Journal of Chromatography A, 1217 (2010) 8016–8025

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Ultra-high-performance liquid chromatography fingerprinting method for chemical screening of metabolites in cultivation broth

Zdeněk Kameník[a,b,*], Franz Hadacek[c], Markéta Marečková[d], Dana Ulanova[a], Jan Kopecký[d], Vladimír Chobot[c], Kamila Plháčková[a], Jana Olšovská[a]

[a] Institute of Microbiology of the Academy of Sciences of the Czech Republic, v.v.i., Vídeňská 1083, 142 20 Prague 4, Czech Republic
[b] Charles University, Faculty of Science, Department of Analytical Chemistry, Albertov 8, 128 40 Prague 2, Czech Republic
[c] University of Vienna, Faculty of Life Sciences, Department of Chemical Ecology and Ecosystem Research, Althanstrasse 14, 1090 Vienna, Austria
[d] Crop Research Institute, Drnovská 507, 161 06 Prague 6, Czech Republic

ARTICLE INFO

Article history:
Available online 19 August 2010

Keywords:
UHPLC
High-throughput fingerprinting
Secondary metabolites
Antibiotics
Cultivation broth
Actinomycetes

ABSTRACT

A fingerprinting method for chemical screening of microbial metabolites, potential antibiotics, in spent cultivation broths is described. The method is based on high-throughput ultra-high-performance liquid chromatography (UHPLC) separation with UV detection (photodiode array detector). Thirteen antibiotic standards and four cultivation broths were used for the method development. The comparison of ten liquid–liquid and solid phase extraction protocols for sample clean-up and pre-concentration revealed that Oasis HLB C18 sorbent gives the best recoveries. The Acquity BEH C18 chromatographic column was chosen for the samples separation with respect to its universality, selectivity, efficiency and robustness. The method is presented by two 3D fingerprints for every sample that was obtained under different, acidic and alkaline, UHPLC conditions. The acidic mobile phase consisted of 0.5% phosphoric acid with methanol and the alkaline mobile phase of 1 mM ammonium formate, pH 9 with acetonitrile. Each pair of 3D fingerprints includes the following physico-chemical information: polarity (retention time), presence and characterization of chromophores (UV spectra), compound concentration (detector response), and acid–base properties (influence of different pH of the aqueous parts of mobile phases on retention times). The sample extraction and method validation were assessed with relative standard deviation (RSD) of 0.5, 5.0 and 20.0% for retention times, peak areas and minor compound peak areas, respectively.

1. Introduction

Bacterial secondary metabolites represent one of the most important sources of bioactive compounds. Almost one half of described antibiotics originate from actinomycetes [1]. Recently, the rate of new antibiotics discovery has declined dramatically and this trend is continuing [2]. In many instances, known compounds are rediscovered [3–5], although Watve et al. estimated that only about 3% of the existing compounds have been described so far [6]. Chemical fingerprinting based on chemical analysis of antibiotics and other secondary metabolites excreted to cultivation broth represents one possible discovery tool exploring a "chemical picture" of the produced set of metabolites without their individual isolation [7]. The applied method that includes both sample preparation and analysis must be universal enough to detect the maximal number of analytes present in the sample. In addition, the fingerprints are to provide physico-chemical information for all unknown compounds (*e.g.* to allow a tentative identification or classification to a specific compound class). Finally, the method should be high-throughput to facilitate screening of a large number of samples. The fingerprints then not only can be used *per se*, but also combined with genetic screening, *e.g.* detection of secondary metabolic genes, operons or clusters. Also, in combination with taxonomical identification the fingerprints may predict horizontal gene transfer among related strains as indication of usefulness for antibiotic production [8].

Chemical fingerprinting always has been based on a chromatographic technique [9,10], and most recently chromatographic techniques hyphenated with various means of detection have been applied: LC–UV [11,12], LC–ELSD [13,14], LC–MS [11,12,14], LC–NMR [11]. Since none of the detectors is fully universal, their advantages and disadvantages must be considered [15–17]. In principle, the information gained by any of the detectors varies in terms of sensitivity and selectivity, but is applicable for obtaining fingerprints. UHPLC represents a current state-of-art liquid chromatographic technique. It approximately enables six times faster analyses than HPLC [18] adding to its suitability for application

* Corresponding author at: Institute of Microbiology of the Academy of Sciences of the Czech Republic, v.v.i., Vídeňská 1083, 142 20 Prague 4, Czech Republic. Tel.: +420 241062616; fax: +420 241062347.
E-mail address: zdenek.kamenik@email.cz (Z. Kameník).

0021-9673/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2010.08.031

in the screening of a large number of samples. Also, selectivity and sensitivity are considerably higher than with standard HPLC columns [19,20]. To date, no UHPLC fingerprinting protocols that specifically is optimized for compounds present in cultivation broth has been introduced and only one publication focusing on UHPLC analysis of cultivation broth is available [18]. Here, we describe a fingerprinting method with UHPLC and photodiode array detection (DAD) that was developed for bacterial secondary metabolites produced in spent cultivation broths. Broths that were obtained from culturing four actinomycetes and a set of 13 antibiotic standards were used to develop a suitable extraction approach and the most efficient UHPLC conditions. The major classes of antibiotics (β-lactames, coumarins, glycopeptides, lincosamides, macrolides, piperidines, polyenes, quinolones, tetracyclines, *etc.*) that cover the existing range of physico-chemical properties (chemical structure, polarity, spectral and acid–base properties) were included into the set of antibiotic standards; Fig. S1 in Supplementary data illustrates their structures.

2. Experimental

2.1. Chemicals

The solvents used as UHPLC mobile phase were of the gradient grade. Acetonitrile (ACN; 99.95%, Biosolve, Netherlands), methanol (MeOH; 99.95%, Chromapur GG) and dichloromethane (DC; min. 99%, Chromapur G) were purchased from Chromservis (Czech Republic). Trifluoroacetic acid (TFA; 99.95%, ULC/MS) was obtained from Biosolve (Netherlands) and formic acid (HCOOH; 99%) from Merck (Germany). *Ortho*-phosphoric acid (H_3PO_4; 99%), ammonium hydroxide (NH_4OH; 29%, A.C.S. reagent) and acetic acid (glacial, min. 99%) were purchased from Sigma–Aldrich (Germany). Diethylether (EE; p.a.) and ethyl acetate (EA; p.a., 99.7%) were obtained from Lach-Ner (Czech Republic). HPLC grade water was prepared by Milli-Q reverse osmosis, Millipore (USA). Amberlite XAD-4 (Amb) was purchased from Supelco (USA) and was used to make a 500 mg-Amberlite-cartridge. Oasis HLB 3cc (50 mg) cartridges (Hlb) were obtained from Waters (USA).

The standard stock solutions of antibiotics were prepared with MeOH or water at a concentration of 1 mg mL^{-1}. Two mixtures A1 and A2 containing six and seven antibiotic standards, respectively, were used for the development of extraction and UHPLC methods.

Mixture A1 contained the following antibiotic standards: cephalosporin C (CEC), penicillin G (PEG) dissolved in water; griseofulvin (GRI), tylosin (TYL), lincomycin A (LIN) dissolved in MeOH; and streptovitacin A (STV) dissolved in 50% MeOH. Standard stock solutions of these antibiotics were mixed and diluted with 50% MeOH so that the final concentration of each compound was 100 μg mL^{-1}.

Mixture A2 contained the following antibiotic standards: novobiocin (NOV), ristocetin (RIS), ofloxacin (OFL) dissolved in water; roxithromycin (ROX), natamycin (NAT), chlortetracycline (CTE) and chloramphenicol (CHL) dissolved in MeOH. Standard stock solutions of these antibiotics were mixed and diluted with 50% MeOH so that the final concentration of each compound was 100 μg mL^{-1}.

CEC, ROX, CHL, LIN, GRI, PEG, TYL, NOV, RIS, OFL, NAT, CTE were obtained from Sigma–Aldrich (Germany) and were of UV grade (>90%). STV was kindly provided by Jaroslav Spížek, Institute of Microbiology of the Academy of Sciences of the Czech Republic, v.v.i. (Czech Republic).

2.2. Cultivation

Four actinomycete strains (E1, E2, E3, and E4) were cultivated. Spores were inoculated in GYM broth (50 mL) (glucose 4 g L^{-1}, yeast extract 4 g L^{-1}, malt extract 10 g L^{-1}, $CaCO_3$ 2 g L^{-1}, pH 7.2) and cultivated in a rotary shaker for 24–48 h at 28 °C. Then, fresh GYM broth (50 mL) was inoculated with 5% of the pre-culture and cultivation continued for 10 days at 28 °C. Cells were centrifuged for 10 min at 4000 rpm and 4 °C. The spent cultivation broth (supernatant) was used for extraction (extracts E1, E2, E3, and E4).

2.3. Fingerprinting method development

2.3.1. Extraction of cultivation broth

Liquid–liquid extraction (*LLE*). The standard mixtures A1 and A2 were diluted 10-fold with water to the concentration of 10 μg mL^{-1} for each antibiotic. Two milliliters of diluted A1 or A2 mixture were mixed with 2 mL of various organic solvents (EE, DC, EA, or EA acidified: EA–acetic acid 95:5, v/v), the emulsion was shaken for 10 min and the organic phase was removed. The procedure was repeated twice and all the three organic fractions were put together, evaporated and reconstituted in 200 μL 50% MeOH so that the theoretical concentration (100 μg mL^{-1}) corresponds with the concentration of the original mixture A1 or A2. This sample was measured by UHPLC under acidic conditions (see Section 2.4.2) and peak areas of the antibiotics were compared with peak areas of antibiotics in the original A1 or A2 mixtures. The recovery was then calculated as the ratio of the respective peak areas.

Solid phase extraction (*SPE*). Hlb and Amb cartridges were conditioned with 3 mL MeOH and equilibrated with 3 mL water. Two milliliters 10-fold diluted A1 or A2 solution was added. The column was then washed with 3 mL water and the absorbed antibiotics were eluted with 1 mL MeOH (fraction F100) or with 1 mL of 40% MeOH (fraction F40) and subsequently with 1 mL 90% MeOH (fraction F90/40). The eluent was evaporated and dissolved in 200 μL 50% MeOH so that the theoretical concentration (100 μg mL^{-1}) corresponded with the original mixture A1 or A2. This sample was analyzed by UHPLC under acidic conditions (see Section 2.4.2). The recovery was assessed as described above for LLE.

The extraction procedures giving the best recoveries were subsequently tested with cultivation broths E1–E4.

2.3.2. UHPLC conditions

The following five UHPLC columns were tested:

- Vision HT C18 column (50 mm × 2.0 mm I.D., particle size 1.5 μm), Grace (HT C18);
- Acquity UPLC BEH Shield RP18 column (50 mm × 2.1 mm I.D., particle size 1.7 μm), Waters (BEH Shield);
- Acquity UPLC BEH C18 column (50 mm × 2.1 mm I.D., particle size 1.7 μm), Waters (BEH C18);
- Acquity UPLC BEH C18 column (100 mm × 2.1 mm I.D., particle size 1.7 μm), Waters (BEH C18 10 cm);
- Acquity UPLC BEH Phenyl column (50 mm × 2.1 mm I.D., particle size 1.7 μm), Waters (BEH Phenyl).

The gradient programs g10, g15, g25, g30, g40 and g60 were used. Isocratic elution of 5% B (organic modifier) was set for 1.5 min (g10 and g15) or 2.0 min (g20 to g60) before the gradient elution. Then, the ratio of solvent B linearly increased from 5% to 100% in 10, 15, 20, 25, 30, 40 and 60 min for gradient programs g10, g15, g25, g30, g40 and g60, respectively. The column was then washed with 100% B for 1.5 min and equilibrated for 1.0 min (5% B).

Section S1 in Supplementary data summarizes the specific UHPLC conditions used during the study.

2.4. Final fingerprinting method

2.4.1. SPE

Hlb cartridge was conditioned with 3 mL MeOH, equilibrated with 3 mL water and then 3 mL cultivation broth was loaded. After that, the cartridge was washed with 3 mL water and absorbed substances were eluted with 1 mL MeOH. The eluent was evaporated to dryness, reconstituted in 200 μL 40% MeOH and centrifuged (13,000 rpm). This sample represented the extract for UHPLC analysis.

2.4.2. UHPLC

The UHPLC analyses were performed on Acquity UPLC system, equipped with the 2996 PDA detection system operating from 194 to 600 nm (Waters). Data were processed with Empower 2 (Waters). The chromatographic conditions were: Acquity UPLC BEH C18 column (50 mm × 2.1 mm I.D., particle size 1.7 μm, Waters); flow rate, 0.4 mL min^{-1}, data sample rate, 20 pts s^{-1}; filter constant, 0.5; injection volume, 5 μL. Every sample was analyzed under acidic and alkaline conditions.

Acidic conditions. Mobile phase consisted of solvent A, 0.5% H_3PO_4 in water, and solvent B, MeOH. Samples were eluted by a linear gradient program (min/%B): 0/5; 1.5/5; 16.5/100 with subsequent column clean-up for 1.5 min (100% B) and equilibration for 1.0 min (5% B). Total analysis time was 19.0 min. The column oven was set to 55 °C. The data were recorded from 210 to 600 nm.

Alkaline conditions. Mobile phase consisted of solvent A, 1 mM ammonium formate pH 9.0, and solvent B, ACN. Samples were eluted by a linear gradient program (min/%B): 0/5; 2.0/5.0; 18.0/65.8 with subsequent column clean-up for 1.0 min (100% B) and equilibration for 1.0 min (5% B). Total analysis time was 20.0 min. The column oven was set to 30 °C. The data were recorded from 194 to 600 nm.

2.5. Data processing

Peak symmetry, selectivity, area, height, and retention time were calculated by Empower 2 (Waters). Symmetry $S = 0.5\,W/F$, where W means peak width at 5% of peak height and F means time from width start point at 5% of peak height to retention time; resolution $R = 1.18\ (t_{R2} - t_{R1})/(W_1 + W_2)$, where t_{R1} and t_{R2} mean retention times of the two assessed peaks, W_1 and W_2 mean peak widths at 50% peak height; peak quality $Q = h/A$, where h represents peak height and A means peak area. S and Q were used for evaluation of the peak shape, whereas R and Q were used to estimate the separation efficiency and selectivity. All antibiotic standards in mixtures A1 and A2 and following unknown compounds in the extracts E1–E4 were assessed: E1a, E1b and E1c (extract E1); E2d and E2e (extract E2); E3f and E3g (extract E3); E4h, E4i and E4j (extract E4). All the unknown compounds as well as antibiotic standards showed characteristic UV spectra that facilitated monitoring of individual compounds under different UHPLC conditions. The UV spectra of the analytes did not differ significantly under acidic and alkaline conditions. Compared compounds in the extracts E1–E4 were selected so that different polarity (retention time) and signal response (minor and major peaks) were always involved.

2.6. Validation and column robustness

SPE extraction and UHPLC analysis of cultivation broths E1–E4 was performed in six replicates using the final conditions. Retention times and peak areas of selected unknown analytes that were detected in the extracts E1–E4 (see Section 2.5) were compared within the six replicates in order to evaluate the fingerprinting method repeatability as defined in the validation guidelines [21].

Five hundred actinomycete spent cultivation broths were extracted and analyzed by UHPLC under final conditions using two different BEH C18 columns, one for the acidic and the other for alkaline conditions. The testing extract E2 was repeatedly injected and analyzed at the beginning and after every 20 samples, *i.e.* 26 injections of E2. The column robustness was assessed by comparison of the 26 chromatograms of E2 and was expressed as repeatability of retention times and areas of selected peaks.

3. Results and discussion

3.1. Development of extraction protocol

Actinomycetes secrete many of the secondary metabolites to their cultivation broth in relatively low concentrations. The cultivation broth is a complex matrix that contains not only bacterial metabolites, but also essential nutrients. Therefore, the sample preconcentration and clean-up prior to the analysis is mandatory.

SPE is generally more universal and repeatable than LLE [22]. The former method also complies with requirements for high-throughput. However, LLE previously has been used widely for natural products extraction [12,23]; therefore both SPE and LLE were evaluated.

LLE was examined with four water non-miscible solvents of different polarity (EE, DC, EA, and acidic EA) and the results summarized in Table 1 indicated that DC and EA yielded the best recovery results though only in case of half of the antibiotic standards more than 50% actually were recovered. In particular, a low recovery was achieved for more polar antibiotics (CEC, RIS, LIN, STV, and OFL).

The SPE method was tested with two sorbents, Amb and Hlb. Amb has been used widely to recover natural products from biomatrices including cultivation broth [23–26]. Hlb was used to extract, for example, LIN from cultivation broth [18] and various antibiotics from wastewater [27]. The nature of Hlb provides many advantages compared to the classical silica-based SPE cartridges, such as low elution volume, higher recovery of polar metabolites, proprietary cleaning process, and minimal drying effect, all factors that contribute to excellent repeatability.

Table 1 summarizes recovery rates of antibiotic standards extracted with Amb and Hlb. Hlb (fraction 100) is capable of extracting all antibiotics except CEC. The recoveries exceeded 50% except for RIS (26.3%) and CTE (46.2%). Amb recovered more antibiotics than any solvent in LLE, however, the recovery rates are much lower than those of Hlb. The extraction of broths E1–E4 also confirmed that Hlb is more efficient than Amb. It mostly provided UHPLC fingerprints that showed peaks of the same compounds but with significantly higher response indicating more efficient recovery (see Fig. 1).

The solvent composition for dissolving of the Hlb extract was optimized. MeOH–water and MeOH–1% acetic acid in ratios 100:0, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, and 30:70 (v/v) were compared. It was found that water and 1% acetic acid yielded the same results whilst the concentration of MeOH much more influenced the recovery of specific compounds. Generally, pure MeOH yielded higher recovery rates of less polar compounds. If the ratio of MeOH was reduced to 40%, the recovery of less polar compounds was the same or slightly reduced, but that of the more polar compounds improved considerably. Therefore, 40% MeOH was used for the reconstitution of the Hlb extract.

3.2. Development of UHPLC conditions

The antibiotic mixtures A1 and A2 and cultivation broth extracts E1–E4 were used for the UHPLC method development. Actinomycete metabolites that are produced in low amounts may be

Table 1
Recovery rates of antibiotics extracted by different liquid–liquid and solid phase extraction techniques.

Antibiotic	CEC	RIS	LIN	STV	OFL	CHL	CTE	PEG	GRI	TYL	NAT	ROX	NOV
Polarity of antibiotic	More polar ↔ Less polar												
Wavelength (nm)	260	199	197	201	295	278	368	197	295	287	304	199	324
LLE													
Diethylether	0.0	0.0	0.0	0.0	2.4	64.2	4.2	0.0	71.3	1.0	1.9	33.5	34.0
Dichloromethane	0.0	0.0	0.0	4.6	39.1	30.9	27.3	0.0	65.3	49.8	0.5	49.9	51.4
Ethylacetate	0.0	0.0	0.0	14.6	2.7	98.6	66.7	53.9	71.4	8.03	0.4	4.7	87.0
Ethylacetate acidified	0.0	0.0	0.0	9.3	0.4	79.1	5.7	37.2	78.9	1.6	1.1	0.0	63.3
SPE													
Amberlite Fraction F100	0.0	0.0	35.3	5.4	12.5	37.9	2.6	0.0	20.3	25.6	19.3	26.5	24.2
Amberlite Fraction F40	0.5	0.0	0.0	0.0	5.1	2.9	4.5	0.0	4.6	5.0	2.0	0.0	3.2
Amberlite Fraction F90/40	0.0	0.0	9.3	2.2	3.6	17.6	0.2	0.0	7.5	7.4	6.0	9.5	12.8
HLB Fraction F100	**0.0**	**26.3**	**90.1**	**84.3**	**90.5**	**95.6**	**46.2**	**60.4**	**95.4**	**87.3**	**91.3**	**91.4**	**94.3**
HLB Fraction F40	0.0	57.2	72.7	76.0	2.0	0.0	7.9	60.1	5.5	1.2	0.0	0.0	0.0
HLB Fraction F90/40	0.0	0.0	18.7	2.7	74.3	86.0	44.8	8.6	86.5	83.5	84.3	76.2	84.0

CEC – cephalosporin C, RIS – ristocetin A, LIN – lincomycin A, STV – streptovitacin A, OFL – ofloxacin, CHL – chloramphenicol, CTE – chlortetracycline, PEG – penicillin G, GRI – griseofulvin, TYL – tylosin, NAT – natamycin, ROX – roxithromycin, NOV – novobiocin; LLE – liquid-liquid extraction, SPE – solid phase extraction.
The bold values refer to the extraction protocol which provided best results and were therefore chosen for the final method (or at least considered for this in case of 0.25% TFA).

easily missed by inappropriate conditions. Therefore, the goal was to develop a method that facilitates detection of the maximal number of analytes in the broth by well-separated peaks with reasonable shape. The conditions chosen for the UHPLC method development reflect those used in published HPLC methods for analysis of natural products, secondary metabolites and antibiotics in crude extracts, cultivation broths, *etc.* [12,18,23,28–30] and emerging trends in separation techniques (*e.g.* sub-2 μm-particle chromatographic columns and alkaline mobile phase).

3.2.1. Linear gradient

The optimal linear gradient elution was developed under UHPLC conditions described in Supplementary data, S1.1. Specifically, the method is aimed at efficient fingerprinting of unknown compounds. Thus, the elution gradient has to run from a very low ratio of organic modifier to its maximum. The only crucial parameter of the gradient is its duration, pronouncing its slope and determining the peak capacity of the analysis. The longer the duration of the gradient, the better the separation is obtained. To comply with high-throughput requirements, however, the analysis time should not be any longer than necessary. Thereby, the gradient duration of g10, g15, g25, g30, g40, and g60 (see Section 2.3.2) were tested. The shorter the gradient time was, the higher the response and *Q* of particular analytes were obtained in the extracts E1–E4. However, the longer the gradient, the better the separation (higher *R* values) was observed. For instance, *Q* of peaks of E1b and E1c compounds (see Fig. 2) were as follows: 0.14 (g60), 0.20 (g40), 0.25 (g30), 0.29 (g25), 0.34 (g20), 0.26 (g15) and 0.33 (g10), whilst *R* for E1b and E1c was following: 1.93 (g60), 1.37 (g40), 1.15 (g30), 1.02 (g25), 0.76 (g20), 0.62 (g15) and 0.42 (g10). The same trend was visible for most compounds in E1–E4. As a compromise, g25 was chosen with respect to *Q* and *R* values as well as to the analysis time that is compatible with high-throughput requirements. Since all compounds present in E1–E4 and all antibiotics in A1 and A2 were eluted within 16 min, the analysis time was shortened without alternation of the gradient slope.

Further modification of the gradient program by replacing ACN as organic modifier by MeOH is described in Section 3.2.3. To sum up, the following gradients were found to be the most suitable: g25 (min/%B): 0/5; 2.0/5.0; 18.0/65.8 when ACN was used and g15 (min/%B): 0/5; 1.5/5; 16.5/100 for MeOH.

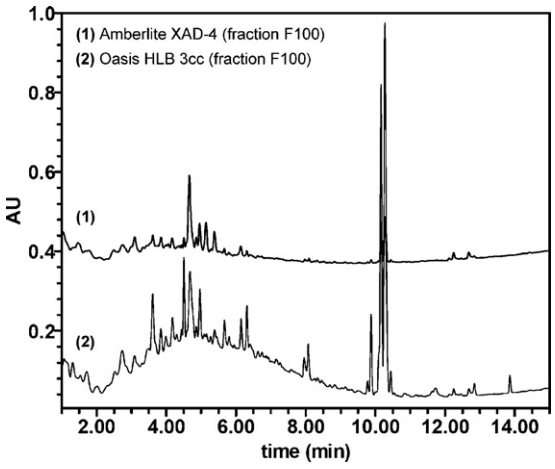

Fig. 1. The comparison of Amberlite XAD-4 and Oasis HLB 3cc for extraction of cultivation broth E1. UHPLC conditions (see also Section 2.4): Acquity UPLC BEH C18 column (50 mm × 2.1 mm I.D., particle size 1.7 μm), mobile phase: solvent A: 0.5% H_3PO_4 in water, and solvent B, MeOH; linear gradient mode (min/%B): 0/5; 1.5/5; 16.5/100 with subsequent column clean-up for 1.5 min (100% B) and equilibration for 1.0 min (5% B); flow rate, 0.4 mL min^{-1}; column temperature, 55 °C; injection volume, 5 μL; UV detection: extracted at maximal wavelengths in the range from 210 to 600 nm (max plot).

3.2.2. Aqueous part of the mobile phase

Various acidic and alkaline modifiers and buffers under UHPLC conditions that are described in Supplementary data, S1.2 were explored: TFA (0.25%, 0.1%, 0.05%, and 0.005%), H_3PO_4 (0.5%, 0.1%, 0.05%, and 0.005%), HCOOH (0.1%), 1 and 5 mM ammonium formate of different pH, water, and ammonium hydroxide (1.2×10^{-2}%). Table 2 summarizes the *Q* values for antibiotic standards that were analyzed with different aqueous parts of the mobile phase.

Pure water as aqueous part of the mobile phase led to an insufficient elution of many antibiotic standards, namely RIS, LIN, PEG, TYL, ROX, and NOV that were not detected in the chromatogram at all. Moreover, most compounds of the extracts E2 and E4 were not separated sufficiently. An addition of acidic additives improved the separation and detection of the most antibiotics compared to pure water. The data in Table 2 show that 0.25% TFA yielded the best *Q* values. However, signals intensity of STV, LIN, PEG and ROX were suppressed significantly, which cannot be deduced from the *Q* value alone. The signal suppression together with increased noise is a crucial disadvantage of TFA. Therefore, 0.5% H_3PO_4 was chosen as a compromise between optimal *Q* and *S* values and peak height. The most convenient solvent turned out to be 0.5% H_3PO_4, also for analyses of the extracts E1–E4. As an example, Fig. 3 compares different aqueous parts of the mobile phase in UHPLC analyses of the extract E2.

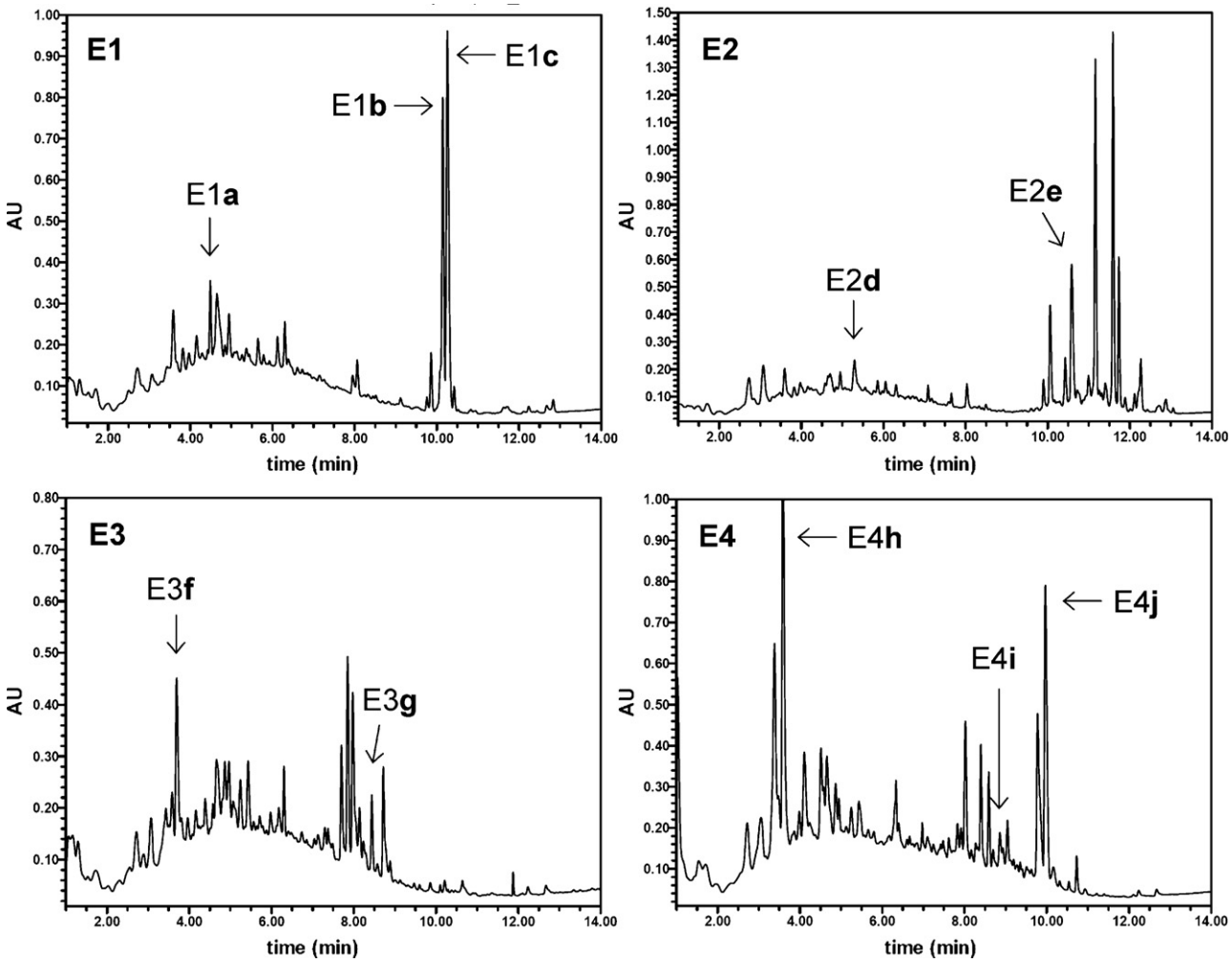

Fig. 2. UHPLC 2D fingerprints of cultivation broth E1, E2, E3 and E4 under acidic conditions. UHPLC conditions (see also Section 2.4): Acquity UPLC BEH C18 column (50 mm × 2.1 mm I.D., particle size 1.7 μm); mobile phase: solvent A, 0.5% H_3PO_4 in water, and solvent B, MeOH; linear gradient mode (min/%B): 0/5; 1.5/5; 16.5/100 with subsequent column clean-up for 1.5 min (100% B) and equilibration for 1.0 min (5% B); flow rate, 0.4 mL min^{-1}; column temperature, 55 °C; injection volume, 5 μL; UV detection: extracted at maximal wavelengths in the range from 210 to 600 nm (max plot). The labels E1a–E4j represent different unknown compounds present in the cultivation broths E1–E4 (see also Section 2.5).

Alkaline conditions generally lowered the separation quality for many antibiotics. Nevertheless, the *Q* values for RIS, LIN, TYL, and ROX were higher with alkaline solvents compared to most acidic solvents tested (see Table 2). Fluctuations in pH may have caused the asymmetric peak shape of some antibiotic standards (STV, PEG, TYL peaks tail or front) and the poorly resolved peaks that were detected in the extracts E3 and E4 when ammonium hydroxide (1.2×10^{-2}%) was used as aqueous part of the mobile phase. In this context, better results were obtained with ammonium formate, which was studied in the pH range from 8.0 to 10.0 with increasing 0.5 steps and identified pH 9.0 as optimum. One mM and 5 mM ammonium formate (pH 9.0) yielded similar *Q* and *S* values; therefore, 1 mM ammonium formate was chosen with respect to extended column life-time.

To sum up, 0.5% H_3PO_4 and 1 mM ammonium formate pH 9.0 were chosen for further development of two parallel UHPLC methods, one under acidic and the other under alkaline conditions. The merit of the latter is the provision of information about analyte acid–base properties.

Apart from UV (DAD), MS detectors are very frequently applied for fingerprinting or profiling since they provide more specific characterization of unknown compounds. Therefore, the applicability of this method for MS detectors was considered. The crucial parameter represents mobile phase composition–H_3PO_4 cannot be used for MS. However, 0.1% TFA and 0.1% HCOOH, that only yielded slightly less optimal results than 0.5% H_3PO_4, offers itself an alternative. One mM ammonium formate (alkaline conditions) as well as 0.1% TFA and 0.1% HCOOH (acidic conditions) are fully compatible with MS [31–33].

3.2.3. Organic part of the mobile phase

For acidic and alkaline aqueous parts of the mobile phase, the organic modifier was studied under UHPLC conditions that are described in Supplementary data, S1.3 and S1.4.

The contribution of the organic content of the mobile phases MeOH–ACN 100:0, 95:5, 50:50, 5:95, and 0:100 (v/v), were only compared under acidic conditions. MeOH yielded much better resolution of several compounds in the extracts E1–E4 compared to ACN and MeOH–ACN mixtures. For example, the *R* value of compounds E1b and E1c was 1.49 compared to 0.86 with ACN. Compared to ACN, however, MeOH increased the analysis time in the same gradient resulting in broader peaks and thus worse *Q* values. Therefore, sharper gradients (g10, g15 and g25) with MeOH as organic modifier were applied. Gradient g15 showed the best results with respect to *Q* and *R* parameters. A higher MeOH absorption cut-off (205 nm), compared to ACN (194 nm), discriminates absorption maxima under 205 nm (*e.g.* LIN, PEG, ROX). However, this is compensated by the simultane-

Table 2
Parameter *Q* for antibiotic standards analyzed by UHPLC with different aqueous parts of mobile phase.

Aqueous part of mobile phase	1.2×10^{-2}% ammonium hydroxide	**1 mM ammonium formate**	5 mM ammonium formate	Water	1 mM ammonium formate	5 mM ammonium formate	0.005% H_3PO_4	0.05% H_3PO_4	0.1% H_3PO_4	**0.5% H_3PO_4**	0.1% HCOOH	0.005% TFA	0.05% TFA	0.1% TFA	**0.25% TFA**
pH	10.5	9.0	9.0	7.3	4.7	4.7	3.1	2.2	2.0	1.6	2.3	3.2	2.3	1.9	1.6
CEC	0.79	**0.34**	0.44	0.30	0.47	0.22	0.23	0.17	0.17	**0.15**	0.19	0.27	0.14	0.19	**0.34**
RIS	0.40	**0.36**	0.29	n/a	0.12	0.09	0.13	0.11	0.11	**0.11**	0.11	0.10	0.29	0.30	**0.40**
LIN	0.38	**0.36**	0.35	n/a	0.13	n/a	0.08	0.15	0.17	**0.19**	0.11	n/s	0.25	0.28	**0.37**
STV	0.20	**0.24**	0.24	0.25	0.24	0.21	0.25	0.25	0.25	**0.24**	0.23	0.25	0.23	0.22	**0.22**
OFL	0.21	**0.38**	0.32	0.06	0.22	0.29	0.22	0.34	0.35	**0.35**	0.27	0.25	0.25	0.35	**0.42**
CHL	0.27	**0.27**	0.26	0.26	0.26	0.25	0.29	0.29	0.29	**0.29**	0.26	0.28	0.25	0.24	**0.25**
CTE	0.12	**0.12**	0.15	0.19	0.30	0.26	0.15	0.21	0.23	**0.27**	0.16	0.21	0.22	0.24	**0.31**
PEG	0.09	**0.17**	0.10	n/a	0.16	0.18	0.30	0.30	0.31	**0.31**	0.24	0.26	0.28	0.27	**0.33**
GRI	0.31	**0.30**	0.29	0.28	0.28	0.28	0.32	0.32	0.32	**0.31**	0.28	0.28	0.27	0.26	**0.31**
TYL	0.11	**0.30**	0.30	n/a	0.16	0.22	0.11	0.15	0.18	**0.26**	0.12	0.11	0.22	0.25	**0.33**
NAT	0.13	**0.28**	0.22	0.28	0.32	0.32	0.11	0.18	0.20	**0.25**	0.14	0.13	0.22	0.23	**0.29**
ROX	0.17	**0.24**	0.21	n/a	0.06	0.10	0.06	0.10	0.12	**0.16**	0.08	0.08	0.16	0.19	**0.27**
NOV	0.17	**0.25**	0.16	n/a	0.16	0.19	0.31	0.31	0.31	**0.33**	0.28	0.28	0.27	0.27	**0.30**

n/a – not present in the chromatogram. For UHPLC conditions see Supplementary data S1.2.
The bold values refer to the mobile phase which provided best results and were therefore chosen for the final method (or at least considered for this in case of 0.25% TFA).

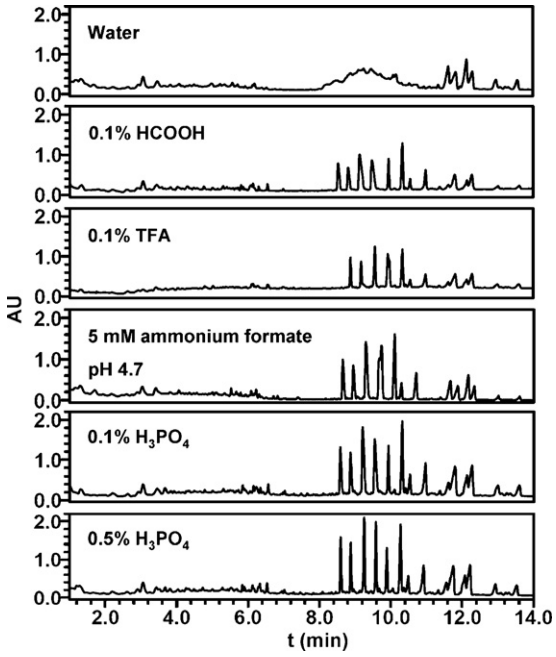

Fig. 3. The comparison of different aqueous parts of the mobile phase for UHPLC analysis of extract E2. UHPLC conditions (see also Supplementary data, S1.2): Acquity UPLC BEH C18 column (50 mm × 2.1 mm I.D., particle size 1.7 μm); mobile phase: solvent B, ACN; linear gradient mode (min/%B): 0/5; 2.0/5.0; 27.0/100 with subsequent column clean-up for 1.5 min (100% B) and equilibration for 1.0 min (5% B); flow rate, 0.4 mL min^{-1}; column temperature, 30 °C; injection volume, 5 μL; UV detection: extracted at maximal wavelengths in the range from 194 to 600 nm (max plot).

ous analysis under alkaline conditions as described in the ongoing text.

MeOH and ACN as organic modifiers tested with 1 mM ammonium formate, pH 9, yielded comparable results. Therefore, ACN was chosen with respect to lower absorption cut-off, which prevents that compounds with low UV absorption may be missed. As a result, MeOH and gradient g15 was used together with acidic conditions (0.5% H_3PO_4) and ACN and gradient g25 with alkaline conditions (1 mM ammonium formate, pH 9.0).

3.2.4. Chromatographic columns

Five chromatographic columns (see Section 2.3.2) were chosen for the fingerprinting method development and they were tested subsequently under UHPLC acidic and alkaline conditions specified in Supplementary data, S1.5 and S1.6.

Under acidic conditions, HT C18 and BEH Shield did not exhibit better *Q* values for any single antibiotic and the separation of the extracts E1–E4 was less efficient compared to the other columns. BEH Phenyl yielded significantly better results, especially concerning the *Q* parameter for PEG. However, similar or slightly better parameters also were achieved with BEH C18 and BEH C18 10 cm columns. The extracts E1 and E2 were more efficiently separated on BEH Phenyl, but fingerprints of the extracts E3 and E4 showed more fully resolved peaks on BEH C18. As an example, the comparison of different chromatographic columns for UHPLC analysis of the extract E3 is presented in Fig. 4. HT C18 and BEH Shield are not stable in pH 9.0. The separation parameters of BEH C18, BEH C18 10 cm and BEH Phenyl columns were very similar under alkaline conditions. BEH C18 10 cm provided similar separation results as BEH C18. However, it extended the analysis time without significantly improving the analysis. The fact that C18 ligand is generally more universally selective than the C6-phenyl ligand recommended the choice of the BEH C18 column for both acidic and alkaline conditions.

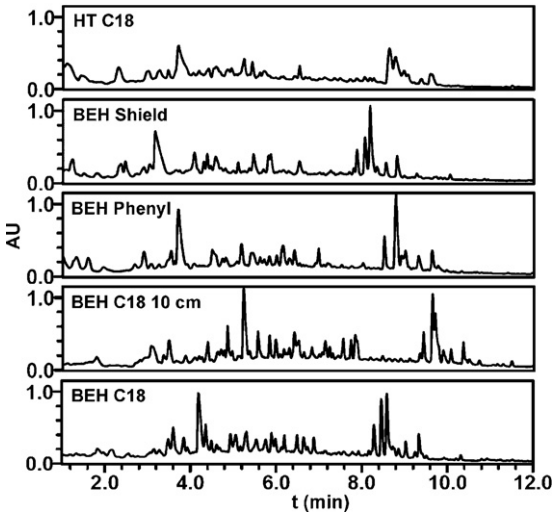

Fig. 4. The comparison of different chromatographic columns for UHPLC analysis of extract E3. UHPLC conditions (see also Supplementary data, S1.5): Mobile phase: solvent A, 0.5% H_3PO_4 and solvent B, MeOH; linear gradient mode (min/%B): 0/5; 1.5/5; 16.5/100 with subsequent column clean-up for 1.5 min (100% B) and equilibration for 1.0 min (5% B); flow rate, 0.4 mL min^{-1}, column temperature, 30 °C; injection volume, 5 μL; UV detection: extracted at maximal wavelengths in the range from 210 to 600 nm (max plot).

3.2.5. Column temperature

The influence of the column temperature on the separations at 30, 40, 50, 55, 60, and 65 °C under UHPLC acidic and alkaline conditions (see Supplementary data S1.7 and S1.8) was tested. The higher the temperature was used, the better the separation was: improved peaks shape and shorter analysis times were obtained. This applied for all extracts and most antibiotics except PEG, which was probably not stable at 60 and 65 °C. To eliminate the risk of losing thermolabile compounds, the temperature of 55 °C was chosen. Under alkaline conditions, increasing temperature did not improve the separation efficiency. Conversely, the peak response of all antibiotics as well as most compounds in E1–E4 was the same or even decreased; therefore, the temperature of 30 °C was applied.

3.3. Validation and column robustness

Repeatability of the fingerprinting method is the only parameter which is necessary to validate with respect to its purpose. The results of the method validation are summarized in Table 3 for acidic UHPLC conditions and in Table 4 for alkaline conditions. In both milieus, retention times are stable with RSD under 0.5% in all cases. As far as the peak area repeatability is concerned, RSD is under 5% with the exception of one minor peak under acidic conditions (E2d, 16.1%) and two minor peaks under alkaline conditions (E1a, 10.6% and E2d, 8.75%). In consideration that the concentration of the minor peaks corresponds to the limits of quantification, the RSD acceptance criteria of 20% are in accordance with the validation guidelines [21].

The BEH C18 column robustness under acidic conditions is sufficient for the analysis of 500 samples as corroborated by the RSD of retention times and areas of selected peaks of the extract E2, which was within the 5% limit for all 26 analyses of the extract E2 (data not shown). However, under alkaline conditions, the column was robust enough only for 180 samples; the RSD values were within 5% for the first 10 analyses of the extract E2. Then, the column separation parameters worsened significantly (data not shown). This may have been caused by precipitation of the sample matrix in high pH. The column robustness under both conditions is sufficient for its purpose, but the sample number limit has to be taken into consideration.

3.4. Fingerprinting method application

This fingerprinting method was designed for the separation of a wide spectrum of unknown compounds focusing on secondary metabolites of bacteria, potential antibiotics. This is why antibiotic standards of various polarity and properties were included in the method development. The developed fingerprinting method is illustrated by two 3D chromatograms (see Figs. 5 and 6). Different appearance of four fingerprints characterizing four different actinomycete strains E1–E4 (see Fig. 2) demonstrates that the method is able to distinguish various bacterial strains on basis of metabolites that they produce and excrete into the cultivation broth. Each

Table 3
Repeatability of the final fingerprinting method (acidic conditions).

Compound[a]	Wavelength (nm)	Retention time (min)	Retention time RSD (%)	Area (mV s)	Area RSD (%)
E1a	220	4.49	0.16	524	4.27
E1b	228	10.1	0.07	2030	1.65
E1c	237	10.3	0.07	3560	1.81
E2d	263	5.29	0.02	1010	16.1
E2e	210	10.6	0.01	2030	2.39
E3f	210	3.69	0.25	1200	3.09
E3g	220	8.44	0.11	476	1.87
E4h	254	3.58	0.09	4500	2.09
E4i	322	8.86	0.02	240	2.12
E4j	284	9.97	0.04	1620	1.49

[a] See Fig. 2.

Table 4
Repeatability of the final fingerprinting method (alkaline conditions).

Compound	Wavelength (nm)	Retention time (min)	Retention time RSD (%)	Area (mV s)	Area RSD (%)
E1a	194	3.85	0.45	670	10.6
E1b + E1c	233	12.2	0.02	6470	1.80
E2d	268	4.32	0.11	100	8.75
E2e	194	8.71	0.08	9220	2.74
E3f	194	4.17	0.04	4030	1.89
E3g	220	9.24	0.02	573	3.63
E4h	238	5.06	0.11	3920	2.27
E4i	322	7.49	0.02	178	3.59
E4j	284	6.73	0.10	813	2.32

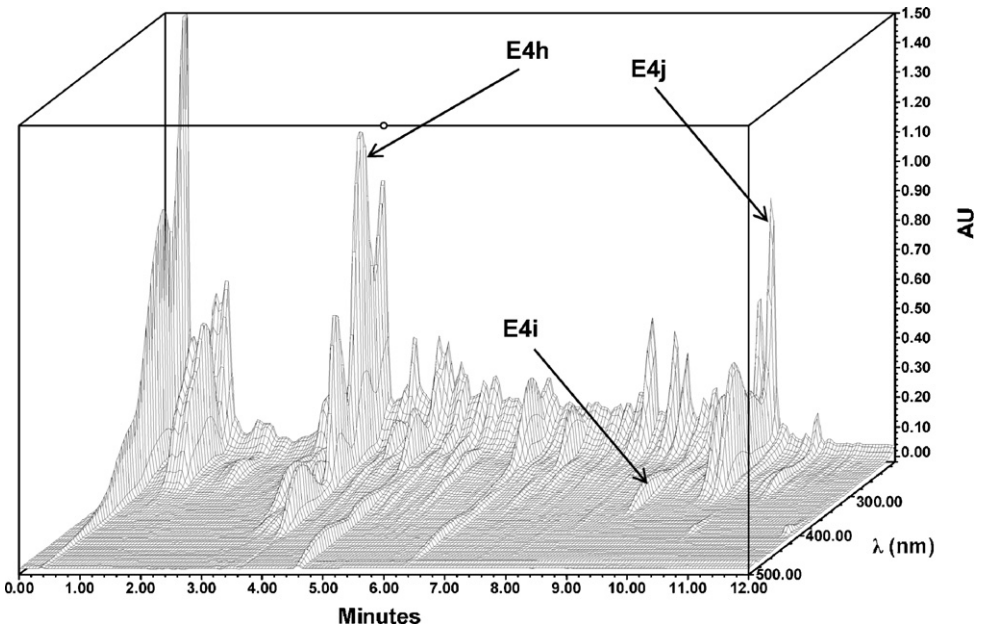

Fig. 5. UHPLC 3D fingerprint of cultivation broth E4 under acidic conditions. UHPLC conditions (see also Section 2.4): Acquity UPLC BEH C18 column (50 mm × 2.1 mm I.D., particle size 1.7 μm); mobile phase: solvent A, 0.5% H_3PO_4 in water, and solvent B, MeOH; linear gradient mode (min/%B): 0/5; 1.5/5; 16.5/100 with subsequent column clean-up for 1.5 min (100% B) and equilibration for 1.0 min (5% B); flow rate, 0.4 mL min^{-1}; column temperature, 55 °C; injection volume, 5 μL; UV detection in the range from 210 to 600 nm.

pair of fingerprints contains physico-chemical information on a set of compounds that is represented by the single peaks in the fingerprint. The detector response reflects the compound concentration and provides the quantitative information whereas the qualitative information consists of retention time, UV spectrum and acid–base properties. The retention time refers to the polarity of the compound and this parameter itself may suggest a tentative classification of the compound into a specific antibiotic group, *e.g.* β-lactames (generally more polar) or macrolides (generally less polar). UV spectra that were obtained for each peak inform about the compound structure in terms of presence and absence of specific chromophores, and together with retention times, they may be used for partial identification or dereplication. The acid–base properties are characterized by the influence of the pH of the mobile phase (aqueous part) on retention times. This is demonstrated by differences in the pair of 3D fingerprints of the extract E4 obtained under both acidic and alkaline conditions (see Figs. 5 and 6, respectively). The fingerprints vary in retention times and even elution order of some compounds indicating their specific acid–base properties. For instance, under acidic conditions, compounds E4 h, E4i and E4j are eluted at 3.6, 8.9, and 10.0 min, whereas, under alkaline conditions, at 6.1, 9.0, and 8.1 min. The retention order of E4i and E4j

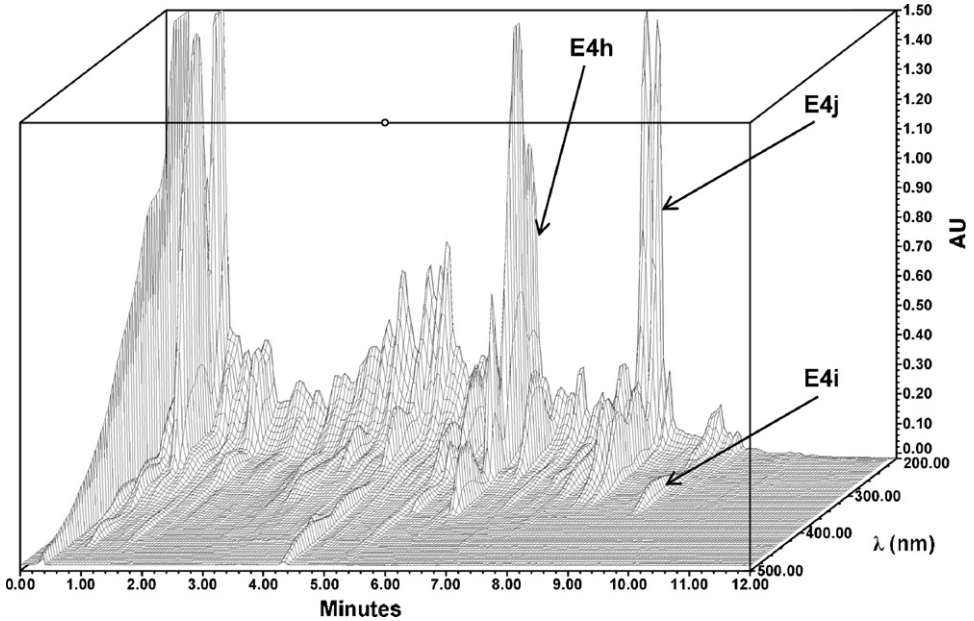

Fig. 6. UHPLC 3D fingerprint of cultivation broth E4 under alkaline conditions. UHPLC conditions (see also Section 2.4): Acquity UPLC BEH C18 column (50 mm × 2.1 mm I.D., particle size 1.7 μm); mobile phase: solvent A, 1 mM ammonium formate pH 9.0, and solvent B, ACN; linear gradient mode (min/%B): 0/5; 2.0/5.0; 18.0/65.8 with subsequent column clean-up for 1.5 min (100% B) and equilibration for 1.0 min (5% B); flow rate, 0.4 mL min^{-1}; column temperature, 30 °C; injection volume, 5 μL; UV detection in the range from 194 to 600 nm.

Table 5
The influence of mobile phase composition on retention times of antibiotics differing in acid–base properties.

Acid–base properties	Antibiotic	pK_a	Retention time[a] (min)	
			Acidic	Alkaline
Acidic	PEG	2.8	7.73	5.27
	CEC	4.8	2.55	0.47
Basic	RIS	7.5	2.70	3.63
	TYL	7.5	8.99	11.43
	LIN	7.8	3.56	7.67
	ROX	9.2	9.96	12.90
	CHL	11.0	4.98	6.21
Amphoteric	OFL	5.7, 7.9	3.92	4.93
	CTE	3.3, 7.6	5.23	5.79
	NOV	4.3, 9.1	12.60	7.57
	NAT	3.8, 7.9	9.20	8.29
Neutral	STV	–	3.86	3.68
	GRI	–	8.62	10.08

PEG – penicillin G, CEC – cephalosporin C, RIS – ristocetin, TYL – tylosin, LIN – lincomycin A, ROX – roxithromycin, CHL – chloramphenicol, OFL – ofloxacin, CTE – chlortetracycline, NOV – novobiocin, NAT – natamycin, STV – streptovitacin A, GRI – griseofulvin.

[a] Retention times were obtained under final acidic and alkaline UHPLC conditions, see Section 2.4.2.

compounds is reversed and the retention time shift of compound E4 h (from 3.6 to 6.1 min) hints its basic properties. More convincing evidence of appearance of the acid–base properties in the fingerprints provide the retention times of the antibiotic standards obtained under acidic and alkaline conditions (see Table 5). The data confirm that all acidic (CEC, PEG) and all basic (RIS, TYL, LIN, ROX, and CHL) antibiotics are more retained on the chromatographic column under their respective corresponding pH conditions. The behavior of amphoteric antibiotics (OFL, CTE, NOV, and NAT) is more complex as it additionally depends on the pK_a values besides of the pH of aqueous part of the mobile phase. Neutral antibiotics (STV and GRI) also show a slight change of retention times, which, in this case, may be affected by other parameters than pH. Generally, the higher the retention times differ, the more probable the prediction is.

Multivariate statistical methods, such as PCA (principal component analysis), may be applied to explore differences and similarities of the fingerprints (after normalization) without consideration of peak identities [34,35]. The other possibility is represented by comparison of particular compounds (peaks) of the fingerprint with data in commercial database (UV spectra) or in-house database of standards measured by the same method (retention times and UV spectra).

The total UHPLC analysis time of 19 and 20 min under acidic and alkaline conditions facilitates a high sample throughput compared to standard HPLC fingerprinting with analysis time usually exceeding 50 min [12,30].

4. Conclusion

The here presented fingerprinting method enables screening of compounds encompassing a broad spectrum of physico-chemical properties including antibiotics of the majority of antibiotic classes. The main prerequisite due to UV detection is the presence of chromophores. Therefore, it is less suitable for aminoglycosides. The major advantage of the presented method is that it facilitates metabolite screening under both acidic and alkaline conditions which provides additional chemical and physical information about the fingerprinted bacterial metabolites: (1) polarity (retention times), (2) structure (presence of chromophores in the range from 194 to 600 nm), (3) concentration (detector response) and (4) acid–base properties (the influence of mobile phase pH on retention times). The fingerprints may be further used for statistical comparison in order to dereplicate already known compounds and strains or to seek correlation between physico-chemical information of the fingerprint and genetic or ecological markers (*e.g.* presence of selected genes responsible for production of antibiotics, locality of the strains origin or their taxonomic identification, *etc.*).

Acknowledgements

This research was supported by Research Projects 2B08064 and MSM 0021620857 of the Ministry of Education, Youth and Sport of the Czech Republic, by Institutional Research Concept AV0Z50200510, and by the Ministry of Agriculture of the Czech Republic, project QH92151 and Institutional Research Concept MZE 0002700604. The financial support of Grant Agency of Charles University in Prague, project SVV 261204 and Ernst Mach grant awarded by Österreichischer Austauschdienst are also greatly acknowledged.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2010.08.031.

References

[1] T. Kieser, M.J. Bibb, M.J. Buttner, K.F. Chater, D.A. Hopwood, Practical Streptomyces Genetics, The John Innes Foundation, Norwich, 2000.
[2] J. Berdy, J. Antibiot. 58 (2005) 1.
[3] M. Goodfellow, A.G. OïDonell, in: H.I. Baumberg S., P.M. Rhodes (Eds.), Microbial Products: New Approaches, Cambridge University Press, Cambridge, 1989, p. 343.
[4] H. Zaehner, H. Fiedler, in: P.A. Hunter, G.K. Darby, N.J. Russell (Eds.), Fifty Years of Antimicrobials: Past Perspective and Future Trends. SGM Symposium 53, Cambridge University Press, Cambridge, 1995, p. 67.
[5] V. Knight, J.J. Sanglier, D. DiTullio, S. Braccili, P. Bonner, J. Waters, D. Hughes, L. Zhang, Appl. Microbiol. Biotechnol. 62 (2003) 446.
[6] M.G. Watve, R. Tickoo, M.M. Jog, B.D. Bhole, Arch. Microbiol. 176 (2001) 386.
[7] T.O. Larsen, J. Smedsgaard, K.F. Nielsen, M.E. Hansen, J.C. Frisvad, Nat. Prod. Rep. 22 (2005) 672.
[8] J.C. Frisvad, B. Andersen, U. Thrane, Mycol. Res. 112 (2008) 231.
[9] A. Taddei, M. Valderrama, J. Giarrizzo, M. Rey, C. Castelli, Res. Microbiol. 157 (2006) 291.
[10] O. Filtenborg, J.C. Frisvad, J.A. Svendsen, Appl. Environ. Microbiol. 45 (1983) 581.
[11] J.L. Wolfender, K. Ndjoko, K. Hostettmann, J. Chromatogr. A 1000 (2003) 437.
[12] K.F. Nielsen, J. Smedsgaard, J. Chromatogr. A 1002 (2003) 111.
[13] P.A. Cremin, L. Zeng, Anal. Chem. 74 (2002) 5492.
[14] G.R. Eldridge, H.C. Vervoort, C.M. Lee, P.A. Cremin, C.T. Williams, S.M. Hart, M.G. Goering, M. O'Neil-Johnson, L. Zeng, Anal. Chem. 74 (2002) 3963.
[15] F. Hadacek, Crit. Rev. Plant Sci. 21 (2002) 273.
[16] J.L. Wolfender, Planta Med. 75 (2009) 719.
[17] G. Lang, N.A. Mayhudin, M.I. Mitova, L. Sun, S. van der Sar, J.W. Blunt, A.L.J. Cole, G. Ellis, H. Laatsch, M.H.G. Munro, J. Nat. Prod. 71 (2008) 1595.
[18] J. Olsovska, M. Jelinkova, P. Man, M. Koberska, J. Janata, M. Flieger, J. Chromatogr. A 1139 (2007) 214.
[19] M.E. Swartz, J. Liq. Chromatogr. Relat. Technol. 28 (2005) 1253.
[20] S.A.C. Wren, P. Tchelitcheff, J. Chromatogr. A 1119 (2006) 140.
[21] Guidance for Industry: Bioanalytical method validation, US Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research, Center for Biologics Evaluation and Research, 2001, available: http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM070107.pdf [cit. April 5, 2010].
[22] R.W. Fedeniuk, P.J. Shand, J. Chromatogr. A 812 (1998) 3.
[23] H.-P. Fiedler, C. Bruntner, A.T. Bull, A.C. Ward, M. Goodfellow, O. Potterat, C. Puder, G. Mihm, Anton. Leeuw. Int. J. Gen. 87 (2005) 37.
[24] E.C. Eckwall, J.L. Schottel, J. Ind. Microbiol. Biotechnol. 19 (1997) 220.
[25] C. Quarta, A. Borghi, L.F. Zerilli, M.T. DePietro, P. Ferrari, A. Trani, G.C. Lancini, J. Antibiot. 49 (1996) 644.
[26] V.R. Hegde, J. Silver, M. Patel, V.P. Gullo, M.S. Puar, P.R. Das, D. Loebenberg, J. Antibiot. 56 (2003) 437.
[27] B. Shao, D. Chen, J. Zhang, Y.N. Wu, C.J. Sun, J. Chromatogr. A 1216 (2009) 8312.

[28] J.R. Tormo, J.B. Garcia, M. DeAntonio, J. Feliz, A. Mira, M.T. Diez, P. Hernandez, F. Pelaez, J. Ind. Microbiol. Biotechnol. 30 (2003) 582.
[29] J. Smedsgaard, J.C. Frisvad, J. Microbiol. Methods 25 (1996) 5.
[30] M. Stadler, T. Henkel, H. Muller, K. Weber, H. Schlecker, J. Chromatogr. A 818 (1998) 187.
[31] H. Kawanishi, T. Toyo'oka, K. Ito, M. Maeda, T. Hamada, T. Fukushima, K. Masaru, S. Inagaki, J. Chromatogr. A 1132 (2006) 148.
[32] S.A.C. Wren, P. Tchelitcheff, J. Pharm. Biomed. Anal. 40 (2006) 571.
[33] A.A.M. Stolker, P. Rutgers, E. Oosterink, J.J.P. Lasaroms, R.J.B. Peters, J.A. van Rhijn, M.W.F. Nielen, Anal. Bioanal. Chem. 391 (2008) 2309.
[34] B. Lavine, J. Workman, Anal. Chem. 82 (2010) 4699.
[35] B. Lavine, J. Workman, Anal. Chem. 78 (2006) 4137.