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Thermal stability assessment of antibiotics in moderate temperature and subcritical water using a pressurized dynamic flow-through system

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ABSTRACT: Thermal degradation of antibiotics has been studied for decades in a broad range of disciplines including food production, agriculture and analytical chemistry. Yet, there is a lack of thermal stability data for many antibiotics. Here we systematically investigated the thermal stability of ten commonly prescribed antibiotics applying a laborsaving automated in-house pressurized dynamic flow-through system. The design of the system allowed a fast access to a large number of data at medium to subcritical water temperatures, ranging from 50-250 °C. The five ß-lactams cefadroxil, cefuroxime, amoxicillin, penicillin V, and penicillin G showed a high degree of stability with a maximum degradation of less than 30 % at 150 °C. The two quinolones ciprofloxacin and norfloxacin showed a very high thermal stability up to 200 °C, as did trimethoprim and sulfamethoxazole. At 250 °C all antibiotics were either partly of fully removed. The tetracycline doxycycline showed a specific removal pattern probably involving both binding to metal surfaces at lower temperatures as well as degradation at increased temperatures.

KEYWORDS: Antibiotics, ß-lactams, cephalosporins, penicillins, fluoroquinolones, trimethoprim, sulfamethoxazole, thermal stability, subcritical water, dynamic system

1 INTRODUCTION

The stability of antibiotics at different temperatures has been researched for several decades in various disciplines. Within the food production sector the presence of antibiotic residues in foodstuffs is of concern due to possible direct allergic and/or toxicity reactions as well as the appearances of resistant strains of bacteria in animals with pathways to humans [1,2]. During manure storage temperature conditions will be of importance since the main part of the administered antibiotics in husbandry will be excreted in feces and finally may end up in soils after application of manure on agricultural lands [3-5]. In analytical chemistry, temperature is often a key parameter to promote the release of analytes from matrix active sites and especially so in the discipline of solid sample extraction [6,7].

Almost 60 years ago Landerkin and Katznelson [8] investigated the stability of six antibiotics in sugar syrup and honey and found a higher stability at 4 °C than at 34 °C. Of the compounds investigated, penicillin and streptomycin were the least and the most stable compound, respectively. Cooking conditions may affect antibiotic residues in meat of various origins, and has received considerable attention since the 1980´s [1,9,10]. Thermal degradation of tetracyclines has been studied at some depth in pork [9,10], chicken [10], sheep [11] and fish [12]. Fedeniuk et al. [9] showed that oxytetracycline (OTC) stability was higher in pork tissue than in aqueous solutions in the interval 60-80 °C. Adding calcium chloride decreased reaction kinetics significantly in both matrices due to complex formation with metals. Comparable stability findings were seen for OTC in salmon tissue at temperatures between 60 °C and 100 °C, with superior stability in tissue than in a parallel aqueous buffer [12]. Additionally, OTC was more stable at pH 3.0 than at pH 6.9. More recent studies examined tetracycline (TC) degradation and the formation of degradation products during microwave heating and boiling of pork and chicken meat [10]. Boiling for 14 min and microwave heating for 6 min reduced the amount of TC to between 56 and 82 %. Similarly, a previous

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investigation on lamb meat showed that boiling for 30 min was necessary to decrease OTC levels by 95 % [11]. Recent studies performed on enrofloxacin (a fluoroquinolone) in chicken meat, showed that this compound is fairly heat tolerable since it could not be degraded fully even after a 3h boiling period [13], indicating major differences between various groups of antibiotics. Liquid food products, primarily milk, have been examined for many years to reveal the effects of various heat treatment procedures on antibiotics [2,14,15]. Several ß-lactams have recently been investigated. Nine spiked ß-lactams were examined by Zorraquino et al. [15] by treating them at five different time-temperature combinations (40-140 °C). A major finding was that medium heating to 60 °C for 30 min, and ultra-high temperature (UHT) treatment to 140 °C for 10 s, only caused slight decreases in antimicrobial activity, while classic sterilization conditions (120 °C) for 20 min showed a considerable inactivation of penicillins (65%) and cephalosporins (90%). Furthermore, five quinolones in milk were investigated in the interval 80-100 °C, finding half-lives ranging from 102-456 min at the highest temperature [2]. This study also showed that ciprofloxacin and norfloxacin were the most sensitive with 12 % degradation at 120 °C for 20 min, while enrofloxacin, flumequine and oxolinic acid were more recalcitrant, being degraded to only 5 % or less. UHT heating to 140 °C for 4 s, had basically no effects on degradation. In this context it should be noted that a comprehensive evaluation of the effects of pH, temperature and buffers has been performed on the ß-lactam cefepime in aqueous solutions at moderate temperatures of 45-75 °C [16]. In this study, maximum stability was obtained in the pH interval 4.6-5.6.

Manure treatment at different temperatures may positively contribute to the removal of various micro-pollutants present in the matrix. Wang and Yates [3] found a faster OTC degradation in manure at 35-45 °C than at 15-25 °C. Correspondingly, Arikan and co-workers [4] detected that OTC degradation in manure occurred within a few days at temperature of 70 °C in the compost. Recent findings on the fate of three TCs and degradation products thereof during manure composting are available in a comprehensive study by Wu and co-workers [5].

Golet et al. [17] investigated temperature effect (50-150 °C) on the extraction efficiency of the two fluoroquinolones (FQ) ciprofloxacin and norfloxacin from sewage sludge. In the interval 50 °C to 100 °C, the extraction was enhanced by temperature, while in the interval 100 °C and 150 °C, efficiency remained constant, but with gradually darker extracts. Sand samples spiked with FQs showed no sign of thermal degradation at 100 °C. Likewise, Göbel et al. [18] found that 100 °C was an optimal extraction temperature for five macrolides, five sulfonamides and trimethoprim. Below 100 °C recoveries were 10 to 20 % lesser for all analytes, while above 100 °C recoveries decreased to between 60 % and 95 %. At the higher temperatures the extracts were also much darker. Decreased recoveries above 100 °C were explained by thermal analyte degradation, yet no specific study was performed to justify this statement. A single spiked sand experiment was performed at 100 °C showing no evidence of thermal degradation of any of the compounds. Other explanations might be plausible such as co-extraction of interfering matrix components causing heavy ion suppression in mass spectrometry as suggested by Díaz-Cruz [19]. In a later study pressurized liquid extraction was used to extract seven macrolides from fish and meat [20]. An optimum temperature of 80 °C was achieved, with a minor reduction in recoveries up to 120 °C, while above 100 °C a cloudy more coloured suspension was seen due to co-extraction of higher molecular compounds possibly hampering the analysis. Once again, decreased recoveries might not necessarily be caused by thermal instability. These arguments may also hold true in a recent study for the extraction of tetracyclines and chloramphenicol from animal feeds applying subcritical water in a temperature range of 60-120 °C [21].

In general, no systematic approach has been performed on the thermal stability of antibiotics during medium and subcritical water treatment covering an extensive range of antibiotic classes. Very few studies are made above 100 °C, and then only for a few seconds. This study aims at expanding the temperature window for antimicrobials. Such fundamental knowledge is crucial in many disciplines dealing with antibiotics. Here ten commonly prescribed antibiotics, covering seven classes of different characteristics, were investigated employing an automated laborsaving in-house pressurized dynamic flow-through system. The system allowed for a fast and systematic testing of antibiotics covering a broad temperature range and a large number of increments from medium to subcritical water temperatures, giving detailed information of thermal stability not previously published.

2 MATERIALS AND METHODS

2.1 CHEMICALS

Methanol (HPLC-grade) was purchased from Sigma-Aldrich (Steinheim, Germany), while sodium hydroxide was obtained from VWR (Stockholm, Sweden). Ultra-pure water was obtained from an OPTIMA water purification system (Elga Ltd, High Wycombe, Buckinghamshire, GBR).

Penicillin G sodium salt, penicillin V potassium salt, sulfamethoxazole, ciprofloxacin and caffeine were delivered by Fluka (Buchs, Switzerland). Amoxicillin, trimethoprim, doxycyclin, cefadroxil, and norfloxacin came from Sigma-Aldrich. Finally,

Fresenius Kabi (Uppsala, Sweden) supplied cefuroxime. Individual antimicrobial stock standard solutions of 300 mg L^{-1} were prepared by dissolving 0.0150 mg of the individual antibiotics in 50 mL methanol, except for the two quinolones where 100 µL of 1 mol L^{-1} NaOH was added to assist in dissolving the compound.

The thermal stability test cell was manufactured in stainless steel column with a volume of 11.8 mL (150 x 10.0 mm i.d.). The cell was carefully packed with stainless steel particles to obtain an inert chromatographic system, with particle sizes in the range 100-300 µm. The size distribution and surface structure of the added particles are shown in Figure 1.

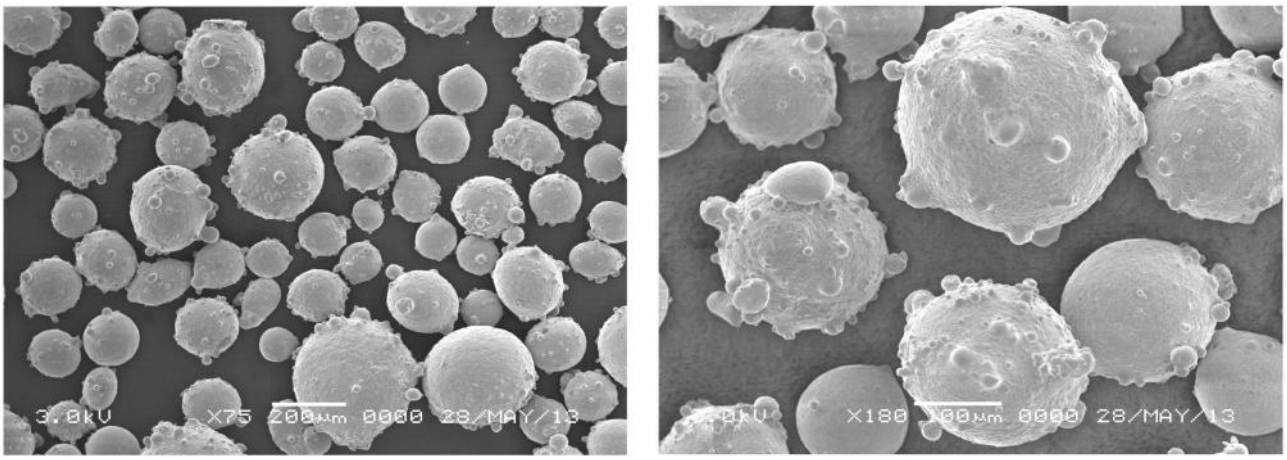

Fig. 1. SEM picture of stainless steel particles showing the size and surface structure of the packing material in the thermal stability test cell.

2.2 INSTRUMENTATION AND BASIC SET-UP

An in-house pressurized dynamic flow-through system was used for thermal stability test as schematically shown in Figure 2. The system was designed on the principles described by Hawthorne et al. for sub- and supercritical water extraction (SHWE) [22].

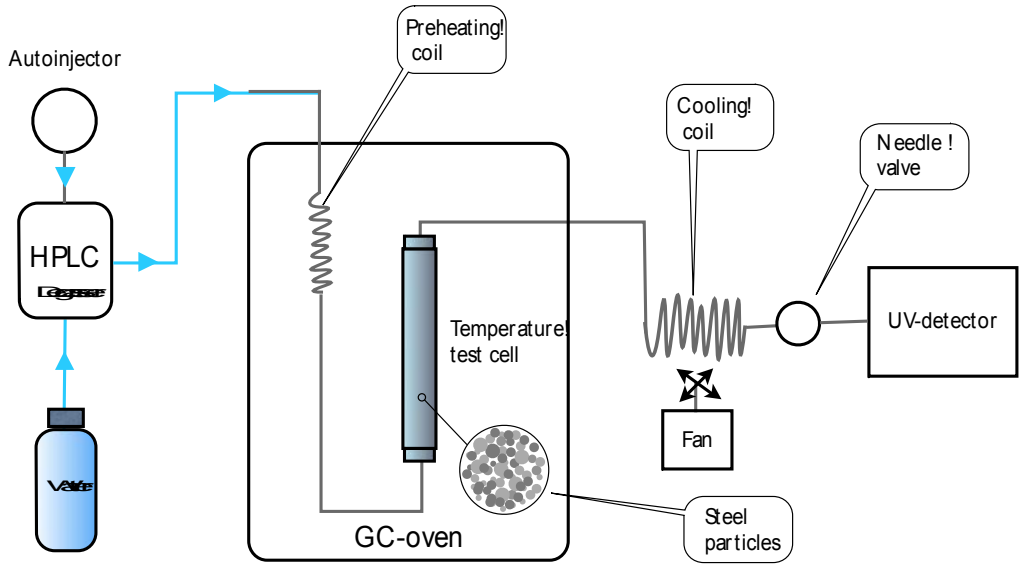

Fig. 2. Schematic picture of the in-house pressurized dynamic flow-through system.

The system consisted of an Agilent 1100 series HPLC instrument (Palo Alto, CA, USA) with an autosampler, vacuum degasser, quaternary pump and a Hewlett-Packard 1050 MWD detector (Palo Alto, CA, USA). The HPLC pump supplied water to the test cell through stainless steel tubing (1/16" o.d. and 0.040" i.d.) with a 1 m preheating coil. Both the preheating coil and the temperature test cell were placed inside a Varian 3400 GC oven (Walnut Creek, CA, USA). The preheating coil

ensured that the water reached operating temperature before entering the test cell. The outlet water flow pressure was controlled by a SSI high-pressure needle two-way valve (Grace Discovery Sciences), pressure held at 23 bars during the whole experiment. Prior to the needle valve a 1 m coil (1/16"-o.d. 0.020" i.d.) was mounted and cooled by air using a common electrical fan (12 V), assuring that the temperature of the water reaching the UV-detector was below 25 °C. Temperature was monitored with thermocouples at the outlet from the GC-oven and after the needle valve. A Hewlett-Packard 1100 Chemstation software evaluated all data acquired (Palo Alto, CA, USA).

2.3 TEMPERATURE TEST PROCEDURE

The thermal stability of all ten antibiotics plus caffeine were tested at 50, 100, 150, 200 and 250 °C. Each individual temperature measurement consisted of the following procedure: A total of 200 µL of each compound was prepared in individual vials and 25 µL of each antibiotic solution was thereafter injected individually into the system. Initially one injections was done without the thermal stability test cell in place, with the oven set at 25 °C to obtain a maximum reference absorbance peak area value for each antibiotic. Thereafter the thermal stability test cell, containing the stainless steel particles, was connected and put into the oven, the pump turned on and the system was allowed to reach the chosen temperature. The autosampler was started when pressure and baseline showed steady conditions. Subsequently two runs followed for each antibiotic and temperature, so that duplicate results could be monitored. After the two runs the thermal stability test cell in place was allowed to cool down, and then disconnected. Finally one more reference value was obtained at 25 °C, which concluded one cycle. The flow rate was set to 2 mL min^{-1} the whole cycle through.

2.4 UV ANALYSIS AND RECOVERY CALCULATIONS

Full absorbance spectra were obtained for all antibiotics in the interval 200-800 nm (spectra not shown). Three wavelengths were applied in all runs based on absorbance maxima studies: 218, 230 and 272 nm. The wavelength giving the highest response was chosen for the individual antibiotics; KOF 272, AMO 230, SUL 272, CEL 230, FEN 218, CEF 218, TRI 272, BEN 218, CIP 272, NOR 272 and DOX 272 nm. For each compound, however, all three wavelengths were recorded simultaneously, and stored. The area of the resulting peaks from all injections was integrated and the absorbance mean was calculated. The relative recovery in % was then calculated as [% compound recovery = $100 \cdot (a / b)$] where a is the absorbance resulting from the injection when the thermal stability test cell was connected and b is the mean absorbance of the two values when no cell was present in the system and temperature is set to 25 °C.

2.5 CALCULATING ADJUSTED RETENTION TIMES IN THE THERMAL STABILITY TEST CELL

In order to get a value of the average time spent by the antibiotics in the test cell, an adjusted retention time was calculated by subtracting the dead time t_0 from the measured retention time t_R. The dead time t_0 was taken as the time needed for the compound to reach the UV detector without the thermal stability test cell in place at a temperature of 25 °C, and was found to be 1.1 min. All compounds, except the quinolones, had basically the same adjusted retention times, which were close to 3.2 min at 50 °C while at 100 and 150 °C they decreased to between 2.7 and 2.8 min. The quinolones CIP and NOR deviated somewhat and showed adjusted retention times of close to 4 min in the temperature interval 50-150 °C. Increasing the temperature to 200 °C caused the adjusted retention times to decrease to 1.8 min for all compounds but CIP and NOR, which both had an adjusted retention time of 3.2 min at this temperature. It should also be noted that both CIP and NOR showed quite dramatic tailing effects from 50 °C to 200 °C giving runtimes up to 15 min for the lower temperatures.

3 RESULTS AND DISCUSSION

3.1 PRE-EXPERIMENTS

Initially the pressurized dynamic flow-through system was developed and various technical constructions were optimized. Thereafter the system performance was tested and evaluated for caffeine (KOF), and all ten antibiotics. These preliminary experiments gave indications of thermal effects for the different antibiotics (data not shown) and served as a basis for the design of a comprehensive thermal stability study in the temperature interval 50-250 °C as outlined below.

3.2 SYSTEMATIC THERMAL STABILITY STUDY

ß-LACTAMS. In all five ß-lactams were investigated, two cephalosporins; cefadroxil (CEL, first generation) and cefuroxime (CEF, second generation) and three penicillins; amoxicillin (AMO), penicillin V (FEN), and penicillin G (BEN). Results for the cephalosporins and the penicillins are shown in Figure 3a and 3b, respectively.

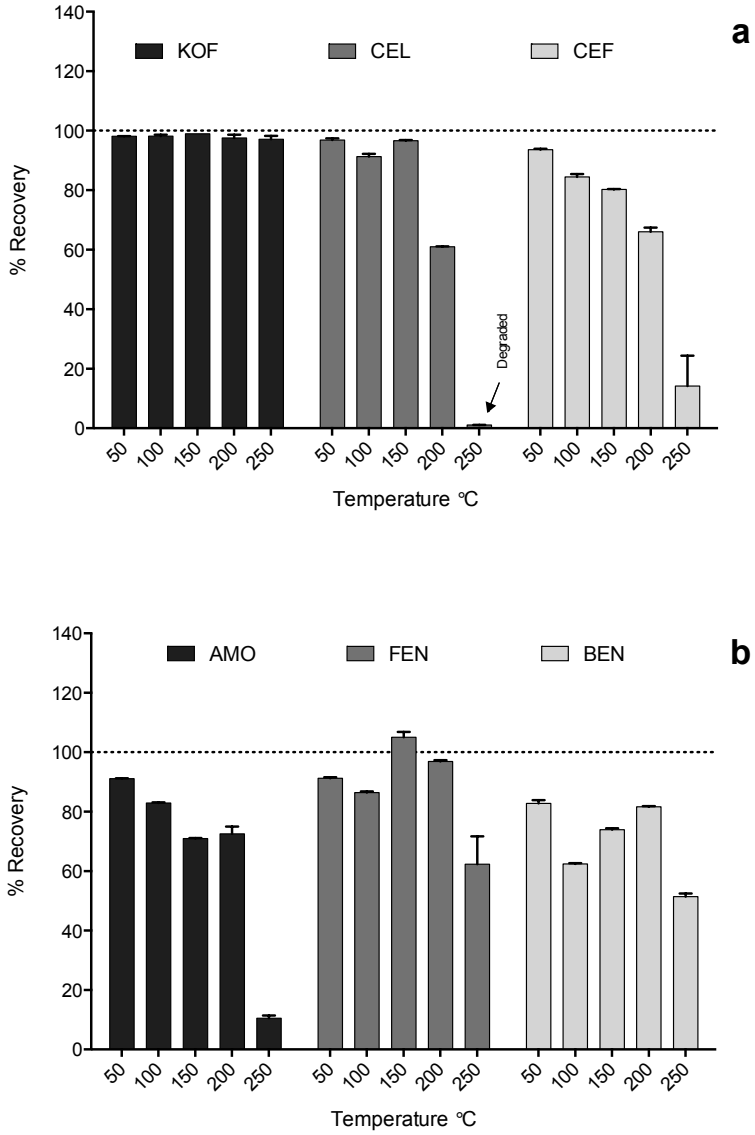

Fig. 3. Thermal stability of caffeine (KOF) and five ß-lactams at medium to subcritical water temperatures presented as amount antibiotic recovered versus a 25 °C test (see Experimental section for details). Investigated compounds covered a) the cephalosporins cefadroxil (CEL) and cefuroxime (CEF) and b) the penicillins amoxicillin (AMO), penicillin V (FEN), and penicillin G (BEN).

Caffeine (KOF) showed to be a thermally stable reference compound throughout the entire temperature series, not even being degraded at 250 °C, while both cephalosporins were almost fully lost at 250 °C. Especially CEL showed poor recoveries, and could hardly be detected. Of the three penicillins AMO was the least thermally stable, with less than 20 % recovered at 250 °C, while FEN and BEN both could be recovered to at least 50 % at the same temperature. Overall, the ß-lactams showed a high degree of stability and a general observation is that none of the antibiotics were degraded more than 29 % at 150 °C. This may explain why Zorraquino et al. [15], who exposed ß-lactams to ultra-high temperature (UHT) treatment at 140 °C for 10 s, only observed minor decreases in antimicrobial activity. An additional notation to be made is that CEF and AMO had a

relatively distinct declining recovery pattern over the entire temperature range, while FEN and BEN showed a fairly constant recovery at 150 °C and 200 °C, before levelling of at 250 °C. When comparing the absorption patterns of the three wavelengths at different temperatures, only minor differences were observed for all 10 molecules except CEF, indicating that the detected compound was the same throughout all temperatures until degradation. In the case of CEF the absorption shifted towards 272 nm as the temperature raised. An explanation to this phenomenon might be a change in the molecular structure resulting in a molecule with a different UV-absorption as the temperature rises. Then as the molecule is exposed to the highest temperature (250 °C), the UV-absorbing moiety is destroyed causing poor recoveries. No instrumentation was available to verify such a molecular change, and was not further investigated.

FLUOROQUINOLONES AND OTHER ANTIMICROBIALS Two fluoroquinolones; ciprofloxacin (CIP) and norfloxacin (NOR) and three other antimicrobials; sulfamethoxazole (SUL), trimethoprim (TRI) and the tetracycline doxycycline (DOX), were investigated. Results for theses five antimicrobials are shown in Figure 4a and 4b, respectively.

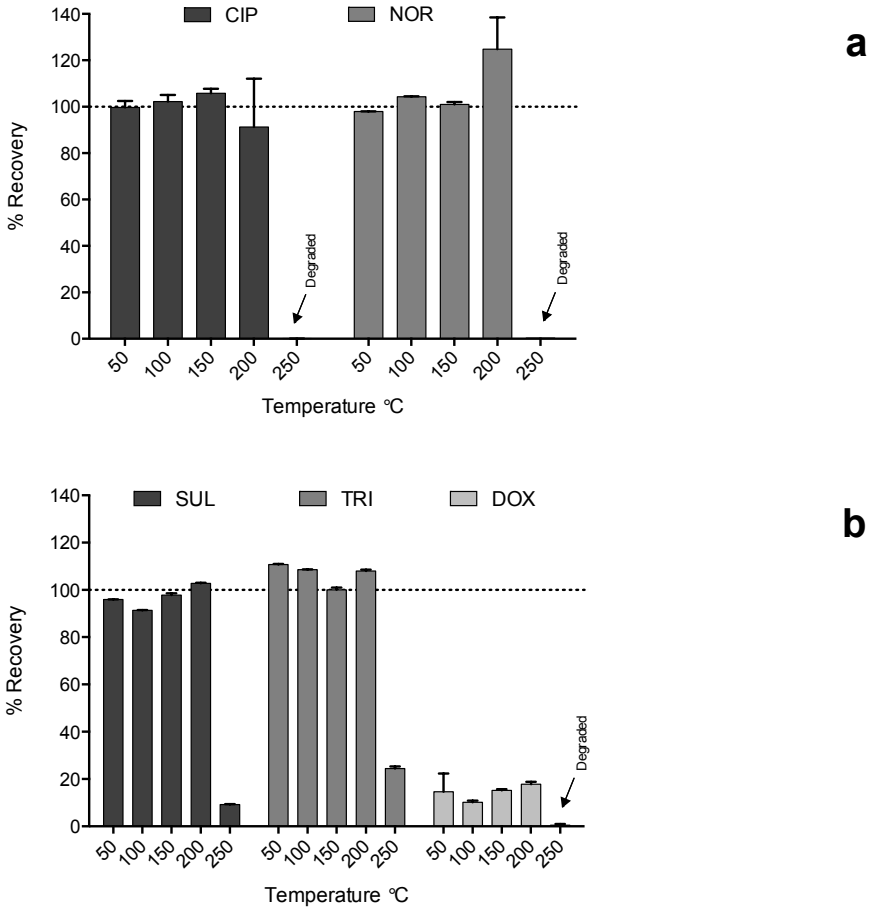

Fig. 4. Thermal stability of two fluoroquinolones and three other antimicrobials at medium and subcritical water temperatures presented as amount antibiotic recovered versus a 25 °C test (see Experimental section for details) Investigated compounds covered a) the fluoroquinolones ciprofloxacin (CIP) and norfloxacin (NOR) and b) sulfamethoxazole (SUL), trimethoprim (TRI) and doxycycline (DOX).

The two quinolones CIP and NOR showed high thermal stability up to 200 °C, with basically no losses, while at 250 °C not even traces of the compounds could be recovered. Almost the same pattern was observed for SUL and TRI, though detectable amounts of both compounds were seen at the highest temperature, with recoveries of 9 % and 24 % for the two compounds, respectively. In a study by Roca and co-workers [2] it was shown that CIP and NOR only were degraded by 12 % at 120 °C when heated for 20 min, while heating to ultra-high temperatures (UHT, 140 °C for 4 s) had nearly no effects on degradation, which is in line with our observations on thermal stability of quinolones.

DOX shows a unique and poor thermal stability pattern never reaching even 20 % in recovery for any of the temperatures, and at 50 °C only 15 % was recovered. This might be explained by both thermal degradation and adsorption of DOX to the

metal particles, since tetracyclines are known to form complexes with metal cations [23]. The latter is supported by a small increase in recovery as the temperature is increased up 200 °C, after which DOX can no longer be recovered. The pH investigations described in the next section further helps explain the behaviour of DOX.

3.3 EFFECTS OF PH

It is well-known that pH might have an effect on the stability of antibiotic compounds. We therefore investigated effects of pH at 4, 7 and 9 fixing the temperature in the middle of the temperature interval to 150 °C. The results for five ß-lactams are shown in Figure 5a while fluoroquinolones and other antimicrobials are shown in Figure 5b.

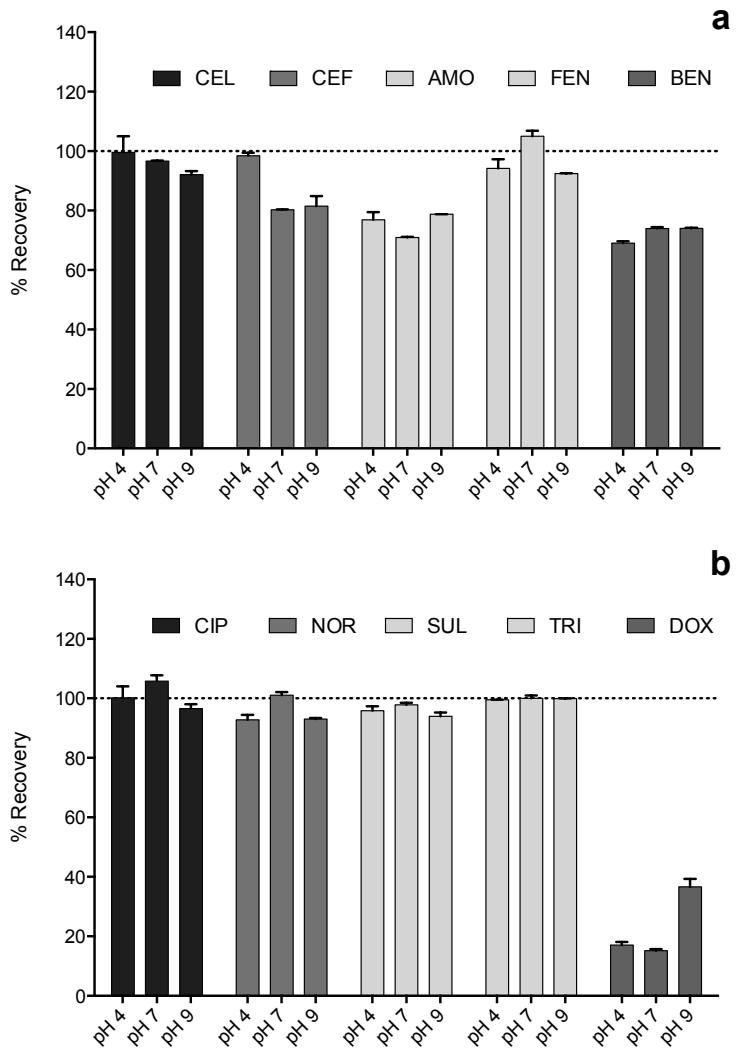

Fig. 5. Thermal stability of a) the five ß-lactams b) the two fluoroquinolones and three other antimicrobials at three different pH, applying a fixed temperature of 150 °C. Results are presented as amount antibiotic recovered versus a 25 °C test (see Experimental section for details).

In general the effects of pH were minor, with only a minor tendency that CEL and CEF were more thermal stability at the lower pH, being fully recovered at a pH of 4. This is comparable to the ß-lactam cefepime investigated by Fubura et al. [16] demonstrating that this compound had its maximum stability in the pH interval 4.6-5.6 in aqueous solutions. Interestingly for the tetracycline DOX, recovery was more than doubled at the highest pH, from around 15 % at pH 4 and 7, to nearly 35 % at pH 9. The explanation to this is not fully clear, but could involve both a higher stability as well as less binding ability and complexation to the metal surface at a pH of 9.

4 CONCLUSION

Thermal stability of antibiotics is of great interest in many disciplines. With the developed in-house pressurized dynamic flow-through system thermal stability of antibiotics could be systematically investigated in the medium to subcritical water temperature range of 50-250 °C. In general ß-lactams showed a high thermal stability and were completely recovered at temperatures up to 150 °C. Concerning fluoroquinolones, trimethoprim and sulfamethoxazole these were even more resistant to degradation surviving temperatures up to 200 °C. The highest temperature investigated was 250 °C where all antibiotics started to more or less vanish from the system. Doxycycline was the only tetracycline investigated and described an explicit pattern of removal, which most likely involved several mechanisms including binding to metal surfaces at lower temperatures and degradation at higher temperatures. The practical implications for food industry is that few if any of the investigated antibiotics can be destroyed at the temperatures applied during food processing. Neither will the heat generated in manure degradation processes affect the stability of the antibiotics during short time. In the field of extraction using hot pressurized solvents, low recoveries are most likely not caused by compound degradation but rather extraction of unwanted components causing matrix effects during final detection.

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