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Differential heat stability of amphenicols characterized by structural degradation, mass spectrometry and antimicrobial activity

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ABSTRACT

Heat stability of amphenicols and the relationship between structural degradation and antimicrobial activity after heating has not been well investigated. Florfenicol (FF), thiamphenicol (TAP), and chloramphenicol (CAP) were heated at 100 °C in water, salt water, soybean sauce and chicken meat for up to 2 h. Degradation and antimicrobial activity of the compounds was evaluated using capillary electrophoresis (CE) with UV-DAD spectrometry, minimum inhibitory concentration (MIC) assay, and gas chromatography with electron impact ionization mass spectrometry (GC–EI-MS). Heat stability of amphenicols in matrices was ranked as water ≥ salt water > soybean sauce > meat, suggesting that heat degradation of amphenicols was accelerated in soybean sauce and was not protected in meat. Heat stability by drug and matrices was ranked as FF > TAP = CAP in water, FF = TAP > CAP in salt water, TAP ≥ FF = CAP in soybean sauce, and TAP ≥ FF = CAP in meat, indicating differential heat stability of amphenicols among the 3 drugs and in different matrices. In accordance with the less than 20% degradation, the MIC against *Escherichia coli* and *Staphylococcus aureus* did not change after 2 h heating in water. A 5-min heating of amphenicols in water by microwave oven generated comparable percentage degradation to boiling in water bath for 30 min to 1 h. Both CE and GC–MS analysis showed that heating of FF produced TAP but not FF amine as one of its breakdown products. In conclusion, despite close similarity in structure; amphenicols exhibited differential behavior toward heating degradation in solutions and protein matrices. Although higher degradations of amphenicols were observed in soybean sauce and meat, heating treatment may generate product with antimicrobial activity (FF to TAP), therefore, heating of amphenicol residues in food cannot always be assumed safe.

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1. Introduction

The widespread use of antibiotics for therapy, disease prevention and growth promotion in food-producing animals raises many concerns over antibiotic residues in food. Antibiotic residues in food have been linked to allergic reactions in sensitive individuals, spread of antibiotic resistance in microorganisms, and alterations in intestinal microflora [1]. Since most food of animal origin is cooked before consumption, it is of interest to know whether residual antibiotics are diminished by the cooking process. To date, insufficient information exists to determine the risk to the consumer from dietary exposure to antibiotic residues in cooked food.

In general, the temperatures achieved during cooking are assumed to degrade antibiotic residues in food. However, ordinary cooking procedures have been found to be unreliable in degrading or inactivating several commonly used veterinary drugs [2]. Various studies have been performed to examine the heat stabilities of antibiotics. The results suggested that aminoglycosides, quinolones, chloramphenicol, clindamycin, novobiocin, trimethoprim, vancomycin, oxacillin [3], and sulfamethazine [4,5] were classified as heat-stable drugs. Tetracyclines and erythromycin were identified as heat-labile while several β-lactams (penicillin G, ampicillin and amoxicillin), nitrofurantoin, polymyxin B and rifampicin were reported as partially heat-labile [3]. It is important to note that antibiotics of the same class may have different

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heat stabilities depending on the type of matrices and heating treatments involved, as reviewed by Moats [2]. Most heat stability studies have been conducted by evaluating the degradation of parent drugs, however the development of degradation products that may also have antimicrobial activity or potential toxicity has been less thoroughly investigated.

Amphenicol antibiotics, namely chloramphenicol (CAP), thiamphenicol (TAP) and florfenicol (FF) are important veterinary antibiotics with broad-spectrum antimicrobial activities. However, due to potential toxicity [6], CAP has been banned from use in food-producing animals in the United States and other countries. Florfenicol, which has not been associated with toxicity in humans, has largely replaced the use of CAP in food animals. Nevertheless, CAP residues are still being detected in pasteurized milk in Brazil [7] as well as in other food products by monitoring programs. Due to their broad antibacterial spectrum, ready availability and low cost, it remains a possibility that amphenicol residues will continue to be found in food products [8]. Information on heat stability of amphenicols is therefore essential for food safety. Very limited information is available in the literature regarding FF and TAP heat stability during cooking. Heat stability of CAP has been investigated in Mueller Hinton broth (MHB) [3], milk [7] and tissues of shrimp [9], beef [10] and pork [4] with variable conclusions on heat stability. Under environmental conditions, FF [11] and TAP [12] were stable at 25 °C, however photodegradation occurred at differing rates in water under different light sources [12]. In a different study, FF was shown to rapidly degrade to FF amine in the deep sediment of marine environments through a biodegradation process [13].

The purpose of the present study was to evaluate heat stability of amphenicols in liquid and protein matrices using multiple procedures including changes in electropherographic profiles, full-spectrum ultraviolet spectrometry, structural integrity, and antimicrobial activity. To the author's knowledge, the relationship between the structural degradation of amphenicols after heating and their antimicrobial activity has not been well investigated. In addition, whether or not heated amphenicols generate degradation products with potential antimicrobial activity is not clear. Results of this study should facilitate the understanding of the risk factors associated with heating of residual amphenicols in food matrices.

2. Experimental

2.1. Study design

Florfenicol, TAP and CAP were evaluated for structural integrity by heating in water, salt water, soybean sauce, and chicken meat. Thermal treatments were performed at a single temperature (100 °C) for 30 min, 1 h, and 2 h. Heat stability was investigated through evaluation of the qualitative and quantitative electropherographic profiles, UV-DAD spectrometry (200–350 nm), antimicrobial activity against test bacteria *Escherichia coli* (*E. Coli*, ATCC 25922) and *Staphylococcus aureus* (*S. aureus*, ATCC 29213), and gas chromatography with electron impact ionization mass spectrometry (GC–EI-MS). For the purpose of the study, a new peak was defined as a peak in which the area was greater than 10% of the total main peak area (non-heated). The degradation of the drug was evaluated by quantifying the reduction in main peak area as well as the appearance of new peak numbers and areas in respective matrices. Data (presented as mean ± SEM) were evaluated for statistical differences among the 4 matrices and among the 3 drugs in each matrix using analysis of variance (ANOVA, SAS 6.12 for Windows; SAS Institute, Cary, NC, USA). Multiple comparisons were conducted using Duncan's multiple comparison procedure. Statistical differences were accepted at $P < 0.05$.

2.2. Chemicals

The standards for FF, TAP, CAP, and sodium chloride, sodium borate decahydrate and sodium dodecyl sulfate (SDS) were obtained from Sigma–Aldrich (St. Louis, MO, USA). FF amine was a gift from Schering Plough International (Union, NJ, USA). Ethyl acetate was supplied by Mallinckrodt Baker, Inc. (Phillipsburg, NJ, USA) and the acetonitrile, methanol and HPLC grade water were purchased from Tedia Company, Inc. (Airfield, OH, USA). The Kikkoman all-purpose soybean sauce (President Kikkoman Inc., Tainan, Taiwan) was purchased from a local supermarket; the nutritional label indicated 14.4 kcal calories, 1.3 g protein, 2.4 g carbohydrate and 826 mg sodium per serving (15 mL). The major ingredients include sugar, soybean, wheat, sodium chloride, alcohol and artificial flavors.

2.3. Sample preparation

For samples heated in water, salt water and soybean sauce, stock solutions at a concentration of 1 mg/mL were prepared by dissolving each amphenicol in double distilled water (DDW). Working standard solutions at concentrations of 50 and 100 μg/mL for each amphenicol were prepared by appropriate dilution of a diluted stock solutions (200 μg/mL) with DDW or salt water (containing 55 mg/mL NaCl, equivalent to the sodium content in the soybean sauce), or with soybean sauce at 1:1 ratio. For thermal treatments, all working solutions were heated in 10 mL glass vials in a water bath (Hipoint, Kaohsiung, Taiwan) at 100 °C for 30 min, 1 h or 2 h. Stabilities of amphenicols in water were also tested after 5 min heating in an 800 W output microwave oven (Sharp R-250 H, Sharp Thai Co., Ltd., Bangkok, Thailand) setting at P100 (100%) power level. All samples were prepared and analyzed fresh to avoid any possible degradation caused by prolonged storage. For samples in meat, chicken thigh muscle was first blended using a blender (Biospec Products Inc., Bartlesville, OK, Switzerland). Each 1 g of blended meat tissue was supplemented with 50 or 100 μL of amphenicol solution (1 mg/mL) to reach final concentrations of 50 and 100 μg/mL, respectively. One milliliter DDW or salt water was added to each gram of sample and mixed thoroughly using a vortex mixer prior to heating in a water bath for the designated lengths of time. After heating treatments, the samples were added to 9 mL ethyl acetate and vortex-mixed for 5 min. The samples were then centrifuged for 10 min at 3000 rpm (1006 g) before the supernatants were collected and evaporated to dryness in a fume hood under a gentle stream of air. The resulting residues were reconstituted with 1 mL of electrophoretic buffer (see below), filtered through 0.22 μm syringe filters (Millipore, Cork, Ireland) and injected for capillary electrophoresis (CE) analysis. An acetonitrile extraction procedure (1:1 to soybean sauce) was necessary to avoid interfering peaks to FF. The acetonitrile/soybean sauce mixture was mixed, evaporated and reconstituted as described above before CE analysis. Blank samples (soybean sauce and meat not supplemented with amphenicol) and drug standards were also prepared for control purposes. All treatments were carried out at least in triplicates ($n = 3$–5).

2.4. Capillary electrophoresis and gas chromatography–mass spectrometry (GC–MS)

The CE analysis was based on a previously reported method [14]. Briefly, the CE analysis was performed using a Beckman P/ACE 5500 system (Beckman Coulter, Inc., Fullerton, CA, USA), coupled to a photodiode array (PDA) detector, and System Gold® software (Beckman Coulter, Inc.). Separations were carried out in an uncoated fused-silica capillary (Beckman Coulter,

Inc.) with a 75-μm internal diameter, and total length of 47 cm (effective length 40 cm). UV detection was performed at 214 nm with a full scan between 200 and 350 nm. The electrophoretic buffer used was sodium borate (pH 9; 50 mM) supplemented with SDS (25 mM). Samples were injected by applying a voltage of 3 kV for 20 s, and separated on 15 kV at 20 °C. Before each analysis, the capillary was rinsed with 0.1 M HCl for 1 min, water for 2 min, 0.1 M NaOH for 2 min and then 2 min with the electrophoretic buffer. Under these conditions, the limit of quantification for all 3 amphenicols was 2.5 μg/mL with good precision (CV < 1.5%) and accuracy (recovery >99.8%) [14]. The working linear range for the current method for quantification was from 1 to 200 ppm, at which concentration ranges the changes in peak area are proportional to the changes in drug concentrations (R^2 = 0.969, 0.998 and 0.986 for FF, CAP and TAP, respectively).

GC–MS analysis was performed on a Finnigan Trace GC Ultra (Thermo Electron Corporation, Milan, Italy) instrument coupled with a Finnigan Trace DSQ MS detector (Thermo Electron Corporation). Prior to GC–MS analysis, all compounds were derivatized by bis(trimethylsilyl) trifluoroacetamide (BSTFA) with 1% trimethylsilyl chloride (TMCS) purchased from Acros Organics (Springfield, NJ, USA). Analytical conditions were modified from that of Shen and Jiang [15]. Briefly, a ZB-5MS (30 m × 0.25 mm i.d., 0.25 μm) capillary column (Phenomenex Inc., Torrance, CA, USA) was used with helium as the carrier gas at the constant flow rate of 1.0 mL/min. For the separation of amphenicols and FF amine, the oven temperature program was as follows: 100 °C for 1 min, raised to 280 °C at 30 °C/min and held for 8 min. The electron impact ionization (EI) mass spectra were obtained at an electron energy of 70 eV and tuned to perfluorotributylamine (PFTBA). The limit of quantification was below 1 μg/mL [16]. The full scan mass spectra were obtained at a mass-to-charge ratio scan range from 150 to 650 u.

2.5. Minimum inhibitory concentration (MIC) method

The MIC tests were performed based on the reference broth liquid microdilution method described by the Clinical and Laboratory Standards Institute (CLSI), formerly the National Committee for Clinical Laboratory Standards (NCCLS) M31-A3 [17]. Briefly, a serial 2-fold dilution of each amphenicol was made in 96-well flat-bottom microdilution plates (Becton Dickinson Labware, Franklin Lakes, NJ, USA) using sterile MHB (Becton Dickinson, Sparks, MD, USA) to obtain the final drug concentration ranges of 0.05–50 μg/mL. *S. aureus* and *E. coli* purchased from Food Industry Research & Development Institute (Hsinchu, Taiwan) were subcultured for 18 h on tryptic soy agar (Acumedia Manufacturers, Inc., Lansing, MI, USA). Bacterial suspensions at approximately 1×10^8 CFU/mL were prepared in sterile saline (0.9% NaCl) and adjusted to a final inoculum of approximately 1×10^6 CFU/mL. Microdilution plates containing 100 μL of 2-fold serial dilutions of amphenicols in each well were inoculated with 100 μL of the final inoculum yielding a final test concentration for each bacterium of approximately 5×10^5 CFU/mL. Following inoculation, the microdilution plates were incubated at 37 °C in an ambient air incubator and the OD values were determined after 18 h with a spectrophotometer at a wavelength of 590 nm. For each drug and treatment, the experiment was carried out in duplicate wells for 3 plates (n = 3). The MIC was defined as the lowest antimicrobial concentration that completely inhibited bacterial growth. In view of the high salt content and perceivable coloration of soybean sauce that may interfere with the OD reading, MIC studies on salt water and soybean sauce were not performed.

3. Results and discussion

3.1. Heat stability of amphenicols

Traditionally, heat stability of antibiotics is evaluated by either measuring decreasing antimicrobial activity to susceptible microbes or by quantifying the reduction of drug concentrations after heating treatments. Utilizing both criteria to assess chemical integrity and its association with antimicrobial activity is seldom done. In this study, a more complete approach with multiple parameters including the electropherographic profiles, main peak reduction, new peak/peak area production, changes in ultraviolet spectrometry, and qualitative characterizations of fragment ions were utilized to assess the structural degradation of amphenicols after heating treatments for various lengths of time in different solutions/matrices and correlating them to changes in the antimicrobial ability. Thermal treatments at one temperature (100 °C) for multiple time lengths and in multiple matrices were employed to simulate stewing conditions and the studied concentration range allowed the changes in peak area to be linearly proportional to the changes in parent drug concentration. In general, amphenicol degradation was apparent following as little as 30 min of heating and was correlated with the length of heating, implying that longer cooking times (ex. stews) may affect the degree of residual drug present in the sample. The 2-h heating time in this study presented a period long enough to encompass most boiling conditions. After heating treatment, the main peak area of all 3 amphenicols was reduced to different degrees and various numbers of new peaks were produced (Fig. 1). Stability was affected to a greater degree by heating in soybean sauce and in meat versus in water and in salt water. The FF, CAP and TAP main peak was reduced by 32–42%, 40–58%, and 66–82% in meat and by 2–9%, 24–42%, and 43–78% in soybean sauce following 30 min, 1 h and 2 h of heating respectively, compared with a reduction of only 5–20% and 8–21% after 2 h of heating in water and salt water (Fig. 2), respectively. Further analysis indicated that while meat and soybean sauce or water and salt water shared similar pattern in heat degradation, TAP degraded 2–4-fold more in water than in salt water. On the other hand, it was not until after 1 h that amphenicols started to show greater than 10% degradation in soybean sauce. In addition to matrix differences, differential degradation profiles were also discovered among amphenicol analogs. FF generated the highest number of new peaks (3 in water after 2 h) while both TAP and CAP produced at most 1 detectable new peak (Figs. 1 and 2). On the other hand, the percentage increase of new peak areas was significantly higher when drugs were heated in water than in salt water (Fig. 2). Based on the changes in peak number and main/new peak area, heat stability of amphenicols in water was ranked as FF > TAP = CAP and in salt water as FF = TAP > CAP while in soybean sauce and meat, it was TAP ≥ FF = CAP. The information derived from these parameters suggested that even with drugs that are very similar in structure, the degradation pattern upon heating can be very different and can be variable in different solutions, therefore prediction of heat stability based on drug class was not reliable for amphenicols. The chemical structure of FF differs from TAP only by the replacement of the C-3 hydroxyl group (–OH) with a fluorine (F), while the only difference between TAP and CAP is the replacement of a sulfomethyl group ($-SO_2CH_3$) with a nitro ($-NO_2$) group. Yet multiple breakdown products were only evident with FF compared to a single major breakdown product in CAP and TAP. The active fluorine group, which is prone to nucleophilic substitution at elevated energy levels, may explain in part the more active breakdown of FF. To elucidate possible causes of higher degradation associated with the soybean sauce, the effect of salt water was investigated and proved that at equivalent salt content the degradation in salt water was comparable to those

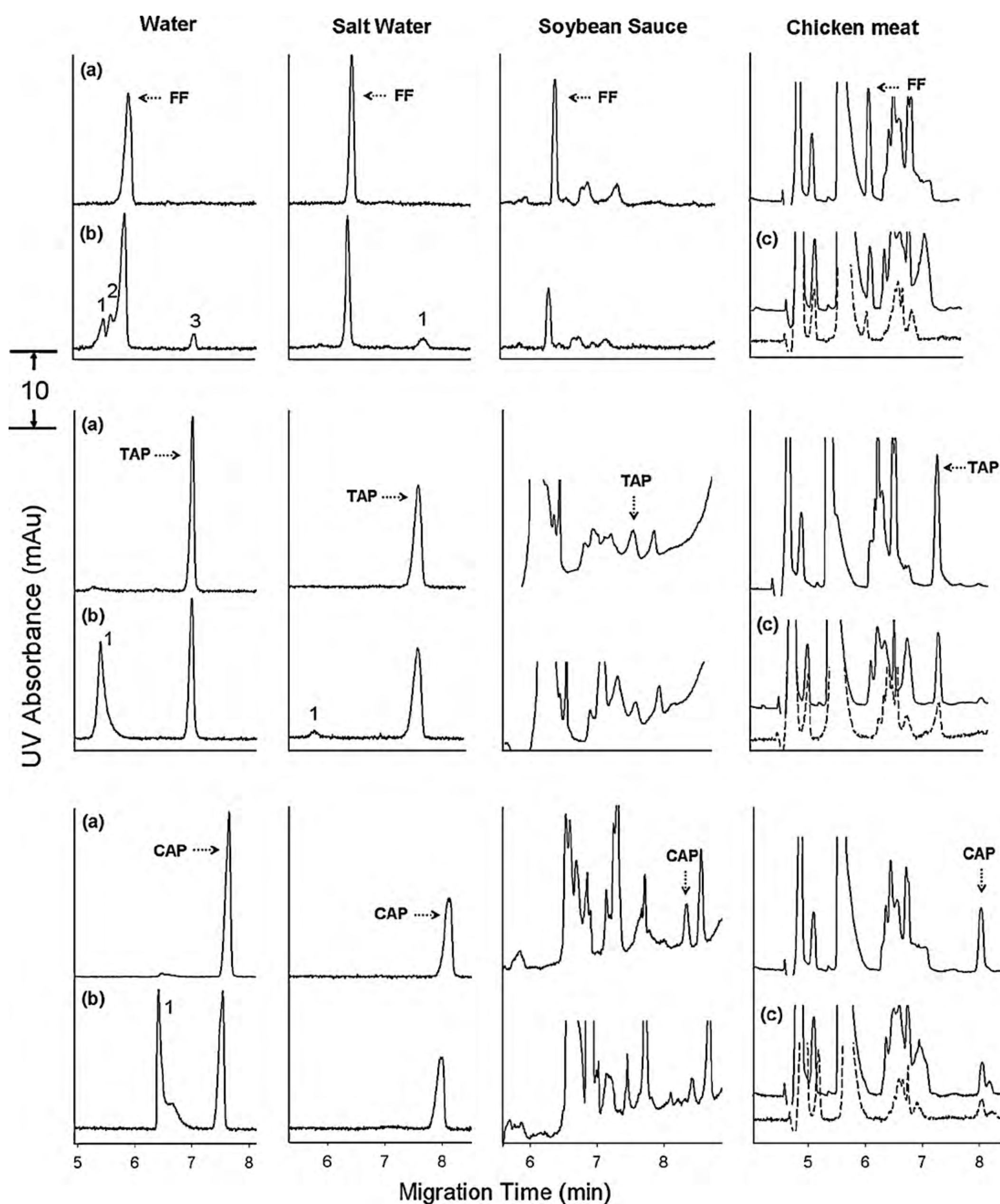

Fig. 1. Typical CE electropherograms of FF, TAP and CAP before (a) and after (b) 2 h of heating (100 °C) in water, salt water (27.5 mg NaCl/mL) and soybean sauce. Heated blank chicken meat spiked with amphenicol (a) and amphenicol-supplemented meat after 1 h of heating (100 °C) (b) is shown on the right column. Dotted line depicts electropherogram after heating of meat in salt water. Numerical numbers indicate the new peaks formed. $n = 3–5$.

in water and was remarkably smaller than that in the soybean sauce, suggesting that higher degradation in the soybean sauce was not directly associated with its salt content or at least not based solely on the salt content. The extent to which the other ingredients in the soybean sauce influence amphenicol degradation remains to be studied, but it is feasible that multiple contributions from more than one ingredient are involved. These results highlight the importance of the other compounds (as stated on the label) to the increased degradation, and indicates that drugs that are conventionally considered heat stable may become highly degradable when cooked with sauces that contain mixtures of salts, food additives and flavors. This information carries very important implications for Asian people and their cooking style because soybean sauce is used extensively in their everyday cooking and is used heavily with brewed or stewed meats. In more western style cooking, salt is often used for seasoning rather than soybean sauce, and it is interesting to note that the presence of salt alone reduced TAP degradation in water (Fig. 2, middle column) but increased FF and CAP degradation in meat (Fig. 1, dotted lines), giving different effects in different matrices. These results again demonstrated the differential heat stability of amphenicol analogs, which showed dependency on matrices and cooking conditions. UV–PDA spectra of the new peaks revealed insignificant changes in spectrum from the original main peak, with only a slight shift of 1–5 nm in the maximum absorption (data not shown) after heating in water and salt water, suggesting that the new degradation products maintained very similar structure with the parent drug. All of the results described above were similar between the tested concentrations (50 or 100 ppm) and at a higher concentration of 200 ppm (data not shown), indicating that drugs degradation is not concentration dependent. Although these concentrations are comparatively higher than possible residue in edible tissue, they demonstrate the

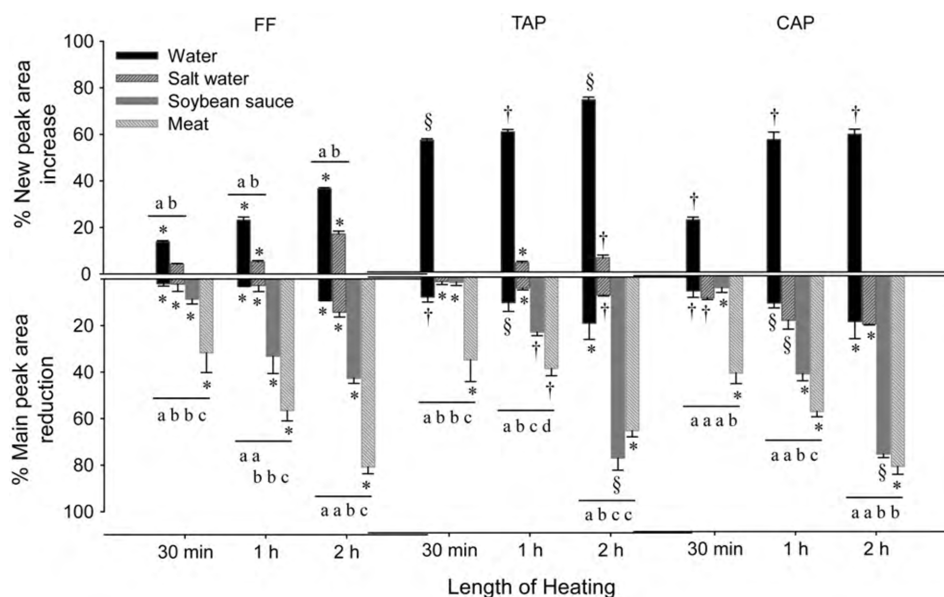

Fig. 2. Percentage main peak area decrease (downward) and percentage new peak area increase (upward) of FF, TAP and CAP after heating (100 °C) in water, salt water (27.5 mg NaCl/mL), soybean sauce and chicken meat. Bars with different letters indicate significant differences among matrices ($P < 0.05$), bars with different symbols (*, † and §) indicate significant differences among amphenicols in the same matrix, $n = 3–5$.

possible breakdown products which are still likely to be produced even at low levels.

One notable result worth emphasizing was that all 3 amphenicols actually degraded more in the presence of meat than in liquid solutions including soybean sauce. Previous study suggested that protein-bound residual drugs were believed to be protected from heat degradation [5], although it has also been postulated that a drug lacking a complex formation with meat matrices will be more vulnerable to heating treatment [18]. In this study, the approximately 9–18-fold higher degradation of FF and 4–6-fold higher degradation of TAP and CAP heated in chicken meat compared with water suggested that the meat matrix may not exhibit a protective effect on all amphenicols. Previous studies had reported that incurred residues of CAP from beef were relatively degraded after cooking [10] while residues in pork were totally degraded at canning [4], further supporting the hypothesis that amphenicols may not be protected in meat. Heating of meat causes inactivation of microbes and denaturation of different proteins as temperature increases. In addition, a decrease in water binding capacity and an increase in pH start to occur between 50 and 65 °C. It is known that the increase in water binding capacity of meat reduces drug degradation [19]. Therefore, it is feasible to suggest that the high degradation of amphenicols in chicken meat might at least partially be attributed to the decrease in the meat's water binding capacity as a consequence of heating. The percentage recoveries of amphenicols at 50 and 100 μg/g in the meat were: 100% and 89% for FF, 100% and 95% for TAP, and 89% and 85% for CAP, respectively.

3.2. Heat stability after microwaving

A smaller scale study investigating heat stability of amphenicols after 5 min of microwaving, another common daily cooking practice, was performed and compared to the boiling of the drugs in water bath. The results suggested that a 5-min heating of amphenicols in water using a microwave oven generated main peak reductions comparable to approximately 30 min to 1 h of heating the drugs in a water bath (Table 1). The decreased main peak produced by microwaving maintained the characteristic spectrum of each amphenicol, indicating the peak represented the parent drug structure. The major difference in degradation patterns between the 2 heating methods was the lack of production of detectable new peaks after microwaving. This may be explained by the fact that microwave heating, in contrast to boiling (or convection heating), cooks or heats food by dielectric heating which uses microwave radiation to heat water and other polarized small molecules. The drugs were degraded by a similar mechanism (boiling) but more rapidly and aided by the dielectric heating of water molecules, thereby explaining the faster but comparable degree of degradation seen with the water bath. At the same time, the degradation products were also subjected to further break down by the facilitated heating process, thus contributing to the chromatographic differences of the 2 heating processes. Nevertheless, the current results show that microwave heating might shorten the time required for breakdown of amphenicols but the degree of degradation was confined within the context of the matrix (water) effect, at least in a short-term heating scenario. This is supported by a recent study in which CAP degradation reached a plateau after 5 min of microwaving [20].

Table 1
Comparison of percentage main peak reduction, percentage new peak increase and number of new peaks produced after heating of amphenicols (100 μg/mL) in water by microwave oven and water bath ($n = 3–5$).

Amphenicols	% main peak reduction	% new peak increase	No. of new peak
FF			
5 min microwave	2.5 ± 0.8	ND	ND
30 min boiling	2.0 ± 0.3	13.8 ± 0.1	2
1 h boiling	3.1 ± 0.1	23.1 ± 0.4	2
TAP			
5 min microwave	6.5 ± 1.7	<5	ND
30 min boiling	8.6 ± 2.1	56.0 ± 0.1	1
1 h boiling	11.0 ± 3.8	60.2 ± 0.2	1
CAP			
5 min microwave	6.7 ± 0.4	<5	ND
30 min boiling	6.5 ± 2.7	22.4 ± 0.5	1
1 h boiling	11.7 ± 2.1	56.9 ± 0.8	1

ND, not detected.

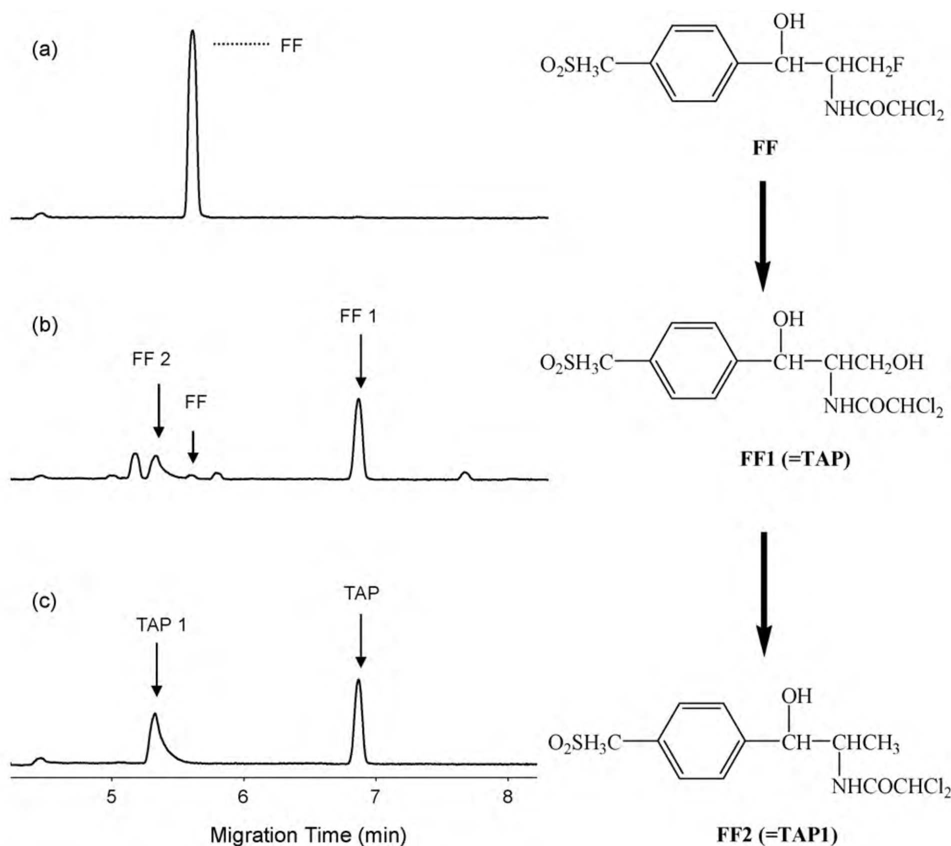

Fig. 3. CE electropherogram of (a) FF before heating, (b) FF after 2 h of heating giving rise to 2 new peaks (FF1 and FF2), and (c) TAP giving rise to 1 new peak (TAP1) after 2 h of heating in soybean sauce (100 °C). The right column shows the structural transformation of FF to TAP.

3.3. Production of TAP after heating of FF

After heating of FF, the CE electropherograms gave rise to one new peak (FF1) which migrated at the same time as TAP peak at 6.9 min. In addition, a second new peak (FF2) migrated at the same time as the new TAP peak (TAP1 at 5.4 min) generated after heating of TAP (Fig. 3). The FF1 and TAP, as well as FF2 and TAP1 showed overlapping UV–PDA spectra (data not shown). These CE results suggested that TAP and TAP1 are likely produced after heating of FF (Fig. 3). Further confirmation by GC–MS revealed that WF3 migrated at the same time and has major mass-to-charge ratios (m/z of 242, 257, 330) identical to that of TAP's (Table 2 and Fig. 4(a and c)), proving TAP was produced from heating of FF in water. TAP was also found to be produced as one of the degradation products of FF after 24 h non-heating exposure in water at 25 °C [11]. This transformation was further examined by GC–MS

Table 2
Qualitative characterization of GC–MS fragments of amphenicols after heating at 100 °C in water and chicken meat (n = 3).

Drugs	New peaks	RT (min)	Relative abundance (%)	m/z of fragment ions and relative abundance (%)
FF		9.6	100	257[a]
Water	WF1	9.8	33	257[a]
	WF2	10.2	8	271(77%), 296[a], 306(36%)
	WF3	10.6	42	242(89%), 257[a], 330(75%)
Chicken	CF1	9.8	24	257[a]
	CF2[b]	10.6	ND	164(76%), 175[a], 188(56%)
	CF3	11.0	10	175(40%), 188(49%), 236[a]
TAP		10.6	100	242(91%), 257[a], 330(78%)
Water	WT1	12.3	18	153[a], 242(72%), 258(56%)
	WT2	10.2	3	271(73%), 296[a], 306(32%)
Chicken	CT1[b]	10.6	ND	164(77%), 175[a], 188(56%)
	CT2	11.4	34	257[a]
CAP		8.8	100	208(41%), 225[a]
Water	WC1	7.7	1	341[a]
	WC2	8.4	3	208(21%), 225(33%), 238[a]
	WC3	9.6	4	153[a], 242(86%)
Chicken	CC1	9.2	34	208(49%), 225[a]

[a] Denotes the base peak (as 100%) in the mass spectrum.
[b] CF2 and CT1 are chicken meat associated products confirmed by heating of bland chicken meat. Relative abundance was not determined (ND).

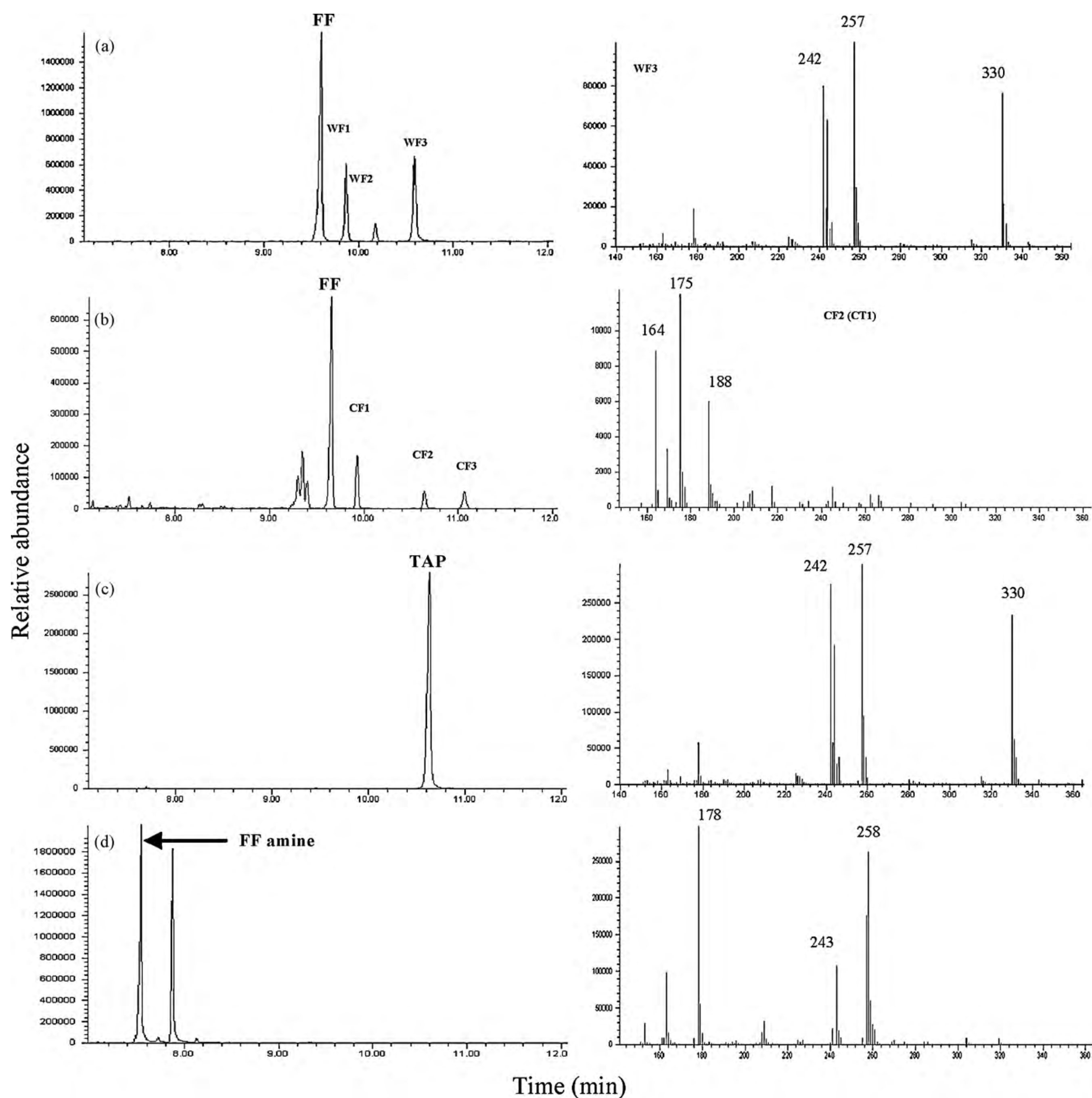

Fig. 4. GC–MS result showing (a) FF new peak after 1 h heating (100 °C) of FF in water (WF1-3) and mass spectra of WF3 (right panel, migration time 10.6 min); (b) 1 h heating (100 °C) of FF in meat (10 ppm, 30 min) and mass spectra of CF2 (right panel, which is the same as CT1); (c) standard TAP migration time and mass spectra; and (d) standard FF amine migration time and mass spectra. Refer to Table 2 for peak legends.

at 10 ppm following 30 min of cooking in chicken meat, however no detectable TAP was produced under these circumstances. This may be due to the relatively low conversion rate of FF (around 4% at 30 min) and quick degradation of TAP (Fig. 2, 40% peak reduction of TAP at 30 min) after possible formation. FF can also rapidly degrade to FF amine in the deep sediment of marine environments through a biodegradation process [13], however, FF amine was not detected after heating of FF in chicken meat for 30 min (Table 2 and Fig. 4(d)), suggesting that heating degradation of amphenicols might either be produced at very low levels or different from its biological metabolism (enzymatic breakdown). The production of TAP upon heating of FF implied that heating of a residual drug might lead to the production of a new compound which may also possess pharmacological properties and possibly accompanying adverse effects, as illustrated in a recent study concerning thermo breakdown of tetracyclines [21]. In this study, the unfavorable structural transformation is relevant to public food safety in that consumers might be exposed not only to residual FF but also to its pharmacologically active degradation products. In this study, even though FF is not used to treat human diseases, its degradation product TAP is, and thus may still be susceptible to the development of microbial resistance through acetyltransferase enzyme inactivation [22]. Findings from the present study suggested that it is unsafe to rely solely on cooking to destroy amphenicol residues in food due to

the possibility of producing pharmacologically active breakdown products.

3.4. Comparison of heat stability to changes in MIC

The original MICs against *E. coli* for non-heated FF, TAP and CAP were 3.125, 50 and 3.125 μg/mL respectively, while the MICs against *S. aureus* were 6.25, 25 and 6.25 μg/mL. After heating of amphenicols in water, the MIC against both *E. coli* and *S. aureus* remain unchanged. Both CE and MIC results clearly demonstrated the marked heat stability of amphenicols in water, which is in agreement with the conclusions of Traub and Leonhard [3] who demonstrated that CAP standard solution did not change its MIC to 5 test bacteria after autoclaving. In this study, comparison of the degree of main peak reduction and the increase in MIC concentration indicated that structural degradation smaller than 20% was not significant enough to elicit changes in antimicrobial activity. However, it should be noted that although amphenicols were considered heat stable in water, their degradation was accompanied by the formation of new components (new peaks), which might possess antimicrobial activity as demonstrated above in the FF degradation. For drugs like amphenicols that are not highly protein bound [23] and highly degraded in the meat (Fig. 2), production of new antimicrobial activity in relation to structural integrity cannot be completely ruled out.

3.5. Qualitative characterization of GC–MS fragments

To complement the data obtained from CE evaluation, thermodegradation of FFs was structurally investigated by GC–MS method using electron impact ionization (GC–EI-MS). The qualitative characterization of FF fragments is shown in Table 2. In comparison to CE/UV detection, the more sensitive GC–MS uncovered a greater number of new peaks of relatively small abundance. Judging by the similarities of fragment ions it was suggested that the mechanistic break down of amphenicols seemed to be similar in the same matrix but different in water than in meat. This was illustrated by the base peak at *m*/*z* 153 and the fragment ion at *m*/*z* 242 in the mass spectra for WT1 and WC3 (TAP and CAP in water) and the base peak at *m*/*z* 175 and the fragment ion at *m*/*z* 188 for CF2/CF3 and CT1 (FF and TAP in chicken meat). Some useful information can be obtained for the closer examination of possible structural transformation after heating. From Table 2, it was clear that WF3 was actually TAP (*m*/*z* 242, 257, and 330). For those with the same base peak ion at *m*/*z* 257, WF1, CF1 and CT2 were most likely the isomers of FF with less structural homology judged by the retention time, as was the case in CC1 and CAP (*m*/*z* 208 and 225). To a certain extent the derived molecular ion of the breakdown compounds could be deduced from the trimethylsilylated amphenicols with various hypothesized breaking point. For example, the peaks with shorter retention time in the chromatogram indicated high polar products, corresponding to more hydroxyl groups in the chemical structure. The ratio between the ion at *m*/*z* 242 and 244 in the mass spectrum indicated two chlorine atoms in the structure from its isotope ratio. The fragment ion at *m*/*z* 257 in the mass spectrum may indicate the formation of $[CH_3SO_2(C_6H_4)CHOSi(CH_3)_3]^+$ ion, while the fragment ion at *m*/*z* 225 indicate the formation of $[NO_2(C_6H_4)CHOSi(CH_3)_3]^+$ ion. The major fragment ions of FF, TAP and CAP, deduced by possible fragmentation pathways have been suggested in He's research [24]. Based on the same concept, the chemical structures of the degradation products in the current study were proposed in Fig. 5 according to their EI mass spectra. The fragment ions were all related to the original drugs, providing qualitative descriptions of the derivative ions after thermodegradation, which was not possible with CE-DAD.

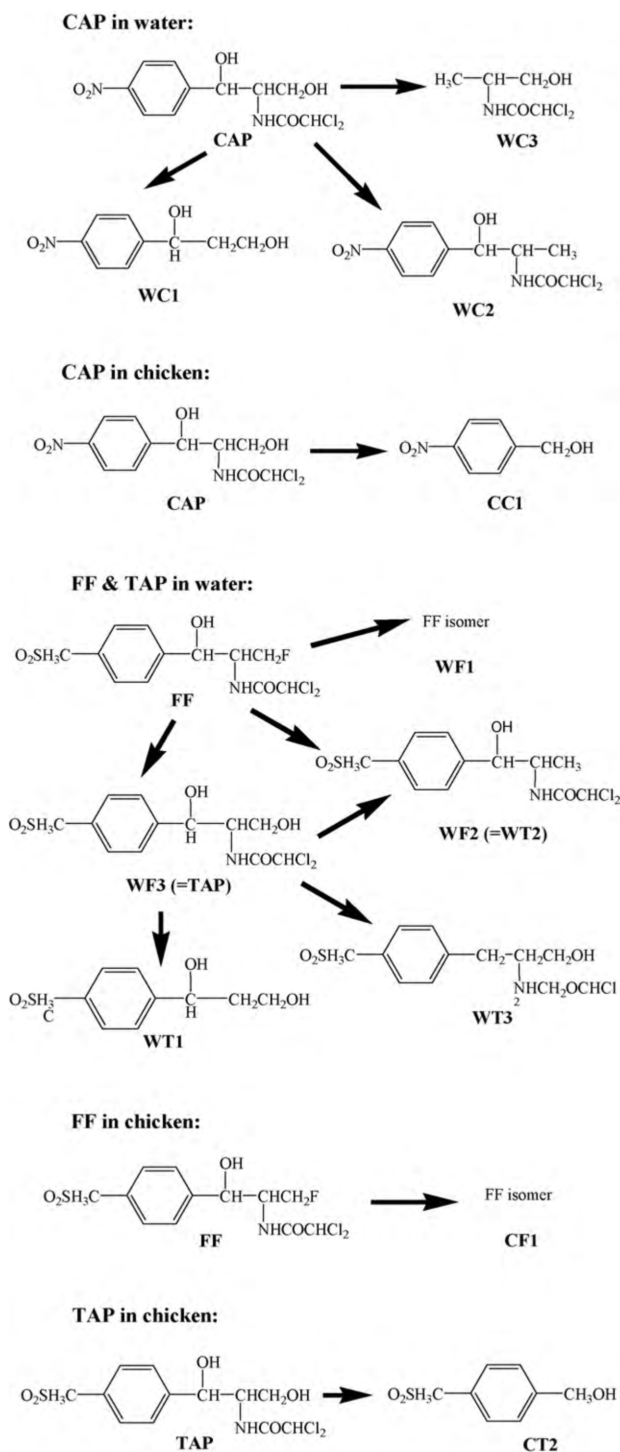

Fig. 5. The proposed chemical structure of degradation products of amphenicols in water and chicken. Refer to Table 2 for abbreviations.

4. Conclusions

Differential heat stability of amphenicols after heating was demonstrated using CE for structural and MIC for functional analysis. Electropherographic profiles and main peak/new peak quantification indicated varied ranking of heat stability of the 3 amphenicols in different solutions/matrices. While identification of all degradation structures was not the main purpose of the current study, qualitative characterization of GC–MS fragments after heating of amphenicols at 100 °C in water and chicken meat

was performed (Table 2 and Fig. 5) and confirmed that TAP but not FF amine can be produced from heating of FF. Amphenicols degraded more when heated in chicken muscle in comparison to heating in water/salt water, challenging the general idea that meat could protect antibiotic residues from heat degradation. Amphenicols also degraded more when heated in soybean sauce, indicating an advantageous effect on reducing drug residues in comparison to use of cooking salt. Moreover, amphenicols degraded faster with microwave heating. This and the above results both carried important implications for Asian and Western cooking styles. The production of TAP as one of the breakdown products of FF implied that water/soy sauce boiling of residual amphenicols in meat may not totally inactivate the antibacterial activity due to possible production of pharmacologically active breakdown products. Therefore, it cannot be assumed that heating of amphenicol residues in food will result in a safe product for human consumption.

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