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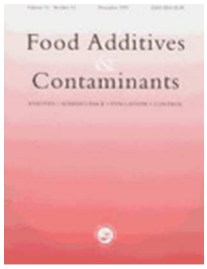

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Abstract:	In the present work, a liquid chromatography- high resolution mass spectrometry method was developed for the screening in meat of a wide range of antibiotics used in veterinary medicine. Full scan mode under high resolution mass spectral conditions using LTQ-Orbitrap mass spectrometer with resolving power 60.000 FWHM was applied for analysis of the samples. Samples were prepared using two extractions protocols prior to LC-MS analysis. The scope of the method focuses on the screening of the following main families of antibacterial veterinary drugs: penicillins, cephalosporins, sulphonamides, macrolides, tetracyclines, aminoglucosides and quinolones. Compounds were successfully identified in spiked samples by their accurate mass and LC retention times from the acquired full-scan chromatogram. An automatic process of the data using ToxId software allows a rapid treatment of the data. Analyses of muscle tissues from real samples collected on antibiotic treated animals have been carried out using the above methodology and antibiotic residues have been identified unambiguously. Further analysis of the data of the real samples allowed the identification of the targeted antibiotic residues but also the non targeted compounds, such as some of their metabolites.

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ABSTRACT

A liquid chromatography- high resolution mass spectrometry (LC-HRMS) method was developed for screening meat for a wide range of antibiotics used in veterinary medicine. Full scan mode under high resolution mass spectral conditions using an LTQ-Orbitrap mass spectrometer with resolving power 60,000 full width at half maximum (FWHM) was applied for analysis of the samples. Samples were prepared using two extraction protocols prior to LC-HRMS analysis. The scope of the method focuses on screening the following main families of antibacterial veterinary drugs: penicillins, cephalosporins, sulfonamides, macrolides, tetracyclines, aminoglucosides and quinolones. Compounds were successfully identified in spiked samples from their accurate mass and LC retention times from the acquired full-scan chromatogram. Automated data processing using ToxId software allowed rapid treatment of the data. Analyses of muscle tissues from real samples collected from antibiotic treated animals was carried out using the above methodology and antibiotic residues were identified unambiguously. Further analysis of the data for real samples allowed the identification of the targeted antibiotic residues but also the non-targeted compounds, such as some of their metabolites.

Keywords: screening; antibacterials, high resolution; mass spectrometry; muscle tissue; residues; LTQ-Orbitrap

INTRODUCTION:

Up to now, for screening residues in food of animal origin, antimicrobial agents used in veterinary medicine are mainly detected by microbiological assays, using the plate test bacterial growth inhibition technique, such as the four-plate test or the STAR test (Kilinc 2007, Pikkemaat 2009, Gaudin 2010). Microbiological methods offer a low cost analysis and do not require expensive equipment. However, due to their mode of detection, even if it is possible to identify one or more families of antibiotics using post-screening microbiological methods, they always lack specificity. It means that using such microbiological methods, it is not possible to discriminate one antibiotic from another. Moreover, they are quite often not sensitive enough to reach the maximum residue limits (MRLs) set by the Commission regulation (EU) 37/2010/EC.

It is well known that for confirmatory purpose in chemical residue testing, mass spectrometry is the technique of choice. A chemical approach based on mass spectrometric detection brings the specificity needed to chemically identify an antibiotic compound, even at the screening step. In the last decade, many analytical methods based on (very high pressure) liquid chromatography coupled to tandem mass spectrometry instruments (VHP) LC-MS/MS have been developed for multi-antimicrobial residue screening. (Granelli 2007, Kaufman, 2007, Bohm 2009, Gaugain-Juhel 2009). The use of HPLC (or U-HPLC) coupled to tandem MS combines chemical separation of analytes with the selectivity and the sensitivity of mass detection achieved in multiple reaction monitoring (MRM) mode. To enhance the confidence in the molecular identification, the analytes are usually detected by monitoring the ionic signal of at least two mass transitions, in combination with determining the chromatographic retention time. This approach is considered as a “pre-target screening” because analytes are pre-selected prior to their mass signal acquisition. This analytical technique in MRM mode needs selection of the compounds supposed to be monitored prior to the analysis and an optimization on each compound is necessary to fully determine the parameters of detection of their mass transitions (i.e. precursor ion, product ions, collision energy and voltages of the source). Consequently, only compounds included in the MRM method will be possibly detected, and other contaminants could never be detected even though they were actually present in the sample.

More recently, new approaches using high resolution mass spectrometry (HRMS) have been reported for screening residual compounds with equipment like time-of-flight mass detectors (TOF) or Orbital trap mass detectors (Orbitrap). These instruments allow full-scan acquisition of all signals obtained from the ionisation source, without pre-selecting any compounds. This approach in screening for trace amounts of chemicals is considered as a “post-target

screening". Analytes are searched after their mass acquisition. The selectivity is obtained from a full scan acquisition of signals by extracting the ion chromatogram of the accurate mass of the target ions, thanks to filters based on narrow mass windows (3 to 20 ppm). This option also offers the possibility to retrospectively analyse the whole set of acquired data, without limitation in the number of compounds to be searched. This post-target approach has been applied recently for screening of marine toxins (Skrabakova 2010, Gerssen, 2011), pharmaceuticals in waste water (Petrovic 2006), veterinary drugs (Kaufmann, 2007 & 2008; Ortelli, 2009; Peters 2009; Stolker 2008) or pesticides (Pico, 2007) in environmental matrices, in food, in biological areas and also for screening drug abuse in horse doping control (Moulard, 2010).

Non-target screening, looking for unknowns without any previous information on their chemical identity, can also be implemented from the full-scan mass acquisition data using the selectivity of high resolution mass spectrometry (HRMS) and adding the power of extractive/statistical software. Processing data from the full-scan chromatograms can eventually lead to extraction and chemical recognition of new biomarkers or trace compounds. This smart approach has recently been used by Hogenboom (2009) for environmental samples to search for emerging water contaminants and by Dervilly-Pinel (2011) to search for metabolic biomarkers adapted to the screening of anabolic steroid treatment in calves. This mass spectrometry-based "metabolomic" strategy opens a new trend in the field of veterinary drug residue control.

In our laboratory, pre-target screening using LC-MS/MS in MRM mode has been developed and validated for the identification of 60 antibiotics all belonging to the main antimicrobial families (i.e. cyclines, penicillins, cephalosporins, macrolides, aminoglycosides, sulfonamides and quinolones) in pig muscle tissues and in cows milk. This method monitors these antimicrobials at their MRL level, employing simple and fast extraction (Hurtaud-Pessel 2008, Gaugain-Juhel 2009). This method is currently being collaboratively assessed in France to be proposed either as a post-screening method leading to formal molecular identification or as an alternative to direct screening with microbiological tests in the national monitoring plan for antibiotic residues in meat products. The objective of the work reported here is first to assess the transfer of this MRM-based method to LC-HRMS and secondly to develop a "post-target" screening method primarily dedicated to penicillins, cephalosporins, sulfonamides, macrolides, tetracyclines, quinolones and aminoglycosides in muscle tissues.

Our work demonstrates that some modifications in sample preparation are necessary to achieve adequate sensitivity of the HRMS signals at the MRL level for some of the tested

compounds. The sensitivity of the method for the whole set of 60 antimicrobials was assessed through analysis of spiked samples. Automatic data processing using specific software (ToxId®) was implemented in order to allow the automatic identification of the compounds through the evaluation of their respective exact mass in combination with their retention times.

Materials and Methods

Reagents

All reagents and solvents used were of analytical grade or HPLC grade. Methanol, trichloroacetic acid (TCA) (analytical grade), formic acid (98-100% for analysis) and ammonium acetate were purchased from Merck (Darmstadt,Germany). Acetonitrile was obtained from Fisher Scientific (St. Quentin Fallavier, France). Heptafluorobutyric acid (HFBA) was obtained from Fluka (St Quentin Fallavier, France). Water was purified using a Milli-Q-System (Millipore, Molsheim, France).

The standards were obtained from different companies:

- marbofloxacin, norfloxacin, ciprofloxacin hydrochloride, enrofloxacin, difloxacin hydrochloride, oxolinic acid, nalidixic acid, flumequine, spiramycin, tylosin tartrate, tilmicosin, erythromycin, josamycin, amoxicillin, ampicillin sodium, penicillin-G sodium (=benzylpenicillin), Penicillin V (= phenoxymethylpenicillinic acid potassium salt), oxacillin sodium, cloxacillin sodium, dicloxacillin sodium, nafcillin sodium, cephapirin sodium, cefquinome sulfate, cefazolin sodium, cefalonium hydrate, cephalexin hydrate, ceftiofur, cefoperazone sodium, oxytetracyclin hydrochloride, chlortetracyclin hydrochloride, tetracyclin hydrochloride, spectinomycin dihydrochloride, streptomycin sulfate, dihydrostreptomycin sesquisulfate trihydrate, kanamycin sulfate, gentamicin sulfate, neomycin trisulfate hydrate, sulfaphenazole, sulfaguanidine monohydrate, sulfadiazin sodium, sulfathiazole, sulfamethazine, sulfamethoxypyridazin, sulfamonomethoxine, sulfadoxine, sulfaquinoxalin sodium, sulfadimethoxin sodium, sulfamethoxazole and sulfamerazine were purchased from Sigma (St Quentin Fallavier, France).

- Sarafloxacin hydrochloride, doxycyclin hyclate, paromomycin sulfate and apramycin sulfate were obtained from Cluzeau Info Labo (Courbevoie, France), danofloxacin mesylate, tulathromycine and tulathromycine marker from Pfizer (Amboise, France), neospiramycin from Wako (Neuss, Germany) and tylvalosin (=3-O-Acetyltylosin) from Eco (London, United kingdom).

Standards solutions

Individual stock standard solutions (0.5 mg/ml) were prepared by dissolving the appropriate amount of each standard into water or methanol according to their solubility: i.e. each penicillin compound in 100% water; each cephalosporins and aminoglycosides compound in water/methanol (1/1; v/v); each compound from tetracycline, macrolide and sulphonamide families in 100% methanol. Each quinolone compound stock solution was prepared in 1N sodium hydroxide/methanol (1/24, v/v). All stock solutions were stored in a dark place at +4°C, except the methanolic solutions which were stored at -20°C. For spiking, dilute composite standard solutions were also prepared in ultra-pure water to obtain the desired concentrations.

A 1mM HFBA and 0.5% formic acid solution was prepared by diluting 0.065 ml of HFBA and 2.5 ml of formic acid (100%) to 500 ml of water. A 0.5% formic acid solution in methanol/acetonitrile (1/1; v/v) was prepared by diluting 2.5 ml of pure formic acid to 500 ml with methanol/acetonitrile (1/1; v/v). These two solutions were employed as the LC mobile phases [A] and [B] respectively.

A 5% TCA solution in acetonitrile was prepared by dissolving 10 g of trichloroacetic acid in a 10 ml volumetric flask and adjusting up to the volume with water, then transferring 2.5 ml of this solution in 45 ml of acetonitrile in a 50 ml volumetric flask and adjusting up to the volume with acetonitrile.

A 5% TCA solution in water was prepared by dissolving 5 g of trichloroacetic acid in a 100 ml volumetric flask and adjusting up to the volume with water. A 2 M ammonium acetate solution was prepared by dissolving 15.4 g of ammonium acetate in 100 ml of water. This solution was then diluted ten times to obtain a 0.2 M solution.

Sample preparation procedures

To allow extraction of all families of studied compounds, two sample preparations were carried out. Twice a 2 g amount of minced muscle tissue per sample was accurately weighed and placed into 16 ml centrifuge tubes. Internal standard solution (200 µl of Sulfaphenazole at 1 $mg.l^{-1}$) and 800 µl of water were added to each tube.

Extraction 1: In the first tube, 8 ml of acetonitrile were added to the sample. After rotary-stirring for 10 min at 100 rd/min and centrifugation at 14,000 g for 5 min, 9 ml of the supernatant were transferred into a clean tube and were evaporated to dryness under a nitrogen stream at 50°C. The remaining residue was dissolved in 0.5 ml of 0.2 M

ammonium acetate, mixed by vortexing and then filtered onto a 0.45 µm PVDF Millex HV (Millipore) filter of 13 mm diameter prior to injection.

Extraction 2: In the second tube, 0.5 ml of 5 % TCA solution in water and 7.5 ml of 5% TCA solution in acetonitrile were added to the sample. After stirring for 10 min and centrifugation at 14,000 g for 5 min, 7.5 ml of the supernatant was transferred into a new tube and 6-7 drops of 12.5 % NH_4OH solution were added for neutralization (pH=7). After centrifugation at 14,000g for 5 min, 7.5 ml of supernatant was transferred for evaporation at 50°C under nitrogen stream until reducing the volume to about 1 ml. At this step, another centrifugation at 14,000 g for 5 min was performed before to continue the evaporation under nitrogen flow at 50°C till about 50-100 µl. The remaining residue was dissolved in 1 ml of water and loaded onto preconditioned C18 solid phase extraction cartridge (Bond-Elut®, 200 mg). After washing the cartridge with 1 ml of water, the elution was carried out with 2 x 0.7 ml of methanol. The methanolic solution was evaporated to dryness under a gentle stream of nitrogen at 50°C and the bottom remaining residue was dissolved in 0.5 ml of 0.2 M ammonium acetate. The final solution was filtered onto a 0.45 µm PVDF Millex HV (Millipore) filter of 13 mm diameter prior to LC injection.

Liquid chromatography-mass spectrometry (LC-LTQ-Orbitrap)

Chromatographic separations were performed on an Accela liquid chromatography U-HPLC system (ThermoFisher, Bremen, Germany) equipped with a RP18e Purospher column (125 x 3 mm; 5 µm particle size) from Merck (Darmstadt, Germany) protected by a RP18e guard column (4 x 4 mm, 5 µm particle size). The column was kept at a temperature of 25°C. The flow rate used was 500 $\mu L.min^{-1}$, and the injection volume was 25 µL. The mobile phase consisted of [A] 1mM HFBA in 0.5 % formic acid solution and [B] 0.5% formic acid solution in Methanol/Acetonitrile (50/50; v/v) The elution gradient was linearly ramped from 10% to 95% of eluent B for 12 min and hold at 95% for 3 min (12-15 min). Then the elution gradient was linearly ramped down to 10 % B for 1 min and maintained for 6 min to allow column conditioning for the next injection.

Mass spectral analysis was carried out on LTQ-Orbitrap mass spectrometer XL MS (Thermofisher, Bremen, Germany) equipped with an electrospray ionization interface (ESI) and operated in the positive ion mode. The instrument was calibrated using the manufacturer's calibration solution consisting of 3 mass calibrators (i.e. the caffeine, the tetrapeptide MRFA and the Ultramark®) to reach mass accuracies in the 1-3 ppm range. Parameters of the ion source were as follows: capillary voltage 35 V, ion spray voltage 4.3 kV, tube lens 125 V, capillary temperature 350 °C, sheath gas flow 40 (arbitrary units),

auxiliary gas flow 10 (arbitrary units) and sweep gas 0 (arbitrary units). Nitrogen was used as the sheath and auxiliary gas in the ion source. The instrument was operated in full scan FTMS over a m/z range of 100-1200 Da at a resolving power of 60,000 (full width at half maximum). The eluent was directed into the source of the mass spectrometer from 1 to 20 min by using a divert valve.

RESULTS AND DISCUSSION

Method development: Sample pre-treatment, liquid chromatography and high resolution mass detection

At the screening step, there are at least two issues of significance for successful implementation of the method: first, the preparation of the sample and second the detection technique. The very first challenge is to develop a generic non-selective extraction able to cover a wide range of compounds of different chemical properties. At the same time this extraction must demonstrate a high rate of efficiency in order to get sufficient sensitivity and to reach the required detection limits. This efficient sample preparation must then be combined to a detection technique which is not restrictive, that means sufficiently fit for all possible compounds and which can lead to a response for all compounds at their required target limit. LC-HRMS can match with these requirements for detection. The full scan MS is not restrictive. The only limitation the mass spectrometer holds is the capacity of the compounds forming ions in the ionization source. Of course, the best settings for the ionizing conditions in the source (temperature of source or capillary, flow of gases...) considering a multi-residue method are not those generally proposed to optimize for specific compounds but those which allow satisfactory medium conditions for ionizing all separated compounds entering into the source. The chromatographic separation of the compounds can also become of strategic importance. In our study, the target compounds were all antibacterial veterinary drugs. Among them, penicillins, cephalosporins, sulfonamides, macrolides, tetracyclines, and quinolones are easily ionizable compounds. Many liquid chromatographic conditions take advantage of a formic acid or an acetic acid solution as the aqueous phase and of MeOH or ACN as the organic phase to separate these compounds through reversed phase LC analytical columns. In opposite, aminoglycosides are not easily separated in these previously notified conditions and it is one of the reasons why some multi-residue methods developed for monitoring antimicrobial veterinary drug residues do not cover aminoside compounds (Kaufman 2008). The use of ion pairing agents diluted into the LC mobile phase is a common way for increasing the retention of these compounds on a reversed phase LC column (Inchauspe 1987). For this purpose, we previously proposed that the separation of all antibacterials could be achieved by adding pentafluoropropionic acid (PFPA) as the aqueous

mobile phase instead of formic or acetic acids (Hurtaud-Pessel 2008, Gaugain-Juhel 2009). In the present method, another ion pairing agent was chosen, the heptafluorobutyric acid (HFBA). It is widely accepted by the LC-MS analysts community that the use of PFPA or HFBA may decrease the sensitivity of signals entering the mass spectrometer detector compared to the use of formic acid or acetic acid. But it is also one of the compromises we proposed to provide a fairly good detection for all the targeted antibacterials.

Starting from the sample preparation previously developed in our laboratory (Hurtaud-Pessel 2008 and Gaugain-Juhel 2009), two extraction routes were finally implemented to cover all the 60 antibacterials. The first extraction with acetonitrile followed by an evaporation step was tested and found suitable for macrolides, sulfonamides, penicillins and cephalosporins. The second extraction with 5% TCA did not fit enough because the sensitivity of the signals was too low for some of the analytes from the tetracycline and the aminoglycoside families. A concentration step was therefore introduced. Extraction in acidic medium, with precipitation of proteins using TCA dissolved in acetonitrile was chosen in order to continue with a concentration step by evaporation of the ACN. Neutralization was then necessary and a further clean-up using SPE was undertaken to reach the target detection level for tetracyclines, aminoglycosides and quinolones.

The list of the monitored compounds is given in table 1. The identification of the compounds is based on their exact mass in positive mode and their corresponding retention time. The high resolving power of the Orbitrap, combined to the high mass accuracy, leads to the requested selectivity to identify a compound using its exact mass. In this method, a resolving power of 60,000 FWHM was chosen for the full scan mass acquisition. This resolution was found excellent even though decreasing it to 30,000 FWHM could also give satisfactory results. When the sample is collected from a complex biological matrix bringing signals to a high background made of a huge number of matrix-generated ions, then specific extracted ion mass chromatogram obtained from the full-scan chromatogram by using a narrow mass window (5 ppm) provides a sharp peak only representative of the asked compound without any other interference. If a higher mass window is used, for example 50 ppm or 100 ppm, then much interfering ions appear on the extracted ion mass chromatogram (fig 1).

Methodology of processing acquired data and concept of validation.

To evaluate the performance of the LC-HRMS screening method developed in our study, some characteristic parameters have been determined. In the field of veterinary drug residues, Commission Decision 2002/657/EC lays down criteria for the validation of analytical methods used for screening or confirmatory purpose. In 2010, a new guideline

dedicated to the validation of screening methods for the monitoring of residues from veterinary medicines has been edited in order to technically complete the Commission Decision 2002/657/EC. According to the Commission Decision 2002/657/EC, the characteristics of performance to be determined specifically for a qualitative screening method are the detection capability of the method also called CCβ, its selectivity/specificity against various interferences and its applicability/ruggedness/stability. Moreover, it is stated that a method utilized for screening purpose should display a false compliant sample rate lower than 5%. CCβ is the concentration at which only ≤ 5% of false compliant results remain possible. In case of analytes with an established regulatory limit (MRL for instance), CCβ must be less or equal to the regulatory limit. During the validation period, to demonstrate that the CCβ of the method is in full accordance with the regulatory/action limit, a minimum of 20 different representative samples and a maximum of 60 of them should be tested depending on the level of sensitivity of the method. The more sensitive the method is, the less number of samples to validate. In our study, no complete validation as stipulated in the guidelines has been implemented yet, , but a pre-validation study was undertaken; Only a small number of different bovine muscle samples (< 5) have been selected when 20-60 different samples should have been taken from different food-producing animal species.

To assess the method, all targeted antimicrobial compounds of table 1 were tested. The compounds were divided into several groups sorted per family and were spiked at a screening target concentration which corresponds to the MRL level or any other level of interest especially for compound bearing no MRL. Four repetitions were performed for each group. An internal standard, the sulfaphenazole was spiked to each sample prior to the extraction, in order to evaluate the extraction efficiency and to control the retention time and the mass accuracy. From these experiments, all retention times were found stable. For example, the relative standard deviation (n=56) calculated for retention time of the sulfaphenazole internal standard is of 0.34 %. The mass accuracy showed also a good stability. The accurate mass measurement of the internal standard sulfaphenazole (*m/z* 315.09102) was operated for all the extracted samples (n=56) and the deviation of the measured accurate mass ranged from -2.0 ppm to 0.03 ppm over a period of 5 days of validation. These mass measurement errors show the high stability of the mass spectrometer and thus allow the use of narrow mass extraction window of 5 ppm. This range of experimental mass errors fits quite well with the specifications of the LTQ-Orbitrap given by the constructor for using external calibration (3 ppm).

The data were further processed with ToxId® software, using a previously created searched list of compounds. This allowed identifying automatically each compound using the

theoretical exact mass with mass windows of 5 ppm, and the expected retention time. All compounds were positively identified in each spiked sample using ToxID when the following criteria were met: RT in accordance with the expected RT, measured accurate mass in accordance with the expected accurate mass with a tolerance of 5 ppm, and peak intensity higher than an arbitrary threshold of 10000. This arbitrary threshold has been established examining chromatograms of blank samples and was the limit chosen to be able to distinguish positive from negative samples. With an intensity lower than 10^4, the peak is considered as an artefact. For sulfonamides, 2 pairs of isobaric compounds sulfamethoxypyridazine and sulfamonomethoxine displaying the same elemental composition, have the same exact mass MH^+ at *m/z* 281.07028. These compounds are differentiated only by their respective retention time at 5.5 min and 6.1 min as shown in figure 2. The same situation occurred with sulfadoxine and sulfadimethoxine at m/z 311.08085 (fig 2), and with tetracycline, epi-tetracycline and doxycycline at m/z 445.16054 (fig 3). Flumequine at m/z 262.08739 and oxolinic acid at m/z 262.07099 are easily differentiated with a resolution of 60,000 FWHM (fig 4). No antibacterial compounds were detected in any of the blank muscle tissue samples.

The sensitivity of the method was very high for macrolides, quinolones and lincosamides, high for sulfonamides and tetracyclines. For penicillins, cephalosporins and aminoglycosides, the sensitivity was lower but no problem of identification occurred except for penicillin V for which a weak signal is observed. Figure 5 displays the intensity of the signal for the whole set of compounds. The arbitrary threshold set at 10000 was the minimum intensity expected for a possible identification using automatic processing with ToxID. The limits of detection (LOD) were calculated from each compound comparing the intensity of the signal obtained for the spiked samples at the target screening concentration to the threshold of 10000 (Table 1).

Applicability to real samples and no target analysis.

The applicability of the method was tested on some incurred samples of muscle tissues collected from cows and swine administered veterinary antibiotic treatments. The same samples were also analysed using the LC-MS/MS method in MRM mode. Samples were extracted, analyzed using the LC-HRMS method and processed using ToxId software. In these different samples, sulfadimethoxine, doxycycline, penicillin G, DHS and tulathromycine were detected both using LC-HRMS method and LC-QqQ method. However, no quantification was made in the various samples as the objective of the method was only for screening, even though quantification using LC-HRMS with the Orbitrap system was feasible. The additional advantage of the LC-HRMS method was the opportunity offered to search for

the presence of additional compounds retrospectively from the full scan spectrum. For example, in one beef muscle, sulfadimethoxine was found and identified using retention time and exact mass. In this sample, comparing chromatograms to the chromatogram of a blank muscle tissue, one other compound was selectively detected at 7.9 min and m/z 353.09142. The identification as N4-acetyl-sulfadimethoxine was further confirmed by CID fragmentation experiments and by comparison with chemical standard. This compound was then added to our ToxId processing file. Even if this compound is not regulated and is not displaying antibacterial activity, its detection in animal tissues is the undoubted evidence of an administration of the parent drug to the animal.

Perspective to further confirmation of chemical structures

From the screening, the further step for definitively confirming an antimicrobial compound has been developed using the LTQ-Orbitrap LC-FTMS instrument. Indeed, the LTQ-Orbitrap XL offers some other possibilities for example to operate fragmentation of a selected precursor ion either in the linear ion trap (CID) or in the High Collision Dissociation cell (HCD). The detection of product ions can also be performed using either the linear ion trap detector or the Orbitrap detector. Therefore at least three possible ways for obtaining further confirmation of a detected compound could be :

1- CID with detection in ion trap leading to low resolution mass measurement of products ions.
2- CID with detection in Orbitrap leading to high resolution mass measurement of products ions
3- HCD with detection in Orbitrap leading to high resolution mass measurement of products ions.

Conclusions

The LC-HRMS method reported here has been successfully pre-evaluated for the screening of at least 63 antimicrobial compounds in muscle tissue. In comparison with the targeted LC-triple quadrupole method currently used for screening in our laboratory, this approach using full-scan mass acquisition offers the possibility to analyse retrospectively the sets of data. The application of the method to real-life contaminated samples showed that veterinary drug metabolites which are a proof of veterinary treatment can easily be searched from the data by extracting the exact mass ion chromatograms. Of course these metabolites have to be confirmed and could further be included in the extending list of searched compounds in our ToxID file. In near future, we intend to open this method to other classes of veterinary drugs, like NSAIDs, antiparasitic or anticcoccidial drugs. There is theoretically no limit in the number of compounds to be acquired. Still the limitation in developing a unique multi-class multi-

residue screening method is the sample preparation, achieving suitable ionization of the compounds and the sensitivity of the signals obtained.

The next issue remaining unsolved is to determine whether the exact mass combined with a retention time are sufficient to unambiguously confirm a compound. Using an LTQ-Orbitrap, fragmentation it is possible either through CID or through HCD devices, and measurement of fragment ions either with low or high resolution. Up to now, there are no criteria laid down in any international Guidelines or in the Commission Decision 2002/657/EC for these new approaches using new HRMS instruments such as TOF or Orbitrap system.

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TABLE 1 List of compounds with their molecular formula, the exact mass of MH^+, their expected retention time and the level of fortification.

Compound Name	class	Molecular formula	Expected RT (min)	Exact mass of MH+ (m/z)	Target screening concentration (µg/kg)	LOD µg/kg
Amoxicillin	Penic	$C_{16}H_{19}N_3O_5S_1$	4.92	366.11182	50	26
Ampicillin	Penic	$C_{16}H_{19}N_3O_4S_1$	6.08	350.11690	50	6
Penicillin G	Penic	$C_{16}H_{18}N_2O_4S_1$	8.75	335.10600	50	11
Penicillin V	Penic	$C_{16}H_{18}N_2O_5S_1$	9.42	351.10092	25	*
Oxacillin	Penic	$C_{19}H_{19}N_3O_5S$	9.63	402.11182	300	82
Cloxacillin	Penic	$C_{19}H_{18}ClN_3O_5S$	9.99	436.07285	300	71
Nafcillin	Penic	$C_{21}H_{22}N_2O_5S$	10.34	405.13222	300	3
Dicloxacillin	Penic	$C_{19}H_{17}Cl_2N_3O_5S$	10.58	470.03387	300	150
Cephapirin	Cepha	$C_{17}H_{17}N_3O_6S_2$	5.01	424.06315	50	6
Ceftiofur	Cepha	$C_{19}H_{17}N_5O_7S_3$	7.7	524.03629	200	4
Cefquinome	Cepha	$C_{23}H_{24}N_6O_5S_2$	5.15	529.13224	50	4
Cephalonium	Cepha	$C_{20}H_{18}N_4O_5S_2$	5.26	459.07914	50 / 100 [a]	10
Cefazolin	Cepha	$C_{14}H_{14}N_8O_4S_3$	5.79	455.03729	50 / 100 [a]	11
Cefalexin	Cepha	$C_{16}H_{17}N_3O_4S$	6	348.10125	200	18
Cefoperazone	Cepha	$C_{25}H_{27}N_9O_8S_2$	6.38	646.14968	50	*
Sulfaphenazole	Sulph	$C_{15}H_{14}N_4O_2S$	7.46	315.09102	100	2
Sulfaguanidine	Sulph	$C_7H_{10}N_4O_2S$	2.61	215.05972	100	40
Sulfadiazine	Sulph	$C_{10}H_{10}N_4O_2S$	4.12	251.05972	100	10
Sulfathiazole	Sulph	$C_9H_9N_3O_2S_2$	4.45	256.02089	100	7
Sulfamerazine	Sulph	$C_{11}H_{12}N_4O_2S$	4.73	265.07537	100	3
Sulphamethoxypyridazine	Sulph	$C_{11}H_{12}N_4O_3S$	5.5	281.07029	100	2
Sulfamonomethoxine	Sulph	$C_{11}H_{12}N_4O_3S$	6.15	281.07029	100	4
Sulfadoxine	Sulph	$C_{12}H_{14}N_4O_4S$	6.43	311.08085	100	1
Sulfaquinoxaline	Sulph	$C_{14}H_{12}N_4O_2S$	7.69	301.07537	100	5
Sulfadimethoxine	Sulph	$C_{12}H_{14}N_4O_4S$	7.56	311.08085	100	1
Sulfamethoxazole	Sulph	$C_{10}H_{11}N_3O_3S$	6.48	254.05939	100	3
Sulfadimerazine	Sulph	$C_{12}H_{14}N_4O_2S$	5.19	279.09102	100	3

Tulathromycin marker	Macro	$C_{29}H_{56}O_9N_2$	6.07	577.40586	100	1
Neospiramycin	Macro	$C_{36}H_{62}N_2O_{11}$	6.86	699.44264	200	2
Spiramycin	Macro	$C_{43}H_{74}N_2O_{14}$	7.22	843.52128	200	1
Tulathromycin	Macro	$C_{41}H_{79}N_3O_{12}$	6.8	806.57365	50 / 100 [a]	1
Tilmicosin	Macro	$C_{46}H_{80}N_2O_{13}$	8.01	869.57332	50	1
Tylosin	Macro	$C_{46}H_{77}NO_{17}$	8.72	916.52643	100	1
Erythromycin	Macro	$C_{37}H_{67}NO_{13}$	8.77	734.46852	200	1
O-acetyltylosin	Macro	$C_{48}H_{79}NO_{18}$	9.08	958.53699	50	1
Josamycin	Macro	$C_{42}H_{69}NO_{15}$	9.79	828.47400	50 / 100 [a]	1
Tyvalosin	Macro	$C_{53}H_{87}NO_{19}$	10.41	1042.59451	50	1
Spectinomycin	Amgly	$C_{14}H_{24}N_2O_7$	3.9	333.16563	300	62
Streptomycin	Amgly	$C_{21}H_{39}N_7O_{12}$	4.6	582.27295	500	307
Dihydrostreptomycin	Amgly	$C_{21}H_{41}N_7O_{12}$	4.65	584.28860	500	6
Kanamycin	Amgly	$C_{18}H_{36}N_4O_{11}$	5.09	485.24533	100	53
Paramomycin	Amgly	$C_{23}H_{45}N_5O_{14}$	5.37	616.30358	500	98
Gentamicin-C1	Amgly	$C_{21}H_{43}N_5O_7$	5.5	478.32353	50 [b]	10
Gentamicin-C1A	Amgly	$C_{19}H_{39}N_5O_7$	5.5	450.29222	- [b]	18
Gentamicin-C2	Amgly	$C_{20}H_{41}N_5O_7$	5.5	464.30787	- [b]	1
Neomycin	Amgly	$C_{23}H_{46}N_6O_{13}$	5.58	615.31956	500	99
Apramycin	Amgly	$C_{21}H_{41}N_5O_{11}$	5.37	540.28753	1000	308
Lincomycin	Linco	$C_{18}H_{34}N_2O_6S$	5.48	407.22103	100	1
Oxytetracycline	Tcyc	$C_{22}H_{24}N_2O_9$	6.12	461.15546	100	2
Tetracycline	Tcyc	$C_{22}H_{24}N_2O_8$	6.35	445.16054	100	1
Chlortetracycline	Tcyc	$C_{22}H_{23}ClN_2O_8$	7.32	479.12157	100	4
Doxycycline	Tcyc	$C_{22}H_{24}N_2O_8$	7.81	445.16054	100	2
Epi-Oxytetracycline	Tcyc	$C_{22}H_{24}N_2O_9$	6.1	461.15546	100	-
Epi-tetracycline	Tcyc	$C_{22}H_{24}N_2O_8$	6.1	445.16054	100	-
Epi-chlorotetracycline	Tcyc	$C_{22}H_{23}ClN_2O_8$	6.5	479.12157	100	-
Marbofloxacin	Quino	$C_{17}H_{19}FN_4O_4$	5.9	363.14631	150	1
Norfloxacin	Quino	$C_{16}H_{18}F_1N_3O_3$	6.08	320.14050	100	1
Ciprofloxacin	Quino	$C_{17}H_{18}F_1N_3O_3$	6.18	332.14050	100	1
Danofloxacin	Quino	$C_{19}H_{20}FN_3O_3$	6.26	358.15615	100	1
Enrofloxacin	Quino	$C_{19}H_{22}FN_3O_3$	6.38	360.17180	100	1
Sarafloxacin	Quino	$C_{20}H_{17}F_2N_3O_3$	6.84	386.13107	200	1

Difloxacin	Quino	$C_{21}H_{19}F_2N_3O_3$	6.83	400.14672	300	1
Oxolinic acid	Quino	$C_{13}H_{11}N_1O_5$	7.65	262.07100	100	1
Nalidixic acid	Quino	$C_{12}H_{12}N_2O_3$	8.93	233.09207	100	1
Flumequine	Quino	$C_{14}H_{12}FNO_3$	9.18	262.08740	200	1

Abbreviation of the class : Penic = penicillins; Cepha = cephalosporins; Sulph = sulphonamides; Macro = macrolides, Amgly = aminoglycosides; Linco = lincosamide; Tcycl= tetracyclines; Quino= quinolones.

[a] two levels of fortification were tested for these compounds.

[b] The spiking solution is prepared from standard of gentamicin containing the three forms C1, C1A and C2.

*: not included in the pre-validation study.

FIGURE CAPTIONS

Figure 1 : Extraction ion chromatograms of ampicillin (MH^+ at m/z 350.11690) spiked in bovine muscle at 50 µg/kg with different extraction mass windows a) extraction window : 200 ppm, b) extraction window : 50 ppm, c) extraction window : 5 ppm

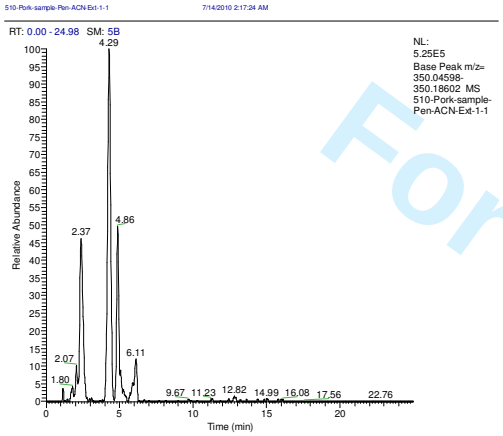
Figure 2 : a) total ion chromatogram obtained from LC-HRMS analysis of a spiked muscle with 12 sulfonamides at 100 µg/kg. b) Extracted ion chromatogram of sulfamethoxypyridazine and sulfamonomethoxine at m/z 281.07028 with extraction window of 5 ppm in spiked muscle at 100 µg/kg c) Extracted ion chromatograms of sulfadimethoxine and suldafoxine at m/z 311.08085 with extraction window of 5 ppm in spiked muscle at 100 µg/kg.

Figure 3 : a) total ion chromatogram obtained from LC-HRMS analysis of a spiked muscle with tetracyclines and epi-tetracyclines compounds at 100 µg/kg. b) Extracted ion chromatogram of ion MH^+ at m/z 281.07028 with extraction window of 5 ppm in spiked muscle at 100 µg/kg, corresponding to tetracycline, epi-tetracycline and doxycycline.

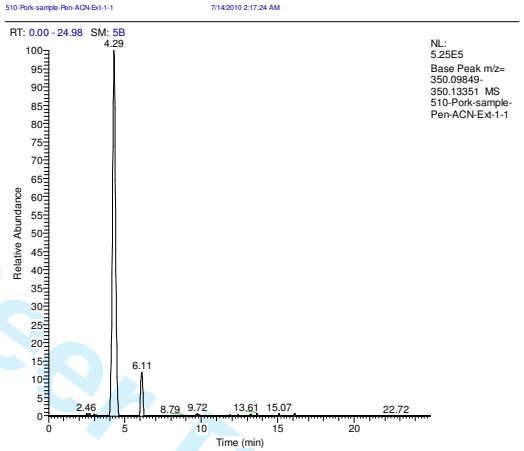
Figure 4 : a) total ion chromatogram obtained from LC-HRMS analysis of a spiked muscle with quinolones compounds at level between 100 and 300 µg/kg. b) Extracted ion chromatogram of flumequine at m/z 262.08739 with extraction window of 5 ppm in spiked muscle at 200 µg/kg, c) Extracted ion chromatogram of oxolinic acid at m/z 262.07099 with extraction window of 5 ppm in spiked muscle at 100 µg/kg.

Figure 5 : Mean signal intensity obtained from each compound spiked in muscle samples (n=4) at level of validation.

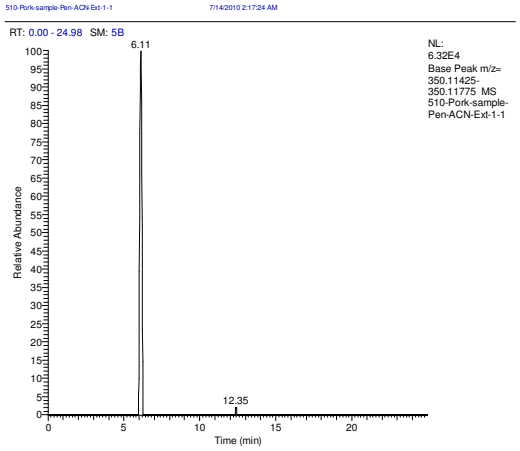
a) extraction mass window : 200 ppm

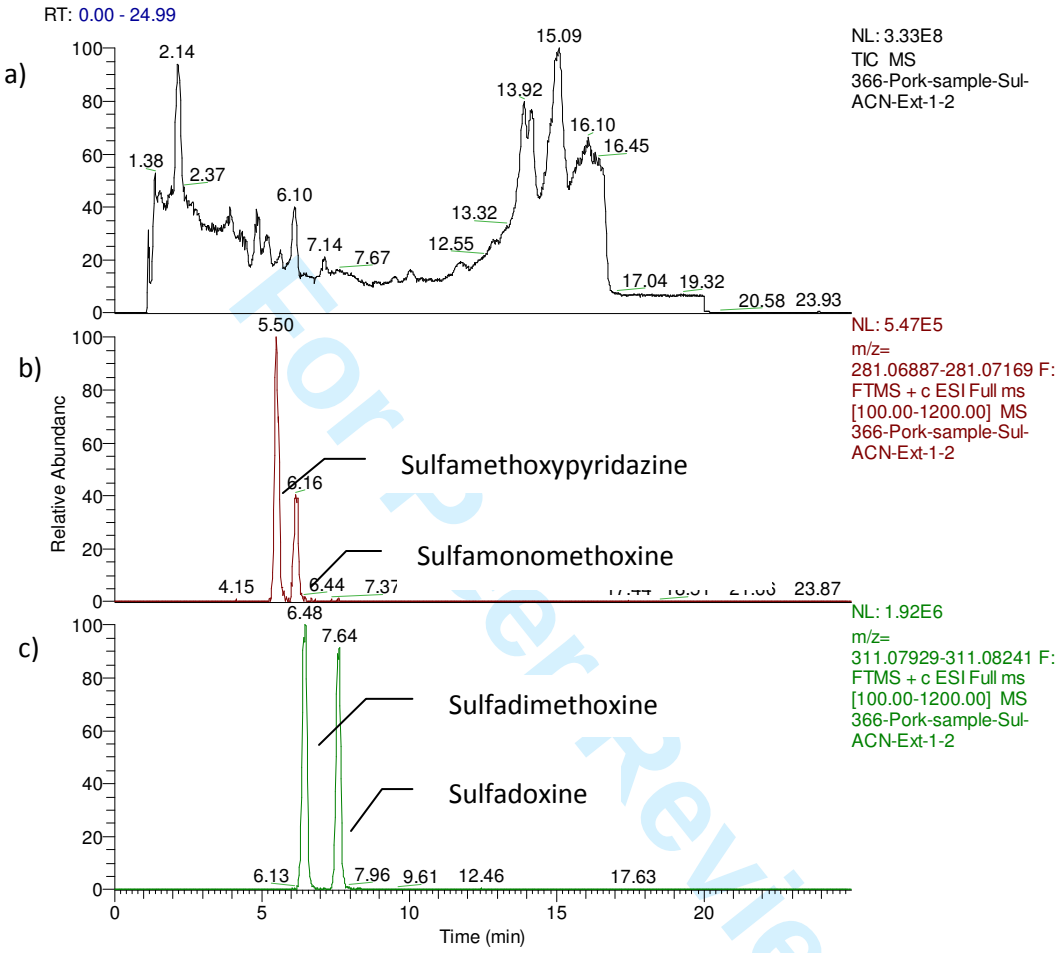


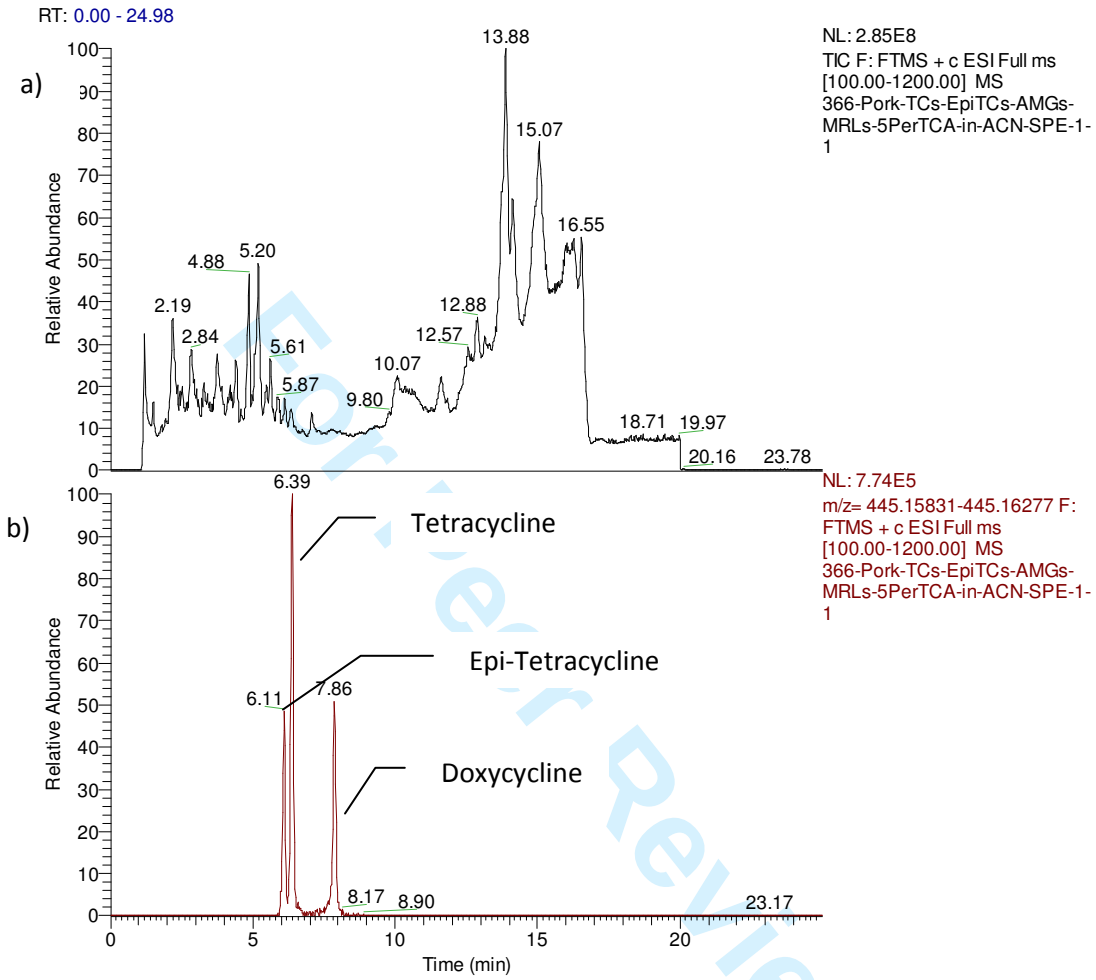
b) extraction mass window : 50 ppm

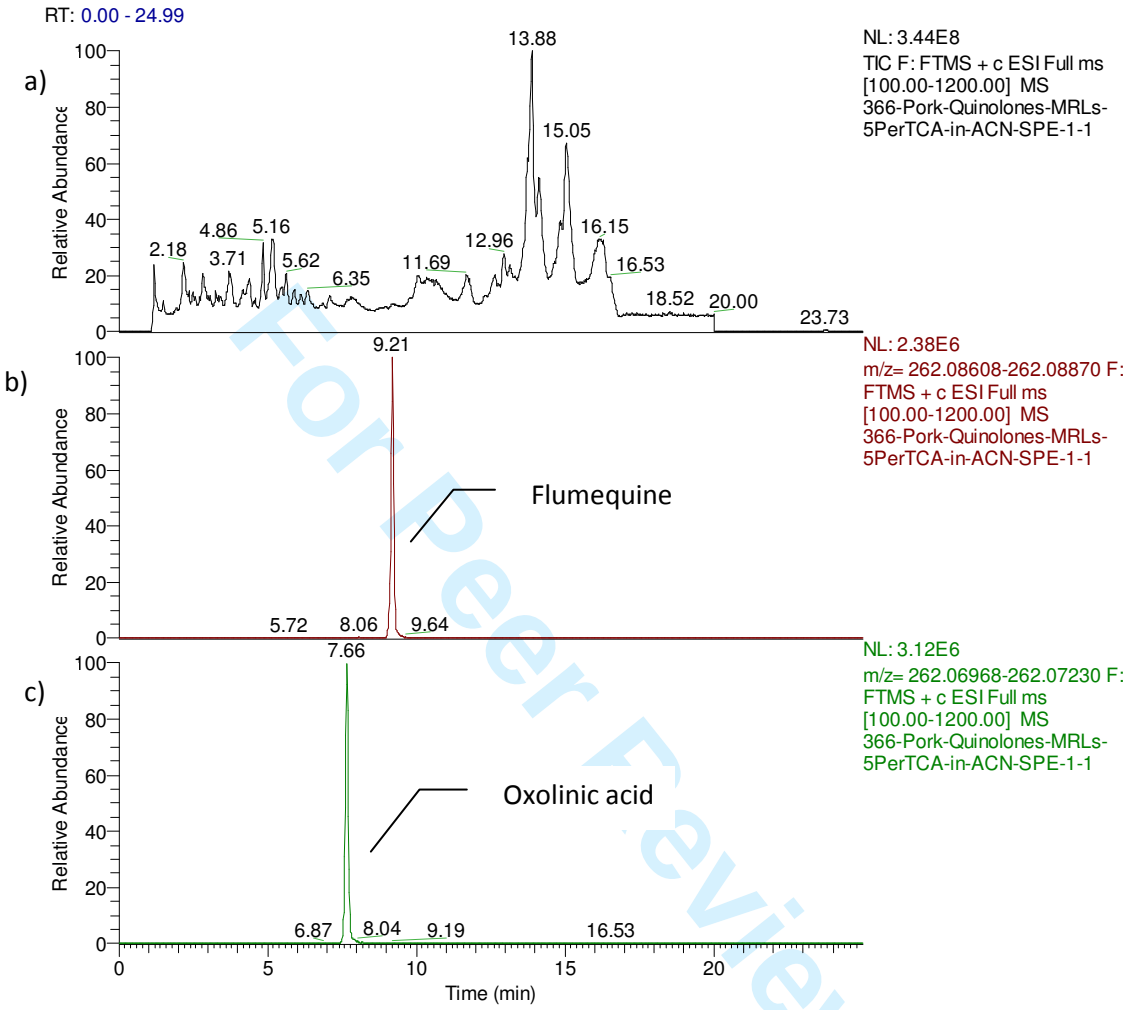


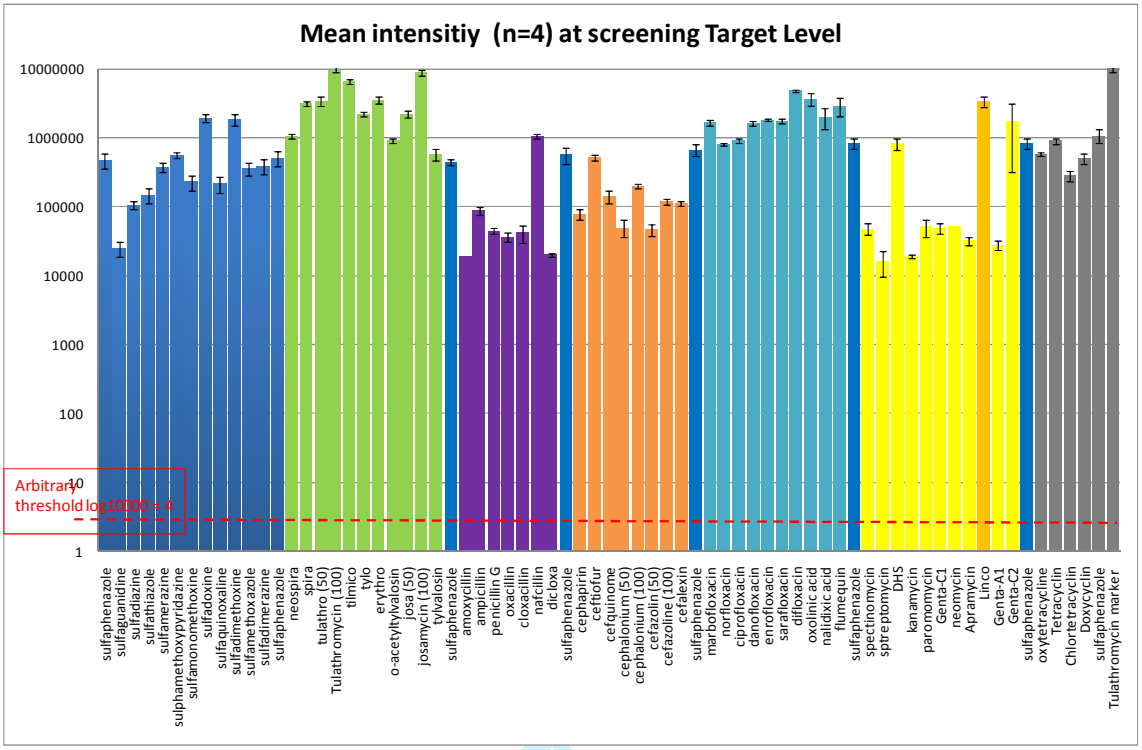
c) extraction mass window: 5 ppm










Mean intensitiy (n=4) at screening Target Level
10000000
1000000
100000
10000
1000
100
10
1
Arbitrary threshold for 1000 = 4
sulfaphenazole
sulfaguanidine
sulfadiazine
sulfathiazole
sulfamerazine
sulphamethoxypyridazine
sulfamonomethoxine
sulfadoxine
sulfaquinoxaline
sulfadimethoxine
sulfamethoxazole
sulfadimerazine
sulfaphenazole
neospira
spira
tulathro (50)
Tulathromycin (100)
tilmico
tylo
erythro
o-acetyltylvalosin
josa (50)
josamycin (100)
tylvalosin
sulfaphenazole
amoxycillin
ampicillin
penicillin G
oxacillin
cloxacillin
nafcillin
dicloxa
sulfaphenazole
cephapirin
ceftiofur
cefquinome
cephalonium (50)
cephalonium (100)
cefazolin (50)
cefazoline (100)
cefalexin
sulfaphenazole
marbofloxacin
norfloxacin
ciprofloxacin
danofloxacin
enrofloxacin
sarafloxacin
difloxacin
oxolinic acid
nalidixic acid
flumequin
sulfaphenazole
spectinomycin
streptomycin
DHS
kanamycin
paromomycin
Genta-C1
neomycin
Apramycin
Linco
Genta-A1
Genta-C2
sulfaphenazole
oxytetracycline
Tetracyclin
Chlortetracyclin
Doxycyclin
sulfaphenazole
Tulathromycin marker