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METHODS AND PROTOCOLS

Development and validation of an ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) method for the quantitative determination of rhamnolipid congeners

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Abstract Rhamnolipids (RLs) are synthesised as a complex mixture of congeners comprising either one or two molecules of rhamnose glycosidically linked to a dimer of 3-hydroxy fatty acids varying in chain length and degree of saturation. Currently, HPLC-MS/MS is the most precise and accurate method for RL determination, while accurate quantification is limited. In this study, a rapid ultra pressure liquid chromatography tandem mass spectrometry (UPLC-MS/MS) method was developed and validated for the rapid and quantification of individual RL congeners. Increased RLs specificity was achieved using tandem mass spectrometry in multiple reaction monitoring (MRM) mode which was used to quantify RL isomer pairs such as Rha-Rha-C_8-C_{10}/Rha-Rha-C_{10}-C_8 which are difficult to resolve chromatographically. UPLC showed an 18-fold reduction in retention time for Rha-Rha-C_{10}-C_{10} (1.07 min) and a 17-fold reduction for Rha-C_{10}-C_{10} (1.36), the major rhamnolipids present, compared to HPLC, with a total run time less than 2.2 min. The results show that the linear range for the main RL congeners (Rha-C_{10}-C_{10} and Rha-Rha-C_{10}-C_{10}) is 0.1 to 100 μg/mL. The LOD and LOQ for Rha-C_{10}-C_{10} is 0.05 and 0.1 μg/mL and for Rha-Rha-C_{10}-C_{10} is 0.1 and 0.5 μg/mL, respectively. The method was validated for linearity, intra- and inter-day precision and accuracy in accordance with FDA guidelines. The method was applied for the quantification of 14 individual RL congeners produced by *Pseudomonas aeruginosa* ST5 and comparison of RLs composition on four different carbon sources. Quantification of the individual congeners showed a conserved congener distribution irrespective of carbon source with a preferential selection for C_{10} β-hydroxyacids as the lipid component of RLs. The only statistically significant differences detected were between actual RL yields on the various carbon sources.

Keywords Rhamnolipids · UPLC-MS/MS · Quantification · Congeners · Triple quadrupole

Introduction

Rhamnolipids (RLs) are low molecular weight glycolipids comprising of a hydrophilic rhamnose group glycosidically linked to the carboxyl end of varying β-hydroxy fatty acids. RLs are synthesised by a variety of microorganisms; however, the genera *Pseudomonas* and *Burkholderia* are the most predominant RL producers. Among these genera, *Pseudomonas aeruginosa* is the best known producer of RLs and with the highest reported yields from strains of this species. RL diversity arises from the heterogeneous congener mixtures synthesised which can vary in alkyl chain length ranging from C_8 to C_{14} (Déziel et al. 1999; Haba et al. 2003; Gunther et al. 2005) and degree of saturation. In *P. aeruginosa*, the two most abundant RL congeners synthesised are Rha-Rha-C_{10}-C_{10} (known as di-RL, α-L-rhamnopyranosyl-α-L-rhamnopyranosyl-β-hydroxydecanoyl-β-hydroxydecanoate) and Rha-C_{10}-C_{10}

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(known as mono-RL, α-L-rhamnopyranosyl-β-hydroxydecanoyl-β-hydroxydecanoate), respectively. Di-RLs are generally the most abundant types of RLs produced (Monteiro et al. 2007; Mata-Sandoval et al. 2001; Samadi et al. 2012; Rahman et al. 2010) with only a few studies reporting mono-RLs as the predominant RL species (Arino et al. 1996; Sim et al. 1997). Some *Pseudomonas* strains have been reported to produce mono-RLs only (Zhang and Miller 1992; Gunther et al. 2005).

RL congener distribution has been shown to be mostly strain dependent but can also be dependent on the phase of the culture with mono-RLs predominantly produced during early stationary phase followed by predominantly di-RLs by the end fermentation (Müller and Hausmann 2011). Mixtures of RLs varying in alky chain length have a direct impact on the properties of the surfactant mixture with each different congener containing slightly different hydrophilic-lipophilic balance (HLB) values thus exhibiting a variety of foaming properties. As a result, RLs find applications as sustainable green replacements for chemical surfactants in bulk markets such as household and personal care industries, namely as detergents in laundry and cleaning applications (Marchant and Banat 2012). For effective detergent compositions, the exact RL ratio of mono- and di-RL congeners must be selected for the different applications. For this reason, the precise chemical composition and purity of RL mixtures produced during the fermentation process must be defined. To date, several analytical methods exist for the identification and relative composition of RL congeners with very few methods available for accurate quantification of RL congeners. These methods range from simple colorimetric methods which measure the rhamnose content of RLs such as the orcinol and anthrone assays (Chandrasekaran and BeMiller 1980; Koch et al. 1991) to detailed structural and compositional analysis using more sophisticated analytical methods such as mass spectrometry (MS), liquid chromatography (LC), nuclear magnetic resonance (NMR), infrared (IR) and high-performance liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS). In the literature, the reported RL production yields can vary from 4 to 100 g/L with most publications still relying on colorimetric methods such as orcinol or anthrone for quantification of RLs. The use of these colorimetric methods overestimates RL yields and is not a true representation of the kinetics of RL production, i.e., carbon source/biomass to RL yield, particularly when these carbon sources are carbohydrate or glycerol based. Perfumo et al. (2013) reported that using the orcinol assay to quantify RLs led to a significant overestimation of actual yields when coupled with the conversion factor to incorporate the fatty acid moiety of RLs. Indeed, even quantifying RLs based on extraction yields can be unreliable due to excess carbon source and other impurities such as HAAs being co-extracted and reported as RLs. Also, using such methods gives no details regarding the structural composition of the sample; as a result, there is a need to have a method that can integrate reliable/accurate quantification with composition and purity analysis.

HPLC-MS/MS is by far the most accurate method for RL identification to date (Costa et al. 2010; Heyd et al. 2008; Haba et al. 2003), yet relatively few studies have validated the method for quantification. While there are a lot of studies characterising RLs using HPLC-MS/MS, few report the actual validation of the method and with the majority citing Déziel et al. (2000) which provided the last known detailed validation of RLs using HPLC-MS/MS. Thus, there is a great need to integrate the structural analysis of RLs with absolute quantification thus allowing for accurate yields to be reported. Using unreliable methods for quantification of RLs has a direct impact on bioprocess optimisation and downstream process. The main aim of this study is to standardise RL quantification using a rapid ultra pressure liquid chromatography tandem mass spectrometry method (UPLC-MS/MS), which could also be used to monitor upstream production. This is the first study to simultaneously develop and validate a rapid UPLC-MS/MS method for the quantification of individual RL congeners from *P. aeruginosa.*

Materials and methods

Reagents and chemicals

HPLC-MS grade methanol, acetonitrile, water and mass spectrometry grade ammonium acetate were obtained from Sigma-Aldrich (Arklow, Ireland). The commercial RL extract JBR425 (Jeneil Biosurfactant Co., LCC, Saukville, USA) was used to generate the RL standards.

UPLC-MS/MS

UPLC separation of RLs was carried out on a Waters ACQUITY® TQD UPLC system using a ZORBAX Eclipse Plus C_{18} UPLC column (21 × 100 mm, 1.8 μm) connected to an inline filter. A binary gradient using HPLC grade H_2O containing 4 mM ammonium acetate as mobile phase A and acetonitrile as mobile phase B was used. The initial conditions were 50 % A:50 % B held for 0.2 min then a linear gradient to 70 % B by 2.0 min followed by change to 90 % B by 2.2 min and held for 0.8 min with a total run time of 3 min. An injection volume of 5 μL and a flow rate of 0.5/min was used throughout. The Waters ACQUITY® UPLC system was connected to a triple quadrupole (TQD) mass spectrometer (Waters Corporation, Milford, MA). Tandem mass spectrometry was carried in negative ESI mode using multiple reaction monitoring following optimisation of MRMs using the IntelliStart software (Waters Crop.). The data was acquired using the MassLynx software 4.1.

Rhamnolipid standards

Rhamnolipid standards used for calibration and validation were obtained from a crude extract of a commercially available rhamnolipid sample (Jeneil, JBR425) as described elsewhere (Smyth et al. 2010). Supernatant was collected, acidified to pH 2.0 with concentrated HCl and extracted three times with an equal volume of ethyl acetate. The organic phase was collected and dried by adding 0.5 g $MgSO_4$ per 100 ml ethyl acetate. The solution was filtered and rotary evaporated to obtain a crude extract containing rhamnolipids. All crude extract yields were measured gravimetrically before further analysis. The extract contained up to 14 different RL congeners; however, only mono-RL C_{10}-C_{10} and di-RL C_{10}-C_{10} were abundantly produced with all other congeners present in low amounts (<5 % total composition). Initial attempts to separate all RL congeners using semi-preparative HPLC (Phenomenex C_{18} Luna, 10 × 250 mm, 10 μm) resulted in limited success as a result of the relatively low abundance of all congeners present in trace amounts. To prepare the RL standards for method validation and quantification, the RL extract was separated into mono and di-RL fractions using normal phase flash chromatography. Fractionation was carried out on a Varian IntelliFlash 310 Flash Chromatography (Analogix Semiconductor Inc., California, USA) system using an Agilent SuperFlash™ SF40–240 g normal phase silica column (40.6 mm × 37.9 cm, 50 μm). A flow rate of 60 ml min^{-1} was used throughout. The column was conditioned with hexane, and 4 g of crude extracted rhamnolipid was dissolved in chloroform and injected onto the column. The column was washed with hexane followed by chloroform to remove lipid material until no further colour was eluted for each solvent. This was then changed to 97:3 (chloroform:methanol) to elute mono rhamnolipids (the mono rhamnolipid band was collected based on visualisation of the column and UV detection). The gradient was subsequently changed to 50:50 (chloroform:methanol) to elute the dirhamnolipids with collection of the visual rhamnolipid band carried out. The purified fractions contained either only mono-RL congeners (R1) or all di-RL congeners (R2). Each fraction was analysed by LC-MS/MS to determine exact sample composition, each congener was quantified based on % peak area of total RL samples (Fig. 1). Since no commercial standards are currently available, a complex purification is required to obtain standards for UPLC-MS/MS quantification. However, once the initial purification of these standards has been completed using the described methods detailed above, these standards should be divided into aliquots and stored at −20 °C. Since extremely small quantities are required for production of a standard curve on the UPLC-MS/MS method, purification on the scale reported above should provide sufficient quantities for UPLC-MS/MS analysis for several years.

LC-MS/MS analysis was performed on a Waters Alliance 2695 HPLC system coupled to a Waters Q-ToF Premier mass spectrometer. Separation was performed on an Agilent poroshell SB-C18, 2.1 × 100 mm, particle size 2.7 μm. Mobile phase A, H_2O (4 mM ammonium acetate) and Mobile phase B, acetonitrile were used for chromatographic separation as follows: 0–15 min 50–70 % mobile phase 2, 15.0–17.5 min 98 % mobile phase 2, 17.5–18.0 min: 50–50 % mobile phase 2, 18–20 min: 50 % mobile phase 2. The column oven temperature was set at 40 °C and an injection volume of 1 μL was used throughout. Q-TOF MS was conducted in negative mode ESI. The source temperature was 120 °C and the desolvation temperature was 350 °C. In each case, nitrogen was used as the desolvation gas (800 L/h) and cone gas (50 L/h). The cone voltage was 15 V and capillary voltage was set at 2.6 kV. Collision induced dissociation (CID) of the analytes, to determine the different lipid chains, was achieved in MS^e mode with argon by ramping up the collision energy from 20 to 40 eV. Samples were analysed in negative mode in the range *m/z* 160–1000.

Due to the complexity of the RL composition, the most abundant RL congener present in each of the purified fractions was analytically weighed to obtain the standard solution concentration of 1 mg/mL. In fraction R1, the deprotonated ion at *m/z* 503 corresponds to Rha-C_{10}-$C_{10,}$ the most abundant congener comprising 83.8 % of the total sample and therefore 1.19 mg/mL of this fraction yields a concentration of 1 mg/mL for Rha-C_{10}-$C_{10.}$ In fraction R2, Rha-Rha-C10-C10 (*m/z* 649) was the most abundant congener comprising 85.1 % (1.175 mg/mL) of the total sample (Table 1) and subsequently 1.175 mg/mL of R2 gives a concentration of 1 mg/mL for Rha-Rha-C10-C10. A dynamic range from 10 to 0.00001 mg/mL of the RL was used to ensure that standard curves could be generated for all congeners including the less abundant RLs by calculation of their quantities present in the standard solutions. All standards were prepared in methanol.

Method validation

The UPLC-MS/MS was validated in accordance with the FDA recommendations for Guidance for Industry—Bioanalytical Method Validation (USFDA, 2001). The method was developed and validated for linearity, intra- and inter-day precision and accuracy. Calibration curves were generated using RL standard stock solutions. A sample of a 12-h *P. aeruginosa* ST5 (NCIMB 30352) culture was used to obtain a matrix sample and extracted using the same procedure for rhamnolipids. This extract contained low levels of rhamnolipids and was deemed the most suitable as a matrix for standard addition of rhamnolipid standards for accuracy determination. Limits of detection (LOD), limit of quantification (LOQ) and the linearly range were determined using a range of different concentrations. Accuracy was determined

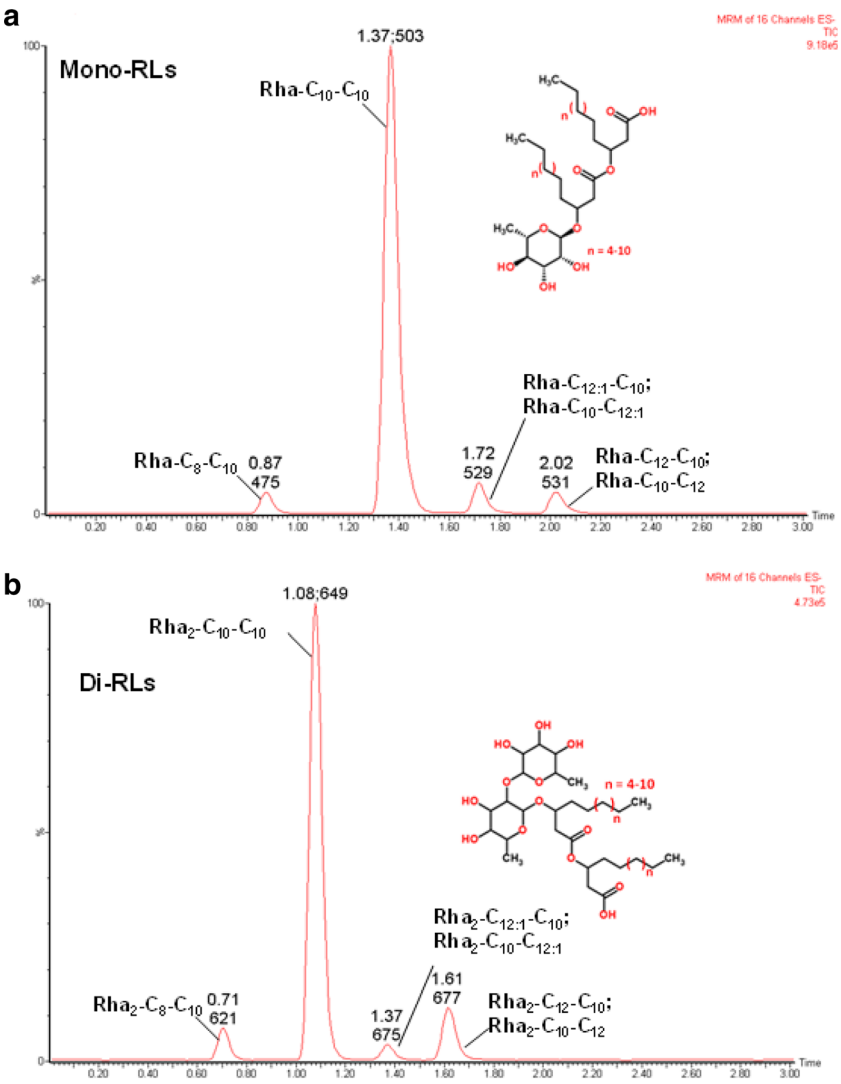

Fig. 1 UPLC-ESI-MS/MS chromatogram for rhamnolipid standards. **a** Mono-RL standard and **b** di-RL

from low, medium and high concentrations for the two main rhamnolipids (Rha-C_{10}-C_{10} and Rha-Rha-C_{10}-C_{10}). For accuracy, values should be within 15 % of the true value and <20 % for values at the lowest limit of quantification (LLOQ). Precision measurement was carried out on the two most abundant RL congeners from each standard fraction, i.e., Rha-C_{10}-C_{10} and Rha-C_{10}–C_{10}. Intra-day precision was calculated from relative standard deviation (RSD) using three determinations per concentration for low, medium and high concentrations in triplicate during the same day. Inter-day precision was calculated from relative standard deviation (RSD) using three determinations per concentration in for low, medium and high concentrations on three separate days.

Application of method

The validated UPLC-MS/MS method was applied to quantify individual RL congeners produced by *P. aeruginosa* ST5 grown on four different carbon sources. *P. aeruginosa* ST5 (NCIMB 30352) was grown on minimal salt media (MSM) ($NaNO_3$ 0.2 %, Na_2HPO_4 0.09 %, KH_2PO_4 0.07 %, $MgSO_4.7H_2O$ 0.04 %, $CaCl_2.2H_2O$ 0.01 %, $FeSO_4.7H_2O$ 0.0001 %) containing either 2 % *w/v* of glucose, glycerol, oleic acid, or hexadecane. Cultures were grown in 100-mL MSM in a 1-L Erlenmeyer flask and incubated at 37 °C for 5 days at 200 rpm. RLs were extracted and crude yields determined from each carbon source as described by Smyth et al. (2010).

Results

Development of rhamnolipid quantification using UPLC-MS/MS

Initially, a HPLC-MS/MS method was developed and optimised for RL separation and characterisation using standard analytical HPLC columns and quadrupole time of flight

Table 1 Composition of RL congeners in purified fractions of commercial extract JBR425 as determined by UPLC-MS/MS

		m/z [M-H]$^-$	% Abundance
Mono-rhamnolipid congeners (R1 fraction)			
1	Rha-C_8-C_{10}	475	3.01
2	Rha-C_{10}-C_8	475	1.64
3	Rha-C_{10}-C_{10}	503	83.8
4	Rha-C_{10}-$C_{12:1}$	529	5.84
5	Rha-$C_{12:1}$-C_{10}	529	0.08
6	Rha-C_{10}-C_{12}	531	4.59
7	Rha-C_{12}-C_{10}	531	1.09
Total			100 %
Di-rhamnolipid congeners (R2 fraction)			
8	Rha-Rha-C_8-C_{10}	621	0.78
9	Rha-Rha-C_{10}-C_8	621	3.73
10	Rha-Rha-C_{10}-C_{10}	649	85.1
11	Rha-Rha-C_{10}-$C_{12:1}$	675	2.50
12	Rha-Rha-$C_{12:1}$-C_{10}	675	0
13	Rha-Rha-C_{10}-C_{12}	677	6.34
14	Rha-Rha-C_{12}-C_{10}	677	1.56
Total			100 %

% Relative abundance was calculated based on the individual congener peak area divided by the sum total area for all RLs for either mono- or di-RL fractions as per current practice for published literature in this area

mass spectrometry (Q-TOF) in full scan mode. Absolute quantification of RLs was not validated using this method, and only semi-quantitative analysis was achieved using % peak area abundance without prior calibration for each of the RL congeners. Analysis of % peak area without external calibration provides only a semi-quantitative assessment of composition and purity. Obtaining sufficient baseline resolution of rhamnolipids on conventional HPLC columns resulted in a relatively long run time, and therefore prior to developing a quantitative method, it was decided to transfer this method to UPLC to overcome this issue. One of the main advantages of UPLC is the reduced run time associated with this technique, and one of the aims of this study was to develop a rapid method for quantitative analysis of RL method. RL separation was optimised with a binary gradient for run time and flowrate ensuring that the shortest run time with baseline resolution of analytes was achieved. While baseline resolution is not required for triple quadrupole mass spectrometry, it was decided that the method that should be optimised was baseline resolution of the major components so that transfer to other analytical techniques using UPLC could be performed.

For a comparative analysis, 0.1 mg/mL of the commercial RL sample was separated with both HPLC (5 μm particle size) and UPLC (1.8 μm). UPLC showed an 18-fold reduction in retention time for Rha-Rha-C_{10}-C_{10} and a 17-fold reduction for Rha-C_{10}-C_{10}, the major rhamnolipids present, compared to HPLC (Fig. 2). In addition, the reduced run time obtained with UPLC combined with a triple quadrupole mass spectrometer provided increased sensitivity and selectivity using multiple reaction monitoring mode (MRM) by only selecting RL-specific congeners and fragment transitions. As a result, only RL compounds are detected thus eliminating other interfering compounds and excess carbon sources that could be present in samples. The use of MRM for analysing RLs allows for direct detection of each individual RL congener present in the sample without requiring baseline separation or removal of non-rhamnolipid components present in the sample. Negative ion mode was used for RL ionisation since ammonium and sodium adducts are frequently present in positive ion mode analysis of rhamnolipids. Due to the selectivity of the UPLC-MS/MS method in MRM mode up to 14 RL congeners were detected and quantified in this study. No further RL congeners were present based on previous analysis of this extract using Q-TOF mass spectrometry. However, UPLC-MS/MS in MRM mode has the capacity for detection and quantitation of several different compounds during each run.

Optimisation of MRM conditions for RL quantification

RLs are produced as a heterogeneous mixture of either mono- or di-RLs varying in the fatty acid chain length and in some cases the degree of unsaturation. Tandem mass spectrometry was used to identify the fatty acid composition for both mono- and di-RL congeners and quantify each structure using triple quadrupole mass spectrometry. These MRM conditions were determined and optimised by tuning the RLs using Waters integrated IntelliStart™ software (Waters Corp., Milford, USA).

Tandem mass spectrometry of the parent ion [M-H]$^-$ RL congeners were identified based on their major fragmentation patterns. The major cleavages depicted in Fig. 1a are shown for *m/z* 503 and 649, respectively, all other RLs fragment in the same manner but yield different *m/z* values based on the number of rhamnose sugar rings and chain length. The main MRM for each congener is based on the loss of the outer fatty acid leaving a major product ion corresponding to the rhamnose moiety glycosidically linked to the remaining fatty acid chain. The resulting product ion allows for the identification of the different RLs with different chain lengths on the outer lipid chain, which then can be used to deduce the composition of the second lipid chain. For example, Rha-C_{10}-C_{10} with an *m/z* at 503 would produce one major product ion at *m/z* 333 [Rha-C_{10}-H]-, corresponding to the loss of the outer lipid chain. Subsequently, subtraction of the rhamnose portion allows the deduction of the composition of the remaining lipid chain. Table 2 summarises the MRMs experimental conditions obtained using the intellistart software for the RL congeners, which were subsequently used for triple quadrupole mass spectrometric quantification utilised in this study.

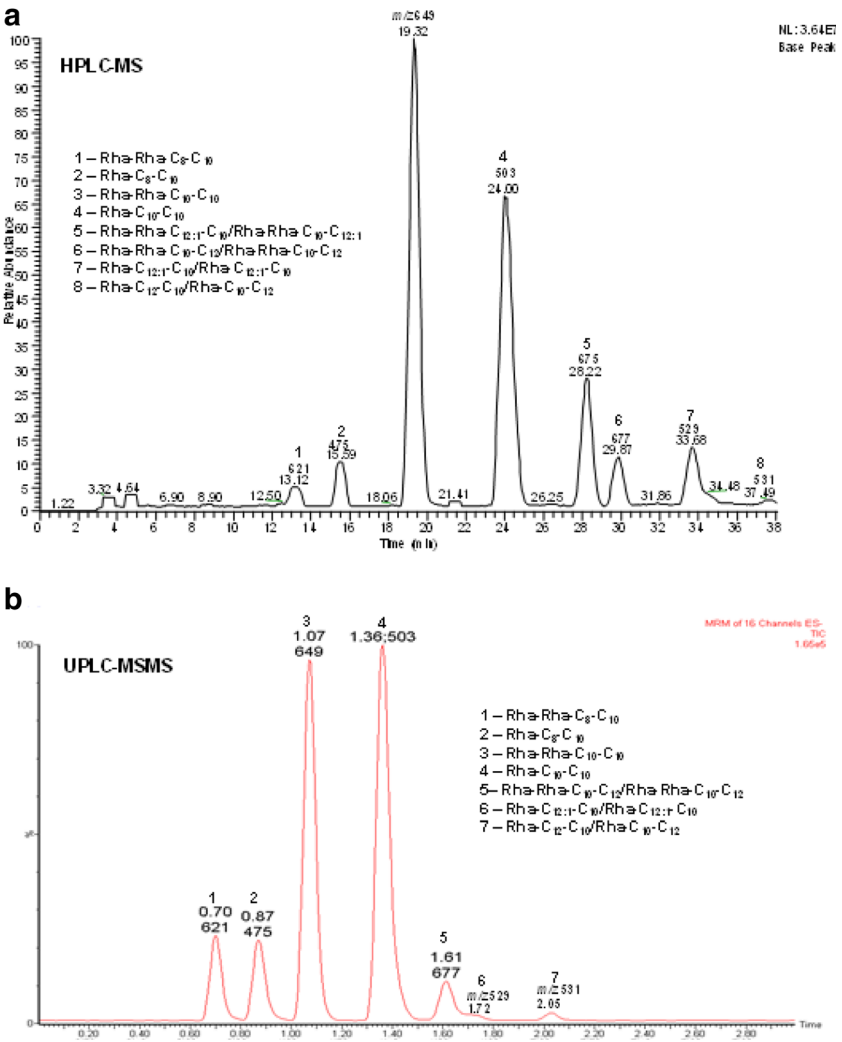

Fig. 2 Comparison of RL separation with HPLC and UPLC. **a** HPLC-MS chromatogram of RL standard and **b** UPLC-MS chromatogram of RL standard

Table 2 UPLC-MS/MS MRM parameters for RL congeners in negative ion mode ionisation

		MS $[M-H]^-$	MS/MS (MRM)	Collision (eV)	Cone voltage (V)
Mono-rhamnolipid di-lipidic congeners					
1	Rha-C_8-C_{10}	475.2	305.1	32	12
2	Rha-C_{10}-C_8	475.2	333.3	32	16
3	Rha-C_{10}-C_{10}	503.3	333.2	34	16
4	Rha-C_{10}-$C_{12:1}$	529.3	333.2	44	14
5	Rha-$C_{121:}$-C_{10}	529.3	359.3	44	14
6	Rha-C_{10}-C_{12}	531.3	333.2	44	16
7	Rha-C_{12}-C_{10}	531.3	361.3	44	14
Di-rhamnolipid di-lipid congeners					
8	Rha-Rha-C_8-C_{10}	621.4	451.3	46	12
9	Rha-Rha-C_{10}-C_8	621.4	479.3	46	20
10	Rha-Rha-C_{10}-C_{10}	649.4	479.3	56	22
11	Rha-Rha-C_{10}-$C_{12:1}$	675.4	479.3	54	24
12	Rha-Rha-$C_{12:1}$-C_{10}	675.4	505.4	54	24
13	Rha-Rha-C_{10}-C_{12}	677.4	479.3	54	22
14	Rha-Rha-C_{12}-C_{10}	677.4	507.4	54	22

Method validation

The results for the linear range, limit of detection (LOD) and limit of quantification (LOQ) are given in Table 3. Most of the RL congeners fell within the linear range of 0.1–100 μg/mL. For each RL congener, a calibration curve with a minimum of six calibration points was used and the linear range was dependent on the abundance of the specific RL quantified. Intra- and inter-day accuracy and precision data were calculated for the two most abundant RL congeners from the mono-RL and Di-RL (Rha-C_{10}-C_{10} and Rha-Rha-C_{10}-C_{10}). Both compounds were analysed at three different concentrations representing low, medium and high (1, 10, and 50 μg/mL). This was carried out by standard addition of known concentrations of rhamnolipids representing low, medium and high concentrations based on the linear standard curve. These standards were added to a 12-h culture of *P. aeruginosa* in which RL had been quantified and analysed for accuracy and precision. Table 4 summarises the precision and accuracy data for both RLs. The %RSD were within the acceptable limit of <20 % with the highest %RSD at 12.32 %. Accuracy within acceptable limits was obtained for both compounds within the range of 89–104 %. Figure S2 and S3 show the MRM UPLC-MS/MS selectivity for individual RL congeners, while Figure S4 displays the TIC for both types of RLs.

Application of the developed UPLC-MS/MS

The validated UPLC-MS/MS method was successfully applied for the quantitation and comparison of individual RL congeners grown on various carbon substrates in *P. aeruginosa* ST5. Table 5 shows the absolute concentrations for each RL congener grown on the various carbon sources. In all carbon substrates, di-RLs are the most predominant; however, Rha-C_{10}-C_{10} and Rha-Rha-C_{10}-C_{10} are by far the most abundant congeners with their relative abundance in the ratio of 30:40 (Mono %:Di %) with minor differences in the ratio between carbon sources (Table S1). This study is the first report of the simultaneous validation and absolute quantification of individual RL congeners using UPLC-MS/MS. With the development of the UPLC-MS/MS method, 12 individual RL congeners were quantified for each carbon source. Rha-Rha-C_{10}-C_{10} and Rha-C_{10}-C_{10} were the most abundant congeners present in cultures grown on all carbon sources ranging from 74 to 87 % of the total % of RL congeners present in each extract. One of the most significant results using this method is that RL production is highly conserved in *P. aeruginosa.* The effect of the various carbon sources on RL production appears to be only significant on the actual yield of RL produced since the absolute RL congener distribution does not vary between the carbon sources (Fig. 3a, b). RL yields are similar when grown on both glucose and glycerol; however, there is a significant increase in RL yield with oleic acid as the main carbon substrate (Fig. 3b) which is consistent with previous reports that RL yield increases on hydrocarbons and various fatty acids (Nitschke et al. 2005). The only exception is RL production on hexadecane as the sole carbon source where <0.1 mg/mg of purified RL was quantified indicating that this carbon source was not used efficiently to produce extracellular RLs. Quantification of the individual congeners showed a conserved congener distribution irrespective of carbon source with a preferential selection for C10 β-hydroxyacids as the lipid component of RLs (Table 5). These findings are in agreement with previous reports that *P. aeruginosa* stringently regulates the fatty acid composition of RLs by the selective affinity of RhlA in the RL biosynthetic pathway for C10 intermediates (Zhu and Rock 2008).

Table 3 Calibration data for all RL congeners analysed

Compound	$[M-H]^-$	MS/MS	Linear range (μg/mL)	R_2	LOD (μg/mL)	LOQ (μg/mL)
Mono-RLs						
Rha-C_{10}-C_{10}	503	333	0.1–100.0	0.9976	0.05	0.1
Rha-C_{10}-C_8	475	333	0.2–50.0	0.9966	0.1	0.2
Rha-C_8-C_{10}	475	305	0.18–71.6	0.9960	0.0358	0.18
Rha-$C_{12:1}$-C_{10}	529	359	0.0475–47.5	0.9924	0.0475	0.0475
Rha-C_{10}-C_{12}	531	333	0.273–54.6	0.9946	0.0546	0.273
Rha-C_{12}-C_{10}	531	361	0.13–130.0	0.9988	0.065	0.13
Di-RLs						
Rha-Rha-C_{10}-C_{10}	649	479	0.5–100.0	0.9933	0.1	0.5
Rha-Rha-C_8-C_{10}	621	451	0.438–87.6	0.9998	0.22	0.438
Rha-Rha-C_{10}-C_8	621	479	0.092–92.0	0.9968	0.046	0.092
Rha-Rha-C_{10}-$C_{12:1}$	675	479	0.15–58.8	0.9947	0.15	0.15
Rha-Rha-C_{10}-C_{12}	677	479	0.373–74.5	0.9975	0.0745	0.373
Rha-Rha-C_{12}-C_{10}	677	507	0.183–91.5	0.9946	0.0915	0.183

Table 4 Precision and accuracy data for the two most abundant RL congeners

	Rha-C_{10}-C_{10}				Rha–Rha-C_{10}-C_{10}			
	Intra-day precision		Inter-day precision		Intra-day precision		Inter-day precision	
	% RSD	% Accuracy	% RSD	% Accuracy	% RSD	% Accuracy	% RSD	% Accuracy
Low	7.58	97.26	8.3	100.89	6.24	99.9	8.02	100.2
Medium	5.73	95.88	6.34	98.23	12.32	82.55	7.3	89.78
High	9.52	104.47	3.54	94.01	9.19	91.02	7.76	90.72

Discussion

Methods for RL characterisation range from simple colorimetric analysis such as orcinol or anthrone to more detailed analytical analysis using MS, HPLC-MS, GC-MS, FITR and NMR (Heyd et al. 2008). However, chromatographic separation of RLs is one the most efficient methods for the analytical characterisation of RLs with HPLC currently the most common method for RL separation. The identification of RLs by HPLC is a well-established process and is often based upon the retention of particular congeners, on a specified column, in comparison to published data. However, the complete separation of RLs in complex matrices results in undesirable run times and a high consumption of mobile phase solvents. This is mainly due to the particle size (5 μm) of the column being used and the upper pressure limits (4000 PSI) of the HPLC system itself. UPLC systems however are designed to use columns with smaller particle sizes (1.8 μm) at higher pressures (10,000 PSI), thus facilitating greater resolution, sensitivity and speed when analysing RL samples. The coupling of UPLC to TQD-MS allows each RL congener to be identified correctly and subsequently quantified based on molecular mass, ms/ms fragmentation as well as retention time, resulting in a more reliable analysis of the extracts.

In this study, an accurate, precise, specific and sensitive UPLC-MS/MS method has been developed and validated for quantification of individual RL congeners. To date, this is the first report of the systematic validation and

Table 5 RL Congener distribution for each carbon substrate

		Glucose		Glycerol		Oleic Acid		Hexadecane	
No.	RL congener	mg/mg	S.D.	mg/mg	S.D.	mg/mg	S.D.	mg/mg	S.D.
Mono-rhamnolipids									
1	Rha-C_{10}-C_{10}	0.2557	0.0158	0.3153	0.0389	0.2464	0.0101	0.0409	0.0171
2	Rha-C_{10}-C_8	0.0214	0.0016	0.015	0.0031	0.0073	0.0009	0	0
3	Rha-C_8-C_{10}	0.0532	0.0045	0.0414	0.0082	0.0098	0.0014	0	0
4	Rha-$C_{12:1}$-C_{10}	0	0	0	0	0	0	0	0
5	Rha-C_{10}-$C_{12:1}$	0.0069	0.0014	0.0104	0.0017	0.0049	0.0003	0.0019	0.0006
6	Rha-C_{10}-C_{12}	0.0064	0.0014	0.0107	0.0024	0.0067	0.0015	0.0011	0.0002
7	Rha-C_{12}-C_{10}	0.0024	0.0005	0.0049	0.0019	0.002	0.0003	0.0004	0.0002
	Total mono-RLs	0.346	0.0252	0.3977	0.0562	0.2771	0.0145	0.0443	0.0181
Di-rhamnolipids									
8	Rha-Rha-C_{10}-C_{10}	0.3326	0.0099	0.3361	0.0693	0.3394	0.0408	0.0426	0.0173
9	Rha-Rha-C_8-C_{10}	0.0119	0.0004	0.0113	0.0027	0.0025	0.0008	0	0
10	Rha-Rha-C_{10}-C_8	0.0351	0.0105	0.0322	0.0053	0.0138	0.0021	0.0002	0.0001
11	Rha-Rha-C_{10}-$C_{12:1}$	0.021	0.0016	0.018	0.0052	0.014	0.0017	0.0031	0.0011
12	Rha-Rha-$C_{12:1}$-C_{10}	0	0	0	0	0	0	0	0
13	Rha-Rha-C_{10}-C_{12}	0.0369	0.0011	0.0242	0.0072	0.0312	0.0017	0.0057	0.0011
14	Rha-Rha-C_{12}-C_{10}	0.009	0.001	0.0052	0.002	0.0065	0.0008	0.0007	0.0002
	Total di-RLs	0.4465	0.0245	0.427	0.0917	0.4074	0.0479	0.0523	0.0198
	Total RLs	0.7835	0.0487	0.8195	0.1459	0.678	0.0616	0.0959	0.0377

Values are given as milligramme of RL per milligramme of purified extract for each carbon source ± S.D. (n = 3 technical replicates only)

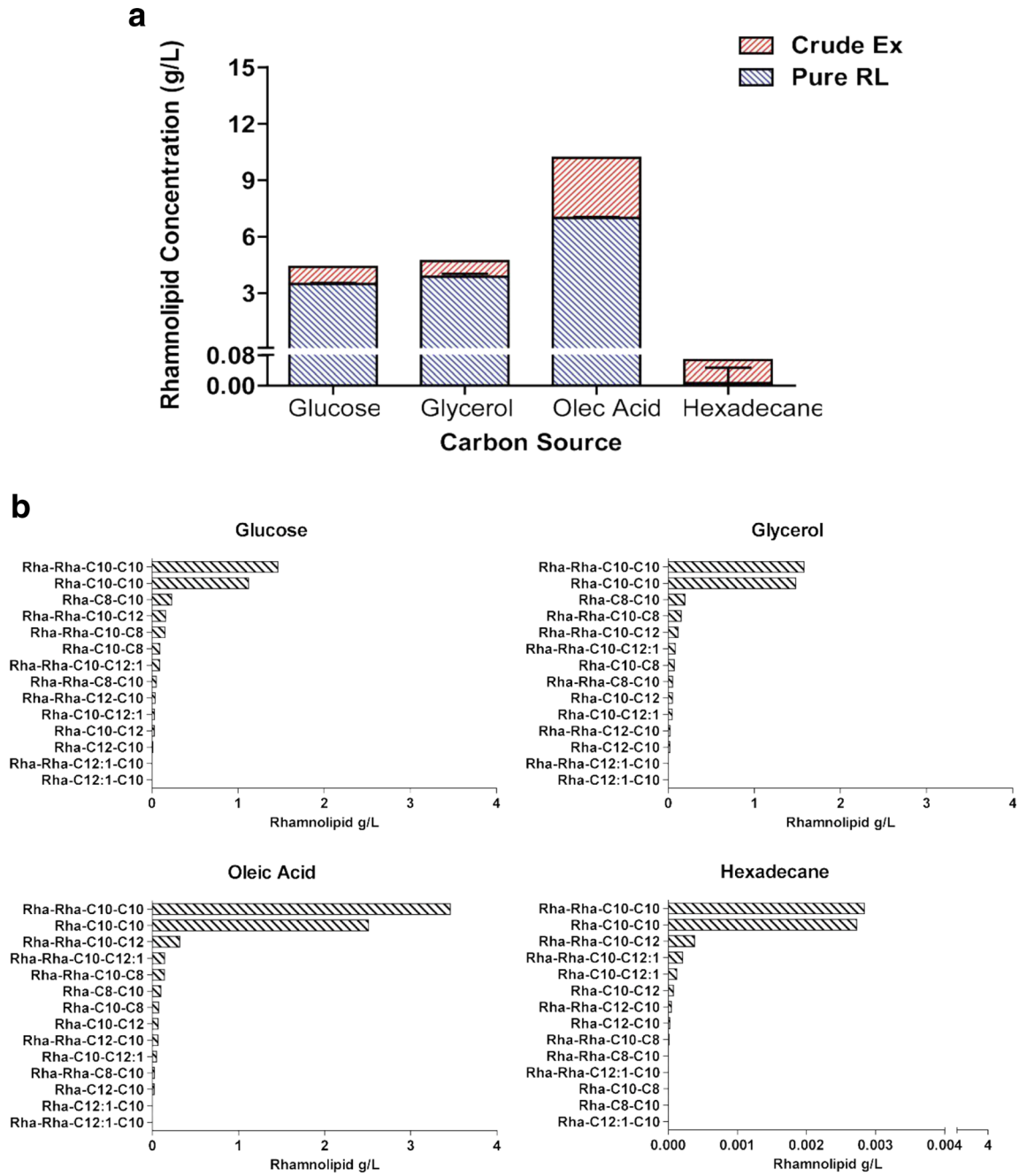

Fig. 3 Rhamnolipid congener distribution is conserved on different carbon substrates. **a** Purified extracts showing the sum total of all RL congeners quantified in the extract. **b** Individual RL congeners for each carbon source. Values are given as grammes per liter of the purified extract as determined from each individual RL calibration curve from the developed UPLC-MS/MS method

quantification of individual RL congeners using UPLC-MS/MS. The method was validated in accordance with the FDA guidelines with regard to sensitivity, precision, accuracy, selectivity, linearity and range. The validated UPLC-MS/MS method presented here is significantly faster (circa 18-fold retention reduction) and more sensitive than the HPLC-MS/MS method previously developed. As a result, a higher throughput of samples can be analysed in shorter run times with reliable and accurate quantification of up 14 individual RL congeners. The specificity of the UPLC-MS/MS method allowed for the accurate and reliable quantification of trace amounts of RL congeners subsequently quantifying 14 RL congeners in less than 3 min. Compared with conventional HPLC-MS analysis ranging from 20 to 50 min run time reported in literature (Neilson et al. 2010; Gunther et al. 2005; Perfumo et al. 2013), UPLC is much more rapid and sensitive allowing for high throughput sampling in less than 3 min.

While RLs are reported to be synthesised in various congeners ranging from C_8 to C_{14} (Déziel et al. 1999), few reports have reliably quantified each congener during production. On all carbon sources analysed, Rha-Rha-C_{10}-C_{10} was the most abundant followed by Rha-C_{10}-C_{10}. The only significant difference observed was in the actual quantities for each congeners on the various carbon source with oleic acid producing the highest quantity of RLs (>7 g/L) and hexadecane producing trace amounts of extracellular RLs (<0.01 g/L). However, the congener distribution for hexadecane was similar to the other carbon sources suggesting that RL production in *P. aeruginosa* is a highly conserved process. RL congener distribution on both hydrophilic and hydrophobic carbon sources showed that *P. aeruginosa* selectively synthesises RLs predominantly with C_{10} fatty acid intermediates. This conserved congener distribution is in agreement with the results published by Zhu and Rock (2008) showing that RhlA has a selective affinity for draining C_{10} intermediates from the FASII pathway as precursors for RL biosynthesis in *P. aeruginosa*. Oleic acid showed the highest yield of RL when compared with glucose and glycerol. The different carbon substrates have a significant impact of RL yield and do not influence the RL congener composition, the differences in yield are probably due to the efficiency of metabolism of the carbon substrate. Glucose and glycerol are metabolised from the central metabolic pathways (i.e., Entner-Dourdoff and FAS II) which is directly influenced by enzyme substrate affinity thereby regulating the metabolic precursor pool for RL biosynthesis. The intermediates from the metabolism of both these carbon sources serve as metabolic precursors for other pathways (rhamnose, LPS, PHA, signal molecules etc.) competing with RL biosynthesis. It is well reported that growth on hydrocarbons and fatty acids increase RL production in *P. aeruginosa* (Nitschke et al. 2005; Li et al. 2011; Lotfabad et al. 2010). Zhang et al. (2012) showed that when *P. aeruginosa* was grown on octadecanoic acid, a combination of de novo fatty acid synthesis and β-oxidation contributed to the lipid precursor pool. They showed RL production increased on this substrate as a result of direct incorporation of β-oxidation intermediate octanoyl-CoA without de novo synthesis. The combination of both β-oxidation and de novo fatty acid synthesis when grown on long-chain fatty acid substrate perhaps increases the total metabolic pool of lipid precursors available for RL biosynthesis thereby increasing the RL yield. To date, it is possible that the overuse of the unreliable colorimetric methods such as the orcinol method coupled with the lack of accurate and reliable validation of precise analytical methods has led to a lack of quantitative understanding of the exact relationship between RL biosynthesis and production in *P. aeruginosa*. It is imperative for more detailed analytical methods to be published for comparison and standardisation of methods between laboratories to reduce inaccurate reports of RL yields.

There is a shift in biosurfactant research away from a more heuristic traditional approach to a more targeted optimisation strategy including development of genetically engineered strains (both metabolically and genetically), optimising metabolic precursor pools and altering genetic regulation (QS) (Müller et al. 2012). Quantitatively understanding the complex interaction between RL genetic regulation and nutritional conditions should allow for the development of a more efficient bioprocess for RL production. The idea of a systems biology approach for understanding and improving RL production has already been suggested by Müller et al. (2012). This newly developed UPLC-MS/MS has been validated in accordance with FDA guidelines specific for individual RL congener quantification. Standardised methods like this will be empirical for a developing 'systems' biology approach to improve the economics of RL bioprocess optimisation.

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Conflict of interest Authors declare that they have no competing interests.

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