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A simple and fast method for the inspection of preservatives in cheeses and cream by liquid chromatography- electrospray tandem mass spectrometry

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ABSTRACT

In this work, a simplified extraction and short time of analysis method for the simultaneous determination of natamycin, nisin and sorbic acid in cheeses and cream by reverse phase liquid chromatography-electrospray-tandem mass spectrometry was developed. Full validation was performed according to the Commission Decision 2002/657/EC criteria and method applicability was checked on several samples, aiming to inspect their compliance with regulatory limits. The method was linear in the concentration ranges of 0–10 mg kg^{-1} (natamycin), 0–25 mg kg^{-1} (nisin) and 0 20 mg kg^{-1} (sorbic acid). Samples of the three most consumed types of cheese (fresh, pasta filata and ripened) in Brazil and cream (ultra high temperature and pasteurized, 20–30% fat content) were assessed. A surprising rate of non-compliance was observed, especially among ripened grated cheeses, since 80% of samples were above the maximum limit for sorbic acid with an average concentration of 2766.3 ± 10.8 mg kg^{-1}. Moreover, a major non-compliance for the cream samples was observed. The proposed method can be applied as an efficient tool for the inspection of preservatives in cheeses and cream.

1. Introduction

The deterioration of food due to microbiological contamination is a worldwide problem. The action of bacteria, yeasts and moulds can generate huge losses in the whole food chain. In addition, pathogenic bacteria such as *Listeria monocytogenes* can contaminate food, constituting serious risk to the consumers health [1].

The addition of antimicrobial preservatives may enable the extension of shelf-life and safety of processed foods, by reducing or even preventing the growth of spoilage and pathogenic microorganisms. Among the antimicrobial agents used to inhibit microorganisms in dairy products are the bacteriocins, the polyene macrolides and the organic acids [2,3]. Nisin (E234) is a heat-stable bacteriocin of the lantibiotics class, produced by certain strains of *Lactococcus lactis*. It is used in dairy products to inhibit Gram-positive spoilage and pathogenic bacteria and their spores [4]. Natamycin (E235) is a polyene macrolide antifungal agent mainly produced by aerobic fermentation of *Streptomyces natalensis* that prevents fungal growth on cheese rind [5]. Sorbic acid or its potassium and calcium salts (E200; E202; E203) are used to prevent yeast and mould contamination in processed foods, such as fermented dairy products, without much interference in the action of bacterial starter cultures [6].

The use of additives such as antimicrobial preservatives in food is strictly regulated by food authorities, given the potential risk to the health and safety of consumers. In Brazil, the use of nisin and natamycin in cheeses should comply the regulatory limits (RL) of 12.5 mg kg^{-1} and 5 mg kg^{-1}, respectively. Moreover, the use of natamycin is allowed only on cheese rind (not exceeding 2 mm depth). A RL of 1000 mg kg^{-1} for sorbic acid has been setlled by inspection authority [7]. On the other hand, preservatives are not allowed for the processing of any type of cream.

Different techniques have been employed for the quantification of preservatives in foods, such as high-performance liquid chromatography-diode array detector (HPLC-DAD) [8], reverse phase HPLC (RP-HPLC) [9,10], HPLC-ultraviolet/visible detector (HPLC-

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UV) [11], micellar electrokinetic chromatography (MEKC) [12], quantitative proton nuclear magnetic resonance spectroscopy (qHNMR) [13], among others. However, liquid chromatography-tandem mass spectrometry (LC-MS/MS) is considered one of the most selective and sensitive techniques for the determination of preservatives in food [14–17].

Due to the lack of analytical data related to the quantification of antimicrobial preservatives in dairy products in Brazil, we developed a simple and fast method method for the simultaneous determination of natamycin, nisin and sorbic acid in cheeses and cream by reverse phase liquid chromatography-tandem mass spectrometry (LC-MS/MS). In this work, we evaluated samples of cream (pasteurized and ultra high temperature) and three of the most consumed types of cheese in Brazil (fresh, pasta filata and ripened). The method applicability was checked on several samples, aiming to inspect their compliance with regulatory limits.

2. Material and methods

2.1. Standards and reagents

Natamycin (CAS no. 7681-93-8) from *S. natalensis* and nisin (CAS no. 1414-45-5) from *L. lactis* were obtained from DuPont Nutrition and Health, (Madison, WI, USA). Sorbic acid (CAS no. 110-44-1) was obtained from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany). Both standards were of analytical grade purity (98%), except nisin (2.5%). All solvents were chromatographic grade. Methanol was supplied by Tedia Co. (Fairfield, OH, USA) and acetonitrile was supplied by Merck KGaA (Darmstadt, Germany). Analytical grade formic acid and acetic acid were obtained from J.T. Baker Chemical Co. (Phillipsburg, NJ, USA). Ultra-pure water was obtained from a Mega Purity water purification system (Billerica, MA, USA). Stock solutions for natamycin and sorbic acid were separately prepared for both standards at 1000 mg L^{-1} in methanol. Stock solution for nisin was prepared at 1000 mg L^{-1} in acetonitrile and aqueous 0.1% formic acid. Working solutions were prepared by diluting all the stock solutions with methanol. Both stock and working solutions were stored at 4 °C.

2.2. Samples and blank samples

Blank samples were prepared for each type of cheese (fresh, pasta filata and ripened) in a food processing plant at Universidade Federal de Santa Catarina. Samples were then freeze-dried at a lyophilizer (Liobrás Comércio e Serviço de Liofilizadores, São Carlos, Brazil) and kept at −18 °C until analysis. The cream blank sample was obtained from a federally inspected factory.

Different Brazilian commercial types of cheese were evaluated in this work: fresh (*Minas Frescal*, $n=54$); pasta filata (*Mozzarella* and *Provolone*, $n=18$); and ripened (*Parmesano*, *Grana*, *Gouda* and *Prato*, $n=20$). Pasteurized and Ultra High Temperature (UHT) cream samples (20–30% fat content, $n=12$), produced by different state or federally inspected dairy factories from seven different Brazilian states, were also assessed.

2.3. Preparation of processed samples

Cheese samples were processed using a grater and a food processor from Oster (Chicago, IL, USA). For the natamycin assay in cheese, the sample was divided into two portions, the first containing only the rind and the second with the internal portion of the homogenized sample. For the analysis of sorbic acid and nisin, the whole homogenized sample was used. Cream samples were homogenized in their original packages and weighed for analysis. The samples were stored in polypropylene tubes at 4 °C until analysis.

2.4. Method of extraction

For the extraction of preservatives, the sample was weighed (2.0 ± 0.1 g) into a 50 mL polypropylene tube. After the addition of 10 mL acetic acid 0.1% in water:methanol (1:9, v/v), the suspension was homogenized with a T18 basic Ultra Turrax (IKA, Staufen, Germany). The suspension was mildly shaken on an orbital shaker (Tecnal Equipamentos para Laboratório, Piracicaba, Brazil) for 20 min and then centrifuged (Thermo Fischer Scientific Inc., Waltham, MA, USA) at 3,488 g for 10 min at 4 °C. The supernatant was transferred to another 15 mL polypropylene tube and then was kept at −18 °C for 1 h. Centrifugation was performed again at 3,488 g for 10 min at 4 °C. Finally, an aliquot of 10 μL of extract was diluted in 990 μL initial mobile phase and transferred to a 1.5 mL polypropylene tube, centrifuged in an ultracentrifuge (Thermo Fischer Scientific Inc., Waltham, MA, USA) at 17,530 g for 10 min. The extract was transferred to an autosampler vial and then injected onto the LC-MS/MS system.

2.5. Instrumental

For LC-MS/MS analysis, a 5500 QTRAP hybrid triple quadrupole-linear ion trap mass spectrometer (Sciex, Framingham, MA, USA), equipped with an electrospray ionization (ESI) source, working in the positive mode (ESI^+) and multiple-reaction monitoring (MRM) mode, was coupled to a 1290 Infinity high-performance liquid chromatography system (HPLC) from Agilent Technologies, Inc. (Santa Clara, CA, USA). The HPLC consisted of degasser, binary pump, auto-sampler and column compartment. The Analyst software (Sciex, Framingham, MA, USA) performed all system control, data acquisition and data analysis. Chromatographic separation was performed using a Zorbax 300SB-CN column (150 mm × 4.6 mm i.d., 5 μm particle size, 300 Å) from Agilent Technologies, Inc. (Santa Clara, CA, USA).

2.6. LC-MS/MS analysis

The mobile phase consisted of aqueous solution with 0.1% formic acid (mobile phase A) and acetonitrile acidified with 0.1% formic acid (mobile phase B). The linear gradient elution was performed as follows: 0–2 min 95% A; 2–4 min 15% A; 4–7 min 10% A; 7–8 min 95% A, and held for 4 min to equilibrate the column. The column was maintained at 35 °C. The flow rate was 0.5 mL min^{-1} and the injection volume was 10 μL. The diverter valve was used as a device to eliminate interferences, directing the flow into the discharge before the elution of the analytes. This valve was kept open during the four initial minutes of each run, avoiding the contamination of the ESI source and thus the separation efficiency of the analytes could be increased.

The optimization of the mass spectrometer in MRM was first obtained by the infusion of the compounds separately in the ESI-MS/MS, in a continuous flow of 10 μL min^{-1} at concentrations of 5–150 μg L^{-1}. The optimization of the ESI source in positive mode was carried out by flow injection analysis (FIA) using the nisin standard at 200 μg L^{-1}, since it was the analyte with lower sensitivity.

2.7. Method validation

Full validation was performed in accordance to the Commission Decision 2002/657/EC criteria [18]. The validation of the method was performed for each analyte using cream and three types of cheese as matrices. Each calibration curve was built with six concentration levels (including zero) using a linear function of concentration (x) *versus* peak area (y). Linearity was evaluated using three replicates per level, in three different days. The