# CHARLES UNIVERSITY IN PRAGUE

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Study programme: Analytical chemistry



**Mgr. Tereza Tylová**


# Liquid chromatography methods for analysis of microbial secondary metabolites of bioactive nature

# Metody kapalinové chromatografie pro analýzu biologicky aktivních mikrobiálních sekundárních metabolitů

## DISSERTATION THESIS


**Supervisor: RNDr. Jana Olšovská, Ph.D.**



**Prague, 2013**

The dissertation thesis summarizes the results obtained in the years 2008-2012 during my Ph.D. studies at the Department of Analytical Chemistry, Faculty of Science of the Charles University in Prague.
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I declare that all results used and published in this thesis have been obtained by my own experimental work, supervised by RNDr. Jana Olšovská, Ph.D. All the references are properly cited and this thesis has not been applied to obtain the same or other academic degree.

Prague, 8.2.2013 Mgr. Tereza Tylová

The presented dissertation thesis is based on following papers that are included as appendices:

**PAPER 1**

Tereza Tylová, Jana Olšovská, Petr Novák, Miroslav Flieger: High-throughput Analysis of Tetracyclines and their Epimers in Liquid Hog Manure Using Ultra Performance Liquid Chromatography with UV Detection; *Chemosphere*, 78 (4), 353-359 (2010).

**PAPER 2**

Tereza Tylová, Miroslav Flieger, Jana Olšovská: Determination of Antibiotics in Influents and Effluents of Wastewater-treatment-plants in the Czech Republic – Development and Application of the SPE and UHPLC-ToFMS Method; *Analytical Methods*, 5 (8), 2110 – 2118 (2013).

**PAPER 3**

Tereza Tylová, Miroslav Kolařík, Jana Olšovská: The UHPLC-DAD Fingerprinting Method for Analysis of Extracellular Metabolites of Fungi of the Genus *Geosmithia* (Acomycota: Hypocreales); *Analytical and Bioanalytical Chemistry*, 400 (9), 2943-2952 (2011).

**PAPER 4**

Tereza Tylová, Zdeněk Kameník, Miroslav Flieger, Jana Olšovská: Comparison of LC Columns Packed with 2.6 µm Core-Shell and sub-2 µm Porous Particles for Gradient Separation of Antibiotics; *Chromatographia*, 74 (1), 19-27 (2011).

**DECLARATION OF CO-AUTHORS**

On behalf of the other co-authors I declare that Mgr. Tereza Tylová contributed substantially to **Paper 1** (her share 70%) and her participation in this paper was following: nearly all experimental work, data evaluation, manuscript preparation.

On behalf of the other co-authors I declare that Mgr. Tereza Tylová contributed substantially to **Paper 2** (her share 80%) and her participation in this paper was following: nearly all experimental work, data evaluation, manuscript preparation.

On behalf of the other co-authors I declare that Mgr. Tereza Tylová contributed substantially to **Paper 3** (her share 80%) and her participation in this paper was following: nearly all experimental work, data evaluation, manuscript preparation.

On behalf of the other co-authors I declare that Mgr. Tereza Tylová contributed substantially to **Paper 4** (her share 45%) and her participation in this paper was following: a half of experimental work, data evaluation, manuscript preparation.

Prague, February 2013 RNDr. Jana Olšovská, Ph.D.

**ABSTRACT (EN)**

This work is focused on development of new analytical methods involving modern solid phase extraction (SPE) for sample purification and pre-concentration, state-of-art ultra high-performance liquid chromatography (UHPLC) for separation of analytes and detection using diode-array detection (DAD) and/or time-of-flight mass spectrometry (ToFMS). The major part of the results has been published in prestigious international journals. The relevant yet unpublished results are included in the thesis as well. Besides a model comparison of various column chemistries used for "ultra-high performance" separations, some meaningful applications were developed. While one part of applications deal with determination of antibiotics as environmental contaminants that are responsible for the development and spread of microbial resistance, the second part is concerned with analyzing and finding of potential natural source of novel antimicrobial agents that can be further used as new antibiotics.

The first part of the thesis was focused on the determination of residual antibiotics in different matrices (liquid hog manure, wastewater, surface water). The mostly prescribed antibiotics in both human and veterinary medicine were chosen as analytes in this study employing analytical methods consisting of UHPLC-DAD and UHPLC-ToFMS together with SPE or liquid-liquid extraction (LLE). The methods were validated and the proper calibration techniques were employed for quantification. The developed procedures were applied for analysis of real environmental samples from different localities in the Czech Republic and the results revealed the occurrence of antibiotics in the majority of the tested samples. The liquid hog manure was positive for tested tetracyclines at concentrations up to 6 mg $kg^{-1}$. All tested water samples were positive for studied antibiotics at concentrations ranging from 5 ng $L^{-1}$ to 1290 ng $L^{-1}$.

The second part of the thesis was focused on development of analytical methods useful for study of secondary metabolites produced by fungi of genus *Geosmithia*. The probability of the employment of the secondary metabolites´ production for the characterization of these fungi was expected. Moreover, these symbionts represent the potential reservoir of bioactive secondary metabolites with various antimicrobial activities. A new UHPLC-DAD-ToFMS method was developed for chemical

fingerprinting of extracellular secondary metabolites in fermentation broth of these fungi and was applied for analysis of 48 *Geosmithia* strains. Various SPE chemistry and LLE procedures were tested resulting in employment of strong cation-exchange mixed-mode polymeric sorbent (Oasis MCX) for extraction of secondary metabolites. Strong correlation between the UHPLC-DAD-ToFMS fingerprints and the taxonomical identity of *Geosmithia* spp. was revealed. These results qualify the method as a chromatographic fingerprinting tool for characterization of fungal strains based of the analysis of their secondary metabolites.

The subsequent chromatographic screening for bioactive secondary metabolites employed both UHPLC-DAD-ToFMS and HPLC-UV methods. Among the tested strains, the secondary metabolites characterized by wide antimicrobial spectrum were revealed and these compounds were determined using the developed analytical methods. This fact confirmed that the introduced LC methods represent a useful tool for the chromatographic screening for bioactive secondary metabolites and that the *Geosmithia* fungi are promising reservoir of interesting antimicrobial compounds.

The third part of the thesis was aimed on the approaches how to improve the efficiency and speed of analysis of microbial secondary metabolites. The recently introduced columns packed with superficially porous particles (represented by Kinetex C18 column) were tested and compared with fully porous particles (represented by Acquity BEH C18 sub-2µm particle column) in UHPLC system under both acidic and alkaline conditions using gradient elution program. The Kinetex C18 column was found to be a suitable alternative to Acquity BEH C18 column. The great advantage of the Kinetex C18 column is the lower column backpressure that enables its use on conventional LC system. However, the limitation of employment of Kinetex C18 column under alkaline conditions was observed with respect to its decreasing performance with the growing number of injections on the column.

**ABSTRAKT (CZ)**

Tato disertační práce je zaměřena na vývoj nových analytických metod sestávajících z extrakce tuhou fází (SPE) použité pro purifikaci a zkoncentrování analytů a jejich stanovení pomocí ultra-vysokoúčinné kapalinové chromatografie (UHPLC) s detektorem diodového pole (DAD) a/nebo hmotnostní detekcí za použití analyzátoru doby letu (ToFMS). Většina předkládaných výsledků byla publikována v prestižních mezinárodních časopisech. Součástí práce jsou také významná dosud nepublikovaná data. Kromě modelových srovnání různých typů kolonových materiálů pro ultra-vysokoúčinné separace představuje práce také významné aplikace vyvinutých metod. Zatímco první část práce je věnovaná stanovení antibiotik jakožto kontaminantů životního prostředí zodpovědných za vývoj a šíření bakteriální rezistence, druhá část se věnuje analýze a hledání biologicky aktivních sekundárních metabolitů z přírodních zdrojů, které mohou být v budoucnu využity jako nová antibiotika.

První část disertační práce je zaměřena na stanovení reziduálních antibiotik v různých matricích (prasečí mrva, odpadní voda, povrchová voda). Jako analyty byla vybrána nejpoužívanější humánní a veterinární antibiotika. Metody sestávající z UHPLC-DAD a UHPLC-ToFMS a dále z SPE nebo extrakce kapalina-kapalina (LLE) byly validovány a pro kvantifikaci byly vybrány vhodné kalibrační techniky. Vyvinuté metody byly použity pro analýzu reálných vzorků z různých lokalit České republiky a výsledky odhalily přítomnost antibiotik ve většině z nich. Prasečí mrva obsahovala tetracyklinová antibiotika v koncentracích až 6 mg $kg^{-1}$. Všechny testované vzorky vod byly pozitivní na antibiotika s naměřenými koncentracemi pohybujícími se od 5 ng $L^{-1}$ do 1290 ng $L^{-1}$.

Druhá část této práce je zaměřena na vývoj analytických metod určených pro studium sekundárních metabolitů produkovaných houbami rodu *Geosmithia*. U těchto hub se předpokládá možnost jejich charakterizace na základě produkce sekundárních metabolitů. Jakožto symbionti jsou tyto houby jsou navíc potenciálním zdrojem biologicky aktivních sekundárních metabolitů. Byla vyvinutá nová UHPLC-DAD-ToFMS metoda pro „chromatografický fingerprinting“ extracelulárních metabolitů ve fermentačním médiu těchto hub a následně byla použita pro analýzu

48 kmenů rodu *Geosmithia*. Pro extrakci analytů byly testovány různé SPE sorbenty a postupy pro LLE s výsledným použitím silného měniče kationtů (Oasis MCX). Výsledky odhalily silnou korelaci mezi „UHPLC-DAD-ToFMS fingerprinty“ a taxonomickou příslušností jednotlivých kmenů. Tyto dosažené výsledky potvrzují, že vyvinutá metoda je vhodná pro účely „chromatografického fingerprintingu“ založeného na analýze produkovaných houbových sekundárních metabolitů.
Dále byly vyvinuty UHPLC-DAD-ToFMS a HPLC-UV metody pro následný „chromatografický screening“ biologicky aktivních sekundárních metabolitů těchto hub. U testovaných kmenů byla zjištěna produkce sekundárních metabolitů se širokým antimikrobiálním spektrem účinnosti a tyto látky byly vyvinutými metodami určeny. Tím bylo potvrzeno, že vyvinuté LC metody jsou vhodné pro „chromatografický screening“ biologicky aktivních sekundárních metabolitů a že houby rodu *Geosmithia* představují slibný zdroj zajímavých antimikrobiálních látek.

Třetí část práce se zabývá možnými přístupy vedoucími ke zvýšení účinnosti a rychlosti chromatografických separací při analýze mikrobiálních sekundárních metabolitů. Byla srovnávána nedávno představená kolona obsahující povrchově porézní částice (reprezentované Kinetex C18 kolonou) s kolonou s plně porézními částicemi (reprezentovány Acquity BEH C18 sub-2µm particle kolonou) na UHPLC systému za použití mobilní fáze o kyselém i zásaditém pH. Bylo zjištěno, že kolona Kinetex C18 je vhodnou alternativou Acquity BEH C18 kolony. Velkou výhodou kolony Kinetex C18 je její nižší zpětný tlak, což umožňuje její použití na běžném HPLC chromatografu. Nicméně byla zjištěna limitace Kinetex C18 kolony v podobě její omezené stability v alkalické oblasti pH.

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## LIST OF ABBREVIATIONS AND SYMBOLS

| | |
|---|---|
| 2D, 3D | two-dimensional, three-dimensional |
| ACN | acetonitrile |
| $\beta$ | gradient slope |
| BEH | bridged ethylene hybrid |
| CE | capillary electrophoresis |
| CFP | chromatographic fingerprinting |
| CID | collision-induced dissociation |
| CIP | ciprofloxacin |
| CLA | clarithromycin |
| CLI | clindamycin |
| CTC | chlortetracycline |
| $CTC_{iso,}$ $CTC_{ep}$ | chlortetracycline isomer, chlortetracycline epimer |
| DAD | diode-array detection |
| DNA, rDNA | deoxyribonucleic acid, ribosomal deoxyribonucleic acid |
| DOX | doxycycline |
| $DOX_{ep}$ | doxycycline isomer, doxycycline epimer |
| ENR | enrofloxacin |
| ERY | erythromycin |
| ESI | electrospray ionization |
| FTMS | Fourier transform mass spectrometry |
| FWHM | full width at half maximum |
| GC | gas chromatography |
| $H$ | separation efficiency |
| HCA | Hierarchical cluster analysis |
| HEPT | Height equivalent of theoretical plate |
| HPLC | high-performance liquid chromatography |
| i.d. | internal diameter |
| i-FIT | isotope predictive filtering |
| ILOQ | instrument limit of quantification |
| ITS | internal transcribed spacer |
| IUPAC | International Union of Pure and Applied Chemistry |
| LC | liquid chromatography |
| LIN | lincomycin |
| $m/z$ | mass-to-charge ratio |
| MEA | malt extract agar |
| MLOQ | method limit of quantification |
| MS, MS/MS | mass spectrometry, tandem mass spectrometry |
| MW | molecular weight |
| NMR | nuclear magnetic resonance |
| NOR | norfloxacin |
| ODS | octadecyl |
| OFL | ofloxacin |
| OXY | oxytetracycline |

| | |
|---|---|
| $P$ | peak capacity |
| PAST | Palaeontological Statistics |
| PC, PCs | principal component, principal components |
| PCA | Principal component analysis |
| PCoA | Principal coordinates analysis |
| ppm | parts per million |
| ROX | roxithromycin |
| RP | reversed phase |
| RP-HPLC | reversed phase HPLC |
| SDD | sulfadimidine |
| SDM | sulfadimethoxine |
| SDZ | sulfadiazine |
| SM, SMs | secondary metabolite, secondary metabolites |
| SMX | sulfamethoxazole |
| STZ | sulfathiazole |
| TC | tetracycline |
| $TC_{iso}$, $TP_{ep}$ | tetracycline isomer, tetracycline epimer |
| TFA | trifluoroacetic acid |
| ToF | time-of-flight mass analyser |
| TYL | tylosin |
| $u_0$ | linear velocity |
| UHPLC | ultra high-performance liquid chromatography |
| UV/VIS | ultraviolet-visible spectrum |
| WHO | World Health Organization |
| WWTPs | wastewater treatment plants |

# 1 PREFACE

Antibiotics are the typical secondary metabolites produced by microorganisms, in particular actinomyces and microfungi. They exhibit antibacterial and antifungal activity and they are widely used as antibiotics in the prevention and treatment of infectious diseases.[1] The consumption of antibiotics rises significantly every year. About 50-90% of the administered pharmaceutical dose is excreted rapidly after the treatment in their parent form or as metabolites that can then enter the environment and cause the development of bacterial resistance. The most important sources of such compounds in the environment are households, wastewater-treatment-plants (WWTPs), hospitals, industrial units and intensive animal husbandry. The development and spread of antibiotic resistance is the greatest threat to successful antibiotic coverage.[2] Therefore, there is a strong need for control of the antibiotic usage, for monitoring of the occurrence of antibiotics in the environment[3-5] and continuing and urgent need for new antibiotics therapeutic agents.[1, 6]

Microbial natural products represent a rich source of biologically active compounds and are an example of molecular diversity with recognized potential in drug discovery and development.[7, 8] Among the microorganisms, fungi cover about 8,000 described microbial species with assumption of existence of several millions species, which is the highest supposed number among the microbial world. This qualifies fungi to have the biggest potential on the field of natural products resources and it looks that the world of fungi is one of the largest reservoirs for isolating further bioactive metabolites.[9] *Geosmithia* (Ascomycota: Hypocreales) are little known but regular and worldwide distributed filamentous symbiotic fungi. As symbionts, they enter complex chemical-based interactions with their hosts and thus possess a diversity of secondary metabolites with various biological activities. Moreover, supposed the production of secondary metabolites to reflect both the phylogenetical and ecological relatedness of their producers. Therefore the study of secondary metabolites can provide the information about the taxonomical identity of the strains and can also lead to discovery of novel biologically active compounds.[10]

Chromatographic techniques in combination with extraction methods provide the opportunity to study the low molecular secondary metabolites in complex matrices in

both high and low concentrations.[11] Moreover, hyphenated techniques such as high-performance liquid chromatography (HPLC) or ultra high-performance liquid chromatography (UHPLC) with diode-array detection (DAD) or with mass spectrometric detection (MS) and tandem mass spectrometric detection (MS/MS) provide multi-dimensional and multi-informational analyses of high sensitivity. They are therefore useful for determination of trace concentrations as well as for chromatographic fingerprinting and screening methods if additional information about unknown compounds is needed.

Nowadays, there is a strong demand on efficiency and speed of analysis in many application areas of liquid chromatography, such as in pharmacy, drug discovery, drug development, food industry, environmental monitoring or clinical analysis. In order to perform rapid procedures, different strategies can be applied. The possibility of employing both UHPLC technique using sub-2 µm column particles and the alternative approach of using superficially porous column particles represent one of the mostly opted approaches in order to achieve fast analyses of high separation efficiency.

## 2 AIMS OF THE THESIS

The thesis is focused on development of analytical methods consisting of chromatographic and extraction procedures for study of microbial secondary metabolites. The microbial secondary metabolites are studied from two different points of view: (a) as classic antibiotics used as pharmaceutical, which is connected with their occurrence in the environment and with development of antimicrobial resistance, (b) as a promising reservoir of bioactive compounds that can be further used as novel antimicrobial agents. In general, the fast analyses of high separation efficiency in the field of liquid chromatography have become of a great importance in antibiotics´ and secondary metabolites´ researches.

**Therefore the main aims of the thesis are**:

**1.** To develop analytical methods consisting of extraction procedures followed by liquid chromatography with both UV/VIS and MS detection for:

- **(a)** Determination of antibiotics in the environmental samples; namely in liquid hog manure, and in influents/effluents of WWTPs. The mostly used and prescribed antibiotics in both human and veterinary medicine should be selected as analytes in this study. The main demand on the method is its versatility for determination of wide spectrum of antibiotics and good method sensitivity. The application of the developed methods for analyses of real samples from different localities in the Czech Republic is a part of the study too. The results will give information about the occurrence of selected antibiotics in the environment of the Czech Republic**. (PAPER 1** and **PAPER 2)**
- **(b)** Study of secondary metabolites produced by fungi of the genus *Geosmithia* since these symbiotic fungi can possess a diversity of secondary metabolites with various biological activities. Firstly, this part is focused on the development of chromatographic fingerprinting method that will serve as a tool for characterization of the fungal strains based on their secondary metabolites´ production. Secondly, this part is aimed on the bioactive secondary metabolites of *Geosmithia* fungi employing chromatographic screening for the novel antimicrobial agents produces by these microorganisms. **(PAPER 3)**

**2.** To focus on the possible approaches of fast analyses of high separation efficiency. This part is aimed on comparison of the performance of superficially porous particles and fully porous sub-2 µm column particles for analysis of low-molecular antibiotics of different chemical properties on UHPLC system. **(PAPER 4)**

# 3 BIOACTIVE MICROBIAL SECONDARY METABOLITES

## 3.1 NATURAL PRODUCTS, SECONDARY METABOLITES

The usual definition of natural products in the widest sense emphasize that they "are chemical compounds included carbon in the molecule that are isolated from diverse living things". These compounds may derive by primary or rather secondary metabolism of living organisms.[9] The metabolism is defined as the sum of all biochemical reactions carried out in an organism. Primary metabolic pathways converge to few products while secondary metabolic pathways diverge to many products.[12] The primary metabolites (polysaccharides, proteins, nucleic and fatty acids) are common in all biological systems. The secondary metabolites (SMs) are, however, low molecular (MW<3000), chemically and taxonomically extremely diverse compounds with obscure function, characteristic mainly to some specific, distinct types of organisms.[9] Plants, fungi, lichen fungi, and actinomycetes are particularly good producers of SMs, whereas yeast, protozoa, and animals are less efficient producers.[13] Higher plant metabolites cover great number of common plant compounds, such as alkaloids, flavonoids, terpenoids, steroids, carbohydrates etc.[9] Microbial SMs include pigments, toxins, effectors of ecological competition and symbiosis, pheromones, enzyme inhibitors, antibiotics etc.[14] The exact definition and the real position and function of microbial SMs are for long time highly discussed topic in the whole area of microbiology.[1, 10] The SMs may evince some kind of biological activity or are without any discovered interaction with other living organisms. The activity may be highly specific, exhibiting usually at low concentration (representing the usual bioactivities), or may be very unspecific (toxic) action. The exact number of bioactive natural products is almost undeterminable but this figure by all means is at least 200 to 250 thousands, including more than 20 thousands microbial metabolites.[9]

## 3.2 BIOACTIVE SECONDARY METABOLITES

Antimicrobial agents are substances that kill or inhibit the growth of prevent damage due to the action of infectious microorganisms. Terms of antimicrobial agents comprises antibacterial, antifungal, antiprotozoal, and antiviral agents.[8]

### 3.2.1 Antibiotics

There exist many definitions of what is antibiotic that were changing during the times. The SMs isolated from microbes and exhibit antimicrobial activity used to be called as antibiotics.[9] The word “antibiotic” was first used by Waksman in 1942[15] and defined as substances produced by microorganisms antagonistic to the growth of life of others in high dilution (the last clause being necessary to exclude such metabolic products as organic acids, hydrogen peroxide and alcohol).[8] Other definition is extended to all of those SMs, which regulate growth processes, replications, and/or exhibit some kind of responding (regulating, inhibiting, stimulating) action to the life cycle of prokaryotic or eukaryotic cells at the biochemical level in minimal concentration. This broadest definition should cover besides the so-called “classical antibiotics” practically all bioactive compounds.[9] At present, the term “antibiotic” refers to molecules that stop microbes, both bacteria and fungi, from growing or kill them outright. Antibiotics include both natural products and manmade synthetic chemicals, designed to block some crucial processes in microbial cells selectively. The antibiotics and similar compounds form perhaps the most interesting, most dynamically increasing, and one of the practically most important groups of the natural products. They are widely used in the human therapy, veterinary, agriculture, scientific research and in the countless other areas.[9]

### 3.2.2 Characterization of antibiotics

The most important, inherent characteristics of the bioactive microbial metabolites are their microbial origin (specific microbial producers), their interaction with the environment (various biological activities), and their unique chemical structures.[9]

#### *3.2.2.1 Producers of antibiotics*

The main producers of antibiotics are soil microorganisms as actinomycetes, moulds and fungi.[16] Antibiotics such as macrolides, lincosamides, tetracyclines, β-lactams, aminoglycosides, and glycopeptides are produced by actinomycetes. Among them, genus *Streptomyces* produces enormous diversity of antibiotics, of which only 1-3% is believed to have been discovered.[17]

#### *3.2.2.2 Modes of action of antibiotics*

At basis of all antimicrobial chemotherapy lies the concept of selective toxicity. This can be achieved in several ways. According to target organism of the antimicrobial agents, antibiotics are mainly classified as antibacterial agents, antifungal agents, antiprotozoal agents and antiviral agents.[8, 18-21] The antibiotics studied in this thesis represent antibacterial agents.

##### *3.2.2.2.1 Antibacterial agents*

The selective action of antibiotics is enabled by structural and metabolic differences of bacterial and mammalian cells. In theory, there are numerous ways in which bacteria can be selectively killed or disabled, namely inhibitors of bacterial cell wall synthesis (e.g. β-lactam agents), inhibitors of bacterial protein synthesis (e.g. **tetracyclines, macrolides, lincosamides**), inhibitors of nucleic acid synthesis (e.g. **fluoroquinolones, sulfonamides**), or agents affecting membrane permeability (e.g. polymixin B, ionophores).[8, 22]

### 3.2.3 Antibiotics classes

Antibiotics can be categorized on the basis of their chemical structure. The major classes include: penicillins, cephalosporins, aminoglycosides, macrolides, sulfonamides, quinolones and fluoroquinolones, tetracyclines, polypeptides, lincosamides. Antibiotics studied in this thesis are further characterized.

#### *3.2.3.1 Tetracyclines*

Tetracyclines are a group of natural products derived from *Streptomyces* spp. and their semi-synthetic derivates based on a hydronaphthacene nucleus containing four fused rings. They exhibit a broad-spectrum of activities including bacteria and protozoa. They are active against many common Gram-positive and Gram-negative bacteria,

*Chlamydia*, *Rickettsia*, etc.; they are distinguished mainly for bacteriostatic action caused by inhibition of bacterial protein synthesis.[8, 23, 24]

The natural products include **chlortetracycline** (CTC), **oxytetracycline** (OTC) and **tetracycline** (TC). **Doxycycline** (DOX) belongs to semi-synthetic derivates.[8] TC and DOX are used in both human and veterinary medicine, while CTC and OXY only in veterinary medicine.[25] For chemical structures, see **Figure 1**.

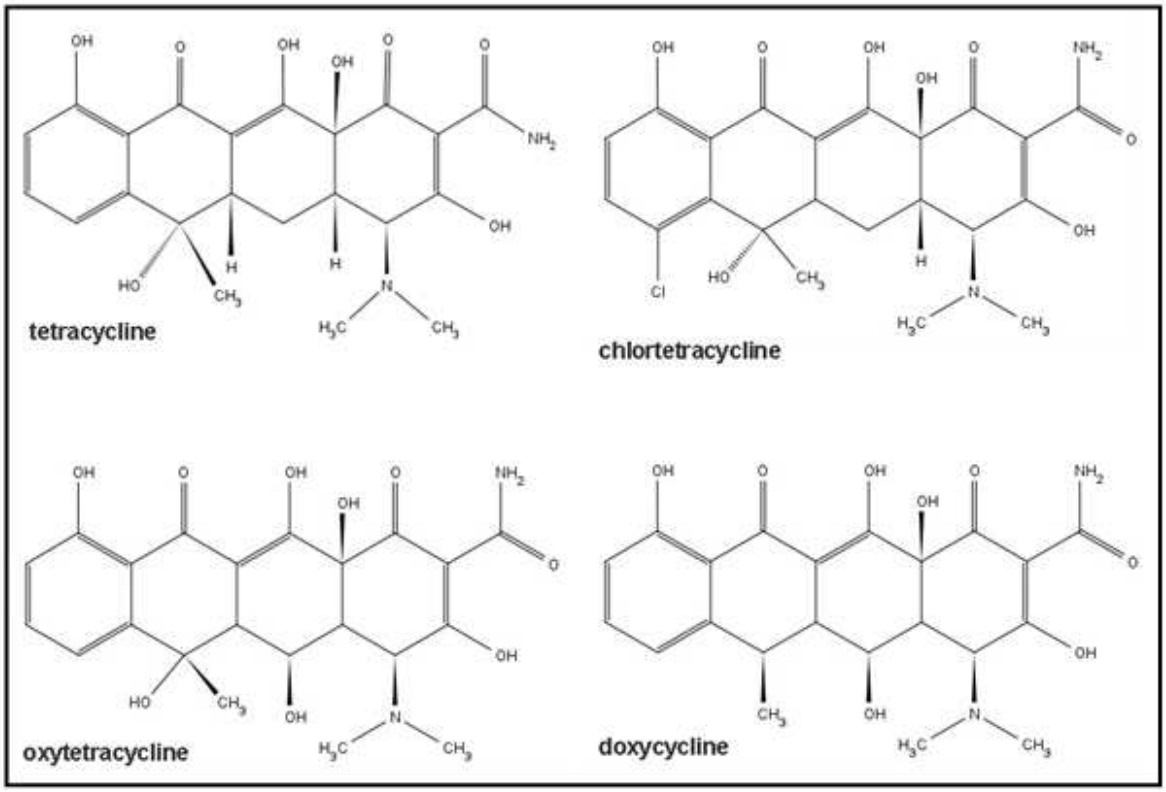


**Figure 1. Chemical structures of tetracycline antibiotics studied in this thesis.**

### *3.2.3.2 Sulfonamides*

Sulfonamides exhibit broad-spectrum antimicrobial activity. Their primary activity is e.g. against susceptible forms of *Streptococcus*, *Staphylococcus aureus*, *Escherichia coli*, and oral anaerobes. Well known members of this group are **sulfathiazole** (STZ), **sulfadiazine** (SDZ), **sulfadimidine** (SDD), sulfafurazole, **sulfadimethoxine** (SDM) or **sulfamethoxazole** (SMX).[8] SMX is highly prescribed in both human and veterinary medicine. All other mentioned sulfonamides are used in veterinary medicine.[25] For chemical structures, see **Figure 2**.

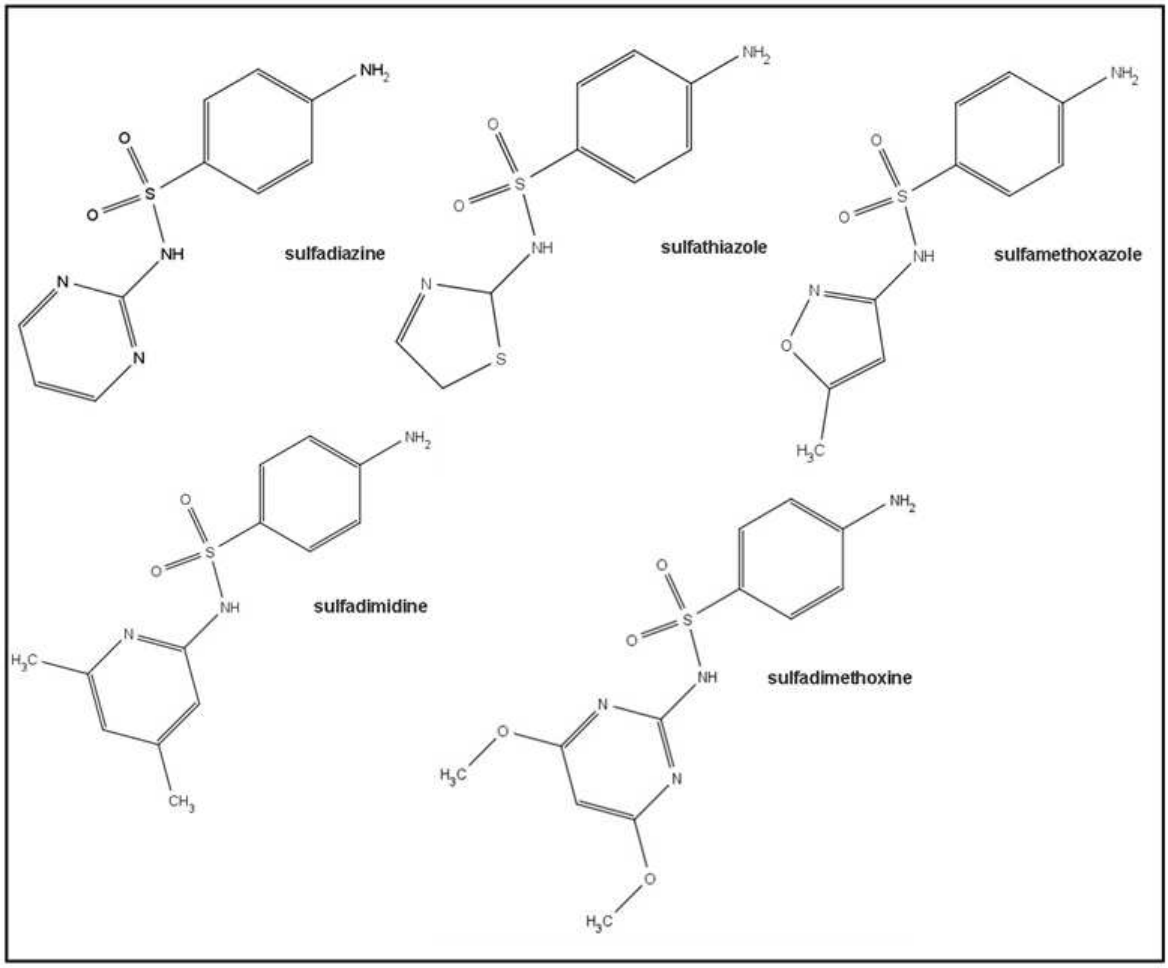


**Figure 2. Chemical structures of sulfonamide antibiotics studied in this thesis.**

### *3.2.3.3 Macrolides*

The macrolides form a large group of closely similar antibiotics produced mostly by *Streptomyces*. They consist of a macrocyclic lactone ring to which typically two sugars, one of amino sugar, are attached. The groups of synthesized members of macrolides contain the most important therapeutic agents characterized by 14-, 15- or 16-membered-ring macrolides. It is, e.g. erythromycin A and its newer derivates roxithromycin or clarithromycin. Concerning antimicrobial activity, the 14-, 15- and 16-membered-ring macrolides share the same antibacterial spectrum including most Gram-positive organisms and both Gram-positive and Gram-negative anaerobes. **Erythromycin** (ERY) is a 14-membered-ring macrolide produced as complex of six components (A to F) by *Saccharopolyspora erythreaus*. Only erythromycin A has been developed for clinical use. **Clarithromycin** (CLA) and **roxithromycin** (ROX) are 14-

membered-ring macrolides derived from erythromycin A; tylosin (TYL) is the 16-membered-ring macrolide.[8]

CLA and ROX are used in human medicine, ERY in both human and veterinary medicine, and TYL in veterinary medicine.[25] For chemical structures, see **Figure 3**.

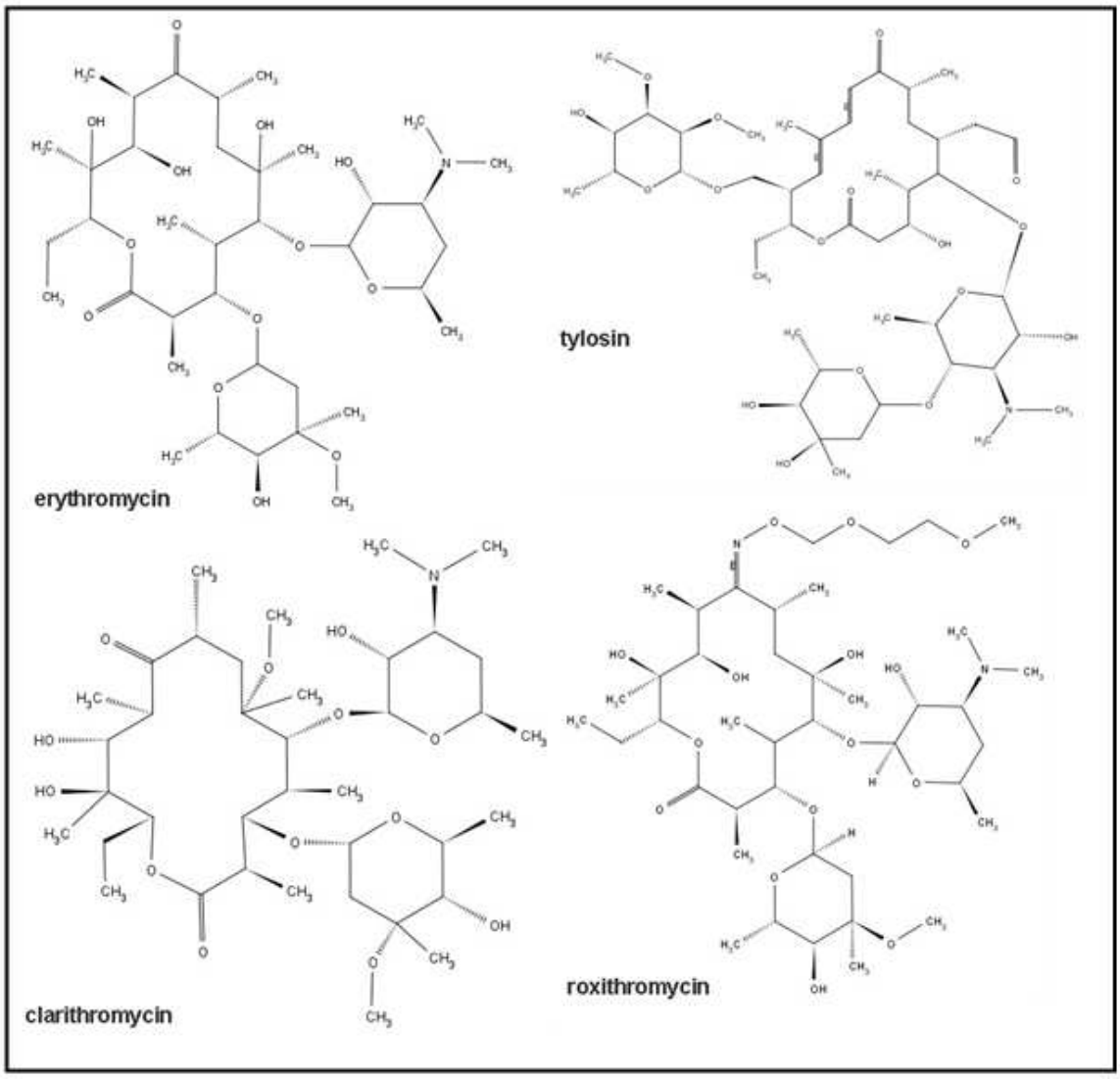


**Figure 3. Chemical structures of macrolide antibiotics studied in this thesis.**

### *3.2.3.4 Lincosamides*

A small group of agents based on a novel structure unlike that of any other antibiotics. The naturally occurring members of the group are lincomycin and celesticetin. Semi-synthetic derivates of lincomycin have been prepared in the hope of improving on its properties; however, they have proved to be less active. The only important exception was the chlorinated derivate, clindamycin. They share an unusual antimicrobial spectrum, being active only against Gram-positive and not against Gram-negative

aerobes, but widely and potently active against anaerobes. They are also active against some mycoplasmas and protozoa.

**Lincomycin** (LIN) is a fermentation product of *Streptomyces lincolnensis.* **Clindamycin** (CLI) is a semi-synthetic chloro-7-dehydrolincomycin of the same antimicrobial spectrum as lincomycin, but it is generally more potent.[8]

LIN and CLI are both human and veterinary medications.[25] For chemical structures, see **Figure 4**.

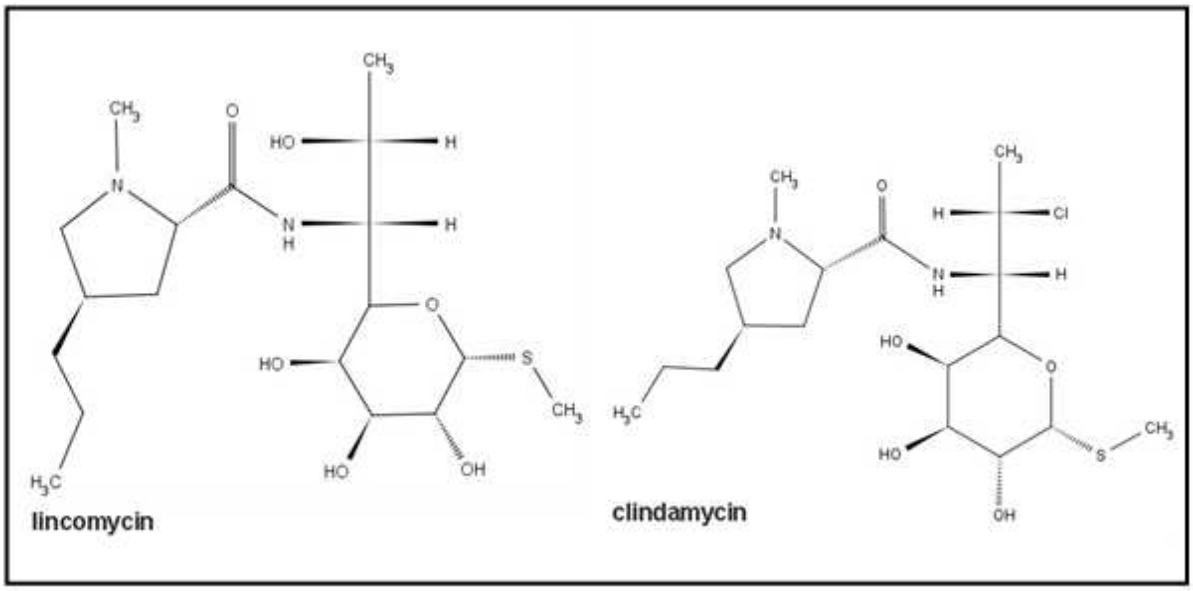


**Figure 4. Chemical structures of lincosamide antibiotics studied in this thesis.**

### *3.2.3.5 Fluoroquinolones*

Fluoroquinolones originated from quinolones, synthetic compounds based on 4-quinolone nucleus. All these compounds are characterized by a dual-ring structure with nitrogen at position 1, a carbonyl group at position 4 and a carboxyl group attached to the carbon at position 3 of the first ring. Due to the increase of bacterial resistance against quionoles, the structure the fluoroquinolones as congeners with fluorine in the 6-position of the molecule with superior properties were developed.[8] **Ciprofloxacin** (CIP) is overall the most potent of the currently available fluoroquinolones against Gram-negative bacteria. **Norfloxacin** (NOR) is particularly active against a wide range of Enterobacteriaceae *(Escherichia coli, Salmonella,* etc.). **Ofloxacin** (OFL) exhibits potent activity against wide range of Enterobacteriaceae including the strains resistant to other members of this group. **Enrofloxacin** (ENR) is effective against broad spectrum of Gram-positive and Gram-negative bacteria (e.g. *Escherichia coli,*

*Enterobacter*. *Salmonella, Chlamydia,* and *Staphylococci*). CIP, NOR, and OFL are used in human medicine, while ENR is veterinary medication.[8] For chemical structures, see **Figure 5**.

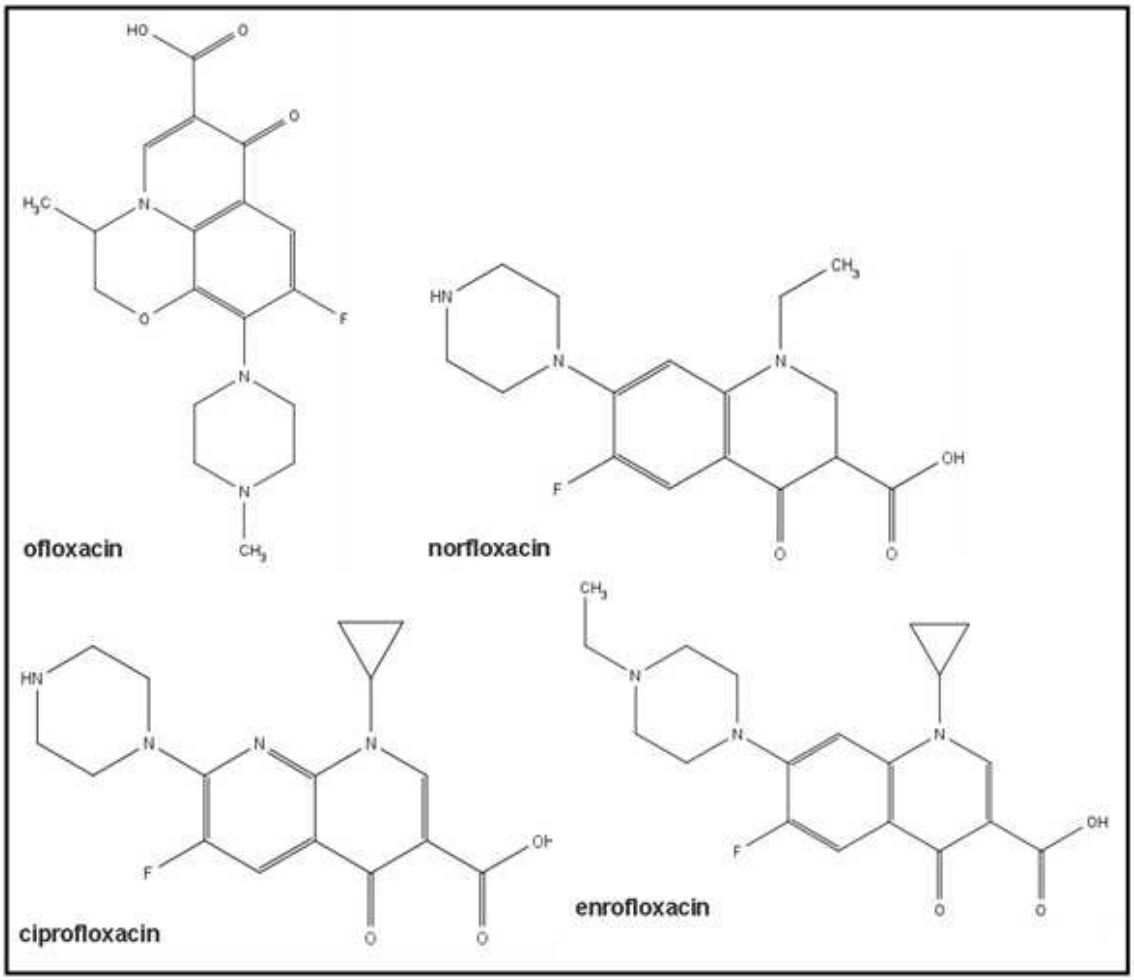


**Figure 5. Chemical structures of fluoroquinolone antibiotics studied in this thesis.**

## 3.3 MICROBIAL RESISTANCE

The microbial resistance represents nowadays serious problem on the field of antibiotic usage. Antibiotic resistance is a type of drug resistance where a microorganism is able to survive exposure to an antibiotic. We can divide the microbial resistance to two main types, at intrinsic (inherent, natural) and acquire resistance.[8]

When quantitative measurements of antimicrobial susceptibility are made, some species are clearly less sensitive to the given antibiotic than others. This is defined as intrinsic resistance and is given by the bacterial species and its characteristic (e.g. Gram-positive and Gram-negative bacteria[8, 26]). The intrinsic resistance defines also the spectrum of antibiotic for the particular agents.[8] On the other hand, some microorganism can become resistant to an antimicrobial medicine to which it was previously sensitive.[27] This is called acquired resistance. The resistant organisms (they include bacteria, viruses and some parasites) are able to withstand attack by antimicrobial medicines, such as antibiotics, antivirals, and antimalarials, so that standard treatments become ineffective and infections persist and may spread to others.[27]

### 3.3.1 Development of acquired resistance (clinical resistance)

The phenomenon of acquired resistance commonly arises when populations of bacteria have grown in the presence of the antibiotic[8] and is a consequence of the great use, particularly misuse, of antimicrobial medicines.[27] The most important sources of antibiotics in the environment are households, wastewater-treatment-plants (WWTP), hospitals, industrial units and intensive animal husbandry.[18] The significantly established resistance levels were also revealed as a result of similar compounds being used in medicine and agriculture. Antibiotic resistance is a growing problem in the treatment of infectious diseases.[19] In particular, bacterial resistance to antibiotics has emerged over the past decades as a major health problem.[28, 29] The most hazardous are hospital infections caused by the bacterial species resistant to the antimicrobial agents, because they often fail to respond to the standard treatment, resulting in prolonged illness and greater risk of death and thus potentially spreading resistant microorganisms to others.[27] The historical observations are that when a new class of

antimicrobial agents is introduced into widespread clinical use, a significant resistance appears in months or years (see **Table 1**).[30] The growth of global trade and travel allows resistant microorganisms to be spread rapidly to distant countries and continents.[27] The evolution and spread of antibiotic resistance is the greatest threat to successful antibiotic coverage.[2] Therefore, it is necessary to choose the strategies how to overcome the antimicrobial resistance.

**Table 1. The development of resistance to selected antimicrobial agents in clinical use.**[21]

| antimicrobial agent | discovery | clinical use | development of resistance |
|---|---|---|---|
| penicillin | 1929 | 1943 | 1940 |
| streptomycin | 1944 | 1947 | 1947 |
| tetracycline | 1948 | 1952 | 1956 |
| erythromycin | 1952 | 1955 | 1956 |

### 3.3.2 Strategies to overcome the antimicrobial resistance

There are several strategies how to overcome the antimicrobial resistance, e.g.:

- control of usage and prescription of antimicrobial agents
- development of new schedules of dosing of these pharmaceuticals
- encouragement of the sensible prescription and usage of antimicrobial agents
- control of the occurrence of antimicrobial agents in the environment
- improvement the processes responsible for disposal of antimicrobial agents exposed into the environment (e.g. in WWTPs)
- searching for novel antimicrobial agents (derivates of known antibiotics, completely novel compounds from natural sources, strategies employing genetic approaches, etc. )

This thesis is focused on strategies dealing with the control of occurrence of antibiotics in the environment and with gaining novel antibiotics from natural sources (microbial SMs with antimicrobial activity). These approaches are described in following chapters.

## 3.4 DETERMINATION OF RESIDUAL ANTIBIOTICS IN THE ENVIRONMENT

Antibiotics represent a group of pharmaceuticals that are widely used in both human and veterinary medicine and their consumption rises significantly every year. About 50-90% of the administered pharmaceutical dose is excreted rapidly after the treatment in their parent form or as metabolites that can then enter the environment and cause the development of bacterial resistance.[31-35] There are three significant field of application of antibiotics for animal husbandry: treatment of infection in livestock, prevention of infection and growth promoters.[36] The most widely used groups of antibiotics in the European Union's animal husbandry are tetracyclines, macrolides, penicilines, aminoglycosides and sulfonamides.[37] Significant growth of antibiotic consumption in the Czech Republic has been observed from the beginning of 1990´s, with the main increase of macrolides, penicilines, and fluoroquinolones,[25, 38-41] and the associated antibiotic resistance grows annually from the 1990´s. This trend is observed in most European countries, but the situation in the Czech Republic is one of the most alarming.[25] Different classes of antibiotics in water samples at concentration ranging from nanograms to micrograms per liter were found e.g. in Spain,[42] France,[31] United Kingdom,[20, 43] Italy,[44] Switzerland,[45, 46] Austria,[47] Sweden,[4, 48, 49] Croatia,[50] and Poland.[20] Concerning the Czech Republic, Seifertova et al. presented a study dealing with fluoroquinolones in wastewater[33] but there is still lack of information about the presence of a wide spectrum of antibiotics in aquatic environment in the Czech Republic. As mentioned above, animal husbandry and WWTPs are important sources of pharmaceuticals in the environment. Although the application of antibiotics as growth promoters in livestock was banned by EU in 2006, there is an apprehension that they are still used for this purpose. Therefore, the control of their application in animal husbandry is obviously necessary. WWTPs contribute also to the exposure of antibiotics into the environment significantly, because the excreted antibiotics are discharged from WWTPs at certain levels due to inadequate removal efficiencies of the plants.[51] This fact shows the need of WWTPs efficiency evaluation and further improvement of the water treatment processes.

## 3.5 GAINING NOVEL ANTIBIOTICS FROM NATURAL SOURCES

### 3.5.1 Approaches to finding novel antimicrobial agents

#### *3.5.1.1 Current methods*

There are two main possible current approaches in the development of novel antibiotics:

- through the discovery of completely novel antibiotic from natural sources
- by the use of derivates of known antibiotics that are frequently prepared by semisynthetic methods and that are not affected by existing resistance mechanisms.

Although completely novel classes of antimicrobial agents were discovered and frequently implemented rapidly into clinical practice throughout the 1940´s and 1950´s, a truly new antibiotic class has not emerged for many years, perhaps since the discovery of the fluoroquinolones in the early 1970´s. In contrast, the modification of known antibiotics has been the major source of new medicinal agents for decades.[19]

#### *3.5.1.2 Genetic approaches*

It is well known that small changes in culture conditions or in the media may result in a big alteration in the biosynthesis of metabolites. The cultivation of producers with unnatural precursors, the random mutagenesis and the use of genetically modified strains are the simplest but promising opportunities to increase the chemical and biological diversity. The mutasynthesis or mutational biosynthesis, namely culturing microbes with unnatural precursors, also produced hundreds of new hybrid molecules (aminoglycosides, glycopeptides, etc.).[9] Combinatorial biosynthesis, the genetic manipulation of enzymes for natural product biosynthesis with the aim to obtain new molecule, is an additional possibility.[9, 52]

#### *3.5.1.3 Novel methods*

Novel methods cover approaches as using non-culturable[1, 53] and non-multiplying bacteria as targets,[53-55] or employing bacteriophages (the viruses that infect bacteria) as antibacterials.[56, 57]

### 3.5.2 Natural products as sources of antimicrobial compounds

Natural products represent a rich source of biologically active compounds and are an example of molecular diversity with recognized potential in drug discovery and development. They play an essential role in many areas in the society, e.g. as nutritional and therapeutic agents to prevent or cure diseases. The World Health Organization (WHO) has estimated that approximately 65% of the world's population rely mainly on plant-derived traditional medicines for their primary health care.[58, 59] By 1990, 80% of drug substances were either natural products or their analogs.[59] Despite changing strategies in natural products research (samples selection and collection, isolation techniques, etc.) the discovery of antibiotics from natural sources decreased, as depicts **Figure 6**. Nevertheless, there is continuing need to isolate unique low molecular weight compounds with biological activities. Natural products resources, including microbial world, are mainly unexplored both in its dimension and in the respect of geographic, ecological and environmental point of view.[9]

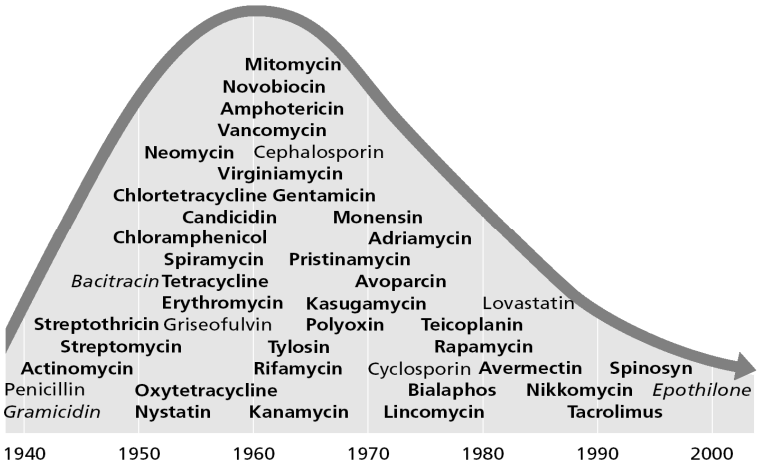


**Figure 6. History and intensity of antibiotics discovery.**[60]

#### *3.5.2.1 Fungal secondary metabolism*

Among the microorganisms, fungi cover about 8,000 described microbial species with assumption of existence of several millions species, which is the highest supposed number among the microbial world. They are the second largest eukaryotic group next to the insects and exceed not only the bacteria and actinomycetes, but the higher plant also in the terms of the possible existing species.[9] In fungi, more than 4,000 fungal

metabolites have been described[10] and 5,000 - 7,000 taxonomic species have been studied with respect to their chemistry. Considering that 6 out of 20 of the most commonly prescribed medications are of fungal origin[61] and only 5% of the fungi have been described[62, 63], fungi offer an enormous potential for new products. It looks that the world of fungi is one of the largest reservoir for isolating further bioactive metabolites.[9]

The fungal kingdom includes many species with unique and unusual biochemical pathways. The products of these pathways include important pharmaceuticals such as penicillin, cyclosporine and statins; potent poisons, including aflatoxins and trichothecenes; and metabolites that are both toxic and pharmaceutically useful, such as ergot alkaloids.[64] The systematic study of fungal SMs began in 1922 under the leadership of Harold Raistrick.[65] However, it was not until the discovery of penicillin that widespread attention was focused on fungal metabolites.[64]

#### *3.5.2.2 Secondary metabolites produced by Geosmithia fungi*

*Geosmithi*a (Ascomycota: Hypocreales) are little known but regular and worldwide distributed filamentous fungi belongs to regular associates of many insect species infesting the phloem or sapwood of various plant genera worldwide (see **Figure 7**).[66-71] These dry-spored symbiotic fungi occur in galleries built by many phloephagous bark beetles (Coleoptera: Curculionidae, Scolytinae). In general, symbiotic fungi enter complex chemical-based interactions with their hosts and thus possess a diversity of SMs with various biological activities.[10] It is supposed that the production of SMs also reflects both the phylogenetical and ecological relatedness of their producers.[70]

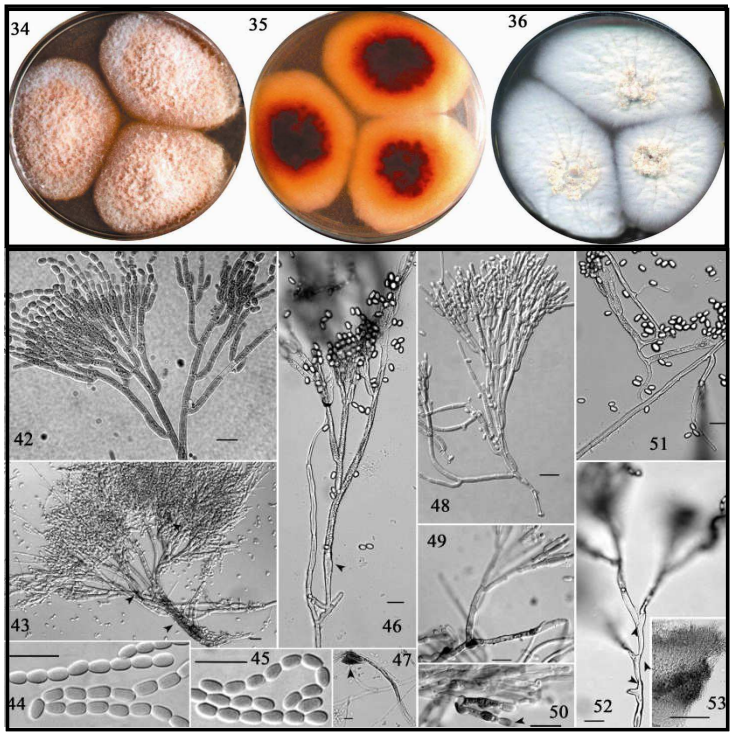


**Figure 7. *Geosmithia obscura*.**[70, 72]
*Top*: Colonies of *G. obscura* (MEA, 14d, 25 °C).
*Down*: Morphological features of *G. obscura*.

The teams of microbiologists from the Institute of Microbiology of the Academy of Sciences of the Czech Republic in cooperation with Culture Collection of Fungi in Prague have collected over 600 *Geosmithia* isolates from beetles from across Europe during the years 1997 - 2005. These isolates were sorted into operational taxonomic units based on their phenotype similarity and phylogeny of internal transcribed spacer (ITS) region of ribosomal DNA (ITS-rDNA sequence).[70] So far, 33 different lineages of *Geosmithia* were found, and some of them were described as new taxa.[69, 71] Twenty lineages appear to be novel taxa with unique genotype and phenotype. Most of *Geosmithia* spp. in our collection are represented by many strains from many locations, which is necessary for quality taxonomic analysis.[70] The study of SMs can provide the information about the taxonomical identity of the strains and can also lead to discovery of novel biologically active compounds. The analytical methods used for study of fungal SMs are detailed described in chapter ***3.6.***

## 3.6 ANALYTICAL METHODS USED FOR STUDY OF SECONDARY METABOLITES

### SEPARATION TECHNIQUES

Since there is often interference with the required measurement by other constituents of the sample, many techniques for separating and concentrating the species of interest have thus been devised.[73, 74] Such techniques are based on the selective transfer of analytes (in a liquid phase) between two immiscible phases and are aimed at exploiting differences in physico-chemical properties between the various components of a mixture. Volatility, solubility, charge, molecular size, shape and polarity are the most useful in this respect. All separation techniques involve one or more chemical equilibria, consequently the resolution achieved can vary greatly according to experimental conditions.[73]

### 3.6.1 Extraction techniques

***Liquid-liquid extraction (LLE)*** is based on solubility differences, where selectivity is achieved by pH control and/or complexation.[74] In practice, one phase is usually aqueous while the other phase is an organic solvent.

***SPE*** is based on distribution of analyte between a solid sorbent and a liquid phase.[73] The sorption process must be reversible[74] and the selectivity of the extraction is achieved by pH control, solvent composition and surface chemistry of the sorbent.[73] SPE is often used for sample clean-up prior to chromatographic analysis and pre-concentration of trace and ultra-trace levels of analytes,[73] and is often useful in both pharmaceutical and environmental applications.[74] This technique nowadays largely replaces LLE because SPE provides major advantages in terms of simplicity, high throughput, robustness, and low solvents consumption. A wide range of SPE sorbent chemistries is now available for various applications, and therefore SPE sample preparation techniques provide improved assay standardization and hence better reproducibility.[75]

The available sorbents cover polar, polymeric, bonded silica, and graphitized carbon sorbents of general applicability as well as functionalized polymeric resins, ion-exchange sorbents, controlled-access sorbents, immunoaffinity sorbents, and molecularly imprinted polymers designed for more specific purposes.[74] The silica or

chemically modified silica used for SPE is similar to the bonded phases used in HPLC but of larger particle size, typically 40–60 μm in diameter. Silica or polymeric resins (e.g. polystyrene/divinylbenzene or *N*-vinylpyrrolidone/divinylbenzene copolymers) with chemically bonded alkyl chains such as octadecyl $C_{18}$ (ODS) or octyl $C_8$, phenyl, amino, ion-exchange groups are widely used. Unmodified silica, florisil (activated magnesium silicate), alumina and charcoal also found applications.[73]

### 3.6.2 Liquid chromatography

**Chromatography**

In general, all chromatographic techniques depend upon the same basic principle, i.e. variation in the rate at which different components of a mixture move through a stationary phase under the influence of a mobile phase.[73]

**High-performance liquid chromatography**

HPLC is suitable for separation of non-volatile substances.[73] At present, reversed-phase HPLC (RP-HPLC) has taken and still takes a dominant position. It is estimated that about 80-90% of HPLC separations are performed using RP-HPLC.[76]

**Stationary phases for HPLC**

Concerning the stationary phases, the prominent position belongs to the microparticulate silicas, but other stationary phases as organic/inorganic hybrid particles, metal oxid-based stationary phases, monolithic columns, and superficially porous particles find their application in the field of RP-HPLC nowadays.[77] The mobile phase is typically pumped at pressures up to about 6000 psi (400 bar), and flow rates of 1-5 mL $min^{-1}$ can be achieved through 10-25 cm columns. Unmodified or chemically modified microparticulate silicas (3, 5 or 10 μm) are preferred for nearly all HPLC applications.[73] The particles, which are totally porous, may be spherical or irregular in shape but it is essential that the size range is as narrow as possible to ensure high column efficiency and permeability. The separation process is performed inside the pores of the fully porous particles. The smaller particles enable faster the inter- and intra-particular mass transfer and improved the separation efficiency.[77]

For separations based on adsorption, unmodified silica, which has a polar surface due to the presence of silanol (Si–OH) groups, is used. Appropriate chemical modification

of the surface by treatment with chloro- or alkoxy-silanes, e.g. $R(CH_3)_2SiCl$, produces bonded-phase packings which are resistant to hydrolysis by virtue of forming siloxane (Si—O—Si—C) bonds. Materials with different polarities and chromatographic characteristics can be prepared. The most extensively used are those with a non-polar hydrocarbon-like surface, the modifying groups, R, being $C_{18}$, $C_8$ or aryl. More polar bonded-phases, e.g. amino-propyl, cyanopropyl (nitrile) and diol; and cation- and anion-exchange materials are also available. Mixed ODS/aminopropyl and ODS/nitrile phases having enhanced selectivity for certain classes of compound have also been produced. Chiral stationary phases for the separation of enantiomers (stereoisomers) are produced as well.[73]

By far, silica is still the most popular substrate to manufacture RP stationary phases. Silica has a high mechanical strength that enables its use under high-pressure conditions encountered in HPLC. Furthermore, this substrate does not swell or shrink when exposed to organic solvents. Finally, its production and bonding chemistry is well understood and can be performed in many different morphologies and is very reproducible.[76] However, the relative chemical and thermal instability are known drawbacks of the silica-based phases.[78] Typically, the $C_{18}$ phase is declared to be stable only in the pH range from 3 to 7-9.[79] Recently, however, substantial progress has been made in the synthesis of RP-HPLC silica-based phases to overcome this limitation, e.g. the improvement of the initial silica material.[76, 77] An alternative approach how to manufacture more stable silica-based phases was the synthesis of organic/inorganic hybrid silica-based stationary phases that was firstly introduced by Unger et al.[80] This concept combines the high efficiency and mechanical stability of the silica together with the extraordinary pH stability of the polymeric sorbent. These types of stationary phases for RP-HPLC are commercially available since 1999.[81-83]

The application of more both chemically and thermal stable oxides like, e.g. aluminum, zirconium and titanium oxides as potential substrates for RP stationary phases has already been investigated. Surface chemistry of these oxides is, however, very different from that of silica because these phases show Lewis basic and acidic activity. Therefore, compared to silica, significantly different retention and selectivity behavior of metal oxide-based RPs can be expected.[76, 78] A numbers of attempts were made to overcome this limitation and further development is expected.[76, 77]

Monolithic columns are another alternative to the conventional packed columns. Monolithic packings consist of one piece of either an organic material or silica-based

continuous homogenous phase instead of a packed bed with individual particles. These columns are usually made by sol-gel technology, which enables formation of highly porous material, containing two types of pores in its structure. Macropores (typically 2 µm) are responsible for a low flow resistance and therefore permit the application of high eluent flow rates, while the mesopores (about 12 nm) verify sufficient surface area for separation efficiency.[84-86] However, compared to conventional packed columns, the achieved efficiency is significantly lower for monoliths.[87]

The development of superficially porous (fused-core or core-shell) particles is often considered as a breakthrough in column technology aimed at reducing analysis times while maintaining column efficiency and requiring relatively low back pressure.[88, 89] The technology of monoliths and superficially porous particles is discussed in more detail in the next chapter.

### 3.6.3 Modern approaches in column chemistry in the last decade (2000-2010)

Efficiency and speed of analysis have become of a great importance in many application areas of liquid chromatography (LC), such as in pharmacy, drug discovery, drug development, environmental monitoring or clinical analysis.[88, 90] In order to perform rapid procedures, different strategies can be applied. The rapid evolution of packed column technology over the last 10 years was marked by the successive appearance of the silica monolithic rods,[87, 91] the sub-2 µm particles,[92, 93] and the superficially porous particles (shell particles).[94, 95] Monolithic columns, for example, were introduced for their potential use at high mobile phase velocities due to decreased mass transfer effects over conventional fully porous particles.[88, 96] Their main drawbacks, however, was low efficiency (100,000 plates per meter[87]), limited number of commercially available columns with dimensions of not full compatibility with mass spectrometry together and high organic solvent consumption.[90]

Therefore, either columns with sub-2 µm particles or shell particles appear to be useful as far as high efficiency and rapid analyses are concerned and studied.

#### *3.6.3.1 Ultra high-performance liquid chromatography using sub-2 µm column particles*

Since the beginning of modern HPLC in the late 1960´s, users have required continually new and improved columns to tackle more difficult separation problems or

to improve their overall productivity and sample throughput, which led to development of more efficient and reliable packing materials. One of the areas in which improvements have been made is in particle size reduction. **Figure 8** depicts a series of *H* (separation efficiency, or height equivalent to theoretical plate [HEPT]) versus *v* (mobile phase linear velocity) curves that showed the influence of particle size of silica gel on column efficiency. These data show that the use of smaller particles results in more efficient separations.[97] The separation efficiency in micrometers as a function of mobile phase velocity is described by the van Deemter equation, shown simplistically in **Equation 1**.

$$H = A + B/\mu + C\mu \qquad \textbf{(1)}$$

where A, B, C are constants and μ is the mobile phase linear velocity (proportional to flow rate), measured in cm $s^{-1}$. The A term is a measure of the packing efficiency and is a function of packing efficiency and particle size. The B term is a function of longitudinal diffusion in the mobile phase, and C term is a function of the mass transfer between the stationary and mobile phase as well as within the mobile phase. Within the C term, there is also a proportional dependency of the particle diameter squared. The minimum of the van Deemter curve represents the ideal flow velocity where maximum column efficiency is obtained.[97]

According to the van Deemter curve, which can be used to investigate chromatographic performance, small column particles size induces an increase in separation efficiency, optimal velocity, and improvements in mass transfer. Therefore, efficient separations can be performed with shorter analysis times when sub-2 μm particles are used[92] (see **Figure 8**). The small particle technology is responsible for significantly higher back pressure. Hence there is a need for suitable instruments that are capable of operation at pressure as high as 15,000 psi (1,000 bar), which gave birth to the UHPLC.[93] These columns are characterized by the achieved efficiencies of at least 300,000 plates per meter but the necessity of the special instrumentation represents the main drawback of UHPLC.[87]

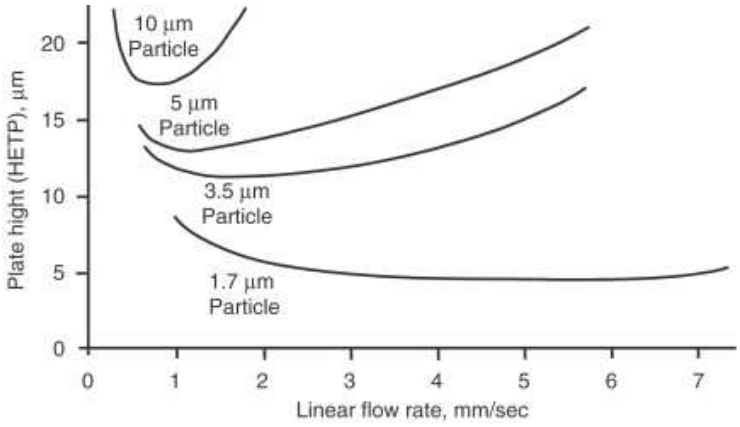


**Figure 8**. **Van Deemter plot for different particle sizes.**
HETP=height equivalent to a theoretical plate.[98]

Acquity BEH columns (bridged ethylene hybrid technology) manufactured by Waters Company belong to the first and mostly used UHPLC columns. These hybrid materials utilizing bridged ethylsiloxane/silica structures exhibit improved column efficiency due to 1.7 µm particles, mechanical strength and stability in wide pH range (1-12). The columns are provided with different bonding chemistries, as $C_8$, $C_{18}$, Phenyl, Shield, etc. The Shield technology is based on incorporation an embedded carbamate group into the bonded phase ligand, which results in the increase of retention for phenolic compounds versus straight chain alkyl columns.[90, 93]

### *3.6.3.2 ALTERNATIVE APPROACH TO UHPLC ANALYSES OF SECONDARY METABOLITES - SUPERFICIALLY POROUS COLUMN PARTICLES*

There was a strong attempt to overcome the limitation of the sub-2 µm particles and to develop separation columns with similar efficiency and short analysis time compared with UHPLC but compatible with conventional HPLC systems. Recently, new column particles composed of solid core surrounded by a thin porous silica layer were introduced as fused-core particles[94] and analogous core-shell particles.[95] The main benefit of the shell particles is the small diffusion path (e.g., 0.5 µm) compared to fully porous particles (e.g., 1.7 µm, 3µm); see **Figure 9**. In theory, decreasing the thickness of the porous layer of porous material should cause a decrease of the C term in the van Deemter plot (see **Figure 8** and **Equation 1**), because the length along which molecules should diffuse decreases. The reduced intra-particle flow path provides superior mass transfer kinetics and better performance at high mobile phase velocities.[87-89] More importantly, e.g. 2.7 µm superficially porous particles produce

only approximately half the backpressure of the 1.8 µm porous particles, which makes possible to use them on conventional HPLC system. This technology is said to be promising alternative to fully porous particles with minimal impact on the separation speed, efficiency, and resolution.[88]

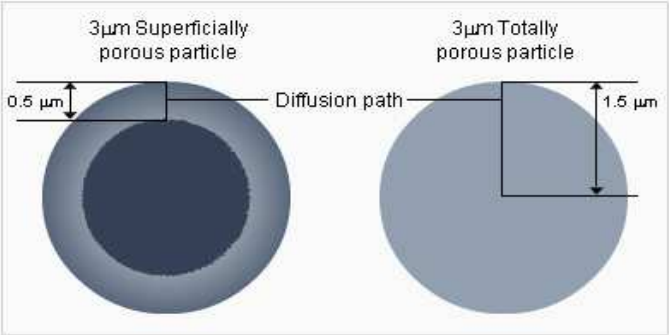


**Figure 9. Comparison of structures of superficially porous and fully porous column particles.**[99]

### 3.6.4 Detection techniques in LC – molecular spectrometric methods

Over the years a large number of LC detectors have been developed. The ultraviolet and visible absorption detector (UV/VIS), and MS detector will be discussed in this section.

#### *3.6.4.1 The UV/VIS and Diode-array detectors*

UV/VIS spectrophotometry is based on the absorption of electromagnetic radiation in the ultraviolet and visible regions (180 - 780 nm) of the spectrum resulting in changes in the electronic structure of ions and molecules.[73, 100] This type of detection belongs to the most widely used technique for liquid chromatography.[73] There are two types of UV detectors, the simple fixed wavelength detectors, and the multi-wavelength or DAD detectors.[100] The DAD detector utilizes a deuterium or xenon lamp that emits light over the UV/VIS spectrum range giving the possibility of recording the full UV/VIS absorption spectra of samples. The UV/VIS spectra increase the reliability of the analyte identification if it is based on comparison of retention times of the standard and the analyte. Moreover, the spectra of complex chromophore systems can classify the analyte to a specific group of compounds with characteristic UV/VIS spectra.[101]

### *3.6.4.2 Mass spectrometry detectors*

Mass spectrometry is an analytical technique that separates and measures the charged particles according to their mass-to-charge ratio (*m/z*) after they are ionized in the ionization source. Ionization methods include e.g. atmospheric pressure chemical ionization, chemical ionization, electrospray ionization (ESI), matrix assisted laser desorption and others. With most ionization methods there is the possibility of creating both positively and negatively charged sample ions.[102, 103]

The generated ions are then separated in mass analyzer, the better known of which include quadrupoles, time-of-flight (ToF) analysers, magnetic sectors, and both Fourier transform (FT) and quadrupole ion traps. The analysers are characterized by different features, including the *m/z* range that can be covered, the mass accuracy, and the achievable resolution, compatibility with ionization methods and others. ToFMS is a relevant for multi-component analyses because these analysers provide high specifity due to both high mass accuracy and high mass resolution, and allow the reconstruction of highly selective accurate mass chromatograms of target residues in complex matrices. The advantage of a ToFMS analyser is its ability to analyze a sample for a theoretically unlimited number of compounds and therefore the LC-ToFMS approach is capable of screening for a large number of analytes with high sensitivity within one run. Furthermore, data can be acquired and reprocessed without any *a priori* knowledge about the presence of certain compounds; that is, no analyte-specific information is required before injecting the sample and the presence of newly identified compounds can be confirmed in previously analyzed sample simply by reprocessing the data.[104, 105] Moreover, accurate mass determination and calculated elemental composition data can be used for structure elucidation as well.[106]

MS/MS detectors consist of more than one analyser and so can be used for structural and sequencing studies. More popular tandem mass spectrometers include those of the quadrupole-quadrupole, magnetic sector-quadrupole, and more recently, the quadrupole-ToF geometries.[102, 103]

Among the detectors employed for monitoring and transmission of the ion current to the data system cover, the more common ones are the photomultiplier, the electron multiplier and the micro-channel plate detectors.[102, 103]

*3.6.4.2.1 Mass spectrometric detection and matrix effects*

Concerning the determination of residual antibiotics in the environmental samples employing the ESI-MS detection technique, the phenomenon known as matrix effect has to be discussed.

In analytical chemistry the matrix effect is defined by IUPAC as "the combined effect of all components of the sample other than the analyte on the measurement of the quality. If a specific component can be identified as causing an effect then this is referred to as interference".[107] When the analysis of a complex real sample is performed, the matrix effect is one of the most relevant drawbacks that can play an important role in the accuracy and precision of a measurement.[74] It can affect the sample preparation step as well as it can influences both qualitative and quantitative analyses if mass spectrometry detection hyphenated to liquid chromatography separation is employed.[74, 108] In ESI-MS quantitative analysis this phenomenon belongs to the significant drawbacks[50, 109] because of the susceptibility of the ESI-MS to organic and inorganic components that are present both in the sample matrix together with the analyte and in the mobile phase.[109] The matrix effects can lead to signal instability reflected in signal suppression or enhancement and can cause erroneous results of quantification.[110-112]

There are several strategies to reduce matrix effect, e.g. selective extraction, effective sample clean-up after the extraction, or improvement of the chromatographic separation. Sometimes, these approaches are not appropriate solutions because they could lead to analyte losses as well as long analyses times.[50, 113] Appropriate calibration technique should be employed to compensate for the matrix effects,[3, 50, 108, 112] such as addition of internal standards, echo-peak technique, calibration using external matrix-matched standards, standard addition, or dilution of sample extracts. The addition of internal standards (isotopically labeled or standards that cannot occur in the analyzed samples) belongs to the most suited, but the main disadvantage of this strategy is the very high costs of the isotopically labeled standards.[50, 108, 112] Calibration using external matrix-matched standards is based on the employment of the standards with the same or similar matrix composition as the analyzed sample. This approach provides practically full compensation of the matrix effects, but, unfortunately the main limitation of this strategy is often the unavailability of the proper blank matrix (material free of residues of target analyte).[108]

Therefore, the calibration approach employing external matrix-matched standards with internal standard addition was used for quantification of residual antibiotics in the influents and effluents of the WWTPs by UHPLC-ToFMS in order to compensate the matrix effects.

### 3.6.5 Chromatographic fingerprinting of SMs and chromatographic screening for novel antimicrobial agents

#### *3.6.5.1 Chromatographic fingerprinting of SMs*

Chromatographic fingerprinting (CFP) is an effective and rational method based on comparison of fingerprints (chromatograms) of the many compounds found in highly complex samples. CFP can provide the whole profile of not only the marker compounds but also the unknown components. These fingerprints can then be used as an effective tool for comparison, classification, or identification of samples based on the production of SMs.[13] Several chromatographic techniques such as HPLC,[114-118] UHPLC,[119] gas chromatography (GC),[120] capillary electrophoresis (CE),[121] and thin layer chromatography (TLC)[122] have been used for fingerprinting. Due to the high complexity of the studied matrix, additional and complementary information is often required. Therefore, hyphenated techniques such as HPLC-DAD, CE-DAD, GC-MS, HPLC-MS, HPLC-DAD-MS/MS, CE-MS, CE-DAD-MS/MS and HPLC with nuclear magnetic resonance detection (NMR) generating multi-dimensional and multi-informational fingerprints are preferred. With additional spectral information, these hyphenated techniques give a much more complete profile of the investigated samples.[118] Since the fungal fermentation broth represents complex matrix, the combination of UHPLC with both DAD and ToFMS detection was found to be a proper instrumentation for the purpose of CFP. UHPLC allows faster separations with higher separation efficiencies[123] and DAD detection provides additional spectral information of the sample components by generating the 3D chromatograms.[118] Moreover, the ToFMS analyser is able to analyze theoretically unlimited number of compounds in the sample with high mass accuracy and high mass resolution.[104]

The purification and pre-concentration of SMs prior the analysis is necessary and can be performed by either LLE or SPE. SPE provides many advantages over LLE and is nowadays preferred for many applications.[75]

Chromatographic and spectrometric data can be treated by multivariate statistical methods as either binary or quantitative data.[13] For the evaluation of similarities or differences of the fungal extracts, the Principal component analysis (PCA), Principal coordinates analysis (PCoA) and Hierarchical cluster analysis (HCA) were used. **PCA** is well known multivariate data analysis approach[116] and is used to explain the maximal variance of the data [115]. Generally, PCA compress the original data, and a new set of variables called principal components (PCs) is obtained. These PCs are linear combinations of the original variables, and are chosen to be orthogonal to each other. PC score – score plots, loading variable plots and biplots are common two dimensional display methods for exploring the data structure. **PCoA** is a more general projection method than PCA. PCoA, also known as Classical scaling, is a metric multidimensional scaling method based on projection, which uses spectral decomposition to approximate a matrix of distances/dissimilarities by the distances between a set of points in few dimensions. It is used to explore and to visualize similarities or dissimilarities of data by finding hypothetical variables (components, coordinates) that account for as much of the variance in a multidimensional data set as possible. These new variables are linear combinations of the original variables. PCoA is a standard method for reducing the dimensionality of morphometric and ecological data.[13, 115] **HCA** has also been applied for fingerprint analysis. In HCA, distances between pairs of samples are calculated and compared. Relatively short distances between samples indicate similarity. The primary purpose of HCA is to present data in a manner that emphasizes natural grouping. Plotting HCA results as a dendrogram facilitates visual recognition of such categories.[115]

#### *3.6.5.2 Chromatographic screening for novel antimicrobial agents*

Nowadays, high-throughput screening techniques are applied in searching for new antimicrobial agents.[59] The success of a screening depends on the application of suitable, sensitive, highly specific and effective, high-throughput method. These methods allow the screening of an almost unlimited number of samples in a very short time.[9] The identification of bioactive compounds from fermentation samples (extracts, whole broths) is one of the most complicated, time consuming steps of the screening protocols. Recently, a great progress in chromatographic isolation methods (LC-DAD-MS, LC-UV, LC-MS, LC-NMR, etc.) has been achieved.[9]

The screening for the bioactive compounds in the fungal fermentation broth consists of the following steps: **(a)** development of the effective extraction technique able to separate and concentrate the potentially active compounds from the fermentation broth; **(b)** bioassay of the obtained extracts; **(c)** tracing the fungal strains with antimicrobial activity; **(d)** detailed study of the selected strain/s using bioassay-guided fractionation; **(e)** determination of the bioactive compound/s and it's/their further identification.

*3.6.5.2.1 Bioassay of fungal SMs in extracts*

The methods used for antimicrobial activity testing includes e.g. broth dilution methods, E-test and mostly used Kirby-Bauer disk diffusion susceptibility test. This method was introduced in 1961 by W. M. M. Kirby and A. W. Bauer. The purpose of this test is to determine the sensitivity or resistance of tested pathogenic organisms to the compound or mixture of compounds with potential antimicrobial activity (e.g. extract obtained from fermentation broth of tested microorganism). The pathogenic organism is grown on Petri dish with agar in the presence of the filter paper impregnated with the tested compound/extract. The incubation on the plates is time and temperature controlled. The presence or absence of pathogenic organism's growth around the disk is an indirect measure of the ability of that compound to inhibit that organism. In order to evaluate the antimicrobial activity, the zone sizes around the filter paper disk are measured after the incubation.[124]

The organisms mostly used for antimicrobial activity testing include both Gram-positive and Gram-negative bacteria (for definition see[8, 26]). ***Kocuria rhizophila*** is the mostly used indicating organism of Gram-positive bacteria,[8] while ***Escherichia coli*** is the mostly used Gram-negative bacteria for antimicrobial tests. ***Saccharomyces cerevisiae***, ***Beauveria bassiana, Penicillinum decumbens,*** and ***Graphium fibriisporum*** are often used eukaryotic representatives of indicating organisms.[125]

*3.6.5.2.2 Bioassay-guided fractionation*

Bioassay-guided fractionation is a procedure of whereby extract is chromatographically fractionated and refractionated until a pure biologically active compound is isolated. Each fraction produced during the fractionation process is evaluated in a bioassay system (e.g. Kirby-Bauer test) and the extract is further fractionated. This method can be successfully employed in drug discovery research

due to its effectiveness to directly link the analyzed extracts and targeted compounds using fractionation procedure that followed with certain biological activity.[126, 127]

*3.6.5.2.3 Methods for identification of biologically active SMs*

The isolated biologically active compounds in pure as possible form are further identified using analytical techniques as MS/MS, NMR, or X-ray crystallography.[9]

# 4 EXPERIMENTAL (for unpublished data)

The experimental sections specify the instrumentation used for development and application of the liquid chromatography methods and describe experimental procedures for the unpublished results. For experimental data concerning the published results, see the respective papers attached in the appendices.

## Instrumentation

**UHPLC analyses** were carried on Waters Acquity UPLC System (Waters, Prague, Czech Republic) consisting of Acquity UPLC Solvent Manager, Acquity UPLC Sample Manager, Acquity UPLC Column Heater/cooler, and Acquity UPLC Diode Array Detector and since 2010 couplet with Waters LCT Premier XE orthogonal accelerated time of flight mass spectrometer (Waters MS, Manchester, UK) with an ESI interface. Data were processed with Empower 2 and MassLynx V4.1 software (Waters, USA).

**HPLC analyses** were performed on Waters HPLC system (Waters, Prague, Czech Republic) equipped with 600 Controller, 717 plus Autosampler, 2487 Dual λ Absorbance Detector and Empower 2 software (Waters, USA) for data processing.

**Off-line MS detection** was carried on an APEX-Ultra FTMS instrument equipped with a 9.4 T superconducting magnet and an Dual II ESI ion source (Bruker Daltonics, Billerica, MA). The interpretation of mass spectra was done using DataAnalysis version 4.0 software package (Bruker Daltonics, Billerica, MA).

## 4.1 DETERMINATION OF RESIDUAL ANTIBIOTICS IN THE ENVIRONMENT

### 4.1.1 High-throughput analysis of tetracyclines and their epimers in liquid hog manure using UHPLC-DAD method

For experimental data, see **PAPER 1** in the appendices.[128] The experimental procedures described in this subsection refer to the results in subsection ***5.1.1***.

### 4.1.2 Determination of antibiotics in influents and effluents of WWTPs in the Czech Republic using SPE and UHPLC-ToFMS method

For experimental data, see **PAPER 2** in the appendices. The experimental procedures described in this subsection refer to the results in subsection ***5.1.2***.

## 4.2 STUDY OF SECONDARY METABOLITES PRODUCED BY *Geosmithia* FUNGI

### 4.2.1 The UHPLC-DAD-ToFMS fingerprinting method for analysis of extracellular metabolites of fungi of the genus *Geosmithia*

For development of SPE and UHPLC-DAD fingerprinting method, see **PAPER 3** in the appendices.[129] The method was updated in 2011 – 2012; namely the ToFMS detection was additionally employed and the set of studied *Geosmithia* strains was enlarged with the total number of 48 strains (see **Table 4** in the Supplementary data). Moreover, the statistical evaluation (PCA, PCoA, HCA) of the UHPL-DAD-ToFMS data was made in 2012. The unpublished experimental procedures are described in this section. The experimental procedures described in this subsection refer to the results in subsection ***5.2.1***.

***UHPLC-DAD-ToFMS method update*:** Due to the better compatibility with the MS detection added, the trifluoroacetic acid (TFA)-water 0.1:99.9 (v/v) used as aqueous component of the mobile phase was replaced with formic acid-water 0.1:99.9 (v/v) under similar gradient elution program (min/ %A): 0/ 95, 15/ 65, 25/ 0, 27/ 0, 30/ 95. The ESI interface of Waters LCT Premier XE ToFMS operated in both positive and negative ion mode. The parameters for ToFMS were as follows: cone voltage, 50 V; capillary voltage, +2500 V (positive), −2500 V (negative); ion source block temperature, 120 °C; nitrogen desolvation gas temperature, 350 °C; desolvation gas flow, 800 L $h^{-1}$; cone gas flow, 50 L $h^{-1}$. Full scan spectra from *m/z* 100 to 1600 were acquired with a scan time of 0.1 s and 0.01 s interscan delay in both W+ and W− optic mode for an operating resolution of 10,000 (FWHM). Data were collected from 1.3 to 27 min and the mass accuracy was maintained by lock spray using Leucine Enkephalin. Other conditions were the same as in the published paper.[129]

For the evaluation of CFP data obtained with UHPLC-DAD-ToFMS method, the PCA, PCoA and HCA were employed.

### 4.2.2 Chromatographic screening for bioactive fungal SMs

The experimental procedures described in this subsection refer to the results in chapter ***5.2.2***.

The *Geosmithia* strains studied in the project dealing with UHPLC-DAD-ToFMS fingerprinting method (chapter ***4.2.1*** and corresponding **PAPER 3** in appendices[129]) were subsequently tested for their potential antimicrobial activity. Kirby-Bauer disk diffusion susceptibility test was employed, using *Kocuria rhizophila* (CCM552), *Escherichia coli* (ATCC3988), *Saccharomyces cerevisiae* (CCM 8191), *Beauveria bassiana* (CCF4422), *Penicillinum decumbens* (CCF4423), and *Graphium fibriisporum* (CCF4421) as indicating organisms. This set of indicating organisms includes both prokaryotic and eukaryotic organisms; among the bacteria both Gram-positive and Gram-negative representatives; which provide the versatile characterization of antimicrobial activity of the tested fungal strains.
Afterwards, the strains with significant antimicrobial activity were selected for further tracking of bioactive SMs employing the method of bioassay-guided fractionation.
For the extraction procedure and UHPLC-DAD-ToFMS method conditions see chapter ***4.2.1*** and Experimental in **PAPER 3.**[129]

**Fungal strains**

Forty eight monosporic strains representing 28 species of the genus *Geosmithia* (see **Table 4** in Supplementary data), maintained at the Institute of Microbiology, Academy of Sciences of the Czech Republic and in Culture Collection of Fungi in Prague were tested for their antimicrobial activity.[129]

#### *4.2.2.1 Kirby-Bauer disk diffusion susceptibility test*

The agar plates for particular indicating organisms were as follows:
*Beauveria bassiana*, *Penicillinum decumbens*, *Graphium fibriisporum*; MEA agar (malt extract, 20 g/L; agar, 20 g/L).
*Kocuria rhizophila, Escherichia coli*; B1 agar (beef extract, 10 g/L; peptone, 10 g/L; NaCl, 5 g/L; agar, 20 g/L).
*Saccharomyces cerevisiae*; F23 agar (yeast extract, 5 g/L; peptone, 4 g/L; glucose 40 g/L; agar, 20 g/L).

All agar plates were dried up before inoculation. Plates were then overlaid with the indication organism (suspended in sterile saline solution and vortexed unless smooth suspension is obtained). The filter paper discs were impregnated with 25 µL of fungal extract and air dried. Disk impregnated with methanol served as a blank control. Afterwards, the impregnated discs were placed onto the agar surface. Growth inhibition zones, indicating the antimicrobial activity of the fungal extract, were detected: after 24 hours of incubation at 30 °C (*Kocuria rhizophila, Escherichia coli;* B1 agar); after 24 hours at 38 °C (*Saccharomyces cerevisiae;* F23 agar); after 48 hours at 24 °C (*Beauveria bassiana, Penicillinum decumbens, Graphium fibriisporum*; MEA agar). Following the incubation the inhibition zones were evaluated and measured.[124]

#### *4.2.2.2 HPLC-UV method for bioassay-guided fractionation*

The fungal strain RJ0258 (*Geosmithia* sp. 9) provided exceptional antimicrobial activity against all indicator organisms and was therefore chosen for further detailed study of the bioactive SMs.

The bioassay-guided fractionation was carried on Waters HPLC system with Gemini C18 analytical column (250 × 4.6 mm i.d., 5 µm; Phenomenex, Torrance, USA). Firstly, the **HPLC-UV grad1** method was employed. The 3.5 minutes lasting fractions were collected, evaporated to dryness at 45 °C and reconstituted in 40 µL of methanol. Twenty five µL were used for Kirby-Bauer antimicrobial activity test employing *Graphium fibriisporum* as indication organism. Fractions were analyzed by UHPLC-DAD-ToFMS method described in chapter ***4.2.1*** in order to control the purity and identity of the fraction. Afterwards, the extract was refractionated using the adjusted gradient **HPLC-UV grad2.** The methods conditions were as follows: **HPLC-UV grad1:** mobile phase (A) formic acid-water 0.1:99.9 (v/v), and (B) ACN; gradient elution (min/%A): 0/ 95, 35/ 65, 65/ 0; flow rate, 1 mL $min^{-1}$; equilibration step, 15 min; column temperature, 25 °C; samples temperature, 10 °C; injection volume, 60 µL, UV 260 nm. **HPLC-UV grad2:** gradient elution (min/%A): 0/ 70, 50/ 50, 55/ 50, 60/ 0; other chromatographic conditions remained the same as for HPLC-UV grad1.

Subsequently, the determined bioactive fractions were isolated on Gemini C18 preparative column (250 × 10 mm i.d., 5 µm; Phenomenex, Torrance, USA) using isocratic elution program **HPLC-UV iso** method: mobile phase composed of

formic acid-water 0.1:99.9 (v/v) and ACN, 50:50 isocratic elution; flow rate, 2 mL $min^{-1}$; analysis time, 60 min; column temperature, 25 °C; samples temperature, 10 °C; injection volume, 120 µL, UV 260 and 310 nm.

#### *4.2.2.3 UHPLC-ToFMS and off-line MS detection of bioactive SMs*

The isolated bioactive fractions were analyzed in order to determine the accurate molecular mass of bioactive compounds, their elemental composition and MS spectra after mass fragmentation. The mass fragmentation was provided by both in-source collision (Waters LCT Premier XE ToFMS) and MS/MS (APEX-Ultra FTMS instrument).

***UHPLC-ToFMS detection:*** The conditions for MS detection were described previously in section ***4.2.1***. The fragmentation using in-source collision-induced dissociation (CID) was achieved by the Aperture I value set to 25V.

In general, for a ToFMS having a mass resolution of about 10,000 (FWHM) and the external calibration the accurate mass measurement is secured.[130] The available applications of Mass Lynx software, namely Elemental Composition editor together with MS spectra acquisition in both ESI+ and ESI− are useful tools for determination of elemental composition of tested compounds. Elemental Composition editor uses a CHNO algorithm, which provides standard functionalities such as mass measurement error (compared to predicted mass), double bond equivalence, isotope predictive filtering (i-FIT), or isotope modeling application for comparison of the theoretical and the measured isotope pattern. The i-FIT value provides an exact numerical comparison of the theoretical and measured isotopic pattern of a compound.[131, 132] Moreover, the in-source collision gives the opportunity to predict the molecular structure of analytes.

***Off-line MS detection*** **(performed by Ing. Petr Novák, Ph.D., ASCR, v.v.i.)*:*** The isolated bioactive fractions were evaporated to dryness and reconstituted in methanol-water-formic acid 50:50:0.1 (v/v/v) were analyzed in negative ion mode by direct infusion on an APEX-Ultra FTMS instrument equipped with a 9.4 T superconducting magnet and an Dual II ESI ion source (Bruker Daltonics, Billerica, MA). The cell was opened for 1.1 msec, accumulation time was set at 0.2 s for MS experiment (1.0 s for MS/MS experiment), and one experiment was collected for each sample where one experiment consists of the average of four spectra. After MS

experiment one MS/MS experiment was carried out from the most intensive ion. The isolation window was set to 3 a.m.u. and the collision energy was kept at -6 V. The acquisition data set size was set to 1M points with the mass range starting at *m/z* 150 a.m.u., resulting in a resolution of 100,000 at *m/z* 400. The instrument was calibrated externally using clusters of arginine resulting in mass accuracy below 1 ppm. The acquired spectra were apodized with a square sine bell function and FT with one zero-fill.

## 4.3 ALTERNATIVE APPROACH TO UHPLC ANALYSES OF SECONDARY METABOLITES - SHELL COLUMN PARTICLES

For experimental data see **PAPER 4** in the appendices.[133]

# 5 RESULTS AND DISCUSSION

This chapter briefly summarizes the published results, for more detailed information see the respective **PAPER 3** in the appendices.[129] Further, the chapter includes the yet unpublished experiments.

## 5.1 DETERMINATION OF RESIDUAL ANTIBIOTICS IN THE ENVIRONMENT

### 5.1.1 High-throughput analysis of tetracyclines and their epimers in liquid hog manure using UHPLC-DAD method

Slight modifications of previously published methods[23, 134] were done in terms of the sample volumes, extraction solution increase and $Na_2EDTA$ solution addition directly to the extraction media. Moreover, the solubility of dry extracts obtained from LLE was tested in three solutions, i.e. 10% methanol in water, 100% methanol, and methanol-acetic acid 99:1 (v/v). The highest solubility was achieved in methanol-acetic acid solution.

The baseline separation of all compounds was observed with the exception of DOX and $DOX_{ep}$ where slight overlay could be observed. The developed UHPLC-UV method was validated in terms of accuracy, precision and selectivity. The analytes were quantified as a sum of peak areas of each antibiotic and its corresponding epimer. The obtained recovery values were 52.4 ± 3.8% (TC), 72.4 ± 5.0% (OTC), 83.8 ± 5.7% (CTC), and 95.9 ± 4.7% (DOX). Instrument limit of quantification (ILOQ) was 8 µg $ml^{-1}$ for all analytes. The determined method limits of quantification (MLOQ) determined with respect to ILOQ and recovery of each analyte were as follows: TC, 1.6 mg $kg^{-1}$; OTC, 1.2 mg $kg^{-1}$; CTC, 1.0 mg $kg^{-1}$; DOX, 0.9 mg $kg^{-1}$ in the liquid hog manure (for abbreviations of the antibiotics, see chapter ***3.2.3***.)

The method was applied for analysis of liquid hog manure samples from 5 different localities in the Czech Republic. One sample was found to contain the monitored antibiotics in concentrations 5.88 mg $kg^{-1}$ (CTC) and 0.99 mg $kg^{-1}$ (DOX). Although the presence of TC and OTC was proved (off-line MS/MS), the concentrations determined were 10 times lower than MLOQ. The UHPLC analysis of the positive sample is shown in **Figure 10**.

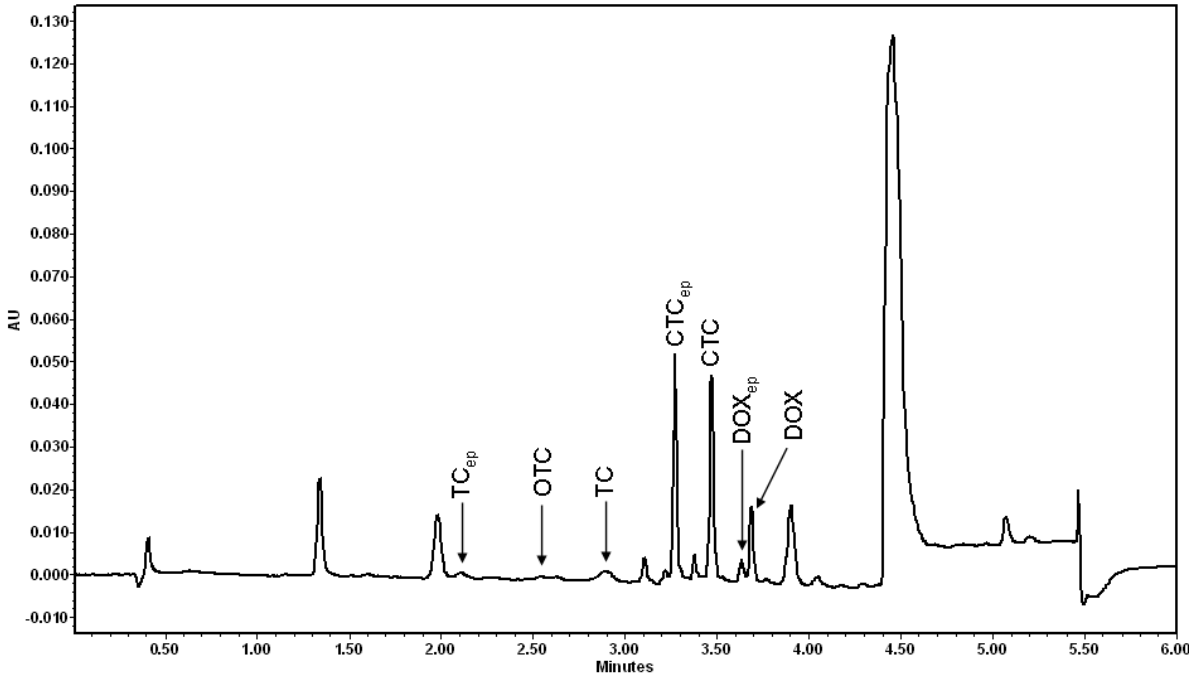


**Figure 10. The UHPLC analysis of positive liquid hog manure sample.**

*Chromatographic conditions*: Acquity UPLC BEH Shield RP18 column (50 × 2.1 mm i.d., 1.7 μm; Waters); mobile phase, (A) formic acid-water 0.1:99.9 (v/v), (B) ACN; gradient elution (min/ %A): 0/ 95, 2.3/ 92, 2.8/ 80, 4.0/ 75, 5.5/ 0, 6.0/ 95; flow rate, 0.4 ml $min^{-1}$; column temperature, 22$^{o}$C; sample temperature, 22 °C; injection volume, 1 μl; UV 350 nm. For abbreviations of antibiotics see chapter *3.2.3.*

### 5.1.2 Determination of antibiotics in influents and effluents of WWTPs in the Czech Republic using SPE and UHPLC-ToFMS method

All 19 tested antibiotics were satisfactorily separated employing the developed UHPLC-ToFMS method during a 25 min gradient elution. The selectivity of the method is demonstrated in

**Figure 11** depicting the chromatographic separation of tested antibiotics in spiked blank water sample (100 ng $L^{-1}$) under the optimized conditions.

The main demand on the method was its utility for a large number of analytes of different physico-chemical characteristics, which is a difficult task during multi-residue analysis.[31] Different SPE sorbents were tested for sample extraction and the recovery using the blank water samples spiked with antibiotics (500 ng $L^{-1}$) was considered to indicate the SPE efficiency. With respect to the acid-base properties of the studied antibiotics, the Oasis HLB, Oasis MCX, and Oasis MAX sorbents were employed for SPE procedure. Macrolides and lincosamides represent weak bases, sulfonamides are weak acids, and tetracyclines and fluoroquinolones are amphoteric drugs that can exhibit both weak acidic and basic character.[135] Although the tested ion-exchange cartridges can be promising sorbents for extraction of particular antibiotic classes (e.g. MCX for macrolides and lincosamides, MAX for fluoroquinolones), none of them was found to be useful for extraction of all analytes in this study.

The Oasis HLB enabled, as the only sorbent, the extraction of all analytes and was therefore used for the maintenance of the method versatility. Subsequently, the sample pH adjustment prior to Oasis HLB method was tested. As it was impossible to find the optimal recoveries for all analytes at a single pH value, it was necessary to employ two extraction procedures differing in sample pH adjustment. The pH 4.5 was proved the most effective for tetracyclines, macrolides, and fluoroquinolones, while pH 7.5 was determined for sulfonamides and lincosamides. Comparing the developed SPE method with previously published studies, the Oasis HLB represents the mostly used sorbent for antibiotics extraction and concentration in water samples.[3, 50, 136-139] The obtained recoveries in this work were in most cases similar[137, 139] or slightly higher[50, 136, 138] than in previously published papers when determined the same analytes in wastewater or surface water. Similar strategy of employment of two extraction procedures for each sample due to recovery improvement was previously described in the literature.[31, 140]

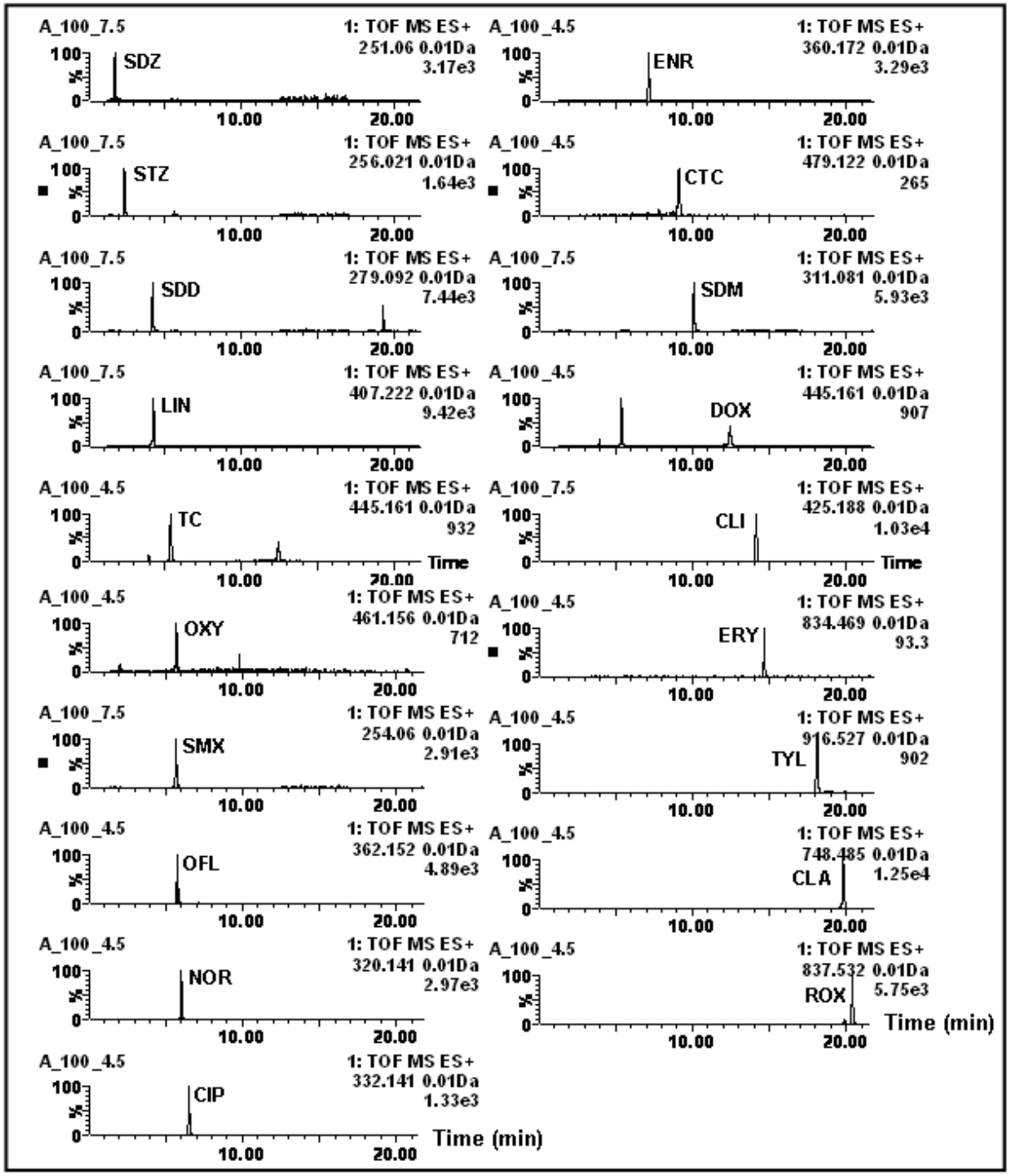


**Figure 11. UHPLC-ToFMS separation of tested antibiotics in spiked blank water sample (100 ng $L^{-1}$) under the optimized conditions.**

*Chromatographic conditions*: Acquity UPLC BEH C18 (50 × 2.1 mm i.d., 1.7 μm; Waters); mobile phase, (A) formic acid-water 0.1:99.9 (v/v), (B) formic acid-methanol 0.1:99.9 (v/v); gradient elution (min/ %A): 0.0/ 95, 10.0/ 75, 15.0/ 60, 21.0/ 45, 21.5/ 45, 23.0/ 0, 25.0/ 95; flow rate, 0.4 ml $min^{-1}$; column temperature, 30°C; sample temperature, 10 °C; injection volume, 3 μl; specific $[M+H]^+$ ions were extracted with 0.01 Da extraction mass window. For abbreviations of antibiotics see chapter ***3.2.3***.

Different aqueous components of the mobile phase, organic modifiers as well as gradient elution steepness were tested during the development of chromatographic method. Moreover, different analytical columns were considered. Concerning the MS detection, the parameters as ionization mode, capillary voltage, cone voltage or needle counter electrode distance were optimized. To compensate matrix effects, which is the main well known and significant drawback in ESI-MS quantitative analysis,[50, 109, 112] the matrix-matched calibration and internal standards´ addition were employed. The usefulness and versatility of the method was documented by achieving MLOQs values up to 10 ng $L^{-1}$ and recoveries >80% for the most analytes differing in their physico-chemical properties. Compared to previously published HPLC-MS/MS method with triple quadrupole analyzer, the MLOQ values that we obtained are often similar or slightly higher,[31, 138, 139] or even lower.[50] These findings demonstrate that, as discussed in a previous article,[105] the UHPLC technique is able to compensate for the lower selectivity of ToFMS instrumentation.

The developed analytical method was used for determination of selected antibiotics in influent and effluent of WWTP from 6 different localities in the Czech Republic. The monitoring study revealed that all analyzed samples were positive for different classes of antibiotics in both influents and effluents of WWTPs. The concentration ranged from 5.0 ng $L^{-1}$ (LIN) to 1287.9 ng $L^{-1}$ (CLA); the detailed results are listed in **Table 2** (for abbreviations see chapter ***3.2.3***).

Analysis of both the influents and effluents revealed the insufficient efficiency of water treatment processes of WWTPs in removal of antimicrobial agents, which causes the release and remaining of antibiotics in the water and leads to the constant increasing of the bacterial resistance.

**Table 2. Antibiotic concentrations (ng $L^{-1}$) in influents and effluents of the WWTPs from 6 different localities in the Czech Republic.**

| locality | A | | B | | C | | D | | E | | F | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | inf.[a] | eff.[a] | inf. | eff. | inf. | eff. | inf. | eff. | inf. | eff. | inf. | eff. |
| **OXY** | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd |
| **CTC** | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd |
| **DOX** | nd | nd | nd | nd | nd | nd | 20.6 | nd | nd | nd | nd | nd |
| **ERY** | nd | nd | 248.6 | 204.2 | 38.2 | 27.4 | 49.3 | 36.3 | 60.3 | nd | 8.7 | 36.3 |
| **TYL** | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd |
| **CLA** | 79.0 | 61.0 | 1287.9 | 289.0 | 952.2 | 422.0 | 989.2 | 273.4 | 237.5 | 85.1 | 1010.4 | 794.2 |
| **ROX** | nd | nd | 74.8 | 35.7 | nd | nd | nd | nd | nd | nd | nd | nd |
| **SDZ** | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd |
| **STZ** | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd |
| **SDD** | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd |
| **SMX** | nd | nd | 33.9 | 272.2 | 150.0 | 681.1 | 550.0 | 438.9 | 796.2 | 544.4 | 102.6 | 377.8 |
| **SDM** | nd | nd | nd | nd | nd | nd | nd | nd | 177.1 | <MLOQ | nd | nd |
| **OFL** | nd | nd | 232.0 | 138.0 | nd | nd | 138.0 | 113.0 | 485.0 | 283.0 | 67.0 | <MLOQ |
| **NOR** | nd | nd | nd | nd | nd | nd | 167.6 | 63.0 | 186.8 | 24.2 | 377.4 | 33.2 |
| **CIP** | nd | nd | 111.0 | 7.53 | nd | nd | 640.6 | 133.6 | 273.8 | 70.7 | 386.6 | 11.8 |
| **ENR** | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd |
| **LIN** | nd | nd | 24.3 | 10.8 | nd | nd | 94.1 | 46.4 | 5.0 | 42.2 | 32.7 | 17.7 |
| **CLI** | nd | nd | 28.6 | 102.1 | 150.7 | 48.4 | 61.9 | 41.9 | 35.2 | nd | 55.3 | 63.6 |

[a] inf. – WWTP influent, eff. – WWTP effluent
nd - antibiotic not detected
for abbreviations of antibiotics see chapter ***3.2.3***

## 5.2 STUDY OF SECONDARY METABOLITES PRODUCED BY *Geosmithia* FUNGI WITH FOCUS ON BIOLOGICAL ACTIVITY

### 5.2.1 UHPLC-DAD-ToFMS fingerprinting method for analysis of extracellular metabolites of fungi of the genus *Geosmithia* (Acomycota: Hypocreales)

The development of the fingerprinting method included several steps; namely optimization of sample pretreatment (extraction procedure), optimization of UHPLC conditions (analytical column, mobile phase, gradient elution program), and MS conditions.

The method used for extraction of SM from fungal fermentation broth was developed using six strains representing six different species of *Geosmithia* spp. (MK1712a, RJ74k, CCF4205, CCF3333, 1259, CCF3555), which differed in the SMs production, and therefore, epitomize a representative set of samples for extraction method development. Five solvents of rising polarity were tested for LLE; namely hexane, diethyl ether, dichloromethane, ethyl acetate, acetic acid-ethyl acetate 1:20 (v/v). Afterwards, different sorbents for SPE were evaluated; namely Amberlite XAD-2, Amberlite XAD-4, Strata C18-E, Strata $NH_2$, Sep-Pack C-18, Oasis HLB, Oasis MAX, Oasis MCX, Oasis WAX, and Oasis WCX. Although LLE belongs to the most frequent used method for extraction of SM from fungal fermentation broth[141-144] this method was not found to be suitable for extraction of SM of *Geosmithia* strains. Among the several tested SPE sorbents, Oasis MCX, Oasis HLB, and Strata C18-E were found to be appropriate sorbents because of the greatest number of SMs presented in the extract. With respect to the peak intensities and shapes the Oasis MCX was finally the sorbent of choice.

The main demands on the new UHPLC-DAD-ToFMS method was to well separate a great number of SM differing in polarity in a relatively short analysis time (about 30 min). Six selected strains of *Geosmithia* spp., namely MK1712a, RJ74k, CCF4205, CCF3333, 1259, CCF3555 were used for UHPLC-DAD-ToF method development. Since the majority of presented SMs showed maximal absorbance at 260 nm, this wavelength was used during the evaluation of the method conditions. Four analytical UHPLC columns, namely Acquity UPLC BEH C18 (50 × 2.1 mm i.d.; 1.7 μm), Acquity UPLC BEH C18 (100 × 2.1 mm i.d.; 1.7 μm), Acquity UPLC BEH Shield RP (50 × 2.1 mm i.d.; 1.7 μm), and Acquity UPLC BEH HILIC (50 x 2.1 mm i.d.; 1.7

μm), all Waters, Czech Republic, were evaluated and compared. The parameters as peaks retention times, peaks shapes and resolution, and the total analysis time were calculated and compared. Acquity BEH C18 column of 100 mm length provides sharper and better separated peaks with higher retention of separated components.

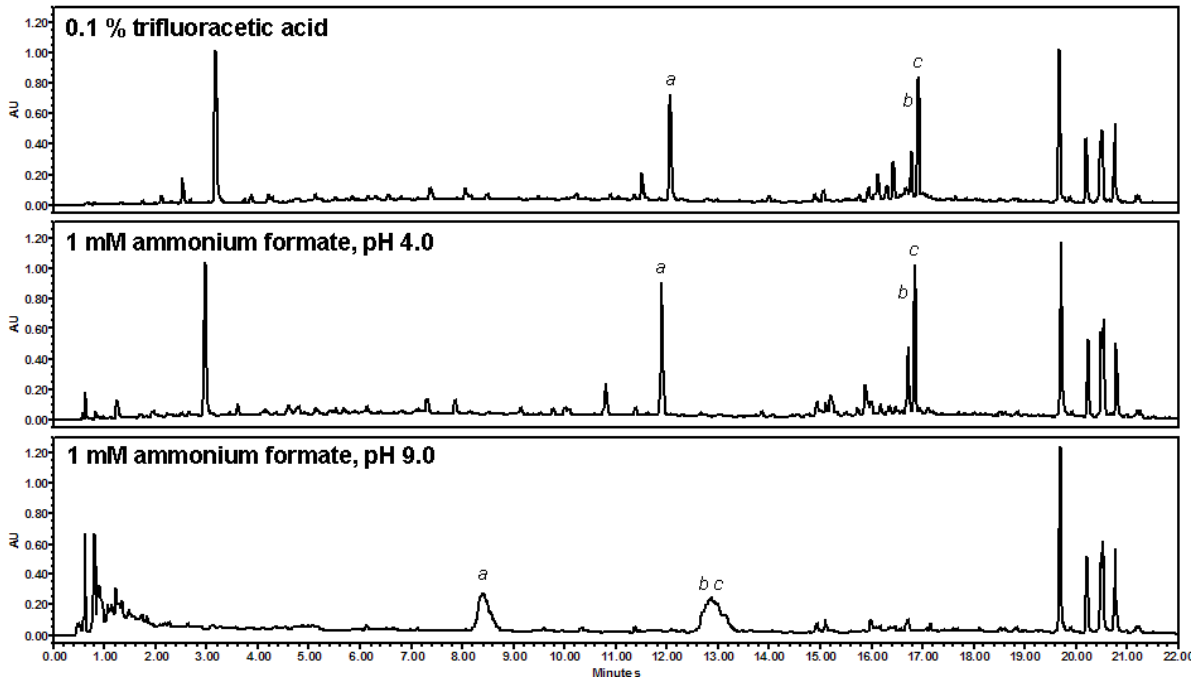


**Figure 12. Different mobile phase composition for UHPLC-DAD analysis of RJ74k strain fermentation broth.**
*a-c*: peaks evaluated during the method development.

*Chromatographic conditions:* Acquity UPLC BEH C18 column (100 × 2.1 mm i.d., 1.7 μm; Waters); mobile phase, (B) ACN; gradient elution (min/ %A): 0/ 95, 15/ 65, 25/ 0, 27/ 0, 30/ 95; flow rate, 0.4 mL $min^{-1}$; column temperature, 25 °C; sample temperature, 10 °C; injection volume, 1 μl;; UV 260 nm.

Several aqueous components of mobile phases such as strongly acidic (0.1% and 0.05% TFA, 0.1% formic acid, 0.1% acetic acid, 0.1% phosphoric acid), acidic (1 mM ammonium formate, pH 4.0) and alkaline (1 mM and 5 mM ammonium acetate, 1 mM ammonium formate, all of pH 9.0) were assessed; the selected results are shown in **Figure 12**. Organic modifiers, gradient elution program and column temperature were tested as well. The used aqueous component of the mobile phase was TFA-water 0.1:99.9 (v/v) for former UHPLC-DAD method (**PAPER 3**[129]), and formic acid-water 0.1:99.9 (v/v) for updated UHPLC-DAD-ToFMS method, due to a better compatibility with the MS detection. For both methods, ACN was employed as organic

modifier and similar gradient was applied, namely (min/%A): 0/95, 15/65, 25/0, 27/0, 30/ 95 (total analysis time of 30 min) with column temperature of 25 °C. Concerning the MS detection, the W optic mode was preferred due to a higher mass accuracy in comparison to V optic mode. The parameters affecting the ESI interface were optimized in direct infusing experiments with the fungal extracts in methanol at a constant flow rate of 5 µL $min^{-1}$ into the analyte probe using the syringe pump. The tested parameters were: capillary voltage (from ±1500 V to ±3000V with 500 V steps) and cone voltage (from 25 V to 50 V with 5 V steps). The final conditions were as follows: cone voltage, 50 V; capillary voltage, +2500 V (positive), −2500 V (negative); ion source block temperature, 120 °C; nitrogen desolvation gas temperature, 350 °C; desolvation gas flow, 800 L $h^{-1}$; cone gas flow, 50 L $h^{-1}$. Full scan spectra from *m/z* 100 to 1600 were acquired with a scan time of 0.1 s and 0.01 s interscan delay in both W+ and W− optic mode for an operating resolution of 10,000 (FWHM). Data were collected from 1.3 to 27 min and the mass accuracy was maintained by lock spray using Leucine Enkephalin. Other conditions remaindered the same as in the published **PAPER 3.**[129]

The developed UHPLC-DAD-ToFMS method generated multi-dimensional and multi-informational chromatographic fingerprints. Every compound in every extract was characterized: **(a)** by its retention time, **(b)** UV/VIS spectral profile (local maxima in UV/VIS; 200-600 nm), **(c)** by MS spectral profile (*m/z* ratio in both ESI+ and ESI−), and **(d)** by peak area. An example of characterization of particular SM is shown in **Figure 13**.

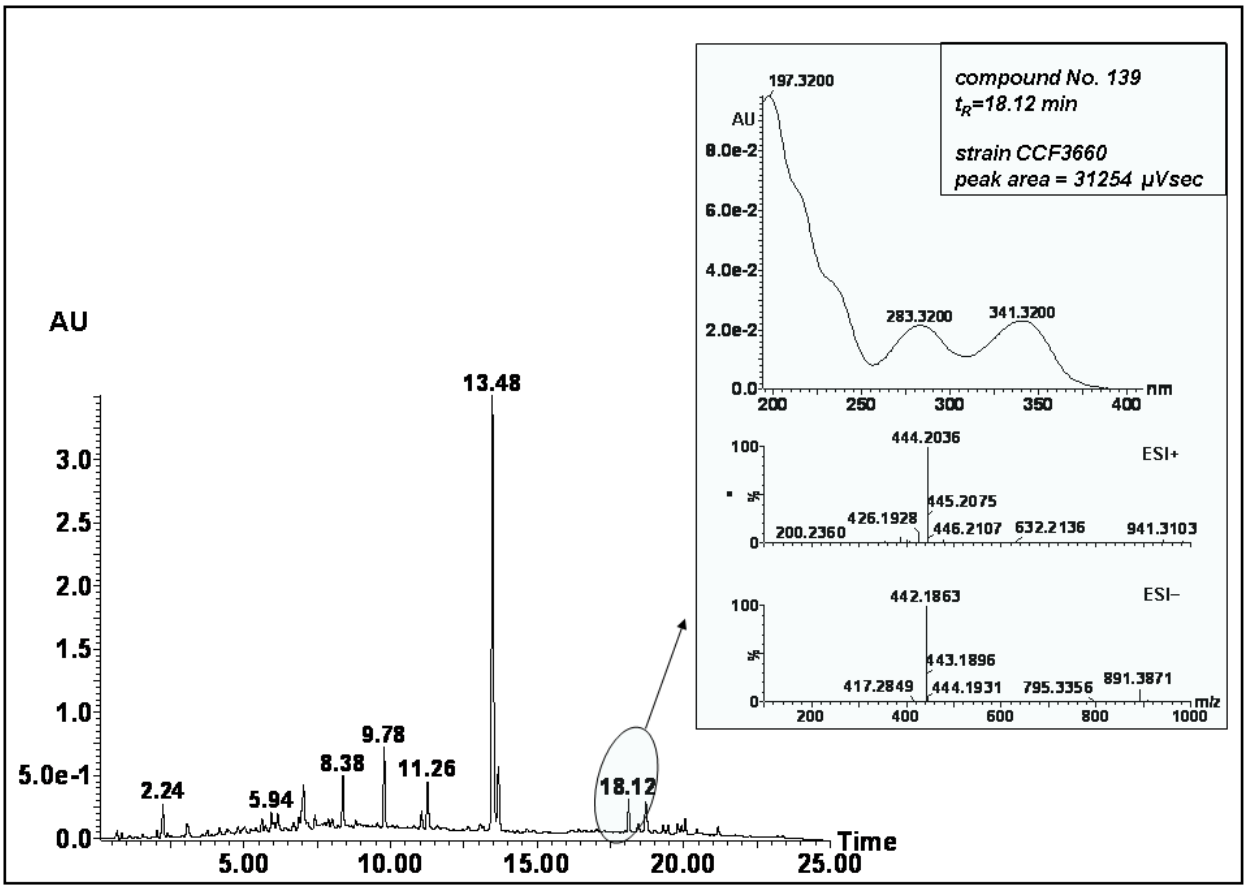


**Figure 13. Characterization of compounds presented in UHPLC-DAD-ToFMS fingerprints.** Every compound is characterized for purpose of statistical analysis by its retention time, UV/VIS spectral profile, MS spectral profile (*m/z* ratio in both ESI+ and ESI−), and peak area.

*Chromatographic conditions:* Acquity UPLC BEH C18 column (100 × 2.1 mm i.d., 1.7 µm; Waters); mobile phase, (A) formic acid-water 0.1:99.9 (v/v), (B) ACN; gradient elution (min/ %A): 0/95, 15/65, 25/0, 27/0, 30/ 95; flow rate, 0.4 mL $min^{-1}$; column temperature, 25 °C; sample temperature, 10 °C; injection volume, 1 µl; UV 260 nm.
*MS detection*: cone voltage, 40 V; capillary voltage, ±2500 V; ion source block temperature, 120 °C; nitrogen desolvation gas temperature, 350 °C; desolvation gas flow rate, 800 L $h^{-1}$; cone gas flow, 50 L $h^{-1}$; W mode; scan time, 0.1 s; interscan delay time 0.01 s.

The developed CFP method was applied for analysis of 48 strains of *Geosmithia* spp. The generated fingerprints revealed the correlation of SMs production with the strain affiliation. As an example, the overlays of chromatograms of four pairs of strains belonging to the same species (extracted at 260 nm), namely *Geosmithia* sp. 8 (MK1712a and MK263), *Geosmithia* sp. 32 (MK1826 and MK1834), or *G. morbida*

(1259 and 1272) and *Geosmithia* sp. 24 (RJ06ka and MK1842) are shown in **Figure 14.** The overall pattern of obtained data therefore suggests obvious correlation of generated UHPLC-DAD-ToFMS chromatograms with taxonomical identity. The results are summarized in **PAPER 3**[129].

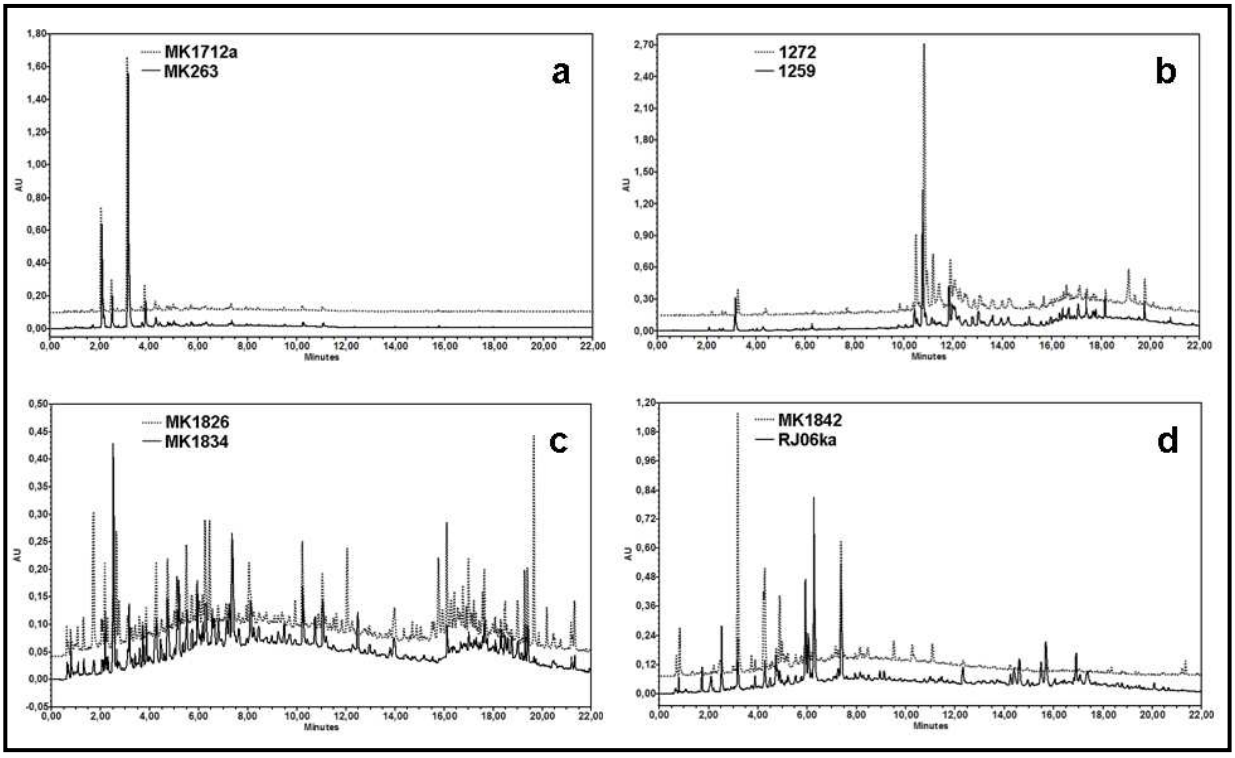


**Figure 14. The overlay of UHPLC-DAD 2D chromatograms of different strains of the genus *Geosmithia*. Overlay of chromatograms of strains MK1712a and MK263 (a); 1272 and 1259 (b); MK1826 and MK1834 (c); MK1842 and RJ06ka (d).**

*Chromatographic conditions:* Acquity UPLC BEH C18 column (100 × 2.1 mm i.d., 1.7 µm; Waters); mobile phase, (A) TFA–water 0.1:99.9 (v/v), (B) ACN; gradient elution (min/ %A): 0/ 95, 15/ 65, 25/ 0, 27/ 0, 30/ 95;flow rate, 0.4 mL $min^{-1}$; column temperature, 25 °C; sample temperature, 10 °C; injection volume, 1 µl; UV 260 nm.

Afterwards, the PCA, PCoA and HCA were used for evaluation of generated UHPLC-DAD-ToFMS data. The CFP data evaluation was performed in PAST statistical software (PAlaeontological STatistics), the data analysis package originally aimed at paleontology but now also popular in many other fields. It includes common statistical, plotting and modeling functions.[145]

The set of 206 major components (peaks in chromatogram refers to SMs) from all 48 tested species of genus *Geosmithia* were chosen for statistical evaluation employing

the PCA, PCoA and HCA. Since only 2D datasets can be analyzed by PCA, PCoA and HCA, the 2D dataset of peaks´ characterization had to be constructed. Therefore, the dataset of all SM found in all studied strains with the specific characterization (**a-d**) were constructed. The statistical analysis of large set fingerprints performs difficult task, especially when spectroscopic detection is used.[146, 147] For instance, shifts of retention times and baseline shifts are known difficulties during the data alignment for the automated statistical computing.[148] Therefore, the manual data handling was used for data evaluation. Although this approach performed long-lasting and difficult task, it was the only possibility how to obtain representative SMs characterization. Finally, for PCA, PCoA and HCA, only binary data matrix was used (1 = present, 0 = not present; concentration expressed as a peak area was not considered), since the variation of SMs concentration in the identical strain was observed for different cultivations in different time and was therefore considered to be irrelevant for data evaluation.

The PCoA analysis of 48 tested strains is depicted in **Figure 15**. The two coordinates of the PCoA plot represent the directions of two highest variations through the data. Therefore, the distinction of the strains rises with their distance along the coordinate. The strains with suffix *-a, -b* (strains CCF4197, CCF3861) refer to the replicate of a single strain used for repeatability testing of cultivation and extraction procedure.

The closeness of the strains in the PCoA plot are is in agreement with the similarity of UHPLC-DAD chromatograms in **Figure 14.** discussed above and in **PAPER 3** in the appendices.[129] For instance, strains 1272 and 1259 (both *G. morbida*, **Figure 14b**) are located in the PCoA plot almost identically, which corresponds with their similar SMs profile. The same findings were obtained for other strains in **Figure 14,** namely MK1712a and MK263 (*Geosmithia* sp. 8), MK1826 and MK1834 (*Geosmithia* sp. 32), MK1842 and RJ06ka and (*Geosmithia* sp. 24). In general, the distribution of the strains in PCoA plot corresponds with their taxonomical identity. However, interesting strain distribution along the Coordinate 1 also revealed the correlation of the data with other characteristics of the particular strains (eg. ecology, distribution). The dashed line in the plot divides the strains into two groups according to their specific host spectrum (see **Figure 15**). “Specialists” refer to strains characterized by a single host plant family, while “generalists” refer to multiple host plant spectrum. These findings enabled tracing of SMs characteristic for the host spectrum of the strains and revealed other utilization of the developed CFP method.

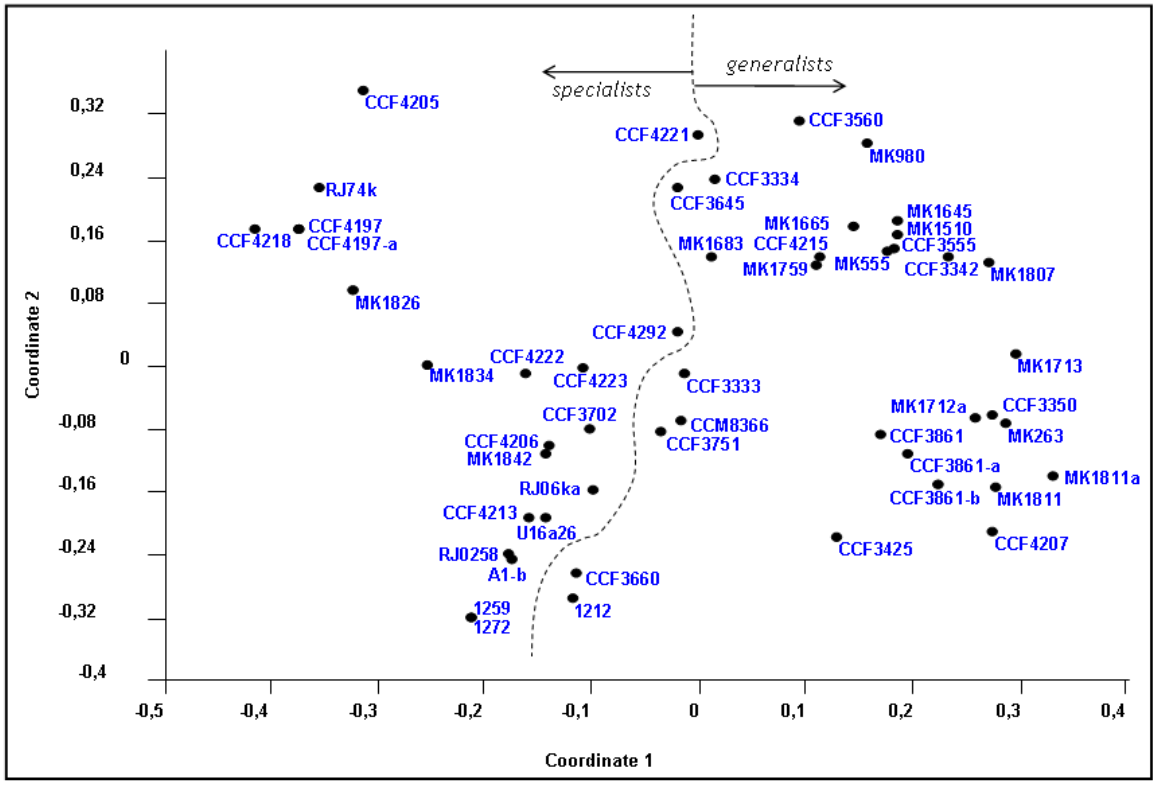


**Figure 15. Principle coordinate analysis (PCoA) of 48 *Geosmithia* strains based on UHPLC-DAD-ToFMS fingerprinting.**

*PCoA conditions*: Euclidian distance; binary data used; general settings in PAST software.
For conditions of UHPLC-DAD-ToFMS analyses, see caption to **Figure 13** and chapter ***4.2.2***.

**Figure 16** depicts the corresponding HCA dendrogram of the studied strains. The primary purpose of HCA is to present data in a manner that emphasizes natural grouping. Relatively short distances between samples indicate similarity. The pairs of strains with similar SMs production (based to the overlay of chromatograms in **Figure 14**) are highlighted in the HCA dendrogram. Their grouping in the HCA demonstrates their similarity of UHPLC-DAD-ToFMS fingerprints and is in agreement with their taxonomical relatedness.

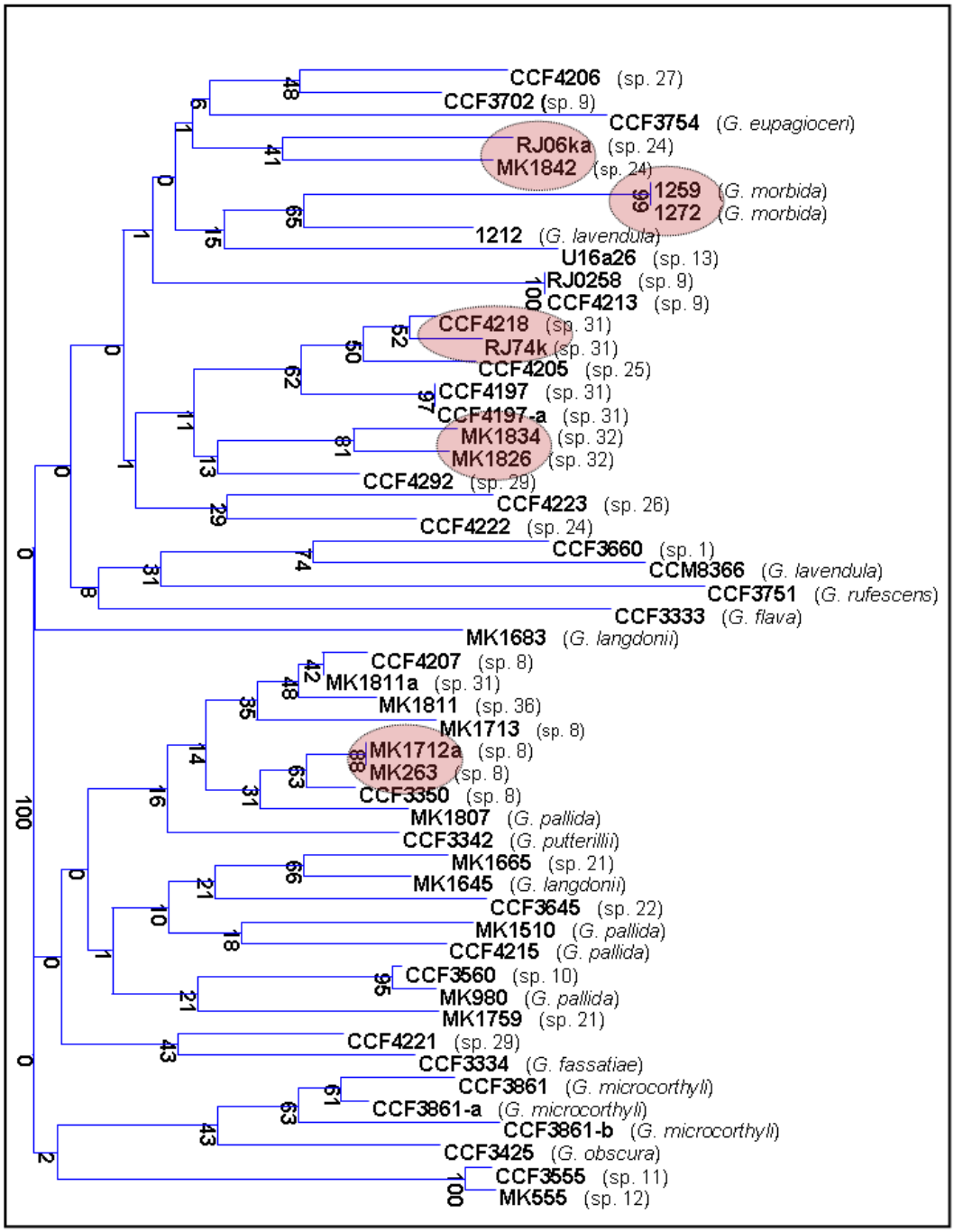


**Figure 16. Hierarchical cluster analysis (HCA) dendrogram representing similarity of 48 *Geosmithia* strains based on UHPLC-DAD-ToFMS fingerprinting.**

*HCA conditions*: neighbor joining (NJ) tree; binary data used; Simpson similarity index; 1000 bootstrap resampling.
For UHPLC-DAD-ToFMS conditions see caption to **Figure 13** and chapter ***4.2.1***. Strains used for demonstration of SMs profile similarity in **Figure 14** are highlighted.

The results from PCA, PCoA and HCA were compared with findings that were obtained by our collaborative group employing the phylogenetical analysis of the rDNA.[70] Both findings were in agreement and the similarity of results proved that the UHPLC-DAD-ToFMS method can be successfully used for CFP analyses of fungal strains and that it is the useful tool for taxonomy and also alternative to the phylogenetical analyses.

Generally, the result from PCoA and HCA analyses verified the correlation of UHPLC-DAD-ToFMS fingerprints with the taxonomical identity of the *Geosmithia* strains.

However, discrepancies in the correlation of UHPLC-DAD-ToFMS fingerprints with taxonomical identity were observed for some of tested strains, e.g. for CCF4206 (*Geosmithia* sp. 27). Based on the taxonomic identity, this strain should be grouped with the taxonomically related species (e.g. MK980 and other *G. pallida* strains). According to the PCoA and HCA plot, the strain CCF4206 shows the similarity of fingerprints e.g. with strains CCF3702 (*Geosmithia* sp. 9) and CCF3754 (*G. eupagioceri*). This, and others, discrepancies are probably caused by not yet understood and complex factors resulting in the evolution of different SMs spectra. In this particular case, the *Geosmithia* sp. 27 (CCF4206) is specifically associated with bark beetles feeding on the family Pinaceae (similarly as *Geosmithia* sp. 24 and 9 on the **Figure 16**), nevertheless taxonomically related species from *G. pallida* group (e.g. MK980) are generalist, distributed on both hardwoods and conifers.

### 5.2.2 Chromatographic screening for bioactive fungal SMs

#### *5.2.2.1 Kirby-Bauer disk diffusion susceptibility test*

Forty eight *Geosmithia* strains were tested for their antimicrobial activity against 5 indicating organisms. In total, 12 strains exhibited antimicrobial activity against at least one of the indication organisms and 36 strains were inactive against all indicating organisms. The overview of strains that revealed antimicrobial activity is listed in **Table 3**; the example of antimicrobial activity testing using Kirby-Bauer disk diffusion susceptibility test depicts **Figure 17**. Concerning bacterial indicating organisms, many strains expressed the antimicrobial activity only against Gram-positive (*Kocuria rhizophila*; e.g. CCF3861, RJ06ka, CCF4205) and were inactive against Gram-negative bacteria (*Escherichia coli*), which is a common phenomenon caused by the structural differences of bacterial cell walls.[8] Strains CCF4213 and RJ0258 (*Geosmithia* sp. 9) exhibited antimicrobial activity against the whole set of indicating organisms, which revealed broad spectrum of their antimicrobial activity and qualified them for further investigation. Since both strains belong to the same species and their SMs production is similar (according to previous UHPLC-DAD-ToFMS fingerprinting method, chapter ***5.2.1*** and **PAPER 3** in the appendices[129]), the strain RJ0258 was chosen for the detailed study using chromatographic screening and bioassay-guided fractionation.

**Table 3. Results of antimicrobial activity testing of *Geosmithia* strains using Kirby-Bauer disk diffusion susceptibility test.**

| **strain No.** | **indicating organism** | | | | | |
|---|---|---|---|---|---|---|
| | ***Kocuria rhizophila*** | ***Escherichia coli*** | ***Saccharomyces cerevisiae*** | ***Graphium fibriisporum*** | ***Beauveria bassiana*** | ***Penicillinum decumbens*** |
| | **antimicrobial activity – inhibition zones (cm)** | | | | | |
| **CCF3334** | 0 | 0 | 0.8 | 0.7 | 1.2 | 0 |
| **CCF3861** | 1.6 | 0 | 0.8 | 0.8 | 0.7 | 0.7 |
| **1272** | 0.8 | 0.8 | 0 | 0.7 | 0.7 | 0.7 |
| **CCF3751** | 0 | 0 | 0.9 | 0.8 | 0.7 | 0.7 |
| **CCF3350** | 0.8 | 0 | 0 | 0 | 0.7 | 0.7 |
| **RJ0258** | **1.1** | **0.9** | **0.9** | **1.4** | **1.3** | **1.0** |
| **CCF4213** | **1.2** | **0.9** | **1.0** | **1.1** | **0.7** | **0.7** |
| **CCF3555** | 0.8 | 0 | 0 | 0.8 | 0.7 | 0 |
| **RJ06ka** | 0.9 | 0 | 0 | 0.8 | 0.8 | 0.7 |
| **CCF4205** | 1.0 | 0 | 0.8 | 0 | 0.9 | 0 |
| **CCF4206** | 0.8 | 0 | 0 | 0 | 0 | 0 |
| **RJ74k** | 0 | 0 | 0 | 0 | 0.7 | 0 |

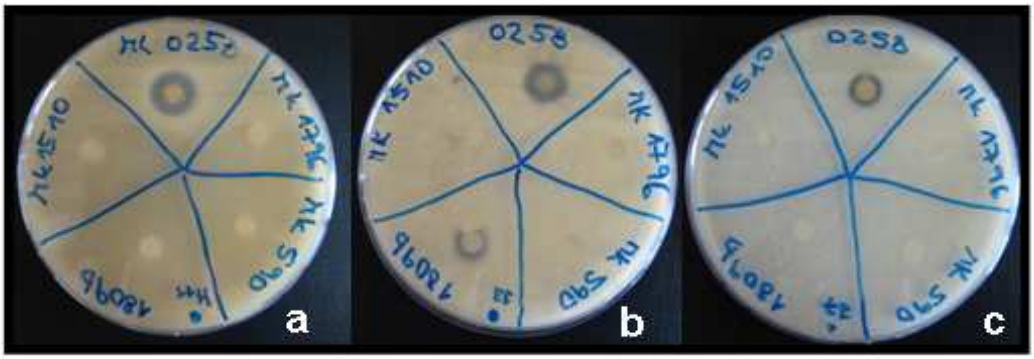


**Figure 17. Example of antimicrobial activity testing of *Geosmithia* strains using Kirby-Bauer disk diffusion susceptibility test.**

Indicating microorganisms: **a**-*Graphium fibriisporum,* **b**-*Beauveria bassiana,* **c**-*Penicillinum decumbens.*

### *5.2.2.2 HPLC-UV bioassay-guided fractionation*

During the ***HPLC-UV grad1*** method development, two analytical columns, namely Gemini C18 column (250 × 4.6 mm i.d., 5 µm) and XTerra Prep RP18 column (150 × 7.8 mm i.d., 5 µm; Waters, Prague, Czech Republic) were evaluated and compared. Since shifts of retention times (compared to UHPLC-DAD-ToFMS analyses) for selected major SMs were observed using XTerra column (data not shown), the Gemini column was finally chosen for the fractionation. Among the aqueous components of the mobile phases, formic acid-water 0.1:99.9 (v/v) and pure water were tested. The formic acid-water 0.1:99.9 (v/v) provided higher peak resolution, better peak shapes and higher retention times. Concerning organic modifiers, ACN provided sharper peaks compared to methanol. The applied gradient elution profile was programmed with regards to peak resolution and the total analysis time. The wavelengths set for dual wavelength UV/VIS detector were 260 and 310 nm respecting the maximal absorbance of the majority of SMs. For final chromatographic conditions see chapter ***4.2.2.2*** in Experimental section and **Figure 18**.

The 3.5 minutes lasting fraction were collected with final number of 23 fractions (see **Figure 18**). Five fractions, marked as FR11 – FR15 exhibited antimicrobial activity (see **Figure 19**) and they were therefore further refractionated using ***HPLC-UV grad2*** method. The gradient elution program was adjusted unless the single peaks were isolated separately. The purity of the fractions was verified by the previously developed UHPLC-DAD-ToFMS method (see chapter ***4.2.1*** in Experimental section).

The final gradient profile of ***HPLC-UV grad2*** method was as follows (min/%A): 0/ 70, 50/ 50, 55/ 50, 60/ 0, followed by 15 min equilibration step (**Figure 20**). Other conditions remained the same as for ***HPLC-UV grad1*** (see chapter ***4.2.2.2*** in Experimental section).

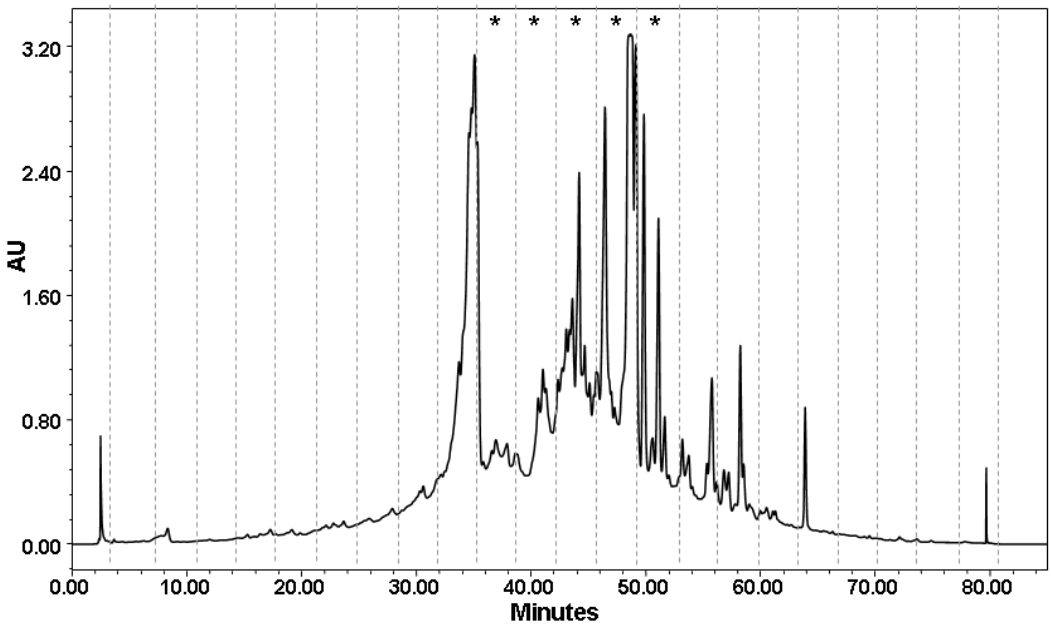


**Figure 18. HPLC-UV fractionation of strain RJ0258 (*HPLC-UV grad1* method).**

*Chromatographic conditions:* Gemini C18 column (250 × 4.6 mm i.d., 5 µm; Phenomenex); mobile phase (A) formic acid-water 0.1:99.9 (v/v), and (B) ACN; gradient elution (min/%A): 0/ 95, 35/ 65, 65/ 0, 75/ 95; flow rate, 1 mL $min^{-1}$; column temperature, 25 °C; samples temperature, 10 °C; injection volume, 60 µL; UV 260 nm. Dashed vertical lines refer to isolated fractions.
* refers to fractions FR11-FR14 with antimicrobial activity according to Kirby-Bauer disc diffusion susceptibility test.

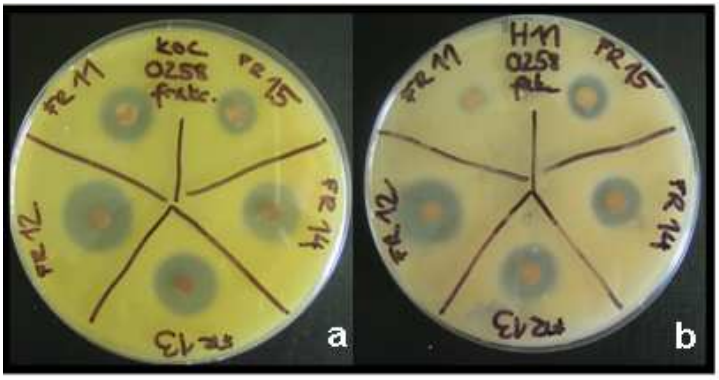


**Figure 19. Antimicrobial activity testing of fraction isolated from *HPLC-UV grad1* method using Kirby-Bauer disk diffusion susceptibility test.**

Indicating microorganisms: **a**-*Kocuria rhizophila* **b**- *Graphium fibriisporum.*

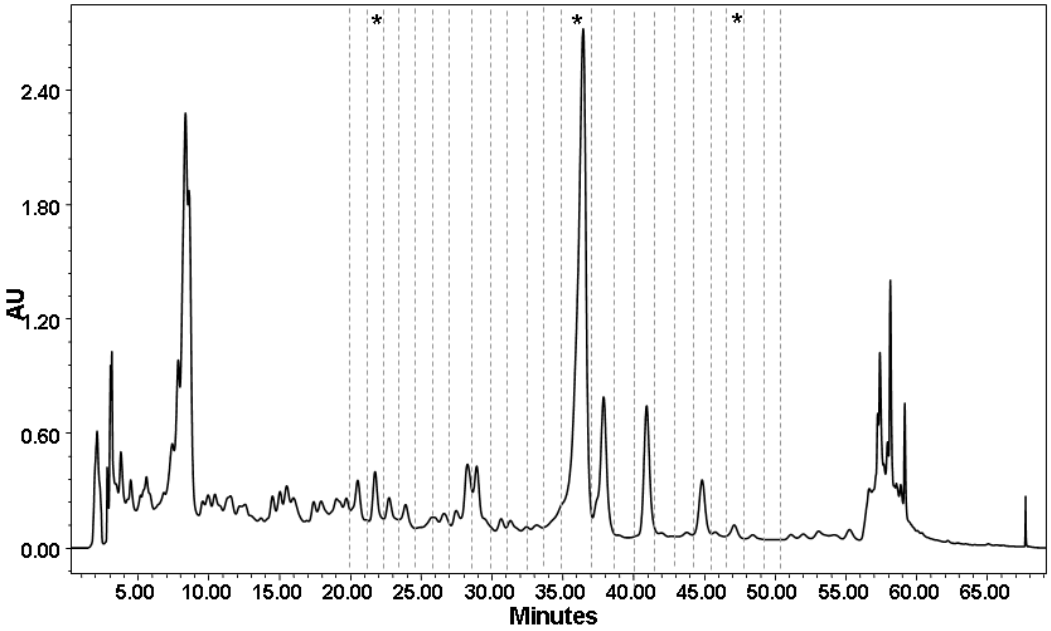


**Figure 20. HPLC-UV refractionation of strain RJ0258 (*HPLC-UV grad2* method).**

*Chromatographic conditions:* gradient elution (min/%A): 0/ 70, 50/ 50, 55/ 50, 60/ 0, 75/ 70 For other chromatographic conditions see caption to **Figure 18**.
Dashed vertical lines refer to isolated fractions.
* refers to fractions with antimicrobial activity according to Kirby-Bauer disc diffusion susceptibility test.

The bioassay-guided refractionation revealed three bioactive SMs of the RJ0258. According to UHPLC-DAD-ToFMS analyses, the major peak with retention time of 36.5 min was the mixture of many co-eluting compounds. This peak was excluded from further detailed study, since it was impossible to optimize the elution program in order to separate these compounds and isolate them separately. Therefore, two SM with retention time of 22.1 min and 47.3 min, respectively, were further isolated using the HPLC-UV preparative analysis and they were marked as **RJ0258-F1** and **RJ0258-F2**.

For subsequent isolation of bioactive SMs **RJ0258-F1** and **RJ0258-F2**, Gemini C18 preparative column (250 × 10 mm i.d., 5 µm) with ***HPLC-UV iso*** method was employed. Since the isocratic elution is generally preferred for preparative HPLC, the mobile phase was composed of formic acid-water 0.1:99.9 (v/v) and ACN 50:50, isocratic elution. Dual λ Absorbance Detector was set at 260 nm (preferred for RJ0258-F1) and 310 nm (RJ0258-F2). Other conditions are described in chapter ***4.2.2.2*** in Experimental section. The analysis is shown in **Figure 21** and the

verification of antimicrobial activity depicts **Figure 22**. The isolation of the selected bioactive SMs was repeated since the amount of 1 mg of each SM was obtained.

The purity of the fractions was confirmed by UHPLC-DAD-ToFMS (see chapter ***4.2.1***) and the SMs were further studied as described in following subsections. The test of bioactivity of isolated SMs was performed, resulting in confirmation of their antimicrobial activity against all employed indicating organisms and their stability during the sample handling (data not shown).

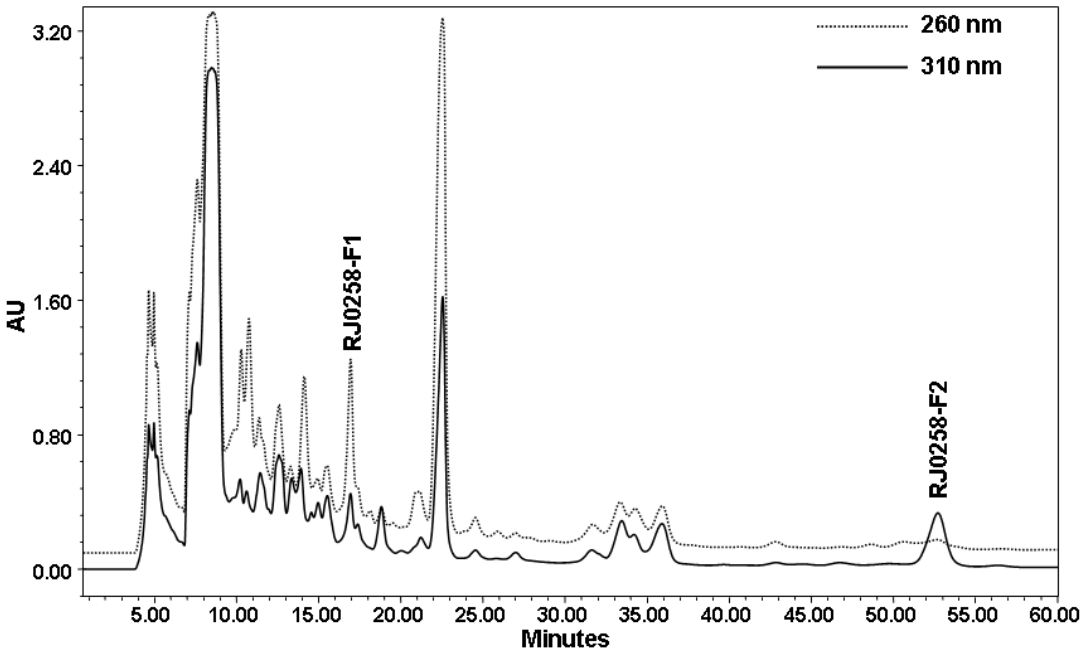


**Figure 21. HPLC-UV preparative analysis of bioactive SMs of strain RJ0258 (*HPLC-UV iso* method).**

*Chromatographic conditions:* Gemini C18 column (250 × 10 mm i.d., 5µm; Phenomenex); mobile phase, formic acid-water 0.1:99.9 (v/v) and ACN 50:50, isocratic elution; flow rate, 2 mL $min^{-1}$; column temperature, 25 °C; samples temperature, 10 °C; injection volume, 120 µL, UV 260 and 310 nm.


**Figure 22. Antimicrobial activity testing of fraction isolated with *HPLC-UV iso* method using Kirby-Bauer disk diffusion susceptibility test.**

Indicating microorganism: *Saccharomyces cerevisiae.*

### *5.2.2.3 Detailed MS and off-line MS/MS study of bioactive SMs*

***UHPLC-ToFMS detection***

The SM **RJ0258-F1** was characterized by MS spectra of *m/z* 325.2020 (positive ion mode; ESI+) and 323.1867 (ESI−), which corresponds with elemental composition of $\mathbf{C_{18}H_{28}O_5}$ with obtained mass error of Δ = 1.5 ppm (ESI+), Δ = 2.8 ppm (ESI−) and i-FIT (norm) error of 0.1 (ESI+) and 0.0 (ESI−).

The formation of the most intensive peak of *m/z* 369.1909 in negative ion mode (see **Figure 23**) was observed, which corresponds with molecular ion adduct with formic acid in mobile phase ($C_{19}H_{29}O_7$ in ESI−; mass error of Δ = −1.1 ppm; i-FIT (norm) error of 2.2). The influence of mobile phase composition on ion adduct formation in obvious from **Figure 24**. The formic acid–water 0.1:99.9 (v/v) and pure water, respectively, were compared as the aqueous components of mobile phase, showing the strong contribution of formic acid to molecular ion adduct formation. If pure water is used as the mobile phase, the molecular ion is preferred and the presence of dimmer of molecular ion $(2M-H)^-$ is also evident. The formation of molecular ion adduct with formic acid even when pure water is employed as the mobile phase was probably caused by trace amounts of formic acid remaining in the UHPLC system.

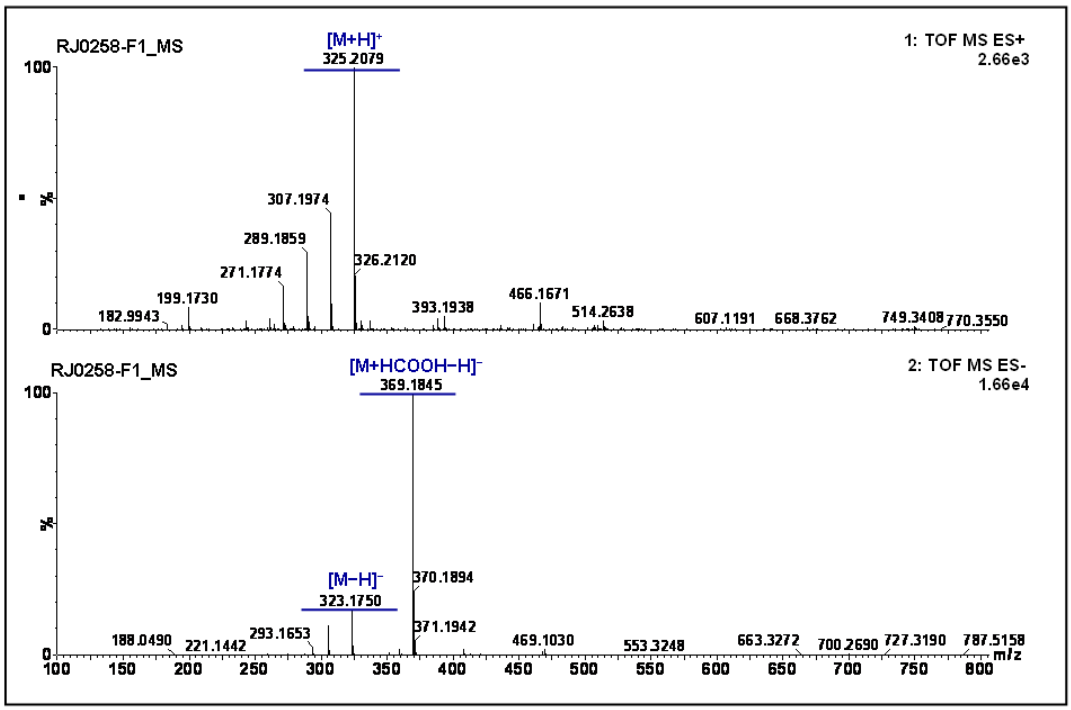


**Figure 23. MS spectra in both ESI+ and ESI− ionization mode of bioactive secondary metabolite RJ0258-F1 produced by RJ0258 *Geosmithia* strain.**

For UHPLC-DAD-ToFMS conditions see caption to **Figure 13** and chapter ***4.2.1***.

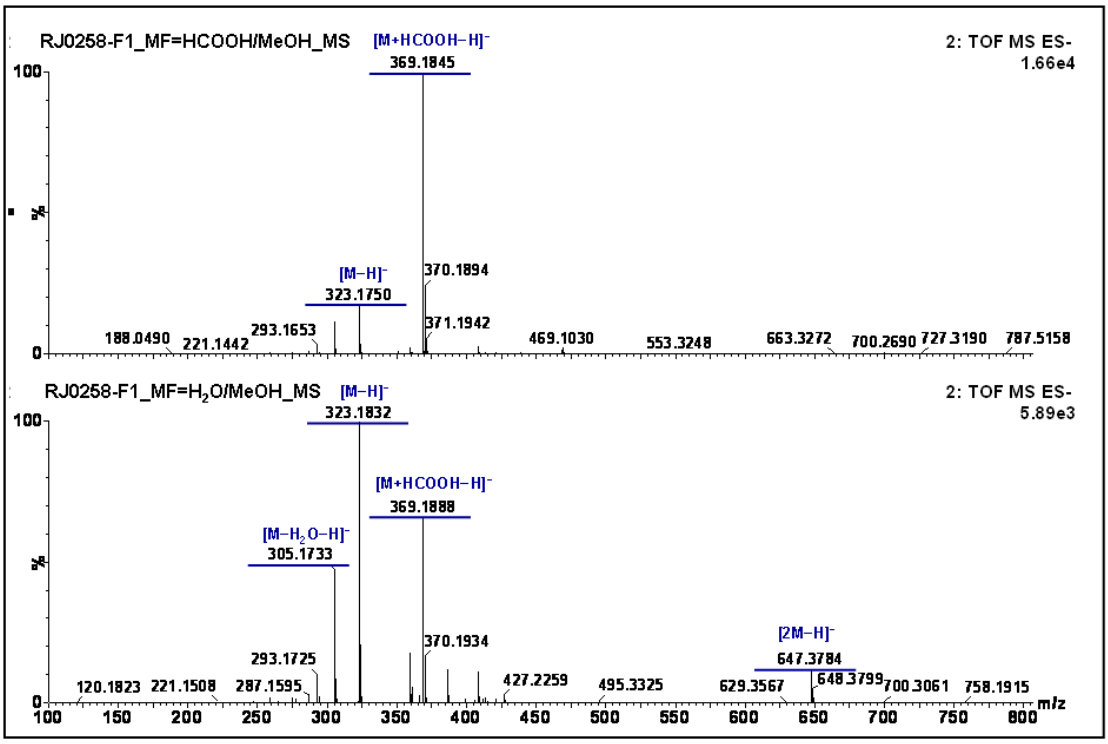


**Figure 24. MS spectra of bioactive secondary metabolite RJ0258-F1 produced by RJ0258 *Geosmithia* strain – comparison of mobile phase used for UHPLC-DAD-ToFMS in ESI− ionization mode.**

*Top*: mobile phase; (A) formic acid–water 0.1:99.9 (v/v), (B) ACN.
*Down*: mobile phase; (A) water, (B) ACN.
For other UHPLC-DAD-ToFMS conditions see caption to **Figure 13** and chapter ***4.2.1***.

The SM **RJ0258-F2** was characterized by MS spectra of *m/z* 307.1903 (ESI+) and 305.1753 (ESI−), as depicts **Figure 25**. This corresponds with elemental composition of $\mathbf{C_{18}H_{26}O_4}$ with obtained mass error of Δ = −2.0 ppm (ESI+), Δ = −2.3 ppm (ESI−) and i-FIT (norm) error of 0.0 (ESI+) and 0.0 (ESI−).

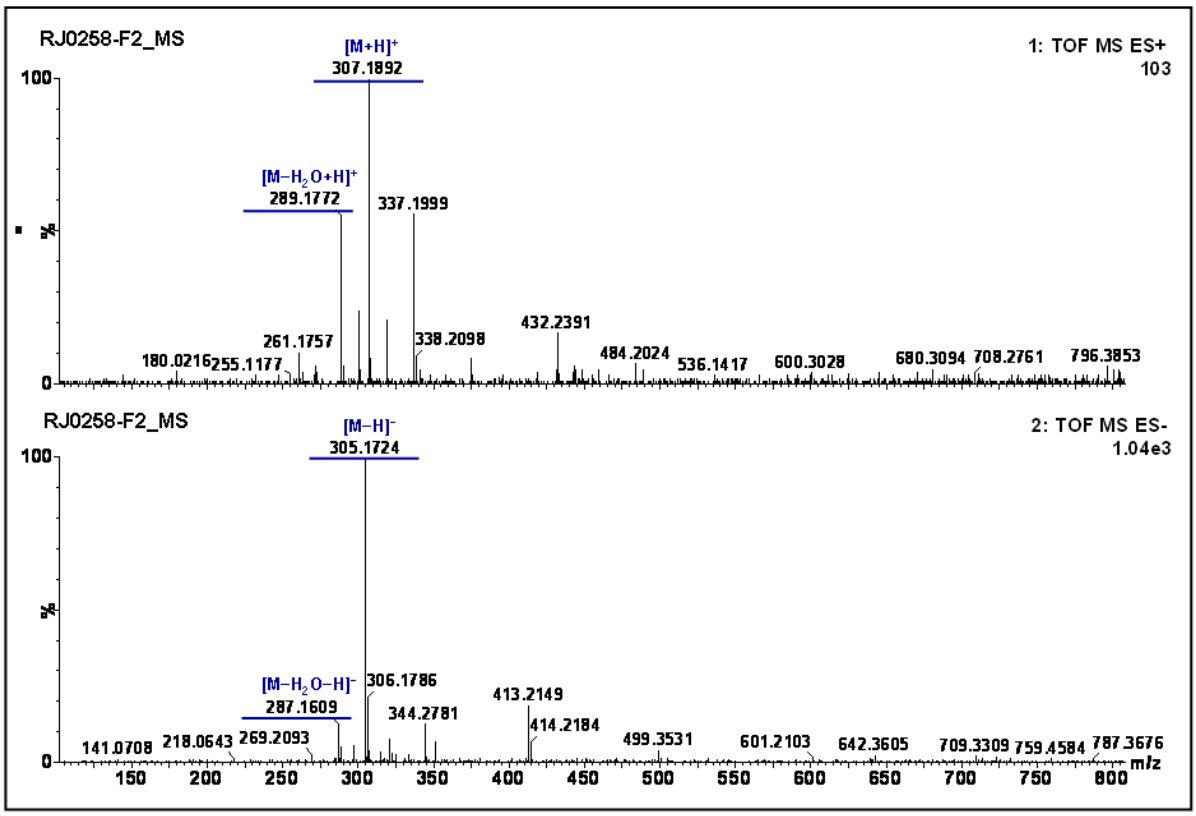


**Figure 25. MS spectra in both ESI+ and ESI− ionization mode of bioactive secondary metabolite RJ0258-F2 produced by RJ0258 *Geosmithia* strain.**

For UHPLC-DAD-ToFMS conditions, see caption to **Figure 13** and chapter ***4.2.1***.

The elemental composition of the bioactive SM was further verified on an APEX-Ultra FTMS instrument by direct infusion. Same findings were obtained resulting in elemental composition of $\mathbf{C_{18}H_{28}O_5}$ (**RJ0258-F1**) and $\mathbf{C_{18}H_{26}O_4}$ (**RJ0258-F2**), respectively. The MS/MS spectra after mass fragmentation are depicted in **Figure 26** and **Figure 27**, respectively. Similar CID MS spectra were also obtained on LCT Premier XE ToFMS when fragmentation using in-source CID was applied (data not shown).

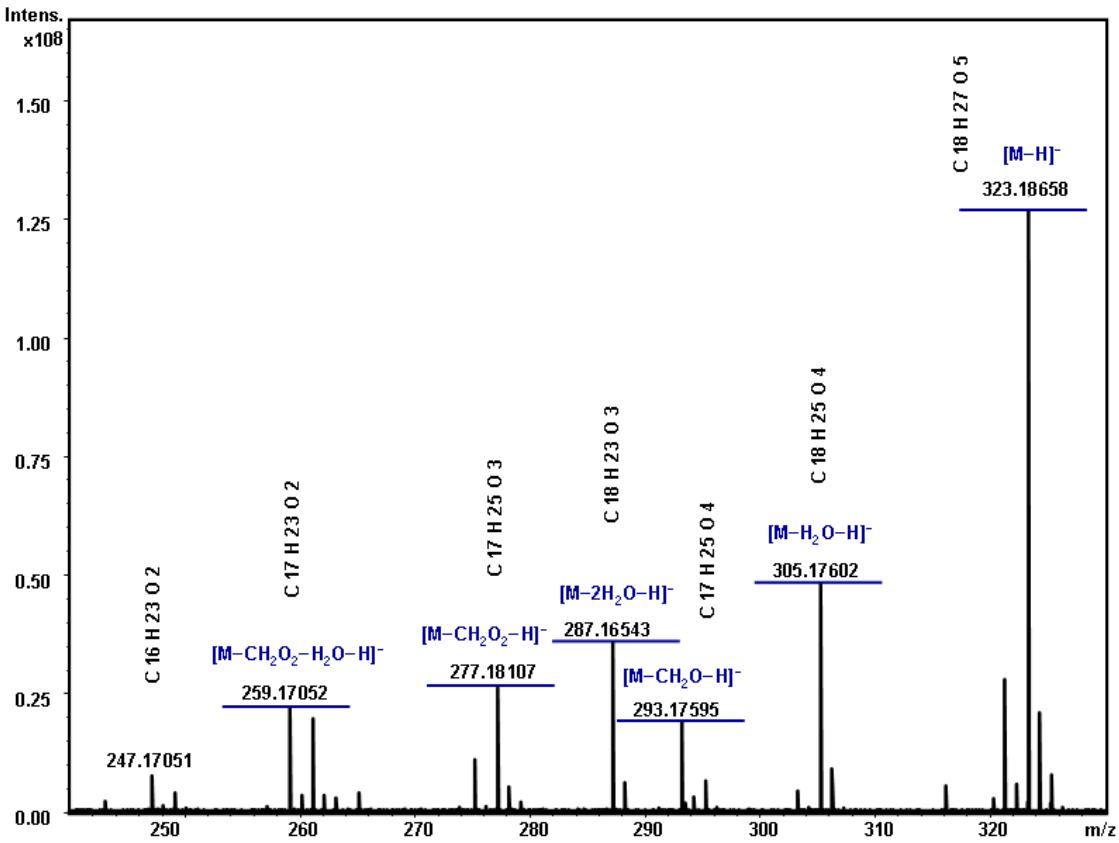


**Figure 26. MS/MS spectrum of bioactive secondary metabolite RJ0258-F1 produced by RJ0258 *Geosmithia* strain.**

*MS/MS detection*: APEX-Ultra FTMS instrument; ESI−; cell opened for, 1.1 msec; accumulation time, 1.0; isolation window, 3 a.m.u.; collision energy, -6 V.
For other conditions see chapter ***4.2.2.3***.

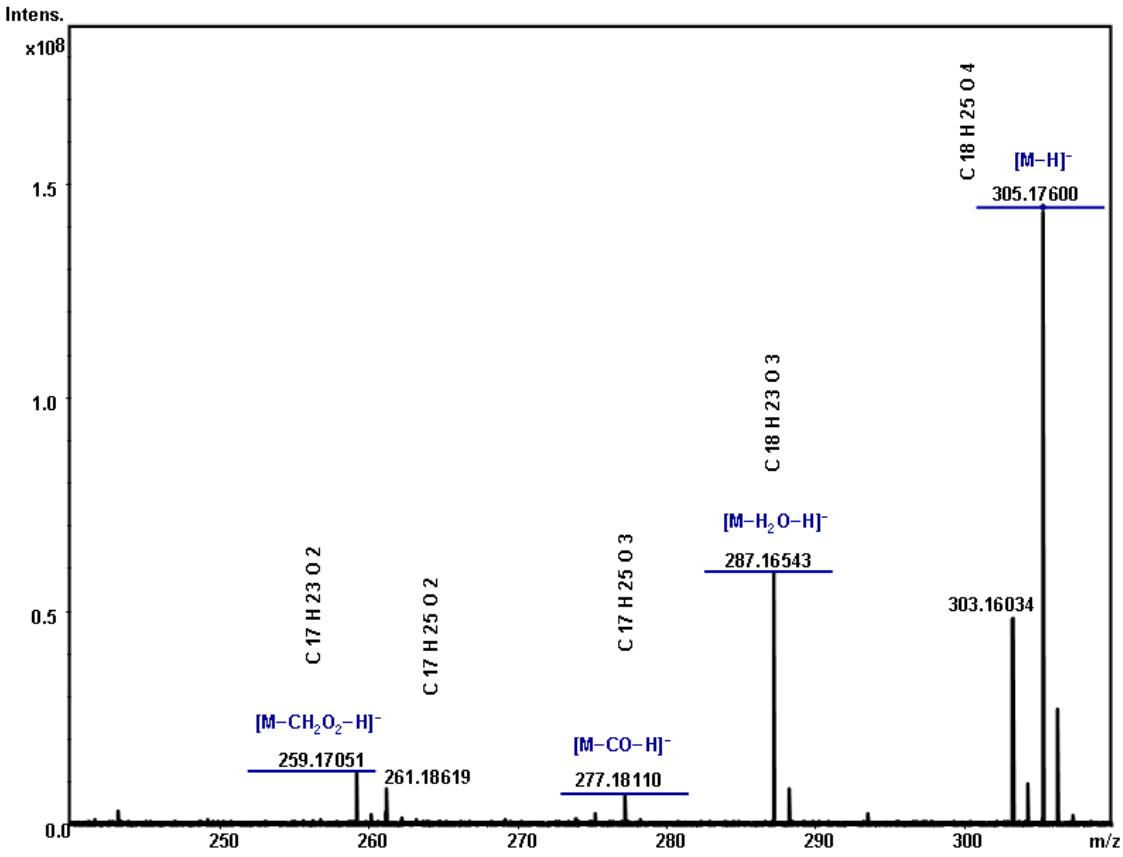


**Figure 27. MS/MS spectrum of bioactive secondary metabolite RJ0258-F1 produced by RJ0258 *Geosmithia* strain.**

For conditions of MS/MS detection, see caption to **Figure 26** and chapter ***4.2.2.3.***

The CFP data revealed that these two bioactive SMs were produced only by strains RJ0258 and CCF4213 and that they were not presented in the blank matrix sample. This is in accordance with the antimicrobial activity testing, using Kirby-Bauer disk diffusion susceptibility test, since no other strain exhibited the antimicrobial activity against all the indicating microorganisms (see **Table 3**). This unique antimicrobial spectrum qualifies these SMs to be the potential novel antibiotics and there is a great demand on their further identification. The utility of the developed extraction, CFP and chromatographic screening methods for tracking of bioactive fungal SMs was therefore proven.

## 5.3 ALTERNATIVE APPROACH TO UHPLC ANALYSES OF SECONDARY METABOLITES - SUPERFICIALLY POROUS COLUMN PARTICLES

The performance of the two tested columns was compared on UHPLC system by evaluation of their experimental peak capacity (*P*). Three curves describing the dependence of experimental *P* on linear velocity of the mobile phase ($u_0$) at three different gradient slopes $\beta/u_0$ constructed for both tetracyclines and macrolides on the two chromatographic columns are depicted in **Figure 28**. Maximal experimental peak capacities for analysis of tetracyclines were 51.8 (Acquity BEH C18 column; 50 × 2.1 mm i.d., 1.7 µm) and 48.4 (Kinetex C18 column; 50 × 2.1 mm i.d., 2.6 µm). This indicated that Kinetex C18 is a suitable alternative to Acquity BEH C18 column for analysis of tetracyclines under acidic conditions. On the contrary, maximal experimental peak capacities for analysis of macrolides on Acquity BEH C18 column were substantially higher (46.7) than that on Kinetex C18 column (36.9). The comparison of analyses of tetracyclines is shown in **Figure 29.**

The curves revealed that experimental *P* values considerably decreased, from certain values, with increasing $u_0$. This phenomenon is in accordance with previously performed experiments,[87] however *P* is according to the theory independent on $u_0$. Additionally, experimental *P* is apparently strongly dependent on gradient slope $\beta/u_0$ so that higher *P* is obtained when lower gradient slope $\beta/u_0$ is applied. Nevertheless, low gradient slope $\beta/u_0$ is in principle connected with long analysis time, which is inconsistent with desired high-throughput analyses. Therefore, the compromise between *P* and gradient slope $\beta/u_0$ represents the crucial task.

Under all conditions compared, higher *P* was achieved on Acquity BEH C18 column for both antibiotic groups with most significant differences observed for macrolides. This was in contrast with the recently published study by Gritti et al.[87] revealing better performance of Kinetex C18 column compared to Acquity BEH C18 column under gradient conditions. However, analytes (proteins) were used in this study.[87] Therefore, the discrepancy can be possibly explained by faster mass transfer in Kinetex C18 column that affects positively the analysis of large molecules, however, the analysis of the low-molecular tetracyclines appears to be influenced by this parameter less significantly.

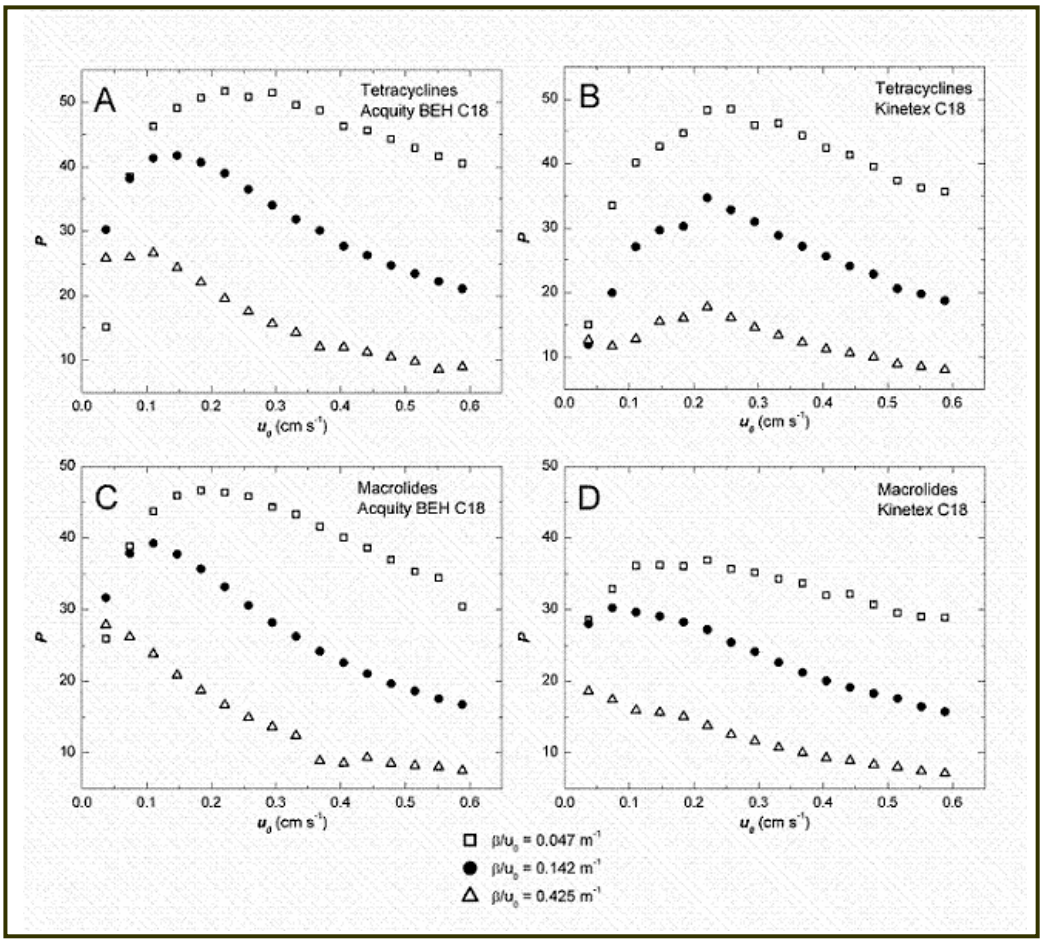


**Figure 28. Experimental peak capacity as a function of linear velocity $u_0$ in gradient separation. (A) Tetracyclines on Acquity C18 column; (B) Tetracyclines on Kinetex C18 column; (C) Macrolides on Acquity C18 column; (D) Macrolides on Kinetex C18 column.**

*Chromatographic conditions:*
*Analytical columns:* Acquity BEH C18 (50 × 2.1 mm i.d., 1.7 µm; Waters), Kinetex C18 (50 × 2.1 mm i.d., 2.6 µm; Phenomenex)
*Tetracyclines*. Mobile phase, TFA-water 99.95:0.05 (v/v) and ACN; gradient started A:B 95:5 (v/v) and changed to A:B 70:30 (v/v) (Δφ = 0.25) during the respective time of gradient $t_g$.; column temperature 40 °C; sample temperature, 10 °C; injection volume, 1 µL; UV 350 nm.
*Macrolides.* Mobile phase, 1 mM ammonium formate pH 9 and ACN; gradient start A:B 80:20 (v/v) and changed to A:B 35:65 (v/v) (Δφ = 0.45) during the respective $t_g$.; column temperature 40 °C; sample temperature, 10 °C; injection volume, 1 µL; UV 194 nm (ROX, CLA), 240 nm (CAM), 286 nm (TYL).

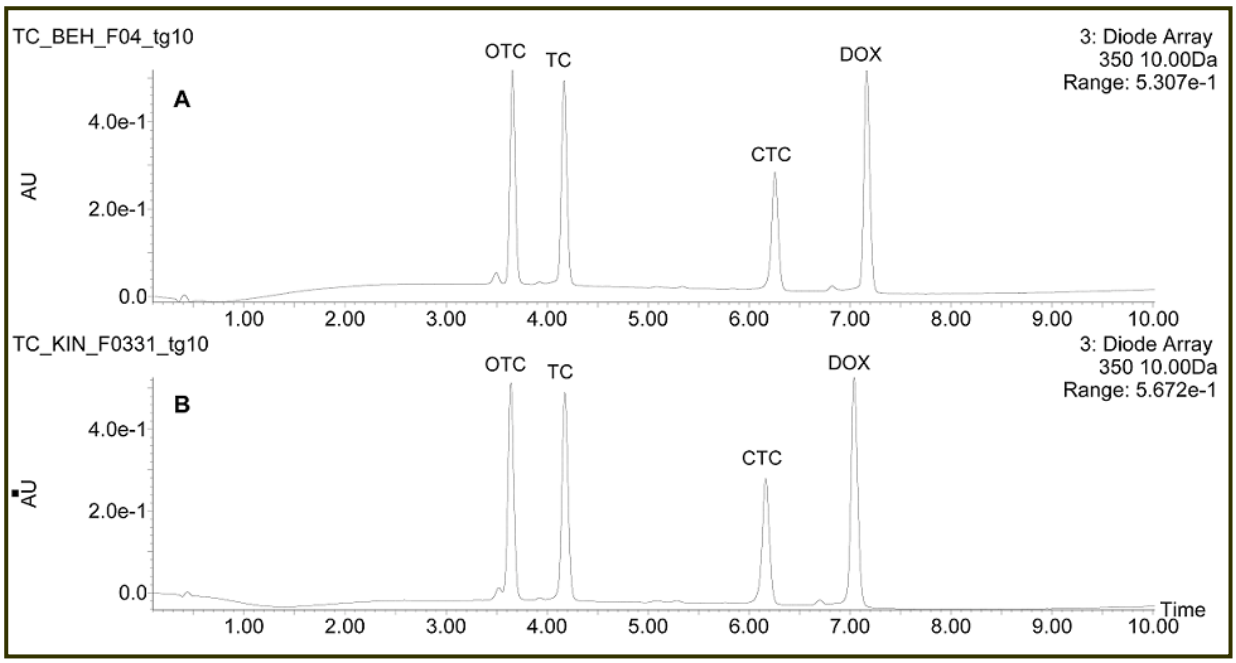


**Figure 29. UHPLC chromatogram of gradient separation of tetracyclines. (A) Acquity BEH C18 column; (B) Kinetex C18 column**

*Chromatographic conditions:* Analytical columns, Acquity BEH C18 (50 × 2.1 mm i.d., 1.7 µm; Waters) and Kinetex C18 (50 × 2.1 mm i.d., 2.6 µm; Phenomenex) Mobile phase, TFA-water 99.95:0.05 (v/v) and ACN; linear velocity $u_0$ = 0.294 cm $s^{-1}$; gradient time $t_g$ = 8.89 min; change of solvent composition during the gradient time $\Delta\varphi$ = 0.25; gradient slope $\beta/u_0$ = 0.142 $m^{-1}$; column temperature 40 °C; sample temperature, 10 °C; injection volume, 1 µl; UV 350 nm.

The maximal backpressure generated on the both tested columns was, as expected, significantly lower on Kinetex C18 column. At $u_0$ = 0.589 cm $s^{-1}$ it was 5,205 psi (under separation conditions for macrolides) and 3,450 psi (for tetracyclines) on Kinetex C18 column, while 8,565 psi (for macrolides) and 8,910 psi (for tetracyclines) was achieved on Acquity BEH C18 column at the same $u_0$. In general, within the whole studied range of $u_0$ the Kinetex C18 column backpressure was lower than 6,000 psi (400 bar), which enables to use this column in HPLC mode.

A decreasing performance of Kinetex C18 column connected with its instability at alkaline conditions was observed during the analysis of macrolides. The worsening performance of Kinetex C18 for CLA and ROX from one injection to another is depicted in **Figure 30**. Unlike Acquity BEH C18 column, Kinetex C18 column exhibited dramatically decreasing performance with growing number of injections for analysis of two macrolides (CLA and ROX). As can be seen, the Kinetex C18 column was after 70 injections incapable of separating CLA and ROX, however, analysis of TYL and CAM including their retention times did not differ considerably. This was

found to be the main drawback of the Kinetex C18 column in comparison to Acquity C18 column.

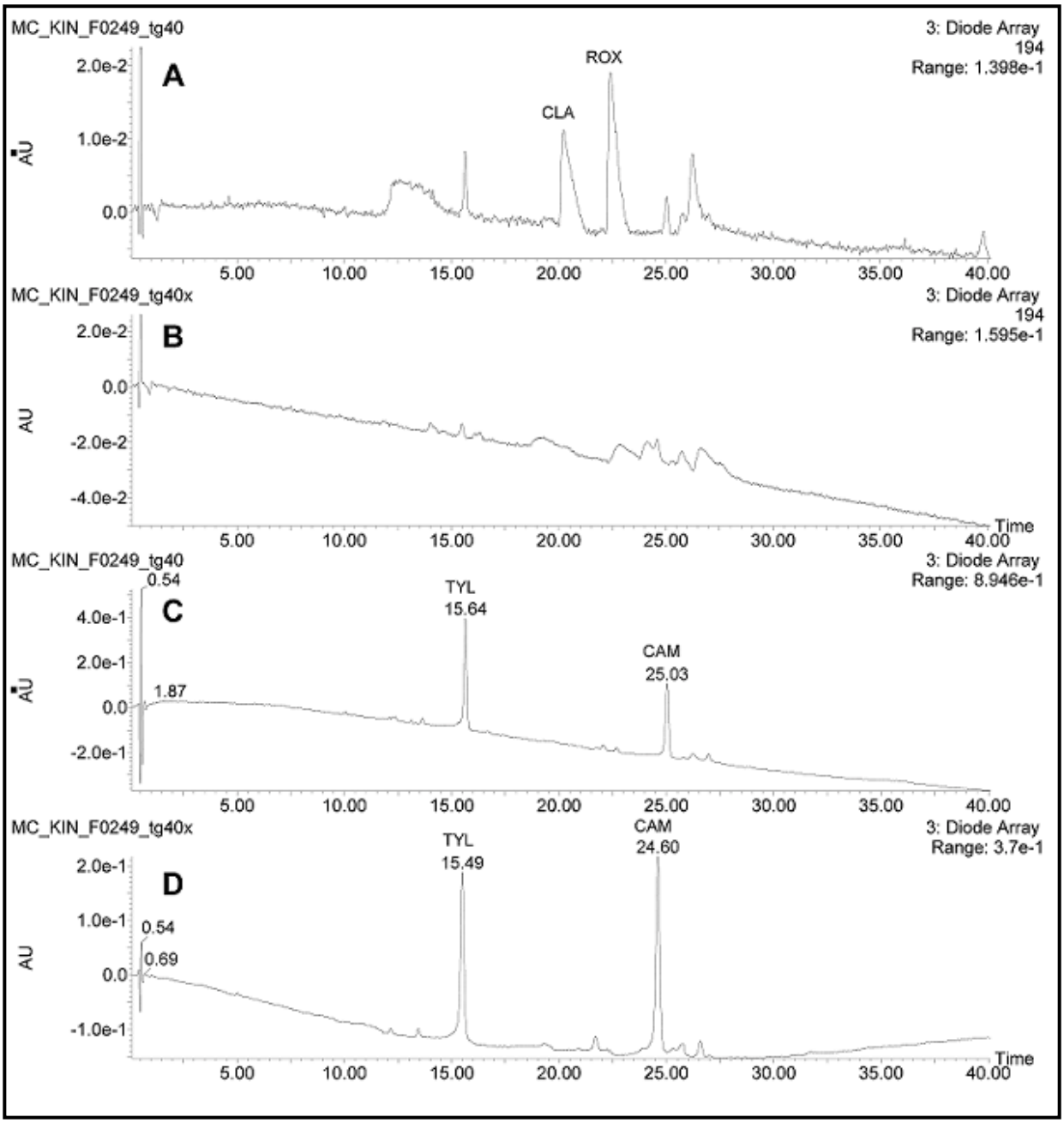


**Figure 30. UHPLC chromatograms of gradient separation of macrolides on Kinetex C18 column. (A), (C) analysis of initial injection; (B), (D) analysis after 70 injections**

*Chromatographic conditions:* Analytical columns, Acquity BEH C18 (50 × 2.1 mm i.d., 1.7 µm; Waters) and Kinetex C18 (50 × 2.1 mm i.d., 2.6 µm; Phenomenex); mobile phase, 1 mM ammonium formate pH 9 and ACN; linear velocity $u_0$ = 0.221 cm $s^{-1}$; gradient time $t_g$ = 40 min; change of solvent composition during the gradient time $\Delta\varphi$ = 0.45; gradient slope, $\beta/u_0$ = 0.047 $m^{-1}$; column temperature, 40 °C; sample temperature, 10 °C; injection volume, 1 µl; UV **A, B** 194 nm; **C, D** extracted from 240 to 286 nm.

# 6 CONCLUSIONS AND FUTURE PERSPECTIVES

Nowadays, in the field of modern analytical chemistry, there is a strong demand on high separation efficiency, high speed of analysis and both high sensitivity and accuracy of detection techniques. Moreover, multi-component analyses employing very complex matrices have to be often performed. It seems that special instrumentation for particular application is necessary. However, this work demonstrates that all these requirements can be accomplished using UHPLC-DAD-ToFMS instrumentation accompanied with appropriate extraction procedure. This instrumentation offers unsurpassed performance for screening purposes; it is also sensitive enough for determination of trace levels of analytes and provides multi-informational data that are useful when unknown components are analyzed. In this thesis, the modern UHPLC methods with DAD and ToFMS detection together with the SPE and LLE pre-separation procedures were developed and applied for study of antibiotics and bioactive microbial secondary metabolites. Both known and unknown secondary metabolites were analyzed in different complex matrices, namely in liquid hog manure, wastewater and river water, and fungal fermentation broth. The utility and versatility of the employed techniques is shown and advocated by achievement of the goals of this thesis.

**The stated aims were accomplished as follows:**

**(a)** The novel analytical method employing UHPLC-DAD and UHPLC-ToFMS techniques along with LLE and SPE extraction procedures were developed and used for determination of residual antibiotics in liquid hog manure, and wastewater and surface water. The mostly used antibiotics in both human and veterinary medicine were chosen as analytes. Proper calibration approaches were employed for quantification. The developed methods were applied for analysis of real environmental samples from different localities in the Czech Republic. All analyses revealed a significant concentration of the selected antibiotics in the tested samples, which means a great threat to the public health because of the associated development of bacterial resistance. The UHPLC-DAD and UHPLC-ToFMS method developed and validated in this thesis is characterized by its versatility, accuracy, selectivity and sensitivity, and can be successfully used for detection of residual antibiotics in different environmental samples.

**(b)** The UHPLC-DAD-ToFMS method employing SPE using Oasis MCX sorbent was developed for analysis of secondary metabolites present in fungal fermentation broth of different strains of the genus *Geosmithia* - symbiotic fungi that are supposed to be strong producers of bioactive SMs. Various column chemistries and SPE sorbents were tested during the method development. The hyphenated UHPLC-DAD-ToFMS technique generates multi-informational fingerprints that provide both chromatographic and spectral information about sample components. The statistical PCA, PCoA and HCA methods were employed for evaluation of UHPLC-DAD-ToFMS data of 48 fungal strains. This pilot analysis proved an interesting coherence between SMs profiles (generated chromatograms) and taxonomical identity, which qualify this method to be useful for chromatographic fingerprinting tool for fungal secondary metabolites and to be an alternative approach to phylogenetical evaluation.

Subsequently, the chromatographic screening for bioactive SMs of these fungi was performed. The Kirby-Bauer disc diffusion susceptibility test selected the strain with exceptional antimicrobial activity that was further studied with the aim to determine SMs of bioactive nature. The method of bioassay-guided fractionation together with UHPLC-DAD-ToFMS method revealed two bioactive SMs with elemental composition of $C_{18}H_{28}O_5$ (RJ0258-F1) and $C_{18}H_{26}O_4$ (RJ0258-F2). It was verified that these compounds exhibit the antimicrobial activity against all used indicating microorganisms and are therefore, characterized by extraordinary antimicrobial spectra. The utility of the developed extraction, CFP and chromatographic screening methods for tracking of bioactive fungal SMs was proven.

**(c)** The novel superficially porous core-shell particles (represented by Kinetex C18 column) were found to be a useful alternative to sub-2 µm column particles (Acquity BEH C18 column) concerning the column efficiency with the advantage of markedly lower generated column backpressure, which enables to use these columns with conventional HPLC instrumentation. However, the main drawback of Kinetex C18 column was the insufficient stability under alkaline conditions when gradient elution was applied. Although the undisputed advantages in terms of comparable efficiency of Kinetex C18 with Acquity BEH C18 column together with its compatibility with HPLC are pronounced, the limitation for analysis under alkaline conditions was obvious. Therefore, the employment under acidic conditions is preferred for Kinetex C18 column.

## REFERENCES


(1) Larsen, T. O.; Smedsgaard, J.; Nielsen, K. F.; Hansen, M. E.; Frisvad, J. C. *Nat Prod Rep* **2005**, *22*, 672-695.

(2) Silver, L. L.; Bostian, K. A. *Antimicrob Agents Chemother* **1993**, *37*, 377-383.

(3) Batt, A. L.; Kostich, M. S.; Lazorchak, J. M. *Anal Chem* **2008**, *80*, 5021-5030.

(4) Bendz, D.; Paxeus, N. A.; Ginn, T. R.; Loge, F. J. *J Hazard Mater* **2005**, *122*, 195-204.

(5) Brown, K. D.; Kulis, J.; Thomson, B.; Chapman, T. H.; Mawhinney, D. B. *Sci Total Environ* **2006**, *366*, 772-783.

(6) Clardy, J.; Fischbach, M. A.; Walsh, C. T. *Nat Biotechnol* **2006**, *24*, 1541-1550.

(7) Demain, A. L.; Fang, A. *Adv Biochem Eng Biotechnol* **2000**, *69*, 1-39.

(8) O´Grady, F.; Lambert, H. P.; Finch, R. G.; Greenwood, D. *Antibiotic and chemotherapy: anti-infective agents and their use in therapy*, 7th ed.; Churchill Livingstone: New York, 1997.

(9) Berdy, J. *J Antibiot (Tokyo)* **2005**, *58*, 1-26.

(10) Dreyfuss, M. M.; Chapela, I. H. *Biotechnology* **1994**, *26*, 49-80.

(11) Kostopoulou, M.; Nikolaou, A. *Trends in Anal Chem* **2008**, *27*, 1023-1035.

(12) Keegan, J.; O´Leary, E. *Primary versus Secondary Metabolites of Microorganisms*, Retrieved December 2, 2012, from: http://www.dcu.ie/~oshead/BE401/lectures/pres4381fa2503270.pdf

(13) Frisvad, J. C.; Andersen, B.; Thrane, U. *Mycol Res* **2008**, *112*, 231-240.

(14) Demain, A. L. *Int Microbiol* **1998**, *1*, 259-264.

(15) Waksman, S. A.; A., L. H. *The actinomycetes*; The Williams and Wilkins: Baltimore, 1962.

(16) Behal, V. *Adv Appl Microbiol* **2000**, *47*, 113-156.

(17) Janata, J.; Najmanova, L.; Novotna, J.; Hola, K.; Felsberg, J.; Spizek, J. *Anton LeeuwInt J G* **2001**, *79*, 277-284.

(18) Nikolaou, A.; Meric, S.; Fatta, D. *Anal Bioanal Chem* **2007**, *387*, 1225-1234.

(19) Wright, G. D. *Chem Biol* **2000**, *7*, R127-132.

(20) Kasprzyk-Hordern, B.; Dinsdale, R. M.; Guwy, A. J. *J Chromatogr A* **2007**, *1161*, 132-145.

(21) Spížek, J. *Antibiotics* – university lecture at Charles University in Prague 2008.

(22) Chopra, I.; Hawkey, P. M.; Hinton, M. *J Antimicrob Chemother* **1992**, *29*, 245-277.

(23) Cooper, A. D.; Stubbings, G. W.; Kelly, M.; Tarbin, J. A.; Farrington, W. H.; Shearer, G. *J Chromatogr A* **1998**, *812*, 321-326.

(24) Debut, Y. *The Veterinary Formulary*; Pharmaceutical Press: London, 1988.

(25) State Institute for Drug Control, SUKL, Retrieved June 10, 2012, from: http://www.sukl.eu/sukl?lang=2.

(26) Science Prof Online. *How to Do a Gram Stain, Test for Gram+ and Gram− Bacteria Identification,* Retrieved at November 29, 2012, from: http://www.scienceprofonline.org/microbiology/gram-stain-test-for-gram-positive-gram-negative-bacteria-id.html.

(27) WHO. *Antimicrobial Resistance*, 2012, Retrieved November 15, 2012, from: http://www.who.int/mediacentre/factsheets/fs194/en/

(28) Davies, J. *Science* **1994**, *264*, 375-382.

(29) Davies, J.; Webb, V. *Emerging infections*; Academic Press: San Diego, 1998.

(30) Walsh, C. *Antibiotics: action, origins, resistance*; ASM Press: Washington, 2003.

(31) Dinh, Q. T.; Alliot, F.; Moreau-Guigon, E.; Eurin, J.; Chevreuil, M.; Labadie, P. *Talanta* **2001**, *85*, 1238-1245.

(32) Halling-Sorensen, B. *Arch Environ Contam Toxicol* **2001**, *40*, 451-460.

(33) Seifrtova, M.; Aufartova, J.; Vytlacilova, J.; Pena, A.; Solich, P.; Novakova, L. *J Sep Sci* **2010**, *33*, 2094-2108.

(34) Seifrtova, M.; Novakova, L.; Lino, C.; Pena, A.; Solich, P. *Anal Chim Acta* **2009**, *649*, 158-179.

(35) Thorsten, C.; Schneider, R. J.; Färber, H. A.; Skutlarek, D.; Meyer, M. T.; Goldbach, H. E. *Acta Hydrochim Hydrobiol* **2003**, *31*, 36-44.

(36) Schlusener, M. P.; Bester, K.; Spiteller, M. *Anal Bioanal Chem* **2003**, *375*, 942-947.

(37) European Commision. *Opinion of the scientific steering committee on antimicrobial resistence,* 1999, Retrieved September 12, 2012, from: http://ec.europa.eu/food/fs/sc/ssc/out50_en.pdf.

(38) Adriaenssens, N.; Coenen, S.; Versporten, A.; Muller, A.; Minalu, G.; Faes, C.; Vankerckhoven, V.; Aerts, M.; Hens, N.; Molenberghs, G.; Goossens, H. *J Antimicrob Chemother* **2011**, *66 Suppl 6*, vi37-45.

(39) Adriaenssens, N.; Coenen, S.; Versporten, A.; Muller, A.; Minalu, G.; Faes, C.; Vankerckhoven, V.; Aerts, M.; Hens, N.; Molenberghs, G.; Goossens, H. *J Antimicrob Chemother* **2011**, *66 Suppl 6*, vi3-12.

(40) Adriaenssens, N.; Coenen, S.; Versporten, A.; Muller, A.; Minalu, G.; Faes, C.; Vankerckhoven, V.; Aerts, M.; Hens, N.; Molenberghs, G.; Goossens, H. *J Antimicrob Chemother* **2011**, *66 Suppl 6*, vi47-56.

(41) Coenen, S.; Ferech, M.; Malhotra-Kumar, S.; Hendrickx, E.; Suetens, C.; Goossens, H. *J Antimicrob Chemother* **2006**, *58*, 418-422.

(42) Carballa, M.; Omil, F.; Lema, J. M.; Llompart, M.; Garcia-Jares, C.; Rodriguez, I.; Gomez, M.; Ternes, T. *Water Res* **2004**, *38*, 2918-2926.

(43) Roberts, P. H.; Thomas, K. V. *Sci Total Environ* **2006**, *356*, 143-153.

(44) Zuccato, E.; Castiglioni, S.; Fanelli, R. *J Hazard Mater* **2005**, *122*, 205-209.

(45) Gobel, A.; Thomsen, A.; McArdell, C. S.; Joss, A.; Giger, W. *Environ Sci Technol* **2005**, *39*, 3981-3989.

(46) Golet, E. M.; Xifra, I.; Siegrist, H.; Alder, A. C.; Giger, W. *Environ Sci Technol* **2003**, *37*, 3243-3249.

(47) Clara, M.; Strenn, B.; Gans, O.; Martinez, E.; Kreuzinger, N.; Kroiss, H. *Water Res* **2005**, *39*, 4797-4807.

(48) Lindberg, R. H.; Wennberg, P.; Johansson, M. I.; Tysklind, M.; Andersson, B. A. *Environ Sci Technol* **2005**, *39*, 3421-3429.

(49) Zorita, S.; Martensson, L.; Mathiasson, L. *Sci Total Environ* **2009**, *407*, 2760-2770.

(50) Gros, M.; Petrovic, M.; Barcelo, D. *Talanta* **2006**, *70*, 678-690.

(51) Leung, H. W.; Minh, T. B.; Murphy, M. B.; Lam, J. C.; So, M. K.; Martin, M.; Lam, P. K.; Richardson, B. J. *Environ Int* **2012**, *42*, 1-9.

(52) Menzella, H. G.; Reeves, C. D. *Curr Opin Microbiol* **2007**, *10*, 238-245.

(53) Coates, A. R.; Hu, Y. *Br J Pharmacol* **2007**, *152*, 1147-1154.

(54) Coates, A.; Hu, Y.; Bax, R.; Page, C. *Nat Rev Drug Discov* **2002**, *1*, 895-910.

(55) Coates, A. R.; Hu, Y. *Drugs R D* **2006**, *7*, 133-151.

(56) Borysowski, J.; Weber-Dabrowska, B.; Gorski, A. *Exp Biol Med (Maywood)* **2006**, *231*, 366-377.

(57) Fischetti, V. A.; Nelson, D.; Schuch, R. *Nat Biotechnol* **2006**, *24*, 1508-1511.

(58) Cragg, G. M.; Grothaus, P. G.; Newman, D. J. *Chem Rev* **2009**, *109*, 3012-3043.

(59) Kvalheim, O. M.; Chan, H. Y.; Benzie, I. F. F.; Szeto, Y. T.; Tzang, A. H. C.; Mok, D. K. W.; Chau, F. T. *Chemometr Intell Lab* **2011**, *107*, 98-105.

(60) Berretti, R. *Discovery and production of antibiotics*, Retrieved at December 2, 2011, from: http://phagetherapylightandshade.blogspot.com/2009/12/examininggenomes.html/

(61) Gloer, J. B. *The Mycota. Environmental and microbial relationships*; Springer-Verlag: New York, 1997.

(62) Hawksworth, D. L. *Mycol Res* **1991**, *95*, 641-655.

(63) Hawksworth, D. L. *Mycol Res* **2001**, *105*, 1422-1431.

(64) Keller, N. P.; Turner, G.; Bennett, J. W. *Nat Rev Microbiol* **2005**, *3*, 937-947.

(65) Raistrick, H. *Proc R Soc Lond B Biol Sci* **1950**, *136*, 481-508, 484 pl.

(66) Kolarik, M.; Freeland, E.; Utley, C.; Tisserat, N. *Mycologia* **2010**, *103*, 325-332.

(67) Kolarik, M.; Kirkendall, L. R. *Fungal Biol* **2010**, *114*, 676-689.

(68) Kolarik, M.; Kostovcik, M.; Pazoutova, S. *Mycol Res* **2007**, *111*, 1298-1310.

(69) Kolarik, M.; Kubatova, A.; Cepicka, I.; Pazoutovta, S.; Srutka, P. *Mycol Res* **2005**, *109*, 1323-1336.

(70) Kolarik, M.; Kubatova, A.; Hulcr, J.; Pazoutova, S. *Microb Ecol* **2008**, *55*, 65-80.

(71) Kolarik, M.; Kubatova, A.; Pazoutova, S.; Srutka, P. *Mycol Res* **2004**, *108*, 1053-1069.

(72) Stodulkova, E.; Kolarik, M.; Kresinova, Z.; Kuzma, M.; Sulc, M.; Man, P.; Novak, P.; Marsik, P.; Landa, P.; Olsovska, J.; Chudickova, M.; Pazoutova, S.; Cerny, J.; Bella, J.; Flieger, M. *Folia Microbiol* **2009**, *54*, 179-187.

(73) Fifield, F. W.; Kealey, D. *Principles and Practice of Analytical Chemistry*, 5th ed.; Blackwell Science: Oxford, 2000.

(74) Mitra, S. *Sample Preparation Techniques in Analytical Chemistry*; John Wiley & Sons, Inc.: Hoboken, 2003.

(75) Li, K. M.; Rivory, L. P.; Clarke, S. J. *Curr Pharm Anal* **2006**, *2*, 95-102.

(76) Claessens, H. A.; van Straten, M. A. *J Chromatogr A* **2004**, *1060*, 23-41.

(77) Sykora, D.; Tesarova, E.; Vosmanska, M.; Zvolankova, M. *Chem Listy* **2007**, *101*, 190-199.

(78) Nawrocki, J.; Dunlap, C.; McCormick, A.; Carr, P. W. *J Chromatogr A* **2004**, *1028*, 1-30.

(79) Snyder, L. R. *Abstr Pap Am Chem Soc* **1997**, *213*, 15-Anyl.

(80) Unger, K. K.; Becker, N.; Roumeliotis, P. *J Chromatogr* **1976**, *125*, 115-127.

(81) Gritti, F.; Perdu, C.; Guiochon, G. *J Chromatogr A* **2008**, *1180*, 73-89.

(82) Guillarme, D.; Russo, R.; Rudaz, S.; Bicchi, C.; Veuthey, J. L. *Curr Pharm Anal* **2007**, *3*, 221-229.

(83) Molikova, M.; Jandera, P. *J Sep Sci* **2010**, *33*, 453-463.

(84) Atia, N. N.; York, P.; Clark, B. J. *J Sep Sci* **2009**, *32*, 2732-2736.

(85) Novakova, L.; Matysova, L.; Solichova, D.; Koupparis, M. A.; Solich, P. *J Chromatogr B* **2004**, *813*, 191-197.

(86) Wang, Y.; Harrison, M.; Clark, B. J. *J Chromatogr A* **2006**, *1105*, 199-207.

(87) Gritti, F.; Guiochon, G. *J Chromatogr A* **2010**, *1217*, 1604-1615.

(88) Abrahim, A.; Al-Sayah, M.; Skrdla, P.; Bereznitski, Y.; Chen, Y.; Wu, N. *J Pharm Biomed Anal* **2010**, *51*, 131-137.

(89) Gritti, F.; Cavazzini, A.; Marchetti, N.; Guiochon, G. *J Chromatogr A* **2007**, *1157*, 289-303.

(90) Novakova, L.; Solichova, D.; Solich, P. *J Sep Sci* **2006**, *29*, 2433-2443.

(91) Minakuchi, H.; Nakanishi, K.; Soga, N.; Ishizuka, N.; Tanaka, N. *Anal Chem* **1996**, *68*, 3498-3501.

(92) Nguyen, D. T.; Guillarme, D.; Rudaz, S.; Veuthey, J. L. *J Chromatogr A* **2006**, *1128*, 105-113.

(93) Swartz, M. E. *J Liq Chromatogr Rel Tech* **2005**, *28*, 1253-1263.

(94) Cunliffe, J. M.; Maloney, T. D. *J Sep Sci* **2007**, *30*, 3104-3109.

(95) Destefano, J. J.; Langlois, T. J.; Kirkland, J. J. *J Chromatogr Sci* **2008**, *46*, 254-260.

(96) Wu, N. J.; Dempsey, J.; Yehl, P. M.; Dovletoglou, A.; Ellison, D.; Wyvratt, J. *Anal Chim Acta* **2004**, *523*, 149-156.

(97) Majors, R. E. *LC GC N Am* **2005**, *23*, 1248-1255.

(98) Bogusz, M. J. *Handbook of Analytical Separations*, 2nd ed.; Elsevier: Amsterdam, 2008.

(99) LC GC Chromacademy. *High efficiency HPLC separations*, 2010, Retrieved September 12, 2012 from: http://www.chromacademy.com/resolver-september2010_High_Efficiency_HPLC_Separations.asp

(100) Scott, R. P. W., Chromatography-online, 2011, Retrieved September 25, 2012, from: http://www.chromatography-online.org/5/contents.html/

(101) Hadacek, F. *Crit Rev Plant Sci* **2002**, *21*, 273-322.

(102) Cvacka, J. *Mass spectrometry and separation techniques* - university lecture at Charles University in Prague 2011.

(103) de Hoffmann, E.; Charette, J.; Stroobant, V. *Mass Spectrometry - Principles and Applications*, 2rd ed.; Wiley & Sons: Chichester, 2002.

(104) Peters, R. J.; Bolck, Y. J.; Rutgers, P.; Stolker, A. A.; Nielen, M. W. *J Chromatogr A* **2009**, *1216*, 8206-8216.

(105) Stolker, A. A. M.; Rutgers, P.; Oosterink, E.; Lasaroms, J. J. P.; Peters, R. J. B.; van Rhijn, J. A.; Nielen, M. W. F. *Anal Bioanal Chem* **2008**, *391*, 2309-2322.

(106) Nielen, M. W. F.; Lasaroms, J. J. P.; Essers, M. L.; Oosterink, J. E.; Meijer, T.; Sanders, M. B.; Zuidema, T.; Stolker, A. A. M. *Anal Bioanal Chem* **2008**, *391*, 199-210.

(107) IUPAC. *IUPAC Gold Book*, Retrieved September 5, 2012, available at: http://goldbook.iupac.org/M03759.html.

(108) Gosetti, F.; Mazzucco, E.; Zampieri, D.; Gennaro, M. C. *J Chromatogr A* **2010**, *1217*, 3929-3937.

(109) Kasprzyk-Hordern, B.; Dinsdale, R. M.; Guwy, A. J. *Talanta* **2008**, *74*, 1299-1312.

(110) Benijts, T.; Dams, R.; Lambert, W.; De Leenheer, A. *J Chromatogr A* **2004**, *1029*, 153-159.

(111) Jahnke, A.; Gandrass, J.; Ruck, W. *J Chromatogr A* **2004**, *1035*, 115-122.

(112) Zrostlikova, J.; Hajslova, J.; Poustka, J.; Begany, P. *J Chromatogr A* **2002**, *973*, 13-26.

(113) Stuber, M.; Reemtsma, T. *Anal Bioanal Chem* **2004**, *378*, 910-916.

(114) Guo, S.; Duan, J. A.; Tang, Y. P.; Su, S. L.; Shang, E. X.; Ni, S. M.; Qian, D. W. J *Pharm Biomed Anal* **2009**, *49*, 1296-1302.

(115) Japon-Lujan, R.; Ruiz-Jimenez, J.; de Castro, M. D. *J Agric Food Chem* **2006**, *54*, 9706-9712.

(116) Ni, Y.; Peng, Y.; Kokot, S. *Chromatographia* **2008**, *67*, 211-217.

(117) Ni, Y. N.; Lai, Y. H.; Brandes, S.; Kokot, S. *Anal Chim Acta* **2009**, *647*, 149-158.

(118) Yan, S. K.; Xin, W. F.; Luo, G. A.; Wang, Y. M.; Cheng, Y. Y. *J Chromatogr A* **2005**, *1090*, 90-97.

(119) Kamenik, Z.; Hadacek, F.; Mareckova, M.; Ulanova, D.; Kopecky, J.; Chobot, V.; Plhackova, K.; Olsovska, J. *j Chromatogr A* **2010**, *1217*, 8016-8025.

(120) Hajimahmoodi, M.; Vander Heyden, Y.; Sadeghi, N.; Jannat, B.; Oveisi, M. R.; Shahbazian, S. *Talanta* **2005**, *66*, 1108-1116.

(121) Atherton, T.; Croxton, R.; Baron, M.; Gonzalez-Rodriguez, J.; Gamiz-Gracia, L.; Garcia-Campana, A. M. *J Sep Sci* **2012**, *35*, 2994-2999.

(122) Gu, M.; Su, Z. G.; Fan, O. Y. *J Liq Chromatogr Rel Tech* **2006**, *29*, 1503-1514.

(123) Bendahl, L.; Sturup, S.; Gammelgaard, B.; Hansen, S. H. *J Anal At Spectrom* **2005**, *20*, 1287-1289.

(124) Bauer, A. W.; Kirby, W. M. M.; Sherris, J. C.; Turck, M. *Am J Clin Path* **1966**, *45*, 493-&.

(125) Brock, T. D.; Madigan, M. T.; Martinko, J. M.; Parker, J. *Biology of Microorganisms*, 7th ed.; Prentice Hall: New Persey, 1994.

(126) Phillipson, D. W.; Milgram, K. E.; Yanovsky, A. I.; Rusnak, L. S.; Haggerty, D. A.; Farrell, W. P.; Greig, M. J.; Xiong, X.; Proefke, M. L. *J Comb Chem* **2002**, *4*, 591-599.

(127) Si, W.; Gong, J.; Tsao, R.; Kalab, M.; Yang, R.; Yin, Y. *J Chromatogr A* **2006**, *1125*, 204-210.

(128) Tylova, T.; Olsovska, J.; Novak, P.; Flieger, M. *Chemosphere* **2010**, *78*, 353-359.

(129) Tylova, T.; Kolarik, M.; Olsovska, J. *Anal Bioanal Chem* **2011**, *400*, 2943-2952.

(130) Williams, J. P.; Lock, R.; Patel, V. J.; Scrivens, J. H. *Anal Chem* **2006**, *78*, 7440-7445.

(131) Carrasco-Pancorbo, A.; Casado-Terrones, S.; Segura-Carretero, A.; Fernandez-Gutierrez, A. *J Chromatogr A* **2008**, *1195*, 107-116.

(132) Peters, R. J. B.; Bolck, Y. J. C.; Rutgers, P.; Stolker, A. A. M.; Nielen, M. W. F. *J Chromatogr A* **2009**, *1216*, 8206-8216.

(133) Tylova, T.; Kamenik, Z.; Flieger, M.; Olsovska, J. *Chromatographia* **2011**, *74*, 19-27.

(134) Hamscher, G.; Sczesny, S.; Hoper, H.; Nau, H. *Anal Chem* **2002**, *74*, 1509-1518.

(135) Zhou, J. L.; Maskaoui, K.; Lafadeju, A. *Anal Chim Acta* **2012**, *731*, 32-39.

(136) Ferrer, I.; Zweigenbaum, J. A.; Thurman, E. M. *J Chromatogr A* **2010**, *1217*, 5674-5686.

(137) Locatelli, M. A.; Sodre, F. F.; Jardim, W. F. *Arch Environ Contam Toxicol* **2011**, *60*, 385-393.

(138) Tong, L.; Li, P.; Wang, Y.; Zhu, K. *Chemosphere* **2009**, *74*, 1090-1097.

(149) Wei, R.; Ge, F.; Huang, S.; Chen, M.; Wang, R. *Chemosphere* **2011**, *82*, 1408-1414.

(140) Li, B.; Zhang, T.; Xu, Z.; Fang, H. H. *Anal Chim Acta* **2009**, *645*, 64-72.

(141) Chen, Y.; Bicker, W.; Wu, J. Y.; Xie, M. Y.; Lindner, W. *J Chromatogr A* **2010**, *1217*, 1255-1265.

(142) Chen, Y.; Zhu, S. B.; Xie, M. Y.; Nie, S. P.; Liu, W.; Li, C.; Gong, X. F.; Wang, Y. X. *Anal Chim Acta* **2008**, *623*, 146-156.

(143) Schmeda-Hirschmann, G.; Hormazabal, E.; Astudillo, L.; Rodriguez, J.; Theoduloz, C. *World J Microb Biot* **2005**, *21*, 27-32.

(144) Stadler, M.; Ju, Y. M.; Rogers, J. D. *Mycol Res* **2004**, *108*, 239-256.

(145) PAST software, Retrieved January 2, 2013, from: http://folk.uio.no/ohammer/past/

(146) Andersen, B.; Nielsen, K. F.; Thrane, U.; Szaro, T.; Taylor, J. W.; Jarvis, B. B. *Mycologia* **2003**, *95*, 1227-1238.

# SUPPLEMENTARY DATA

**Table 4. Overview and characterization of 48 genus *Geosmithia* species that were analyzed using developed chromatographic fingerprinting method.**

| strain No. | species identification | reference |
|---|---|---|
| CCF3754 | *G. eupagioceri* | [67] |
| CCF3334 | *G. fassatiae* | [69] |
| CCF3333 | *G. flava* | [71] |
| MK1645 | *G. langdonii* | [70] |
| MK1683 | *G. langdonii* | [69] |
| CCM8366 | *G. lavendula* | [68] |
| 1212 | *G. lavendula* | [68] |
| CCF3861 | *G. microcorthyli* | [67] |
| 1259 | *G. morbida* | [66] |
| 1272 | *G. morbida* | [66] |
| CCF3425 | *G. obscura* | [69] |
| MK1510 | *G. pallida* (sp. 2) | [70] |
| CCF4215 | *G. pallida* (sp. 5) | unpublished |
| MK980 | *G. pallida* (sp. 5) | [70] |
| MK1807 | *G. pallida* (sp. 23) | unpublished |
| CCF3342 | *G. putterillii* | [70] |
| CCF3751 | *G. rufescens* | [67] |
| CCF3660 | *Geosmithia* sp. 1 | [70] |
| CCF4207 | *Geosmithia* (sp. 8) | unpublished |
| MK1712a | *Geosmithia* (sp. 8) | [70] |
| MK1713 | *Geosmithia* (sp. 8) | [70] |
| MK263 | *Geosmithia* (sp. 8) | [70] |
| CCF3350 | *Geosmithia* (sp. 8) | [70] |
| RJ0258 | *Geosmithia* (sp. 9) | [70] |
| CCF4213 | *Geosmithia* (sp. 9) | [70] |
| CCF3702 | *Geosmithia* (sp. 9) | unpublished |
| CCF3560 | *Geosmithia* (sp. 10) | [70] |
| CCF3555 | *Geosmithia* (sp. 11) | [70] |
| MK555 | *Geosmithia* (sp. 12) | [70] |
| U16a26 | *Geosmithia* (sp. 13) | [70] |
| MK1665 | *Geosmithia* (sp. 21) | [68] |
| MK1759 | *Geosmithia* (sp. 21) | [68] |
| CCF3645 | *Geosmithia* (sp. 22) | [68] |
| RJ06ka | *Geosmithia* (sp. 24) | unpublished |
| MK1842 | *Geosmithia* (sp. 24) | unpublished |
| CCF4222 | *Geosmithia* (sp. 24) | unpublished |
| CCF4205 | *Geosmithia* (sp. 26) | unpublished |
| CCF4223 | *Geosmithia* (sp. 26) | unpublished |
| CCF4206 | *Geosmithia* (sp. 27) | unpublished |
| CCF4292 | *Geosmithia* (sp. 29) | [67] |
| CCF4221 | *Geosmithia* (sp. 29) | unpublished |
| CCF4197 | *Geosmithia* (sp. 31) | unpublished |
| CCF4218 | *Geosmithia* (sp. 31) | unpublished |
| MK1811a | *Geosmithia* (sp. 31) | unpublished |
| RJ74k | *Geosmithia* (sp. 31) | unpublished |
| MK1826 | *Geosmithia* (sp. 32) | unpublished |
| MK1834 | *Geosmithia* (sp. 32) | unpublished |
| MK1811 | *Geosmithia* (sp. 36) | unpublished |

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## APPENDICES (PAPERS 1-4)

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# High-throughput analysis of tetracycline antibiotics and their epimers in liquid hog manure using Ultra Performance Liquid Chromatography with UV detection

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ABSTRACT

Antibiotics contained in animal manure can contaminate soil, groundwater and eventually surface and drinking water. To reduce the usage of antibiotics in livestock industry the EU banned their application as growth promoters in 2006. Even though the antibiotics are still used for this purpose and therefore it is necessary to control their applications.

An Ultra Performance Liquid Chromatography method (UPLC) with UV detection for determination of tetracycline (TC), oxytetracycline (OTC), chlortetracycline (CTC), and doxycycline (DOX) including their epimers in the liquid hog manure was developed. The antibiotics were extracted with ethyl acetate and separated on UPLC BEH Shield RP18 column. The validated method was selective for all analytes and system suitability was assessed. Calibration curves ranged from 7.8 to 250.0 μg $mL^{-1}$ with determination coefficient of 0.9999. The method limits of quantification ranged from 0.9 to 1.6 mg $kg^{-1}$. Recoveries were 52.4 ± 3.8%, 72.4 ± 5.0%, 83.8 ± 5.7% and 95.9 ± 4.7% for TC, OTC, CT, and DOX, respectively. The method was used for the determination of TC, OTC, CT, and DOX in liquid hog manure samples.



## 1. Introduction

Antibiotics represent a large group of pharmaceuticals with still growing consumption in both human and veterinary medicine (McEvoy et al., 2004). There are three significant field of application of antibiotics for animal husbandry: treatment of infection in livestock, prevention of infection and growth promoters (Schlüsener et al., 2003). About 50–90% of the administered pharmaceutical dose is excreted rapidly after the treatment (Kroker, 1983). Little is known about the behavior, concentration and the fate of antibiotics in manure and soil (Nowara et al., 1997; Haller et al., 2002; Schlüsener et al., 2003). Excreted antibiotics may be mobile in soil and could be transported to the ground and surface water and consequently to the drinking water (Schlüsener et al., 2003; Thorsten et al., 2003). Regarding former studies, the presence of antibiotics in the environment can cause the development of antibiotics-resistant bacteria and can have an adverse effect on the water environment and animals (Lu et al., 2004). Their potential presence in source drinking water could have unknown health effect on humans and animals due to the chronic low-level exposure to these substances over the lifetime (Batt and Aga, 2005).

The most widely used groups of antibiotics in the European Union's animal husbandry are tetracyclines, macrolides, penicillines, aminoglycosides and sulfonamides (European Commision, 1999).

Tetracyclines both natural and semisynthetic form a large group of products produced mainly by *Streptomyces* spp. They have a broad-spectrum of activities including inhibition of many common Gram-positive and Gram-negative bacteria, chlamydia, rickettsie, etc.; they are distinguished mainly for bacteriostatic action caused by inhibition of protheosynthesis (Cooper et al., 1998; Debut, 1988).

Extraction procedures and chromatographic methods for determination of tetracyclines (oxytetracycline, tetracycline, chlortetracycline, and doxycycline) in liquid manure have been previously published. For extraction of tetracyclines liquid–liquid extraction (LLE) using various extraction solution and pressurized-liquid extraction (PLE) as well as solid-phase extraction (SPE) were described. Extraction solutions for LLE were, e.g. ethyl acetate (Hamscher et al., 2002), citric buffer (Kühne et al., 2000), mixture of acidified ACN and EDTA-McIlvaine buffer (Hu et al., 2008; Martínez-Carballo et al., 2007) mixture of citric acid, oxalic acid, methanol and water (Wang and Yates, 2008) or buffered methanol–water mixture (Aust et al., 2008). For PLE citric acid followed by mixture of methanol, water and citric acid (Jacobsen and Halling-Sørensen, 2006) was used as the extraction solution. SPE was developed either as the only extraction step (Thorsten et al., 2003; Kemper et al., 2008) or as the clean-up and pre-concentration step after LLE (Jacobsen and Halling-Sørensen, 2006; Martínez-Carballo et al., 2007; Aust et al., 2008; Hu et al., 2008).

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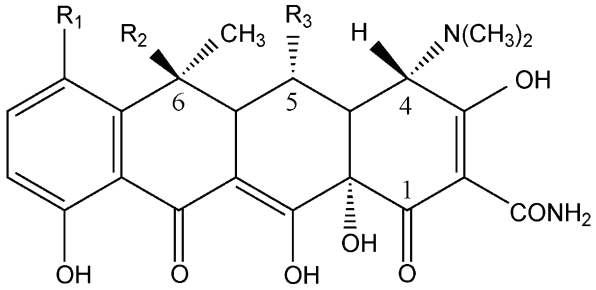


**Fig. 1.** Structures of tetracyclines.

| | $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|
| TC | H | OH | H |
| OTC | H | OH | OH |
| CTC | Cl | OH | H |
| DOX | H | H | OH |

Determination of tetracyclines was performed on HPLC-UV (Hu et al., 2008; Kühne et al., 2000; Thorsten et al., 2003; Wang and Yates, 2008) or on HPLC–MS/MS (Aust et al., 2008; Hamscher et al., 2002; Jacobsen and Halling-Sørensen, 2006; Kemper et al., 2008; Martínez-Carballo et al., 2007) system.

To date no studies about determination of tetracyclines in any matrix using Ultra Performed Liquid Chromatography (UPLC) has been published. UPLC is a modern separation technique providing considerable high-throughput analysis compared to HPLC. UPLC allows separations on column materials at high pressures up to 100 MPa using sub-2 μm particles, which yields significantly higher separation efficiencies and shorter run times compared to HPLC technique (Bendahl et al., 2005).

In this study, the validated UPLC method with LLE pre-concentration for analysis of tetracycline antibiotics, i.e. tetracycline (TC), oxytetracycline (OTC), chlortetracycline (CTC), doxycycline (DOX) and their epimers ($TC_{ep}$, $CTC_{ep}$, and $DOX_{ep}$) in liquid manure is described (for structures see Fig. 1). Because both the LLE procedure and UPLC analysis were performed under acidic conditions, the formation of the epimers was considered (Cooper et al., 1998; Halling-Sørensen et al., 2002; Hamscher et al., 2002; Skúlason et al., 2003). The developed method was applied for analysis of liquid hog manure samples from five different localities in the Czech Republic.

## 2. Experimental

### 2.1. Chemicals, reagents, and glassware

TC was purchased from Spofa (Prague, Czech Republic), OTC from VUAB (Roztoky u Prahy, Czech Republic), CTC from Sigma–Aldrich (Steinheim, Germany) and DOX from Calbiochem (San Diego, USA). The acetonitril (ACN) used as the chromatographic mobile phase was LC/MS grade and was obtained from Chromservis (Prague, Czech Republic). Methanol HPLC grade used for standards and manure samples preparation was purchased from Merck (Darmstadt, Germany). Ethyl acetate p.a. and citric acid were purchased from Lach-Ner (Neratovice, Czech Republic). Acetic acid and $Na_2EDTA$ were purchased from Sigma–Aldrich (Steinheim, Germany).

To avoid formation of complexes of tetracyclines with metal ions, proteins, and silanol groups (Thorsten et al., 2003; Batt and Aga, 2005) all glassware used was rinsed with saturated solution of $Na_2EDTA$ in methanol–water (50:50, v/v) and air-dried before use.

### 2.2. Standards and samples preparation

#### 2.2.1. Stock solution

Individual antibiotic standards (1 mg) were diluted in 1 mL of methanol–acetic acid (99:1, v/v) and stored at −20 °C. Stock mixture solution was prepared by mixing of equal volumes of TC, OTC, CTC, and DOX standard solutions to final concentration of 250.0 μg $mL^{-1}$ and were stored at −20 °C for maximum of 14 d (see Section 3.3.6).

#### 2.2.2. Manure sampling and pretreatment

Liquid manure samples were collected (1 L amber glass bottles) in five different animal farming areas (one sample from each area) in the Czech Republic in autumn 2008 and stored at −20 °C before use.

#### 2.2.3. Spiked matrix preparation

Liquid manure (5 g) free of targeted antibiotics (blank) was spiked with stock solution (0.5 mL) to required concentration for validation tests (see Section 2.6) and kept at 4 °C for period of 3 d prior to analysis (simulation of natural conditions).

### 2.3. Liquid–liquid extraction

LLE procedure was similar to previously published one (Cooper et al., 1998; Hamscher et al., 2002). Briefly, 6 mL of citrate buffer (pH 4.7) was vortexed intensively with 5 g of liquid manure sample. Then 1 mL of $Na_2EDTA$ solution (5 mg $mL^{-1}$) was added and vortexed intensively again for 1 min. After that 35 mL of ethyl acetate was added, vortexed intensively for 1 min, slightly for 15 min and centrifuged. The organic phase was separated and the procedure was repeated with another 1 mL of $Na_2EDTA$ solution and 35 mL of ethyl acetate. The organic phase was evaporated to dryness and reconstituted in 0.5 mL of methanol–acetic acid (99:1, v/v).

### 2.4. UPLC

UPLC analysis with UV detection was carried out on a Waters Acquity UPLC System ("W", Czech Republic, Prague) consisted of Acquity UPLC Solvent Manager, Acquity UPLC Sample Manager, Acquity UPLC Column Heater/cooler, Acquity UPLC Diode Array Detector (PDA, set at 200–400 nm, 350 nm used for chromatogram extraction). Empower 2 software was used for data processing. The analyses were performed on Acquity UPLC BEH Shield RP18 column (50 × 2.1 mm i.d.; particle size, 1.7 μm; Waters); flow rate, 0.4 mL $min^{-1}$; column temperature, 22 °C. The temperature of the sample manager was set to 10 °C for analysis of standards and 22 °C for manure samples to avoid matrix precipitation (for details see Section 3.2.2). The mobile phase A, formic acid–water (0.1:99.9, v/v); B, ACN; gradient elution (min/%A): 0/95, 2.3/92, 2.8/80, 4.0/75, followed by 1.5 min equilibration step (total run time 5.5 min). For analysis of manure samples the gradient procedure was supplemented with 1.0 min wash step with 100% ACN.

### 2.5. Off-line MS detection

UPLC fractions evaporated to dryness and reconstituted in methanol–water–formic acid (50:50:0.1, v/v/v) were analyzed by direct infusion on an APEX-Ultra FTMS instrument equipped with a 9.4 T superconducting magnet and an Dual II electrospray ionization (ESI) ion source (Bruker Daltonics, Billerica, MA). The cell was

opened for 1.3 ms, accumulation time was set at 0.2 s, and one experiment was collected for each sample where one experiment consists of the average of four spectra. The acquisition data set size was set to 1 M points with the mass range starting at m/z 150 a.m.u., resulting in a resolution of 100 000 at *m*/*z* 400. The instrument was externally calibrated using clusters of arginine resulting in mass accuracy below 1 ppm. The acquired spectra were apodized with a square sine bell function and Fourier transformed with one zero-fill. The interpretation of mass spectra was done using Data Analysis version 4.0 software package (Bruker Daltonics, Billerica, MA).

### 2.6. Method validation

#### 2.6.1. Selectivity

Selectivity of the analytical method was verified by comparison of chromatograms of blank liquid manure, standard mixture (8.0 μg $mL^{-1}$), and spiked (2.0 mg $kg^{-1}$) liquid manure (see Section 2.2.3) samples (CDER/CBER, 2001).

#### 2.6.2. Calibration curves

Calibration curves over linear range from 7.8 to 250.0 μg $mL^{-1}$ for individual antibiotic standards were determined. Stock standard solution was diluted to required concentration with methanol-acetic acid (99:1, v/v). Three sets of calibration standards were prepared; each point of calibration curve represents arithmetic means of three values.

#### 2.6.3. ILOQ and MLOQ

Instrument limit of quantification (ILOQ) was determined as the lowest point of the calibration curves with a precision (expressed as RSD %) less than 20% and accuracy of 80–120% in six replicates.

Method limit of quantification (MLOQ) was determined as the lowest amount of tetracyclines that can be quantified in extracted sample of liquid hog manure after LLE with respect to ILOQ, enrichment factor, and recovery of each target analyte. MLOQ was expressed in mg $kg^{-1}$ units (CDER/CBER, 2001).

#### 2.6.4. Accuracy and precision

To evaluate the accuracy and precision of the assay, quality control samples were prepared at concentrations of 250.0, 60.0 and 8.0 μg $mL^{-1}$ for each standard. For accuracy and precision, six replicates of quality control samples at each concentration were assayed. Relative standard deviation (RSD %) was taken as a measure of precision, and the percentage difference between determined and expected amounts was considered a measure of accuracy (CDER/CBER, 2001).

#### 2.6.5. Recovery

The recoveries were determined at two different concentration levels, 25.0 and 2.0 mg $kg^{-1}$, and calculated by comparing the peak areas of the analytes in the extract from the spiked liquid hog manure and their standard solutions in methanol–acetic acid (99:1, v/v) (four replicates for each concentration) (CDER/CBER, 2001).

#### 2.6.6. Stability

Stock solutions and spiked liquid manure samples extracted by LLE method were tested for short and long term stability. Samples were divided into required number of parts, stored at room temperature (RT, short term stability) or at −20 °C (long term stability) and analyzed after 0, 2, 4, 6, 8, 12, and 24 h, or 0, 1, 2, 3, 4, 7, and 14 d, respectively. Two concentration levels, 10.0 and 125.0 μg $mL^{-1}$ (standard mixture) and 2.0 and 25.0 mg $kg^{-1}$ (liquid manure) were tested. Each sample was analyzed in triplicate (CDER/CBER, 2001).

## 3. Result and discussion

### 3.1. Liquid–liquid extraction

Slight modifications of previously published methods (Cooper et al., 1998; Hamscher et al., 2002) were done to achieve a greater enrichment factor for tested compounds. Namely, the volumes of sample, citrate buffer and ethyl acetate were increased; $Na_2EDTA$ solution was added directly to the extraction media to suppress the formation of antibiotic complexes. Further, the solubility of dry extracts obtained from LLE was tested in three solutions, i.e. 10% methanol in water, 100% methanol, and methanol-acetic acid (99:1, v/v). The highest solubility was achieved in methanol–acetic acid solution (data not shown).

### 3.2. UPLC

#### 3.2.1. Detection

Tetracyclines have a very characteristic UV spectrum with three maxima, TC (221, 271, 258 nm), CTC (233, 270, 370 nm), OTC (219,

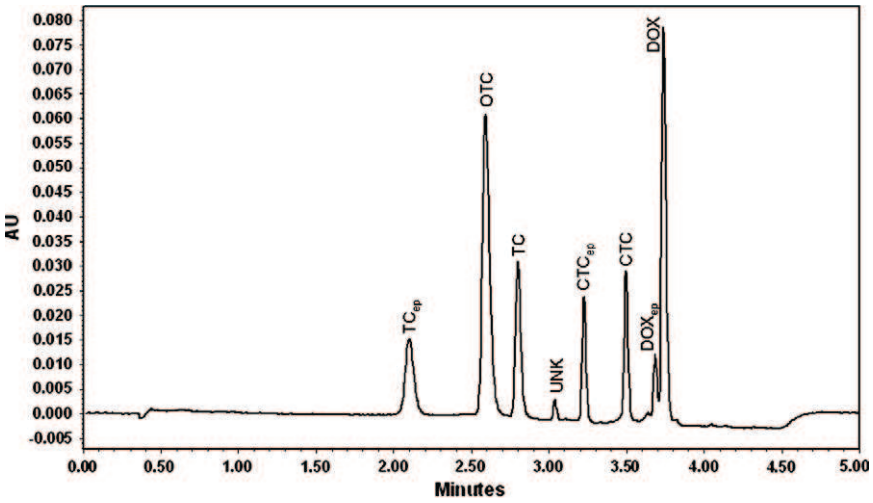


**Fig. 2.** UPLC separation of antibiotic standard mixture including epimers. Chromatographic conditions: acquity UPLC BEH Shield RP18 column (50 × 2.1 mm i.d., 1.7 μm); column temperature, 22 °C; injection volume, 1 μL; flow rate, 0.4 mL $min^{-1}$; mobile phase, formic acid–water (0.1:99.9, v/v) (A) and acetonitrile (B); gradient elution (min/%A): 0/95, 2.3/92, 2.8/80, 4.0/75; UV 350 nm.

270, 355 nm), DOX (220, 278, 351). Therefore 350 nm was used for both identification and quantification of antibiotics thorough this study. Furthermore, this wavelength proved to be advantage due to the fact, that no interferences of a very complex matrix were observed.

### 3.2.2. Method development

Two analytical UPLC columns (Acquity UPLC BEH C18 and Acquity UPLC BEH Shield RP18) in three mobile phases differing in strength of organic acid (acetic acid, trifluoroacetic acid, formic acid, all 0.1%, v/v in water) and two mobile phases differing in pH of ammonium acetate buffer solution (1.0 mM; pH 4.0, 9.0) were tested during the procedure development. In all cases the acetonitrile was used as an organic modifier. The best separation was achieved on Acquity UPLC BEH Shield RP18 column with gradient elution in mobile phase A, formic acid–water (0.1:99.9, v/v) and B, ACN. The gradient elution was further optimized for analysis of liquid manure samples where washing step (100% ACN, 1 min) was added to remove all matrix content from the column.

UPLC analysis of standard mixture including epimers (TC, $TC_{ep}$, OTC, CTC, $CTC_{ep}$, DOX, and $DOX_{ep}$) is shown in Fig. 2. The baseline separation of all compounds was achieved with the exception of DOX and $DOX_{ep}$ where slight overlay could be observed. Unknown peak (UNK) originated from OTC standard. The identities of all peaks were confirmed by off-line MS analysis. Retention and separation parameters, i.e. peak symmetry, resolution, and efficiency (expressed as EP plate count) are summarized in Table 1.

The temperature of autosampler was set to 22 °C due the manure sample matrix precipitation at lower temperature. This was observed in repeated analysis of one among the five manure samples where the peak areas of target analytes decreased rapidly with decreasing temperature of the sample. While the peak areas at 22 °C were 100%, the areas at 5 °C were about 3%. This event was not observed with standard solutions.

## 3.3. Method validation

### 3.3.1. Selectivity

The developed method shows a very good selectivity for all studied compounds which is demonstrated in Fig. 3. No interfering components of sample matrix were detected by UV detection under chromatographic conditions used.

### 3.3.2. Calibration curves

The calibration curves of tetracyclines were prepared over a linear range from 7.8 to 250.0 μg $mL^{-1}$ at six concentration levels, 7.8, 15.6, 31.3, 62.5, 125.0 and 250.0 μg $mL^{-1}$. Samples were prepared from the stock solution by subsequent dilution. The analytes were quantified as a sum of peak areas of each antibiotic and its corresponding epimer. The resulting regression equations were: TC (TC + $TC_{ep}$), $y = 3.2 \times 10^3 x - 8.4 \times 10^2$; OTC, $y = 3.2 \times 10^3 x + 8.1 \times 10^2$; CTC (CTC + $CTC_{ep}$), $y = 2.7 \times 10^3 x - 2.6 \times 10^3$; and DOX (DOX + $DOX_{ep}$), $y = 3.8 \times 10^3 x - 3.9 \times 10^3$. Determination coefficients for all regressions reached the value of 0.9999.

### 3.3.3. ILOQ and MLOQ

ILOQ was determined as 8.0 μg $mL^{-1}$ with following values of accuracy: TC, 99.2 ± 0.4% and RSD 3.5%; OTC, 97.7 ± 0.1% and RSD

**Table 1**
Retention characteristics and system suitability of UPLC method for determination of tetracyclines. Chromatographic conditions: acquity UPLC BEH Shield RP18 column (50 × 2.1 mm i.d., 1.7 μm); column temperature, 22 °C; injection volume, 1 μL; flow rate, 0.4 mL $min^{-1}$; mobile phase, formic acid–water (0.1:99.9, v/v) (A) and acetonitrile (B); gradient elution (min/%A): 0/95, 2.3/92, 2.8/80, 4.0/75; UV 350 nm.

| Analyte | RT (min) | Resolution | EP plate count | Symmetry factor | RT repeatability (RSD %) | Area repeatability (RSD %) |
|---|---|---|---|---|---|---|
| $TC_{ep}$ | 2.05 | – | 5451 | 1.03 | 0.1 | 3.2 |
| OTC | 2.59 | 5.15 | 11 442 | 1.14 | 0.1 | 1.5 |
| TC | 2.86 | 2.79 | 12 023 | 1.07 | 0.1 | 2.6 |
| UNK | 3.19 | 4.87 | – | – | 0.1 | – |
| $CTC_{ep}$ | 3.27 | 2.3 | 130 226 | 1.00 | 0.0 | 7.8 |
| CTC | 3.47 | 5.42 | 132 101 | 1.06 | 0.1 | 2.6 |
| $DOX_{ep}$ | 3.60 | 2.00 | 120 722 | 0.98 | 0.1 | 0.7 |
| DOX | 3.68 | 3.23 | 114 336 | 1.19 | 0.1 | 2.5 |

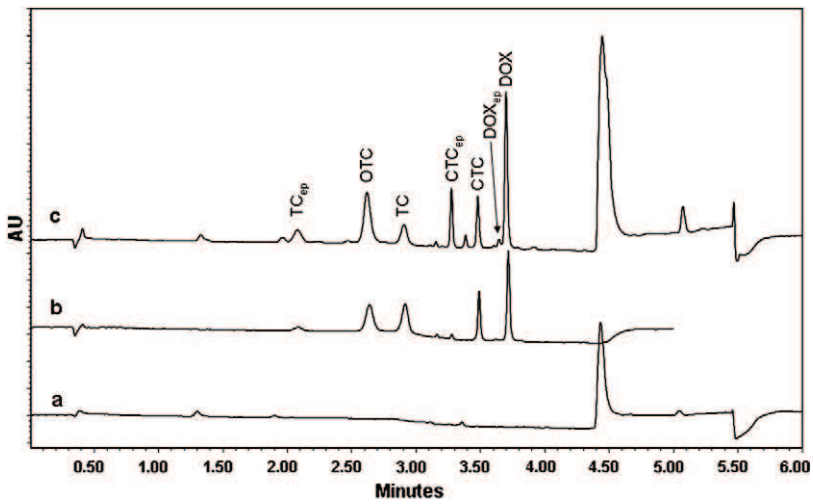


**Fig. 3.** The UPLC chromatograms overlay of blank liquid manure (a), standard mixture at concentration of 8.0 μg $mL^{-1}$ (b) and spiked liquid manure at concentration of 2.0 mg $kg^{-1}$ (c). For chromatographic conditions, see Fig. 2.

1.3%; CTC, 103.6 ± 0.6% and RSD 4.7%; DOX, 102.1 ± 0.1% and RSD 1.5%.

The determined MLOQ values were as follows: TC, 1.6 mg $kg^{-1}$; OTC, 1.2 mg $kg^{-1}$; CTC, 1.0 mg $kg^{-1}$; DOX, 0.9 mg $kg^{-1}$ in the liquid hog manure (all analyses were in quadruplicate).

### 3.3.4. Accuracy and precision

Results of accuracy and precision are summarized in Table 2. Results confirm that developed analytical method is accurate and precise.

### 3.3.5. Recovery

The individual antibiotic recovery values of the assay were 52.4 ± 3.8% (TC), 72.4 ± 5.0% (OTC), 83.8 ± 5.7% (CTC), and 95.9 ± 4.7% (DOX) at all concentrations studied. Detailed results of recovery determination are shown in Table 2. Lower recoveries of antibiotics from liquid manure are caused by the complexity of the matrix.

### 3.3.6. Stability

The stabilities of TC, OTC, CTC, and DOX during the sample storage and processing procedure under conditions described above (see Section 2.6.6) were evaluated. No significant degradations were found (data not shown). Total content of individual antibiotics was calculated as a sum of the peak areas of each target compound and its corresponding epimer. The epimerization process at −20 °C was not observed and the content of epimers (resulted from sample handling) remains constant during the stability test (14 d). On the other hand, the epimerization was much faster when samples were stored at RT and data are summarized in Table 3. TC and CTC reach the equilibrium (epimer ratio was about 1:1) after 12 h. Both TC and CTC form epimers on C(4) (Cooper et al., 1998; Halling-Sørensen et al., 2002). The epimerization of DOX was slower than that of TC and CTC. The content of $DOX_{ep}$ reached about 15% within 24 h. This could result from possible stabilization of the structure by formation of intramolecular hydrogen bond between H of hydroxyl group on C(5) (proton donor) and disubstituted N (proton acceptor) on C(4), i.e. (–C(5)–O–H ← $N(CH_3)_2$–C(4)–) (see Fig. 1). However, DOX was known to form epimer also on C(6) (Skúlason et al., 2003), which was probably the epimer found under condition used. No epimerization was found for OTC, which structure could be again stabilized by hydrogen bond formation as in the case of DOX.

**Table 3**
Content of TC, CTC, and DOX epimers (rel %) in standard mixture of antibiotics stored at room temperature; solvent, methanol–acetic acid (99:1, v/v).

| Time (h) | Analyte | | |
|---|---|---|---|
| | $TC_{ep}$ (%)[a] | $CTC_{ep}$ (%)[b] | $DOX_{ep}$ (%)[b] |
| 0 | 11 | 9 | 1 |
| 6 | 44 | 42 | 4 |
| 12 | 49 | 47 | 5 |
| 24 | 52 | 48 | 14 |

[a] $TCep(\%) = \frac{AreaTCep}{AreaTCep+AreaTC} \cdot 100$.
[b] Same equation for $CTC_{ep}$ (%) and $DOX_{ep}$ (%).

### 3.4. HPLC and UPLC method comparison

In comparison with formerly developed HPLC methods (Hamscher et al., 2002; Thorsten et al., 2003; Jacobsen and Halling-Sørensen, 2006; Hu et al., 2008) the most significant advantage of the new UPLC method is run time reduction. While the analysis time of HPLC methods were 25 min (Hamscher et al., 2002; Jacobsen and Halling-Sørensen, 2006; Hu et al., 2008), and 45 min (Thorsten et al., 2003), a total analysis time of the presented UPLC method is 6.5 min. This value assures high-throughput analysis, which is essential for residual antibiotic monitoring.

The high selectivity of the sub-2 μm particles chromatographic column used in UPLC method allows complete (baseline) separation of all tetracyclines and their epimers formed during extraction under acidic conditions. This fact substantially increases the accuracy and reproducibility of the described method. It is evident from our results that the tetracycline antibiotic epimerization significantly affects final quantification (Section 3.3.6.).

In general the determined MLOQs of our UPLC procedure are about the same as previously described for other HPLC methods, i.e. 1.0–2.0 mg $kg^{-1}$.

### 3.5. Off-line MS antibiotic detection

Tetracyclines and their epimers structures were confirmed by off-line MS detection after isolation of appropriate peaks during UPLC analysis. This determination was proved in both standard mixture and positive liquid hog manure sample. In the HR-MS spectra of the standard tetracyclines samples, the peaks of *m/z* 445.16047 ($\Delta$ = 0.2 ppm), 445.16055 ($\Delta$ = 0.0 ppm), 461.15600 ($\Delta$ = −0.2 ppm), 479.12181 ($\Delta$ = −0.7 ppm), 479.12147 ($\Delta$ = 0.2 ppm), and 445.16054 ($\Delta$ = 0.0 ppm) corresponding to the pseudomolecular ions $[C_{22}H_{25}N_2O_8]^+$ (TC), $[C_{22}H_{25}N_2O_8]^+$ ($TC_{ep}$), $[C_{22}H_{25}N_2O_9]^+$ (OTC), $[C_{22}H_{24}ClN_2O_8]^+$ (CTC), $[C_{22}H_{24}N_2O_8]^+$ ($CTC_{ep}$), and $[C_{22}H_{25}N_2O_8]^+$ (DOX), respectively, were observed. The HR-MS spectra of the isolated fractions from the positive liquid hog manure sample showed the peaks of m/z 445.16070 ($\Delta$ = −0.4 ppm), 445.16093 ($\Delta$ = −0.8 ppm), 461.15546 ($\Delta$ = −0.8 ppm), 479.12207

**Table 2**
Accuracy, precision and recovery of the UPLC method for determination of tetracyclines.

| Analyte | Nominal concentration (μg $mL^{-1}$) | Accuracy (%)[a] | Precision RSD (%)[a] | Nominal concentration (mg $kg^{-1}$) | Recovery (%)[b] |
|---|---|---|---|---|---|
| TC | 250.0 | 100.2 ± 6.8 | 1.8 | 25.0 | 52.4 ± 3.8 |
| | 60.0 | 109.0 ± 3.1 | 3.0 | | |
| | 8.0 | 99.2 ± 0.4 | 3.5 | 2.0 | 53.8 ± 1.5 |
| OTC | 250.0 | 103.0 ± 1.4 | 0.7 | 25.0 | 72.4 ± 5.0 |
| | 60.0 | 102.5 ± 3.8 | 5.6 | | |
| | 8.0 | 97.7 ± 0.1 | 1.3 | 2.0 | 76.9 ± 2.7 |
| CTC | 250.0 | 100.4 ± 6.1 | 1.7 | 25.0 | 84.2 ± 3.4 |
| | 60.0 | 109.3 ± 1.2 | 1.9 | | |
| | 8.0 | 103.6 ± 0.6 | 4.7 | 2.0 | 83.8 ± 5.7 |
| DOX | 250.0 | 104.0 ± 6.1 | 2.4 | 25.0 | 95.9 ± 4.7 |
| | 60.0 | 107.9 ± 1.6 | 2.6 | | |
| | 8.0 | 102.1 ± 0.1 | 1.5 | 2.0 | 99.6 ± 5.7 |

[a] Mean value of six measurements.
[b] Mean value of four measurements.

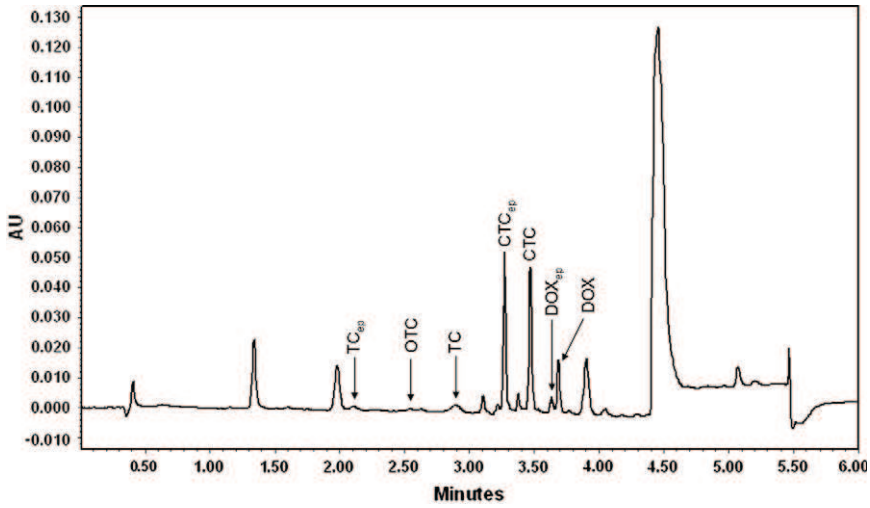


**Fig. 4.** The UPLC analysis of positive liquid hog manure sample. For chromatographic conditions, see Fig. 2.

($\Delta$ = −1.0 ppm), 479.12193 ($\Delta$ = −0.8 ppm), and 445.16093 ($\Delta$ = −0.9 ppm) corresponding to the pseudomolecular ions $[C_{22}H_{25}N_2O_8]^+$, $[C_{22}H_{25}N_2O_8]^+$, $[C_{22}H_{25}N_2O_9]^+$, $[C_{22}H_{24}ClN_2O_8]^+$, $[C_{22}H_{24}N_2O_8]^+$, and $[C_{22}H_{25}N_2O_8]^+$, respectively. It could be thus concluded that the isolated fractions contained TC, $TC_{ep}$, OTC, CTC, $CTC_{ep}$, and DOX.

### 3.6. Method application

Liquid hog manure samples from five different localities were analyzed using the developed assay. One sample was found to contain the monitored antibiotics in concentrations 5.88 mg $kg^{-1}$ (CTC) and 0.99 mg $kg^{-1}$ (DOX, both values were recalculated for 100% recovery).

Although the presence of TC and OTC was proved (off-line MS) the concentrations determined were 10 times lower then MLOQ. The UPLC analysis of the positive sample is shown in Fig. 4.

The tetracyclines concentration in positive liquid hog manure samples published in previous articles ranged from 0.6 to 173.2 mg $kg^{-1}$ (Hu et al., 2008), from 0.1 to 4.0 mg $kg^{-1}$ (Hamscher et al., 2002), from 0.061 to 15.7 mg $kg^{-1}$ (Jacobsen and Halling-Sørensen, 2006) or were about 0.1 mg $kg^{-1}$ (Thorsten et al., 2003). These concentration values correspond with concentrations found in positive manure sample in the Czech Republic. These results confirmed that the MLOQ reached by our method is sufficient for predicted antibiotics concentrations (from tenths to hundreds of mg $kg^{-1}$). The novel UPLC method with UV detection is recently used for monitoring of tetracycline antibiotics in liquid hog manure samples.

## 4. Conclusions

An assay for determination of four tetracycline antibiotics (TC, OTC, CTC, and DOX) and their epimers in liquid hog manure is described. Parameters reached, i.e. selectivity, sensitivity, analysis time, etc. qualify this assay for high-throughput analysis of liquid hog manure samples to control the usage of antibiotics as growth promoters in animal farming. The necessity of the development of such a control process is well documented by finding of one positive sample in our study. Further, the UPLC analytical method could be directly applied for LC/MS analysis of the antibiotic in other matrices without any modification due to the MS compatibility of the mobile phase used.

## Acknowledgments

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## References

Aust, M.-O., Godlinski, F., Travis, G.R., Hao, X., McAllister, T.A., Leinweber, P., Thiele-Bruhn, S., 2008. Distribution of sulfamethazine, chlortetracycline and tylosin in manure and soil of Canadian feedlots after subtherapeutic use in cattle. Environ. Pollut. 156, 1243–1251.

Batt, A.L., Aga, D.S., 2005. Simultaneous analysis of multiple classes of antibiotics by ion trap LC/MS/MS for assessing surface water and groundwater contamination. Anal. Chem. 77, 2940–2947.

Bendahl, L., Stürup, S., Gammelgaard, B., Hansen, S.H., 2005. UPLC-ICP-MS – a fast technique for speciation analysis. Anal. Atom. Spectrom. 20, 1287–1289.

CDER/CBER, US Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research, Center for Biological Evaluation and Research, 2001. Guidance for Industry: Bioanalytical method validation. <http://www.fda.gov/CDER/GUIDANCE/4252fnl.pdf>.

Cooper, A.D., Stubbings, G.W.F., Kelly, M., Tarbin, J.A., Farrington, W.H.H., Shearer, G., 1998. Improved method for the on-line metal chelate affinity chromatography-high-performance liquid chromatographic determination of tetracycline antibiotics in animal products. J. Chromatogr. A 812, 321–326.

Debut, Y., 1988. The Veterinary Formulary. Pharmaceutical Press, London.

European Commision, 1999. Opinion of the Scientific Steering Committee on Antimicrobial Resistance. <http://ec.europa.eu/food/fs/sc/ssc/out50_en.pdf>.

Haller, M.Y., Müller, S.R., McArdell, Ch.S., Alder, A.C., Suter, M.J.-F., 2002. Quantification of veterinary antibiotics (sulfonamides and trimethoprim) in animal manure by liquid chromatography–mass spectrometry. J. Chromatogr. A 952, 111–120.

Halling-Sørensen, B., Sengeløv, G., Tjørnelund, J., 2002. Toxicity of tetracyclines and tetracycline degradation products to environmentally relevant bacteria, including selected tetracycline-resistant bacteria. Arch. Environ. Contam. Toxicol. 42, 263–271.

Hamscher, G., Sczesny, S., Höper, H., Nau, H., 2002. Determination of persistent tetracycline residues in soil fertilized with liquid manure by high-performance liquid chromatography with electrospray ionization tandem mass spectrometry. Anal. Chem. 74, 1509–1518.

Hu, X.-G., Luo, Y., Zhou, Q.-X., Xu, L., 2008. Determination of thirteen antibiotics residues in manure by solid phase extraction and high performance liquid chromatography. J. Anal. Chem. 36, 1162–1166.

Jacobsen, A.M., Halling-Sørensen, B., 2006. Multi-component analysis of tetracyclines, sulfonamides and tylosin in swine manure by liquid chromatography–tandem mass spectrometry. Anal. Bioanal. Chem. 384, 1164–1174.

Kemper, N., Färber, H., Skutlarek, D., Krieter, J., 2008. Analysis of antibiotic residues in liquid manure and leachate of dairy farms in Northern Germany. Agric. Water Manage. 95, 1288–1292.

Kroker, R., 1983. Aspekte zur Ausscheidung antimikrobiell wirksamer Substanzen nach der chemotherapeutischen Behandlung von Nutztieren. Wiss. Umwelt 4, 305–308.

Kühne, M., Ihnen, D., Möller, G., Agthe, O., 2000. Stability of tetracycline in water and liquid manure. J. Vet. Med. A 47, 379–384.
Lu, H.-T., Jiang, Y., Li, H.-B., Chen, F., Wong, M.-H., 2004. Simultaneous determination of oxytetracycline, doxycycline, tetracycline and chlortetracycline in tetracycline antibiotics by high-performance liquid chromatography with fluorescence detection. Chromatographia 60, 259–264.
Martínez-Carballo, E., González-Barreiro, C., Scharf, S., Gans, O., 2007. Environmental monitoring study of selected veterinary antibiotics in animal manure and soils in Austria. Environ. Pollut. 148, 570–579.
McEvoy, G.K., Miller, J.A.D., Snow, E.K., 2004. AHFS Drug Information. The American Society of Health-System Pharmacists, Bethesda, Maryland.
Nowara, A., Burhenne, J., Spiteller, M., 1997. Binding of fluoroquinolone carboxylic acid derivatives to clay minerals. J. Agric. Food Chem. 45, 1459–1463.
Schlüsener, M.P., Bester, K., Spiteller, M., 2003. Determination of antibiotics such as macrolides, ionophores and tiamulin in liquid manure by HPLC–MS/MS. Anal. Bioanal. Chem. 375, 942–947.
Skúlason, S., Ingólfsson, E., Kristmundsdóttir, T., 2003. Development of a simple HPLC method for separation of doxycycline and its degradation products. J. Pharm. Biomed. Anal. 33, 667–672.
Thorsten, Ch., Schneider, R.J., Färber, H.A., Skutlarek, D., Meyer, M.T., Goldbach, H.E., 2003. Determination of antibiotic residues in manure, soil and surface waters. Acta Hydrochim. Hydrobiol. 31, 36–44.
Wang, Q., Yates, S.R., 2008. Laboratory study of oxytetracycline degradation kinetics in animal manure and soil. J. Agric. Food Chem. 56, 1683–1688.

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# Determination of antibiotics in influents and effluents of wastewater-treatment-plants in the Czech Republic – development and application of the SPE and a UHPLC-ToFMS method

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A method consisting of solid phase extraction followed by ultra-high performance liquid chromatography combined with time-of-flight mass spectrometry (UHPLC-ToFMS) has been developed for the determination of multiple-class antibiotics in influents and effluents of wastewater-treatment-plants (WWTPs). The nineteen analyzed antibiotics belong to the 5 most prescribed antibiotic classes in the Czech Republic, namely tetracyclines, macrolides, sulfonamides, fluoroquinolones, and lincosamides. The matrix-matched calibration technique with internal standard addition for each antibiotic group was employed for quantification. Two extractions employing an Oasis HLB cartridge and two injections were needed for each sample due to the different physico-chemical properties of the tested antibiotics. Prior to the extraction on the Oasis HLB SPE cartridge, the samples were adjusted to pH 4.5 and 7.5. The usefulness and versatility of the method was documented by achieving method limits of quantification up to 10 ng $L^{-1}$ and recoveries >80% for most analytes. The method was used for analysis of water samples of WWTPs from 6 localities in the Czech Republic in order to reveal the occurrence of selected antimicrobial agents and assess the efficiency of WWTPs in the removal of these antibiotics. All tested samples were positive for antibiotics with concentrations ranging from 5 ng $L^{-1}$ to 1290 ng $L^{-1}$. Antibiotics were present in both influent and effluent water samples, which documented the poor removal efficiency of the WWTPs.

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## 1 Introduction

Antibiotics represent a group of pharmaceuticals that are widely used for treatment of various bacterial infections in both human and veterinary medicine and their consumption rises significantly every year. About 50–90% of the administered pharmaceutical dose is excreted rapidly after the treatment in their parent form or as metabolites that can then enter the environment and cause the development of bacterial resistance.[1–4] The most important sources of such compounds in the environment are domestic sewage, wastewater-treatment-plants (WWTPs), hospitals, industrial units and intensive animal husbandry.[5] Significant growth of antibiotic consumption in the Czech Republic has been observed from the beginning of 1990s, with the main increase of macrolides, penicillins, and fluoroquinolones.[6–10] The associated antibiotic resistance has grown annually from the 1990s, and beginning in 2000 the increase in antibiotic resistance of some important infection agents is very significant. This trend is observed in the majority of European countries, but the situation in the Czech Republic is one of the most alarming.[6]

Various classes of antibiotics in water samples were found at concentration ranging from nanograms to micrograms per liter *e.g.* in Spain,[11,12] France,[3] UK,[13,14] Italy,[15] Switzerland,[16,17] Austria,[18] Sweden,[19–21] Croatia,[22] and Poland.[13] Concerning the Czech Republic, Seifrtova *et al.* presented a study dealing with fluoroquinolones in wastewater,[1] but there is still a lack of information about the presence of a wide spectrum of antibiotics in aquatic environment in the Czech Republic. As mentioned above, WWTPs are important point sources of pharmaceutical entry into the environment, because the excreted antibiotics are discharged from WWTPs at certain levels presumably due to inadequate removal efficiencies in the plants.[23]

The liquid chromatography-tandem mass spectrometry (LC-MS/MS) is the most frequent method of choice for the determination of trace concentrations of pharmaceuticals in aquatic environment. For instance, HPLC coupled to triple quadrupole (QqQ) has recently been employed in many studies.[3,15,22,24–26] Another approach introduced in other papers was the use of an

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ion trap (IT).[27,28] The advantage of UHPLC over conventional HPLC when connected with QqQ was demonstrated in other related studies.[13,29] The UHPLC generates narrow peaks (improving sensitivity), facilitates resolving the analytes and matrix interferences (crucial for complex matrices such as wastewater) and brings shorter analysis times.[30] Due to the recent innovations of this technology,[31,32] time-of-flight (ToF) analyzers find their application in the determination of residual antibiotics in complex matrices. This instrumentation provides high specificity due to both high mass accuracy and high mass resolution, and allows the reconstruction of highly selective accurate mass chromatograms of target residues in complex matrices. The full scan data permit the testing of any *a posteriori* hypotheses by extracting the desired exact mass chromatogram. The higher resolution provided by UHPLC is an important factor compensating for the fact that the selectivity of ToFMS instrumentation is generally less than that provided by monitoring MS → MS transitions.[33] A fast method development is an advantage of the UHPLC-ToFMS technology. A method consisting of UHPLC coupled to hybrid quadrupole time-of-flight MS (QToFMS) has recently been introduced,[30,34] revealing the potential of this instrumentation for rapid screening of antibiotics in wastewater and surface water with higher certainty of compounds' identity provided by fragment ions.

Concerning the extraction techniques, the solid phase extraction (SPE) during the determination of antibiotics in wastewater and surface water was employed in the majority of published articles using different sorbent chemistries.[13,15,22,24–27,46,47] Among them, the copolymeric sorbent Oasis HLB belongs to the most frequent.[22,24–27,46,47]

The aim of this article is to demonstrate that the UHPLC-ToFMS instrumentation can be successfully used for determination of a wide spectrum of antibiotics at trace levels in influents and effluents of WWTPs, as it was employed previously for screening of antibiotics in other complex matrices as meat or urine.[31,32] The matrix effects, which are the major drawback of LC-MS and LC-MS/MS, especially in combination with electrospray ionization (ESI), require a special calibration technique for their compensation described in previous studies.[22,35,36]

Nineteen most prescribed antibiotics of diverse physical and chemical characteristics used in both human and veterinary medicine were used for method optimization.[6,8–10,36,37] The selected antibiotics belong to 5 different antibiotic classes, namely tetracyclines, macrolides, sulfonamides, fluoroquinolones, and lincosamides. The SPE was employed for extraction and concentration of the antibiotics. The utility and sensitivity of the newly developed UHPLC-ToFMS method was demonstrated by monitoring the residual antibiotics in the influent and effluent of WWTPs from 6 localities in the Czech Republic.

The main novelty of this study is the use of UHPLC-ToFMS instrumentation for determining multiple-class antibiotics in wastewater and treated water in the Czech Republic employing the matrix-matched calibration technique with internal standard addition for each antibiotic.

## 2 Experimental

### 2.1 Chemicals, reagents, and glassware

Tetracycline (TC) was purchased from Spofa (Prague, Czech Republic); oxytetracycline (OTC) from VUAB (Roztoky u Prahy, Czech Republic); chlortetracycline (CTC), erythromycin (ERY), roxithromycin (ROX), tylosin (TYL), sulfadimethoxine (SDM), sulfadimidine (SDD), sulfadiazine (SDZ), sulfamethoxazole (SMX), sulfathiazole (STZ), enrofloxacin (ENR), norfloxacin (NOR), lincomycin (LIN), clindamycin (CLI), ofloxacin-D3 (OFL-D3), sulfadimethoxine-D6 (SDM-D6) from Sigma-Aldrich (Steinheim, Germany); doxycycline (DOX) from Calbiochem (USA); ciprofloxacin (CIP) from Krka (Novo Mesto, Slovenia); clarithromycin (CLA) from Dr Ehrenstorfer (Augsburg, Germany). Ofloxacin (OFL), oleandomycin (OLE), granaticin (GRA), and butyllincomycin (BULIN) were kindly provided by Prof. Jaroslav Spížek, Institute of Microbiology of the Academy of Sciences of the Czech Republic, v.v.i., Czech Republic. All antibiotics were of +95% purity except for CTC (90%). Acetonitrile (ACN), methanol and trifluoroacetic acid used as the chromatographic mobile phase were LC/MS grade and were obtained from Biosolve (Valkenswaard, The Netherlands). Ammonium hydroxide A.C.S. reagent (29% aqueous $NH_4OH$ solution) and disodium salt of ethylenediaminetetraacetic acid ($Na_2EDTA$) were purchased from Sigma-Aldrich (Steinheim, Germany), and formic acid (98–100%) was from Merck (Darmstadt, Germany). Ultrapure water was prepared using the Milli-Q water purification system (18.2 MΩ; Millipore; Billerica, USA). The SPE cartridges Oasis HLB, Oasis MAX, and Oasis MCX (all 60 mg) were purchased from Waters, Czech Republic.

SPE 12-position Vacuum Manifold (Phenomenex; Torrance, USA) and Visiprep™ Large Volume Sampler (Sigma-Aldrich; Steinheim, Germany) were used for the SPE procedure. The Acquity UPLC BEH C18 (50 × 2.1 mm i.d.; 1.7 μm) analytical column was purchased from Waters, Prague, Czech Republic.

To avoid formation of complexes of tetracyclines and macrolides with metal ions, proteins, and silanol groups, all glassware used was rinsed with saturated solution of $Na_2EDTA$ (0.3 M) in methanol–water (50 : 50, v/v) and air-dried before use.[27,39]

The antibiotics that exist in their parent form as well as isomers and/or degradation products were quantified as a sum of all their forms present in the sample. This involves isomers of tetracyclines: $TC_{iso}$ and $CTC_{iso}$,[28,40] macrolides' degradation products: ERY–$H_2O$ existing as two isomers (ERY–$H_2O_{iso1}$ and ERY–$H_2O_{iso2}$) and as ERY–$H_2O$ + $H_2$, and aglycones $ERY_{ag}$, $TYL_{ag}$, $CLA_{ag}$, $ROX_{ag}$.[41,42] The tested antibiotic forms are listed in Table 1. GRA, OLE, SDM-D6, OFL-D3, and BULIN were employed as internal standards. SDM-D6 and OFL-D3 are isotopically labeled; GRA, OLE, and BULIN are not commercially available and therefore cannot occur in real samples.

### 2.2 Sample preparation and SPE

The water samples (raw influents and final effluents of WWTPs) were collected from 6 different localities in the Czech Republic into methanol pre-washed 1 L amber glass bottles rinsed with the sample water on site. Sampling was provided by qualified

 

**Table 1** List of tested antibiotics including their isomers and degradation products with corresponding retention times and acquisition parameters for UHPLC-ToFMS analysis

| Antibiotic | Abbreviation | $R_t$ (min) | $[M + H]^{+a}$ ($m/z$) | Average mass error[c] (ppm) |
|---|---|---|---|---|
| Tetracycline | TC | 5.45 | 445.161 | 5.8 |
| Tetracycline isomer | $TC_{iso}$ | 4.11 | 445.161 | 3.4 |
| Oxytetracycline | OXY | 5.88 | 461.156 | 6.0 |
| Chlortetracycline | CTC | 9.46 | 479.122 | 2.5 |
| Chlortetracycline isomer | $CTC_{iso}$ | 7.39 | 479.122 | 6.1 |
| Doxycycline | DOX | 12.72 | 445.161 | 5.4 |
| Erythromycin | ERY | 17.71 | 734.469 | 1.6 |
| Erythromycin–$H_2O$ isomer1 (degr.[b]) | ERY–$H_2O_{iso1}$ | 18.65 | 716.459 | 3.0 |
| Erythromycin–$H_2O$ isomer2 (degr.[b]) | ERY–$H_2O_{iso2}$ | 20.66 | 716.459 | 5.2 |
| Erythromycin–$H_2O$ + $H_2$ (degr.[b]) | ERY–$H_2O$ + $H_2$ | 19.61 | 718.459 | 6.5 |
| Erythromycin aglycon (degr.[b]) | $ERY_{ag}$ | 17.74 | 576.375 | 2.8 |
| Tylosin | TYL | 18.19 | 916.527 | 0.8 |
| Tylosin aglycon (degr.[b]) | $TYL_{ag}$ | 18.41 | 742.430 | 7.0 |
| Clarithomycin | CLA | 19.81 | 748.485 | 1.6 |
| Clarithromycin aglycon (degr.[b]) | $CLA_{ag}$ | 19.99 | 590.390 | 3.0 |
| Roxithromycin | ROX | 20.59 | 837.532 | 1.1 |
| Roxithromycin aglycon (degr.[b]) | $ROX_{ag}$ | 20.62 | 679.438 | 6.5 |
| Sulfadiazine | SDZ | 1.91 | 251.060 | 3.8 |
| Sulfathiazole | STZ | 2.57 | 256.021 | 1.2 |
| Sulfadimidine | SDD | 4.33 | 279.092 | 3.7 |
| Sulfamethoxazole | SMX | 5.94 | 254.060 | 3.0 |
| Sulfadimethoxine | SDM | 10.27 | 311.081 | 1.7 |
| Ofloxacin | OFL | 6.10 | 362.152 | 3.4 |
| Norfloxacin | NOR | 6.39 | 320.141 | 0.8 |
| Ciprofloxacin | CIP | 6.83 | 332.141 | 2.7 |
| Enrofloxacin | ENR | 7.57 | 360.172 | 3.7 |
| Lincomycin | LIN | 4.43 | 407.222 | 3.8 |
| Clindamycin | CLI | 14.37 | 425.188 | 1.7 |
| **Internal standard** | | | | |
| Granaticin | GRA | 13.71 | 445.114 | 2.2 |
| Oleandomycin | OLE | 15.84 | 688.427 | 2.1 |
| Sulfadimethoxine-D6 | SDM-D6 | 10.15 | 317.120 | 4.1 |
| Ofloxacin-D3 | OFL-D3 | 5.80 | 365.172 | 5.2 |
| Butyllincomycin | BULIN | 7.24 | 421.238 | 4.2 |

[a] Ion used for detection in the UPLC-ToFMS with ESI+ ionization; mass to charge ratio. [b] Degradation product. [c] Average absolute mass error obtained as a mean value of 6 measurements (antibiotics mixture in the sample matrix; 200 ng $mL^{-1}$).

personnel using a standard sampling field protocol.[43] The samples are marked anonymously in accordance with the pledge of secrecy. Information about localities, inhabitants, or treatment process characterization is therefore unfortunately missing. The samples were filtered through a 2 μm glass microfiber filter (Whatman; Maidstone, UK) and stored at 4 °C.

The chemistries of the SPE cartridges used were:

Oasis HLB; copolymeric sorbent, hydrophilic *N*-vinylpyrrolidone and lipophilic divinylbenzene; retention, reversed-phase and polar interactions; application, acidic, neutral and basic compounds.

Oasis MCX; strong cation-exchange mixed-mode polymeric sorbent built upon HLB copolymer; retention, reversed phase and cation-exchange (sulfonic acid content); application, bases.

Oasis MAX; strong anion-exchange mixed-mode polymeric sorbent built upon HLB copolymer; retention, reversed phase and anion-exchange (quaternary amine content); application, acids.

The SPE using Oasis HLB cartridges was employed for extraction of the samples; namely real, spiked, and blank water samples (water with an absence of any tested antibiotics). Prior to extraction, the water was filtered through a 0.45 μm nylon membrane (Millipore; Billerica, USA). $Na_2EDTA$ (500 mg $L^{-1}$) was added as a chelating agent. Each water sample was treated using two methods differing in sample pH adjustment, namely pH 4.5 (adjusted by formic acid) and pH 7.5 (by $NH_4OH$). The pH 4.5 was preferred for tetracyclines, macrolides, and fluoroquinolones, while pH 7.5 was more appropriate for sulfonamides and lincosamides (see Section 3.1). Both sets of samples were then treated using a similar procedure as follows. The Oasis HLB was conditioned with 2 mL of methanol followed by equilibration with 2 mL of Milli-Q water. Two hundred fifty mL of water sample was passed through the cartridge at a flow rate of 3 mL $min^{-1}$ and the sorbent was then rinsed with 2 mL of Milli-Q water. Afterwards, the cartridge was air-dried for 5 min, the analytes were then eluted with 1.2 mL of methanol and the extracts were evaporated to dryness. The extracts of raw or spiked WWTP influents/effluents were reconstituted in 200 μL of methanol–water (50 : 50, v/v) and 50 μL of internal standards' mixture (500 ng $mL^{-1}$) was added. The blank water sample extracts were dissolved in 250 μL of methanol–water (50 : 50, v/v) and used for preparation of matrix-matched calibration standards (see Section 2.4). Two extracts differing in pH adjustment prior to SPE were obtained for each sample, and both extracts were injected after centrifugation into the UHPLC-ToFMS system and both were analyzed under similar conditions described later in the text.

The same protocol as described in the previous paragraph was used for other extraction sorbents with specific conditions as follows:

*Oasis MAX*: sample pH, >8; wash solvent, $NH_4OH$–water (5 : 95, v/v); elution solvent I, methanol; elution solvent II, formic acid–methanol (2 : 98, v/v).

*Oasis MCX*: sample pH, <3; wash solvent, formic acid–water (2 : 98, v/v); elution solvent I, methanol; elution solvent II, $NH_4OH$–methanol (5 : 95, v/v).

### 2.3 UHPLC-ToFMS analysis

UHPLC-ToFMS analysis was carried out on a Waters Acquity UPLC System (Waters, Prague, Czech Republic) consisting of Acquity UPLC Solvent Manager, Acquity UPLC Sample Manager, and Acquity UPLC Column Heater/cooler coupled with Waters LCT Premier XE orthogonal accelerated ToFMS (Waters MS; Manchester, UK) with an ESI interface operating in positive ion mode, using the following parameters: cone voltage, 25 V;

capillary voltage, +3000 V; ion source block temperature, 120 °C; nitrogen desolvation gas temperature, 350 °C; desolvation gas flow, 800 L $h^{-1}$; cone gas flow, 50 L $h^{-1}$. Full scan spectra from *m*/*z* 100 to 1200 were acquired with a scan time of 0.1 s and 0.01 s interscan delay. Mass accuracy was maintained by lock spray using leucine enkephalin, and Mass Lynx V4.1 software was employed for data processing. After acquisition, the specific $[M + H]^+$ ions (see Table 1) were extracted with 0.01 Da extraction mass window. Separations were performed on an Acquity UPLC BEH C18 column (50 × 2.1 mm i.d.; 1.7 μm) with the mobile phase flow rate of 0.4 mL $min^{-1}$, column temperature of 30 °C, and injection volume of 3 μL. The mobile phase consisted of (A) formic acid–water (0.1 : 99.9, v/v), and (B) formic acid–methanol (0.1 : 99.9, v/v) using gradient elution program (min/%A): 0.0/95, 10.0/75, 15.0/60, 21.0/45, 21.5/45, followed by 1.5 min wash step with 100% B and 2.0 min equilibration step.

### 2.4 Method validation

Individual antibiotic standards (2 mg) were diluted in 1 mL of methanol–water (50 : 50, v/v) and the stock solution was prepared by mixing individual standard solutions to the final concentration of 100 μg $mL^{-1}$. The stock solution of internal standards (100 μg $mL^{-1}$) was prepared in an identical manner.

The matrix-matched calibration technique with internal standards addition was employed for quantification. The calibration solutions were prepared by subsequent dilution of the stock solution into the blank water sample extracts (WWTP influents/effluents with an absence of any tested antibiotics) and by internal standard addition. For each antibiotic group, an internal standard was used, namely GRA (tetracyclines), OLE (macrolides), SDM-D6 (sulfonamides), OFL-D3 (fluoroquinolones), and BULIN (lincosamides). Two series of calibration solutions were prepared using the blank water samples with pH adjustment prior to SPE of 4.5 (tetracyclines, macrolides, fluoroquinolones) and 7.5 (sulfonamides, lincosamides), respectively. The concentration of analytes ranged from the instrument limit of quantification (ILOQ) of each analyte to 500 ng $mL^{-1}$; the concentrations of internal standards were 100 ng $mL^{-1}$ in each sample. Three sets of calibration standards were prepared. The ILOQ was determined as the lowest point of the calibration curve with a precision (closeness of agreement between independent measurements of a quantity under the same conditions; expressed as RSD%) less than 20% and trueness (percentage difference between determined and expected amounts) of 80–120% in six replicates. The method limit of quantification (MLOQ) was determined as the lowest amount of each antibiotic that can be quantified in water extract after SPE with respect to ILOQ, enrichment factor, and recovery of each analyte. MLOQ was expressed in ng $L^{-1}$ units.[44]

The repeatability of the instrument was determined as the system suitability assessment using 6 replicated injections of the antibiotic mixture in the sample matrix (200 ng $mL^{-1}$) by measurement of retention time and peak area. RSD% was taken as a measure of repeatability. To evaluate the trueness and precision of the assay, quality control samples (antibiotic mixture in the sample matrix) were prepared at concentrations of 500, 50 and 5 ng $mL^{-1}$ in replicates of 6 for each concentration.[44]

The blank water samples spiked at concentration levels of 500 and 60 ng $L^{-1}$, in replicates of 4 for each concentration, were treated by the SPE procedure and used for recovery tests. The recoveries were expressed as the percentage ratio of peak areas before and after extraction.[43]

The antibiotic mixture in extracted blank sample (100 ng $mL^{-1}$) and extract of spiked water (500 ng $L^{-1}$) were tested for both short and long term stability. Samples were divided into the required number of parts, stored at room temperature (RT, short term stability) or at −20 °C (long term stability) and analyzed after 0, 2, 4, 6, 8, 12, and 24 hours, or after 0, 1, 2, 3, 4, 7 and 14 days, respectively. Stability was expressed as the percentage ratio of peak areas of all forms of each analyte.[44]

## 3 Results and discussion

### 3.1 Sample preparation and SPE

The recovery using the blank water samples spiked with antibiotics (500 ng $L^{-1}$) was used for determination of SPE efficiency, which is a key parameter of final multi-residue analysis. Moreover, the main demand on the developed method was its utility for a large number of analytes of different physicochemical characteristics.[3] With respect to the acid–base properties of the studied antibiotics, the Oasis HLB, Oasis MCX, and Oasis MAX sorbents were tested for SPE procedure development. Macrolides and lincosamides represent weak bases, sulfonamides are weak acids, and tetracyclines and fluoroquinolones are amphoteric drugs that can exhibit both weak acidic and basic characters.[45] The SPE protocols for all three sorbents are described in Section 2.2.

Great differences in recoveries were observed using tested SPE sorbents. Selected results of SPE development are shown in Table 2. The Oasis MCX was found to be effective for macrolides[13,15] (except for TYL) with recoveries >73% and for lincosamides[15] with recoveries >48%, which is in agreement with their weak basic character. Unfortunately, this sorbent was absolutely unusable for other antibiotic groups such as fluoroquinolones and tetracyclines (recoveries 0%). Concerning the MAX sorbent, macrolides and lincosamides were eluted in elution step I (100% MeOH), which reveals that only hydrophobic and polar interactions were employed for retention of these compounds on the MAX sorbent. This also corresponds with the weak basic character of macrolides and lincosamides. Furthermore, it was found that the Oasis MAX cartridge was the most fitting for fluoroquinolones (recoveries >80%), which is caused by its weak acidic character. To the best of our knowledge, the MAX sorbent has never been used for extraction of fluoroquinolones from water samples. Tetracyclines and sulfonamides, as weak acids, exhibited retention on the MAX sorbent as well. However, the recoveries for tetracyclines were lower than 62% and for sulfonamides lower than 23%.

Although the tested ion-exchange cartridges can be promising sorbents for extraction of particular antibiotic classes (*e.g.* MAX sorbent for fluoroquinolones), we did not find them useful for extraction of all analytes under study.

 

As the only sorbent, Oasis HLB permitted the extraction of all analytes and was therefore used for determining the versatility of the method. Due to the weak acid–base character of the tested antibiotics, the dependence of the sample pH on the retention for the HLB sorbent was expected. Therefore, the sample pH adjustment prior to the Oasis HLB method was subsequently tested in the range from 3 to 9 with 0.5 unit steps and a strong dependence of recovery on sample pH was revealed. Since it was impossible to find optimal conditions for all analytes at a single pH value, it was necessary to employ the two extraction procedures differing in sample pH adjustment. Therefore, the pH 4.5 was chosen for tetracyclines, macrolides, and fluoroquinolones, while pH 7.5 was used for sulfonamides and lincosamides. On comparing the developed SPE method with previously published studies, the Oasis HLB represents the most used sorbent for antibiotic extraction and concentration in water samples.[22,24,25,27,46,47] The obtained recoveries were in most cases similar[24,47] or slightly higher[22,25,46] than in previously published papers. A similar strategy of two extraction procedures for each sample resulting in recovery improvement was previously described in the literature.[48,49]

Finally, the versatility of the extraction procedure was verified by achieving >80% recoveries for all antibiotics (except for TYL and SDZ, see Table 2). Moreover, after slight modification the method could be used for determination of the other added antibiotics when required.

**Table 2** Overview of SPE recoveries (%) for studied antibiotics using different extraction sorbents and sample pH adjustment during the method development

| | Oasis HLB | | | | | Oasis MAX | | Oasis MCX | |
|---|---|---|---|---|---|---|---|---|---|
| | pH | | | | | | | | |
| Antibiotic | 3.0 | 4.5 | 7.5 | 9.0 | Antibiotic | Elution solvent[a] | | Elution solvent[b] | |
| TC | 97.8 | 94.4 | 55.2 | 32.4 | TC | I | 2.4 | I | 0.0 |
| | | | | | | II | 52.5 | II | 0.0 |
| OTC | 100.3 | 95.0 | 71.3 | 36.8 | OTC | I | 3.8 | I | 0.0 |
| | | | | | | II | 62.7 | II | 0.0 |
| CTC | 92.3 | 86.5 | 48.2 | 10.7 | CTC | I | 0.0 | I | 0.0 |
| | | | | | | II | 39.4 | II | 0.0 |
| DOX | 88.8 | 90.2 | 68.5 | 16.6 | DOX | I | 0.0 | I | 0.0 |
| | | | | | | II | 49.2 | II | 0.0 |
| ERY | 70.1 | 90.1 | 84.5 | 64.9 | ERY | I | 76.7 | I | 0.0 |
| | | | | | | II | 3.4 | II | 76.2 |
| TYL | 55.6 | 60.4 | 55.2 | 51.5 | TYL | I | 65.0 | I | 0.0 |
| | | | | | | II | 3.8 | II | 11.7 |
| CLA | 77.1 | 96.2 | 93.8 | 66.2 | CLA | I | 70.5 | I | 0.0 |
| | | | | | | II | 2.0 | II | 73.1 |
| ROX | 60.1 | 93.6 | 85.4 | 49.5 | ROX | I | 76.1 | I | 0.0 |
| | | | | | | II | 0.9 | II | 78.0 |
| SDZ | 48.1 | 64.7 | 74.9 | 51.7 | SDZ | I | 0.0 | I | 0.0 |
| | | | | | | II | 7.1 | II | 8.2 |
| STZ | 45.8 | 67.9 | 89.3 | 85.2 | STZ | I | 0.0 | I | 0.0 |
| | | | | | | II | 6.3 | II | 34.0 |
| SDD | 32.7 | 66.0 | 92.7 | 54.1 | SDD | I | 0.0 | I | 0.0 |
| | | | | | | II | 18.0 | II | 96.4 |
| SMX | 41.2 | 63.0 | 90.0 | 79.2 | SMX | I | 0.0 | I | 0.0 |
| | | | | | | II | 22.8 | II | 33.2 |
| SDM | 43.6 | 72.2 | 88.8 | 87.3 | SDM | I | 0.0 | I | 0.0 |
| | | | | | | II | 14.7 | II | 16.0 |
| OFL | 98.6 | 100.0 | 88.9 | 80.4 | OFL | I | 0.0 | I | 0.0 |
| | | | | | | II | 80.9 | II | 0.0 |
| NOR | 103.3 | 104.4 | 83.6 | 71.9 | NOR | I | 0.0 | I | 0.0 |
| | | | | | | II | 84.3 | II | 0.0 |
| CIP | 97.9 | 106.3 | 81.8 | 63.0 | CIP | I | 0.0 | I | 0.0 |
| | | | | | | II | 91.6 | II | 0.0 |
| ENR | 98.0 | 98.0 | 86.8 | 81.5 | ENR | I | 0.0 | I | 0.0 |
| | | | | | | II | 89.0 | II | 0.0 |
| LIN | 35.5 | 32.5 | 92.5 | 99.2 | LIN | I | 95.8 | I | 0.0 |
| | | | | | | II | 0.0 | II | 48.7 |
| CLI | 44.5 | 38.3 | 89.6 | 98.0 | CLI | I | 94.7 | I | 0.0 |
| | | | | | | II | 0.0 | II | 52.5 |

[a] Elution solvents: elution solvent I, 100% methanol; elution solvent II, formic acid–methanol (2 : 98, v/v). [b] Elution solvents: elution solvent I, 100% methanol; elution solvent II, $NH_4OH$–methanol (5 : 95, v/v). For other conditions see Experimental section.

### 3.2 UHPLC-ToFMS analysis

**3.2.1 Chromatographic separation.** Formic acid, acetic acid and trifluoroacetic acid (all 0.1 and 0.05%), and 1 mM ammonium formate (pH 4.0 and 9.0) were tested among the aqueous components of the mobile phase. ACN, methanol, formic acid–ACN (0.1 : 99.9, v/v), and formic acid–methanol (0.1 : 99.9, v/v) were evaluated as organic modifiers. The gradient elution program was employed in view of the different polarities of the analytes. The standard stock solution (100 ng $mL^{-1}$) and spiked water extract (500 ng $L^{-1}$) were used for developing the chromatographic method. The final conditions were chosen with respect to the efficiency of the separation of all analytes and the matrix components, analyte peak shapes, and total analysis time. Methanol was found to be an optimal organic modifier with respect to the peak resolution and ESI ionization. Basic additives, such as ammonium formate, suppressed the signal in the ESI+ interface, while acidic additives provided signal increase, as described previously.[13] Based on these findings, the composition of the mobile phase was (A) formic acid–water (0.1 : 99.9, v/v), and (B) formic acid–methanol (0.1 : 99.9, v/v). Subsequently, we tested the effect of column temperature (ranging from 25 °C to 45 °C with 5 °C steps) in combination with the gradient steepness on the separation of analytes.

All 19 tested antibiotics were satisfactorily separated during a 25 min gradient elution (50 min in total considering that two injections per sample are needed). The selectivity of the method is demonstrated in Fig. 1 depicting the chromatographic separation of tested antibiotics in spiked blank water sample (100 ng $L^{-1}$) under optimal conditions. The high mass accuracy of the method is accomplished by achieving average absolute mass error up to 10 ppm (see Table 1).

The detailed method conditions are described in Section 2.3.

**3.2.2 Optimization of MS parameters.** A standard solution of 200 ng $mL^{-1}$ prepared in methanol–water (50 : 50, v/v) was used to optimize the MS parameters. The ESI+ mode was employed, because all antibiotic forms showed intense protonated molecular ions $[M + H]^+$ with higher peak intensities compared to ESI−. The different solvents for sample dilution, namely methanol–water (20 : 80, v/v), methanol–water (50 : 50, v/v), and 100% methanol were compared. The highest peak intensities were obtained with methanol–water (50 : 50, v/v).

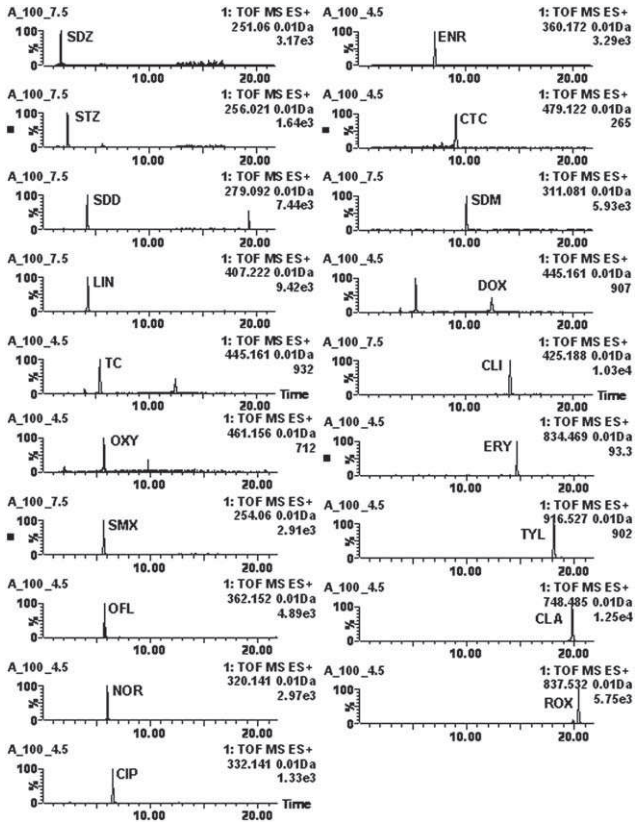


**Fig. 1** UHPLC-ToFMS separation of tested antibiotics in spiked blank water sample (100 ng $L^{-1}$; WWTP influent) under optimal conditions.

The parameters affecting the ESI interface were optimized in direct infusing experiments with standard solution of 200 ng $mL^{-1}$ in methanol–water (50 : 50, v/v) at a constant flow rate of 5 μL $min^{-1}$ into the analyte probe using the syringe pump. The tested parameters were: capillary voltage (from +1500 V to +3000 V with 500 V steps), cone voltage (from 25 V to 50 V with 5 V steps), and needle counter electrode distance (from 2 mm to 4 mm with 0.5 mm steps). The optimal conditions are given in Section 2.3.

Matrix effects caused by the susceptibility of ESI-MS to organic and inorganic components both in the sample matrix and in the mobile phase can lead to signal suppression or enhancement and can cause erroneous results during quantification.[22,35,36] Therefore, the matrix-matched calibration and addition of internal standards were employed to compensate the signal instability. The main drawback of this approach is the absolute absence of a blank matrix.[50] The extracted influent/effluent of WWTP was therefore used as the blank matrix solution for matrix-matched calibration. Since the analyses proved the presence of a low concentration of CLA in this sample, the peak area of CLA (originating from the sample itself) was subtracted during method validation (calibration, recovery tests).

### 3.3 Method validation

The matrix-matched calibration curves were prepared over a linear range from ILOQ of each analyte to 500 ng $mL^{-1}$. The concentration levels were 3.91, 7.81, 15.63, 31.25, 62.5, 125, 250, and 500 ng $mL^{-1}$ (the lowest point depended on the ILOQ of each analyte). The analytes were quantified as a sum of peak areas of each antibiotic and its corresponding isomers or degradation products.[27,51] ILOQ, which complied with precision (expressed as RSD%) of less than 20% and trueness of 80–120%, varied from 3.9 to 15.6 ng $mL^{-1}$ for all analytes. With respect to ILOQ, recovery, and concentration factor, the corresponding MLOQ ranged from 3.68 to 18.07 ng $L^{-1}$. The MLOQs were verified by analysis of blank water samples spiked at a corresponding concentration (4 samples in total). Except for CTC, DOX, and SDZ, the MLOQ values were up to 10 ng $L^{-1}$. For the parameters of calibration curves and detailed ILOQ and MLOQ see Table 3. Compared to the previously published HPLC-MS/MS method with QqQ analyzer, the MLOQ values we obtained are often similar or slightly higher,[3,46,47] or even lower.[22] These findings demonstrate that, as discussed in a previous article,[33] the UHPLC technique is able to compensate for the lower selectivity of ToFMS instrumentation.

The RSD% values of retention times and peak areas of the antibiotics did not exceed 0.54% and 4.34%, respectively, which verified the instrumental repeatability. The trueness and precision of the method (including the ILOQ) were confirmed, with trueness values from 94.53% to 107.14% and with the precision <8.78% for all tested antibiotics. The detailed results of recovery determination and corresponding RSD% are shown in Table 3. Although different recoveries in influents and effluents of the WWTP could be expected due to the difference in the matrix, control tests revealed a similar recovery for all compounds. This fact was probably caused by using of an internal standard, which compensated for the matrix effect. While the recoveries at both concentration levels were similar, the arithmetical mean was taken as the average recovery. Except for TYL and SDZ, good average recoveries ranging from 86.50% to 106.30% were obtained.

All tested antibiotics were found to be stable, with the percentage ratio of peak areas ranging from 93.80% to 110.67% and from 95.81% to 109.23% for short term and long term stability, respectively.

### 3.4 Method application

The method was used for determination of selected antibiotics in influent and effluent of WWTP from 6 different localities in the Czech Republic. All tested samples were anonymous. A monitoring study revealed that all analyzed samples were positive for various classes of antibiotics. The concentration in both influents and effluents of WWTPs ranged from 5.0 ng $L^{-1}$ (LIN) to 1287.9 ng $L^{-1}$ (CLA); the detailed results are listed in Table 4. These concentration values correspond to those previously found in other European countries.[3,13–22,52] In some cases, the antibiotic concentrations in effluents are higher than in influents. This phenomenon is caused by the fact that both samples (influents and effluents) were collected at the same time and therefore the effect of the treatment process does not show. In general, the antibiotics so determined and their concentrations are in agreement with the frequency of their use.[6,8–10,37,38] For instance, CLA was found in a concentration ranging from 61.0 to 1287.9 ng $L^{-1}$ in all samples, which

 

**Table 3** Analytical parameters of the UHPLC-ToFMS method with SPE

| | Calibration curve[a] | | | Recovery | | | |
|---|---|---|---|---|---|---|---|
| Antibiotic | Linear range (ng mL$^{-1}$) | $R^2$ | ILOQ (ng mL$^{-1}$) | pH[b] | Recovery[c] (%) | RSD (%) ($n$ = 8) | MLOQ[d] (ng L$^{-1}$) |
| TC[e] | 500–15.63 | 0.9996 | 7.81 | 4.5 | 94.4 | 0.81 | 8.27 |
| OXY | 500–7.81 | 0.9984 | 7.81 | 4.5 | 95.0 | 1.79 | 8.22 |
| CTC[f] | 500–15.63 | 0.9993 | 15.63 | 4.5 | 86.5 | 2.15 | 18.07 |
| DOX | 500–15.63 | 0.9989 | 15.63 | 4.5 | 90.2 | 0.51 | 17.33 |
| ERY[g] | 500–3.91 | 0.9962 | 3.91 | 4.5 | 90.1 | 0.43 | 4.34 |
| TYL[h] | 500–3.91 | 0.9986 | 3.91 | 4.5 | 60.4 | 6.56 | 6.47 |
| CLA[i] | 500–3.91 | 0.9985 | 3.91 | 4.5 | 96.2 | 0.86 | 4.06 |
| ROX[j] | 500–3.91 | 0.9970 | 7.81 | 4.5 | 93.6 | 1.00 | 8.34 |
| SDZ | 500–7.81 | 0.9995 | 7.81 | 7.5 | 74.9 | 7.82 | 10.43 |
| STZ | 500–7.81 | 0.9991 | 7.81 | 7.5 | 89.3 | 2.31 | 8.75 |
| SDD | 500–7.81 | 0.9979 | 7.81 | 7.5 | 92.7 | 2.77 | 8.43 |
| SMX | 500–7.81 | 0.9991 | 7.81 | 7.5 | 90.0 | 3.75 | 8.68 |
| SDM | 500–7.81 | 0.9978 | 7.81 | 7.5 | 88.8 | 3.41 | 8.80 |
| OFL | 500–7.81 | 0.9999 | 7.81 | 4.5 | 100.0 | 3.03 | 7.81 |
| NOR | 500–3.91 | 0.9996 | 3.91 | 4.5 | 104.4 | 5.95 | 3.75 |
| CIP | 500–3.91 | 0.9990 | 3.91 | 4.5 | 106.3 | 4.84 | 3.68 |
| ENR | 500–3.91 | 0.9996 | 3.91 | 4.5 | 98.0 | 7.09 | 3.99 |
| LIN | 500–3.91 | 0.9997 | 3.91 | 7.5 | 92.5 | 1.42 | 4.23 |
| CLI | 500–3.91 | 0.9997 | 3.91 | 7.5 | 89.6 | 2.29 | 4.36 |

[a] Calibration curves over linear range from ILOQ of each antibiotic to 500 ng mL$^{-1}$ using an internal standard matrix-matched calibration. [b] pH adjustment of the water sample prior to solid phase extraction. [c] Average recovery obtained as a mean value of 8 measurements (4 measurements at both 500 ng L$^{-1}$ and 60 ng L$^{-1}$). [d] Obtained with respect to ILOQ, enrichment factor, and average recovery. [e] Quantified as a sum of peak areas of ERY, ERY–$H_2O_{iso1}$, ERY–$H_2O_{iso2}$, ERY–$H_2O$ + $H_2$, and $ERY_{ag}$. [f] Quantified as a sum of peak areas of TYL and $TYL_{ag}$. [g] Quantified as a sum of peak areas of CLA and $CLA_{ag}$. [h] Quantified as a sum of peak areas of ROX and $ROX_{ag}$.

corresponds with its growing consumption in human medicine in the past decade.[7] Five out of 6 samples were positive for SMX, a frequently prescribed antibiotic in both human and veterinary medicine.[37,38,53] The repetitive occurrence of LIN and CLI observed in the water samples is in accordance with their large consumption in both human and veterinary medicine.[7,37,53] Although tetracyclines belong to highly consumed antibiotics in the veterinary medicine,[37,53] they were absent in the tested samples. This can be explained by their great tendency to form complexes with metal ions, proteins, and silanol groups;[2,39] they

**Table 4** Antibiotic concentrations (ng L$^{-1}$) in influents and effluents of the WWTPs from 6 different localities in the Czech Republic

| | A | | B | | C | | D | | E | | F | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Locality | Inf.[a] | Eff.[a] | Inf. | Eff. | Inf. | Eff. | Inf. | Eff. | Inf. | Eff. | Inf. | Eff. |
| TC | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd |
| OXY | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd |
| CTC | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd |
| DOX | nd | nd | nd | nd | nd | nd | 20.6 | nd | nd | nd | nd | nd |
| ERY | nd | nd | 248.6 | 204.2 | 38.2 | 27.4 | 49.3 | 36.3 | 60.3 | nd | 8.7 | 36.3 |
| TYL | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd |
| CLA | 79.0 | 61.0 | 1287.9 | 289.0 | 952.2 | 422.0 | 979.2 | 273.4 | 237.5 | 85.1 | 1010.4 | 794.2 |
| ROX | nd | nd | 74.8 | 35.7 | nd | nd | nd | nd | nd | nd | nd | nd |
| SDZ | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd |
| STZ | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd |
| SDD | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd |
| SMX | nd | nd | 33.9 | 272.2 | 150.0 | 681.1 | 550.0 | 438.9 | 796.2 | 544.4 | 102.6 | 377.8 |
| SDM | nd | nd | nd | nd | nd | nd | nd | nd | 177.1 | <MLOQ | nd | nd |
| OFL | nd | nd | 232.0 | 138.0 | nd | nd | 138.0 | 113.0 | 485.0 | 283.0 | 67.0 | <MLOQ |
| NOR | nd | nd | nd | nd | nd | nd | 167.6 | 63.0 | 186.8 | 24.2 | 377.4 | 33.2 |
| CIP | nd | nd | 111.0 | 7.53 | nd | nd | 640.6 | 133.6 | 273.8 | 70.7 | 386.6 | 11.8 |
| ENR | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd |
| LIN | nd | nd | 24.3 | 10.8 | nd | nd | 94.1 | 46.4 | 5.0 | 42.2 | 32.7 | 17.7 |
| CLI | nd | nd | 26.8 | 102.1 | 150.7 | 48.4 | 61.9 | 41.9 | 35.2 | nd | 55.3 | 63.6 |

[a] Inf. – WWTP influent, eff. – WWTP effluent, nd – antibiotic not detected.

are therefore more likely to be found in water sediments.[54] The only exception was DOX, which was detected in one sample at a concentration of 20.6 ng $L^{-1}$. The reason was probably its extremely high prescription also in human medicine.[37,38] Therefore there is a likelihood that the real concentration of DOX and other tetracyclines in the environment is much higher.

It follows from the analysis of both the influents and effluents of the WWTPs that the efficiency of water treatment processes is often very poor. The low efficiency of the WWTPs together with the 50–90% excretion of the administered pharmaceutical dose after the treatment[1–4] causes the release and retention of the antibiotics in the water. This phenomenon leads to a constant increase of bacterial resistance and, therefore, a strong need for improvement of the water treatment processes is obvious.

## 4 Conclusions

A UHPLC-ToFMS method was developed for determination of the most prescribed antibiotics in the influents and effluents of WWTPs in the Czech Republic. The validated procedure is characterized by its versatility, trueness, and sensitivity. These parameters, together with the potential of fast UHPLC-ToFMS method development, qualify this assay for screening of trace levels of antibiotics in wastewater and treated water. The method employs the SPE using Oasis HLB extraction sorbent for extraction and concentration of the analytes. Due to the different physico-chemical properties of the tested antibiotics, two extractions and injections were needed for each sample.

The monitoring study revealed a significant concentration of antibiotics in the influents and effluents of WWTPs in the Czech Republic. Close attention should be paid to this phenomenon, because the associated development of bacterial resistance represents a threat to the public health. These repeated alarming findings call for urgent reduction of antibiotic use and especially for the need for significant improvement of the wastewater treatment processes.

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## References

1 M. Seifrtova, J. Aufartova, J. Vytlacilova, A. Pena, P. Solich and L. Novakova, *J. Sep. Sci.*, 2010, **33**, 2094–2108.
2 C. Thorsten, R. J. Schneider, H. A. Färber, D. Skutlarek, M. T. Meyer and H. E. Goldbach, *Acta Hydrochim. Hydrobiol.*, 2003, **31**, 36–44.
3 Q. T. Dinh, F. Alliot, E. Moreau-Guigon, J. Eurin, M. Chevreuil and P. Labadie, *Talanta*, 2011, **85**, 1238–1245.
4 B. Halling-Sorensen, *Arch. Environ. Contam. Toxicol.*, 2001, **40**, 451–460.
5 A. Nikolaou, S. Meric and D. Fatta, *Anal. Bioanal. Chem.*, 2007, **387**, 1225–1234.
6 State Institute for Drug Control, SUKL, June 2012, http://www.sukl.eu/sukl?lang=2.
7 S. Coenen, M. Ferech, S. Malhotra-Kumar, E. Hendrickx, C. Suetens and H. Goossens, *J. Antimicrob. Chemother.*, 2006, **58**, 418–422.
8 N. Adriaenssens, S. Coenen, A. Versporten, A. Muller, G. Minalu, C. Faes, V. Vankerckhoven, M. Aerts, N. Hens, G. Molenberghs and H. Goossens, *J. Antimicrob. Chemother.*, 2011, **66**(suppl. 6), vi37–45.
9 N. Adriaenssens, S. Coenen, A. Versporten, A. Muller, G. Minalu, C. Faes, V. Vankerckhoven, M. Aerts, N. Hens, G. Molenberghs and H. Goossens, *J. Antimicrob. Chemother.*, 2011, **66**(suppl. 6), vi3–12.
10 N. Adriaenssens, S. Coenen, A. Versporten, A. Muller, G. Minalu, C. Faes, V. Vankerckhoven, M. Aerts, N. Hens, G. Molenberghs and H. Goossens, *J. Antimicrob. Chemother.*, 2011, **66**(suppl. 6), vi47–56.
11 M. Carballa, F. Omil, J. M. Lema, M. Llompart, C. Garcia-Jares, I. Rodriguez, M. Gomez and T. Ternes, *Water Res.*, 2004, **38**, 2918–2926.
12 P. Vazquez-Roig, V. Andreu, M. Onghena, C. Blasco and Y. Pico, *Anal. Bioanal. Chem.*, 2011, **400**, 1287–1301.
13 B. Kasprzyk-Hordern, R. M. Dinsdale and A. J. Guwy, *J. Chromatogr., A*, 2007, **1161**, 132–145.
14 P. H. Roberts and K. V. Thomas, *Sci. Total Environ.*, 2006, **356**, 143–153.
15 E. Zuccato, S. Castiglioni and R. Fanelli, *J. Hazard. Mater.*, 2005, **122**, 205–209.
16 A. Gobel, A. Thomsen, C. S. McArdell, A. Joss and W. Giger, *Environ. Sci. Technol.*, 2005, **39**, 3981–3989.
17 E. M. Golet, I. Xifra, H. Siegrist, A. C. Alder and W. Giger, *Environ. Sci. Technol.*, 2003, **37**, 3243–3249.
18 M. Clara, B. Strenn, O. Gans, E. Martinez, N. Kreuzinger and H. Kroiss, *Water Res.*, 2005, **39**, 4797–4807.
19 D. Bendz, N. A. Paxeus, T. R. Ginn and F. J. Loge, *J. Hazard. Mater.*, 2005, **122**, 195–204.
20 R. H. Lindberg, P. Wennberg, M. I. Johansson, M. Tysklind and B. A. Andersson, *Environ. Sci. Technol.*, 2005, **39**, 3421–3429.
21 S. Zorita, L. Martensson and L. Mathiasson, *Sci. Total Environ.*, 2009, **407**, 2760–2770.
22 M. Gros, M. Petrovic and D. Barcelo, *Talanta*, 2006, **70**, 678–690.
23 H. W. Leung, T. B. Minh, M. B. Murphy, J. C. Lam, M. K. So, M. Martin, P. K. Lam and B. J. Richardson, *Environ. Int.*, 2012, **42**, 1–9.
24 M. A. Locatelli, F. F. Sodre and W. F. Jardim, *Arch. Environ. Contam. Toxicol.*, 2011, **60**, 385–393.
25 I. Ferrer, J. A. Zweigenbaum and E. M. Thurman, *J. Chromatogr., A*, 2010, **1217**, 5674–5686.
26 EPA Method 1694, *EPA-821-R-08-002*, Pharmaceuticals and personal care products in water, soil, sediment, and biosolids by HPLC/MS/MS, 2007.
27 A. L. Batt, M. S. Kostich and J. M. Lazorchak, *Anal. Chem.*, 2008, **80**, 5021–5030.
28 G. Hamscher, S. Sczesny, H. Hoper and H. Nau, *Anal. Chem.*, 2002, **74**, 1509–1518.
29 B. Shao, D. Chen, J. Zhang, Y. Wu and C. Sun, *J. Chromatogr., A*, 2009, **1216**, 8312–8318.

 

30 M. Ibáñez, C. Guerrero, J. V. Sancho and F. Hernández, *J. Chromatogr., A*, 2009, **1216**, 2529–2539.
31 A. Kaufmann, P. Butcher, K. Maden and M. Widmer, *Anal. Chim. Acta*, 2007, **586**, 13–21.
32 A. Kaufmann, P. Butcher, K. Maden and M. Widmer, *J. Chromatogr., A*, 2008, **1194**, 66–79.
33 A. A. M. Stolker, P. Rutgers, E. Oosterink, J. J. P. Lasaroms, R. J. B. Peters, J. A. van Rhijn and M. W. F. Nielen, *Anal. Bioanal. Chem.*, 2008, **391**, 2309–2322.
34 R. Diaz, M. Ibanez, J. V. Sancho and F. Hernandez, *Anal. Methods*, 2012, **4**, 196–209.
35 B. Kasprzyk-Hordern, R. M. Dinsdale and A. J. Guwy, *Talanta*, 2008, **74**, 1299–1312.
36 J. Zrostlikova, J. Hajslova, J. Poustka and P. Begany, *J. Chromatogr., A*, 2002, **973**, 13–26.
37 European Medicines Agency, *Trends in sales of veterinary antimicrobial agents in nine European countries, reporting period 2005–2009*, 2011, http://www.ema.europa.eu/docs/en_GB/document_library/Report/2011/09/WC500112309.pdf.
38 S. Coenen, N. Adriaenssens, A. Versporten, A. Muller, G. Minalu, C. Faes, V. Vankerckhoven, M. Aerts, N. Hens, G. Molenberghs and H. Goossens, *J. Antimicrob. Chemother.*, 2011, **66**(suppl. 6), vi57–70.
39 J. Zhu, D. D. Snow, D. A. Cassada, S. J. Monson and R. F. Spalding, *J. Chromatogr., A*, 2001, **928**, 177–186.
40 B. Halling-Sorensen, G. Sengelov and J. Tjornelund, *Arch. Environ. Contam. Toxicol.*, 2002, **42**, 263–271.
41 R. Hirsch, T. Ternes, K. Haberer and K. L. Kratz, *Sci. Total Environ.*, 1999, **225**, 109–118.
42 S. Omura, *Macrolide Antibiotics: Chemistry, Biology, and Practice*, Academic Press, San Diego, 2nd edn, 2002.
43 ASTM, *Standard Practice for Sampling Water*, ASTM Annual Book of Standards, ASTM, Philadelphia, 1980.
44 CDER/CBER, US Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research, Center for Biologics Evaluation and Research, 2001, Guidance for Industry: Bioanalytical method validation: http://www.fda.gov/CDER/GUIDANCE/4252fnl.pdf.
45 S. Babić, A. J. M. Horvat, D. M. Pavlović and M. Kaštelan-Macan, *Trends Anal. Chem.*, 2007, **26**, 1043–1061.
46 L. Tong, P. Li, Y. Wang and K. Zhu, *Chemosphere*, 2009, **74**, 1090–1097.
47 R. Wei, F. Ge, S. Huang, M. Chen and R. Wang, *Chemosphere*, 2011, **82**, 1408–1414.
48 Q. T. Dinh, F. Alliot, E. Moreau-Guigon, J. Eurin, M. Chevreuil and P. Labadie, *Talanta*, 2001, **85**, 1238–1245.
49 B. Li, T. Zhang, Z. Xu and H. H. Fang, *Anal. Chim. Acta*, 2009, **645**, 64–72.
50 F. Gosetti, E. Mazzucco, D. Zampieri and M. C. Gennaro, *J. Chromatogr., A*, 2010, **1217**, 3929–3937.
51 T. Tylova, J. Olsovska, P. Novak and M. Flieger, *Chemosphere*, 2010, **78**, 353–359.
52 M. Carballa, F. Omil, J. M. Lema, M. Llompart, C. Garcia, I. Rodriguez, M. Gomez and T. Ternes, *Water Sci. Technol.*, 2005, **52**, 29–35.
53 European Medicines Agency, *European Surveillance of Veterinary Antimicrobial Consumption*, 2012, http://www.ema.europa.eu/ema/index.jsp?curl=pages/regulation/document_listing/document_listing_000302.jsp&mid=WC0b01ac0580153a00&jsenabled=true.
54 S. C. Kim and K. Carlson, *Anal. Bioanal. Chem.*, 2007, **387**, 1301–1315.

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ORIGINAL PAPER

# The UHPLC-DAD fingerprinting method for analysis of extracellular metabolites of fungi of the genus *Geosmithia* (Acomycota: Hypocreales)

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**Abstract** A new simple ultra-high-performance liquid chromatography method with diode array detection (UHPLC-DAD) was developed for chemical fingerprinting analysis of extracellular metabolites in fermentation broth of *Geosmithia* spp. The SPE method employing Oasis MCX strong cation-exchange mixed-mode polymeric sorbent was chosen for extraction of the metabolites. The analyses were performed on an Acquity UPLC BEH C18 column (100×2.1 mm i.d.; particle size, 1.7 μm; Waters) using a gradient elution program with an aqueous solution of trifluoroacetic acid and acetonitrile as the mobile phase. The applicability of the method was proved by analysis of 38 strains produced by different species and isolated from different sources (hosts). The results revealed the correlation of obtained UHPLC-DAD fingerprints with taxonomical identity.

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**Keywords** Ultra-high-performance liquid chromatography · Diode array detection · Solid-phase extraction · Extracellular secondary metabolites · *Geosmithia* spp. · Chromatographic fingerprinting

## Introduction

Filamentous fungi of the genus *Geosmithia* (Ascomycota: Hypocreales) are little known although they represent frequent and worldwide broad type symbionts of bark beetles (Coleoptera: Curculionidae, Scolytinae). They include common associates of bark beetles and ambrosia beetles [1] and recently recognized plant parasitic species, *Geosmithia morbida* [2]. In general, symbiotic fungi enter complex chemical-based interactions with their hosts and thus possess a diversity of secondary metabolites (SM) with various biological activities, which makes them a proper target for bioprospecting [3]. It is supposed that the production of SM will reflect both the phylogenetical and ecological relatedness of their producers.

A collection of strains from 22 published species [2] and at least 20 more unpublished species of the genus *Geosmithia* is maintained at the Institute of Microbiology, Academy of Sciences of the Czech Republic and in Culture Collection of Fungi in Prague. For updating of taxonomic overview of these strains and/or for studying the diversity and ecological role of SM, chromatographic fingerprinting (CFP) can be employed. CFP is an effective and rational method based on comparison of fingerprints (chromatograms) of the many compounds found in highly complex samples. CFP can provide the whole profile of not only the marker compounds but also the unknown components [4, 5]. These fingerprints can then be used as an effective tool for comparison, classification, or identification of samples

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and have found widespread use in, e.g., chemotaxonomic characterization of microorganisms [6], in quality assessment [7–9], or in flavor research [10].

Several chromatographic techniques such as high-performance liquid chromatography (HPLC) [7, 8, 11–14], ultra-high-performance liquid chromatography (UHPLC) [5], gas chromatography (GC) [15], capillary electrophoresis (CE) [16], and thin-layer chromatography (TLC) [17] have been used for fingerprinting. Due to the high complexity of the studied matrix, additional and complementary information is often required. Therefore in 2000, Luo et al. proposed a solution of multi-dimensional and multi-informational fingerprint by hyphenated techniques such as HPLC with diode array detection (DAD), CE-DAD, GC with mass spectrometric detection (MS), HPLC-MS, HPLC-DAD-MS/MS, CE-MS, CE-DAD-MS/MS, and HPLC with nuclear magnetic resonance detection (NMR) [18]. With additional spectral information, these hyphenated techniques give a much more complete profile of the investigated samples [14]. Among all the detection techniques of hyphenated instruments, tandem mass spectrometry is generally considered as one of the best tools for CFP due to its selectivity and powerful ability of structural qualification [19–21]. On the other hand, DAD is widely applied to simultaneous determination [22], peak purity checking [23], chromatographic discrepancy correction [24], and identification of chromatographic peaks [9]. Moreover, both HPLC-DAD and UHPLC-DAD belong to very common hyphenated instruments. Therefore, both these techniques represent useful methods for CFP of SM produced in fungal fermentation broth.

Since the fungal fermentation broth contains many compounds that have to be separated during the analysis, the analysis run time generally needed on HPLC is about 1 h [7, 8, 13]. In comparison, the UHPLC allows faster separations on column materials at high pressures of up to 100 MPa using sub-2 μm particles yielding higher separation efficiencies and shorter run times [25] and represent, therefore, more effective technique for CFP.

The reproducible extraction method represents an integral part of the final CFP assay, because purification and pre-concentration of SM with diverse physicochemical properties presented in the complex matrix is needed in order to obtain representative fingerprints [26]. Liquid–liquid extraction (LLE) belongs to the most frequent methods used for fungal fermentation broth sample preparation [26–30]. Solid-phase extraction (SPE) was used for fungal SM extraction less often and was employed either directly [31–34] or as the second extraction step that followed after LLE [35–37]. To the best of our knowledge, SPE has never been used as an original method for extraction of SM from fungal fermentation broth for the CFP analysis.

The aim of this study was to develop an analytical method for CFP analysis of *Geosmithia* spp. Firstly, the fast and simple SPE method was employed for extraction of a wide spectrum of extracellular SM presented in fungal fermentation broth. Secondly, an UHPLC method with DAD detection was developed for the analysis of fungal extracts. The obtained UHPLC-DAD fingerprints were then evaluated to reveal, e.g., taxonomical, host specific, or ecological relatedness of secondary metabolites production.

## Experimental

### Chemicals and materials

Acetonitrile (ACN), methanol (MeOH), and trifluoroacetic acid (99.95%; TFA) were of LC/MS grade and were obtained from Biosolve (the Netherlands). Hexane, diethyl ether, dichloromethane, and ethyl acetate were all p.a. grade and were purchased from Lach-Ner (Czech Republic). Acetic acid (99+%) and ammonium hydroxide A.C.S. reagent (29% aqueous $NH_4OH$ solution) were obtained from Sigma-Aldrich (Germany), and formic acid (98–100%) was purchased from Merck (Germany). Ultrapure water was prepared using Milli-Q water purification system (18.2 MΩ, Millipore, USA).

The SPE sorbents used for sample extraction were: Oasis HLB, Oasis MAX, Oasis MCX, Oasis WAX, Oasis WCX, and Sep-Pack C-18 (all were purchased from Waters, Czech Republic); Amberlite XAD-2 and Amberlite XAD-4 (both from Supelco, USA); and Strata C18-E and Strata $NH_2$ (both from Phenomenex, USA). SPE 12-position vacuum manifold was obtained from Phenomenex (USA).

The analytical UHPLC columns used were: Acquity UPLC BEH C18 (50×2.1 mm i.d.; 1.7 μm), Acquity UPLC BEH C18 (100×2.1 mm i.d.; 1.7 μm), Acquity UPLC BEH Shield RP (50×2.1 mm i.d.; 1.7 μm), and Acquity UPLC BEH HILIC (50×2.1 mm i.d.; 1.7 μm); all purchased from Waters.

### Fungal strains, cultivation conditions

Thirty-eight monosporic strains representing 28 species were used (Table 1). Cultures were maintained on malt agar slants (malt extract, 20 g/l and agar, 20 g/l) and cultivated on malt extract medium (malt extract, 20 g/l; glucose, 20 g/l; and peptone 1 g/l). The submerged cultivations were done

**Table 1** Overview and characterisation of 38 genus *Geosmithia* species analyzed using developed chromatographic fingerprintig method

| Strain No. | Species identification | Reference |
|---|---|---|
| CCF3754 | *G. eupagioceri* | [1] |
| CCF3334 | *G. fassatiae* | [38] |
| CCF3333 | *G. flava* | [39] |
| MK1683 | *G. langdonii* | [38] |
| CCF3861 | *G. microcorthyli* | [1] |
| 1259 | *G. morbida* | [2] |
| 1272 | *G. morbida* | [2] |
| CCF3425 | *G. obscura* | [38] |
| MK1510 | *G. pallida* sp. 2 | [39] |
| RJ137m | *G. pallida* sp. 5 | Unpublished |
| MK1807 | *G. pallida* sp. 23 | Unpublished |
| CCF3342 | *G. putterillii* | [40] |
| CCF3751 | *G. rufescens* | [1] |
| CCF3660 | *Geosmithia* sp. 1 | [37, 40] |
| MK1712a | *Geosmithia* sp. 8 | [40] |
| MK263 | *Geosmithia* sp. 8 | [40] |
| CCF3350 | *Geosmithia* sp. 8 | [40] |
| RJ10m | *Geosmithia* sp. 9 | [40] |
| CCF3560 | *Geosmithia* sp. 10 | [40] |
| CCF3555 | *Geosmithia* sp. 11 | [40] |
| U16A26 | *Geosmithia* sp. 13 | [40] |
| MK1665 | *Geosmithia* sp. 21 | [41] |
| MK1759 | *Geosmithia* sp. 21 | [41] |
| RJ06ka | *Geosmithia* sp. 24 | Unpublished |
| MK1842 | *Geosmithia* sp. 24 | Unpublished |
| MK1796 | *Geosmithia* sp. 26 | Unpublished |
| RJ0919 | *Geosmithia* sp. 27 | Unpublished |
| MK1820 | *Geosmithia* sp. 29 | [1] |
| RJ15ak | *Geosmithia* sp. 31 | Unpublished |
| MK1793 | *Geosmithia* sp. 31 | Unpublished |
| MK1811a | *Geosmithia* sp. 31 | Unpublished |
| RJ74k | *Geosmithia* sp. 31 | Unpublished |
| MK1834 | *Geosmithia* sp. 32 | Unpublished |
| MK1826 | *Geosmithia* sp. 32 | Unpublished |
| MK1835 | *Geosmithia* sp. 33 | Unpublished |
| MK1827b | *Geosmithia* sp. 33 | Unpublished |
| MK1811 | *Geosmithia* sp. 36 | Unpublished |
| MK1806 | *Geosmithia* sp. 37 | Unpublished |

in 250-ml Erlenmeyer flasks on a rotary shaker (3.4 Hz) for the period of 14 days at 24 °C in the dark. All strains are maintained in the Culture Collection of Fungi (CCF codes) or in the laboratory of the senior author (others). The blank matrix sample was prepared under same conditions with the absence of fungal inoculum.

## Sample preparation

After the 14-day cultivation, the fermentation broth was centrifuged (15 min at 4,000×*g*) and then filtered through 2 μm glass microfibre filter (Whatman, UK). Prior to SPE, the pH of the samples was adjusted to 3 with formic acid (98–100%). SPE was performed using 60 mg Oasis MCX cartridges. The sorbent was conditioned with 2 mL of MeOH followed by equilibration with 2 mL of Milli-Q water; 50 mL of fermentation broth was passed through the cartridge at the flow rate of 3 ml $min^{-1}$, and the sorbent was then rinsed with 2 mL of water and 2 mL of formic acid–water (1:99, *v*/*v*). Afterwards, the cartridge was air-dried and the SM were eluted using 2 mL of MeOH. The MeOH extracts were evaporated to dryness and reconstituted in 100 μL of MeOH.

## UHPLC instrumentation and chromatographic conditions

The UHPLC-DAD analyses were carried out on Waters Acquity UPLC System (Waters, Prague, Czech Republic) consisting of Acquity UPLC Solvent Manager, Acquity UPLC Sample Manager, Acquity UPLC Column Heater/cooler, and Acquity UPLC Diode Array Detector (set at 200–600 nm). Empower 2 software was used for data processing. Analyses were performed on Acquity UPLC BEH C18 column (100×2.1 mm i.d.; particle size, 1.7 μm; Waters) with the mobile phase flow rate of 0.4 ml $min^{-1}$ and column temperature of 25 °C. The mobile phase consisted of (A) TFA–water (0.1:99.9, *v*/*v*) and (B) ACN with the gradient elution (min/% A): 0/95, 15/65, 25/0, and 27/0, followed by a 2-min equilibration step. The samples were kept at 5 °C, and the injection volume was 1 μL.

## Method reproducibility and repeatability

Validation tests of reproducibility and repeatability were performed to ensure the validity of the developed method [42]. Ten major and well-separated peaks in sample CCF3333 were selected and used for validation tests. The reproducibility of the chromatographic data was investigated by system suitability evaluation that was assessed by six replicated injections of an identical CCF3333 sample. The repeatability of the analytical method was assessed by analysis of four independently prepared samples of CCF3333 fermentation broth. The reproducibility and repeatability of the method were expressed as repeatability of retention times and peak areas (relative standard deviation (RSD%)).

### Sample stability

Samples MK1712a, RJ74k, MK1835, CCF3333, 1259, and CCF3555 were selected for sample stability determination in order to obtain information about stability of various unknown compounds produced by different species (see Table 1). Samples were divided into identical aliquots, stored at room temperature (short-term stability) or at −20 °C (long-term stability), and analyzed after 0, 2, 4, 8, 12, and 24 h or after 0, 1, 2, 3, 4, 7, and 14 days, respectively. In each sample, ten major peaks were selected and their peak areas repeatability (RSD%) was investigated.

## Results and discussion

### Sample preparation optimization

The extraction method was developed using six strains representing six different species of *Geosmithia* spp. (MK1712a, RJ74k, MK1835, CCF3333, 1259, and CCF3555; see Table 1), which differ in the SM production, and therefore epitomize the representative set of samples for extraction method development. Five solvents of rising polarity were tested for LLE; namely hexane, diethyl ether, dichloromethane, ethyl acetate, acetic acid-ethyl acetate (1:20, *v*/*v*). Afterwards, different sorbents for SPE were evaluated; namely Amberlite XAD-2, Amberlite XAD-4, Strata C18-E, Strata $NH_2$, Sep-Pack C-18, Oasis HLB, Oasis MAX, Oasis MCX, Oasis WAX, and Oasis WCX. The comparison of extracted samples with un-extracted samples analyzed directly after filtration and centrifugation of the crude fermentation broth was used in order to verify that the loss of monitored major SM was minimal.

An example of comparison of LLE and SPE procedures for sample RJ74k is shown in Figs. 1 and 2, respectively. Although LLE belongs to the most frequented method used for extraction of SM from fungal fermentation broth [26–30, 37], this method was not found to be suitable for extraction of SM of *Geosmithia* strains. It is obvious from comparison of LLE and SPE procedures in Figs. 1 and 2 that, unlike the SPE methods, none of the solvents used for LLE was able to extract both polar and less-polar compounds in one extraction step. The early eluting peaks ($t_R$=1.5 min) in diethyl ether, ethyl acetate, and acetic acid-ethyl acetate (1:20, *v*/*v*) fractions, respectively, represent solvent peaks and did not originate from the fermentation broth. This fact was confirmed by analysis of the blank solvent sample (pure solvent). Moreover, in general, the peak shapes and their intensity in retention window ranging from 16 to 21 min are evidently much better using SPE than with LLE. SPE proved to be a method characterized by high throughput, robustness, and low solvent consumption. A wide range of SPE resin chemistries is now available for various applications, and therefore SPE provide improved assay standardization and hence better reproducibility [43]. The comparison of several tested SPE sorbents is shown in Fig. 2. Oasis MCX, Oasis HLB, and Strata C18-E were found to be appropriate sorbents because of the greatest number of SM presented in the extract. Finally, the Oasis MCX sorbent was chosen as the best alternative with respect to the peak intensities and shapes (for extraction protocol see "Sample preparation.").

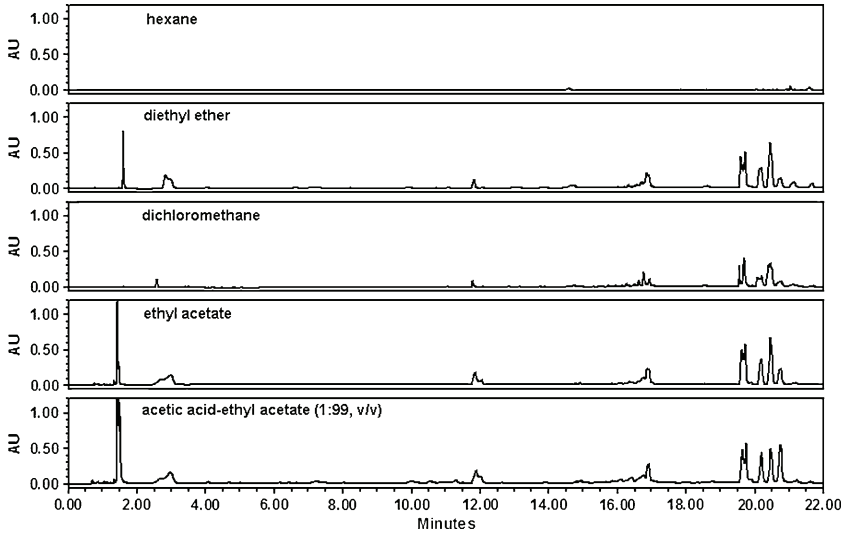


**Fig. 1** The UHPLC-DAD analyses of extracts obtained with liquid–liquid extraction of RJ74k fermentation broth using different extraction solvents. *Chromatographic conditions:* Acquity UPLC BEH C18 column (100×2.1 mm); mobile phase (**A**) TFA–water (0.1:99.9, *v*/*v*) and (**B**) ACN; flow rate, 0.4 mL $min^{-1}$; column temperature, 25 °C; sample temperature, 10 °C; injection volume, 1 μl; gradient elution (min/% A), 0/95, 15/65, 25/0, 27/0; UV, 260 nm

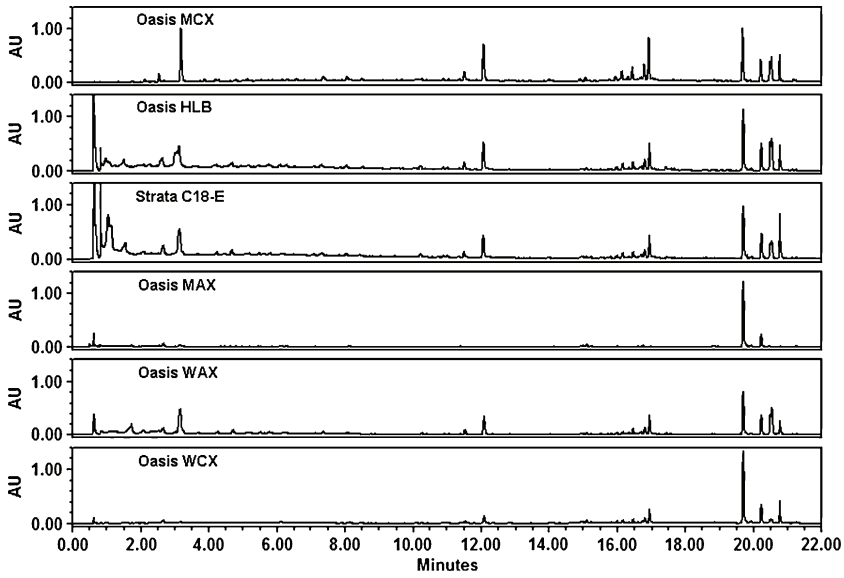


**Fig. 2** The UHPLC-DAD analyses of extracts obtained with solid-phase extraction of RJ74k fermentation broth using different extraction sorbents. For chromatographic conditions, see Fig. 1

The generic Oasis MCX method for extraction of basic compounds (http://www.waters.com/webassets/cms/library/docs/lcSP.pdf) consists of two elution steps; namely MeOH (elute 1) and $NH_4OH$–MeOH (5:95, *v*/*v*; elute 2). In case of extraction of SM from *Geosmithia* fermentation broth, the elute 2 was finally eliminated, because $NH_4OH$–MeOH fraction did not contain any compounds absorbing in the wavelength range from 200 to 600 nm (data not shown). According to the generic Oasis MCX method (http://www.waters.com/webassets/cms/library/docs/lcSP.pdf), the presence of weaker acids and neutral compounds is expected in elute 1, thus SM extracted from *Geosmithia* fermentation broth are probably of this character. This presumption is supported by the fact that the pH values of all tested fermentation broths ranged from 3.35 to 5.48, while the pH of the blank matrix sample was 5.52.

## UHPLC-DAD method optimization

The main demands on the new UHPLC-DAD method was to separate well a great number of SM differing in polarity in a relatively short analysis time (about 30 min). Six selected strains of *Geosmithia* spp., namely MK1712a, RJ74k, MK1835, CCF3333, 1259, and CCF3555 were used for UHPLC-DAD method development. Since the majority of the presented compounds (SM) showed maximal absorbance at 260 nm, this wavelength was used during the evaluation of the method conditions.

Four analytical UHPLC columns, namely Acquity UPLC BEH C18 (50×2.1 mm i.d.; 1.7 μm), Acquity UPLC BEH C18 (100×2.1 mm i.d.; 1.7 μm), Acquity UPLC BEH Shield RP (50×2.1 mm i.d.; 1.7 μm), and Acquity UPLC BEH HILIC (50×2.1 mm i.d.; 1.7 μm) were evaluated and compared. The length of the column was found to be the most significant parameter affecting the quality of the analysis. All 50-mm length columns had approximately comparable results, while the Acquity BEH C18 column of 100-mm length provides sharper and better separated peaks with higher retention of separated components. The parameters as peaks retention times, peaks shapes and resolution, and the total analysis time were calculated and compared (data not shown). The example of comparison of tested columns is shown in Fig. 3.

Several aqueous components of mobile phases such as strongly acidic (0.1% and 0.05% TFA, 0.1% formic acid, 0.1% acetic acid, and 0.1% phosphoric acid), acidic (1 mM ammonium formate, pH 4.0) and alkaline (1 and 5 mM ammonium acetate, 1 mM ammonium formate, and all of pH 9.0) were assessed; the selected results are shown in Fig. 4. Although the analyses using 0.1% TFA and 1 mM ammonium formate of pH 4.0 as mobile phase, respectively, were comparable, better peak shapes and longer retention times of early eluting peaks were observed with 0.1% TFA. When alkaline mobile phase was used, the decrease in quality on analysis was evident. For instance, while the selected peaks *a*, *b*, and *c* were sharp and well separated in strongly acidic and acidic mobile phase, under alkaline conditions the significant increase of peak widths was observed. Moreover, the peaks *b* and *c* coeluted using alkaline mobile phase (Fig. 4).

Concerning organic modifiers, ACN and MeOH were tested. ACN provided sharper peaks than MeOH (data not shown). Since very similar results were obtained for the

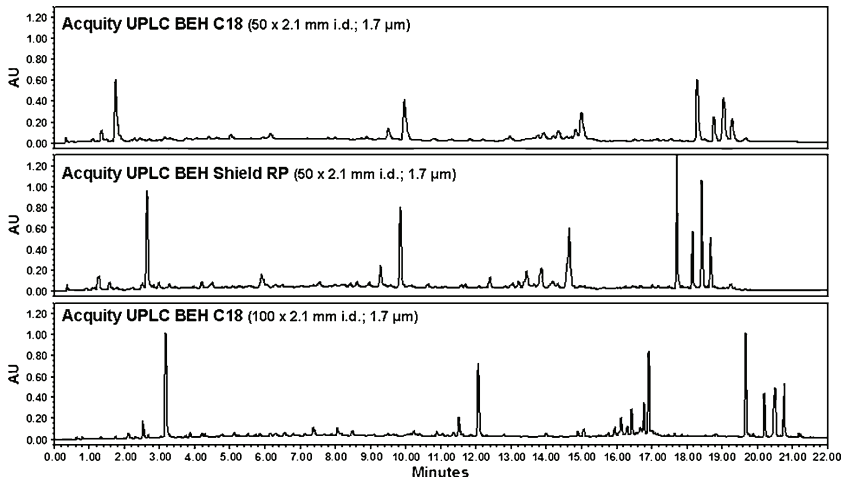


**Fig. 3** Different analytical columns used for UHPLC-DAD analysis of RJ74k strain fermentation broth. For other chromatographic conditions, see Fig. 1

other strains, the mixture of TFA–water (0.1:99.9, *v*/*v*) with ACN was chosen as the final mobile phase.

Several gradient programs based on multiple stepwise increase of the ACN ratio were applied and final gradient (min/% A): 0/95, 15/65, 25/0, 27/0 was chosen with respect to resolution of major compounds, peak shapes, and total analysis time (29 min), which was about half that used in the HPLC fingerprinting methods recently described [7–9, 13, 14]. Similar analysis time shortening was obtained by Liu et al. [5] after an optimization of UHPLC method for CFP analysis of 20 bioactive components in plant material.

The effect of column temperature on analysis was tested in the range from 25 °C to 45 °C with 5 °C steps. The analysis time decreased with rising column temperature, which led to a worse separation of the first eluting peaks. For this reason, the column temperature of 25 °C was chosen as a final condition.

## Method reproducibility and repeatability

The results of reproducibility and repeatability tests are listed in Table 2. The RSD% values of retention times and peak areas of ten selected peaks (Fig. 5) in six replicated injections (reproducibility) ranged from 0.01% to 0.05% and from 0.41% to 0.75%, respectively.

The RSD% values of retention times and peak areas of ten selected peaks in four independently prepared CCF3333 samples (repeatability) did not exceed 0.25% and 1.59%, respectively. These results confirm that the developed analytical method is reproducible and repeatable, and therefore, it is applicable for CFP analyses.

## Sample stability

The corresponding RSD% values of ten selected peak areas in each chromatogram were less than 3.20% and 3.91% in short-

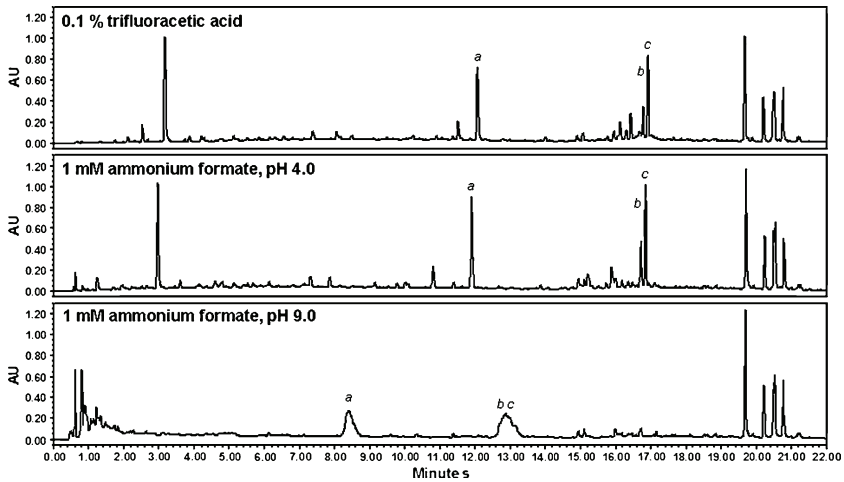


**Fig. 4** Different mobile phase composition for UHPLC-DAD analysis of RJ74k strain fermentation broth. *a*–*c* Peaks evaluated during the method development. For other chromatographic conditions, see Fig. 1

**Table 2** The reproducibility and repeatability of the analytical method demonstrated as repeatability of retention times and peak areas of ten selected peaks in strain CCF3333

| Peak no. | $t_R$ (min) | UHPLC system suitability[a] | | Method repeatability[b] | |
|---|---|---|---|---|---|
| | | $t_R$ repeatability (RSD%) | Area repeatability (RSD%) | $t_R$ repeatability (RSD%) | Area repeatability (RSD%) |
| 1 | 2.562 | 0.05 | 0.74 | 0.22 | 1.10 |
| 2 | 3.208 | 0.02 | 0.41 | 0.25 | 1.22 |
| 3 | 4.330 | 0.03 | 0.67 | 0.11 | 1.59 |
| 4 | 6.582 | 0.04 | 0.58 | 0.08 | 0.93 |
| 5 | 10.421 | 0.02 | 0.54 | 0.05 | 1.33 |
| 6 | 10.751 | 0.02 | 0.62 | 0.05 | 0.96 |
| 7 | 12.257 | 0.02 | 0.58 | 0.06 | 0.72 |
| 8 | 13.235 | 0.03 | 0.75 | 0.05 | 0.88 |
| 9 | 13.580 | 0.02 | 0.63 | 0.05 | 1.57 |
| 10 | 17.079 | 0.01 | 0.53 | 0.04 | 0.76 |

[a] System suitability was assessed by repetitive analysis of strain CCF3333 sample ($n$=6)

[b] Method repeatability was assessed by analysis of independently prepared samples of CCF3333 fermentation broth ($n$=4)

For chromatographic conditions and peak numbering, see Figs. 1 and 5, respectively

term and long-term stability tests, respectively, which indicated that all tested samples were stable (data not shown).

## Method application

The developed UHPLC-DAD fingerprinting method was used for analysis of 38 strains of *Geosmithia* spp. and blank matrix sample. Generated 3D chromatograms represent multi-dimensional and multi-informational chromatographic fingerprints and every compound in the extract is thus characterized by its retention time (corresponding to its polarity), spectral profile (UV/VIS spectra of wavelengths in the range from 200 to 600 nm), and concentration (peak area). The peaks originating strictly from the matrix were identified using analysis of blank matrix sample and were excluded from SM data file.

The example of 3D chromatograms of strains CCF3333 and CCF3660 (the known producer of hydroxylated antraquinones [44]) are shown in Fig. 6. These strains belong to different species (*Geosmithia flava* and *Geosmithia* sp. 1, respectively; see Table 1) and the production of different SM are obvious according to presented compounds and their additional spectral information.

Figure 7 shows the overlay of 2D chromatograms of strain RJ74k and MK1793 belonging to the same *Geosmithia* sp. 31 (see Table 1). For the simplicity, the chromatograms were extracted at 260 nm and their overlay reveals the production of similar SM. This statement was proved by correspondence

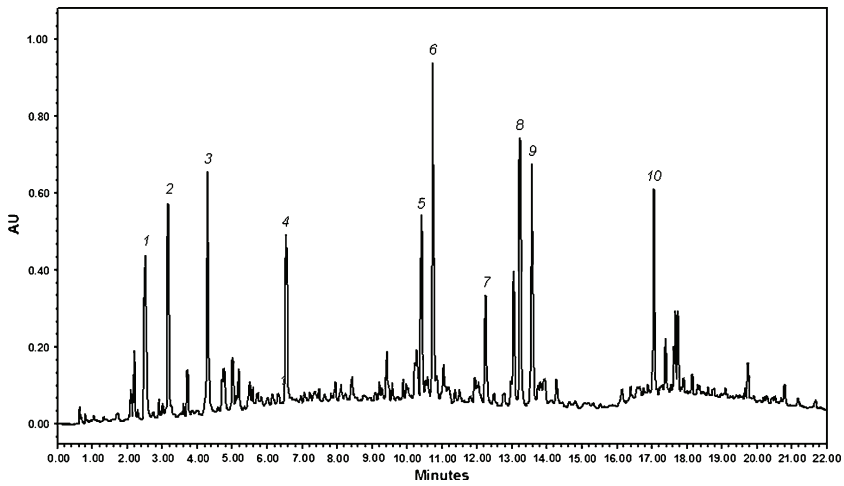


**Fig. 5** The UHPLC-DAD analysis of CCF3333 strain fermentation broth under optimal conditions. *1–10* Peaks selected and used in method reproducibility and repeatability tests. For chromatographic conditions, see Fig. 1

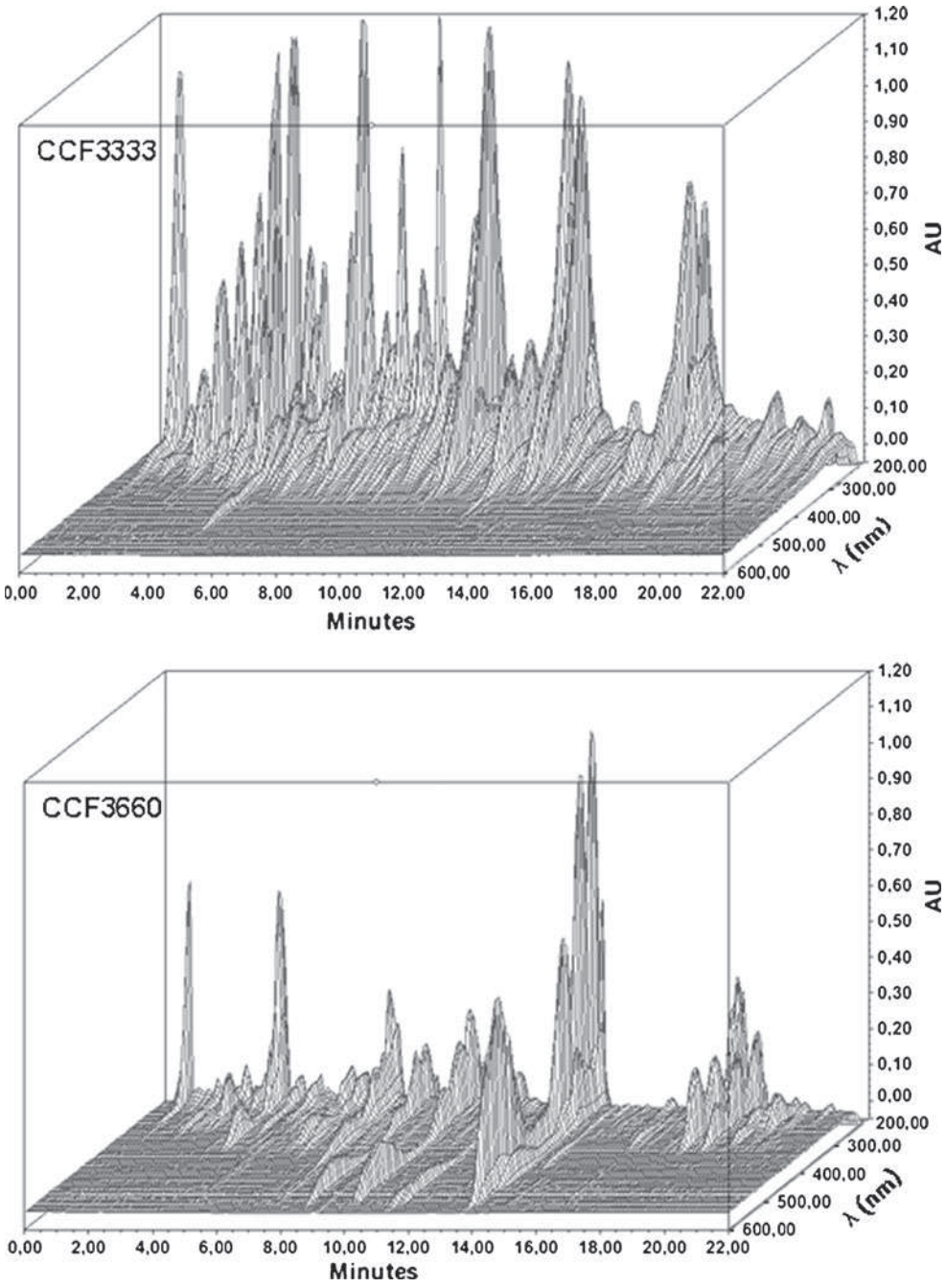


**Fig. 6** UHPLC-DAD 3D chromatograms of strain CCF3333 and CCF3660 fermentation broths. UV detection in the range from 200 to 600 nm, and for other chromatographic conditions, see Fig. 1

of retention times and spectral characteristics of 12 selected major SM (for $\lambda_{max}$ see Fig. 7). The same correlation was observed among all tested strains, for instance in *Geosmithia* sp. 8 (MK1712a and MK263), *Geosmithia* sp. 32 (MK1826 and MK1834), *G. morbida* (1259 and 1272), and *Geosmithia* sp. 24 (MK1842 and RJ06ka); the overlays of chromatograms extracted at 260 nm are shown in Fig. 8. The overall pattern of obtained data therefore suggests obvious correlation of generated 3D chromatograms with taxonomical identity.

It is supposed that the compounds having general occurrence within the genus or compounds correlating with the particular ecological trait can be traced by this method. According to the frequency of production some compounds were found to be characteristic for the whole genus. For instance, one of the most frequent SM with $t_R$= 6.273 min and $\lambda_{max}$ (206, 261, 319) nm was presented in 24 out of 38 species. On the other hand, some SM were found to be characteristic only for a selected species; e.g., peak with $t_R$=11,831 min and $\lambda_{max}$ (200, 256, 321) nm presented only in strains of *G. morbida*, or peak with $t_R$=12.537 min with $\lambda_{max}$ (262, 321) nm found strictly in strains belonging to *Geosmithia* sp. 9. Such characteristic SM with taxonomical information is supposed to be responsible for the

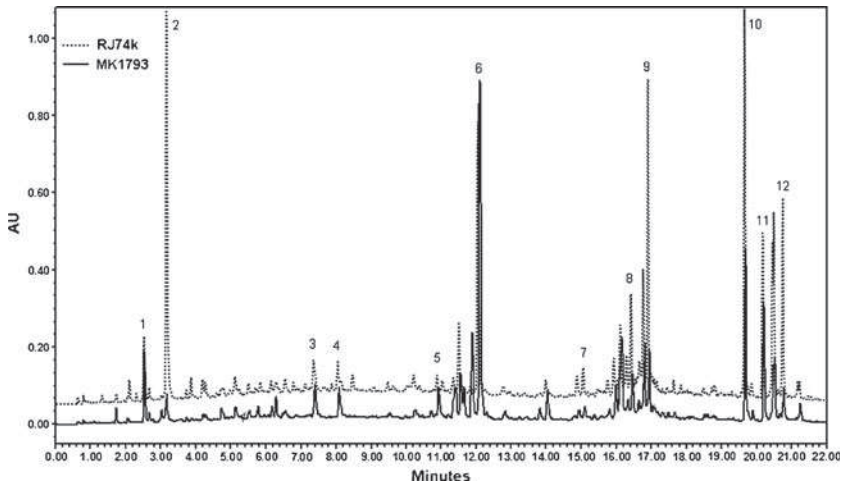


**Fig. 7** The overlay of UHPLC-DAD 2D chromatograms of strains RJ74k and MK1793. *1–12* Selected major peaks with spectral characteristics. UV, 260 nm; for other chromatographic conditions, see Fig. 1

| Peak No. | $t_R$ (min) | $\lambda_{max}$ (nm) MK1793 | $\lambda_{max}$ (nm) RJ74k |
|---|---|---|---|
| 1 | 2.529 | (215,253) | (217,253) |
| 2 | 3.184 | (200,256) | (200,254) |
| 3 | 7.402 | (212,220,282) | (212,219,282) |
| 4 | 8.092 | (207,269) | (210,266) |
| 5 | 10.931 | (218,269) | (216,269) |
| 6 | 12.103 | (267) | (267) |
| 7 | 14.938 | (250,336) | (250,339) |
| 8 | 16.462 | (266,420) | (266,420) |
| 9 | 16.942 | (265) | (265) |
| 10 | 19.687 | (265) | (265) |
| 11 | 20.208 | (264) | (264) |
| 12 | 20.772 | (265) | (265) |

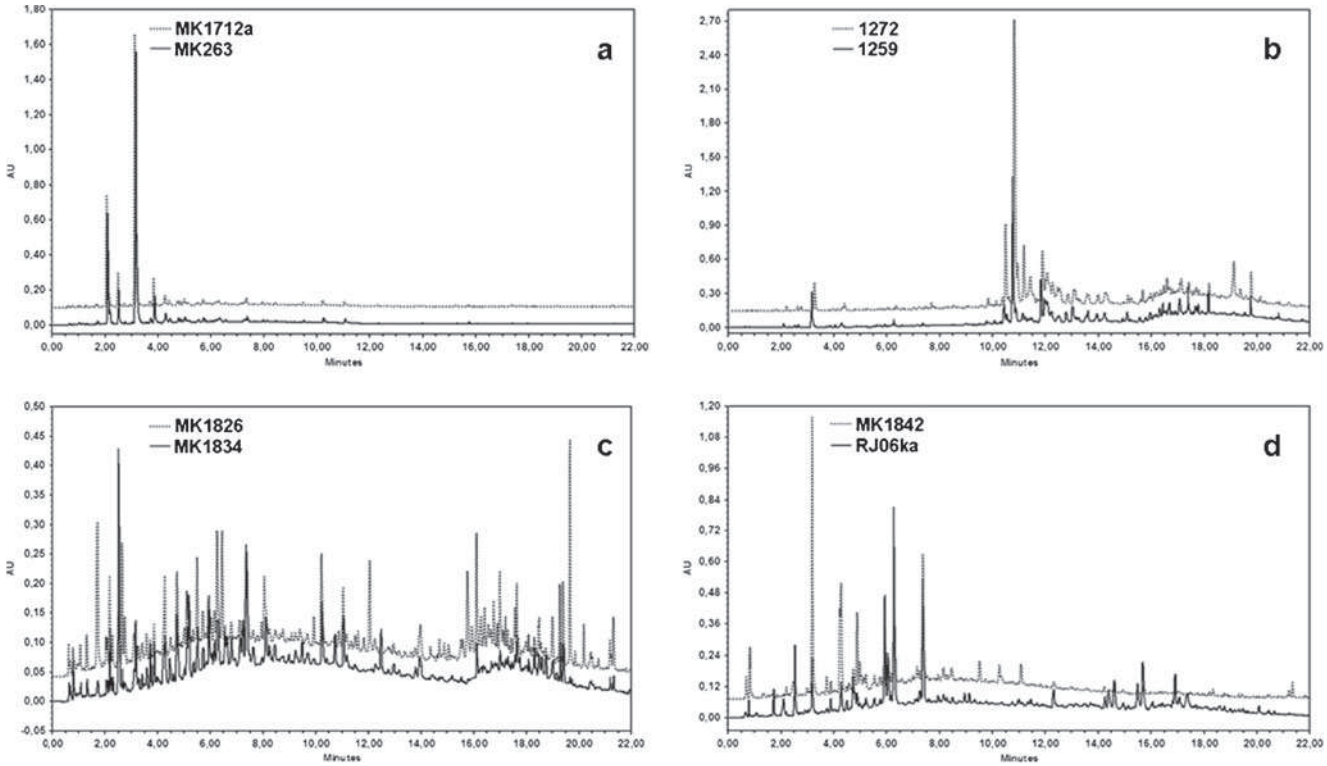


**Fig. 8** The overlay of UHPLC-DAD 2D chromatograms of different strains of the genus *Geosmithia*. Overlay of chromatograms of strains MK1712a and MK263 (**a**); 1272 and 1259 (**b**); MK1826 and MK1834 (**c**); MK1842 and RJ06ka (**d**). UV, 260 nm; for other chromatographic conditions, see Fig. 1

specific behavior of the species. Therefore, these SM will be studied in detail and will serve as reference compounds for the particular species.

## Conclusions

The UHPLC-DAD method employing SPE using Oasis MCX sorbent was developed for analysis of SM present in fungal fermentation broth of different strains of the genus *Geosmithia*. The method represents a fast, simple, and reproducible assay that is useful for high-throughput analysis of fungal samples. Moreover, the hyphenated UHPLC-DAD technique generates multi-informational fingerprints that provide both chromatographic and spectral information about sample components. The pilot analysis of 38 strains proved an interesting coherence between SM production and taxonomical identity, which qualify this method to be useful for CFP analyses of fungal SM.

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## References

1. Kolařík M, Kirkendall LR (2010) Fungal Biol 114:676–689
2. Kolařík M, Freeland E, Utley C, Tisserat N (2011) Mycologia, doi:10.3852/10-124
3. Dreyfuss MM, Chapela IH (1994) Fungi as producers of secondary metabolites. In: Gullo VP (ed) Discovery of natural products with therapeutic potential. Butterworth-Heinemann, Stoneham
4. Hansen ME, Andersen B, Smedsgaard J (2005) J Microbiol Meth 61:295–304
5. Liu M, Li Y, Chou G, Cheng X, Zhang M, Wang Z (2005) J Chromatogr A 1157:51–55
6. Andersen B, Nielsen KF, Thrane U, Szaro T, Taylor JW, Jarvis BB (2003) Mycologia 95:1227–1238
7. Guo S, Duan J-A, Tang Y, Su S, Slang E, Ni S, Qian D (2009) J Pharm Biomed Anal 49:1296–1302
8. Ni Y, Peng Y, Kokot S (2008) Chromatographia 67:211–217
9. Lian H, Wei Y (2007) Talanta 71:264–269
10. Counet C, Ouwerx C, Rosoux D, Collin S (2004) J Agric Food Chem 52:6243–6249
11. Chen Y, Yan Y, Xie M-Y, Nie S-P, Liu W, Gong X-F, Wang Y-X (2008) J Pharm Biomed Anal 47:469–477
12. Japón-Luján R, Ruiz-Jiménez J, Luque de Astro MD (2006) J Agric Food Chem 54:9706–9712
13. Ni Y, Lai Y, Brandes S, Kokot S (2009) Anal Chim Acta 647:149–158
14. Yan S-K, Xin W-F, Luo G-A, Wang Y-M, Cheng Y-Y (2005) J Chromatogr A 1090:90–97
15. Hajimahmoodi M, Heyden YV, Sadeghi N, Nahnat B, Oveisi MR, Shahbazian S (2005) Talanta 66:1108–1116
16. Luhua Z, Bingren X, Xiying T (2010) J Chromatogr Sci 48:417–420
17. Gu M, Su ZG, Fan OY (2006) J Liq Chromatogr Relat Technol 29:1503–1514
18. Luo GA, Wang YM, Cao J (2000) Chin Trad Pat Med 22:395–401
19. Qu J, Wang YM, Luo GA, Wu ZP (2001) J Chromatogr A 928:155–162
20. Qu J, Wang YM, Luo GA (2001) J Chromatogr A 919:437–441
21. Bao YW, Li C, Shen HW, Nan FJ (2004) Anal Chem 76:4208–4216
22. Garcinuno RM, Fernandez-Herbando P, Camara C (2004) J Chromatogr A 1043:225–229
23. Wiberg K, Anderson M, Hagman A, Jacobsson SP (2004) J Chromatogr A 1029:13–20
24. Comas E, Gimeno RA, Ferr J, Marc RM, Borrull F, Rius FX (2002) Anal Chim Acta 470:163–173
25. Bendahl L, Strup S, Gammelgaard B, Hansen SH (2005) Anal Atom Spectrom 20:1287–1289
26. Stadler M, Ju Y-M, Rogers JD (2004) Mycol Res 108:239–256
27. Chen Y, Zhu S-B, Xie M-Y, Nie S-P, Liu W, Li Ch, Gong X-F, Wang Y-X (2008) Anal Chim Acta 623:146–156
28. Chen Y, Bicker W, Wu JY, Xie MY, Lindner W (2010) J Chromatogr A 1217:1255–1265
29. Schmeda-Hirschmann G, Hormazabal E, Astudillo L, Rodriquez J, Theoduloz C (2005) World J Microb Biot 21:27–32
30. Hu F-L, He Y-Q, Juany B, Li Ch-R, Fan M-Z, Li Z-Z (2009) Diod Chem 116:198–201
31. Stoppacher N, Reithner B, Omann M, Zeilinger S, Krska R, Schuhmacher R (2007) Rapid Commun Mass Spectrom 21:3963–3970
32. MacEachern-Keith GJ, Wagner Butterfield LJ, Incorvia Mattina MJ (1997) Anal Chem 69:72–77
33. Gaspari F, Paian Y, Mainini M, Losi D, Ron EZ, Marinelli F (2005) J Appl Microbiol 98:429–439
34. Brunati M, Dohas JL, Sponga F, Civilisto I, Losi D, Göttlich E, de Hoog S, Genilloud O, Marinelli F (2006) Amino Acids 30:435–443
35. Szczęsna-Antczak M, Antczak T, Piotrowicz-Wasiak M, Ryska M, Binkowska N, Bielecki S (2006) Enzyme Microb Technik 39:1214–1222
36. Krause C, Kirschbaum J, Brückner H (2006) Amino Acids 30:435–443
37. Stodůlková E, Kolařík M, Křesinová Z, Kuzma M, Šulc M, Man P, Novák P, Maršík P, Landa P, Olšovská J, Chudíčková M, Pazourová S, Černý J, Bella J, Flieger M (2009) Folia Microbiol 54:179–187
38. Kolařík M, Kubátová A, Čepička I, Pažoutová S, Šrůtka P (2005) Mycol Res 109:1323–1336
39. Kolařík M, Kubátová A, Pažoutová S, Šrůtka P (2004) Mycol Res 108:1053–106
40. Kolařík M, Kubátová A, Hulcr J, Pažoutová S (2008) Microb Ecol 55:65–80
41. Kolařík M, Kostovčík M, Pažoutová S (2007) Mycol Res 101:1298–1310
42. CDER/CBER, US Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research, Center for Biological Evaluation and Research (2001) Guidance for Industry: Bioanalytical method validation. Available at: http://www.fda.gov/CDER/GUIDANCE/4252fnl.pdf. Accessed 25 Oct 2010
43. Li KM, Tibory LP, Clarke SJ (2006) Curr Pharm Anal 2:95–102
44. Stodůlková E, Man P, Kolařík M, Flieger M (2010) J Chrom A 1217:6296–6302

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ORIGINAL

# Comparison of LC Columns Packed with 2.6 μm Core-Shell and Sub-2 μm Porous Particles for Gradient Separation of Antibiotics

**Tereza Tylová · Zdeněk Kameník · Miroslav Flieger · Jana Olšovská**

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**Abstract** The recently introduced Kinetex C18 column packed with core-shell 2.6 μm particles is declared to provide similar efficiency and short analysis as Acquity BEH C18 column with 1.7 μm porous particles. Unlike Acquity BEH C18 column, Kinetex C18 column exhibited lower column backpressure making this column compatible to conventional LC systems. The performance of Kinetex C18 column (2.1 × 50 mm) and Acquity BEH C18 column (2.1 × 50 mm) for gradient separation of tetracyclines under acidic conditions (oxytetracycline, tetracycline, chlortetracycline, and doxycycline) and macrolides under alkaline conditions (tylosin, clarithromycin, roxithromycin, and carbomycin) was studied. The columns were compared by evaluation of their experimental peak capacity and its dependence on linear velocity and gradient slope. The maximal experimental peak capacities for analysis of tetracyclines were 51.8 (Acquity BEH C18 column) and 48.4 (Kinetex C18 column). This indicated that Kinetex C18 was a suitable alternative to Acquity BEH C18 column for the analysis of tetracyclines under acidic conditions. On the contrary, the maximal experimental peak capacities for analysis of macrolides on Acquity BEH C18 column was higher (46.7) than that on Kinetex C18 column (36.9). Moreover, application of Kinetex C18 column for the analysis of macrolides under alkaline conditions was limited with respect to its decreasing performance with growing number of injections on the column.

**Keywords** Column liquid chromatography · Sub-2 μm particles · Acquity BEH C18 column · Core-shell particles · Kinetex C18 column · Macrolides · Tetracyclines

## Introduction

High performance liquid chromatography represents nowadays the most widespread separation technique applied commonly in pharmacology, toxicology, clinical analysis as well as various research fields [1–4].

The efficiency of chromatographic separation can be described by van Deemter equation. The comparison of van Deemter curves for column particles of different size reveals that the smaller particles are used the more effective separation is obtained. This statement led to the development of sub-2 μm particles [5] giving birth to ultra high-performance liquid chromatography (UHPLC). Acquity BEH C18 column packed with 1.7 μm particles is one of the most often used UHPLC sub-2 μm particles columns. The particles based on BEH technology (bridged ethylene hybrid) provide excellent mechanical robustness, chemical stability in wide pH range, high separation efficiency, and short analysis time. On the other hand, particles of this size are responsible for significantly higher back pressure. Therefore, UHPLC columns are not compatible with conventional HPLC systems and their use is thus unavoidably connected with the employment of special instrumentation capable of dealing with the pressure up to 15,000 psi [6]. A great deal of attempts was done in order to overcome this disadvantage and develop separation columns with similar efficiency and short analysis time

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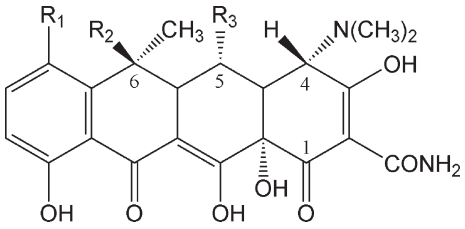


| | $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|
| TC | H | OH | H |
| OTC | H | OH | OH |
| CTC | Cl | OH | H |
| DOX | H | H | OH |

**Fig. 1** Structures of analyzed tetracyclines

compared with UHPLC, but compatible with HPLC systems working usually up to 6,000 psi (400 bar).

These requirements are accomplished in recently introduced technologies fused-core particles [7, 8] and analogous core-shell particles [9, 10]. The particles of Kinetex C18 column (core-shell technology) are composed of a solid core (1.9 μm) surrounded by a thin porous silica layer (0.35 μm). This technology enables that the analyte can diffuse only into the pores of the thin porous layer. The particle size together with porous silica layer provides separation efficiency and analyses time similar to UHPLC, but with lower column backpressure making these columns compatible to conventional LC systems [9, 10].

The first comparative study [11] of fused-core silica and sub-2 μm particles for fast separations in pharmaceutical process development revealed that fused-core packed columns have the same or slightly better separation parameters at a much lower column backpressure. Recently, Gritti et al. [12] have compared Kinetex C18 column and the sub-2 μm particles Acquity BEH C18 column for analysis of large molecule mixtures and have obtained similar peak capacities for both columns. Even though, several publications have investigated Kinetex C18 performance [13–15], there is a lack of studies comparing this column with conventional sub-2 μm particles columns for specific applications in gradient mode.

The aim of this study was to compare the performance of Kinetex C18 column and Acquity BEH C18 column for analysis of low-molecular antibiotics of different chemical properties on UHPLC system.

Both tested columns are declared to be stable in a wide pH range: pH 1–12 for Acquity BEH C18 and pH 1.5–10 for Kinetex. Therefore, the columns were tested under acidic as well as alkaline conditions.

The testing compounds represent antibiotics frequently used in human and veterinary medicine, which currently belong to potential residual environmental pollutants [16]. Tetracyclines (see Fig. 1), namely oxytetracycline (OTC), tetracycline (TC), chlortetracycline (CTC), and doxycycline (DOX), were separated under acidic conditions, whereas macrolides (see Fig. 2), namely tylosin (TYL), clarithromycin (CLA), roxithromycin (ROX), and carbomycin (CAM) were analyzed under alkaline conditions.

## Experimental

### Chemicals, Standards

TC was purchased from Spofa (Prague, Czech Republic), OTC from VUAB (Roztoky u Prahy, Czech Republic), TYL from Fluka (Steinheim, Germany), DOX from Calbiochem (San Diego, USA) and CTC, CLA, ROX, and CAM from Sigma–Aldrich (Steinheim, Germany). Acetonitrile (ACN), methanol, and trifluoracetic acid (99.95%; TFA) were of LC/MS grade and were obtained from Biosolve (Netherlands), and formic acid (98–100%) was purchased from Merck (Germany). Ammonium hydroxide A.C.S. reagent (29% aqueous $NH_4OH$ solution) was purchased from Sigma–Aldrich (Steinheim, Germany). Ammonium formate was prepared of formic acid of the respective molarity and ammonium hydroxide was added until the required pH value was obtained.

### Standard Stock Solution Preparation

Standard stock solutions were prepared with methanol at a concentration of 1 mg $mL^{-1}$. Tetracycline standard mixture was prepared by mixing equal volumes of TC, OTC, CTC, and DOX standard stock solutions resulting in a final concentration of individual compounds of 100.0 μg $mL^{-1}$. Macrolide standard mixture consisted of CLA, ROX, TYL, and CAM methanolic solutions spiked into 50% methanol to the concentrations of 500, 300, 50 and 50 μg $mL^{-1}$, respectively.

### Chromatographic System

Chromatographic analyses were carried out on the Acquity UPLC™ system equipped with 2996 Photo Diode Array (PDA) Detector (cell volume, 500 nL, optical path length, 10 mm). The dwell volume of the UHPLC system was 0.17 mL and was measured as described in [17]. The capillary connecting chromatographic column and PDA detector was 24 cm long with internal diameter of 177.8 μm. MassLynx V4.1 software was used for data processing. Samples were separated on Acquity BEH C18

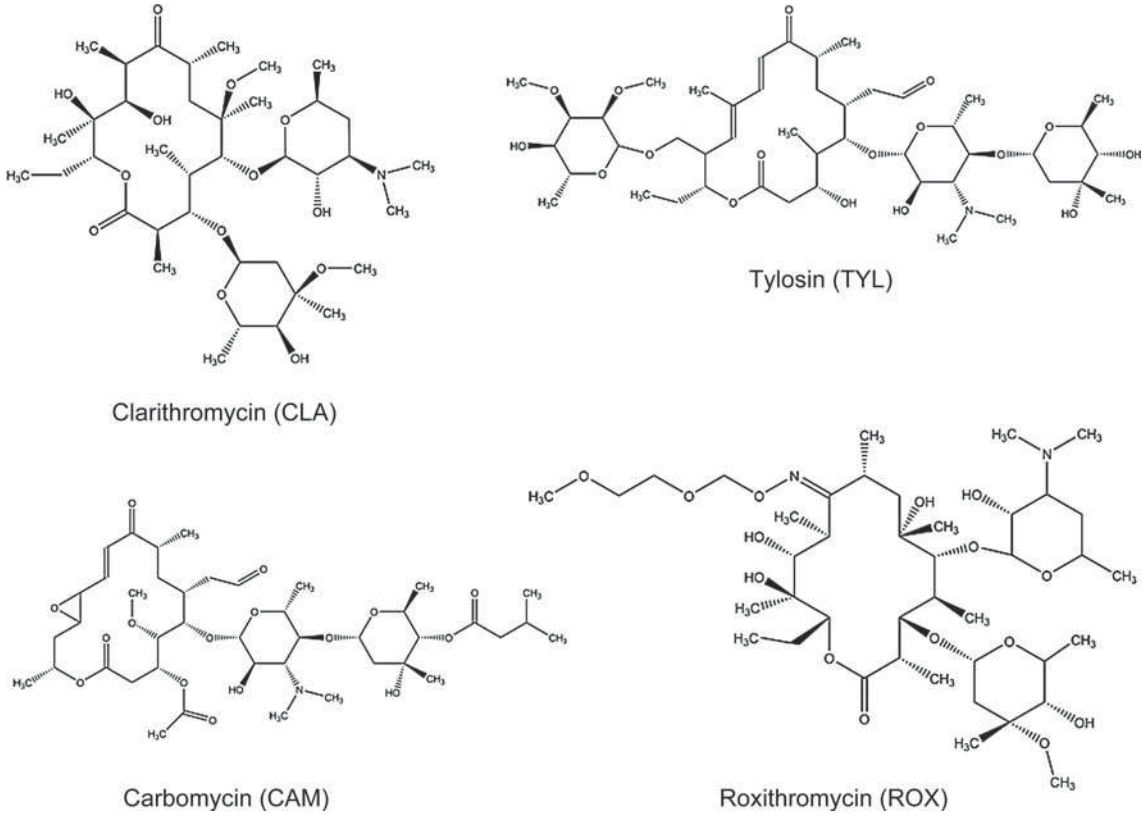


**Fig. 2** Structures of analyzed macrolides

column (50 × 2.1 mm i.d., particle size; 1.7 μm, Waters) and Kinetex C18 column (50 × 2.1 mm i.d., particle size; 2.6 μm, Phenomenex). One microliter of sample was injected by Acquity Sample Manager in partial loop with needle overfill mode (the loop volume was 10 μL).

## Chromatographic Conditions

### *Tetracyclines*

Tetracyclines were separated on both columns at 40 °C using the mobile phase consisted of solvent A, TFA-water (0.05:99.95, *v/v*) and solvent B, ACN, injection volume was 1 μL. The gradient started at solvent composition A:B 95:5 (*v/v*) and changed to A:B 70:30 (*v/v*) ($\Delta\varphi = 0.25$) during the respective time of gradient $t_g$. The PDA detector was operating in the range from 200 to 400 nm with the sampling rate of 10 spectra per second and the chromatograms were extracted at 350 nm.

### *Macrolides*

Macrolides were separated on both columns at 40 °C using the mobile phase consisted of solvent A, 1 mM ammonium formate pH 9 and solvent B, ACN, injection volume was 1 μL. The gradient started at solvent composition A:B 80:20 (*v/v*) and changed to A:B 35:65 (*v/v*) ($\Delta\varphi = 0.45$). The PDA detector was operating in the range from 194 to 400 nm with the sampling rate of 10 spectra per second. The chromatograms for further processing were extracted at wavelengths of 194, 194, 240, and 286 nm for ROX, CLA, CAM, and TYL, respectively.

## Comparison of Column Performance under Gradient Conditions

The chromatographic column performance under gradient conditions was characterized by experimental peak capacity ($P$), which was calculated as follows:

$$P = 1 + \frac{t_N - t_1}{(1/N)\sum_{i=1}^{i=N} \omega_i} \quad (1)$$

where $t_N$ and $t_1$ represent the gradient elution times of the most and less retained peak, respectively, $N$ is the number of peaks selected for the calculation ($N = 4$ for tetracycline and 2 for macrolide analyses), and $\omega_i$ is the baseline peak width of the $i^{th}$ peak measured as follows:

$$\omega_i = \frac{2\left(t_{1/2,r,i} - t_{1/2,f,i}\right)}{\sqrt{1.38629}} \quad (2)$$

where $t_{1/2,r,i}$ and $t_{1/2,f,i}$ are the experimental elution times of the rear and front parts of the peak measured at half of its height [12].

In order to make an appropriate comparison of columns performances the linear gradient was applied with constant intrinsic gradient steepness, $G$, defined as [18, 19]:

$$G = S\beta t_0 \quad (3)$$

where $S$ is the slope of the relationship between the logarithm of the retention factor measured under isocratic conditions ($k$) and the organic solvent concentration ($\varphi$) in the case of linear solvent strength retention model (LSSM), $\beta$ represents gradient slope, and $t_0$ is the column hold-up time. The LSSM describes the retention behavior and the dependence of the retention factor of the compound on the mobile phase composition under gradient elution conditions [18, 20, 21].

According to the equation:

$$S = \Delta logk/\Delta\varphi, \quad (4)$$

the values of $S$ for given compound, column and separation conditions were obtained from two isocratic measurements with $\varphi$ varied for tetracyclines and macrolides, respectively, on both tested columns [18].

Since the total column porosity $\varepsilon_T$ of the tested columns is different ($\varepsilon_T = 0.654$ and 0.542 for Acquity BEH C18 and Kinetex C18, respectively [12]), the volumetric flow rate $F$ for both columns was adjusted with respect to their $\varepsilon_T$:

$$F = u_0 \pi r^2 \varepsilon_T \quad (5)$$

where $r$ is the internal column radius, $u_0$ is the linear velocity dependent on the column length ($L$), and $t_0$:

$$u_0 = \frac{L}{t_0}. \quad (6)$$

The change of solvent composition during the gradient, $\Delta\varphi$, was deliberately kept constant at 0.25 for tetracycline and 0.45 for macrolide analyses on both columns. $P$ was measured for 16 values of $u_0$ ranging from 0.037 to 0.589 cm $s^{-1}$ with 0.037 cm $s^{-1}$ steps. These measurements were performed for three different $\beta/u_0 = 0.047$, 0.142, and 0.425 $m^{-1}$, where $\beta$ depends on time of gradient $t_g$ as follows:

$$\beta = \frac{\Delta\varphi}{t_g}. \quad (7)$$

Table 1 summarizes linear velocities $u_0$ with corresponding volumetric flow rates $F$ at the two columns and times of gradient $t_g$ for the three tested values of $\beta/u_0$.

**Table 1** Summary of parameters employed during the Acquity BEH C18 and Kinetex C18 columns comparison

| | Acquity BEH C18 | Kinetex C18 | $\beta/u_0$ ($m^{-1}$) | | |
|---|---|---|---|---|---|
| $u_0$ (cm $s^{-1}$) | $F$ (mL $min^{-1}$) | | 0.047 | 0.142 | 0.425 |
| | | | $t_g$ (min) | | |
| 0.037 | 0.050 | 0.041 | 240.00 | 80.00 | 26.67 |
| 0.074 | 0.100 | 0.083 | 120.00 | 40.00 | 13.33 |
| 0.110 | 0.150 | 0.124 | 80.00 | 26.67 | 8.89 |
| 0.147 | 0.200 | 0.166 | 60.00 | 20.00 | 6.67 |
| 0.184 | 0.250 | 0.207 | 48.00 | 16.00 | 5.33 |
| 0.221 | 0.300 | 0.249 | 40.00 | 13.33 | 4.44 |
| 0.258 | 0.350 | 0.290 | 34.29 | 11.43 | 3.81 |
| 0.294 | 0.400 | 0.331 | 30.00 | 10.00 | 3.33 |
| 0.331 | 0.450 | 0.373 | 26.67 | 8.89 | 2.96 |
| 0.368 | 0.500 | 0.414 | 24.00 | 8.00 | 2.67 |
| 0.405 | 0.550 | 0.456 | 21.82 | 7.27 | 2.42 |
| 0.441 | 0.600 | 0.497 | 20.00 | 6.67 | 2.22 |
| 0.478 | 0.650 | 0.539 | 18.46 | 6.15 | 2.05 |
| 0.515 | 0.700 | 0.580 | 17.14 | 5.71 | 1.90 |
| 0.552 | 0.750 | 0.622 | 16.00 | 5.33 | 1.78 |
| 0.589 | 0.800 | 0.663 | 15.00 | 5.00 | 1.67 |

## Result and Discussion

### Development of UHPLC Methods

#### *Tetracyclines*

During the UHPLC method development, three mobile phases differing in the strength of organic acids (acetic acid, trifluoroacetic acid, formic acid in water) were tested. In all the cases, the ACN was used as an organic modifier. The best separation of all analytes on both columns was achieved with formic acid–water (0.05:99.95, *v*/*v*) as solvent A, and ACN as solvent B of the mobile phase.

The significant effect of the column temperature on separation of tetracyclines was observed and studied in the range from 30 to 60 °C with 5 °C steps. The higher the temperature was the lower retention times of analytes were achieved. At lower temperatures (30, 35 °C), the poor resolution of early-eluting peaks (OTC, TC) was obtained, while at higher temperatures (from 45 to 60 °C), the later-eluting analytes (CTC, DOX) were only partially separated. This phenomenon was observed on both Acquity BEH C18 and Kinetex C18 columns. Therefore, the temperature of 40 °C was chosen as a compromise parameter for both columns.

#### *Macrolides*

Strongly acidic (0.1% trifluoracetic acid), acidic (1 mM ammonium formate, pH 4.75) and alkaline (1 mM ammonium formate, pH 9) aqueous parts of mobile phases were

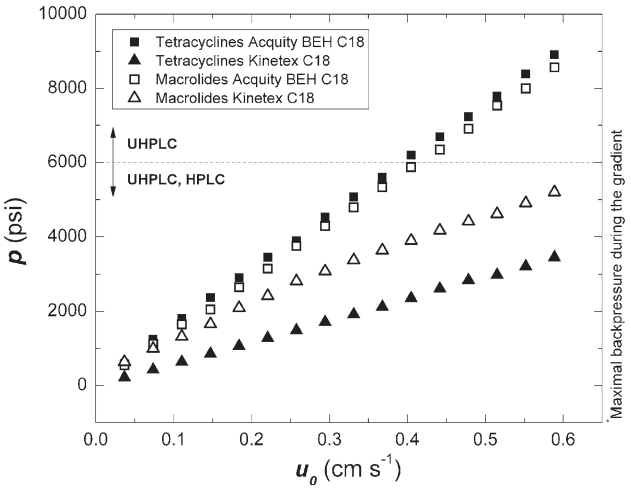


**Fig. 3** The dependence of maximal column backpressure $p$ on linear velocity $u_0$ for Acquity BEH C18 and Kinetex C18 columns

tested for macrolides separation. The alkaline conditions were the most convenient, which corresponds with previously published data [22]. ACN as an organic modifier was chosen with respect to low cut off, so it enables sensitive detection of CLA and ROX, which absorb at 194 nm. The effect of column temperature on macrolides analysis was tested in the range from 30 to 60 °C with 5 °C steps. The increasing temperature improved significantly the separation of the analytes and peaks symmetry in the whole tested range. However, considering the alkaline conditions (pH 9) employed for the separation, the temperature of 40 °C was applied with respect to column life-time.

## Comparison of the Column Performances on UHPLC System

The dependence of maximal column backpressure achieved during the gradient on $u_0$ in the range from 0.037 to 0.589 cm $s^{-1}$ is shown in Fig. 3. The maximal backpressure on Acquity BEH C18 column achieved at $u_0 = 0.589$ cm $s^{-1}$ was 8,565 psi (macrolides) and 8,910 psi (tetracyclines), while on Kinetex C18 column, it reached 5,205 psi (macrolides) and 3,450 psi (tetracyclines) at the same $u_0$. In the whole studied range of $u_0$, the Kinetex C18 column backpressure was lower than 6,000 psi (400 bar), which enables to use this column in HPLC mode.

Further, the performance of the two tested columns was compared with UHPLC system by evaluation of their experimental $P$. As described earlier, the intrinsic gradient steepness $G$ has to be kept constant for a fair comparison of the columns performance. The crucial parameter determining $G$ is parameter $S$ (see Eq. 4). It was revealed that the values of $S$ are slightly higher for Kinetex C18 than for Acquity BEH C18 column for all analytes with maximal difference of 13.8%. However, this difference affects the values of $G$ only negligibly as shown in Table 2. Since this variation influences the experimental peak capacity insignificantly [12], the parameter $S$ was considered to be comparable for both columns and therefore did not need to be taken into account for the columns performance comparison. Three curves describing the dependence of experimental $P$ on $u_0$ at three different gradient slopes $\beta/u_0$ constructed for both tetracyclines and macrolides on the two chromatographic columns are depicted in Fig. 4. The curves revealed that experimental $P$ considerably decreased with increasing $u_0$. Even though $P$ is according to the literature independent on $u_0$, this phenomenon is in accordance with previously performed experiments [12]. Additionally, experimental $P$ is apparently strongly dependent on gradient slope $\beta/u_0$ so that higher $P$ is obtained when lower gradient slope $\beta/u_0$ is applied. Nevertheless, low gradient slope $\beta/u_0$ is in principle connected with long analysis time, which is inconsistent with desired high-throughput

**Table 2** Parameters $S$ and $G$ for tetracyclines and macrolides on Acquity BEH C18 and Kinetex C18 columns

| | Acquity BEH C18 | | | | Kinetex C18 | | | |
|---|---|---|---|---|---|---|---|---|
| | | $\beta/u_0$ ($m^{-1}$) | | | | $\beta/u_0$ ($m^{-1}$) | | |
| | $S_i$ | 0.047 | 0.142 | 0.425 | $S_i$ | 0.047 | 0.142 | 0.425 |
| | | $G = S\beta t_0$ | | | | $G = S\beta t_0$ | | |
| Tetracyclines | | | | | | | | |
| OTC | 2.609 | | | | 2.628 | | | |
| TC | 2.127 | 0.0039 | 0.0116 | 0.0347 | 2.196 | 0.0040 | 0.0121 | 0.0364 |
| CTC | 1.030 | | | | 1.141 | | | |
| DOX | 0.778 | | | | 0.884 | | | |
| Macrolides | | | | | | | | |
| TYL | 1.822 | | | | 1.825 | | | |
| CLA | 0.819 | 0.0039 | 0.0118 | 0.0354 | 0.880 | 0.0041 | 0.0124 | 0.0372 |
| ROX | 0.759 | | | | 0.846 | | | |
| CAM | 0.305 | | | | 0.347 | | | |

$G$ values were obtained from the arithmetic mean of $S_i$ values for tetracyclines or macrolides

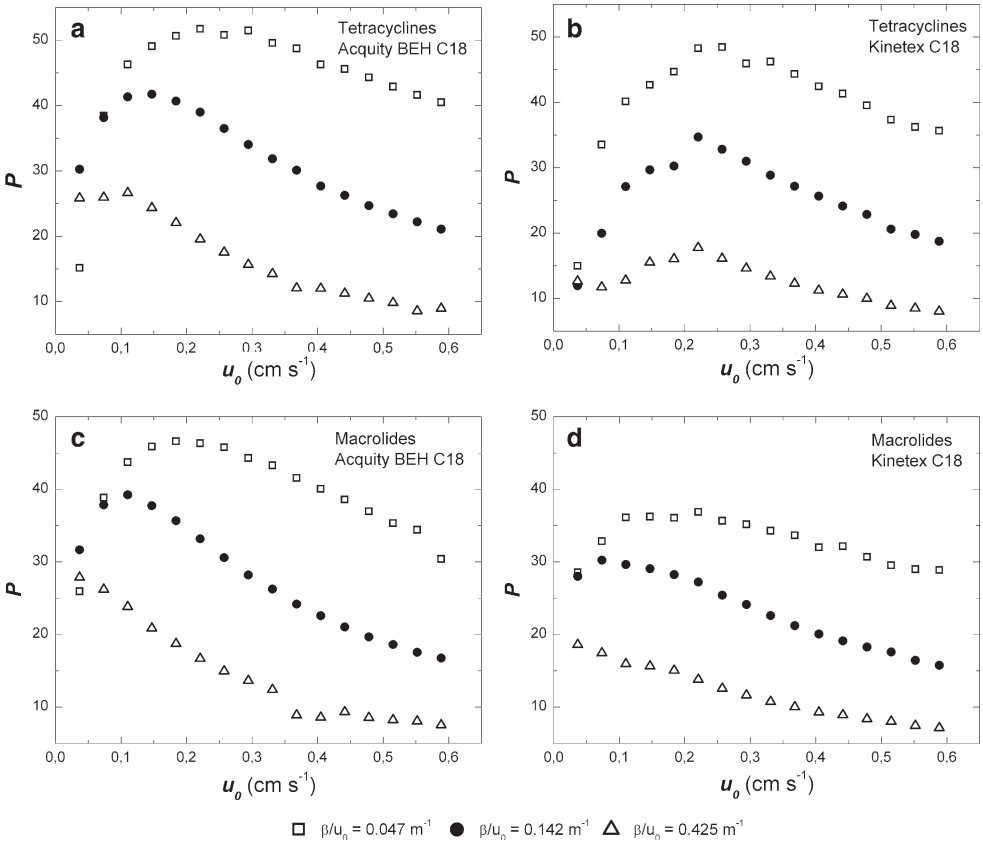


**Fig. 4** Experimental peak capacity as a function of linear velocity $u_0$ in gradient separation. **a** Tetracyclines on Acquity C18 column; **b** tetracyclines on Kinetex C18 column; **c** macrolides on Acquity C18 column; **d** macrolides on Kinetex C18 column

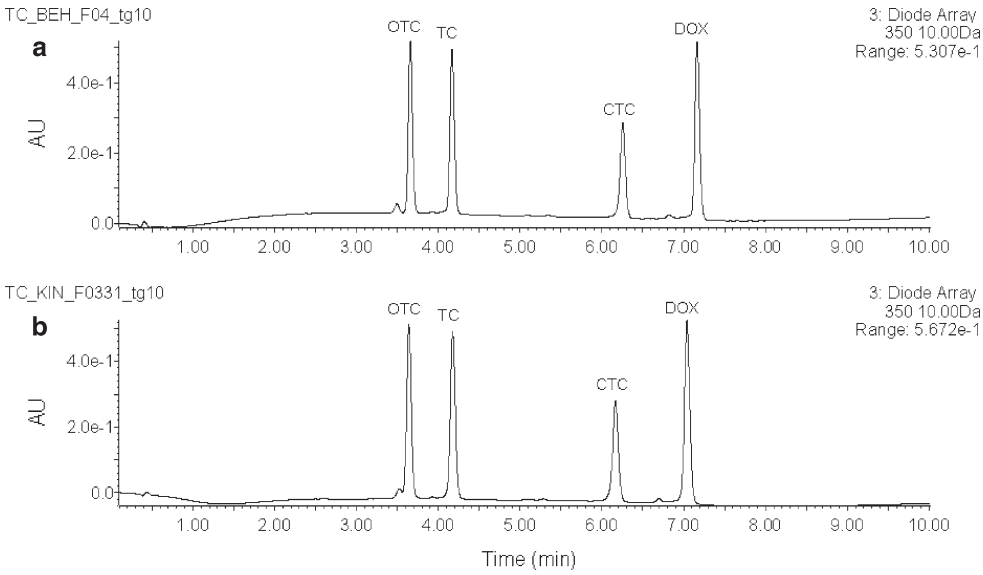


**Fig. 5** UHPLC chromatogram of gradient separation of tetracyclines. **a** Acquity BEH C18 column; **b** Kinetex C18 column. Chromatographic conditions: mobile phase: 0.05% trifluoracetic acid and acetonitrile; column temperature 40 °C; linear velocity $u_0 = 0.294$ cm s$^{-1}$; gradient time $t_g = 8.89$ min; change of solvent composition during the gradient time $\Delta\varphi = 0.25$; gradient slope $\beta/u_0 = 0.142$ m$^{-1}$; injection volume 1 μL; UV: 350 nm

analyses. Therefore, the compromise between $P$ and gradient slope $\beta/u_0$ represents the crucial task.

*Tetracyclines*

The analyses of tetracyclines on Acquity BEH C18 and Kinetex C18 columns employing $u_0 = 0.294$ cm s$^{-1}$ and $\beta/u_0 = 0.142$ m$^{-1}$ as a compromise between experimental $P$ and analysis time are shown in Fig. 5.

For all applied conditions, all four analytes were baseline separated with good peak symmetry. The only exception with poor peak symmetry was found on both columns at $u_0 = 0.037$ cm s$^{-1}$ and gradient slope $\beta/u_0 = 0.047$ m$^{-1}$ for CTC and DOX, which also

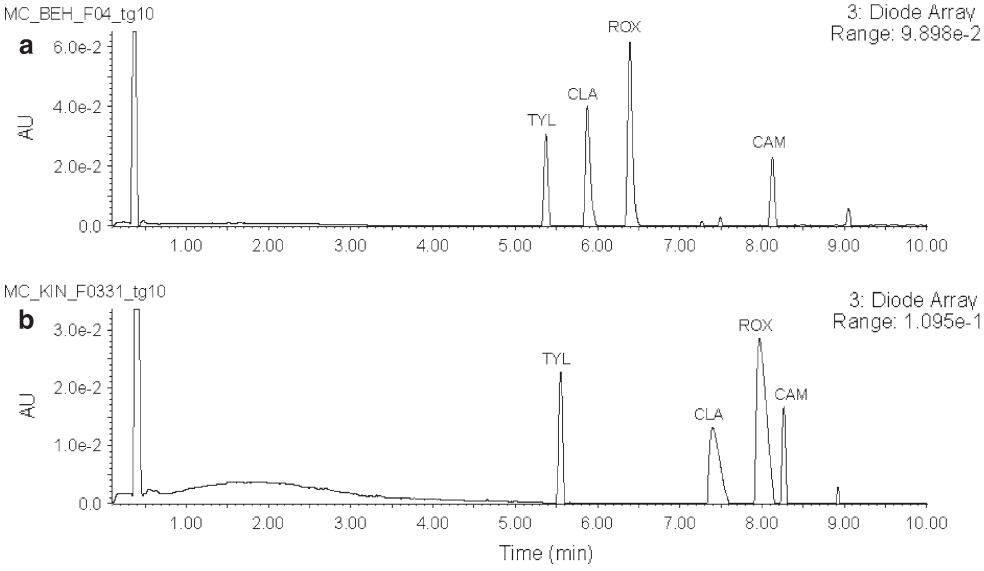


**Fig. 6** UHPLC chromatogram of gradient separation of macrolides. **a** Acquity BEH C18 column; **b** Kinetex C18 column. Chromatographic conditions: mobile phase: 1 mM ammonium formate pH 9 and acetonitrile; column temperature 40 °C; linear velocity $u_0 = 0.294$ cm s$^{-1}$; gradient time $t_g = 8.89$ min; change of solvent composition during the gradient time $\Delta\varphi = 0.45$; gradient slope $\beta/u_0 = 0.142$ m$^{-1}$; injection volume 1 μL; UV: extracted at maximal wavelength of the analytes-maxplot

resulted in a very low peak capacity at this $u_0$ and $\beta/u_0$.

The maximal experimental $P$ of Acquity BEH C18 column was 51.8 (at $u_0 = 0.221$ cm s$^{-1}$ and $\beta/u_0 = 0.047$ m$^{-1}$) and for Kinetex C18 was 48.4 (at $u_0 = 0.221$ cm s$^{-1}$ and $\beta/u_0 = 0.047$ m$^{-1}$). In general, the experimental $P$ of Acquity BEH C18 column was for tetracycline analysis found to be slightly higher than that of Kinetex C18 column. On the contrary, Gritti et al. [12] recently published results revealing better performance of Kinetex C18 column compared with Acquity BEH C18 column under gradient conditions. However, proteins–analytes of different properties were used in their study. Therefore, the discrepancy can be possibly explained by faster mass transfer in Kinetex C18 column that affects positively the analysis of large molecules, but the analysis of the low-molecular weight tetracyclines appears to be influenced by this parameter less significantly.

Interestingly, the maximal experimental $P$ for Kinetex C18 column was for all the three $\beta/u_0$ achieved at same value of $u_0 = 0.221$ cm s$^{-1}$. On the other hand, the maximal experimental $P$ for Acquity BEH C18 column was at higher $\beta/u_0$ ratios shifted to lower $u_0$: $u_0 = 0.110$ cm s$^{-1}$ for $\beta/u_0 = 0.425$ m$^{-1}$, $u_0 = 0.147$ cm s$^{-1}$ for $\beta/u_0$ 0.142 m$^{-1}$, and $u_0 = 0.221$ cm s$^{-1}$ for $\beta/u_0 = 0.047$ m$^{-1}$ (see Fig. 4a, b). The steepness of the experimental $P$ decrease with increasing $u_0$ was for both columns very similar.

*Macrolides*

The chromatograms of macrolides analyses on both columns at $u_0 = 0.294$ cm s$^{-1}$ and $\beta/u_0 = 0.142$ m$^{-1}$ representing a reasonable compromise between experimental $P$ and time of gradient $t_g$ are shown in Fig. 6.

Under all $u_0$ and gradient slopes $\beta/u_0$ applied, macrolides were well separated on Acquity BEH C18 column with good peak symmetry rates for all peaks. Kinetex C18 column also separated macrolides sufficiently; however, the peak symmetry rates of CLA and ROX were not optimal. Unlike TYL and CAM, retention times of CLA and ROX on both columns differed significantly. Moreover, worsening performance of Kinetex C18 for CLA and ROX from one injection to another was observed. Figure 7 depicts analysis of macrolides on Kinetex C18 column ($u_0 = 0.221$ cm s$^{-1}$ and $\beta/u_0 = 0.047$ m$^{-1}$) and the same analysis on the same column after 70 injections. The Kinetex C18 column was after 70 injections incapable of separating CLA and ROX; however, analysis of TYL and CAM including their retention times did not differ considerably. This phenomenon observed on two newly employed Kinetex C18 columns is apparently connected with alkaline pH of the mobile phase and with the two specific analytes—CLA and ROX. The cause of this phenomenon and whether it has a more general relevance remains unclear and requires further investigation. With respect to this fact, CLA and ROX were excluded from the evaluation of the columns performance by experimental $P$ and only TYL and CAM were in case of both columns considered.

The maximal experimental $P$ of Acquity BEH C18 column was 46.7 (at $u_0 = 0.184$ cm s$^{-1}$ and $\beta/u_0 = 0.047$ m$^{-1}$) and for Kinetex C18 was 36.9 (at $u_0 = 0.221$ cm s$^{-1}$ and $\beta/u_0 = 0.047$ m$^{-1}$). The experimental $P$ of Acquity BEH C18 column was up to $u_0 = 0.294$ cm s$^{-1}$ for all gradient slopes $\beta/u_0$ substantially higher than that for Kinetex C18 column. However, the differences between experimental $P$ at higher $u_0$ were negligible (see Fig. 4c, d). In other words, the steepness of

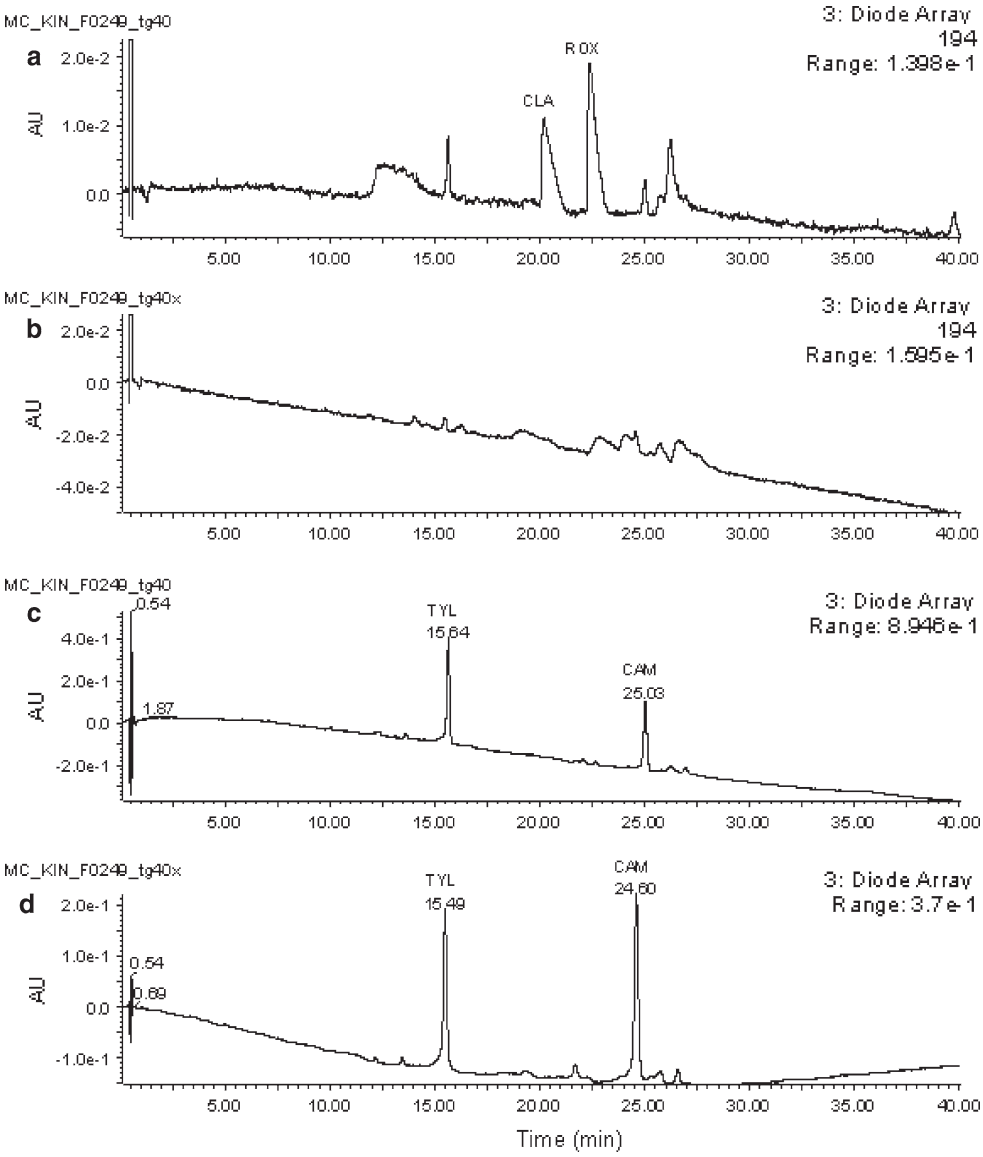


**Fig. 7** UHPLC chromatogram of gradient separation of macrolides on Kinetex C18 column. **a**, **c** analysis of initial injection; **b**, **d** analysis after 70 injections. Chromatographic conditions: Mobile phase: 1 mM ammonium formate pH 9 and acetonitrile; column temperature 40 °C; linear velocity $u_0 = 0.221$ cm s$^{-1}$; gradient time $t_g = 40$ min; change of solvent composition during the gradient time $\Delta\varphi = 0.45$; gradient slope $\beta/u_0 = 0.047$ m$^{-1}$; injection volume 1 μL; UV: **a**, **b** 194 nm; **c**, **d** extracted from 240 to 286 nm

experimental $P$ decrease with increasing $u_0$ is much flatter in case of Kinetex C18 column.

## Conclusion

The chromatographic performance of Kinetex C18 and Acquity BEH C18 columns in gradient separation of low-molecular tetracycline and macrolide antibiotics on UHPLC system was tested by evaluation of experimental peak capacity $P$ and its dependence on linear velocity $u_0$ for three gradient slopes $\beta/u_0$. Under all conditions, higher $P$ was achieved on Acquity BEH C18 column for both antibiotic groups; however, the difference was dependent on specific parameters and was more pronounced for macrolides. The markedly lower column backpressure generated on Kinetex C18 column during the gradient elution confirmed its compatibility with conventional HPLC system. On the other hand, unlike Acquity BEH C18 column, Kinetex C18 column exhibited dramatically decreasing performance with growing number of injections for analysis of two macrolides (CLA and ROX). This phenomenon is connected to alkaline conditions (pH 9) but was not observed neither for the other macrolides (TYL and CAM) nor tetracyclines analyzed under acidic conditions. In conclusion, Kinetex C18 column represents a convenient alternative to Acquity BEH C18 column for analysis of tetracyclines under acidic conditions, but exhibited substantial limitations for analysis of macrolides under alkaline conditions.

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## References

1. Stroh JG, Petucci CJ, Brecker SJ, Nogle LM (2008) J Sep Sci 31:3698–3703
2. Pragst F, Herzler M, Erxleben BT (2004) Clin Chem Lab Med 42:1325–1340
3. Vogeser M, Seger C (2008) Clin Biochem 41:649–662
4. Klein EJ, Rivera SL (2000) J Liq Chromatogr Relat Technol 23:2097–2121
5. Mellors JS, Jorgenson J (2004) Anal Chem 76:5441–5450
6. Swartz ME (2005) J Liq Chrom 28:1253–1263
7. Cunliffe JM, Maloney TD (2007) J Sep Sci 30:3104–3109
8. Way WK, Brandes H (2008) LC GC North Am Suppl S:64–64
9. DeStefano JJ, Langlois TJ, Kirkland JJ (2008) J Chromatogr Sci 46:254–260
10. Koerner P, Mathews T (2010) LC GC North Am. Suppl. S:55–59
11. Abrahim A, Al-Sayah M, Skrdla P, Bereznitski Y, Chen Y, Wu N (2010) J Pharm Biomed Anal 51:131–137
12. Gritti F, Guiochon G (2010) J Chromatogr A 1217:1604–1615
13. Gritti F, Leonardis I, Shock D, Stevenson P, Shalliker A, Guiochon G (2010) J Chromatogr A 1217:1589–1603
14. Gritti F, Leonardis I, Abia J, Guiochon G (2010) J Chromatogr A 1217:3819–3843
15. Oláh E, Fekete S, Fekete J, Ganzler K (2010) J Chromatogr A 1217:3642–3653
16. Batt AL, Aga DS (2005) Anal Chem 77:2940–2947
17. Gomis DB, Nunez NS, Garcia EA, Abrodo PA, Alvarez MDG (2006) J Liq Chromatogr Relat Technol 29:1861–1875
18. Snyder LR, Dolan JW (2007) High-performance gradient elution—the practical application of linear-solvent-strength model. Wiley, New Jersey, pp 15–18
19. Snyder L (1986) High performance liquid chromatography–advances and perspectives. Elsevier, Amsterdam
20. Snyder LR, Dolan JW, Gant JR (1979) J Chromatogr 165:3–30
21. Dolan JW, Gant JR, Snyder LR (1979) J Chromatogr 165:31–58
22. Olsovska J, Kamenik Z, Cajthaml T (2009) J Chromatogr A 1216:5774–5778