Studies conducted by Marin et al. (2003) showed that calcium propionate, sodium benzoate and potassium sorbate were effective to inhibit some bakery products isolates in pH 4.5 when applied at 0.3% concentration.

Concentration of sorbate benzoate and propionate at 0.003% was not effective at any pH value, whereas 0.03% has demonstrated a satisfactory result in pH 4.5, but negative in pH 6.0 up to 7.5 in bread samples originating from Spain. None of preservatives have shown any effect in product shelf-life in high pH close to 7.0. Potassium sorbate was effective at 0.03% concentration (MARIN et al., 2002).

Guynot et al. (2005) showed similar results in pH 4.5 – 6.5. Potassium sorbate was the most effective one preventing deterioration by fungi at 0.3% concentration regardless water activity. At the same concentration, calcium propionate and sodium benzoate were effective at low water activity (aw) values only.

Suhr & Nielsen (2004) evaluated different preservative concentrations, pH and water activity of mould growth on rye bread. At 0.3% concentration of calcium propionate, aw 0.97 and pH 4.8 all fungi were inhibited for a period of two weeks, except for Penicillium roqueforti, Penicillium commune and Eurotium rubrum. This study demonstrated that P. roqueforti was stimulated by the presence of preservative, with growth and water activity increase. In aw 0.80 only Eurotium species were capable to grow within 30 days. The higher pH and aw, the shorter the time for sample deterioration. Calcium propionate was less effective than sorbate and sodium benzoate.

Brazilian law establishes limits for sorbic acid and its salts (sodium, calcium and potassium) of 0.1g/100 g in bakery product and cookies (BRASIL, 1999).

Sorbic and benzoic acid and its salts have been widely used in food industry. A study developed by Tlouni & Toledo (2002) evaluating several food categories as soft drinks, yogurts, fruit juices, margarines and cheese obtained undetected levels and 804 mg/L for benzoic acid, and undetected levels to 1371 mg/L for sorbic acid. Only one sample exhibited level above the maximum allowed by Brazilian law. In a subsequent paper, the same authors conducted an estimative of taking such preservatives in Brazil considering such food.
Estimative of ingesting benzoate and sorbate acids varied from 0.3 to 0.9 and from 0.2 to 0.3 mg/Kg body weight/day respectively, below ADI (Acceptable Daily Ingestion) established by JECFA, which is 5 mg/kg body weight for benzoic acid and 25 mg/kg body weight for sorbic acid and its salts (WHO, 1997).

Using substitutes for these preservatives in food has been supported by some authors, who have been associating benzoate and sorbate ingestion with the occurrence of several adverse effects in animals and human beings. Metabolic acidosis and seizures have been observed in animals and human beings when fed at high doses of benzoic acid (WHO, 1997), and several studies have reported the development of allergic reactions to benzoate in human beings (JUHLIN et al., 1972; RADEMAKER & FORSYTH, 1989). Regarding sorbates, few cases of intolerance and emergence of allergies and hives have been noted (WALKER, 1990).

Ethanol is a strong bactericide that has been recently used due to its effect against microorganisms. Katsinis et al. (2008) have tested the synergistic effect of preservatives on bread shelf-life with ethanol sprinkle at 0.5%. Sample shelf-life was extended in 43.5% and 38.5% using ethanol with potassium sorbate and calcium propionate, respectively, increasing preservation efficacy. The best results were achieved with the use of ethanol + potassium sorbate, followed by ethanol + calcium propionate, potassium sorbate and calcium propionate. Doulia et al. (2000) demonstrated an increase in bread shelf-life using only ethanol 0.5% and 3.5% in bread slices.

II.3.3. Polyelev amicrobial in food

Natamycin also known as Pimarin as a polyelev amicrobial produced by actinomycete bacterium Streptomyces natalensis and it is used against fungal infections. It is also widely used in food industry to prevent contamination by fungi in cheese and other non-sterile food such as cured meat (ANISCO, 2007).

Basilisco et al. (2001) evaluated the effect of several antifungal on the inhibition of species Phoma graminata isolated from cheese and plant ambient,
verifying that parabens and polyene antifungal at 2.0 and 0.5% concentrations respectively have showed the best effects against this fungus. Fente-Sampayo et al. (1995) when studying fungi of the same gender reported that potassium sorbate 750 ppm and polyene antifungal 25 ppm were the minimum concentrations to inhibit Phoma sp isolated from cheese.

The use of polyene antifungal to control strains of Aspergillus carbonarius that produce ochratoxin A was reported by Medina et al. (2007). Ochratoxin A is a secondary metabolite generated by Aspergillus ochraceus, Aspergillus carbonarius and Penicillium verrucosum, liable for nephrotoxic and carcinogenic effects in animals (IARC, 1993). The authors (MEDINA et al., 2007) have tested polyene antifungal effect on Aspergillus carbonarius mycelium and on ochratoxin A production in fresh grapes extract under different temperature (15, 20 and 25°C) and water activity (0.94 – 0.96) conditions. In preliminary studies performed by these same authors, there was an inhibition of fungal growth using concentrations > 0.5 μg/mL in Malt-Extract Agar medium, and > 0.25 μg/mL in medium with grape fruit. In this paper, the authors suggested that polyene antifungal at 50-100 μg/mL is effective to inhibit Aspergillus carbonarius growth and ochratoxin A production under several ambient conditions in which this species may grow, facilitating reduction of consumers’ exposure to this toxin.

Mann & Beuchat (2008) have studied the use of polyene antifungal combined with other antifungal to inhibit fungal species responsible for producing 1,3-pentadiene compound (undesirable odor of hydrocarbon) resulting from sorbic acid degradation. Every fungus tested (Penicillium brevicompactum, Penicillium roqueforti, Paecilomyces variotii, Aspergillus niger and Cephalosporium fragrans) was capable to grow at 21°C in Parmesan Cheese Agar with potassium sorbate (3500 μg/ml), calcium propionate (3000 μg/ml), or polyene antifungal (20 μg/ml). On the other hand, when potassium sorbate (250 – 1000 μg/ml), calcium propionate (250 – 1000 μg/ml), and/or polyene antifungal (10 – 18 μg/ml) were combined, they were capable to completely inhibit mould growth in the same medium.

Parameters that influence polyene antifungal performance have been studied by several authors. According to Thoma & Kübler (1997) and Lück et al.
(1997), these antifungal are very sensitive to ultraviolet light compared with other antifungals. Gutteridge et al. (1983) confirmed this theory verifying that exposure to UV light has caused a reduction on biological activity greater than air and ferrous ion oxidation. The influence of UV irradiation and formation of cyclodextrins complexes were factors analyzed by Koontz et al. (2003) to assess polyene antimicrobial stability. Since cyclodextrin act as host molecules, they could form complexes with other molecules protecting them from degradation caused by light, oxidation, heat, and alkaline and acid conditions. The authors compared free and complexed antimicrobial acting with different cyclodextrins (β, HP-β and γ). In addition, different atmosphere conditions (air and 75% N₂(25%CO₂), aqueous or methanolic solution, as well as different luminosity conditions (UV light and darkness) were tested. Polyene antimicrobial contents were evaluated by CLAE on 0, 1, 3, 7, and 14 days time points. Results demonstrated a reduction on antifungal content in aqueous solution with an increase of storage time at 4°C in the absence of light. Nevertheless, the compound was considered stable since 92.2% was maintained after 14 days under such conditions. There was no influence of atmosphere in compound contents during the period studied. Polyene antimicrobial complex with cyclodextrins was significantly more stable than this complex in free status. Antimicrobial degradation was not detected after 24h of storage in darkness, at room temperature, and on pure methanol solution. On the other hand, when a 20 mg/L solution of compound in water, both in free and cyclodextrins complexed forms, was exposed to intense fluorescent light (1000 lx), the opposite situation occurred. In this case, antimicrobial was completely degraded after 24h. As per Dekker & Ark (1959), this antimicrobial degradation was dependant on absorption wavelength, which has occurred with greater intensity between 300 and 350 nm.

It is known that polyene antibiotics as nystatin and filipin act on ergosterol found in fungi membrane, contributing to an irreversible interaction that causes a leakage in the cell membrane and leads up to cell death. Bacteria not containing ergosterol are not affected by this compound. However, its action method has not been completely elucidated (WELSCHER et al., 2008).
II.4. MATERIAL AND METHODS

II.4.1. MATERIAL

The following preservatives were used in white bread:
- Potassium sorbate, powder, brand CAAL (as Sorbic Acid), purchased by Danisco do Brasil;
- Natamycin, powder (Natamex®), provided by Danisco do Brasil.

II.4.2. METHODS

II.4.2.1. VALIDATION OF ANALYTICAL METHODOLOGY FOR DETERMINATION OF NATAMYCIN IN BREAD

II.4.2.1.1. Sample preparation

a) Samples for analytical methodology validation (for natamycin)

White bread samples from bread processing company brand of this study and knowingly natamycin-free were used and collected in local market. A cutting mold was used to remove areas with 40 cm² and 5 mm depth from bread sides. Such 40 cm² samples were weighted and used for methodology validation analyses.

b) Samples for sprinkler system consistency validation

Bread samples sprinkled with natamycin and used to validate the sprinkler were provided non-sliced to Chemical Laboratory (CCQA/ITAL) in three different occasions. Each bread side was denominated with letters A to F, as shown on Figure 16, and a cutting mold was used to remove an area of 40 cm² with 5 mm depth from each side.
c) Samples originating from industrial bread processing

Samples originating from industrial processing have summed up 30 loaves sprinkled with natamycin at 3 μg/cm² concentration and 30 loaves at 6 μg/cm² concentration. Three samples sprinkled with sorbic acid were collected for preservative analysis. For each natamycin concentration, 15 samples were randomly selected and analysed, as well as 3 samples sprinkled with potassium sorbate. For these determinations, bread samples were totally homogenized and then weighted at appropriate quantity for analyses that were performed in duplicate.

Since analyses were performed in the entire bread sample, theoretical natamycin concentration was calculated on bread surface adopting total bread weight (500 g) and a surface area of 1480 cm² as parameters (Figure 17).
II.4.2.1.2. Analysis of natamycin migration to the 2nd layer

In order to verify natamycin migration to the 2nd layer, placed 5 mm below bread surface, two sides (E and F) of three bread samples sprinkled at 6.0 μg/cm² concentration were analyzed, as shown in Figure 18. The 6.0 μg/cm² concentration was defined in a critical analysis meeting with Danisco company, considering that should no migration be observed in this level, at natamycin concentration of 3.0 μg/cm² it probably would not occur either.

Figure 18. 5 mm layer of bread surface.

Natamycin standard

A secondary natamycin standard with 88.99% purity supplied by Danisco company was used.

II.4.2.1.3. Validation of natamycin analytical methodology

To validate analytical methodology for natamycin determination in white bread linearity, limit of detection, precision and accuracy were used as evaluation parameters.

Linearity corresponds to method capacity to provide results directly proportional to analytes concentration or mass range. According to IUPAC (International Union of Pure and Applied Chemistry) in a technical document published by Thompson et al. (2002), calibration curve must have six or more concentration points, which should be analyzed in duplicate. In this paper, calibration curve was built with 6 points injected in duplicate at a concentration range of 0.3 to 3.0 μg/mL.
Method precision was provided by coefficients of variation from analyses performed in 5 replicates of natamycin-free white bread sample spiked with natamycin standard.

Accuracy was determined through recovery obtained from analyses of natamycin-free white bread sample spiked with natamycin standard at 1.5, 3.0, and 6.0 µg/cm² level.

Limit of detection was calculated in accordance with INMETRO [National Institute of Metrology, Standardization and Industrial Quality] (2007), using the following formula:

Limit of detection (LOD) = $t \cdot s$

where:
$t =$ abscissa of Student distribution dependent on sample size
$t = 3.75$ for 5 replicates

$s =$ standard deviation of natamycin sample added at the lowest level (1.5 µg/cm²).

II.4.2.2. NATAMYCIN DETERMINATION

Methodology used was validated and standardized to determine natamycin in white bread using parameters suggested by INMETRO (2007). Natamycin extraction was performed with methanol followed by extract dilution and subsequent clean-up by cooling, and separation was conducted by high performance liquid chromatography with ultraviolet detector. These procedures are described below.

As per IDF Standard 140 A methodology (1992), ten grams of sample (or 40 cm²) were weigh in a 250 mL Erlenmeyer flask. 100 mL of methanol (HPLC grade) were added and the flask was stirred in an orbital shaker for 1 hour and 30 minutes. The extract obtained was diluted with 50 mL of ultrapure water (MilliQ®) and put in a freezer with temperature around -20°C for approximately 1 hour for fat precipitation. Then the extract was taken from freezer and immediately filtered in common filter paper, discarding the first 10 mL. Extract
was filtered again in 0.45 μm pore size membrane and injected into the chromatographer.

Methanol:water:acetic acid (60:40:5) was used as mobile phase at a flow rate of 1 mL/min flow. An analytical column of octadecysilyl stationary phase (C18) with 5 μm particle size and 4 x 250 mm of dimension was used. The injection volume was 20 μL were injected and detection was performed by ultraviolet (λ – 303 nm).

Identification was done by comparison of retention times of peaks obtained in samples and standards.

For natamycin quantification in samples and spiked control samples, an external standardization was used with a calibration curve, where peak area referring to natamycin content in the sample is compared with the equation obtained from the linear regression (peak area ratio versus natamycin concentration).

II.4.2.3. DEFINITIVE TESTS IN INDUSTRIAL LINE

II.4.2.3.1. Bread processing

Bread was industrially processed in a company of São Paulo/SP metropolitan region following commercial proprietary formulation of the company. A completely automatic processing method (Sponge and Dough) was used to manufacture bread according to the process flowchart exhibited in Figure 19.

This process may be divided into three stages: 1) sponge or liquid ferment; 2) dough mixture and development; 3) fermentation and baking.
Figure 19. Flowchart of bread process.

a) Liquid sponge

Liquid sponge phase was composed of a yeast suspension in water and other ingredients, including flour, at a percentage varying from 0 to 70% (the specific information belongs to the company). Liquid sponge was fermented at controlled temperature from 27 to 29°C for approximately 2 hours.
b) Dough mixture and development

Every ingredient was added and mixed to liquid sponge in a continuous mixer specifically designed. Ideal dough development has occurred in mixer where dough has acquired structure, elasticity and extensibility enough to keep the gases produced during growth phase. This structure obtainment was achieved through mechanical work of the dough for 40 to 50 seconds.

c) Fermentation and baking

The dough was put into appropriate molds and taken to continuous fermentation chamber with strict temperature and humidity control for approximately 40 to 50 minutes. To conclude fermentation stage, dough was baked in a continuous tunnel-type oven, then following by conveyor belt for sprinkle stage of preservatives which concentrations are shown in Table 13. Breads were submitted to continuous cooling system in conveyor belts and packed into company's polyethylene bags.

Table 13. Preservative type and concentration used on each assay

<table>
<thead>
<tr>
<th>Assay</th>
<th>Preservative Type</th>
<th>Concentration</th>
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<tbody>
<tr>
<td>1</td>
<td>-</td>
<td>0.0</td>
</tr>
<tr>
<td>2</td>
<td>Sorbic Acid</td>
<td>0.1 g/kg</td>
</tr>
<tr>
<td>3</td>
<td>Natamycin</td>
<td>3 μg/kg</td>
</tr>
<tr>
<td>4</td>
<td>Natamycin</td>
<td>6 μg/kg</td>
</tr>
</tbody>
</table>

For each treatment in industrial scale 180 bread samples were processed and transported for storage chambers of Instituto de Tecnologia de Alimentos [Food Technology Institute] (ITAL)-Campinas/SP. Cereal Chocotec was liable for storing bread Control, Sorbic acid and Natamycin (3 and 6 μg/cm³) in acclimation chambers, as well as for distributing the product on dates scheduled for stability follow-up through physical, microbiological and sensory analyses in the respective analytical labs in charge.
II.4.2.4. ANALYSES PERFORMED IN BREAD ORIGINATING FROM DEFINITIVE TESTS IN THE COMPANY

II.4.2.4.1. Natamycin analysis

Methodology described on item 2.2.2.3 was used. Natamycin content was determined in bread sprayed with 3.0 μg/cm² and 6.0 μg/cm². 30 samples of each level were collected, of which 15 were randomly chosen. All analyses were performed in duplicate.

II.4.2.4.2. Sorbic acid analysis

Sorbic acid analyses were conducted by high performance liquid chromatography with ultraviolet detection based on methodology already validated for bread and published by Bui & Cooper (1987). Methodology involves quantitative extraction with acetone and acetic acid 1% followed by clarification with potassium and zinc salts. Separation was performed by high performance liquid chromatography with ultraviolet (261 nm), respectively, and quantification was performed by external standardization.

II.4.2.4.3. Microbiological analysis

Microbiological analyses were conducted for 30 days in samples with natamycin at 3 and 6 μg/cm² dosages, as well as Control and Sorbic acid samples stored at 23 and 35°C. Commercial samples of A, B, and C brands purchased by Danisco company on local market were kept at room temperature and analyzed for the presence of mould and yeast for 12 days.

For mould and yeast analyses, bread was homogenized through its complete maceration, and after this step an aliquot of 25 g was collected for analysis. For serial decimal dilution, a peptonated water solution 0.1% was used and Dichloran Glycerol 18% medium was applied for plating. Plates were incubated at 25°C for 5 days and results reading was done in CFU/g (Colonies-Forming Unit per gram) according to Downes & Ito, 2001 methodology. Analyses were performed in triplicate, considering each unit (package) as a replicate.
II.4.2.4.4. Visual evaluation

A daily visual evaluation of bread was performed by Cereal Chocotec and CCQA for 30 days to check the presence of visible mould on the surface of bread stored in acclimated chambers at 23°C and 35°C.

Commercial samples of A, B, and C brands were maintained at room temperature and daily monitored for 12 days with respect to mould presence.

II.4.2.4.5. Texture analysis

Bread firmness was determined by texturometer SMS (Figure 20), model TA-XT2i (Godalming/Surrey, UK), as per method 74-09 (AACC, 2000) with the purpose of evaluating the influence of preservatives in maintaining texture characteristics of white bread. For this analysis, probe SMS P/36R platform HDI/90 was used under the following operating conditions: measure force in compression, pre-test speed: 1.0 mm/s, test speed: 1.7 mm/s, post-test speed: 10.0 mm/s, and penetration distance 40%. Samples with natamycin at 6 μg/cm² dosage stored only at 23°C without preservative sprinkle (control) and with standard market preservative (sorbic acid) on days 1, 12, and 28 were analyzed. In this study, texture and sensory analyses were not performed for 3 μg/cm² dosage, upon Danisco company’s request. Texture results were obtained from using three bread samples of each treatment and 10 central slices of each loaf, totaling 30 readings for each sample.

Instrumental firmness test results were statistically evaluated by a comparison of averages by Tukey’s test (SAS, 1997).
II.4.2.4.6. Sensory Analysis

Sensory Characteristics Description Test

Control, Potassium sorbate and Natamycin 6 μg/cm² bread samples kept only at 23°C were evaluated on days 1, 12, and 28 after manufacturing for sensory characteristics of aspect, odor, texture/ sensation in mouth, and taste by a team of evaluators from Lafise-CCQA/ ITAL selected for sensory acuity. Only on day 28 Natamycin 3 μg/cm² sample was assessed by the same team of evaluators following Danisco company's request and aiming to compare this sample with control sample.

Test of Difference from Control

As defined in a critical analysis meeting with Danisco company, in case of perception of a difference on any sample compared to Control, a Test of Difference from Control must be applied. As on day 12, samples with Sorbic acid and Natamycin 6 μg/cm² showed some difference from Control as per evaluators' perception, who described sensory characteristics of each sample. On the next day, a test of difference from control was applied to evaluate odor...
and taste of each sample compared with Control (C). On day 28, the same samples and also a Natamycin 3 μg/cm² one were evaluated for odor and taste and compared with Control (C).

On both dates (days 13 and 28), samples were assessed by a team of 20 evaluators selected for sensory acuity. The following Scale of Difference from Control with 6 points was used:

1 = Superior to C in odor or taste;
2 = Equal to C in odor or taste;
3 = Inferior to C in odor or taste, with no odd odor or taste detected;
4 = Inferior to C, with mild odd odor or taste;
5 = Inferior to C, with moderate odd odor or taste;
6 = Inferior to C, with strong odd odor or taste.

For the assessment each evaluator received, in addition to Control identified as such, three (or four) samples coded with 3-digit random numbers with Control also coded between the samples. Samples presentation followed a balanced complete block design.

Tests were conducted in individual cubicles equipped with Compusense Five program version 4.8, and red lightening to blind occasional visual differences between the samples.

Results from evaluators’ assessments who rated codified Control with scale values equal or superior to 4 were excluded, whereas results obtained in correct judgments (rare 1, 2, or 3 of scale for codified Control) were submitted to an analysis of variance and comparison of averages by Dunnett’s test indicated to compare samples with a standard (ABNT, 1995).

II.5. RESULTS AND DISCUSSION

II.5.1. RESULTS OF VALIDATION OF ANALYTICAL METHODOLOGY FOR DETERMINATION OF NATAMYCIN IN BREAD

• Linearity – Calibration Curve

Linearity was determined by constructing calibration curves with standard solutions of natamycin in the range of 0.3 to 3.0 ng/mL. Two injections were made at each level.
Means areas were used to elaborate the calibration curve. Coefficient of determination (R²) was 0.9949 and the equation obtained was: \( y = 6922x - 333.29 \), where \( y \) was the area obtained in chromatogram and \( x \) was natamycin concentration in \( \mu g/mL \). Correlation coefficient of calibration curves were always higher than 0.99 and shows linearity of detector response at concentrations studied.

In Table 14 below, values of concentrations and areas obtained from standard are exhibited, and Figure 21 shows mean calibration curve of natamycin.

<table>
<thead>
<tr>
<th>Table 14. Areas obtained in several natamycin concentrations analyzed by high performance liquid chromatography</th>
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<tbody>
<tr>
<td><strong>Concentration (µg/mL)</strong></td>
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<tr>
<td>----------------------------</td>
</tr>
<tr>
<td>0.3</td>
</tr>
<tr>
<td>0.5</td>
</tr>
<tr>
<td>1.0</td>
</tr>
<tr>
<td>1.5</td>
</tr>
<tr>
<td>2.0</td>
</tr>
<tr>
<td>3.0</td>
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</table>

Angular coefficient (a) | 7000.85       | 6843.17     | 6922.02   |
Linear coefficient (b)  | -492.35       | -184.22     | -333.29   |
Correlation coefficient (r) | 0.9944       | 0.9977     | 0.9974    |

SD = standard deviation estimaive; CV = Coefficient of variation (%)