

ENSURING THE STABILITY OF NATAMYCIN ON SHREDDED CHEESE

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### ABSTRACT

Natamycin is an antimycotic compound that is widely used in the cheese industry to increase the shelf life of cheeses, especially shredded cheeses, by inhibiting the growth of molds. Natamycin is applied to the surface of cheese as an aqueous suspension or as a powder. However, natamycin is not readily water soluble making it harder to distribute evenly over shredded cheese. Natamycin is degraded by ultraviolet (UV) light at wavelengths of 350 nm and below. Typical packaging applications do not provide adequate UV protection causing natamycin to degrade.

This work was undertaken to determine the efficacy of UV absorber film to prevent UV light degradation of natamycin on the surface of shredded cheese. Current accepted methods to determine concentration of natamycin were evaluated for appropriateness in natamycin degradation studies. The use of cyclodextrins to increase water solubility was tested to see if a uniform distribution of natamycin over the shredded cheese could be done effectively. Furthermore, a known application of mold was performed to see how well natamycin and each of its applications could prevent visible mold growth from occurring. The International Dairy Federation recognizes two methods to quantify natamycin on shredded cheese: high performance liquid chromatography (HPLC) and spectrophotometry. Concentrations of natamycin in aqueous suspensions were determined using both methods. Results show that spectrophotometry is flawed when quantifying the amount of active natamycin because the method gives erroneously high results. The amount of active natamycin is not accurately quantified using spectrophotometric techniques because it cannot separate the active form from the inactive form of natamycin.

Polymer packages containing a UV absorber (11.4% light transmission at 350 nm) allow significantly less UV-associated degradation of natamycin than those packages that lacked a UV protectant (90.0% light transmission at 350 nm) ( $p < 0.05$ ). Incorporating a UV absorber into a package helps protect natamycin and its various complexes from UV light degradation, which can increase the shelf life of shredded cheese. However, even with a UV absorber, natamycin is still able to degrade.

Natamycin was complexed with different cyclodextrins to help better solubilize natamycin -  $\beta$ -cyclodextrin, hydroxy-propyl  $\beta$ -cyclodextrin and  $\gamma$ -cyclodextrin. Using cyclodextrins to apply natamycin more uniformly onto shredded cheese did not significantly increase the consistency of distribution ( $p < 0.05$ ). Variability was uniform throughout all treatments with the exception of HPBCD complex. After 27 days, all of the UV packages treated with each of the cyclodextrin treatments containing shredded cheese began to show visible mold growth. Those packages stored in total darkness remained mold free through the duration of the experiment ending on day 62.

When untreated with natamycin and an initial concentration of  $10^1$ - $10^2$  spores/gram of *Penicillium roqueforti*, shredded cheese remained free from visible mold growth for 24 days in total darkness at 4°C. Samples treated with one of the natamycin treatments were able to remain mold free for at least 9 more days, showing visible signs of mold growth at day 33. There was no statistical difference between the treatments of dry natamycin, aqueous suspension natamycin,  $\beta$ -cyclodextrin-natamycin complex, and  $\gamma$ -cyclodextrin-natamycin complex ( $p < 0.05$ ). However, there was a difference with the use of hydroxy-propyl  $\beta$ -cyclodextrin-natamycin complex. Hydroxy-propyl  $\beta$ -cyclodextrin-natamycin complex allowed the shredded cheese to last for 41 days, 17 days longer than the control sample.

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## CHAPTER 1: INTRODUCTION

### Cheese Industry & Obstacles

With a creamy texture and 1 gram or less of carbohydrates per ounce, cheese has been growing in popularity (4). According to Hains (5), cheese is consumed in 98% of U.S. households and 83% of restaurants have cheese on the menu. As consumers become busier, the demand for convenience foods, such as shredded cheese, has escalated greatly. In 1998, retail shredded cheese sales and dollar sales increased compared to the previous year.

Increased surface area of the shredded cheese allows mold to have more surfaces on which to grow allowing the cheese to spoil quicker (3). Manufacturers have tried to limit the growth of mold by modifying the atmosphere within the package. Oxygen is required for mold growth and replacing the atmosphere of a package with nitrogen and other inert gases prior to package sealing, mold growth is inhibited (1). Changing the atmosphere of the package protects the cheese while on the grocer's shelves but once purchased by the consumer, further protection is needed. When the consumer first opens the package of shredded cheese, the modified atmosphere packaging is lost allowing oxygen to enter the package giving mold the oxygen required for growth.

To help further protect the cheese, an antimycotic such as sorbic acid or natamycin is added. Natamycin kills germinating mold spores before the consumer can see visible mold growth. Without the added protection of natamycin, the cheese is able to spoil quicker in the consumer's refrigerator resulting in returned product. With shredded cheese accounting for nearly a quarter of all the cheese sold in the United States (2), cheese processors are investigating ways to increase shelf life after the consumer has released the modified atmosphere while still keeping a high quality product.

### Sorbic Acid and Cheese Preservation

Many different types of antimicrobials are used to help extend the shelf life of cheese. Sorbic acid is commonly used in the cheese industry. This straight chained,  $\alpha$ ,  $\beta$ -unsaturated *trans-trans*, 2, 4-hexadienoic monocarboxylic aliphatic acid with the molecular formula of  $\text{CH}_3\text{-CH=CH-CH=CH-COOH}$  (7) may be used as a free acid or as a salt when

combined with potassium or calcium. Potassium sorbate has the highest water solubility (138g potassium sorbate/100g water), while calcium sorbate is less soluble (1.2g potassium sorbate/100g water) (6).

In order for sorbic acid to be effective against a cell, it must be able to penetrate the cell wall, which requires the chemical to dissociate and enter the cell. The amount of dissociation is dependent on the pH of the environment. The closer to neutral, the harder it is for sorbic acid to dissociate. Sorbic acid works best at a pH close to the pka (4.75). At this pH, sorbic acid is 50% undissociated (7). Once in the cell interior, sorbic acid is able to destroy the cell. However, some microorganisms are able to change the structure of sorbic acid into a chemical compound that is not harmful. *Penicillium* species are able to deplete sorbate and are able to grow. *P. puberulum*, *P. roqueforti*, and *P. cyclopium* are some of the *Penicillium* species that are able to degrade sorbic acid into 1,3-pentadiene, rendering it harmless (6). Cheeses are a good target for the use of sorbic acid due to the high pH range and sorbic acids ability to target molds. Concentrations of 0.05-0.07% are typically used for cheese preservation (6).

## **Research Objectives**

The objectives of this research are: (a) to determine if a UV absorber incorporated into a polymer package can help maintain the stability of natamycin on shredded cheese under fluorescent lighting; (b) to determine if the shelf life of shredded cheese can be extended while removing the modified atmosphere from the package; (c) to quantify the effect of the delivery system of natamycin as a dry powder, an aqueous suspension, and as an aqueous solution when complexed with cyclodextrin; and (d) to quantify the effect of the initial mold population in shredded cheese on the time duration until visible mold growth occurs for different concentrations of natamycin.

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## CHAPTER 2: LITERATURE REVIEW

### Natamycin

#### *History*

In 1955, a *Streptomyces* strain was found to have natural antibiotic production. The strain was isolated near the town of Pietermaritzburg in Natal, South Africa, which led to the name *Streptomyces natalensis* (42). This antimycotic was originally named pimaricin, named after the city of discovery, Pietermaritzburg. However, the World Health Organization created a regulation that any antibiotic produced by this strain of organism must have a name ending with "...mycin" changing the name to natamycin (*natalensis*). In 1959, researchers had discovered that many different strains of *Streptomyces* have the ability to produce natamycin. Today, natamycin is most commonly produced commercially by either *Streptomyces natalensis* or *Streptomyces gilvosporeus* (6, 37).

#### *Production*

Patents protect the different ways that natamycin is produced commercially and in laboratory settings. Most methods are based on the same principles that are characteristic to natamycin particularly the principal of solubility. Natamycin is not readily soluble in water but it is soluble in other aqueous compounds. Natamycin may be extracted from a culture broth using butanol after acidification to a pH of 3.0. Once extracted and purified, the natamycin is precipitated from the extract and then spray dried to an activity level of 950 µg/mg (37).

#### *Properties*

Natamycin contains a chromophore of four conjugated double bonds classifying it as a polyene antifungal antibiotic. Natamycin, shown in Figure 2-1, contains a 25-carbon lactone ring that contains this chromophore of double bonds. The compound is a white, tasteless and odorless powder and in the solid state, natamycin is crystalline. Temperatures

of ~200°C will decompose the crystals while temperatures below 120° C have no effect on the antimycotic activity. Considered a polyene macrolide, natamycin has a molecular weight of 665.75 and the empirical formula of C<sub>33</sub>H<sub>47</sub>NO<sub>13</sub> (7, 37, 40).

Natamycin is stable in both a dry and aqueous states when stored at room temperature to 37°C. Natamycin becomes degraded when cleavage of the chromophore structure occurs. Light of all types can be detrimental to the compound, particularly ultraviolet (UV) light with wavelengths of 350 nm and below. Under UV light, the tetraene structure is quickly inactivated and there is a significant loss in antimicrobial activity (37, 40). If the packaging material allows UV light to penetrate, degradation of the compound will occur. Because of this, natamycin is typically stored in amber glass to give it photoprotection from the UV wavelengths (45).

Solubility of natamycin depends on the type of solvent used. In polar organic solvents, natamycin tends to dissolve readily, while in water natamycin is practically insoluble (37). The limited water solubility has a lot to do with the structure of natamycin. The lactone ring contains three free hydroxyl groups as well as a zwitterion. On the opposite side of the structure, a chromophore of four double conjugated bonds is found. Water solubility is decreased with the increasing number of these hydrophobic natured bonds (47). The exact solubility of natamycin is unknown. While some report a maximum solubility of up to 50-100 mg/L (15, 42), others report a maximum 30 mg/L (6), a difference of up to 70 mg/L. Based on the structure and nature of the compound, 30 mg/L seems to be the more agreed upon solubility.

The pH of the solvent can also influence the solubility and the stability of natamycin. Natamycin is stable at pH values of 5.0-9.0. At extreme pH ranges, degradation of natamycin is rapid. However, natamycin becomes soluble allowing the compound to have an increased antifungal activity if only for a short time (37, 40).

#### *Mechanism of Antimycotic Action*

Yeast and fungi cells have eukaryotic membranes composed of lipids, phospholipids, proteins, and sterols. The primary sterol in fungal cells is ergosterol. Polyene antibiotics such as natamycin are able to bind irreversibly to ergosterol, which causes the cell membrane to be disrupted (6, 37, 40). This high affinity for ergosterol is due

to the hydrophobic region of the sterol. Due to the high hydrophobicity of natamycin, this region of the sterol is an attractive site allowing the compound to break the phospholipid-sterol interaction that keeps the membrane stable. Research suggests that once many of the sterols located on the membrane have been reached by the compound, a central hydrophilic pore is created in the membrane allowing the potassium ions and other small molecules to begin rapidly leaking from the cell membrane (23), eventually causing cell lysis.

Bacteria are prokaryotic cells and do not contain sterols in their outer membrane. Due to the lack of sterols, natamycin is not effective in eliminating unwanted bacteria from products. While natamycin is effective in eliminating mold growth, it is not effective in removal of toxins produced by molds (40). This is essential to understand so that the mold can be eliminated before toxin growth occurs. It is important to note that while natamycin is effective on young dividing fungal cells, it is not effective against fungal spores. It is not until the fungal spores begin to germinate and divide does the natamycin take effect (34).

#### *Quantification of Natamycin*

Quantification of natamycin can be done using both HPLC and spectrophotometric methods according to the International Dairy Federation (28). HPLC analysis uses a reverse C8 column with UV detection. This method can determine the degradation products that have a shorter retention time. When degradation products are formed, the HPLC method has formation of multiple peaks allowing the degradation products to be separated from the intact natamycin (17). This is helpful when quantifying the amount of natamycin once exposed to UV light. When natamycin is in the presence of UV light, degradation products have a shorter retention time and can form up to seven separate peaks while pure natamycin unaffected by UV light forms a single peak (17, 46).

Quantification of natamycin can be analyzed based on the spectral characteristics of the compound using spectrophotometry. Biological activity and the intact tetraene structure are directly related. This correlation can be determined based on the baseline between the absorbance at 311 nm and 329 nm or calculated using equation 1:

$$E = E_{317} - \frac{2}{3}(E_{322}) - \frac{1}{3}(E_{329})$$

1

This equation takes into consideration the cheese matrix that interferes with the absorption wavelengths (17, 27)

While the International Dairy Federation suggests using the spectrophotometric method in analyzing the amount of natamycin on cheese, it does not separate the amount of actual amount of biologically active natamycin from the degraded natamycin. Brik (6) discovered that if natamycin becomes inactivated by an extreme pH, such as at a pH of 12, then using the baseline method shows no decrease in amount of natamycin present. This could be the result of a natamyoic acid formed by the saponification of natamycin. Using spectrophotometry in stability analyses of natamycin may not be the most effective way to quantify the amount of biologically active natamycin present after degradation has occurred.

### *Toxicology*

Studies have been conducted to determine if natamycin or its degradation products are toxic to humans. Mutagenicity has been studied using the Ames test with various bacterial strains (40). The degradation products of the commercial product Devlocid®, a compound containing 50% natamycin and 50% sodium carbonate, are aponatamycin, dinatamycinolidediol, and mycosamine. All of the degradation products as well as natamycin tested negative in each of the mutagenicity tests performed. It has been shown in many of the studies that neither natamycin nor the degradation products were absorbed into the body. In addition to all of the studies, natamycin has proved itself over the years by controlling mold growth in various food products while maintaining a safe reputation for human consumption (40).

### *Uses in the Food Industry*

Natamycin has many characteristics that make it applicable for use in the food industry. The compound gives no adverse effects on the consumer, does not effect the quality characteristics of the product, is effective at a low dosage, and remains active for a long duration of time if protected from light (40). Natamycin can also be used in a variety of

food products such as cheese, sausages, fruits, and an assortment of beverages.

Natamycin has been successful in a variety of cheeses ranging from soft to hard. The compound has also been effective when applied as an aqueous polymer dispersion or when placed in brine solutions used for cheese dips (40). As for sausage studies, natamycin prevents mold spoilage during both the process and storage of the product. Sausages treated with 0.2% natamycin have shown greatly improved protection from spoilage molds than other antimycotic agents used. Fruits such as strawberries, cranberries, apples, pears, and raspberries can be treated with natamycin either in the fields or shortly after harvest giving the fruit an increased shelf life (40).

Numerous beverages such as juice, beer, wine, lemonade, and iced tea are affected by mold growth every year before the consumer ever opens the package. With the addition of 1-5 mg/L of natamycin, these beverages are able to stay fresh longer until desired by the consumer. Natamycin also works well with beverages with high pH values. Currently, the pH is lowered in these beverages so that sorbic acid can be used to prolong the growth of fungal organisms. However, sorbic acid develops an off flavor at a low pH making it a less desired alternative (40).

### *Regulatory Status*

On December 1, 1998, the Food and Drug Administration (FDA) amended the food additive regulations to include using a safe dry form of natamycin in cheeses for cut and sliced cheese only. It stated that no more than 20 mg/L of the additive could remain on the final product and could be applied by dipping or spraying using an aqueous solution containing 200-300 mg/L of the additive (10). This however, did not help clarify the use of the antimycotic on grated and shredded cheeses to the food industry (48). On March 8, 2001, the FDA responded to the petition filed by Cultor Food Science, Inc., DSM Food Specialties and Protein Technologies International by stating that natamycin could not exceed 20 mg/L and this limitation would not restrict the process of application nor the physical form of the cheese, whether it be sliced, cut, or grated (11).

## Cyclodextrins and their Molecular Inclusion Complexes

### *History*

Villiers first discovered cyclodextrins in 1891. At first, he had only discovered both  $\alpha$  and  $\beta$ -cyclodextrins, and he called these compounds “cellulosine” because of their similarities with cellulose. Years later, Schardinger began studying *Bacillus macerans*, an organism thought to cause food poisoning. However, in his published report it was noted that the organism had produced two crystalline byproducts that resembled “cellulosine” with no signs of food toxin production. Schardinger characterized these crystalline dextrins as compounds that seemed to form complexes with different types of organic compounds. In the 1930s, Freudenberg began to study the dextrins more closely. He and his colleagues came to the conclusion that these compounds were comprised of maltose units containing  $\alpha$ -1,4-glycosidic linkages (43).

Later, in the 1940s through the 1950s, more cyclodextrins were discovered including  $\gamma$ -cyclodextrin and other larger cyclodextrins, all which could form inclusion complexes. In the 1960s, cyclodextrins could be prepared on a large laboratory-scale. Most of their chemical and physical properties had been determined, however much was still unknown concerning their toxicity. It was not until the late 1970s that it was determined that cyclodextrins were not harmful or toxic unless complexed with a hazardous material or if the compound itself contained impurities (43). This led to a large increase in the number of studies performed with cyclodextrins so that they could be incorporated into many different chemical aspects. Today, the original  $\alpha$ ,  $\beta$ , and  $\gamma$ -cyclodextrins are used along with a variety of other cyclodextrin derivatives in a number of various industries (43).

### *Physical and Chemical Properties*

Cyclodextrins (CD) are characterized as crystalline, homogeneous, and non-hygroscopic compounds. All are torus-like macro-rings comprised of glucopyranose units. The three main types of cyclodextrins are  $\alpha$  ( $\alpha$ -CD),  $\beta$  ( $\beta$ -CD), and  $\gamma$ -cyclodextrins ( $\gamma$ -CD) each containing 6, 7, and 8 glucopyranose units respectively. With an increased number of glucopyranose units on a CD, the properties of the structure become more rigid and less

water-soluble (43).

The ring structure of a CD resembles a conical cylinder and has alternating hydrogen atoms and glycosidic oxygen bridges lining the inner cavity. These unpaired electrons on the glycosidic oxygen bridges create a non-polar effect allowing water molecules to form a polar-non-polar interaction. Because of this unstable relationship, when given the chance, water will leave the cylinder when a more appropriate compound is introduced allowing for the CD to readily form a complex with other compounds through noncovalent bonds (21, 43). On the outer edges of the compound is where the hydroxyl groups lay giving cyclodextrins their water solubility characteristics as well as the ability to react with polar groups to form hydrogen bonds (26).

#### *Preparation of Cyclodextrin Inclusion Complexes*

Creating a CD inclusion complex can be done in a variety of ways. They can be formed in solution, suspension, percolation, kneading, melting, heating in a closed container, or cogrinding (44). These methods have their advantages and disadvantages but all have the same purpose: to obtain a strong and stable complex with the guest compound of choice. Complexation in solution will be the focus in this paper.

Water is found inside the CD complex until a new, more hydrophobic molecule is introduced. A guest molecule that is less polar than water and one that can fit tightly into the CD cavity will form a good inclusion complex (43, 44). Of all the CDs available, the most widely used is  $\beta$ -CD because of its adaptable cavity and how easily it is produced (26, 44).

Complexation in solution for CD complexes is relatively simple. The guest compound and CD of choice ( $\alpha$ ,  $\beta$ , or  $\gamma$ -CD) are placed together in a beaker with a certain amount of water depending on the guest compound. Once the two are placed together, stirring is done with the addition of heat. Research has shown that stirring alone works, however, extra agitation in conjunction with the stirring can also increase the amount of complexation that takes place. Once the compounds are complexed, drying is a common method for storage. The compounds can be dried in different ways. Vacuum evaporation, spray-drying, freeze-drying, fluid bed dryers, tray dryers, and hot air ovens are all common practices in the industry (26, 44).

### *Strength of Cyclodextrin Complexes*

The strength of the CD complex depends on the compound that is being complexed within the CD. An increased interaction of the inner walls of the CD with the desired compound creates a stronger and more desired complexation interaction. The forces that hold these complexes together consist of van der Waals forces, hydrophobic interactions, and dipole-dipole interactions. While these forces together give a good and stable complex, the interaction between the guest compound and the CD are not chemically bound and can be separated at any time (26).

The types of groups along the guest compound can also promote a stronger bond between the guest and host compounds. According to Szente (44), the greater the number of methyl or ethyl groups along the guest molecule, the stronger the interaction between the guest and the CD because of the CD hydrophilic characteristic.

### *HPLC Analysis*

HPLC can be used to quantify the guest molecule within the cyclodextrin complex once it is dissociated from the complex into the mobile phase (44).  $\beta$ -CD is not readily water-soluble and methanol further decreases the solubility. This makes  $\beta$ -CD a limiting factor when quantifying the amount of the guest molecule in the complex. Solubility of  $\beta$ -CD can be increased with various cosolvent/water mixtures (14) such as a methanol/water/acetic acid (v/v/v) mixture.

### *Uses in the Food Industry*

CDs are used in a variety of ways in the food industry as food-processing aids to add wanted flavor compounds and to remove unwanted flavors. The use of CDs has grown and reached 20-30% while 80-90% of that growth is within the food industry (24). The primary CD used in the food industry is  $\beta$ -CD because it is not only the easiest CD to produce, it is also the easiest to purify (25). Hashimoto (24) summarizes the many applications of CDs in the food industry. One application for CDs is protection against oxidation, light and heat to which many compounds are susceptible. Products that use CDs for this application consist

of spices, nuts and seeds, vinegar and oil. CDs are also used to change the perception of offensive tastes and odors. Lastly, CDs aid in improving food quality as well as advances in technology in the food industry. Progress has been made in the food industry in improving emulsification, stabilizing sweeteners that are easily degraded, prolonged storage, and prevention of turbidity in high quality juices (24).

### *Regulatory Status*

In 1997, the Food and Drug Administration (FDA) received a petition from Wacker Bicochem Corporation asking that  $\gamma$ -CD be considered for Generally Regarded as Safe (GRAS) status for the use as an emulsifier, stabilizer, carrier, and formulation aid. The FDA granted GRAS status of  $\gamma$ -CD and made it GRAS Notice No. 000046 (18). In 2000, the FDA received another petition from Wacker Biochem Corporation asking that  $\beta$ -CD be considered for GRAS status for use as a flavor carrier and protectant. The FDA accepted this claim and stated that the compound be considered GRAS Notice No. GRN 000074 based on the company's submission, not on FDA testing (19).

## **Ultraviolet Light Absorber Packaging**

### *Effects on Plastics & Products*

Ultraviolet (UV) light negatively impacts plastics and can effect physical properties such as brightness, color, opacity, and can cause cracks along the surface. Chemical damage can occur by the formation of carbonyl groups, carboxylic acids, and hydroperoxides, all which degrade the plastic material. To decrease the degradation rate of plastics, UV absorbers can be added to the plastics to stabilize them with success demonstrated in polyethylene (PE), polypropylene (PP) and polyethylene terephthalate (PET) (20, 22)

While there has been an increase in methods of protecting plastic integrity, more focus has been on how to protect the contents within the packaging. Today there has been a rise in the amount of products offered in plastic, but more specifically there has been an increase in clear plastics available on the market. Clear plastics allow the consumer to see the product within the packaging but do not offer protection from light exposure during

storage. Many food products and their ingredients are light sensitive and can easily degrade under both visible and UV light changing the products color, flavor or nutritional value (2).

### *Protection Methods*

Barrier layers put into plastic packaging protect products in different ways. While some layers focus on oxygen scavenging, others focus primarily on light. These layers can absorb or reflect the light that penetrates the plastic. Absorption of light is dependent on the thickness of the package itself. Equation 2 shows the Beer-Lambert law giving the relationship of UV absorption along with sample thickness (38):

$$I = I_0 e^{-kx} \quad 2$$

where:

- I = intensity of light transmitted by material
- $I_0$  = intensity of incident light
- k = absorbance of packaging material
- x = thickness of packaging material

The amount of light absorbed is based on both the amount of light as well as the characteristics of the packaging material (22, 38).

Taking into consideration the Beer-Lambert law, characteristics are generalized for plastics. Characteristically a plastic might be able to absorb wavelengths up to 300 nm. However, the thickness might be too thin to obtain the maximum amount of absorption. Clear plastics are able to reduce the amount of UV light that can penetrate within a package with various degrees. PET is a common plastic used in the food industry due to its clarity, gloss, shatter-resistance and variety of forms and shapes that it can create. PET however offers limited protection of products within the packaging, up to 300 nm. The ideal package would block all UV radiation, up to 400 nm while allowing the contents of the package to still be seen by the consumer (36, 16). This would protect most food products that are in danger of UV destruction from 200-380 nm. This range would protect different types of

vitamins and other compounds in food products that are sensitive to UV light. UV absorbers can be added to a variety of plastic polymers such as PET, PP and HDPE during their manufacturing process (2).

### *Penicillium roqueforti*

#### *Classification & Characteristics*

The classification of *Penicillium* is as follows:

Kingdom - Fungi

Phylum - Ascomycota

Order - Eurotiales

Genus - *Penicillium*

The phylum Ascomycetes can reproduce both sexually and asexually (8). Sexually producing Ascomycetes are known as teleomorphs. These teleomorphs produce ascus (pl. asci) through mitosis in what are called ascocarps. The fungi in this phylum can be further classified by their ascocarp characteristics. The fungi in the order Eurotiales produce spherical closed ascocarps referred to as cleistothecia, which protect the spores until they are released. Within the ascocarp, the ascus breaks releasing the spores. The spores then break the outside cleistothecia allowing them to be spread into the outside environment. While some of the genera within the group are teleomorphic, many more are anamorphs that produce asexually (8).

Anamorphs produce conidia within aerial hyphae known as conidiophores above the substratum. Within the order, many of the genera can be further classified by the asexual stage characterizations. *Penicillium* forms conidial heads that appear to look like brushes or fingers. The conidiophores have blunt ends and upon each end, conidia are able to form (9, 30).

*Penicillium roqueforti* falls into place as a species in the genus *Penicillium*. *P. roqueforti* is related to other anamorphs such as *Aspergillus* and *Paecilomyces*. This family of organisms is well known for decaying fields of grain and foods in the refrigerator while others are responsible for making the most expensive cheeses worldwide (1). The colonies of the

*Penicillium* group are typically green-yellow or green but the colors can range from brown to yellow as well. The velvety colonies are covered with dense, soft and silky looking hairs. *P. roqueforti* has the ability to grow in 5% lactic acid as well as 0.5% acetic acid showing that the organism can survive in a low pH (5, 33, 41).

While the species of *Penicillium* can exhibit different characteristics, they can show similar growth responses on G25N medium at 5°C and 37°C indicating how closely related all of the species within the genus actually are. *P. roqueforti* is related closely with other *Penicillium* species: *olivicolor*, *purberulum*, *aurantiogriseum*, *viridicatum*, and *crustosum* (35). In recent years, Boysen et al. (5) discovered that *P. roqueforti* actually contains three different species. By analyzing different DNA sequences and the secondary metabolites produced by each species, three separate species arose: *P. roqueforti*, *P. paneum* and *P. expansum*. While *P. roqueforti* has the ability to produce the PR toxin, the other two species cannot. However, *P. paneum* and *P. expansum* produce patulin, a mutagenic, immunotoxic and neurotoxic toxin. Seeing how the relation of one species in a genus is related to another can explain why the organism possesses some of the same characteristics as the larger group while still containing individual characteristics of their own.

#### *Laboratory Methods*

*P. roqueforti* is a hardy mold that can grow rapidly at refrigeration temperatures. This characteristic explains why the organism can spoil cheeses when stored at proper refrigeration temperatures. The organism can also be cultured on different agars at different temperatures. Two of the most common agars used are Malt Extract Agar (MEA) and Czapek Yeast Autolysate Agar (35). Incubation temperatures can range from 5°C to 37°C for 7 days. On these types of agars at 25°C, the *P. roqueforti* colonies can reach up to 70mm in diameter (35) and yield the typical colony color and form mentioned above. Once properly isolated, a MEA slant containing *P. roqueforti* can be stored for up to 8 months at 4°C (41).

## *Toxicity*

*P. roqueforti* has the ability to produce a variety of toxins that are of concern to humans. The most lethal toxin of primary concern is a secondary metabolite known as the PR toxin ( $C_{18}H_{20}O_6$ ) (32). The toxin is soluble in organic acids and insoluble in water, dilute acid, and dilute alkali. The PR toxin has an LD<sub>50</sub> value of 6 mg/L in mice (32, 33). The other toxin of high concern is a secondary metabolite known as roquefortine C. The LD<sub>50</sub> value of roquefortine is 340 mg/L in mice and has been reported to be the cause of death of dogs (32). Roquefortine C can cause neurotoxic problems in mice and can be found in commercial blue cheeses in levels up to 7 mg/L. While these toxic compounds can be formed by *P. roqueforti*, it is not characteristic to see these toxins every time the organism is present. It is also common to see a difference in the amount of toxin production within individual strains of the organism (49).

## *Uses in the Food Industry*

*P. roqueforti* is most common strain of mold used during the ripening of blue cheeses. During blue cheese ripening, the organism releases proteolytic enzymes, which soften the curd giving the cheese the proper body texture. Further into the ripening process, milkfats are hydrolyzed by lipases developing the cheeses characteristic flavor. Other flavors are developed further in the ripening stage by the formation of secondary alcohols from methyl ketone groups within the cheese. *P. roqueforti* is also known for the production of Roquefort cheeses which are similar to blue cheese (31).

## **Cheddar Cheese**

### *Ingredients & Production*

Cheddar cheese consists of 37% water, 33% fat, 24.9% protein, 1.3% lactose, 3.7% ash, and 1.6% added salt. The combination of these ingredients makes cheddar cheese a well preserved food product. The moisture content of cheddar by law must fall between 34-39% (13) allowing some microorganisms to grow, especially molds.

Cheddar cheese is produced more efficiently now giving the cheese better quality causing the demand for cheddar to increase. Milk standardization is easier to consistently obtain batch to batch using ultrafiltration (29). The use of ultrafiltration allows the proper amount of protein and fat content to be added to the milk but does not change the lactose content, which can cause problems later in the cheese making process. This allows for a higher quality cheese and is permitted in cheeses without a standard of identity. However, the FDA has allowed cheddar cheese makers to use ultrafiltrated milk as long as it is declared on the label. The types of cultures used in cheeses are also more consistent due to the decrease in bacteriophage infections (29).

A typical process for cheddar contains about 14 steps (13) allowing many different opportunities for mold contamination to occur. When cheddar is shredded, the increased surface area and the shredding step can lead to mold contamination from the processing environment (4). According to the Code of Federal Regulations (12), any cheese that is manufactured using unpasteurized milk or any unpasteurized milk ingredients must be stored for at least 60 days at a temperature no less than 35°F. Once the cheese process has been completed, cheddar is ripened. The duration of the ripening process is dependent on the degree of sharpness desired. This ripening time can be as short as 2 weeks for a mild cheese or as long as 12 months for a sharper cheese (13).

## List of Abbreviations and Terms

|                |   |
|----------------|---|
| CD             | cyclodextrin  |
| CFR            | Code of Federal Regulations                             |
| FAO            | Food and Agriculture Organization of the United Nations |
| GRAS           | Generally Recognized as Safe                            |
| HP $\beta$ -CD | hydroxy-propyl $\beta$ -cyclodextrin                    |
| HPLC           | high performance liquid chromatography                  |
| LD50           | lethal dose 50%   |
| MEA            | Malt Extract Agar                                       |
| PDA            | photodiode array  |
| PE             | poly(ethylene)  |
| PET            | poly(ethylene terephthalate)                            |
| UV             | ultraviolet   |
| $\beta$ -CD    | $\beta$ -cyclodextrin                                   |
| $\gamma$ -CD   | $\gamma$ -cyclodextrin                                  |

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Figures

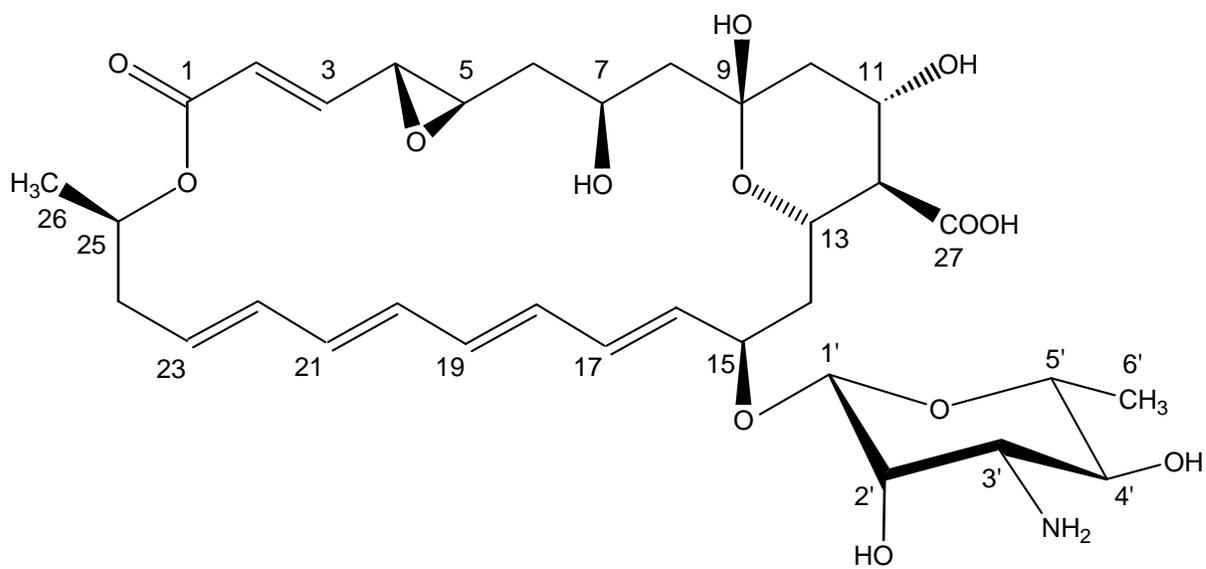


Figure 2-1. Chemical Structure of Natamycin.

### CHAPTER 3: NATAMYCIN METHODS OF QUANTIFICATION & THE STABILITY OF NATAMYCIN EXPOSED TO FLUORESCENT LIGHTING

#### Abstract

Natamycin is an antimycotic compound that is widely used in the cheese industry to increase the shelf life of cheeses, especially shredded cheeses, by inhibiting the growth of molds. However, natamycin is degraded by ultraviolet (UV) light at wavelengths of 350 nm and below. Typical cheese packaging applications do not provide adequate UV protection causing the natamycin to degrade readily.

The International Dairy Federation recognizes two methods to quantify the amount of active natamycin on shredded cheese: high performance liquid chromatography (HPLC) and analysis by spectrophotometry. Experimental samples consisting of 20 mg/L aqueous natamycin suspension were prepared by sealing the suspensions into a polymer packaging and placing them under  $1000 \pm 50$  lux of light in a 4°C cooler. Some of the polymer packages contained a UV absorber allowing 11.4% transmittance at 350 nm, and others did not contain a UV absorber, allowing 90.0% transmittance at 350 nm, and quantified using HPLC and spectrophotometric methods.

Comparison of the two methods after 48 hours of light exposure with no UV protection showed that spectrophotometry is flawed when quantifying the amount of active natamycin because the method gives erroneously high results (spectrophotometric -  $16.7 \pm 0.84$  mg/L, HPLC - to  $3.1 \pm 1.6$  mg/L). The amount of active natamycin is not accurately quantified using spectrophotometric techniques because it cannot separate the active form from the inactive form of natamycin.

Polymer packages containing a UV absorber showed degradation of natamycin after 48 hours ( $13.7 \pm 1.4$  mg/L of natamycin) but had significantly less natamycin than those samples taken from the polymer packages without a UV absorber ( $3.1 \pm 1.6$  mg/L of natamycin). Incorporating a UV absorber into a package helps protect natamycin from UV light degradation.

#### KEYWORDS

natamycin, polyene macrolide, antimycotic, ultraviolet absorber, mold inhibitor, high performance liquid chromatography, spectrophotometer

## Introduction

Natamycin was discovered in the 1950s and ever since has been commonly used in the food industry to prevent undesired yeast and mold growth in various food products. The pharmaceutical industry uses natamycin to treat many types of dermatological diseases. In the food industry, natamycin is used in juices, meat products and a variety of different cheeses (17). The cheese industry uses natamycin in hopes that once the consumer releases the modified atmosphere packaging, the product will be protected from visible mold growth. Shredded cheese is more susceptible to mold growth because of both the increased surface area of the product as well as the additional exposure occurring before the final packaging (1). The United States allows up to 20 mg/L of natamycin to be used on shredded cheese (5).

The solubility of natamycin tends to be extremely low in water while relatively high in polar organic solvents. Much of the solubility of natamycin is dependent on the structure itself. Containing a chromophore of four conjugated double bonds and three free hydroxyl groups, the lactone ring of natamycin displays low water solubility (19). According to Raab (14), natamycin exhibits a 0.005-0.01% soluble percentage in water at 25°C but a 18.5% solubility in glacial acetic acid (15).

Natamycin is highly sensitive to ultraviolet (UV) light. The exact wavelength that natamycin degrades under is not exactly known but natamycin should be protected from wavelengths of 350 and below in order to remain active (8). During inactivation, the tetraene structure is reconfigured (Figure 3-1) causing natamycin to lose the antimycotic mechanism used to target yeast and molds. The rate in which natamycin degrades under UV light has been suggested to be 24 hours regardless if in suspension or in solution (14, 17).

The International Dairy Federation (IDF) has proposed two different methods for the quantification of natamycin on cheese: spectrophotometry and high performance liquid chromatography (HPLC) (9). Natamycin has characteristic absorption peaks at 290, 304 and 318 nm. These peaks can be used to quantify natamycin being aware that certain types of inactivation of natamycin may or may not change the UV absorbance of the compound based on the amount of intact tetraene chromophore remaining (14). HPLC methods include detection of the compound at a 304 wavelength, the maximum absorbance rate for

natamycin and are able to separate out the individual peaks of the inactive and active form of natamycin. Both methods are inexpensive and rapid making them more readily used by the cheese industry to ensure the proper amount of natamycin is being applied onto cheese products (4).

This research was performed to compare the current accepted methods of quantification to determine their appropriateness in quantifying biologically active natamycin. In addition, the efficacy of UV absorber film to prevent UV light degradation of natamycin on the surface of shredded cheese was also analyzed.

## **Materials & Methods**

**Materials.** Natamycin of 90.5% purity was supplied by DSM Food Specialties (Delft, The Netherlands). Proprietary, multilayer, polymer packaging with and without a UV light absorber were supplied by Cryovac (Duncan, SC). The packaging without a UV absorber contained all cheese samples. Those samples that were protected from UV light were also surrounded by the UV protective packaging (Figure 3-2).

**Treatment of Samples.** A stock solution containing 200 mg/L of natamycin was prepared in HPLC grade methanol so that the natamycin was in a true solution. Both packaging types (no UV absorber and UV absorber packaging) containing 450 mL of deionized water (pH - 6.1) received 50 mL of the natamycin stock solution transferred via syringe. Pressure sensitive tape was used to seal the holes that were formed when each sample was taken. All samples were performed in triplicate.

The samples were exposed to continuous lighting by Cool White 40-watt T12 fluorescent bulbs (GE Lighting, Cleveland, OH) mounted 16 cm above the samples to deliver an illumination intensity of  $1000 \pm 50$  lux. Light intensity measurements were measured using a foot candle/lux meter (Extech Instruments, Waltham, MA). These samples were stored at 4°C for 48 hours with samples taken at 0, 1, 2, 3, 4, 5, 6, 24 and 48 hours. Samples consisted of 10 mL aliquots and of this aliquot, 4 mL were divided into two HPLC vials and 6 mL were divided into two spectrophotometric cuvettes for quantification.

**Spectrophotometric Analysis of Different Polymer Packaging.** Differential

spectrophotometry was performed using a Shimadzu UV-2101PC UV-VIS scanning spectrophotometer (Shimadzu Scientific Instruments, Inc., Columbia, MD) to determine the spectral scan of each of the polymer films and their overlay. Sidewall cutouts of the multilayer packages containing UV absorber were prepared. Each sample of polymer was placed in a UV cuvette and qualitatively analyzed by a spectral scan between 200-500 nm to determine specific light transmittance at specific wavelengths (Figure 3-2).

**Spectrophotometric Analysis of Natamycin.** Differential spectrophotometry was performed using a Shimadzu UV-2101PC UV-VIS scanning spectrophotometer (Shimadzu Scientific Instruments, Inc., Columbia, MD) to determine the quantity of natamycin within each sample. The spectral scan was between the wavelengths of 280-340 nm. Each sample was placed in a UV cuvette and the absorbencies measured. An external standard curve was constructed using USP Reference Standard Natamycin (91.7% purity) to quantify free natamycin content in all samples.

**HPLC Analysis of Natamycin.** Analyses were performed with Agilent 1100 series HPLC autosampler, containing a controller and a photodiode array detector (PDA) (Palo Alto, CA). An Agilent rapid resolution HT 4.6 X 50mm, 1.8  $\mu$ m Eclipse XDB-C8 reverse phase analytical column (Palo Alto, CA) was used at ambient temperature. The mobile phase system employed was methanol-water-acetic acid, 60:40:5, v/v/v. The samples were eluted in isocratic mode in the mobile phase for 6 minutes. The flow rate was 0.2 mL/min, the injection volume was 10  $\mu$ L, and the detection wavelength by PDA was 304 nm. The wavelength range of the PDA was 260-360 nm. An external standard curve was constructed using USP Reference Standard Natamycin (91.7% purity) to quantify free natamycin content in all samples. Agilent 1100 series software was used for data management.

**Statistical Methods.** Data was analyzed to detect differences between HPLC and spectrophotometric methodology and between packaging with and without the incorporation of a UV absorber. Both analyses were compared using a split-plot design with a p-value of <0.05. All statistical analyses were performed using SAS statistical software version 9.1, 2001 (SAS Institute Inc., Cary, NC). Least squared means were used to

determine significance between means.

## Results & Discussion

**HPLC vs. Spectrophotometric Analyses.** Figure 3-3 shows the comparison of the HPLC and spectrophotometric methods analyzing the degradation of natamycin without the protection of a UV absorber allowing 90.0% light transmission at 350 nm. After 48 hours at 4°C exposed to  $1000 \pm 50$  lux, natamycin concentrations in the aqueous suspension measured using the HPLC method have decreased to  $3.1 \pm 1.6$  mg/L while the spectrophotometric method quantifies the amount of active natamycin at a concentration of  $16.7 \pm 0.84$  mg/L. While both HPLC and spectrophotometric methods yield a significant loss of natamycin after 48 hours of light exposure, there is a significantly greater loss of natamycin shown by the HPLC method.

Figure 3-3 shows the comparison of the two methods analyzing the degradation of natamycin in an aqueous suspension incorporating a UV absorber allowing 11.4% light transmission into the polymer packaging at 350 nm. After 48 hours at 4°C exposed to  $1000 \pm 50$  lux, natamycin concentrations using the HPLC method have decreased to approximately  $13.7 \pm 1.4$  mg/L while the spectrophotometric method quantifies the natamycin at a significantly higher concentration of  $20.4 \pm 0.83$  mg/L. Once again, both methods, HPLC and spectrophotometry, show significant amount of loss after 48 hours, however, there is a significantly greater loss in natamycin concentration shown by the HPLC method. Regardless of packaging type, there was a difference of quantification of both HPLC and spectrophotometric methods.

Quantification of natamycin can be performed using spectrophotometry except in the case of stability studies suggests Brik (2). During degradation, correlation between microbial activity and tetraene content is not apparent using a one-point spectrophotometer method at 303 nm. Using base line absorption (equation 1) the measurement is based on the maximum of 303 nm as well as the minima at 295 and 311 nm.

$$A_{303} - \frac{A_{295} + A_{311}}{2} \quad 1$$

Quantification using the base line absorption method more appropriately matches other quantification methods (2). It is not until natamycin is further broken down that the spectrophotometric method begins to show significant degradation (Figure 3-3). Because spectrophotometric methods are not able to separate the amount of inactive natamycin from the active form, it should not be used as a quantification method of active natamycin.

**No UV Absorber vs. UV Absorber using HPLC & Spectrophotometric Quantification.** Figure 3-3 shows the direct comparison of using a polymer package containing a UV absorber allowing 11.4% light transmission at 350 nm versus using a polymer package allowing 90.0% light transmission at 350 nm quantified by using the HPLC and the spectrophotometric method. Over 48 hours, degradation of natamycin is observed in both packaging types. At time 48 hours however, the amount of natamycin remaining in the UV absorber packaging is significantly higher than the samples that were unprotected using both methods of quantification (HPLC , UV -  $13.7 \pm 1.4$  mg/L, NUV -  $3.1 \pm 1.4$  mg/L; spectrophotometer, UV -  $20.4 \pm 0.83$  mg/L, NUV -  $16.5 \pm 0.84$  mg/L). Using a UV absorber does help protect the natamycin from degradation by UV wavelengths. The ability for a package to absorb UV light is based on the intensity of light, duration of exposure, percent transmittance and the natural characteristics of the polymer (16).

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Figures

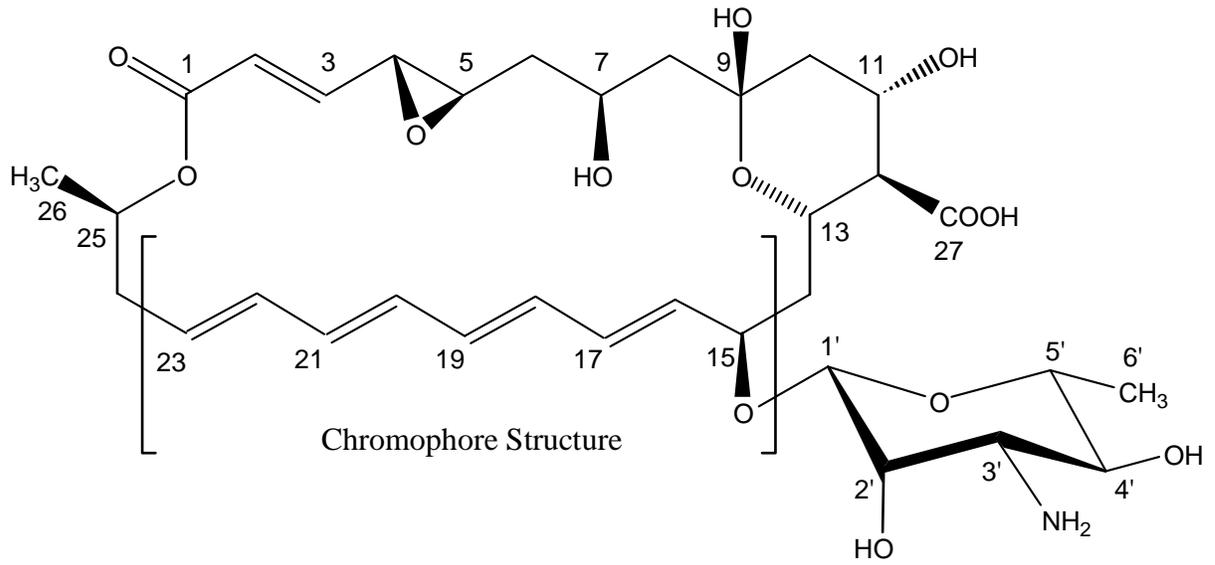
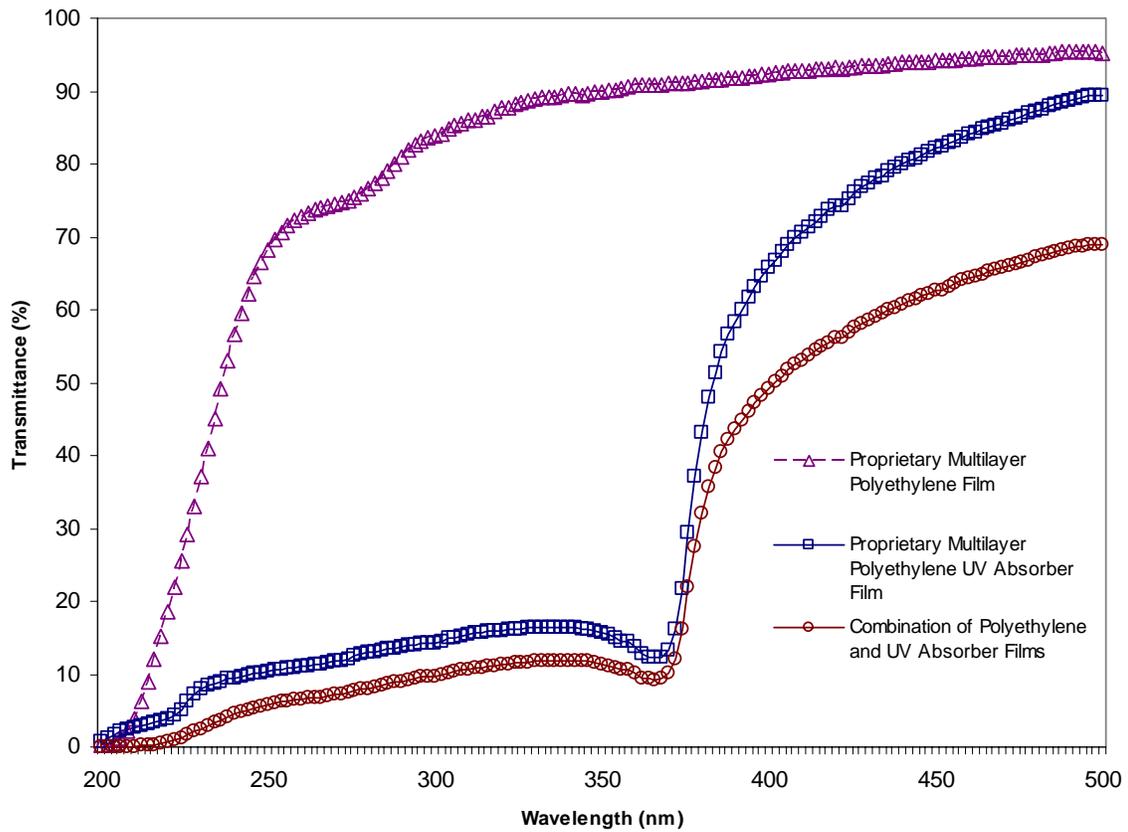
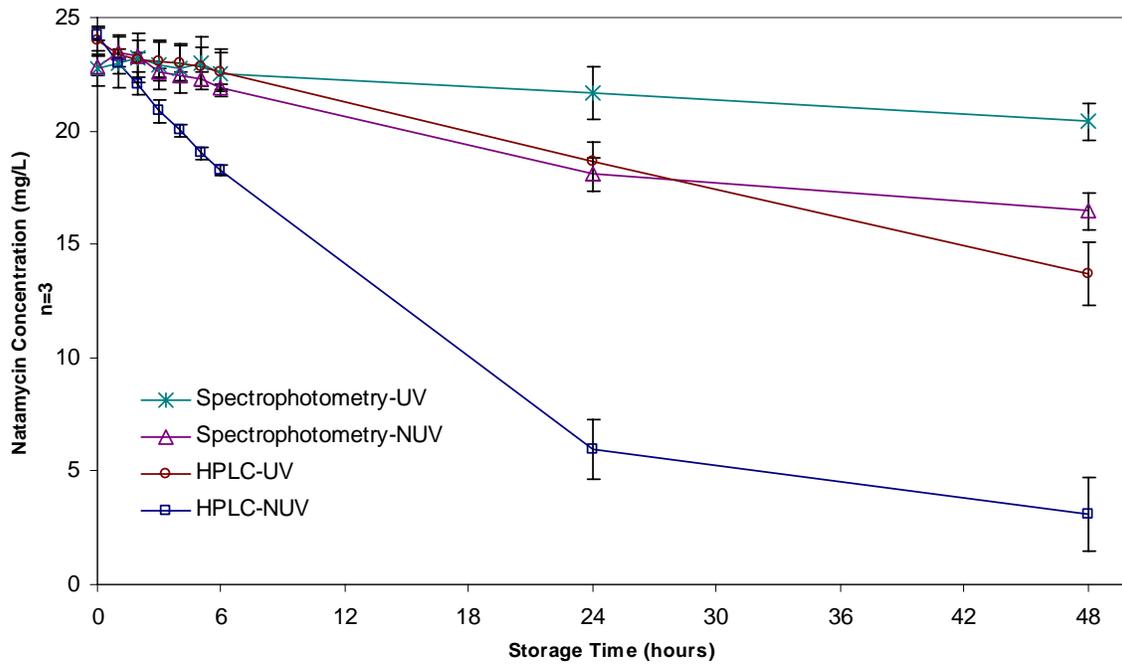


Figure 3-1. Natamycin structure with emphasis on chromophore structure.



**Figure 3-2.** Light transmission of various films used. At 350 nm, the UV absorber combination allows 11.4% light transmission while the film without a UV absorber allows 90.0% light transmission.



**Figure 3-3.** Comparison of the International Dairy Federation methods, HPLC and spectrophotometry, for quantifying active natamycin in an aqueous suspension while comparing the use of a UV absorber (11.4% light transmission at 350 nm) and no UV absorber (90.0% light transmission at 350 nm) after 48 hours at 4°C exposed to 1000 ± 50 lux.

## CHAPTER 4: STABILITY OF NATAMYCIN AND ITS COMPLEXES ON SHREDDED CHEESE EXPOSED TO FLUORESCENT LIGHTING

### Abstract

Natamycin is an antimycotic compound that is widely used in the cheese industry to increase the shelf life of cheeses, especially shredded cheeses, by inhibiting the growth of molds. Natamycin is applied to the surface of cheese as an aqueous suspension or as a powder because it is not readily water soluble. This quality makes it harder to distribute natamycin evenly over shredded cheese. The use of cyclodextrins to increase water solubility was tested to see if a uniform distribution of natamycin over the shredded cheese could be done more effectively. Natamycin is degraded by ultraviolet (UV) light at wavelengths of 350 nm and below. The use of a UV absorber within the polymer packaging was used to determine if the natamycin stability could be better ensured from UV light degradation.

Natamycin was complexed with different cyclodextrins to help solubilize natamycin –  $\beta$ -cyclodextrin, hydroxy-propyl  $\beta$ -cyclodextrin and  $\gamma$ -cyclodextrin. Using cyclodextrins to apply natamycin more uniformly did not have a significant influence on shelf life extension ( $p < 0.05$ ). By adding a UV absorber into the packaging, natamycin was able to extend the shelf life of shredded cheese from 9 days to 27 days under  $1000 \pm 50$  lux. On day 30, all of the UV absorber packages containing shredded cheese began to show visible mold growth. Those samples stored in total darkness remained mold free through the duration of the experiment (62 days).

### KEYWORDS

natamycin, polyene macrolide, cyclodextrin, mold, mold inhibitor, ultraviolet light absorber,  $\beta$ -cyclodextrin, hydroxy-propyl  $\beta$ -cyclodextrin,  $\gamma$ -cyclodextrin, HPLC

## Introduction

Cheddar cheese is made up of 37% water, 33% fat, 24.9% protein, 1.3% lactose, 3.7% ash, and 1.6% added salt. While these ingredients make cheddar cheese naturally preserved, the moisture content falls between 34-39% (3) allowing mold to grow and cause spoilage. In 2003, 8,598 million pounds of cheddar cheese were produced (10). Cheese has become so popular in the United States that 98% of households have cheese in their refrigerators and 83% of restaurants have cheese on the menu (7).

During the processing of shredded cheese, the shredding step can increase the incidence of mold contamination due to the increased exposure of the cheese in the processing plant (1). Produced by *Streptomyces natalensis*, natamycin is an antifungal agent that has the ability to inhibit yeast and mold growth on shredded cheese (20). The United States allows natamycin to be used up to a maximum of 20 mg/L on shredded cheese (2, 19). This polyene macrolide is used to protect mold growth from occurring once the consumer releases the modified atmosphere packaging.

Polar organic solvents tend to dissolve natamycin readily, however, natamycin is practically insoluble in water (15). Natamycin exhibits 0.005-0.01% solubility in water at 25°C (15, 16). The structure of natamycin has much to do with the lack of solubility in water. The 25-carbon lactone ring contains three free hydroxy groups, a zwitterion and a chromophore of four conjugated double bonds. The higher the number of double bonds within the chromophore, the more insoluble the compound is in water (25).

The lack of solubility can complicate the application methods used in the cheese industry due to the possibility of clogging the spray nozzles (18). The introduction of cyclodextrins (CDs) can help to increase the solubility of natamycin allowing the compound to be spread more homogeneously. CDs are doughnut shaped which allow the guest molecule (i.e. natamycin) to fit inside the middle of the CD structure. CDs possess a hydrophobic core and a hydrophilic outer layer allowing for easier complexation of a guest molecule that tends to be hydrophobic (6, 21). The introduction of natamycin into various cyclodextrins can increase the solubility up to 105-fold, as shown with hydroxy-propyl beta cyclodextrin (11).

Natamycin is highly sensitive to ultraviolet (UV) light at wavelengths 350 nm and below. Inactivation occurs when the tetraene structure is lost, without which, natamycin no

longer has the ability to bind to the sterols on yeast and mold cell walls. Depending on the conditions presented, natamycin can degrade in 24 hours (15, 19) making it ineffective for protecting cheese products from molding. UV absorbers can easily be added to packaging at various levels or by incorporating a multilayered package structure. By incorporating one UV absorber into a food package blocking at 360 nm, 90% UV light reduction protects a large number of food products (4). The objective of this research was to determine if the shelf life of shredded cheese could be extended without a modified atmosphere and to quantify the effect of the natamycin delivery method as a dry powder, an aqueous suspension, and as an aqueous solution complexed with various types of cyclodextrins ( $\beta$ -cyclodextrin, hydroxy-propyl  $\beta$ -cyclodextrin, and  $\gamma$ -cyclodextrin).

## Materials & Methods

**Materials.** Natamycin of 90.5% purity was supplied by DSM Food Specialties (Delft, The Netherlands).  $\beta$ -cyclodextrin ( $\beta$ -CD), food grade; hydroxy-propyl  $\beta$ -cyclodextrin (HP  $\beta$ -CD), pharmaceutical grade (5.3-5.4 DS); and  $\gamma$ -cyclodextrin ( $\gamma$ -CD) were provided by Cerestar (Hammond, IN). Proprietary, multilayer, polymer packaging with and without a UV light absorber were supplied by Cryovac (Duncan, SC). The packaging without a UV absorber was used for all cheese samples. Those samples that were protected from UV light using a UV absorber packaging were surrounded by the UV protective package (Figure 4-1). Cheddar cheese was obtained commercially from US Food Service (Roanoke, VA). Dichloran Rose Bengal Agar (DRBC) was supplied for enumeration of mold due to environmental contamination by Difco (Sparks, MD).

**Preparation of Inclusion Complexes.** Inclusion complexes of natamycin with  $\beta$ -CD, HP  $\beta$ -CD, and  $\gamma$ -CD were obtained by complexation in an aqueous solution. Solutions of 16 mM  $\beta$ -CD, 70 mM HP  $\beta$ -CD, and 70 mM  $\gamma$ -CD were prepared in HPLC water. The concentrations chosen were based on their relatively high complexation efficiency based on previous phase solubility studies (12). Natamycin was added in great excess of its intrinsic solubility at concentrations of 2.0, 6.0 and 5.0 g/L to the  $\beta$ -CD, HP  $\beta$ -CD, and  $\gamma$ -CD solutions respectively.

These solutions were ultrasonicated in a VWR Aquasonic 250D water bath (VWR

Scientific Products, West Chester, PA) in a frequency range of 38.5-40.5 kHz for 5 minutes to increase the complexation efficiency. The suspensions were protected from light by wrapping in aluminum foil and then stirred rapidly for 48 hours to achieve solubility equilibrium. Suspension batches were filtered through a 0.45- $\mu$ m nylon membrane to obtain clear aqueous solutions of natamycin and each cyclodextrin type. Sample filtrate was quantified by UV differential spectrophotometry. These solutions were then lyophilized in a laboratory freeze-dryer (Virtis, Gardiner, NY). The resulting samples were flaky powders of low density, so an electric shaker (Janke & Kunkel, Satufen, Germany) was used on a high setting to compact the sample powders. The natamycin content (w/w) in its cyclodextrin complexes as a solid dry powder was approximately 2.8% for  $\beta$ -CD, 2.4% for HP  $\beta$ -CD, and 2.1% for  $\gamma$ -CD.

**Treatment of Cheese Samples.** Cheddar cheese was shredded by an electric cheese shredder (Hallde Machine, Type RG-7, 1725 rpm, #45 cutter, Paxton Corp., Shelton, CT). After shredding, four bags containing 500 g of cheese was separated into Ziploc™ bags containing 2% w/w cellulose (10g) and shaken for 1 minute. After shaking, 2% w/w water (10g) was sprayed onto the shredded cheese with an atomizer. Included in the 2% water spray were the appropriate amounts of each treatment (aqueous suspension of natamycin,  $\beta$ -cyclodextrin, hydroxy-propyl  $\beta$ -cyclodextrin, and  $\gamma$ -cyclodextrin complexes) to deliver a final concentration of 20 mg/L. A fifth bag containing 500 g of cheese was prepared differently for the dry powder natamycin application. The dry natamycin treatment was added with the cellulose (10g) and to stay consistent, 2% w/w (10g) water was added to the dry natamycin treatment as well.

All treatments were shaken again for one minute and then divided into smaller polymer packaging that lacked a UV absorber. Each package contained 10 g of shredded cheese, the amount used to quantify natamycin. One third of these cheese samples were placed directly under the fluorescent lighting of  $1000 \pm 50$  lux. Another third were packaged further into the polymer packaging containing a UV absorber. The final third were placed in total darkness. All cheese packages were stored at 4°C and those exposed to light were exposed to continuous lighting by Cool White 40-watt T12 fluorescent bulbs (GE Lighting, Cleveland, OH) mounted 16 cm above the samples to receive an illumination

intensity of  $1000 \pm 50$  lux. Light intensity measurements were performed with a foot candle/lux meter (Extech Instruments, Waltham, MA). Packages that did not contain a UV absorber were sampled every day for a total of 10 days. Packages that contained a UV absorber were sampled every three days for a total of 27 days. Another set of packages were placed in total darkness and sampled every three days for 27 days and then once a week until day 62. All treatments, samples and packaging types were performed in triplicate.

**Evaluation of Environmental Mold Contamination.** Environmental air samples were taken from the area that shredding took place using a Microbio MB2 (Spiral Biotech Inc., Norwood, MA) at an airflow of 100 L/min. These samples were enumerated on DRBC and incubated for 7 days at room temperature. This was performed each time shredding occurred to determine the amount of environmental contamination that could potentially contaminate the cheese during the shredding process.

**Extraction of Natamycin from Cheese Samples.** The following procedure is a modified version of the International Dairy Federation (IDF) method of extracting natamycin from shredded cheese samples (9). Sample size was doubled along with the amount of methanol and water needed for the extraction. The rest of the method follows the IDF method accordingly.

A 10 g sample of cheese was taken and placed into an Erlenmeyer flask containing 100 mL of methanol. The samples were then shaken for 90 minutes in a Precision Scientific 360 Orbital Shaker (Midland, ON, Canada) shaking at a speed of approximately 220 rpm. Samples were diluted with 50 mL of distilled water followed by cooling to between  $-15$  °C and  $-20$  °C for 60 minutes to precipitate the majority of the fat. The samples were then cold filtered using filter paper with a coarse porosity, a diameter of 15.0 cm and a fast flow rate. This step was followed by further filtration using a  $0.45$   $\mu\text{m}$  and a  $0.22$   $\mu\text{m}$  syringe filter membrane. Natamycin content in the filtrate was then determined by HPLC in duplicate.

**HPLC Analysis of Natamycin.** Analyses were performed with Agilent 1100 series HPLC autosampler, containing a controller and a photodiode array detector (PDA) (Palo

Alto, CA). An Agilent rapid resolution HT 4.6 X 50mm, 1.8  $\mu\text{m}$  Eclipse XDB-C8 reverse phase analytical column (Palo Alto, CA) was used at ambient temperature. The mobile phase system employed was methanol-water-acetic acid, 60:40:5, v/v/v. The samples were eluted in isocratic mode in the mobile phase for 6 minutes. The flow rate was 0.2 mL/min, the injection volume was 10  $\mu\text{L}$ , and the detection wavelength by PDA was 304 nm. The wavelength range of the PDA was 260-360 nm. An external standard curve was constructed using USP Reference Standard Natamycin (91.7% purity) to quantify free natamycin content in all samples. Agilent 1100 series software was used for data management.

**Statistical Methods.** Data was analyzed to detect differences between packaging with and without the incorporation of a UV absorber and differences between the application methods of natamycin onto shredded cheese. Both analyses were compared using a split-plot design with a p-value of  $<0.05$ . All statistical analyses were performed using SAS statistical software version 9.1, 2001 (SAS Institute Inc., Cary, NC). Least squared means were used to determine significance between means.

## Results & Discussion

**Initial Environmental Mold Counts.** After taking an enumeration of mold during the shredding process, it was determined that while mold is naturally in the environment and has the potential to contaminate cheese surfaces during shredding, the level of contamination was not detectable with the sampler used. Environmental contamination of the cheese did not add significant levels of mold spores to the shredded cheese samples.

**Effect of UV Light on Natamycin Application Methods.** All treatments contained  $20 \pm 3.9$  mg/L of natamycin applied homogenously over the cheese except for HPBCD complex, which started with  $17 \pm 5.1$  mg/L, significantly less than the rest of the treatments. This is important to note as this will be left out of the discussion unless otherwise noted. After 9 days of  $1000 \pm 50$  lux light intensity and no UV protection, significant loss of natamycin was observed in all treatments compared to the original amount ( $p < 0.05$ ) (Figures 4-2 - 4-6). The greatest loss was in those samples that were not protected from light,  $8.4 \pm 1.1$  mg/L (90.0% light transmittance at 350 nm) followed by those that were

protected by a UV absorber,  $10.8 \pm 1.5$  mg/L (11.4% light transmittance at 350 nm). The least amount of degradation occurred in those cheese samples that were in darkness,  $16.9 \pm 2.1$  mg/L. There is significant evidence by day 9 that the most protection comes from those samples in total darkness.

Samples exposed to UV light at day 9 were stopped based on the previous natamycin stability study indicating that after 48 hours of unprotected storage, natamycin had degraded to  $3.1 \pm 1.6$  mg/L (23). UV absorber and total darkness treatments were continued until visible mold growth was observed. At day 27, the samples remaining under light and in darkness did not exhibit any visible mold growth. The amount of natamycin remaining on the samples showed a significant difference between the UV absorber packages stored under  $1000 \pm 50$  lux and total darkness treatments. In total darkness,  $15.1 \pm 3.0$  mg/L of natamycin remained while on the UV absorber packaging samples only  $6.6 \pm 2.2$  mg/L of natamycin was active suggesting the best method for ensuring natamycin stability over time on shredded cheese would be to protect it from the most amount of light possible. On day 30, the next sampling period for both treatments, visible mold growth was observed on those samples stored with a UV absorber. The darkness samples did not exhibit visible mold growth during the duration of the experiment (day 62).

**Variation of Natamycin on Shredded Cheese.** During the course of the experiment, variation of natamycin on shredded cheese still occurred even with better application methods. BCD complex shows this variability by having a  $2.4 \pm 1.4$  mg/L increase in natamycin concentration from day 8 to day 9 (Figure 4-4). While a homogeneous spread of the natamycin is highly desired, the reality is that many variables create a heterogeneous spread of the natamycin. Variability can occur because of the type of method used to distribute natamycin, the droplet size of the application method, the uneven surface area of the shredded cheese and the arrangement of the cheese within the package. Some shreds of cheese protect other shreds by the way that they are arranged in the package. Variability is also observed because only one side of the package faces the light, thereby protecting the unexposed surface of the cheese. These variables can lead to what appears to be an increase in natamycin concentration simply because the compound is being protected more in one sample compared to another.

**Effect of No UV Absorber Protection on all Natamycin Treatments.** While degradation is observed when natamycin is not protected from UV light, the rate of degradation is different between natamycin treatments (Figure 4-7). Over the course of 9 days, every treatment had significant loss of natamycin ( $20.1 \pm 1.5$  mg/L at day 0 and  $8.4 \pm 1.1$  mg/L by day 9). The two treatments that showed the most significant loss were BCD ( $8.9 \pm 1.4$  mg/L) and GCD complex ( $5.1 \pm 0.5$  mg/L).

UV light is able to penetrate through the unprotected packaging to degrade the natamycin. In the previous degradation study, aqueous suspensions of natamycin yielded significantly lower concentrations after 48 hours,  $3.1 \pm 1.6$  mg/L, about 5.3 mg/L lower than the natamycin cheese stability study (23). This can be explained by the variability reasons mentioned previously. In an aqueous suspension, light is able to penetrate through the packaging exposing the entire suspension to light. Shredded cheese however is not transparent and does not allow light penetration through the product allowing exposure to only the top layer of cheese. This protects the natamycin on the cheese within the package but leaves the top layer of cheese more susceptible to mold growth due to the decrease in natamycin concentration. The structural change that occurs during degradation of natamycin begins after the *trans*- bonds change to *cis*- bonds. As *cis*- bonds, the chromophore structure is weakened. These bonds begin breaking causing the tetraene structure to change into a triene structure (5, 24). While little is known about the exact reaction occurring, research suggests that it resembles a retro-aldol cleavage accompanied by polymerization (13).

**Effect of UV Absorber Protection on all Natamycin Treatments.** When protected from UV light, natamycin in theory should remain active and intact. Figure 4-8 shows the degradation each treatment went through during the 27 days of storage. At day 9, no significant difference is observed between dry natamycin, aqueous natamycin, BCD and HPBCD complex ( $7.1 \pm 2.0$  mg/L). GCD complex however has significantly decreased in concentration to  $5.2 \pm 2.4$  mg/L. Around the final storage day, the average of all the treatment concentrations is  $6.7 \pm 0.8$  mg/L. Packaging containing a UV absorber helps protect the natamycin concentration for longer, but at day 27, the type of treatment no longer becomes significant. By day 30, mold growth was visible on all treatments.

Natamycin degradation on shredded cheese when packaged with a UV absorber can be explained due to the transmission of light that is able to penetrate the package (11.4% light transmission at 350 nm, Figure 4-1).

Water, being a less complex matrix in comparison to shredded cheese (23), shows natamycin degradation because of UV light penetration. However, with the protection of a UV absorber, degradation on shredded cheese may be caused by flavins (i.e. riboflavin, vitamin B2). In 500 grams of cheddar cheese, there is about 1.875 mg of riboflavin (26). Some researchers suggest that a triplet-triplet transfer is involved (22). In this reaction, riboflavin is excited by light around 445 nm causing it to pass on energy to natamycin. Because natamycin is a sensitive compound, in this state the tetraene structure can decompose causing natamycin to degrade. While other mechanisms of action have been theorized, there has been no conclusive evidence that this mechanism or others actually cause the degradation of natamycin by photosensitizers such as riboflavin (14, 22). However, it is known that when riboflavin and natamycin are together in total darkness, there are no signs of degradation of natamycin (8).

**Effect of Total Darkness on all Natamycin Treatments.** Storage of each treatment in total darkness, showed the best method for protecting natamycin on shredded cheese. During storage in total darkness, there is initial degradation observed with each of the treatments. Active compounds and initial yeast and mold that are present on the cheese can explain the initial degradation of natamycin, a loss of about 3.4 mg/L (Figure 4-9). Once natamycin levels began to stabilize over all the treatments, the lowest concentrations observed were GCD ( $12.8 \pm 2.7$  mg/L) and HPBCD ( $10.6 \pm 3.9$  mg/L) complex. These concentrations were not significantly different from the other treatments ( $14.9 \pm 2.2$  mg/L). After 62 days of storage, no mold growth was observed on any of the treatments. When natamycin remains active on shredded cheese, mold growth is slowed without the use of modified atmosphere packaging.

**Effect of Natamycin Application Methods on Shredded Cheese.** The amount of variability at time zero suggests that HPBCD complex has greater variability in distribution (a difference of 4.7 mg/L in concentration of natamycin) than any other treatment (Table 1).

Between all of the other treatments, there is no significant difference in distribution variability between the subsamples taken. The standard deviations of HPBCD complex range from 1.9 – 8.7 mg/L while the range of all of the other treatments combined range from 0.1 – 5.4 mg/L. Though cyclodextrins help to increase the solubility of natamycin (11) they may not help even distribution of natamycin over shredded cheese.

When natamycin is complexed with cyclodextrins, the ratio of natamycin to cyclodextrin is not 1:1. This causes the cyclodextrins to act as a bulking agent. By having such an excess of cyclodextrins with natamycin, the distribution of actual natamycin may be subject to chance. While the cyclodextrins may be uniformly distributed onto the cheese, the natamycin may not be uniformly distributed within the complex. During complexation, only a certain percentage of natamycin is able to bind with the cyclodextrins (Table 1). The rest of the complex consists of cyclodextrin illustrating its bulking agent ability. Variability of distribution of the natamycin within the complex can lead to an increased variability on the cheese. The complex that exhibits the most variability is HPBCD-natamycin complex. HPBCD-natamycin complex exhibited the best increase in natamycin solubility, 152-fold (11) again explaining that while increased solubility of the natamycin complex can be achieved, the dispersal of natamycin may not necessarily spread more homogeneously.

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## Figures

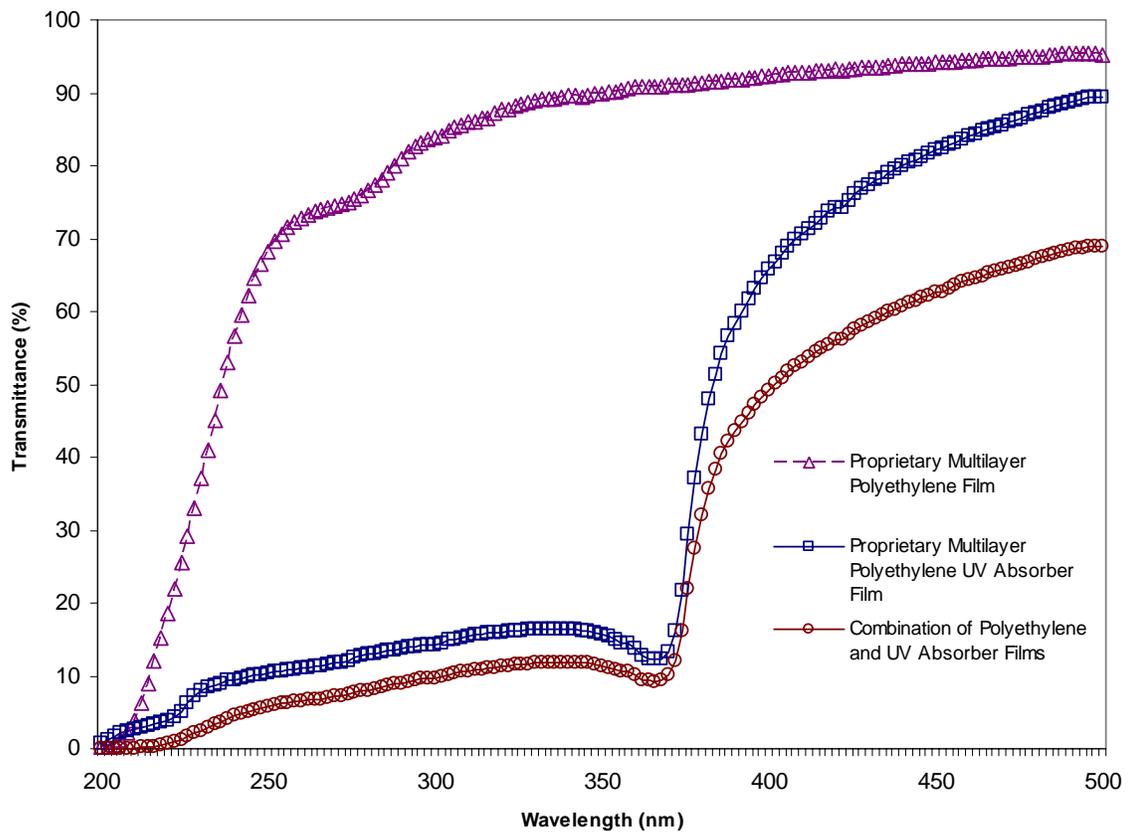
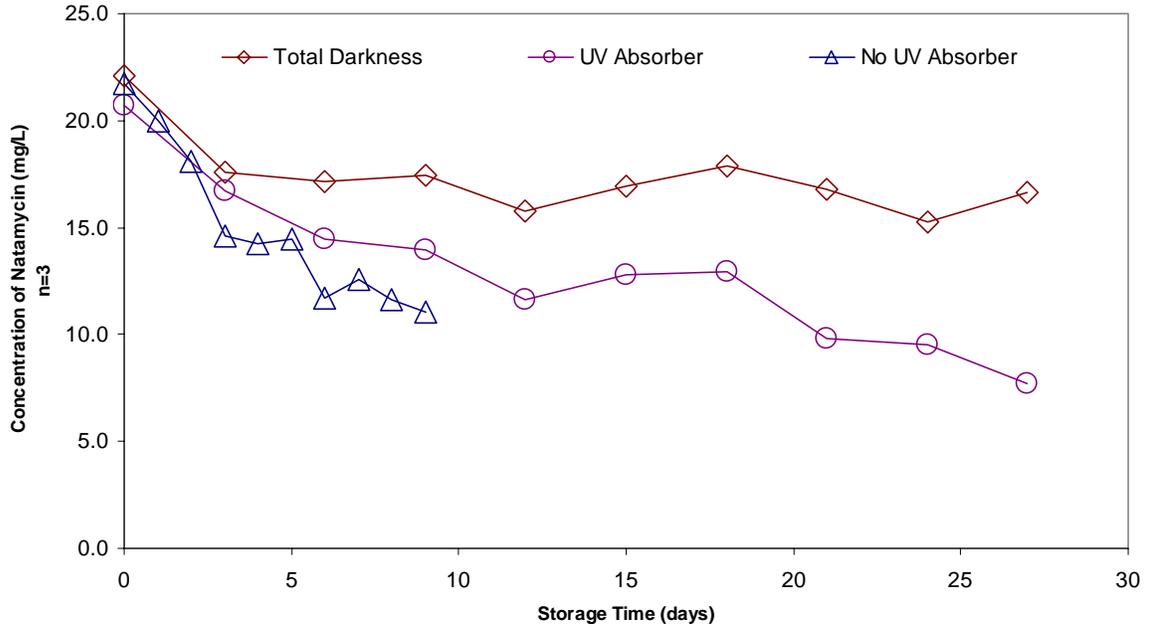
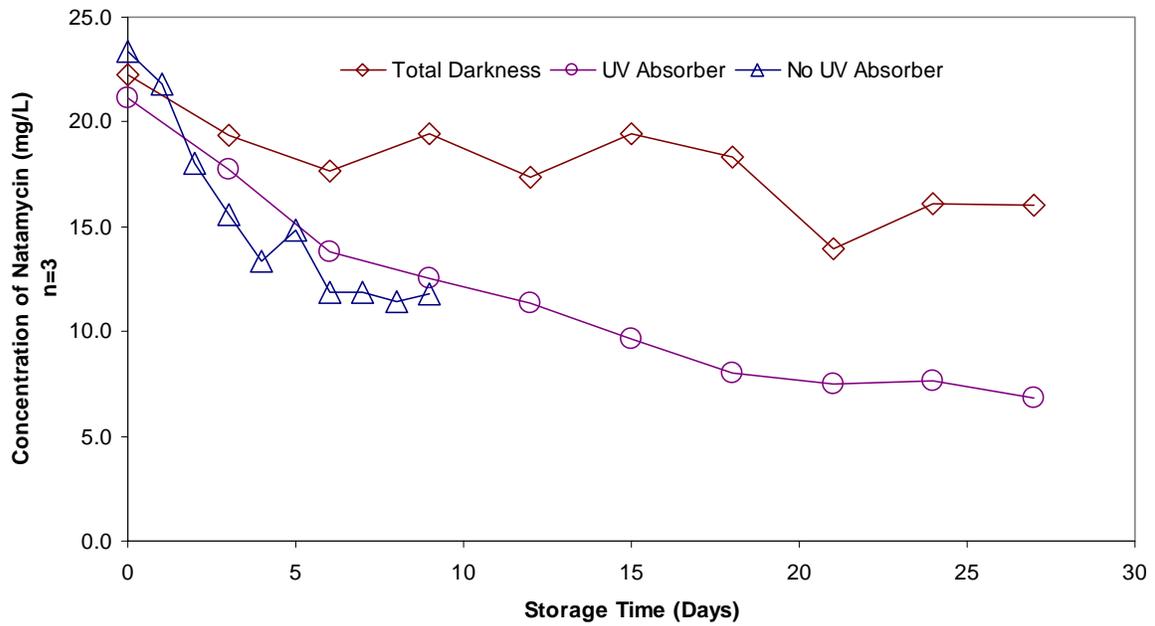


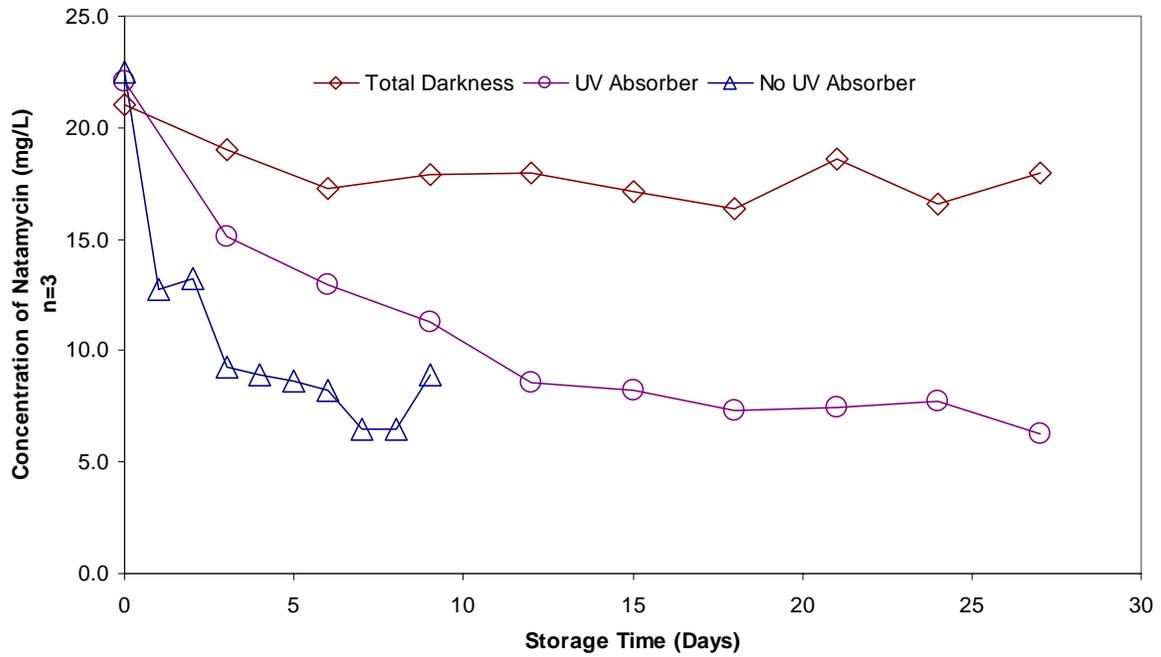
Figure 4-1. Light transmission of various films used.



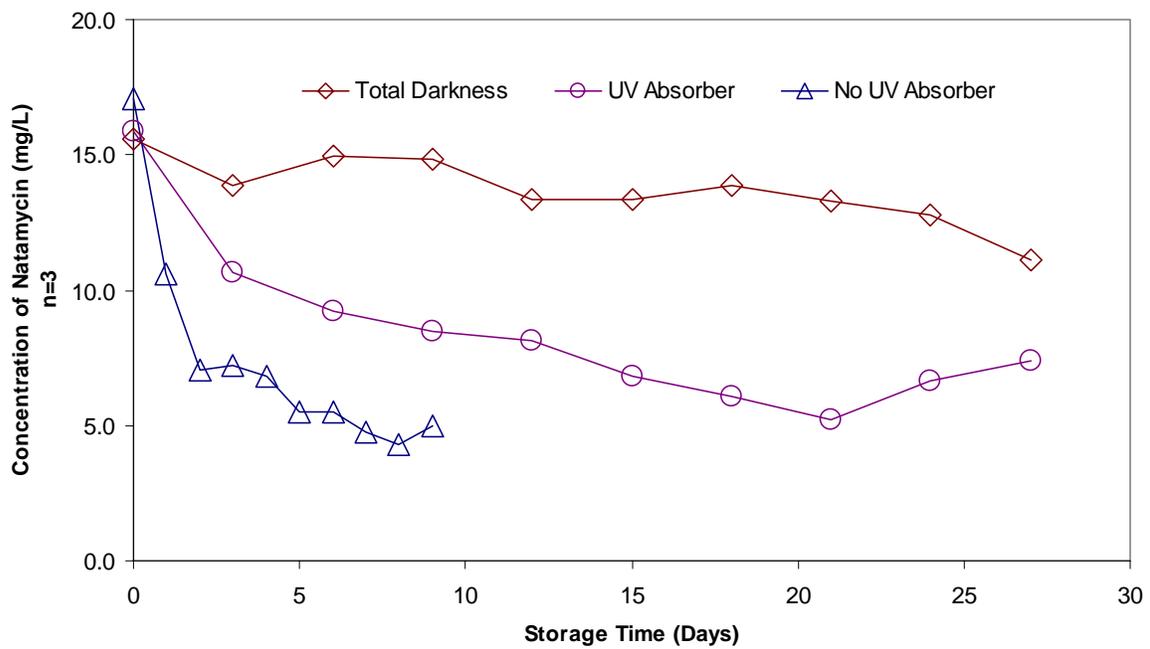
**Figure 4-2.** Concentration of natamycin applied as a dry powder on shredded cheese in different packaging types and storage conditions over time: No UV absorber and UV absorber under  $1000 \pm 50$  lux at  $4^{\circ}\text{C}$  & no UV protection under 0 lux at  $4^{\circ}\text{C}$ .



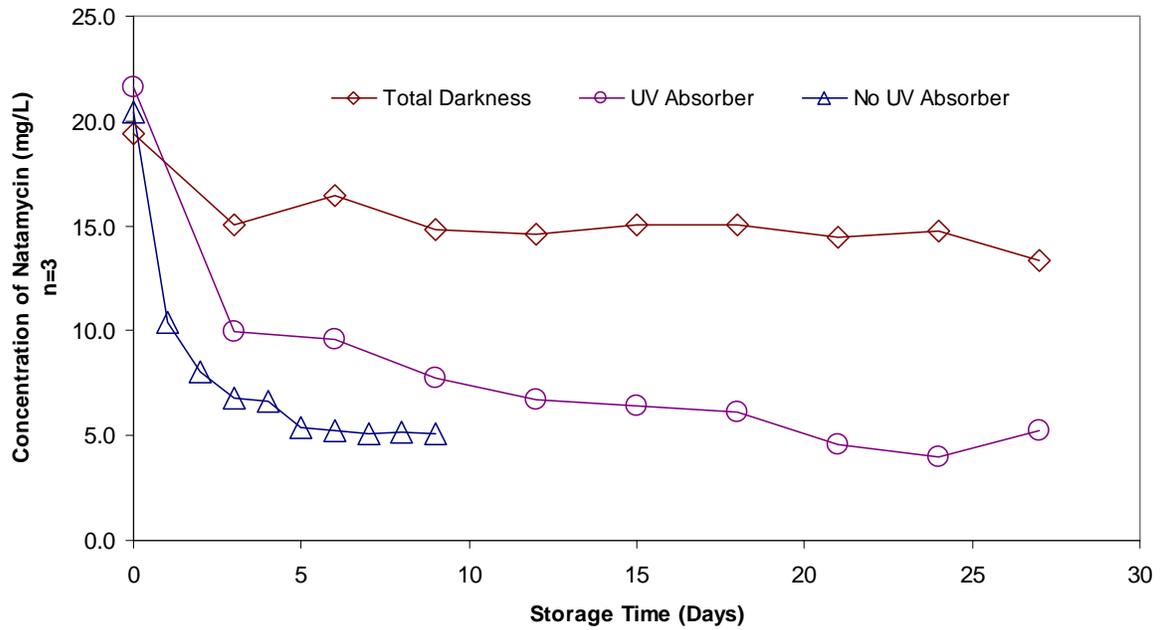
**Figure 4-3.** Concentration of natamycin applied as an aqueous suspension on shredded cheese in different packaging types and storage conditions over time: No UV absorber and UV absorber under  $1000 \pm 50$  lux at  $4^{\circ}\text{C}$  & no UV protection under 0 lux at  $4^{\circ}\text{C}$ .



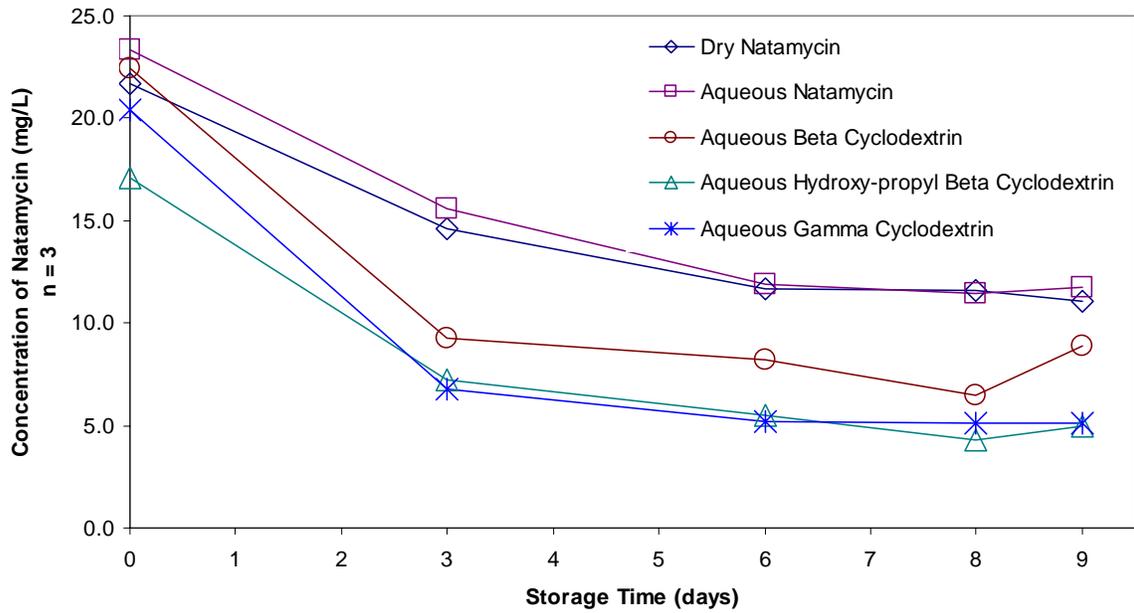
**Figure 4-4.** Concentration of natamycin applied as an aqueous suspension complexed with  $\beta$  cyclodextrin on shredded cheese in different packaging types and storage conditions over time: No UV absorber and UV absorber under  $1000 \pm 50$  lux at  $4^\circ\text{C}$  & no UV protection under 0 lux at  $4^\circ\text{C}$ .



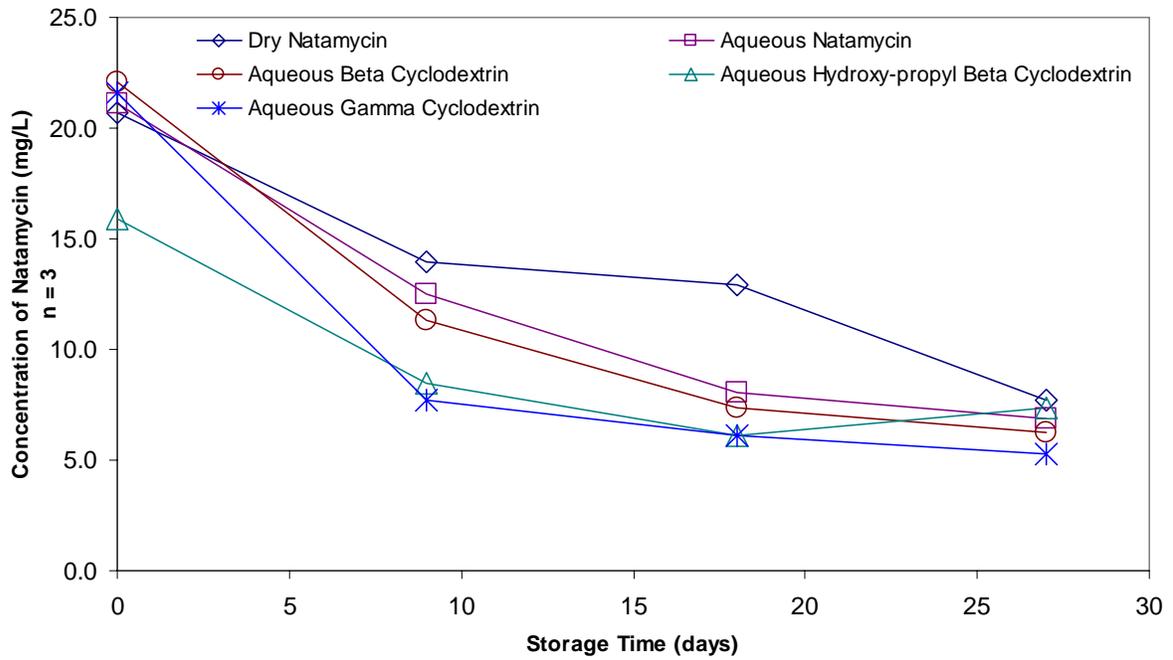
**Figure 4-5.** Concentration of natamycin applied as an aqueous suspension complexed with hydroxy-propyl  $\beta$  cyclodextrin on shredded cheese in different packaging types and storage conditions over time: No UV absorber and UV absorber under  $1000 \pm 50$  lux at  $4^{\circ}\text{C}$  & no UV protection under 0 lux at  $4^{\circ}\text{C}$



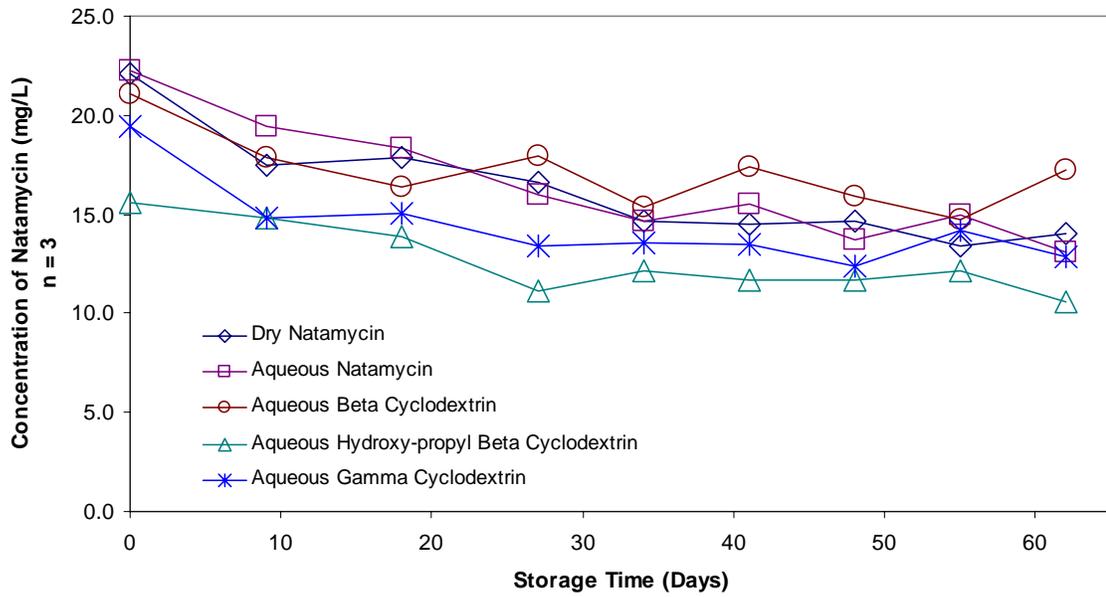
**Figure 4-6.** Concentration of natamycin applied as an aqueous suspension complexed with  $\gamma$  cyclodextrin on shredded cheese in different packaging types and storage conditions over time: No UV absorber and UV absorber under  $1000 \pm 50$  lux at  $4^\circ\text{C}$  & no UV protection under 0 lux at  $4^\circ\text{C}$ .



**Figure 4-7.** Concentration of natamycin treatments and its cyclodextrin complexes on shredded cheese stored without UV absorber packaging under  $1000 \pm 50$  lux at  $4^{\circ}\text{C}$  for 9 days.



**Figure 4-8.** Concentration of natamycin treatments and its cyclodextrin complexes on shredded cheese stored using UV absorber packaging under  $1000 \pm 50$  lux at  $4^{\circ}\text{C}$  for 27 days.



**Figure 4-9.** Concentration of natamycin treatments and its cyclodextrin complexes on shredded cheese stored under 0 lux at 4°C for 62 days.

## Tables

**Table 4-1.** Treatment standard deviations at time 0 for all types of packaging and storage conditions. Target concentrations on shredded cheese were targeted at 20 mg/L. Natamycin percentages in complexes.

| Treatment | Mean<br>(mg/L) | Natamycin<br>Content<br>(w/w)) |
|-----------|----------------|--------------------------------|
| Dry Nat   | 21.5 ± 2.53    | --                             |
| Nat       | 22.3 ± 2.73    | --                             |
| BCD       | 21.9 ± 2.72    | 2.8%                           |
| HPBCD     | 16.2 ± 4.70    | 2.4%                           |
| GCD       | 20.5 ± 2.96    | 2.1%                           |

## CHAPTER 5: EVALUATION OF INITIAL MOLD POPULATIONS IN DETERMINING SHELF LIFE OF SHREDDED CHEESE

### Abstract

Natamycin is an antimycotic compound that is widely used in the cheese industry to increase the shelf life of cheeses, especially shredded cheeses, by inhibiting the growth of molds. Mold can contaminate cheese during one of the many processes it goes through before final packaging. Natamycin is used to help preserve the cheese once the consumer releases the modified atmosphere packaging. This research was performed to determine the efficacy of different natamycin applications (dry, aqueous, and  $\beta$ -cyclodextrin-natamycin complex, hydroxy-propyl  $\beta$ -cyclodextrin-natamycin complex and  $\gamma$ -cyclodextrin-natamycin complex) on shredded cheese when a known concentration of mold spores ( $10^1$ - $10^2$  spores/gram) were applied before each of the treatments.

When untreated with natamycin and an initial concentration of  $10^1$ - $10^2$  spores/gram of *Penicillium roqueforti*, shredded cheese remained free from visible mold growth for 24 days in total darkness at 4°C. Samples treated with any one of the natamycin treatments were able to remain mold free for at least 9 more days, showing visible signs of mold growth at day 33. There were no statistical difference between the treatments of dry natamycin, aqueous suspension natamycin,  $\beta$ -cyclodextrin-natamycin complex, and  $\gamma$ -cyclodextrin-natamycin complex ( $p < 0.05$ ). However, hydroxy-propyl  $\beta$ -cyclodextrin-natamycin complex allowed the shredded cheese to last for 41 days, 17 days longer than the control sample and longer than any of the other treatments.

The use of natamycin in any type of treatment can help to increase the shelf life of shredded cheese for up to 9 days longer. The use of hydroxy-propyl  $\beta$ -cyclodextrin-natamycin complex can increase that shelf life even further, up to 17 days past cheese that has been untreated.

### KEYWORDS

natamycin, *Penicillium roqueforti*, antimycotic, ultraviolet absorber, mold inhibitor, high performance liquid chromatography

## Introduction

Cheddar cheese consists of 37% water, 33% fat, 24.9% protein, 1.3% lactose, 3.7% ash, and 1.6% added salt. The nature of these ingredients help to preserve cheddar cheese however, the moisture content falls between 34-39% (5) allowing mold to grow and cause spoilage. Shredded cheese has an increased exposure and surface area, which increases the concern of mold growth and contamination (2). In 2003, 8,598 million pounds of cheddar cheese was produced (10).

Natamycin is an antimycotic commonly employed in the cheese industry to protect shredded cheese from molding. The United States regulates the amount of natamycin on shredded cheese to 20 mg/L (4, 17). The solubility of natamycin in water is low allowing only a maximum of 0.01% of the compound to suspend itself at 25°C (15, 16). This lack of solubility can become an obstacle for the cheese industry when it comes to applying natamycin onto shredded cheese. The introduction of cyclodextrins can help increase the solubility of natamycin allowing the compound to be spread more homogeneously.

Cyclodextrins (CDs) are doughnut shaped which allow the guest molecule to fit inside the middle of the CD structure. The outer layer of the CD structure is hydrophilic while the inner section tends to be more hydrophobic. This allows for easier complexation of a guest molecule that tends to be hydrophobic (7, 19). The use of CDs can increase the solubility up to 105-fold, as seen with hydroxyl-propyl beta cyclodextrin (11). This increased solubility may help in the application of natamycin onto shredded cheese products for a more homogenous distribution of the antimycotic.

*Penicillium roqueforti* is a mold that commonly spoils a variety of food products. Being able to grow at refrigeration temperatures, *P. roqueforti* spoils cheeses while stored properly (14). Spoilage is seen on cheeses when the colonies become green-yellow, green, or brown. Colonies are covered with dense, velvety soft and silky looking hairs. Having the ability to grow in 5% lactic acid as well as 0.5% acetic acid shows that the organism can survive in a low pH environment (3, 13, 17).

The incorporation of natamycin onto shredded cheese can effectively inhibit the growth of *Penicillium roqueforti* if the natamycin is protected from degradation. Natamycin has the ability to bind irreversibly to the ergosterol on the *P. roqueforti* cell wall causing the cell membrane to slowly leak potassium ions and other small molecules. Eventually these

molecules begin to rapidly leak from the cell eventually causing cell lysis (8, 17). The objective of this research was to determine the effect of a high initial mold population on the amount of natamycin applied to the shredded cheese. The shelf life was determined based on visible mold growth on the shredded cheese after storage in total darkness.

## Materials & Methods

**Materials.** *Penicillium roqueforti* Thom (ATCC 52322) (ATCC, Manassas, VA) was obtained. Dichloran Rose Bengal Agar (DRBC) was supplied by Difco (Sparks, MD). Yeast and mold petrifilm was supplied by 3M (Somerville, NJ). Natamycin of 90.5% purity was supplied by DSM Food Specialties (Delft, The Netherlands).  $\beta$ -cyclodextrin ( $\beta$ -CD), food grade; hydroxy-propyl  $\beta$ -cyclodextrin (HP  $\beta$ -CD), pharmaceutical grade (5.3-5.4 DS); and  $\gamma$ -cyclodextrin ( $\gamma$ -CD) were provided by Cerestar (Hammond, IN). Proprietary, multilayer, polymer packaging without a UV light absorber were supplied by Cryovac (Duncan, SC). Cheddar cheese was obtained commercially from US Food Service (Roanoke, VA).

**Preparation of Solid Complexes.** Solid inclusion complexes of natamycin with  $\beta$ -CD, HP  $\beta$ -CD, and  $\gamma$ -CD were obtained by complexation in an aqueous solution. Solutions of 16 mM  $\beta$ -CD, 70 mM HP  $\beta$ -CD, and 70 mM  $\gamma$ -CD were prepared in HPLC water. The concentrations chosen were based on their relatively high complexation efficiency based on previous phase solubility studies (12). Natamycin was added in great excess of its intrinsic solubility at concentrations of 2.0, 6.0 and 5.0 g/L to the  $\beta$ -CD, HP  $\beta$ -CD, and  $\gamma$ -CD solutions respectively.

These solutions were ultrasonicated in a VWR Aquasonic 250D water bath (VWR Scientific Products, West Chester, PA) in a frequency range of 38.5-40.5 kHz for 5 minutes to increase the complexation efficiency. The suspensions were protected from light by wrapping in aluminum foil and then stirred rapidly for 48 hours to achieve solubility equilibrium. Suspension batches were filtered through a 0.45- $\mu$ m nylon membrane to obtain clear aqueous solutions of natamycin and each cyclodextrin type. Sample filtrate was quantified by UV differential spectrophotometry. These solutions were then lyophilized in a laboratory freeze-dryer (Virtis, Gardiner, NY). The resulting samples were flaky powders of low density, so an electric shaker (Janke & Kunkel, Satufen, Germany) was used on a high

setting to compact the sample powders. The natamycin content (w/w) in its cyclodextrin complexes as a solid dry powder was approximately 2.8% for  $\beta$ -CD, 2.4% for HP  $\beta$ -CD, and 2.1% for  $\gamma$ -CD.

**Isolation of *Penicillium roqueforti*.** *Penicillium roqueforti* was received in pellet form in a frozen state. Spores were placed in 9 mL tubes containing a solution of 0.1% peptone and 1% Tween. The spores were pour plated using DRBC and incubated at room temperature (25 °C) for 7 days. The spores were harvested from the DRBC using sterile water and 1% Tween. DRBC plates were washed three consecutive times using enough of the water solution to cover the plate. This was repeated three times on each plate to dislodge as many spores as possible. Spores were placed into a sterile centrifuge tube and centrifuged at 19,883  $\underline{G}$  for 20 minutes. Supernatant was decanted and spores were frozen at approximately -18°C for 24 hours. Spores were then freeze-dried in a laboratory freeze-dryer (Virtis, Gardiner, NY) for 48 hours. Spores were weighed out (0.01 g) and combined with 100 g of cellulose creating a cellulose spore stock. Further dilutions were made with cellulose as needed.

**Treatment of Cheese Samples with Mold Spores and Treatments.** Cheddar cheese was shredded by an electric cheese shredder (Hallde Machine, Type RG-7, 1725 rpm, #45 cutter, Paxton Corp., Shelton, CT). After shredding, four bags containing 100 g of cheese was separated into Ziploc™ bags containing 2% w/w cellulose (2g) and manually shaken for 1 minute. The 2% w/w cellulose contained *P. roqueforti* at a concentration of approximately  $10^1$ - $10^2$  CFU/g. After shaking, 2% w/w water (2g) was sprayed onto the shredded cheese with an atomizer. Included in the 2% water spray were the appropriate amounts of each treatment (aqueous suspension of natamycin,  $\beta$ -cyclodextrin, hydroxypropyl  $\beta$ -cyclodextrin, and  $\gamma$ -cyclodextrin complexes) to deliver a final concentration of 20 mg/L onto the cheese. The fifth bag containing 100 g of cheese was prepared differently. The dry natamycin treatment was added with the cellulose (2g) that again contained *P. roqueforti*. To stay consistent with the other treatments, 2% w/w (10g) water was added to the dry natamycin treatment. All treatments were shaken again for one minute. Cheese packages were placed in total darkness and sampled at time 0 and again once visible mold

growth was present. All treatments, samples and packaging types were performed in triplicate.

**Extraction of Natamycin from Cheese Samples.** The following procedure is a modified version of the International Dairy Federation (IDF) method of extracting natamycin from shredded cheese samples (9). Sample size was doubled along with the amount of methanol and water needed for the extraction. The rest of the method follows the IDF method accordingly.

The natamycin present in treated shredded cheese samples was extracted using methanol. A 10 g sample of cheese was taken and placed into an Erlenmeyer flask containing 100 mL of methanol. The samples were then shaken for 90 minutes in a Precision Scientific 360 Orbital Shaker (Midland, ON, Canada) shaking at a speed of approximately 220 rpm. Samples were diluted with 50 mL of distilled water followed by cooling to between - 15 °C and - 20 °C for 60 minutes to precipitate the majority of the fat. The samples were then cold filtered using filter paper with a coarse porosity, a diameter of 15.0 cm and a fast flow rate. This step was followed by further filtration using a 0.45 µm and a 0.22 µm syringe filter membrane. Natamycin content in the filtrate was then determined by HPLC in duplicate.

**Enumeration of Mold Populations on Cheese Samples.** Enumeration methods were taken from the Bacteriological Analytical Manual (21). Each sample contained 25 g of shredded cheese. Once cheese was placed in a stomacher bag, 50 mL of water were added and the sample was stomached for 2 minutes. Dilutions were made using 0.1% peptone water and plated on yeast and mold petrifilm.

**HPLC Analysis of Natamycin.** Analyses were performed with Agilent 1100 series HPLC autosampler, containing a controller and a photodiode array detector (PDA) (Palo Alto, CA). An Agilent rapid resolution HT 4.6 X 50mm, 1.8 µm Eclipse XDB-C8 reverse phase analytical column (Palo Alto, CA) was used at ambient temperature. The mobile phase system employed was methanol-water-acetic acid, 60:40:5, v/v/v. The samples were eluted in isocratic mode in the mobile phase for 6 minutes. The flow rate was 0.2 mL/min,

the injection volume was 10  $\mu$ L, and the detection wavelength by PDA was 304 nm. The wavelength range of the PDA was 260-360 nm. An external standard curve was constructed using USP Reference Standard Natamycin (91.7% purity) to quantify free natamycin content in all samples. Agilent 1100 series software was used for data management.

**Statistical Methods.** Data was analyzed to detect differences between the control samples with no natamycin treatment to each of the various natamycin treatments. Analyses were compared using a one-way ANOVA with a p-value of  $<0.05$ . Statistical analyses were performed using SAS statistical software version 9.1, 2001 (SAS Institute Inc., Cary, NC).

## Results & Discussion

**Effect of Treatments on Shredded Cheese.** While there was visible mold growth seen on each of the samples, there was no significant decrease in the amount of natamycin present on the shredded cheese. Table 1 shows the initial natamycin concentrations compared to the final concentrations at the different days the samples were taken. The most significant loss in natamycin concentration is with HPBCD complex with a loss of  $\sim 3$  mg/L from the original amount. This concentration is lower at the final sampling day because HPBCD complex was able to prevent visible mold growth the longest extending the shelf life of the shredded cheese 17 days past the control sample which did not receive any natamycin treatment.

Every natamycin treatment was able to protect the cheese significantly longer than the control, on average 12 days longer, however, HPBCD complex was able to give the cheese protection the longest. This could be related to the solubility that HPBCD complex exhibits. The addition of HPBCD can increase the solubility of natamycin up to 152-fold (12). This can help distribute the natamycin more evenly over shredded cheese. A prior study indicated that HPBCD complex may have increased variability in application when distributed over shredded cheese (a standard deviation of 1.9 – 8.7 mg/L over 27 days). That study analyzed the different application methods of natamycin and how it was distributed throughout the cheese. The sub-samples of HPBCD illustrated the most variability in comparison to the other application methods (20). This may help increase the

shelf life of the shredded cheese after release of the modified atmosphere packaging.

Visible mold growth is observed even when the average of natamycin quantified is above the minimum inhibitory concentration (MIC). This relates to the distribution of the natamycin treatments on shredded cheese. While the overall concentration of natamycin on the shredded cheese may be at the target level of 20 mg/L, one small area of the cheese may have significantly less and even perhaps below the MIC of 4 mg/L (6). If this occurs, that one area of shredded cheese is susceptible to mold growth, which can spoil the rest of the cheese. More needs to be done to ensure the distribution of natamycin over shredded cheese is spread more evenly so that it may be more effective in preventing mold growth. The overall effectiveness of natamycin is based on its ability to be properly distributed over the shreds of cheese.

**Effect of *Penicillium roqueforti* on Shredded Cheese.** The germination of *Penicillium roqueforti* was slowed by at least 9 days with the use of a natamycin treatment on shredded cheese. The use of HPBCD complex was able to stop visible mold growth from occurring for up to 41 days, 17 days past the control sample, which was significantly longer than any of the other natamycin treatments. The use of the other treatments were also significantly longer than the control, the shortest being 9 days better and the longest being 12 days longer. Overall, there were no significant differences between the dry natamycin, aqueous suspension natamycin, and GCD complex; however, BCD complex was able to stand out significantly when compared to the aqueous suspension of natamycin by protecting the shredded cheese for up to 3 days longer.

Table 2 shows the differences in mold and yeast counts on each of the treatments at both the initial time compared to the final sample day. The amount of mold that was enumerated on the HPBCD complex was significantly lower than each of the other treatments except for BCD complex. The same amount of mold spores were placed onto the cheese samples but HPBCD complex was able to stop the mold from becoming visible to the consumer. With the addition of a natamycin treatment, shredded cheese is protected longer from visible mold growth.

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## Tables

| Treatment                 | Initial Day | Natamycin mg/L | Final Day | Natamycin mg/L |
|---------------------------|-------------|----------------|-----------|----------------|
| Dry Natamycin             | 0.0         | 21.14          | 35.0      | 21.12          |
| Natamycin                 | 0.0         | 19.82          | 33.0      | 18.92          |
| b-CD-Natamycin Complex    | 0.0         | 21.42          | 36.3      | 18.46          |
| HP-b-CD-Natamycin Complex | 0.0         | 20.62          | 41.0      | 17.99          |
| g-CD-Natamycin Complex    | 0.0         | 19.84          | 34.7      | 17.05          |

**Table 5-1.** Natamycin and its various complexes applied on shredded cheese stored in total darkness at 4°C sampled at time 0 and when visible mold growth was present.

| Treatment                 | Initial Day | Initial Mold Count (CFU/g) | Final Day | Final Mold Count (CFU/g) |
|---------------------------|-------------|----------------------------|-----------|--------------------------|
| Control                   | 0.0         | 9.88E+01                   | 24.0      | 1.5E+05                  |
| Dry Natamycin             | 0.0         | 9.88E+01                   | 35.0      | 1.1E+05                  |
| Natamycin                 | 0.0         | 9.88E+01                   | 33.0      | 1.2E+05                  |
| b-CD-Natamycin Complex    | 0.0         | 9.88E+01                   | 36.3      | 8.4E+04                  |
| HP-b-CD-Natamycin Complex | 0.0         | 9.88E+01                   | 41.0      | 5.6E+04                  |
| g-CD-Natamycin Complex    | 0.0         | 9.88E+01                   | 34.7      | 4.7E+05                  |

**Table 5-2.** *Penicillium roqueforti* counts on shredded cheese stored in total darkness at 4°C sampled at time 0 and when visible mold growth was present.

## VITA

Vanessa Elizabeth Teter was born on September 1, 1982 to Stephen and Elizabeth Teter in Elkins, WV. At age 4, Vanessa and her family moved to Virginia where she has lived for the majority of her life. In 2000, Vanessa graduated from Courtland High School. She attended Longwood College for a year and then transferred to Virginia Tech in the fall of 2001.

While at Virginia Tech, Vanessa became active on the crew team as well as the Food Science Club. As a member of the crew team, Vanessa was picked as team captain and became the club secretary in the fall of 2002. While a member of the Food Science Club, she participated on the College Bowl Team as captain for two years. In the summer of 2003, she studied abroad in Paris, France where she finished her French minor. In 2004, Vanessa graduated from Virginia Tech receiving her Bachelor's in Food Science and Technology. She received the Alpha Zeta Outstanding Senior award in the department of Food Science. During that summer, she interned at the USDA in Washington, D.C. and traveled to Italy after receiving the Italian Packaging Award.

That following fall, she began working towards her Master's degree in Food Science under Dr. Joseph Marcy. Vanessa joined IFT in the spring of 2003 and IAFP in the summer of 2005. She has remained active in the Food Science Club, became President for the second term in 2005-2006, and continued her participation on the College Bowl Team as a graduate competitor as well as captain. As President of the Food Science Club, she heavily pushed for a Food Product Development Team and with the help and expertise of Mark Kline, she helped start the first Product Development Team at Virginia Tech.