



Targeted and untargeted quantification of quorum sensing signalling molecules in bacterial cultures and biological samples via HPLC-TQ MS techniques

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Abstract

Quorum sensing (QS) is the ability of some bacteria to detect and to respond to population density through signalling molecules. QS molecules are involved in motility and cell aggregation mechanisms in diseases such as sepsis. Few biomarkers are currently available to diagnose sepsis, especially in high-risk conditions. The aim of this study was the development of new analytical methods based on liquid chromatography-mass spectrometry for the detection and quantification of QS signalling molecules, including N-acyl homoserine lactones (AHL) and hydroxyquinolones (HQ), in biofluids. Biological samples used in the study were *Pseudomonas aeruginosa* bacterial cultures and plasma from patients with sepsis. We developed two MS analytical methods, based on neutral loss (NL) and product ion (PI) experiments, to identify and characterize unknown AHL and HQ molecules. We then established a multiple-reaction-monitoring (MRM) method to quantify specific QS compounds. We validated the HPLC-MS-based approaches (MRM-NL-PI), and data were in accord with the validation guidelines. With the NL and PI MS-based methods, we identified and characterized 3 and 13 unknown AHL and HQ compounds, respectively, in biological samples. One of the newly found AHL molecules was C12-AHL, first quantified in *Pseudomonas aeruginosa* bacterial cultures. The MRM quantitation of analytes in plasma from patients with sepsis confirmed the analytical ability of MRM for the quantification of virulence factors during sepsis.

Keywords Quorum sensing molecules · Homoserine lactones · Hydroxyquinolones · *Pseudomonas aeruginosa* · Mass spectrometry · Triple quadrupole

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Abbreviations

3-oxo-C12-AHL	N-(3-Oxododecanoyl)-L-homoserine lactone
AHLs	N-Acyl homoserine lactones
AI	Autoinducer
C4-AHL	N-Butanoyl-L-homoserine lactone
C7 HQ (or PQS)	2-Heptyl-3-hydroxy-4(1H)-quinolone
HPLC	High-performance liquid chromatography
HPLC-TQMS	High-performance liquid chromatography-triple quadrupole mass spectrometry
HRMS	High-resolution mass spectrometry
HQs	Hydroxyquinolone signalling molecules
IS	Internal standard
LB	Luria-Bertani
LLOQ	Lower limit of quantification
LTQ	Linear trap quadrupole
M9	Mineral medium

MOF	Multi-organ failure
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MW	Molecular weight
NL	Neutral loss
PI	Product ion
RT	Room temperature
QS	Quorum sensing
TQ MS	Triple quadrupole mass spectrometer
UHPLC	Ultra-high-performance liquid chromatography
ULOQ	Upper limit of quantification

Introduction

Bacteria have the ability to interact with each other through a complex language called “quorum sensing” (QS) [1–3]. Literally, QS means “detection of the quorum”, and it refers to the ability of bacteria to monitor their population density, and consequently to control their gene expression, through the control of the amount of specific molecules, called autoinducers (AIs), in their living environment [4–6]. AIs are small and diffusible molecules produced by bacteria, released and accumulated in the extracellular environment. When many AIs are produced and stored, and their concentration reaches a threshold level (the quorum), the bacterial population is able to activate or to repress target genes [7]. This mechanism enables the survival of a bacterial population in a constantly changing environment (temperature, pH and osmotic concentration variations, and nutrient availability) thanks to the synthesis of new proteins. QS-mediated changes are energetically expensive, and they are advantageous only when cells have reached a high-density population [8, 9].

Gram-positive and Gram-negative bacteria use different means of communication [10]. Gram-positive bacteria produce oligopeptides as QS autoinducer molecules, while Gram-negative bacteria use others QS signal molecules [11–13]. The most abundant and common are N-acyl homoserine lactones (AHLs) [14, 15]. These molecules are characterized by a γ -lactone cycle which is N-acylated in the α position and an acyl chain (indicated as R-chain in Fig. 1). Chain length is the signal specificity factor for

bacteria, and the chain typically contains between 4 and 16 carbon atoms. The presence of an oxo or a hydroxy group linked to the third carbon atom of the chain is a further element of distinction.

In some human opportunistic pathogens, like *Pseudomonas aeruginosa*, the secretion of the most abundant AHL QS signalling molecules N-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-AHL) and N-butanoyl-L-homoserine lactone (C4-AHL) depends on the regulatory circuit systems *Las* or *Rhl* [16, 17]. In bacteria, those systems control the expression of different virulence genes in a population density-dependent manner.

In addition to AHLs, Gram-negative bacteria such as *Pseudomonas aeruginosa* use a hydroxyquinolone molecule (HQ), the 2-heptyl-3-hydroxy-4(1H)-quinolone (known as PQS or C7 HQ), as a QS signalling compound [18, 19]. The basic structure of quinolone molecules consists of a bicyclic ring. The length of the acyl chain (marked as R-chain in Fig. 2) ranges between seven and 11 carbon atoms; the presence of a hydroxy or a carbonyl group in position 1 of the bicyclic ring is a further element of distinction.

As described above, AHLs and HQs are the most abundant QS signalling molecules produced by Gram-negative bacteria. Many studies in the literature have aimed to describe and quantify well-known or new QS molecules in bacterial culture or human samples [20–35]. There are two main methods for obtaining the measurements: biosensors [21–23, 26, 30, 31] and methods based on liquid chromatography coupled to (high-resolution) mass spectrometry [20, 23–25, 27–29, 32–36]. Biological biosensors reach very low sensitivity, with a pg/mL (fmol) limit of detection, and are highly specific for a single QS (AHL or HQ). However, they miss unknown molecules, and the quantitation demonstrates low accuracy [32]. On the contrary, mass spectrometry-based methods, both targeted and untargeted, offer accurate quantitation of AHL and HQ compounds. During the past few years, many LC-MS methods have been developed that exploit several stationary and mobile phases, using both targeted and untargeted MS approaches. Some studies showed a very low limit of quantitation (pg/mL) and good selectivity, optimizing a short liquid chromatography separation both for AHLs, furthermore enantiomeric, and for HQs [27–29, 33–36]. Other research groups have developed extended chromatography separation runs to

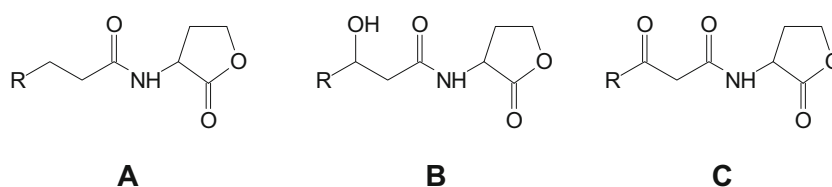
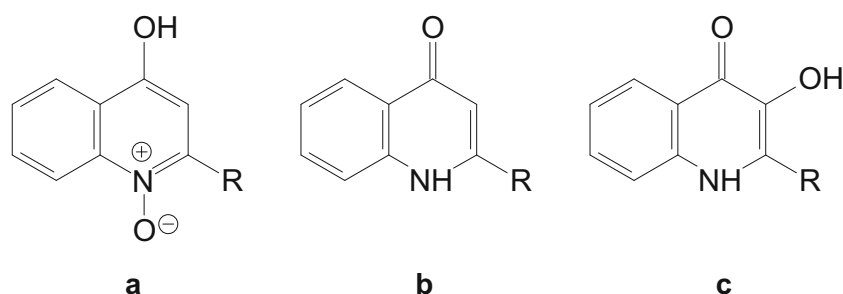


Fig. 1 N-Acyl homoserine lactone generic structure. **a** Non-substituted N-acyl homoserine-L-lactone (C_n-HSL) acyl chain; **b** N-(3-hydroxyacylhomoserine)-L-lactone (3-OH-C_n-HSL) acyl chain; **c** N-(3-

oxoacylhomoserine)-L-lactone (3-oxo-C_n-HSL) acyl chain. The length is variable, generally $n = 4–14$

Fig. 2 Generic structure of quinolone signalling molecules. **a** 2-Alkyl-4-hydroxyquinolone N-oxide with a variable chain length (C7–C9); **b** 2-alkyl-4(1H)-quinolone alkyl chain (the chain length is variable, C7–C11); **c** 2-alkyl-3-hydroxy-4(1H)-quinolone



provide the quantitation of a higher number of molecules [24] or to identify new AI molecules with an untargeted high-resolution MS approach [32].

Summarizing, there are a huge number of QS molecules produced by bacteria, and the identification, characterization and accurate quantitation of peculiar and unknown QS are still needed.

The purpose of this work was to develop and validate HPLC-TQ MS chemical class-specific methods able to identify and quantify quorum sensing molecules (AHLs and HQs) in different matrices, such as bacterial cultures and biological plasma samples. In order to characterize and identify unknown AHL and HQ signalling molecules, the aim of the research was the development of an MS analytical method based on neutral loss (NL) and product ion (PI) experiments. A tandem mass spectrometry method based on a multiple reaction monitoring (MRM) approach was developed for the quantitation of AHLs and HQs in bacterial cultures and biological plasma samples. To ensure greater reliability of analytical data, fragmentation pathways of analytes of interest and the exact mass of detected unknown molecules were confirmed by high-resolution mass spectrometry (LTQ-Orbitrap). The developed MRM method was firstly applied to quantify the QS molecules in *Pseudomonas aeruginosa* cultures obtained from two different strains [37], and with our NL and PI methods we aimed to elucidate whether other QS molecules were involved in the protection mechanism used by bacteria. Secondly, the MRM method was applied to pathology plasma samples (before and after hemoperfusion) of patients affected by sepsis-related multi-organ failure (MOF), which is associated with high mortality. To our knowledge, this study is the first to provide a tandem mass spectrometry quantitation of both AHLs and HQs in plasma samples from patients with sepsis.

Materials and methods

Chemicals

Analytical standards (purity >98%) of 2-heptyl-3-hydroxy-4(1H)-quinolone (C7 HQ), N-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-AHL), N-hexanoyl-L-

homoserine lactone-D3 (ND3) and N-butanoyl-L-homoserine lactone (C4-AHL) were purchased from Merck KGaA (Darmstadt, Germany). Stock solutions were prepared with a concentration of 1000 mg/L using methanol and were stored at $-4\text{ }^{\circ}\text{C}$ until use. Further dilutions were obtained in 0.1% formic acid in water/acetonitrile 60:40. All aqueous solutions were prepared with HPLC-grade water from a Milli-Q Academic System (Millipore, Burlington, MA, USA). Ethyl acetate HPLC-MS grade, acetonitrile and methanol hyper-grade for LC-MS, and formic acid were purchased from VWR International (Radnor, PA, USA).

Instrumentation

Separation and analysis of all analytes and samples were achieved upon an HPLC-TQ MS platform. HPLC consisted of a Shimadzu Nexera X2 Ultra High Performance Liquid Chromatography (UHPLC) system (Shimadzu, Kyoto, Japan) coupled for identification and quantitation to a QTRAP 5500 system (Sciex, Darmstadt, Germany). The triple quadrupole was equipped with a Turbo VTM Source (ESI mode) which utilized nitrogen and air as sheath and reagent gas, respectively. To verify the exact mass values, we used an LTQ-Orbitrap high-resolution mass spectrometer (Thermo Scientific, Waltham, MA, USA) within mass accuracy of ± 3 ppm and resolution of 30 k.

HPLC parameters

To investigate AHL and HQ signalling molecules in biological samples, different chromatographic gradients were carried out. However, HPLC methods had several features in common. The analytical column used was a Phenomenex Luna C18 reversed-phase column (150×2.1 mm i.d., $3\text{ }\mu\text{m}$ particles). Sample injection volume was $10\text{ }\mu\text{L}$. Autosampler and oven temperatures were set at $15\text{ }^{\circ}\text{C}$ and $45\text{ }^{\circ}\text{C}$, respectively, for the duration of all analyses.

For AHLs, HPLC-TQ MS analysis eluents were formic acid 0.1% in water (solvent A) and in acetonitrile (solvent B). Flow rate was set at $200\text{ }\mu\text{L}/\text{min}$. For bacterial cultures, the mixture percentage changed from 40% solvent B to 100% solvent B during the first 35 min, maintained for 10 min, and then the column returned to the starting conditions. For

plasma samples, the HPLC run started from 40% solvent B, increased to 100% solvent B in 19 min, maintained for 10 min, and then returned to the starting conditions. Bacterial cultures were more complex than plasma samples and required us to obtain a satisfactory chromatographic separation with slower solvent variation during gradient elution (see Electronic Supplementary Material [ESM] Fig. S1).

Chromatographic separation for the MRM and PI analysis of C7 HQ in bacterial cultures and plasma samples was achieved using 2-picolinic acid 2 mM/formic acid 0.1% in water (solvent C) and acetonitrile 0.1% formic acid (solvent D). 2-Picolinic acid acted as a bidentate chelator, preventing peak distortion caused by C7 HQ, an iron chelator molecule [33]. Gradient started from 20% solvent D, up to 100% solvent D in 12 min; then the column returned to the starting conditions. Flow rate was 250 $\mu\text{L}/\text{min}$.

MS settings

The LC effluent was delivered to the Turbo VTM Source (ESI positive ionization mode) using nitrogen as the sheath (GS1) and curtain (CUR) gas and air as reagent (GS2) gas, respectively. The mass spectrometer parameters were as follow: CUR 26 arbitrary units (arb), GS1 45 arb, GS2 50 arb, ion spray voltage 5.5 kV and ion spray temperature 500 °C.

Considering all the possible MS experiments developed, each sample was analysed five times: MRM-NL-PI for AHL analysis, and MRM-PI for HQ analysis. The MRM, NL and PI parameters are listed in Table 1. For MRM acquisition, we selected one qualitative and one other quantitative (bold in Table 1) transition for each analyte. The instrument parameters are listed in ESM Tables S1 and S2.

Bacterial cultures and biological samples

Two *Pseudomonas aeruginosa* strains were selected as representatives of bacterial cultures: wild-type PAO1 strain and its isogenic mutant RhII defective in the synthesis of C4-AHL previously obtained [37, 38].

Different *Pseudomonas aeruginosa* PAO1 and RhII bacterial cultures were prepared and analysed: (i) wild-type bacterial culture grown in Luria-Bertani (LB) broth (rich in nutrients), WT-LB; (ii) wild-type bacterial culture grown in the mineral medium (M9) culture (low in nutrients), WT-M9; (iii) RhII mutant bacterial culture grown in the LB broth, RhII-LB; and (iv) RhII mutant bacterial culture grown in the M9 culture, RhII-M9.

For the application of the MRM-NL-PI methods developed, we analysed plasma samples from healthy people and patients affected by MOF. All the patients involved in the present study expressed their consent and their will based on their awareness of the proposed study of their biological fluids, freely deciding whether to accept. All the procedures followed in the work were carried out in accordance with the ethical standards of our institutional, the national research committee and with the Code of Ethics of the World Medical Association (1964 Helsinki declaration).

Sample preparation and enrichment

All the bacteria media were prepared as described by Orlandi et al. [37]. Bacterial cultures were centrifuged for 10 min at 12,000 rpm and RT, and the supernatants were then collected and filtered (Minisart RC15 \varnothing 0.20 mm; Sartorius) before extraction (see below).

Samples from patients with MOF were taken during a weekly dialysis session, and they were collected before (t_0), after 2 h (t_{2h}) and after 24 h (t_{24h}) of hemoperfusion, and those from healthy people during a draw in the morning. Plasma was separated from blood with an ultracentrifuge and then stored refrigerated at 4 °C until use. Samples were processed and analysed within 2 days.

Before extraction of the analytes from samples, 200 μL of bacterial culture medium or plasma was spiked with ND3 internal standard (IS) to a final concentration of 200 $\mu\text{g}/\text{L}$. The internal standard was used to evaluate injection only. All of the analytical validation was done on the basis of external standard calibration. Samples were extracted twice in

Table 1 Multiple reaction monitoring (MRM), neutral loss (NL) and product ion (PI) scan parameters for AHL and HQ analysis (bolded transitions were used as quantitative ones)

	Analyte	Precursor ion $[M + H]^+$	Product ion $[M + H]^+$	Molecule family	MS mode	Δm (Da)
MRM	3-oxo-C12-AHL	298.2	102.2	AHL	NL	101.0
		298.2	197.2		PI	102.0
	C4-AHL	172.1	102.2	HQ	PI	175.0
		172.1	71.1			
	C7 HQ	260.0	188.0			
		260.0	147.0			
	ND3	203.2	102.1			
		203.2	74.1			

succession with 1 mL of ethyl acetate [39]. After each addition of organic solvent, the sample was centrifuged at $5000 \times g$ for 5 min at RT, and the organic fractions were collected and dried under a gentle stream of N_2 with heating at 40 °C. Finally, the residue was reconstituted in 100 μ L of 0.1% formic acid in water/acetonitrile 60:40.

HPLC-TQ MS method validation

The validation procedure was performed on the HPLC-TQ MS platform, according to the European Medicines Agency (EMA) and Eurachem guidelines [40, 41]. The calibration curves were run using a matrix free of quorum sensing molecules (plasma samples from healthy people) by performing the standard addition method. The absence of analytes of interest within the matrix used (QS-free matrix) for the method validation was verified through the LTQ-Orbitrap high-resolution power platform.

For method validation, different parameters were evaluated: selectivity, recovery, carry-over, intra-run accuracy and precision, limit of quantitation (LOQ), lower LOQ (LLOQ), upper LOQ (ULOQ), stability to freeze-thaw cycle and calibration model. The last one, in particular, was evaluated using a stepwise approach as described elsewhere [42], and the linearity of the calibration curves using an R routine developed by Desharnais et al. [43, 44]: Firstly, the heteroscedasticity of data points was tested by performing an *F*-test on the variance of the area ratios at the lowest and highest calibration levels. The heteroscedasticity study was also integrated with the Levene test (in the version modified by Brown and Forsythe). Then, a partial *F*-test was used to evaluate whether the calibration model followed a linear or quadratic trend [45]. The goodness of fit of the calibration model was finally evaluated by studying the normality of the standardized residuals (with the Cramer von Mises test) and by performing the back-calculation on the averaged signal from four replicates [43, 44].

For the validation of AHL molecules, C4-AHL and 3-oxo-C12-AHL were selected as molecule class representatives, whereas C7 HQ was used as a representative of HQ molecules. For all the analyses, ND3 was used as injection standard.

Selectivity (SEL%) was evaluated by comparing the chromatograms of six individual QS-free matrices and could be below 20%. Recovery (REC%) was evaluated by relating the responses of analytes in the extracted samples to those solubilized in the injection solvent. The experiments were conducted at LLOQ and ULOQ concentrations, depending on the analyte. REC% was evaluated only for the MRM approach. The carry-over effect (CO%) was studied by comparing the signal of the molecule of interest in QS-free matrix after the injection of the highest concentration point of the calibration curve. Values could be below 20% of the LLOQ

value. Accuracy (BIAS%) and precision (variation coefficient CV%) of intraday (repeatability) were calculated in QS-free matrix samples at three different concentrations carried out five times. Values could be below 15%. Limit of quantitation (LOQ) was determined by ten times the signal-to-noise ratio, expressed as the absolute value of analyte concentration. LLOQ and ULOQ were expressed as the experimentally lower and upper measured analyte concentrations, respectively. Freeze-thaw stability (STAB%) was evaluated by comparing freshly prepared processed samples frozen at -20 °C for three cycles and thawed at RT in order to determine the freeze-thaw stability of the AHL and HQ standards. The experiments were evaluated at two concentrations, LLOQ and ULOQ. STAB% ranged between 85% and 115%.

Results

Validation results

Validation results are summarized in Table 2 and presented in ESM Section 2, “Validation tables and results”. All parameters conformed to those suggested by EMA and Eurachem guidelines.

Selectivity of each MS approach was satisfactory with a value always below 20%, ranging between 0.50 and 16.5%. No isomeric or isobaric interfering compound co-eluted with the analytes, and no ion suppression was observed for the AHL and HQ standard molecules.

The recovery calculated for the MRM approach ranged between 34% and 61%. This was presumably correlated with the presence of esterase enzymes in the plasma matrices, and quorum sensing molecules, being esters, underwent partial hydrolysis. However, using the calibration curves obtained in plasma matrices, we overcame the issue.

Carry-over effect measurements demonstrated, for all MS methods, that in a QS-free matrix sample, the area under the curve (AUC) of the analytical standards was lower than 20% of the corresponding LLOQ area.

The precision and accuracy of intraday runs were below 20% for the selected curve calibration points.

LOQ values for analytes ranged between 0.09 and 0.457 μ g/L, and the MRM method showed the best LLOQ value (0.4 μ g/L).

Stability test parameters always fell within the acceptable limits, and based on the results, the average stability % value at each concentration level ranged between 85% and 117%. Therefore, the plasma matrix was similarly stable at operative condition temperatures as in freezing and thawing cycles without affecting the concentration of the analyte.

The study of the calibration model provided concordant results for all the analytes. Concerning the study of heteroscedasticity, the *F*-test and the Levene test provided

Table 2 Validation parameters for 3-oxo-C12-AHL, C4-AHL and C7 HQ obtained in MRM, NL, PI HPLC-MS approaches (NM = not measured)

Validation parameter	Conc. ($\mu\text{g/L}$)	3-oxo-C12-AHL			C4-AHL			C7 HQ	
		MRM	NL	PI	MRM	NL	PI	MRM	PI
SEL%		8.12	0.50	1.10	5.65	1.63	2.54	16.5	4.73
REC%	LLOQ	45.5	NM	NM	51.0	NM	NM	49.9	NM
	ULOQ	53.7	NM	NM	60.9	NM	NM	34.3	NM
CO%		7.10	0.57	1.29	8.90	1.65	0.87	16.1	2.98
I-R BIAS%	LLOQ	1.65	3.66	2.40	0.61	2.72	3.59	2.11	2.28
	50.0	9.88	7.77	3.05	5.32	1.84	4.32	2.27	8.68
	ULOQ	1.61	3.63	4.97	0.23	0.80	6.98	4.14	2.27
I-R CV%	LLOQ	12.4	0.87	12.9	18.4	25.0	13.7	26.5	11.9
	50.0	17.1	8.87	5.91	10.8	19.1	16.6	18.4	1.72
	ULOQ	14.3	4.98	12.0	15.6	20.9	18.1	20.7	5.84
LOQ	$\mu\text{g/L}$	0.090	0.293	0.117	0.271	0.457	0.066	0.151	0.385
LLOQ	$\mu\text{g/L}$	0.40	5.00	1.00	0.40	5.00	1.00	0.40	1.00
ULOQ	$\mu\text{g/L}$	400	400	300	400	400	300	200	300
STAB%	LLOQ	106	89.5	86.0	97.3	91.1	88.9	115	90.2
	ULOQ	101	88.6	90.2	102	86.9	95.5	117	96.2

consistent results for all but two calibration sets, namely C4-AHL-NL and C4-AHL-PI, for which the Levene test suggested a homoscedastic trend (ESM Table S5). In both cases, a more conservative approach was preferred, and the weight was applied as suggested by the routine from Desharnais. The weight for the heteroscedasticity was equal to $1/x^2$ in all cases. Furthermore, all the calibration curves were confirmed to be linear, with p values for the partial F -test above the significance limit of 0.05. The goodness of the calibration models was proved by the good results provided by the Cramer von Mises test (p values always not significant) and the back-calculation (deviations far below $\pm 25\%$ for all the models). Finally, also the R-squared values ($R^2 > 0.990$) demonstrated how our model explained all of the variation in the response variable around its mean.

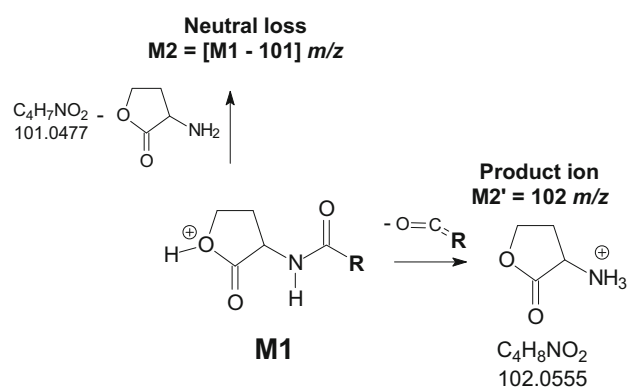
Sample results

With the development of three different MS approaches, we detected and quantified both unknown and known AHL and HQ molecules in biological samples.

For AHL unknown structures, we used the NL and PI approaches. In NL experiments, precursor and product ions were monitored for the loss of 101 Da which corresponds to 2-amino- γ butyrolactone. In PI mode, Q3 was set to detect only m/z 102 corresponding to the elemental composition of protonated 2-amino- γ butyrolactone (Fig. 3).

For new molecules belonging to the HQ family, we used a PI methodology selecting as product ion the protonated radical ion 2-methyl-3-hydroxyquinolin-4-one with an m/z ratio of 175 (Fig. 3).

AHL



HQ

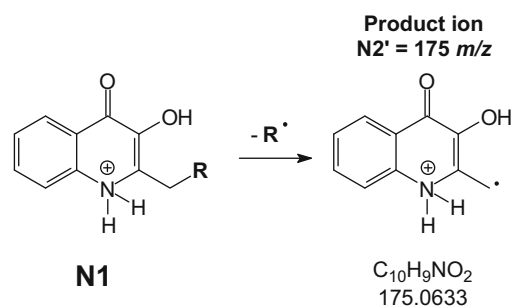


Fig. 3 TQ MS/MS selective acquisition modes. M1 is a generic structural formula of a protonated molecule belonging to the N-acyl homoserine lactones (AHLs) family. R varies between 4 and 16 carbon atoms. M2 is the product ion formed after the neutral loss of a 2-amino- γ butyrolactone molecule (MW 101 Da). M2' is the product ion with m/z 102. Analogously, N1 is a general structural formula of compounds belonging to the hydroxyquinolone signalling molecule (HQ) family. R varies between 7 and 11 carbon atoms. N2' is the product ion with m/z 175

Unknown AHL and HQ signalling molecules, identified and characterized in an untargeted approach using NL and PI modes, were confirmed by the use of the HRMS tool (LTQ-Orbitrap), and to better describe them we studied the fragmentation pathways of analytical standards (see “Discussion” section).

Two unknown AHL species were detected both in *P. aeruginosa* bacterial cultures and patients’ plasma samples: 3-oxo-C10-AHL ($C_{14}H_{23}NO_4$, m/z 270.1705) and C12-AHL ($C_{16}H_{30}NO_3$, m/z 284.2226). One other, C6-AHL ($C_{10}H_{17}NO_3$, m/z 200.1287), was present in bacterial cultures only (see ESM for chemical and structural formulas, Table S3).

In the bacterial cultures, 13 unknown HQ molecules were identified by the PI approach and confirmed by HRMS analysis: C2-HQ ($C_{11}H_{11}NO_2$, m/z 190.0868), C3-HQ ($C_{12}H_{13}NO_2$, m/z 204.1024), C4-HQ ($C_{13}H_{15}NO_2$, m/z 218.1181), C5-HQ ($C_{14}H_{17}NO_2$, m/z 232.1337), C6-HQ ($C_{15}H_{19}NO_2$, m/z 246.1494), C6:1-HQ ($C_{15}H_{17}NO_2$, m/z 244.1337), C7:1-HQ ($C_{16}H_{19}NO_2$, m/z 258.1494), C8-HQ ($C_{17}H_{23}NO_2$, m/z 274.1807), C8:1-HQ ($C_{17}H_{21}NO_2$, m/z 272.1650), C9-HQ ($C_{18}H_{25}NO_2$, m/z 288.1936), C9:1-HQ ($C_{18}H_{23}NO_2$, m/z 286.1807), C11-HQ ($C_{20}H_{29}NO_2$, m/z 316.2276) and C11:1-HQ ($C_{20}H_{27}NO_2$, m/z 314.2120) (see ESM for chemical and structural formulas, Table S4).

The detected compounds were then semi-quantified in bacterial cultures using the validated calibration curves of C4-AHL and 3-oxo-C12-AHL, depending on chain length and on hydroxylation grade, to quantify AHL molecules, and of C7 HQ to quantify HQ compounds. For the quantification of both AHL and HQ molecules, we used the calibration curves obtained with the PI MS methods because of their lower LLOQ (1 $\mu\text{g/L}$ for PI vs. 5 $\mu\text{g/L}$ for NL). The results are listed in Tables 3 (AHL) and 4 (HQ).

Finally, the known analytes (C4-AHL, 3-oxo-C12-AHL and C7 HQ) were quantified in bacterial cultures and patients’ plasma samples with the validated HPLC-TQ MRM MS method, and the results are shown in Tables 5 and 6.

Table 3 *Pseudomonas aeruginosa* culture concentrations (expressed in $\mu\text{g/L}$) of the unknown AHL molecules semi-quantified using the linear equation of the PI validated MS approach

Concentration ($\mu\text{g/L}$)			
	C6-AHL	3-oxo-C10-AHL	C12-AHL
WT-LB	6.57	0.62	0.13
WT-M9	ND	0.72	0.70
RhlI-LB	ND	0.52	ND
RhlI-M9	ND	2.89	ND

WT wild type, RhlI mutant bacterium, M9 mineral medium, LB Luria-Bertani broth, ND not detectable (<LLOQ).

Table 4 *Pseudomonas aeruginosa* culture concentrations (expressed in $\mu\text{g/L}$) of the unknown HQ molecules semi-quantified using the linear equation of the PI validated MS approach

Concentration ($\mu\text{g/L}$)						
	C2 HQ	C3 HQ	C4 HQ	C5 HQ	C6 HQ	C6:1 HQ
WT-LB	ND	0.76	3.96	16.11	38.54	0.33
WT-M9	ND	ND	ND	ND	ND	ND
RhlI-LB	11.15	98.76	33.14	232.45	145.45	17.02
RhlI-M9	ND	ND	ND	ND	ND	ND
	C7:1 HQ	C8 HQ	C8:1 HQ	C9 HQ	C9:1 HQ	C11 HQ
WT-LB	24.07	81.44	1.93	182.57	26.98	1.57
WT-M9	ND	ND	ND	ND	ND	ND
RhlI-LB	2.00	45.75	3.61	12.46	7.40	ND
RhlI-M9	ND	ND	ND	ND	ND	ND
	C11:1 HQ					
WT-LB	28.60					
WT-M9	ND					
RhlI-LB	1.48					
RhlI-M9	ND					

WT wild type, RhlI mutant bacterium, M9 mineral medium, LB Luria-Bertani broth, ND not detectable (<LLOQ)

Discussion

Method validation discussion

The three acquisition techniques showed different LLOQ values and linearity ranges, and MRM was the one which allowed the wider calibration range, with an LLOQ equal to 0.4 $\mu\text{g/L}$ (for PI and NL, LLOQ was 1 $\mu\text{g/L}$ and 5 $\mu\text{g/L}$, respectively). It is known that the MRM approach has a better sensitivity with respect to NL and PI [46], moreover balanced by the better diagnostic possibilities of these different approaches.

Independently from the MS/MS, the class of the targeted molecule and the calibration range, the heteroscedasticity tests showed concordant results. Both the *F*-test and the Levene test on the variance of the calibration replicates demonstrated the need for a $1/x^2$ weight. The only exceptions are the NL and PI experiments performed for C4-AHL, for which the Levene

Table 5 Analyte concentrations ($\mu\text{g/L}$) measured in bacterial cultures of *Pseudomonas aeruginosa* with the MRM approach

Concentration ($\mu\text{g/L}$)			
	C4-AHL	3-oxo-C12-AHL	C7 HQ
WT-LB	5.36	3.66	357
WT-M9	nd	2.07	nd
RhlI-LB	nd	2.17	736
RhlI-M9	nd	1.16	nd

Table 6 Analyte concentrations ($\mu\text{g/L}$) measured in plasma samples of patients with multi-organ failure (MOF) pathology with the MRM approach

Concentration ($\mu\text{g/L}$)						
	MOF#1			MOF#2		
	C4-AHL	3-oxo-C12-AHL	C7 HQ	C4-AHL	3-oxo-C12-AHL	C7 HQ
t_0	6.90	0.52	14.1	4.70	0.51	3.21
t_{2h}	6.40	nd	10.1	3.70	0.55	4.17
t_{24h}	6.10	nd	5.70	3.80	0.49	1.99
	MOF#3			MOF#4		
	C4-AHL	3-oxo-C12-AHL	C7 HQ	C4-AHL	3-oxo-C12-AHL	C7 HQ
t_0	5.40	0.56	2.85	5.80	0.56	2.85
t_{2h}	5.00	0.54	2.75	5.20	0.55	2.75
t_{24h}	4.60	0.51	2.50	5.70	0.52	2.50
	MOF#5			MOF#6		
	C4-AHL	3-oxo-C12-AHL	C7 HQ	C4-AHL	3-oxo-C12-AHL	C7 HQ
t_0	6.70	0.56	17.2	5.10	0.55	4.52
t_{2h}	6.00	0.53	12.1	4.00	0.51	3.96
t_{24h}	5.90	0.52	5.09	5.50	nd	4.16

test suggested a homoscedastic trend. This was discordant with the t -test output and also with the visual inspection of the replicates, evidencing a progressively greater variance for replicates of the higher calibration levels. Hence, a more conservative approach was preferred and a weight applied. Furthermore, the verification of the linearity trend by means of a partial F -test confirmed the linearity within the selected calibration range, with all the p values above the cut-off limit of 0.05. The goodness of the selected calibration model was finally verified by high p values for the study of the normality of the standardized residuals and the back-calculation at all the calibration levels (ESM Tables S5 and S6).

Sample results discussion

Before starting with the identification and characterization of unknown AHL and HQ molecules (presented in the section “Sample results” and in ESM Tables S1 and S2), an investigation using HRMS of fragmentation pathways of analytical standards was carried out. As representatives of the AHL class, we selected 3-oxo-C12-AHL and C4-AHL, and C7 HQ from the HQ family. ND3 as previously described was used as IS. CID MS² fragmentation schemes of C4-AHL, 3-oxo-C12-AHL, C7 HQ and ND3 are presented in Fig. 4. As mentioned before, AHL molecules shared the product ion m/z 102, and C7 HQ showed the peculiar product radical ion m/z 175 common for the HQ family.

Since the analysis of bacterial cultures and plasma samples of patients were completed only 2 days after sampling and handling, and samples were stored refrigerated at $-4\text{ }^\circ\text{C}$, no other pathogens should develop [33]. In this situation, the unknown AHLs and HQs detected with the developed MS

methods resulted from *P. aeruginosa* bacteria or patients with sepsis.

The chromatographic runs proposed here for AHL molecules were much longer if compared with other analytical methods. Ortoni and co-workers [27] used a 2.9-min-long separation run to quantify 26 AHLs in bacterial cultures. Readell et al. [36], with an acetonitrile isocratic run of 10 min, successfully separated six racemic L/D homoserine lactones. Struss et al. [29] and Hoang et al. [35] developed a 17-min reversed-phase HPLC method to quantify AHL in sputum of cystic fibrosis patients and bacterial cultures, respectively. In particular, the group led by Hoang [35] presented a green analytical method with the use of supercritical-fluid chromatography (SFC), and performed an in-depth optimization of the method. Unfortunately, SFC-TOF (Time of Flight) is quite an expensive technology and requires a very skilled operator. Although these methods showed a very low limit of quantitation (pg/mL), with good selectivity and robustness, they quantified well-known molecules belonging to the AHL family (using analytical standards) [27, 35, 36] or a limited number of analytes [29].

When the number of molecules increases and, more significantly, when unknown compounds should be identified and characterized, the duration of the separation run is increased in order to avoid analyte co-elution or ion suppression. The developed chromatographic run lasted 35 min, which is comparable to that proposed by Kumari et al. [24] for the detection of ten AHLs. An untargeted high-resolution mass spectrometry method for the identification of novel AHLs in eight different bacterial cultures grown under different conditions was proposed by Patel et al. [32]. They used a C18 column and a Q Exactive high-resolution mass spectrometer, and within

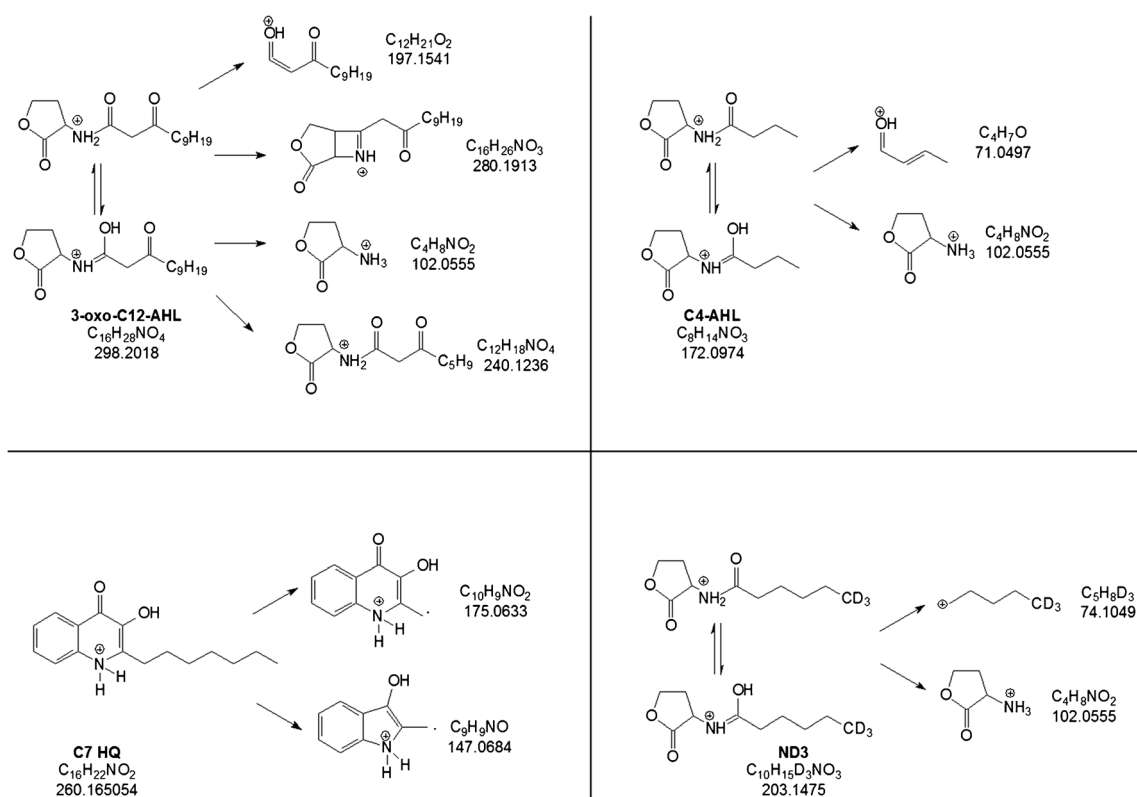


Fig. 4 MS² fragmentation pathways of N-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-AHL), N-butanoyl-L-homoserine lactone (C4-AHL), 2-heptyl-3-hydroxy-4(1H)-quinolone (C7 HQ) and N-

hexanoyl-L-homoserine lactone-D3 (ND3). Product ion m/z 102 and product radical ion m/z 175 are the characteristic fragmentation products of molecules belonging to AHL and HQ families, respectively

13 min they detected 23 AHLs, some of them never described in the literature. Their method showed little overlapping and tailing of chromatographic peaks; nevertheless, they achieved a very low limit of detection for all analytes and performed the identification of unexpected AHL molecules using a labelled approach. As with our method, the MS/MS method for the identification of AHLs was based on a peculiar product ion with m/z 102.

The HQ separation run was shorter than that for AHLs, and could be compared to those developed by Maurer et al. [28] and Brewer et al. [34]. It lasted 12 min (Maurer 9' and Brewer 8'), and the separation obtained with the use of 2-picolinic acid was satisfactory (ESM Fig. S2). Although Maurer and co-workers [28] used a mixture of acetic anhydride-pyridine to derivatize C7-HQ, and the Brewer team [34] employed EDTA as mobile aqueous phase, we preferred to follow the Turpenney recommendation [33] in order to obtain a good shape of chromatographic peaks and consequently a possibly more accurate quantification of the HQ compounds. Therefore, we ran the chromatographic separation with the use of 2-picolinic acid. Indeed, the EDTA molecule is not suggested for MS analysis, since it is a non-volatile salt and gives an ion suppression effect, and derivatization is not a quantitative operation. On the contrary, 2-picolinic acid is highly volatile and a bidentate chelating agent, and as

Turpenney noted, the use of formic acid together with 2-picolinic acid improved the ESI positive ionization of the studied molecules [33].

As previously presented, we were able to detect and identify 13 HQs in bacterial cultures and plasma samples. Among all the possible QS molecules, the most highly expressed (high concentration) was the C7 HQ. In minimal medium poor in nutrients (W9), the C7 HQ species was the only one detected in both the wild-type and the RhlI derivative bacterial cultures. This finding supports the hypothesis that the knockout mutant, unable to produce C4-AHL, intensified the production of this signal molecule to promote bacterial cell growth. As for AHL compounds, cells grown in LB broth showed the most abundant content of HQ molecules with respect to M9 medium. The detection of HQ molecules in biological samples is very important because their concentration is related to the expression of many virulence factors and to the regulation of iron usage inside the cells [47].

In addition to the importance of a good chromatographic separation, the developed NL and PI MS approaches also played a fundamental role in the identification of unknown compounds. The unknown species C6-AHL was detected in bacterial cultures only, with an empirical formula of C₁₀H₁₇NO₃, and accurate m/z of 200.1287 (confirmed with the LTQ-Orbitrap system) is in agreement with Alayande et al.

[48]. The authors grew *Pseudomonas aeruginosa* PAO1 in LB medium and evaluated the levels of AHL by LC-MS/MS analysis. This work is among the very few on *P. aeruginosa* that showed the detection of C6-AHL signal molecule production, and the only report where C6-AHL was detected in an amount greater than that of 3-oxo-C12-AHL. They found for the first time that among the various signal molecules, C4-AHL and C6-AHL played the most important signalling role in the QS system of *P. aeruginosa* PAO1. Moreover, the search for C6-AHL showed the presence of three chromatographic peaks presumably corresponding to three isomers. Even the known C4-AHL molecule ($C_8H_{13}NO_3$, m/z 172.0974) was detectable as two different isomers when monitored using the NL approach. However, the ability to discriminate between the isomers would require a more detailed investigation using for example an NMR technique to obtain structural information. The analysis of HQ molecules did not show any isomeric species.

Also, 3-oxo-C10-AHL was recognized in bacterial cultures, and its amount was rather high, especially in RhII-W9 samples. This indicates that in the absence of C4-AHL, bacteria can use this other molecule to communicate. Similar results were obtained by Patel et al. [32] in *Erwinia carotovora* culture grown in nutrient-rich medium.

Last but not least, we quantified C12-AHL in bacterial samples, to our knowledge for the first time. In fact, other research groups discovered novel AHL molecules in bacterial cultures [27, 32, 35]. For example, Patel and co-workers [32] measured 24 AHLs in eight bacterial cultures using a high-resolution MS approach; they recognized C12-AHL in others cultures, but not in *Pseudomonas aeruginosa*. The same was found for the study by Hoang et al. [35] that quantified C12-AHL in the Gram-negative endophytic bacterium *Paraburkholderia* spp. As previously underlined, C7 HQ, 3-oxo-C10-AHL and finally, C12-AHL could be the alternative controlled factors expressed by mutant *P. aeruginosa* bacteria responsible for their high tolerance to photodynamic therapy and photo-oxidative stress induced by the therapy. These results lay the foundations for more in-depth studies on the *lasI* and the *rhII* QS signal systems.

An accurate quantitation of the molecules was achieved for bacterial cultures WT-W9, WT-LB, RhII-W9 and RhII-LB, and for human plasma samples using the NL and PI validated MS method. We decided to validate the analytical method in plasma QS-free matrix (belonging to healthy volunteers) because the ultimate goal of the study was to quantify QS molecules in plasma of patients affected by MOF. Many other research groups have validated their analytical approaches in bacterial cultures [28, 34, 35]. A comprehensive method for QS compound quantitation in human samples is lacking [23–25, 29]. Furthermore, we studied the *Pseudomonas aeruginosa* QS molecule production because this is one of the most common bacteria found to infect patients with sepsis

[49, 50]. Data shown in Table 6 indicate that C4-AHL was detectable in all the samples within a concentration range of 4–7 $\mu\text{g/L}$, 3-oxo-C12-AHL in a range of 0–0.6 $\mu\text{g/L}$, and finally, C7-HQ in a range between 2 and 18 $\mu\text{g/L}$. A general tendency toward a decrease in QS molecule concentration was evidenced, showing that hemoperfusion of plasma of patients with MOF was effective in the removal of QS compounds. The monitoring of QS analytical standard molecules in hemoperfused plasma samples from patients with MOF confirmed the ability of the MRM approach to quantify virulence factors during sepsis with good sensitivity.

Conclusions

In conclusion, selective, sensitive and robust HPLC-TQ MS methods were developed and applied to wild-type and mutant *P. aeruginosa* bacterial cultures and to biological plasma samples. Thanks to untargeted NL and PI and targeted MRM methods, different AHL and HQ molecules were identified, characterized and quantified.

The HPLC-TQ MS/MS analytical procedure was validated in MRM, NL and PI scan mode. Experimental data indicated that the method was suitable for the detection of low concentrations of AHLs and HQs in bacterial cultures of *Pseudomonas aeruginosa* and biofluids in early stages of sepsis-related multi-organ failure. Using the NL and PI scan methods, it was possible to discover a new kind of species whose presence within the sample was not predictable at the beginning of the analysis. The presence of high concentrations of C7 HQ, 3-oxo-C10-AHL and C12-AHL in mutant strains of *P. aeruginosa* knocked for the production of C4-AHL could be the starting point for a better understanding of how bacteria exploit the controlled factors to survive and proliferate. The MRM approach was suitable for the detection of low AHL and HQ levels within less abundant samples. The comparison between bacterial cultures and plasma samples highlighted even more why high-sensitivity methods are essential for plasma, given the concentration of a few micrograms per litre of the molecules of interest within the sample.

The three validated approaches were demonstrated to be reproducible, repeatable, robust and sensitive enough to quantify QS in real biological samples and, we think, to have evidenced the possibility of applying them for solving practical analytical problems with known limitations of sensitivity. The LTQ-Orbitrap-HRMS platform has been confirmed as an indispensable tool for investigating the fragmentation pathways and confirming the detection of unexpected QS molecules.

In an on-going study in patients with MOF, we are demonstrating the ability of hemoperfusion to reduce the QS amount, underlining the physiopathological implications of these findings. Here we have presented the usefulness of the studied LC-MS approaches and applied them to real samples.

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Availability of data and material The data reported in this manuscript that support the findings of this study are available on request from the corresponding author on reasonable request. The data are not publicly available as they contain patients' personal information that could compromise their privacy.

Compliance with ethical standards

Disclosure of potential conflicts of interest The authors declare that they have no conflict of interest.

Research involving human participants and/or animals As assurance that ethical and professional principles were followed in each step of the present work, the research was approved by the proper university human research ethics committee (protocol number 0114479 approved 29th November 2017 at AOU Città della Salute e della Scienza di Torino). All procedures described in studies were in accordance with the ethical standards of our institutional and/or national research committee and with the Code of Ethics of the World Medical Association (1964 Helsinki declaration) and its later amendments or comparable ethical standards.

Informed consent All healthy volunteers and patients who took part in the study gave their written informed consent before participation. All studies were performed in accordance with the ethical standards provided in the Declaration of Helsinki. The ethical approval was granted by the Research Ethical Committee (protocol number 0114479 approved the 29th of November 2017 at AOU Città della Salute e della Scienza di Torino).

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