High-resolution mass spectrometry applied to the study of metabolome modifications in various chicken tissues after amoxicillin administration

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Abstract

The performance of high resolution accurate mass spectrometry (HRMS) operating in full scan MS mode was investigated for the quantitative determination of amoxicillin (AMX) as well as qualitative analysis of metabolomic profiles in tissues of medicated chickens. The metabolomic approach was exploited to compile analytical information on changes in the metabolome of muscle, kidney and liver from chickens subjected to a pharmacological program with AMX. Data consisting of *m/z* features taken throughout the entire chromatogram were extracted and filtered to be treated by Principal Component Analysis. As a result, it was found that medicated and non-treated animals were clearly clustered in distinct groups. Besides, the multivariate analysis revealed some relevant mass features contributing to this separation. In this context, recognizing those potential markers of each chicken class was a priority research for both metabolite identification and, obviously, evaluation of food quality and health effects associated to food consumption.

Key Words: Amoxicillin, Linear Trap Quadrupole-Orbitrap mass spectrometry, metabolomics, food biomarkers, multivariate analysis.

1. Introduction

Poultry are one of the most consumed foods by humans and their intakes have been positively associated with some healthy effects because of their dietary relevance. Prior to commercialization, comprehensive quality controls of poultry products are necessary to ensure a high level of protection of human health. For this purpose, regulation concerning the control of food from animal origin is enshrined in the Annex I of Commission Regulation 37/2010 (Commission Regulation (EU) No 37/2010, 2010). In the cited text, legislated MRLs of antibiotics allowed for veterinary use are given, including those of β-lactamic drugs.

Nowadays, there is an increasing interest in monitoring regulated compounds and their metabolites. However, beyond the quantification of exogenous components, researchers have been pointed out the need of studying the influence of such xenobiotics on the endogenous metabolism from the evaluation of changes in metabolite levels (e.g., up- and down-regulations). As a result, metabolic modifications generated “in vivo” might be of great interest in the research of new potentially toxic or healthy compounds and conclusions extracted can be applied to further studies on food regulations (García-Reyes, Hernando, Molina-Díaz, & Fernández-Alba, 2007). At this point, considering the scarce information about metabolomic alteration caused to the use of antibiotics in veterinary and human medicine, thorough studies to assess possible biomarkers of the pharmaceutical treatments are increasingly demanded.

In this work, the metabolic profile of amoxicillin (AMX) has been studied. AMX is a penicillin drug sometimes administered to farm animals due to its high antimicrobial activity. As a result, AMX might be detected in biological fluids and tissues of animals subjected to therapeutic treatment (De Baere, Cherlet, Baert, & De Backer, 2002;

Reyns, De Boever, Schauvliege, Gasthuys, Meissonnier, Oswald, De Backer, & Croubels, 2009).

High-resolution mass spectrometry (HR-MS) has become the current approach of choice to face some challenges raised in metabolomic studies. In particular, mass spectrometry time-of-flight (MS-TOF), and more recently, linear ion trap quadrupole-Orbitrap MS (LQT- Orbitrap MS), both coupled to liquid chromatography, have proved their excellent performances for metabolomic research (Berendsen, Gerritsen, Wegh, Lameris, Van Sebille, Stolker, & Nielen, 2013; Bousova, Senyuva, & Mittendorf, 2013; Hurtaud-Pessel, Jagadeshwar-Reddy, & Verdon, 2011; Szultka, Krzeminski, Szeliga, Jackowski, & Buszewski, 2013; Zubarev, & Makarov, 2013). Qualitative analysis corresponding to exact mass measurements and elemental composition assignment are fundamental for a more feasible characterization of small pharmacologically active substances (Hermo, Gómez-Rodríguez, Barbosa, & Barrón, 2013; Pérez-Parada, Agüera, Gómez-Ramos, García-Reyes, Heinzen, & Fernández-Alba, 2011). In parallel, MS^n experiments have been exploited successfully to confirm fragmentation routes and to elucidate of structures of target and unknown compounds (Nägele, & Moritz, 2005). However, the assessment of metabolic changes in biological matrices is a complex task, and full scan chromatograms may result in an excellent source of high quality data to evaluate variations in the chemical composition in a comprehensive manner without losing statistically significant information. Nowadays, the most used strategy for data treatment relies on Principal Component Analysis (PCA), Partial Least Squares Discriminant Analysis (PLS-DA) and related methods (Marquez, Albertí, Salvà, Saurina, & Sentellas, 2012). Such chemometric methods allow noise filtering and the concentration of information into a reduced number of latent variables. Raw data from HPLC-MS measurement of samples is taken to construct a data matrix focused on the

differentiation of positive and blank samples. As a result, PCA-based methods have demonstrated to be highly efficient to extract and visualize the useful information using, for instance, scatter plots of samples (scores plot) and variables (loadings plot) on the principal components (PCs) (Serrano-Lourido, Saurina, Hernández-Cassou, & Checa, 2012). The most discriminat MS features, characterized by their retention time and *m/z* values, may result in chemical markers to define the different categories or classes of samples.

One of the aims of this study was to improve the AMX detection in different biological tissues using LTQ-Orbitrap mass spectrometry, to quantify the active compound in several positive chicken muscle, liver and kidney samples from animals medicated with AMX. Besides, multivariate analysis with PCA was exploited to study the distribution of samples and variables, to associate veterinary treatments with *m/z*, and to find reliable indicators, drug related compounds and up- and down-regulated endogenous metabolites in the biological tissues.

2. Experimental

2.1. Reagents and materials

Unless specified, all reagents were of analytical grade. Amoxicillin (AMX) was supplied by Sigma-Aldrich (St. Louis, MO, USA) and piperacillin (PIP), used as internal standard (IS) was supplied by Fluka (Buchs, Switzerland). Acetonitrile (MeCN, MS grade), ammonium acetate, ammonia, formic acid, potassium dihydrogenphosphate, methanol (MeOH) and sodium hydroxide were from Merck (Darmstadt, Germany). Ultrapure water was generated by the MilliQ system of Millipore (Billerica, MA, USA).

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

Nylon microcon centrifugal filter membranes of 45 µm pore size (Millipore) were used to filter the extracts before the injection into the chromatographic system.

2.2. Preparation of standard solutions

Individual stock solutions of AMX and PIP (IS) were prepared at a concentration of 100 µg ml^{-1} by dissolving the exactly weighed quantity of each compound in MilliQ water. The working solutions used to spike the chicken tissue samples were prepared from the individual stock solutions by appropriate dilution to obtain concentrations of 10, 5, 1 and 0.5 µg ml^{-1} AMX. For the extraction procedures, 50 mM dihidrogenphosphate solution (adjusted to pH 5 with sodium hydroxide 0.1 M) and hydroorganic solutions consisting of MeCN:H_2O (91:9, v:v) and MeCN:MeOH (50:50, v:v) were also prepared.

2.3. Instrumentation

The LC-ESI-LTQ-Orbitrap MS method was carried out using an Accela HPLC system from Thermo Fisher Scientific (Hemel Hempstead, UK) equipped with an autosampler injector, a thermostatically controlled column compartment and a linear ion trap quadrupole-Orbitrap-mass spectrometer LTQ-Orbitrap-MS from Thermo Fisher Scientific (Hemel Hempstead, UK). The analytical column was a reversed-phase Pursuit UPS 2.4 µm (50 x 2.0 mm) C18 column from Agilent Technologies (Waldbronn, Germany).

Auxiliary equipment was as follows: A CRISON 2002 potentiometer (± 0.1 mV) from Crison S.A. (Barcelona, Spain) using a CRISON 5203 combination pH electrode was used to measure the pH of the buffers. A centrifuge 460R of Hettich Zentrifugen (Tuttlingen, Germany) was used to perform the extractions and obtain the final extracts. SPE was carried out on a SUPELCO vacuum manifold for 24 cartridges connected to a SUPELCO vacuum tank (Bellefone, PA, USA). TurboVap LV system with nitrogen stream was used for the evaporation of the extracts from Caliper LifeSciences (Hopkinton, MA, USA).

2.4. Procedures

2.4.1. Sample preparation procedures

Blank and positive samples

An amount of 4 g (± 0.1 mg) of minced chicken muscle (blank or positive) or 2 g (± 0.1 mg) of minced chicken kidney and liver was introduced into a 50 ml centrifuge tube (Macarov, Tong, Martínez-Huélamo, Hermo, Chirila, Wang, Barrón, & Barbosa, 2012). The I.S., PIP, was added at a concentration of 300 µg kg^{-1}. Analytes were extracted from the muscle tissue with 2 ml water (1 ml for kidney and liver tissues) by shaking for 1 min. Then, 20 ml MeCN were added to muscle (10 ml to kidney and liver) in order to precipitate the proteins. Extracts were shaken for 1 min and the resulting mixtures were centrifuged at 3500 rpm for 5 min at 20 ºC. Subsequently the organic solvent (MeCN) was eliminated by evaporation under nitrogen current at 35ºC. To improve the retention of penicillins on the SPE cartridge, 25 ml of 50 mM dihydrogenphosphate at pH 5.0 solution were added to the final muscle extracts (12.5 ml to liver and kidney).

Spiked samples

An amount of 4 g (± 0.1 mg) of minced blank chicken muscle or 2 g (± 0.1 mg) of chicken kidney and liver was introduced into a 50 ml centrifuge tube (Macarov, Tong, Martínez-Huélamo, Hermo, Chirila, Wang, Barrón, & Barbosa, 2012). Samples were directly spiked with suitable volumes of AMX working solutions to provide the desired concentrations. PIP was also added at a concentration of 300 µg kg^{-1}. The samples were allowed to stand in the dark for 20 min at room temperature to promote the interaction between the antibiotics and chicken matrix. The recovery of AMX and its metabolites was as detailed above for blank and positive samples.

2.4.2. Solid phase extraction (SPE)

ENV+ Isolute cartridges were activated with 2 ml of MeOH, 2 ml of MilliQ water and 2 ml of 50 mM dihydrogenphosphate (pH 5) solution. Sample extracts, as prepared in 2.4.1, were passed through the SPE system. Cartridges were then cleaned with 3 ml of dihydrogenphosphate and 1 ml of MilliQ water. The analytes were eluted with 4 ml of MeCN:MeOH (50:50; v:v). The samples were evaporated to dryness at 35^{o}C under current of nitrogen. 200 µl of MilliQ water were added to muscle (100 µl to kidney and liver) in order to redissolve the residue. The samples were stored in a freezer at -80 ºC until analysis. Prior to injection into the chromatographic system, samples were thawed and filtered with microcon filter.

2.4.3. Liquid chromatography-mass spectrometry (LC-ESI-LTQ-Orbitrap MS)

LC-MS conditions were established by multiple injection of individual standard of AMX using a Pursuit UPS C18 column. The separation was carried out under the elution gradient given in Table 1 using 5 mM ammonium acetate adjusted at pH 2.5

with formic acid and MeCN as the eluents. The flow rate the mobile phase was maintained at 0.3 ml min^{-1} and the injection volume was 10 µl.

The ESI-LTQ-Orbitrap MS conditions were optimized by automatic gain control (AGC) by direct infusion of 100 µg ml^{-1} AMX at 10 µl min^{-1} which mixed with a stream of 5 mM ammonium acetate (pH 2.5) and MeCN (92:2, v:v) flowing at 0.3 ml min^{-1}. The ESI source was used in positive mode to acquire mass spectra in profile mode with a setting of 30000 resolution at *m/z* 400. To ensure the accurate mass measurements, the instrument was calibrated every two days (external calibration) by direct infusion of 5 µl min^{-1} of PGG calibration solution (formulation: caffeine (2 µg ml^{-1}), MRFA (1 µg ml^{-1}), Ultramark 1621 (0.001%) and n-butylamine (0.0005%) in aqueous solution of MeCN (50%), MeOH (25%) and HAc (1%)) in the scan range of *m/z* 100-2000. Operational parameters were as follows: source voltage, 5 kV; sheath gas, 50 (arbitrary units); auxiliary gas, 20 (arbitrary units); sweep gas, 0.01 (arbitrary units); and capillary temperature, 300ºC. Default values were used for other acquisition parameters (Fourier transform (FT) AGC target $5 \cdot 10^{-5}$ for MS mode and $5 \cdot 10^{-4}$ for MS^n mode). Operating in MS^n mode, the Orbitrap resolution set at 15000 at *m/z* 400. The C-trap capacity was set at normalized collision energy of 35% and an activation time of 10 ms. All extracted ion chromatograms (XICs) were based on a 5 ppm mass window. Chromatographic data acquisition, peak integration, and quantification were performed using Xcalibur 2.1.0 QualBrowser and QuanBrowser (using Genesis peak detection algorithm) software (Thermo Fisher Scientific).

2.4.4. Quality parameters

Quality parameters established to AMX in the different biological tissues were as follows: limit of detection (LOD), limit of quantification (LOQ) and calibration curve in order to determine the drug concentration in the three tissues. The LOD, defined as the lowest concentration that the analytical method can reliably differentiate from background level, was estimated for a signal-to-noise ratio of 3 from the chromatograms of spiked chicken tissue samples at the lowest analyte concentration tested. Similarly, the LOQ was determined for a S/N of 10 (Commission Regulation (EU) No 657/2010, 2010).

Calibration curves were constructed using analyte/IS peak area ratios versus analyte/IS concentration ratios, at 300 $\mu g\ kg^{-1}$ IS.

Matrix effects in chicken muscle, liver and kidney were evaluated by comparison of slopes on two sets of samples, namely: (i) calibration curve with analyte standard solutions in water and (ii) calibration curves corresponding to the different biological tissues (both series were subjected to the sample treatment procedure as explained in sections 2.4.1 and 2.4.2).

2.5. Application to real samples

Blank chicken tissue samples were purchased from retail markets in Barcelona (Spain). Meat was minced, homogenized and stored at -20^{o}C until sample treatment.

The live chickens were medicated according to the pharmacological administration protocol fit for human consumption. The veterinary protocol was applied to broilers in the chicken producer farm “Pondex S.A.”. Animals were treated with AMX dissolved in water at dose of 19 $mg\ kg^{-1}$ on 4 consecutive days. Blank, 2-day treated, 4-day treated and post-treatment (recovery) samples were defined in four classes as follows:

Class A, blank prior to AMX administration: 2 broiler chickens randomly selected (specimens 1 and 2), which had not ever been treated with antibiotics, were sacrificed.

Class B corresponded to 3 broiler chickens (specimens 3, 4 and 5) slaughtered the second day during the pharmacological treatment (48 h treatment).

Class C corresponded to 3 broiler chickens (specimen 6, 7 and 8) sacrificed the fourth day during the pharmacological treatment (96 h treatment).

Class D corresponded to 2 broilers chickens (specimen 9 and 10) animals sacrificed 4 days after suspending the veterinary treatment (e.g., 96 h treatment + 96 h wait).

All animals were handle and sacrificed according to the ethical protocols of the chicken producer farm. In any case, biological tissues to be studied (muscle, liver and kidney) were taken and the resulting samples were refrigerated at -80ºC until performing sample treatment as specified in section 2.4. For each type of tissue (muscle, liver and kidney), three independent extraction replicates of the 10 specimens were analyzed. Each extract was injected twice.

2.6. Data analysis

Raw LC-MS data consisted of MS spectra taken throughout the entire chromatographic domain for each type of matrix (muscle, liver and kidney). Such data was exported to Matlab (Mathworks) to be further analyzed by Principal Component Analysis (PCA) and related tools using PLS_Toolbox 3.5 (Eigenvector Research, Inc., Manson, WA 98831) for Matlab.

The overall procedure for extracting LC-MS data, providing compatible file formats, applying data pretreatments and removing irrelevant variables according to a statistic criterion was as follows: (1) The raw files containing mass spectra was first converted

into a mzXML file using Xtract program from Xcalibur software. (2) Free code software packages mzMine (Katajamaa, & Oresic, 2005) and XCMS (Smith, Want, O'Maille, Abagyan, & Siuzdak, 2006) were used to process mzXML files to obtain suitable data matrices to be analyzed by PCA under MATLAB environment. Apart from format transformation, typical preprocessing tools in both time (chromatographic) and *m/z* (spectral) domains were applied to improve the data quality. Peak alignment on the time domain was very important to correct the small, or sometimes moderate, variations in the retention time of analytes among runs (otherwise, a given analyte could be seen erroneously as two or more contributions). For this chromatographic method, the shifting tolerance was 15 s and peak width variations between 15 and 45 s were allowed. Due to the high accuracy and resolution in the *m/z* domain, mass tolerances of 5 ppm were considered. A preliminary discrimination between significant and irrelevant peak features relied on establishing threshold values (tunable as a function of given experimental conditions such as instrument performance and complexity of the sample matrix). In the present case, the minimum signal-to-noise ratio for taking or rejecting peaks was set to 15 and the intensity threshold for accepting *m/z* peaks was 1000 counts. After this step, data was reintegrated and peaks of common features among the different samples were grouped taking into account the tolerance values defined above. As a result, a table or data matrix of intensity (counts) areas was obtained in which each row corresponded to a given sample and each column corresponded to a feature (defined by retention time and *m/z*). 6 LC-MS runs (i.e., 3 independent extracts × 2 replicate injections) were available for each specimen and each type of tissue. As a result, dimensions of the corresponding data matrices of muscle, liver or kidney were $s \times f$, being s the number of runs (60 of blanks and positive samples + some standards) and f the number of features (typically ranging from 2000 to 5000, depending on the data set).

Prior to PCA analysis, some additional filters were applied to reduce the data dimensions, discarding noisy and irrelevant chromatographic and spectral ranges. In the chromatographic domain, time windows containing disturbing peaks of the death volume (t_R < 0.20 min), cleaning and conditioning periods (t_R > 8 min) as well as those regions containing mainly blank contributions were cleared. Analogously, in the MS dimension, spectral ranges with poor analytical information were also suppressed from the data set (*m/z* < 100). Once the working chromatographic and spectral ranges were delimited, the number remaining features (characterized by retention time and *m/z*) was dramatically reduced using a statistic criterion based on searching for the most abundant ions in positive samples in comparison with blanks (and vice versa). Hence, at a significance level of 0.01, features occurring at significantly higher concentrations in one class with respect to the other (e.g., blanks versus positive samples) were taken as potential discriminate features to be used in PCA analysis. Conversely, compounds/features occurring at similar levels in blank and positive samples were discarded for analysis.

3. Results and discussion

3.1. Optimization of MS conditions

The most significant advantage of the Orbitrap mass spectrometry instrumentation versus others high resolution technologies is the higher resolving power of the instrument (Zubarev, & Makarov, 2013). This capability combined with high mass accuracies permits narrower mass windows to be defined and, thus, higher selectivities and sensitivities are accomplished (Kaufmann, & Butcher, 2006).

To ensure appropriate sensitivity for AMX and its metabolites, the main parameters of the LTQ-Orbitrap mass spectrometer were optimized. For this purpose, 10 µl (maximum injection capacity) of 10 mg l^{-1} AMX standard solution were injected into the LC-MS system. Resolution was the most important parameter of HRMS to be optimized in order to achieve appropriate compound discrimination through the MS domain. It is important to notice that resolution has significant effects on the method performance, affecting figures like selectivity or reproducibility (because the number of data scans throughout chromatographic peaks strongly depends on resolution). Here, two typical resolution values (60000 and 30000 FWHM) were tested using the single-stage FT-MS and FT-MS/MS. The selection of resolution values depends on the mass accuracy obtained, that it is often deteriorated in complex matrices due to the occurrence unresolved interferences (Zubarev, & Makarov, 2013). For example, for a biological matrix with AMX at a level of 75 µg kg^{-1}, the number of points for the peak changed from 185 to 320 maintaining the S/N when the resolution decreased from 60000 to 30000. In order to avoid losing peaks of compounds present at low concentrations, near LODs and LOQs, we choose resolution values of 30000 to continue the studies in biological samples.

In the MS^n mode, the optimum resolution and collision-induced dissociation (CID) values to fragment the precursors by high-energy C-trap dissociation with normalized collision energy were 15000 and 30%, respectively.

3.2. Qualitative and quantitative assessment

3.2.1 Sensitivity

328 The presence of AMX in the samples was tested by FTMS and MS^n experiments
329 monitoring the molecular ion and the neutral loss of NH_3, respectively, along with other
330 complementary characteristics such as retention time, accurate mass, molecular formula,
331 mDa of error between the mass found and the accurate mass.

332 To establish the LOD and LOQ of AMX in biological samples using HR Orbitrap MS,
333 chicken muscle, liver and kidney samples spiked with drug at different levels were treated
334 as explained in section 2.4 (Commission Regulation (EU) No 657/2010, 2010). LOD in
335 muscle and kidney tissues were < 10 and 10 µg kg^{-1} and LOQ were 15 and 25 µg kg^{-1}
336 respectively. These values are lower than the MRL established by the Council Regulation
337 37/2010. In contrast, for liver samples, the sensibility was lower so that LOD and LOQ
338 values were 75 and 100 µg kg^{-1} respectively, thus, giving higher values compared with the
339 MRL established in the European normative (Commission Regulation (EU) No 37/2010,
340 2010). These poorer limits were attributed to the higher complexity of the liver samples
341 leading to a severe matrix effect on AMX signals (see below).

342

343 *3.2.3 Matrix effect*

344 The influence of tissue matrices on the sensitivity was evaluated by comparison of slopes
345 of standard calibration curve in each biological matrix after sample preparation with that
346 established in water as is reported by Macarov et al. (2012). As shown in Table 2, signal
347 suppression in the three tissues was noticeable. A F tests concluded that differences in the
348 calibration curves by LC-MS between in chicken matrices and water were statistically
349 significant ($F_{cal}(16.45) > F_{tab}(7.70)$) so matrix effects were significant. Differences among
350 slopes between the three tissues (muscle, kidney and liver), were also found. These
351 differences were attributed to variations in efficacy of the sample treatment to remove

matrix interferences such as proteins, lipids and other endogenous components as is described by Stolker & Th. Brinkman (2005). Differences in the final extract compositions were responsible for a significant matrix effect in the electrospray source mass spectrometry.

3.2.4 Recovery

Recoveries, established by comparing the analytical results of sample extracts spiked with AMX after the extraction procedure, were around 45% for AMX in the three tissues.

3.2.5 Quantification of AMX in treated biological samples

The method was applied to determine AMX concentrations in tissues of 10 broiler chickens under the after therapeutic protocol defined in section 2.5. AMX was quantified form calibration curves establish for each chicken matrix spiked with different AMX concentration levels in the range LOQ - 200 µg kg^{-1} (each concentration level was prepared and assayed by duplicate). For the analysis of chicken samples, two independent replicate extractions were prepared and injected into the LC-MS system. The mean tissue concentration-time curves of AMX for each sample class (blank, treated and recovery samples) are presented in Figure 1. Detailed pharmacokinetic data for all animals enrolled in the pharmaceutical program shows that the absorption (time and amount) of AMX is significantly different depending on the tissue analyzed. The drug concentrations in muscle were lower than in liver or kidney, according to studies by Reyns, De Boever, De Baere, De Backer & Croubels (2008). Besides, as shown in the Figure 1, the AMX behavior in the two organs was quite different. AMX absorption was faster in liver than in kidney. Considering the AMX levels established in the Commission Regulation 37/2010 (Commission Regulation (EU) No 37/2010, 2010) in the three tissues studied, kidney and

liver samples were no-compliant, giving AMX concentrations above the MLR (except for specimen 8 in liver matrix).

In the analysis of Class D samples (specimens 9 and 10), corresponding to animals sacrificed 4 days after the drug treatment finished, no residues of the active substance and the main metabolites were detected by LC-MS. In this case, the waiting period applied was enough to excrete and eliminate the drug by urinary tract. Although no antibiotic/metabolite residues were found in these specimens, differences in metabolomic behavior compared to specimens 1 and 2 were encountered (see section 3.3 for more details).

Regarding to the main AMX metabolites, amoxicilloic acid (AMA) was not detected in any case. Amoxicillin diketopiperazine-2',5'-dione (DIKETO), in contrast, was found in chicken kidney samples of classes B and C as can be observed in the chromatograms (Figure 2) where the extracted ions corresponding to AMX and DIKETO (with the same theoretical *m/z* 366.1118) for 48h (Figure 2A) and 96 h (Figure 2B) of the pharmacological treatment are shown. In addition, the MS^2 spectrum of AMX and DIKETO is also shown (Figure 2C and 2D respectively). In Figure 2D, peaks characteristic of 207.0767, 160.0430 and 114.0377 indicated a common fragments corresponding to loss of thiazolidinic ring ($C_6H_{10}O_2NS$, *m/z* 160.0430) giving an *m/z* 207.0767, and loss of carboxyl group combined with loss of H_2O observing the *m/z* 114.0377. Although it was clearly evidenced that the tissue amounts of DIKETO increased with the intake of antibiotic, concentrations could not be calculated experimentally since DIKETO standards were not available commercially (Hermo, Gómez-Rodríguez, Barbosa & Barrón, 2013).

3.3 Effect of pharmaceutical treatment on the metabolome

To our knowledge, only Sun et al. (2013) have reported a study to evaluate the effects of pharmacological treatment with Penicillin V on the metabolome analyzing rat urine and plasma samples. These authors have made samples classification by PLS-DA to evaluate metabolome differences between control and medicated samples. Based on the reviewed literature, we have not found similar studies with administration of antibiotics, in general, or AMX, in particular, in food of animal origin.

Data obtained as explained in section 2.6 was analyzed by PCA. Gross data matrices created for the three tissues contained all extracted features under the following conditions: chromatographic peak width, from 15 to 60 s; signal-to-noise threshold, 50; count threshold in m/z peaks, 1000 counts; mass accuracy tolerance, 5 ppm; time peak shifting tolerance, 15 s; *m/z* peak shifting tolerance, 0.01. Under these circumstances, gross matrices contained about 4000 – 4500 count intensity values of peak features, defined by their *m/z* and t_R. Extracted data features were further sorted according to their ability to discriminate among the predefined classes, here blank or non-treated (class A,) and treated (classes B to D) chicken specimens. A probability value $p < 0.01$ was chosen as the threshold to separate features considered as potentially discriminant (e.g., present in one of the classes and absent in the other or present at higher levels in one of the classes with respect to the other) from those occurring at similar concentrations in blank and treated classes. After statistic filtering, about 1200 features were kept for further PCA treatment while the rest (approx. ~ 4000 variables) were excluded as they were common to the two groups.

The analysis of both gross and reduced data matrices led to analogous results in the sample maps, thus indicating that even exploratory studies without assuming any sample category were sufficient to obtain well organized distributions of samples as a function of the drug treatment. Anyway, from the point of view of simplicity, models

resulting from statistically reduced data sets were more easily interpreted and the underlying meaningful variance concerning relevant variables was more efficiently recovered.

The study of data sets from the three chicken matrices (muscle, liver and kidney) was carried out following the same approach. Here, the case of liver samples is resolved in details as an example of the overall procedure based on the interpretation of chemometric results. Data was first pretreated by autoscaling to minimize the contribution of high intensity *m/z* peaks with respect to minor signals. Figure 3 shows the results corresponding to the study of liver samples by PCA. 3 PCs were able to retain 60% of variance so that plots of scores (Figure 3A) and loading (Figure 3B) on PC1, PC2 and PC3 resulted in great system to find out the behavior of samples and variables and their relationships with the predefined chicken classes.

The first revision of the scatter plots of scores showed a great reproducibility of replicates (Figure 3A). It was found that extraction replicates of the same specimen appeared in very close positions, thus indicating that the analytical procedure was highly reproducible in terms of extraction and LC-MS analysis. It was also observed that inter-class differences (i.e., among individuals of different classes) were much more marked than within a class. This was consistent to the fact that specimens belonging to the same class displayed highly similar scores on PC1 and PC2 so they appeared in the same area. Hence, quite compact class clusters were observed. The evolution of the metabolomic behavior was clearly visualized from the position of class groups on the plot of scores (Figure 3A). Sample clusters appeared from right to left as follows: class A, blanks $\Rightarrow$ class B, AMX treatment (2 days) $\Rightarrow$ class C, AMX treatment (4 days) $\Rightarrow$ class D, AMX treatment finished (4 days). PC3 and further significant PCs described subtle changes such as variability among chicken samples belonging to the same class.

449 The map of variables resulting from the scatter plot of loadings was studied in a similar
450 way. The distribution of features on PC1 vs PC2 vs PC3 may reveal the most
451 characteristics variables dealing with each class. Anyway, the number of variables
452 included in the data matrix was enormous (~ 1200 data points) so, certainly, not all of
453 them were attributable to relevant chemical markers. Indeed, it should be mentioned that
454 even after suppressing the non-discriminat variables from the gross matrix (see above) a
455 lot of the remaining features were chemically meaningless. This is a quite frequent
456 drawback of the metabolomic approach since random signal contributions, just
457 generated by chance, may appear when dealing with large series of samples. As a result,
458 discriminating among actual sample descriptors (i.e., those associated to chemical
459 components) and random features is a very complex and time-consuming task.

460 In order to try to identify other AMX related compounds from clouds of close features,
461 probable and equivocal phase I and phase II metabolites were estimated for AMX,
462 AMX penicilloic acid, AMX penilloic acid and DIKETO using METEOR software
463 (Lhasa Ltd., https://www.lhasalimited.org/). More than 100 chemical candidates from
464 diverse biotransformation processes were proposed from the parent drug plus the
465 mentioned metabolites. The exact molecular masses of the corresponding protonated
466 molecular ions $[M+H]^+$ were compared with the experimental *m/z* values of the retained
467 features to try to confirm (or discard) the identity of such compounds. In case of
468 doubts, additional criteria were considered including, logP, count intensity, MS spectra,
469 chromatographic peak shape, etc. However, none of the tentative drug related
470 metabolites could be assigned. As a result, it was concluded that the extracted features
471 might be mainly associated to endogenous chemical components rather than AMX
472 species. According to the MS data, it was also noticed that the overall chemical
473 behavior of each sample class was very different. Hence, despite AMX and metabolite

levels were undetectable in some samples the tracks in metabolome modifications were certainly dramatic. Even in the case of class D, corresponding to chicken slaughtered 4 days after stopping the veterinary treatment, sets of *m/z* features were completely different to those corresponding to non-treated chickens. This finding suggested that a waiting period of 4 days was not sufficient to remove any sign of metabolic alterations as a consequence of the AMX administration. Thus, even when the drug was not detected analytically, endogenous compounds underwent changes from normal levels of as a consequence of the drug treatment by up- and down regulations.

The behavior described here for the specific case of liver samples was similar to that found for the other biological matrices. For muscle and kidney tissues, samples were clearly grouped according to the predefined classes. The evolution in the metabolism from blank, 2-day treated, 4-day treated and post-treatment animals was evidenced in the corresponding plots of scores. The structures of the loading representations were complex because the huge amount of features retained. The *m/z* biomarkers which are mostly changed due to pharmacological treatment and used to characterize the clusters are shown in the Table 3. When data corresponding to kidney were studied, only the *m/z* 366.1117 found in chicken kidney, class B and C, has been unequivocal identification as the main metabolite of AMX (diketopiperazin-2,5-dione). This finding was consistent with the simultaneous interpretation of score and loading plots, the so-called bi-plot analysis, as treated samples (class C) were also located in the equivalent relative position (i.e., centre/left of the plot of scores). Reasonably, other data points appearing in the same area should correspond to contributions displaying more intensity in treated samples than in blanks. Tentative identification of the markers in the METLIN database, assigning positive charge $[M+H]^+$ and an experimental error lower than ±5ppm, proposes the best probably elemental composition and the associate error in

these assignations, following the methodology describe by Cajka, Danhelova, Zachariasova, Riddellova, Hajslova (2013). Such features could not be associated to putative metabolites estimated from *in-silico* programs. Most of the components that could be characteristic of the different classes might correspond to endogenous compounds with altered (increased or decreased) concentrations with respect to the blank class. Besides, as above, blank and post-treatment samples were not coincident which indicated that differences in the metabolomic profiles still arose.

4. Conclusions

A study concerning to metabolomics profile in different tissues from broiler chickens subject to a pharmacological program with AMX have been successfully classified by combination of high resolution accuracy mass spectrometry data and PCA. The ability of the data processing to visualize and to interpret metabolomics data on the base of the relationships of mass features have allowed to extract valuable information about the food metabolome quality. In this context, the loadings and scores plot have shown a real contribution and changes in the chemical behavior depend on the chicken muscle, liver and kidney. The significant metabolite clusters were characterized by a several mass features as a consequence the administration of AMX to the animals. These biomarkers corresponding to the endogenous metabolites, but however, in all biological tissues have also observed the active compound in the samples corresponding to broiler chickens slaughtered the second and fourth day during the pharmacological treatment (48 and 96 h), and solely one of the main metabolite of AMX, DIKETO, formed via exogenous metabolism, only in chicken kidney from the class B and C samples. The absorption of AMX in liver and kidney tissues was upper than muscle, giving concentration level of AMX in liver and kidney above the MRL established by European Commission excepted specimen 8 in liver.

In addition, data corresponding to the quality parameters and matrix effect studies for all tissues were included in this research article. The reported results prove the LC-HRMS method allow the determination of AMX in chicken muscle and kidney under the MRLs fixed. The poorer sensitivity obtained in chicken liver were attributed to the higher complexity (matrix effect) of the liver samples and the ineffective samples treatment to remove the components matrix. Nonetheless, the applied methodology proves an excellent

way to produce bioinformation to discriminate whether there is pharmacological adulteration in food analysis.

Acknowledgements

The authors are gratefully acknowledged the financial support of the *Ministerio de Educación y Ciencia* of the Spanish Ministry (Project CTQ2010-19044/BQU). M.P.H. would like to thank her position as *Investigador Postdoctoral BRD* from the University of Barcelona and the *Centres Científics i Tecnològics* of the University of Barcelona (CCiTUB) for the use of the LC-LTQ-Orbitrap MS. We also wish to acknowledge M. Rey and A. Villa, from PONDEX S.A. factory, Juneda (Lleida), for kind donation of the treated chicken samples used in the study.

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FIGURE CAPTIONS

Figure 1. Concentration-time profile of AMX after pharmaceutical administration in tissues of chicken muscle, liver and kidney.

Figure 2. Extracted Ion Chromatogram of AMX and DIKETO from medicated chicken kidney samples slaughtered A) 48 h and B) 96 h of pharmacological treatment. High resolution accurate mass spectra of C) AMX and D) DIKETO.

Figure 3. A) Scores plot and B) loadings plot from the medicated chicken liver samples under pharmacological treatment.

Table 1. LC-LTQ-Orbitrap MS optimized separation gradient.

Time (min)	**%H_2O**[a]	**%MeCN**
0	98	2
3	98	2
4	75	25
5	75	25
5.5	65	35
6.5	65	35
7	20	80
7.6	20	80
7.7	98	2
18	98	2

[a]5 mM ammonium acetate adjusted at pH 2.5 with formic acid

Table 2. Calibration curves for AMX in chicken muscle, kidney and liver analysing by LC-LTQ-Orbitrap MS.

	Standard curve (in water)	Calibration curve (in matrix)
Muscle	y = 0.128 C – 0.003 (r = 0.985)	y = 0.048 C – 0.003 (r = 0.994)
Kidney	y = 0.099 C – 0.004 (r = 0.963)	y = 0.068 C – 0.007 (r = 0.985)
Liver	y = 0.127 C – 0.004 (r = 0.994)	y = 0.014 C – 0.005 (r = 0.985)

y = area AMX/IS radio; C = concentration of AMX/IS radio.

Table 3. *m/z* cluster biomarker after pharmacological treatment with AMX in chicken tissues.

Biological tissue	Classes	R_t (min)	*m/z*	Elemental composition
Liver	Class B	4.09	338.1096	Unknown
		6.09	255.0618	$C_{10}H_{10}N_2O_6$ (2 ppm)[a]
	Class C	0.73	362.1183	$C_{17}H_{19}N_3O_4S$ (3 ppm)
		0.81	489.1436	Unknown
		5.41	332.1802	$C_{14}H_{25}N_3O_6$ (4 ppm)
		6.97	307.2368	Unknown
		5.36	245.1851	$C_{12}H_{24}N_2O_3$ (3 ppm)
		1.47	329.1803	$C_{14}H_{24}N_4O_5$ (4 ppm)
		5.33	377.1806	$C_{18}H_{24}N_4O_5$ (3 ppm)
	Class D	5.81	391.1598	$C_{18}H_{22}N_4O_6$ (3 ppm)
Kidney	Class B and C	1.42	252.1224	$C_{13}H_{17}NO_4$ (2 ppm)
		5.58	366.1117[b]	$C_{16}H_{19}N_3O_5S$ (0 ppm)
		6.74	176.0705	
	Class D	5.25	584.2914	$C_{27}H_{37}N_9O_6$ (4 ppm)
		7.81	282.1493	Unknown
		6.74	408.1317	Unknown
Muscle	Class B, C and D	2.26	295.1289	$C_{14}H_{18}N_2O_5$ (0 ppm)
		3.40	307.1651	Unknown

[a]Error obtained in the assignation of the elemental composition
[b]Diketopiperazine-2,5-dione ($C_{16}H_{19}N_3O_5S$)

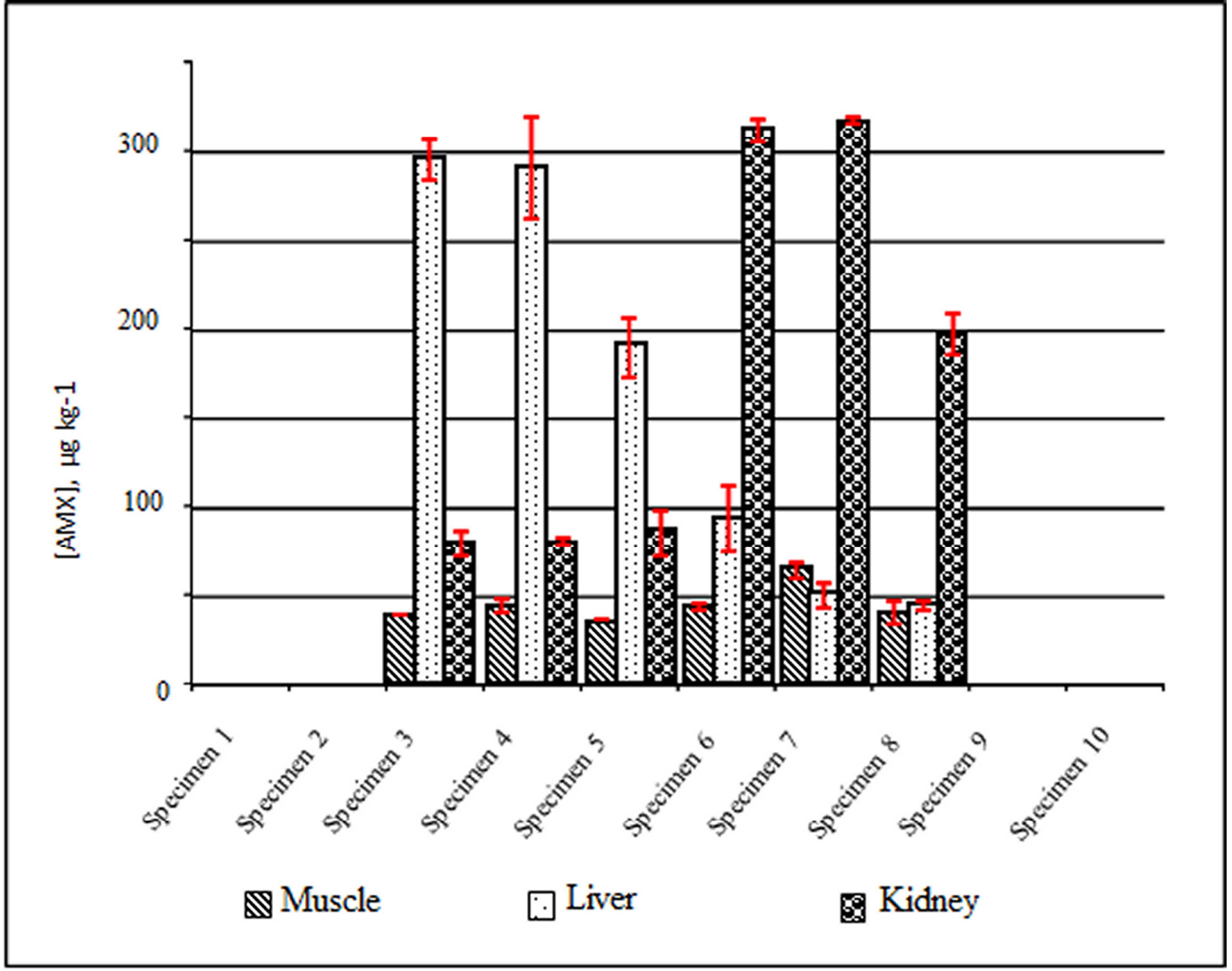
300
200
100
0
[AMX], µg kg-1
Specimen 1
Specimen 2
Specimen 3
Specimen 4
Specimen 5
Specimen 6
Specimen 7
Specimen 8
Specimen 9
Specimen 10
Muscle
Liver
Kidney

Figure 1.

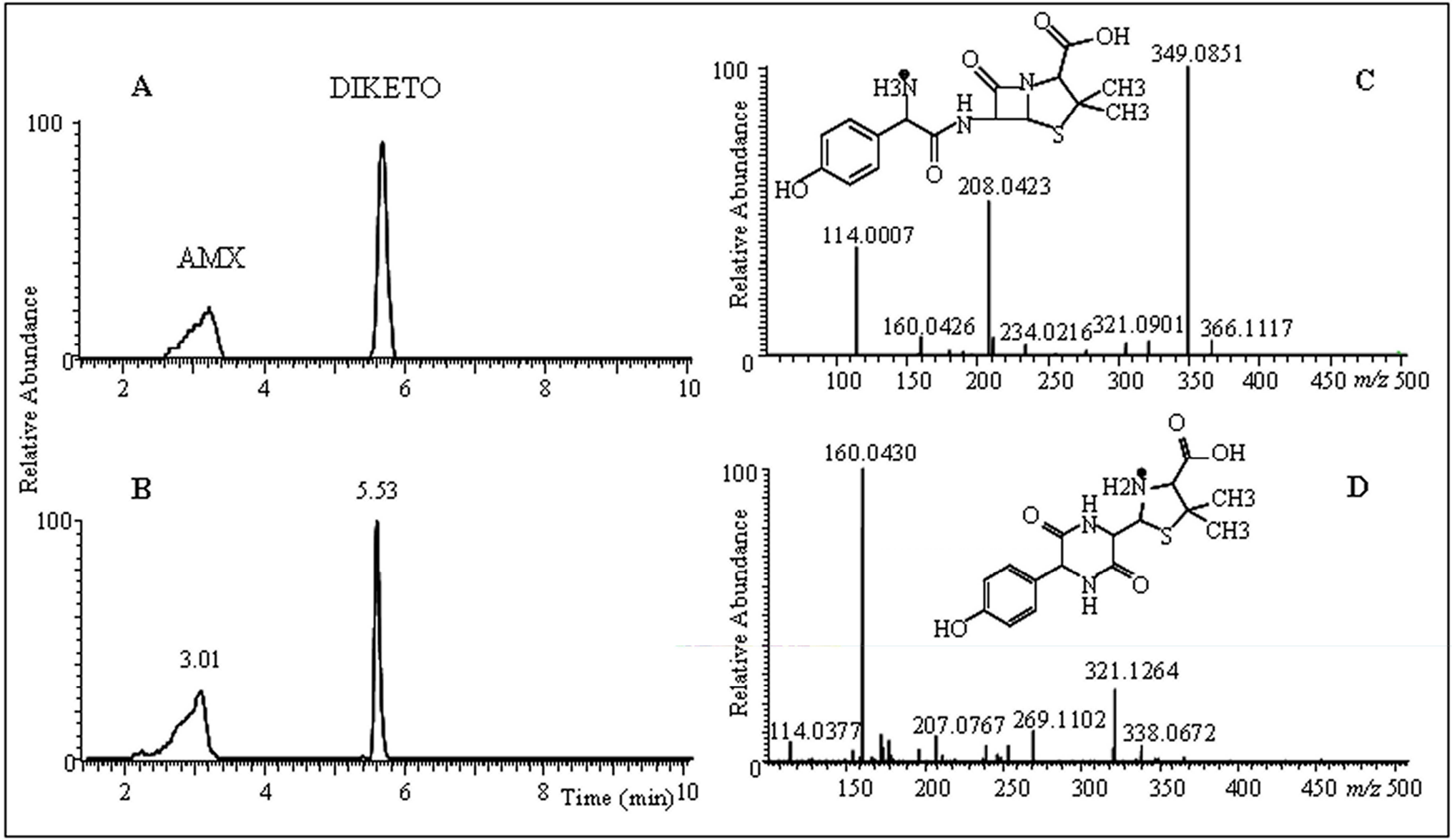
A
DIKETO
AMX
100
0
Relative Abundance
2
4
6
8
10
B
5.53
3.01
Time (min)
C
349.0851
208.0423
114.0007
160.0426
234.0216
321.0901
366.1117
100
150
200
250
300
350
400
450 m/z 500
D
160.0430
321.1264
114.0377
207.0767
269.1102
338.0672

Figure 2.

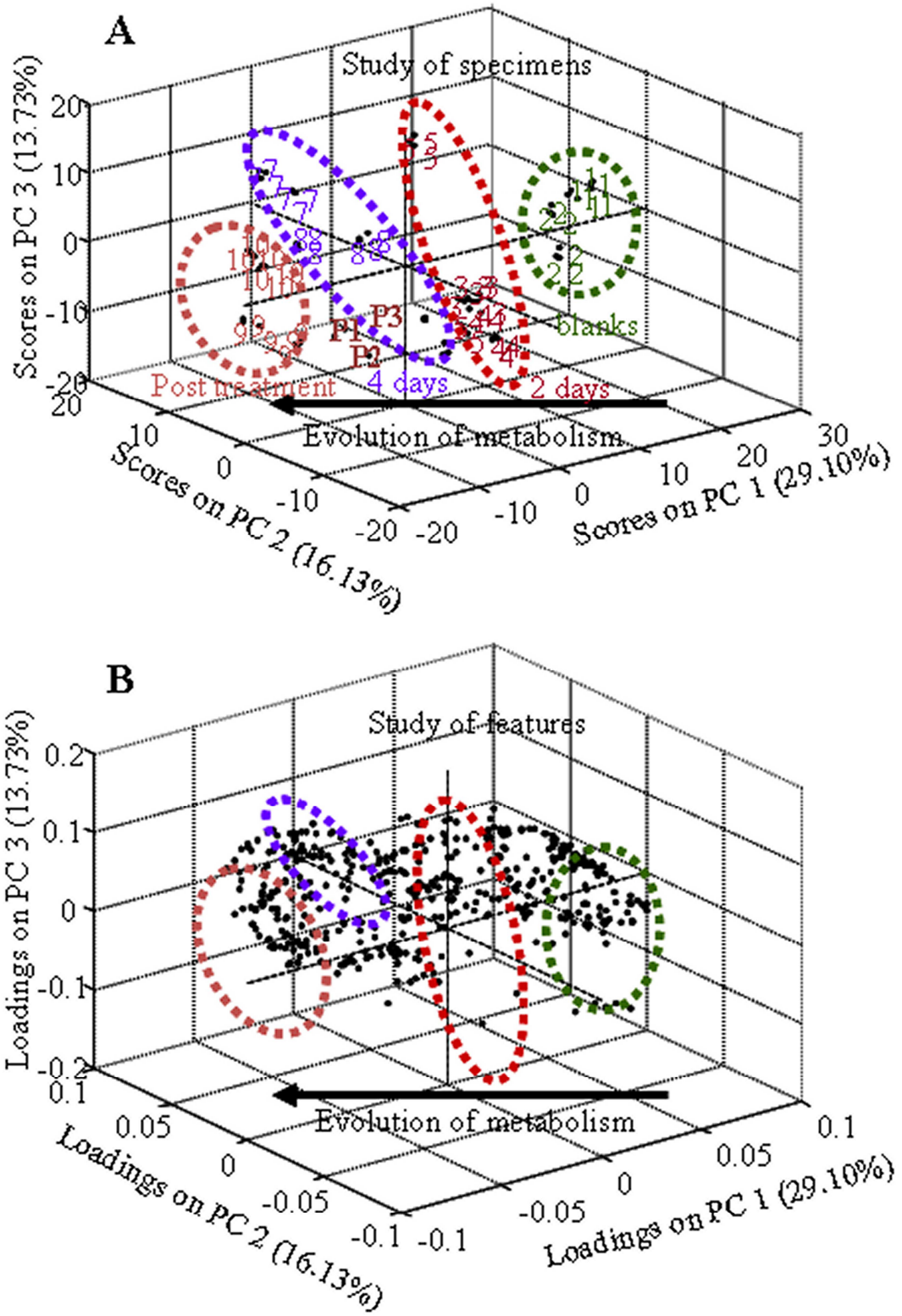
A
Study of specimens
Scores on PC 3 (13.73%)
Scores on PC 2 (16.13%)
Scores on PC 1 (29.10%)
P1
P2
P3
Post treatment
4 days
2 days
blanks
Evolution of metabolism
B
Study of features
Loadings on PC 3 (13.73%)
Loadings on PC 2 (16.13%)
Loadings on PC 1 (29.10%)
Evolution of metabolism

Figure 3.