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RESEARCH PAPER

Development and Validation of Liquid Chromatographic Method with DAD for the Quantification of Natamycin In Dairy Products

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ABSTRACT

In this paper high performance liquid chromatography method was developed for the determination of natamycin in dairy product in Turkish market. Chromatographic separation was achieved on a C8 column (150 mm x 4.6 mm x 5 µm 100 mm) with a mobile phase of methanol : water : acetic acid (12:8:1 v/v/v), at 1ml/min flow rate with DAD detection at 303 nm. Natamycin was analysed simultaneously using HPLC-UV method of ISO 9233+2, 2007(IDF 140-2, 2007).

The method was found linear over the concentration ranges of 0.01-0.8 mg/L. Correlation coefficients (r) of the regression equations were greater than 0.999. The LOD and LOQ values for UV and DAD were found 0.321 mg/kg and 0.355 mg/kg; 0.321 mg/kg and 0.388 mg/kg for cheese samples respectively. The relative recoveries obtained for the spiked cheese samples were between 90 and 110 %. Also the interday precision of the method was found as 1.874% and 3.442 % RSD values for UV and DAD dedections. From the results of the present study using HPLC-DAD indicated that it is not reasonable to attribute the determination of natamycin in cheese. Therefore, the difference of the validation parameters were found insignificant for both detections. However, It was succesfully applied to determination of 46 different cheese products and finding dealing with the presence of natamycin in cheese samples were presented.

Keywords: HPLC-DAD, validation, natamycin, antifungal agent

1. INTRODUCTION

Natamycin (NAT) is produced during fermentation by the bacterium *Streptomyces natelensis* and is used in the food additive for the prevention of mold contamination in meats, cheese and fruits (1). NAT is classified as a polyene macrolide antibiotic and specifically as a tetraene antibiotic because of its four conjugated double bonds. The mycosamine moiety (3-amino-3,6-dideoxy-*D*-mannose) of NAT at the C15 position is a six-membered pyranose ring. NAT forms a cylindrical structure due to the alignment of the hydroxyl groups of its amphipathic chain towards each other (Figure 1) (2). NAT has an empirical formula of $C_{33}H_{47}NO_{13}$ and a molar mass of 665.73 g/mol. The solubility of NAT is 20-50 mg/L in water. It has good stability in foods provided that pH is in the range from 5 to 9 (3). NAT is used in the food industry as an antifungal preservative in cheeses, it has E number E235 (4). According to Turkish Food Codex, NAT can be used for the surface treatment of semi-hard and semi-soft 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 (5). Therefore determination of NAT in food samples especially in dairy products is necessary.

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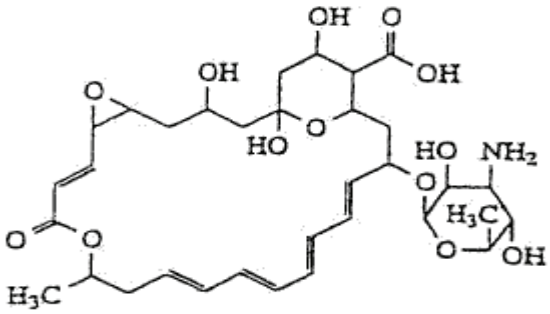


Figure 1. Chemical structure of NAT (EFSA 2007)

A number of analytical methods have been reported for the detection and quantification of natamycin including spectrophotometric (6, 7), derivative spectrophotometric (8) and liquid chromatographic methods (9-14). Some of these studies are with respect to compounds in some food while others are with regard to compound in drugs or biological samples (15, 16).

Natamycin analysis in foods were based on organic solvents extraction followed by UV dedection or further HPLC seperation with UV dedections.

Rybinska determined high performance liquid chromatography (HPLC) for NAT determination in routine control of ripening cheeses. In the method the antibiotic is extracted from the studied sample with a 2:1 methanol/ water solution, freezing of contaminants at -18^{0}C and determination of HPLC using a RP C8 column and UV detection. In case of low concentrations of the antibiotic the extract was condensed by extraction to solid phase (SPE) (10).

Guarino determined a single method, based on RP-HPLC with UV detection, was developed with the aim of simultaneously quantifying four preservatives in cheeses: benzoic acid, sorbic acid, NAT and lysozyme. The preservatives were extracted from different cheeses by using the same procedure, and separated by a single RP-HPLC gradient elution showing good resolution, in a short time. Recoveries were always higher than 91%; RSD ranged from 1% to 7%. Quantitation was performed in reference to a matrix matched calibration curve(13).

Passeiro *et al* extracted from food samples by using methanol acidified with acetic acid. The chromatographic separation was performed on a reversed-phase Kromasil ODS (C18) (150 × 3.20 mm i.d., 5 μm particle size) and the analysis was completed within 6 min. The method was validated in terms of linearity, limits of detection and quantification, repeatability and recovery (14).

Although UV spectrum of NAT shows three major absorbtion peaks in the range 290 - 320 nm, 303 nm is the wavelength commonly used to quantify the antifungal, because of spectral interferences from other compounds (Fig 1)(14). Therefore the identification of NAT peak was made by HPLC - DAD system because of its degredation in food although it suffers from various problem (2). According to proposed method the levels of NAT in dairy product manufactured by small- and large-scale brands in Turkey, were analyzed finding dealing with the presence of NAT in dairy samples are presented.

2. EXPERIMENTAL

2.1 Apparatus and chromatographic conditions

A Waters Bondapak C8 column (150 mm x 4.6 mm, 5μm) was used in Agilent 1200 with setting of UV and DAD detector at 303 nm. The mobile phase consisted of methanol, water and acetic acid (12:8:1; v/v/v), flow rate was 1.0 mL/min, temperature 25^{o}C. Injection volume was 20 μl and detector wavelength was 303 nm.

2.2 Chemicals and Reagents

All chemicals and reagents were of analytical grade and water was distilled and filtered through a membrane filter (0.45 μm). NAT powder was obtained from (Dr. Ehrenstorfer, 16208400, 97.0 %). Methanol (HPLC grade, Merck, 106018 ≥99.8 %) acetonitrile (HPLC grade, Sigma, 27225, 99.8-100.5 %) Acetic acid was used to prepare the diluted solution and mobile phase. Cheese samples from Turkey producers were obtained from trade network. All stock and working solutions were protected from light and stored in the fridge at about 4^{o}C.

2.3 Preparation of Standard Solution

Stock solution of NAT (0.5 mg/mL) was prepared by dissolving in methanol. 50 mg of pure NAT ($C_{33}H_{47}NO_{13}$) was dissolved in methanol in a 100 ml one-mark volumetric flask, marked with water, mixed and protected from light. The concentration ranges of NAT were (0.01 mg/mL - 0.8 mg/mL). The calibration curve for HPLC analysis was

constructed by plotting the ratio of the peak area of the natamycin. The linearity plots were constructed and the acceptable fit to linear.

2.4 Preparation of cheese rind and cheese interior for HPLC analysis

Handmade cheese (without NAT) varieties were used for validation and application studies. Test samples were prepared according to ISO 9223 + 2, 2007.

A cheese sample (5 ± 0.010g) was weighed into conical flask and added 100,0 ml of methanol. Sample was stirred for 90 min in a magnetic stirrer. 50,0 ml of deionized water was added and placed the conical flask in the freezer for about 60 min. Cold extract was filtered through a folder filter paper (Macherey-Nagel 100 751/60/030). Filtrate was warmed on room temperature. A portion of the filtrate was filtered through a membrane microfilter of pore size 0.45 µm (Minisart RC25 17765) and than 0.20 µm (Minisart RC25).

The minimum amount of test solution (filtrate) required is 20 µl per injection for direct chromatographic measurement. Plot the peak area or peak height was obtained for each solution on the ordinate against the NAT concentration, in micrograms per millilitre, on the abscissa.

3. RESULTS AND DISCUSSION

NAT is an antimicrobial food additive against yeast and moulds. In this reason, the use of NAT has increased in food industry. This paper presents rapid and simple methods for the determination of NAT in cheese samples by HPLC-DAD. We planned this work the identification of NAT peak by HPLC - DAD system because of its degredation in food (2) The proposed method gives a good resolution within a short analysis time. The simultaneous determination of the cheese samples was performed on a C8 column of (150 mm x 4.6 mm) dimension and 5µm of particle size. A mixture of methanol, water and acetic acid (12:8:1; v/v/v), as mobile phase with flow rate of 1 mL/min. The obtained results demonstrated a good usefulness of HPLC for routine control of NAT content in ripening cheeses

3.1 Method Validation

According to ISO+9223-2, 2007 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 stability of the chromatographic system was tested before each stage of validation. 20 replicate injection of sample preparation were injected and number of theoretical plats, tailing factor and relative standart deviation of peak area were determined. System suitability test results were reported in Table 1.

The components of the cheese samples did not show any interference at 303 nm and no detector signal was produced during the analysis.

Method precision (repeatability) was evaluated by assaying ten sets of test samples, the same day (intra day precision) and method precision were also determined by another person under the same experimental conditions.

Six solutions were prepared containing 0.01 mg/L; 0.1 mg/L; 0.2 mg/L; 0,6 mg/L; 0,8 mg/L NAT concentrations respectively. Each solution was injected in duplicate. Linearity was evaluated by linear regresion analysis. Table 2 includes the calibration data and related validation parameters. The regression analysis was performed, shows the equation: y=88.669 x + 0.2894 Correlation coefficient was 0.9991. Both the standard NAT mixture and the sample showed good linearity in the tested range. By applying linear regression analysis, the slope, intercept and regression standard deviation were calculated.

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

Detector	Retention time(min)	Peak area	Tailing factor	Peak width	Theoretical plates(N)
UV	3.27±0.2	35.7±0.01	0.94±0.01	0.13±0.01	10123±456
DAD	2.98±0.2	10.3±0.01	0.82±0.01	0.18±0.01	4385±383

Table 2. Statistical evaluation of the calibration data of NAT

	NAT (DAD)	
Linearity	Concentration	Peak area
Standard solution	0.01	1.072
	0.1	9.668
	0.2	17.814
	0.4	36.028
	0.6	52.197
	0.8	72.049
Slope	88.669	
SE of slope	1.219	
Intercept	0.2894	
SE of intercept	0.0544	

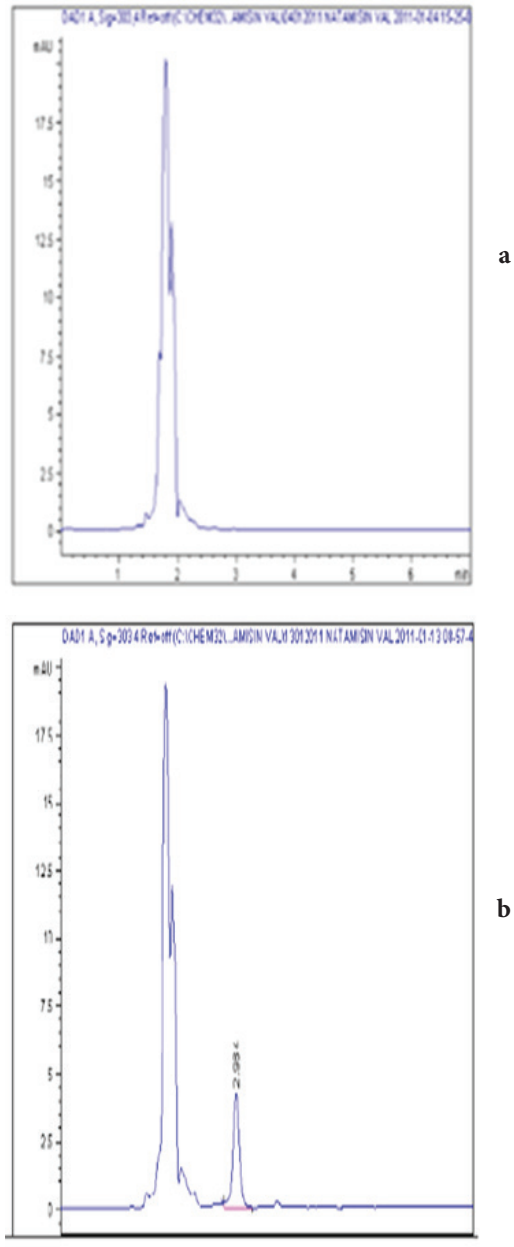
Each value is the mean of 3 experiment

It is the lowest concentration of analyte in the sample that can be determined with the acceptable precision and accuracy under the determined experimental conditions. The solution was injected three times and the signal and the noise for each injection were recorded. Each signal to noise ratio was then calculated and averaged. The concentration of the solution was used to determine the dedection limit if the average S/N ratio is between 3 and 10. Also LOQ value was calculated from the calibration curve using equation LOQ = 10 xSD / b (Where, SD = Standard Deviation of intercepts of calibration and b = Slop of corresponding calibration curve). LOD and LOQ were found 0.321 mg/kg and 0.388 mg/kg for cheese samples respectively.

Fig 2b shows a typical chromatogram obtained for analysis of spiked NAT in cheese. As shown in Fig 2b the substances formed well shaped, NAT peak were well separated from the mobile phase front.

The accuracy of the developed method was carried out by adding the known amount of NAT pure drug to the pre analyzed cheese samples and subjected to the proposed method. Results of recovery study are shown in Table 3. The study was done at 3mg/kg and 6 mg/kg of test concentration levels. All the results indicate that the method is highly accurate. A specimen HPLC DAD chromatogram of a sample solution is shown in Fig 3.

Precision of the assay was determined by intra-day and intermediate assay of the developed method. Intra-day analysis refers to the use of the analytical procedure within a laboratory over a short period of time that was evaluated by assaying 10 sample solutions for each analyst, at the final concentration corresponding to 0.6 mg/kg of NAT during the



a

b

Fig 2. Chromatogram obtained from the mobile phase(a) and cheese (b)

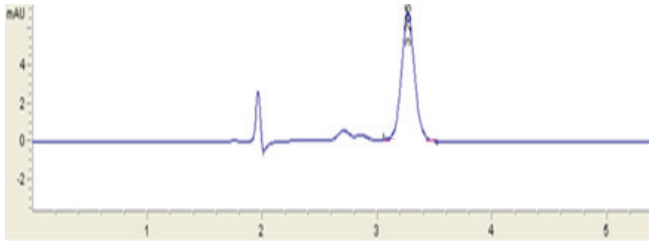


Figure 3. NAT peak for UV detector

same day. Intermediate assay was carried out by two different analyst. The interday precision (repeatability) of the method was found as 1.874 % and 3.442 % RSD values for UV and DAD dedections. Recovery studies were also carried out to determine accuracy and precision of the proposed method. The recovery procedure was carried out by spiking already analyzed samples of cheeses with known concentrations of standart solution of NAT. The results of the recovery analysis are shown in Table 3. According to the student's t-test the computed t-value (t = 0.093) is less than the table value and and accuracy parameter has been validated.

Table 3. Accuracy and precision study of NAT samples

	Parameters					
Detector	**Mean Assay (mg/kg)**	**Standart Deviation**	**RSD (%)**	**LOD (mg/kg)**	**LOQ (mg/kg)**	**Recovery (%)**
UV	0.53	0.018	1.874	0.321	0.355	109
DAD	0.54	0.051	3.442	0.321	0.388	92

Analysis of food preparations were carried out according to steps mentioned in sample preparation part. The results were fitting with the label claims for each of the substances. The results of the proposed method were compared with HPLC-UV method. The results showed that F values are not more than 4.41 which is the theoretical value for N=10 at 95% confidence limit, indicating no significant difference between the mean contents of the NAT obtained by two different analysts. The data gained by recovery studies were summarized in Table 3

A total of 46 cheese samples purchased from the supermarkets were analyzed to evaluate the NAT contents and results are listed in Table 4. NAT was detected in all cheese samples but the level of NAT in the one cheese sample was detected above limit of detection .

Table 4. NAT content in cheese samples

Sample no	NAT level	Sample no	NAT level
1	< LOQ	24	< LOQ
2	< LOQ	25	< LOQ
3	< LOQ	26	< LOQ
4	< LOQ	27	< LOQ
5	< LOQ	28	< LOQ
6	< LOQ	29	< LOQ
7	< LOQ	30	< LOQ
8	< LOQ	31	< LOQ
9	< LOQ	32	< LOQ
10	< LOQ	33	< LOQ
11	< LOQ	34	< LOQ
12	< LOQ	35	< LOQ
13	< LOQ	36	< LOQ
14	< LOQ	37	< LOQ
15	< LOQ	38	< LOQ
16	< LOQ	39	< LOQ
17	< LOQ	40	< LOQ
18	< LOQ	41	< LOQ
19	< LOQ	42	< LOQ
20	< LOQ	43	< LOQ
21	< LOQ	44	< LOQ
22	< LOQ	45	< LOQ
23	< LOQ	46	1.66

4. CONCLUSION

The RP-HPLC method is enabled in determining of NAT in the spiked handmade cheese samples and 46 commercial cheese samples with good accuracy and precision.The developed method gives a good resolution in 7 min. The relative standart deviations (RSD%) obtained from replicate measurements at a concentration of 0.3 mg/kg for intra-day (n=10) and interdays (n=10) precions were 1.874 and 3.442% respectively. Retention times were 3.27 and 2.98 min for UV and DAD respectively. ISO 9233+2, 2007(IDF 140-2, 2007). Reference method was used fo determination of cheese samples selected C_8 (150 mm x 4.6 mm, 5 µm) column and DAD detectors at the same stationary phase. Proposed method is suitable for quality control laboratories. High recovery shows that the method is free from the interferences of the commonly used additives in the cheese products. Obtained results using both detector were compared and proved the system confidence.

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Peynir örneklerinde Natamisin'in nicel analizi için HPLC-DAD yöntemi geliştirilmesi ve validasyonu

ÖZ

Bu çalışma kapsamında süt ürünlerinde yüksek performanslı sıvı kromatografisi kullanılarak bir analitik yöntemi geliştirildi. Kromatografik ayırmalar C8 kolon (150 mm x 4.6 mm x 5 µm) metanol : su : asetik asit (12:8:1 v/v/v) mobil fazı ile 1 mL/dak akış hızında ve DAD dalga boyunda 303 nm'de çalışılmıştır. Natamisin analizi için geliştirilen yöntemde ISO 9233+2, 2007 yöntemi kullanılmıştır. Yapılan doğrusallık çalışmalarında natamisin için 0,01-0,8 mg /kg aralıklarında elde edilen regresyon eşitliklerinin korelasyon katsayıları 0.999'dan yüksek bulunmuş ve yöntemlerin doğrusallığı gösterilmiştir. Geri kazanım sonuçları % 90-110 aralığında bulunmuştur. The LOD and LOQ değerleri UV and DAD dedektörleri için sırasıyla 0.321 mg/kg and 0.355 mg/kg; 0.321 mg/kg and 0.388 mg/kg bulunmuştur. Ayrıca yöntemin UV ve DAD için güniçi kesinliği %1.874 ve %3.442 bulunmuştur. Çalışma sonuçları HPLC DAD kullanımının natamisin tayinine katkısı olmadığını gösterdi. Bu nedenle validasyon parametrelerinin her iki dedektör için farklılığının önemsiz olduğu bulundu. Fakat yöntem ISO 9233+2, 2007 (IDF 140-2, 2007 yöntemine göre valide edildi ve 46 peynir örneğinin tayininde uygulandı ve peynir örneklerindeki natamisin varlığına ilişkin bulgular sunuldu.

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