

Simultaneous quantitative profiling of *N*-acyl-L-homoserine lactone and 2-alkyl-4(1*H*)-quinolone families of quorum-sensing signaling molecules using LC-MS/MS

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Abstract An LC-MS/MS method, using positive mode electrospray ionization, for the simultaneous, quantitative and targeted profiling of the *N*-acyl-L-homoserine lactone (AHL) and 2-alkyl 4-(1*H*)-quinolone (AQ) families of bacterial quorum-sensing signaling molecules (QSSMs) is presented. This LC-MS/MS technique was applied to determine the relative molar ratios of AHLs and AQS produced by *Pseudomonas aeruginosa* and the consequences of mutating individual or multiple QSSM synthase genes (*lasI*, *rhlI*, *pqsA*) on AHL and AQ profiles and concentrations. The AHL profile of *P. aeruginosa* was dominated by *N*-butanoyl-L-homoserine lactone (C4-HSL) with lesser concentrations of *N*-hexanoyl-L-homoserine lactone (C6-HSL) and 3-oxo-substituted longer chain AHLs including *N*-(3-oxodecanoyl)-L-homoserine lactone (3-oxo-C10-HSL) and *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL). The AQ profile of *P. aeruginosa* comprised the C7 and C9 long alkyl chain AQS including 2-heptyl-4-hydroxyquinoline (HHQ), 2-nonyl-4-hydroxyquinoline, the “*Pseudomonas* quinolone signal” (2-heptyl-3-hydroxy-4-

quinolone) and the *N*-oxides, 2-heptyl-4-hydroxyquinoline *N*-oxide and 2-nonyl-4-hydroxyquinoline *N*-oxide. Application of the method showed significant effects of growth medium type on the ratio and the nature of the QSSMs synthesized and the dramatic effect of single, double and triple mutations in the *P. aeruginosa* QS synthase genes. The LC-MS/MS methodology is applicable in organisms where either or both AHL and AQ QSSMs are produced and can provide comprehensive profiles and concentrations from a single sample.

Keywords Bioanalytical methods · Mass spectrometry · HPLC · *Pseudomonas aeruginosa* · *N*-acyl-L-homoserine lactones · 2-alkyl-4-(1*H*)-quinolones

Introduction

Bacteria produce a range of secondary metabolites which facilitate competition with other species and colonization of diverse ecological niches. Quorum-sensing signaling molecules (QSSMs) are low molecular weight diffusible molecules which act as a mean of intercellular communication to coordinate bacterial behaviors such as secondary metabolite production, biofilm development, swimming and swarming motility and virulence [1]. Two main classes of bacterial QSSMs are the *N*-acyl-L-homoserine lactones (AHLs) and the 2-alkyl-4-(1*H*)-quinolones (AQS) and both consist of large families of lipid-like molecules of closely related structures with a range of acyl/alkyl chain lengths [1, 2]. AHL-dependent QS systems encompass a family of more than 30 different QSSMs which differ in the length (from C4 to C18), degree of unsaturation (none, one or two

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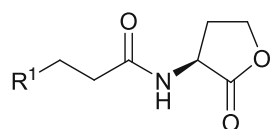
double bonds) and substitution at C3 (which can be 3-oxo, 3-hydroxy or unsubstituted) of the acyl side chain [3] (Fig. 1).

In the human opportunistic pathogen, *Pseudomonas aeruginosa*, QS depends on two AHL regulatory circuits (*las* and *rhl*) closely linked to an AQ system. In the *las* system, the *lasI* gene product mainly directs the synthesis of *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL), which interacts with the transcriptional regulator LasR to activate target promoters. In the *rhl* system, RhII directs the synthesis of *N*-butanoyl-L-homoserine lactone (C4-HSL), which interacts with the cognate regulator RhIR and activates target gene expression. Apart from C4-HSL and 3-oxo-C12-HSL, RhII and LasI are also known to be responsible for the synthesis of additional AHLs including C6-HSL [4] and 3-oxo-C8-HSL, 3-oxo-10-HSL and 3-oxo-C14-HSL [5], respectively.

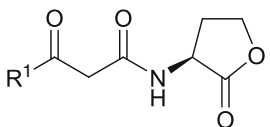
The *las* and *rhl* systems are hierarchically connected and regulate the timing and production of multiple virulence factors [6, 7]. In addition to 3-oxo-C12-HSL and C4-HSL, *P. aeruginosa* releases two major AQ signal molecules, the Pseudomonas Quinolone Signal (PQS; 2-heptyl-3-hydroxy-4(1*H*)-quinolone) and its immediate biosynthetic precursor, 2-heptyl-4-hydroxyquinoline (HHQ) into the extracellular milieu, the synthesis and bioactivity of which are inter-linked with AHL-dependent QS [7].

The biological activities of AQS including PQS include antimicrobial activity [8–10] e.g., inhibition of the growth of *Staphylococcus aureus* and other Gram-positive bacteria [11, 12]. Some AQS also act as iron chelators [13–15] and as immune modulators [16, 17]. *P. aeruginosa* releases a multitude of AQS belonging to structurally related families characterized by the presence of a hydrogen or hydroxyl group at the 3 position, variations in length and the degree

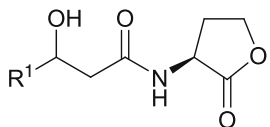
Fig. 1 Chemical structures of AHL and AQ families of bacterial quorum sensing molecules



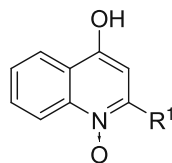
(1) Unsubstituted *N*-acylhomoserine-L-lactone (C_n-HSL) acyl chain length (even) n = 4-14



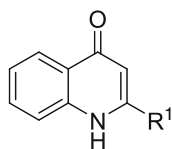
(2) *N*-(3-Oxoacyl)homoserine-L-lactone (3-oxo-C_n-HSL) acyl chain length (even) n = 4-14



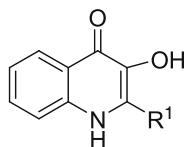
(3) *N*-(3-Hydroxyacyl)homoserine-L-lactone (3-OH-C_n-HSL) acyl chain length (even) n = 4-14



(4) 2-Alkyl-4-hydroxyquinoline *N*-oxide (HQNO, NQNO) alkyl chain length (odd) R¹ = C7,9



(5) 2-Alkyl-4(1*H*)-quinolone (HHQ, NHQ, UHQ) alkyl chain length (odd) R¹ = C7-11



(6) 2-Alkyl-3-hydroxy-4(1*H*)-quinolone (PQS, C9 PQS, C11 PQS) alkyl chain length (odd) R¹ = C7-11

of saturation of the 2-alkyl side chain as well as *N*-oxide derivatives of these variants [18]. In the context of QS, the most important *P. aeruginosa* Aqs are PQS and HHQ which regulate the expression of multiple virulence genes [15, 19–21]. Aqs similar to those produced by *P. aeruginosa* have also been reported in *Burkholderia* species [22, 23]. Since many different bacteria produce multiple QSSMs, there is a need to profile their nature and concentrations in the same sample to provide comprehensive information relating to both QS regulatory mechanisms and the biosynthesis and metabolism of QSSMs.

Until relatively recently, the analysis of Aqs and AHLs in bacterial culture has been indirect and often problematic: PQS and specific AHLs were semi-quantified by thin-layer chromatography and densitometric analysis [24, 25] by an indirect biological assay with a luminescent bacteria biosensor systems or a combination of these [26–28]. The TLC biosensor method is simple and widely used but it cannot separate some individual QSSMs and can give rise to false negative/positive results. In addition, although the biosensor systems can respond to a range of QSSMs, the response is differential such that the most intense response does not necessarily correspond with the most abundant compound present [27, 28]. Consequently, it is difficult to determine the exact molar ratios of the different QSSMs produced by the same organism using this approach. Measurement of unknown QSSMs in bacterial culture supernatants preferably requires a targeted method using mass spectrometry detection and the use of synthetic calibration standards to provide accurate quantification. Although several LC-MS/MS methods have been developed to measure either AHLs [29–35] or Aqs [23, 36, 37] few of the methods have been fully validated to accepted international bioanalytical standards [38] and there are no existing LC-MS/MS methods which can simultaneously quantify the full range of both AHL and AQ classes of QSSMs in bacterial cultures.

In this work, we have developed and validated an LC-MS/MS method to measure a wide range of QSSMs and applied it to study the production of AHLs and Aqs in *P. aeruginosa* wild-type and QS mutant cultures after growth in either a rich complex medium (LB) or in a chemically defined medium suitable for metabolomic studies.

Experimental

Chemicals

The following AHL standards and derivatives were used: *N*-butanoyl-L-homoserine lactone (C4-HSL), *N*-hexanoyl-L-homoserine lactone (C6-HSL) *N*-octanoyl-L-homoserine lactone (C8-HSL), *N*-decanoyl-L-homoserine lactone

(C10-HSL), *N*-dodecanoyl-L-homoserine lactone (C12-HSL) and *N*-tetradecanoyl-L-homoserine lactone (C14-HSLs), *N*-(3-oxobutanoyl)-L-homoserine lactone (3-oxo-C4-HSL), *N*-(3-oxohexanoyl)-L-homoserine lactone (3-oxo-C6-HSL), *N*-(3-oxooctanoyl)-L-homoserine lactone (3-oxo-C8-HSL), *N*-(3-oxodecanoyl)-L-homoserine lactone (3-oxo-C10-HSL), *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL), ring opened form of 3-oxo-C12-HSL (OR 3-oxo-C12-HSL), *N*-(3-oxotetradecanoyl)-L-homoserine lactone (3-oxo-C14-HSL), *N*-(3-hydroxybutanoyl)-L-homoserine lactone (3-hydroxy-C4-HSL), *N*-(3-hydroxyhexanoyl)-L-homoserine lactone (3-hydroxy-C6-HSL), *N*-(3-hydroxyoctanoyl)-L-homoserine lactone (3-hydroxy-C8-HSL), *N*-(3-hydroxydecanoyl)-L-homoserine lactone (3-hydroxy-C10-HSL), *N*-(3-hydroxydodecanoyl)-L-homoserine lactone (3-hydroxy-C12-HSL), *N*-(3-hydroxytetradecanoyl)-L-homoserine lactone (3-hydroxy-C14-HSL). Regarding Aqs the standards used were: 2-heptyl-4-hydroxyquinolone (HHQ), 2-nonyl-4-hydroxyquinolone (NHQ), 2-undecyl-4-hydroxyquinolone (UHQ), 2-heptyl-3-hydroxy-4(1*H*)-quinolone (PQS), 2-hydroxy-2-nonyl-4(1*H*)-quinolone (C9-PQS), 2-hydroxy-2-undecyl-4(1*H*)-quinolone (C11-PQS), 2-heptyl-4-hydroxyquinolone *N*-oxide (HQNO), 2-nonyl-4-hydroxyquinolone *N*-oxide (NQNO). All standards were synthesized in-house [39, 40]. Methanol, acetonitrile, acetic acid, and ethyl acetate were purchased from Fisher Scientific (Loughborough, UK). Formic acid (MS grade) and ethylenediaminetetraacetic acid (EDTA) were obtained from Sigma-Aldrich (Poole, UK) and ultra-pure water (>18 MΩ/cm) from an Elga Maxima water purification system (Elga Ltd., High Wycombe, UK) were used. Individual solutions of QSSM standards were prepared in acetonitrile and stored at –80 °C at a concentration of approximately 1 mg/ml and were appropriately further diluted in acetonitrile to prepare other stock solutions and create calibration.

Synthesis of *N*-(pentanoyl-d₉)-L-homoserine lactone

The internal standard, *N*-pentanoyl-L-homoserine lactone-d₉ (C5-HSL-d₉), was synthesized as follows. To a solution of L-homoserine lactone hydrochloride (2 mmol) in water (4 mL) was added a solution of pentanoic-d₉ acid (2.2 mmol) in 1,4-dioxane (4 mL) followed by triethylamine (2.4 mmol) and *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (3 mmol). The mixture was stirred at room temperature overnight and concentrated to remove dioxane by rotary evaporation. The remaining aqueous solution was extracted with ethyl acetate (×3); the extracts were pooled and washed once with a saturated sodium chloride solution. After drying over MgSO₄, the ethyl acetate was removed by rotary evaporation to obtain C5-HSL-d₉ as a white crystalline solid in 79% yield. The product was 99.4% pure by RP-

HPLC (t_R 4.49 min, Kromasil KR100-5 C8; 250 mm \times 4.6 mm; isocratic 40% acetonitrile) 1H NMR ($CDCl_3$) δ 2.15 (1H, m, ring 4 α -H), 2.91 (1H, m, ring 4 β -H), 4.31 (1H, m, ring 5 α -H), 4.48 (1H, td, 5 β -H), 4.56 (1H, m, ring 3-H), 5.94 (1H, bs, NH).

Media and culture methods

The *P. aeruginosa* wild-type strain PAO1 and the isogenic *lasI*, *rhlI*, *pqsA* single and *rhlI lasI* double mutants were constructed as described previously [22, 41, 42] and were grown at 37 °C in a 2-L all-glass fermenter (Sartorius Stedim Biotech, Aubagne, France) in either LB broth or a chemically defined medium (CDM). The basal CDM used in this study contained 20 mM D-glucose, 3 mM KCl, 3 mM NaCl, 12 mM $(NH_4)_2SO_4$, 3.2 mM $MgSO_4 \cdot 7H_2O$, 0.02 mM $FeSO_4 \cdot 7H_2O$, 1.2 mM K_2HPO_4 , and 50 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS; all purchased from Sigma-Aldrich, Gillingham, UK) buffered to a pH of 7.2 (Ombaka et al., 1983 [43]).

Extraction of quorum-sensing signaling molecules from spent culture medium

Samples of culture medium (1.0 ml) were centrifuged at (8,500 \times g) for 10 min. A volume of 5 μ l of 10 μ M d_9 C5-HSL used as internal standard was added with 1.0 ml of acidified (0.01% acetic acid) ethyl acetate to the culture supernatant, the mixture was vortex-mixed for 1 min and the organic phase was removed. The extraction procedure was repeated twice and the pool of ethyl acetate extracts was evaporated to dryness. The dried samples were stored at -80 °C until use and reconstituted in 50 μ l methanol immediately prior to LC-MS/MS analysis.

LC-MS/MS analysis of QSSMs

Five microliters of the extracts was injected for LC-MS/MS analysis at a flow rate of 0.45 mlmin⁻¹ (Shimadzu series 10 AD VP, Columbia, MD, USA) equipped with binary pumps, a vacuum degasser, a SIL-HTc autosampler and column oven (Shimadzu, Columbia, MD, USA) using a Phenomenex Gemini Column C18, 150 \times 2 mm (5 μ m particle size) maintained at 50 °C. The HPLC system used as the mobile phase A constituted by 0.1% formic acid (Sigma-Aldrich, Gillingham, UK) and 200 μ M EDTA (Sigma-Aldrich, Gillingham, UK) in water and a mobile phase B constituted by 0.1% formic acid (Fisher Scientific, Loughborough, UK) in acetonitrile (Fisher Scientific, Loughborough, UK). The mobile phase A was sonicated for 30 min and filtered through 0.45 μ m nylon Whatman disk filters (Maidstone, UK) prior to use. The gradient profile was as follows: isocratic for 1 min, a linear gradient

from 10% to 50% B over 0.5 min, then a further gradient from 50% to 99% B over 4 min followed by 99% B for 1.5 min, at a flow rate of 0.45 ml/min. The column was re-equilibrated for a total of 2.9 min.

All mass spectrometry (MS) experiments were conducted on a 4000 QTRAP hybrid triple-quadrupole linear ion trap mass spectrometer (Applied Biosystem, Foster City, CA, USA) equipped with a TurboIon source used in positive ion electrospray mode. A Windows XP (Microsoft, Redmond, WA, USA) workstation running Analyst (version 1.4.1) was used for data acquisition and processing. MRM parameters (precursor and product ion pairs, declustering potential, collision cell exit potential and collision energy) were optimized by software automation while infusing at 50 μ l min⁻¹ with the MS peak widths set to 0.7 Th. Source parameters were optimized during infusion experiments at the working flow rate and were as followed: curtain, Gas 1 and 2 were 20, 30, and 10, respectively. The ion source potential was 5,000 V and the source was held at 450 °C. Quantification was performed using Analyst 1.4.1, in Quantitate mode.

Calibration and validation

Eight-point calibration lines were prepared for each analyte by adding known amounts of each QSSM to 1.0 ml sterile medium (range 0.2–20 μ M final concentration, 10–1,000 nM in the medium) and extracting as using the same method as described above. The recovery of analytes from medium was determined by adding a standard mixture of all AHLs, and AQs at low (20 nM), medium (0.1 μ M), and high (1 μ M) final concentrations. The recovery was calculated by comparing the response ratios of spiked extracted medium with a standard mixture prior to extraction. To determine inter- and intra-day precision and accuracy, AHLs and AQs were spiked into sterile CDM at a low, mid-range and high concentrations. Precision was calculated from the relative standard deviation (RSD) of the replicates ($n=5$), and accuracy was calculated by direct comparison of mean measured levels of spiked analytes with expected concentrations for unextracted standards (precision and accuracy defined according to [38]). The ratios of LC-MS/MS peak areas of the analyte/internal standard were calculated and used to construct calibration curves of peak area ratio against analyte concentration using unweighted linear regression analysis.

The lower limit of detection (LLOD) was determined using serial dilutions of the AHL and AQ mixture, without spiking into a bacterial matrix, and was defined as the concentration at which a signal/noise ratio of 3:1 was achieved. The lower limit of quantification (LLOQ) was defined as the concentration at which a signal/noise ratio of 10:1 was achieved. The matrix effect (ionization suppres-

sion) was expressed as the ratio of the mean peak area of the analytes spiked after extraction to the mean peak area of the same standard spikes without matrix, multiplied by 100. A value of >100% indicates ionization enhancement, and a value of <100% indicates ionization suppression. Guidance for bioanalytical method validation was followed wherever possible [38].

Application of the method

The developed and validated methodology was utilized to determine extracellular QSSM metabolite pools produced by *P. aeruginosa* PAO1 and its corresponding QS mutants. The *P. aeruginosa lasI*, *rhlI* and *pqsA* single mutants, the *lasI rhlI* double mutant, as well as the *lasI rhlI pqsA* triple mutant were constructed previously by homologous recombination and grown by batch culture or in a fermenter in either LB or CDM. For each strain or growth condition, a total of six biological replicates were

collected and extracted with acidified ethyl acetate as described above.

Results and discussion

LC-MS/MS method development

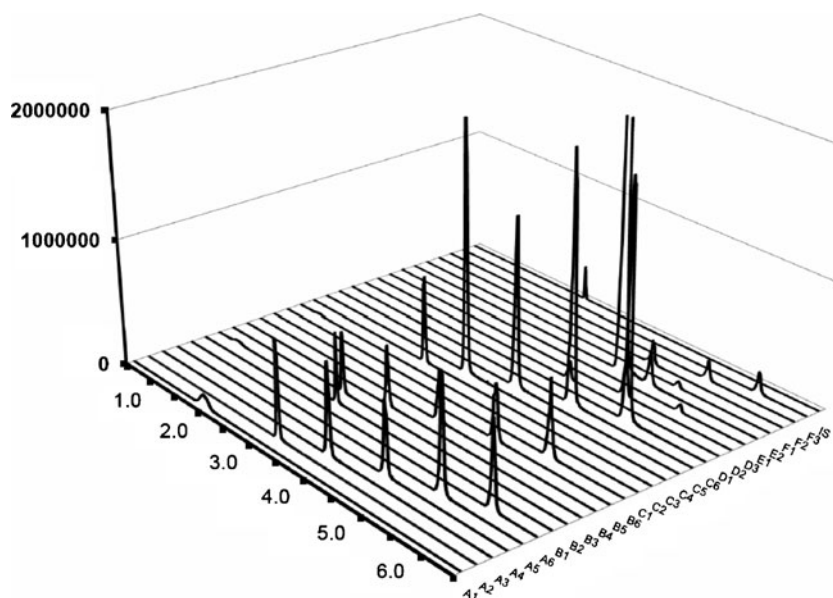
The analysis of two structurally distinct families of QSSMs requires fast and selective analysis. The choice of +ESI mode was based upon a greater sensitivity for both AHL and AQ analytes and was relatively straightforward since all the analytes demonstrated protonated $[M+H]^+$ species as the dominant pseudo-molecular ion. The MS parameters (precursor and product ions used for MRM transitions and corresponding optimized voltages) are listed in Table 1, which shows common product ion fragments could be used for individual families of analytes: m/z 102 for AHL, m/z 159 for AQ and m/z 175 for PQS. All analytes were eluted

Table 1 Selected precursor and product ion m/z values, retention times and mass spectrometer parameters used for QSSM analytes

QSSM analyte	Retention time (min)	Precursor ion (m/z)	Product ion (m/z)	Cone Voltage	Collision Energy (V)
C4-HSL	2.6	172.1	102.1	26	15
C6-HSL	3.4	200.1	102.1	31	15
C8-HSL	3.9	228.1	102.1	26	15
C10-HSL	4.6	256.1	102.1	31	17
C12-HSL	5.3	284.1	102.1	31	17
C14-HSL	6.0	312.1	102.1	45	19
3-oxo-C4-HSL	1.3	186.1	102.1	26	15
3-oxo-C6-HSL	3.1	214.1	102.1	26	15
3-oxo-C8-HSL	3.5	242.1	102.1	26	17
3-oxo-C10-HSL	4.0	270.1	102.1	26	17
3-oxo-C12-HSL	4.7	298.1	102.1	30	19
3-oxo-C14-HSL	5.4	326.2	102.1	30	21
3-OH-C4-HSL	1.3	188.1	102.1	26	15
3-OH-C6-HSL	3.1	216.1	102.1	26	15
3-OH-C8-HSL	3.4	244.1	102.1	31	17
3-OH-C10-HSL	3.9	272.1	102.1	35	17
3-OH-C12-HSL	4.5	300.1	102.1	35	19
3-OH-C14-HSL	5.2	328.2	102.1	21	19
HHQ	4.0	244.1	159.1	31	20
NHQ	4.6	272.1	159.1	81	27
UHQ	5.4	300.1	159.1	81	27
HQNO	4.0	260.1	159.1	71	32
NQNO	4.7	288.1	159.1	96	20
PQS	4.4	260.1	175.1	71	22
C9-PQS	5.1	288.1	175.1	111	25
C11-PQS	5.8	316.1	175.1	116	35
C5-HSL-d ₉ ^a	3.3	195.1	103.1	40	15

^a Internal standard

Fig. 2 Extracted ion LC-MS/MS chromatograms of a mixture of standard AHL and AQ molecules showing separation. In sequence, A1–6: unsubstituted AHL (alkyl chain length, C4 to C14), B1–6: 3-oxo-substituted AHLs (C4 to C14), C1–C6: 3-hydroxy-substituted AHLs (C4 to C14), D1–3, AQ analogs (C7 to C11), E1 and E2 *N*-oxides HQNO and NQNO (C7 and C9), F1–3, 2-alkyl-3-hydroxy-4-quinolones (PQS analogs, C7 to C11), *IS* internal standard



between 1.3 and 6.0 min, providing a rapid separation with a total run time of 9 min, including re-equilibration time. All 27 analyte standards were completely resolved by the LC-MS/MS method (Fig. 2).

During method development, a problem was encountered with extremely poor peak shapes for the PQS series of analytes. This manifested itself as peaks with elongated tails, and in extreme cases the peaks were smeared over almost the entire chromatogram, resulting in raised baselines and unquantifiable peaks. The peak shape of the PQS analytes was noticeably improved when they were injected at higher concentrations ($>100 \mu\text{M}$). This problem has been noted previously and the authors overcame it to a certain extent by using densely linked or modified LC column packings [37]. We evaluated many alternative stationary phases for these PQS analytes, but all suffered to a greater or lesser extent from the same poor chromatographic peak shapes, especially when the concentration fell below $1 \mu\text{M}$. We theorized that the poor chromatographic behavior might be linked to the known metal chelating properties of the PQS molecules [13], which might encourage unwanted strong interactions with the underlying silica based column packing material. We decided to investigate the use of a more acidic mobile pH since low pH has been observed to decrease the extent of PQS chelation with iron [15] and the addition of competing soluble metal chelators to the mobile phase. Acetylacetone, a volatile metal chelating agent compatible with MS analysis, improved the PQS peak shape when added to the mobile phase, but the peak shape was still not suitable for quantitative analysis (Fig. 3). Although acetylacetone did improve the peak shape of PQS analytes in the short term, this improvement was not stable over a period of several weeks and the peak shape gradually reverted to the original smeared peak. The solution was

found in the addition of the metal chelating agent EDTA ($200 \mu\text{M}$) and an acidic mobile phase pH which improved PQS peak shape (Fig. 3) even at the lowest measurable concentrations (20 nM and lower). However, the addition of non-volatile EDTA introduced two potential problems, that of ionization suppression (hence possible reduction of sensitivity) and that of potential contamination of the MS source with involatile material leading to high background or signal loss during extended analytical runs. However, only relatively minor signal suppression by EDTA (peak area reduced by 20%) was observed for the AQ analytes which ionize very readily, although suppression was more pronounced for the AHLs which showed significant peak area reduction of up to 80%. The effect on AHLs was most pronounced for the early eluting analytes (1–3 min) and

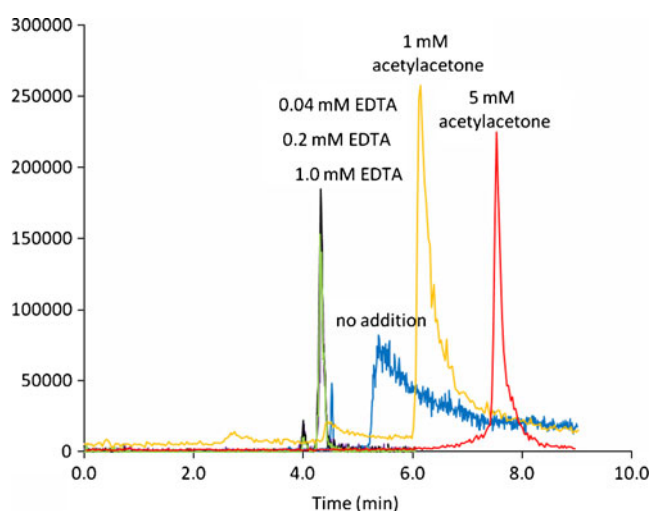


Fig. 3 The effect of mobile phase additives acetyl acetone and EDTA on the chromatographic peak shape of PQS

Table 2 Validation results: calibration, intra- and inter-day precision, accuracy and recovery of assay method determined at three concentrations (L=20 nM, M=0.1 μM and H=1.0 μM) in spiked bacterial extract

Analyte	Intra-day (n=6)						Inter-day (n=6)						Ionization suppression (%)					
	Precision (RSD%)			Accuracy (%)			Precision (RSD%)			Accuracy (%)			Recovery (%±SD)			Recovery (%±SD)		
	L	M	H	L	M	H	L	M	H	L	M	H	L	M	H	L	M	H
C4-HSL	12.1	7.5	8.8	98.8	102.2	106.7	6.5	14.3	4.6	97.0	90.6	100.8	81.5±5.7	71.2±3.5	76.8±5.8	82.3	84.7	100.1
C6-HSL	14.2	13.8	8.7	92.7	113	96.1	6.3	13.6	6.6	97.3	109.6	100.4	72.7±1.1	90.8±5.8	97.5±7.9	83.0	88.8	94.4
C8-HSL	11.0	10.0	11.9	96.8	96.8	101.1	16.4	11.3	4.9	99.0	94.7	105.4	69.3±5.5	78.3±3.8	94.2±3.6	76.7	93.8	98.9
C10-HSL	3.9	12.1	5.8	97.9	97.9	101.6	14.5	11.1	10.8	92.7	96.7	104.8	71.8±6.9	86.7±8.7	96.1±6.1	89.7	89.1	94.9
C12-HSL	9.4	10.8	11.0	104.1	104.1	95.4	10.1	9.1	8.1	95.9	94.9	95.6	67.6±4.3	88.3±6.8	89.0±7.8	87.3	90.3	100.3
C14-HSL	12.3	9.4	9.0	111.4	106	99.2	10.23	5.9	9.2	91.3	95.4	92.5	58.0±5.6	93.9±8.3	74.9±4.9	79.1	88.8	97.0
3-oxo-C4-HSL	9.9	9.4	4.7	97.6	109.4	96.8	5.9	6.9	8.8	127.3	101.2	101.6	24.3±8.0	43.3±1.7	38.1±1.8	102.5	87.6	88.9
3-oxo-C6-HSL	11.8	11.7	10.1	97.3	97.3	97.3	5.0	12.8	2.1	106.2	96.3	100.2	74.9±11.9	61.2±1.3	87.5±7.5	90.9	88.2	100.1
3-oxo-C8-HSL	6.1	12.2	5.8	107.2	107.2	97.3	6.1	8.8	5.2	91.3	97.6	99.3	75.4±8.1	80.2±3.8	97.7±11.6	88.2	88.4	93.8
3-oxo-C10-HSL	7.1	6.3	9.0	95.8	95.8	96.6	7.0	15.1	5.0	88.8	84.8	99.4	77.9±11.7	78.6±3.5	91.0±6.2	85.2	91.8	96.4
3-oxo-C12-HSL	8.5	11.5	5.9	94.1	94.1	104.8	19.0	6.3	4.1	108.0	92.7	101.4	73.2±9.3	76.3±3.4	93.1±7.3	85.9	94.5	95.2
3-oxo-C14-HSL	10.3	11.1	7.4	99.2	99.2	100.4	11.5	5.7	4.5	107.6	93.3	98.6	58.6±5.7	70.6 ± 3.5	81.2±3.0	83.2	90.9	91.2
3-OH-C4-HSL	24.0	14.4	11.6	107.8	107.8	100.0	16.8	12.3	2.8	91.6	58.2	92.7	53.2±5.3	13.4±8.0	33.2±6.3	84.0	82.0	98.4
3-OH-C6-HSL	5.8	12.0	8.2	105.3	113.5	103.3	5.4	6.7	4.7	96.8	92.3	99.4	55.6±7.9	58.4±3.2	71.8±6.7	91.0	85.6	98.4
3-OH-C8-HSL	13.2	5.8	10.9	109.1	113.0	101.4	5.0	10.8	3.5	101.3	96.6	103.9	90.4±8.9	82.8±2.9	97.2±6.9	73.5	86.6	98.0
3-OH-C10-HSL	4.1	14.0	8.9	100.6	102.3	99.1	3	7.3	2.3	106.8	104.6	100.1	74.2±5.4	93.9±2.9	98.3±6.0	82.7	92.0	93.2
3-OH-C12-HSL	9.1	7.9	6.3	100.1	104.7	97.3	9.4	9.4	2.6	111.4	105.6	101.1	70.4±10.1	94.0±3.4	97.9±8.4	68.0	107.3	93.6
3-OH-C14-HSL	10.0	5.2	6.0	101.4	101.4	100.0	9.6	7.5	6.4	106.2	105.3	99.4	73.7±3.2	90.8±7.1	84.2±10.1	73.3	104.0	91.2
HHQ	12.7	4.2	4.6	100.8	112.3	105.7	8.1	12.1	13.3	104.2	108.8	98.3	93.2±2.9	90.4±12.9	97.1±3.7	80.8	82.4	95.4
NHQ	5.5	5.3	5.3	96.4	112.3	106.5	6.6	5.5	3.4	95.1	99.6	99.1	81.3±2.5	89.8±2.9	95.3±2.5	75.9	81.0	92.4
UHQ	6.5	3.9	7.1	99.4	99.4	99.5	7.3	3.3	4.9	107.5	99.0	99.7	67.9±4.6	80.1±16.3	89.1±3.6	78.4	81.3	93.1
HQNO	10.4	10.4	8.2	102.2	102.2	98.2	6.6	11.8	11.2	88.1	90.4	95.6	43.9±3.7	66.0±8.9	92.5±6.3	68.7	84.7	92.4
NQNO	14.0	7.1	4.9	96.1	91.1	105.3	12.3	12.1	11.8	100.5	100	104.2	41.4±4.0	66.1±14.6	91.9±4.6	81.0	82.6	89.8
PQS	8.0	15.4	4.5	106.8	100.6	108.8	14.0	11.8	4.3	90.6	95.3	99.3	26.0 ± 7.0	61.9±1.4	93.3±4.4	75.9	86.2	88.4
C9-PQS	10.0	7.8	6.4	115.5	95.5	99.7	9.1	12.8	4.2	98.1	93.1	100.5	23.8±4.0	69.3±2.1	82.4±5.4	80.7	79.6	90.3
C11-PQS	12.2	8.9	9.9	90.6	102.7	101.5	11.9	8.2	10.0	96.6	95	99.2	15.3 ± 4.8	56.1±5.4	72. ± 5.6	60.2	83.6	94.7

these showed a greater degree of ionization suppression. Contamination of the MS source by EDTA was shown to be a relatively insignificant effect; after a typical long analytical run of 150 injections, signals remained on average at 84% of the first injection. To ensure that this small drop-off in MS response did not affect the quantitative performance of the method analytical runs were limited to 120 injections before rinsing the outer MS stainless steel gas cone to remove deposits. In addition, potential contamination of the source was further reduced by the use of a flow diverter during times not critical to data acquisition.

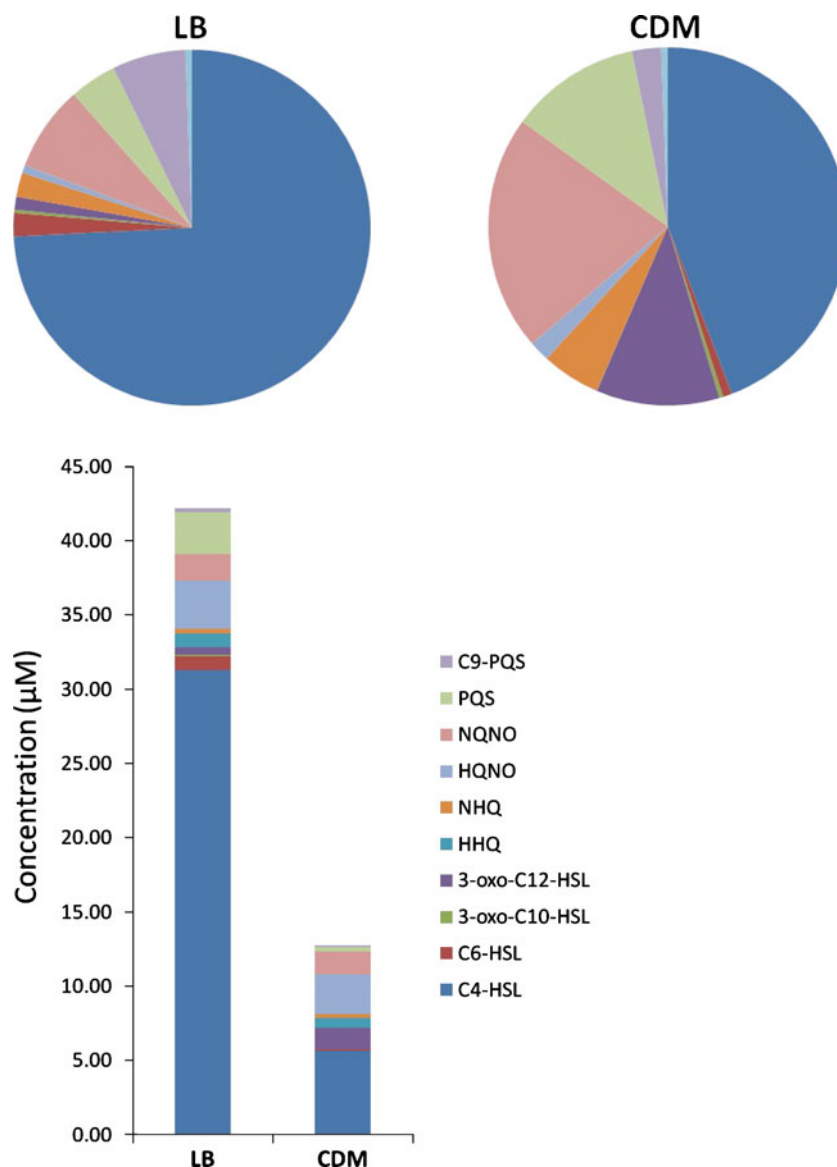
Quantitative aspects: validation of method

Table 2 summarizes the method validation data and the results confirm that the method is sensitive, precise and

accurate and provides satisfactory recovery, for all analytes making the method suitable for quantitative profiling of AHLs and AQS. The linearity of the method was confirmed by analysis of six-point calibration lines for each. Non-linearity of calibration was observed for some analytes at higher concentrations but this was not a problem in practice since measured concentrations in real samples were always within the range of the linear calibration. The intra-day precision was between 3.9% and 14%, and the inter-run precision between 2.3% and 19%. Intra-run accuracy established at the L, M and H concentrations was between 90.6% and 113.5% with inter-run accuracy between 85% and 127%.

The measured recoveries of the AHLs and AQS in general gave values of greater than 70% and showed the suitability of the method in extracting these varied analytes from the complex culture medium. However, at the lowest

Fig. 4 Relative molar proportions (*pie charts*) and quantitative profiling (*bar graphs*) of AHLs and AQS produced by *P. aeruginosa* wild-type strain grown in LB and CDM growth medium



validation concentration (20 nM) the recoveries of 3-oxo-C4-HSL, 3-OH-C4-HSL and the PQS series (PQS, C9-PQS and C11-PQS) were noticeably lower than that of the other analytes, perhaps due to their more polar nature resulting in reduced partitioning into the extraction solvent. However, the precision, accuracy and other validation parameters were all within the required specification for these analytes, confirming that the method provides quantitative measurements despite the low recovery. It is possible that the losses at very low concentrations of the PQS family were a result of metal chelating activity causing adsorption to glass or plastic surfaces during the extraction process (although this was not investigated further).

The measured values of matrix effect were in generally in the range 90–100% (where 100%=no ionization suppression) indicating very little matrix effect at the L and M validation concentrations. The L concentration, as would be expected, showed a greater effect but 23 of 26 analytes showed ion suppression values of better than 70% at this level. LLOQ of the AHLs and AQ families were 1.6 and 0.6 nM, respectively.

Biological applications: AHL and AQ profiling of *P. aeruginosa* wild-type and QS mutants

P. aeruginosa produced a wide range of QSSMs, the nature of which varies in acyl/alkyl chain length as well as the level of substitution. When grown in LB, the *P. aeruginosa* PAO1 parent strain produced primarily C4-HSL (31 μ M) and 3-oxo-C12-HSL (0.5 μ M) but also C6-HSL (0.9 μ M) and 3-OH-C4-HSL (0.5 μ M; Fig. 4, Table 3). These profiles and concentrations of AHLs are in line with those found in a previously published study [35]. A similar profile was observed following growth in CDM although the overall AHL concentrations were lower than in LB: C4-HSL (6 μ M) and 3-oxo-C12-HSL (1.4 μ M) C6-HSL (0.7 μ M) and 3-OH-C4-HSL (0.1 μ M; Fig. 4, Table 3). Similarly, total AQ concentrations in CDM (5.6 μ M) were lower than those observed in LB (9.4 μ M) with a notable 10-fold higher concentration of PQS in LB compared with CDM. The concentration of PQS generally documented after growth in LB corresponds to concentrations 10 to 100-times higher (5–10 μ M) [19] than in CDM. In LB batch culture, PQS has been detected in the logarithmic phase and a *P. aeruginosa* culture contains 5–10 μ M PQS which increased up to 25 μ M in stationary phase [19]. A recent study showed that the AQ biosynthetic pathway and the anthranilate degradation pathway, which both feed into the TCA cycle for energy metabolism, draw from the same pool of intracellular metabolite [44, 45]. Therefore, in CDM, the metabolite pools directing PQS synthesis may be drawn into the TCA cycle to sustain cell growth at the cost of AQ synthesis.

Table 3 Changes in measured concentrations of AHLs and AQS in *P. aeruginosa* culture in LB medium or CDM

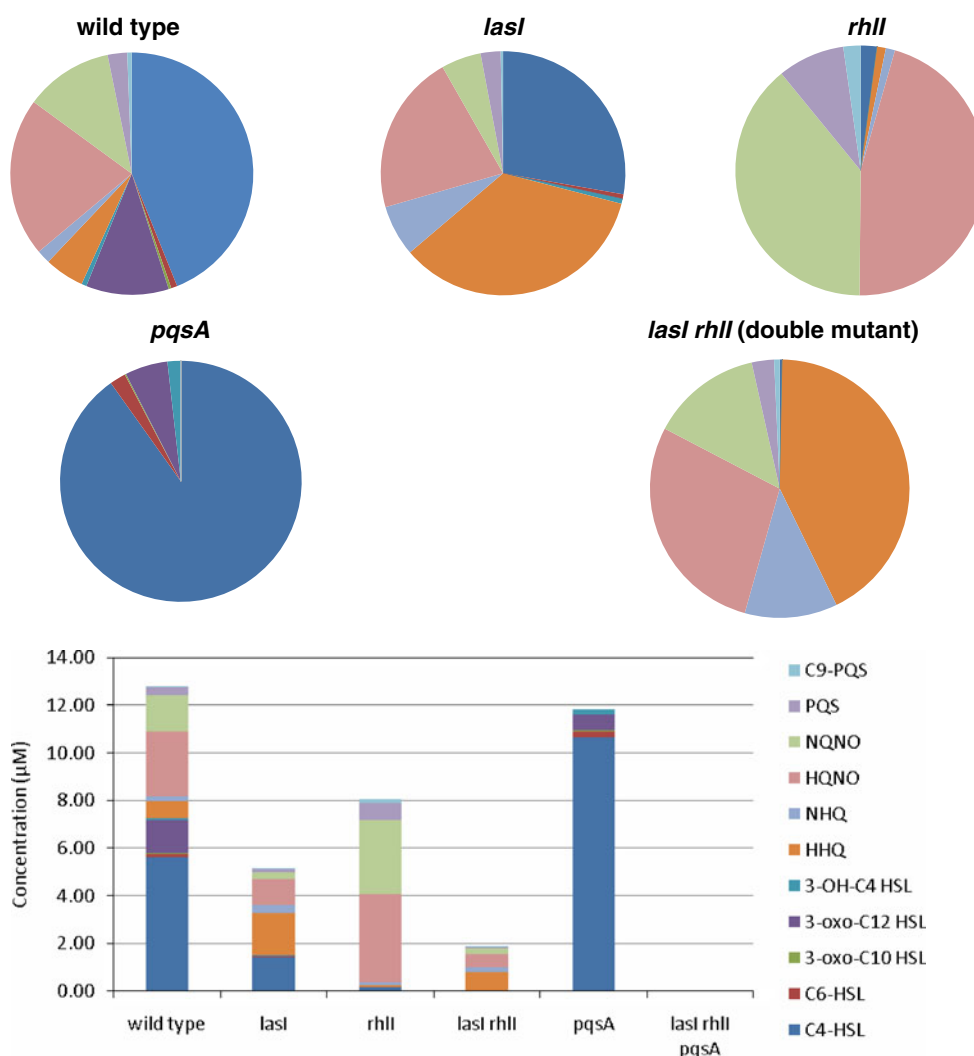
QSSM analyte	QSSM concentration (μ M)			
	LB Medium		CDM	
	Mean	SD	Mean	SD
C4-HSL	31.31	2.17	5.63	0.41
C6-HSL	0.89	0.04	0.10	0.03
C8-HSL	0.02	0.00	nq	–
C10-HSL	nq	–	nq	–
C12-HSL	nq	–	nq	–
C14-HSL	nq	–	nq	–
3-oxo-C4-HSL	nq	–	nq	–
3-oxo-C6-HSL	nq	–	nq	–
3-oxo-C8-HSL	0.04	0.00	nq	–
3-oxo-C10-HSL	0.12	0.01	0.05	0.02
3-oxo-C12-HSL	0.50	0.06	1.41	0.12
3-oxo-C14-HSL	nq	0.00	nq	–
3-OH-C4-HSL	0.47	0.03	0.09	0.09
3-OH-C6-HSL	0.01	0.00	nq	–
3-OH-C8-HSL	nq	–	nq	–
3-OH-C10-HSL	nq	–	nq	–
3-OH-C12-HSL	nq	–	nq	–
3-OH-C14-HSL	nq	–	nq	–
HHQ	0.93	0.04	0.68	0.08
NHQ	0.29	0.03	0.23	0.07
UHQ	0.01	0.00	0.02	0.02
HQNO	3.28	0.37	2.71	0.90
NQNO	1.80	0.11	1.50	0.22
PQS	2.81	0.32	0.33	0.07
C9-PQS	0.25	0.03	0.08	0.07
C11-PQS	0.01	0.00	nq	–

nq not quantifiable (AHLs<1.6 nM; AQS<0.6 nM)

SD standard deviation

Analysis of the QSSM profiles of mutants of *P. aeruginosa* grown in CDM showed that disruption of one QS system (AHL or AQ biosynthetic genes) impacts on the synthesis of the QSSMs from other QS systems. Examples of extracted ion LC-MS/MS chromatograms are given in Electronic Supplementary Material Fig. S1. The full data from this series of experiments is shown in Electronic Supplementary Material Table S1 and Fig. 5. The disruption of the *pqsA* gene resulted in a twofold increase in C4-HSL and C6-HSL and a significant reduction in 3-oxo-C12-HSL when compared with the wild type. Furthermore, a mutation in *rhII* resulted in a large increase in HQNO, NQNO, and PQS levels while a mutation in *lasI* resulted in a significantly increased level of HHQ, NHQ, the disappearance of PQS congeners and a large reduction in C4-

Fig. 5 Pie charts illustrating the relative molar proportions of the major AHLs and AQs identified in *P. aeruginosa* cultures: *P. aeruginosa* (wild type), *las I* mutant, *rhII* mutant, *pqsA* mutant, *las I rhII* double mutant. Bar graphs show quantitative representation of data showing complete removal of all QSSMs with the *las I rhII pqsA* triple mutant *P. aeruginosa*. QSSM concentrations ≤ 20 nM have been excluded for clarity



HSL levels (Fig. 5, Electronic Supplementary Material Table S1). It has been previously established that both the *las* and *rhI* systems exert an influence on the *pqs* system and that the *pqs* system affects the synthesis of C4-HSL and 3-oxo-C12-HSL (reviewed by Heeb et al. [46]. McKnight et al. [20] showed that PQS positively regulates *rhII* expression, thus influencing C4-HSL accumulation. It has also been observed that *pqsABCDE* expression depends on the ratio between the two AHL molecules, 3-oxo-C12-HSL having a positive effect, and C4-HSL having a negative effect [47] this has also been corroborated by [48] who showed that whereas LasR has a positive impact on *pqsR* expression and hence AQ production, RhII has the opposite effect.

The increase in HHQ and NHQ in the *lasI* mutant can be explained by the positive control of *pqsH* (which encodes the mono-oxygenase required for the conversion of HHQ and NHQ to PQS and C9-PQS) by LasR/3-oxo-C12-HSL. Similar results have been obtained previously [49–51]).

Conclusions

A selective and rapid method for the simultaneous analysis of the two main classes of QSSMs has been developed, validated and shown to be applicable to obtain good quality experimental data from *P. aeruginosa*. In addition to this application, the method has been successfully applied to measure AHL and AQ profiles in both complex and defined culture media under a range of experimental situations. Although this methodology was conceived for profiling studies in relation to the biosynthesis of QSSMs, the methodology could be easily modified to facilitate studies on biological samples other than bacterial cultures. We have started to exploit this technology for the analysis QSSM levels in the body fluids of patients with cystic fibrosis who are colonized with *P. aeruginosa* or *Burkholderia* spp., both of which produce complex mixtures of AHLs and AQs [23, 52]. Therefore, the technology presented in this manuscript will be a valuable investigative tool for metabolic profiling in many environments including the clinic.

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