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Research Article

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A Novel and Rapid HPLC Method for Determination of Natamycin in Turkish Cheese Samples

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ABSTRACT

This study was aimed at developing a single RP-HPLC method for determination of Natamycin in 20 different cheese samples purchased from local Turkish supermarkets. Chromatographic separation was achieved on a X-Terra RP18 column (250 mm x 4.6 mm x 5 µm) with a mobile phase of acetonitrile: water (30:70 v/v/v), at 0.8 mL/min flow rate with DAD detection at 305 nm. In twenty cheese samples, Natamycin was analyzed by using sample preparation method of ISO 9233-2, 2007 (IDF 140-2, 2007). The results of analysis have been fully validated statistically and recovery studies confirmed the accuracy of the proposed method. The precision (intra-day & inter-day) of method was found within limits (RSD < 2%). The sensitivity of the method was assessed by determining limit of detection and limit of quantification. Findings dealing with the presence of Natamycin in cheese samples are presented.

Key words: *Natamycin, HPLC, Turkish Cheese.*

INTRODUCTION

Natamycin is a naturally occurring antimicrobial agent that is generally produced by the bacterium *Streptomyces natalensis*. Given a generally recognized as safe (GRAS) status, it is considered as a natural preservative by the European Union (EEC No. 235). Natamycin is usually applied at concentrations between 1 and 10 mg/L. Natamycin also known as pimaricin or tennectin is a polyene macrolide antimycotic produced by submerged fermentation of strains of *Actinomycetes* such as *Streptomyces natalensis*, *Streptomyces chattanogenesis*, and other closely related species. Due to its inherent property of being safe to humans, it is widely used against yeast and moulds [1].

Natamycin has an empirical formula of $C_{33}H_{47}NO_{13}$ and a molar mass of 665.73 g/mol (Figure 1). The structure of this compound was first discovered in 1958 and then it was developed. This compound belongs to the group of polyene macrolide antifungals, compounds having a carbon atomic ring coated with lactonization, and four conjugated double bonds. It classifies a tetraene as antimycotic. The structure of Natamycin is closely related to other antimycotics such as Nystatin, Rimodisin and Amphotericin [2].

Because the molecule contains an acidic and a basic group, it is amphoretic and the isoelectric point is 6.5. It is a chemically stable compound and has prolonged effectiveness. Solid natamycin and natamycin suspensions are quite stable to heat. Even heating for several hours at 100 ^{o}C causes only a slight decrease of its activity.

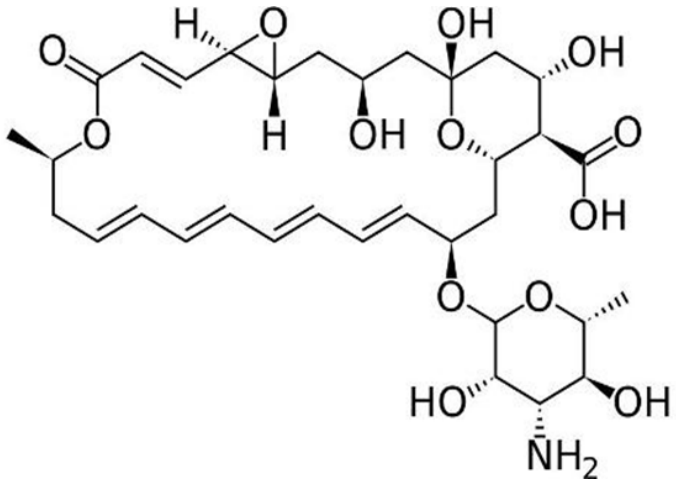


Figure 1. The chemical structure of Natamycin

The solubility of Natamycin is 20-50 mg/L in water. However, it is known that Natamycin is not soluble in high alcohols, ethers, esters, aromatic or aliphatic hydrocarbons, chlorinated hydrocarbons, ketones, dioxane, cyclohesanol and various oils. In literature, it was seen that glacial acetic acid, methyl pyrrolidone, dimethyl formamide, dimethyl sulfoxide, glycerol, and propylene glycol are good solvent for Natamycin [3]. Natamycin is used as an antifungal preservative in the food industry and in cheese and it has E number (E235) [4]. According to Turkish Food Codex [5], Natamycin can be used for the surface treatment of semi-hard and semisoft cheese at a maximum level of 1 mg/dm^2 in the outer 5 mm of the surface and should not be detectable at 2 mm depth [6]. Therefore, the detection of Natamycin is particularly important in dairy foods. Several studies have reported for the determination of Natamycin in cheese samples [7-13].

The basis of this study is to develop an efficient, simple, economical, new, rapid, accurate, reproducible, precise, and fully validated RP-HPLC method with good detection ranges for estimation of Natamycin in cheese samples.

The values obtained as a result of the validation of the method indicate the accuracy of the method. Finally, the method was applied to 20 commercial cheese samples purchased from local Turkish markets in order to provide effective quality control in selected cheese samples.

MATERIALS AND METHODS

Chemicals and Material

All chemicals in this study were used without further purification. The certified standard of Natamycin and acetic acid glacial were obtained from Sigma-Aldrich (St. Louis, Missouri, USA). HPLC grade acetonitrile (ACN) and methanol (MeOH) were purchased from Merck. Ultrapure water, with conductivity lower than 0.05 µS/cm was obtained with a Milli-Q system (Millipore, Bedford, MA, USA).

MeOH was used as a solvent for Natamycin and stock solutions of compound was prepared by dissolving in MeOH (50 mL) to give a 100 mg/L solution. All solutions were protected from light and all solutions were stored at 4º C for short time usage and at -20º C for long-term usage.

Apparatus

The LC analysis was carried out on an Agilent 1260 series HPLC system with ternary solvent pump, online degasser, automatic injection system, column heater and multi wavelength detector was used. UV detection was performed at 305 nm. Analyses were run at a flow rate of 0.8 mL.min^{-1}. Several columns were tested; Ascentis RP Amide (3.5 µm, 150 mm x 4.6 mm ID), Gemini C18 110 Aº (250 mm x 4.6 mm x 3µm), YMC Pack ODS-AM (5 µm, 250 mm x 4.6 mm ID) and X- Terra C-18 (5 µm, 250 mm x 4.6 mm ID). Finally, because of the peak shape and analysis time, X-Terra RP-18 (250 × 4.60 mm ID × 5 µm) column was selected as stationary phase at 30°C.

Preparation of Cheese Samples for HPLC Analysis

The developed method was applied on many commercial cheese samples. These were purchased from local Turkish markets. The cheese samples were prepared according to ISO 9223-2, 2007. A cheese sample (10.00 ± 0.01 g) was weighed in a conical flask and 100 ml of methanol was added to this sample. The mixture was stirred for 90 minutes in a magnetic stirrer than 50.0 ml of deionized water was added to this solution. It was

put in the freezer for about 60 min. The cold extract was filtered through filter paper. (Macherey-Nagel 100 751/60/030). The solution was allowed to stand at room temperature. A portion of the filtrate was filtered through a membrane micro filter of pore size 0.45 µm (Minisart RC25 17765) and then 0.20 µm (Minisart RC25). The resulting filtrate was used for 20 µL per injection for chromatographic analysis. Plot the peak area was obtained for each solution on the ordinate against the Natamycin concentration, in milligrams per liter, on the abscissa.

RESULTS AND DISCUSSION

Natamycin is an antimicrobial food additive against yeast and moulds. For this reason nowadays, the use of Natamycin has increased especially in food industry. This paper presents rapid and simple methods for the determination of Natamycin in 20 cheese samples by HPLC-DAD. The improved HPLC method has a good resolution within a short analysis time. For determination of Natamycin in the cheese samples, C18 column of 250 mm × 4.6 mm ID dimension and 5µm of particle size was used. The mobile phase used during the analysis was 30% (*v/v*) ACN-water containing 0.1 (*v/v*) glacial acetic acid. By this chromatographic conditions, analysis time is about six minutes (Fig 2).

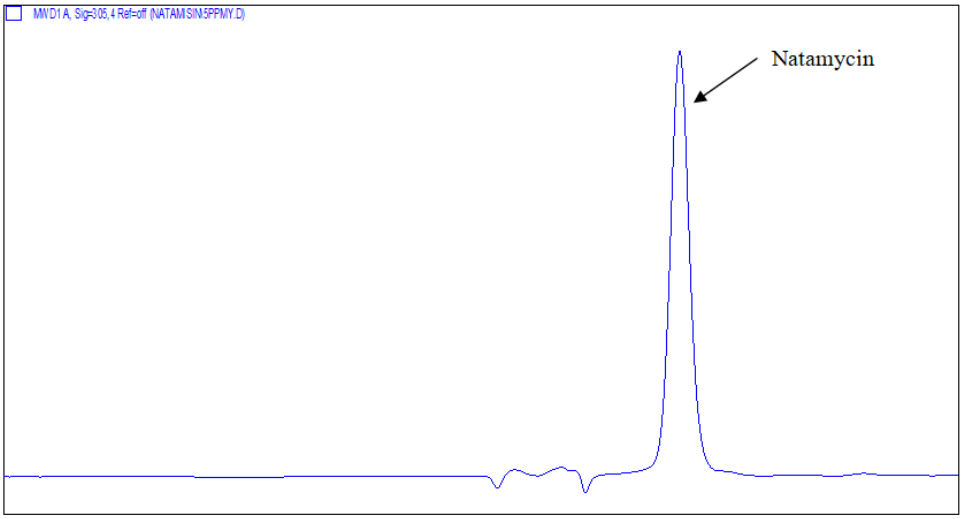


Figure 2. Chromatogram of a 10 µg/mL Natamycin standard. The eluent was monitored at 305 nm. Mobile phase consist of ACN:water (30:70, *v/v*) mixture containing 0.1 (*v/v*) acetic acid.

The effluent was monitored at 305 nm. Natamycin contains strong chromophores and shows the most intense absorption at 303 nm [12]. The components of the cheese samples did not show any interference at 305 nm during the analysis. According to ISO 9223-2, 2007 [4], preparation of test sample system suitability test can be defined as a test to ensure that the method can generate results of acceptable accuracy and precision. The requirements for the system suitability are usually designed after method development and validation have been completed. The system suitability test results were given in Table 1.

Table 1. System suitability parameters of the proposed RP-LC method

Parameters	DAD
Retention time (min)	5.89 ± 0.20
Theoretical plates (N)	3585 ± 183
Tailing factor	0.91 ± 0.01
Peak wavelength	0.12 ± 0.01

A set of Natamycin standards were tested to determine the validation parameters [14]. The linearity was calculated by plotting the peak area versus concentration of the compounds. Seven solutions were prepared containing 0.50 µg.mL^{-1}, 1.00 µg.mL^{-1}, 5 µg.mL^{-1}, 10 µg.mL^{-1}, 25 µg.mL^{-1}, 40 µg.mL^{-1} and 60.00 µg.mL^{-1} natamycin concentration, respectively. Each solution was injected in duplicate. The calibration curves were obtained by linear least squares regression. The validation data are reported in Table 2. The method exhibited

good linearity based on a correlation coefficient > 0.999 for Natamycin. The LOD and LOQ were calculated as LOD = 3.3s m^{-1} and LOQ = 10s m^{-1}, where s is the standard deviation of response and m is the slope of the corresponding calibration curve [15, 16].

Table 2. Statistical evaluation of the calibration data of Natamycin by HPLC- DAD method

	Natamycin
Linearity range (µg mL^{-1})	0.50 – 60.00 (n=7)
Slope	164.920
SE of Slope	1.661
Intercept	-21.537
SE of Intercept	48.415
Correlation coefficient (*r*)	0.999
Detection limit (LOD) (µg.mL^{-1})	0.0088
Quantitation limit (LOQ) (µg.mL^{-1})	0.0269

Repetitive analyzes of standard solutions containing 1-25 µg.mL^{-1} Natamycin were performed to evaluate the precision and reproducibility of the method. Natamycin was analyzed in consecutive days with five replicates. Repeatability and reproducibility were characterized by mean recovery and RSD and the results are summarized in Table 3. As deduced from Table 3, there was no significant difference for the assay, as tested by within-day (intra-day) and between-days (inter-day). The results supported good precision of the method.

Table 3. Summary of repeatability (intra-day) and reproducibility (inter-day) precision data for Natamycin by HPLC-DAD

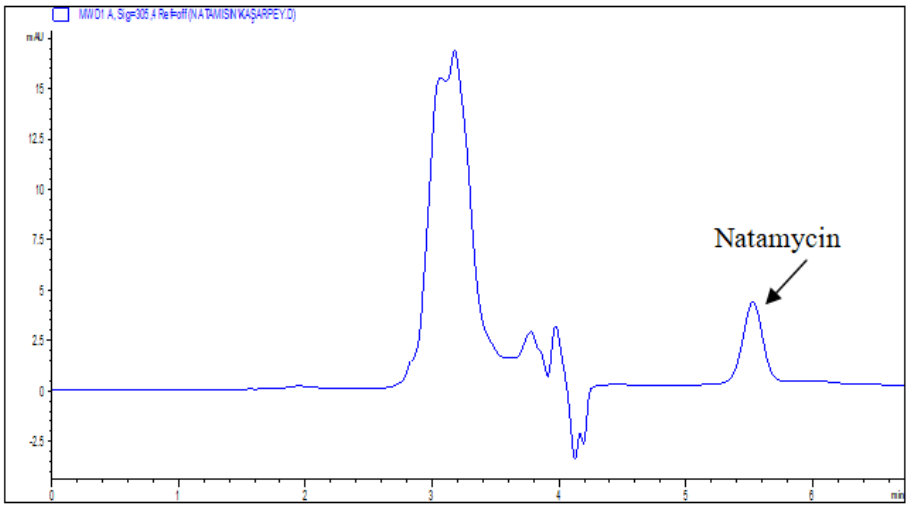
Compound Concentration (µg.mL^{-1})	Intra-day	Inter-day
	Mean Recovery* % ± RSD %	Mean Recovery* % ± RSD %
1.0	100.013 ± 0.741	100.225 ± 0.561
25.0	100.024 ± 0.032	99.918 ± 0.381

*Each value is obtained from five experiments (n=5)

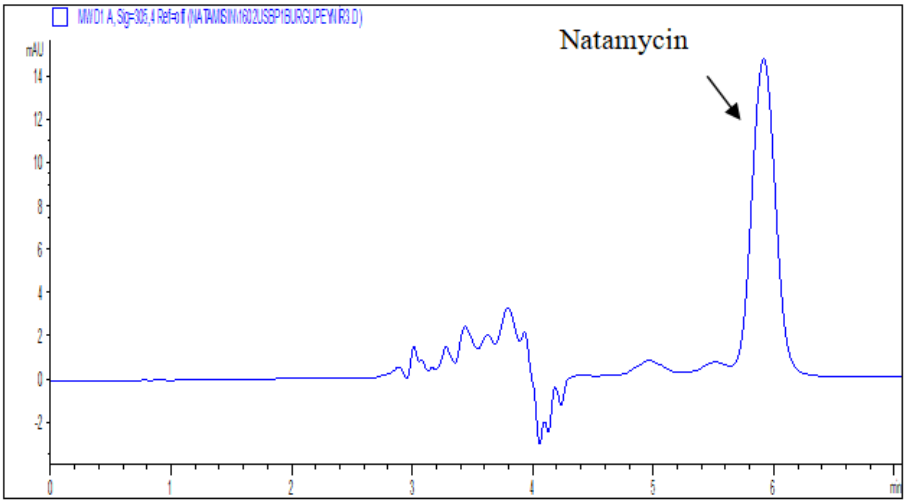
The method was finally tested to evaluate the content of Natamycin in several kinds of commercial cheeses and data are shown in Table 4. The calculation of the amount of Natamycin was performed using the matrix matched calibration curve. In Fig 3. Kashar and white cheese chromatograms were given as an example. It was found that kashar cheeses contain minumun Natamycin concentration.

Table 4. Natamycin content in cheese samples collected from local markets

Sample		**Sample**	
Kashar cheese 1	0.22 mg kg^{1-}	White Cheese 11	1.14 mg kg^{1-}
Kashar cheese 2	0.30 mg kg^{1-}	White Cheese 12	1.15 mg kg^{1-}
Kashar cheese 3	<LOQ	White Cheese 13	1.00 mg kg^{1-}
Kashar cheese 4	<LOQ	White Cheese 14	0.65 mg kg^{1-}
Fresh cheese 5	0.93 mg kg^{1-}	White Cheese 15	0.63 mg kg^{1-}
Fresh cheese 6	3.85 mg kg^{1-}	White Cheese 16	0.84 mg kg^{1-}
Fresh cheese 7	3.93 mg kg^{1-}	White Cheese 17	1.25 mg kg^{1-}
Fresh cheese 8	3.82 mg kg^{1-}	White Cheese 18	0.97 mg kg^{1-}
Fresh cheese 9	3.30 mg kg^{1-}	White Cheese 19	1.73 mg kg^{1-}
White Cheese 10	1.30 mg kg^{1-}	White Cheese 20	2.06 mg kg^{1-}



(a)



(b)

Figure 3. Chromatogram of (a) kashar cheese (b) white cheese samples

For the accuracy of the developed method, known amount of Natamycin was spiked to the cheese sample and analyzed by the developed method. The study was done at 5 mg/kg and 10 mg/kg of test concentration levels. Recoveries % were determined on n = 10 analyzed samples for each spiking level. The results were found 98.7 and 98.9 with low level of RSD values at 0.371 and 0.299 respectively. As a result, sufficient precision and accuracy could be achieved to by this developed method for analysis of cheese samples. The high recovery values obtained show that the method is not affected by the matrix effects in cheese samples.

CONCLUSION

In this study, a novel and fast HPLC method for the determination of Natamycin in 20 commercial cheese samples was developed with good accuracy and precision. The analysis is completed in about 6 minutes. ISO 9233-2, 2007 (IDF 140-2, 2007) reference method was used for preparation of cheese samples. X Terra C18 (250 mm x 4.6 mm, 5 µm) column and DAD detectors were selected at the same stationary phase. The proposed HPLC method of Natamycin is suitable for quality control laboratories. High recovery values indicate that the method is independent of the detrimental effects of additives commonly used in cheese samples. This study has lower detection limit, higher recovery values compared with literature data.

Natamycin has been used for decades in food industry. It was also approved in the European Union and known as E235. In Turkey, few companies use modern techniques in the production of cheese; generally, yeast and moulds can occur in cheese samples after opening and storage; also, use of basic technology could cause problem in the shelf life of cheese. The action of natamycin does not destroy other microorganisms, meaning

that it does not alter the food's natural maturing process. Unlike other antimicrobial agents, it does not affect the appearance, taste or color of the products. Because of these reasons, most of the producers prefer Natamycin as a preservative to prevent the occurrence of yeast and moulds in different kind of cheese. However, according to Turkish Food Codex, only surface applications of hard and semi-hard class cheeses are allowed to use Natamycin. A total of twenty commercial cheese samples purchased from the local supermarkets were analyzed to evaluate the Natamycin contents. Natamycin was detected in eighteen samples and it is not acceptable according to Turkish codex.

Conflict of Interest

There was no conflict of interest between authors.

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