Rapid screening of antibiotics in foods by HPTLC-FLD/EDA/MS

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For my dear parents & my dear wife

One person's meat is another one's poison.

− Gaius Julius Caesar (100-44 B.C.)

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Abbreviation list

General terms

HPTLC	High-performance thin-layer chromatography
MRLs	Maximum residue limits
EDA	Effect-directed assay
PCU	Population correction unit
ESVAC	European Surveillance of Veterinary Antimicrobial Consumption
EMA	European Medical Agency
EFSA	European Food Safety Authority
MS	Mass spectrometry
MSD	Mass selective detection
ESI	Electrospray ionization
GC	Gas chromatography
HPLC	High-performance liquid chromatography
λ	Wavelength
FDA	Food and Drug Administration
LOD	Limit of detection
LOQ	Limit of quantitation
m/z	Mass to charge ratio
QuEChERS	Quick, easy, cheap, effective, rugged and safe
RSD	Relative standard deviation
USDA	United States Department of Agriculture

Chemical terms

TC	Tetracycline
SA	Sulfonamide
FQ	Fluoroquinolone
AG	Aminoglycoside
ML	Macrolide
AP	Amphenicol
PC	Penicillin
OTC	Oxytetracycline
CTC	Chlortetracycline
DC	Doxycycline

GMC	Gentamicin
DSMC	Dihydrostreptomycin
SMC	Streptomycin
NOMC	Neomycin
TLS	Tylosin
ERTC	Erythromycin
SPMC	Spiramycin
CAP	Chloramphenicol
TAP	Thiamphenicol
SDX	Sulfadoxin
SDZ	Sulfadiazine
SMZ	Sulfamethazine
SNMD	Sulfanilamide
SMTZ	Sulfamethiozole
SPDZ	Sulfachloropyridazine
STAZ	Sulfathiazole
SPD	Sulfapyridine
SMRZ	Sulfamerazine
SIXZ	Sulfisoxazole
SQLX	Sulfaquinoxaline
SCTD	Sulfacetamide
PCG	Penicillin G
OXC	Oxacillin
CF	Ciprofloxacin
EF	Enrofloxacin
MF	Marbofloxacin
EDTA	Ethylenediamine tetraacetic acid

CHAPTER I

General introduction

1 Problem statement

1.1 Antibiotic residues in food: origin and seriousness

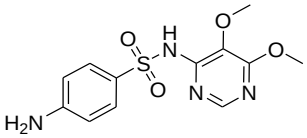
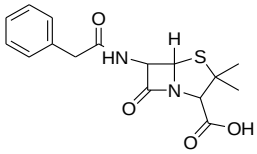
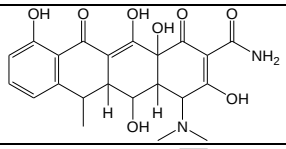
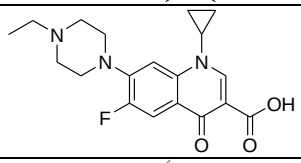
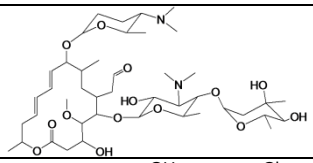
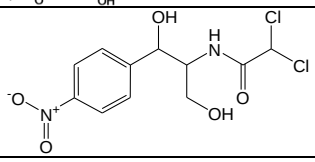
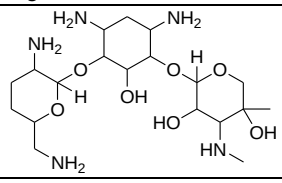
Antibiotics refer to a group of compounds, either naturally obtained or chemically synthesized, showing ability to destroy or inhibit the growth of bacteria. Since the introduction of sulfonamides in 1930s and later penicillin in 1940s, the mortality claimed by many infectious diseases was dramatically reduced. Inspired by the initial success of such “miracle medicine”, tireless efforts were dedicated to search for new antibiotics of better efficacy and broader action-spectrum. So far, antibiotics have derived a big family consisted of a large variety of compounds. However, most of them belong to a few major classes, *e.g.* tetracyclines, TCs, penicillins PCs, aminoglycosides AGs, macrolides MLs, sulfonamides SAs, fluoroquinolones FQs and amphenicols APs, respectively, as summarized in **Table 1**.

In the last decade, however, there was a trend to strike out antibiotics in prescription for human beings, out of growing concerns on their serious negative impacts on health. Instead, increasing amount of antibiotics is being used in livestock husbandry, in order to treat and prevent diseases that are frequently encountered in high density rearing. In addition to their therapeutic uses, antibiotics are also excessively administrated to animals to promote feed efficiency and weight gain.

Though the application of veterinary antibiotics remarkably enhanced animal well-being and contributed to a fruitful food supply, problems associated with their usage are however becoming increasingly severe. The direct consequence of administrating antibiotics to food-producing animal is the emerging of residues in edible tissues. Consumption of such animal foods would directly threat consumers, acutely provoking allergic reactions or chronically effecting organ systems. Moreover,

residues of antibiotics have technological implication in the manufacturing of dairy products, by interfering the normal fermentation reactions [1].

Table 1 Basic information of the major antibiotics groups.

Group	Representative structures	Action mechanism	Action spectrum
SAs		Competitive inhibitors of the enzyme dihydropteroatesynthetase involved in folate synthesis	Gram+
PCs		Biosynthesis inhibitors by preventing the formation of peptidoglycan cross-links in the bacterial cell wall	Gram+
TCs		Protein synthesis inhibitors by binding to the 30S subunit of microbial ribosomes	Broad
FQs		Inhibitors by preventing DNA from unwinding and duplicating	Broad
MLs		Protein synthesis inhibitors by preventing peptidyltransferase from adding the peptidyl attached to tRNA to the next amino acid	Gram+
APs		Protein biosynthesis inhibitors similar to macrolides	Broad
AGs		Protein synthesis inhibitors, ribosomal translocation inhibitors, bacterial cell membrane integrity interrupters	Gram-

On the contrary, an indirect threaten due to veterinary antibiotics usage is however prone to be overlooked. This effect seems hardly imperceptible, because only chronically exposing to antibiotics accelerates the spread of gene fragments encoding drug-resistance in bacteria [2-5]. Bacteria acquired such capacity therefore become less sensitive to antibiotics. There have been already substantial evidences supporting

the role of veterinary antibiotics in this process. For instance, 336 *Listeria* strains from ready-to-eat meat products and meat-processing environment were evaluated [3]. The investigation revealed that *Listeria* strains isolated from ready-to-eat meat products displayed significantly higher overall antimicrobial resistance (31.3%) than those from the environment (13.4%). In the European Union (EU), it was estimated that drug-resistant pathogens were responsible for about 25000 human deaths annually. Apart from avoidable death, this also related to additional healthcare costs and productivity losses of at least 1.5 billion € [6].

In EU, considerable works had been dedicated to monitoring sales data of veterinary antibiotics. Their legal basis is the "Copenhagen Recommendations" in 1998, which initiated the concept "good practice in the use of antimicrobial agents" [7]. Thereafter, the European Surveillance of Veterinary Antimicrobial Consumption (ESVAC) project was started by the European Medicine Agency (EMA) in September 2009, following the request from the European commission to develop a database on the consumption of veterinary antimicrobial agents in the member states [8].

Against this background, EMA collected and calculated the distribution of sold antibiotics in target biomass that is in terms of the population correction unit (PCU, *i.e.* the estimated weight at treatment of livestock and of slaughtered animals). As shown in **Fig. 1**, this work sheds light on gross profiles concerning consumption of different antibiotics, individually and as a whole. Meanwhile, it is interesting to notice that the so-called "old" antibiotics belonging to tetracyclines, sulfonamides and β-lactams were dominantly used in EU, accounting for almost 70% of the total veterinary antibiotics consumption in most member states. This figure also revealed a fact that the usage of veterinary antibiotics has reached a formidable level (>50 mg/kg biomass), even in a highly regulated market like EU.

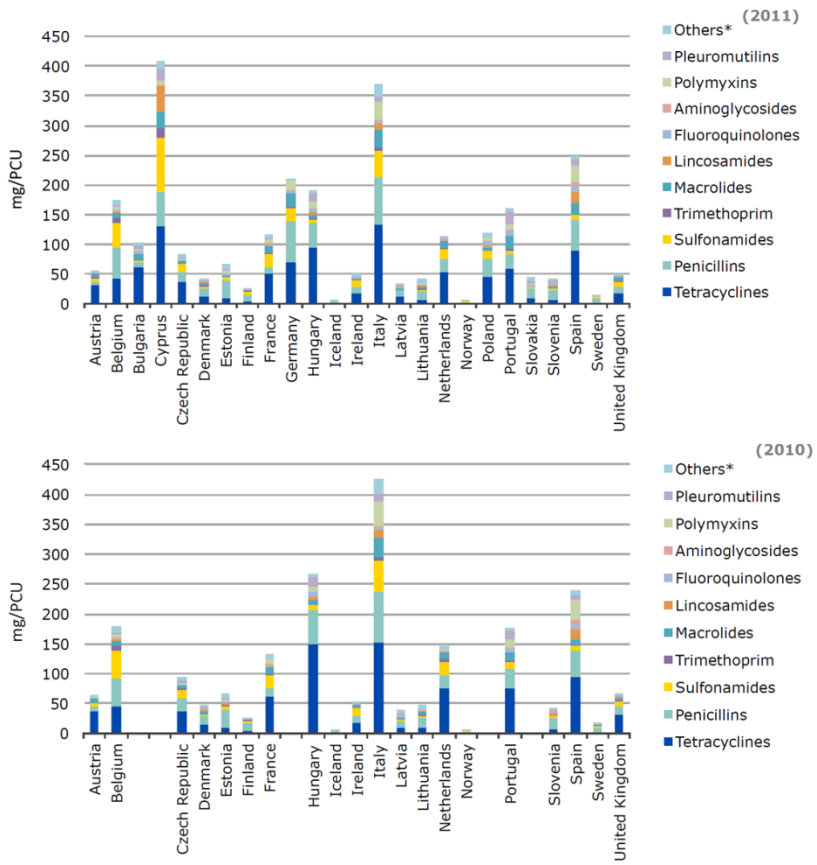


Fig. 1 Sales of food-producing species in mg/PCU, of the various veterinary antibiotics families, within 25 EU member states in 2011 [8].

1.2 Regulations on antibiotic residues in food: a policy review

Alerted by these serious consequences, a worldwide campaign has been launched against the illegal usage of veterinary antibiotics. Apart from the numerous national regulations, there have been considerable international efforts to harmonize standards for veterinary drug residues [9]. Remarkably, the United States Department of Agriculture (USDA) offers a comprehensive database of international regulations to common agriculture drugs, including veterinary antibiotics [10]. Summing up, all developed and several emerging economics have well-established, legal binding procedures for evaluating application for marketing authorizations. However, it was noticeable that the tolerance levels toward antibiotic residues in foods are surprisingly

different; besides, the implementation forces of such legislations vary from region to region. In comparison, the situations in EU are the most representative.

In EU, a tight framework consisted of series of amendable legislations was established, aiming to coordinate and harmonize the numerous domestic laws within each member states. In this framework, the latest tolerance limits, in terms of maximum residue levels (MRLs), for individual antibiotics in different animal food categories was set by Council Regulation 37/2010 [11]. In comparison to governmental issues in other regions, this is the most detailed and strictest standard for antibiotic residues in food (see **Fig. 2** and **Table 2**), which therefore serves as the technical criteria assessing the state-of-the-art methods in this field.

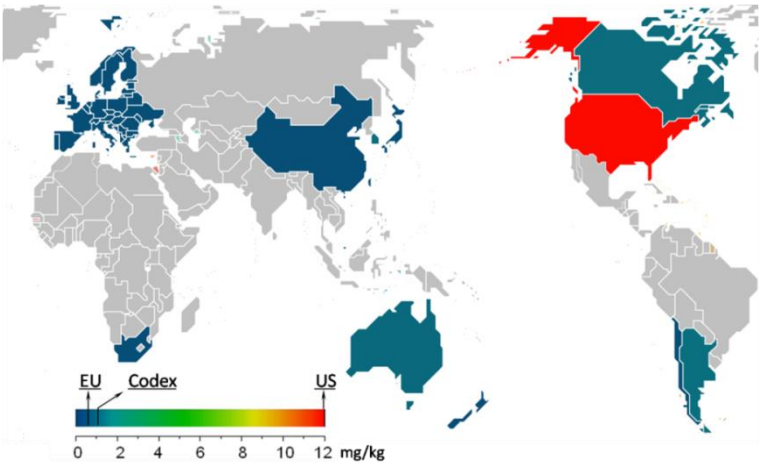


Fig. 2 A comparison on tolerance limits to veterinary antibiotic residues, an example for tetracyclines residues in bovine kidney. Data were collected from [10-12].

Different from the US FDA-style concentration, controlling laboratories in EU are not obligated to a fixed method for residue determination. Instead, they are free to adopt any methods considered the most accurate and sensitive. Nevertheless, the performances of analytical methods employed and interpretation of results generated must comply with a specified criteria: Council Directive 96/23/EC [13] and Commission Decision 2002/657/EC [14].

To implement the regarding legislation framework in EU, tight and well-organized cooperation and monitoring programs have been launched between the EU member states. As required by article 31 of Regulation (EC) No 178/2002 [15], all these monitoring results are eventually summarized by European Food Safety Authority (EFSA), forming an annual report. This report clearly outlined the reality and tendency of veterinary antibiotics residual contamination, providing valuable information essential to identify risk factors regarding different antibiotics species and food categories.

As laid down by EU Commission Decision 97/747/EC [16], a minimum requirement for sampling frequency in proportion to the slaughtered animals must be fulfilled. Especially in Germany, a detailed regulation on sampling rates of animal products against antibiotics residues was issued [17].

Consequently, huge numbers of samples (>120000, data from Germany were *not* included) were therefore collected and analyzed for antibiotic residues every year within EU, as depicted in **Fig. 3**. Interestingly, the overall non-compliant rates stayed remarkably and constantly low (<0.5%, except honey samples).

Particularly noticeable in Germany, a more straightforward strategy in compliance with the national legislation was implemented. Therefore, all analyses are carried out by microbial inhibition tests. In these cases, samples showing marked inhibition should be sufficiently rejected, saving any confirmation by a physicochemical method. Despite of these differences, the contamination profile detected in Germany (**Fig. 4**) is quite familiar to that of the entire EU, revealing an enormous contrast between sampling numbers (>260000) and positive results (on average <0.3%). In sum, screening tasks of such positive rate is analogous to finding a needle in haystacks

Table 2 An overview on the residual regulation on studied antibiotics; abbreviation: NL is not listed and NP is not permitted. Values outside brackets are EU MRL values [11], inside brackets are US MRL values [10].

Groups	Species	MRLs indifferent animal-derived foods (mg/kg)			
		Liver	Kidney	Muscle	Milk
TCs	All species and epimers	0.3(6)	0.6(12)	0.1(2)	0.1(0.3)
SAs	All species	0.1(0.1)	0.1(0.1)	0.1(NL)	0.1(0.1)
MLs	ERTC	0.2(0.1)	0.2(0.1)	0.2(NL)	0.04(NL)
	SPMC	0.5(NL)	1.5(NL)	0.2(NL)	0.15(NL)
	TLS	0.1(0.2)	0.1(0.2)	0.1(0.2)	0.05(0.05)
AGs	DSMC	0.5(0.5)	1(2)	0.5(NL)	0.2(NL)
	NOMC	0.5(3.6)	5(7.2)	0.5(1.2)	1.5(NL)
	SMC	0.5(NL)	1(NL)	0.5(0.5)	0.2(NL)
	GMC	0.2(NL)	0.75(NL)	0.05(0.1)	0.1(NL)
PCs	OXC	0.3(NL)	0.3(NL)	0.3(NL)	0.03(NL)
	PCG	0.05(NL)	0.05(NL)	0.05(NL)	0.004(NL)
APs	CAP	NP(NL)	NP(NL)	NP(NL)	NP(NL)
	TAP	0.05(NL)	0.05(NL)	0.05(NL)	0.05(NL)
FQs	EF/CF	0.2(NL)	0.3(NL)	0.1(0.3)	0.1(NL)
	MF	0.15(NL)	0.15(NL)	0.15(NL)	0.075(NL)

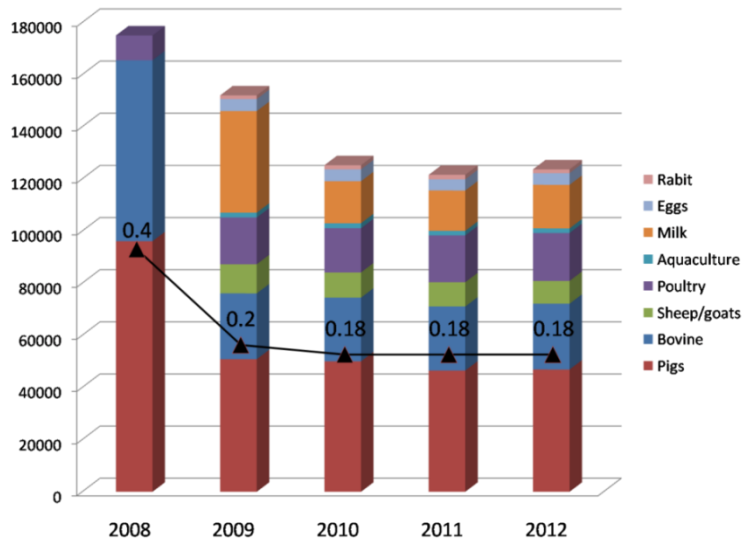


Fig. 3 Number of targeted/suspected samples analyzed within EU member states (*not* include Germany) and the average percentage (%) of non-compliant samples -▲-. Data were collected from [18-22].

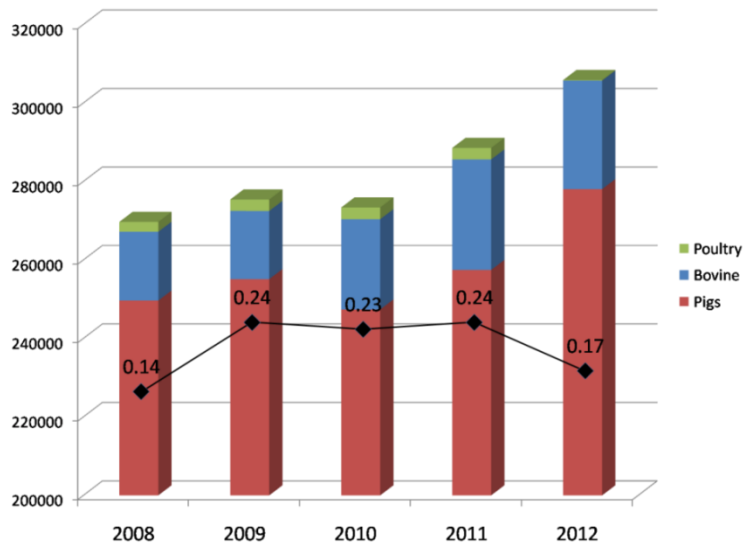


Fig. 4 Number of targeted/suspected samples analyzed in Germany by the microbial inhibition tests and the average percentage (%) of non-compliant samples -◆-. *Note*: food categories like horse, poultry and rabbit with sample numbers less than 1000 were not accounted. Data were collected from [18-22].

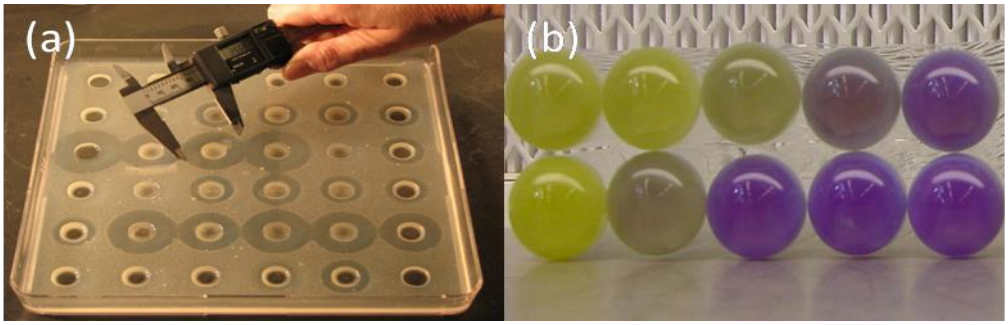


Fig. 5 (a) Example of a *Bacillus cereus* plate assay for the detection of tetracyclines in urine; (b) a *B. Stearothermophilus* tube test with a bromocresol purple pH indicator [26] (Pictures are with kind permission of reuse).

Besides, further interpretation of the statistics data revealed that the residues species of antibiotics in animal foods cover broadly. Their diversities in chemical structure and action mechanism increase the difficulty involved in finding generic analytical procedures for their detection. That is why some compromises were normally made in the practice. For instance, a few EU member states still employ microbial inhibition assays for the crude screening of antibiotics residues in foods. As depicted in **Fig. 5**, these methods are reputed for superior simplicity. However, they had been proved not meeting the latest EU MRLs, especially concerning some "difficult" antibiotics like tetracyclines [23-25]. Therefore, novel methods/strategies striking better balance between simplicity and sensitivity is highly desired in screening antibiotic residues in animal foods.

2 Screening-oriented assays by HPTLC and hyphenated detections

For a long time, the role of HPTLC in residue analysis seemed insignificant. In this field, methods based on HPLC-MS are traditionally favored for determining multi class antibiotics residues in food [27-35]. However, the direct hyphenation of multi detection modes to HPTLC subverts this concept. The advantages of HPTLC over

HPLC are not only the analysis of many samples in parallel but also the compatibility to novel effect-directed assays (EDA). Moreover, sample cleanup and hyphenation efforts to HPTLC are markedly eased, because of its open nature [36]. This is extremely suitable for the screening of antibiotics residue.

2.1 HPTLC-fluorescence densitometry

Fluorescence densitometry (FLD) is a very efficient tool for quantitating depositions on HPTLC plates. Compared with UV-Vis densitometry, FLD is not only superior in specificity, but also enables high detection sensitivity (to ng/zone level), which is highly attractive in residue analysis. A couple of antibiotics are readily subject to this detection mode. For instance, tetracyclines and quinolones possessing rigid π-π conjugate structures emit intensive fluorescence when excited (see **Table 1**). Apart from that, another important antibiotics group sulfonamides containing aniline group can be easily gifted intensive fluorescence character, by reacting with the fluorescent precursor fluram, shown in **Fig. 6**.

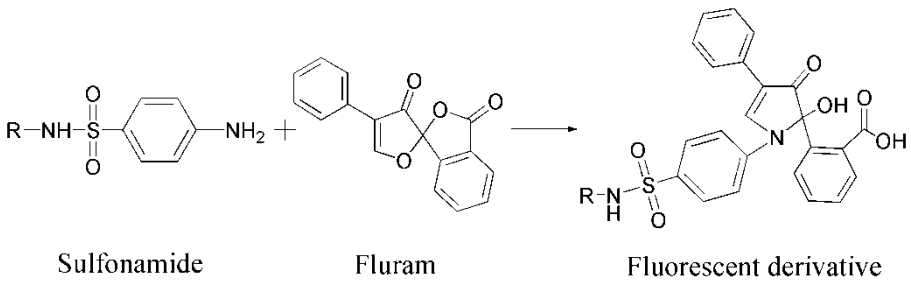


Fig. 6 Mechanism of sulfonamide derivatization reaction with fluram [37].

The fluorescence property of antibiotics, either natural or artificial, had already been employed in HPLC methods for their residues analysis [28, 37-45]. However, the use of HPTLC helps to ease the laborious steps of sample cleanup and pre-column

derivatization that normally take hours. Moreover, the plate itself offers an ideal platform for post-separation derivatization, while all the targets were in the waste bottle in the case of column chromatography.

2.2 HPTLC-bioluminescence bioautography

Hyphenation of EDAs to HPTLC opens another horizon in residue analysis. Such analytical strategy is particularly suitable to HPTLC, because organic solvents which inactivate or kill biosensors are readily evaporated before detection, which is however hardly compatible to HPLC/GC. A significant advantage of this strategy is its intrinsic capability to disclose the relation between chemical information and corresponding bio-activity. Besides, unparalleled high sensitivity can be achieved in this detection mode, even in the sub-ng to pg range [46-50].

So far, various bioassays based on enzymes and living organisms have been successfully coupled to HPTLC [50-56]. Among them, marked interests were shown to a luminescent bacterium, *Aliivibrio fischeri* (Gram negative) that is a useful visual marker [57, 58]. *Aliivibrio fischeri* is a natural marine bacterium, which luminizes 490 nm light under suitable conditions (**Fig. 7a**). Its bioluminescence is regulated by an autoregulator, termed “lux autoinducer”. At a cell density of 10^{10}-10^{11} mL^{-1}, the autoinducer accumulates, triggering chemiluminescent reaction catalyzed by a specific enzyme, luciferase (**Fig. 7b**). Since its respiratory chain is tightly linked to the energy-consuming luminescence system, any compounds interfering the cytoplasm metabolism may result in luminescence change [53, 58, 59].

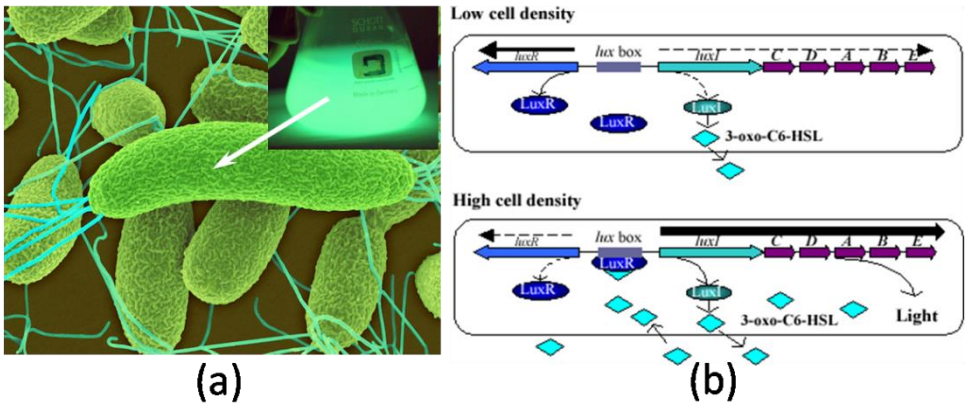


Fig. 7 (a) Micrographs of *Aliivibrio Fischeri* [60]; (b) bioluminescence mechanism [61].

HPTLC-bioluminescence bioautography based on *Aliivibrio fischeri* proved to be a robust and effective probe to estimate bioactive components. In contrast to other high-throughput systems (*e.g.* agar tube/plate tests), HPTLC enables a chromatographic separation prior to bioassays. This avoids false results due to interferences from antagonistic, synergistic effects or matrix noises. More importantly, this method suffers little "target-restrict". In traditional spectroscopic methods, detection principally depends on only a few targeted chemical characters (*e.g.* fluorescence and UV-absorption), which is therefore more or less group specific; while in bioautography, any compounds effecting the bioluminescence of *Aliivibrio fischeri* can be readily detected, regardless of their chemical differences. In sum, this strategy enables a very cost-effective alternative to physicochemical methods in large-scale antibiotics residues screening tasks, where the main purpose is to identify samples that require additional chemical confirmation.

2.3 HPTLC-mass spectrometry

In the last decade, a couple of smart devices/strategies were developed for efficiently linking HPTLC and mass spectrometry (MS) [47, 62-70]. Particularly, the

elution-head based TLC-MS interface manufactured by CAMAG attracted marked interests. Analysis in this way enables direct and rapid chemical elucidation on HPTLC with relative ease, offering concrete and conclusive identification of the substances concerned. This is a particular concern for the antibiotics residue analysis. Not limited to direct mass spectrometry analysis, sampling through this manner also facilitates many other powerful structural elucidation tools, *e.g.* nuclear magnetic resonance and infrared spectroscopy [71, 72].

3 Research objectives

Base on the above-mentioned points, the objective of this study is to develop methods on HPTLC media for the rapid and accurate screening of multi veterinary antibiotics residues in food matrices. Therefore, a multidisciplinary approach is designed through integrating HPTLC separation, fluorescence densitometry, bioautography, and mass spectrometry detection, aiming to accelerate and ease screening procedures. To achieve this objective, the following points were addressed:

• Apply the "quick, easy, cheap, effective, rugged and safe" (QuEChERS) strategy [72] for extracting target antibiotic residues from food matrices;

• Optimize fluorescence densitometry for the determination of tetracyclines and fluoroquinolones residues; reduce ion-suppression effect caused by EDTA deposited on HPTLC plates when hyphenated to mass spectrometry (Chapter II);

• Optimize fluorescence derivatization and densitometry for the determination of sulfonamides; improve the parameters of HPTLC-MS for target compounds identification on fluram derivatized plates (Chapter III);

• Improve the bioautography by *Aliivibrio fischeri* (DSM No. 7151) [74] to targeted antibiotics on HPTLC media; circumvent the interferences due to bioactive matrix by applying novel chromatographic strategy (Chapter IV).

To achieve these goals, a group of 25 first-line veterinary antibiotics, including tetracycline, oxytetracycline, doxycycline, chlortetracycline, enrofloxacin, ciprofloxacin, marbofloxacin, sulfadoxin, sulfadiazine, sulfamethazine, sulfanilamide, sulfamethiozole, sulfachloropyridazine, sulfathiazole, sulfapyridine, sulfamerazine, sulfisoxazole, sulfaquinoxaline, sulfacetamide, spiramycin, erythromycin, tylosin, neomycin, gentamicin, dihydrostreptomycin, thiamphenicol, chloramphenicol, penicillin G, and oxacillin (chemical structures see **Appendix**), were targets for HPTLC detection. In addition to parameters optimization, the reliability of methods was evaluated through spiking experiments. Principally guided by the Commission Decision 2002/657/EC [14], real samples (porcine meat and bovine milk) were screened to guarantee the precision and accuracy of the established methodology.

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CHAPTER II

Planar chromatography mediated screening of tetracycline and fluoroquinolone antibiotics in milk by fluorescence and mass selective detection

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Key words

HPTLC-FLD, HPTLC-ESI/MS, tetracyclines, fluoroquinolones, milk

Abstract

A rapid and efficient method for preliminary screening of four tetracyclines (tetracycline, chlortetracycline, oxytetracycline, doxycycline) and three fluoroquinolones (enrofloxacin, ciprofloxacin, marbofloxacin), mostly detected in milk, by high-performance thin-layer chromatography–fluorescence detection and electrospray ionization mass spectrometry (HPTLC-FLD-ESI/MS) is highlighted. The optimized separation of the target antibiotics on ethylenediamine tetraacetic acid modified silica gel plates showed marked benefits for screening purposes. Besides, selective and sensitive densitometry in fluorescence mode was established with excitation at 366 nm for the tetracyclines, 300 nm for enrofloxacin and ciprofloxacin, and 280 nm for marbofloxacin. Limits of detection (LOD) and quantitation (LOQ) with 95% confidence were in the range of 12-25 and 45-95 µg/kg, respectively, in milk samples. Recoveries of target antibiotics from milk samples spiked at three critical levels (50, 100 and 150 µg/kg) ranged from 76 to 105%. More importantly, a mass selective detection (MSD) was established as additional tool for confirmatory purposes. Using the elution-head based TLC-MS interface, the optimized elution flow consisting of acetonitrile/ammonium formate buffer (9/1, v/v) at a rate of 0.3 mL/min enabled time-dependent resolution of analytes from the major interfering compounds, thus circumventing serious ion suppression effects. The established MSD assay also offered high sensitivity (25 µg/kg) for confirmation, meeting Commission Regulation (EU) No 37/2010.

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1 Introduction

The abuse of antibiotics in animal husbandry has led to serious problems for human health associated with bacterial resistances and food safety. Therefore, stringent regulations on their maximum residue limits (MRLs) have increasingly been legislated around the world [1,2]. In light of this background, monitoring antibiotic residues in food stuffs have attracted a great deal of attention during the last decade. Thus, various approaches for the detection and quantification of antibiotics have been reported in the literature [3-8]. Most of these studies focused on similar procedures, i.e., extraction of the homogenized sample by organic solvents (acetonitrile or methanol), solid-phase extraction (SPE) for clean-up, pre-concentration step, derivatization if required, chromatographic separation by high-performance liquid chromatography (HPLC) coupled to mass spectrometry (MS) [8-12]. However, extensive maintenance of the chromatography system is needed to ensure reproducible chromatograms and MS sensitivity. On the other hand, a thorough clean-up of extracts is of great importance to prevent matrix effects, which may markedly undermine throughput and comparability of methodologies. In comparison, planar chromatography, namely high performance thin-layer chromatography (HPTLC) shows marked advantages like parallel separation of many samples. Additionally, sample clean-up can be omitted or greatly reduced, because the plate is disposable, and the chromatography itself is effective solid phase purification [13,14]. Apart from that, with the growing demands in antibiotics analysis, marked interests have been shown to direct couple HPTLC separation with mass spectrometry (MS) analysis. This combination has been the state of the art technique, offering strong confirmation of positive findings that cannot be distinguished by spectrophotometric detections. A literature survey revealed that several reports have been published

describing the developments in HPTLC-MS, as well as their potential and limits [15-22]. For tetracyclines, different MS devices and ionization methods have been published for HPTLC-MS assays. Oka et al. [18-20] applied fast atom bombardment MS combined with a special sample condensation technique on C8 HPTLC plates, which allowed the detection of TCs at 50 µg/kg milk. Apart from that, Crecelius et al. [21] reported a matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) technique employing graphite suspensions on normal phase silica plates modified by ethylenediamine tetraacetic acid (EDTA), which enabled the detection of <10 µg pure standards per zone. MALDI-TOF MS was also used by Meisen et al. [22], who in terms of detectability found C18 plates superior to EDTA modified silica plates. Also just working with standards of TCs, they reported an approximate detection limit of 5 ng/zone. In any case, the applied equipment is generally not available in a routine laboratory of residue analysis, while the recently introduced elution-head based TLC-MS interface can be coupled to any LC-MS system for a rapid and in-situ analysis of residues separated by HPTLC.

In light of this background, an HPTLC method for the simultaneous analysis of four tetracyclines (TCs) and three fluoroquinolones (FQs), which are the most problematic antibiotics residues in European Union [23], was developed and optimized. In keeping up with the concept of a simple, fast, reliable and highly reproducible principles, EDTA modified silica gel HPTLC plates, densitometry in fluorescence mode, and the TLC-MS interface were integral parts of the method. First of all, efforts were made to optimize the parameters of HPTLC separation and fluorescence detection (FLD), which plays a key role in screening tests associated with large number of samples. Besides, marked improvement for the direct coupling of HPTLC and MS was achieved by using an elution-head based extraction step that showed both fine

extractability and tolerance to interferences. The performance of the proposed method was further validated with milk samples. With these methodology progresses, a cost-efficient high-throughput screening of target antibiotics can be realized.

2 Materials and methods

2.1 Chemicals and materials

The antibiotic standards (enrofloxacin EF, marbofloxacin MF, ciprofloxacin hydrochloride CF, tetracycline hydrochloride TC, chlortetracycline hydrochloride CTC, oxytetracycline hydrochloride OTC, and doxycycline hydrochloride DC, all with >95% purity certified by HPLC) were purchased from Sigma-Aldrich (Deisenhofen, Germany). HPTLC glass plates (20 cm × 10 cm) pre-coated with silica gel 60 (No. 1.05641.0001) were from Merck (Darmstadt, Germany). For preliminary experiments, other HPTLC plates with amino silica or RP18 layers were obtained from Merck (Darmstadt, Germany) and Machery Nagel (Dueren, Germany).

Disodium ethylenediaminetetraacetate dihydrate (Na_2-EDTA), anhydrous magnesium sulfate and the HPLC grade solvents chloroform, acetonitrile, and methanol were purchased from Sigma-Aldrich, anhydrous sodium acetate, acetic acid, and ammonium hydroxide solution (25%) from Fluka (Neu-Ulm, Germany). All chemicals were of analytical grade. Ultra pure water was prepared by a Synergy system (Millipore, Schwalbach, Germany). BONDESIL-PSA (40 µm) was obtained from Varian (Darmstadt, Germany). Bovine milk samples (1.5% and 3.5% fat content) of both organic and conventional sources were purchased in a local supermarket.

2.2 Stock solutions and sample preparation

Stock solutions of standards were prepared by dissolving antibiotics in methanol at the concentration of 1 mg/mL. Concerning the poor solubility of FQs, their stock solutions were alkalized by adding 0.5% (v/v) aqueous ammonium hydroxide solution (25%). The stock solutions were further diluted by methanol to a working concentration of 0.01 mg/mL.

The extraction procedure followed the QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) strategy [3,24]. Milk samples (10 g) were weighed into 50-mL polyethylene centrifuge tubes (Sarstedt, Germany), to which 10 mL of an EDTA solution (0.1 mol/L, pH=8.0), 10 mL acetonitrile, and 0.1 mL acetic acid (5% in water) were added. The mixtures were vigorously shaken followed by treatment in an ultrasonic bath for 10 min. Thereafter, 4.0 g anhydrous magnesium sulphate and 1.0 g anhydrous sodium acetate were added; the mixtures were vigorously shaken for 1 min and centrifuged for 5 min at 4000 × *g*. The supernatant was collected and evaporated under a stream of nitrogen; the residue was dissolved in 1 mL acetonitrile. After nylon membrane filtration (0.45 µm), the extracts were ready for HPTLC analysis.

2.3 HPTLC

HPTLC glass plates were pre-developed with methanol, dried at 100 ^{o}C for 20 min, cut in two plates of 10 cm × 10 cm, and stored in a desiccator. A TLC immersion device (CAMAG, Muttenz, Switzerland) was used to achieve an Na_2-EDTA modification of silica gel plates at an immersion speed of 3 cm/s and an immersion time of 5 s; the dipping solution was an aqueous Na_2-EDTA solution (100 g/L adjusted to pH 8.0 by a 20% sodium hydroxide solution). After modification, plates were dried at 120 ^{o}C for 1 h. Appropriate volumes of standard solutions resulting in 5,

15, 30, 50 and 80 ng/zone, and sample extracts (20 µL) were sprayed as 6-mm bands onto the plate by an automatic TLC sampler ATS4 (CAMAG) with 8 mm distance from the button, 14 mm from the left side, and 8 mm distance between the tracks. The plates were allowed to dry shortly, followed by development in the automatic developing chamber ADC 2 (CAMAG) with chloroform/methanol/ammonium hydroxide solution (25%), 60/35/5 (v/v/v) to a migration distance of 45 mm from the lower edge. Chamber saturation was obtained by placing 20 mL mobile phase together with a piece of filter paper into the second trough. The operation time was about 35 min, including chamber saturation (10 min), plate pre-conditioning (10 min), and drying (2 min).

For plate documentation, the DigiStore 2 (CAMAG) consisting of a Reprostar 3 illuminator with a Baumer Optronic DXA252 digital camera was used with the following settings: 2000 ms (366/254 nm) at a gain of 1. For quantitation, the plates were densitometrically measured by a TLC scanner 3 (CAMAG) prior to MS analyses. Tracks were scanned in remission fluorescence mode with the following constant parameters: slit dimension 3 × 0.3 mm, optical system optimized for maximum light, scanning speed 20 mm/s, data resolution 50 µm/step. The excitation wavelength (mercury lamp) for TCs was 366 nm with K400 filter, for EF and CF 280 nm with K320 filter, and for MF 300 nm with K400 filter. Fluorescence excitation spectra were recorded in the range 200-400 nm (deuterium/wolfram lamp); slit dimension 3 x 0.3 mm, optical system optimized for maximum resolution, spectrum scan speed 50 nm/s, data resolution 10 nm/step, optical filter K400. All HPTLC instruments were controlled by WinCats 1.4.5 software.

2.4 HPTLC-mass spectrometry

The HPTLC-MS equipment consisted of an Agilent (Waldbronn, Germany) 1100 modular HPLC system with a quaternary pump, vacuum solvent degasser unit, and a TLC-MS interface (CAMAG) coupled to an Agilent G1956B MSD single quadrupole mass spectrometer equipped with an electro spray ionization (ESI) interface, and was operated by ChemStation B.02.01 SR2 software (Agilent). After densitometry, the zones of interest were slightly marked with a soft pencil under UV illumination (366 nm). Through the TLC-MS interface equipped with an oval shaped elution head, the analyte was extracted from the plate by a flow (0.3 mL/min) provided by the HPLC pump, consisting of 90% acetonitrile and 10% ammonium formate buffer (10 mM + 2% methanol) and lasting for 30 s. The mass spectrometer settings were: capillary voltage 3.0 kV, skimmer voltage 35 V, lens 2.5 V, quadrupole temperature 100 ^{o}C, drying gas temperature 200 ^{o}C, drying gas flow rate 10 L/min and nebulizer gas pressure 25 psig. Total ion current chronograms in full scan mode were recorded from *m/z* 200 to 700 using a fragmentor voltage of 100 V, gain 1, threshold 100, and step size 0.25.

3 Results and discussion

3.1 HPTLC method

Among the various HPTLC layer types, silica gel is the most economic choice for screening purposes. However, the analytes (especially for TCs) displayed strong tendency to form chelate complexes with alkaline earth and transition metal ions, leading to serious tailing effects. The results of preliminary experiments on different layer materials directed our focus on RP18 silica gel and normal silica gel modified by EDTA. The former, with mobile phases containing auxiliary acid (oxalic acid) enabled separation of the target compounds. However, this method was precluded due

to its high cost and significant fluorescence quenching effects on the analytes. Therefore, silica gel-EDTA offering considerable fluorescence enhancement was selected as the stationary phase. As the result of screening tests with different established solvent systems [21,22,25-28], a ternary system of chloroform/methanol/ammonium hydroxide (60/35/5, v/v/v) was identified to give full separation of the seven analytes, as shown in **Fig. 1**. Besides, the applied EDTA concentration for plate modification was found of great importance. By comparison, it was proven that a 10% EDTA solution is necessary for the complete resolution of TCs and FQs (**Fig. 1S**). Moreover, the equilibrium between the plate layer and the chamber atmosphere is highly important for the chromatographic performance as well. Plates that did not equilibrate with the chamber atmosphere caused serious edge effects, leading to distorted zones (**Fig. 1S**). Therefore, to achieve strict control of working conditions, chromatography was carried out with an automated development chamber enabling standardized and reproducible actions of each step.

3.2 Screening and quantitation by HPTLC-FLD

Benefitting from the intensive fluorescence of target compounds, even eye-inspection of plate images offers straightforward judgments (semi-quantitative) at residue levels. Nevertheless, fluorescence densitometry that is much more precise and sensitive was optimized for a quantitative screening [25,26]. Therefore, the fluorescence excitation spectra of the analytes were first recorded to identify the optimal parameters (**Fig. 2)**. Apparently, TCs uniformly exhibit the maximum emission at an excitation wavelength of 366 nm, while optimal wavelengths of excitation for EF/CF and MF were 280 and 300 nm, respectively. These excitation wavelengths showed marked sensitivity and selectivity to the corresponding analyte, as depicted in **Fig. 3**. It is

interesting to note that the UV light options of the TLC scanner show different performances with signal quality. Compared to the deuterium lamp, the mercury lamp offered remarkably better sensitivity, therefore being favored for quantitation.

3.3 Validation

To ascertain reliability in routing screening, quantitative determination with bovine milk matrices was validated. As summarized in **Table 1**, calibration curves (25-400 μg/kg) were established with fine linearity (**Fig. 2S**) and acceptable signal-to-noise ratios within the range that are well below the MRLs. LODs and LOQs were established according to a DIN method with at least 95% confidence [29]. Apparently, quantitation around the MRLs can be done with high statistical reliability. Additionally, precision and accuracy of the method was evaluated according to Council directive 96/23/EC [30]. Since every step of planar chromatography was automatically accomplished, satisfactory with-in laboratory reproducibility (<9%, inter-day) was achieved, revealing good method stability. Based on these data, the detection capability (CCβ) at permission limit was determined as follows, $CC\beta = CC\alpha + 1.64 \times SD_{(at\ MRL)}$, in which $CC\alpha = MRL + 1.64 \times SD_{(at\ MRL)}$. For further evaluation of the method's accuracy, recoveries and repeatabilities of the analytes spiked at 50, 100, and 150 μg/kg were evaluated. As shown in **Table 2**, recoveries obtained from the target compounds spiked into full milk displayed fine consistency, in a relative narrow range of 76-105% with acceptable repeatabilities (<10.5%, intra-day).

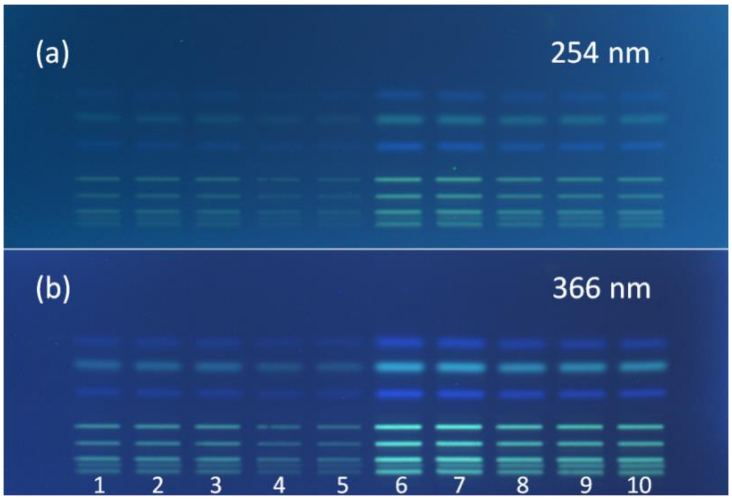


Fig. 1 HPTLC separation of the target antibiotics on an EDTA modified silica gel plate, visualized under 254 nm (A) and 366 nm (B).Track assignments: (1) and (8) whole milk (3.5% fat) spiked at 100 and 200 µg/kg, (2) and (9) skimmed milk (1.5% fat) spiked at 100 and 200 µg/kg , (3) and (10) whole milk (bio, 3.5% fat) spiked at 100 and 200 µg/kg, (4) and (5) standards of 10 ng/zone (6) and 50 ng/zone (7). Standards, hRF: OTC 7, CTC 11, TC 17, DC 23; CF 38, MF 49, EF 58.

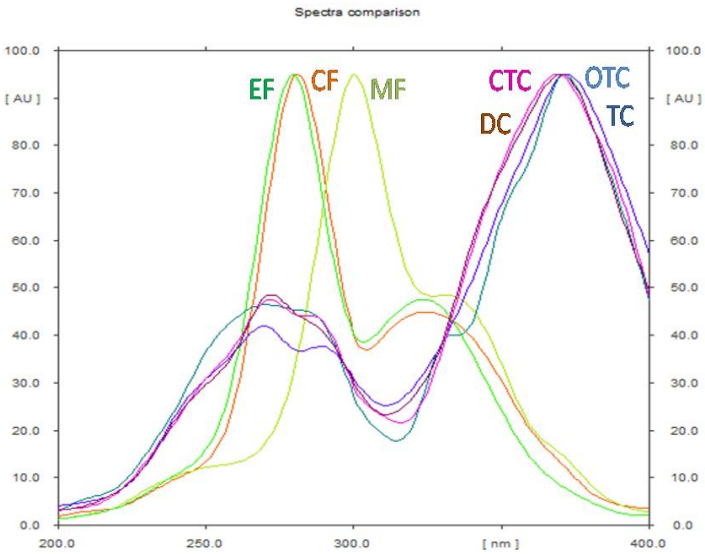


Fig. 2 Fluorescence excitation spectra of the target antibiotics: oxytetracycline OTC, tetracycline TC, doxycycline DC, chlortetracycline CTC, enrofloxacin EF, marbofloxacin MF and ciprofloxacin CF.

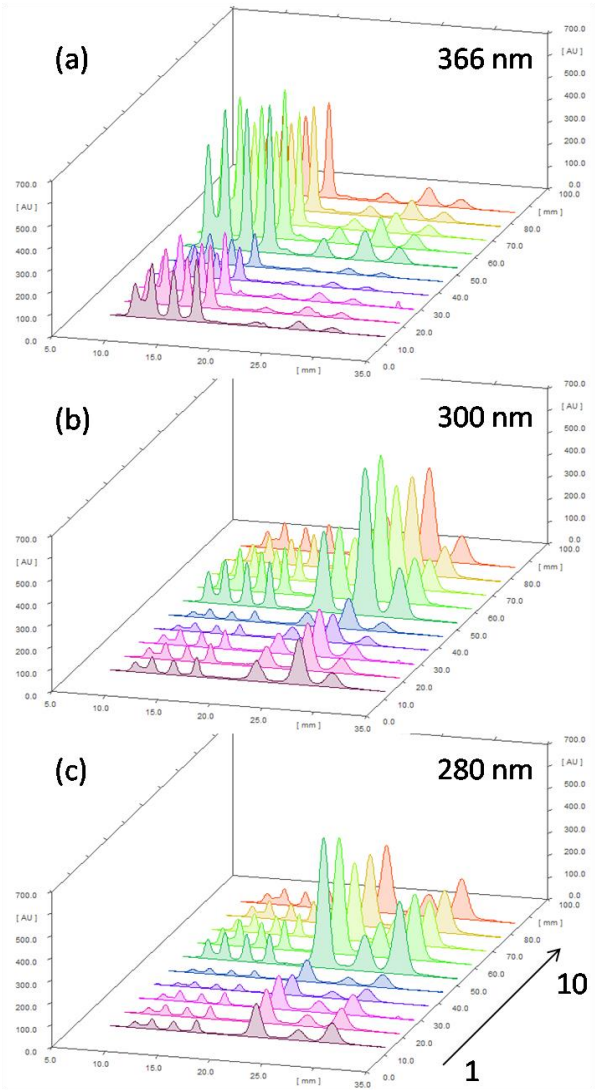


Fig. 3 Fluorescence densitograms of the target antibiotics excited at 280 nm (A), 300 nm (B), and 366 nm (C). Track assignment identical to **Fig. 1**.

3.4 Post-screening confirmation by HPTLC-MS

3.4.1 Selectivity optimization of the extraction flow

The hyphenation of planar chromatography and mass spectrometry can be really helpful in confirming the screening results, offering highly relevant molecular information of the responsible compounds. In this work, the direct combination of HPTLC and electrospray ionization mass spectrometry was facilitated by the TLC-MS interface. Target compounds are eluted from the HPTLC plate and directly introduced into the ion source. That way, both extraction of target compounds and

separation of background impurities responsible for undesired effects (like ion suppression) are enabled. Therefore, in a first attempt the performance of different eluents was tested. Mixtures of methanol and buffer (ammonium formate) were proved to be not suitable for extraction, since they showed no discrimination between analyte and interfering compounds. The co-eluted impurities (mainly EDTA) led to rather strong ion suppressions that could not be compensated by background subtraction (**Fig. 3S**) and clearly visible deposits in the ion source. Marked improvements were achieved by using acetonitrile as eluent, since it hardly dissolves EDTA salts. Therefore, the extraction flow was further optimized, regarding acetonitrile/buffer ratios (**Fig. 4S**). The use of pure acetonitrile suffered from both tailing extraction profiles and poor ionization intensity. The addition of 10% ammonium formate buffer significantly improved the ionization efficiency. As depicted in **Fig. 4**, target antibiotics were immediately eluted from the plate, which took about 0.25 min. Hereafter, the background impurities characterized by *m/z* 227 and a series of signals with a distance of 68 amu were eluted (0.25-0.65 min), and finally EDTA characterized by *m/z* 293 $[M+H]^+$ and *m/z* 315 $[M+Na]^+$. However, further increasing the buffer concentration did not show any improvement, but contrarily tailing effects appeared again, which might be attributed to increasing co-elution of EDTA.

3.4.2 Diagnostic ion species defining

With the optimized conditions, elution procedure can be considered a micro-chromatography taking place inside the elution head. This time-resolved mass detection offers a sufficient “window space” specifically for target compounds in the elution stream, as shown in **Fig. 5** and **Fig. 5S**.

Table 1 Quantitative data of HPTLC-FLD for the target antibiotics.

Analyte	MRL μg/kg[a]	Detection Parameters	LOD[b] [μg/kg]	LOQ[b] [μg/kg]	CCα[c] [μg/kg]	CCβ[c] [μg/kg]	Calibration[d] [μg/kg]	*S/N* at 25 μg/kg[e]	Linear regression[d]		
									Slope	Intercept	R^2
OTC	100	366/<400nm	25	95	107	115	25-400	54	88	519	0.999
CTC	100	366/<400nm	18	67	110	120	25-400	72	133	520	0.999
TC	100	366/<400nm	16	64	108	116	25-400	87	141	687	0.999
DC	100	366/<400nm	25	93	108	116	25-400	80	151	777	0.999
EF	100	280</320nm	20	77	114	128	25-400	22	134	86	0.999
MF	75	300</400nm	19	61	87	99	25-400	61	327	194	0.999
CF	100	366/<400 nm	12	45	112	124	25-400	65	252	410	0.999

[a] Values for bovine milk samples in China and European Union [1].

[b] LOD and LOQ were calculated according to a DIN method with at least 95% confidence [29].

[c] $CC\alpha=MRL+1.64\times SD_{(at\ MRL)}$, $CC\beta=CC\alpha+1.64\times SD_{(at\ MRL)}$; SD values are from 18 replicates (spiked at 1 MRL) within three days [30].

[d] Linear regression was based on 5 matrix-matched points of serial concentrations (25, 75, 150, 250 and 400 μg/kg).

[e] values were peak height of signal.

Table 2 Recoveries and repeatabilities for the target antibiotics spiked into full milk.

Analyte	Recovery [%][a]			Repeatability [% RSD][b]		
	50µg/kg	100µg/kg	150µg/kg	50µg/kg	100µg/kg	150µg/kg
OTC	88	88	76	8.1	7.9	7.3
CTC	78	89	77	6.4	6.5	8.3
TC	83	89	77	7.6	6.6	8.1
DC	91	90	78	8.4	3.1	7.8
EF	105	93	84	7.5	3.2	10.5
MF	102	93	83	6.8	3.0	8.7
CF	97	84	76	8.0	3.6	8.9

[a] mean values of 6 replicates.

[b] RSD (%) values for mean results corresponding to each analyte with 6 replicates.

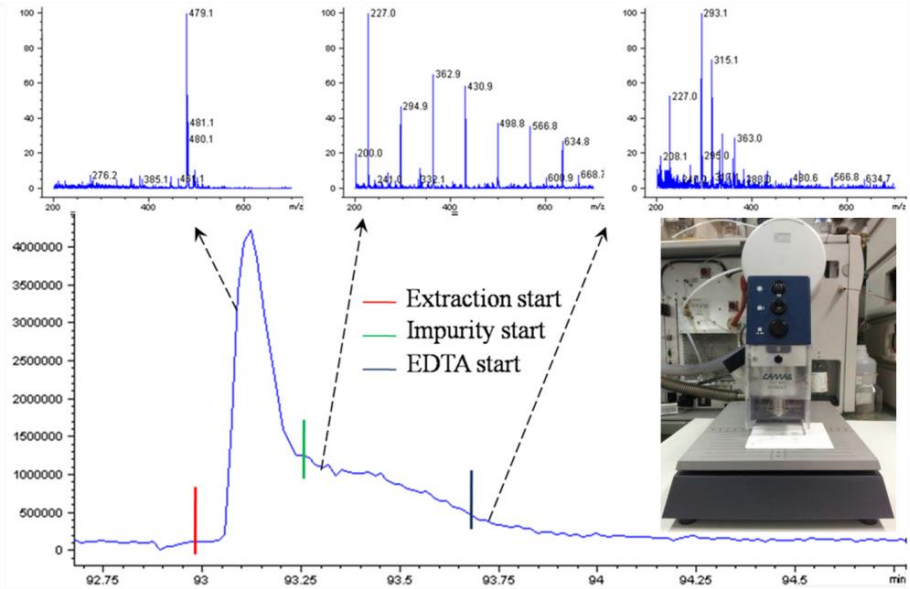
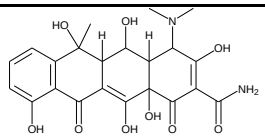
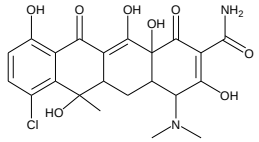
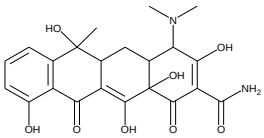
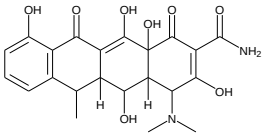
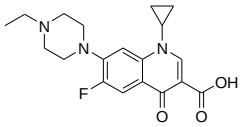
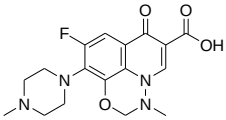
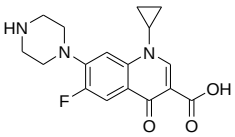


Fig. 4 Typical HPTLC-MS elution profile of target compounds from the EDTA modified silica gel plate, recorded as total ion current in the ESI positive mode, exemplarily shown for a doxycycline standard. Elution was performed with acetonitrile/ammonium formate buffer (90/10) at a flow rate of 0.3 mL/min. Mass spectra were extracted at the top of the peak, the beginning of the shoulder, and at the tail, respectively; inserted is a photography of the TLC-MS interface setup.

Table 3 HPTLC-MS data obtained for the target antibiotics.

Analyte	Elementary composition	Elemental composition	diagnostic ions (m/z)	
			ESI positive	ESI negative
OTC		$C_{22}H_{24}N_2O_9$	461.1 $[M+H]^+$ 483.1$[M+Na]^+$	459.1 $[M-H]^-$ 481.1$[M-2H+Na]^-$
CTC		$C_{22}H_{24}N_2O_8$	479.1 $[M+H]^+$ 501.1$[M+Na]^+$	477.1 $[M-H]^-$ 499.0$[M-2H+Na]^-$
TC		$C_{22}H_{24}N_2O_8$	445.1 $[M+H]^+$ 467.1$[M+Na]^+$	443.1 $[M-H]^-$ 465.1$[M-2H+Na]^-$
DC		$C_{22}H_{23}ClN_2O_8$	445.1 $[M+H]^+$ 467.1$[M+Na]^+$	443.1 $[M-H]^-$ 465.1$[M-2H+Na]^-$
EF		$C_{19}H_{22}FN_3O_3$	360.2 $[M+H]^+$ 382.2$[M+Na]^+$	394.1 $[M+HCOO]^-$
MF		$C_{17}H_{19}FN_4O_4$	363.1 $[M+H]^+$ 385.1$[M+Na]^+$	407.1 $[M+HCOO]^-$
CF		$C_{17}H_{18}FN_3O_3$	332.1 $[M+H]^+$ 354.1$[M+Na]^+$	376.1 $[M+HCOO]^-$

On this basis, diagnostic ions were established for target compounds. As shown in **Fig. 6S**, full-scan mass spectra recorded in the ESI positive mode generally provided the protonated molecules (partly accompanied by sodium adducts) with highest abundances for both TCs and FQs. In the ESI negative mode, the TCs produce the deprotonated molecules, but only with very low intensities; while the FQs noticeably showed a strong signal of formate adducts. Despite of these differences, the

characteristic signals from both mass polarities are supposed to be of value for qualitative confirmations (**Table 3**).

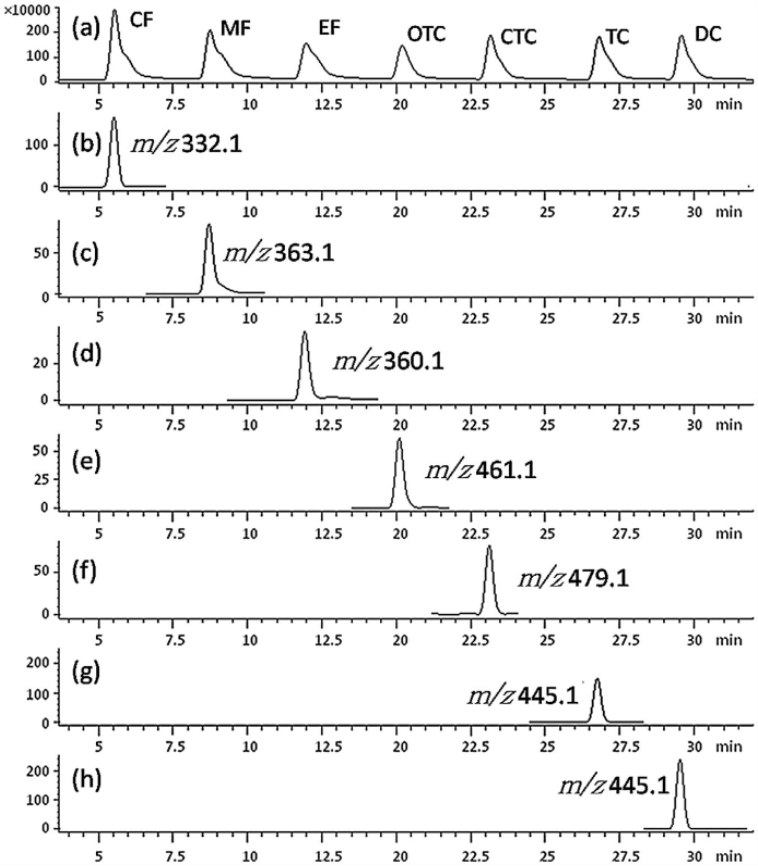


Fig. 5 HPTLC-MS elution profiles of the seven antibiotic standards recorded as total ion current chronograms in the ESI positive mode (a) and the extracted ion tracks (b-h) of the respective protonated molecules. Each elution was carried out in intervals of about 1.5 min.

From a practical point of view, the major problem for HPTLC-MS as confirmatory tool is associated with the location of the target zone. Therefore, 5 ng/zone that is just visible under UV light (366 nm) was used as the critical concentration for assessing the sensitivity. Measurements were carried out in parallel on pure and matrix-matched standard tracks. As shown in **Fig. 6** and **Fig. 7S**, the protonated molecules could readily be identified, even at the presence of milk matrix.

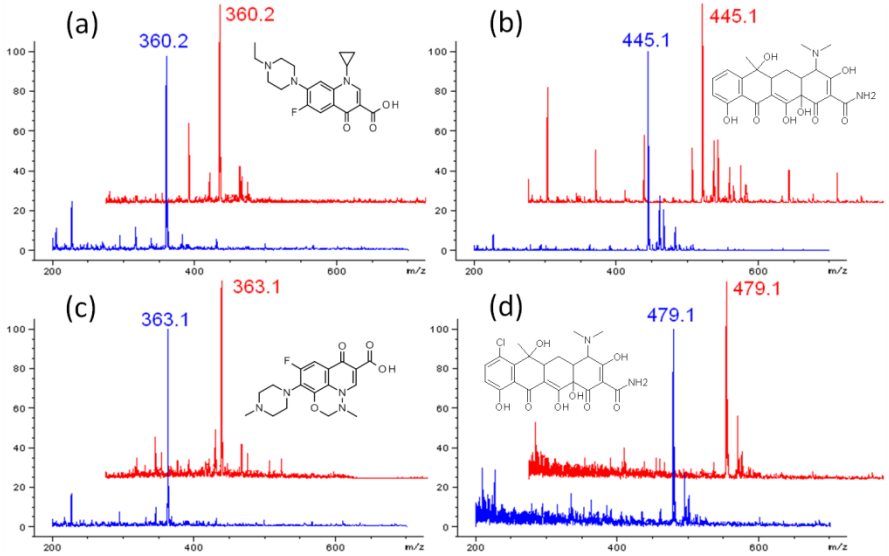


Fig. 6 Assessment of matrix-effects on the full-scan mass spectra (ESI+) of fourrepresentative analytes, enrofloxacin (a), tetracycline (b), marbofloxacin (c), and chlortetracycline (d), at threshold concentrations (25 μg/kg); pure standards in front, matrix-matched standards in background.

4 Conclusions

The developed method based on a simple and rapid extraction without any further clean-up of extracts, HPTLC separation, densitometry, and HPTLC-MS analyses was systematically optimized. Improved densitometry measurements in fluorescence mode offer the primary identification and quantitation of target compounds, which is extremely suitable for screening purposes. Besides, the introduction of an elution-head based extraction step coupling HPTLC and MS enables the clear confirmation of positive findings of TCs and FQs in milk from EDTA modified silica gel plates by a standard ESI-MS system. Apart from that, the most important merit of the established method is its simplicity and rapidity as compared to GC and HPLC analyses. Assuming 15 samples applied together with a set of calibration standards on a 20 cm × 10 cm plate, the chromatographic run time per sample is about 2 min, consuming only 2 mL of solvents. Because the plates are disposable, the sample preparation steps can be greatly simplified and little attention is required to the

maintenance of instruments and stationary phase. Particularly in MS detection, only zones of interests were extracted, while the major part of matrix compounds stay on the plate, saving a lot of MS maintenance. Hence, this method can be considered a highly efficient tool for preliminary screening, whereupon only the few percent of positive findings have to be confirmed by LC-MS/MS analyses.

5 Acknowledgements

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7 Supplementary data

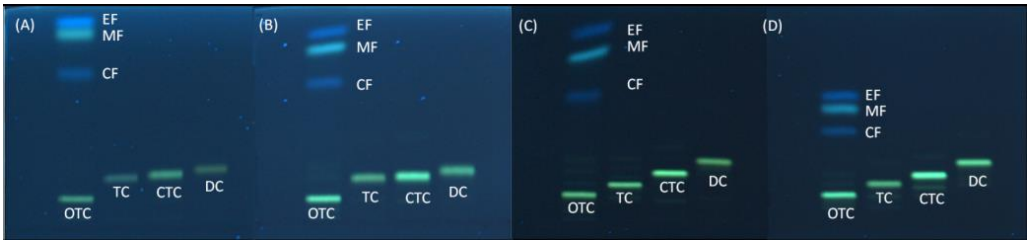

Fig. 1S The chromatographic resolution of the target antibiotics on silica 60 plates modified with an aqueous solution of Na_2-EDTA at concentrations of 4% (A), 6% (B), and 10% (C), respectively. Effect of pre-conditioning on the chromatographic behavior of fluoroquinolones (D).

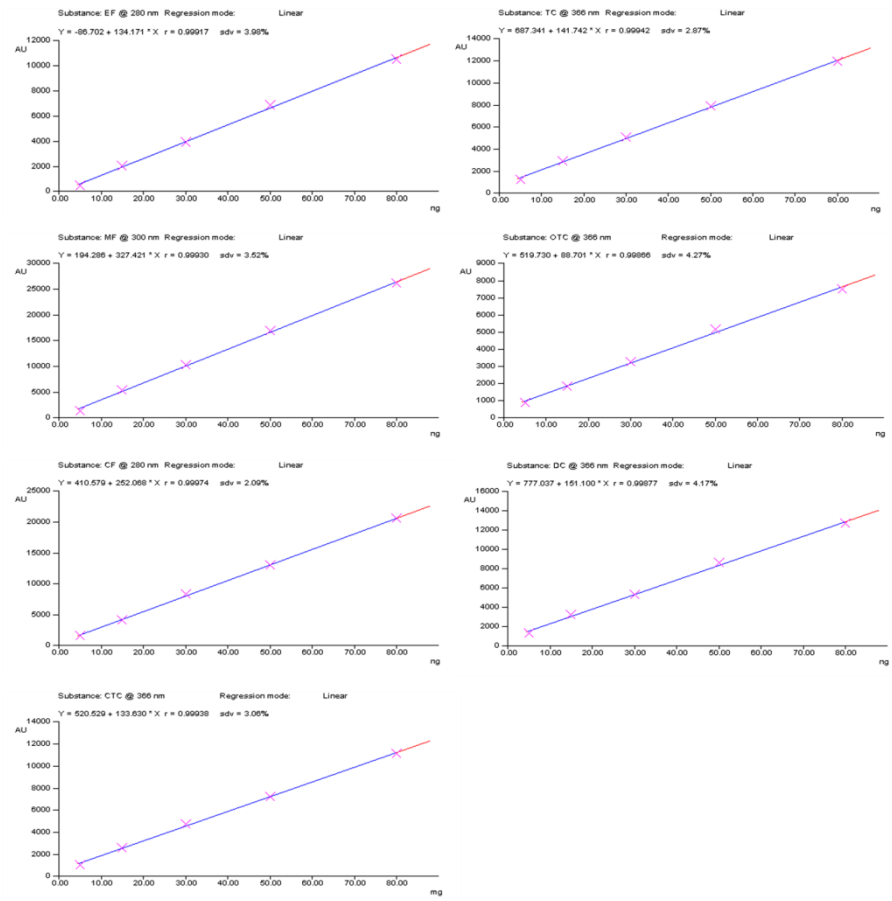

Fig. 2S Linear regressions of the seven antibiotics under study, based on five matrix-matched points of serial concentrations (25, 75, 150, 250 and 400 µg/kg).

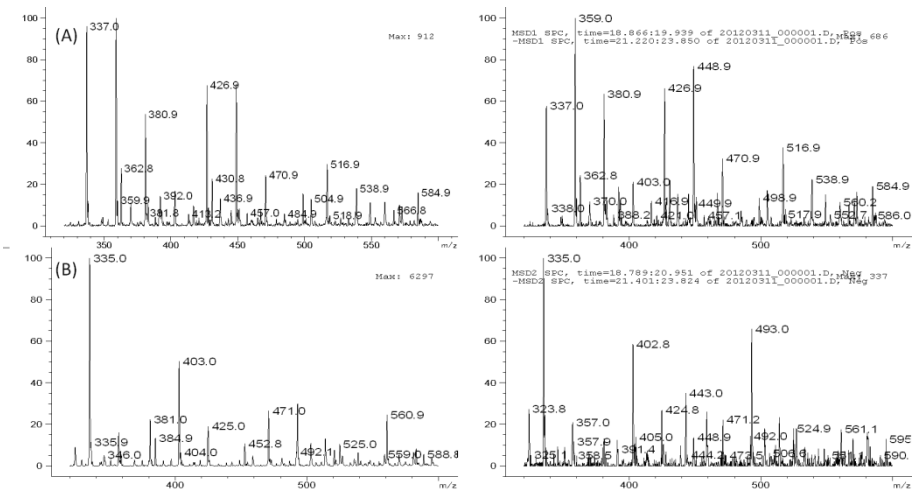

Fig. 3S Mass spectra of CTC eluted by methanol/buffer (90/10) at a flow rate of 0.2 mL/min, recorded in ESI^+ (A) and ESI^- (B), and the background subtracted results (right).

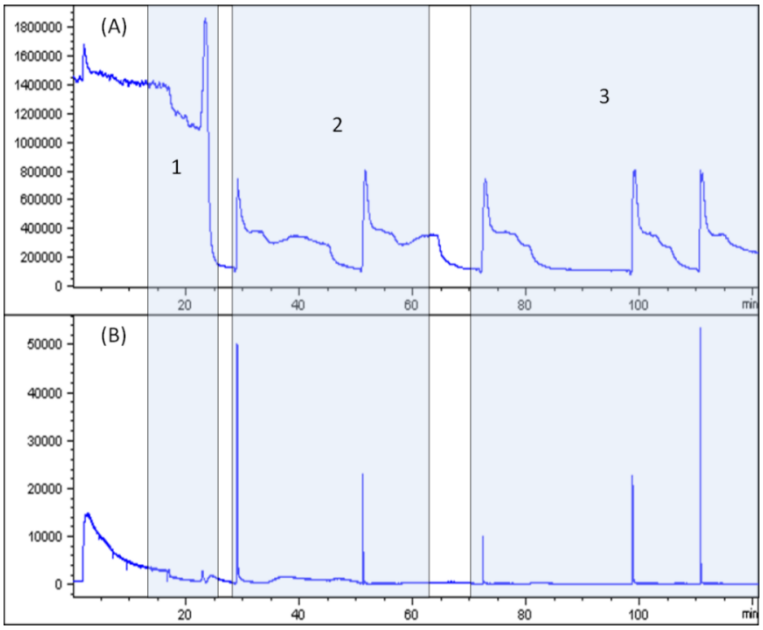

Fig. 4S Flow formula optimization for target compound elution by the TLC-MS interface, exemplarily shown for chlortetracycline recorded as total ion current (100-700 *m/z*) (A) and in selected ion monitoring 461.0 *m/z* (B); flow conditions: acetonitrile (1), acetonitrile/buffer (80/20) (2), acetonitrile/buffer (90/10) (3).

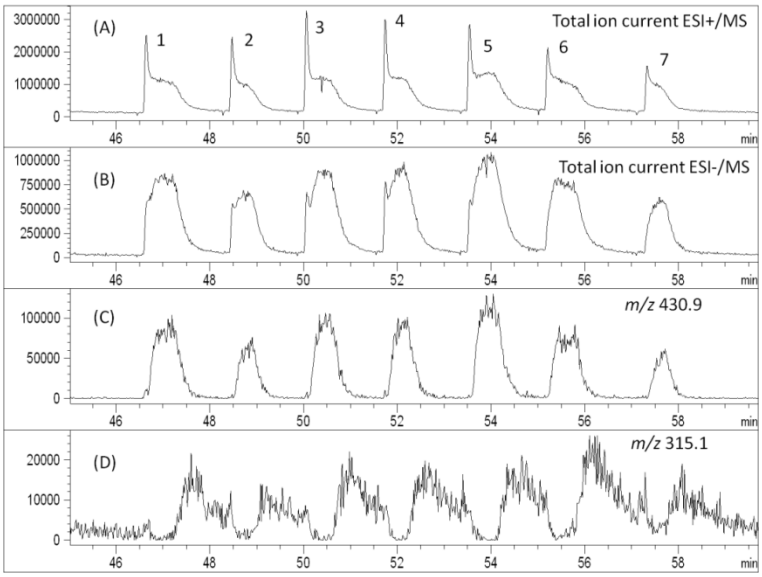

Fig. 5S TLC-MS elution profiles of studied antibiotics including enrofloxacin (1), marbofloxacin (2), ciprofloxacin (3), oxytetraycyline (4), chlortetracycline, tetracycline (6), and doxycycline (7), in total ion current chronograms in (A) ESI^+/MS and (B) ESI^-/MS, and extracted ion chronograms (in ESI^+/MS) of the main background impurities (C-D).

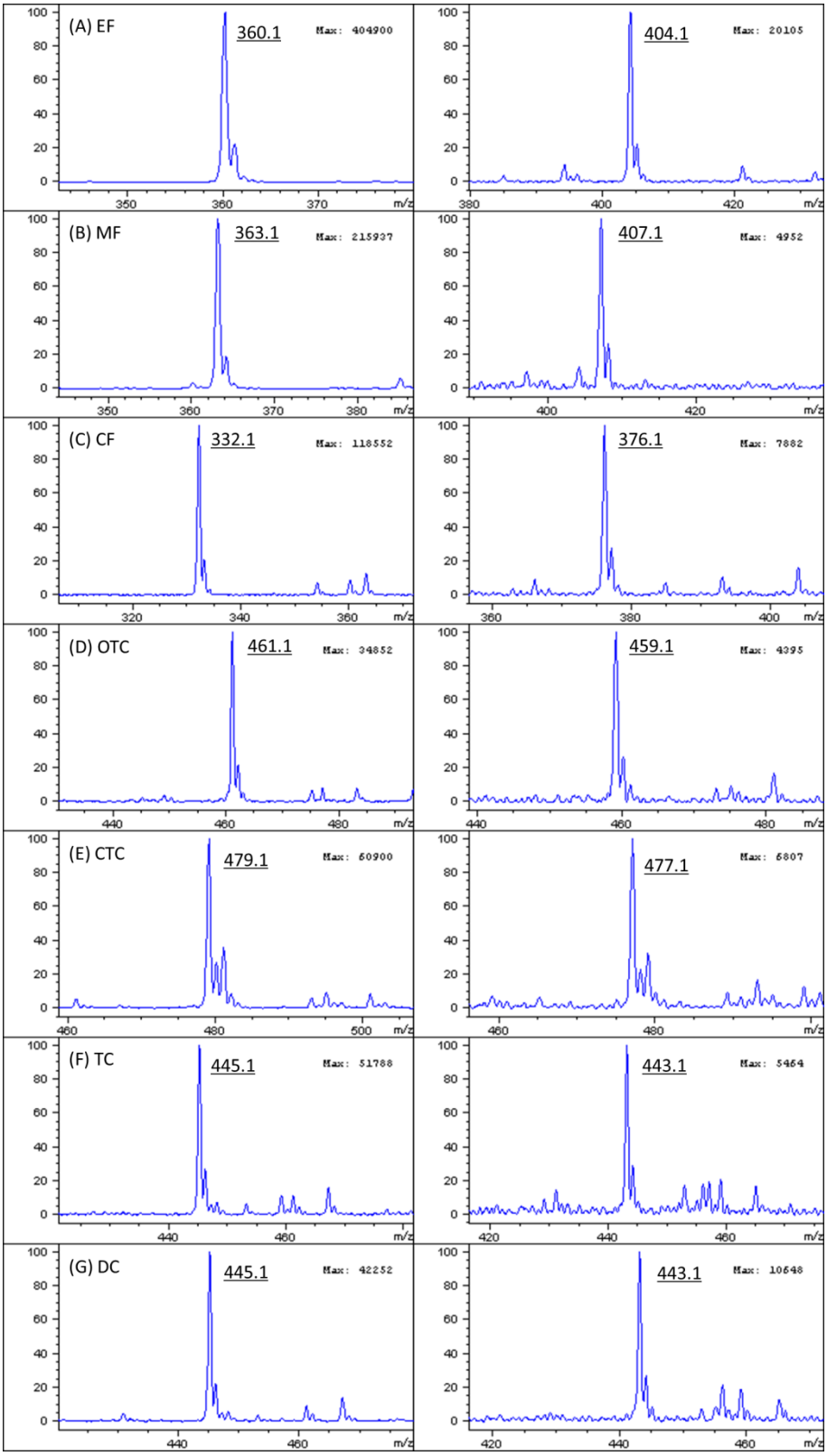

Fig. 6S Mass spectra (partly shown) of TCs and FQs, separated on an EDTA modified silica gel plate, recorded in both ESI positive (left) and ESI negative mode (right): enrofloxacin (A), marbofloxacin (B), ciprofloxacin (C), oxytetraycyline (D), chlortetracycline (E), tetracycline (F), and doxycycline (G).

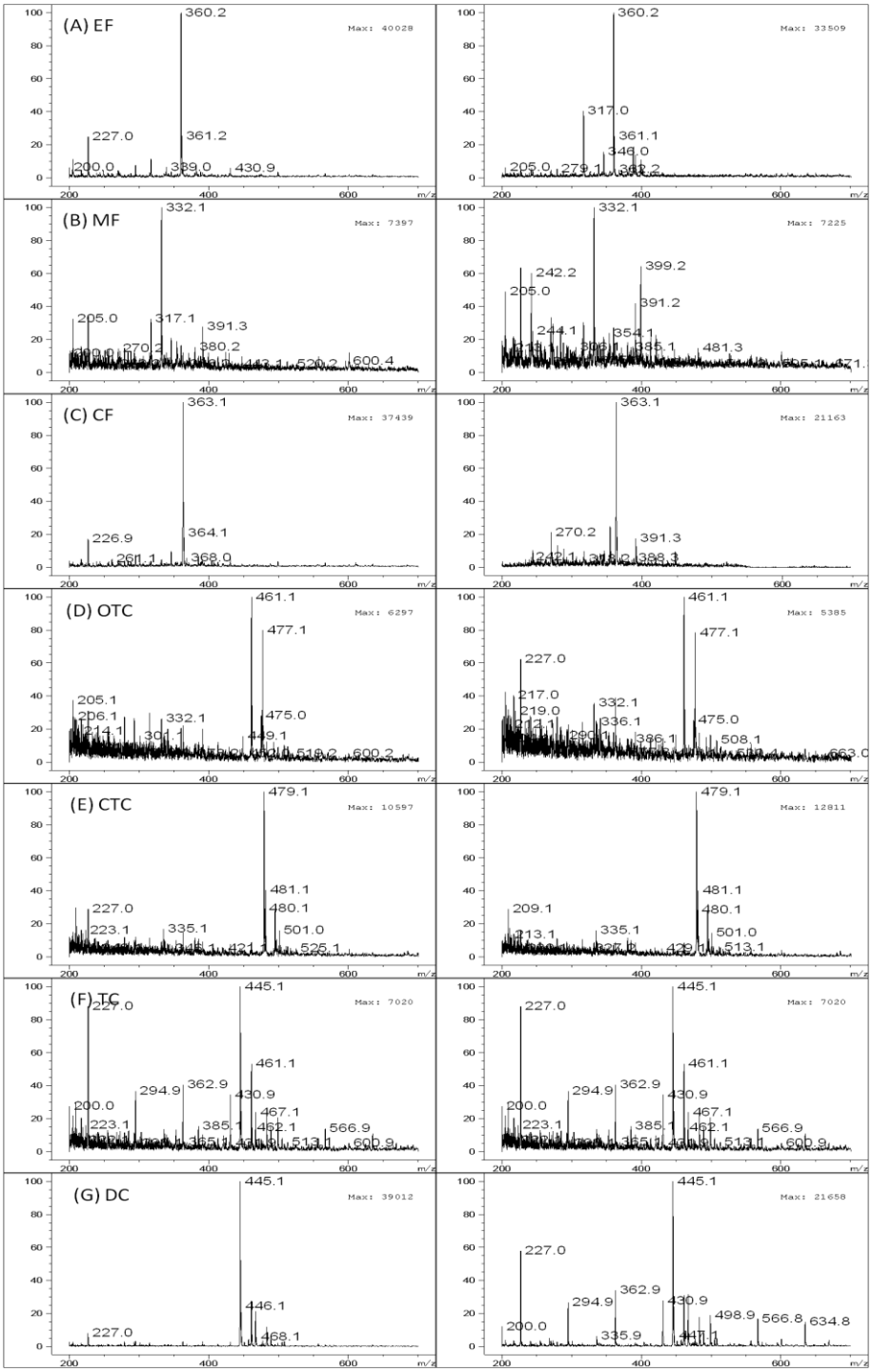

Fig. 7S TLC-ESI$^+$/MS spectra of target compounds at 5 ng/zone: (A) enrofloxacin, (B) ciprofloxacin, (C) marbofloxacin, (D) oxytetracycline, (E) chlortetracycline, (F) tetracycline, (G) doxycycline; pure standards (left), in the presence of whole full milk matrix (right).

CHAPTER III

Rapid and selective determination of multi-sulfonamides by high-performance thin layer chromatography coupled to fluorescent densitometry and electrospray ionization mass detection

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Key words

Sulfonamides, Fast screening, HPTLC, Fluorescence densitometry, TLC-MS interface, Mass spectrometry

Abstract

In the European Union (EU), sulfonamides are among the most widely administrated groups of antibiotics in animal husbandry. Therefore, monitoring their residues in edible animal tissues plays an important role in the EU food safety framework. In this work, a simple and efficient method for the rapid screening of twelve prior sulfonamides frequently prescribed as veterinary drugs by high-performance thin-layer chromatography (HPTLC) was established. Sample extracts obtained with acetonitrile were tenfold concentrated and applied to HPTLC without any further cleanup. Following separation and fluram derivatization, sensitive and selective quantitation of the analytes can readily be accomplished with fluorescent densitometry. Limits of detection and quantitation were 15-40 and 35-70 µg/kg, respectively. Additionally, a confirmative detection by HPTLC-electrospray ionization mass spectrometry (HPTLC-ESI/MS) was optimized, offering straightforward identification of target zones. Therefore, the risk of potential false positive findings can efficiently be reduced. The method was validated to meet the enforced commission regulation (EU) No. 37/2010, regarding different matrix complexities (bovine milk, porcine liver and kidney).

1 Introduction

Sulfonamides (SAs) are referred to a group of synthetic compounds characterized by a common *p*-amino-benzene sulfonamide moiety. In the last decade, this drug group was among the most commonly used antibiotics in veterinary medication and to a lesser extent for human prescriptions. According to the data analyses generated from EU surveillance programs of ten major EU member states on the sales of veterinary antibacterial agents, SAs almost were in the second position, right after tetracyclines [1]. It is notable that this type of compounds shows considerable stability, so that they cannot easily be converted to safe degradation products by metabolic processes [2,3]. Due to their well documented adverse effects like acute allergies for instance, strict regulations regarding SAs maximum residues level (MRL) have been established in the EU and many other countries [4-6]. To enforce the administrative demands, most EU member states collaborate and consolidate extensive monitoring and surveillance programs. In these programs, the fundamental question is how to satisfy the demands of high throughput, sensitivity and cost-efficiency in a large-scale screening of SAs residues, which is especially challenging in animal products in view of the vast number of matrix compounds. In Germany, for example, ≥2% of all commercially slaughtered calves and ≥0.5% of all other commercially slaughtered hoofed animals must be officially sampled and analyzed for residues, according to a national regulation [7]. Practically noteworthy, remarkably low rates of non-compliant samples were revealed by the EU monitoring programs. Concerning SAs, for instance, non-compliant results exemplarily only accounted for 0.08% for the categories bovines and pigs, as reported by EU member states in 2010 [8]. Thus, a great effort was undertaken to analyze the huge amount of compliant samples, keeping in mind that only low percentages of samples were positive.

Reviews on the methods dealing with residue analysis of SAs revealed that a large variety of techniques have been reported in this field, each showing advantages and limitations in specific aspects [9-18]. Though displaying remarkable merits, the efficiency of LC-MS methods with SAs screening is far from satisfactory, because they highly demand sample clean-up. On the other hand, microbial growth inhibition assays, generally playing a key role in veterinary drug screening, almost lack in sensitivity, not being able to detect residues of SAs at the tolerance limits. This dilemma leads to the likelihood of misinterpretation and false-negative reports [8,19].

Against this background, the potential of the modern HPTLC was deeply underestimated among the reviews. On the contrary, HPTLC has become a full-scale analytical technology and a highly valuable platform for chemical screening, efficiently linked to plate image inspection and sensitive detection methods. Therefore, HPTLC should be highly appreciated for screening purposes. Additionally, direct linking with mass spectrometry provides a powerful analytical tool to substantially expand the scope of detection that can be coupled to HPTLC [20-23]. Especially, the elution-head based TLC-MS interface shows considerable improvements in various aspects. This simple and easily controlled sampling approach can be readily applied in routine screening, offering straight forward identification of zones and, therefore, rapid confirmation of suspected positive-findings [24,25].

In this study, an HPTLC method integrated with fluorescence densitometry (FLD) and elution-head based electrospray ionization mass spectrometry (ESI/MS) was developed and optimized for the preliminary screening of twelve representative SAs at their EU MRLs. In this regard, the aim was to provide timely and reliable screening data from complex matrices, including quantitation and confirmation. The established

method was successfully applied to spiked bovine milk and porcine kidney and liver, which are the hot-spots and headaches of traditional LC-MS approaches.

2 Experimental

2.1 Chemicals and materials

Analytical standards of studied SAs (sulfadoxin SDX, sulfadiazine SDZ, sulfamethazine SMZ, sulfanilamide SNMD, sulfamethiozole SMTZ, sulfachloropyridazine SPDZ, sulfathiazole STAZ, sulfapyridine SPD, sulfamerazine SMRZ, sulfisoxazole SIXZ, sulfaquinoxaline SQXL, sulfacetamide SCTD), all with a purity >95%, and acetonitrile, ethylacetate, and methanol, all HPLC grade, and magnesium sulfate of analytical purity were purchased from Sigma-Aldrich (Steinheim, Germany). Fluram of 98% purity, ammonium hydroxide solution (28%), ammonium formate, and anhydrous sodium acetate of analytical purity were from Fluka (Darmstadt, Germany). Ultra pure water was prepared by a Synergy system (Millipore, Schwalbach, Germany).

HPTLC silica gel 60 F_{254} plates (20 cm × 10 cm) No.1.05641.0001 were supplied by Merck (Darmstadt, Germany). Before using, all plates were washed by pre-developing with methanol, dried in an oven at 120 ^{o}C for 20 min, wrapped in aluminum foil, and stored in a glass container to prevent contamination.

2.2 Standard solutions

Separate stock solutions of each SA (0.05 mg/mL) were prepared in methanol and stored at -20 ^{o}C. Working solutions for spiking and calibration were freshly prepared by equally mixing 200 µL of the stock solutions within the same sub-group (group 1:

SPD, SMRZ, SIXZ, STAZ, SQLX, SMTZ; group 2: SNMD, SDZ, SDX, SPDZ, SMZ, SCTD), resulting in concentrations of 0.01 mg/1.2mL.

2.3 Sample preparation

Blank bovine milk, porcine kidney and liver samples of organic sources were purchased in a local supermarket. Kidney and liver samples were manually sliced before extraction. For extraction, 10 g food samples were homogenized with 10 mL water in a MediFASTH 2 homogenizer (Sam-Sol, Bahlingen a.K., Germany) for 2 min. Artificial contamination of blank homogenates was achieved by adding 0.5, 1 and 2 μg analytes (namely, 60, 120,or 240 μL working solutions, respectively), resulting in 0.5-, 1-, or 2-folds the MRL (100 μg/kg). The homogenates were transferred into 50-mL polyethylene centrifuge tubes (Sarstedt, Germany) and extracted with 10 mL acetonitrile. After vigorously shaking by hand for 1 min, 4 g anhydrous magnesium sulfate and 1 g sodium acetate were added. The tubes were immediately shaken for another min and subjected to centrifugation at 4000 × *g* for 5 min, while the temperature was controlled at 15 ^{o}C. The supernatants were pipetted into ampoules and evaporated at room temperature under a stream of nitrogen. The final residues were redissolved with 1 mL acetonitrile, followed by nylon membrane filtration (0.45 μm) prior to HPTLC sampling.

2.4 HPTLC

Appropriate volumes of standard mix solutions resulting in 5, 15, 25, 40 and 50 ng/band and sample extracts (20 μL) were applied as 6-mm bands by the Automatic TLC sampler 4 (CAMAG, Muttenz, Switzerland). The sampling started 15 mm from the left side and 8 mm from the bottom of plates with the automatically setting of

band distance. Application conditions: filling speed 10 μL/s, dosage speed 150 μL/s, rinsing (with methanol) vacuum time 4 s, filling vacuum time 1 s, and rinsing cycles 1. The plates were developed with a mobile phase consisting of 8 mL ethyl acetate, 2 mL methanol and 0.1 mL 28% ammonium hydroxide solution. An automatic developing chamber (ADC 2, CAMAG) was used with the following settings: 30 s pre-drying, 1 min humidity control (33% relative humidity with saturated magnesium chloride), 5 min tank saturation with mobile phase, 5min plate pre-conditioning, 60 mm migration distance, 3 min post-chromatography drying. To remove residual ammonia completely, the plates were dried at 100 ^{o}C on a TLC Plate Heater III (CAMAG) for 5 min, and then cooled to room temperature for 2 min. Post-chromatographic derivatization was performed by dipping the plate into a solution of fluram (10 mg in 100 mL acetone) using a TLC Immersion Device III (CAMAG) with a vertical speed of 2 cm/s and 2 s immersion time. Thereafter, the plate was heated at 100 ^{o}C for 5 min on the plate heater.

Digital documentation of the developed plates was carried out with a TLC Visualizer (CAMAG) both before and after derivatization under 254 and 366 nm, respectively. Images of 0.10 mm/Pixel resolution were captured by a Baumer Optronic DXA 252 digital camera. Then, the derivatized plates were densitometrically evaluated with a TLC Scanner 4 (CAMAG) in fluorescence mode with the general settings: slit dimension 3.00 × 0.30 mm (Micro), optical system for maximum light, scanning speed 20 mm/s, data resolution 50 μm/step. Parameters for fluorescence excitation spectrum recording: deuterium/wolfram lamp, scanning range 250-450 nm, optical filter K540; parameters for quantitation: mercury lamp, λ_{ex} 400 nm, optical filter K400. Data acquisition and processing was done winCATS software, version 1.4.5.2027 (CAMAG).

2.5 HPTLC-mass spectrometry

Zones of interest were located under UV light illumination at 254 nm and 366 nm for non-derivatized and derivatized plates, respectively, and marked with a soft pencil. Through the TLC-MS interface (CAMAG) equipped with an oval shaped elution head, analytes on the plates were extracted with eluent provided by a quaternary 1100 HPLC pump (Agilent) at the rate of 0.2 mL/min for 60 s. For non-derivatized plates, the eluent consisted of acetonitrile/20 mM ammonium formate buffer (7/3, v/v), and for derivatized plates of methanol/20 mM ammonium formate buffer (7/3, v/v). The mass spectrometric evaluation of zone extracts was simultaneously performed with a G1956B MSD single quadrupole mass spectrometer equipped with an electrospray ionization (ESI) interface (Agilent, Waldbronn, Germany), operated by ChemStation B.02.01 SR2 software. Full scan MS data acquisition was carried out in both positive and negative mode with following settings: capillary voltage 3.0 kV, skimmer voltage 35 V, lens 2.5 V, quadrupole temperature 100 ^{o}C, drying gas temperature 250 ^{o}C, drying gas flow rate 10 L/min and nebulizer gas pressure 25 psig. Spectra were recorded in the ranges of m/z 150-400 (non-derivatized plates) and m/z 400-650 (derivatized plates), with fragmentor voltage 100 V, gain 1, threshold 100, and step size 0.25.

3 Results and discussion

3.1 HPTLC method

Among various tested stationary phases, the separation of SAs was found to be optimal on normal silica gel plates that are the most economic choice for screening. As the result of trials for mobile phase optimization, a solvent system consisting of ethyl acetate/methanol/ammonium hydroxide solution (28%) offered the best

performance for the targets to be separated from co-extracted matrix compounds (**Fig. 1**). Therefore, the optimized chromatography system was further evaluated by being transferred to matrix-matched runs. Considering the limited separation number, the twelve target SAs were evenly divided into two sub-groups (group 1: SPD, SMRZ, SIXZ, STAZ, SQLX and SMTZ; group 2: SNMD, SDZ, SDX, SPDZ, SMZ and SCTD). As shown in **Fig. 2**, a broad window space for the target compounds was established with the selected mobile phase, while the major interferences from the food matrix were either left behind or pushed to the solvent front. Apparently, all target compounds can be resolved with insignificant background interferences.

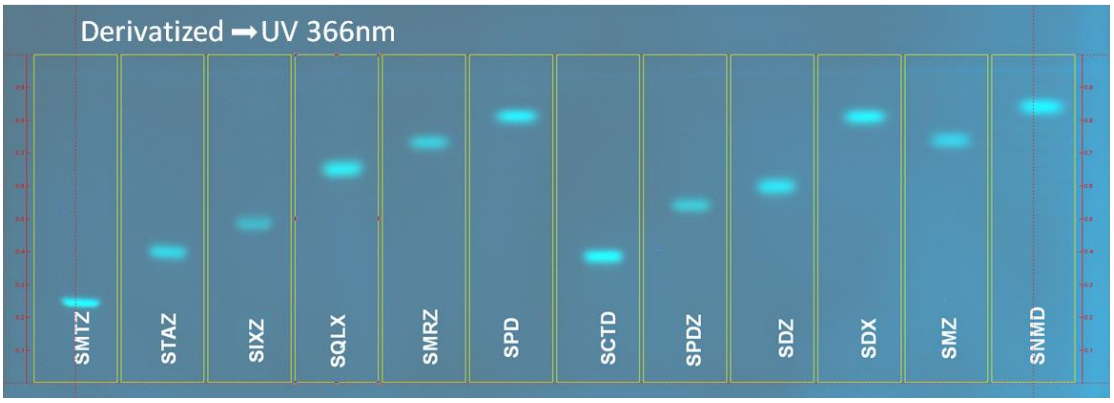


Fig. 1 Separation of studied SAs (40 ng/zone) on a HPTLC silica F_{254} plate derivatized by fluram; standards hR_f: SMTZ 20, STAZ 30, SIXZ 34, SQLX 42, SMRZ 47, SPD 53, SCTD 19, SPDZ 28, SDZ 31, SMZ 39, SDX 46, SNMD 53.

3.2 Derivatization and quantitation by HPTLC-FLD

Although the presence of SAs at >20 ng/zone could be visually detected under UV illumination (254 nm), reliable location and quantitation of the target zones was vulnerable by food matrix compounds that were UV absorptive as well (**Fig. 2a and b**). To improve both sensitivity and selectivity, fluram as a highly specific reagent was used to fluorescently label the common *p*-amino-benzene sulfonamide group of the

SAs. The molecular mechanism behind the derivatization has been well elucidated, but the optimal reaction conditions reported in literature were rather inconsistent [9, 26-29]. In consideration of the usage of ammonium hydroxide solution as tailing reducer in the mobile phase, plates after development must be sufficiently heated to completely remove ammonia residues. Besides, it was found that a short heating (at 100 ^{o}C) drastically accelerated the derivatization being complete within a few minutes. The derivatized SAs selectively displayed greenish fluorescence on a blue background, when inspected under UV 366 nm (**Fig. 2c and d)**.

To identify the optimal parameters for fluorescence densitometry, the fluorescence excitation spectra of the derivatized SAs were profiled. From **Fig. 3a and b**, it becomes apparent that they uniformly exhibit maximum emission at an excitation wavelength of 400 nm, which was not affected by possibly co-migrated food matrix compounds. Accordingly, 400 nm was used as excitation wavelength and the resulting fluorescence signal as quantifier for FLD evaluation. As shown in **Fig. 3c and d**, detected signals of analytes showed high signal-to-noise ratios at the levels of interest.

3.2.1 Method validation

In this study, the enforced EU commission regulation No. 37/2010 pre-defining tolerance limits of veterinary drug residues were used as criteria for assessing method performances [4]. Three critical levels of 50, 100 and 200 μg/kg that were 0.5-, 1- and 2-fold the MRL (100 μg/kg) specified for all SAs were examined as target concentrations. First, precision performances of the method were calculated according to Council directive 96/23/EC [29]. As shown in **Table 1**, the standardized HPTLC procedure resulted in satisfactory within-lab reproducibilities of <15% RSD. Based on these data, the detection capability (CCβ) at permission limit was determined as

follows, CCβ=CCα+1.64×$SD_{(at\ MRL)}$, in which CCα=MRL+1.64×$SD_{(at\ MRL)}$. As the result, relatively low CCα (103-118 μg/kg) and CCβ (107-136 μg/kg) values were achieved regardless of the food matrices. Therefore, screening results generated with this method are statistically reliable.

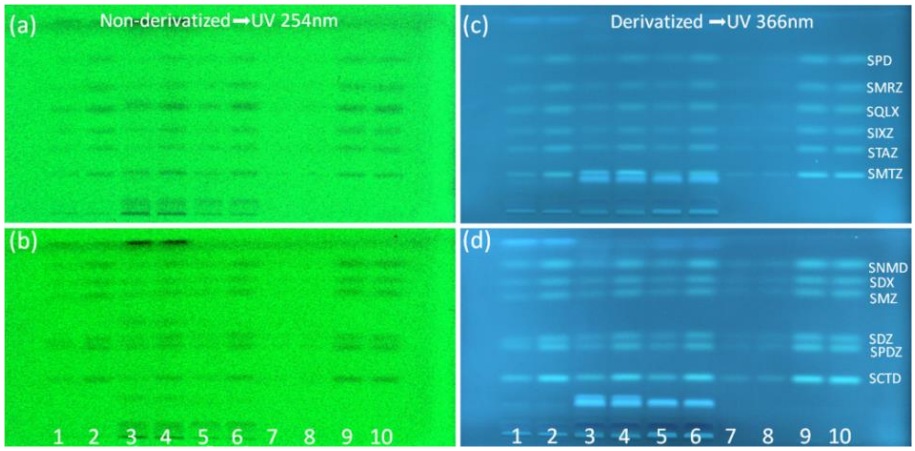


Fig. 2 Matrix-matched chromatography of target compounds: subgroup 1 (a and c) and subgroup 2 (b and d), with track assignment: 1-2 spiked milk, 3-4 spiked liver, 5-6 spiked kidney (spiked levels 100 and 200 μg/kg, respectively), 7-8 pure standards (10 ng/band), and 9-10 pure standards (50 ng/band).

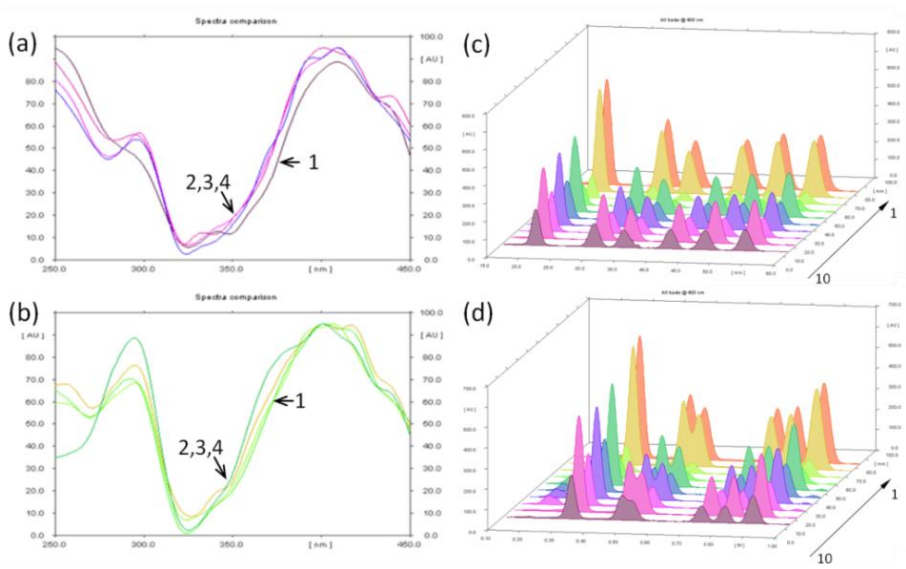


Fig. 3 Fluorescence excitation spectra of two representative analytes, SQLX (a) and SIXZ (b), as pure standards (1) and matrix matched (milk, kidney, liver) standards

(2-4); fluorescence densitograms of SAs subgroup 1 (c) and subgroup 2 (d). Track assignment is identical to **Fig. 2**.

Besides, linear regressions based on five levels (25-250 μg/kg, namely 5-50 ng/band) covering concentrations of interest were established (**Fig. 1S**), from which the LODs and LOQs were calculated according to a DIN method with 95% confidence [30] and are summarized in **Table 2**. It is apparent that the method offers calibration at the most critical levels with good linearity, providing acceptable signal-to-noise ratios even at the lowest point.

Method accuracy was further evaluated by determining recoveries from different food samples of animal origin. Standards of SAs were spiked into sample homogenates, resulting in the three target concentrations of 50, 100 and 200 μg/kg. The determined recoveries obtained from FLD detection, summarized in **Table 3**, were 73-95%, 71-102%, and 83-109% for milk, kidney and liver samples, respectively. Apparently, recovery rates were nearly independent from spiking levels, and insignificant variation could be observed between the different animal matrices.

Table 1 Precision performances of the HPTLC-FLD determination at the threshold level of 100 μg/kg in different food matrices.

Analyte	Within-lab reproducibility[a]			CCα [μg/kg][b]			CCβ [μg/kg][b]		
	Milk	Kidney	Liver	Milk	Kidney	Liver	Milk	Liver	Kidney
SMTZ	7.5	11.8	9.7	108	109	114	116	119	128
STAZ	5.8	7.5	9.9	110	109	106	121	118	113
SIAZ	7.2	7.2	4.4	110	118	116	112	136	133
SQLX	10.5	14.3	8.6	115	119	113	130	138	126
SMRZ	3.8	8.1	5.1	105	110	107	110	120	114
SPD	7.9	7.4	4.9	109	109	106	119	119	113
SCTD	2.7	11.3	7.4	103	117	112	107	134	124
SCPD	9.4	12.2	8.2	106	106	108	113	112	117
SDZ	5.4	4.7	6.1	111	109	106	122	119	112
SDX	8.5	6.7	4.1	115	115	115	130	130	122
SMZ	11.0	10.3	7.4	112	117	113	124	134	126
SNMD	6.3	8.1	6.1	108	110	109	116	121	119

[a] Values are RSD % of 6 matrix-matched runs from different plates at days

[b] $CC\beta = CC\alpha + 1.64 \times SD_{(at\ MRL)}$, in which $CC\alpha = VL + 1.64 \times SD_{(at\ MRL)}$; MRL for target compounds is 100 μg/kg [29].

Table 2 Quantitative performances for the studied SAs determined by HPTLC-FLD.

Analyte	LOD[a]		LOQ[a]		Calibration[b]	Linearity[b]	
	[μg/kg]	[ng/zone]	[μg/kg]	[ng/zone]	[μg/kg]	Regression function	R^2
SMTZ	30	6	60	12	25-250	y=321x-331	0.999
STAZ	35	7	65	13	25-250	y=232x-291	0.998
SIXZ	35	7	65	13	25-250	y=204x-738	0.998
SQLX	35	7	65	13	25-250	y=163x-97	0.998
SMRZ	35	7	70	14	25-250	y=210x-349	0.998
SPD	40	8	70	14	25-250	y=200x-287	0.997
SCTD	30	6	55	11	25-250	y=366x-463	0.998
SPDZ	15	3	50	10	25-250	y=232x-293	0.999
SDZ	30	6	55	11	25-250	y=216x-494	0.999
SDX	30	6	55	11	25-250	y=226x-83	0.999
SMZ	30	6	50	10	25-250	y=237x-179	0.999
SNMD	30	6	55	11	25-250	y=382x+88	0.999

[a] Values were established according the DIN method [30] with at least 95% confidence.

[b] Linear regression functions are based on serial concentrations of 25, 75, 125, 200, and 250 μg/kg.

Table 3 Accuracy performances (expressed as recoveries at different spiking levels) for the HPTLC-FLD determination; values are means of six replicates.

Analytes	Spiked[ug/kg]	Recoveries [%]		
		Milk	Kidney	Liver
SMTZ	50	115±4	88±4	83±5
	100	86±5	79±6	89±9
	200	86±8	77±9	86±8
STAZ	50	113±1	93±1	92±4
	100	83±7	78±6	85±4
	200	85±6	74±5	89±5
SIXZ	50	99±3	82±2	79±3
	100	87±7	94±11	106±10
	200	89±7	85±12	99±6
SQLX	50	105±1	82±5	82±1
	100	87±9	83±12	93±8
	200	84±5	76±5	87±5
SMRZ	50	108±1	86±2	85±4
	100	83±3	79±6	87±4
	200	86±4	74±4	88±5
SPD	50	105±3	104±5	105±4
	100	84±6	80±6	91±4
	200	85±5	77±5	90±5
SCTD	50	92±1	85±1	88±1
	100	87±2	93±11	99±7
	200	94±7	84±8	96±6
SPDZ	50	89±3	85±3	81±2
	100	75±4	83±4	88±5
	200	85±4	76±6	91±5
SDZ	50	93±3	82±3	85±3
	100	79±7	87±6	92±4
	200	87±4	79±6	92±6
SDX	50	92±7	82±4	79±3
	100	83±9	91±9	93±7
	200	89±9	81±9	91±7
SMZ	50	93±4	85±3	82±3
	100	78±7	87±11	98±8
	200	90±6	82±9	95±6
SNMD	50	91±3	86±1	83±2
	100	77±5	81±7	94±6
	200	87±6	76±7	94±6

3.3 HPTLC-MS analysis

3.3.1 Diagnostic ion species defining

In addition to fluorescence densitometry measurements, mass spectra of zones of interest were recorded from the developed plates (**Fig. 4a and b**). First, mass spectra of standards were recorded in both positive and negative ESI mode (**Fig. 4c**). Regarding the sensitivity of detection at the MRL (20 ng/zone), two eluents were optimized for non-derivatized and derivatized plates separately. The obtained mass spectra are exemplarily shown in **Fig. 5** (additionally in **Fig. 2S and 3S**), while the diagnostic ions are summarized in **Table 4**. From the non-derivatized plates, protonated molecules $[M+H]^+$ and sodium ion adducts $[M+Na]^+$ were the most pronounced signals in the ESI positive mode, while in the negative mode, deprotonated molecules were generally produced. These mass signals should be clear evidences allowing specific identification of SAs. However, the weak detectability of the non-derivatized SAs under UV 254 nm hindered eye-directed location of zones, which is quite easier after fluram derivatization. On derivatized plates, a mass increase of 278 amu was expected, resulting from the reaction of the SAs with fluram. However, both in ESI positive and negative mode, a neutral loss of water was observed in most cases (**Table 4**). As less sampling amount was required while the target zones can be much more clearly located, derivatized plates are to be favored for confirmation purposes of suspicious findings. In both cases (non-derivatized and derivatized), signals from the positive ESI mode was generally more sensitive than those from the ESI negative mode. Secondly, mass spectra were recorded from the respective zones of spiked milk, kidney and liver samples after development and derivatization. The diagnostic ions (**Table 4**) clearly could be identified, but the sodium adducts almost turned to be the most intensive mass signals (**Fig. 4S**).

Table 4 HPTLC-MS data (diagnostic ions) obtained from both non-derivatized and derivatized SAs.

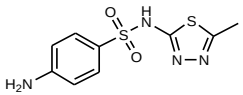
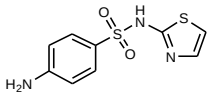
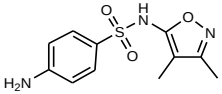
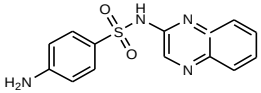
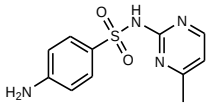
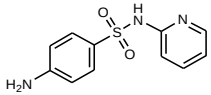
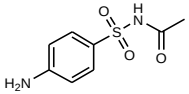
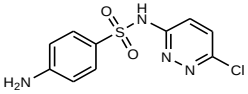
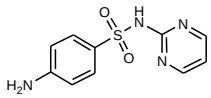
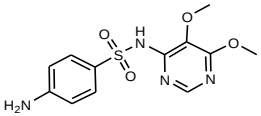
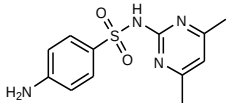
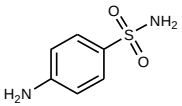
Analyte	Chemical structure	Chemical formula	ESI^+/MS		ESI^-/MS	
			Non-derivatized	Derivatized	Non- derivatized	Derivatized
SMTZ		$C_9H_{10}N_4O_2S_2$	293.0$[M+Na]^+$ 315.0$[M+2Na-H]^+$	531.0$[M+H-H_2O]^+$ 553.0$[M+Na-H_2O]^+$	269.0$[M-H]^+$	529.0$[M-H-H_2O]^-$
STAZ		$C_9H_9N_3O_2S_2$	278.0$[M+Na]^+$ 300.0$[M+2Na-H]^+$	516.0$[M+H-H_2O]^+$ 538.0$[M+Na-H_2O]^+$	254.0$[M-H]^+$	514.0$[M-H-H_2O]^-$
SIXZ		$C_{11}H_{13}N_3O_3S$	268.0$[M+H]^+$ 290.0$[M+Na]^+$	528.0$[M+H-H_2O]^+$ 550.0$[M+Na-H_2O]^+$	266.0$[M-H]^+$	526.0$[M-H-H_2O]^-$
SQLX		$C_{15}H_{16}N_4O_2S$	301.0$[M+H]^+$ 323.0$[M+Na]^+$	561.0$[M+H-H_2O]^+$ 583.0$[M+Na-H_2O]^+$	299.0$[M-H]^+$	559.0$[M-H-H_2O]^-$
SMRZ		$C_{11}H_{12}N_4O_2S$	265.0$[M+H]^+$ 287.0$[M+Na]^+$	525.0$[M+H-H_2O]^+$ 547.0$[M+Na-H_2O]^+$	263.0$[M-H]^+$	523.0$[M-H-H_2O]^-$
SPD		$C_{11}H_{11}N_3O_2S$	250.0$[M+H]^+$ 272.0$[M+Na]^+$	510.0$[M+H-H_2O]^+$ 532.0$[M+Na-H_2O]^+$	248.0$[M-H]^+$	508.0$[M-H-H_2O]^-$
SCTD		$C_8H_{10}N_2O_3S$	237.0$[M+Na]^+$ 259.0$[M+2Na-H]^+$	475.0$[M+H-H_2O]^+$	213.0$[M-H]^+$	473.0$[M-H-H_2O]^-$
SPDZ		$C_{10}H_9ClN_4O_2S$	307.0$[M+Na]^+$ 329.0$[M+2Na-H]^+$	545.0$[M+H-H_2O]^+$ 563.0$[M+H]^+$	283.0$[M-H]^+$	543.0$[M-H-H_2O]^-$ 561.0$[M-H]^-$

Table 4 Continued

Analyte	Chemical structure	Chemical formula	ESI^+/MS		ESI^-/MS	
			Non-derivatized	Derivatized	Non- derivatized	Derivatized
SDZ		$C_{10}H_{10}N_4O_2S$	251.0$[M+H]^+$ 273.0$[M+Na]^+$	511.0$[M+H-H_2O]^+$ 533.0$[M+Na-H_2O]^+$	249.0$[M-H]^+$	527.0$[M-H]^-$ 509.0$[M-H-H_2O]^-$
SDX		$C_{12}H_{14}N_4O_4S$	311.0$[M+H]^+$ 333.0$[M+Na]^+$	571.0$[M+H-H_2O]^+$ 593.0$[M+Na-H_2O]^+$	309.0$[M-H]^+$	569.0$[M-H-H_2O]^-$ 587.0$[M-H]^-$
SMZ		$C_{12}H_{14}N_4O_2S$	279.0$[M+H]^+$ 301.0$[M+Na]^+$	539.0$[M+H-H_2O]^+$ 561.0$[M+Na-H_2O]^+$	277.0$[M-H]^+$	537.0$[M-H-H_2O]^-$
SNMD		$C_6H_8N_2O_2S$	195.0$[M+Na]^+$	433.0$[M+H-H_2O]^+$ 455.0$[M+Na-H_2O]^+$	171.0$[M-H]^+$	449.0$[M-H]^-$ 431.0$[M-H-H_2O]^-$

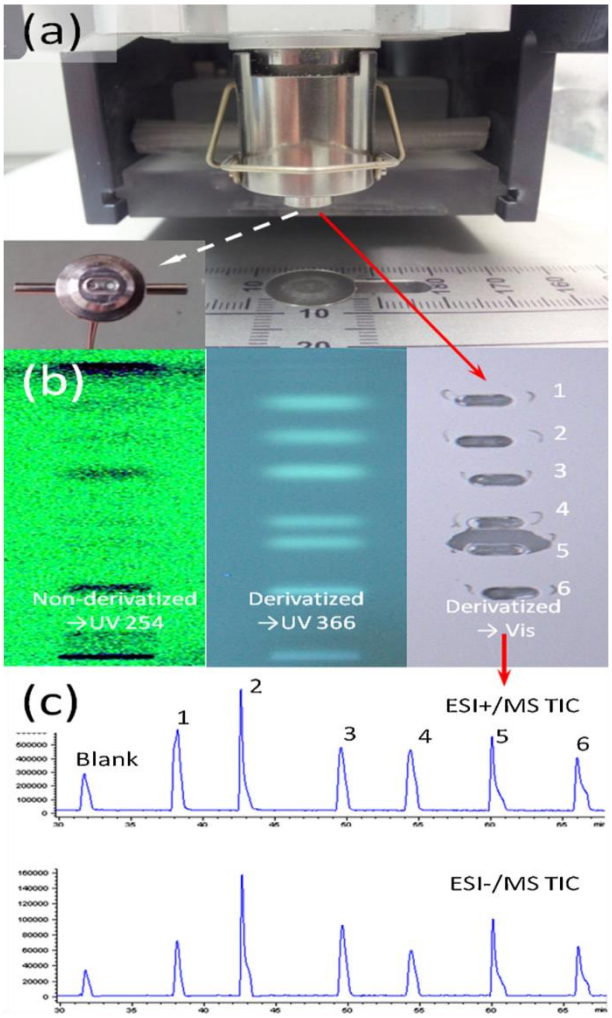


Fig. 4 Illustration of the elution head of the TLC-MS interface (inserted is the profile of its cross-section) (a); extraction marks on HPTLC plates (b), and the corresponding total ion current chronograms (c) in the ESI positive and negative mode.

3.3.2 Identification of unknown

Despite the selective derivatization, some minor interferences due to co-extracted matrix compounds could be observed in blank samples. These zones of unknowns obviously pose a problem for the interpretation of results, but clearly could be identified as false positives by HPTLC-MS (**Fig. 5S**). The comparison of the obtained mass spectra of the suspicious zones from kidney and liver extracts and the nearly co-migrating SMTZ unequivocally excluded the presence of SMTZ (**Fig. 6**). Therefore, the possibility a contamination of blank samples could easily be precluded.

In a practical perspective, the rapid availability of mass spectra directly from HPTLC plates implied a great reduction of energy and work, because independent HPLC-MS data are assayed in parallel.

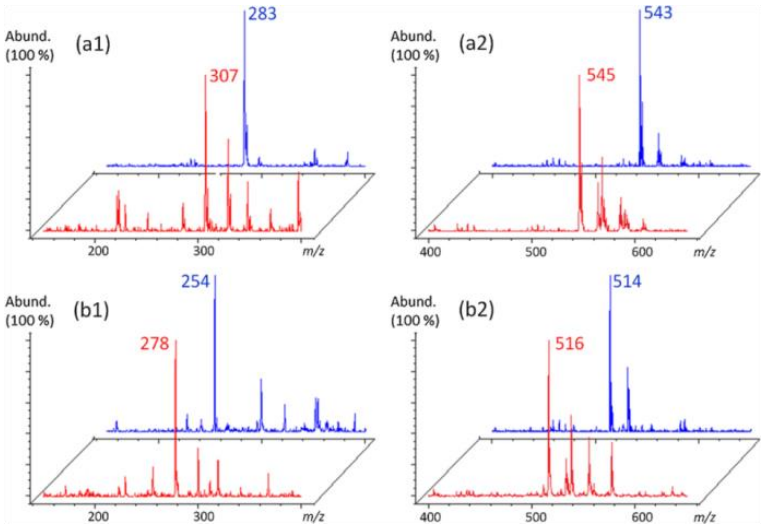


Fig. 5 HTLC-mass spectra of target compounds, exemplary shown for SPDZ (a) and STAZ (b) standards, from non-derivatized (1) and fluram derivatized (2) plates; ESI positive in the front, ESI negative behind.

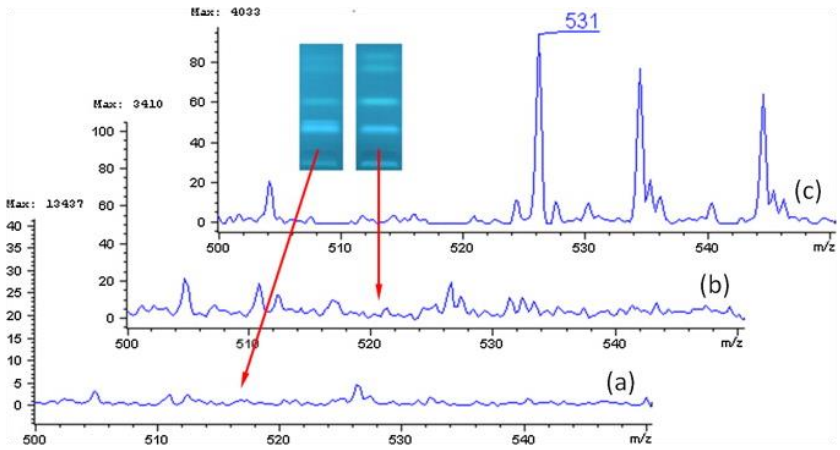


Fig. 6 Comparison of mass spectra (ESI positive) of the suspected zones of blank kidney (a) and liver (b) extracts with the spectrum of the SMTZ standard at 20 ng/zone (c).

4 Conclusions

The proposed HPTLC method integrating multi-detection dimensions featured a screening-oriented strategy for the qualitative and quantitative analysis of SAs in foods of animal origin, showing high throughput and cost-efficiency. The whole screening procedure can be decentralized as several independent steps, without strict time constraints with each other. Therefore, the depth of the measurements can be subjectively controlled, saving time and energy with the majority of compliant samples. Compared to traditional LC-MS methods, the simplicity and efficiency of the method implied a great reduction of work with scaled-up screening tasks. Summarizing, this screening-oriented method satisfactorily answer the problems that might be encountered in practice, efficiently integrating all tasks together on HPTLC media. Thus, it is an attractive alternative for the rapid screening of residues of SAs in animal-derived samples like milk, kidney and liver.

5 Acknowledgements

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7 Supplementary data

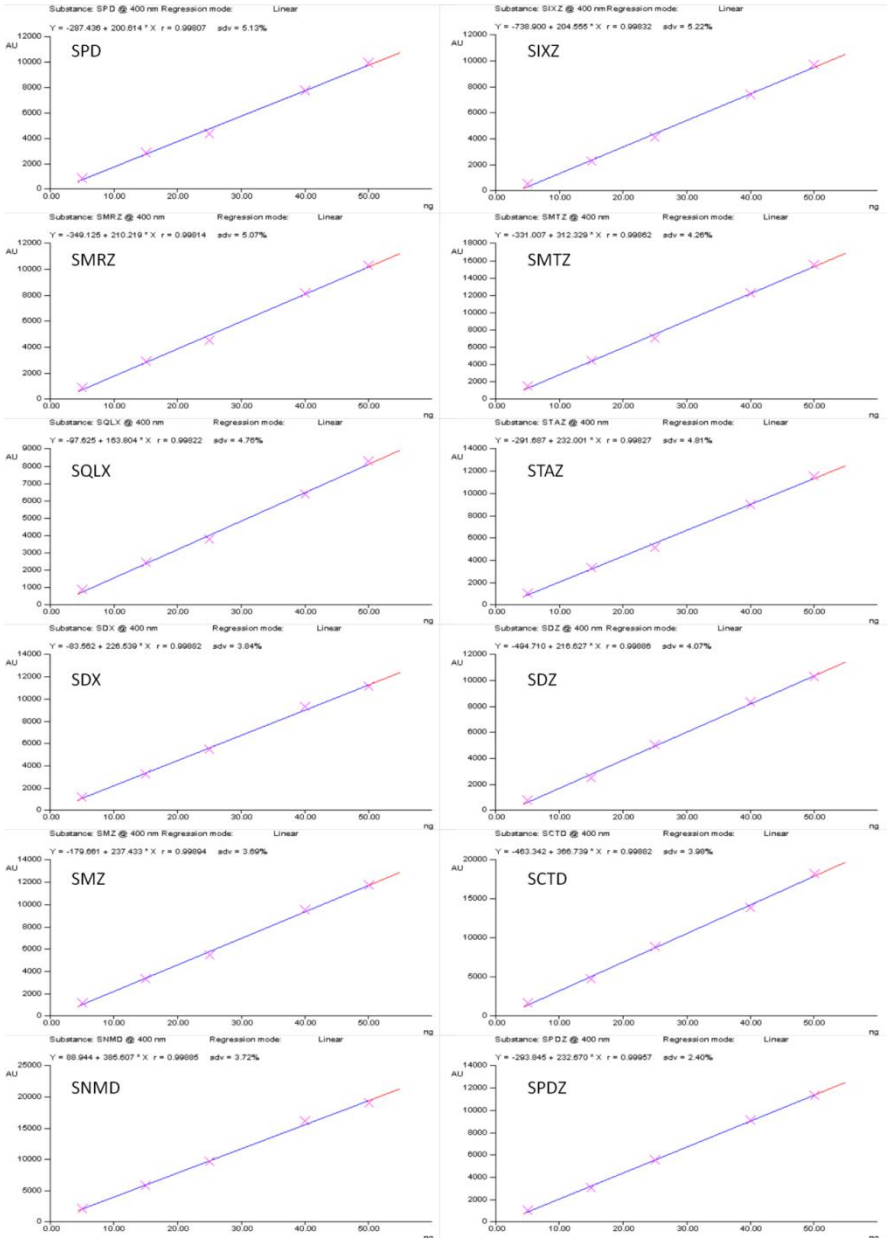

Fig. 1S Calibration curves of studied SAs, measured by densitometry in fluorescence mode.

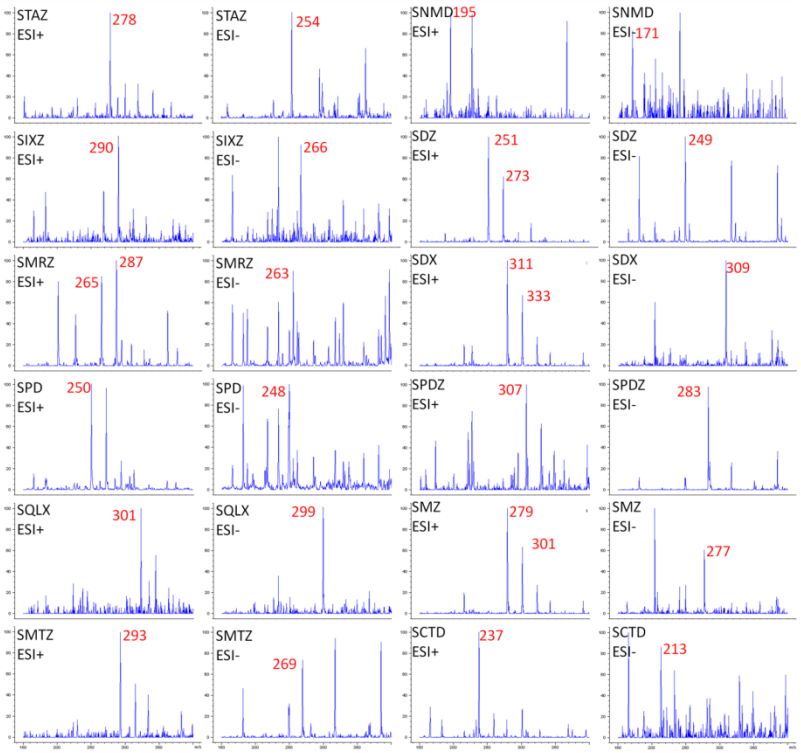

Fig. 2S Mass spectra of studied SAs standards (20 ng/zone), obtained from non-derivatized plates.

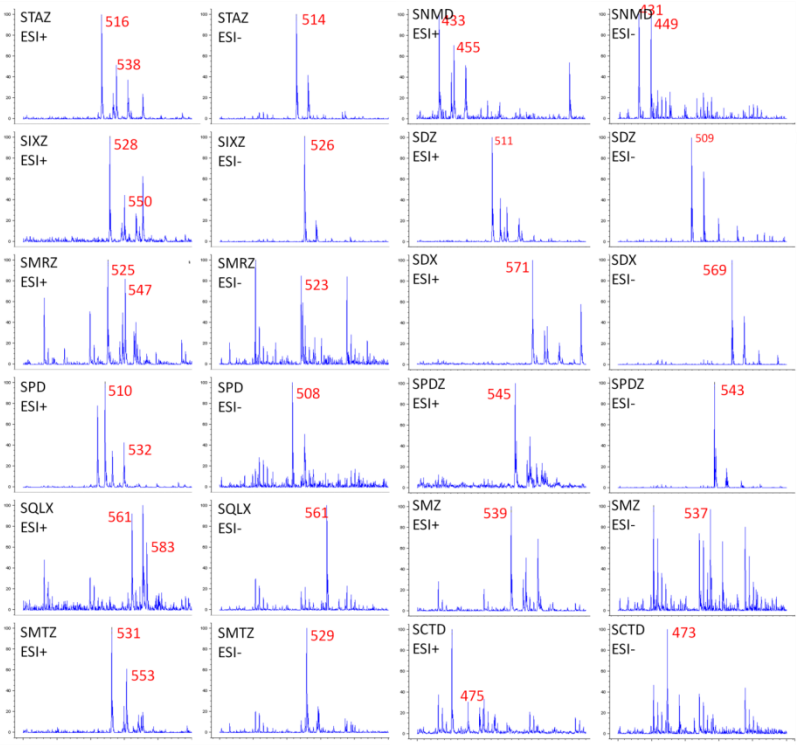

Fig. 3S Mass spectra of studied SAs standards (20ng/zone) as fluram derivatives.

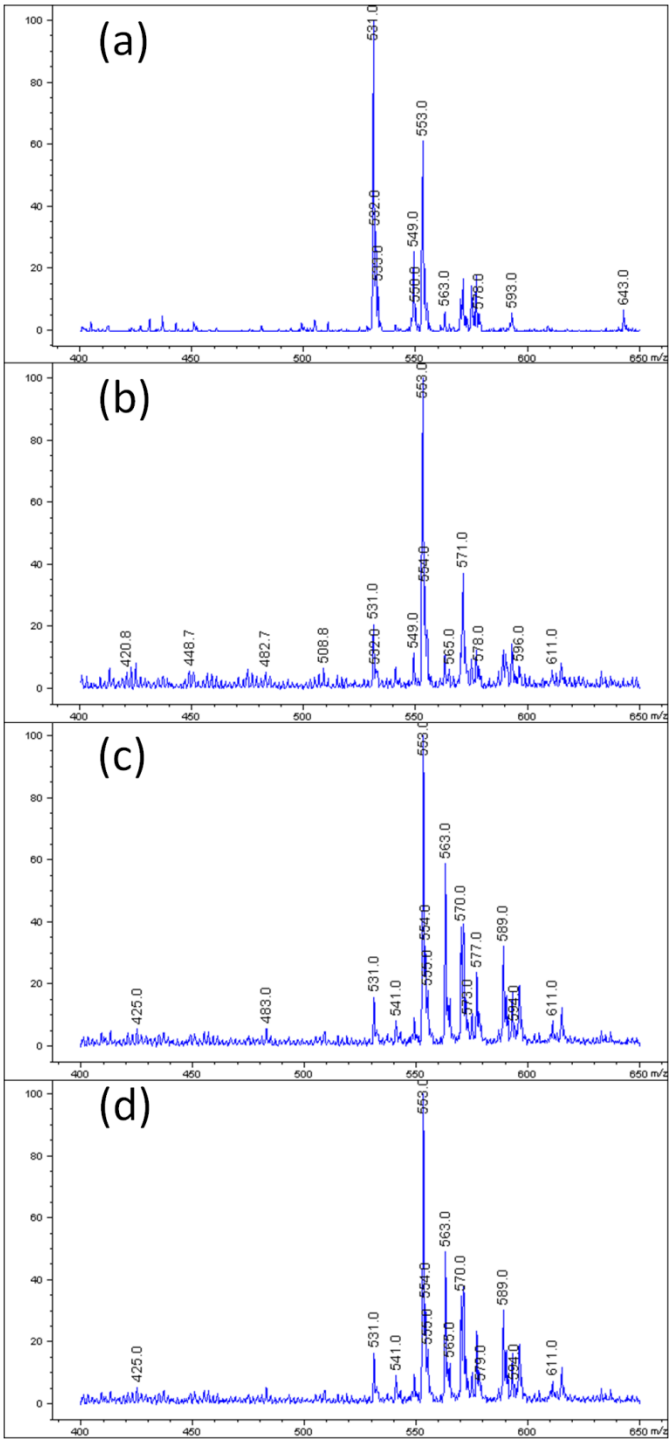

Fig. 4S Comparison mass spectra (ESI$^+$/MS) obtained from pure standards (a) and spiked milk (b), liver (c) and kidney (d) extracts after development and fluram derivatization, exemplarily shown for SMTZ (20 ng/zone).

CHAPTER IV

High-performance thin-layer chromatography screening of multi class antibiotics in animal food by bioluminescent bioautography and electrospray ionization mass spectrometry

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Key words

Multi-antibiotics screening, Aliivibrio fischeri, TLC-bioluminescent bioautography, TLC-mass spectrometry

Abstract

The world-wide usage and partly abuse of veterinary antibiotics resulted in a pressing need to control residues in animal-derived foods. Large-scale screening for residues of antibiotics is typically performed by microbial agar diffusion tests. This work employing high-performance thin-layer chromatography (HPTLC) combined with bioautography and electrospray ionization mass spectrometry introduces a rapid and efficient method for a multi-class screening of antibiotic residues. The viability of the bioluminescent bacterium *Aliivibrio fischeri* to the studied antibiotics (16 species of 5 groups) was optimized on amino plates, enabling detection sensitivity down to the strictest maximum residue limits. The HPTLC method was developed not to separate the individual antibiotics, but for cleanup of sample extracts. The studied antibiotics either remained at the start zones (tetracyclines, aminoglycosides, fluoroquinolones, and macrolides) or migrated into the front (amphenicols), while interfering co-extracted matrix compounds were dispersed at hR_f 20-80. Only after a few hours, the multi-sample plate image clearly revealed the presence or absence of antibiotic residues. Moreover, molecular information as to the suspected findings was rapidly achieved by HPTLC-mass spectrometry. Showing remarkable sensitivity and matrix-tolerance, the established method was successfully applied to milk and kidney samples.

1 Introduction

Veterinary antibiotics play a key role against animal infectious diseases in modern intensive livestock husbandry and also may be used as growth promoters, which is not allowed any more in the European Union (EU) since 2006 [1,2]. Indications of abuses not only directed food safety authorities' attention to antibiotics, but they also received considerable public concern. High levels of residues of antibiotics in animal-derived foods not only provoke allergic reactions, but also promote development of pathogen resistances. Therefore, governments all over the world are intensifying their efforts to control veterinary antibiotics usage and issuing increasingly stringent regulations on maximum residue limits (MRLs) (**Table 1S** and **2S**). Compared with other market regions like the United States (US), the EU shows remarkably strict tolerance to residues of most antibiotics. For instance, the EU MRLs for tetracyclines (in kidney) that have been recognized as the most problematic antibiotics are only 5% of the US MRLs [3,4]. Particularly noteworthy, there is no mandatory method laid down by the EU food safety authorities. Therefore, each EU member state is free to adopt the methods considered the most competitive and suitable [5].

Nevertheless, efficient screening assays are indispensable to the framework enforcing administrative measurements, because huge numbers of samples in proportion to the productivities must officially be monitored for antimicrobial residues [6,7]. The annual report for 2010 on the implementation of EU national residue monitoring programs revealed that only 299 samples (0.23%) were eventually confirmed non-compliant, among the total of 128,698 investigations [8]. Therefore, fulfilling surveillance tasks directly by LC-MS approaches [9-12] involving sophisticated

devices and laborious steps implies a great input of time and energy, keeping in mind of rather low positive rates.

Practically, screening tasks are preferably done by microbial assays that are characteristically of high cost-efficiency [13]. As a trigger of further evaluations, these assays responsing “yes/no” efficiently preclude the majority of compliant samples, thus substantially condensing the workload for subsequent instrumental confirmatory. Yet, it has been shown that traditional sensor strains are not often adequate to meet the EU legislations, especially regarding some prior antibiotic groups [5,14]. While being highly appreciated for their specificity and high throughput, enzyme-linked immunoassays suffers from the restricted detection spectrum, not being able to sense multi-group antibiotics. Besides, misinterpretation (false negative/positive) of results is prone in either case, because assays in agar or microtiter plates are not compatible with chromatographic tools.

Against this background, marked interest has been shown to HPTLC-bioautography that offers an ideal platform for screening tests. The basic principle of an HPTLC strategy is the separation of target and matrix compounds with the subsequent application of a microorganism that plays a key role in targeting further intensive evaluation of positive samples after the crude screening [15-18]. Apparently, this is a tailored strategy for screening, because it substantially opens and bridges the possibility for each individual technique. Among the microorganisms used for bioautography, the bioluminescent bacteria *Aliivibrio fischeri* showed exciting potential. The luminescence of the bacteria is a specific indicator of analytes with bioactive relevance, offering image-giving results that are desired in screening. Being natural marine bacteria, the cost-efficiency and versatility of *A. fischeri* have widely been acknowledged [19].

The aim of the present work was to explore an alternative way for the crude screening of multi-antibiotics in food, using *A. fischeri* for a HPTLC-bioluminescent bioautography assay. For the first time, the toxicity of 22 first-line veterinary antibiotics from seven prior groups to the *A. fischeri* was investigated and optimized on different HPTLC layers. In addition, an HPTLC clean-up of QuEChERS extracts was applied to overcome matrix interferences from real samples like bovine milk and porcine kidney.

2 Experimental

2.1 Material and reagents

Twenty-two analytical standards with purity >90% from seven antibiotic groups were purchased from Sigma-Aldrich (Steinheim, Germany): tetracyclines TCs (tetracycline TC, oxytetracycline OTC, doxycycline DC, chlortetracycline CTC), fluoroquinolones FQs (enrofloxacin EF, ciprofloxacin CF, marbofloxacin MF), macrolides MLs (erythromycin ERTC, spiramycin SPMC, tylosin TLS), aminoglycosides AGs (gentamicin GMC, neomycin NOMC, dihydrostreptomycin DSMC, streptomycin SMC), amphenicols APs (chloramphenicol CAP, thiamphenicol TAP), penicillins PCs (penicillin G PCG, oxacillin OXC), and sulfonamides SAs (sulfadoxin SDX, sulfamethazine SMZ, sulfadiazine SDZ, sulfaquinoxaline SQLX). Methanol and acetonitrile (HPLC grade), yeast extract, and peptone were obtained from Sigma-Aldrich. All other chemicals (analysis grade) were from Merck (Darmstadt, Germany). Ultra pure water was prepared by a Synergy system (Millipore, Schwalbach, Germany).

HPTLC layers pre-coated on glass backs were provided by Merck: silica gel 60 F_{254} (Silica F_{254}), silica gel 60 (Silica), silica gel 60 NH_2 (Amino), silica gel 60 NH_2 F_{254S}

(Amino F_{254}), silica gel 60 RP-18W (RP-18W), silica gel 60 RP-2 F_{254S} (RP-2 F_{254S}), silica gel 60 CN F_{254S} (Cyano F_{254S}), silica gel 60 DIOL F_{254S} (Diol F_{254S}). All plates were washed by pre-developing with methanol, followed by drying at 120 ^{o}C for 20 min on a TLC plate heater (CAMAG, Muttenz, Switzerland). The plates were wrapped in aluminum foil and stored in a desiccator to prevent contamination.

2.2 Standard solutions

Individual stock and working solutions of the studied antibiotics were prepared by dissolving each standard in a proper solvent based on its solubility (**Table 3S**). All solutions were kept at -20 ^{o}C.

2.3 Sample extraction

Bovine milk and porcine kidney (manually pre-sliced) of organic source in Germany were purchased from local supermarket. Initially, 10 g raw samples were homogenized in a high-speed blender (MediFASTH, Switzerland) for 2 min. Spiking of the blank homogenates was done by adding working solutions of standards, resulting in levels around the EU MRLs (**Table 2S**). Then the homogenates were transferred into 50-mL polyethylene tubes containing 200 mg Na_2-EDTA, 10 mL water, and 10 mL acetonitrile. After shaking for one min, 4 g anhydrous magnesium sulfate and 1 g sodium acetate were added, followed by shaking for another min and centrifugation for 5 min at 4000 × *g*. Afterwards, the supernatants were collected, filtered through 0.45 µm nylon filters, transferred into ampoules and evaporated under streams of nitrogen at ambient temperature using a vapotherm basis mobil I system (Barkey, Leopoldshöhe, Germany). Finally, the residues were redissolved with 1 mL

acetonitrile/water (1+1, v/v) and filtered through 0.45 µm nylon filters into 1.5 mL autosampler vials.

2.4 Bacterial suspension preparation

A deep-frozen *A. fischeri* strain (Gram negative, DSM No. 7151), formerly known as *Vibrio fischeri*, was purchased from Leibniz Institute German Collection and Microorganisms and cell cultures (DSMZ, Düsseldorf). The modified preparation of the bacterial suspension was principally based on procedure for the toxicity assay for water and sewage samples (DIN EN ISO 11248-1, section 5) [20]. Briefly, a single bacterial colony from a nutrition agar plate was seeded into flasks containing 50 mL liquid medium (30 g/L NaCl, 6.1 g/L $NaH_2PO_4 \cdot H_2O$, 2.75 g /L KH_2PO_4, 0.204 g/L $MgSO_4 \cdot 7\ H_2O$, 0.5 g/L $(NH_4)\ H_2PO_4$, 3 ml/L glycerol, 5 g/L peptone, and 0.5 g/L yeast extract, adjusted to pH7 with sodium hydroxide solution (25%), and sterilized at 120 ^oC for 20 min). The suspension was incubated at 20±3 ^oC on a rotary shaker set to 100 rpm. After 12 h incubation, when the optimal optical density (OD 600) was determined to approximately 0.5, the ready-to-use bacterial suspension was prepared by diluting the harvest liquid with an equal volume of fresh medium.

2.5 High-performance thin-layer chromatography (HPTLC)

After preparation, 20 µL of blank and spiked sample extracts were applied as 5-mm bands onto amino F_{254S} plates, using an Automatic TLC Sampler 4 (ATS 4, CAMAG), 8 mm from the button, distance from the left-side 15 mm, with automatic tracks intervals. Application conditions: filling speed 15 µL/s, dosage speed 150 nL/s, rinsing (methanol) vacuum time 6 s, filling vacuum time 1 s, and rinsing cycles 1.

Plate development was performed in an Automatic Developing Chamber 2 (ADC 2, CAMAG) with a 20 × 10 cm twin-trough chamber. The following parameters were used, leading to a standardized planar chromatography: 30 s pre-drying, 1 min humidity control (to 33% relative humidity), 5 min tank saturation, 5 min preconditioning, 60 mm migration distance, 10 min post-chromatographic drying. The mobile phase consisted of methanol/acetonitrile (4+6, v/v).

2.6 Plate readout and signal interpretation

The dried plates were dipped into the bacteria suspension with a TLC immersion device (CAMAG) for 1 s at a vertical speed of 2 mm/s. Before measured by a cooled CCD camera (Bioluminizer, CAMAG), the plates were incubated for 3 h in a closed plastic chamber saturated with a damp filter paper. Afterward, plate images were captured with exposure time of 10 s, sequence display delay 250 ms, and automatic gain and offset. Semi-quantitative analysis was principally based on the protocols for image (grayscale mode) processing [21].

2.7 HPTLC-mass spectrometry

Referring to a parallel plate visualized by bioluminescence assay, zones of interest on the dry plates were aimed by a red lesser beam, facilitated by an *x-y* coordinate crosshairs on the TLC-MS interface (CAMAG). Through an oval-shape extraction head plunger, the analyte was extracted from the plates with proper eluents: for TCs, FQs, APs and MLs, acetonitrile/10 mM aqueous ammonium formate (70/30); for AGs, acetonitrile/0.03% aqueous formic acid (70/30). The eluents were provided by an HPLC pump (HP 1100, Agilent Waldbronn, Germany) at a flow rate of 0.2 mL/min, lasting 60 s. The TLC-MS interface was coupled to a G1956B MSD

single-quadrupole mass spectrometer (Agilent) with an electrospray ionization interface. The mass spectrometer were operated with the following settings: drying temperature 250 ^{o}C; drying gas rate 10L/min; capillary voltage 4.0 kv; nebulizing gas 30 bar; fragmentor voltage 100 V; gain 1; threshold 1; step-size 0.05; time filter off; scan data storage full. For data acquisition and processing, LC/MSD ChemStation B.02.01-SR1 (260) software (Agilent) was used.

3 Results and discussion

3.1 Plate selection and treatment

In previous works, TLC-bioluminescence assay were almost performed on normal phase silica gel plates. However, there is a broad range of layer materials commercially available, of which the potential "matrix-effects" to the microorganism were still missing. Therefore, initial experiments were carried out to study the bio-compatibility of various plate layers associated with the sensitivity of the detection of antibiotics. As shown in **Table 1**, markedly different effects of layer material on the bioluminescence were disclosed. Reversed phase layers like RP-2 and RP-18, cyano and diol layers showed strong antipathy to bioluminescence, leading to rather dark backgrounds shortly after dipping. Contrarily, brilliant bioluminescence background was generally observed on normal phase silica and amino plates.

However, the EU MRLs for most antibiotics are rather low; thus the common acute bio-luminescent assay failed to produce inhibition patterns. Therefore, efforts were then focused on enhancing the susceptibility of the test organism to antibiotics at trace levels. According to the work of Froehner [22], two factors, metabolic activity and exposure time, may tightly be related to the viability of *A. fischeri* towards antibiotics at concentrations far below lethal levels. Accordingly, two modifications were

introduced for the HPTLC-bioluminescent bioautography. Instead the usual incubation of 2 days [20], the bacterial test suspension was used after 12 h of incubation, when it showed an optical density (OD 600) of about 0.5 (**Fig. 1S**). The microorganisms generally were in the logarithmic phase providing the most reproductive potential. Additionally, 50% fresh medium was added to the harvested suspension to provide continuous nutrition for the bacteria.

Regarding to the EU MRLs for antimicrobial residues in bovine milk and porcine kidney samples, two concentrations of interest were investigated as thresholds for method optimization (**Table 2**). The MRLs for other sample categories like meat and eggs generally were within this range. Observations over a long-term incubation revealed that only small changes were detectable within the initial 30 min, on both normal phase silica and amino plates. Extending the incubation to 3 h resulted in a dramatic enhancement of the response to most antibiotics, however only on the amino F_{254S} plates (**Table 1**). Time-dependent toxicity of analytes (shown in **Fig. 2S**) during 3 h are qualitatively summarized in **Table 2**. It was apparent that the viability of *A. fischeri* was strongly group-specific. As to be expected, PCs and SAs, which merely affect Gram positive microorganisms, showed no effect on the test organism at all, even at concentrations higher the MRLs. On the contrary, significant inhibition effects of the rest 5 antibiotic groups (16 species) were observed. Further extending the incubation time did not improve the inhibition intensities, but just resulted in a deteriorated background. Therefore, 3 h incubation was preferred for screening applications. Therefore, the developed system is rather time-saving, as compared to traditional microbial assays, like the EU 4-plates assay [23] that requires days of incubation. For the non-detectable SAs, there is another chance for a rapid screening by HPTLC [24].

3.2 QuEChERS-HPTLC clean-up

The optimized HPTLC-bioluminescence detection was further applied to assaying two representative samples, bovine milk and porcine kidney. Despite of initial sample treatment by the QuEChERS strategy designed for the extraction of animal tissues [25, 26], a large variety of matrix components were inevitably co-extracted (**Fig. 1a-c**). Moreover, the co-extractives represented considerable endogenous compounds that were of bioactive relevance as well. As depicted in **Fig. 1d**, these natural inhibitors resulted in significant dark zones. A possible solution to prevent matrix interferences is the use of solid phase extraction that was widely integrated into QuEChERS approaches. However, the traditional SPE clean-up seemed not suitable for multi-group antibiotics analysis, because it generally leads to a loss in analytes due to its inherent selectivity [27].

The unique advantages of HPTLC, however, offer the possibility to perform the clean-up directly on the plate. In this case, analyte loss due to sorbent selectivity is excluded, because all compounds of an extract stay on the plate after development, either at the origin or at higher positions. Nevertheless, two crucial points had to be taken into consideration. First, serious tailing effects of matrix/analytes on plates occurred during migration, leading to rather spreaded zones. The zones of matrix inhibition, for instance, occupied nearly half of the track space in either sample investigated. Meanwhile, the diffusion effect on plates after dipping into bioluminescent suspension further expanded zones, resulting in round spots instead the line-shaped bands applied onto the plates. These problems associated with the bioluminescence assay on HPTLC media implied that clear resolution of all targets and interfering matrix within the limited track space was impossible. To circumvent these problems, an interference-free development strategy, the “window separation”,

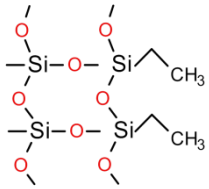
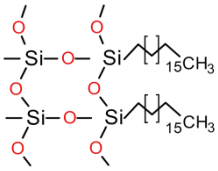
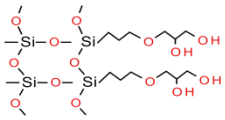
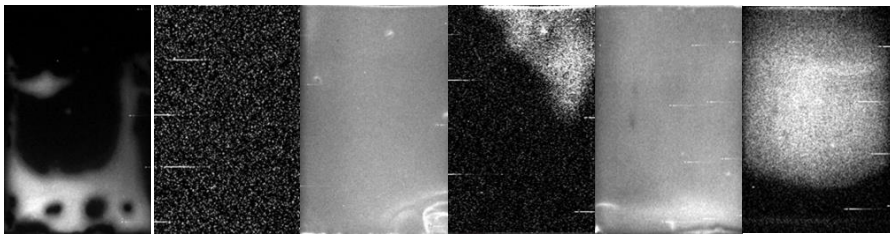
was developed. Through trials of different mobile phases, a compromise was successfully achieved with a formula consisting of methanol/acetonitrile (4+6, v/v) with a migration distance of 60 mm. As shown in **Fig. 2a1** and **b1**, the interfering matrix causing prominent inhibition effect was pushed to the middle of the tracks. Meanwhile, the target compounds were focused in the sharp zones located at both ends of the tracks, resulting in two windows that are nearly free from interferences. Therefore, the bioluminescent pattern within the window spaces can be a specific indicator if any noteworthy inhibitor is presented in a sample, through comparison with standards and blank outcomes.

3.3 HPTLC-bioluminescent pattern evaluation

The image-giving HPTLC-bioluminescent bioautography enables specific and rapid readout of plates. However, the detection zones of antibiotics at the MRLs were of preliminary importance for a crude screening, with a special attention on potential matrix interferences. Compared to other bioluminescence detection devices [28,29], the bioluminizer not only directly facilities eye inspection, but also offers in-depth interpretation of the detected signals, based on digital processing of the tracks (**Fig. 2 a2**,**a3**,**b2** and **b3**). For instance, the blank kidney extract resulted in a small inhibition zone near the front, which led to confusing discrimination from APs. By transforming the grayscale images into palette mode, the potential differences in signal intensities could clearly be discerned. With this mode, the noises due to matrix became significantly distinguishable from the signals caused by target compounds at levels of interest, offering convincing evidences for decision-making. Therefore, such a visual-appealing interpretation mode should be more favored for eye-guided screening.

Table 1 Characterization of the bioluminescence compatibility with different HPTLC layer materials after different incubation times. To all plates (not developed) TC (left) and OTC (right) were applied at 40 (lower zone) and 20 (upper zone) ng/zone, only detectable on the amino plates.

(a) Group 1

	RP-2 F_{254}[a]		RP18W F_{254}[a]		$DiolF_{254S}$[b]	
Layer material						
Incubation time (min)	5	30	5	30	5	180
Images						
Detectability	NO		NO		NO	

(b) Group 2

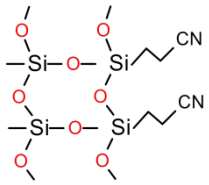
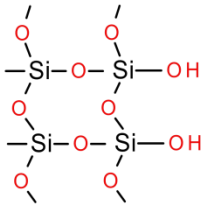
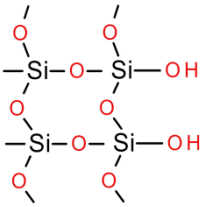
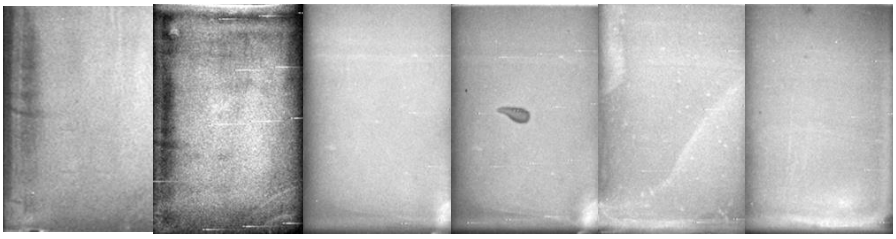
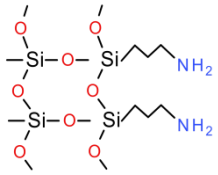
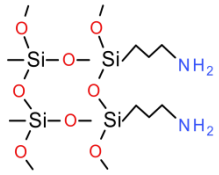
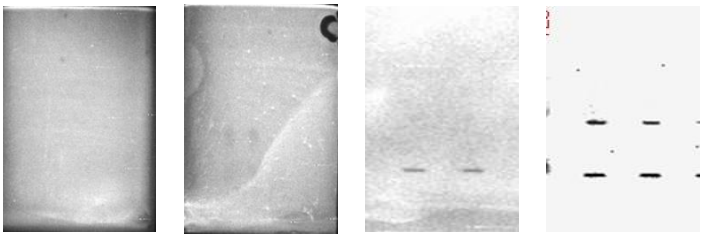
	$CyanoF_{254S}$[a]		Silica gel 60		Silica gel 60 F_{254}[a]	
Layer material						
Incubation time (min)	30	180	30	180	30	180
Images						
Detectability	NO		NO		NO	

Table 1 Continued

(c) Group 3				
	Amino		AminoF$_{254S}$[b]	
Layer material				
Incubation time (min)	30	180	30	180
Images				
Detectability	NO		YES	

[a] Zn_2SO_4:Mn as fluorescence indicator.

[b] $MgWO_4$ as fluorescence indicator.

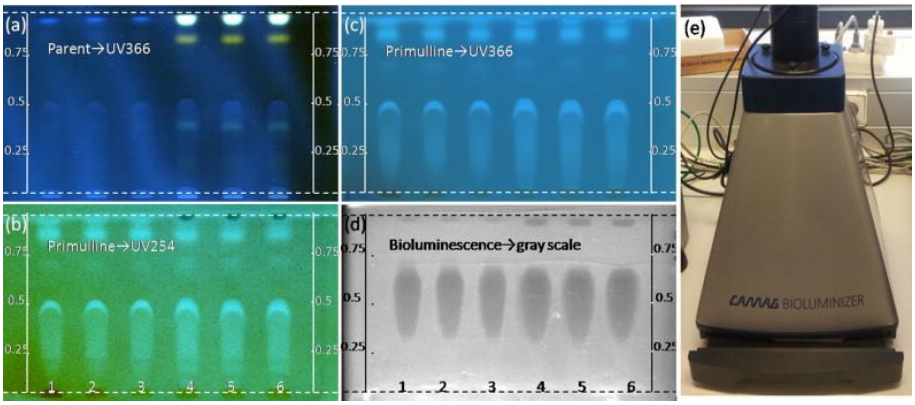


Fig. 1 Visualization of co-extracted matrix interferences under 366 nm (a), after primuline derivatization under 366 (b) and 254 nm (c), and the bioluminescent bioautography pattern (d) detected by the Bioluminizer (e). Track assignment: 1-3 blank milk extract, 4-6 blank kidney extract.

Table 2 Qualitative estimation of HPTLC-bioluminescence signal intensity at antibiotic concentrations of interest after different incubation times.

Analytes	Critical conc. [mg/kg][a]	Time dependent inhibition[b]				
		0.5 h	1 h	2 h	3 h	7 h
TC	0.1/0.6	-/M	W/M	M/S	S/S	S/S
OTC	0.1/0.6	-/M	W/M	M/S	S/S	S/S
DC	0.1/0.6	-/M	W/M	M/S	S/S	S/S
CTC	0.1/0.6	-/M	W/M	M/S	S/S	S/S
SDX	0.1/0.5	-/-	-/-	-/-	-/-	-/-
SMZ	0.1/0.5	-/-	-/-	-/-	-/-	-/-
SDZ	0.1/0.5	-/-	-/-	-/-	-/-	-/-
SQLX	0.1/0.5	-/-	-/-	-/-	-/-	-/-
PCG	0.1/0.5	-/-	-/-	-/-	-/-	-/-
OXC	0.1/0.5	-/-	-/-	-/-	-/-	-/-
GMC	0.1/0.75	W/W	W/S	S/S	S/S	S/S
NOMC	01.05.2005	W/W	W/S	S/M	S/S	S/S
DSMC	0.2/1	W/W	W/S	S/S	S/S	S/S
SMC	0.2/1	W/W	M/M	S/S	S/S	S/S
ERTC	0.05/0.2	N/N	N/N	S/S	S/S	S/S
SPMC	0.2/1	N/N	N/N	S/S	S/S	S/S
TLS	0.05/0.1	N/N	N/N	S/S	S/S	S/S
CAP	0.02/0.05	M/M	S/S	S/S	S/S	S/S
TAP	0.02/0.05	M/M	S/S	S/S	S/S	S/S
MF	0.1/0.15	M/M	S/S	S/S	S/S	S/S
CF	0.1/0.3	N/N	W/W	S/S	S/S	S/S
EF	0.1/0.3	N/N	W/W	S/S	S/S	S/S

[a] The values separated by a slash refer to the EU MRLs for milk and kidney, respectively.

[b] Abbreviation used: — is no effect, W is weak inhibition, M is moderate inhibition, Sis strong inhibition.

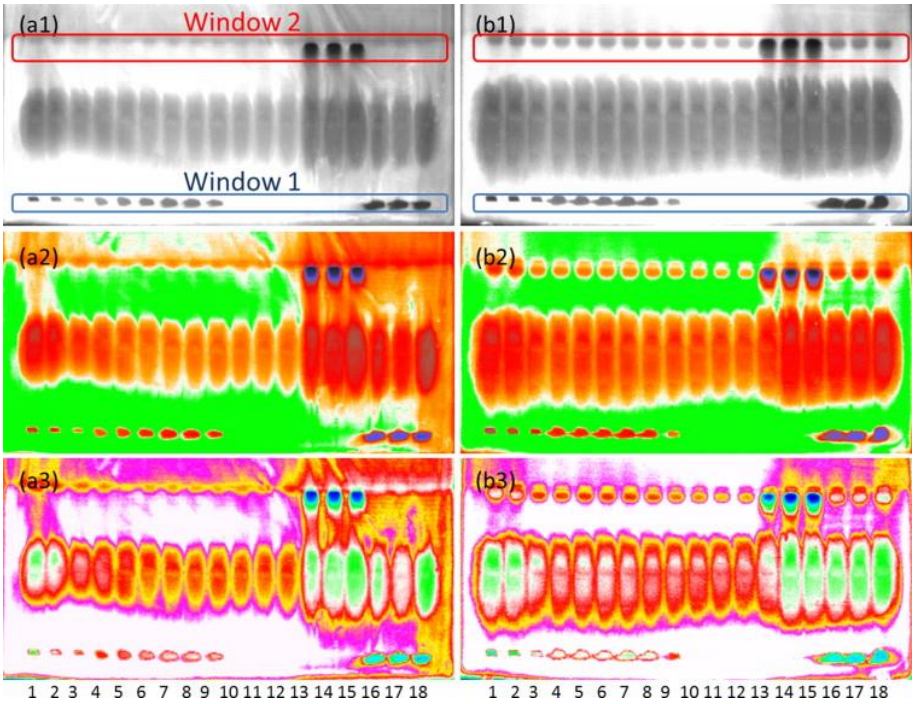


Fig. 2 HPTLC-bioluminescence assay of representative antibiotics spiked into milk (a) and kidney (b), shown in gray-scale mode (a1, b1), palette mode 1 (a2, b2), and palette mode 2 (a3, b3). Track assignment and spiking levels: 1-3 OTC 0.05 mg/kg, 4-6 GMC 0.1 mg/kg, 7-9 ERTC 0.05 mg/kg, 10-12 blank, 13-15 CAP 0.01 mg/kg, 16-18 CF 0.025 mg/kg.

Furthermore, the screening results can be rapidly evaluated by video-scan, providing semi-quantitative evaluation of results, as shown in **Fig. 3** (additionally in **Table 4S**). It was apparent that a blank background was achieved in the lower window, in which most analytes are located. In this case, a strong positive result should be sufficient to reject the sample or to conduct an LC-MS analysis for confirmation, respectively. On the contrary, stronger interferences were observed in the upper window near the front. However, in any case, the signal differences between blank and spiked tracks were significant, which agreed well with the visual impression. Noteworthy, the proposed HPTLC-bioluminescent bioautography showed outstandingly nice detection features for TCs, FQs and APs, for which, however, other microbial tests like the Premi®test

[5] are almost “blind”. This means a marked improvement in antibiotics screening, since compounds from these groups pose the most threats in terms of either frequency or harmness [30].

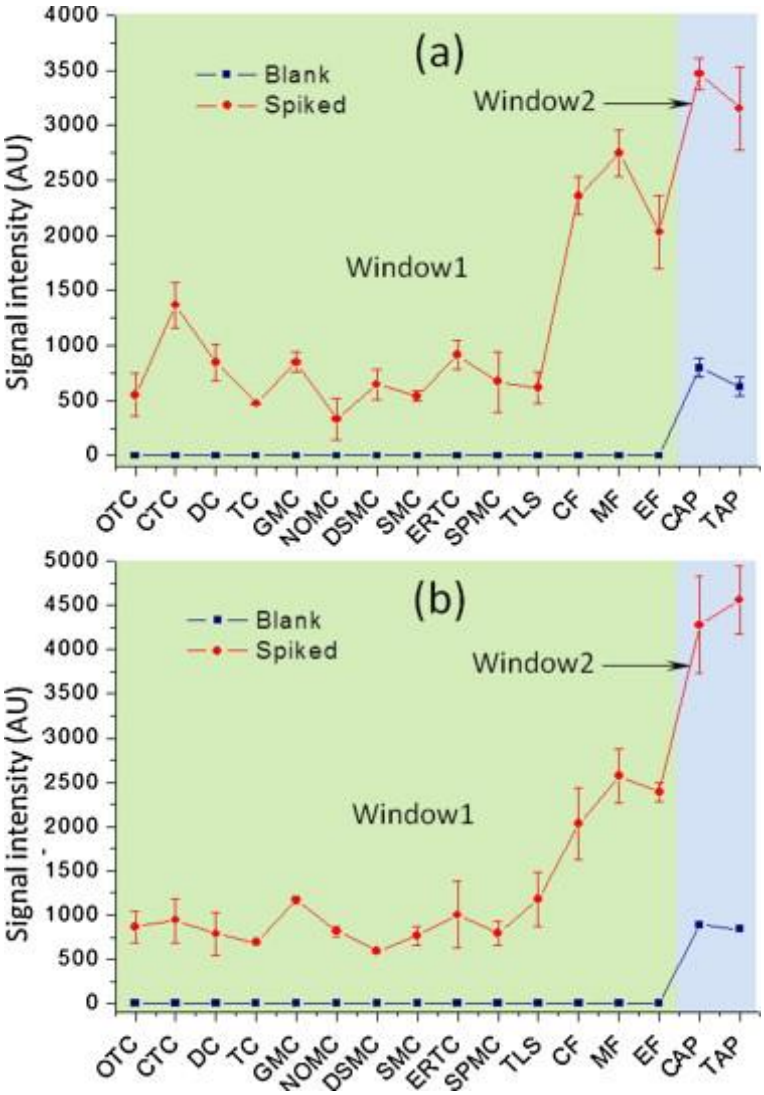


Fig. 3 Evaluation of the bioluminescence detectability of the studied antibiotics spiked into milk (a) and kidney (b) at levels of interest: TCs 0.1/0.05 mg/kg, GMC 0.1/0.2 mg/kg, NOMC 1/2 mg/kg, DSMC 0.1/0.2 mg/kg, SMC 0.1/0.2 mg/kg, ERTC 0.05/0.1 mg/kg, SPMC 0.1/0.2 mg/kg, TLS 0.05/0.1 mg/kg, FQs 0.025/0.05 mg/kg, APs 0.01/0.01 mg/kg. (The values separated by a slash refer to the spiked concentrations in milk and kidney, respectively).

3.4 HPTLC-MS

Although showing marked simplicity, the bioluminescence assay only discloses the presence of bioactive compounds, lacking in chemical specificity. Traditionally, an independent LC-MS method must be established in parallel to certify the suspicions targeted by biosensors [15,22]. However, linking TLC plates directly to mass spectrometry proved a solution to remedy this deficiency, saving additional work [24,31-34]. Therefore, samples positively detected by HPTLC-bioluminescent bioautography were applied onto a new HPTLC plate, developed and analyzed by mass spectrometry, applying the TLC-MS interface (**Fig. 4a**). Targeting the zones was not a great problem, because analytes were restricted in the fixed windows that can be readily aimed at the assistance of a laser-cross and the *x-y* coordinate crosshairs. As summarized in **Table 3**, characteristic ions of the analytes were formed by electrospray ionization, which enables the identification of suspicions.

As mentioned before, the application of 20 μL kidney extract resulted in inhibition zones that may be confused with APs, but HPTLC-MS offered a shortcut to confirm or preclude the presence of APs (**Fig. 4b**). The inspection of the extracted ion chronogram indicated that the suspicious zone of the kidney blank extract did not match the signals of APs. Further investigation into the full-scan spectra revealed that characteristic ions of sufficient abundance were produced despite of the presence of co-elutions (**Fig. 5**). Therefore, this detection dimension might be a valuable tool for rapid reorganization confusing inhibitions encountered in real application.

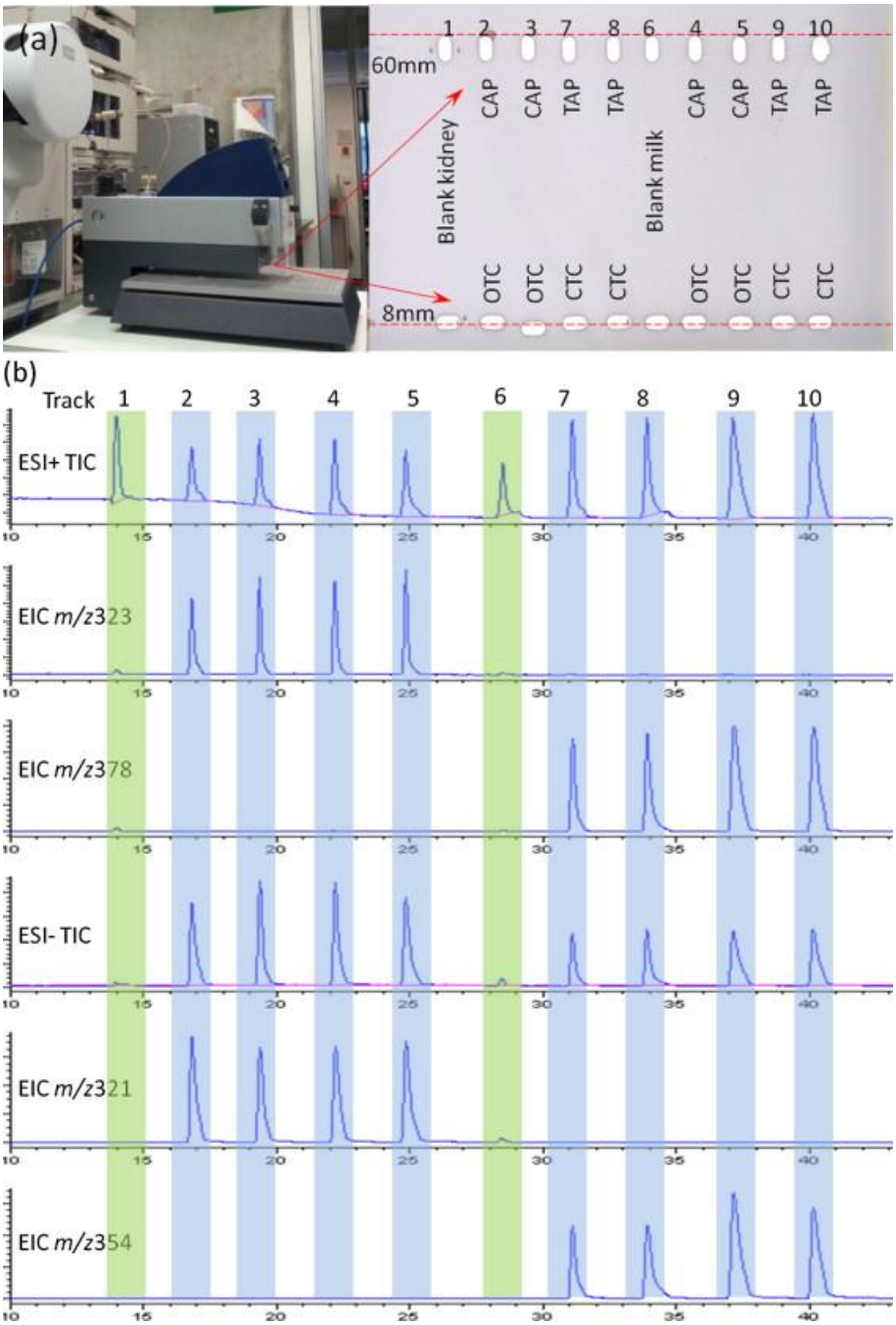


Fig. 4 Illustration of the TLC-MS interface set-up and the extraction marks on a plate (a), and the obtained total ion current (TIC) and extracted ion current (EIC) chronograms in both ESI positive and ESI negative (b), exemplarily shown for APs.

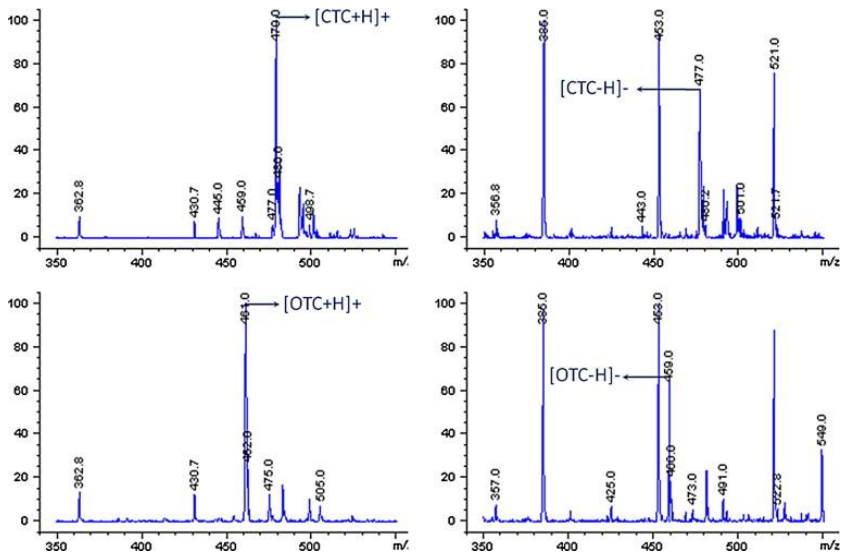


Fig. 5 Mass spectra of kidney matrix-matched analytes (20 ng/zone), exemplarily shown for CTC and OTC, obtained from the plates in both ESI positive (left) and ESI negative mode.

4 Conclusions

For the first time, HPTLC-bioluminescent bioautography combination was applied for sensing trace level antibiotics in animal food matrix. The proposed method that features an attractive screening-oriented solution for most prior antibiotics assaying satisfactorily answers this question, even faced demanding technique challenges (strict MRLs). More importantly, the nature *A. fischeri* was proved suitable for trace analysis under specified conditions, saving troubles raised by disputable genetically modified organism [35,36]. The visual appealing interpretation of the detected signals (bioluminescence bioautography pattern and mass spectra) directly on the HPTLC media enable a novel image-giving evaluation based mode for crude screening. In addition, automatic actions facilitate throughout the analysis procedures, which are highly desired in practice. Even the interfacing of HTPLC-MS that is the most labor-intensive step is expected to be fully automated by intelligent devices in the near future [37-41].

Table 3 HPTLC–MS data obtained for the target antibiotics (the most intensive ions are in bold font).

Analytes	Elemental composition	ESI^+/MS	ESI^-/MS
OTC	$C_{22}H_{24}N_2O_9$	**483.0[M+Na]$^+$**	459.0[M-H]$^-$
		461.0[M+H]$^+$	481.0[M+Na-2H]$^-$
TC	$C_{22}H_{24}N_2O_8$	**467.0[M+Na]$^+$**	443.0[M-H]$^-$
		445.0[M+H]$^+$	465.0[M+Na-2H]$^-$
DC	$C_{22}H_{24}N_2O_8$	**467.0[M+Na]$^+$**	465.2[M+Na-2H]$^-$
		445.0[M+H]$^+$	
CTC	$C_{22}H_{23}ClN_2O_8$	**479.0[M+H]$^+$**	477.0[M-H]$^-$
		501.0[M+Na]$^+$	499.0[M+Na-2H]$^-$
TLS	$C_{46}H_{77}NO_{17}$	**916.5[M+H]$^+$**	914.3[M-H]$^-$
ERTC	$C_{37}H_{67}NO_{13}$	**734.2[M+H]$^+$**	779.3[M+Na-2H]$^-$
SPMC	$C_{43}H_{74}N_2O_{14}$	**843.5[M+H]$^+$**	841.5[M-H]$^-$
			863.3[M+Na-2H]$^-$
GMC	$C_{21}H_{43}N_5O_7$	**478.3[M+H]$^+$**	—
NOMC	$C_{23}H_{46}N_6O_{13}$	**637.0[M+Na]$^+$**	—
SMC	$C_{21}H_{39}N_7O_{12}$	**582.0[M+H]$^+$**	—
DSMC	$C_{21}H_{41}N_7O_{12}$	**584.0[M+H]$^+$**	—
CAP	$C_{11}H_{12}Cl_2N_2O_5$	323.0[M+H]$^+$	**321.0[M-H]$^-$**
		345.0[M+Na]$^+$	367.0[M+HCOO]$^-$
TAP	$C_{12}H_{15}Cl_2NO_5S$	378.0[M+Na]$^+$	**354.0[M-H]$^-$**
		373.0[M+NH_4]$^+$	400.0[M+HCOO]$^-$
MF	$C_{17}H_{19}FN_4O_4$	**363.0[M+H]$^+$**	407.0[M+HCOO]$^-$
			424.2[M+HCOO]$^-$
EF	$C_{19}H_{22}FN_3O_3$	**360.0[M+H]+**	404.0[M+HCOO]$^-$
CF	$C_{17}H_{18}FN_3O_3$	**332.0[M+H]$^+$**	376[M+HCOO]$^-$

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7 Supplementary data

Table 1S Overview of the online sources of MRL information from major national authorities.

Countries	Authorities	Links for detailed regulation inform	Issue year
China	Ministry of agriculture of People's Republic of China	http://www.gzahi.gov.cn/news/1000_31_1001_3771.html	2012
EU	The council of European Union	http://eur-lex.europa.eu/JOHtml.do?uri=OJ:L:2010:015:SOM:EN:HTML	2010
US	Food and drug administration	http://www.mrldatabase.com/	2001
Canada	Heath Canada	http://www.hc-sc.gc.ca/dhp-mps/vet/mrl-lmr/index-eng.php	2013
Australia	Australian government depart of agriculture	http://www.daff.gov.au/agriculture-food/nrs/nrs-australian-and-overseas-mrl-database/cattle-sheep-pigs/international_beef_maximum_residue_limits_mrls	2010
New Zealand	New Zealand minister for food safety	http://www.foodsafety.govt.nz/index.htm	2011
Japan	Japan food chemical research foundation	http://www.m5.ws001.squarestart.ne.jp/foundation/search.html	2013

Table 2S Overview of the basic information (mode of action, violation profile and residual regulation) of studied antibiotics.

Groups	Species	Action mechanism and spectrum	MRLs violation rate [%][a]	MRLs for different animal-derived foods [mg/kg][b]			
				Liver	Kidney	Muscle	Milk
TCs	All species including epimers	Protein synthesis, inhibitor binding to the 30S subunit of microbial ribosomes; Broad spectrum active	< 5	0.3(6)	0.6(12)	0.1(2)	0.1(0.3)
SAs	All species	Competitive inhibitors of the enzyme dihydropteroatesynthetase involved in folate synthesis; Gram+ active	<2	0.1(0.1)	0.1(0.1)	0.1(NL)	0.1(0.1)
MLs	ERTC	Protein synthesis, inhibitors by preventing peptidyltransferase from adding the peptidyl attached to tRNA to the next amino acid; Gram+ active.	<2	0.2(0.1)	0.2(0.1)	0.2(NL)	0.04(NL)
	SPMC			0.5(NL)	1.5(NL)	0.2(NL)	0.15(NL)
	TLS			0.1(0.2)	0.1(0.2)	0.1(0.2)	0.05(0.05)
AGs	DSMC	Protein synthesis inhibitor, ribosomal translocation inhibitor, bacterial cell membrane integrity interrupter; Gram- active.	<5	0.5(0.5)	1(2)	0.5(NL)	0.2(NL)
	NOMC			0.5(3.6)	5(7.2)	0.5(1.2)	1.5(NL)
	SMC			0.5(NL)	1(NL)	0.5(0.5)	0.2(NL)
	GMC			0.2(NL)	0.75(NL)	0.05(0.1)	0.1(NL)
PCs	OXC	Biosynthesis inhibitor by preventing the formation of peptidoglycan cross-links in the bacterial cell wall; Gram+ active.	<20	0.3(NL)	0.3(NL)	0.3(NL)	0.03(NL)
	PCG			0.05(NL)	0.05(NL)	0.05(NL)	0.004(NL)
APCs	CAP	Protein biosynthesis inhibitor similar to macrolides; broad-spectrum antibacterial	<1	NP(NL)	NP(NL)	NP(NL)	NP(NL)
	TAP			0.05(NL)	0.05(NL)	0.05(NL)	0.05(NL)
FQs	EF/CF	Inhibitor by preventing DNA from unwinding and duplicating; broad-spectrum active.	<3	0.2(NL)	0.3(NL)	0.1(0.3)	0.1(NL)
	MF			0.15(NL)	0.15(NL)	0.15(NL)	0.075(NL)

a) Data were collected from [8]

b) Outside bracket-EU and China MRL values, inside bracket-US tolerance limits; abbreviation used: NL not listed, NP not permitted.

Table 3S Stock/working solutions preparation for studied antibiotics standards.

Group	Species	Abbr.	MRLs [mg/kg][a]		Solvent	Stock→work con. [mg/mL]	Spiked [mL][b]	
			Milk	Kidney			Milk	Kidney
TCs	Tetracycline	TC	0.1(0.3)	0.6(12)	MeOH	0.1→0.01	0.05	0.1
	Oxytetracycline	OTC	0.1(0.3)	0.6(12)	MeOH	0.1→0.01	0.05	0.1
	Doxycycline	DC	0.1(0.3)	0.6(12)	MeOH	0.1→0.01	0.05	0.1
	Chlortetracycline	CTC	0.1(0.3)	0.6(12)	MeOH	0.1→0.01	0.05	0.1
SAs	Sulfadoxin	SDX	0.1(0.1)	0.1(0.1)	MeOH	0.1→0.01	N/A	N/A
	Sulfamethazine	SMZ	0.1(0.1)	0.1(0.1)	MeOH	0.1→0.01	N/A	N/A
	Sulfadiazine	SDZ	0.1(0.1)	0.1(0.1)	MeOH	0.1→0.01	N/A	N/A
	Sulfaquinoxaline	SQLX	0.1(0.1)	0.1(0.1)	MeOH	0.1→0.01	N/A	N/A
PCs	Penicillin G	PCG	0.004(0.05)	0.05(NL)	MeOH	0.05→0.01	N/A	N/A
	Oxacillin	OXC	0.03(0.01)	0.3(NL)	MeOH	0.05→0.01	N/A	N/A
AGs	Gentamicin	GMC	0.1(NL)	0.75(0.4)	MeOH/H_2O 90/10	0.1→0.01	0.1	0.2
	Neomycin	NOMC	1.5(NL)	5(7.2)	MeOH/H_2O 90/10	0.5→0.05	0.2	0.4
	Dihydrostreptomycin	DSMC	0.2(NL)	1(2)	MeOH/H_2O 90/10	0.1→0.02	0.05	0.1
	Streptomycin	SMC	0.2(NL)	1(2)	MeOH/H_2O 90/10	0.1→0.02	0.05	0.1
MLDs	Erythromycin	ERTC	0.04(NL)	0.2(0.1)	MeOH	0.1→0.01	0.05	0.1
	Spiramycin	SPMC	0.2(NL)	1(NL)	MeOH	0.1→0.02	0.05	0.1
	Tylosin	TLS	0.05(0.05)	0.1(0.2)	MeOH	0.05→0.01	0.05	0.1
APs	Chloramphenicol	CAP	NP(NL)	NP(NL)	MeOH	0.01→0.001	0.1	0.1
	Thiamphenicol	TAP	0.05(NL)	0.05(NL)	MeOH	0.01→0.001	0.1	0.1
FQs	Marbofloxacin	MF	0.075(NL)	0.15(NL)	MeOH/NH_40H[c] 99.9/0.1	0.1→0.01	0.025	0.05
	Ciprofloxacin	CF	0.1(NL)	0.3(NL)	MeOH/NH_40H[c] 99.9/0.1	0.1→0.01	0.025	0.05
	Enrofloxacin	EF	0.1(NL)	0.3(NL)	MeOH/NH_40H[c] 99.9/0.1	0.1→0.01	0.025	0.05

a) Outside the brackets-EU MRLs; inside -US tolerance limits; abbreviation used: NL not listed, NP not permitted.

b) N/A not applied.

c) NH_40H: aqueous ammonium hydroxide solution (28%).

Table 4S Evaluation of potential matrix effect on the screening performances, regarding different animal tissues.

Analytes	Milk				Kidney			
	Vc[a] [mg/kg]	Blank Signal[b]	Spiked Signal[b]	RSD% *n*=3	Vc[a] [mg/kg]	Blank Signal[b]	Spiked Signal[b]	RSD % *n*=3
OTC	0.05	0	553	35	0.1	0	866	20
CTC	0.05	0	1368	15	0.1	0	941	27
DC	0.05	0	848	20	0.1	0	784	31
TC	0.05	0	478	2	0.1	0	691	3
GMC	0.1	0	847	11	0.2	0	1169	3
NOMC	1	0	331	58	2	0	813	7
DSMC	0.1	0	647	21	0.2	0	593	3
SMC	0.1	0	541	8	0.2	0	764	14
ERTC	0.05	0	914	14	0.1	0	1002	37
SPMC	0.1	0	672	41	0.2	0	794	16
TLS	0.05	0	619	23	0.1	0	1178	26
CF	0.025	0	2361	7	0.05	0	2032	20
MF	0.025	0	2748	8	0.05	0	2568	12
EF	0.025	0	2033	16	0.05	0	2392	4
CAP	0.01	800	3470	4	0.01	887	4276	13
TAP	0.01	625	3154	12	0.01	836	4557	8

a) Vc-validation concentration.

b) pixels gray-scale intensities of digital images.

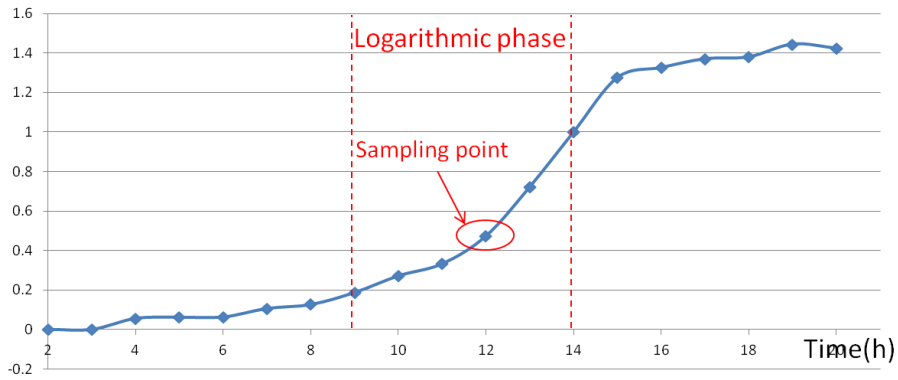


Fig. 1S Growth curve of *Aliivibrio fischeri*, measured as optical density at 600 nm.

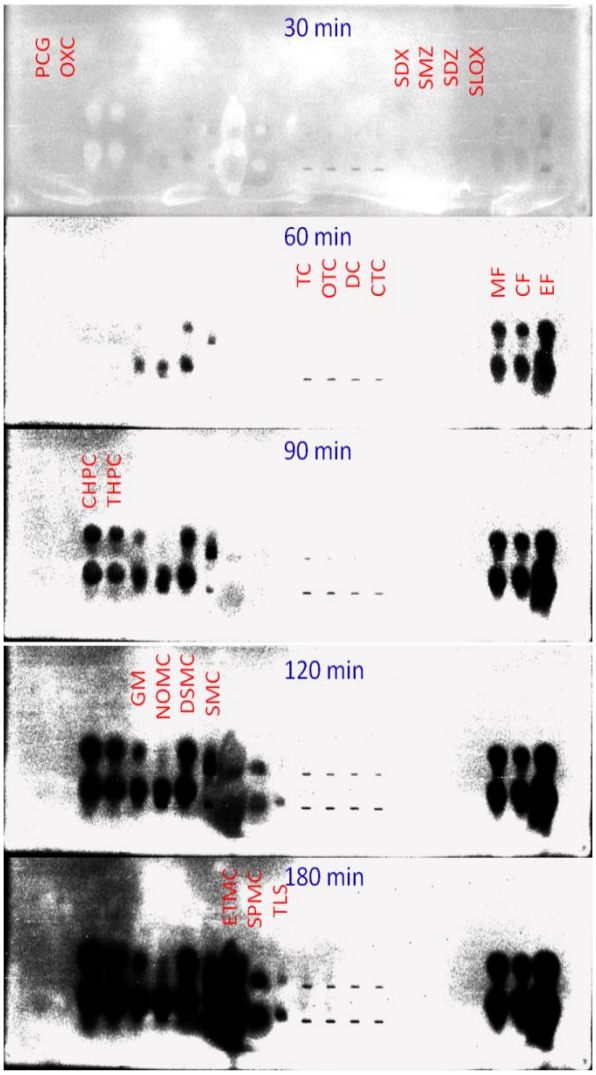


Fig. 2S Cytotoxicity of studied antibiotics to *Aliivibrio fischeri* bioluminescence in the initial 3 hours of incubation. Standards of antibiotics were spotted at MRLs of milk (upper line) and of kidney (lower line) on an undeveloped amino F_{254S} plate.

CHAPTER V

Summary

Summary

Nowadays, the usage and partly abuse of veterinary antibiotics resulted in a very pressing need to control residues in foods of animal origin. Particularly, the increasingly demanding MRL issues and the huge number of samples to be monitored raised great challenges in this field. Microbial growth inhibition assays are traditionally employed for screening purposes, while sophisticated HPLC-MS methods are alternatively used or only used for confirmation purposes. To substitute the time consuming growth inhibition assays, HPTLC as a platform hyphenated to multi detection modes was employed in this study for the development of a high throughput, sensitive and cost-efficient screening-oriented methodology for antibiotics residues.

The first step was focused on tetracyclines and fluoroquinolones, which are the most problematic antibiotics in the European Union and account for the most of the used veterinary antibiotics. To prevent strong tailing effects, the separation was optimized on normal-phase silica gel plates modified with ethylenediamine tetraacetic acid (EDTA). Besides, selective and sensitive fluorescence densitometry was optimized to achieve best signal/noise ratios. Under these conditions, limits of detection (LODs) and quantitation (LOQs) were in the range 12-25 and 45-95 µg/kg, respectively. Recoveries from milk samples, spiked at 50, 100 and 150 µg/kg and extracted by a modified QuEChERS procedure, ranged from 76 to 105%. To circumvent the ion suppressions due to EDTA, HPTLC-mass spectrometry (HPTLC-MS) was optimized, allowing the selective confirmation of positive findings, also offering high sensitivity of 25 µg/kg, and meeting Commission Regulation (EU) No. 37/2010.

In the second step, sulfonamides were targeted, which are the secondly most administered veterinary antibiotics in the European Union. Separation of twelve most

important sulfonamides was achieved on HPTLC silica gel plates, followed by fluram derivatization and sensitive and selective quantitation by fluorescent densitometry. LODs and LOQs were determined to 15-40 and 35-70 µg/kg, respectively. Samples of bovine milk, porcine liver and kidney were extracted according to the "QuEChERS" strategy. Additionally, a confirmative detection by HPTLC-MS was optimized, offering straightforward identification of target zones. The method was validated to meet the enforced Commission Regulation (EU) No. 37/2010.

Finally, a more universal screening method based on HPTLC-bioautography was developed for most of the first-line veterinary antibiotics. A comprehensive HPTLC plate test revealed that the bio-compatibility of different plate layer materials to the applied bioluminescent bacteria (*A. fischeri* DSM No. 7151) was surprisingly different. It was then discovered that both bright bioluminescent background and significant inhibition zones of antibiotics can only be achieved on HPTLC amino F_{254S} plates.

In this case, HPTLC was not used for the chromatographic separation of individual antibiotics extracted with acetonitrile, but in terms of planar solid phase extraction to separate bioactive matrix compounds and to focus the analytes within two distinct target zones of different polarity. Together with HPTLC-MS for identification and confirmation purposes, the developed procedure enabled the rapid, sensitive and efficient multi-class screening of antibiotic residues (16 species of 5 groups, except sulfonamides and penicillins, which only affect Gram positive bacteria). The multi-sample plate images provided the results within a few hours. Thanks to the high sensitivity and the great matrix tolerance, the established method was successfully applied to bovine milk and porcine kidney samples, each spiked at the EU MLRs.

CHAPTER VI

Zusammenfassung

Zusammenfassung

Der enorme Einsatz und partielle Missbrauch von Antibiotika in der Tiermedizin führte zu dringend notwendigen Maßnahmen, um Rückstände in tierischen Lebensmitteln zu kontrollieren. Insbesondere die steigend anspruchsvollen Rückstandshöchstgehalte sowie die geforderten zunehmenden Probenzahlen stellen eine große Herausforderung dar. Mikrobielle Hemmhof-Assays werden traditionell zum Proben-Screening eingesetzt, während anspruchsvolle HPLC-MS Methoden häufig alternativ oder nur zur Absicherung positiver Befunde verwendet werden. Um die zeitintensiven Hemmhof-Assays zu ersetzen, wurde in dieser Arbeit die HPTLC gekoppelt mit Multi-Detektionsmethoden eingesetzt, um eine schnelle und kostengünstige Screening-orientierte Methodik für Antibiotika-Rückstände zu entwickeln.

Der erste Teil der Arbeit konzentrierte sich auf zwei Gruppen von "schwierigen" Antibiotika, Tetracycline und Fluorchinolone, die zu den häufigst eingesetzten veterinärmedizinischen Antibiotika gehören. Unter Vermeidung von Tailing-Effekten wurde die HPTLC-Trennung auf Normalphasen-Kieselgelplatten, modifiziert mit Ethylendiamintetraessigsäure (EDTA), optimiert. Außerdem wurde eine selektive und empfindliche Fluoreszenz-Densitometrie genutzt, um beste Signal/Rausch-Verhältnisse zu erreichen. Nachweis- und Bestimmungsgrenzen lagen im Bereich von 12-25 und 45-95 µg/kg. Wiederfindungen aus Milchproben, dotiert auf 50, 100 und 150 µg/kg und extrahiert mit einer modifizierten QuEChERS-Methode, ergaben sich zu 76-105%. Zur Absicherung positiver Befunde wurde die Massenspektrometrie (HPTLC-MS) bezüglich Ionensuppressionen durch EDTA dahingehend optimiert, dass eine empfindliche Detektion von 25 µg/kg möglich war und die Vorgaben der Verordnung (EU) Nr. 37/2010 erfüllt wurden.

Im zweiten Schritt galt das Interesse den Sulfonamiden, den zweithäufigst eingesetzten veterinärmedizinischen Antibiotika. Die Trennung erfolgte auf HPTLC-Kieselgelschichten und nachfolgender Fluram-Derivatisierung. Dies erlaubte eine selektive und sehr sensitive Quantifizierung der zwölf bedeutendsten Sulfonamide durch Fluoreszenz-Densitometrie. Nachweis- und Bestimmungsgrenzen ergaben sich zu 15-40 und 35-70 µg/kg. Die Extraktion von Milch-, Leber- und Nierenproben erfolgte mit Acetonitril (analog "QuEChERS"). Zur Bestätigung positiver Proben wurde erneut die HPTLC-MS Kopplung zur einfachen Identifizierung der Zielzonen optimiert. Die Methode wurde hinsichtlich der Vorgaben der Verordnung (EU) Nr. 37/2010 für Milch sowie Schweine-Leber und -Nieren validiert.

Schließlich wurde eine universelle Screening-Methode mittels HPTLC-Bioautographie für die meisten der First-Line Tier-Antibiotika entwickelt. Ein umfangreicher HPTLC-Plattentest zeigte, dass die Bio-Kompatibilität der verschiedenen Schichtmaterialien mit den eingesetzten Leuchtbakterien (*Aliivibrio fischeri* DSM-Nr. 7151) überraschend unterschiedlich war. Nur auf HPTLC-Amino F_{254S} Platten zeigten die Bakterien die optimale Biolumineszenz und damit signifikante Hemmzonen für Antibiotika.

Nach Extraktion mit Acetonitril wurde die HPTLC hier nicht zur Auftrennung der einzelnen Antibiotika optimiert, sondern im Sinne einer planaren Festphasenextraktion zur Abtrennung ebenfalls bioaktiver Matrixkomponenten und zur Fokussierung der Antibiotika in zwei unterschiedlich polare Zielzonen. Zusammen mit der HPTLC-MS Kopplung lieferte diese Methode ein schnelles, empfindliches und effizientes Multi-Class-Screening von Antibiotika-Rückständen (16 Wirkstoffe aus 5 Gruppen, außer Sulfonamide und Penicilline, welche nur

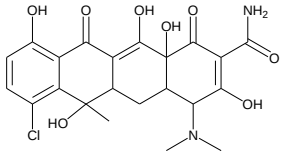
Gram-positive Bakterien hemmen). Ein Plattenbild lieferte innerhalb weniger Stunden das Ergebnis für viele Proben. Dank hoher Empfindlichkeit und großer Matrixtoleranz wurde die Methode erfolgreich auf Milch- und Schweinenieren-Proben angewendet, dotiert auf die EU-Rückstandshöchstgehalte.

CHAPTER VII

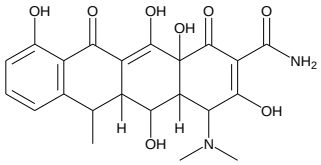
Appendix

Appendix Chemical structures of studied antibiotics.

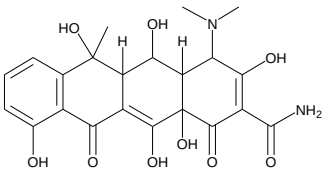
Tetracyclines (TCs)



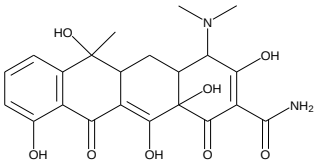
Chlortetracycline (CTC)



Doxycycline (DC)

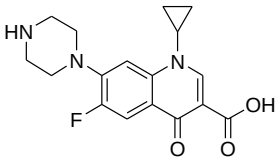


Oxytetracycline (OTC)

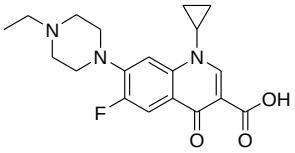


Tetracycline (TC)

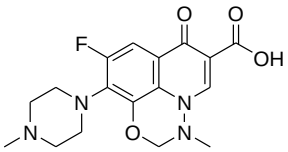
Fluoroquinolones (FQs)



Ciprofloxacin (CF)

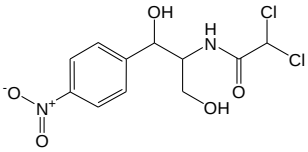


Enrofloxacin (EF)

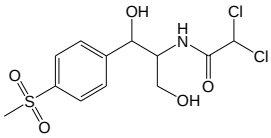


Marbofloxacin (MF)

Amphenicols (APs)

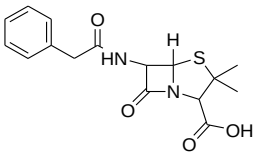


Chloramphenicol (CAP)

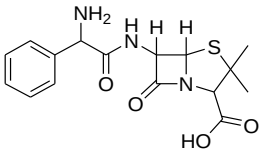


Thiamphenicol (TAP)

Penicillins (PCs)

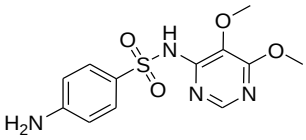


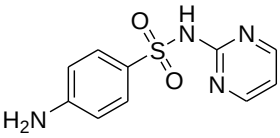
Penicillin G (PCG)

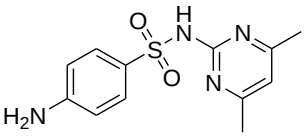


Oxacillin (OXC)

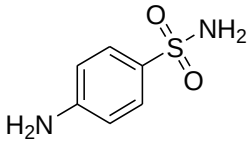
Sulfonamides (SAs)



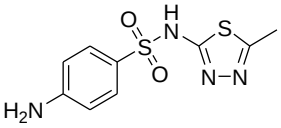




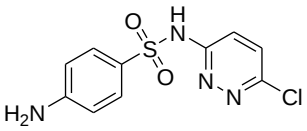
Sulfadoxin (SDX)



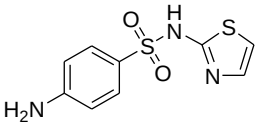
Sulfadiazine (SDZ)



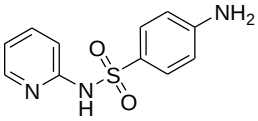
Sulfamethazine (SMZ)



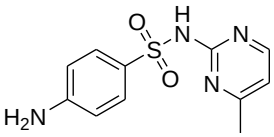
Sulfanilamide (SNMD)



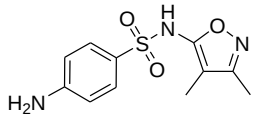
Sulfamethiozole (SMTZ)



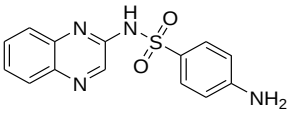
Sulfachloropyridazine (SPDZ)



Sulfathiazole (STAZ)



Sulfapyridine (SPD)

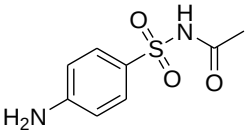


Sulfamerazine (SMRZ)

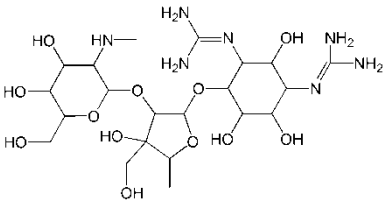
Sulfisoxazole (SIXZ)

Sulfaquinoxaline (SQLX)

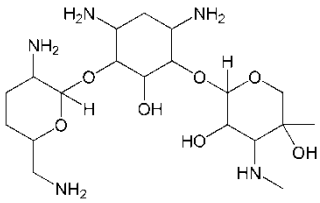
Sulfacetamide (SCTD)



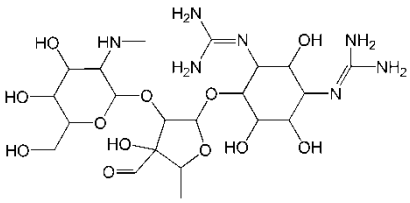
Aminoglycosides (AGs)



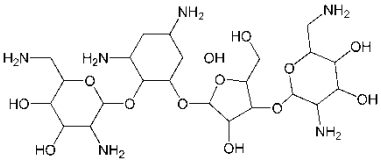
Dihydrostreptomycin (DSMC)



Gentamicin (GMC)

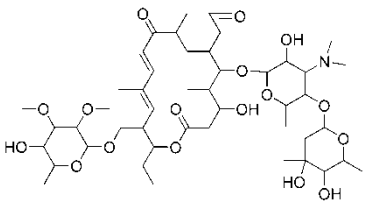


Streptomycin (SMC)

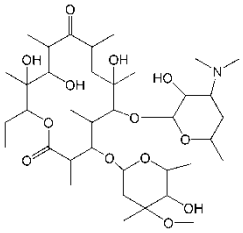


Neomycin (NOMC)

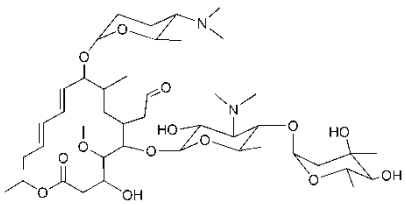
Macrolides (MLs)



Tylosin (TLS)



Erythromycin (ERTC)



Spiramycin (SPMC)