

SCIENTIFIC OPINION

Scientific Opinion on the use of natamycin (E 235) as a food additive¹

EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS)^{2,3}

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ABSTRACT

Following a request from the European Commission to EFSA, the Scientific Panel on Food Additives and Nutrient Sources added to Food (ANS) was asked to provide a scientific opinion on the safety in use of natamycin (E 235) as a food additive, and on the issue of antimicrobial resistance to natamycin. Natamycin is a fungicide of the polyene macrolide group. According to Directive 95/2/EC, natamycin may be used for the surface treatment of semi-hard and semi-soft cheese and dry, cured sausage at a maximum level of 1 mg/dm² in the outer 5 mm of the surface. The SCF in 1979 considered that the database was adequate to conclude that natamycin does not give rise to safety concern, but inadequate to establish an ADI. JECFA assigned an ADI of 0.3 mg/kg bw/day (1968, 1976, 2002). The Panel considered that the available data are not sufficiently robust for the purpose of deriving an ADI because of the limitations of the present database on natamycin (design of the animal studies, limited number of animals, lack of a carcinogenicity study) and in view of the inadequate description of the human data. The highest potential exposure to natamycin was below 0.1 mg/kg bw/day for children at the 97.5th percentile. Given that natamycin is very poorly absorbed, the Panel considers that this conservative estimate would provide an adequate margin of safety from the effect level seen from the long-term animal studies and the human study used by JECFA to establish an ADI. The Panel considered that the proposed use levels of natamycin are not of safety concern if it is only used for the surface treatment of the rind of semi-hard and semi-soft cheese and on the casings of certain sausages. The Panel concluded that there was no concern for the induction of antimicrobial resistance.

KEY WORDS

Natamycin, pimaricin, antibiotics, E 235, CAS 7681-93-8, antibiotic resistance.

1 On request from the European Commission, Question No EFSA-Q-2006-009, adopted on 26 November 2009.

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3 Acknowledgement: The Panel wishes to thank the members of the Working Group B on Food Additives and Nutrient Sources for the preparation of this opinion: D. Boskou, R. Charrondiere, B. Dusemund, D. Gott, T. Hallas-Møller, A. Hearty, J. König, D. Parent-Massin, I.M.C.M. Rietjens, G.J.A. Speijers, P. Tobback, T. Verguieva, R.A. Woutersen. Furthermore, the Panel wishes to thank the members of the former Additives Working Group of the former Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food for the preparation of this opinion: W. Grunow, K. Hulshof, C. Leclercq, W. Mennes, F. Toldra.

Suggested citation: EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS); Scientific Opinion on the use of natamycin (E 235) as a food additive. EFSA Journal 2009;7(12):1412 [25 pp.]. doi:10.2903/j.efsa.2009.1412. Available online: www.efsa.europa.eu

SUMMARY

Following a request from the European Commission to the European Food Safety Authority, the Scientific Panel on Food Additives and Nutrient Sources added to Food (ANS) was asked to provide a scientific opinion on the safety in use of natamycin (E 235) as a food additive. In addition, EFSA should address the issue of antimicrobial resistance to natamycin.

Natamycin (pimaricin) is a fungicide of the polyene macrolide group. According to Directive 95/2/EC, natamycin may be used for the surface treatment of semi-hard and semi-soft cheese and dry, cured sausage at a maximum level of 1 mg/dm² in the outer 5 mm of the surface, corresponding to 20 mg/kg.

The Scientific Committee for Food (SCF) in 1979 did not establish an Acceptable Daily Intake (ADI) but considered that in relation to the uses of natamycin on cheese and sausages, the database was adequate and did not give rise to safety concern.

The Joint FAO/WHO Expert Committee on Food Additives (JECFA) reviewed the safety of pimaricin (natamycin) in 1968, 1976 and 2002 and assigned an ADI of 0.3 mg/kg body weight (bw)/day.

Information available on the metabolism of natamycin suggests that natamycin is not absorbed to a significant extent from the gastrointestinal tract and is rapidly excreted in faeces either unchanged or as degradation products.

In toxicological studies, the effects observed in animals were a decrease in food intake with a decrease in the rate of body weight gain, gastrointestinal irritation and diarrhoea. Dogs are the most sensitive species to these effects.

Three subchronic toxicity studies with natamycin are available, two in the rat and one in the dog. In the first study, no modifications of haematological and biochemical parameters and organ weights were noted. In the second rat study, decreases of mean food intake and mean body weight have been observed. The No-Observed-Adverse-Effect Level (NOAEL) is considered to be 45 mg/kg bw/day. In the third study, dogs were exposed for 3 months to natamycin. Transient diarrhoea and slight body weight loss have been observed. The NOAEL is considered to be 12 mg/kg bw/day.

Two long-term studies are available, a 2-year chronic toxicity study in the rat and a 2-year chronic toxicity study in the dog. In the rat study, decrease of food intake and reduced growth rate were seen only at the highest dose group. The data showed that the numbers and types of tumours were not significantly different in any of the natamycin-treated groups compared with the untreated control animals. The NOAEL of this study is considered to be 22.4 mg/kg bw/day. In the dog study, the highest dietary concentration induced obesity among the animals. Dietary levels of 6.25 mg/kg bw/day, or less did not affect body weight gain. The NOAEL of this study is considered to be 6.25 mg/kg bw/day.

Natamycin bears a structural alert for genotoxicity since the molecule contains an epoxide ring. However, in the light that:

- the induction of chromosomal aberrations observed in a recent study was accompanied by cytotoxicity,
- there are *in vitro* studies on mutagenicity in bacteria and mammalian cells and on chromosomal aberrations in mammalian cells which were performed in compliance with GLP and were negative,
- no substance-related neoplastic effects were observed in the long term studies,

the Panel considered that the available data do not raise concern with respect to genotoxicity of natamycin.

In a three-generation study of reproductive toxicity in the rat, at the highest dose, an increased number of fetuses born dead, and a decreased number of animals born alive surviving at 21 days in F1 generation, was described. The NOAEL of this study amounts to 50 mg/kg bw/day.

A developmental toxicity study has been performed in female rats from the second litter of the F1 generation of the three-generation reproductive toxicity study. No adverse effects on nidation or maternal or fetal survival were found. The number of abnormalities seen in the soft or skeletal tissues did not differ from that occurring spontaneously in controls. The NOAEL of this study amounts to 50 mg/kg bw/day. In a rabbit developmental study on mated female Dutch belted rabbits, the maternal mortality rates were 0, 5, 9 and 19% in the 4 treatment groups (0, 5, 15 or 50 mg/kg bw/day), respectively. A significant increase in extra sternbrae was noted in groups treated at 15 and 50 mg/kg bw/day, but was considered as normal variation by the Panel. The NOAEL of this study is considered to be 15 mg/kg bw/day due to maternal toxicity at the higher dose level.

A clinical study in humans performed in 1960 showed that natamycin, used for systemic mycoses, induced nausea, vomiting and diarrhoea. Anorexia, nausea, vomiting and flatulence were observed at different doses in different patients. The Panel considered that this study is too limited to derive a NOAEL.

In 1968, JECFA established an ADI of 0.3 mg/kg bw/day based on these human data. The level causing no toxicological effects in man was estimated to be 200 mg/per/day, equivalent to 3 mg/kg bw/day. Given that this dose was derived from human data, an uncertainty factor equal to 10 has been used to calculate the ADI. In 2002 JECFA confirmed this ADI.

Because of the limitations in the present database on natamycin (design of the animal studies, limited number of animals, lack of a carcinogenicity study) and in view of the inadequate description of the human data, the Panel considered that an ADI could not be established from these data.

The highest potential exposure to natamycin was at the 97.5th percentile below 0.1 mg/kg bw/day for children and below 0.05 mg/kg bw/day for adults, derived from the high level consumption of cheese (assuming solely a rind treatment with natamycin) and dried, cured sausages.

Given that natamycin is very poorly absorbed, the Panel considers that this conservative estimate would provide an adequate margin of safety from the effect level seen from the long-term studies in animals and the human study used by JECFA to establish an ADI. The Panel considered that the proposed use levels of natamycin are not of safety concern if it is only used for the surface treatment of the rind of semi-hard and semi-soft cheese and on the casings of certain sausages.

The Panel noted that natamycin is used in the food industry as an antifungal preservative in cheeses and sausages. Natamycin is a polyene antibiotic. The mechanism of action for polyene antibiotics is binding to sterols (principally ergosterol) in the fungal cell membrane. Bacteria are insensitive to polyene antibiotics because their membrane lacks sterols. Furthermore, induction of natamycin-resistant mutants in yeast is reported to be difficult. The Panel concluded that there was no concern for the induction of antimicrobial resistance.

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BACKGROUND AS PROVIDED BY THE EUROPEAN COMMISSION

Natamycin is authorised for food preservation in the European Union by Directive 95/2/EC⁴ on food additives other than colours and sweeteners. Natamycin is permitted for the surface treatment of hard, semi-hard and semi soft cheese and dried, cured sausages. Specific purity criteria for natamycin are laid down in Directive 2008/84/EC⁵.

The Scientific Committee on Food (SCF) in 1979 evaluated the safety of natamycin and considered its use acceptable for the surface treatment of the rind of whole pressed cheese and for casings of certain sausages (SCF, 1979). At that time the SCF recommended that the residues of natamycin in food at the time of sale, expressed in terms of surface area of the casing or rind, should not exceed 1 mg/dm² and that they should not be present at a depth of greater than 5 mm in the food.

The Scientific Committee on Food adopted an opinion on antimicrobial resistance in 28 May 1999. On the basis of this opinion, the Commission adopted on 20 June 2001 a communication on a Community strategy against antimicrobial resistance. Action 9 listed in the Communication is to review the use of two antimicrobial agents in food.

The two substances mentioned are nisin (E 234) and natamycin (E 235).

Therefore, in addition to the toxicological review of natamycin, the issue of antimicrobial resistance should also be addressed.

TERMS OF REFERENCE AS PROVIDED BY THE EUROPEAN COMMISSION

In accordance with Article 29 (1) (a) of Regulation (EC) N° 178/2002⁶, the European Commission asks the European Food Safety Authority to provide a scientific opinion on the safety in use of natamycin. In addition, EFSA should address the issue of antimicrobial resistance to natamycin.

⁴ European Parliament and Council Directive 95/2/EC of 20 February 1995 on food additives other than colours and sweeteners. OJ No L 61, 18. 3. 1995, p. 1.

⁵ Commission Directive 2008/84/EC of 27 August laying down specific purity criteria on food additives other than colours and sweeteners

⁶ Regulation (EC) No 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety.

ASSESSMENT

1. Introduction

The present opinion deals with the safety of natamycin when used for the surface treatment of the rind of semi-hard and semi-soft cheese and on the casings of certain sausages requiring maturation before marketing.

2. Technical data

2.1. Identity of the substance

Natamycin (pimaricin) is a fungicide of the polyene macrolide group. It has a molecular mass of 665.725 g/mol. The CAS Registry Number of natamycin is 7681-93-8 and the molecular formula is $C_{33}H_{47}NO_{13}$. The primary structure of natamycin consists of a large lactone ring of 25 carbon atoms (Figure 1). The lactone ring is linked to a mycosamine moiety, m-amino-sugar, by a glycosidic linkage. Natamycin is classified as a polyene macrolide antibiotic and specifically as a tetraene antibiotic because of its four conjugated double bonds. The mycosamine moiety (3-amino-3,6-dideoxy-D-mannose) of natamycin at the C15 position is a six-membered pyranose ring. Natamycin forms a cylindrical structure due to the alignment of the hydroxyl groups of its amphipathic chain towards each other (Figure 1). The exterior of the cylinder is completely non-polar.

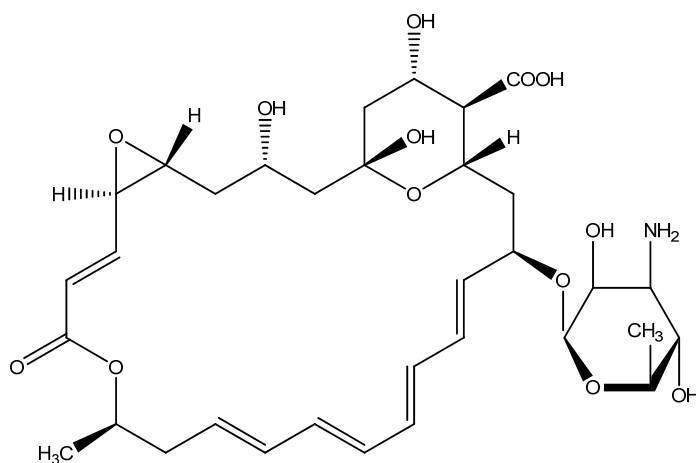


Figure 1: Natamycin

The solubility of natamycin is 20-50 mg/L in water. Natamycin is soluble in glacial acetic acid, methylpyrrolidone, dimethylformamide, dimethylsulfoxide, glycerol and propylene glycol. Natamycin is insoluble in higher alcohols, ethers, esters, aromatic or aliphatic hydrocarbons, chlorinated hydrocarbons, ketones, dioxane, cyclohexanol and various oils (Raab, 1972).

Mycosamine is a major product of hydrolysis of natamycin (Figure 2).

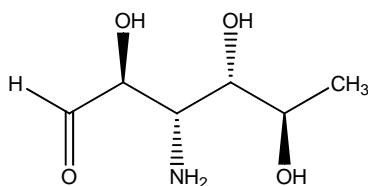


Figure 2: Mycosamine

The petitioner indicates that mycosamine and traces of 13-hydroxy-2,4,6,8,10-tetradeca pentaen-1-al (Figure 3) have been identified in pharmaceutical or industrial natamycin preparations.

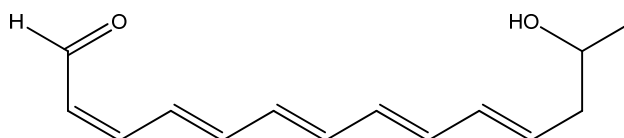


Figure 3: 13-hydroxy-2,4,6,8,10-tetradeca pentaen-1-al

Evidence for the existence of decomposition products of natamycin with an intact lactone ring was obtained when an attempt was made to degrade natamycin at a low pH. In an aqueous 5% weight/volume suspension at pH 1.5, natamycin lost its biological activity completely after having been kept in the dark for 2 months at room temperature, or for 2 weeks at 40°C. From the reaction mixture, the aglycon apo-natamycin (Figure 4) was isolated as a light yellow amorphous substance in a rather high yield. In the degradation reaction, two moles of natamycin gave rise to one mole of apo-natamycin and one mole of the mycosamine

Apo-natamycin contains one natamycin- and one natamycinolide-moiety with each of the epoxy group (at C4 – C5) hydrolysed (Brik, 1976).

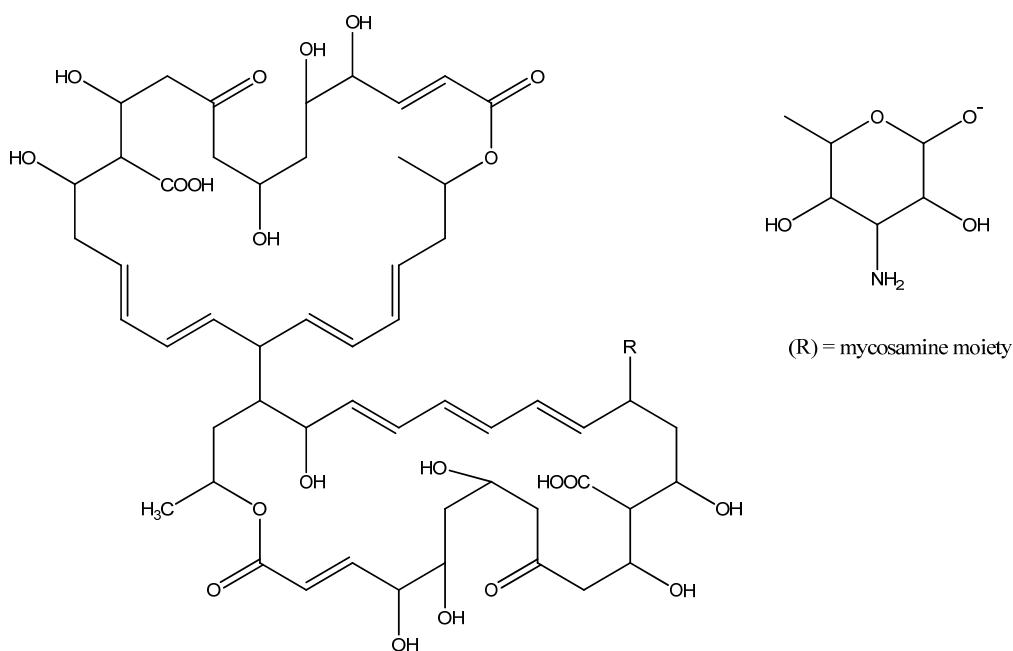


Figure 4: Apo-natamycin

According to Brik (1976), more drastic acid degradation of natamycin eliminates the aminosugar, with formation of the dimer of the hypothetical aglycone of natamycin natamycinolide (in Figure 4, with R=OH). In this dimer, the epoxy groups are also hydrolysed.

2.2. Specifications

Specifications have been defined in Directive 2008/84/EC on purity criteria on food additives others than colours and by the Joint FAO/WHO Expert Committee on Food Additives (JECFA, 2006) (Table 1).

Natamycin is a fungicide of the polyene macrolide group, and is produced by natural strains of *Streptomyces natalensis* or of *Streptococcus lactis*.

Table 1: Specifications for natamycin according to Commission Directive 2008/84/EC and JECFA (JECFA, 2006)

Purity	Commission Directive 2008/84/EC	JECFA (2006)
Loss on drying	Not more than 8% (over P ₂ O ₅ , in vacuum at 60°C to constant weight)	Not more than 8% (60°C, over P ₂ O ₅ , pressure less than 5 mmHg)
Sulphated ash	Not more than 0.5%	Not more than 0.5%
Arsenic	Not more than 3 mg/kg	-
Lead	Not more than 5 mg/kg	Not more than 2 mg/kg
Mercury	Not more than 1 mg/kg	-
Heavy metals (as Pb)	Not more than 10 mg/kg	-
Microbiological criteria	Not more than 100cfu/g	-

2.3. Manufacturing process

Natamycin is produced by submerged aerobic fermentation by *Streptomyces natalensis* and related species. Fermentation is conducted for several days, and the antibiotic is isolated either by broth extraction or by extraction of the mycelium. Dried natamycin recovered from the fermentation broth is white to cream-coloured and has little or no odour or taste; in the crystalline form it is very stable. Optimisation of nutrients in the fermentation media for natamycin production by *S. natalensis* in submerged batch culture has been performed (Farid *et al.*, 2000). *S. natalensis* is absent from the final product. During the extraction procedure the natamycin is dissolved and filtered through a membrane. The membrane is not permeable to the organism and the concentration of the solvent is high enough to kill the organism.

2.4. Mode of action and antimicrobial resistance

Natamycin is used in the food industry as an antifungal preservative in cheeses and sausages. The preservative is effective at concentrations between 1 and 10 mg/kg (Thomas and Delver-Broughton, 2003; Stark, 2004). In general, yeasts are more sensitive than moulds.

The antifungal activities of natamycin and other polyenes (i.e. amphotericin B) are due to their binding to the cell membrane sterol, ergosterol, which is the principal sterol in fungal membranes. Natamycin has a large lactone ring with a rigid lipophilic chain containing conjugated double bonds and a flexible

hydrophilic portion bearing several hydroxyl groups. It is probable that the hydrophobic region complexes with ergosterol in the membrane forming a polar pore through which small ions such as K^+ , H^+ , amino acids and other metabolites can pass freely, disrupting the cell's ionic control and killing the cell (Hamilton-Miller, 1974; Deacon, 1997).

Bacteria are insensitive to these antibiotics because their membranes lack sterols and are therefore naturally resistant to natamycin. Reported Minimum Inhibitory Concentrations (MICs) of natamycin for bacteria are higher than 250 mg/L. Animal cell membranes have cholesterol as their major membrane sterol, for which natamycin has a much lower specificity than ergosterol.

Induction of natamycin-resistant mutants in fungi is reported to be difficult (Athar and Winner, 1971). Such mutants invariably show reduced metabolic and growth rates *in vitro*, and in the absence of polyenes readily revert to normal metabolism, growth, and sensitivity to natamycin. *Candida* strains resistant to nystatin contain less ergosterol than sensitive ones (Athar and Winner, 1971; Safe *et al.*, 1977). It is generally accepted that there is a potential risk of development of resistance among fungal flora as a consequence of prolonged, repeated application of natamycin. However, such studies indicate that the level of resistance would be low. *C. albicans* resistance to natamycin has been induced after 25 passages in media with increasing concentrations of natamycin. This resistance developed gradually, and the MIC increased from 2.5-12 to 12-50 mg/L. JECFA in 1976 reported that the selection of natamycin-resistant strains *in vitro* has not induced cross-resistance to other polyenes (JECFA, 1976).

Surveys in cheese warehouses and in dry sausage factories where a 50% natamycin preparation had been used for up to 10 years showed no change in the composition or the sensitivity of the contaminating fungal flora (de Boer and Stolk-Horsthuis, 1977; de Boer *et al.*, 1979; Hoekstra and Van der Horst, 1998). De Boer and Stolk-Horsthuis (1977) attempted to induce tolerance in strains of fungi by transferring each culture 25-31 times in media containing concentrations of natamycin equal to and greater than the MIC. Following multiple transfers, the MIC increased in only 8 of 26 strains by a maximum of 4 mg/L. The overall lack of increased resistance was due to the lethal (fungicidal) mode of action, and the compound's instability.

The human gastrointestinal flora may be exposed to trace quantities of ingested natamycin residues. The intestinal microflora is predominated by bacterial species, whereas yeast and fungal species are only ca. 0.001% of the total flora. Several studies in experimental animals indicate that natamycin and any potential degradation products do not express antibiotic activity in the colon. There is no experimental evidence of fungi acquiring resistance to natamycin. As bacteria are not affected by polyenes, and fungi are found in low quantities in the intestinal tract, the consequences of exposure to ingested traces of natamycin could be considered as minimal.

2.5. Therapeutic applications in humans and animals

The antifungal properties of natamycin were originally used in the development of products for the treatment of topical fungal disorders. Historically natamycin has been used for treatment of infections of the eye, hair, mucous membranes, nails, and skin involving organisms of the genera *Candida* (candidiasis), *Epidermophyton*, *Microsporium* and *Trichophyton* (tinea; ringworm).

Although originally introduced in a number of countries, natamycin-containing drugs for common fungal infections have become nearly obsolete. However, the transition to newer treatment modalities is not complete worldwide. In some countries, natamycin is still in use. Nonetheless, global sales of natamycin for pharmaceutical use, as recorded by IMS Health (MIDAS), showed a decline of 39% between 1997 and 2000 (JECFA, 2002). The drug encyclopaedia Martindale shows no new additions to its uses section for natamycin from 1972 to 2002 (Martindale 1972, 1977, 1989, 2002).

The only significant remaining human therapeutic use for natamycin is in the treatment of fungal keratitis. A review of the scientific literature since 2002 continues to support this conclusion.

In Europe, a veterinary medicine containing natamycin is available. Although natamycin is still used, newer modalities, e.g. the antimycotic azole agents, are preferred.

2.6. Methods of analysis in foods

Methods of analysis in foods were based on organic solvent extraction followed by UV detection or further HPLC separation with UV detection. The detection limits can reach 0.05 to 0.25 mg/kg (de Ruig *et al.*, 1987; Riedl *et al.*, 1984; Fletouris *et al.*, 1995; Tuinstra and Traag, 1982; Luf and Brandl, 1986; Maruyama *et al.*, 1988). An enzyme immunoassay with anti-natamycin antibody from rabbit was developed (Maertlbauer *et al.*, 1990). Cross-reaction with related antimicrobials (amphotericin B and nystatin) was <0.001% (equivalent to 0.1 mg/kg) and the detection limit was reported to be 0.005 mg/dm², with a recovery of 76 to 84%.

2.7. Stability, reaction and fate on food

Natamycin shows good stability in foods provided that pH is in the range from 5 to 9 (Raab, 1972). It is less stable in foods outside this pH range (Stark, 2004). Natamycin is sensitive to inactivation by oxidants such as peroxides, chlorine and heavy metals (Raab, 1972).

2.8. Case of need and proposed uses

Natamycin is proposed for use for the surface treatment of the rind of semi-hard and semi-soft cheese and on the casings of certain sausages requiring maturation before marketing.

Natamycin is approved under Directive 95/2/EC (Annex III Part C) for the surface treatment of semi-hard and semi-soft cheese and dry, cured sausages at a maximum level of 1 mg/dm² surface (not present at a depth of 5 mm).

2.9. Existing authorisations and evaluations

According to Directive 95/2/EC, natamycin may be used for the surface treatment of semi-hard and semi-soft cheese and dry, cured sausage at a maximum level of 1 mg/dm² in the outer 5 mm of the surface, corresponding to 20 mg/kg. According to the definitions in the Codex General Standard for Cheese (CODEX Stan A-6-1978, rev1-1999, amended 2003), the term 'cheese surface' is used for the outside layer of cheese or parts of cheese, even in the sliced, shredded or grated form. The term includes the outside of whole cheese, disregarding whether a rind had been formed or not (CODEX STAN, 2003).

The SCF reviewed natamycin in 1979 and concluded as follows:

"1. Natamycin has a limited but important use in human medicine and is therefore not acceptable as a food additive for general use in and on foodstuffs."

2. *Its use for the surface treatment of the rind of whole pressed cheese (semi-hard) ripened under aerobic conditions e.g. Gouda and Edam, and on the casings of certain sausages requiring maturation before marketing is acceptable, provided that:*

- *the substance is applied only to the final product*
- *the residues of natamycin in food at a time of sale, expressed in relation to the surface area of the casing or rind, do not exceed 1 mg/dm² and that they will not be present at a depth greater than 5 mm.*

3. *The use of natamycin on the casings of these foods shall be clearly indicated by suitable labelling.*

4. *The position should be reviewed if there is any significant increase in the range of therapeutic uses.”*

However, the SCF in 1979 did not establish an ADI but considered that in relation to the uses of natamycin on cheese and sausages, the database was adequate and did not give rise to safety concern. Neither natamycin nor its principal degradation products are absorbed from the digestive system.

JECFA has reviewed the safety of natamycin (pimaricin) in 1968, 1976 and 2002 and assigned an ADI of 0.3 mg/kg bw/day. The review concluded that “*New information was available on the effects of breakdown products and the development of microbial resistance to the antimycotic if it is used for food preservation. While the Committee expressed a general concern about the use of therapeutic agents in food, it agreed that the data on natamycin showed that problems were unlikely to arise from microbial resistance*”.

This was confirmed by JECFA in 2002, as more recent publications had not conflicted with earlier studies.

On the issue of resistance to antibiotics, JECFA (2006) noted that although use of natamycin as an antifungal agent in food may result in exposure of the endogenous flora to trace quantities of antimicrobial residues, bacteria in the human gastrointestinal tract are not affected by polyenes, and the Committee concluded that disruption of the colonization barrier is not a concern. Fungi are found in much smaller amounts than bacteria in the human gastrointestinal tract, and the negative results in studies of acquired resistance indicate that the selection of natamycin-resistant fungi is not an issue.

2.10. Exposure

The petitioner provided exposure estimates based on an assessment made by the JECFA in 2002. These calculations were based on the consumption of natamycin in ‘a wider range of cheeses and meats’ and partly at higher use levels than currently approved in the EU. The mean potential dietary exposure was 0.014 mg/kg body weight (bw) per day for UK consumers, and 0.015 and 0.01 mg/kg bw/day for children and people aged more than 10 years in Germany. For high consumers (97.5th percentile) these estimates were 0.041, 0.051 and 0.031 mg/kg bw/day, respectively.

The Panel noted that JECFA re-evaluated the exposure to natamycin at its sixty-seventh meeting in 2006 (JECFA, 2007). Refined estimates of dietary exposure were also based on individual consumption surveys from the UK and Germany, with a focus on children aged 1.5-4.5 years and 4-10 years, respectively (Gregory *et al.*, 1995, 2000; Heseker *et al.*, 1994). Children generally have higher food intake than adults, when expressed on a body weight basis, and therefore represent the group with the highest potential exposure to natamycin per kg body weight. The high level exposure estimates (97.5th percentile) for consumers only were presented separately for cheese (assumed use level 40

mg/kg) and cured meat comminuted such as salamis and other dried sausages (use level 20 mg/kg). The Panel used this information to make an estimated exposure using the present EU authorised use levels, corresponding to 20 mg/kg in both cheese and dry, cured sausages.

As shown in Table 2, the estimated high level exposure to natamycin from cheese was 0.04 mg/kg bw/day in the UK and 0.03 mg/kg bw/day in Germany for children, and 0.02 mg/kg bw/day in the UK and 0.025 mg/kg bw/day in Germany, for adults. The estimated high level exposure to natamycin from dry, cured sausages was 0.04 and 0.03 mg/kg bw in children, and 0.006 and 0.02 mg/kg bw in adults in UK and Germany, respectively.

The highest potential exposure to natamycin was at the 97.5th percentile below 0.1 mg/kg bw/day for children and below 0.05 mg/kg bw/day for adults, derived from the high level consumption of cheese (assuming solely a rind treatment with natamycin) and dried, cured sausages. If cheese were treated with natamycin after grating or shredding, the surface on which the treatment is applied would increase significantly. For instance, assuming a surface of 42 cm²/cm³ and grated to pieces of 1 cm x 0.1 cm x 0.1 cm with a density of 1g/cm³, the theoretical maximal concentration level in the rated cheese would be 420 mg/kg.

Table 2: Estimated dietary exposure to natamycin, based on individual food consumption data

Country	Food category	Use level (mg/kg)	Children ¹ at the 97.5 th percentile		Adults at the 97.5 th percentile	
			Food consumption (g/day)	Dietary exposure (mg/kg bw/day)	Food consumption (g/day)	Dietary exposure (mg/kg bw/day) ²
UK	Cheese	20 ³	28	0.04	62	0.02
Germany	Cheese	20	40	0.03	74	0.025
UK	Cured meat comminuted ⁴	20	30	0.04	19	0.006
Germany	Cured meat comminuted	20	43	0.03	64	0.02

¹UK: 1.5-4.5 years (body weight of 15 kg); Germany: 4-10 years (body weight of 25 kg)

² based on a body weight of 60 kg

³ all cheeses other than cream cheese are included, as well as cheeses used in recipes

⁴ salamis and other dried sausages

3. Biological and toxicological data

3.1. Absorption, distribution, metabolism and excretion

3.1.1. Animals

After an oral 50 mg/kg bw dosage of ¹⁴C-natamycin to rats, virtually no radioactivity could be demonstrated outside the gastrointestinal tract by whole-body autoradiography. It may be assumed that the label is distributed uniformly over the large ring system which is made by ¹⁴C-acetate units. At 1 hour, radioactivity was solely concentrated in the oesophagus, stomach and small intestine. At 2 hours, there was some radioactivity in the caecum as well. At 4 hours, it reached the colon. At 8 hours, it concentrated in the intestine, but the stomach still contained radioactive material. At 24 hours, the radioactivity in the gastrointestinal tract was found to have decreased considerably, the largest

concentration was noted in the caecum and colon. Radioactivity was still detected in the stomach and not in the small intestine.

After oral administration, the majority of the radiolabel was eliminated in the faeces within 24 hours. Traces of radioactivity in the liver, kidneys and fatty tissue were only visible following extremely long exposures of the autoradiographic plates (150 days), which was indicative of an extremely low absorption of natamycin from the gastrointestinal tract (Blankwater and Hesse, 1979).

In dogs, after oral administration of ^{14}C -natamycin, the radioactivity is mainly found in the faeces. It may be assumed that the label is distributed uniformly over the large ring system which is made by ^{14}C -acetate units. Less than a few percentages of the dose applied are found in the urine. No essential differences appeared whether the ^{14}C -natamycin was administered via a capsule, as a suspension or via cheese. If the presence of the radioactivity in the urine is the result of absorption, this can be considered to be very low. The authors considered that the low level of radioactivity found in the urine could be caused, partly or in total, by contamination with the radioactivity eliminated via the faeces (Hesse and Meier, 1980). Following intravenous administration of ^{14}C -natamycin in the dog, radioactivity was predominantly excreted via bile. The authors concluded based on both the oral and intravenous data that a maximum of 5% of the radioactivity was absorbed.

Products formed in stomach in acid conditions are likely to be similar to degradation products as described by Brick (1976). Approximately 50% natamycin is broken down in 1 hour in simulated gastric juice, and losses from the stomach of 33-43% and 0-31% occurred in fasted and non-fasted rats respectively (Morgenstern and Muskens, 1976).

3.1.2. Humans

Little information is available on the absorption, distribution, excretion, or metabolism of natamycin in humans. Less than 1 mg natamycin/L (LOD) could be detected in the blood following the ingestion of 500 mg by human subjects (Anonymous, 1968).

3.2. Toxicological data

3.2.1. Acute oral toxicity

The LD_{50} values of natamycin after oral administration are reported to be greater than 1400 mg/kg bw for mouse (Ottens, 1965), 2700 and 4700 mg/kg bw in male and female rats and 1400 mg/kg bw in the rabbit (Levinskas *et al.*, 1966). The LD_{50} for female guinea-pigs is reported to be 450 mg/kg bw (Struyk *et al.*, 1958).

3.2.2. Short-term and subchronic toxicity

Three subchronic toxicity studies are available, two in the rat and one in the dog.

In the first rat study, in which rats (15 males and 15 females per group) were fed natamycin at levels of 0 or 500 mg/kg diet, equivalent to 45 mg/kg bw/day, for 94 to 96 days, there were no significant differences in haematological parameters, organ histology and mean body weight gain of animals

receiving 500 mg/kg natamycin in the diet compared to their respective controls (Hutchison *et al.*, 1966).

The second rat study was carried out using dose levels of 0, 125, 500, 2000 or 8000 mg/kg diet, (equivalent to 0, 10, 45, 190 and 750 mg/kg bw/day for 94 to 96 days, 20 male and 20 female Carworth Farms rats per group). Haematological findings and organ weights were within normal limits, and no gross or microscopic lesions were found that could be attributed to natamycin. After 3 months on test, food consumption of males and females of the highest dose group was approximately 23% and 17% less respectively than that of rats receiving a control diet, while at the 190 mg/kg bw/day level, mean food intake was decreased about 5% for both sexes. Males and females at the highest dose level (750 mg/kg /bw/day), had mean body weights averaging 54% and 67% of their respective controls. At the 190 mg/kg bw/day level, animals averaged about 85% of the mean body weight of their controls (Levinskas *et al.*, 1966).

The Panel concludes that the No-Observed-Adverse-Effect Level (NOAEL) of this study was 45 mg/kg bw/day.

Natamycin was administered to Beagle dogs, (2 male and 2 female per group) in doses of 0, 12, and 25 mg/kg bw/day for 3 months. Clinical findings reported were a transient diarrhoea, recorded mainly in the high-dose group, which lasted in one male for 39 days, and in two females for 8 or 10 days, respectively, and a slight body weight loss in the high dose group. A transient diarrhoea was considered by the authors of the study to be the result of a local bowel irritation (van Eeken *et al.*, 1984).

The Panel noticed that the NOAEL of this study was 12 mg/kg bw/day.

3.2.3. Genotoxicity

The mutagenic potential of natamycin (a 50% suspension of natamycin in water), some of its degradation products (i.e. apo-natamycin, natamycinolidediol and mycosamine hydrochloride), and nitrite with or without a 50% suspension of natamycin in water, have been evaluated in *Bacillus subtilis*, *Salmonella typhimurium* (TA1535, TA1538, TA98 and TA100) and *Escherichia coli* (WP2 trp⁻ and his mutant WP2 uvrA⁻) without exogenous metabolic activation, except for the 50% suspension of natamycin in water. No statistical analyses were reported. The author reported that no positive responses were observed in the spot tests in any of the 3 test systems, except a slight positive response observed with nitrite alone and with the 50% suspension of natamycin in water and nitrite. The authors concluded that the slight positive effect of nitrite is not enhanced by the 50% suspension of natamycin in water (Khoudokormoff, 1977).

The Panel noted that the study's protocol would not match the current standards.

Bone-marrow preparations of 5 male and 5 female rats selected at random from litters produced by the F0, F1 and F2 from the three-generation study in Wistar rat (dosed 0, 5, 15, 50 or 100 mg natamycin/kg bw/day for 11 weeks) were examined. Animals were given colchicine 3 to 4 hours before sacrifice in order to induce metaphase arrest. The number of abnormalities in the metaphase chromosomal preparations of test groups did not differ significantly from that in sham-treated controls (Cox *et al.*, 1973).

According to a report of the European Agency for the Evaluation of Medicinal Products (EMEA) (1998), the mutagenicity of natamycin has been tested in a set of GLP-compliant studies. A bacterial mutation assay in *S. typhimurium* strains TA1535, TA1537, TA1538, TA98, TA100 with and without metabolic activation (S9 mixed from Arochlor 1254-induced liver preparations), a mouse lymphoma

mutation assay at the TK locus with and without metabolic activation and a chromosomal aberration assay with Chinese hamster ovary (CHO) cells *in vitro* have been carried out. In none of the experiments was there any observed evidence that natamycin had mutagenic potential.

In 2009, Rencüzoğullari *et al.*, investigated the effects of natamycin on chromosome aberrations (CAs), sister chromatid exchanges (SCEs), and micronucleus (MN) formation in human lymphocytes. The human lymphocytes were treated with 13, 18, 23 and 28 µg/mL of natamycin for 24 and 48 hours. According to the authors, natamycin increased the SCE frequency at the highest concentration for 48 hours only; however, it increased the structural CA and MN frequency at all concentrations when compared to control and at all concentrations, except the lowest concentration (13 µg/mL), when compared to solvent control. Natamycin showed a cytotoxic effect as indicated by decrease in the replication index, mitotic index, and nuclear division index (NDI), especially at the highest concentrations for two treatment periods. The Panel considered the results from the SCE and MN assays negative, since the effects observed were very weak. The results of the CA assay were of limited relevance, since these effects were accompanied by cytotoxicity.

3.2.4. Chronic toxicity and carcinogenicity

Two long-term toxicity studies are available, a 2-year chronic toxicity study in the rat and a 2-year chronic toxicity study in the dog.

The rat study (35 male and 35 female Carworth Farms rats per group), was carried out at dietary levels of 0, 125, 250, 500 or 1000 mg/kg diet, equivalent to 4.5, 11.0, 22.4 and 46.3 mg/kg bw/day in males, and 7.58, 15.4, 30.4 and 63.7 mg/kg bw/day in females. Natamycin had no effect on the survival of the rats. Decreased food intake and reduced growth rate were seen only at the highest dose group. After 6 months on test, females fed 500 mg/kg of natamycin had a significant increase in hemoglobin value. Since there were no significant differences in mean hemoglobin concentration at other times or among animals fed higher level of natamycin, this difference was considered to be of no consequence. Means haematocrits at all times did not differ significantly from corresponding control values. Total and differential leukocyte counts at each period did not indicate any deviations from normal values. The data showed that the numbers and types of tumours, which were mainly mammary gland adenocarcinoma, pituitary chromophobe adenoma and uterine and vaginal polyps, were not significantly different in any of the natamycin-treated groups compared with the untreated control animals (Levinskas *et al.*, 1963, 1966).

The Panel concludes that the NOAEL of this study amounts to 22.4 mg/kg bw/day, given the decrease of food intake and the reduced growth rate at the highest level.

The Beagle dog study (3 males and 3 females per group) was carried out at dietary levels of 0, 125, 250 or 500 mg/kg diet. Body weights increased steadily from the start of the experiment until the 15th month of the trial. The daily dose was then reduced by one-sixth because of excessive obesity among the animals at the highest dose; there was a marked reduction in body weight of all dogs. After dose reduction, two males and one female were unable to maintain an adequate body weight. Dietary levels of 250 mg/kg or less, did not affect body weight gain or its maintenance. Periodic determinations of haematologic and clinical chemistry values did not reveal any alterations which could be ascribed to feeding of natamycin. Males fed 125 and 250 mg natamycin/kg diet had mean liver weights at autopsy which were significantly lower than the mean liver weights of the controls. Since mean liver weight of both sexes fed 500 mg/kg diet did not differ significantly from the corresponding value for their respective controls, and since there is no indication of a dose-response effect, the authors concluded that feeding of natamycin did not affect liver-to-body weight ratios (Levinskas *et al.*, 1966).

The Panel concludes that the NOAEL of this study was 250 mg/kg diet, equivalent to 6.25 mg/kg bw/day.

3.2.5. Reproductive and developmental toxicity

3.2.5.1. Studies of reproductive toxicity

A study of reproductive toxicity has been performed in the rat, at dietary levels of 0 or 1000 mg/kg diet. Fertility, gestation, lactation and viability indices were similar to or better than those of the controls. There was a low incidence of abnormalities among pups in this study, but none were considered treatment-related by the authors (Levinskas *et al.*, 1963; 1966).

The Panel concludes that this study is too limited to derive a NOAEL.

In a three-generation study of reproductive toxicity in the rat (0, 5, 15, 50 or 100 mg/kg bw/day for 11 weeks, 10 males and 20 females per group, Wistar), animals exposed to 100 mg/kg bw/day had an increased number of fetuses born dead, and a decrease in the number of animals born alive surviving at 21 days in F1 generation. Pup weight at 21 days was also depressed for the second generation. Fertility, gestation, viability and lactation indices were within normal limits for both litters of all three generations. Based on growth and reproduction data, the highest dose level of 100 mg/kg bw/day of natamycin in the diet of rats is considered an effect level. Natamycin dietary doses of 5, 15 and 50 mg/kg bw/day had no effect on growth, reproduction and on gross and microscopy pathology (Cox *et al.*, 1973).

The Panel concludes that the NOAEL of this study amounts to 50 mg/kg bw/day.

3.2.5.2. Developmental studies

A developmental toxicity study has been performed in female Wistar rats from the second litter of the F1 generation of the three-generation study of reproductive toxicity. They were reared to maturity and mated with untreated control males. The 20 pregnant females/group were given the same dose as their parents (0, 5, 15 or 50 mg/kg bw/day of natamycin according to their original group) by intragastric intubation on days 6 to 18 of gestation, and were killed and examined on day 20. No adverse effects on nidation or maternal or fetal survival were found. The number of abnormalities seen in the soft or skeletal tissues did not differ from that occurring spontaneously in controls (Cox *et al.*, 1973).

The Panel concludes that the NOAEL of this study amounts to 50 mg/kg bw/day.

In a rabbit developmental study, a 50% suspension of natamycin in water was administered by gavage to mated female Dutch belted rabbits (0, 5, 15 or 50 mg/kg bw/day on days 6 to 18 of gestation, 20-26 females per group). The maternal mortality rates were 0% (0/20), 5% (1/20), 9% (2/22), and 19% (5/26) in the 4 groups, respectively. The cause of death was not indicated in the report. There were no significant differences in pregnancy, implantation, number of live fetuses, number of dead fetuses or number of resorptions per dam between any test group and the control. Fetuses were evaluated for skeletal anomalies. The abnormalities noted in fetuses whose dams received natamycin at 5, 15 and 50 mg/kg bw/day consisted of skeletal anomalies generally regarded as spontaneous variations rather than malformations. A significant increase in extra sternbrae was noted in groups treated at 15 and 50 mg/kg bw/day in 5 litters out of 7, and 3 litters out of 14 respectively.

The Panel considered as indicated by the petitioner, that extra sternebrae is a common variant in developmental toxicity studies, particularly in the presence of maternal toxicity as in this study, and is not considered to be indicative of a teratogenic effect of natamycin, since there were no other significant skeletal effects that could be ascribed to treatment (Knickerbocker and Re, 1978, 1979).

The Panel considered that the incidence of mortality at the level of 5 and 15 mg/kg bw/day could be expected in a normal rabbit colony and therefore considered that only the dose level of 50 mg/kg bw/day provided conclusive evidence of toxicity. The Panel derived a NOAEL of 15 mg/kg bw/day based on maternal toxicity.

3.2.6. Human data

In a study by Newcomer *et al.* (1960), natamycin has been administered orally to 10 patients suffering mycosis. The doses given to the patients varied from 25 to 1000 mg/person/day for 20 to 180 days. The treatment caused anorexia, nausea and vomiting at doses of 200 mg/person/day and above. At a level of 50 mg/person/day flatulence was described. Only one of the 10 patients, individually administered 25-75 mg/person/day during 70 days did not report any adverse effect of treatment. In three cases, the treatment was stopped because of toxicity. One patient tolerated a level of 400 mg/day without gastro-intestinal troubles, but could not exceed this dose. According to the authors, one patient reported anorexia, nausea and vomiting at a level of 50 mg/day. JECFA allocated the ADI of 0.3 mg/kg bw/day from this study in 1968, considering that the level causing no toxicological effects in man was 200 mg/per/day, equivalent to 3 mg/kg bw/day, with an uncertainty factor of 10.

The Panel considers that this study is too limited to derive a NOAEL.

Natamycin has been used for over 40 years to treat vaginal candidiasis, including during early pregnancy. An early study found no effect of treatment on congenital abnormalities (Patel, 1973). A large case-control study in Hungary covering births from 1980-1996 (Czeizel *et al.*, 2003) included 22843 pregnancies resulting in a congenital abnormality and 38151 pregnancies with normal outcomes. Among these, there were 62 cases and 98 controls that had been treated with natamycin during pregnancy by intravaginal tablet of 25 mg/day, once or twice per day, for a minimum of at least 2 days. There was no increase in fetal abnormalities following maternal treatment with natamycin at any time during pregnancy (Odds Ratio 1.1, 95% Confidence Interval 0.8-1.5), nor was there any increase when the data for treatment during the susceptible period of the second or third month of pregnancy was analysed (Odds Ratio 0.9, 95% Confidence Interval 0.4-1.8).

The Panel notes that these data do not raise any concern, but that they cannot be used for the risk assessment of natamycin as a food additive.

4. Discussion

The information on the toxicokinetics of natamycin suggests that natamycin is not absorbed to any significant extent from the gastrointestinal tract and is excreted in the faeces. After oral administration of ¹⁴C-natamycin to rats, virtually no radioactivity could be demonstrated outside the gastrointestinal tract by whole-body autoradiography, and most of the radiolabel was eliminated in the faeces within 24 hours. Products formed in acidic conditions of the stomach are likely to be the same as degradation products obtained in acidic conditions *in vitro* (Brik, 1976).

Three subchronic toxicity studies with natamycin are available, two in the rat and one in the dog. In the first rat study, no modifications of haematological, biochemical parameters and organ weight were noted. In the second rat study, decreases of mean food intake and mean body weight have been observed. The NOAEL is considered to be 45 mg/kg bw/day. In the third study, dogs were exposed for 3 months to natamycin. Transient diarrhoea and slight body weight loss have been observed. The NOAEL is considered to be 12 mg/kg bw/day.

Two long-term studies are available, a 2-year chronic toxicity study in the rat and a 2-year chronic toxicity study in the dog. In the rat study, decrease of food intake and reduced growth rate were seen only at the highest dose group. The data showed that the numbers and types of tumours were not significantly different in any of the natamycin-treated groups compared with the untreated control animals. The NOAEL of this study is considered to be 22.4 mg/kg bw/day. In the dog study, the highest dietary concentration induced obesity among the animals. Dietary levels of 6.25 mg/kg bw/day or less, did not affect body weight gain. The NOAEL of this study is considered to be 6.25 mg/kg bw/day.

In a three-generation study of reproductive toxicity in the rat, at the highest dose an increased number of fetuses born dead, and a decreased number of animals born alive surviving at 21 days in F1 generation was described. The NOAEL of this study amounts to 50 mg/kg bw/day.

A developmental toxicity study has been performed in female rats from the second litter of the F1 generation of the three-generation study of reproductive toxicity. No adverse effects on nidation or maternal or fetal survival were found. The number of abnormalities seen in the soft or skeletal tissues did not differ from that occurring spontaneously in controls. The NOAEL of this study amounts to 50 mg/kg bw/day. In a rabbit developmental study on mated female Dutch belted rabbits, the maternal mortality rates were 0, 5, 9, and 19% in the 4 treatment groups (0, 5, 15 or 50 mg/kg bw/day), respectively. A significant increase in extra sternbrae was noted in groups treated at 15 and 50 mg/kg bw/day, but considered as normal variation by the Panel.

The NOAEL of this study is considered to be 15 mg/kg bw/day due to maternal toxicity at the higher dose level.

Natamycin bears a structural alert for genotoxicity since the molecule contains an epoxide ring. However, in the light that:

- the induction of chromosomal aberrations observed in a recent study was accompanied by cytotoxicity,
- there are *in vitro* studies on mutagenicity in bacteria and mammalian cells and on chromosomal aberrations in mammalian cells which were performed in compliance with GLP and were negative,
- no substance-related neoplastic effects were observed in the long term studies,

the Panel considered that the available data do not raise concern with respect to genotoxicity of natamycin.

A clinical study in humans performed in 1960 showed that natamycin, used for systemic mycoses, induced nausea, vomiting and diarrhoea. Anorexia, nausea, vomiting and flatulence were observed at different doses in different patients. The Panel considered this study too limited to derive a NOAEL.

In 1968, JECFA established an ADI of 0.3 mg/kg bw/day based on these human data. The level causing no toxicological effects in man was estimated to be 200 mg/per/day, equivalent to 3 mg/kg bw/day. Given that this dose was derived from human data, an uncertainty factor equal to 10 has been used to calculate the ADI. In 2002, JECFA considered that the results of the developmental study

performed by Knickerbocker and Re (1978, 1979) were difficult to interpret owing to maternal mortality, problems associated with gavage of rabbits, and because the digestive system of rabbits is sensitive to antibiotics. However, JECFA, in agreement with the general consensus on the significance of extra sternebrae, considered that there was evidence that the extra sternebrae observed in fetuses at the intermediate (15 mg/kg bw/day) and high (50 mg/kg bw/day) doses of natamycin were variations rather than malformations. In view of the known sensitivity of the rabbit to gastrointestinal disturbance from antibiotics and the evidence of maternal toxicity in this study, JECFA confirmed in 2002 the ADI of 0.3 mg/kg bw/day.

Because of the limitations in the present database on natamycin (design of the animal studies, limited number of animals, lack of a carcinogenicity study) and in view of the inadequate description of the human data, the Panel considered that an ADI could not be established from these data.

The highest potential exposure to natamycin was at the 97.5th percentile below 0.1 mg/kg bw/day for children and below 0.05 mg/kg bw/day for adults, derived from the high level consumption of cheese (assuming solely a rind treatment with natamycin) and dried, cured sausages. If cheese were treated with natamycin after grating or shredding, the surface on which the treatment is applied would increase significantly. For instance, assuming a surface of 42 cm²/cm³ and grated to pieces of 1 cm x 0.1 cm x 0.1 cm with a density of 1g/cm³, the theoretical maximal concentration level in the rated cheese would be 420 mg/kg.

The Panel noted that natamycin is used in the food industry as an antifungal preservative in cheeses and sausages. Natamycin is a polyene antibiotic. The mechanism of action for polyene antibiotics is binding to sterols (principally ergosterol) in the fungal cell membrane. Bacteria are insensitive to polyene antibiotics because their membrane lacks sterols. Furthermore, induction of natamycin-resistant mutants in yeast is reported to be difficult.

CONCLUSIONS

The Panel considered that the available data are not sufficiently robust for the purpose of deriving an ADI because of the limitations of the database on natamycin (design of the animal studies, limited number of animals, lack of carcinogenicity study) and in view of the inadequate description of the human data.

The highest potential exposure to natamycin was at the 97.5th percentile below 0.1 mg/kg bw/day for children and below 0.05 mg/kg bw/day for adults, derived from the high level consumption of cheese (assuming solely a rind treatment with natamycin) and dried, cured sausages.

Given that natamycin is very poorly absorbed, the Panel considers that this conservative estimate would provide an adequate margin of safety from the effect level seen from the long-term studies in animals and the human study used by JECFA to establish an ADI. The Panel considered that the proposed use levels of natamycin are not of safety concern if it is only used for the surface treatment of the rind of semi-hard and semi-soft cheese and on the casings of certain sausages.

The Panel concluded that there was no concern for the induction of antimicrobial resistance.

DOCUMENTATION PROVIDED TO EFSA

1. A Review of the Safety of Natamycin. December 2005. Submitted by Cantox Health Sciences International.

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GLOSSARY [AND/OR] ABBREVIATIONS

ADI	Acceptable Daily Intake
ANS	Scientific Panel on Food Additives and Nutrient Sources added to Food
bw	body weight
CA	Chromosome Aberrations
CAS	Chemical Abstract Service
cfu	colony-forming units
EFSA	European Food Safety Authority
EMA	European Medicines Agency
FAO/WHO	Food and Agriculture Organization/World Health Organization
GLP	Good Laboratory Practice
HPLC	High-Performance Liquid Chromatography
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LOD	Limit Of Detection
MN	Micronucleus
NDI	Nuclear Division Index
NOAEL	No-Observed-Adverse-Effect Level
SCE	Sister Chromatid Exchanges
SCF	Scientific Committee for Food
UV	Ultra-Violet