# Chapter 30

# Liquid Chromatography/Mass Spectrometry for the Identification and Quantification of Rhamnolipids

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## Abstract

Rhamnolipids (RL) are surface-active glycolipids produced by *Pseudomonas aeruginosa*. They are always produced by this bacterium as a complex mixture of congeners, each composed of one or two rhamnose molecules linked to a dimer of 3-hydroxyfatty acids with a chain length of 8–12 carbons. Increasing interest for RL drives the need for efficient analytical methods to characterize these mixtures of molecules.

High-performance liquid chromatography (HPLC) coupled with tandem mass spectrometry (MS/MS) is a very precise and relatively high-throughput method for the identification of each congener and their quantification in bacterial cultures. Using $^{13}$C-labeled RL as internal standards can further enhance the precision of the quantification. Collision-induced dissociation (CID) experiments by MS/MS is a powerful tool for the detection and identification of structural variations in RL produced by various *Pseudomonas* strains or by a specific strain under different culture conditions. CID even allows the discrimination between isomers with subtle structural variations, like Rha-$C_8$-$C_{10}$ and Rha-$C_{10}$-$C_8$, which are almost inseparable chromatographically. We are presenting here the detailed protocols for HPLC/MS and HPLC/MS/MS analysis of RL and their lipid precursors, the 3-(3-hydroxyalkanoyloxy)alkanoic acids (HAA), directly in bacterial culture supernatants.

**Key words** HPLC, Reversed phase, Tandem mass spectrometry, Electrospray negative ionization, Rhamnolipids, Congeners, HAA, $^{13}$C-labeled RL, Identification, Quantification

## 1 Introduction

While the structures and the biosynthetic pathway of rhamnolipids (RLs) produced by *Pseudomonas aeruginosa* were initially described in considerable details [1–3], their exact biological role remained poorly understood for a long time. Over the years, many functions were attributed to these unique glycolipids with surface-active properties [4]. They were at first described as a heat-stable hemolysin [5, 6]. Then, their role in the assimilation of hydrocarbons was investigated extensively [7–10]. Their involvement in infections as virulence factors and as immunomodulators was described [11–14] and further defined over recent years [15–17]. In the mid-1990s, the genes required for the biosynthesis of RLs were

Alain Filloux and Juan-Luis Ramos (eds.), *Pseudomonas Methods and Protocols*, Methods in Molecular Biology, vol. 1149, DOI 10.1007/978-1-4939-0473-0_30, 

identified [18], including those responsible for their regulation, the RhlR/RhlI quorum sensing system [19, 20]. In the following years, the role of RLs and of their biosynthetic precursors 3-(3-hydroxyalkanoyloxy)alkanoic acids (HAAs) in bacterial surface motility, especially swarming [21–24], and also twitching [25, 26], emerged as an important function that is increasingly linked to biofilm development [27–30].

Nowadays, the primary interests for RLs among scientists investigating *P. aeruginosa* stem from their involvement in surface motility/biofilm development and their role as virulence factors. Concurrently, these biosurfactants are still attracting a lot of attention as alternatives to synthetic surfactants and a great deal of literature is devoted to the development of production processes for their industrial applications [31, 32]. Since we know that the level of rhamnosylation and the length of the side chains impact the function and activity of these tensioactive compounds [22, 24], it is important to know which molecules are produced out of the more than 50 congeners known [33] and in which quantity.

A number of methods have been developed and used over the years to characterize the presence of RLs [32, 34]. These include (a) the drop-collapsing test, which indirectly indicates the presence of a surfactant in a liquid as revealed by a reduction in surface tension [35]; (b) the methylene blue agar plate, which allows the semiquantitative on-plate detection of the production of anionic amphiphilic molecules by growing bacteria [36]; (c) the colorimetric quantification of rhamnose after hydrolysis of RL, using orcinol, as an indirect measurement [10, 37]; (d) chromatographic separation of the more abundant congeners by thin-layer [38] or liquid chromatography (LC) [39] using various detection methods; and, finally, (e) mass spectrometry (MS), as a very precise and efficient technique [33, 40]. We describe here how to use LC/MS to directly detect and quantify RLs/HAAs in bacterial cultures.

## 2 Materials

The chromatography eluents consist of high-performance liquid chromatography (HPLC)-grade solvents. The aqueous solutions are prepared using Milli-Q water with a resistivity of 18 MΩ cm at 25 °C. Otherwise, reagents are of analytical or ACS grade. Prepare and store all reagents at room temperature (unless otherwise indicated). Diligently follow all waste disposal regulations.

### 2.1 Bacterial Strain, Culture Conditions, and Sample Preparation

1. *Pseudomonas aeruginosa*, strain PA14 [41], is the RL-producing bacterium used for the presented example. Nonetheless, any RL-producing *Pseudomonas* strain would be applicable with this protocol. PA14 strain is preserved in glycerol stock (15 % v/v) and stored at −80 °C.

2. Tryptic soy broth (TSB) medium (Difco) or any other rich medium for overnight culture of frozen cells.
3. In principle, any typical culture medium is appropriate to perform the cultures for RL production. An optimized mineral salts medium (MSM) with glycerol as the carbon source is used for the presented protocol. MSM has the following composition: $Na_2HPO_4$ (0.9 g/L), $KH_2PO_4$ (0.7 g/L), $CaCl_2{\cdot}2H_2O$ (0.1 g/L), $MgSO_4{\cdot}7H_2O$ (0.4 g/L), $NaNO_3$ (2 g/L), tryptone (1 g/L, Fisher Scientific), trace element solution (TES, 2 mL/L), pH 7 ± 0.1. Composition of TES is as follows: $FeSO_4{\cdot}7H_2O$ (2 g/L), $MnSO_4{\cdot}H_2O$ (1.5 g/L), $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ (0.6 g/L). Store TES at 4 °C. Glycerol is added as the carbon source at 15 g/L (*see* **Note 1**).
4. Sterile borosilicate glass test tubes (18 × 150 mm).
5. Incubator adjusted at 34 °C and equipped with a TC-7 roller drum (New Brunswick Scientific).
6. Polypropylene 1.5 mL microtubes and pipette tips.
7. Mini centrifuge to achieve 10,000 × *g*.
8. Freezer at −20 °C.

### 2.2 HPLC/MS Solutions and Standards

1. Concentrated ammonium hydroxide, ACS grade (28–30 %).
2. Ammonium acetate solution: 100 mM ammonium acetate solution in Milli-Q water, pH 8.55. Weigh 1.9271 g of ammonium acetate in 200 mL Milli-Q water, adjust the pH to 8.55 by the addition of concentrated ammonium hydroxide (28–30 %) (*see* **Note 2**), and complete the volume to 250 mL using a volumetric flask. Store at 4 °C.
3. Mobile phase, solvent A: 2 mM ammonium acetate in Milli-Q water, pH 8.0–8.5. In a 500 mL volumetric flask, add 10 mL of 100 mM ammonium acetate solution, then add 400 mL of Milli-Q water, adjust the pH with concentrated ammonium hydroxide (28–30 %) to 8.0–8.5, and then complete the volume to 500 mL with Milli-Q water (*see* **Note 3**).
4. Mobile phase, solvent B: 2 mM ammonium acetate in acetonitrile, pH 8.0–8.5. In a 500 mL volumetric flask, add 10 mL of 100 mM ammonium acetate solution, then add 400 mL acetonitrile, adjust the pH with ammonium hydroxide (28–30 %) to 8.0–8.5, and then complete the volume to 500 mL with acetonitrile (*see* **Note 4**).
5. Internal standard (IS) stock solutions: $^{13}$C-labeled mono-RL (Rha-$C_{10}$-$C_{10}$; $^{13}C_{26}H_{48}O_9$, with a mass shift of 26 Da) and di-RL (Rha-Rha-$C_{10}$-$C_{10}$, $^{13}C_{32}H_{58}O_{13}$ with a mass shift of 32 Da) (*see* **Note 5**). These $^{13}$C-labeled RLs are produced by PA14 fed with dextrose-$^{13}C_6$ as sole carbon source, extracted from the cultures with ethyl acetate, and purified using 1 mm thin-layer chromatography (TLC) plates (Partisil PK6F,

Whatman) developed with methanol:acetic acid:ethyl acetate (10:1:89). TLC-purified HAA-$^{13}C_{20}$ with a mass shift of 20 Da is prepared from an ethyl acetate extract of HAAs produced by a *rhlB* mutant strain of PA14 fed with dextrose-$^{13}C_6$ as sole carbon source. All internal standard solutions are prepared in ACS-grade methanol at 5,000 mg/L for labeled RL and 2,500 mg/L for labeled HAAs. Store all internal standard solutions at −20 °C.

6. HPLC vials: Screw capped, borosilicate glass vials 11.6 × 32 mm.

### 2.3 HPLC/MS Instrumentation

1. HPLC instrument (Alliance HT, 2795 Separation Module, Waters) equipped with a C8 reversed phase column (4.6 × 150 mm Eclipse XDB-C8, Agilent, USA, particle size 5 μm) with a C8 guard column (*see* **Note 6**).
2. The HPLC is coupled to the MS (Quattro Premier XE, triple quadrupole, Micromass) through a Tee splitter with a fixed 10:1 split ratio (Agilent). This is achieved by fitting the third outlet of the splitter with a tube of internal diameter and length such that 10 % of the initial flow goes to the electrospray ionization interface of the MS operated in negative ionization mode.
3. Nitrogen is used as the cone and desolvation gas. Argon is used as collision gas in collision-induced dissociation (CID) experiments or in cases quantification is performed in multiple reaction monitoring (MRM) mode.
4. The MassLynx™ software with its QuanLynx™ function (version 4.1, Waters) is used for automatic peak integration.

## 3 Methods

RLs are rhamnosylated β-hydroxy fatty acids initially isolated from *P. aeruginosa* cultures. Currently, nearly 60 RL homologues have been identified in a few bacteria with β-hydroxy fatty acid of various lengths and unsaturation level [4]. The di-RL (Rha-Rha-$C_{10}$-$C_{10}$) and mono-RL (Rha-$C_{10}$-$C_{10}$) are the most abundant congeners produced by *P. aeruginosa* (Fig. 1). This bacterium also releases the di-lipidic precursors of RL (HAAs) in the supernatant, also produced as a series of homologues with carbon chains varying from $C_8$ to $C_{12}$ [42]. The most abundant of these HAA congener is 3-(3-hydroxydecanoyloxy)-decanoic acid ($C_{10}$-$C_{10}$) (Fig. 1).

HPLC coupled with MS is a very powerful tool for the separation, identification, and quantification of RL congeners directly in bacterial culture supernatants. Moreover, tandem MS/MS even allows the discrimination of HPLC-inseparable structural isomers such as Rha-$C_{10}$-$C_8$ from Rha-$C_8$-$C_{10}$, according to their fragmentation patterns.

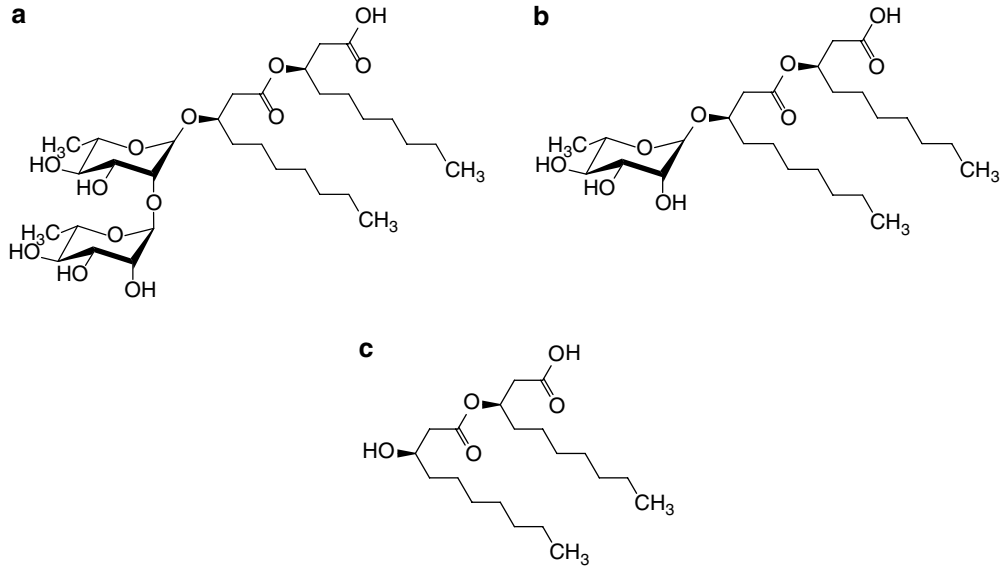


**Fig. 1** Chemical structure of the two rhamnolipids produced by *P. aeruginosa*: (**a**) di-RL (Rha-Rha-$C_{10}$-$C_{10}$), (**b**) mono-RL (Rha-$C_{10}$-$C_{10}$), and their di-lipid precursor (**c**) 3-(3-hydroxydecanoyloxy)decanoic acid ($C_{10}$-$C_{10}$) (HAA)

HPLC separation is performed using a mobile-phase gradient of water to acetonitrile, each buffered with 2 mM ammonium acetate using a reversed-phase column. Mass spectrometric analyses of RL and HAA are performed under negative electrospray ionization (ESI) mode, which generates $[M–H]^-$ pseudomolecular ions corresponding to the mass of the neutral molecule minus 1 Da as a result of the loss of one proton. Mass spectrometers analyze ions according to their mass divided by their charge (*m/z*), but since only one charge is applied under the conditions used, the *m/z* ratio is equal to the mass of the pseudomolecular ion. The usefulness of ESI is that it is considered as a soft method of ionization with little fragmentation of the primary pseudomolecular ion $[M–H]^-$.

The HPLC/MS method described here is for RL produced by *P. aeruginosa*, with particular emphasis on the two most abundant RL congeners Rha-Rha-$C_{10}$-$C_{10}$ (*m/z* 649) and Rha-$C_{10}$-$C_{10}$ (*m/z* 503). This protocol is also valid for other RL homologues produced by *Pseudomonas* species with β-hydroxy fatty acid chain lengths ranging from $C_8$ to $C_{12}$, taking into consideration that 14 Da must be added to their masses for every additional methylene unit ($–CH_2–$) and subtracted by 2 Da for each additional unsaturation. In addition to their pseudomolecular ions, RL can be identified using tandem MS/MS in which the pseudomolecular ion is fragmented by colliding with argon, generating fragments of specific masses (Fig. 2).

Depending on their structure, RL congeners will produce fragment ions with different *m/z* values according to their number of rhamnose (one or two) or β-hydroxy fatty acid moiety (one or two), the length of their carbon chains, or the presence of

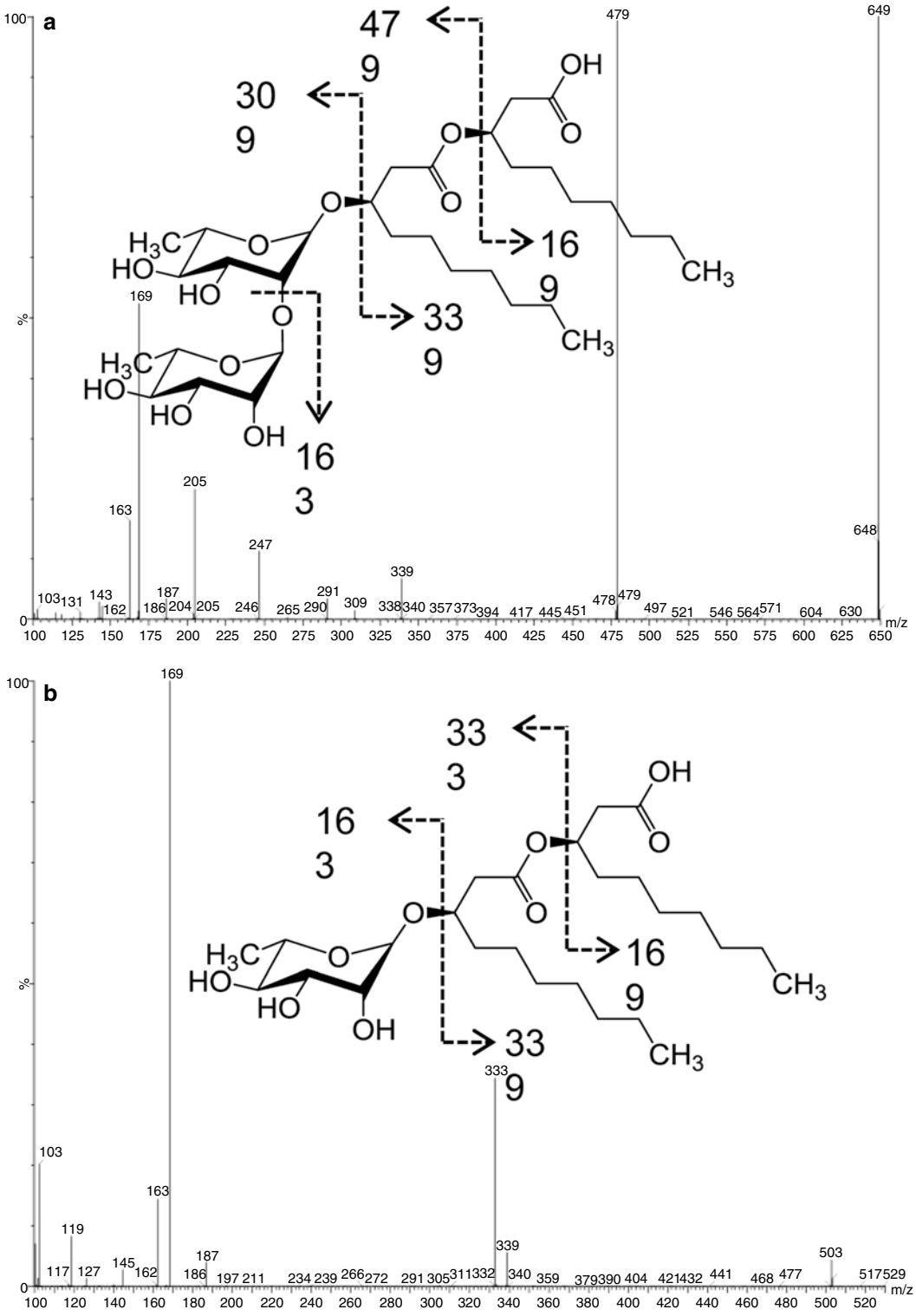


**Fig. 2** Common fragment ions produced upon fragmentation of (**a**) di-RL (Rha-Rha-$C_{10}$-$C_{10}$) and (**b**) mono-RL (Rha-$C_{10}$-$C_{10}$) using tandem MS/MS

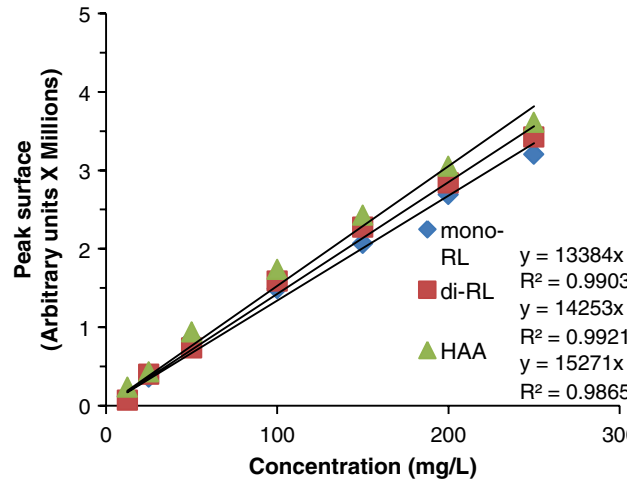


**Fig. 3** Range of concentrations showing linearity of MS detection for di-RL, mono-RL, and HAAs

unsaturations or substitutions in the β-hydroxy fatty acid moiety. The masses of these fragment ions can be used to tentatively identify structural changes in new RL congeners.

In addition to the structural information it provides, HPLC/MS also allows precise quantification of these congeners by comparing the peak area of the pseudomolecular ion relative to that of an internal standard added at a known concentration during sample preparation. In this approach, RL can be quantified in the culture supernatants after undergoing chromatographic separation, although quantification can even be performed by direct infusion without prior chromatographic separation [40]. However, because in direct infusion mode other components of the matrix will also compete for the charges to be applied on RL molecules, the sensitivity of MS detection and hence precision of quantification are considerably improved by chromatographic separation.

Under the conditions described in the methods presented here, the linear detection range of mono- and di-RL lies between 10 and 250 mg/L in the full scan mode and that of HAAs lies between 10 and 150 mg/L (Fig. 3). Accordingly, to achieve precise quantification, the supernatants should be diluted when RL concentrations exceed this range.

Detection limits below 10 mg/L can be achieved using an alternative scanning mode called "MRM". In MRM, tandem MS/MS is used to fragment selected pseudomolecular ions of RL congeners and monitor the intensity of a selected fragment ion, from which the concentration of the parent ion can be deduced. MRM provides a much better signal-to-noise ratio than the full scan mode. However, MRM requires the prior determination of the ions to fragment and the fragment to monitor. Thus, novel RLs with unexpected structures will not be detected resulting in a potential loss of information

that would have been provided in full scanning mode. Therefore, MRM is advised only when maximal sensitivity is needed for detection and quantification of a limited number of predetermined RL congeners. Otherwise, full scanning mode will provide sufficient sensitivity for detection and quantification of a wider range of molecules that can be detected in negative ionization mode.

An important advantage of the method described here is that it analyzes RLs and HAAs directly in culture supernatants without any prior extraction steps (typically with organic solvents), thus avoiding the inevitable losses encountered during purification. Nevertheless, if an even greater sensitivity is required or the culture medium is not amenable to direct injection (e.g., high concentrations of interfering exoproducts), RLs can be extracted from the culture medium with ethyl acetate, the solvent evaporated to dryness, and the residue dissolved in a minimum amount of a 30:70 water:acetonitrile solution.

For quantification purposes, it is imperative that a fixed concentration of an internal standard be added in the sample to analyze in order to compensate for the frequently encountered day-to-day or run-to-run variations in the sensitivity of the MS detector and hence the peak area-based calculations of RL concentrations. The internal standards used in the presented method are fully $^{13}$C-labeled Rha-Rha-$C_{10}$-$C_{10}$ and Rha-$C_{10}$-$C_{10}$, which are directly added in the culture sample prior to centrifugation to act as reference for all di- and mono-RL congeners, respectively. Similarly, the fully $^{13}$C-labeled HAA, $^{13}C_{10}$-$^{13}C_{10}$, is used as internal standard for quantification of all HAA homologues. These internal standards are ideal for precise RL quantification as they possess essentially identical physicochemical, chromatographic, and ionization properties to natural RL while remaining discriminable by the mass analyzer. With an elution program of 20 min, this HPLC/MS method allows the quantification of RL and HAAs at a rate of three samples per hour. Higher throughput can be achieved using isocratic gradients. The preparation of the samples for RL and HAA analysis and details of HPLC/MS method setup are described below.

### 3.1 Rhamnolipid Production and Sample Preparation for HPLC/MS Analysis

1. Transfer 1 mL of a PA14 preculture (*see* **Note 7**) grown overnight in TSB (200 rpm, $OD_{600}$ of 5.0–6.0) into a 1.5 mL microtube and centrifuge at 10,000 × *g* for 2 min. Discard the supernatant, add 1 mL of sterile MSM–glycerol, vortex, and centrifuge at 10,000 × *g* for 2 min. Repeat the washing with MSM–glycerol twice (*see* **Note 8**). Resuspend the cell pellet in 1 mL of MSM–glycerol, and measure the $OD_{600}$. Transfer a calculated volume (~100 μL) of this washed cell suspension into a borosilicate test tube containing 5 mL of MSM–glycerol to achieve an initial $OD_{600}$ of 0.1.
2. Incubate the inoculated MSM–glycerol test tube under rotation (200 rpm) at 34 °C for 3–4 days (*see* **Note 9**).

3. Transfer about 500 µL of culture broth into a 1.5 mL microtube, and record the $OD_{600}$ reading. Centrifuge at 10,000 × *g* for 10–15 min to remove cells and debris. Carefully transfer 250 µL of the supernatant into another 1.5 mL microtube. This supernatant can be stored at –20 °C until the day of analysis as RLs remain stable for unlimited time at this temperature.
4. Prepare the sample diluent solution containing 52 mg/L of each of the two IS, di-RL-$^{13}C_{32}$ and mono-RL-$^{13}C_{26}$, in 30:70 v/v water:acetonitrile. Add 480 µL of this IS-containing solution into an HPLC borosilicate vial, add 20 µL of sample supernatant (*see* **Note 10**), and vortex for 10 s. It is recommended to prepare a stock solution of IS (52 mg/L) to be distributed to each vial as this takes less time and avoids sample-to-sample variations in IS concentration. The final concentration of each IS. in the vial will be 50 mg/L, and the supernatant will have been diluted 25-fold.

### 3.2 Quantitative Analysis of RL Using HPLC/MS

1. Ensure the stability of the HPLC conditions (tubing, injector, and separation column) as well as the MS sensitivity (*see* **Note 11**).
2. Place the HPLC borosilicate vials in the HPLC tray chamber, and set the injection volume at 20 µL.
3. Set the mobile-phase flow rate to 400 µL/min with a 20-min gradient of water (solvent A) and acetonitrile (solvent B). Set the gradient as follows: 0 min, 30 % A + 70 % B; 16 min, 10 % A + 90 % B; 17 min, 30 % A + 70 % B; and 20 min, 30 % A + 70 % B.
4. Verify that the split ratio at the Tee splitter is fixed at 10:1, so that the flow at the mass spectrometer is at 40 µL/min.
5. Configure the mass spectrometer tuning parameters according to the following: Negative ESI mode; capillary voltage 3.0 kV; cone voltage 20 V; source and desolvation temperatures 120 and 150 °C, respectively; and cone and desolvation gas ($N_2$) flow 15 and 100 L/h, respectively.
6. Set the MS method to scan over a *m/z* range of 130–800 every 1.7 s (for an instrument calibration scan speed of 400 Da/s). A characteristic total ion chromatogram (TIC) and selected ion chromatograms are generated using the MassLynx 4.1 software (Fig. 4).
7. For quantitation of a specific RL congener, display the single-ion chromatogram corresponding to the pseudomolecular ions $[M–H]^-$, such as the *m/z* ions 649 and 503, which correspond to the pseudomolecular ions of natural di-RL (Rha-Rha-$C_{10}$-$C_{10}$) and mono-RL (Rha-$C_{10}$-$C_{10}$), respectively. Similarly, display the ion chromatogram of the masses of $^{13}C$-labeled RL acting as internal standards, having *m/z* of 681 and 529 for di-RL-$^{13}$ C32 and mono-RL-$^{13}C_{26}$, respectively (Fig. 4) (*see* **Note 12**).

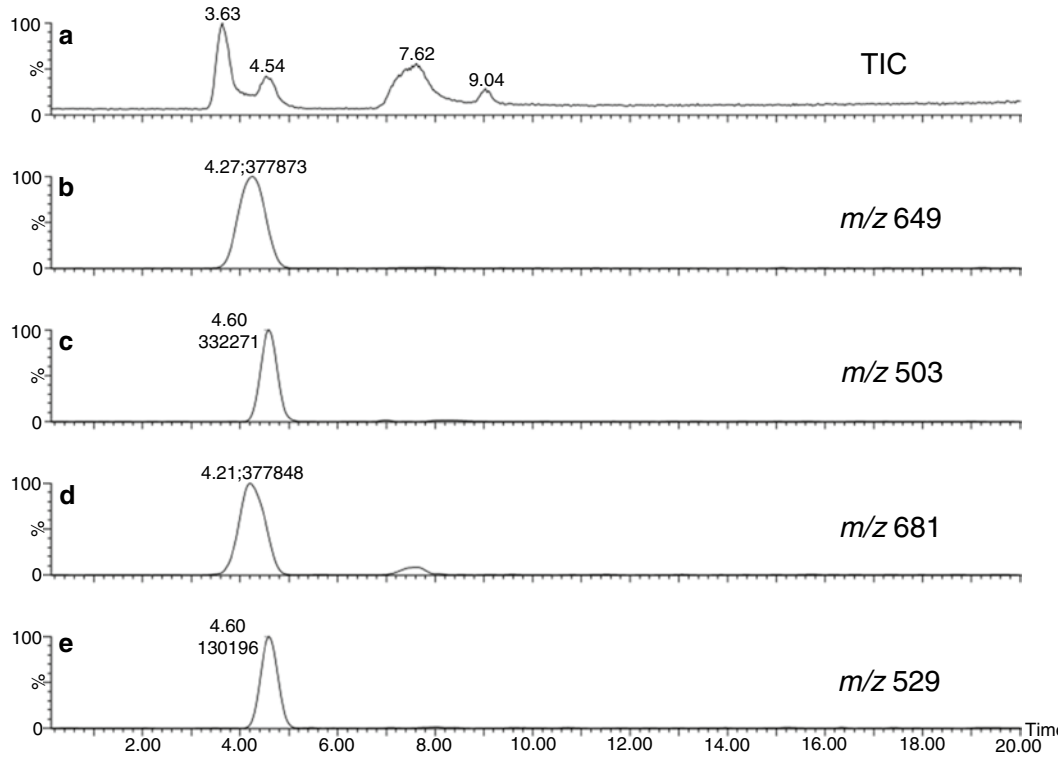


**Fig. 4** Chromatograms of the supernatant of *P. aeruginosa* PA14 after 3 days of cultivation in MSM–glycerol at 34 °C. (**a**) Total ion chromatogram (TIC). (**b**) The *m/z* 649 ion chromatogram corresponding to $[M–H]^-$ of di-RL (Rha-Rha-$C_{10}$-$C_{10}$) with retention time ($R_t$) at 4.27 min. (**c**) The *m/z* 503 ion chromatogram corresponding to $[M–H]^-$ of mono-RL (Rha-$C_{10}$-$C_{10}$) with $R_t$ at 4.6 min. (**d**) The *m/z* 681 ion chromatogram corresponding to $[M–H]^-$ of $^{13}$C-labeled di-RL-$^{13}C_{32}$ which acts as internal standard for di-RL. (**e**) The *m/z* 528 ion chromatogram corresponding to $[M–H]^-$ of $^{13}$C-labeled mono-RL-$^{13}C_{26}$ which acts as internal standard for mono-RL

8. Integrate the analyte and internal standard peaks using MassLynx 4.1 integration function, and use the peak areas to calculate the analyte concentration using the following formula:

$$C_T = \mathrm{DF} \times \left(C_S \times A_T \,/\, A_S\right)$$

where $C_T$ is the concentration of the RL congener in mg/L, and DF is the dilution factor which corrects for the 25-fold dilution of the culture supernatant as described in **step 4** under Subheading 2.1. $C_S$ is the concentration of corresponding internal standard ($^{13}$C-labeled RL), 50 mg/L in this case. $A_T$ is the peak area of the RL congener to quantify. $A_S$ is the peak area of the corresponding internal standard (*see* **Note 13**).

### *3.3 Identification of New RL Homologues Using HPLC–Tandem MS/MS*

Tandem MS/MS is useful to identify a new RL congener observed in different strains of *P. aeruginosa* cultures or under different culture conditions [4]. Tandem MS/MS is also very useful for isotope tracking for the study of the biosynthesis of RL. The following

protocol illustrates the identification of the homologues of the two major RL congeners produced by *P. aeruginosa*.

1. Prepare samples as described in Subheading 3.1, and then follow the HPLC/MS **steps 1–6** in Subheading 3.2.
2. Identify the pseudomolecular ions $[M–H]^-$ of the RL congener of interest.
3. Conduct a CID experiment to fragment the ion of interest. Use argon as the collision gas at a pressure of $3.28 \times 10^{-3}$ mBar. Scan for fragment ions at a mass range of *m/z* 100–530 for mono-RL homologues and of *m/z* 100–650 for di-RL. Set collision energy at 20 eV for both molecules (*see* **Note 14**).
4. Display the spectrum of the fragment ions, identify the fragment ions characteristic to RL, and tentatively identify the structural changes according to the fragmentation pathways presented in Fig. 2.

### 3.4 Quantitative Analysis of HAAs Using HPLC/MS

1. Prepare samples as described in Subheading 3.1, but add labeled HAA ($^{13}C_{10}$-$^{13}C_{10}$) as internal standard at a final concentration of 25 mg/L. The sample supernatant should be diluted to get the concentration within the linear range of detection.
2. Follow **steps 1–6** under Subheading 3.2.
3. For quantitation of a given HAA congener, display the ion chromatogram of the pseudomolecular ions $[M–H]^-$ of interest. For example, the *m/z* 357 ion corresponds the $[M–H]^-$ of 3-(3-hydroxydecanoyloxy)-decanoic acid, $C_{10}$-$C_{10}$. Display also the ion chromatogram of the internal standard, $^{13}C_{10}$-$^{13}C_{10}$, at *m/z* 377.
4. Integrate the analyte and internal standard peak areas, and calculate the analyte concentration using the following formula:

$$C_T = DF \times \left( C_S \times A_T / A_S \right)$$

where $C_T$ is the concentration of the HAA congener in mg/L, and DF is the dilution factor which corrects for the 25-fold dilution of the culture supernatant as described in **step 4** under Subheading 2.1. $C_S$ is the concentration of corresponding internal standard ($^{13}C_{10}$-$^{13}C_{10}$) at 25 mg/L. $A_T$ is the peak area of the HAA congener. $A_S$ is the peak area of corresponding internal standard.

## 4 Notes

1. Dissolve the MSM's ingredients in the listed order using a magnetic stirrer. Sterilize by autoclaving at 121 °C for 20 min. Take the medium out of the autoclave immediately after sterilization.

If the medium appears cloudy while hot, shake well in order to avoid any precipitation. The medium turns clear when it cools down to room temperature. The composition of MSM might have to be changed if a different source of carbon is used. For instance, if dextrose is provided instead of glycerol, higher buffering capacity is required (e.g., MSM–100 mM phosphate), as assimilation of dextrose by *P. aeruginosa* results in significant medium acidification to a pH as low as 3, which inhibits RL production.

2. About 425 μL of ammonium hydroxide (28–30 %) solution is required to achieve pH 8.55 per 250 mL of 100 mM ammonium acetate solution.
3. A few drops of concentrated ammonium hydroxide (28–30 %) will suffice. We find that it is better to use a freshly prepared solvent A as, upon exposure to air, the pH of the solution tends to decrease to below 7.0 over time, probably due to dissolution of atmospheric $CO_2$. Microbial contamination can also be a problem upon long-term storage at room temperature. Solution A should not be used when older than 24 h. It is important to ensure a pH above 8.0 throughout all the HPLC/MS runs as this can affect the RL retention times and the efficiency of the electrospray negative ionization.
4. Solvent B can be used for up to 1 week after preparation as its pH does not decrease much upon storage. Yet, the pH should be checked and readjusted to 8.0–8.5 before use, if required.
5. If $^{13}$C-labeled RLs as internal standards are not available, 5,6,7,8-tetradeutero-4-hydroxy-2-heptylquinoline (HHQ-$d_4$) or 16-hydroxyhexadecanoic acid has been used [43]. However, in this case a specific response factor must be calculated, as done previously [40].
6. A C18 reversed-phase column with the corresponding guard column can be used alternatively [40].
7. Besides strain PA14, other *P. aeruginosa* strains also produce RLs. Some other *Pseudomonas* and a few *Burkholderia* species produce RLs as well [4].
8. Inoculation is performed using washed cells instead of directly inoculating from the overnight TSB culture broth in order to avoid carryover from the rich nutritive ingredients of TSB into the nitrogen-limited MSM medium.
9. For *P. aeruginosa*, the optimal temperature for RL production is close to 30 °C and for growth at 37 °C. We typically select 34 °C as a compromise. At this temperature, in MSM–glycerol at 200 rpm, PA14 reaches the stationary phase at the third day of incubation and RL production peak at the fourth day; under these conditions, RL start to be degraded by the bacteria afterwards.

10. It is important that the supernatant sample and the internal standard solutions be at room temperature before mixing. Culture supernatants are typically diluted to bring the RL concentration within the linear range of detection (Fig. 3). When *P. aeruginosa* PA14 is cultivated under optimized conditions, for instance in the MSM–glycerol described here, it can achieve RL yields higher than 2,000 mg/L. However, cultivation in commonly used rich media (e.g., LB, TSB) will not require dilution, since, in these media, concentrations in the range of 100 mg/L are typically expected.
11. Attaining appropriate MS detector sensitivity is critically important, especially when switching from the positive ionization in previous analyses to the negative ionization mode. This can be assessed by first injecting 2–3 blank samples containing only the internal standard and monitoring the increase in the peak area of the IS that usually attains its maximum after three runs.
12. The response factor of mono-RL-$^{13}C_{26}$ can be directly used for the quantification of all the mono-RL congeners [4]. Similarly, the response factor of di-RL-$^{13}C_{32}$ is directly used for quantification of all di-RL congeners [4].
13. Make sure that the peak area of the analyte does not exceed the linear range for the estimation of concentration to be valid.
14. The mass range of fragment ions should be adjusted based on the predicted structural changes, e.g., in case of isotopically enriched RL. Collision energy should also be optimized if incomplete or no fragmentation is obtained; this can be carried out by varying the value upward or downward by 5 eV increments.

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