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Rapid method to determine natamycin by HPLC-DAD in food samples for compliance with EU food legislation

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ABSTRACT

Natamycin is an antibiotic belonging to the group of polyene macrolides extensively employed as additive to prevent the microbial deterioration in food. The Annex III of the Directive 95/2/EC fixes in 1 mg/dm^2 the maximum level of the antifungal allowed in the final product.

Reliable and sensitive methods for the analysis of natamycin are required to guarantee compliance with food legislation as well as to improve consumer protection.

In the present paper, a simple and rapid high performance liquid chromatographic method with diode-array detection (HPLC-DAD) to determine natamycin in food samples was developed. Natamycin was extracted from food samples by using methanol acidified with acetic acid. The chromatographic separation was performed on a reversed-phase Kromasil ODS (C18) (150 × 3.20 mm i.d., 5 μm particle size) and the analysis was completed within 6 min. The method was validated in terms of linearity, limits of detection and quantification, repeatability and recovery. Satisfactory repeatability (R.S.D. ($n = 10$) < 4%, excellent sensitivity (LOD: 0.01 μg/mL; LOQ: 0.05 μg/mL) and appropriate recoveries were achieved.

With the proposed method natamycin levels were determined in different food items. Data showed that two samples exceeded the limit established by the Annex III of the Directive 95/2/EC. Natamycin was also detected in samples in which its use is not allowed. The results were confirmed by LC-MS/MS using electrospray ionization (ESI) in positive mode.

1. Introduction

Surface of foods, particularly, cheese and sausage, are prone to contamination by micoorganisms. This contamination causes deterioration in food, which diminishes food quality and consumers acceptance. In order to inhibit or retard the growth of the microorganisms and consequently enhance the food safety and extend the shelf-life of the food products different systems have been developed. Some of the most commonly used include polymeric films additivated with antimicrobial agents or methods that involve a direct application on the food surface such as, dipping, spraying or brushing (Fajardo et al., 2010; Hanušová, Dobiáš, & Klaudisová, 2009; Koontz & Marcy, 2003; Koontz, Marcy, Barbeau, & Duncan, 2003; Pintado, Ferreira, & Sousa, 2010; Türe, Eroğlu, Soyer, & Özen, 2008).

Among the antimicrobial substances employed by the food industry, natamycin has been proven effective in controlling microbial growth. Natamicyn or pimaricin is an antibiotic belonging to the group of polyene macrolides produced by actinomycetes *Streptomyces natalensis* (Koontz & Marcy, 2003; Moreira de Oliveira, Ferreira Soares, Magela Pereira, & de Freitas Fraga, 2007) "these antibiotics" act by binding to sterols, especially ergosterol, in the fungal cell membrane (EFSA, 2009). Several studies have reported the use of natamycin for the surface treatment of cheeses and dry sausages (Hanušová et al., 2010; Moreira de Oliveira et al., 2007; Var, Erginkaya, Güven, & Kabak, 2006).

The European Union through Annex III Directive 95/2/EC stated the use of natamycin as an additive. The maximum levels of the antifungal allowed in the final product should not exceed 1 mg/dm^2 and should not be present at a depth greater than 5 mm (EU Parliament and Council Directive 95/2, 1995).

The European Food Safety Authority (EFSA) by request of the European Commission published a scientific opinion on the use of natamycin as food additive. The Scientific Panel on Food Additives and Nutrient Sources added to Food (ANS) concluded that the levels

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of natamycin allowed for the surface treatment of the rind of semi-hard and semi-soft cheese and on the casings of certain sausages were not of safety concern (EFSA, 2009).

Recently, it was published that German authorities had detected natamycin in wines from Argentina. Its use in wines is not allowed in the EU. The sale of the wines was prohibited and withdrawn from the market. Because the antifungal is used as a cleaning product in wine cellars, natamycin could be present in the wine as a result of an accidental contamination.

Regarding the analytical methods to determine natamycin in food samples, the process generally involves the extraction from the sample by using organic solvents followed by a spectrophotometric (dos Santos Pires et al., 2008) or chromatographic analysis with UV detection (Koontz et al., 2003; EFSA, 2009).

dos Santos Pires et al. (2008) determine natamycin spectrophotometrically in sliced mozzarella cheese samples after extraction with a mixture of acetonitrile-phosphoric acid, (4:1 v/v).

Koontz et al. (2003) quantify the antifungal by reversed-phase high-performance liquid chromatography (RP-HPLC). The separation was performed on a (4.6 × 150 mm, 5 μm) Waters Spherisorb C8 column and using a gradient system composed by (A) methanol-water-acetic acid, (60:40:5, v/v/v), (B) 100% water; and (C) 100% methanol. Detection was carried out at 304 nm.

Recently, liquid chromatography coupled to mass spectrometry has appeared as a powerful analytical tool to determine the additive at very low concentrations in wine samples (Mariño Repizo, Dante Martínez, Olsina, Cerruti, & Raba, 2012; Roberts, Scotter, Godula, Dickinson, & Charlton, 2011).

This study aims to develop a simple and rapid method to determine natamycin in food samples that can be used for quality control. The proposed method was validated with regard to linearity, limit of detection, repeatability and recovery. In the second part of the work, a survey on the presence of natamycin in different types of cheese, sausages and wines purchased in local supermarkets was performed.

LC-MS/MS using electrospray ionization (ESI) in positive mode was used to confirm the results.

2. Materials and methods

2.1. Reagents

Standard of pimaricin or natamycin (CAS no 7681-93-8), from Streptomyces chattanoogensis, minimum 95% (HPLC) was supplied by Sigma (Steinheim, Germany). The chemical structure and physicochemical properties of natamycin are presented in Table 1. The information was obtained from ChemIDplus Advanced (United States National Library of Medicine). All reagents were of analytical quality. Acetonitrile hypergrade for LC-MS, methanol and acetonitrile HPLC grade and acetic acid were from Merck (Darmstadt, Germany); formic acid solution puriss. p.a. for HPLC, 50% in water was from Fluka (Steinheim, Germany); water used for all solutions was obtained from Milli-Q water purification system (Millipore) (Bedford, MA, USA).

2.2. Standard solution preparation

Stock standard solutions were prepared by dissolving an accurate quantity of natamycin in methanol acidified with 0.001% (v/v) acetic acid in a volumetric flask. The flask was shaken until a homogenous solution was formed. The stock standard solution had a concentration of 100 μg/mL. Finally, the solution was diluted with methanol acidified with 0.001% (v/v) acetic acid until the desired concentration was achieved.

2.3. Apparatus

2.3.1. HPLC–UV analysis

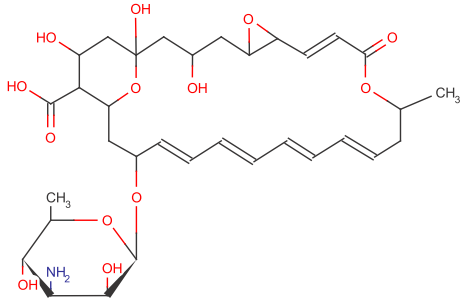
An HPLC HP1100 system (Hewlett–Packard, Waldbronn, Germany) equipped with a quaternary pump, a degassing device, an autosampler, a column thermostatting system, a diode-array detector (DAD), and Agilent Chem-Station for LC and LC/MS systems software was used. Separation was performed on a Kromasil ODS (C18) (150 × 3.20 mm i.d., 5 μm particle size) column thermostatted at 25 °C. Acetonitrile (A) and Milli-Q water (B) were used as mobile phase. Samples were eluted in gradient mode. The gradient elution programme is shown in Table 2. Three selected wavelengths were set in DAD detector, 291.4, 304.4 and 319.4 nm, corresponding to the three absorption peaks of the characteristic natamycin spectrum (Fig. 1A). The injection volume was 20 μL.

2.3.2. LC/ESI/MS/MS analysis

An LC-MS/MS system comprising an Accela autosampler, a column oven and Accela 1250 pump fitted with a degasser, coupled to a triple quadrupole mass spectrometer TSQ Quantum Access max controlled by Xcalibur was used (Thermo Fisher Scientific, San José, CA, USA).

MS data were acquired in the positive ion mode employing electrospray ionization (ESI). Mass spectra were monitored in the mass range *m/z* 100–800. Optimized MS/MS detector settings were: Spray voltage 3000 V, vaporizer temperature 350 °C, ion transfer tube temperature 350 °C. Nitrogen was used as sheath gas (pressure 20 psi) and as auxiliary gas (pressure 5 arbitrary units), Argon was used as the collision gas (1.5 mTorr) and tube lens voltage was 69 V.

Table 1
Chemical structure and physicochemical characteristics of natamycin.

Chemical structure	Physico chemical characteristics	
	Formula	$C_{33}H_{47}NO$
	CAS No	7681-93-8
	MW	665.7
	Melting point	290 °C[a]
	log P (octanol–water)	−3.670[b]
	Water solubility	4100 mg/L (21 °C)

[a] Experimental.
[b] Estimated.