**Determination of natamycin (food additive in cheese production) by liquid chromatography-electrospray tandem mass spectrometry**

T Radicevic¹, S Jankovic¹, S Stefanovic¹, D Nikolic¹, J Djinovic-Stojanovic¹, D Spiric¹ and S Tankovic²

¹ Institute of Meat Hygiene and Technology, Kacanskog 13, 11000 Belgrade, Serbia  
² Veterinary Office of Bosnia and Herzegovina, Marsala Tita 9a, 71000 Sarajevo, Bosnia and Herzegovina

E-mail: tatjana.radicevic@inmes.rs

**Abstract.** Natamycin is a polyene macrolide antifungal agent produced by aerobic fermentation of *Streptomyces natalensis* that prevents fungal growth on cheese surface. Commission Regulation EU 1129/2011 establishes a Union list of food additives and the use of natamycin (E235) in production of hard, semi-hard and semi-soft cheese, and lays down maximum residue limit (MRL) of 1 mg/dm² surface. It also stipulates that natamycin is not to be present at a depth of 5 mm and deeper. The aim of this study was to present the analytical method for determination of natamycin in cheese by reverse phase liquid chromatography-electrospray tandem mass spectrometry. Method validation was performed according to Commission Decision 2002/657/EC. The method is linear in the concentration ranges of 0-5 mg/dm², with the limit of detection (LoD) of 0.13 mg/dm². The performance of the method was successfully verified by participating in a proficiency study.

1. **Introduction**

Natamycin (pimaricin), whose formula is shown in Figure 1., is a fungicide of the polyene macrolide group. It shows activity against yeasts and filamentous fungi such as *Candida spp.*, *Aspergillus spp.*, *Cephalosporium spp.*, *Fusarium spp.*, but is not effective against gram-positive and gram-negative bacteria.

In the food industry natamycin is used as an additive (E235) for the preservation of cheese and fermented meat against yeasts and moulds. The mechanism of action of natamycin against moulds is based on inhibition of amino acids and glucose transport through fungal cell membranes due to its specific binding to sterols, principally ergosterol in fungal cell membranes [1]. As it has no effect on bacteria, the starter cultures in fermented food remain active. Natamycin is preferable to other preservatives because it is odorless and colorless and has no adverse effect on the taste of food. The use of natamycin as food additive for surface treatment of hard, semi-hard and semi-soft cheese is regulated by Commission Regulation EU 1129/2011 [2]. The maximum residue level (MRL) is set to 1 mg/dm² surface and its presence at a depth of 5 mm and deeper is prohibited. Customs Union Technical Regulation on Safety of Milk and Dairy products - TR TS 033/2013 sets the same MRL [3]. In 2009, EFSA - the European Food Safety Authority, EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS), issued a Scientific opinion on the use of natamycin (E235) as a food additive.
additive [4]. The opinion of the Panel is that proposed use levels of natamycin are not of safety concern if it is only used for the surface treatment of semi-hard and semi-soft cheese and on the casings of certain sausages [4]. The Panel also concluded that there was no concern for the induction of antimicrobial resistance. In 2012, the German Federal Institute for Risk Assessment (BfR) issued a statement that they follow the conclusion of EFSA. Surveys in cheese warehouses and in dry sausage factories where natamycin had been used for up to nine years showed no change in the composition or sensitivity of the contaminating fungal flora [5,6].

However, existence of MRL in relevant legislative, calls upon development and validation of reliable analytical methodology for determination of natamycin in foods for the purposes of regulatory controls.

Therefore, the aim of this work was the development of sensitive, simple and rapid method for the determination of natamycin in cheese by reverse phase liquid chromatography tandem mass spectrometry.

2. Materials and Methods

Natamycin (CAS No. 7681-93-8) analytical standard was purchased from Sigma-Aldrich (St. Louis, USA). Water, methanol, acetonitrile were all HPLC grade and purchased from Sigma-Aldrich. Formic acid LC grade was from Merck (Merck KGaA, Darmstadt, Germany). Stock solution of natamycin, c = 1.00 mg/mL was prepared in methanol and stored at 4 ºC.

Natamycin was analysed using Shimadzu UHPLC instrument consisting of LC-30AD pumps, CTO-30A column oven, DGU-20A degasser, SIL-30AC autosampler and CBM-20A system controller coupled to triple quadrupole mass spectrometer LCMS-8040 via an ESI interface (Shimadzu, Europa, Duisburg, Germany). The instrument was controlled by LabSolution software. The analytical column used for separation was Kinetex 50 x 2.1 mm 2.6µ C-18 100Å with UltraGuard cartridge (Phenomenex, Torrens, CA, USA). The oven temperature was set to 40ºC. The chromatographic separation was achieved in isocratic mode using 60% of water acidified with 0.1% formic acid (mobile phase A) and 40% of acetonitrile acidified with 0.1% formic acid (mobile phase B) at flow rate of 0.30 mL/min. Electrospray ionization (ESI) was used in positive mode, with the following parameters: probe voltage 4kV, block heat (BH) temperature 400 ºC, desolvation line (DL) temperature 250 ºC, interface temperature 350 ºC, nebulizing and drying gas flow were 3 and 15 L/min respectively. Argon was used as collision gas. The precursor and product ions for natamycin, and collision energies are presented in Table 1.

| Compound | Precursor ion (m/z) | Product ions (m/z) | Collision energies (eV) | Ionization mode |
|----------|--------------------|-------------------|------------------------|----------------|
|          | 503.2              | 12                |                        |                |

Figure 1. Structural formula of natamycin
Prior to analysis of natamycin in cheese, the cheese sample was divided in two portions, the first containing only the rind (5 mm thick), while the second portion was the layer below the specified depth. From each of the two portions, two samples of 10 g were cut (40x40x5mm). Samples were carefully sliced and cut in small pieces, extracted with 50 mL of 90% methanol in water acidified with 0.01% acetic acid and homogenized with UltraTurrax (T 25 basic, IKA Werke, Germany). The extract (2 mL) was removed and stored at -18 ºC for one hour in order to precipitate proteins and lipids. After that, 50µL of extract was transferred to HPLC vial and diluted up to 1mL with the initial mobile phase, and 10µL was injected into LC-MS/MS system. Quantification was carried out using matrix extracted calibrations curves at four levels. With every batch, blank cheese samples were fortified at four different levels with standard solution and subjected to the full extraction procedure.

Validation was performed in accordance to the Commission Decision 2002/657/EC [9]. Each calibration curve was constructed with five concentration levels (including zero) and was fitted to a linear equation within the 0-5 mg/dm$^2$ range. Linearity was evaluated on three different days. The average regression coefficient ($R^2$) was 0.99957, which was satisfactory. The acceptance criteria were that the average regression coefficient ($R^2$) should be greater than 0.996. Other validation parameters (decision limit $CC_{\alpha}$, detection capability $CC_{\beta}$, accuracy, repeatability, reproducibility, measurement uncertainty) were determined based on the procedure given in the software ResVal for the validation of the analytical methods made in EURL RIKILT, Wageningen, The Netherlands. A total of four experiments were performed for four days. The batch of samples was made up of following samples:
- five calibration level samples (including zero) - fortified blank sample of cheese at 0.0, 0.5, 1.0, 2.0 and 5.0 mg/dm$^2$
- seven blank samples
- seven fortified blank samples at half of the required validation level (0.5 mg/dm$^2$)
- seven fortified blank samples at the required validation level (1.0 mg/dm$^2$)
- seven fortified blank samples at one and a half of the required validation level (1.5 mg/dm$^2$)

After validation, method was used in routine laboratory work and forty samples of hard cheese supplied from producers, were analysed using this method.

3. Results and discussion

Regarding the analytical methods for the determination of natamycin in food samples, the methods usually involves extraction of natamycin from the sample using organic solvents followed by high-performance liquid chromatography-diode array detection (HPLC-DAD) or liquid chromatography-tandem mass spectrometry (LC-MS/MS) [7,8]. In this work we presented a method with a simple extraction step coupled with efficient separation and highly selective and sensitive detection system. Appropriate extraction with high recovery rate was achieved with 90% methanol in water acidified with 0.01% acetic acid. Addition of the freezing step led to adequate protein and lipid precipitation, so no additional clean–up procedure were required. A suitable chromatographic separation was achieved with a reversed phase (C-18) column and in isocratic elution using 60% of water acidified with 0.1% formic acid and 40% of acetonitrile acidified with 0.1% formic acid at flow rate of 0.30 mL/min. We monitored protonated molecular ion [MH]$^+$ of 666.4 m/z and three fragments of 503.2, 485.2 and 137 m/z respectively, as presented in Table 1. The most intense fragment of 503.2 m/z was used for quantification. The ratio of abundance of these three fragments was used to conclusively identify natamycin.

Typical chromatograms of blank and cheese sample fortified with natamycin are shown in Figure 2.
As can be seen there were no interfering peaks at the retention time of natamycin, so matrix endogenous compounds did not affect the method specificity. The limit of detection and quantification were 0.13 mg/dm$^2$ and 0.26 mg/dm$^2$ respectively, showing that the developed method had sufficient sensitivity to detect natamycin at the regulatory level (1 mg/dm$^2$). The decision limit CC$\alpha$ and the detection capability CC$\beta$ were 1.09 mg/dm$^2$ and 1.18 mg/dm$^2$ respectively. The CC$\alpha$ is the limit from which the sample is considered non-compliant with a probability of $\alpha$ error and CC$\beta$ is the lowest content of the analyte which can be quantified with probability of $\beta$ error [9]. These limits should be considered in decision making when non-compliant samples are detected. The other validation parameters are shown in Table 2. The results of accuracy, repeatability and within laboratory reproducibility expressed as relative standard deviation (RSD) were satisfactory. Extended measurement uncertainty was 8.5%.

Table 2. Validation parameters

| Compound | Fortified level (mg/dm$^2$) | Accuracy (%) | RSD repeatability (%) | RSD reproducibility (%) |
|----------|-----------------------------|--------------|------------------------|-------------------------|
|          | 0.5                         | 99.4         | 3.5                    | 4.7                     |
| Natamycin| 1.0                         | 102.4        | 5.1                    | 5.3                     |
|          | 1.5                         | 101.0        | 1.9                    | 1.9                     |

The performance of the method was verified by participating in proficiency testing organized by DRRR – Deutsches Referenzbüro für Ringversuche und Referenzmaterialen RVEP 180534 in 2018. A total of 21 laboratories participated in this study, and two samples of cheese were analysed. Z-score values obtained by our laboratory were 0.23 and 0.58 respectively. No false positive nor false negative results were obtained.
This method was applied in everyday laboratory work for the analysis of natamycin in cheese samples. Forty samples of hard cheese, supplied from the producers, were analysed and results are displayed in Table 3.

| Table 3. Concentration of natamycin (mg/dm²) in hard cheese supplied from the producers |
|-------------------------------------------------------------|
| | Rind Internal (≥5mm depth) |
|-----------------|-----------------|
| min.            | < LoD           |
| max.            | 0.670           |
| average         | 0.455           |
| Compliant samples | Hard-cheese (n=36) | < LoD |
| No.1            | 6.3             |
| No.2            | 2.4             |
| No.3            | 1.67            |
| No.4            | 4.22            |
| Non-compliant samples | Hard-cheese (n=4) | < LoD |
| No.1            | 6.3             |
| No.2            | 2.4             |
| No.3            | 1.67            |
| No.4            | 4.22            |

Each sample of cheese was divided into two portions as had been described above and subsequently analysed. Of 40 samples of hard-cheese, in four samples (10%) concentration of natamycin exceeded maximum residue level (MRL) in the rind. However, no detectable quantities of natamycin were found in the internal layer, ≥5mm depth. These samples were declared non-compliant. In 36 samples that were declared compliant, concentrations ranged from non-detectable to 0.670 mg/dm². These results were comparable to those found by Molognoni et.al in Brazil [8]. In the samples were natamycin exceeded MRL in the rind, it could be due to unsuitable application of natamycin onto cheese surface. If hard cheeses are preserved by natamycin-soaked wrapping foil, it has to be removed prior to consumption.

4.Conclusion
The method for determination of natamycin in cheese is simple, rapid and has sufficient sensitivity to detect natamycin at the regulatory level, so the method is suitable for routine laboratory work, regulatory controls and efficient tool for compliance with European food legislative.

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