to determine the optimal conditions for the analysis of nisin by RP-HPLC to check the quality of commercial nisin samples during long-term storage without the need for additional purification.

## Materials and methods

*Chemicals and reagents*

The commercial nisin samples were purchased from four different producers: N1 - Xi'an International

Healthcare Factory Co., Ltd. Shaanxi, China (date/series: 04.2016); N2 - Hangzhou Yeastar Biotech Co., Ltd. Zhejiang, China (date/series: 12.2021); N3 - Henan Senchi Biological Technology Co., Ltd. Henan, China (date/series: 11.2021); N4 - Aurora Industry Co., Ltd. Liaoning, China (date/series: 03.2021). Purity of nisins was 1.5 - 2.5%.

*Instrumentation*

The HPLC equipment consisted of Milichrom A-02 HPLC system (Envirochrom). The LC column was ProntoSIL 120-5 C18 AQ (Bischoff Anal. GmbH, Germany) and obtained from Analitica LLC (Kharkiv, Ukraine). A gradient mobile phase was employed: buffer A: [4M $LiClO_4$ – 0.1M $HClO_4$]: $H_2O$=1:19; and buffer B: 100 % $CH_3CN$ were used. The gradient started from 23 to 28 % of buffer B in 25 min. The flow rate was held constant at 0.1 mL $min^{-1}$. A typical gradient used in analytical separations of peptides was from 23 to 28 % buffer B in buffer A, linear 15- 50 min. In all experiments, the columns were thermostated at 40 °C. Other conditions for separation also were used (see description under Figures). Multiwave UV detector was used for detection of fractions at 230 nm wavelength.

*Sample preparation*

10 mg of nisin sample and 10 mg of ascorbic acid were diluted with 700 µL water and 300 µL acetonitrile and centrifuged for 3 min. The solvent was removed from the isolated nisin fractions by rotary evaporation. The solid sample was dissolved in 200 µL of the mobile buffer A, and 50 µL was injected into the LC injector.

## Results and discussion

In an earlier article was shown that commercial nisin preparation contained several minor components [2]. Six fractions were isolated by preparative RP-HPLC and their chemical structure was determined by two-dimensional NMR spectroscopy and electrospray mass spectrometry as described below. The different components were identified as [2-hydroxy-AlaS] nisin (fraction I), (Tle4-amide, pyruvyl- Leu6Ides-Dha5-nisin (fraction 2), [Met(0)21 I nisin (fraction 3), and [Ser33] nisin (fraction 4). Fraction 5 was native nisin A and fraction 6 appeared to be nisin-(I-32)-peptide amide, which has already been described by Chan et al. (1989). An additional component, obtained by freeze drying a solution of nisin A from 0.1 M HCI, was identified as [2-hydroxy-AlaS] nisin-(I - 32)- peptide amide.

In commercial samples, a mixture of nisin A and nisin Z is mainly represented in concentrations of 2.5-5.0 %. The remaining products are denatured protein and peptides, but they are slightly soluble and precipitate during centrifugation if the sample preparation/dissolution of nisin is carried out in a 0.5 % acidic medium. In fact, the dissolution of nisin in 0.5 % acetic acid leads to the final denaturation of the accompanying proteins and the possibility of their separation by centrifugation. In this case, the nisins remain in solution. The same technology can be used to obtain dosage forms of nisin from industrial nisin. In our case, a solution of 1% ascorbic acid was used as a preservative and solution stabilizer. The same denaturation of accompanying proteins was observed, but it will no longer be necessary to purify the solution before introducing into the dosage form, due to the fact that ascorbic acid is a stabilizer/reducer against oxidation and a preservative against microorganisms/ fungi at concentrations above 0.5 %.

In all cases, nisin was a gray powder, which gave opalescence when dissolved in ascorbic acid solution. After centrifugation at 8000 rpm (9 g) for 15 min, an amorphous precipitate was observed in the Eppendorf tubes. 5 µl of the supernatant from each sample was taken for further PR-HPLC analysis. At the initial stage of the research, most fresh N2 sample was analyzed under the conditions of a standard chromatograph database. As eluents, buffer A: [4M $LiClO_4$ – 0.1M $HClO_4$]: $H_2O$=1:19; and buffer B: 100 % $CH_3CN$ were used. A typical gradient used in analytical separations was from 0 to 100 % buffer B in buffer A, up to 45 min. In all experiments, the columns were thermostated at 40 °C.

Figures 1-4 show the chromatograms of samples N1, N2, N3, N4 in conditions selected for the oligo-peptide fractions.

As follows from the data in Figure 2, the peaks on the plateau 1 and 2 are more pronounced than on the chromatogram of the expired sample N1. The remaining minor peaks of accompanying impurities do not differ.

Analysis of the chromatogram in Figure 3 proves that the plateau and peaks 1 and 2 are more pronounced than in the overdue sample N1 (Figure 1).

The plateau and peaks 1 and 2 (presented in Figure 4) are identical to those in samples N2 and N3 from other producers, but differ from the peaks in chromatogram profile of expired sample N1 (Figure 1). Thus, the asymmetry of the two absorption peaks of nisins A and Z can be used to check the quality and shelf life of commercial nisin samples without the need for additional purification. In other articles, a chromatogram of pure nisin and its retention time is given, but the separation conditions are far from optimal and do not correspond to the gradient region for peptide fractions.

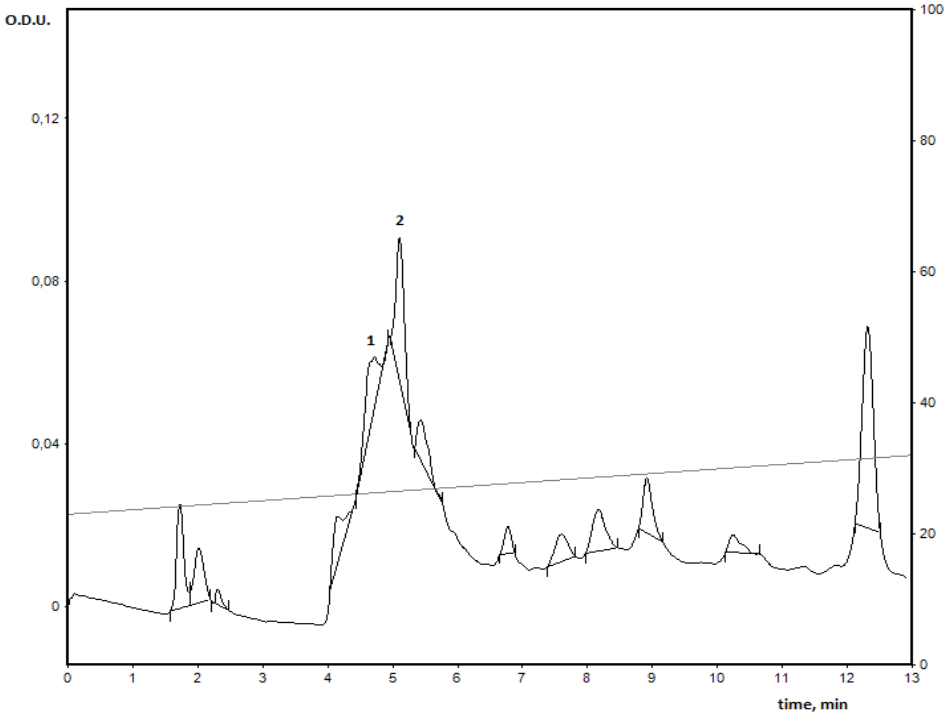


**Fig. 1.** HPLC chromatogram profile of sample N1. Condition for analysis: column ProntoSIL 120-5 C18 AQ (Bischoff Anal. GmbH, Germany); buffer A: [4M $LiClO_4$ – 0.1M $HClO_4$]:$H_2O$=1:19; and buffer B: 100 % $CH_3CN$ were used; gradient buffer B from 23 to 28 % B, linear up to 12 min; column was thermostated at 40 °C. UV detector was used for detection of fractions at 230 nm wavelength.

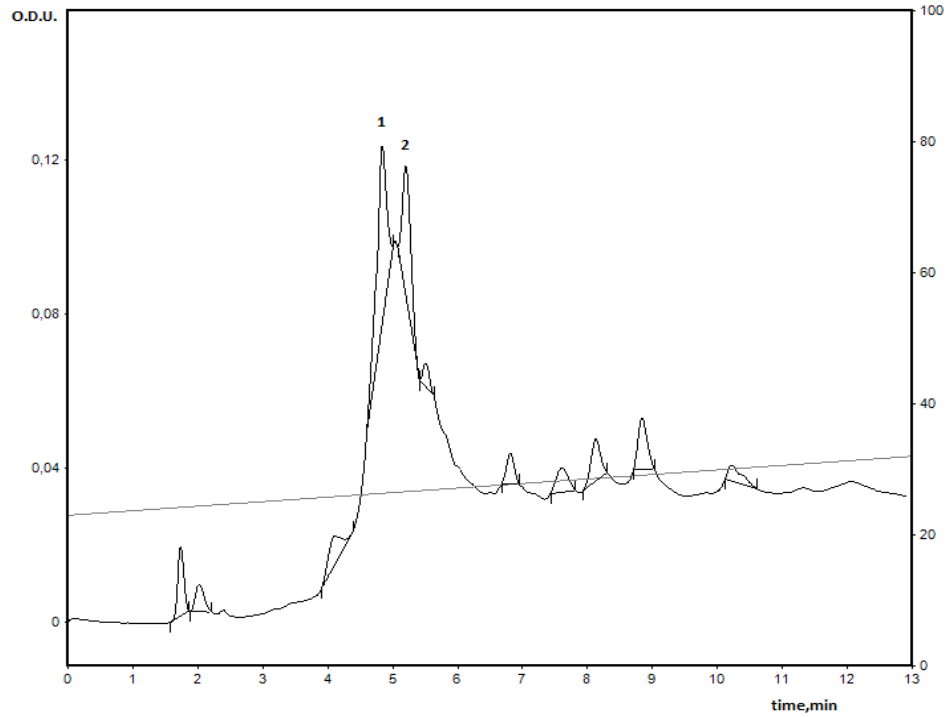


**Fig. 2.** HPLC chromatogram profile of sample N2. Condition for analysis: column ProntoSIL 120-5 C18 AQ (Bischoff Anal. GmbH, Germany); buffer A: [4M $LiClO_4$ – 0.1M $HClO_4$]:$H_2O$=1:19; and buffer B: 100 % $CH_3CN$ were used; gradient buffer B from 23 to 28 % B, linear up to 12 min; column was thermostated at 40 °C. UV detector was used for detection of fractions at 230 nm wavelength.

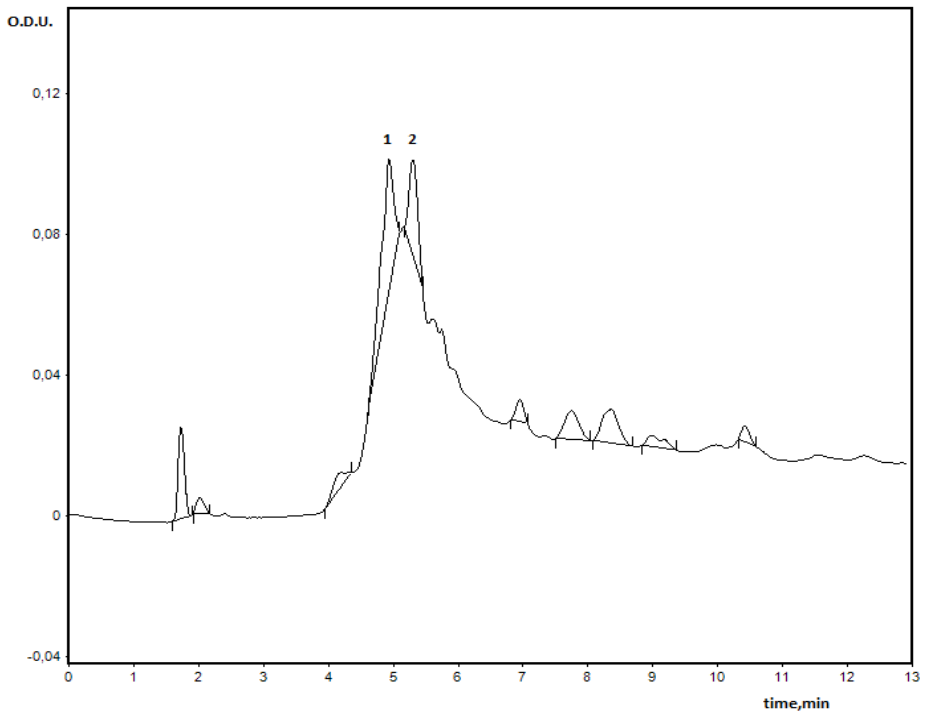


**Fig. 3.** HPLC chromatogram profile of sample N3. Condition for analysis: column ProntoSIL 120-5 C18 AQ (Bischoff Anal. GmbH, Germany); eluents: buffer A: [4M $LiClO_4$ – 0.1M $HClO_4$]:$H_2O$=1:19; and buffer B: 100 % $CH_3CN$ were used; gradient buffer B from 23 to 28 % B, linear up to 12 min; column was thermostated at 40 °C. UV detector was used for detection of fractions at 230 nm wavelength.

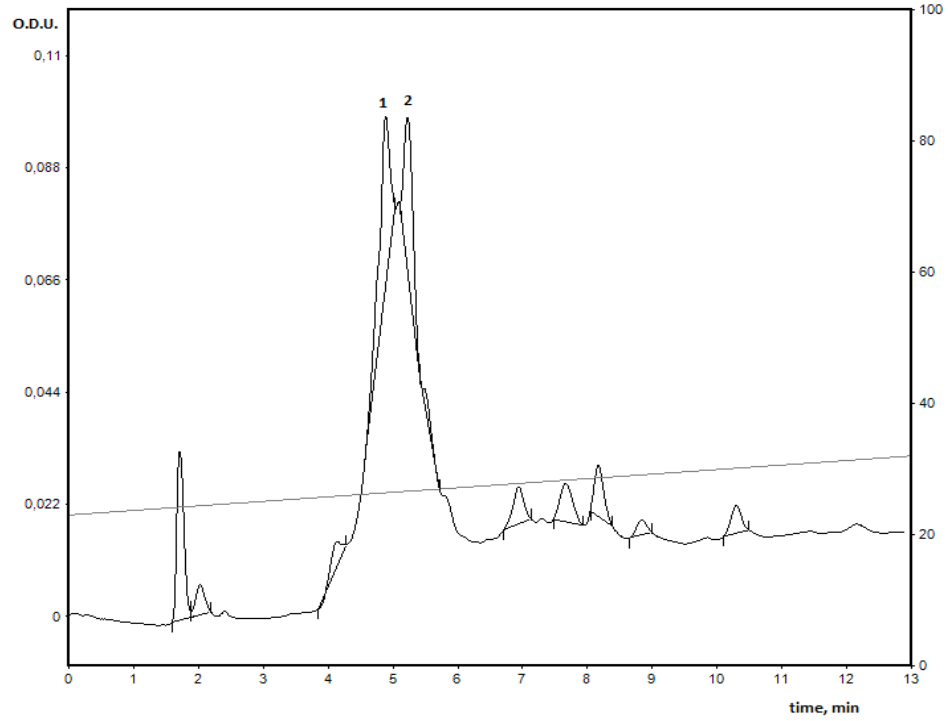


**Fig. 4.** HPLC chromatogram profile of sample N4. Condition for analysis: column ProntoSIL 120-5 C18 AQ (Bischoff Anal. GmbH, Germany); eluents: buffer A: [4M $LiClO_4$ – 0.1M $HClO_4$]:$H_2O$=1:19; and buffer B: 100 % $CH_3CN$ were used; gradient buffer B from 23 to 28 % B, linear up to 12 min; column was thermostated at 40 °C. UV detector was used for detection of fractions at 230 nm wavelength.

## Conclusions

1. The optimal conditions for RP-HPLC analysis of nisin is a gradient from 23 to 28% acetonitrile (buffer B) when used as solvents: buffer A: [4M $LiClO_4$ – 0.1M $HClO_4$]: $H_2O$=1:19; and buffer B: 100 % $CH_3CN$ with a retention time of 12 min.

2. The expired nisin sample N1 differs from the non-expired samples N 2-4 by the presence of asymmetric nisin A/Z peaks with significant degradation of the nisin A peak.

## Conflict of Interest

Is absent

## Statement of Human and Animal Right

Not applicable

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