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Effect of Heating on the Stability of Quinolones in Milk

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Nowadays, the possible public health risk associated with the presence of quinolone residues and other antibiotics in milk is well-known, but there is a lack of information about the effect milk processing temperatures have on the presence of antimicrobial residues. The aim of this work was to determine the effect of different temperatures and heating times on the concentration of quinolones in milk by employing liquid chromatographic equipment analysis with fluorescence detection. In order to determine the thermo-stability of these compounds, the first-order kinetic model was applied, and the activation energies, half-lives, and percentages of degradation of each compound were calculated. Results showed that quinolones are very resistant to different heat treatments with maximum losses of concentration of 12.71% for ciprofloxacin and 12.01% for norfloxacin at 120 °C and 20 min. The high stability of quinolones represents a significant risk to human health because the residues of these antibiotics can remain in milk after heat treatment and, therefore, can reach the dairy industry and consumers.

KEYWORDS: Quinolones; milk; heat treatments; first-order kinetic model; thermo-stability

INTRODUCTION

Quinolones and fluoroquinolones are a large, powerful, and expanding group of synthetic antimicrobial compounds whose main action is the inhibition of bacterial DNA-gyrase within the bacterial cell (*3*). In the last 10 years, these compounds have been used in veterinary medicine to treat various diseases as they are highly active against a wide range of Gram-negative and Gram-positive bacteria, but mainly for the treatment and prevention of cattle mastitis (*20*).

The abuse or misuse of quinolones, and other antimicrobial agents in veterinary medicine, can constitute a serious public health risk associated with the presence of residues in foods of animal origin, and often induce problems in the manufacturing of products (*17*).

In recent years, some studies have shown that the excessive therapeutic use of quinolones in humans may be related with allergic reactions and the emergence of resistance to *Campylobacter* and *Salmonella* (*1*, *19*, *22*), indicating that the use of these antibiotics in food producing animals can have a direct impact on public health (*8*, *25*). To ensure control over the presence of quinolone residues in foodstuffs of animal origin, Regulations 37/2010/CE of the European Union (*6*) sets maximum residue limits (MRLs) for some of these substances in milk (enrofloxacin and ciprofloxacin, 100 μg kg^{-1}; flumequine, 50 μg kg^{-1}; and marbofloxacin, 75 μg kg^{-1}).

Microbiological methods based on the inhibition of *Geobacillus stearothermophilus* are most frequently used for the screening analysis of milk in farms and dairy industries. These methods detect β-lactam antibiotics efficiently and in some cases tetracyclines, but are not capable of detecting quinolones at or near MRL levels. Also, some other methods, such as protein receptor-binding, inmuno-enzimatic tests, etc. are employed routinely at the farm level and in the dairy industry because they are fast and simple to use, but the majority of them are specific and detect merely β-lactam and sometimes tetracycline residues.

Only a few methods for the detection of quinolones in milk are available, such as a bioassay based on the inhibition of *Escherichia coli* as part of multiresidue microbiological systems (*9*, *16*) and some protein receptor-binding tests (e.g., Rosa Charm Enroflox). These methods are not generally used in quality control schemes at the farm and dairy industry levels, which means that the presence of quinolones in milk often remains unchecked.

Moreover, in the past 15 years, the online combination of chromatography with different detectors has developed into a widely applicable detection system for antimicrobial residue analysis in foodstuffs. Current methods for detecting quinolones in biological matrixes are based on liquid chromatography (LC), mainly with fluorescence, ultraviolet, mass spectrometry, and tandem mass spectrometry (MS/MS) detection (*4*, *5*, *7*, *11*, *12*, *14*, *23*, *26*). These methods allow for the detection and quantification of antimicrobial agents with high selectivity and sensitivity. However, they are slow and costly and also require highly qualified personnel for their use. That is why these methodologies are generally only used by quality control and public health laboratories but not for screening in farms and the dairy industry.

Milk in the dairy industry is subjected to different thermal treatments before marketing to ensure its quality and preservation.

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However, there is a lack of scientific research regarding the effect milk processing temperatures can have on the presence of antimicrobial residues (*3*).

The relevance of the probable heat inactivation of quinolone residues in milk for food safety makes it necessary to carry out studies that evaluate the effect of processing of these compounds. Therefore, in a previous study we analyzed heat treatments in different groups of antibiotics employing bioassays with various microorganism tests, and we studied certain time−temperature combinations (*28*−*30*). However, we considered whether it is necessary to continue with this study applying quantitative techniques such as HPLC to establish kinetic models of degradation concerning the most frequently used antibiotics in cows.

Therefore, the aim of this work is to establish a kinetic model of degradation of quinolones by heating and estimate the effect of different heat treatments on the concentrations of ciprofloxacin, enrofloxacin, norfloxacin, flumequine, and oxolinic acid in milk.

MATERIALS AND METHODS

Chemicals and Reagents. Acetonitrile and methanol were of HPLC-grade; 10 M potassium hydroxide, 0.05 M potassium phosphate buffer at pH 7.4, 0.02 M potassium phosphate buffer at pH 3, 2 M sodium hydroxide, and 25% liquid ammonia were of analytical-reagent grade (Merck KGaA, Darmstadt, Germany). Ultrahigh purity water was obtained from a Milli-Q system (Millipore Corp., Bedford, MA).

Standard Solutions. Five quinolones were analyzed: ciprofloxacin, enrofloxacin, norfloxacin, flumequine, and oxolinic acid purchased from Sigma (Sigma; European Pharmacopeia, Strasbourg Cedex, France).

Individual quinolone stock standard solutions were prepared in methanol at 1 ng kg^{-1} after correcting for purity and stored at 4 °C in the dark for no longer than one month. Two standard working solutions were prepared every day before analysis which were detected in two separate HPLC runs by means of their fluorescence, one with ciprofloxacin, enrofloxacin, and norfloxacin, and another with flumequine and oxolinic acid.

Calibration standards were prepared at concentrations of 20, 50, 100, 300, and 600 ng kg^{-1} for ciprofloxacin, enrofloxacin, and norfloxacin, and 30, 150, 300, 600, and 1200 ng kg^{-1} for flumequine and oxolinic acid.

Sample Preparation and Heat Treatments. Spiked milk samples were prepared by fortifying commercial UHT milk with a quinolone standard working solution in order to obtain samples with 1500 μg kg^{-1}. This concentration was chosen due to the sensitivity and linearity of the detector to respond to quinolones ranging from 10 to 5000 μg kg^{-1} which permits the detection of losses of concentration above the detection limit. Then, milk samples were heated for 0, 30, 60, 90, 120, 150, and 180 min at 80 and 100 °C in a water bath and for 0, 10, 20, 30, and 40 min at 120 °C in an autoclave. The samples were allowed to stand for 15 min at room temperature before extraction as described below.

Extraction Procedure. The extraction and purification of quinolones from milk samples was conducted using a procedure similar to that reported by Delepine et al. (*7*). In this procedure, 2 g of spiked milk sample was mixed with 20 mL of 0.05 M potassium phosphate buffer at pH 7.4. The prepared solutions were homogenized for 10 min in an ultrasonic bath and centrifuged for 10 min at 4000 rpm and 15 °C. The supernatant liquid was then filtered through a 0.80 μm Millex filter (Millipore Corporation, USA). Aliquots of 15 mL of each filter solution were purified with SPE Discovery DSC-18 cartridges (Waters, Mildford, MA), previously conditioned with 3 mL of methanol and 3 mL of water. Quinolones were eluted with 5 mL of 25% methanol/ammonia (75:25). The collected solutions were evaporated at 40 °C under a nitrogen stream until dry, and finally, residues were resuspended in 1 mL of phosphate buffer at pH 7.4.

HPLC Analysis. The quinolones in milk samples were determined by means of a liquid chromatographic equipment analysis system made up of a separation module Alliance Waters 2695 equipped with a Waters 2475 fluorescent detector using a Luna C_{18} column (5 μm, 250 × 4.6 mm) and a Phenomenex C_{18} precolumn (5 μm; 4 × 3 mm). The system and acquisition of data were controlled by the software Empower Pro Millennium 40 (Waters, Mildford, MA). The isocratic mobile phase consisted of a mixture of 0.02 M potassium phosphate buffer, pH 3 (83% A), and acetonitrile (17% B) at a flow rate of 0.3 mL min^{-1}. The fluorescence detector operated at an excitation of 294 nm and an emission wavelength of 514 nm for 0−11.75 min for the detection of enrofloxacin, ciprofloxacin, and norfloxacin. An excitation of 280 and an emission wavelength of 450 nm for 11.75 and 30 min were used for flumequine and oxolinic acid.

Statistical Analysis. The first-order kinetic model was applied for the statistical analysis of the thermal degradation of quinolones in the following way (*2*):

$$\frac{\partial[C]}{\partial t} = -k_1 \cdot [C] \qquad \text{(eq 1)}$$

where $\partial[C]/\partial t$ is derived from the concentration of quinolones related to time, k_1 is the degradation rate constant, and $[C]$ is the concentration of each compound in the milk sample. By integrating equation (eq 1), we get:

$$\ln[C] = \ln[C_0] - k_1 \cdot t \qquad \text{(eq 2)}$$

For each temperature, the effect heating has on the logarithmic transformations of the concentration of quinolones in milk is adjusted by means of the linear regression model using the PROC REG procedure of the SAS statistical program (*21*).

According to the theory postulated by Arrhenius, the degradation rate constant (k_1) depends on temperature and can be expressed as follows:

$$k_1 = A \cdot e^{-Ea/R \cdot T} \qquad \text{(eq 3)}$$

where A is the frequency factor, e is the base of the natural logarithms (e = 2.7182), Ea is the activation energy, R is the universal gas constant (R = 8.315 J mol^{-1} K), and T is absolute temperature (K). Using a logarithmic transformation of the expression (eq 3), the following is obtained:

$$\ln k_1 = \ln A - \frac{Ea}{R \cdot T} \qquad \text{(eq 4)}$$

The application of the linear regression model to the logarithmic transformations of the degradation rate constant based on the inverse values of the absolute temperatures allows the values of A and Ea to be calculated. To do this, the PROC REG procedure of SAS was used (*21*).

Finally, using equations (eq 2) and (eq 3) we can estimate the percentages of degradation of each quinolone for the dairy heat treatments by the following equation:

$$\%\text{degradation} = \frac{C_0 - C}{C_0} \cdot 100 = (1 - e)\left[A \cdot e^{\left(-\frac{Ea}{R \cdot T}\right)}\right] \cdot t \cdot 100 \qquad \text{(eq 5)}$$

RESULTS AND DISCUSSION

Table 1 shows the equations calculated by applying the first kinetic model (eq 2). For each quinolone, it can be seen that parameter k_1 (degradation rate constant) increases as the temperature rises. This demonstrates that at higher temperatures these molecules show greater heat inactivation.

It can also be seen that enrofloxacin (0.00067, 0.00081, and 0.00338), flumequine (0.00050, 0.00064, and 0.00170), and oxolinic acid (0.00051, 0.00083, and 0.00184) are more heat stable than ciprofloxacin (0.00113, 0.00158, and 0.00880) and norfloxacin (0.00092, 0.00129, and 0.00855) as they present lower values of their k_1 coefficients after the same heat treatments at 80, 100, and 120 °C, respectively. The fit obtained by applying the first kinetic model is good, as the determination coefficient ranges between 73.03% (for norfloxacin at 80 °C) and 98.84% (for norfloxacin at 120 °C).

Figure 1 shows the effect the heating time has on the concentration of quinolones in milk at different temperatures. When compared to the other temperatures tested, a greater heat inactivation of the quinolones was observed every time they were heated at 120 °C. A greater degradation of ciprofloxacin and norfloxacin was also observed, compared to that of the rest of the quinolones.

Table 1. First-Order Equations of Quinolone Concentrations at Different Temperatures[a]

temperature (° C)	kinetic model of first order	R^2
ciprofloxacin		
80	ln [ciprofloxacin] = 7.2129 − 0.00113·t	0.733
90	ln [ciprofloxacin] = 7.2880 − 0.00158·t	0.944
100	ln [ciprofloxacin] = 7.2911 − 0.00880·t	0.988
enrofloxacin		
80	ln [enrofloxacin] = 7.2778 − 0.00067·t	0.884
90	ln [enrofloxacin] = 7.3166 − 0.00081·t	0.926
100	ln [enrofloxacin] = 7.3128 − 0.00338·t	0.985
flumequine		
80	ln [flumequine] = 7.3166 − 0.00050·t	0.955
90	ln [flumequine] = 7.2893 − 0.00064·t	0.900
100	ln [flumequine] = 7.3254 − 0.00170·t	0.907
norfloxacin		
80	ln [norfloxacin] = 7.2268 − 0.00092·t	0.730
90	ln [norfloxacin] = 7.2929 − 0.00129·t	0.908
100	ln [norfloxacin] = 7.2914 − 0.00855·t	0.988
oxolinic acid		
80	ln [oxalinic acid] = 7.2829 − 0.00051·t	0.814
90	ln [oxalinic acid] = 7.3190 − 0.00053·t	0.959
100	ln [oxalinic acid] = 7.3298 − 0.00184·t	0.906

[a] t: time (min). R^2: determination coefficient.

Applying the linear regression model to the logarithmic transformations of the Arrhenius expression (eq 4) allows us to calculate the coefficients ln A and Ea/R, which are shown in **Table 2**. These coefficients present greater values for ciprofloxacin and norfloxacin, which points to the more unstable nature of these molecules when compared to flumequine and oxolinic acid, as may be seen in **Table 2** and **Figure 1**.

From the previous linear regression and the first-order kinetic model (**Tables 1** and **2**), the values of activation energy (Ea) and half-lives ($t_{1/2}$) of the quinolones were estimated, as shown in **Table 3**. Once again, it can be seen that ciprofloxacin and norfloxacin are of a more unstable nature when compared to flumequine and oxolinic acid, as they have shorter half-lives.

In the case of quinolones, no activation energy and half-life values have been calculated by other authors; therefore, the results obtained in this study cannot be compared. Nevertheless, studies have been carried out into the thermo-stability of other antimicrobials, especially β-lactam antibiotics, which demonstrate that these compounds are more unstable than most antimicrobial substances such as quinolones and tetracyclines, with shorter half-lives (*10*, *28*) and higher activation energy values (*15*, *18*, *24*, *27*).

Equation 5 was used to calculate degradation percentages of quinolones in HTST pasteurized milk (72 °C, 15 s, high temperature−short time), sterilized milk at 120 °C, 20 min, and UHT sterilization (140 °C, 4 s, ultrahigh temperature), most frequently used treatments in the dairy industry. The coefficients from **Tables 1** and **2** were used to this end. The estimated degradations (**Table 4**) show that quinolones are very resistant to the different heat treatments used in the dairy industry with very low degradation rates (< 6% in all cases) and maximum values of 12.71% for ciprofloxacin and 12% for norfloxacin in the case of common sterilization at 120 °C and 20 min.

In an earlier study by the same group of researchers (*28*), higher degradation percentages were obtained when they used the microbiological method based on the inhibition of *E. coli* ATCC 11303, with a loss of antimicrobial activity in milk of 18% (enrofloxacin), 17% (flumequine), and 32% (norfloxacin) for treatment at 120 °C and 20 min. The differences found between the two studies are probably caused by the different analytical techniques employed in each case. We must remember that results obtained by the HPLC confirmation technique allow us to quantify the losses of concentration, whereas with microbiological methods, antimicrobial activity losses are calculated. Despite the differences found, if one takes into account the uncertainties in each of the techniques employed, the results are quite similar.

Another study carried out on the effect of cooking on enrofloxacin residues in chicken tissue using a validated LC-MS (*13*)

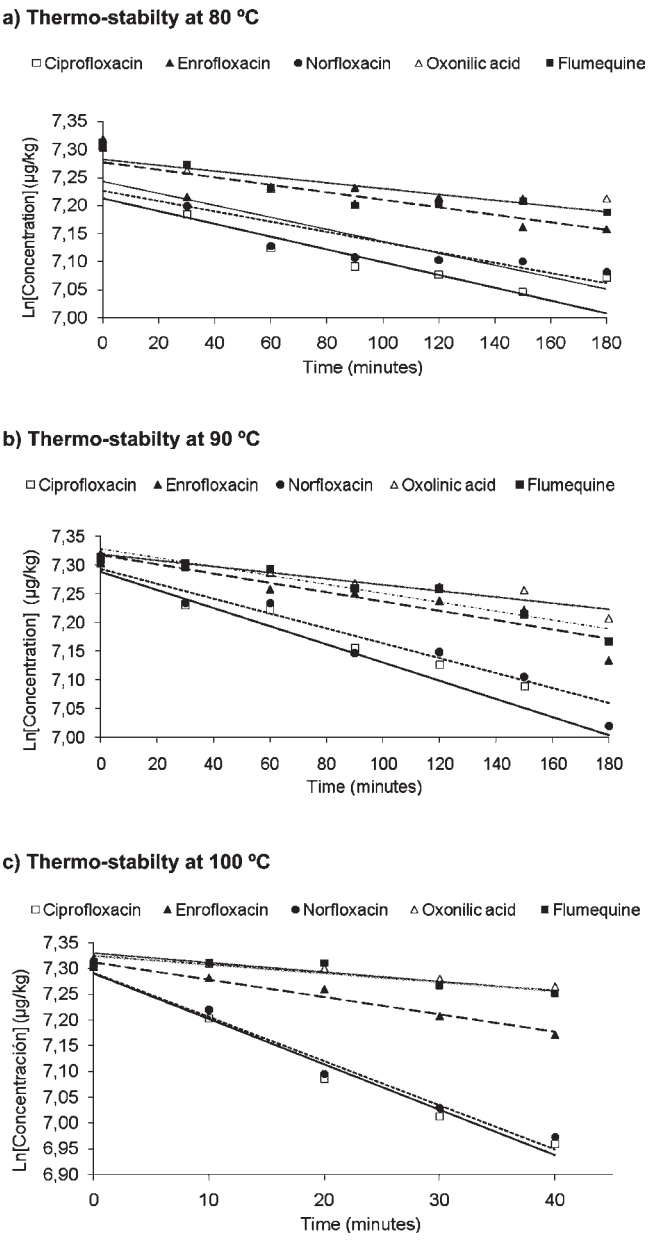

Figure 1. Variation of the concentration of quinolones at different temperatures and heating times.

Table 2. Summary of Estimates of Parameters in the Arrhenius Equation[a]

quinolones	ln [$k_{Quinolne}$] = ln A − Ea/R × (1/T)	R^2
ciprofloxacin	ln [$k_{Ciprofloxacin}$] = 12.882 − 7024 × (1/T)	0.919
enrofloxacin	ln [$k_{Enrofloxacin}$] = 8.135 − 5524 × (1/T)	0.901
flumequine	ln [$k_{Flumequine}$] = 2.065 − 3362 × (1/T)	0.934
norfloxacin	ln [$k_{Norfloxacin}$] = 14.391 − 7641 × (1/T)	0.858
oxolinic acid	ln [$k_{Oxalinic\ Acid}$] = 4.446 − 4310 × (1/T)	0.858

[a] k, first order kinetic constant; A, frecuency factor for the reaction; Ea, activation energy; R, universal gas constant (R = 8,315 J/mol K); T, absolute temperature in K; R^2, determination coefficient.

Table 3. Activation Energy (*Ea*) and Half-Lives ($t_{1/2}$) of Quinolones in Milk at Different Temperatures[a]

		$t_{1/2}$ (min)		
quinolones	*Ea* (kJ mol^{-1})	80 °C	90 °C	100 °C
ciprofloxacin	58.21	772	266	102
enrofloxacin	45.85	1271	549	258
flumequine	27.90	1203	722	456
norfloxacin	63.42	981	307	108
oxalinic acid	35.77	1631	848	471

[a] *Ea*, activation energy; $t_{1/2}$, half-life.

Table 4. Percentages of Degradation of Quinolones in Milk: Estimates for Different Dairy Industry Treatments

quinolones	72 °C, 15 s	120 °C, 20 min	140 °C, 4 s
ciprofloxacin	0.01	12.71	0.11
enrofloxacin	0.01	5.22	0.04
flumequine	0.01	2.99	0.02
norfloxacin	0.01	12.01	0.11
oxolinic acid	0.01	2.90	0.02

concluded that cooking procedures did not affect enrofloxacin residues, which remained stable during heating at different cooking processes (microwaving, roasting, boiling, grilling, and frying). These results show the thermal stability of enrofloxacin, as shown in this work. However, the results are not comparable because foodstuff and heat treatment used in both studies are different, as well as the analysis of data obtained.

In conclusion, the high stability of quinolones represents a significant human health risk as the residues of these antibiotics can remain in milk after dairy processing and, therefore, can reach consumers. Current control systems use specific and microbiological methods to detect β-lactam and tetracycline antibiotics with little sensitivity to quinolones. Only some countries include in milk monitoring schemes a screening test for the detection of quinolones at MRL levels (e.g., based on *E. coli*). For this reason, it would be desirable to improve the control screening system used for the detection of these molecules in foodstuffs.

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