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Analytical Methods

Development of a LC–MS/MS method for the simultaneous determination of sorbic acid, natamycin and tylosin in *Dulce de leche*

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ABSTRACT

A simple extraction, rapid routine method for the simultaneous determination of sorbic acid, natamycin and tylosin in *Dulce de leche*, a traditional South American product, by liquid chromatography–tandem mass spectrometry has been developed and fully validated. The limits of detection were set to 24.41 mg kg^{-1} (sorbic acid), 0.10 mg kg^{-1} (natamycin) and 2 μg kg^{-1} (tylosin). Recoveries ranged from 95% to 110%. Proportionally, internal standardization was more efficient than external standard, resulting in a smaller measurement of uncertainty. In total, 35 commercial samples from Brazil, Argentina and Uruguay have been assessed. The proposed method was tested on other dairy desserts, demonstrating to be versatile. Although tylosin was not detected in any sample, a high rate of non-compliance was found, with 67.39% of samples above the maximum allowed for sorbic acid and a maximum concentration of 2105.36 ± 178.60 mg kg^{-1}. In two samples, natamycin was irregularly found.

1. Introduction

Dulce de leche (DL) is a dairy product obtained from the concentration of fluid milk by heat treatment with ingredients added, especially sucrose. It is widely consumed in South America, especially in Argentina, Uruguay, Brazil and Chile (Zalazar & Perotti, 2011). DL is also used as a food ingredient in bakery and ice cream products (Demiate, Konkel, & Pedroso, 2001).

The use of additives such as antimicrobial preservatives in food is strictly regulated by national and international authorities, given the potential risk to the health and safety of consumers. The Brazilian regulation allows the use of the antimicrobial preservatives sorbic acid and natamycin in DL at concentrations of 600 mg kg^{-1} and 5 mg kg^{-1}, respectively, to prevent the action of yeasts and moulds. Natamycin is an antifungal agent produced during fermentation by *Streptomyces natalensis*, which can be added to the surface of DL and cannot be detected in the inner part of the product (Brasil. Ministério da Agricultura, 1996).

In the '60 s, tylosin, a macrolide antibiotic, was also used in foods in order to prevent the growth of coagulase-positive *Staphylococcus* sp. and *Clostridium botulinum* (Denny, Sharpe, & Bohrer, 1961; Greenberg & Silliker, 1962). Currently, the use of tylosin in foods is not allowed worldwide. Studies have shown that regular consumption of foods containing tylosin may cause undesirable effects, such as allergic manifestations, cytotoxic, carcinogenic, mutagenic and nephrotoxic effects, reproductive disorders and the development of microorganisms resistant to antibiotics used in human therapy (FAO, 2015; Nisha, 2008). Brazilian regulation and the Codex Alimentarius determine the tylosin maximum residue level (MRL) of 100 μg L^{-1} in milk (Brasil. Ministério da Agricultura & de 15 de julho de, 2015; Codex Alimentarius Commission, 2009).

Several methods have been developed using various analytical techniques for the identification and quantification of antimicrobials preservatives, such as high-performance liquid chromatography with diode array detector (HPLC-DAD) (ISO 9231:2008; Paseiro-Cerrato et al., 2013), ultraviolet–visible detector (HPLC-UV) (Guarino, Fuselli, La Mantia, & Longo, 2011; ISO 9233:2008), micellar electrokinetic chromatography (MEKC) (Soliman & Donkor, 2010), among others. Liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) is considered a selective and sensitive technique that has been widely employed for the simultaneous determination of antimicrobial preservatives in food (Fuselli et al., 2012; Ortelli, Cognard, Jan, & Edder, 2009).

The use of antimicrobial preservatives in food has been widely assessed, but for the first time in Brazil a research focusing on

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the quantitative determination of natamycin, tylosin and sorbic acid in DL is being presented. In Spain, Paseiro-Cerrato et al. (2013) analyzed 26 food samples, which included dairy products, detecting natamycin in samples with levels higher than allowed. In Romania, Gradinaru, Popescu, and Solcan (2011) investigated the presence of tylosin residues in milk during three years. It was found that the tylosin values were above the MRL established by the European Union in a significant fraction of the study. Thus, the need for greater control of the use of these antimicrobials in food is evident. In order to improve the controllability and monitor DL marketed in South America, this study aimed to develop and validate an analytical method by LC–MS/MS for the simultaneous determination of sorbic acid, natamycin and tylosin.

2. Materials and methods

2.1. Standards, reagents and blank samples

Natamycin (CAS n° 7681-93-8) from *Streptomyces natalensis* was obtained from Danisco (DuPont Nutrition and Health, MA, USA), sorbic acid (CAS n° 110-44-1) and tylosin (CAS n° 1401-69-0) were obtained from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany). Robenidine hydrochloride (CAS 25875-50-7), used as internal standard for natamycin and tylosin, was obtained from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany). Both standards were analytical grade (⩾98% purity) and 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 was obtained from J.T. Baker Chemical Co. (Phillipsburg, NJ, USA) and ultrapure water was obtained from a MegaPurity water purification system (Billerica, MA, USA). Stock solutions were separately prepared for both standard chemicals at 1000 mg L^{-1} in methanol. Working solutions were prepared by diluting each individual stock solution with methanol. Stock solutions and working solution were stored at 4 °C. Blank samples were produced in the Food Processing Plant of the Federal University of Santa Catarina and stored at −18 °C prior to use.

2.2. Extraction of antimicrobial preservatives from DL

DL (2.0 ± 0.1 g) was weighed into polypropylene centrifuge tubes and 5 mL of formic acid 0.1% in water:methanol (1:9, v/v) were added to each tube. The tubes were mildly shaken on an orbital shaker (Tecnal Equipamentos para Laboratório, Piracicaba, Brazil) for 20 min and then centrifuged (Thermo Fischer Scientific Inc., Waltham, MA, EUA) at 4000 rpm for 10 min at 4 °C. The supernatant was transferred to another polypropylene tube and then was kept at −18 °C for 1 h, then centrifugation was performed again at 4000 rpm for 10 min at 4 °C. Finally, an aliquot of 100 μL of extract was diluted in mobile phase and transferred to a 1.5 mL microtube, centrifuged in an ultracentrifuge (Thermo Fischer Scientific Inc., Waltham, MA, USA) at 13,000 rpm for 10 min. The extract was transferred to an autosampler vial and then injected onto the LC–MS/MS system.

2.3. LC–MS/MS analysis

Liquid chromatography was performed on an Alliance 2795 HPLC system (Waters Corporation, Milford, MA, USA), coupled to a Quattro Micro triple quadrupole mass spectrometer (Waters/Micromass UK Ltd., Manchester, UK) with positive electrospray ionization (ESI) interface in multiple-reaction monitoring (MRM) mode. Chromatographic separation was performed using a Venusil XPB C_{18} column (50 mm × 2.1 mm i.d., 3 μm particle size) (Bonna-Agela Technologies Inc., Wilmington, DE, USA) with a guard column (4.0 mm × 3.0 mm i.d., 5 μm particle size) (Phenomenex Inc., Torrance, CA, USA) maintained at 30 °C by an automatic column heater. The mobile phase consisted of aqueous solution of with 0.1% formic acid (mobile phase A) and methanol 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; 7–9 min 95% A and held for 4 min to equilibrate the column. The flow rate was 0.3 mL min^{-1} and the injection volume was 10 μL. High purity nitrogen was used as desolvation gas at flow-rate of 600 L h^{-1}. Argon was used as collision gas. Source and desolvation temperatures were 130 °C and 450 °C, respectively. All system control, data acquisition and data analysis were performed by the MassLynx version 4.1 software (Waters Corporation, Milford, MA, USA).

2.4. Method validation and measurement uncertainty (MU)

The method validation was performed in accordance to the Commission Decision 2002/657/EC criteria (European Commission. Commission Decision, 2002). Additionally, matrix effects, limit of detection (LOD) and limit of quantification (LOQ) were also evaluated according to the Brazilian Ministry of Agriculture, Livestock and Food Supply (Brasil, 2011). The MU was calculated according to the Guide to the Expression of Uncertainty in Measurement (ISO/IEC Guide 98-3:2008).

2.4.1. Calibration curves, linearity and matrix effect

The calibration curve was prepared 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. Robenidine was used as internal standard for the quantification of natamycin and tylosin. The acceptance criterion was that the average regression coefficient (R^2) should be >0.95. Analysis of variance was carried out to determine whether there were significant differences between days at 5% significance level. Statistical analysis was performed using the Microsoft Office (Excel 2010) software.

Matrix effect was assessed by preparing three types of calibration curves. Curve type I, called 'solvent', was prepared by diluting the standard solution in the mobile phase initial composition. Curve type II, or 'tissue standard curve', was prepared by adding the standard solution to extracts of blank samples after extraction. Curve type III, called 'matrix matched', was prepared by fortifying blank sample before extraction with the desired amounts which were extracted and analyzed as conventional samples. Additionally, effects from matrix co-extractives were investigated by comparing slopes obtained in the linear calibration curves according to Hoff et al. (2015).

2.4.2. Selectivity/specificity

Specificity was checked by the analysis of 20 samples in order to evaluate the possible endogenous matrix interferences. The results were assessed by the presence of interfering substances in the surrounding of analytes retention times compared to fortified blank sample.

2.4.3. Recovery and precision

The recovery and precision were determined by linking experiments. Blank samples (18 aliquots) were fortified at 0.5, 1 and 1.5 times the regulatory limit of each analyte (six replicates per level). The evaluation was performed using the coefficient of variation (CV) and the recovery rate. Repeatability was assessed in terms of intra-day and inter-day precision, considering different analysts (n = 2) and days of analysis (n = 3). Analysis of variance was carried

out to determine whether there were significant differences between days and analysts.

2.4.4. Analytical limits

Both decision limit (CC_α) and detection capability (CC_β) were calculated from the measurement uncertainty at interest levels. CC_α was calculated as the measurement uncertainty of the regulatory level. CC_β was calculated as the measurement uncertainty of the MRL, using random effects data obtained in Section 2.4.3. The limits of detection (LOD) were established as the analyte concentration that has a signal three times above the signal/noise ratio and the limits of quantification (LOQ) as ten times above the signal/noise ratio with acceptable precision.

2.4.5. Ruggedness

The ruggedness was evaluated by the Youden fractional factorial design approach (Youden & Steiner, 1975). Seven factors that could influence the routine method were slightly varied. These factors have been identified with letters and consisted of: acid concentration in the mobile phase, acid concentration in the extraction, extract's freezing time, proportion of initial mobile phase to dilute an aliquot of the extract, extract's stirring time, temperature of chromatographic column and concentration of organic solvent in extraction.

2.4.6. Extract's stability

Extracts were processed in triplicate for each period of 1, 5, 10 and 15 days under different temperature storage conditions (25 °C, 4 °C and −18 °C). Analysis of variance at 5% significance level was carried out to determine whether there were significant differences between concentrations of the extracts at each period and the freshly prepared extract.

2.4.7. Measurement uncertainty (MU)

The measurement uncertainty was calculated using two models. Firstly, an internal standard (IS) for the quantification of natamycin and tylosin was used. Secondly, for the quantification of sorbic acid, an external standard (ES) was used. The sources of uncertainty identified were: weighing, dilution, interpolation of the analytical signal in the calibration curve and uncertainty associated to the precision of the method. Uncertainties were calculated considering the regulatory level for each compound.

The equations for calculating the concentrations of preservatives in mg kg^{-1}, using IS or ES were given respectively as:

$$C_{preservatives(IS)} = \frac{\left(\frac{A_{analyte}}{A_{IS}} - b\right) \cdot C_{IS} \cdot V}{a.m} + R\&R$$

where $C_{preservatives\ IS}$: concentration of analyte in stock solution (mg kg^{-1}); m: weight of sample (g); $A_{analyte}$: area of the analyte (area unit); A_{IS}: area of IS (area units); C_{IS}: IS concentration in extractive solution (mg kg^{-1}); V: extractive solution volume (mL); a: angular coefficient (area units. mg^{-1} kg); b: linear coefficient (area units) and R&R: intra-day and inter-day precision (dimensionless).

$$C_{preservatives(ES)} = \frac{L \cdot V}{m} + R\&R$$

where $C_{preservatives\ ES}$: concentration of analyte in stock solution (mg kg^{-1}); m: weight of sample (g); V: extractive solution volume (mL); L: result from the linear calibration curve (mg kg^{-1}) and R&R: intra-day and inter-day precision (dimensionless).

2.4.7.1. Uncertainty contribution from the sample weighing. The standard uncertainty regarding weighing took into consideration the error of eccentricity and those displayed by the balance. This uncertainty was calculated using the uncertainty in the maximum error accepted by the laboratory, considering the resolution of the balance. This uncertainty was calculated using the equation:

$$u^2(m(Ex_t, M_t, Ex, M)) = \left(\frac{\partial m}{\partial Ex_t}\right)^2 \cdot (\sigma Ex_t)^2 + \left(\frac{\partial m}{\partial M_t}\right)^2 \cdot (\sigma M_t)^2 + \left(\frac{\partial m}{\partial Ex}\right)^2 \cdot (\sigma Ex)^2 + \left(\frac{\partial m}{\partial M}\right)^2 \cdot (\sigma M)^2$$

where u(m): standard uncertainty the weighing; Ex: eccentricity error value (g); M: the mass value which has been measured in the balance (g); *t*: index to represent the tare analytical process; $(\sigma x_i)^2$: output standard uncertainty and $\frac{\partial m^2}{\partial x_i}$: sensitivity coefficients.

2.4.7.2. Uncertainty contribution from the dilution. The standard uncertainty regarding the dilution was calculated taking into account uncertainties, errors and performance data of volumetric instruments. This source of uncertainty was calculated using the equation:

$$u^2(V(V_{pip}, V_{final})) = \left(\frac{\partial D}{\partial V_{pip}}\right)^2 \cdot (\sigma V_{pip})^2 + \left(\frac{\partial D}{\partial V_{final}}\right)^2 \cdot (\sigma V_{final})^2$$

where u(V): Standard uncertainty the dilution; V_{pip}: pipetted value (mL); V_{final}: volumetric final volume (mL); $(\sigma x_i)^2$: output standard uncertainty and $\frac{\partial V^2}{\partial x_i}$: sensitivity coefficients.

2.4.7.3. Uncertainty contribution from the calibration curve (L, a, b). The standard uncertainty related to the concentration of the sample as an interpolation result from the calibration curve (L) by the method of least squares using ES was calculated by:

$$u(L) = \frac{S_y}{b} * \sqrt{\frac{1}{N} + \frac{1}{n} + \frac{(C - \bar{x})^2}{\sum_{i=1}^{N}(x_i - \bar{x})^2}}$$

where u(L): standard uncertainty regarding the calibration curve; S_Y: residual standard deviation (mg kg^{-1}); b: angular coefficient (area unit mg^{-1} kg); n: number of measurements for the calibration; C: determined preservative concentration (mg kg^{-1}); N: number of measurements to determine C; x: mean value of the different calibration standards; n: number of measurements and *i*: index for the number of measurements to obtain the calibration curve.

The standard uncertainty of the angular (b) and linear (a) coefficients, used in IS model, was calculated according to the following equations:

$$u(a) = \frac{\sigma^2 \sum x_i^2}{N \sum x_i^2 - (\sum x_i)^2}$$

$$u(b) = \frac{N\sigma^2}{N \sum x_i^2 - (\sum x_i)^2}$$

where the equation format is $\Delta Y_i = Y_i - a - bx$; $\sigma = \frac{\sum \Delta(Y_i)^2}{N-2}$ and N: number of measurements to determine.

2.4.7.4. Uncertainty associated with the concentration (C_{IS}) and areas (A_i). The standard uncertainty of the analyte areas (Ai) was calculated according to the Section 2.4.7.3. For the standard uncertainty regarding the C_{IS}, a model composed by standard purity, weighing and final volume of the stock solution was used, according to the equation:

$$u^2(C_{IS}(m, p, V_{final})) = \left(\frac{\partial C_{IS}}{\partial m}\right)^2 \cdot (\sigma m)^2 + \left(\frac{\partial C_{IS}}{\partial p}\right)^2 \cdot (\sigma p)^2 + \left(\frac{\partial C_{IS}}{\partial V_{final}}\right)^2 \cdot (\sigma V_{final})^2$$

where u(C_{IS}): standard uncertainty regarding the concentration (mg kg^{-1}); p: uncertainty regarding the purity of IS (%); m: standard uncertainty related to weighing (g); V_{final}: volumetric final volume (mL); $(\sigma x_i)^2$: output standard uncertainty and $\frac{\partial C_{IS}}{\partial x_i}^2$: sensitivity coefficients.

2.4.7.5. Uncertainty associated to R&R. This source of uncertainty was calculated using the standard deviation related to repeated measurements of the standard through intra-day and inter-day precision.

$$u^2(R\&R) = \left(\frac{\partial R\&R}{\partial Rep}\right)^2 \cdot (\sigma Rep)^2 + \left(\frac{\partial R\&R}{\partial Repro}\right)^2 \cdot (\sigma Repro)^2$$

where $u(R\&R)$: standard uncertainty regarding the repeatability combined with intra-day and inter-day precision; $(\sigma Rep)^2$: standard deviation of repeatability; $(\sigma Repro)^2$: standard deviation of inter-day precision and $\frac{\partial R\&R}{\partial x_i}^2$: sensitivity coefficients = 1.

2.4.7.6. Combined and expanded standard uncertainty. The combined standard uncertainty was determined by combining the correlated output standard uncertainties in the overall measurement model. The calculation was performed according to the propagation of uncertainty using the equation:

$$u_c^2(y) = \sum_{i=1,n}\left(\frac{\partial f}{\partial x_i}\right)^2 u^2(x_i) + 2\sum_{i=1,n-1}\sum_{j=i+1,n=1}\left(\frac{\partial f}{\partial x_i}\right)^2\left(\frac{\partial f}{\partial x_j}\right)^2 u^2(x_i)u^2(x_j)r(x_i x_j)$$

where u_c : combined standard uncertainty; y : measuring; $\frac{\partial f}{\partial x_i}, \frac{\partial f}{\partial x_j}$ – sensitivity coefficients; $u^2(x_i)eu^2(x_j)$: uncertainty standard output of each variable of the overall measurement model and r: estimated correlation coefficient.

The expanded uncertainty (U) was calculated by multiplying the combined standard uncertainty by the coverage factor (k):

$$U = k \cdot u_c(y)$$

where U: standard uncertainty expanded; $u_c(y)$: combined standard uncertainty and k: coverage factor.

2.5. Method applicability

Method applicability was evaluated by analyzing 35 real samples of DL from 29 commercial brands, processed by inspected establishments in Argentina (n = 3), Uruguay (n = 2) and Brazil (n = 30). For the determination of natamycin, aliquots were removed from the surface and the internal content, after homogenization. For determination of tylosin and sorbic acid, samples were withdrawn from the homogenized content. Analyzes were performed in duplicate.

2.5.1. Scope extension

In order to extend the scope to other dairy products, the proposed method was tested on commercial samples of DL variations: DL with fruits (strawberry, coconut and plum) added (n = 3), DL with chocolate (n = 1) and DL with whey (n = 2). Samples of condensed milk (n = 4), lactose-free (n = 1) and sweetened (n = 1) variations were also analyzed. To confirm the method performance for these matrices, precision (repeatability), recovery (by fortification of blank samples) and linearity were assessed.

3. Results and discussion

3.1. Extraction of antimicrobial preservatives from DL

DL is a complex matrix with high concentrations of carbohydrates, proteins and fat. Thus, the use of organic solvent in high concentration has been required to reduce the extraction of endogenous interferents. The use of methanol with 0.1% formic acid decreased the extraction of interferents and provided a better recovery of the analytes. The procedure, combined with the freezing step, led to satisfactory protein precipitation and, therefore, more clear extracts were obtained. Additional procedures for fat extraction with nonpolar solvents were not required.

When using the LC–MS/MS system, the diverter valve was used as a device to eliminate the interference of carbohydrates, directing the flow into the discharge before the elution of the analytes. This valve was kept open during the five initial minutes of each run, avoiding the contamination of the ESI source and increasing the separation efficiency of the analytes, as also observed by Bretanha, Piovezan, Sako, Pizzolati, and Micke (2014).

3.2. Optimization of LC–MS/MS analysis

Different methods using LC–MS/MS for the analysis of sorbic acid have been previously reported. Ammonium acetate, combined with acetic acid and trifluoroacetic acid (TFA), has been used as additives for the mobile phase (Fuselli et al., 2012; Gören et al., 2015). For natamycin and tylosin analysis, the use of TFA, formic acid, acetic acid and ammonium acetate has been reported (Bourdat-Deschamps, Leang, Bernet, Daudin, & Nélieu, 2014; Chitescu, Oosterink, de Jong, & Stolker, 2012; Fuselli et al., 2012; Ortelli et al., 2009). In this work, we used formic acid as an additive for analyzing sorbic acid, tylosin and natamycin. This is the first study using this method which identifies and quantifies these three compounds simultaneously in DL. Several tests were performed using organic solvents and additives to get the best chromatographic conditions, as a result the use of 0.1% formic acid in methanol provided the best condition.

The optimization of the mass spectrometer was performed to obtain maximum sensitivity. According to the Commission Decision 2002/657/EC, to satisfy the criteria for confirmation and quantification of substances such as natamycin, sorbic acid and tylosin by LC–MS/MS, precursor ions and their fragments are necessary. With this goal, the infusion of the compounds (200 μg L^{-1}) was performed in methanol:aqueous solution of 0.1% formic acid 1:1 (v/v) for searching protonated molecular ions [MH]+ as precursors. We monitored two fragments. The more intense fragment was used for quantification and the other for confirmation. Optimization of the parameters for each monitored transition in the ESI-MS/MS was performed (Table 1).

Table 1
Values of the optimized optimal multiple reaction monitoring (MRM) parameters using electrospray ionization (ESI) positive mode for the determination of preservatives in *Dulce de leche* by liquid chromatography–tandem mass spectrometry.

Analyte	Retention time (min)	Precursor ion (*m*/*z*)	Product ion (*m*/*z*)	Collision energy (V)	Cone voltage (V)
Sorbic acid	5.90	113.2 (1^+)[a]	95.0	10	23
Sorbic acid		113.2 (1^+)[a]	66.9	20	23
Natamycin	6.02	666.2 (1^+)[a]	503.3	20	15
Natamycin		666.2 (1^+)[a]	485.0	20	15
Tylosin	5.85	916.4 (1^+)[a]	174.3	40	50
Tylosin		916.4 (1^+)[a]	145.1	42	50
Robenidine (IS)	6.20	334.0 (1^+)[a]	155.0	20	25

[a] Molecule ionization state.

3.3. Method validation

Regardless of the analytical technique employed, the reliability of the generated results must be verified by validation procedures (NBR ISO/IEC 17025:2005). In this work, we developed a method for the analysis of preservatives in DL performing a full validation, in which all parameters were in accordance with the criteria established by the Commission Decision 2002/657/EC.

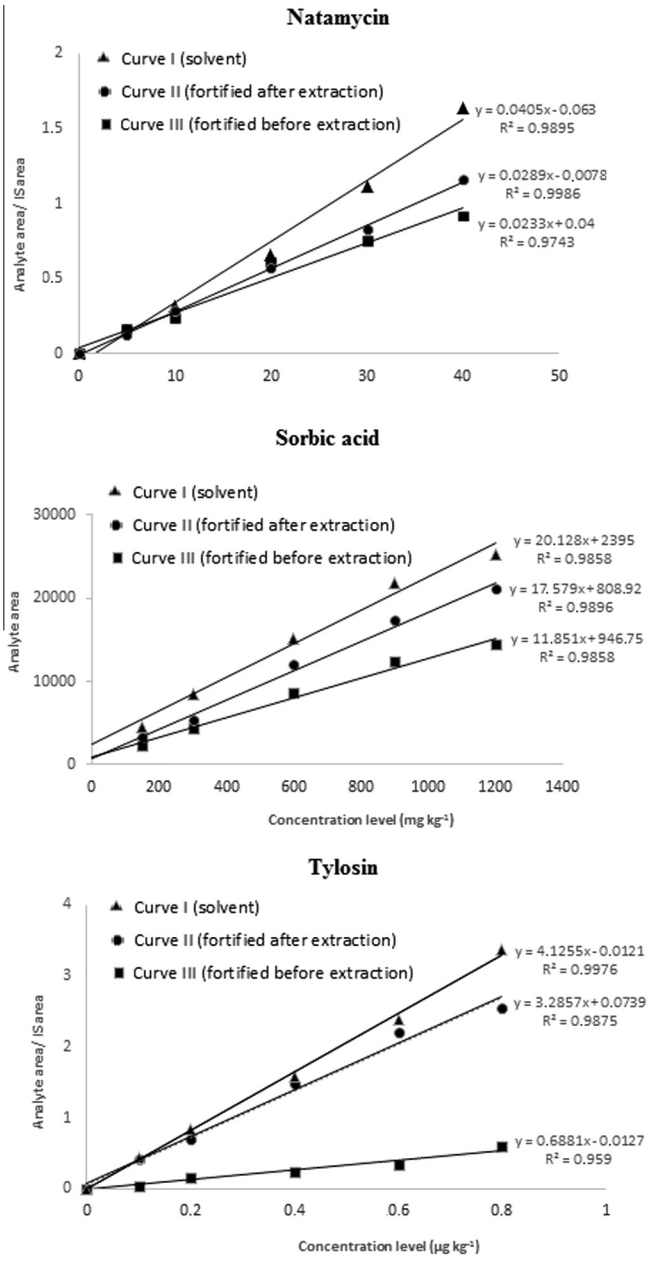

Fig. 1. Graphical plot of the three calibration curves types used to matrix evaluation in *Dulce de leche* by liquid chromatography–tandem mass spectrometry.

3.3.1. Linearity and matrix effect

The method was linear in the concentration ranges of 0–200 µg kg^{-1} (tylosin), 0–10 mg kg^{-1} (natamycin) and 0–1200 mg kg^{-1} (sorbic acid). The curves showed no significant difference in slope and in intercept among the studied days ($p > 0.05$) and the values of the regression coefficients were satisfactory ($R^2 > 0.95$). The study of the different calibration curves showed interference in the analyte response when the matrix was present. Type I curve (solvent), when compared to type III (fortified before extraction), proved to have a different inclination (Fig. 1). The matrix effect was confirmed when the type II curve (fortified after extraction) also showed difference in slope when compared to the curve I, in accordance with the calculation presented by Hoff et al. (2015), who found ME values lower than 0.9. Thus, matrix-matched calibration curves were adopted.

3.3.2. Selectivity/specificity

No interference was observed at the retention times of the analyte and the internal standard, showing that the method was not affected by endogenous compounds.

3.3.3. Recovery, precision and analytical limits

The results of accuracy and recovery were satisfactory with CV <16% and recoveries in the range of −20% to +10%. The results are presented in Table 2. There was no significant difference between the results of accuracy and recovery when analysts and test days were varied ($p > 0.05$).

The CC_α is the limit from which one can conclude that a sample is non-compliant with a probability of α error and CC_β is the lowest content of the substance that can be quantified in a sample with a probability of β error (European Commission, 2002). In this work, the CC_β was established only for tylosin compound, because it is the only veterinary drug residue studied. The CC_α was established for all compounds, as they all have a regulatory limit. Low limits of detection and quantification were obtained, showing that the developed method has good sensitivity.

3.3.4. Ruggedness

According to the criteria of Youden test, the chromatographic method proved to be robust when subjected to small variations. The greatest effect was observed for natamycin (0.6) and tylosin (0.3) when the freezing time was altered in the sample preparation. It can be inferred that the deficiencies in the sample clean-up provide signal suppression, reducing the concentration of the analytes without affecting the ruggedness.

3.3.5. Extract's stability

The stability study demonstrated that, by the fifth day, all extracts were stable in any temperature, as shown in Fig. 2. In extracts containing natamycin and sorbic acid, kept at room temperature until the tenth day, a significant decrease in the concentration was noticed, compared to the fresh extract. On the

Table 2
Recovery, interday precision and analytical limits for the determination of preservatives in *Dulce de leche* by liquid chromatography–tandem mass spectrometry.

Analyte	Recovery (%) and repeatability (% CV)			Interday precision (% CV)			CC_α (mg kg^{-1})	CC_β (mg kg^{-1})	LOD (mg kg^{-1})	LOQ (mg kg^{-1})
	0.5RL[b]	1.0RL[b]	1.5RL[b]	0.5RL[b]	1.0RL[b]	1.5RL[b]				
Sorbic acid	96 (2.9)[a]	105 (2.0)[a]	101 (3.9)[a]	7.5	6.0	8.2	687.21	–	24.41	29.05
Natamycin	98 (5.0)[a]	95 (5.3)[a]	98 (4.7)[a]	15.8	10.4	14.0	5.54	–	0.10	0.28
Tylosin	96 (5.0)[a]	110 (3.9)[a]	109 (9.4)[a]	15.8	11.3	17.5	0.12	0.15	0.002	0.007

[a] Values in brackets represent the coefficients of variation (% CV).
[b] Fortifications to achieve concentrations of 0.5, 1.0 and 1.5 times the regulatory level (RL).

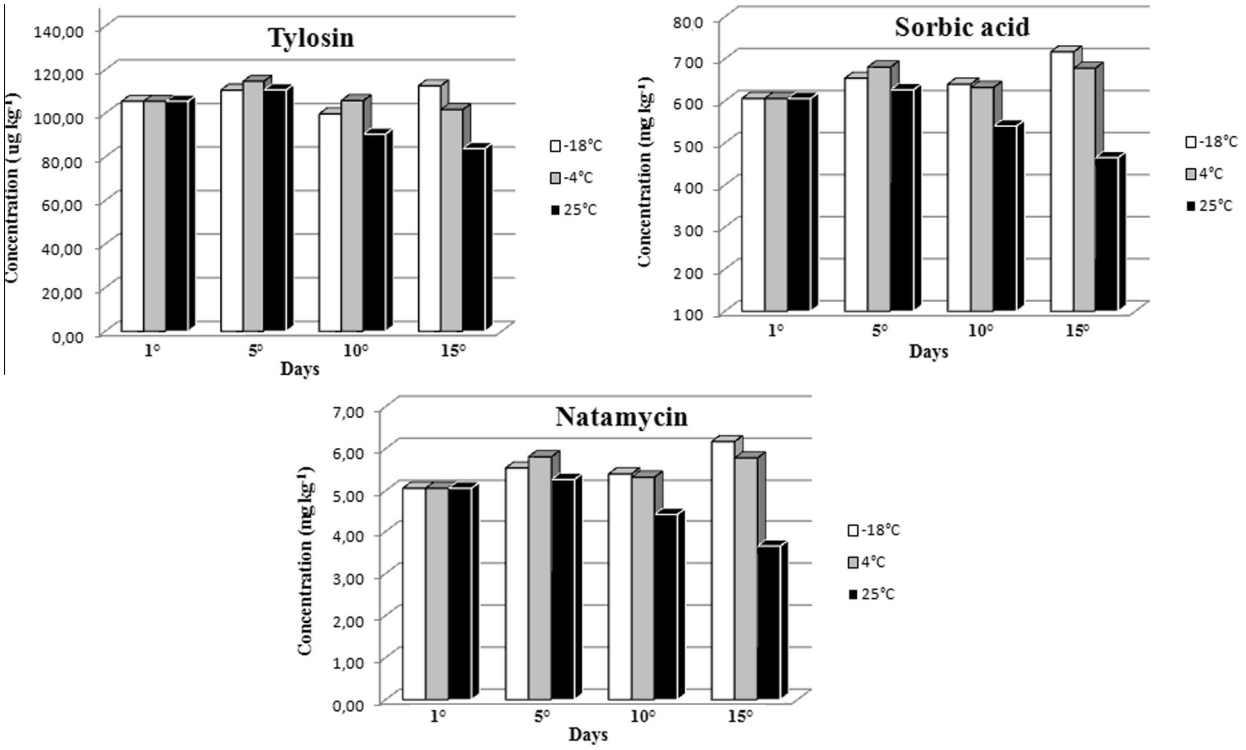

Fig. 2. Graphical plot of the extracts stability test for the determination of preservatives in *Dulce de leche* by liquid chromatography–tandem mass spectrometry.

Table 3
Quantification of preservatives in commercial *Dulce de leche* samples by liquid chromatography–tandem mass spectrometry.

Dulce de leche samples	Preservative content (mg kg^{-1} ± MU[a])			
	Sorbic acid	Natamycin (surface)	Natamycin (internal)	Tylosin
1	730.53 ± 107.02	<LOQ	<LOQ	<LOQ
2	1187.04 ± 124.82	<LOQ	<LOQ	<LOQ
3	828.49 ± 108.50	<LOQ	<LOQ	<LOQ
4	758.64 ± 107.10	<LOQ	<LOQ	<LOQ
5	1610.58 ± 149.59	<LOQ	<LOQ	<LOQ
6	998.93 ± 109.20	<LOQ	<LOQ	<LOQ
7	2105.36 ± 178.60	<LOQ	<LOQ	<LOQ
8	<LOQ	<LOQ	<LOQ	<LOQ
9	471.63 ± 100.70	<LOQ	<LOQ	<LOQ
10	1016.59 ± 124.67	<LOQ	<LOQ	<LOQ
11	1013.66 ± 124.65	<LOQ	<LOQ	<LOQ
12	998.81 ± 109.20	<LOQ	<LOQ	<LOQ
13	936.19 ± 109.90	<LOQ	<LOQ	<LOQ
14	<LOQ	<LOQ	<LOQ	<LOQ
15	809.54 ± 108.32	<LOQ	<LOQ	<LOQ
16	660.23 ± 107.22	<LOQ	<LOQ	<LOQ
17	640.38 ± 107.19	13.91 ± 0.72	10.86 ± 0.69	<LOQ
18	621.54 ± 107.09	<LOQ	<LOQ	<LOQ
19	1280.06 ± 149.59	<LOQ	<LOQ	<LOQ
20	1028.42 ± 149.20	<LOQ	<LOQ	<LOQ
21	719.27 ± 106.87	<LOQ	<LOQ	<LOQ
22	713.80 ± 106.90	<LOQ	<LOQ	<LOQ
23	192.24 ± 100.96	<LOQ	<LOQ	<LOQ
24	849.60 ± 108.60	<LOQ	<LOQ	<LOQ
25	1627.0 ± 149.60	<LOQ	<LOQ	<LOQ
26	1746.02 ± 149.72	<LOQ	<LOQ	<LOQ
27	227.11 ± 100.96	<LOQ	<LOQ	<LOQ
28	897.93 ± 108.91	<LOQ	<LOQ	<LOQ
29	1017.74 ± 124.67	<LOQ	<LOQ	<LOQ
30	586.28 ± 107. 06	12.17 ± 0.70	9.20 ± 0.56	<LOQ
31	762.10 ± 107.13	<LOQ	<LOQ	<LOQ
32	451.01 ± 100.68	<LOQ	<LOQ	<LOQ
33	856.43 ± 108.61	<LOQ	<LOQ	<LOQ
34	762.00 ± 107.13	<LOQ	<LOQ	<LOQ
35	816.02 ± 108.48	<LOQ	<LOQ	<LOQ

[a] Measurement uncertainty (MU) calculated using an effective degree of freedom that corresponds to a probability of coverage factor of approximately 95.45%. LOQ: limit of quantification.

other hand, tylosin has remained stable ($p > 0.05$). All extracts prepared on the 15th day and submitted to room temperature showed a significant decrease in the concentration ($p < 0.05$).

3.4. Analysis of real samples

Although being a traditional product in Latin America, the analysis of preservatives in DL still lacks research demonstrating its conformity. In this work, the method applicability was tested on several commercial DL samples (Table 3). It is observed a high rate of non-compliance, with 71% of samples above the maximum allowed for sorbic acid and an average concentration of 920.95 mg kg^{-1}. Several studies have turned attention to the control of sorbic acid in foods. Tfouni and Toledo (2002) analyzed various foods in Brazil, also detecting non-compliance in the concentrations of sorbic acid in some dairy products. Recently, Gaze et al. (2015) assessed several compounds in Brazilian DL samples, detecting sorbic acid in most of them. Although a small number of samples have been examined, non-compliant labeling for this preservative was reported. However, recent reports have not been focusing on the use of sorbic acid in DL under a quantitative point of view. The sorbic acid is considered safe for human consumption, with an acceptable daily intake (ADI) of 25 mg per kg^{-1} per body weight (JECFA, 2002a). Our findings show high concentrations of sorbic acid, which demonstrate the need for a stricter control of the use of this additive in the processing of DL. Despite its low toxicity, cases of idiosyncratic intolerance to sorbic acid, as well as hives and allergies, were reported (Deuel, Calbert, Anisfeld, & Blunden, 1954; Hannuksela & Haahtela, 1987; Juhlin, 1981; Walker, 1990).

The analysis of natamycin has been widely researched in dairy products (Fuselli et al., 2012; Guarino et al., 2011; Ortelli et al., 2009; Paseiro-Cerrato et al., 2013). However, no reports focusing on the use of natamycin in DL were found. In this research, only two samples had quantifiable results for natamycin in the surface of the product and both were above the maximum allowed. Moreover, since natamycin was internally found in DL, it seems to be improperly added to the inner matrix of these samples. This non-compliance seems to be an isolated problem, because the samples were from the same factory, although there was an interval of three months between the manufacturing dates. The natamycin is considered safe for human consumption with an ADI of 0.3 mg kg^{-1} (JECFA, 2002b). Toxicity studies using natamycin oral administration in animals warned of care to misuse this compound. The use of natamycin may be related to changes in the

Table 4
Standard uncertainty values in the preservatives determination in *Dulce de leche* by liquid chromatography–tandem mass spectrometry using internal standard (IS) and external standard (ES).

[a]Type	Measurement variable	Quantity value	Uncertainty value	Divisor	[b]u(xi)	Input unity	Distribution	[c]Ci	[d]u(yi)	Output unity	[e]V_{eff}	Contribution
Internal standard model												
B	Weight u(m)	2.1000	0.0010	2.02	0.0005	g	t-Student	−680.27	0.3358	mg kg^{-1}	∞	0.52%
B	Volume u(V)	5.00	0.0020	2.02	0.00	mL	t-Student	285.71	0.0000	mg kg^{-1}	∞	0.00%
A	Calibration curve u(L)	600.00	15.9872	2.23	7.17	mg kg^{-1}	t-Student	2.38	17.0694	mg kg^{-1}	12.09	26.66%
A	Variability u (R&R)	653.18	46.6180	1.00	46.62	–	t-Student	1.00	46.6180	–	17.00	72.82%
					Combined uncertainty (u_c)=	49.65		V_{eff}=	17.13			
					Expanded uncertainty (U)=	107.24						
					Coverage factor (k)=	2.16						
External standard model												
B	Weight u(m)	2.1000	0.0010	2.02	0.0005	g	t-Student	4.866	0.0023	mg kg^{-1}	∞	0.65%
B	Volume u(V)	5.00	0.0000	2.02	0.0000	mL	t-Student	2.044	0.0000	mg kg^{-1}	∞	0.00%
B	Analyte area u (A)	83.00	0.0112	2.00	0.0056	Area unit	t-Student	0.137	0.0008	mg kg^{-1}	17.00	0.23%
B	IS concentration u(C_{IS})	0.25	0.0002	2.00	0.0001	mg kg^{-1}	t-Student	40.874	0.0047	mg kg^{-1}	∞	1.33%
B	IS area u(A_{IS})	101.00	0.0092	2.00	0.0046	Area unit	t-Student	−0.113	0.0005	mg kg^{-1}	17.00	0.14%
A	Linear coefficient u (a)	0.0836	0.0107	1.73	0.0062	Area unit	Rectangular	−13.843	0.0854	mg kg^{-1}	17.00	24.13%
A	Angular coefficient u (b)	0.043	0.0002	1.73	0.0001	Area unit mg^{-1} kg	Rectangular	237.639	0.0317	mg kg^{-1}	17.00	8.95%
A	Variability u (R&R)	4.62	0.2286	1.00	0.2286	–	t-Student	1.00	0.2286	–	12.00	64.58%
					Combined uncertainty (u_c)=	0.25		V_{eff}=	15.89			
					Expanded uncertainty (U)=	0.55						
					Coverage factor (k)=	2.23						

[a] Type A – Method of evaluation of uncertainty by the statistical analysis of series of observations. Type B – Method of evaluation of uncertainty by means other than the statistical analysis of series of observation.
[b] u(xi) Input standard uncertainty.
[c] Ci – Sensitivity coefficient.
[d] u(yi) Output standard uncertainty.
[e] V_{eff} = Effective degrees of freedom.

immune response and genotoxic effects in rodents (Martínez et al., 2013).

Tylosin was not detected in any sample. DL is obtained by the concentration of fluid milk, making it a suitable factor to increase the concentration of this residue if present in the raw material. It can be suggested that there is an adequate control of the use of the substance in the milk used for the processing of DL.

3.4.1. Scope extension

The method was tested on other dairy desserts, demonstrating to be versatile. The performance criteria applied in different matrices were satisfactory, with acceptable precision values (CV < 16%), recovery rates (about 99.5%) and adequate linearity (average $R^2 > 0.95$). The samples showed no quantifiable results for natamycin and tylosin. However, high concentrations of sorbic acid were again observed in commercial samples of DL with fruits, chocolate and whey. In these samples, the sorbic acid concentration ranged from 762.00 ± 107.13 mg kg^{-1} to 1,128.88 ± 127.55 mg kg^{-1}. Only one sample of condensed milk were quantificable for sorbic acid (772.49 ± 107.23 mg kg^{-1}). Although a regulatory limit of sorbic acid is not established for this product, this concentration is considered high if compared to the DL regulation. However, due to the rigorous heat treatment in condensed milk processing, lower concentrations of sorbic acid are expected.

3.5. Measurement uncertainty (MU)

The measurement uncertainty provided analysts detailed knowledge of the method and more reliable measures. In this study, the method presented in ISO/IEC Guide 98-3:2008 was successfully adapted to calculate the measurement uncertainty of the chromatographic method. As it can be seen in Table 4, the greatest contribution of uncertainties was the intra-day and inter-day precision (variability). Since no quantifiable results for tylosin were achieved, uncertainty was assessed for natamycin and sorbic acid only. Uncertainty values obtained were around 11% and 17% compared to CC_α values of 10% and 14% for natamycin and sorbic acid, respectively. The MU values were higher than the CC_α values, in addition to the contribution of random effects, "type B" uncertainties as calibration certificates, errors and resolutions inherited from equipments (ISO/IEC Guide 98-3:2008, 2008). Proportionally, the result of MU using the model with IS was smaller than with the ES. In this work, the internal standardization corrected more effectively the major source of uncertainty that was variability, determined from recovery. In liquid chromatography, the internal standardization technique is more effective than the external standard, correcting loss of the analyte in the sample extraction procedure (Ding, Peng, Ma, & Zhang, 2015; Pigini, Cialdella, Faranda, & Tranfo, 2006; Zenkevich & Makarov, 2007).

4. Conclusions

The method developed in this work has simplified extraction and shortened the time of chromatographic analysis (about ten minutes) for simultaneous determination of natamycin, sorbic acid and tylosin in DL and other dairy desserts. All validation parameters met the recommendations of the Commission Decision 2002/657/EC. Proportionally, internal standardization was more efficient than external standard method, resulting in a smaller measurement uncertainty. The method was used to analyze several commercial samples, demonstrating its applicability. The occurrence of non-compliance in 67.39% of the samples demonstrates the importance of controlling the use of preservatives in dairy products. Thus, the proposed method can be an efficient tool for the inspection of natamycin, tylosin and sorbic acid in DL.

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