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Cheese — Determination of nisin A content by LC-MS and LC-MS-MS

Fromage — Détermination de la teneur en nisine A par CL-SM et CL-SM-SM

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Foreword

IDF (the International Dairy Federation) is a non-profit organization representing the dairy sector worldwide. IDF membership comprises National Committees in every member country as well as regional dairy associations having signed a formal agreement on cooperation with IDF. All members of IDF have the right to be represented on the IDF Standing Committees carrying out the technical work. IDF collaborates with ISO in the development of standard methods of analysis and sampling for milk and milk products.

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All work was carried out by the Joint ISO-IDF Action Team on *Food additives and vitamins* of the Standing Committee on *Analytical methods for additives and contaminants* under the aegis of its project leader, Mr. T. Berger (CH).

Cheese — Determination of nisin A content by LC-MS and LC-MS-MS

1 Scope

This Technical Specification specifies a method for the quantitative determination of the nisin A content in cheese.

The method is suitable for measuring low levels of nisin A with a quantification limit of 1 mg/kg.

NOTE Nisin is a peptide produced by some bacteria (e.g. *Lactococcus lactis* subsp. *Lactis)* inhibiting or destroying other microorganisms. It is widely used as a natural preservative for foods, e.g. vegetables, cheese, meat, and cacao. In cheese making, nisin is used to prevent late blowing. Its use is restricted to maximum levels in the final product. Nisin appears in two forms, nisin A and nisin Z, which differ in one amino acid. This method is applicable to the determination of nisin A only.

2 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

2.1
nisin A content

mass fraction of substance determined by the procedure specified in this Technical Specification

NOTE The nisin A content is expressed in milligrams per kilogram.

3 Principle

The sample is grated and extracted with dilute formic acid at 80 °C. After ultracentrifugation, interfering proteins are separated by means of filtration through an ultrafiltration (UF) membrane. In the purified extract, nisin A is separated using a polymeric stationary phase and detected using mass spectrometry (with MS-MS as an option).

4 Reagents and reference substances

Use only reagents of recognized analytical grade and distilled water or water of at least equivalent purity, unless otherwise specified.

4.1 Bovine serum albumin (BSA) stock solution. Dissolve 10 mg of BSA (purity > 96 % mass fraction), in 10 ml water.

4.2 Bovine serum albumin (BSA) buffer solution. Mix 80 ml water with 20 ml of acetonitrile (4.6), 0,5 ml of formic acid (4.3), 0,01 ml of trifluoracetic acid (4.5) and 1 ml of BSA stock solution (4.1).

4.3 Formic acid (HCOOH).

4.4 Formic acid solution, ρ_{HCOOH} = 5 g/l. Pipette 0,41 ml of formic acid (4.3) into a 100 ml one-mark volumetric flask (5.12). Make up to the mark with water and mix.

4.5 **Trifluoracetic acid** (CF_3COOH).

4.6 **Acetonitrile** (CH_3CN), "pure".

4.7 **Methanol** (CH_3OH).

4.8 **Nisin A**, of purity > 95 % mass fraction[1)].

5 Apparatus

Usual laboratory equipment and, in particular, the following.

5.1 **Laboratory centrifuge**, capable of producing a radial acceleration of at least 3 000*g*.

5.2 **Ultracentrifuge**, capable of producing a radial acceleration of 20 800*g*.

5.3 **Ultrafiltration membrane**, of pore size 30 kD[2)].

5.4 **Membrane filter**, of pore size 0,22 µm[3)].

5.5 **Balance**, capable of weighing to the nearest 10 mg, with a readability of 1 mg.

5.6 **Analytical balance**, capable of weighing to the nearest 0,1 mg, with a readability of 0,01 mg.

5.7 **Water bath**, capable of shaking and maintaining a temperature of 80 °C ± 2 °C.

5.8 **Ultrasonic bath**, capable of shaking and maintaining a temperature of 80 °C ± 2 °C.

5.9 **Cheese grater**, with openings of size approximately 2 mm.

5.10 **LC-MS equipment**.

5.10.1 **Elution gradient pumping system**, capable of operating at 0,25 ml/min.

5.10.2 **Manual** or **automatic injector**, capable of injecting volumes of 5 µl.

5.10.3 **Column heater**, capable of maintaining a column temperature of 40 °C ± 2 °C.

5.10.4 **Column, reversed phase**, PLRP-S, 300 Å[4)], 3 µm, 150 mm × 2 mm.

5.10.5 **Mass spectrometer detector**, capable of operating in ion mode ESI+ at *m*/*z* 839,6.

5.11 **LC-MS-MS equipment** (optional).

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1) Ambicin N is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO or IDF of this product.

2) Millipore Centricon YM-30 is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO or IDF of this product.

3) Millipore Millex-GV PVDF 0,22 µm is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO or IDF of this product.

4) PLRP-S, 300 Å is the trade name of a product supplied by Polymer Lab Ltd. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO or IDF of the product named. Equivalent products may be used if they can be shown to lead to the same results.

5.11.1 Elution gradient pumping system, capable of delivering a flow rate of 0,2 ml/min.

5.11.2 Manual or **automatic injector**, capable of injecting volumes of 10 µl.

5.11.3 Column heater, capable of maintaining a column temperature of 30 °C ± 2 °C.

5.11.4 Column for reversed phase chromatography, PLRP-S, 300 Å[4)], 3 µm, 150 × 2 mm.

5.11.5 Mass spectrometer detector, capable of operating in ion mode ESI+ MS-MS at m/z 672/672, 672/811, 672/649, 840/840.

5.12 One-mark volumetric flasks, of capacity 100 ml, ISO 1042[2] class A.

6 Sampling

A representative sample should have been sent to the laboratory. It should not have been damaged or changed during transport or storage.

Sampling is not part of the method specified in this Technical Specification. A recommended sampling method is given in ISO 707|IDF 50[1].

7 Procedure

7.1 Preparation of the nisin A standard solution

7.1.1 Nisin A standard stock solution, ρ_{nA} = 100 mg/l.

Weigh, to the nearest 0,01 mg, 10,00 mg nisin A (4.8) into a 100 ml one-mark volumetric flask (5.12). Make up to the mark with formic acid solution (4.4) and mix.

Prepare the nisin A standard stock solution daily.

7.1.2 Nisin A standard working solution, ρ_{nA} = 300 µg/l.

Pipette 300 µl of nisin A standard stock solution (7.1.1) into a 100 ml one-mark volumetric flask (5.12). Make up to the mark with BSA buffer solution (4.2) and mix. The standard working solution thus obtained contains 300 µg of nisin A per litre.

Prepare the nisin A standard working solution daily.

7.2 Extraction of the test portion

Before weighing, grate the test sample with the cheese grater (5.9).

Weigh, to the nearest 0,01 g, 2,50 g of test sample into a 100 ml one-mark volumetric flask (5.12). Add 70 ml of water and 0,5 ml of formic acid (4.3) and mix.

Put the flask either in the water bath (5.7) at 80 °C and shake it for 30 min or in the ultrasonic bath (5.8) at 80 °C and shake it for 10 min. After cooling to room temperature, make up to the mark with water and mix.

NOTE Soft cheese can be grated after freezing.

7.3 Filtration of the test portion with UF membrane

Pipette approximately 1,5 ml of the extract into a 1,5 ml tube (e.g. Eppendorf) and centrifuge using the ultracentrifuge (5.2) at 20 800*g* for 10 min.

Determine the tare of the collecting container for the ultrafiltration membrane (5.3) on the analytical balance (5.6). Place the collecting container on the ultrafiltration membrane and set the analytical balance to zero.

Filter 0,6 ml to 0,7 ml of the centrifuged extract through a membrane filter (5.4) into the tared ultra filtration membrane.

Weigh the amount of the extract on the ultrafiltration membrane using the analytical balance. Centrifuge the ultrafiltration membrane in the laboratory centrifuge (5.1) at 3 000*g* for 45 min. Determine the gross mass of the filter container.

Supplement the net mass of filtrate with water to the original amount weighed with a correction for the peptide distribution in the ultrafiltration membrane.

NOTE Generally, the amount of water added is approximately 15 % of the extract used for centrifugation. Consult the information provided by the membrane manufacturer.

Transfer the filtrate thus obtained into an HPLC vial and measure.

7.4 LC-MS and LC-MS-MS determination

7.4.1 Elution solvents for LC-MS

Use the following elution solvents:

Eluant A: pipette 2,5 ml of formic acid (4.3) and 0,05 ml of trifluoracetic acid (4.5) into 500 ml water.

Eluant B: mix 350 ml of acetonitrile (4.6) with 150 ml water, 2,5 ml of formic acid (4.3) and 0,05 ml of trifluoracetic acid (4.5).

7.4.2 Elution solvents for LC-MS-MS

Optionally, the following elution solvents can be used if LC-MS-MS is chosen:

Eluant C: mix 50 ml acetonitrile (4.6) with 450 ml water and 2,5 ml formic acid (4.3).

Eluant D: mix 400 ml acetonitrile (4.6) with 100 ml water and 2,5 ml formic acid (4.3).

7.4.3 LC-MS and LC-MS-MS conditions

Table 1 — Preferred conditions

Conditions	LC-MS			LC-MS-MS		
Injection volume, µl	5			10		
Column	PLRP-S[4] 150 mm × 2 mm, 300 Å, 3 µm					
Column temperature, °C	40			30		
Mass detector	Ion mode ESI+ Sample 500 °C Needle 3,5 kV			Ion mode ESI+ Nitrogen 8 l/min 350 °C		
Detection	*m/z* 839,6[a] Span 0,5 Dwell time 1,0 min Cone 60 V			*m/z* Q1/*m/z* Q3 672/672[b] 672/811[c] (most selective transition) 672/649 840/840[b]		
Flow rate, ml/min	0,25			0,20		
Nisin A retention time, min	~13			~5,7		
Gradient[d]	Min.	%A	%B	Min.	%C	%D
	0	70	30	0	85	15
	13	50	50	1	85	15
	13,1	0	100	4	65	35
	17	0	100	6	65	35
	17,1	70	30	9	10	90
	23	70	30	12	10	90
				12,1	85	15
				16	85	15
On the whole, the MS or MS-MS parameters are critical and shall be carefully controlled to achieve sensitivity and repeatability because of different analytical factors. Optimize the instrument to produce the highest signal for nisin.						

[a] Higher sensitivities for ions *m/z* 1 118,9+3 ($[M+3H]^{3+}$) or *m/z* 1 677,7+2 ($[M+2H]^{2+}$) were observed in other laboratories and could be used alternatively.

[b] Identical *m/z* signal, equipment for MS-MS has to be optimized.

[c] Due to a lower ion charge, a higher *m/z* signal occurs in Q3.

[d] The elution gradient might require slight modification in order to achieve the resolution shown in Figure 1.

7.4.4 LC-MS calibration

An example of a chromatogram of the nisin A standard solution is given in Figure 1. The chromatogram was obtained by measuring the nisin A standard working solution (7.1.2) three times. The example shows that calibration is linear for the whole measuring range (see Figure 2). The equipment linearity, however, should be checked regularly.