Quantitative analysis of multi-components via a single marker

Methods
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Direct enantioselective gradient reversed-phase ultra-high performance liquid chromatography tandem mass spectrometry method for 3-hydroxy alkanoic acids in lipopeptides on an immobilized 1.6 μm amylose-based chiral stationary phase

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3-Hydroxy fatty acids are important chiral building blocks of lipopeptides and metabolic intermediates of fatty acid oxidation, respectively. The analysis of the stereochemistry of such biomolecules has significant practical impact to elucidate and assign the enzymatic specificity of the biosynthesis machinery. In this work, a new mass spectrometry compatible direct chiral ultra high performance liquid chromatography separation method for 3-hydroxy fatty acids without derivatization is presented. The application of amylose tris(3,5-dimethylphenyl carbamate) based polysaccharide chiral stationary phase immobilized on 1.6 μm silica particles (CHIRALPAK IA-U) allows the enantioseparation of 3-hydroxy fatty acids under generic electrospray ionization mass spectrometry friendly reversed phase gradient elution conditions. Adequate separation factors were achieved with both acetonitrile and methanol as organic modifiers, covering hydrocarbon chain lengths between C\textsubscript{6} and C\textsubscript{14}. Elution orders were derived from rhamnolipid (R-95) of which enantiomerically pure or enriched (R)-3-hydroxy fatty acids were recovered after ester hydrolysis. The S-configured acids consistently eluted before the respective R-enantiomers. The method was successfully applied for the elucidation of the absolute configuration of 3-hydroxy fatty acids originating from a novel lipopeptide with unknown structure. The work furthermore demonstrates that gradient elution is a viable option also in enantioselective (ultra)high performance liquid chromatography, even for analytes with modest separation factors, although less commonly exploited.

KEYWORDS
3-hydroxy fatty acids, chiral stationary phases, enantioselective lipidomics, non-ribosomal peptide synthetase, polysaccharide selectors

Article Related Abbreviations: 3-OH-FA, 3-hydroxy fatty acid; CSP, chiral stationary phase; NRPS, non-ribosomal peptide synthetase

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1 | INTRODUCTION

Lipopeptides are amphiphilic compounds containing either a linear or cyclic peptide headgroup attached to a linear, branched, or even cyclic lipid moiety of varying length (typically C₆-C₁₈) [1,2]. In fact, they have emerged as a new class of antibiotics against drug resistant bacteria, due to their efficacy and biodegradability [3, 4]. More than once, genome sequence research has led to the identification of lipopeptides by encoding orphan gene clusters, which are a potential source for novel compounds and potential bioactive agents [5–7]. Isolation and identification of these compounds, synthesized by nonribosomal peptide synthetases (NRPSs) [5, 8, 9], is therefore paramount for their accessibility and applicability as drugs as well as for their biological testing.

Even though signature sequences within the adenylation domains of NRPSs are adequate to specify the amino acid composition of the oligopeptide, complementary methods are required for experimental verification (Marfey’s, Edman, NMR, MS/MS sequencing). Full structural characterization requires to address the stereochemistry of the constituent amino acids and of the, often substituted fatty acid side chain(s). Accessing information regarding the absolute configuration at the chiral center of the fatty acid side chain substituents, which is commonly a 3-hydroxy fatty acid (3-OH-FA) [5, 6], by enantioselective LC is still a challenge [10–15].

Polysaccharide-based chiral stationary phases (CSPs) appreciate tremendous popularity, which is mainly attributed to their wide range of versatility [16–18]. Traditionally theses phases were predominantly used in normal phase mode, but actually have multimodal applicability. Their recent immobilization (instead of coating) on 1.6 μm particles has afforded their implementation for fast and efficient UHPLC separations [19, 20]. Their compatibility with virtually all mobile phases comprising supercritical fluid, polar organic, hydrophilic interaction, and RP elution modes is maintained and selectivity with non-standard solvents often enhanced or complementary, making these CSPs rather universal [10, 17, 18, 21-23]. The most prominent derivatives are based on amyllose and cellulose aryl esters and carbamates with methyl, chloro, or mixed methyl/chloro substituents [24]. Through these structural variations a large number of broadly selective chiral columns are obtained. Changes in enantioselectivity can often be achieved by replacing one with the other. Enantioselectivity can be tuned by the backbone stereochemistry of the polysaccharide, the functional group by which the aryl moiety is attached and by substituents on the aromatic ring which influence the electron density in the aromatic moiety and hydrogen donor-acceptor properties of the functional (ester, carbamate) group [22, 25]. The carbamate functionality additionally allows some flexibility of the aromatic moiety to maximize π-π interactions. Overall, a combination of electrostatic interactions, steric discrimination and the docking into chiral (binding) cavities governs the chiral recognition process [10, 26, 27].

The direct separation of hydroxylated aliphatic acids on polysaccharide based CSPs has been reported for polyunsaturated oxygenated fatty acids [14, 19] as well as 3-hydroxyalkanoic acids after pre-column derivatization with 3,5-dimethylphenylisocyanate [28]. The latter, based on 3,5-dimethylphenyl carbamate derivative of cellulose coated silica, resolved a homologous series of 3-OH-FAs (R < S) ranging from C₆ to C₁₈, albeit requiring runtimes over 100 min due to loss of resolution with gradient elution and relatively large particle sizes (5 μm) [28]. Up to date the successful direct enantioreolution for underivatized 3-hydroxy alkanoic acids employing these polysaccharide phases was not reported, although other approaches based on derivatization [29] or utilizing ion-exchange CSPs were successfully implemented for such aliphatic alkanoic acids [30–32].

To our knowledge, we herein report the first direct UHPLC enantioseparation of 3-hydroxy alkanoic acids on an amylose tris(3,5-dimethylphenyl carbamate) based polysaccharide CSP immobilized on 1.6 μm silica particles (CHIRALPAK IA-U) for UHPLC-ESI-MS/MS applications. RP type gradient elution allowed to analyze a homologous series in one run within about 20 min and revealed perfect ESI-MS compatibility. Subsequently, the new method was employed for the enantioselective analysis of 3-hydroxy fatty acids released from a recently discovered novel class of lipopeptides. Assignment of absolute configurations of the 3-hydroxy fatty acid side chain of the lipopeptide was realized by use of rhamnolipid (R-95) hydrolysate as enantiomer standard.

2 | MATERIALS AND METHODS

2.1 | Materials

The standards 3-hydroxybutyric acid (3-OH-FA (4:0)) sodium salt, (±)-3-hydroxyhexanoic acid (3-OH-FA (6:0)), (±)-3-hydroxyoctanoic acid (3-OH-FA (8:0)), (±)-3-hydroxydecanoic acid (3-OH-FA (10:0)), (±)-3-hydroxydecanoic acid (3-OH-FA (12:0)), (±)-3-hydroxy myristic acid (3-OH-FA (14:0)), and rhamnolipid (R-95), di-rhamnolipid dominant (Rha), were obtained from Sigma Aldrich (Steinheim, Germany). Solvents and additives used for MS-detection were of LC-MS grade. Methanol (MeOH), acetonitrile (ACN), and acetic acid (AcOH) were obtained from Carl Roth (Karlsruhe, Germany).
TABLE 1  Optimized parameters for ESI MS/MS selected reaction monitoring transitions

| Name                  | Q1  | Q3  | Dwell time (ms) | CE | DP  |
|-----------------------|-----|-----|-----------------|----|-----|
| 3-OH-FA (4:0)         | 103 | 59.1| 50              | −15| −80 |
| 3-OH-FA (6:0)         | 131.1| 59.1| 50              | −15| −80 |
| 3-OH-FA (8:0)         | 159.1| 59.1| 50              | −15| −80 |
| 3-OH-FA (10:0)        | 187.1| 59.1| 50              | −15| −80 |
| 3-OH-FA (12:0)        | 215.2| 59.1| 50              | −15| −80 |
| 3-OH-FA (14:0)        | 243.2| 59.1| 50              | −15| −80 |
| 3-OH-FA (12:0) [M+1-H]− | 132.1| 59.1| 50              | −15| −80 |
| 3-OH-FA (14:0) [M+1-H]− | 160.1| 59.1| 50              | −15| −80 |

2.2 Sample preparation of standards

Due to differing solubility in water, as a consequence of varying chain length, 3-OH-FA (4:0), 3-OH-FA (6:0), and 3-OH-FA (8:0) were dissolved in H₂O, while 3-OH-FA (10:0), 3-OH-FA (12:0), and 3-OH-FA (14:0) were dissolved in MeOH/H₂O (6:4, v/v), both at a concentration of 2 μg/mL.

2.3 Rhamnolipid hydrolysis

For acidic hydrolysis, 5 mg of rhamnolipid (R-95) was suspended in 0.5 mL 2.7 M H₂SO₄ in a screw-capped glass vial. A volume of 0.5 mL of CHCl₃ was added and the obtained biphasic system was heated at 110°C for 140 min. The chloroform layer containing the fatty acid was collected, evaporated to dryness, and subsequently, solubilized in 1 mL MeOH.

For alkaline hydrolysis, a stock solution of 5 mg of rhamnolipid (R-95) in 0.5 mL MeOH (10 mg/mL) was prepared. Stock solution (50 μL) and a methanolic solution of 2N NaOH (50 μL) were each added to 900 μL of a solution of THF/MeOH (9:1, v/v) and stirred for 2 h at room temperature (∼25°C). The solvents were then removed under vacuum, the residue diluted with 200 μL of water and acidified with 0.1 M HCl to pH 2–3. The solution was then extracted thrice with 200 μL ethyl acetate, the combined organic layers evaporated to dryness and reconstituted with 100 μL MeOH/H₂O (3:7, v/v). The solution was diluted tenfold (H₂O) for LC-MS analysis.

2.4 Lipopeptide hydrolysis

The lipopeptide was first dissolved in MeOH to obtain the stock solution (10 mg/mL). An aliquot of 50 μL (corresponding to 500 μg) was added up to 1 mL with a solution of 6 M deuterated hydrochloric acid (DCl/D₂O, 1:1, v/v) in a screw-capped glass vial and heated for 24 h at 110°C. The hydrochloric acid was evaporated in an EZ-2 high performance evaporator from GeneVac (Ipswich, UK). The residue was extracted with 200 μL of a mixture of water and chloroform in a ratio of 1:1 (v/v). The chloroform layer containing the 3-hydroxyalkanoic acid was evaporated to dryness using the Genevac, and the residue reconstituted with 100 μL MeOH. The solution was dissolved tenfold with H₂O for RP and MeOH for HILIC measurements. The aqueous layer was likewise evaporated to dryness and used for amino acid analysis (reported elsewhere).

2.5 Instrumentation

Chiral chromatographic separation was performed on an Agilent 1290 Infinity UHPLC system (Waldbronn, Germany) equipped with a binary pump (G4220A), a column thermostat (G1316A), and a PAL autosampler (CTC Analytics AG, Switzerland). The separations were performed on a CHIRALPAK IA-U column (100 × 3.0 mm, 1.6 μm). The mobile phases comprised water (MP-A) and acetonitrile (MP-B), both containing 0.1% (v/v) acetic acid. The following gradient was applied if not otherwise stated: 0–2 min 10% MP-B, 2–20 min 10–100% MP-B, 20–22 min 100% MP-B, 22–22.1 min 100–10% MP-B, and 22.1–25 min 10% MP-B. The flow rate was 300 μL/min, the column temperature 40°C, and the injection volume 10 μL.

MS detection was performed on an AB SCIEX API 4000 MS/MS mass spectrometer equipped with a TurboIon-Spray (SCIEX, Ontario, Canada) in selected reaction monitoring (SRM) mode. The parameters of the selected reaction monitoring transitions, including dwell time, collision energy, and delustering potential (DP), were optimized for each compound individually and are displayed in Table 1. The total cycle time was 385 ms. All measurements were run in negative polarity mode. The cell exit potential was set to −15 V, the entrance potential to −10 V, the ion source voltage to −4500 V, the temperature to 400°C, the nebulizer gas and heater gas pressures to 30 psi, the curtain gas
to 35 psi, and the collisionally activated dissociation gas to 6 psi. PeakView 2.2 software was used for data analysis.

3 RESULTS AND DISCUSSION

3.1 Generation of enantiomeric standards by rhamnolipid hydrolysis

The full structural characterization of chiral building blocks from natural products requires besides the determination of the chemical composition also their stereochemical analysis. What lipopeptides are concerned, the amino acid sequence and their configuration as well as fatty acid carbon chain length, hydroxyl substitution and stereochemistry are structural variables which need to be clarified. Herein we use the coupling of MS with enantioselective UHPLC as a powerful tool for the structural analysis of the 3-hydroxyalkanoic acid side chains of lipopeptides. MS provides the fatty acid information, while the stereoconfiguration of the 3-hydroxyl group can be derived from known elution orders on an enantioselective chiral column.

For enantioselective analysis, enantiomeric elution orders are usually derived by comparison with enantiomerically pure (internal) standards with known configuration. However, due to their unavailability for the full series of 3-hydroxyalkanoic acids, they were generated as enantiomerically enriched references by hydrolysis of the glycolipid rhamnolipid (R-95) which is a bacterial surfactant produced by Pseudomonas aeruginosa [33]. This sample was reported to contain the corresponding abundant C<sub>10</sub> and C<sub>12</sub> (R)-3-hydroxy fatty acids (corresponding to (3R)-OH-FAs) [34, 35].

According to literature, rhamnolipid hydrolysis is performed under acidic conditions [36], probably due to a desired release of further lipid sub classes, such as the recently discovered fatty acid esters of hydroxy fatty acids [37]. However, for pure 3-hydroxy fatty acid hydrolysis the reaction kinetics governing ester hydrolysis of fatty acyl esters is substantially faster than the corresponding acidic approach [38, 39]. Two methods of hydrolysis were therefore compared, acidic with H<sub>2</sub>SO<sub>4</sub> and basic (saponification) using NaOH. Although only the ester bonded fatty acid chain is cleaved with alkaline conditions (Figure 1A), the yield was found to be >100 and >1000 times higher, for 3-OH-FA (10:0) and 3-OH-FA (12:0), respectively, and therefore quantitative. Nevertheless, acidic hydrolysis should be preferred for glycoside bond cleavages, e.g. for releasing the di-rhamnose moiety. The results in Figure 1B clearly document that alkaline ester hydrolysis is more efficient for release of 3-OH-FAs from rhamnolipid than acidic hydrolysis (cf. black vs. red traces). Furthermore, the results in Figure 1B confirm the presence of 3-OH-FA

![Figure 1](image-url)
Separation of a homologous series of 3-hydroxy alkanoic acids originating from commercial standards (black trace) and rhamnolipid (R-95) hydrolysates (red trace). Both acetonitrile (ACN) and methanol (MeOH) were used as organic modifiers (MP-B, 0.1% AcOH, v/v) in a RP gradient containing water as MP-A (0.1% AcOH, v/v). From these results we can conclude that the rhamnolipid (R-95) should be a useful standard for pinpointing the elution orders for wider series of 3-OH-FAs and for supporting the identification of the stereochemistry of 3-OH-FA building blocks in lipopeptides. An alkaline hydrolysis protocol is recommended for this purpose prior to analysis.

### 3.2 Enantioseparation of 3-hydroxy fatty acid homologues

Chiral recognition processes and hence column selection are usually hardly predictable, especially for polysaccharide-based chiral selectors and CSPs. Recently, 3-hydroxyalkanoic acids were resolved into enantiomers on cellulose tris(3,5-dimethylphenyl carbamate) coated CSP after precolumn derivatization with 3,5-dimethylphenyl isocyanate, generating carbamate derivatives [23]. The goal in the present work, however, was to avoid a derivatization step. Polysaccharide phases exhibited also enantioselectivity for hydroxylated polynsaturated fatty acids and oxylipins [19]. This was, however, not demonstrated yet for non-derivatized 3-hydroxyalkanoic acids.

In our recent work, CHIRALPAK IA-U was, to some degree, successfully applied for the structurally similar oxylipins [19] and was therefore tested first. Operated in RP elution mode, a separation of the hydroxy fatty acids according to increasing carbon number and hydrophobicity including their enantioseparation becomes feasible in a single run. At the same time, the mobile phase volatility assures ESI-MS compatibility. Therefore, a straightforward linear gradient from 10 to 100% organic modifier was applied for the separation of the homologous series of racemic 3-OH-FA standards (Figure 2, black traces). Both ACN and MeOH were applied as organic modifiers (MP-B, 0.1% AcOH) and water as MP-A (0.1% AcOH), resulting in a successful enantioseparation for all 3-OH-FAs (C6, C8, C10, C12, C14), except for 3-hydroxybutyric acid (C4). The corresponding chromatographic data are reported in Table 2. It can be seen that separation factors in gradient elution mode (ratio of net retention factors of the two enantiomers) are very similar for both organic modifiers, ACN and MeOH, ranging typically between 1.02 and 1.15. However,
peak shapes are narrower for ACN than for MeOH, which are in agreement with the findings for oxylipin separations on CHIRALPAK IA-U [19], most probably due to its lower viscosity and better mass transfer properties. The separation clearly benefits from the high efficiency of the 1.6 μm CSP which allows baseline resolutions in spite of modest separation factors. Resolution values increased with carbon chain length and were between 1.05 and 3.25 with ACN and between 1.09 and 2.16 for MeOH (Table 2). Early eluted 3-OH-FA (6:0) was not fully baseline separated in gradient elution mode (Rs ∼ 1.1). Assuming that through the gradient the separation factor could be compromised, a series of experiments under isocratic conditions was devised. The results corroborated that under isoeutoptic conditions no significant improvement in Rs can be achieved upon switching from gradient to isocratic separations (see Figure 3 and Table 2). In order to achieve full baseline separation for 3-OH-FA (6:0), conditions with significantly longer retention need to be adjusted, at expense of analysis times. 3-OH-FA (4:0) was not retained, therefore no enantioselectivity was observed. Some further optimizations under isocratic elution conditions were undertaken for 3-OH-FA (4:0). Minor changes in mobile phase composition and temperature were examined under isocratic elution for ACN (5, 10, 12.5, 15, 20, 25, 30, and 35% organic modifier) and MeOH (5, 10, 30, and 60% organic modifier) between 20 and 40°C (at 10% organic modifier in intervals of 5°C), but retention was not increased significantly to allow sufficient interaction with the CSP (data not shown). Since it was not contained in our sample, no further attempts were made to separate 3-OH-FA (4:0) enantiomers.

Finally, gradient elution with ACN was deemed favorable for fast separations producing narrower peaks, which is beneficial regarding detection sensitivities. Enantioseparation factors large enough to conclude of the absolute configurations of the hydroxyl fatty acid side chains were obtained for all test compounds, except 3-OH-FA (4:0), within a runtime of 20 min. It was found highly adequate for the present application and similar ones because lipopeptides typically exhibit fatty acid chain lengths varying from C6 to C18 [1]. Fortunately, successful separation conditions for derivatized and underivatized enantiomers of 3-OH-FA (4:0) have been reported for anion exchange-type quinine- and quindine-derived CSPs [31, 32], which provides a good alternative for this analyte if it becomes relevant.

### 3.3 Determination of elution orders

The identity of enantiomers is fully characterized only once the absolute configuration is known. Rhamnolipid (R-95) is known to contain 3-hydroxyfatty acids in R-configuration [40]. For rhamnolipid, alkaline hydrolysate, peak abundances for R-3-OH-FA (10:0) and R-3-OH-FA (12:0) are significantly higher (note, scale is ×10^6 cps), while minor concentrations of R-3-OH-FA (8:0) and R-3-OH-FA (14:0) are present as well and can be used as

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**Table 2** Chromatographic parameters obtained by chiral LC-MS 3-hydroxy fatty acids

| Sample                | ACN  | MeOH   |
|-----------------------|------|--------|
|                       | $t_\text{R}(S)$ (min) | $t_\text{R}(R)$ (min) | $\alpha^*$ | $R_\text{S}$ |
| 3-OH-FA (4:0)         | 1.85 | –      | –          | –          |
| 3-OH-FA (6:0)         | 2.97 | 3.14   | 1.15       | 1.05       |
| 3-OH-FA (8:0)         | 7.27 | 7.50   | 1.04       | 1.80       |
| 3-OH-FA (10:0)        | 10.37| 10.62  | 1.03       | 1.86       |
| 3-OH-FA (12:0)        | 12.76| 13.06  | 1.03       | 2.17       |
| 3-OH-FA (14:0)        | 14.85| 15.29  | 1.03       | 3.25       |
| Lipopeptide hydrolysate | 7.28 | 7.50   | 1.04       | 1.86       |
| 3-OH-FA (8:0)#        | –    | 7.49   | –          | –          |
| 3-OH-FA (10:0)#       | –    | 10.61  | –          | –          |
| 3-OH-FA (12:0)#       | –    | 13.05  | –          | –          |
| 3-OH-FA (14:0)#       | –    | 15.26  | –          | –          |
| 3-OH-FA (6:0), isocratic 10%B | 3.09 | 3.27   | 1.14       | 1.01       |
| 3-OH-FA (6:0), isocratic 12.5%B | 2.62 | 2.73   | 1.14       | 0.72       |
| 3-OH-FA (6:0), isocratic 15%B | 2.39 | 2.47   | 1.14       | 0.54       |
| 3-OH-FA (6:0), isocratic 30%B | –    | –      | –          | –          |

*Calculated for gradient conditions, except where stated otherwise.

#Obtained from rhamnolipid hydrolysis.

$t_0 = 1.85.$
3.4 Applicability: Side chain elucidation of a novel lipopeptide

The applicability of the new method was then demonstrated on a real lipopeptide sample with unknown structure. In order to access the stereoconfiguration of the lipopeptide side chain of this research sample from a genome-driven drug discovery study for which the structure, including stereochemistry of the constituents, has to be fully elucidated, it has to be released from the oligopeptide. Total acidic hydrolysis with HCl is the most efficient and method of first choice [36]. Structure elucidation of the compounds was accomplished by a combination of achiral LC-ESI-HR-MS and multidimensional NMR experiments, inferring the FA sidechain constituting of 3-OH-FA (8:0). However, the stereoconfiguration of the fatty acid sidechain remained inaccessible using the above-mentioned approaches.

Consequently, the cyclic lipopeptide was hydrolyzed to release the corresponding 3-OH-FA, its identity and stereoconfiguration was confirmed and concluded, respectively, by enantioselective UHPLC using the method described above employing CHIRALPAK IA-U as the chiral column prior ESI-MS detection. Using the same method for the racemic 3-OH-FA standard, fatty acids hydrolyzed from the rhamnolipid were analyzed alongside that released from the cyclic lipopeptide (Figure 4). Significant, but minor amounts of the S isomer were found in the sample in addition to the predominantly R configured isomer. According to our knowledge, natural lipopeptides containing fatty acid side chains of both configurations have not been reported, which is why the occurrence of the S isomer likely occurred due to racemization. However, the large excess of the R-enantiomer confirms that the NRPS biosynthesis generates the R-configuration of the 3-OH-FA in the lipopeptide of the research sample. This finding is in agreement with our results for the FA moiety of poaeamide [36, 41]. However, it has to be emphasized that the S-configuration has also been reported for 3-OH-FA in lipopeptides, e.g. one related to orfamide A [5]. It
Clearly demonstrates that the stereochemistry cannot be simply assumed, but must be experimentally derived. The proposed method is relatively generic and straightforward to apply.

4 CONCLUDING REMARKS

A direct simple enantioselective UHPLC-ESI-MS/MS assay was established for the experimental assignment of both the carbon chain length and stereochemistry of 3-hydroxy fatty acids ranging from hexanoic to myristic acid. The analysis can be performed without sample derivatization using amylose tris(3,5-dimethylphenyl carbamate) based polysaccharide CSP immobilized on 1.6 μm silica particles (CHIRALPAK IA-U). The elution order was consistently S < R for all reference compounds found in rhamnolipid (R-95). Enantioselectivity may also be expected for the corresponding 3-hydroxy alkanoic acids with carbon chain length > C_{14} since resolution improved with carbon chain length from C_{6} to C_{14}. In contrast, for short chain 3-0H-FAs (< C_{6}) a different selector e.g. quinine or quinidine carbamates is a viable option [30–32]. In general, the CHIRALPAK IA-U column has great potential to resolve hydroxy fatty acid enantiomers emerging as secondary metabolites from different natural sources, such as rhamnolipids [35], and biomarkers of inflammatory processes derived from polyunsaturated fatty acids [19]. Gradient elution outperformed isocratic separation as it allows to analyze the full series in a single run within 20 min and at no significant expense for separation factors.

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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REFERENCES

1. Götte S, Stallforth P. Structure elucidation of bacterial nonribosomal lipopeptides. Org Biomol Chem. 2020;18:1710–27.
2. Youssef NH, Duncan KE, McInerney MJ. Importance of 3-hydroxy fatty acid composition of lipopeptides for biosurfactant activity. Appl Environ Microbiol. 2005;71:7690–5.
3. Nemati R, Dietz C, Anstadt E, Clark R, Smith M, Nichols F, Yao X. Simultaneous determination of absolute configuration and quantity of lipopeptides using chiral liquid chromatography/mass spectrometry and diastereomeric internal standards. Anal Chem. 2017;89:3583–9.
4. Schneider T, Müller A, Miess H, Gross H. Cyclic lipopeptides as antibacterial agents - potent antibiotic activity mediated by intriguing mode of actions. Int J Med Microbiol. 2014;304:37–43.
5. Gross H, Stockwell VO, Henkels MD, Nowak-Thompson B, Loper JE, Gerwick WH. The genomsitopic approach: a systematic method to isolate products of orphan biosynthetic gene clusters. Chem Biol. 2007;14:53–63.
6. Kirchner N, Cano-Prieto C, Schulz-Finke C-A, Gütschow M, Ortlieb N, Moschyn J, Niedermeier TJ, Horak J, Lämmerhofer M, van der Voort M, Raaijmakers JM, Gross H. Discovery of Thanafactin A, a linear, proline-containing octalipopeptide from pseudomonas sp. SH-C52, motivated by genome mining. J Nat Prod. 2021;84:101–9.
7. Jahanshah G, Yan Q, Gerhardt H, Patat Z, Lämmerhofer M, Piant I, Josten M, Sahil H-G, Silby MW, Loper JE, Gross H. Discovery of the cyclic lipopeptide gacamide a by genome mining and repair of the defective GacA regulator in Pseudomonas fluorescens PFO-1. J Nat Prod. 2019;82:301–8.
8. Raaijmakers JM, de Bruijn I, de Kock MJ. Cyclic lipopeptide production by plant-associated Pseudomonas spp.: diversity, activity, biosynthesis, and regulation. Mol Plant-Microbe Interact. 2006;19:699–710.
9. Nybroe O, Sørensen J, in: Ramos, J.-L. (Ed.), Pseudomonas: Volume 3 Biosynthesis of Macromolecules and Molecular Metabolism. Boston, MA: Springer US; 2004:147–72.
10. Lämmerhofer M. Chiral recognition by enantioselective liquid chromatography: Mechanisms and modern chiral stationary phases. J Chromatogr A. 2010;1217:814–56.
11. Ilisz I, Bajtai A, Lindner W, Péter A. Liquid chromatographic enantiomer separations applying chiral ion-exchangers based on Cinchona alkaloids. J Pharm Biomed Anal. 2018;159:127–52.
12. Scriba GKE. Chiral recognition mechanisms in analytical separation sciences. Chromatographia. 2012;75:815–38.
13. Sardella R, Ianni F, Lisanti A, Marinuzzi M, Scorzoni S, Natalini B. The effect of mobile phase composition in the enantioseparation of pharmaceutically relevant compounds with polysaccharide-based stationary phases. Biomed Chromatogr. 2014;28:159–67.
14. Ianni F, Saluti G, Galarini R, Fiorito S, Sardella R, Natalini B. Enantioselective high-performance liquid chromatography analysis of oxygenated polyunsaturated fatty acids. Free Radicals Biol Med. 2019;144:35–54.
15. Scriba GKE. Chiral recognition in separation science – an update. J Chromatogr A 2016;1467:56–78.
16. Jakubec P, Douša M, Nováková L. Supercritical fluid chromatography in chiral separations: evaluation of equivalency of polysaccharide stationary phases. J Sep Sci. 2020;43:2675–89.
17. Zhang T, Nguyen D, Franco P. Enantiomer resolution screening strategy using multiple immobilised polysaccharide-based chiral stationary phases. J Chromatogr A 2008;1191:214–22.
18. Thunberg L, Hashemi J, Andersson S. Comparative study of coated and immobilized polysaccharide-based chiral stationary phases and their applicability in the resolution of enantiomers. J Chromatogr B Analyt Technol Biomed Life Sci. 2008;875:72–80.
19. Cebo M, Fu X, Gawaz M, Chatterjee M, Lämmerhofer M. Enantioselective ultra-high performance liquid chromatography-tandem mass spectrometry method based on sub-2μm particle polysaccharide column for chiral separation of oxypills and its application for the analysis of autooxidized fatty acids and platelet releasates. J Chromatogr A 2021;44:1875–1883.

20. Berger TA. Preliminary kinetic evaluation of an immobilized polysaccharide sub-2μm column using a low dispersion super-critical fluid chromatograph. J Chromatogr A 2017;1510:82–8.

21. Ikai T, Okamoto Y. Structure control of polysaccharide derivatives for efficient separation of enantiomers by chromatography. Chem Rev. 2009;109:6077–101.

22. Chankvetadze B. Recent developments on polysaccharide-based chiral stationary phases for liquid-phase separation of enantiomers. J Chromatogr A 2012;1269:26–51.

23. Colombo M, Ferretti R, Zanitti L, Cirilli R. Direct separation of the enantiomers of ramoseoter on a chlorinated cellulose-based chiral stationary phase in hydrophilic interaction liquid chromatography mode. J Sep Sci. 2020;43:2589–93.

24. Matarashvili I, Chelidze A, Dolidze G, Kobidze G, Zaqashvili N, Dadianidze A, Bacsay I, Felinger A, Farkas T, Chankvetadze B. Separation of enantiomers of chiral basic drugs with amyllose- and cellulose-phenylcarbamate-based chiral columns in acetonitrile and aqueous-acetonitrile in high-performance liquid chromatography with a focus on substituent electron-donor and electron-acceptor effects. J Chromatogr A 2020;1624:461218.

25. Chankvetadze B. Recent trends in preparation, investigation and application of polysaccharide-based chiral stationary phases for separation of enantiomers in high-performance liquid chromatography. TrAC, Trends Anal Chem. 2020;122:115709.

26. Scriba GK, Chiral Separations. Boston, MA: Springer; 2019:1–33.

27. Scriba GKE. Chiral recognition in separation sciences. Part I: Polysaccharide and cyclodextrin selectors. TrAC, Trends Anal Chem. 2019;120:115639.

28. Abdel-Mawgoud AM, Lépine F, Déziel E. A chiral high-performance liquid chromatography–tandem mass spectrometry method for the stereospecific analysis of enoyl-coenzyme A hydratases/isomerases. J Chromatogr A 2013;1306:37–43.

29. Tsai Y-C, Liao T-H, Lee J-A. Identification of 1,3-hydroxybutyrate as an original ketone body in rat serum by column-switching high-performance liquid chromatography and fluorescence derivatization. Anal Biochem. 2003;319:34–41.

30. Liu S-L, Oyama T, Miyoshi Y, Sheu S-Y, Mita M, Ide T, Lindner W, Hamase K, Lee J-A. Establishment of a two-dimensional chiral HPLC system for the simultaneous detection of lactate and 3-hydroxybutyrate enantiomers in human clinical samples. J Pharm Biomed Anal. 2015;116:80–5.

31. Ianni F, Pataj Z, Gross H, Sardella R, Natalini B, Lindner W, Lämmerhofer M. Direct enantioseparation of undervatized aliphatic 3-hydroxyalkanoic acids with a quinine-based zwitterionic chiral stationary phase. J Chromatogr A 2014;1363:101–8.

32. Calderón C, Lämmerhofer M. Chiral separation of short chain aliphatic hydroxycarboxylic acids on cinchonan carbamate-based weak chiral anion exchangers and zwitterionic chiral ion exchangers. J Chromatogr A 2017;1487:194–200.

33. Varjani SJ, Upasani VN. Critical review on biosurfactant analysis, purification and characterization using rhamnolipid as a model biosurfactant. Bioresour Technol. 2017;232:389–97.

34. Tiso T, Zauter R, Tulke H, Leuchte B, Li W-J, Behrens B, Wittgens A, Rosenau F, Hayen H, Blank LM. Designer rhamnolipids by reduction of congener diversity: production and characterization. Microb Cell Fact. 2017;16:225.

35. Behrens B, Engelen J, Tiso T, Blank LM, Hayen H. Characterization of rhamnolipids by liquid chromatography/mass spectrometry after solid-phase extraction. Anal Bioanal Chem. 2016;408:2505–14.

36. Gerhardt H, Sievers-Engler A, Jahanshah G, Pataj Z, Ianni F, Gross H, Lindner W, Lämmerhofer M. Methods for the comprehensive structural elucidation of constitution and stereochemistry of lipopeptides. J Chromatogr A 2016;1428:280–91.

37. Ma Y, Kind T, Vaniya A, Gennity I, Fahrmann JF, Fiehn O. An in silico MS/MS library for automatic annotation of novel FAHFA lipids. J Cheminf. 2015;7:53.

38. Ault A. Telling it like it is: teaching mechanisms in organic chemistry. J Chem Educ. 2010;87:937–41.

39. Theodorou V, Skobridis K, Tzakos AG, Raoussis V. A simple method for the alkaline hydrolysis of esters. Tetrahedron Lett. 2007;48:8230–3.

40. Déziel E, Lépine F, Milot S, Villemur R. Mass spectrometry monitoring of rhamnolipids from a growing culture of Pseudomonas aeruginosa strain 57RP. Biochim Biophys Acta, Mol Cell Biol Lipids. 2000;1485:145–52.

41. Zachow C, Jahanshah G, de Bruijn I, Song C, Ianni F, Pataj Z, Gerhardt H, Pianet I, Lämmerhofer M, Berg G, Gross H, Raaijmakers JM. The novel lipopeptide poaeamide of the endophyte Pseudomonas poae RE*1-1-14 is involved in pathogen suppression and root colonization. Mol. Plant-Microbe Interact 2015;28:800–10.

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