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Thermostability of Oxytetracycline, Tetracycline, and Doxycycline at Ultrahigh Temperatures

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The thermostability parameters of three tetracycline antibiotics at high and ultrahigh temperatures (110–140 °C) as well as the influence of treatment medium pH and water activity on their thermotolerance have been investigated. The thermal degradation of the three antibiotics followed a first-order reaction kinetic within the 1.5–2 $\log_{10}$ cycles investigated. A linear relationship was observed between the log of the D_T values and the treatment temperature. The temperature dependence of the D_T values was similar for the three molecules ($z = 28 \pm 2$ °C). D_T values of doxycycline were approximately 1.5 and 3 times higher than those of tetracycline and oxytetracycline, respectively. Changes in the treatment medium pH (7.0–4.0) and water activity (0.99–0.93) scarcely varied the antibiotics' thermal stability. Only when doxycycline was heat-treated at pH 4.0 did its thermal resistance increase by 3 times. The thermostability parameters obtained would allow the effect of different cooking and sterilization procedures to be estimated. Whereas low-temperature−long-time treatments (conventional sterilization) would destroy >98% of the initial concentration of the residues of the three antibiotics, high-temperature−short-time treatments (UHT) would leave unaltered residues in the 50–90% range.

KEYWORDS: Oxytetracycline; tetracycline; doxycycline; heat treatments; inactivation kinetics; conventional sterilization; UHT treatments

INTRODUCTION

Antibiotics are used in veterinary medicine due to their therapeutic value and in animal production as additives to enhance growth and food efficiency. Their use may lead to the occurrence of residues in foods when sufficient time for the residues to dissipate is not allowed (*2*).

The absence of these antibiotic residues is of utmost importance in understanding their effects on public health. There is a growing public concern due to the possible development of antibiotic resistance in intestinal microorganisms that may affect humans (*3*). Moreover, some technological processes of food transformation, such as fermentation, can be inhibited by the presence of drug residues, leading to important economic losses (*19*).

It has been the interest of many researchers to evaluate if residues of antibiotics can be destroyed by cooking procedures, pasteurization processes, or canning (*14–16, 25–31*). Most of the earlier studies were based on the evaluation of loss of microbiological activity after heat treatment by using the end point method. However, this procedure did not allow degradation kinetics to be established to obtain the thermostability parameters, which restricts the possibilities of a mathematical calculation of the effects of conventional thermal treatments on antibiotic residues. Moats (*18*) summarized the major findings and their conclusions.

Later, chromatographic methods of adequate sensitivity were developed for determining the occurrence of residues in foods (*1, 5, 10, 11, 14, 15*). As shown by previous data, most of the studies have focused on the effects of several culinary treatments in typical animal food products. Under these conditions, the cold spot in solid foods experiences a lengthy heating "lag" phase and, therefore, it is difficult to assess the actual exposure of residues to heat. Because current data do not generally allow the accurate determination of z values [increase in heating temperature (°C) necessary to reduce decimal reduction times to 10%] for each residue, it is not possible to integrate the whole heating treatment and to compare the thermostability of each drug residue quantitatively.

On the other hand, according to their aims, most studies have been performed at cooking temperatures, generally lower than 100 °C (*14, 15, 25*). There are no data available obtained in liquid media at ultrahigh temperatures, where the "lag" phases of heating would have been avoided. Knowledge of thermostability parameters [D_T (decimal reduction time) and z values] at high and ultrahigh temperatures is important, because it would allow us to predict the effect of several cooking and techno-

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logical treatments on antibiotic contamination that might reach consumers, if safety barriers are overcome.

Tetracycline antibiotics are widely used in the prevention and treatment of infectious diseases and as food additives for animal growth promotion. As a consequence, tetracycline residues are commonly detected in foods (*2, 20*). The thermal stability of tetracycline antibiotics in animal food products has been the subject of previous studies (*14–16, 30, 31*). However, to the best of our knowledge, the thermal degradation kinetics of tetracycline antibiotics above 120 °C has not been studied yet.

Our research group has designed and performed an adequate instrument to determine microbial, enzymatic, and also antibiotic thermal destruction over a wide range of temperatures (*6, 7*). The thermoresistometer avoids common problems, such as the difficulty to work at ultrahigh temperatures, the lack of homogeneity during the heat treatment, or the existence of prolonged lag phases of heating. Its use provides an important advantage to obtain reliable heat resistance data in liquid media at ultrahigh temperatures.

The objective was to determine the thermostability parameters of three tetracycline antibiotics (oxytetracycline, tetracycline, and doxycycline) at high and ultrahigh temperatures, as well as the influence of treatment medium pH and water activity on their thermotolerance. These data could be useful to reinterpret some published data and to compare them. It was also expected that these data might be used in the agricultural and food industries to predict the effect of heating or cooking treatments. To simplify their use, the data were obtained through a methodology similar to the one successfully used to determine the kinetics of heat-labile microorganisms, nutrients, and food quality factors following a thermal process, and they were expressed with similar parameters (D_T and z) (*4, 33*).

MATERIALS AND METHODS

Chemicals and Apparatus. The oxytetracycline (oxytetracycline hydrochloride), tetracycline (tetracycline hydrochloride), and doxycycline (doxycycline hyclate) used in this investigation were purchased from Sigma-Aldrich (Steinheim, Germany) and were stored according to the manufacturer's instructions.

A stock solution (50 mg/mL) of each antibiotic was prepared using distilled water (doxycycline and oxytetracycline) and 0.1 M acetic acid (Sigma-Aldrich) (tetracycline) as diluents. All stock solutions were prepared immediately before use. Samples for the calibration curves and thermostability experiments were prepared by diluting these stock solutions to working standards of 5000, 1000, 500, 100, 50, and 10 ng/mL.

Citrate−phosphate McIlvaine buffer of pH 7.0, 5.5, and 4.0 (*9*), as well as the same buffer of pH 7.0 with NaCl (Sigma-Aldrich) added to obtain reductions in the water activity to 0.96 and 0.93, were used as treatment media. Water activity was measured by the dew point method (model CX-1; Decagon Devices, Inc., Pullman, WA).

HPLC analysis grade acetonitrile was supplied by Labscan (Dublin, Ireland). Analytical grade 2-monohydrate oxalic acid was from Panreac (Barcelona, Spain).

The HPLC system was a HP 1100 chromatograph (Agilent Technologies, Karlsruhe, Germany), including a binary pump, a diode array and multiple-wavelength detector (DAD), and a manual sample valve injector with a 20 μL loop and was controlled by HP Chemstation software.

Antibiotic Thermostability Experiments. Heat treatments were carried out in a thermoresistometer TR-SC, as previously described (*6, 7*). Once the temperature (110, 120, 130, and 140 °C) of the heat treatment medium (350 mL) had attained stability (±0.05 °C), it was inoculated with 0.2 mL of the stock solution. At preset intervals, samples of 0.2 mL were collected and resuspended in 1.8 mL of the mobile phase and analyzed by HPLC, obtaining an initial concentration of approximately 1150 ng/mL. At least four different heating times were used for each thermostability determination. All samples were stored at −80 °C until required for HPLC analysis.

HPLC Analysis. This procedure was based on the method of Oka et al. (*21*) and optimized in our laboratory. The HPLC determination of tetracyclines was carried out in reversed-phase chromatography with a photodiode array detector operated at 357 nm. A 20 μL volume sample was manually injected. The separation was performed on an ACE C18 column (5 μm, 250 mm × 4 mm i.d.). Analytes were eluted in a linear gradient with 0.01 M aqueous oxalic acid (pH 3) (solvent A) and acetonitrile (solvent B) as a mobile phase. The gradient program (solvent B) was as follows: 0 min, 10%; 3 min, 12%; 13 min, 20%; 23 min, 25%; 25 min, 15%, at a flow rate of 1 mL/min, at room temperature.

Calibration standards at different levels were prepared in mobile phase. The external standard (ESTD) technique was used for quantification. Detection limits were 15 ng/mL for oxytetracycline and tetracycline and 90 ng/mL for doxycycline.

Thermostability Parameters. The traditional model based on the first-order kinetics was used to describe degradation curves (*4*). Degradation curves were obtained by plotting the $\log_{10}$ of the residual antibiotic fraction versus their corresponding heating times at a constant temperature. Decimal reduction times (D_T value = minutes of heating at T temperature for the antibiotic concentration to drop 10-fold or one $\log_{10}$ cycle) were calculated from the slope of the portion of the linear section of the degradation curves. z values [increase in the temperature (°C) of treatment for D_T value to decrease 10-fold or one $\log_{10}$ cycle] were calculated from the slope of the decimal reduction time curves (DRTC, also called the phantom TDT curve) (*33*) obtained by plotting the $\log_{10}$ D_T versus its corresponding heating temperature.

The error bars in the figures indicate the 95% confidence limits for the data points obtained from at least two independent experiments. The determination coefficients (R^2), 95% confidence limits, and the statistical differences ($P = 0.05$) were calculated by GraphPad PRISM software (GraphPad Software, Inc., San Diego, CA). The significance of differences between the D_T values for each antibiotic at the different treatment medium pH values and water activities was determined by ANOVA (Excel, Microsoft Corp., Seattle, WA).

RESULTS

Figure 1 shows a chromatogram of the three tetracycline antibiotics assayed. Chromatographic conditions resulted in a good analytical resolution with the following elution pattern: oxytetracycline > tetracycline > doxycycline. No significant differences were observed when tetracycline antibiotics were suspended in citrate−phosphate McIlvaine buffer of pH 7.0, 5.5, and 4.0 and water activity of 0.99, 0.96, and 0.93 (data not shown).

Figure 2 shows the degradation curves of the tetracycline antibiotics suspended in citrate−phosphate buffer of pH 7.0 at a constant temperature (120, 130, or 140 °C). As can be observed, doxycycline was the most thermotolerant antibiotic and oxytetracycline the least at the temperatures tested. This figure has been included to illustrate the shape of the degradation curves within the 1.5–2 $\log_{10}$ cycles investigated. For the temperature range tested (110–140 °C), the thermal degradation of the three tetracyclines was nearly linear. Thus, the traditional model based on a first-order inactivation kinetic adequately fitted the degradation curves of the three antibiotics investigated (**Figure 2**). D_T values were calculated from the regression lines that described the degradation curves of each antibiotic at every temperature investigated.

Figure 3 shows the relationship between the treatment temperature and the log of D_T values of the three antibiotics heat-treated in citrate−phosphate buffer of pH 7.0 (DRT curves). This figure allows us to compare the thermal tolerance of the three antibiotics within the temperature range investigated. As seen in the figure, D_T values decreased with increased temper-

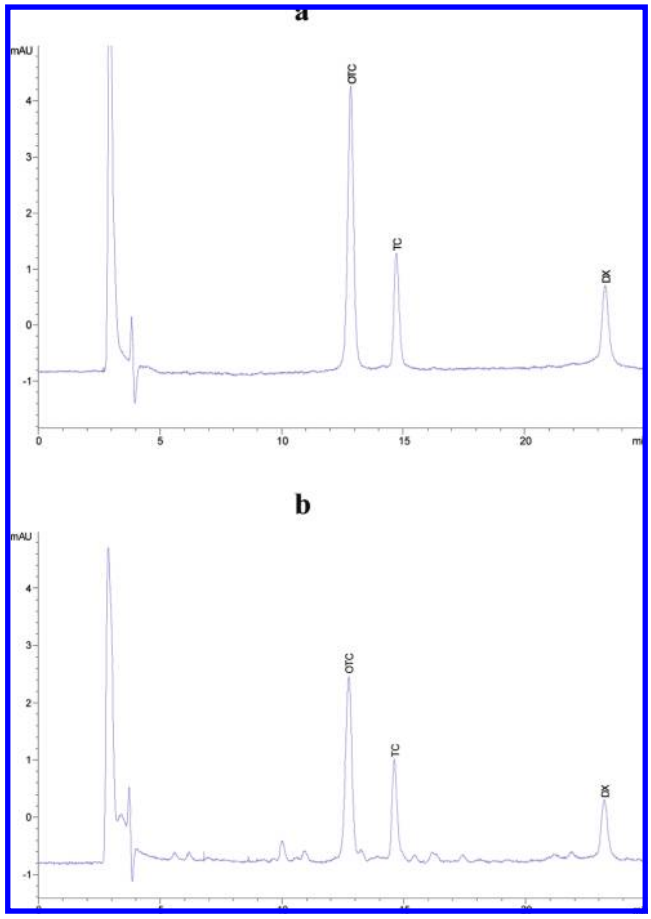

Figure 1. Comparison of chromatograms of oxytetracycline (OTC), tetracycline (TC), and doxycycline (DX) before (**a**) and after (**b**) a heat treatment in citrate−phosphate buffer of pH 7.0 for 1, 2.5, and 3 min, respectively, at 130 °C.

ature. A linear relationship was also observed between the log of the D_T value and the treatment temperature. The z values, equations, and correlation coefficients of DRT curves of the three antibiotics are shown in **Table 1**. No statistically significant differences ($P > 0.05$) were detected among the z values for each antibiotic ($z = 28 \pm 2$ °C). Therefore, the temperature dependence of the D_T values was similar for the three molecules. Oxytetracycline was the most sensitive tetracycline, showing a D_{140} of <1 min. At any temperature investigated, the D_T values of doxycycline were approximately 2 and 4 times higher than those of tetracycline and oxytetracycline, respectively. The Arrhenius activation energy constant of the three antibiotics heat-treated in citrate−phosphate buffer of pH 7.0 was 24.5 ± 2 kcal/mol.

The influence of the treatment medium pH and water activity on the thermostability of the three antibiotics was studied at 130 °C. The degradation kinetics under these treatment conditions again followed the traditional model based on a first-order inactivation (data not show). **Table 2** shows the D_{130} values obtained when antibiotics were heat-treated in citrate−phosphate buffer of pH 7.0, 5.5, and 4.0 and water activity of 0.99, as well as in citrate−phosphate buffer of pH 7.0 and water activity of 0.96 and 0.93. Most of the treatment conditions assayed did not cause any significant variation in the thermal stability of the three antibiotics. Only the reduction of the treatment medium pH from 5.5 to 4.0 caused a statistically significant increase ($P < 0.05$) in the thermal resistance of doxycycline: its D_{130} value increased by approximately 3 times at pH 4.0.

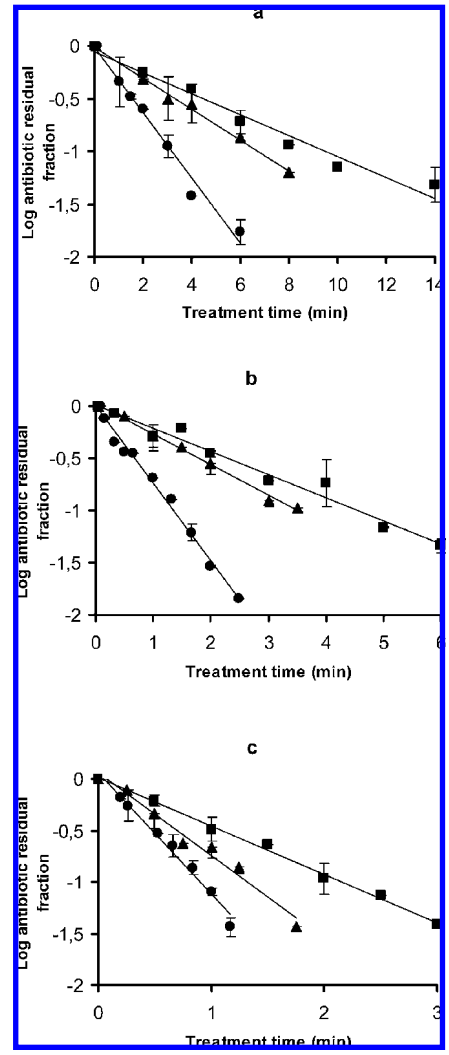

Figure 2. Degradation curves of oxytetracycline (●), tetracycline (▲), and doxycycline (■) suspended in citrate−phosphate buffer of pH 7.0 after heat treatments at constant temperature: (**a**) 120 °C; (**b**) 130 °C; (**c**) 140 °C. Linear regressions are shown as solid lines, and 95% confidence limits are shown as error bars.

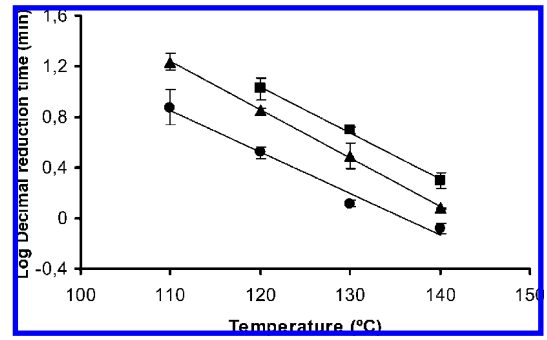

Figure 3. DRT curves of oxytetracycline (●), tetracycline (▲), and doxycycline (■) suspended in citrate−phosphate buffer of pH 7.0 after heat treatments at different temperatures. Linear regressions are shown as solid lines, and 95% confidence limits are shown as error bars.

DISCUSSION

In this investigation, the use of the thermoresistometer TR-SC has allowed us to evaluate and compare the heat resistance of three tetracycline antibiotics at high and ultrahigh temperatures, avoiding lag phases of heating. Thus, reliable data have been obtained to adequately describe the inactivation kinetics

Table 1. z Values, Equations, and Determination Coefficients of DRT Curves of Oxytetracycline, Tetracycline, and Doxycycline

antibiotic	z (°C)	DRT curve eq	R^2
oxytetracycline	30.3	$\log D_T = 4.45 - 0.033T$	0.983
tetracycline	26.3	$\log D_T = 5.45 - 0.038T$	0.999
doxycycline	27.8	$\log D_T = 5.38 - 0.036T$	0.997

and to calculate the thermostability parameters that define the inactivation of these antibiotics at ultrahigh temperatures.

Our results have demonstrated that the thermal degradation of the three tetracyline antibiotics follows a first-order reaction kinetic within 1.5–2 $\log_{10}$ cycles. The existence of an exponential relationship between the $\log_{10}$ of terramycin residual antimicrobial activity and treatment time was first suggested by Shahani (*30*). However, in that case, the heat treatments were applied at temperatures below 100 °C, the degradation curves represented the loss of antimicrobial residual activity against treatment time, and most of them dropped by <0.5 $\log_{10}$ cycles. Later, Kitts et al. (*15*), using chromatographic analysis in the detection of the residual antibiotic concentration, also observed the existence of a pseudo-first-order inactivation kinetic when farmed salmon was contaminated with oxytetracycline. In this study, the temperature range was 60–100 °C and the degradation curves dropped by <1 $\log_{10}$ cycle. To the best of our knowledge, none of the published studies has described the inactivation kinetics of any antibiotic at temperatures above 100 °C and the degradation curves were not more than 1 $\log_{10}$ cycle. Thus, the D_T values obtained in this study cannot be compared.

An exponential relationship has also been demonstrated between decimal reduction times and treatment temperatures (DRT curve). This relationship was also suggested in the 1950s (*29–31*). However, the DRT curves were obtained within the range of 60–100 °C, so it is unknown whether the linearity could be maintained at higher temperatures.

The z values obtained for tetracyline antibiotics at temperatures above 100 °C (28 ± 2 °C) were lower than those obtained in previous studies at lower temperatures: 48 °C for terramycin (oxytetracycline) (*30*) and 42.6 °C for oxytetracycline heat-treated in buffer of similar pH (*15*). These results would indicate that the linearity would not be maintained at high and ultrahigh temperatures, suggesting that the temperature dependence of the thermostability of tetracycline antibiotics might be greater at high and ultrahigh temperatures. Nevertheless, the variety of methodologies employed in heat inactivation experiments and in the detection of the antibiotic residual concentration might explain these differences. In any case, these results demonstrate that any extrapolation from previous results obtained at temperatures below 100 °C would introduce some errors in the calculation of the thermostability parameters at ultrahigh temperatures.

Thermal stability of microorganisms, proteins, or enzymes may be greatly influenced by the effect of several environmental factors (*33*). The greatest increases in heat resistance have been observed when certain characteristics of the treatment medium, especially pH and water activity, have been modified (*13, 22, 23*). For example, *Listeria monocytogenes* might increase its heat resistance as much as 100-fold (*32*). Antibiotics have been demonstrated to be more stable in foods than in water or buffer at temperatures below 100 °C (*15, 18*). Although the different methodologies employed make it difficult to draw clear conclusions, the thermotolerance increase seems to be twice the amount. The pH and water activity of most foods are within the 7–4 and 0.99–0.93 ranges, respectively. Our results have demonstrated that any variation of these physicochemical characteristics of the treatment medium within the range established would not substantially modify the thermostability parameters of the tetracycline antibiotics investigated. Only when doxycycline was heat-treated in citrate−phosphate buffer of pH 4.0 did its thermal resistance increase by approximately 3 times. These results are in agreement with those of Kitts et al. (*15*), who described double resistance when oxytetracycline was treated in buffer at pH 3.0 in comparison with pH 6.9.

Table 2. Decimal Reduction Time Values at 130 °C of Oxytetracycline (OTC), Tetracycline (TC), and Doxycycline (DX) Suspended in Treatment Media of Different pH Values and Water Activities[a]

	D_{130} (min)		
treatment medium	OTC	TC	DX
pH 7.0/a_w = 0.99	1.31a	3.16a	4.94a
pH 5.5/a_w = 0.99	1.28a	4.10a	6.37a
pH 4.0/a_w = 0.99	1.72a	4.82a	15.6b
pH 7.0/a_w = 0.96	2.42a	4.90a	4.19a
pH 7.0/a_w = 0.93	2.14a	4.17a	3.91a

[a] D_T values of each antibiotic with different letters indicate significant differences ($P < 0.05$) among treatments.

Table 3. Estimation of the Percentage of Residues of Oxytetracycline (OTC), Tetracycline (TC), and Doxycycline (DX) from Thermostability Parameters Shown after Low-Temperature−Long-Time or High-Temperature−Short-Time Treatments

heat treatment	T/time	% OTC	% TC	% DX
conventional	118 °C/30 min	<1	<1	<1
sterilization	121 °C/20 min	<1	<1	1.3
UHT treatments	135 °C/15 s	56	76	84
	140 °C/ 7 s	60	77	85

In general, microorganisms show low z values, in the range of 5–10 °C, but most protein and vitamin molecules show very high values, around 20–40 °C (*17*). These differences in the dependency of the thermal tolerance of food contaminants and components led to the design of high-temperature−short-time treatments (*12, 24*). UHT treatments that are carried out by heating and cooling liquid food in flow and have the advantage of achieving the required destruction of thermoresistant spores with minimum nutritional and quality losses. In solid or packaged foods, heat exchange is much less efficient, and this results in much longer heating times and lower temperatures, requiring conventional sterilization processes in autoclaves. Under these conditions, vitamin, protein, and enzyme degradation increased and so did browning. Tetracycline antibiotics showed z values very close to those obtained for proteins and enzymes, so they are also more affected by low-temperature−long-time treatments than high-temperature−short-time treatments. As a result, tetracycline residues are also much more heat stable at ultrahigh temperatures than foodborne bacteria or spores of public health concern.

Table 3 shows the percentage of antibiotic residues after several heat treatments commonly applied by industries, consumers, and researchers (*8, 12, 24*). The quantification of the residues was made by using the thermostability parameters obtained for each antibiotic. As shown in the table, low-temperature−long-time treatments (conventional sterilization) would cause the degradation of 98% of the initial concentration of doxycycline and would reduce the presence of tetracycline and oxytetracycline to negligible amounts (theoretically <0.01%). UHT treatments would reduce by >40% the initial concentration of oxytetracycline, by >30% the amount of tetracycline and by <20% the amount of doxycycline. Therefore, the most

effective treatment would be the conventional sterilization (121 °C/20 min) rather than UHT treatments. Taking into account the increase in thermal stability of doxycycline observed when treated at pH 4.0, and assuming a z value similar to that obtained at pH 7.0, a conventional sterilization would cause the degradation of maximum 80% of the initial concentration, and the UHT treatment would leave intact almost 95% of the initial concentration. Nevertheless, it should be noted that the breakdown products formed from tetracyclines during heat treatments have not been described yet. Further investigation is needed to clearly define if these breakdown products might still cause an allergic response in sensitive individuals (*18*).

In conclusion, the degradation curves of the three tetracycline antibiotics obtained at high and ultrahigh temperatures have demonstrated that antibiotic destruction follows a first-order reaction kinetic. The degradation curves were examined within 1.5–2 $\log_{10}$ cycles, and the D_T values were obtained at ultrahigh temperatures. The thermostability parameters obtained in this study have allowed us to calculate the efficacy of different sterilization procedures. Regarding sterilization, whereas low-temperature−long-time treatments (conventional sterilization) would destroy >98% of the initial concentration of the residues of the three antibiotics, high-temperature−short-time treatments (UHT) would leave unaltered residues in the 50–90% range.

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