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Formation of anhydrotetracycline during a high-temperature treatment of animal-derived feed contaminated with tetracycline

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Abstract

Residues of tetracyclines and their epimers were frequently found in bones of slaughtered animals, and also in meat and bone meals for animal feed that had been heat treated according to EU legislation. The aim of this study was to examine the formation of anhydrotetracycline and 4-epianhydrotetracycline, as toxic degradation products of tetracycline, during a heat treatment. Meat and bone meals containing bound tetracycline residues were heat treated at 100 and 133 °C for up to 45 min, extracted with hydrochloric acid and subsequently analyzed for tetracycline, 4-epitetracycline, anhydrotetracycline and 4-epianhydrotetracycline, using HPLC and LC–MS–MS. Small amounts of anhydrotetracycline and 4-epianhydrotetracycline were even found in the pre-treatment control samples. The heat treatments led to a significant increase of the amounts of these degradation products. During the most rigorous heat treatment, at 133 °C for 45 min, the mean percentage decrease of the concentrations of tetracycline and 4-epitetracycline was 50%, while the mean percentage increase of the concentrations of anhydrotetracycline and 4-epianhydrotetracycline was 533%.

Keywords: Anhydrotetracycline; 4-Epianhydrotetracycline; Tetracycline, Heat treatment; HPLC-UV; HPLC-DAD; LC-ESI-MS-MS

1. Introduction

Tetracycline residues are frequently to be found in bones of slaughtered animals that have passed meat inspection (Kühne, Wegmann, Kobe, & Fries, 2000). These residues may lead to the development of antibiotic resistance in pathogens and are therefore a potential risk for the production of meat and bone meals for animal feeding. The heat treatment in rendering plants was thought to be adequate to destroy possible contamination from the raw material (European Scientific Steering Committee, 1999). Nevertheless, in commercially available meat and bone meals in Germany, tetracycline residues in concentrations of up to 3.5 mg kg^{-1} (Körner, Kühne, & Wenzel, 2001) were found. Bound tetracycline residues in bone particles seemed to be quite heat-resistant even during a rigorous heat treatment at 133 °C and 3 bars for 45 min. A complete destruction of tetracycline (TC) and chlortetracycline (CTC) after a heat treatment at 133 °C for up to 45 min could not be demonstrated in artificially contaminated bone meals, but there was a significant decrease of detectable TC and CTC residues, by approximately 50 and 100%, respectively (Kühne, Körner, & Wenzel, 2001). Both Körner et al. (2001) and Kühne et al. (2001) determined only the parent substances and their 4-epimers.

The question arose as to whether any other degradation products of tetracyclines were formed during the heat treatment.

Possible degradation products, besides the 4-epimers of the parent substances (Rogalski, 1985), are iso-derivatives (Kennedy, McCracken, Carey, Blanchflower, & Hewitt, 1998), N-desmethyltetracycline (Zurhelle, Müller-Seitz, & Petz, 2000), and anhydrotetracycline (ATC) (Yuen & Sokoloski, 1977; Sokoloski, Mitscher, Yuen, Jnvarkal, & Hoener, 1977; Walton, Howlett, & Selzer, 1970), together with their epimers.

The toxicological significance of ATC and 4-epianhydrotetracycline (e-ATC) has been discussed (Klimova & Ermolova, 1976).

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The aim of the present study was to examine the formation of ATC and e-ATC in a heat treatment similar to that used in rendering plants.

2. Materials and methods

2.1. Preparation of meat and bone meals

The visible bones of pig carcasses were scanned with UV light at 366 nm for tetracyclines in accordance with Kühne, Wegmann, Kobe, and Fries (2000). If there was any obvious fluorescence, the long bones were thoroughly prepared (separation of fat and periosteum). The bones were cut longitudinally and the ends removed with a bone saw (MKB 548, MaDo, Dornhan, Germany). Then the marrow was removed under running water with a brush. The remaining solid substance was milled to particles <4 mm using a bone mill (P 42, Siepmann, Dorsten, Germany). The bone splinters were mixed with an intermediate product from a German rendering plant (125 g bone splinters and 375 g intermediate product) in aluminium containers. The intermediate product consisted of material that had already been heated in accordance with the obligatory heat treatment at 133 °C / 3 bars / 20′ and, as a result of that, freed of part of the water.

The containers, filled with 500 g of the mixture, were covered with aluminium foil after the removal of a sample of approximately 80 g for pre-treatment analysis. They were then heated in an autoclave at 133 °C for 20, 30 and 45 min, respectively, and at 100 °C for 20 and 30 min. Each time, four containers were heated at the same temperature–time combination. Into one of these four samples, a digital thermometer (GTH 1150, Greisinger electronic, Regenstauf, Germany) was installed before the heating. The temperature was observed and documented during the whole experiment. Cooling down of the samples took place overnight in the closed autoclave. At a temperature of approximately 27 °C, the samples were taken out of the autoclave, defatted, dried for 4 h and then stored at −18 °C until the analysis.

2.2. Extraction procedures

The extraction was performed with 1 M hydrochloric acid (Körner et al., 2001). To estimate the extent of the formation of ATC and e-ATC during a sample treatment with hydrochloric acid, standard solutions of TC (24 μg TC ml^{-1}) were incubated with different concentrations of hydrochloric acid.

The clean-up with a chelating sepharose column used in this study followed Farrington, Tarbin, Bygrave, and Shearer (1991). Up to 5 g of the sample was filled into a centrifugation tube and 20 ml of 1 M hydrochloric acid (Roth, 4625.2) were added. The samples were stirred with an Ultraturrax (Art-Miccra D-8; Art Labortechnik, Müllheim, Germany) tissue materials for approximately 10 s. The box was covered with aluminium foil and stored overnight at +8 °C. The next day the samples were centrifuged at 8000 ***g*** for 10 min. The supernatant was passed through a paper filter and the pH adjusted to pH 4.0 with sodium hydroxide (Roth, 8655.2). After another centrifugation, at 5000 ***g*** for 10 min, the combined supernatants were transferred to a chelating sepharose column.

2.2.1. Preparation of the chelating sepharose column

Five millilitres of chelating sepharose suspension (Sigma; I-4510) were thoroughly mixed and placed in a 200×20 mm glass column. After settling and flow from the excessive liquid, the column was dried using a water pump. Twenty millilitres of copper sulphate were passed through the column, followed by 15 ml of succinate buffer. The column was stored at 4 °C prior to use. Each column was used only once.

The column was equilibrated at a flow-rate of $<$ 4 ml min^{-1}. Then the column was first rinsed with 10 ml of distilled water and 30 ml of methanol, and again with another 10 ml of distilled water. The tetracyclines were eluted using 40 ml of EDTA succinate buffer (succinate buffer with 37.2 g EDTA l^{-1}). This was repeated with 10 ml of the same buffer.

The eluates were transferred to a C 18 endcapped column (Macherey & Nagel; 703014; Düren, Germany) that had been conditioned with 5 ml of methanol and equilibrated with 5 ml of distilled water. After rinsing with 5 ml of distilled water, the column was dried and retained compounds were eluted with 5 ml of methanol. The eluates were evaporated to dryness and dissolved in 1 ml of the mobile phase for HPLC.

2.3. Determination of TC and 4-epitetracycline (e-TC)

The HPLC system was comprised of the following apparatus and materials:

HPLC pump	Type 64, Knauer, Berlin, Germany
Degasser	Knauer
Precolumn	CC 8/4 Lichrospher 100-5 RP-18, Macherey & Nagel
Column	CC 250/4 Lichrospher 100-5 RP-18, Macherey & Nagel
UV detector	SPD 10A, Shimadzu, Duisburg, Germany. The UV detector was maintained at 360 nm.
DAD detector	SPD-M6A, Shimadzu
Integrator	C-R5A Chromatopac, Shimadzu
Mobile phase	50% acetonitrile (Roth, Karlsruhe, Germany; 7330.1) and 50% 0.01 M oxalic acid (Merck; 495), pumped at 0.9 ml min^{-1} at room temperature.

For the calibration curves, the following substances were dissolved in distilled water, these standards (1 mg ml^{-1}) being kept at −40 °C: tetracycline hydrochloride (Sigma, Deisenhofen, Germany; T-3258); 4-epi tetracycline hydrochloride (e-TC; Acros Chimika, Schwerte, Germany; 23312-1000). The working standards were prepared each day in concentrations of 0.5, 1, 5 and 10 μg ml^{-1} by dilution in mobile phase. The absolute recovery was determined each day by examining blank fish-meal samples spiked with standard solutions.

2.4. Calculation of the results

A calibration curve was produced with peak area plotted against concentration. The amount of tetracyclines in each sample was then calculated from the measured peak area. This figure was corrected for sample weight, injection volume and mean percentage recovery. Positive results were confirmed by UV spectra in the wavelength range of 290–390 nm.

2.5. Determination of ATC and e-ATC

The HPLC system was similar to that mentioned above. Instead of acetonitrile and oxalic acid 35% acetonitrile was used (Roth, Karlsruhe, Germany; 7330.1) and 65% phosphoric acid (0.5%), pumped at 0.9 ml min^{-1}, for the separation of ATC and e-ATC.

For the calibration curves, the following substances were dissolved in distilled water, these standards (1 mg ml^{-1}) being kept at −40 °C: Anhydrotetracycline hydrochloride (Acros Chimika, Nr. 23313-1000, Schwerte, Germany); 4-epianhydrotetracycline hydrochloride (Acros Chimika, Schwerte, Germany; 23311-1000) The working standards were prepared each day in concentrations of 0.5, 1, 5 and 10 μ gml^{-1} by dilution in mobile phase.

The absolute recovery was established and the results calculated as described before for TC and e-TC.

2.6. Confirmation of positive results

The identity of ATC and its 4-epimer was confirmed by LC-ESI-MS-MS. The eluates of the substances, tentatively identified as ATC and e-ATC were manually collected and diluted 1:1 with acetonitrile, and an aliquot was then transferred to an LC–MS–MS system.

The HPLC system used was a gradient system, consisting of a Thermoquest P4000 pump, an AS3000 autosampler, a Puresil C18 column (5 μm, 4 × 150 mm, Waters Corporation, USA), operated at 23 °C. The flow rate of 1 ml min^{-1} was split 1:10 before entrance to the mass spectrometer. The mobile phase consisted of 0.5% formic acid in water (solvent A), and acetonitrile (solvent B). After each run the column was rinsed for 6 min with 99% acetonitrile and re-equilibrated for 12 min with solvent A. The injection volume was 20 μl for fractions 1 and 2.

ESI–MS–MS (Electrospray ionization tandem mass spectrometry) was carried out using an LCQ Ion Trap with an electrospray ionization source (Finnigan Mat, San Jose, USA). The source polarity was set at positive and the spray needle voltage was 5 kV. Sheath gas flow was set at 100 units and the auxiliary gas flow was turned off. The capillary temperature was 150 °C. In the MS–MS analysis, MH^+ ions were trapped at m/z 427 and fragmented with a relative collision energy of 22%.

2.7. Statistical analysis of the results

The results were analyzed with the "SAS—Statistical Analysis System". The significance of the influence of the temperature and time of the heat treatment on tetracycline concentrations was analyzed. The values of significance (P) were calculated and assigned as follows: $P < 0.01$ highly significant, $P < 0.05$ significant, $P > 0.05$ not significant.

3. Results

3.1. Chromatography and determination

With the use of a Lichrospher 100-5 RP-18 column (250 mm) and acetonitrile-0.01 M oxalic acid (50:50, v/v) as mobile phase, sharp and symmetrical peaks for TC and e-TC were obtained under isocratic conditions. The retention times were 4.8 and 6.2 min for e-TC and TC, respectively. For the separation of ATC and e-ATC a column with a length of only 125 mm was used and acetonitrile-phosphoric acid (0.5%) (35:65, v/v) as mobile phase. The retention times were 7.9 and 9.3 min for e-ATC and ATC, respectively. The absolute recoveries were 65±7.2% for TC, 63±6.6% for e-TC, 71±4.4% for ATC and 65±3.9% for e-ATC.

The separation of e-ATC and ATC was achieved in a gradient LC–MS–MS system that has already been described (Hamscher, Sczesny, Abu-Qare, Höper, & Nau, 2000). The retention times were 8.8 and 9.3 min for e-ATC and ATC, respectively. The MS–MS spectra were very similar for e-ATC and ATC with the most

Table 1
Anhydrotetracycline (ATC) and tetracycline (TC) concentrations after an incubation of 24 μg ml^{-1} TC with HCl (%)

	ATC (∑ ATC and e-ATC)	TC (∑ TC and e-TC)
0.25 M	2.9	97.1
0.5 M	9.1	90.9
1 M	28.3	71.7
2 M	68.6	31.4
4 M	97.7	2.3

abundant daughter ion at m/z 410 corresponding to the loss of NH_3 from the molecular ion (Fig. 1).

3.2. Formation of ATC in HCl

The incubation of TC with HCl led to a significant reduction of TC concentrations and an increasing formation of ATC (Table 1).

3.3. TC, e-TC, ATC and e-ATC determinations

Before heat treatment, the mean TC concentrations (as the sum of TC and e-TC) were 894 μg kg^{-1} and the mean ATC concentrations (as the sum of ATC and e-ATC) 96 μg kg^{-1}, respectively (Table 2). After heat-treatment at 100 °C, TC and e-TC concentrations were found to be higher than before the heat treatment, with an increase of detectable total TC concentrations of between 35 and 72%. The mean levels of increase were 51% for the 20-min treatment and 63% for the 30-min treatment. The increase was highly significant for TC and slightly significant for e-TC. The length of the heat-treatment did not significantly affect the concentrations. For ATC and e-ATC, a slightly significant increase in detectable concentrations could also be observed after the heat treatments at 100 °C. The mean increase for

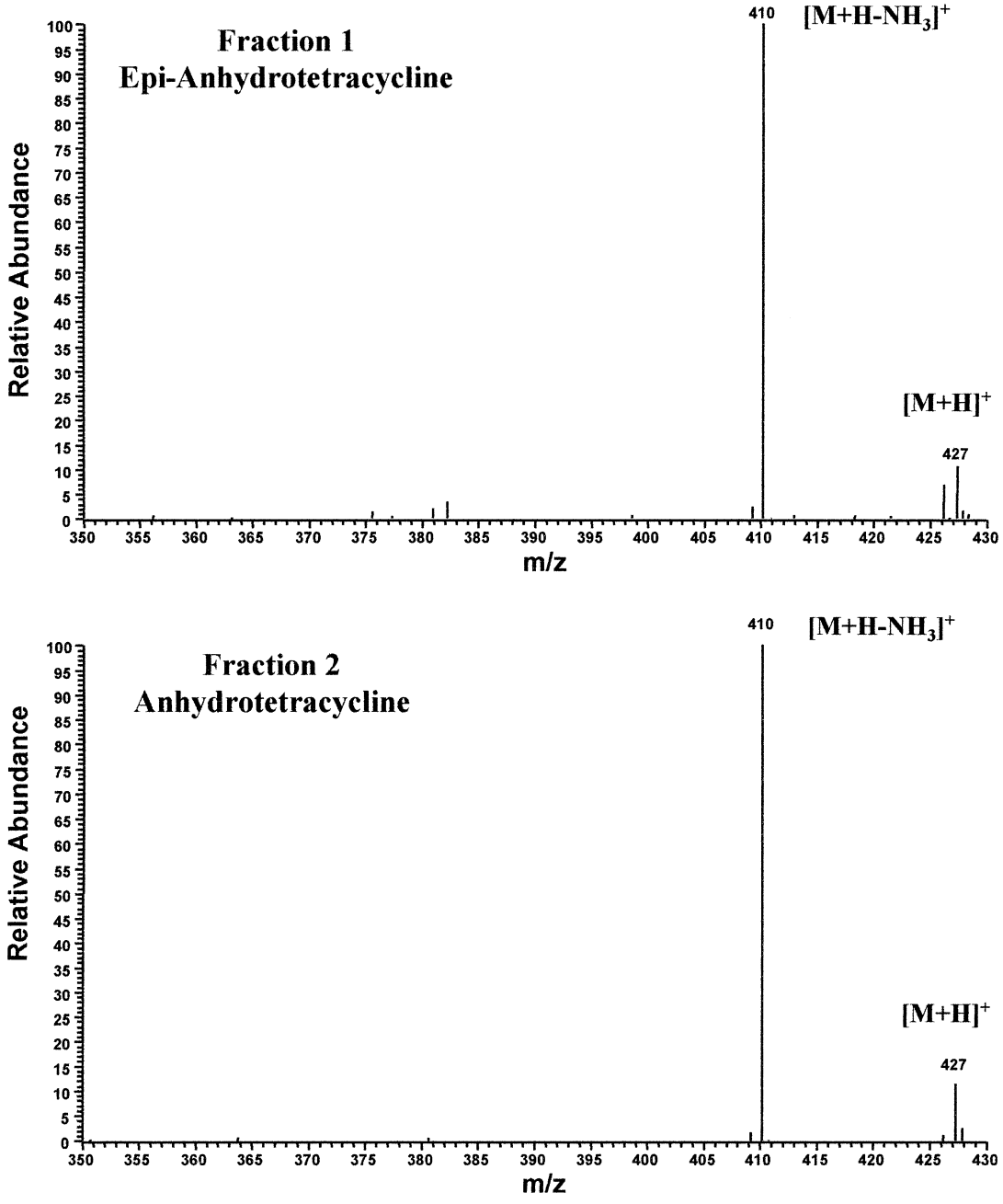

Fig. 1. ESI tandem mass spectra of 4-epianhydrotetracycline and anhydrotetracycline, obtained after rechromatography of fractions 1 and 2.

Table 2
Effect of heat treatments on tetracycline (TC), e-TC, anhydrotetracycline (ATC) and e-ATC concentrations in a meat and bone meal ($n=4$ per treatment; mean and S.D.; μg kg^{-1})

	Before treatment					After treatment				
	TC	e-TC	ATC	e-ATC	∑	TC	e-TC	ATC	e-ATC	∑
100 °C										
20 min	602±211	244±98	47±27	34±21	927±212	1005±241	293±114	77±13	45±12	1379±226
30 min	525±186	209±121	41±15	20±9	795±184	922±165	260±99	122±50	42±21	1346±166
133 °C										
20 min	819±145	351±132	68±32	45±27	1283±456	409±99	163±80	403±168	194±76	1169±155
30 min	607±245	248±174	66±41	37±29	958±389	327±87	116±56	382±200	184±151	1009±139
45 min	616±199	250±123	41±12	54±18	961±502	310±121	122±68	427±233	162±118	1021±181

total ATC was 50% for the 20-min treatment and 103% for the 30-min treatment. The difference between the treatments was highly significant. e-TC and e-ATC could be found in all samples. The mean percentage of epimerization was 29% for TC and 42% for ATC in pre-treatment samples, and 22% for TC and 36% for ATC after the heat-treatment. The differences in epimerization between TC and ATC were slightly significant. There were no significant differences between pre- and post-treatment samples, nor was the duration of heat treatment significant. After the heat treatment at 133 °C, TC was found in concentrations of between 432 and 565 μg kg^{-1}. These concentrations were, significantly, very much lower than before the heat-treatment. The mean decrease was 50%. At the same time, the ATC and e-ATC concentrations increased very significantly. The mean percentage increase was 533%. e-TC and e-ATC could be found in all samples. The mean percentage of epimerization was 28% for TC and 33% for ATC. There were no significant differences between pre- and post-treatment samples, nor was the duration of heat-treatment significant. The differences in the epimerization rate, between TC and ATC, were slightly significant.

4. Discussion

Studies on the stability of tetracyclines in food during storage and cooking have been performed since as early as 1959. These experiments used microbiological methods of analysis (Escanilla, Carlin, & Ayres, 1959; Katz, Fassbender, & Dowling, 1973), but HPLC methods were used in recently published research (Rose, Bygrave, Farrington, & Shearer, 1996). All authors found that tetracyclines are not very heat resistant. Nevertheless, Honikel, Schmidt, Woltersdorf and Leistner (1978) stated that they found bound residues of tetracyclines in bones to be stable at 130 °C. Our results are in accordance with their findings.

A complete destruction of bound TC residues during a heat-treatment of 133 °C for up to 45 min could not be demonstrated, but there was a significant decrease in detectable residues by approximately 50%. In this study, the heating at 100 °C led to an increase of detectable TC concentrations in comparison with the pre-treatment analysis. This fact indicates that 100 °C does not affect the chemical integrity of the tetracyclines but increases the effectiveness of extraction. This might be due to a possible weakening of the tetracycline calcium complexes in the bone particles. The time factor did not have a significant effect on the detectable TC concentrations. An explanation might be the slow cooling off of the samples, for the autoclave could not to be opened before the temperature had fallen below 50 °C. This made active cooling of the samples impossible. Another factor could have been the defatting of the samples before extraction. After defatting, the samples had to be dried for 4 h at 103 °C.

For the determination of ATC and e-ATC, a different mobile phase and determination wavelength had to be used. For confirmation, a sensitive and highly selective LC–ESI–MS–MS method, recently developed for the detection of TC residues in soil was applied (Hamscher et al., 2000). With these methods, considerable concentrations of ATC and e-ATC could be detected, even in pre-treatment samples. Compared with the TC concentrations, the ATC concentrations were quite low in these samples. In another approach, we demonstrated the formation of ATC during an incubation of TC standard solutions with HCl. The ATC amounts in pre-treatment samples were most probably a result of the extraction of the samples with HCl. This conclusion accords with the results of Walton et al. (1970), who found ATC and e-ATC in acidic preparations of TC. The ATC and e-ATC concentrations increased significantly after the heat treatment. The increase was most significant in the samples that had been treated at 133° C and 3 bars for 45 min, according to European legislation (Council of the European Community, 1999). There is little available knowledge about the formation of ATC. One mechanism suggested, is the possible effect of chemical treatment on the dehydration of TC to ATC. Walton et al. (1970)

found that citric acid increases the tendency of TC to degrade to ATC, and Sokoloski et al. (1977) and Rogalski (1985) found the same with mineral acids.

A possible effect of temperature was discussed by Loseva and Shukailo (1971), who studied the formation of ATC and e-ATC from TC under various temperature conditions. No data could be found demonstrating the impact of high temperature on degradation products of tetracyclines, except the studies of Rose et al. (1996), who examined the stability of oxytetracycline and the formation of epimers and apo-oxytetracycline.

Calculating the total tetracycline concentrations in the post-treatment samples, we found that TC was not destroyed at all during the 133° C treatment, but was considerably degraded to ATC.

ATC is much more toxic than TC. The no-observed-effect levels (NOEL) for ATC in different tests for embryotoxicity, immuno-depressive action and teratogenic effects were 4.8 to 100 times lower than that of TC (Klimova & Ermolova, 1976).

The main effect of tetracyclines that is of interest here is their antibiotic activity. ATC is three times less effective than TC for the most relevant bacteria. This means that a microbiological determination of ATC residues in feed or food is almost impossible. Additionally, the microbiological determination of TC in feed is difficult if the TC is bound to macromolecules, as in bone particles (Kühne & Körner, 2001). Nevertheless, there is an effect on micro-organisms. Bertrand, Postle, Wray, and Reznikoff (1984) found that ATC is a stronger inducer of TC resistance than the parent drug, and Degenkolb, Takahashi, Ellestad, and Hillen (1991) gave some molecular explanations for this fact during their experiments with Tet repressors.

5. Conclusions

The results of the present study provide clear evidence that the toxic degradation products ATC and e-ATC could be found in material such as meat and bone meal that contains TC and has been treated at high temperature. Even the application of 133 °C did not destroy TC in the material, but resulted in a significant degradation to ATC. It is therefore highly recommended that samples which have been treated with high temperatures or acidic solutions and which contain tetracyclines should also be tested for anhydro-derivatives.

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