

ORIGINAL ARTICLE

# Quantification, migration and decline of natamycin in blue cheeses

*Quantificação, migração e declínio de natamicina em queijos azuis*

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## Abstract

This study aimed to quantify the concentration of natamycin in the rind and verify its migration in Gorgonzola type cheese, as well as evaluating the decline of natamycin in Blue cheese during maturation. Eight samples of Gorgonzola cheese were purchased in supermarkets of which six were produced in different regions of Brazil, one in Argentina and one in Italy. The rinds of four of the eight cheese samples presented natamycin concentrations above 5 mg/kg, three of these samples being from Brazil and one from Argentina. With the exception of the Italian cheese, all the samples showed natamycin inside the cheese mass (up to 6 mm from the rind). To evaluate the rate of decline during the ripening of Blue cheeses, samples were collected from an industry as from day 0 (day of manufacture) up to the 45 days of ripening. The results showed that, after 45 days of ripening for cheese immersed in a 3% natamycin solution, the concentration in the rind was 4 times the amount allowed by the legislation (5 mg/kg). However, for immersion in a 1.5% natamycin solution, after 25 days of ripening, the antifungal concentration was in accordance with the standards established by the legislation.

**Keywords:** Ripening; Gorgonzola cheese; *Penicillium roquefort*; Antifungal; Pimaricin.

## Resumo

Este estudo teve por objetivo quantificar a concentração de natamicina na casca e verificar sua migração em queijos tipo Gorgonzola, além de avaliar o declínio da natamicina em queijo Azul, ao longo da maturação. Para isso, foram adquiridas oito amostras de queijo tipo Gorgonzola em supermercados, sendo seis produzidos em diferentes regiões do Brasil, um na Argentina e um da Itália. Na casca, quatro das oito amostras de queijo apresentaram concentração de natamicina acima de 5 mg/kg, sendo, destas, três provenientes do Brasil e uma da Argentina. Com exceção do queijo italiano, todas as amostras apresentaram natamicina na massa do queijo (até 6 mm da casca).



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Para avaliar a taxa de declínio ao longo da maturação em queijos Azuis, estes foram coletados em uma indústria a partir do dia zero (dia da fabricação) até os 45 dias de maturação. Os resultados mostraram que, com o banho de natamicina a 3%, aos 45 dias de maturação, sua concentração na casca era igual a quatro vezes o valor permitido pela legislação (5 mg/kg). No entanto, com banho de 1,5% de natamicina, aos 25 dias de maturação, a concentração do antifúngico encontrava-se de acordo com os padrões estabelecidos pela legislação.

**Palavras-chave:** Maturação; Queijo gorgonzola; *Penicillium Roquefort*; Antifúngico; Pimaricina.

## 1 Introduction

Blue cheeses are characterized by the growth of the fungus *Penicillium roquefort* inside the cheese, either naturally or by its introduction. The blue mould grows in the air spaces, creating veins and releasing free fatty acids, resulting in the characteristic aroma and flavour, and in the appearance and in the moist, soft and crumbly texture after ripening (Fernández-Salguero, 2004; Banjara et al., 2015), besides important chemical changes such as proteolysis and the formation of bioactive amines (Moreira et al., 2018).

For the production of Gorgonzola type cheese, the milk is mixed with the yeast and with the fungus *Penicillium roquefort*. After coagulation, the curd is cut and salted and natamycin applied in a concentration of approximately 0.2% to 0.4%, to avoid contamination with yeast on the cheese surface. After 24 hours, the cheeses are drilled and stored in a ripening chamber, where they are frequently turned until completing the ripening period (Furtado, 2013).

Natamycin or Pimaricin, is a natural antibiotic used as a preservative in the food industry, more specifically in the cheese industry (Kallinteri et al., 2013). In Brazil, its use is permitted in cheeses up to a maximum limit of 1 mg/dm<sup>2</sup> or 5 mg/kg on the surface, and it should not be detected at depths of 2 mm and should be absent inside the cheese mass (Brasil, 1996).

In the cheese, the natamycin operates on the surface, preventing the proliferation of *Penicillium roquefort* and other opportunistic fungi on the outside of the cheese, but does not interfere with its growth on the inside of the mass, where the growth is desired (Oliveira et al., 2006).

According to the European Food Safety Authority (2009), the use of natamycin in cheeses and processed meats is safe since it does not lead to microbial resistance and is absorbed and subsequently eliminated intact or as its degradation products mycosamine, aponatamycin or di-nathaminoldediol (Venturini, 2012). Studies with humans showed no adverse effects on the health, its ingestion being safe up to 0.3 mg kg<sup>-1</sup> day<sup>-1</sup> (Expert Committee on Food Additives, 2017). According to Suloff et al. (2003), daily oral doses of 300 to 400 mg resulted in nausea, vomiting and diarrhoea.

The addition of natamycin to unauthorized products or in quantities above those stipulated by the legislation is characterized as fraud by adulteration (Evangelista, 2008). Studies have shown the quantification of natamycin in cheeses in quantities above the recommended levels in the rinds (Paseiro-Cerrato et al., 2013) and have also shown the migration of natamycin to the inside of the cheese mass, where it should be absent (Vierikova et al., 2013).

Thus this study aimed to quantify the concentration of natamycin in the rind and verify internal migration of the preservative in Gorgonzola type cheeses sold in South-Western Paraná and in Western Santa Catarina, both in Brazil, besides evaluating the rate of decline during the ripening of Blue cheese.

## 2 Material and methods

### 2.1 Sampling

Samples of eight different brands of Gorgonzola type cheese, available to consumers in municipalities in South-Western Paraná and Western Santa Catarina, Brazil, were obtained. Of the eight Gorgonzola brands, six (6) were of national origin and two (2) imported, one from Argentina and the other from Italy (a legitimate Gorgonzola Dolce cheese). The samples were codified with letters from the alphabet (A, B, C, D, E, F, G and H) so as to preserve the identity of each brand. Three samples from the same batch of each brand under study were acquired, giving a total of 24 samples. The samples were packed, transported in thermal boxes, and the analyses carried out in triplicate. With the exception of sample H, all the samples indicated the presence of natamycin on the label.

A partnership was subsequently established with a Blue cheese manufacturer in South-Western Paraná, which provided 3 samples of Blue cheese from the same vat and batch, which had been immersed in a 3% natamycin solution for 2 seconds. These samples were analysed after 0, 35 and 45 days of ripening. Three more samples were later provided, where each cheese had been immersed in a 1.5% natamycin solution for 2 seconds and then maintained side by side in a ripening chamber. A wedge of each cheese was removed after 0, 7, 14, 25, 35 and 45 days to determine the behaviour of the natamycin during ripening.

### 2.2 Quantification of natamycin and the evaluation of internal migration and decline during ripening

The methodology developed by Fletouris et al. (1995) was used to quantify the natamycin, in each case taking a 5 grams sample of the 2 mm thick surface cheese to extract the additive. The natamycin was extracted from the cheese with the use of 25 mL of a mixture of acetonitrile with 1 mol/L phosphoric acid (4:1). Further samples were removed every 2 mm (measured with the aid of a pachymeter) to follow migration into the internal part of the cheese. The cheese samples were mixed with the solvents, stirred for 1 minute using a magnetic stirrer, and filtered through filter paper for scanning using a UV visible spectrophotometer (Thermo Scientific, Evolution 201) at 316 nm, reading to the third derivative. A nine-point calibration curve was prepared using a commercial natamycin solution (Delvocid - 50% lactose and 50% natamycin) of known concentration. The results were obtained in  $\mu\text{g/mL}$  and transformed into  $\text{mg/kg}$  using Equation 1, and the limits of detection and quantification determined using Equations 2 and 3, respectively, according to the methodology of the National Health Surveillance Agency (*Agência Nacional de Vigilância Sanitária*) (Brasil, 2003). The decline of natamycin with time was monitored by quantification of the antifungal agent during ripening of the Blue cheese.

$$\text{Natamycin concentration (mg / kg)} = (\text{Natamycin concentration } (\mu\text{g / mL}) \times 25) / \text{Sample weight} \quad (1)$$

$$\text{Limit of Detection (LD)} = (\text{Standard deviation of ten blank measurements} \times 3) / \text{Angular coefficient of the calibration curve} \quad (2)$$

$$\text{Limit of Quantification (LQ)} = (\text{Standard deviation of ten blank measurements} \times 10) / \text{Angular coefficient of the calibration curve} \quad (3)$$

### 2.3 Data treatment

The analysis of variance (ANOVA) and Tukey's test were used to compare the results obtained for the concentration of natamycin in the different brands of Gorgonzola type cheese before and after ripening of the Blue cheese. The Student t-test was used to verify concentrations of natamycin considered to be greater than that established by the Brazilian legislation.

A confidence interval of 95% was used for all the above-mentioned tests, and the ACTION<sup>®</sup> software, version 2014, was used to apply the analyses (Action, 2014).

### 3 Results and discussion

#### 3.1 Quantification of natamycin and evaluation of internal migration in Gorgonzola type cheeses

In accordance with Brazilian legislation (Brasil, 1996), natamycin is accepted as an additive in cheeses, but its presence may only be detected on the surface at a limit of 5mg/kg in the rind, and there should be no migration to the interior of the cheese, where it should not be detected as from a depth of 2 mm.

Table 1 shows the results obtained for the concentrations of natamycin (mg/kg) in Gorgonzola type cheeses sold in South-Western Paraná and Western Santa Catarina, Brazil.

**Table 1.** Concentrations of natamycin (mg/kg) in the rind and inside Gorgonzola type cheeses sold in South-Western Paraná and Western Santa Catarina, Brazil.

ORIGIN	BRAND	LAYERS			
		2 mm	4 mm	6 mm	8 mm
**Paraná	A	20.43±0.72 c <sup>*A</sup>	6.86 ± 3.61 c <sup>*B</sup>	1.52 ± 0.40 c <sup>*B</sup>	<QL
**Minas Gerais	B	10.40 ± 2.56 c <sup>*A</sup>	4.65 ± 1.21 c <sup>*B</sup>	2.06 ± 0.76 c <sup>*B</sup>	1.33 ± 0.79 a <sup>*B</sup>
**Minas Gerais	C	2.80 ± 0.64 d <sup>A</sup>	2.26 ± 0.40 c <sup>*A</sup>	1.60 ± 0.30 c <sup>*A</sup>	<QL
**RGS	D	2.39 ± 0.34 d <sup>A</sup>	2.37 ± 0.29 c <sup>*A</sup>	1.82 ± 0.33 c <sup>*A</sup>	1.40 ± 0.19 a <sup>*A</sup>
**Minas Gerais	E	2.70 ± 1.58 d <sup>A</sup>	3.77 ± 2.24 c <sup>*A</sup>	2.31 ± 1.50 bc <sup>*A</sup>	3.63 ± 3.24 a <sup>*A</sup>
**Minas Gerais	G	73.26 ± 11.11 a <sup>*A</sup>	36.17 ± 7.22 a <sup>*B</sup>	25.98 ± 0.93 a <sup>*B</sup>	18.07 ± 1.49 b <sup>*B</sup>
Argentina	F	45.68 ± 14.05 b <sup>*A</sup>	19.96 ± 7.55 b <sup>*A</sup>	6.31 ± 2.88 b <sup>*A</sup>	2.62 ± 1.98 a <sup>*B</sup>
Italy	H	<QL	<QL	<QL	<QL

\*\*Brazilian states. RGS: Rio Grande do Sul. The results represent the mean ± standard deviation of three repetitions analysed in triplicate. The same lower-case letters in the same column represent equal means according to Tukey's test ( $p > 0.05$ ). The same upper-case letters in the same line represent equal means according to the Tukey's or Kruskal Wallis tests ( $p > 0.05$ ). \*means above that permitted by the legislation (5 mg/kg in the rind or absent in the cheese) according to the Student t test at a level of 5% significance. QL: 1.2 mg/kg.

For the rind (up to a depth of 2 mm), the cheeses evaluated in this study presented natamycin concentrations that varied from 2.39 to 73.26 mg/kg, with samples A, B, F and G presenting the highest concentrations, greater than the permitted value for this food. Thus, of the eight brands of Gorgonzola type cheese evaluated, 50% presented a quantity of natamycin in the rind above that established by the Brazilian legislation (Brasil, 1996).

For the same depth in the external rind of Gorgonzola type cheeses, Oliveira et al. (2006) found a concentration of 2.58 mg/kg after 45 days of ripening, using spectrophotometry to the third derivative.

For the cheese itself, all the Gorgonzola brands evaluated, except the brand identified by the letter H (the legitimate Italian Gorgonzola), obtained results below the quantification limit (QL: 1.2 mg/kg) in all the layers. The Gorgonzola samples identified as C, D and E presented no decline in any of the layers, while sample F only presented a significant decline ( $p < 0.05$ ) at a depth of 8 mm.

Nevertheless, the results showed that there was migration of natamycin in brands A, B and G, presenting a decline in concentration in the cheese in relation to the rind. Although this reduction was found, it should be considered that these results diverge from that defined in the Brazilian legislation (Brasil, 1996), which states that the internal layers of these products should be free of this preservative.

The migration of the preservative into the cheese verified in the majority of the samples may be related to the needling process, which should be carried out after salting and the application of the antifungal agent, to

allow for the escape of the carbonic gas resulting from fermentations and the renovation of oxygen (Furtado, 2013).

Furtado (2013) indicates that the antifungal treatment in aqueous solution by immersion or aspersion should use a solution containing 3 to 5 g of antifungal agent per litre. The different results found here show there was probably a variation in the quantities of antifungal agent used by the manufacturers. Another factor which may contribute to natamycin migration in this type of cheese is the porosity of the rind, which was thicker than 2 mm at various points.

Other studies with cheeses have also demonstrated the migration of natamycin into the cheese. Vierikova et al. (2013) recorded the presence of natamycin in 80% of semi soft cheese samples, at a depth of 5 mm inside the cheese.

Paseiro-Cerrato et al. (2013) analysed natamycin in 26 samples of cheeses (soft, cured, semi-cured, Roquefort and Camembert) and natamycin was detected in quantities above the recommended values (1 mg/dm<sup>2</sup>) in the rinds of cured (1.4 mg/dm<sup>2</sup>) and semi-cured (4.9 mg/dm<sup>2</sup>) cheeses and also inside the soft cheeses (0.42 – 5.9 mg/dm<sup>2</sup>), where its addition is not permitted.

### 3.2 Decline of natamycin during the ripening of Blue cheese

The decline of natamycin in Blue cheese was carried out on cheeses immersed in a 3% natamycin solution (method used in the industry).

The analyses were done at three distinct times, the first being on the day the antifungal agent was applied (day 0); the second after 35 days of ripening and the last one after 45 days of ripening. Table 2 shows the results of these analyses.

**Table 2.** Natamycin concentrations (mg/kg) in the rinds and inside the Blue cheeses during ripening.

DAYS	LAYERS			
	2 mm	4 mm	6 mm	8 mm
0	45.33 ± 1.79 <sup>a*</sup>	4.01 ± 0.97 <sup>a*</sup>	1.38 ± 0.13 <sup>a*</sup>	<DL
35	19.91 ± 7.22 <sup>b*</sup>	3.31 ± 2.91 <sup>a*</sup>	1.32 ± 0.12 <sup>a*</sup>	<QL
45	20.69 ± 0.47 <sup>b*</sup>	4.45 ± 0.06 <sup>a*</sup>	1.96 ± 0.52 <sup>a*</sup>	<QL

The results represent the mean ± standard deviation of three repetitions analysed in triplicate; \*Mean above that permitted by the legislation (5 mg/kg in the rind or absent in the cheese) according to the Student t test at a level of 5% significance; The same letters in the same column represent no significant difference according to the Tukey test; DL: 0.24 mg/kg; QL: 1.2 mg/kg.

The application of a 3% natamycin solution produced unsatisfactory results, considering that at the end of 45 days of ripening, the samples presented concentrations in the rind more than four times higher than that permitted by Brazilian legislation (Brasil, 1996), besides presenting high concentrations of the antifungal agent inside the cheese (layers at 4 and 6 mm).

The concentration of antifungal agent in the rind declined from the beginning of ripening up to 35 days, and then remained stable. There was no reduction in the natamycin content with ripening in the internal layers of the cheese.

Based on these results, the cheese manufacturer opted to reduce the concentration of the natamycin solution to 1.5%, meet the requirements of the legislation with respect to the maximum concentration of natamycin permitted in the product. The Table 3 shows the results of the evaluation of natamycin when used at a concentration of 1.5% during ripening.

Using the reduced concentration of natamycin (1.5%), an accentuated decline was observed in the natamycin concentration in the cheeses evaluated. After 25 days of ripening, the cheeses already showed a concentration of the antifungal agent within that specified by the legislation for human consumption.

**Table 3.** Natamycin concentrations (mg/kg) in the rinds and inside the Blue cheeses during ripening.

DAYS	LAYERS			
	2 mm	4 mm	6 mm	8 mm
0	12.10 ± 4.04 <sup>a*</sup>	2.00 ± 0.71*	<QL	<DL
7	12.08 ± 2.28 <sup>a*</sup>	2.18 ± 0.45*	<QL	<QL
14	10.18 ± 1.17 <sup>a*</sup>	2.87 ± 1.24*	<QL	<QL
25	4.44 ± 1.02 <sup>b</sup>	<LQ	<DL	<DL
35	4.28 ± 0.36 <sup>b</sup>	<LQ	<DL	<DL
45	3.63 ± 0.68 <sup>b</sup>	1.60 ± 0.06*	<QL	<DL

The results represent the mean ± standard deviation of three repetitions analysed in triplicate; \*mean above that permitted by the legislation (5 mg/kg in the rind or absent in the cheese) according to the Student t test at a level of 5% significance; The same letters in the same column represent no significant difference according to the Tukey test; DL: 0.24 mg/kg; QL: 1.2 mg/kg.

According to Reys et al. (2002), the factors which interfere in the natamycin concentration and the way it declines with time are the initial concentration, the application method (before or after salting, immersion or different types of packaging), the type of product to which it is applied (soft cheese, hard cheese, salami), the immersion time, the ripening conditions and the ripening time of the product. According to these authors, the rind is an important protector against the migration of natamycin to the centre of the cheese, especially when applied via aqueous solution immersion.

Considering that natamycin was found inside both the Gorgonzola type cheese and the Blue cheese due to migration towards the centre of the cheese, the use of a biofilm, where the antifungal agent migrates slowly into the rind of the cheese, is an alternative to be considered.

Oliveira et al. (2007) produced a cellulose film with added natamycin to wrap Gorgonzola type cheese in, which presented good results for microbiological control with 2% natamycin, and resulting in antifungal levels in accordance with the legislation as well as offering a product with good quality and food safety to the population.

The antimicrobial efficiency of biofilms is great due to efficient migration, which is greater the more tightly the packaging is wrapped around the product (Hanusová et al., 2012). Lantano et al. (2014) suggested that the migration of the substance was related to the percentage of organic component wrapped in the packaging.

## 4 Conclusion

Of the samples of Gorgonzola type cheese evaluated, four (50%) presented a natamycin concentration in the rind at quantities above that established by the Brazilian legislation. Natamycin was only not detected in one of the cheese samples, which shows that the majority of the manufacturers were not complying with the legislation in force, confirming there was migration of the antifungal agent due to the porosity of the rind and the characteristic texture of this type of cheese.

The natamycin concentration declined in the Blue cheeses with ripening, due to degradation. A concentration of 1.5% natamycin in the immersion solution was considered to be safe for consumer health, since after 25 days of ripening the values found in the cheese rinds were below the value recommended by the Brazilian legislation, and no natamycin was detected in the cheese itself.

Based on the results for natamycin, it is important to make the manufacturers aware of the correct way to apply the product as well as the minimum quantities necessary. New studies testing the application of natamycin in Blue cheese by methods such as spraying or at different concentrations of the antifungal agent, may complement this study, allowing for new possibilities of use with acceptable results, still meeting the legislation.

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