METABOLOMIC ASSAYS OF AMOXICILLIN, CEPHAPIRIN AND CEFTIOFUR IN CHICKEN MUSCLE. APPLICATION TO TREATED CHICKEN SAMPLES BY LIQUID CHROMATOGRAPHY QUADRUPOLE TIME-OF-FLIGHT MASS SPECTROMETRY.

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ABSTRACT

The aim of this study was to identity metabolites and transformation products (TPs) in chicken muscle from amoxicillin (AMX), cephapirin (PIR) and ceftiofur (TIO), which are antibiotics of the β-lactam family. Liquid chromatography coupled to quadrupole time-of-flight (QqTOF) mass spectrometry was utilized due to its high resolution, high mass accuracy and MS/MS capacity for elemental composition determination and structural elucidation.

Amoxicilloic acid (AMA) and amoxicillin diketopiperazine (DKP) were found as transformation products from AMX. Desacetylcephapirin (DAC) was detected as a metabolite of PIR. Desfuroylceftiofur (DFC) and its conjugated compound with cysteine (DFC-S-Cys) were detected as a result of TIO in contact with chicken muscle tissue. The metabolites and transformation products were also monitored during the in vivo AMX treatment and slaughtering period. It was found that two days were enough to eliminate AMX and associated metabolites/transformation products after the end of administration.

Keywords: Amoxicillin, cephapirin, ceftiofur, chicken muscle, quadrupole time-of-flight mass spectrometry, β-lactams, metabolites, transformation products.

INTRODUCTION

Penicillins and cephalosporins are β-lactamic antibiotics that are widely used in veterinary medicine (for livestock farming and bovine milk production) to prevent and treat bacterial infections (respiratory, urinary and skin infections). They can also be illegally used as growth promoters. The improper use of antibiotics may result in undesirable residues in edible animal tissues, which are a hazard for human health, causing allergic reactions in some individuals and, more importantly, reducing the efficacy of antibiotics to treat diseases, due to the occurrence of new strains of antibiotic-resistant bacteria [1].

In order to regulate the use of these substances, and ensure a high level of protection for human health, the European Union has laid down a set of policies and measures, including the establishment of maximum residue limits (MRLs) for these antibiotics in food. A list of permitted substances with MRLs, is available in Annex I of Commission Regulation 37/2010 [2-3]. In addition, EU Commission Decision 2002/657/EC [4] sets requirements about the performance of analytical methods for determining veterinary drug residues in food [1, 5-8].

However, this legislation generally does not include metabolites or transformation products (TPs) that may be formed by pH shock (from 1.2 in the stomach to 8.0 in the colon), temperature excursion, or interaction with biological substances. TPs need to be identified in order to understand the associated toxicity or harmful effects.

Factors in sample treatment [5,9-13] that could affect the stability of antibiotics and lead to the formation of artificial degradation products may also need to be evaluated to avoid misleading results. Efforts need to be made to minimize degradation during sample preparation.

Liquid chromatography-mass spectrometry (LC-MS) instruments with high resolution and high accuracy, such as liquid chromatography coupled to quadrupole time-of-flight mass spectrometry (LC-QqTOF MS/MS), have been used for the identification or structural characterisation of unknown compounds associated with β-lactam antibiotics [5, 14-20].

In this report, we studied the effect of pH, temperature and incubation time on the formation of metabolites and TPs from amoxicillin, cephapirin and ceftiofur in chicken muscle. The metabolites and TPs formed in AMX-treated chicken were also investigated. Liquid chromatography coupled with electrospray quadrupole time-of-flight mass spectrometry (LC-QqTOF MS/MS) was used.

EXPERIMENTAL

2.1. Reagents and materials

Amoxicillin (AMX) was supplied by Sigma-Aldrich (St. Louis, MO, USA); cephapirin (PIR), ceftiofur (TIO) and piperacillin (PIP, internal standard, IS) were supplied by Fluka (Buchs, Switzerland).

All reagents were of analytical grade. Merck (Darmstadt, Germany) supplied hydrochloric (HCl), acetic (HAc), dichloroacetic and formic (HFor) acids, acetonitrile (MeCN, MS grade), ammonium acetate, ammonia (NH_3), potassium dihydrogenphosphate, methanol (MeOH) and sodium hydroxide. Ultrapure water was generated by a MilliQ system (Millipore, Billerica, MA, USA).

The SPE cartridges used were ENV+ Isolute (3 cm^3/ 200 mg) from Biotage AB (Uppsala, Sweden).

We used 45 μm nylon microcon centrifugal filter membranes (Millipore) to filter the extracts before injection into the chromatographic system.

2.2. Preparation of standard and working solutions

Individual AMX, PIR, TIO and PIP (IS) stock solutions were prepared at a concentration of 100 μg mL^{-1} in MilliQ water. Individual working solution used to spike the chicken muscle sample was prepared by diluting the stock solution to a concentration of 30 μg mL^{-1}.

Buffers between pH 1.5 and 8 were prepared to make the antibiotic working solutions, in order to investigate the generation of TPs at various pH. pH 1.5 was obtained with 0.1% dichloroacetic acid buffer adjusted with 0.1 M HCl, pH 2 and 2.5 buffers were made with 0.1% HFor adjusted with 0.1 M hydrochloric acid; pH 3.0 and 3.5 buffers

with 0.1 % HAc adjusted with 0.1 M HCl; pH 4.5 and 5.5 buffers were made with 0.1% HAc adjusted with 0.1 M NH_3; pH 6.5 and 8.0 buffer were made with 10 mM ammonium acetate adjusted with 0.1 M NH_3.

We adjusted 50 mM phosphate solutions to pH 5.0 and 8.5 with 0.1 M sodium hydroxide. Aqueous/organic solution H_2O:MeCN (9:91; v:v) and organic MeCN:MeOH (50:50; v:v) were also prepared.

2.3. Biological samples

Chicken muscle meat was purchased from retail markets in Barcelona (Spain). The meat was minced, homogenized and stored at -20 ºC until sample preparation.

2.4. Instrumentation

LC-QqTOF analysis was performed on an Agilent 1200 RRLC binary pump chromatographic system (Waldbronn, Germany) with an autosampler injector, a thermostatically controlled column compartment coupled with an hybrid quadrupole time-of-flight QSTAR Elite mass spectrometer from Applied Biosystems (Concord, Ontario, Canada).

Chromatographic separation was achieved on a Luna C18 column (50 x 2.0 mm, 3 µm, 100 Å) from Phenomenex (Torrance, CA, USA).

SPE was carried out on a SUPELCO vacuum manifold, connected to a SUPELCO vacuum tank (Bellefone, PA, USA).

Auxiliary apparatuses were: a CRISON 2002 potentiometer (± 0.1 mV) (Crison S.A., Barcelona, Spain) equipped with a CRISON 5203 combination pH electrode from Orion Research (Boston, MA, USA); a centrifuge 460R from Hettich Zentrifugen (Germany); and a TurboVap LV system from Caliper LifeSciences, with a stream of nitrogen

(Hopkinton, MA, USA). An analytical balance with a precision ±0.1 mg and a vortex-mixer were also used.

2.5. Procedures

2.5.1. Preliminary stability studies

The stability of AMX, PIR and TIO solutions at different pH and repeated freeze-thawing cycles was tested. Stock solutions were diluted in each buffer solution (pH values from 1.5 to 8) to obtain 10 µg mL^{-1} and analysed after 1 and 3 freeze-thawing cycles.

We also evaluated the stability of each antibiotic in aqueous/organic solutions (H_2O:MeCN and MeCN:MeOH) used in the sample treatment, at three different temperatures; 25, 35 and 45ºC.

The influence of pH and incubation time was studied at 4 residence times of 0.3 (20 minutes), 6, 12 and 24 hours within the pH range of 1.5 and 8.

2.5.2. Sample preparation procedures

Spiked chicken muscle samples were prepared by mincing 4 g (± 0.1mg) of blank chicken muscle and mixing with the desired volume of individual working solution (30 µg mL^{-1}) of antibiotics to obtain a final concentration of 1000 µg kg^{-1} for AMX and 500 µg kg^{-1} for PIR and TIO.

For sample extraction, 2 mL of MilliQ water and 20 mL of MeCN were mixed with 4g of muscle sample. The mixture was then shaken for 2 min. followed by centrifugation at 3500 rpm for 5 min.. The supernatant was evaporated in a TurboVap system at 35ºC to remove the organic solvent, followed by an SPE clean up. The ENV+ Isolute cartridge was first activated with 2 mL of MeOH, 2 mL of MilliQ water and 2 mL of 50 mM phosphate buffer at pH 5. The muscle extract was then loaded to the cartridge and

154 washed with 3 mL of hydrogenphosphate buffer (pH 5) and 1 mL of MilliQ water. The
155 targeted analytes were eluted with 4 mL of MeCN:MeOH (50:50; v:v) and dried at 35ºC
156 under nitrogen stream. The residue was then reconstituted in 200 µL of MilliQ water
157 and stored at -80ºC, which was filtered prior to LC-MS injection.

158 ***2.5.3. Chromatography conditions***

159 LC used a binary linear gradient elution at a flow rate of 0.3 mL min^{-1}. Mobile phase A
160 was water containing 0.1% of HFor and mobile phase B was MeCN containing 0.1% of
161 HFor with the following elution profile: 0 to 2.5 min, 1% mobile phase B; 2.5 to 3.5
162 min, mobile phase B increased to 25% and held to 4.5 min; 4.5 to 5 min, mobile phase
163 B increased to 35% and held to 10 min.; 10 to 10.1 min, mobile phase B increased to
164 90%. The column temperature was 20 ºC and the injection volume was 20 µL.

165 ***2.5.4. ESI-QqTOF system***

166 The instrument was calibrated daily with a standard solution of rennin (1 pmol μL^{-1})
167 using characteristic ions *m/z* 879.9723 and 110.0713. Mass spectra were acquired over
168 the *m/z* 100-1100 range at a scan rate of 1s per spectrum. The instrument provided a
169 typical resolution of 10000 at *m/z* 879.9723. To further ensure the mass accuracy,
170 continuous calibration was carried out with phthalate at *m/z* 391.2843 and 149.0233.
171 The ESI-QqTOF parameters were optimized using AMX (3 mg L^{-1} in water), due to its
172 relatively lower response compared with that of PIR and TIO. The turbo ionspray
173 source worked in positive ion and full spectrum mode. The optimized parameters were
174 as follows: declustering potential (DP) 50 V, focusing potential (FP) 200 V, turbo
175 ionspray voltage 5500 V, Q1 transmission window 50.1% for *m/z* 80 and 49.9% for *m/z*
176 190, declustering potential 2 (DP2) 10 V, gas source 1 (GS1) 50 (arbitrary units), gas
177 source 2 (GS2) 50 (arbitrary units), curtain gas (CUR) 50 (arbitrary units), ion release
178 delay (IRD) 6 (arbitrary units), ion release width (IRW) 5 (arbitrary units) and

179 temperature (TEM) 400ºC. A collision energy (CE) of 20 V was applied to obtain
180 MS/MS spectrum for the information dependant acquisition (IDA) and product ion scan
181 (PIS).

182 All the acquisition and data analyses were performed using Analyst QS version 2.0
183 (Applied Biosystems, PE Sciex, Concord, Ontario, Canada).

184

RESULTS AND DISCUSSION

3.1. Stability of AMX, PIR and TIO in solution

According to some reports, AMX has chemical stability issues [10,21-23]. In this investigation, the impacts of pH, freeze-thaw cycles, solvents and evaporation temperature on the stability of AMX, PIR and TIO was studied to provide guidance for sample treatment and give insights into the fate of these antibiotics in metabolic processes.

- *Effect of pH and freeze-thaw cycles*

Stability of the antibiotics was investigated within the pH range close to that of animal digestion. AMX was reported to show chemistry stability problems [10,21-23].

The solutions of individual AMX, PIR and TIO in buffer of pH between 1.5 and 8 at a concentration of 10 mg L^{-1} were used. Samples were frozen at -80ºC for 72 h and then thawed at room temperature for analysis. Figure 1 shows a total ion chromatogram of the AMX samples exhibiting different profiles depending on the pH. Figure 1 also depicts EICs of the molecular ions and MS/MS spectra. AMX became more stable in the least acidic condition. From pH 3 to 8, AMX remained stable (peak 1). The presence of *m/z* 349.0869 that corresponded to a loss of ammonia and a product ion at *m/z* 160.0426 corresponding to subsequent cleavage is common for AMX and other β-lactam antibiotics [24-26]. However, at pH 1.5, AMX was totally degraded to AMX-1 (peak 2) with an ion at *m/z* 384.1224 ($[M+H]^+$, $[C_{16}H_{22}N_3O_6S]^+$). Losses of ammonia, and CO_2 from the protonated molecular ion produced ions at *m/z* 367.0968 and 340.1330. This suggests that AMX-1 has a structure of amoxycilloic acid (AMA) [25-27]. The presence of four chromatographic peaks with the same mass ion of 384.1224 indicates the possible formation of stereoisomers. However, only two of them, AMA

(5S,6R and 5R,6R), were reported [25-27]. At pH 2, AMX was only partially converted to AMA, which also contains stereoisomers but with a different distribution, possibly due to the impact of pH. Only a low level of AMA was formed at pH 2.5. The MS/MS fragment ions observed are listed in Table 1. AMX was also found to react with buffer components (formic acid, acetic acid and NH_3) to form different adducts that exhibit chromatographic peaks at different retention times (peaks 3, 4 and 5) with MS ions at *m/z* 412.1197, 426.1304 and 383.1400 respectively. MS/MS losses of HCOOH, CH_3COOH and NH_3 revealed that they are the corresponding adducts.

LC-MS chromatograms of PIR samples (Figure 2) revealed that PIR is also unstable in acidic media. In the pH range of 1.5 to 2, PIR-1 was observed with an ion at *m/z* 364.0249 ($[M+H]^+$, $[C_{15}H_{14}N_3O_4S_2]^+$) as a TP (Table 1), which is *m/z* 60 units less than the *m/z* of PIR. This indicates that PIR-1 may be generated from PIR by loss of the lateral chain. The MS/MS ions of PIR-1 at *m/z* 226.0255, 152.0175 and 112.0235 are consistent with a low resolution MS/MS, previously described for cephapirin lactone (PLA) in bovine milk [28]. At pH 3 and above, PIR remained stable.

TIO seems to be the most stable one among the three antibiotics studied within the pH range tested. TIO exhibits ions at *m/z* at 524.0354 ($[M+H]^+$) and 241.0408, which corresponds to the fragmentation of the β-lactam ring. At pH 8, TIO-1, a minor degradation product, was observed with an ion at *m/z* 430.0322 ($[C_{14}H_{16}N_5O_5S_3]^+$) and an MS/MS fragmentation ion at *m/z* 241.0410, which is attributed to fragmentation of the β-lactam ring of the parent compound. The mass spectrum for TIO-1 matched desfuroylceftiofur (DFC), which has been obtained in milk [29].

Table 1 shows the parent compounds (AMX, PIR and TIO), TPs, molecular ions and fragmentation ions observed in IDA mode, and the pH at which the TPs were formed.

The number of freeze-thaw cycles could also affect the stability of the antibiotics. Three freeze-thaw cycles were carried out on the antibiotic buffer solutions of different pH. The chromatograms of AMX at pH 1.5 show five peaks of TPs. MS/MS showed that three are AMA stereoisomers, while the other two are new transformation product isomers of AMX-2, which exhibited ion at *m/z* 340.1338 ($[M+H]^+$, $[C_{15}H_{22}N_3O_4S]^+$). MS/MS ions were found at *m/z* 323.1076 corresponding to the loss of NH_3, *m/z* 189.0706 via fragmentation of the amide group, and *m/z* 160.0433 generated by the cleavage of the five membered thiazolidine ring from the molecular ion. This suggests that AMX-2 matches the structure of amoxicillin penilloic acid (AMP) [26]. The results are shown in Table 1.

After three freeze-thaw cycles, PIR generated more new TPs: PIR-2, PIR-3 and PIR-4, in addition to PLA. The LC-MS chromatograms are presented in Figure 2. PIR-2 was observed in the whole pH range with an ion at *m/z* 382.0539 ($[M+H]^+$, $[C_{15}H_{16}N_3O_5S_2]^+$). MS/MS fragmentation of the ion of $[M+H]^+$ gave the product ions as shown in Table 1. The MS/MS spectrum and fragmentation pathway are presented in Figure 3A. PIR-2 matched the structure of desacetylcephapirin (DAC). PIR-3 was also observed in the whole pH range, with an ion at *m/z* 366.0584 ($[M+H]^+$, $[C_{15}H_{16}N_3O_4S_2]^+$). MS/MS fragmentation ions are shown in Table 1. The ion at *m/z* 209.0335 is generated by cleavage of the β-lactam group, while cleavage of the β-lactam and the six-membered ring gave ions at *m/z* 253.0136. The loss of CO from the ion at *m/z* 253.0136 produced the ion at *m/z* 226.0247. PIR-3 has the structure of dihydrogenated PLA (diH-PLA). PIR-4 only appeared at pH below 3. PIR-4 has the same molecular ion as that of PIR-2, but a different mass spectral pattern (Figure 3). MS/MS spectra of PIR-4 are shown in Figure 3B, with a proposed structure that

matches that of hydrolysed PLA (Hydro-PLA). No new TPs were observed from TIO after three freeze-thaw cycles.

-Effect of solvents and evaporation temperature

We used a procedure reported in literature [30] with the mixture of MeCN:H_2O and MeCN:MeOH to extract the antibiotics from biomatrix. Accordingly, stability of the antibiotics in MeCN:MeOH (50:50; v:v) and H_2O:MeCN (9:91;v:v) were evaluated with evaporation temperatures of 25, 35 and 45ºC. MeCN:MeOH (50:50; v:v) at 35ºC was found to be the best combination. In H_2O:MeCN (9:91;v:v) especially at higher evaporation temperature, AMX showed degradation. In addition to AMA, another degradation product AMX-3 was detected with *m/z* at 366.1143 ($[M+H]^+$, $[C_{16}H_{20}N_3O_5S]^+$) . The MS/MS ions are shown in Table 1. The ions at *m/z* 207.0783 and 160.0447 correspond to fragments via cleavage of the five-membered thiazolidine ring. AMX-3 matched the structure of amoxicillin diketopiperazine (DKP). Figure 4A shows the abundance of AMX and its TPs at various temperatures. AMX decreased while AMA and DKP increased especially at high temperatures. Figure 4B shows the abundance of PIR and DAC at the evaluated temperatures. Formation of DAC was observed. However, the level of DAC did not change substantially by increasing the temperature, although the PIR signal decreased significantly, which may be due to other unknown side reactions.

Figure 4C shows the change in abundance of TIO. Although the TIO signal decreased at higher temperatures no TPs were detected.

The degradation of antibiotics may explain the low recovery values reported in the literature for the determination of AMX and PIR in food of animal origin [9,30]. In this

investigation, 35 ºC was selected as the most suitable extract evaporation temperature, to decrease the evaporation time without significant degradation of the antibiotics.

3.2. Optimisation on biological samples

- *Impact of the pH of SPE eluent on recovery*

The pH of the SPE eluent had a significant impact on the recovery of the antibiotics, particularly AMX, which had a recovery of 13% at pH 8.5, but 46% at pH 5.0. At pH 5.0, PIR and TIO had recoveries of 94% and 72%, respectively. Accordingly, we selected a pH of 5.0 for the eluent, which was adjusted using phosphate buffer for the biological sample analysis.

- *Effect of pH and incubation time on stability*

The effect of pH and the incubation time of the antibiotics with the chicken muscle matrix was studied. Four pH (1.5, 2.0, 6.5, 8.0) with four incubation time points (0.3, 6, 12, 24 hr) were evaluated. For AMX during an incubation time of 6 hr or less at all of pH tested, only a low level of DKP was detected, along with AMX as a major peak. Neither AMP nor AMA were detected under these conditions. A slight splitting of the AMX EIC peak was observed, possibly due to a slow kinetic isomerisation process. After 12 hr, the intensity of the AMX peak decreased, with no further increase in DKP. After 24 hr, AMX completely disappeared. AMA and AMP were not observed, while a new compound (AMX-4) was detected with *m/z* at 252.1224 ($[M+H]^+$, Table 1). The concentration changes of AMX and AMX-4 with incubation time at pH 2 are shown in Figure 5A. Similar behaviour was also observed at pH 1.5, 6.5 and 8. The structure of AMX-4 remains to be determined.

PIR became much less stable when it was incubated with chicken muscle. Even at 0.3 hr, a high level of DAC was detected as the most abundant compound in all samples, independent of pH. DAC was observed as a result of interaction between PIR and solvents, but incubation with chicken muscle facilitated its formation. Splitting in the DAC peak that increased with time indicated potential isomerisation. PLA or its derivatives Hydro-PLA and diH-PLA were not observed. At 6 hr or more, PIR disappeared in almost all samples with DAC as the principal species. No new TPs were detected.

TIO is the most stable of the three antibiotics. However, the incubation of TIO with chicken muscle tissue facilitated the formation of DFC. Figure 5B shows the concentration change of TIO with time at pH 8. At 0.3 hr, a low level of DFC was detected. At 6 hr, the level of TIO decreased with a concomitant increase in DFC. However, at 12 hr TIO still decreased, but DFC was lower too. A new compound, TIO-2, with an ion at *m/z* 549.0314 ($[M+H]^+$, $[C_{17}H_{20}O_7N_6S_4]^+$) was detected in all of the samples. The change of TIO-2 over time is presented in Figure 5B. At 24 hr, both TIO and TIO-2 decreased while DFC showed a constant level. Similar phenomena were observed at all of the pH tested. This suggests that TIO-2 may be an adduct of DFC with cysteine (DFC-S-Cys), which is still a TP of TIO. The suggested structure of TIO-2 is shown in Table 1, where the associated mass errors were below 5 ppm in all compounds, except for AMX-3 and TIO-2, in which the errors were slightly higher, possibly due to the low concentration.

3.3. Application to treated chicken

The established methodology was applied to the analysis of muscle samples from chickens that had been treated with AMX. The treatment involved a daily dose of 14 mg

331 kg^{-1} for 4 days. The chickens were slaughtered on the third day of treatment and 48 hr
332 after the 4 day treatment. The muscle samples were obtained and stored at -80ºC prior to
333 analysis. Blank control samples were obtained from untreated chicken. AMX was
334 detected in the 3 day samples, but not AMA or DKP. A new compound was detected,
335 AMX-5, with an ion *m/z* 209.0932 ($[M+H]^+$, $[C_{10}H_{13}O_3N_2]^+$). The MS/MS fragment ion
336 at *m/z* 192.0663 may be due to the loss of NH_3. AMX-5 matched the structure of
337 amoxicillin aldehyde (AMX-ALD), as presented in Table 1. In the samples taken 48 hr
338 after 4 day treatment, none of the AMX, other metabolites or TPs were detected. This
339 indicates that 48 hr after the end of AMX treatment was long enough to metabolize and
340 eliminate administered AMX. After storage for 10 months at -80ºC, no AMX or TPs
341 were detected in any of samples, including the three day samples.
342

CONCLUSIONS

Solution stability studies with a pH range between 1.5 and 8.0 revealed that AMX and PIR are unstable in strongly acidic media, whereas TIO only suffered a partial degradation at pH 8. AMA, PLA and DFC were the main TPs from AMX, PIR and TIO respectively. New TPs were observed after multi-freeze-thaw cycles of the solutions containing the antibiotics, including AMP from AMX and DAC, diH-PLA and Hydro-PLA from PIR. Incubation of AMX, PIR, and TIO with chicken muscle matrix for different times (0.3 hr, 6 hr, 12 hr and 24 hr) at different pH (1.5, 2.0, 6.5 and 8.0) were performed. DKP, AMX-4, DAC, DFC, and an adduct of DFC with cysteine were observed as TPs. In the muscle samples from chicken treated in vivo with AMX for 4 days, AMX-ALD was observed as a new TP. No AMX residues or any TPs were detected in the samples 48 hr after AMX treatment.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the financial support of the Spanish Ministry of Economy and Competitiveness (Project CTQ2010-19044/BQU). M.P.H. would like to thank the University of Barcelona for her position as *Investigador Postdoctoral BRD.* We also wish to acknowledge M. Rey and A. Villa, from PONDEX S.A. poultry farm, Juneda (Lleida), for their kind donation of medicated chicken samples.

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467

FIGURE CAPTIONS

Figure 1. Effect of pH on the formation of transformation products of AMOX. EIC and MS/MS spectra of the observed compounds.

Figure 2. Effect of pH and freeze-thaw cycles on the formation of transformation products of PIR. EIC and MS/MS spectra of the observed compounds.

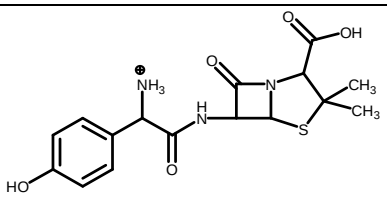
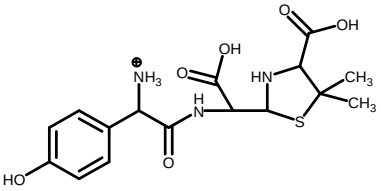
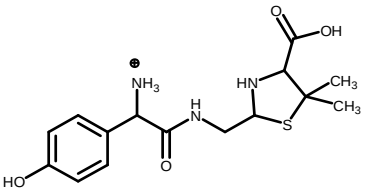
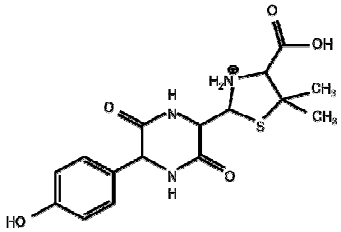
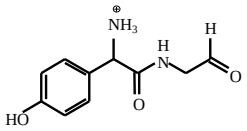
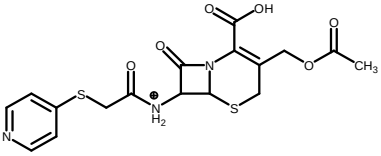
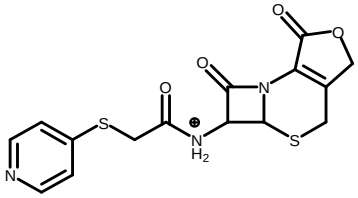
Figure 3.Comparison between the mass spectra fragmentation pattern of PIR-2 and PIR-4.

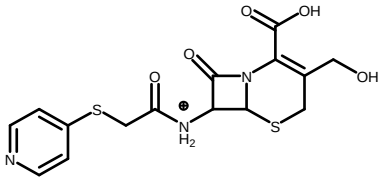
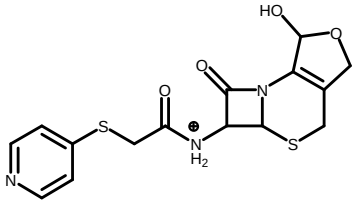
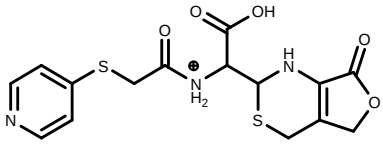
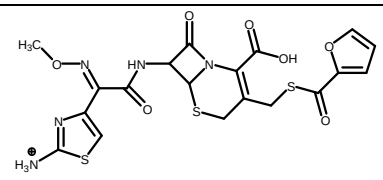
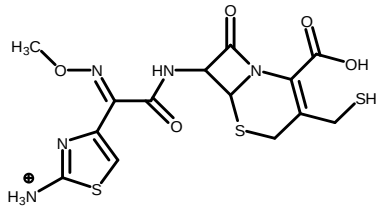
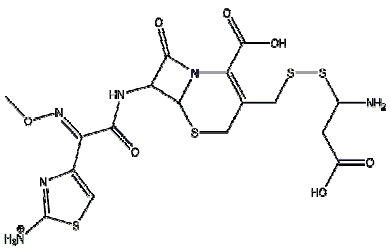
Figure 4. Effect of aqueous/organic mixture H_2O:MeCN (9:91;v:v) and temperature on A) AMX, B) PIR, C) TIO and the corresponding TPs.

Figure 5. Effect of the contact time between antibiotics and matrix on the formation of TPs. A) AMX at pH 2. B) TIO at pH 8.

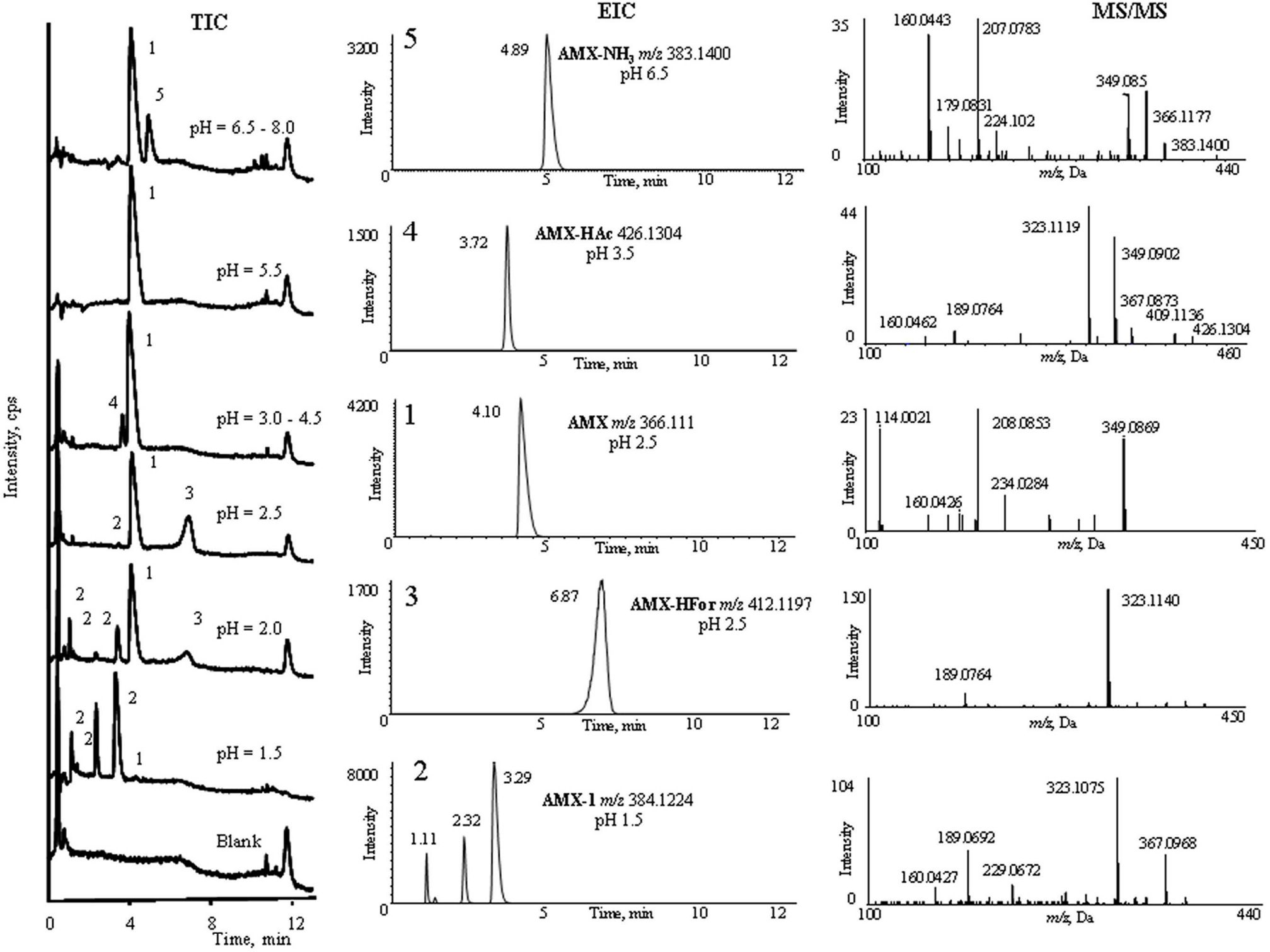
Symbols:

Table 1. Transformation products and metabolites found in pH, freeze-thaw (f-t) and biological sample studies.

Compound	Conditions	$[M+H]^+$ exp	Fragments	Suggested structure	Suggested name[a]	$[M+H]^+$ theo	Error (ppm)
AMX	pH 1.5-8.0	366.1134 $[C_{16}H_{20}N_3O_5S]^+$	349.0869 $[C_{16}H_{17}N_2O_5S]^+$ 208.0853 $[C_{10}H_{12}N_2O_3]^+$ 160.0426 $[C_6H_{10}NO_2S]^+$		AMX	366.1118	4.3
AMX-1	pH 1.5-2.5	384.1222 $[C_{16}H_{22}N_3O_6S]^+$	367.0968 $[C_{16}H_{19}N_2O_6S]^+$ 340.1330 $[C_{15}H_{22}N_3O_4S]^+$ 323.1075 $[C_{15}H_{19}N_2O_4S]^+$ 189.0692 $[C_7H_{13}N_2O_2S]^+$ 160.0427 $[C_6H_{10}NO_2S]^+$		AMA	384.1224	-0.5
AMX-2	pH 1.5 3 f-t cycles	340.1338 $[C_{15}H_{22}N_3O_4S]^+$	323.1076 $[C_{15}H_{19}N_2O_4S]^+$ 235.0755 $[C_8H_{15}N_2O_4S]^+$ 189.0706 $C_7H_{13}N_2O_2S]^+$ 160.0433 $[C_6H_{10}NO_2S]^+$		AMP	340.1326	+3.5
AMX-3	Aqueous-organic solvent- Contact with biological matrix	366.1143 $[C_{16}H_{20}N_3O_5S]^+$	207.0783 $[C_{10}H_{11}N_2O_3]^+$ 160.0447 $[C_6H_{10}NO_2S]^+$		DKP	366.1118	+6.8
AMX-4	Contact with biological matrix	252.1224	206.1171 135.0677	n.a.	n.a.	n.a.	n.a.
AMX-5	Treated chicken	209.0932 $[C_{10}H_{13}O_3N_2]^+$	192.0663 $[C_{10}H_{10}O_3N]^+$		AMX-ALD	209.0921	+5.4
PIR	pH 1.5-8.0	424.0639 $[C_{17}H_{18}N_3O_6S_2]^+$	364.0422 $[C_{15}H_{14}N_3O_4S_2]^+$ 320.0530 $[C_{14}H_{14}N_3O_2S_2]^+$ 292.0591 $[C_{13}H_{14}N_3OS_2]^+$		PIR	424.0632	+1.7
PIR-1	pH 1.5-2.0	364.0249 $[C_{15}H_{14}N_3O_4S_2]^+$	226.0255 $[C_9H_{10}N_2OS_2]^+\cdot$ 152.0175 $[C_7H_6NOS]^+$ 112.0235 $[C_5H_6NS]^+$		PLA	364.0420	+2.4

Compound	Conditions	$[M+H]^+$ exp	Fragments	Suggested structure	Suggested name[a]	$[M+H]^+$ theo	Error (ppm)
PIR-2	pH 1.5-8.0 3 f-t cycles-Contact time with biological matrix	382.0539 $[C_{15}H_{16}N_3O_5S_2]^+$	364.0271 $[C_{15}H_{14}N_3O_4S_2]^+$ 320.0486 $[C_{14}H_{14}N_3O_2S_2]^+$ 292.0578 $[C_{13}H_{14}N_3OS_2]^+$ 253.0087 $[C_{10}H_9N_2O_2S_2]^+$ 226.0235 $[C_9H_{10}N_2OS_2]^+$ 152.0157 $[C_7H_6NOS]^+$ 112.0227 $[C_5H_6NS]^+$		DAC	382.0526	+3.4
PIR-3	pH 1.5-8.0 3 f-t cycles	366.0584 $[C_{15}H_{16}N_3O_4S_2]^+$	253.0136 $[C_{10}H_9N_2O_2S_2]^+$ 226.0247 $[C_9H_{10}N_2OS_2]^+$ 209.0335 $[C_9H_9N_2O_2S]^+$ 152.0176 $[C_7H_6NOS]^+$ 112.0228 $[C_5H_6NS]^+$		diH-PLA	366.0577	+1.9
PIR-4	pH 1.5-2.5 3 f-t cycles	382.0546 $[C_{15}H_{16}N_3O_5S_2]^+$	338.0657 $[C_{14}H_{16}N_3O_3S_2]^+$ 294.0548 $[C_{12}H_{12}N_3O_4S]^+$ 227.0358 $[C_9H_{11}N_2O_3S]^+$ 152.0183 $[C_7H_6NOS]^+$ 126.0381 $[C_6H_8NS]^+$ 112.0239 $[C_5H_6NS]^+$		Hydro-PLA	382.0526	+5.2
TIO	pH 1.5-8.0 3 f-t cycles	524.0354 $[C_{19}H_{18}N_5O_7S_3]^+$	241.0408 $[C_8H_9N_4O_3S]^+$		TIO	524.0363	-1.7
TIO-1	pH 8.0-Contact with biological matrix	430.0322 $[C_{14}H_{16}N_5O_5S_3]^+$	241.0410 $[C_8H_9N_4O_3S]^+$		DFC	430.0308	+3.2
TIO-2	Contact with biological matrix	549.0314 $[C_{17}H_{21}N_6O_7S_4]$	n.a.		DFC-S-Cys	549.0349	-6.4

([a]) Key names: AMX, Amoxicillin; AMA, Amoxicilloic acid; AMP, Amoxicillin penilloic acid; DKP, Amoxicillin diketopiperazine; AMX-ALD, Amoxicillin aldehyde; PIR, Cephapirin; PLA, Cephapirin lactone; DAC, Desacetilcephapirin; diH-PLA, Hidrogenated cephapirin lactone; Hydro-PLA, Hydrolized cephapirin lactone; TIO, Ceftiofur; DFC, Desfuroylceftiofur; DFC-S-Cys, Desfuroylceftiofur-S-cysteine; n.a., non-assigned.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EIC
MS/MS
Intensity, cps
Time, min
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pH = 5.5
pH = 3.0 - 4.5
pH = 2.5
pH = 2.0
pH = 1.5
Blank
5
AMX-NH3 m/z 383.1400
pH 6.5
4.89
4
AMX-HAc 426.1304
pH 3.5
3.72
1
AMX m/z 366.111
pH 2.5
4.10
3
AMX-HFor m/z 412.1197
pH 2.5
6.87
2
AMX-1 m/z 384.1224
pH 1.5
3.29
2.32
1.11
160.0443
207.0783
349.085
179.0831
224.102
366.1177
383.1400
323.1119
349.0902
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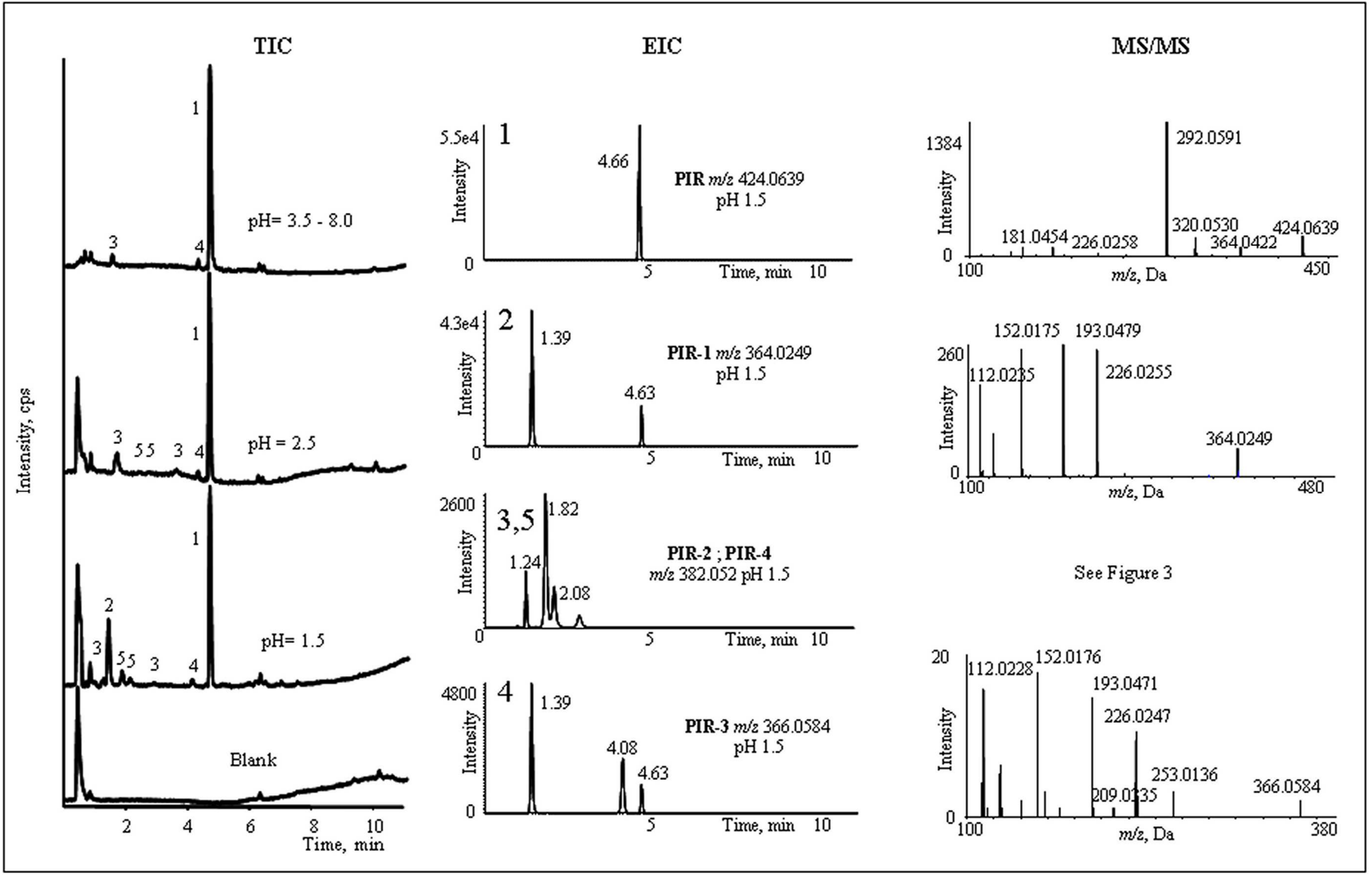
m/z, Da

Figure 1.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EIC
MS/MS
Intensity, cps
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pH = 2.5
pH= 1.5
Blank
Time, min
Intensity
Time, min
PIR m/z 424.0639
pH 1.5
PIR-1 m/z 364.0249
pH 1.5
PIR-2 ; PIR-4
m/z 382.052 pH 1.5
PIR-3 m/z 366.0584
pH 1.5
m/z, Da
See Figure 3

Figure 2.

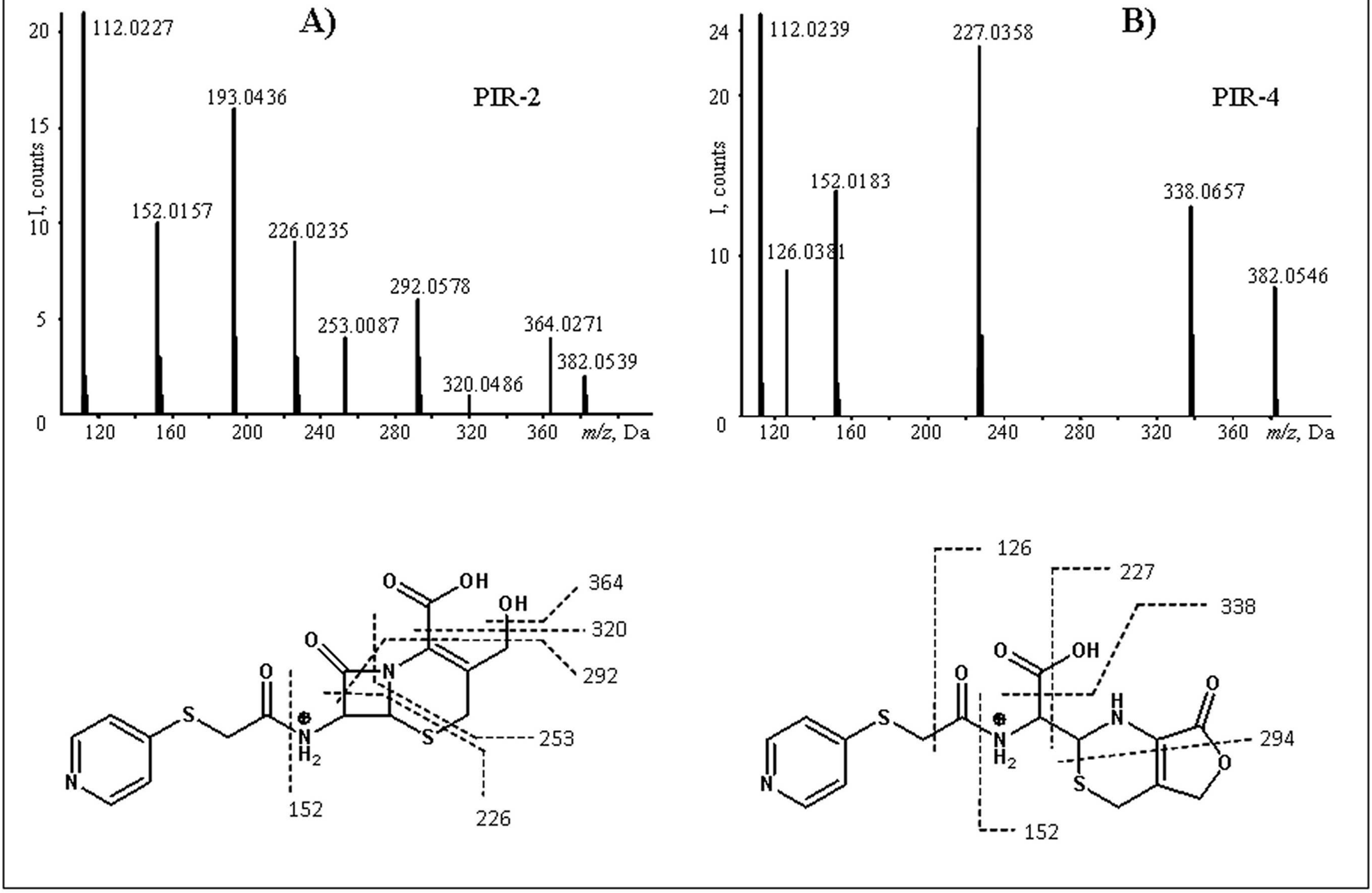

A)
PIR-2
I, counts
m/z, Da
112.0227
193.0436
152.0157
226.0235
292.0578
253.0087
364.0271
382.0539
320.0486
B)
PIR-4
112.0239
227.0358
152.0183
338.0657
126.0381
382.0546
364
320
292
253
152
226
126
227
338
294
152

Figure 3.

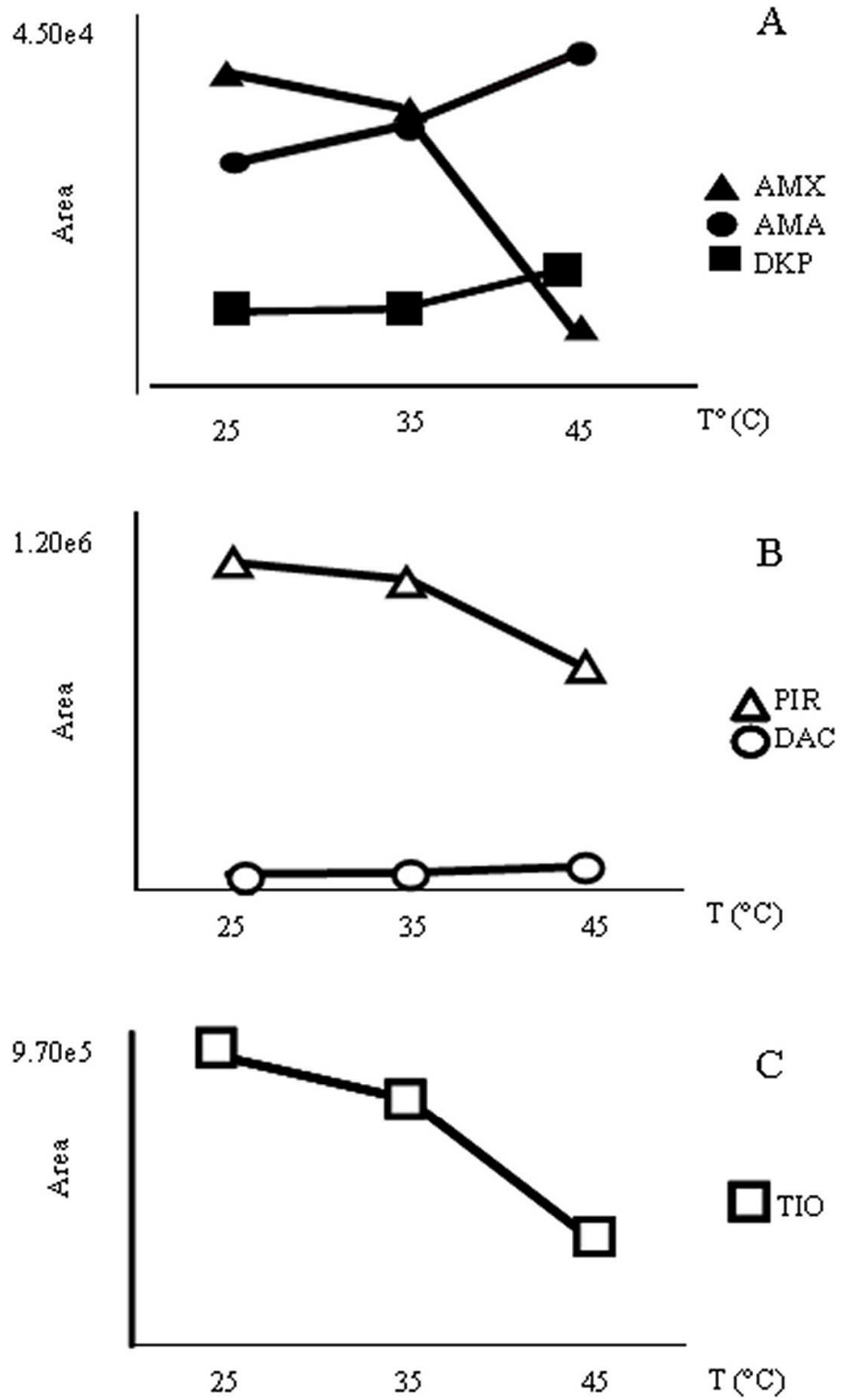
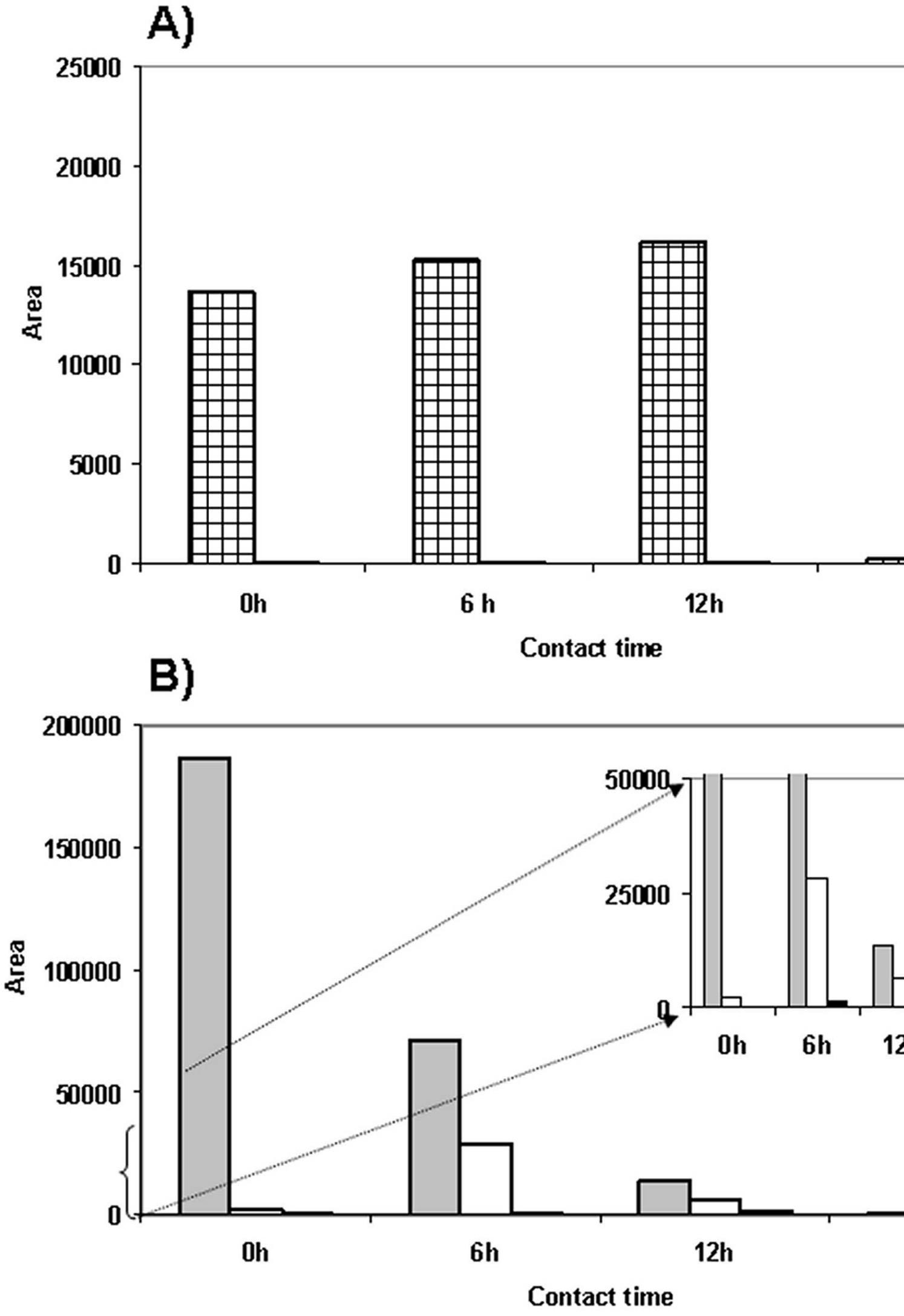

4.50e4
Area
A
AMX
AMA
DKP
25
35
45
T° (C)
1.20e6
Area
B
PIR
DAC
25
35
45
T (°C)
9.70e5
Area
C
TIO
25
35
45
T (°C)

Figure 4.


A)
25000
20000
15000
10000
5000
0
Area
0h
6 h
12h
Contact time
B)
200000
150000
100000
50000
0
Area
50000
25000
0
0h
6h
12h
Contact time