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(Article begins on next page)

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Targeted and untargeted quantification of quorum sensing signaling 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 signaling 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 in biofluids of QS signaling molecules like N-acyl homoserine lactones (AHL) and hydroxyquinolones (HQ). Biological samples used in the study were *Pseudomonas aeruginosa* bacterial cultures and plasma from septic patients. 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. Then we settled 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 accordance with the validation guidelines. With the NL and PI MS based methods we identify and characterize in biological samples 3 and 13 unknown AHL and HQ compounds respectively. One of the new-found AHL molecules was C12-AHL firstly quantified in *Pseudomonas aeruginosa* bacterial cultures. The MRM quantitation of analytes in plasma from septic patients confirmed the ability of MRM analysis to quantify virulence factors during sepsis.

Keywords

Quorum sensing molecules, Homoserine lactones, Hydroxyquinolones, *Pseudomonas aeruginosa*, Mass Spectrometry, Triple Quadrupole

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-TQ MS: High Performance Liquid Chromatography- Triple Quadrupole Mass Spectrometry

HRMS: High Resolution Mass Spetrometry

HQs: Hydroxyquinolone signaling 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

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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

1. Introduction

Bacteria have the ability to interact each other through a complex language called "Quorum Sensing" (QS) [1-3]. Literally, QS means "detection of the quorum" and it is referred to the ability of bacteria to monitor their density of population 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 extra cellular environment. When many AIs are produced and stored, and their concentration gains a threshold level (the quorum), the bacterial population is able to activate or to repress target gene [7]. This mechanism allows the survival of 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 reached high density population [8, 9].

Gram-positive and Gram-negative bacteria use different communication-ways [10]. Gram-positive bacteria produce oligopeptides as QS autoinducers 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 α position and an acyl-chain (indicated as R-chain in Fig.1). Chain length is the signal specificity factor for bacteria and, generally, the chain contains between 4 and 16 carbon atoms. Furthermore, the presence of an -oxo or a -hydroxy group linked to the 3rd carbon atom of the chain is a further element of distinction.

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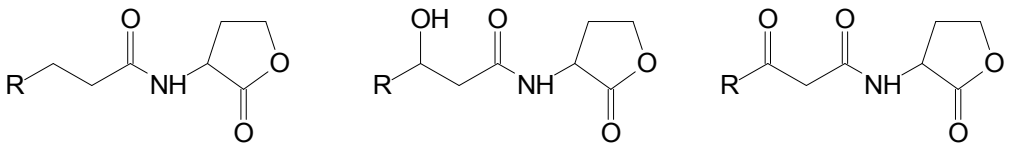

Figure 1: N-acyl homoserine lactone generic structure. A: Non-substituted N-acyl homoserine-L-lactone (Cn-HSL) acyl chain; B: N-(3-Hydroxyacylhomoserine)-L-lactone (3-OH-Cn-HSL) acyl chain; C: N-(3-Oxoacylhomoserine)-L-lactone (3-oxo- Cn-HSL) acyl chain. The length is variable, generally n=4-14

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

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 QS signaling compound [18, 19]. The basic structure of quinolone molecules consists in a bicyclic ring. The length of the acyl-chain (marked as R-chain in Fig.2) ranges between 7 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.

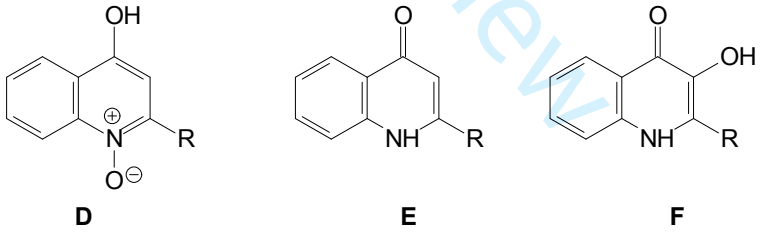

Figure 2: Quinolone signaling molecules generic structure. D: 2-alkyl-4-hydroxyquinolone N-oxide with a chain length variable (C7-C9); E: 2-alkyl-4(1H)-quinolone alkyl chain (the length chain is variable, C7-C11); F: 2-alkyl-3-hydroxy-4(1H)-quinolone.

As described, AHLs and HQs are the most abundant QS signaling molecules produced by Gram negative bacteria. Many studies are present in literature with the aim to describe and quantify well-known or new QS molecules in bacterial culture or human samples [20-35]. There are two main methods to obtain the measurements: biosensors [21-23, 26, 30, 31] and methods

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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 one single QS (AHL or HQ). However, they miss unknown molecules and the quantitation shows low accuracy [32]. On the contrary, mass spectrometry based methods, both targeted and untargeted, offer accurate quantitation of AHL and HQ compounds. During the last years, many LC-MS methods were developed exploiting several stationary and mobile phases, and both targeted and untargeted MS approaches. Some studies showed a very low limit of quantitation (pg/mL) and a good selectivity optimizing a short liquid chromatography separation both for AHLs, furthermore enantiomeric, and for HQs [27-29, 33-36]. Other research groups have been developed extended chromatography separation runs to provide the quantitation of a higher number of molecules [24] or to identify new AIs molecules with untargeted high resolution MS approach [32].

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

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 AHLs and HQs signaling molecules, the aim of the research was the development of MS analytical method based on neutral loss (NL) and product ion (PI) experiments. A tandem mass method based on 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 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 if other QS molecules were involved in protection mechanism used by bacteria. Secondly, the MRM method was applied to pathological plasma samples (before and after hemoperfusion) of patients affected by sepsis-related multi-organ failure (MOF) pathology, which is associated with high mortality. To our knowledge this

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study is the first that provide a tandem/mass spectrometry quantitation both of AHLs and HQs in plasma samples coming from patients with sepsis.

2. Materials and methods

2.1. 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 (Rome, Italy). Stock solutions were prepared with a concentration of 1000 mg/L using methanol and stored at -4 °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 MilliQ System Academic (Millipore, Milan, Italy). Ethyl acetate for HPLC-MS grade, acetonitrile and methanol hyper grade for LC-MS, and formic acid were purchased from VWR International (Milan, Italy).

2.2. Instrumentation

Separation and analysis of all analytes and samples were achieved upon a 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 V™ Source (ESI mode) which utilized nitrogen and air as sheath and reagent gas, respectively. Furthermore, to verify the exact mass values, we used the high resolution mass spectrometer LTQ-Orbitrap (Thermo Scientific, Bremen, Germany) within mass accuracy of ± 3 ppm and resolution of 30k.

2.3. HPLC parameters

To investigate AHL and HQ signaling 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 reverse-phase (150 × 2.1 mm i.d., 3 µm particles). Sample injection volume was 10 µL. Autosampler and oven temperatures were set at 15 °C and 45 °C respectively for all the duration of the 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 µL/min. For bacterial cultures, the mixture percentage changed from 40% solvent B to 100% solvent B during the first 35 min,

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maintained for 10 minutes and then the column went back to the starting conditions. For plasma sample, HPLC run started from 40% solvent B, increased to 100% solvent B in 19 min, maintained for 10 minutes and then the column went back to the starting conditions. Bacterial cultures were more complex than plasma samples and required to obtain a satisfactory chromatographic separation a 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 went back to the starting conditions. Flow rate was 250 µL/min.

2.4. MS settings

The LC effluent was delivered to Turbo V™ Source (ESI positive ionization mode) using nitrogen as 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 AHLs analysis, and MRM-PI for HQs analysis. The MRM, NL and PI parameters were listed in table 1. For MRM acquisition we selected one qualitative and one another quantitative (bold in Table 1) transition for each analyte. The instrument parameters were listed in ESM Tables S1 and S2.

	Analyte	Precursor ion $[M+H]^+$	Product Ion $[M+H]^+$
MRM	3-oxo-C12-AHL	**298.2**	**102.2**
		298.2	197.2
	C4-AHL	**172.1**	**102.2**
		172.1	71.1
	C7 HQ	**260.0**	**188.0**
		260.0	147.0
	ND3	**203.2**	**102.1**

Molecule family	MS Mode	Δm (Da)
AHL	*NL*	101.0
	PI	102.0
HQ	*PI*	175.0

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	203.2	74.1

Table 1: Multiple Reaction Monitoring (MRM), Neutral Loss (NL) and Product Ion (PI) scan parameters for AHLs and HQs analysis (bolded transitions were used as quantitative ones).

2.5. *Bacterial cultures and biological samples*

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

Different *Pseudomonas aeruginosa* PAO1 and RhlI 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) RhlI- mutant bacterial culture grown in the LB broth, RhlI-LB; and IV) RhlI- mutant bacterial culture grown in the M9 culture, RhlI-M9.

For the application of the MRM-NL-PI methods developed, we analysed plasma samples belonging to 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 research upon their biological fluids, freely deciding whether to accept it or not. All the procedures followed in the work have been 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).

2.6. *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 RT and then the supernatants were collected and filtered (Minisart RC15 Ø 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 hours (t_{2h}) and 24 hours (t_{24h}) of hemoperfusion, and from healthy people during a draw in the morning. Plasma was separated from blood with an ultra-centrifugation and then stored refrigerated at 4 °C until use. Samples were processed and analysed within two days.

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Before extracting the analytes from samples, 200 µL of bacterial cultures medium or plasma were spiked with ND3 internal standard (IS) with a final concentration of 200 µg/L. The internal standard was used to evaluate injection only. All of the analytical validation was done on the bases of external standard calibration. Successively, samples were extracted twice with 1 mL of ethyl acetate [39]. After each addition of organic solvent, the sample was centrifuged at 5,000 *g* × 5 min RT and the organic fractions were collected and dried under a gentle stream of N_2 heating at 40° C. Finally, the residue was reconstituted in 100 µL of 0.1% formic acid in water/acetonitrile 60:40.

2.7. HPLC-TQ MS methods validation

The validation procedure was performed upon the HPLC-TQ MS platform, according to the European Medicine Agency (EMA) and Eurachem guidelines [40, 41]. The calibration curves were run using a matrix free from quorum sensing molecules (plasma samples from healthy people) by performing standard addition method. The absence of analytes of interest within the matrix used (QS-free matrix) for the methods validation was verified through LTQ-Orbitrap high resolving 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 schematized elsewhere [42] and linearity of calibration curves using a R routine developed by Desharnais *et al.* [43, 44]: firstly, the heteroscedasticity of data points was tested 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 if the calibration model follows a linear or quadratic trend [45]. The goodness 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 the four replicates [43, 44].

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

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Selectivity (SEL%) was evaluated by comparing the chromatogram 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 with those solubilized in solvent of injection. The experiments were conducted at LLOQ and ULOQ concentration, depending on 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 LLOQ value. Accuracy (BIAS%) and precision (variation coefficient CV%) of intraday (repeatability) were calculated in QS-free matrix samples at three different concentration repeated 5 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. Lower LOQ (LLOQ) and Upper LOQ (ULOQ) were expressed as experimentally lower and upper measured analyte concentration. Freeze-thaw stability (STAB%) was valued by comparing freshly prepared processed samples freezing at -20 °C for three cycles and thawing at RM in order to depict freeze-thaw stability of the AHLs and HQs standards. The experiments were evaluated at two concentrations, LLOQ and ULOQ. STAB% could be comprised between 85% and 115%.

3. Results

3.1. Validation results

Validation results were summarized in table 2 and presented in ESM Section "2. Validation tables and results". All parameters were conformed with those suggested by EMA and Eurachem guidelines.

Validation parameter	Conc. (µg/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	n.m.	n.m.	51.0	n.m.	n.m.	49.9	n.m.
	ULOQ	53.7	n.m.	n.m.	60.9	n.m.	n.m.	34.3	n.m.
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

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	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	µg/L	0.090	0.293	0.117	0.271	0.457	0.066	0.151	0.385
LLOQ	µg/L	0.40	5.00	1.00	0.40	5.00	1.00	0.40	1.00
ULOQ	µg/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

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

Selectivity of each MS approach was satisfactory with a value always below 20%, comprised 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 AHLs and HQ standard molecules.

The recovery calculated for MRM approach ranged between 34% and 61%. These was presumably correlated with the presence of esterase enzymes in the plasma matrices and quorum sensing molecules, being esters, underwent to a 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 QS-free matrix sample the analytical standards area under the curve (AUC) was lower than 20% of the corresponding LLOQ area.

Precision and accuracy of intra-day run were below 20% for the selected curve calibration points.

LOQ values for analytes ranged between 0.09 to 0.457 µg/L, respectively and the MRM method showed the best value of LLOQ (0.4 µg/L).

Stability test parameters had always fell within the acceptable limits and based on the results, average stability % value at each concentration level was in the range between 85% and 117%. Therefore, plasma matrix was stable as at operative conditions temperatures as to 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 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 the cases, a more

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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 ±25% for all the models). Finally, also the R-squared values (R^2> 0.990) demostrated how our model explained all of the variation in the response variable around its mean.

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3.2. Samples results

With the development of three different MS approaches, we detected and quantified both unknown and known AHLs and HQs 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-gamma butyrolactone. In PI mode, Q3 was set to detect only *m/z* 102 corresponding to the elemental composition of protonated 2-amino-gamma butyrolactone (Fig. 3).

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

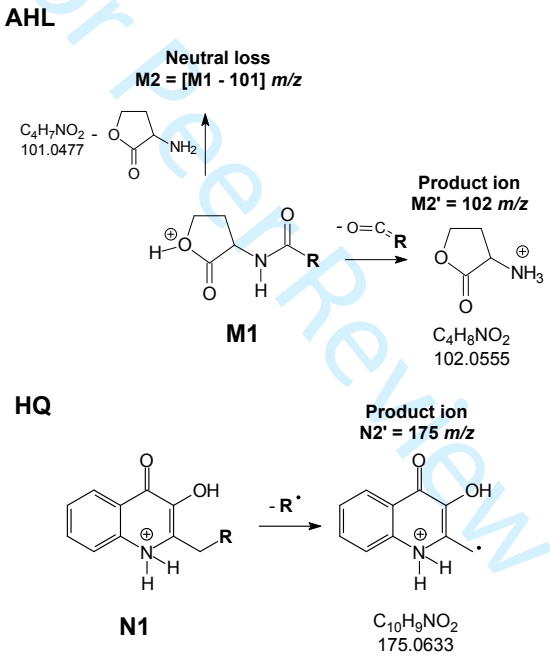

Figure 3: TQ MS MS selective acquisition modes. M1 is a generic structural formula of a protonated molecule belonging to N-acyl homoserine lactones (AHLs) family. R varies between 4 and 16 carbon atoms. M2 is the product ion formed after the neutral loss of a 2-amino-gamma butyrolactone molecule (MW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

Unknown AHL and HQ signaling molecules, identified and characterized in an untargeted approaches using NL and PI modes, were confirmed by the use of HRMS tool (LTQ

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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 identify by 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 AHLs molecules, and of C7 HQ to quantify HQs compounds. For both quantifications of AHLs and HQs molecules we used the calibration curves obtained with PI MS methods because of their lower LLOQ (1 µg/L for PI vs. 5 µg/L for NL). The results were listed in Tables 3 (AHL) and 4 (HQ).

	Concentration (µg/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

Table 3: *Pseudomonas aeruginosa* cultures concentrations (expressed in µg/L) of the unknown AHL molecules semi-quantified using linear equation of PI validated MS approach. WT: wilde type; RhlI: mutant bacterium; M9: mineral medium; LB: Luria-Bertani broth; nd: not detectable (<LLOQ).

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	Concentration (µg/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					

Table 4: *Pseudomonas aeruginosa* cultures concentrations (expressed in µg/L) of the unknown HQ molecules semi-quantified using linear equation of PI validated MS approach. WT: wilde type; RhlI: mutant bacterium; M9: mineral medium; LB: Luria-Bertani broth; nd: not detectable (<LLOQ).

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.

	Concentration (µg/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 5: Analytes concentration (µg/L) measured in bacterial cultures of *Pseudomonas aeruginosa* with MRM approach.

	Concentration (µg/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

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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

Table 6: Analytes concentration (µg/L) measured in plasma samples of patients with multi-organ failure (MOF) pathology with MRM approach.

4. Discussion

4.1. Method validation discussion

The three acquiring techniques showed different LLOQ values and linearity ranges and MRM was the one which allowed the wider calibration range, with a LLOQ equal to 0.4 µg/L (for PI and NL LLOQ was 1 µg/L and 5 µg/L, respectively). It is known that the MRM approach has a better sensitivity 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 of a $1/x^2$ weight. The only exceptions are the NL and PI experiments performed for C4-AHL, for which the Levene test suggested a homoscedastic trend. This was discordant with the T-test output and also with the visual inspection of the replicates, by which it is evident 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 values of the p-value for the study of the standardised residuals normality and the back calculation at all the calibration levels (ESM Tables S5 and S6).

4.2. Sample results discussion

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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 AHLs class we selected 3-oxo-C12-AHL and C4-AHL, and C7 HQ from HQs family. ND3 as previously described was used as IS. CID MS^2 fragmentation schemes of C4-AHL, 3-oxo-C12-AHL, C7 HQ and ND3 are presented in Figure 4. As mentioned before, AHLs 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.

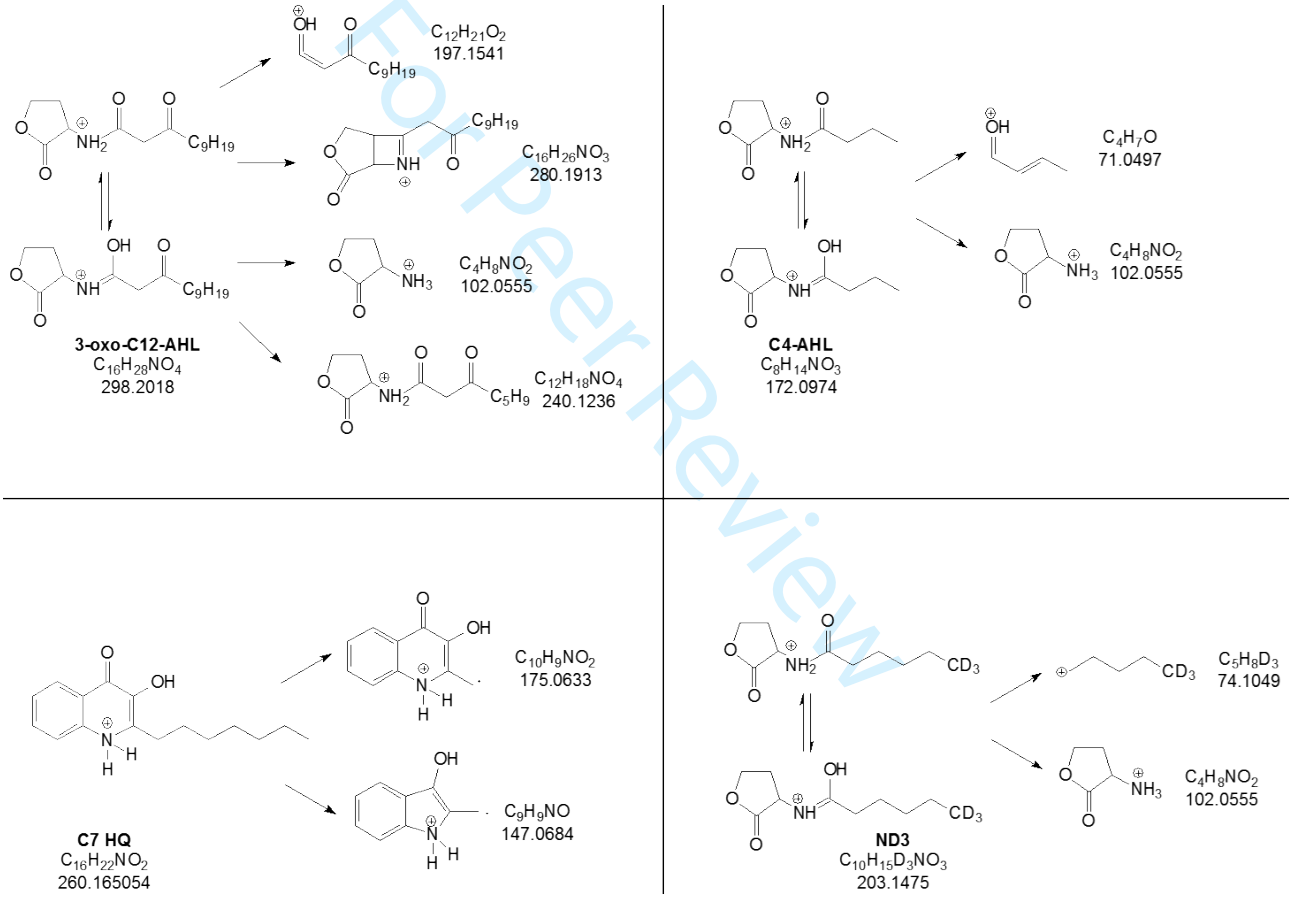

Figure 4: MS^2 fragmentation pathways of, N-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-AHL), N-butanoyl-L-homoserine lactone (C4-AHL), 2-heptyl-3-hydroxy-4(1H)-quinolone (C7 HQ) and N-hexanoyl-L-homoserine lactone-D3 (ND3). Product ion *m/z* 102 and product radical ion *m/z* 175 are the characteristic fragmentation products of molecules belonged to AHLs and HQs family respectively.

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Since the analysis of bacterial cultures and plasma of patients samples were completed only two days after sampling and handling, and samples were stored refrigerated at -4 °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 here proposed for AHL molecules were quite longer if compared with others analytical methods. Ortoni and co-workers [27] used a 2.9 minutes long separation run to quantify 26 AHLs in bacterial cultures. Readel *et al.* [36] with an acetonitrile isocratic run of 10 minutes successfully separated six racemic L/D homoserine lactones. Struss [29] and Hoang [35] teams developed a 17 minutes reverse phase HPLC methods to quantify AHL in sputum of cystic fibrosis patients and bacterial cultures, respectively. In particular, Hoang group [35] presented a green analytical method with the use of supercritical-fluid chromatography (SFC); they performed a profound optimization of the method. Unfortunately, SFC-TOF is quite expensive technology and requires very skilled operator. Although these methods showed very low limit of quantitation (pg/mL), good selectivity and robustness, they quantified well-known molecules belonged to 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 separation run lengthens in order to obtain no analytes co-elution or ion suppression. The developed chromatographic run lasted 35 minutes, time comparable with those 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 growth under different condition was proposed by Patel *et al.* [32]. They used a C18 column and a Q-exactive high resolution mass spectrometer and within 13 minutes they recognized 23 AHLs, some of them never described in literature. Their method showed little overlapping and tailing of chromatographic peaks, nevertheless they achieved very low limit of detection for all analytes and performed the identification of unexpected AHL molecules using a labelled approach. The MS/MS method for the identification of AHLs was based, as for our, on peculiar product ion with *m/z* 102.

The HQs separation run was shorter than AHLs, and it could be comparable to those developed by Maurer [28] and Brewer [34] groups. It lasted 12 minutes (Maurer 9'and Brewer 8') and the separation obtained with the use of 2-picolinic acid was satisfactory (ESM Fig. S2).

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Although Maurer and co-workers [28] used a mixture of acetic anhydride–pyridine to derivatize C7-HQ, and Brewer team [34] employed EDTA as mobile aqueous phase, we preferred to follow the Turnpenny recommendation [33] in order to obtain a good shape of chromatographic peaks and consequently a possible and accurate quantification of the HQ compounds. So, we run the chromatographic separation with the use of 2-picolinic acid. Indeed, EDTA molecule is not suggested for MS analysis since it is a non-volatile salt and gives 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 Turpnenny declared, 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 expressed (high concentration) was the C7 HQ. In minimal medium poor in nutrients (W9), the C7 HQ species was the only one detected both in the wild type and the RhlI- derivative bacterial cultures. This finding could support the hypothesis that the knock-out mutant, unable to produce C4-AHL, intensified the production of this signal molecule to bear bacterial cell growth. As for AHLs compounds, cells grew in LB broth showed most abundant content of HQs molecules 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, also the developed NL and PI MS approaches played a fundamental role in the identification of unknown compounds. The unknown species C6-AHL detected in bacterial cultures only, with empirical formula $C_{10}H_{17}NO_3$, and accurate *m/z* 200.1287 (confirmed with 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 C4-AHL and C6-AHL among the various signal molecules played the most important signaling role in 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)

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was detectable as two different isomers when monitored using NL approach. However, the possibility to discriminate between the isomers would require a more detailed investigation using for example NMR technique to obtain structural informations. The analysis of HQ molecules didn't show any isomeric species.

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

Last but not least, we quantified C12-AHL in bacterial samples, to our knowledge for the first time. In fact, others 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 for the study of Hoang *et al*. [35] that quantified C12-AHL in the Gram-negative endophytic bacterium *Paraburkholderia* sp. 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 of their high tolerance to the 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 *rhlI* QS signal systems.

An accurate quantitation of the molecules was achieved for bacterial cultures WT-W9, WT-LB, RhlI-W9 and RhlI-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 others research groups validated their analytical approaches in bacterial cultures [28, 34, 35]. A comprehensive method for QS compounds quantitation in human samples is lacking [23-25, 29]. Furthermore, we studied the *Pseudomonas aeruginosa* QS molecules production because this is one of the most common bacteria giving infection in patients with sepsis [49, 50]. Data shown in table 6 evidence that C4-AHL was detectable in all the samples within a concentration range of 4-7 µg/L, 3-oxo-C12-AHL in a range of 0-0.6 µg/L and finally C7-HQ in a range between 2 and 18 µg/L. A general tendency of QS molecules concentration to decrease was evidenced showing that hemoperfusion of plasma of patients with MOF was effective in removing of QS compounds. The monitoring of QS

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analytical standard molecules in hemoperfused plasma samples from MOF patients confirmed the ability of MRM approach to quantify virulence factors during sepsis with a good sensitivity.

5. Conclusions

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

HPLC-TQ MS/MS analytical procedure has been validated in MRM, NL and PI scan mode. Experimental data indicated that the method was suitable for the detection of low concentration of AHLs and HQs in bacterial cultures of *Pseudomonas aeruginosa* and biofluids in early stages of the sepsis-related multi-organ failure illness. Using the NL and PI scan methods, it was possible to discover 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. MRM approach was suitable for the detection of low AHLs and HQs levels within less abundant samples. The comparison between bacteria cultures and plasma samples highlighted even more why high sensitivity methods are mandatory for plasma, given the concentration of few µg/L of the molecules of interest within the sample.

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

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

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For Peer Review

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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 insurance that principles of ethical and professional have been followed in each step of the present work, the research was approved by the proper university human research ethics committee (protocol number 0114479 approved the 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 November 2017 at AOU Città della Salute e della Scienza di Torino).

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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 due to their content in personal patients' information that could compromise their privacy.

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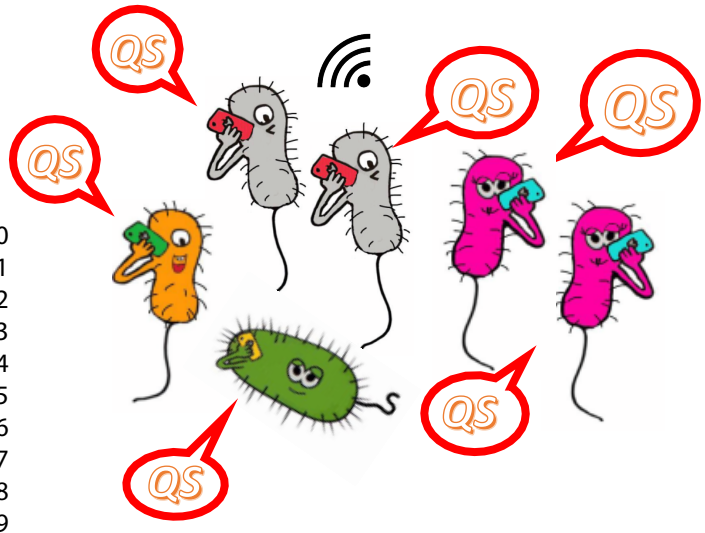
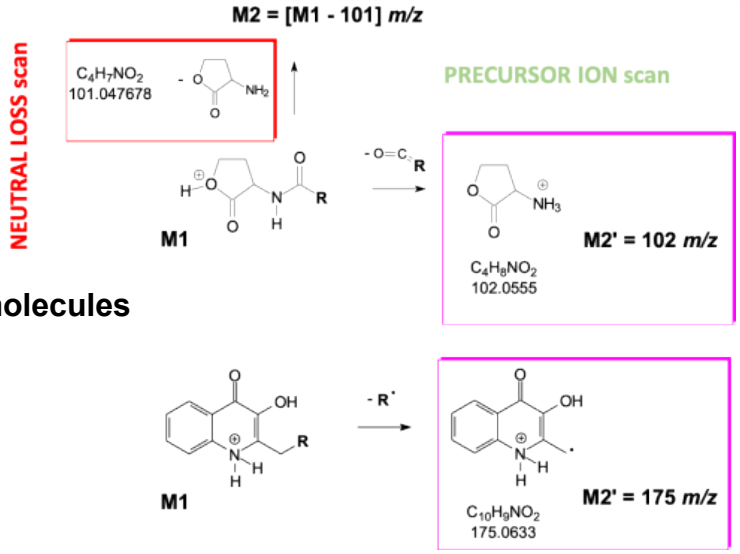


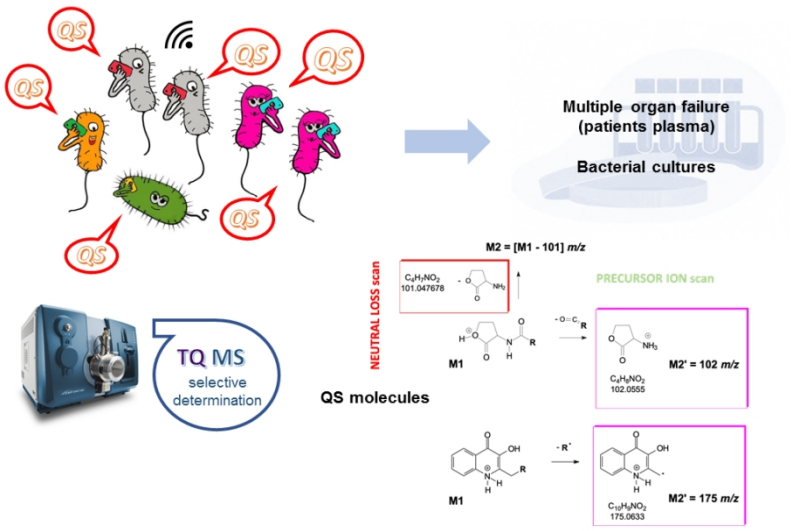
Multiple organ failure (patients plasma)

Bacterial cultures

TQ MS
selective determination

QS molecules





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Analytical and Bioanalytical Chemistry

Electronic Supplementary Material

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

Federica Dal Bello, Michael Zorzi, Riccardo Aigotti, Davide Medica, Vito Fanelli, Vincenzo Cantaluppi, Eleonora Amante, Viviana Teresa Orlandi, Claudio Medana

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1. Figures

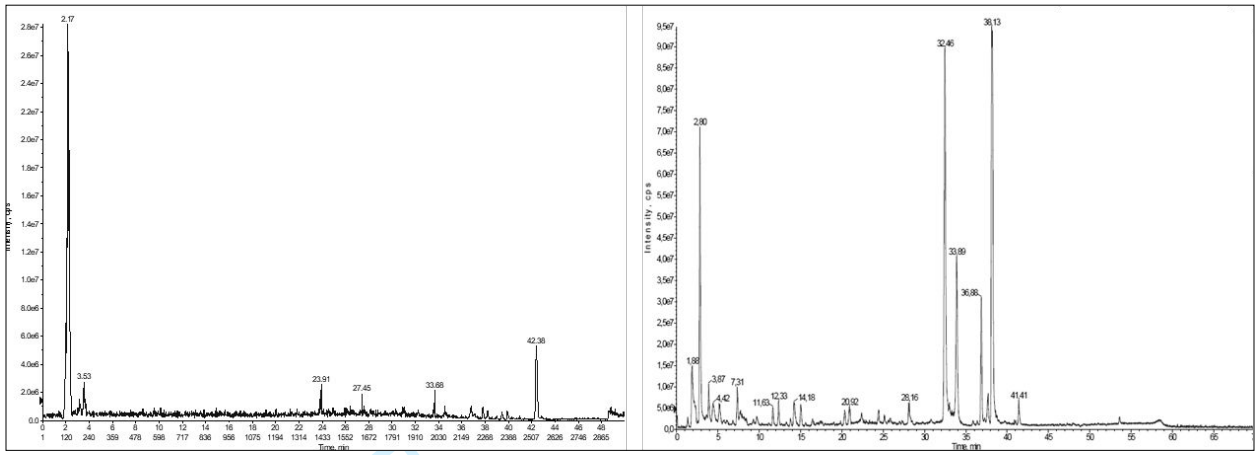

Fig. S1 Chromatograms acquired with NL MS approach for AHLs signalling molecules analysis. On the left it was presented the separation of few AHL compounds in a sample of patient plasma. As discussed in the main manuscript, since the sample was poor in AHL detection, the elution gradient was of 48 minutes and no overlapping peaks were observed. On the contrary, the right panel shows the NL AHLs separation in a sample of *Pseudomonas aeruginosa* wild type grown in Luria Bertani (LB) broth. Here, many AHL molecules were detected and the gradient separation was slower compared with plasma sample in order to obtain a satisfactory separation of peaks

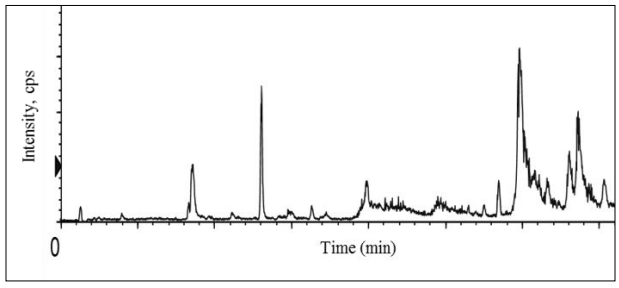

Fig. S2 Chromatogram acquired with PI MS approach for HQs signalling molecules analysis using 2-picolinic acid as aqueous mobile phase

For Peer Review

2. Validation tables and results

*The acronym AUC means “area under the curve”.

2.1. 3-oxo-C12-AHL (N-(3-oxododecanoyl)-L-homoserine lactone)

2.1.1. Calibration curves AUC and equation

-MRM

Conc. (µg/L)	AUC cal. curve 1	AUC cal. curve 2	AUC cal. curve 3	AUC cal. curve 4
0.4	4.91E+04	4.81E+04	6.58E+04	5.75E+04
1	1.12E+05	1.19E+05	1.60E+05	1.40E+05
5	6.21E+05	7.30E+05	9.11E+05	5.80E+05
10	1.27E+06	1.48E+06	1.88E+06	1.12E+06
50	6.46E+06	7.94E+06	1.00E+07	7.06E+06
100	1.07E+07	1.30E+07	1.62E+07	1.25E+07
200	2.30E+07	2.84E+07	3.52E+07	2.67E+07
300	3.56E+07	4.25E+07	5.04E+07	40117103
400	4.56E+07	5.57E+07	6.81E+07	5.41E+07

Linear regressive analysis using a weighting factor of $1/x^2$: y= 141900x-2609

-NL

Conc. (µg/L)	AUC cal. curve 1	AUC cal. curve 2	AUC cal. curve 3	AUC cal. curve 4
5	2.67E+06	2.72E+06	2.69E+06	2.72E+06
10	4.12E+06	4.52E+06	4.88E+06	4.99E+06
50	2.31E+07	2.65E+07	2.80E+07	2.95E+07
100	4.03E+07	5.12E+07	4.83E+07	4.79E+07
200	9.90E+07	9.17E+07	9.99E+07	1.08E+08
300	1.41E+08	1.40E+08	1.41E+08	1.45E+08
400	1.90E+08	1.98E+08	2.14E+08	2.14E+08

Linear regressive analysis using a weighting factor of $1/x^2$: y= 490900x + 152400

-PI

Conc. (µg/L)	AUC cal. curve 1	AUC cal. curve 2	AUC cal. curve 3	AUC cal. curve 4
1	8.40E+05	8.57E+05	9.19E+05	6.66E+05
5	3.55E+06	7.69E+05	4.71E+06	4.13E+06
10	7.33E+06	5.67E+06	7.45E+06	6.54E+06
50	3.47E+07	3.59E+07	40318826	3.59E+07
100	7.27E+07	6.98E+07	78447022	7.07E+07
200	1.41E+08	1.37E+08	1.54E+08	1.50E+08
300	1.83E+08	2.30E+08	2.58E+08	2.25E+08

Linear regressive analysis using a weighting factor of $1/x^2$: y= 709400x + 93520

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2.1.2. Selectivity (SEL%)

- MRM

Sample	AUC	Average AUC	SEL%	8.12
QS-free_matrix_001	3.45E+03			
QS-free_matrix_002	4.67E+03	4.41E+03		
QS-free_matrix_003	5.12E+03			
STD_400 ppt_001	4.91E+04			
STD_400 ppt_002	4.81E+04	5.43E+04		
STD_400 ppt_003	6.58E+04			

-NL

Sample	AUC	Average AUC	SEL%	0.50
QS-free_matrix_001	1.14E+04			
QS-free_matrix_002	1.54E+04	1.34E+04		
QS-free_matrix_003	1.33E+04			
STD_5 ppb_001	2.67E+06			
STD_5 ppb_002	2.72E+06	2.69E+06		
STD_5 ppb_003	2.69E+06			

-PI

Sample	AUC	Average AUC	SEL%	1.10
QS-free_matrix_001	9.94E+03			
QS-free_matrix_002	8.77E+03	9.58E+03		
QS-free_matrix_003	1.00E+04			
STD_1 ppb_001	8.40E+05			
STD_1 ppb_002	8.57E+05	8.72E+05		
STD_1 ppb_003	9.19E+05			

2.1.3. Recovery (REC%)

- MRM

Sample	AUC	Average AUC
STD_0.4ppb_solv_01	1.26E+05	1.09E+05
STD_0.4ppb_solv_02	9.87E+04	
STD_0.4ppb_solv_03	1.02E+05	
STD_400ppb_solv_01	1.02E+08	9.87E+07
STD_400ppb_solv_02	9.74E+07	
STD_400ppb_solv_03	9.63E+07	
STD_0.4ppb_pls_01	5.00E+04	4.95E+04
STD_0.4ppb_pls_02	5.00E+04	

For Peer Review

STD_0.4ppb_pls_03	4.86E+04	
STD_400ppb_pls_01	5.34E+07	5.30E+07
STD_400ppb_pls_02	5.54E+07	
STD_400ppb_pls_03	5.03E+07	
REC%	**LLOQ**	**45.5**
	ULOQ	**53.7**

2.1.4. Carry-over (CO%)

	Sample	**AUC**		
MRM	STD_400 ppb	4.56E+07	**CO%**	**7.10**
	QS-free_matrix	4.67E+03		
	STD_400 ppt	6.58E+04		
NL	STD_400 ppb	1.90E+08	**CO%**	**0.57**
	QS-free_matrix	1.54E+04		
	STD_5 ppb	2.69E+06		
PI	STD_300 ppb	1.83E+08	**CO%**	**1.29**
	QS-free_matrix	9.94E+03		
	STD_1 ppb	7.69E+05		

2.1.5. Intra-day accuracy (BIAS%) and precision (CV%)

-MRM

Nominal conc. (µg/L)	Real conc. (back-calculated, µg/L)				CV%	BIAS%
	Cal. curve 1	Cal. curve 2	Cal. curve 3	Cal. curve 4		
0.4	0.36	0.36	0.48	0.42	12.4	1.65
1	0.81	0.85	1.15	1.00	14.0	4.92
5	4.39	5.16	6.44	4.11	18.0	0.50
10	9.00	10.5	13.3	7.88	20.0	1.51
50	45.5	56.0	70.6	49.8	17.1	9.88
100	75.5	91.8	114	88.2	15.1	8.16
200	162	200	248	189	15.6	0.15
300	251	300	355	283	12.8	0.96
400	322	392	480	381	14.3	1.61

For Peer Review

-NL

Nominal conc. (µg/L)	Real conc. (back-calculated, µg/L)				CV%	BIAS%
	Cal. curve 1	Cal. curve 2	Cal. curve 3	Cal. curve 4		
5	5.13	5.23	5.17	5.24	0.87	3.66
10	8.07	8.90	9.63	9.85	7.64	9.72
50	46.8	53.6	56.6	59.8	8.87	7.77
100	81.8	104	98.0	97.2	8.60	4.99
200	201	187	203	221	5.96	1.45
300	287	284	287	296	1.53	4.00
400	387	404	435	435	4.98	3.63

-PI

Nominal conc. (µg/L)	Real conc. (back-calculated, µg/L)				CV%	BIAS%
	Cal. curve 1	Cal. curve 2	Cal. curve 3	Cal. curve 4		
1	1.05	1.08	1.16	0.81	12.9	2.40
5	4.88	0.95	6.51	5.69	47.3	10.9
10	10.2	7.86	10.4	9.09	10.8	6.63
50	48.7	50.5	56.7	50.4	5.91	3.05
100	102	98.3	111	99.5	4.62	2.56
200	199	193	217	211	4.62	2.51
300	258	324	364	317	12.0	4.97

2.1.6. LOD and LOQ

-MRM

Sample	**AUC**		
QS-free_matrix_001	3.45E+03		
QS-free_matrix_002	4.67E+03		
QS-free_matrix_003	5.12E+03		
QS-free_matrix_004	5.02E+03		
QS-free_matrix_005	4.11E+03	Slope	1.42E+05
QS-free_matrix_006	4.53E+03	Intercept	-2.61E+03
Average AUC	4.48E+03		
Std. dev QS-free matrix	5.69E+02		**Conc. (µg/L)**
10std. Dev. + Average AUC	1.02E+04	**LOQ**	**0.090**

For Peer Review

-NL

Sample	AUC		
QS-free_matrix_001	4.58E+03		
QS-free_matrix_002	4.69E+03		
QS-free_matrix_003	4.82E+03		
QS-free_matrix_004	3.52E+03		
QS-free_matrix_005	4.11E+03	Slope	4.91E+05
QS-free_matrix_006	4.53E+03	Intercept	1.52E+05
Average AUC	4.37E+03		
Std. dev QS-free matrix	4.42E+02		**Conc. (µg/L)**
10std. Dev. + Average AUC	8.80E+03	**LOQ**	**0.293**

-PI

Sample	AUC		
QS-free_matrix_001	3.45E+03		
QS-free_matrix_002	4.67E+03		
QS-free_matrix_003	5.12E+03		
QS-free_matrix_004	5.02E+03		
QS-free_matrix_005	4.11E+03	Slope	7.09E+05
QS-free_matrix_006	4.53E+03	Intercept	9.35E+04
Average AUC	4.48E+03		
Std. dev QS-free matrix	5.69E+02		**Conc. (µg/L)**
10std. Dev. + Average AUC	1.02E+04	**LOQ**	**0.117**

2.1.7. Freeze-thaw stability (STAB%)

-MRM

	AUC T_0	AUC $T_{freeze-thaw}$	Conc. (µg/L) T_0	Conc. (µg/L) $T_{freeze-thaw}$	STAB%	AVAREGE STAB%
LLOQ (0.4 µg/L)	1.23E+05	1.23E+05	0.88	0.88	100	106
	5.54E+04	4.81E+04	0.41	0.36	115	
	5.43E+04	5.57E+04	0.40	0.41	102	
ULOQ (400 µg/L)	5.54E+07	5.63E+07	390	397	102	101
	5.55E+07	5.58E+07	392	393	100	
	5.60E+07	5.68E+07	394	400	101	

For Peer Review

-NL

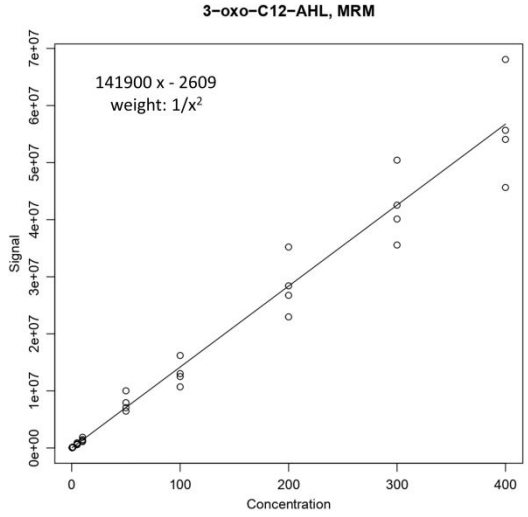
	AUC T_0	AUC $T_{freeze-thaw}$	Conc. (µg/L) T_0	Conc. (µg/L) $T_{freeze-thaw}$	STAB%	AVAREGE STAB%
LLOQ (5 µg/L)	2.78E+06	2.41E+06	5.35	4.60	86.0	89.5
	2.61E+06	2.59E+06	5.01	4.96	98.9	
	2.61E+06	2.21E+06	5.01	4.18	83.5	
ULOQ (400 µg/L)	1.98E+08	1.80E+08	403	367	91.1	88.6
	2.05E+08	1.82E+08	417	371	88.9	
	2.06E+08	1.77E+08	420	360	85.8	

-PI

	AUC T_0	AUC $T_{freeze-thaw}$	Conc. (µg/L) T_0	Conc. (µg/L) $T_{freeze-thaw}$	STAB%	AVAREGE STAB%
LLOQ (1 µg/L)	9.88E+05	7.20E+05	1.26	0.88	70.0	86.0
	6.59E+05	6.61E+05	0.80	0.80	100	
	8.14E+05	7.24E+05	1.02	0.89	87.6	
ULOQ (300 µg/L)	2.23E+08	2.13E+08	315	300	95.2	90.2
	1.99E+08	1.86E+08	281	261	93.2	
	1.87E+08	1.54E+08	264	217	82.2	

2.1.8. Figure of average of four calibration curves

-MRM



For Peer Review

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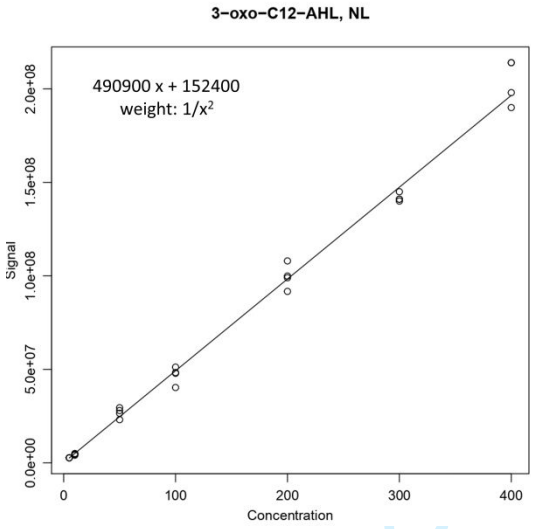

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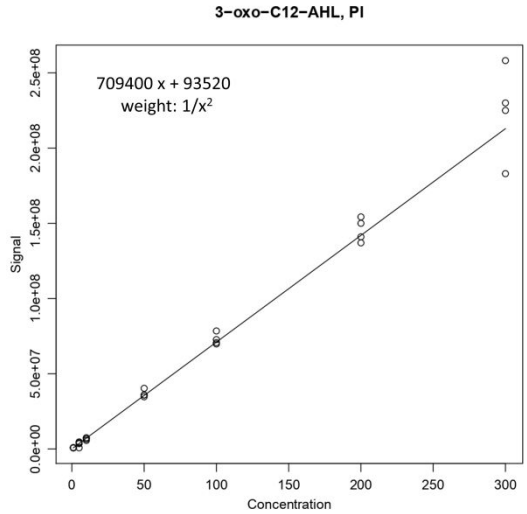

For Peer Review

2.2. C4-AHL (N-butanoyl-L-homoserine lactone)

2.2.1. Calibration curves AUC and equation

-MRM

Conc. (µg/L)	AUC cal. curve 1	AUC cal. curve 2	AUC cal. curve 3	AUC cal. curve 4
0.4	5.78E+03	6.18E+03	8.73E+03	7.13E+03
1	1.28E+04	1.40E+04	2.08E+04	1.75E+04
5	9.27E+04	1.00E+05	6.08E+04	8.16E+04
10	1.42E+05	1.48E+05	1.79E+05	1.61E+05
50	7.10E+05	7.58E+05	8.93E+05	9.20E+05
100	1.29E+06	1.44E+06	1.64E+06	1.59E+06
200	2.43E+06	2.89E+06	3.54E+06	2.58E+06
300	3.77E+06	4.18E+06	5.53E+06	5.03E+06
400	4.89E+06	5.68E+06	7.38E+06	6.83E+06

Linear regressive analysis using a weighting factor of $1/x^2$: y= 15520x + 783.5

-NL

Conc. (µg/L)	AUC cal. curve 1	AUC cal. curve 2	AUC cal. curve 3	AUC cal. curve 4
5	1.00E+05	7.95E+04	1.39E+05	1.06E+05
10	1.52E+05	1.52E+05	2.28E+05	1.77E+05
50	7.58E+05	7.22E+05	1.14E+06	8.73E+05
100	1.44E+06	1.40E+06	2.42E+06	1.75E+06
200	2.89E+06	2.91E+06	4.41E+06	3.40E+06
300	4.18E+06	4.11E+06	6.29E+06	4.86E+06
400	5.68E+06	5.55E+06	9.07E+06	6.77E+06

Linear regressive analysis using a weighting factor of $1/x^2$: y= 16740x + 20230

-PI

Conc. (µg/L)	AUC cal. curve 1	AUC cal. curve 2	AUC cal. curve 3	AUC cal. curve 4
1	1.68E+05	1.61E+05	1.16E+05	1.49E+05
5	8.05E+05	1.31E+06	5.64E+05	8.64E+05
10	1.34E+06	2.12E+06	1.24E+06	1.55E+06
50	7.62E+06	8.29E+06	5.21E+06	7.91E+06
100	1.50E+07	1.61E+07	1.11E+07	1.79E+07
200	3.02E+07	2.98E+07	2.22E+07	3.32E+07
300	4.47E+07	4.44E+07	2.99E+07	5.09E+07

Linear regressive analysis using a weighting factor of $1/x^2$: y= 151400x + 2447

For Peer Review

2.2.2. Selectivity (SEL%)

- MRM

Sample	AUC	Average AUC	SEL%	5.65
QS-free_matrix_001	4.82E+02			
QS-free_matrix_002	4.85E+02	7.44E+02		
QS-free_matrix_003	1.27E+03			
STD_400 ppt_001	1.88E+04			
STD_400 ppt_002	1.20E+04	1.32E+04		
STD_400 ppt_003	8.73E+03			

-NL

Sample	AUC	Average AUC	SEL%	1.63
QS-free_matrix_001	1.75E+03			
QS-free_matrix_002	1.65E+03	1.76E+03		
QS-free_matrix_003	1.88E+03			
STD_5 ppb_001	7.95E+04			
STD_5 ppb_002	1.39E+05	1.08E+05		
STD_5 ppb_003	1.06E+05			

-PI

Sample	AUC	Average AUC	SEL%	2.54
QS-free_matrix_001	5.94E+03			
QS-free_matrix_002	4.77E+03	5.58E+03		
QS-free_matrix_003	6.03E+03			
STD_1 ppb_001	7.64E+04			
STD_1 ppb_002	1.71E+05	2.20E+05		
STD_1 ppb_003	4.13E+05			

For Peer Review

2.2.3. Recovery (REC%)

Sample	AUC	Average AUC
STD_0.4ppb_solv_01	1.59E+04	1.42E+04
STD_0.4ppb_solv_02	1.42E+04	
STD_0.4ppb_solv_03	1.26E+04	
STD_400ppb_solv_01	1.59E+07	1.49E+07
STD_400ppb_solv_02	1.24E+07	
STD_400ppb_solv_03	1.63E+07	
STD_0.4ppb_pls_01	6.45E+03	7.26E+03
STD_0.4ppb_pls_02	7.86E+03	
STD_0.4ppb_pls_03	7.46E+03	
STD_400ppb_pls_01	1.03E+07	9.05E+06
STD_400ppb_pls_02	7.24E+06	
STD_400ppb_pls_03	9.65E+06	
REC%	**LLOQ**	**51.0**
	ULOQ	**60.9**

2.2.4. Carry-over (CO%)

	Sample	**AUC**		
MRM	STD_400 ppb	6.93E+06	**CO%**	**8.90**
	QS-free_matrix	4.82E+02		
	STD_400 ppt	5.41E+03		
NL	STD_400 ppb	6.77E+06	**CO%**	**1.65**
	QS-free_matrix	1.65E+03		
	STD_5 ppb	1.00E+05		
PI	STD_300 ppb	1.26E+05	**CO%**	**0.87**
	QS-free_matrix	1.27E+03		
	STD_1 ppb	1.46E+05		

For Peer Review

2.2.5. Intra-day accuracy (BIAS%) and precision (CV%)

-MRM

Nominal conc. (µg/L)	Real conc. (back-calculated, µg/L)				CV%	BIAS%
	Cal. curve 1	Cal. curve 2	Cal. curve 3	Cal. curve 4		
0.4	0.32	0.35	0.51	0.41	18.4	0.61
1	0.77	0.85	1.29	1.08	20.2	0.03
5	5.93	6.39	3.87	5.20	17.8	6.49
10	9.12	9.49	11.5	10.3	8.93	0.96
50	45.7	48.8	57.5	59.2	10.8	5.32
100	83.2	92.4	105	102	9.05	4.43
200	157	186	228	166	14.9	8.57
300	243	270	357	324	15.0	0.61
400	315	366	475	440	15.6	0.23

-NL

Nominal conc. (µg/L)	Real conc. (back-calculated, µg/L)				CV%	BIAS%
	Cal. curve 1	Cal. curve 2	Cal. curve 3	Cal. curve 4		
5	4.76	3.54	7.11	5.14	25.0	2.72
10	7.87	7.87	12.4	9.38	19.7	6.64
50	44.1	41.9	66.8	50.9	19.1	1.84
100	84.5	82.1	143	103	23.7	3.19
200	171	173	262	202	18.2	1.03
300	249	245	375	289	18.1	3.67
400	338	331	541	403	20.9	0.80

-PI

Nominal conc. (µg/L)	Real conc. (back-calculated, µg/L)				CV%	BIAS%
	Cal. curve 1	Cal. curve 2	Cal. curve 3	Cal. curve 4		
1	1.10	1.05	0.75	0.97	13.7	3.59
5	5.30	8.63	3.71	5.69	30.5	14.3
10	8.83	14.0	8.19	10.2	21.8	2.97
50	50.3	54.8	34.4	52.3	16.6	4.32
100	99.3	106	73.4	118	16.6	0.67
200	199	197	147	220	14.0	4.97
300	295	293	198	336	18.1	6.98

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2.2.6. LOD and LOQ

-MRM

Sample	AUC		
QS-free_matrix_001	4.82E+02		
QS-free_matrix_002	4.85E+02		
QS-free_matrix_003	1.27E+03		
QS-free_matrix_004	4.72E+02		
QS-free_matrix_005	1.27E+03	Slope	1.55E+04
QS-free_matrix_006	1.36E+03	Intercept	7.84E+02
Average AUC	8.88E+02		
Std. dev QS-free matrix	4.10E+02		**Conc. (µg/L)**
10std. Dev. + Average AUC	4.99E+03	**LOQ**	**0.271**

-NL

Sample	AUC		
QS-free_matrix_001	4.82E+02		
QS-free_matrix_002	4.85E+02		
QS-free_matrix_003	1.27E+03		
QS-free_matrix_004	1.36E+03		
QS-free_matrix_005	3.65E+03	Slope	1.67E+04
QS-free_matrix_006	4.02E+02	Intercept	2.02E+04
Average AUC	1.27E+03		
Std. dev QS-free matrix	1.13E+03		**Conc. (µg/L)**
10std. Dev. + Average AUC	1.26E+04	**LOQ**	**0.457**

-PI

Sample	AUC		
QS-free_matrix_001	1.27E+03		
QS-free_matrix_002	1.36E+03		
QS-free_matrix_003	3.65E+03		
QS-free_matrix_004	4.02E+02		
QS-free_matrix_005	2.47E+02	Slope	1.51E+05
QS-free_matrix_006	1.25E+03	Intercept	2.45E+03
Average AUC	1.36E+03		
Std. dev QS-free matrix	1.11E+03		**Conc. (µg/L)**
10std. Dev. + Average AUC	1.25E+04	**LOQ**	**0.066**

For Peer Review

2.2.7. Freeze-thaw stability (STAB%)

-MRM

	AUC T_0	AUC $T_{freeze\text{-}thaw}$	Conc. (µg/L) T_0	Conc. (µg/L) $T_{freeze\text{-}thaw}$	STAB%	AVAREGE STAB%
LLOQ (0.4 µg/L)	7.24E+03	6.59E+03	0.42	0.37	90.0	97.3
	6.72E+03	6.95E+03	0.38	0.40	104	
	7.27E+03	7.13E+03	0.42	0.41	97.9	
ULOQ (400 µg/L)	6.09E+06	6.32E+06	392	407	104	102
	6.16E+06	6.19E+06	37	399	100	
	6.18E+06	6.23E+06	398	401	101	

-NL

	AUC T_0	AUC $T_{freeze\text{-}thaw}$	Conc. (µg/L) T_0	Conc. (µg/L) $T_{freeze\text{-}thaw}$	STAB%	AVAREGE STAB%
LLOQ (5 µg/L)	9.10E+04	8.02E+04	4.23	3.58	84.8	91.1
	8.93E+04	9.30E+04	4.13	4.34	105	
	9.22E+04	8.01E+04	4.30	3.58	83.2	
ULOQ (400 µg/L)	6.84E+06	5.91E+06	407	352	86.4	86.9
	6.79E+06	5.91E+06	404	352	87.0	
	6.83E+06	5.95E+06	407	354	87.1	

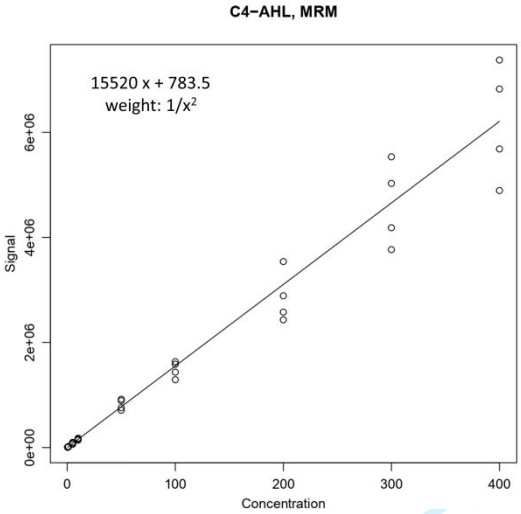
-PI

	AUC T_0	AUC $T_{freeze\text{-}thaw}$	Conc. (µg/L) T_0	Conc. (µg/L) $T_{freeze\text{-}thaw}$	STAB%	AVAREGE STAB%
LLOQ (1 µg/L)	1.56E+05	1.41E+05	1.01	0.92	90.3	88.9
	1.61E+05	1.40E+05	1.04	0.91	86.9	
	1.60E+05	1.44E+05	1.04	0.93	89.6	
ULOQ (300 µg/L)	4.27E+07	4.07E+07	282	269	95.4	95.5
	4.26E+07	3.99E+07	281	263	93.6	
	4.22E+07	4.11E+07	278	272	97.6	

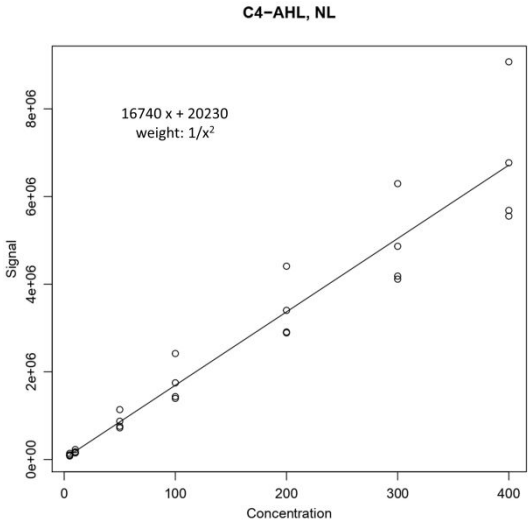
For Peer Review

2.2.8. Figure of average of four calibration curves

-MRM

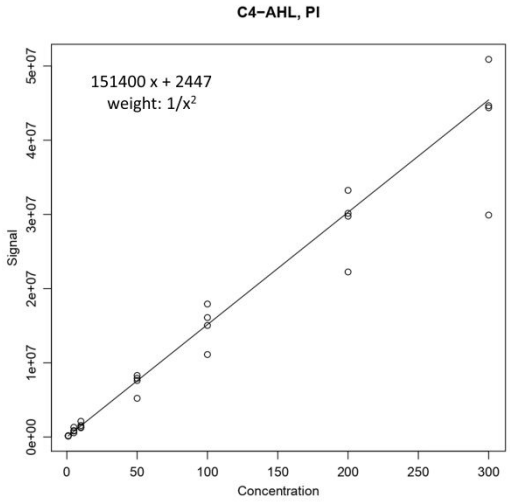


-NL



For Peer Review

-PI



2.3. C7 HQ (2-heptyl-4-hydroxyquinoline)

2.3.1. Calibration curves AUC and equation

-MRM

Conc. (µg/L)	AUC cal. curve 1	AUC cal. curve 2	AUC cal. curve 3	AUC cal. curve 4
0.4	8.02E+03	5.06E+03	5.07E+03	8.51E+03
1	1.83E+04	1.63E+04	1.14E+04	2.31E+04
5	8.53E+04	5.54E+04	6.20E+04	8.01E+04
10	1.99E+05	1.19E+05	1.21E+05	1.46E+05
50	9.01E+05	6.42E+05	6.58E+05	9.74E+05
100	1.81E+06	1.30E+06	1.28E+06	2.02E+06
200	3.72E+06	2.65E+06	2.51E+06	4.06E+06

Linear regressive analysis using a weighting factor of $1/x^2$: y= 15500x + 594.3

-PI

Conc. (µg/L)	AUC cal. curve 1	AUC cal. curve 2	AUC cal. curve 3	AUC cal. curve 4
1	2.56E+05	2.27E+05	2.69E+05	2.30E+05
5	8.24E+05	7.36E+05	6.35E+05	8.15E+05
10	1.69E+06	1.24E+06	1.64E+06	1.47E+06
50	8.32E+06	8.00E+06	7.98E+06	8.12E+06
100	1.43E+07	1.62E+07	1.55E+07	1.43E+07
200	2.95E+07	3.05E+07	3.05E+07	2.90E+07
300	4.47E+07	4.62E+07	4.12E+07	4.00E+07

Linear regressive analysis using a weighting factor of $1/x^2$: y= 146300 x + 96020

For Peer Review

2.3.2. Selectivity (SEL%)

- MRM

Sample	AUC	Average AUC	SEL%	16.5
QS-free_matrix_001	1.01E+03			
QS-free_matrix_002	1.37E+03	1.19E+03		
QS-free_matrix_003	1.19E+03			
STD_400 ppt_001	8.02E+03			
STD_400 ppt_002	5.07E+03	7.20E+04		
STD_400 ppt_003	8.51E+03			

-PI

Sample	AUC	Average AUC	SEL%	4.73
QS-free_matrix_001	9.14E+03			
QS-free_matrix_002	1.13E+04	1.08E+04		
QS-free_matrix_003	1.20E+04			
STD_1 ppb_001	1.98E+05			
STD_1 ppb_002	2.57E+05	2.29E+05		
STD_1 ppb_003	2.31E+05			

2.3.3. Recovery (REC%)

Sample	AUC	Average AUC
STD_0.4ppb_solv_01	1.52E+04	1.26E+04
STD_0.4ppb_solv_02	1.13E+04	
STD_0.4ppb_solv_03	1.12E+04	
STD_200ppb_solv_01	1.12E+07	1.03E+07
STD_200ppb_solv_02	9.86E+06	
STD_200ppb_solv_03	9.74E+06	
STD_0.4ppb_pls_01	5.98E+03	6.28E+03
STD_0.4ppb_pls_02	6.85E+03	
STD_0.4ppb_pls_03	6.01E+03	
STD_200ppb_pls_01	3.65E+06	3.52E+06
STD_200ppb_pls_02	2.95E+06	
STD_200ppb_pls_03	3.95E+06	
REC%	**LLOQ**	**49.9**
	ULOQ	**34.3**

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2.3.4. Carry-over (CO%)

	Sample	AUC		
MRM	STD_200 ppb	2.59E+06	**CO%**	**16.1**
	QS-free_matrix	1.37E+03		
	STD_400 ppt	8.51E+03		
PI	STD_300 ppb	4.35E+07	**CO%**	**2.98**
	QS-free_matrix	7.04E+03		
	STD_1 ppb	2.36E+05		

2.3.5. Intra-day accuracy (BIAS%) and precision (CV%)

-MRM

Nominal conc. (µg/L)	Real conc. (back-calculated, µg/L)				CV%	BIAS%
	Cal. curve 1	Cal. curve 2	Cal. curve 3	Cal. curve 4		
0.4	0.48	0.29	0.29	0.51	26.5	2.11
1	1.14	1.01	0.70	1.45	25.3	7.09
5	5.46	3.54	3.96	5.13	17.6	10.5
10	12.8	7.61	7.80	9.36	22.3	6.41
50	58.1	41.4	42.4	62.8	18.4	2.27
100	117	83.8	82.6	130	20.1	3.21
200	240	171	162	262	20.7	4.14

-PI

Nominal conc. (µg/L)	Real conc. (back-calculated, µg/L)				CV%	BIAS%
	Cal. curve 1	Cal. curve 2	Cal. curve 3	Cal. curve 4		
1	1.10	0.89	1.19	0.92	11.9	2.28
5	4.97	4.38	3.68	4.91	11.6	11.5
10	10.9	7.80	10.6	9.36	12.6	3.55
50	56.2	54.0	53.9	54.9	1.72	8.68
100	97.1	110	105	97.3	5.34	2.34
200	201	208	208	197	2.19	1.68
300	305	315	281	273	5.84	2.27

For Peer Review

2.3.6. LOD and LOQ

-MRM

Sample	AUC		
QS-free_matrix_001	1.43E+03		
QS-free_matrix_002	1.01E+03		
QS-free_matrix_003	9.14E+02		
QS-free_matrix_004	1.13E+03		
QS-free_matrix_005	1.20E+03	Slope	1.55E+04
QS-free_matrix_006	9.03E+02	Intercept	5.94E+02
Average AUC	1.10E+03		
Std. dev QS-free matrix	1.83E+02		**Conc. (µg/L)**
10std. Dev. + Average AUC	2.93E+03	**LOQ**	**0.151**

-PI

Sample	AUC		
QS-free_matrix_001	1.20E+04		
QS-free_matrix_002	7.04E+03		
QS-free_matrix_003	1.37E+04		
QS-free_matrix_004	1.51E+04		
QS-free_matrix_005	1.46E+04	Slope	1.46E+05
QS-free_matrix_006	1.38E+04	Intercept	9.60E+04
Average AUC	1.27E+04		
Std. dev QS-free matrix	2.71E+03		**Conc. (µg/L)**
10std. Dev. + Average AUC	3.98E+04	**LOQ**	**0.385**

2.3.7. Freeze-thaw stability (STAB%)

-MRM

	AUC T_0	AUC $T_{freeze-thaw}$	Conc. (µg/L) T_0	Conc. (µg/L) $T_{freeze-thaw}$	STAB%	AVAREGE STAB%
LLOQ (0.4 µg/L)	8.02E+03	8.51E+03	0.48	0.51	107	115
	5.07E+03	6.67E+03	0.29	0.39	136	
	7.06E+03	7.25E+03	0.42	0.43	103	
ULOQ (200 µg/L)	3.72E+06	4.06E+06	240	262	109	118
	2.51E+06	3.23E+06	161	209	129	
	2.65E+06	3.04E+06	171	196	115	

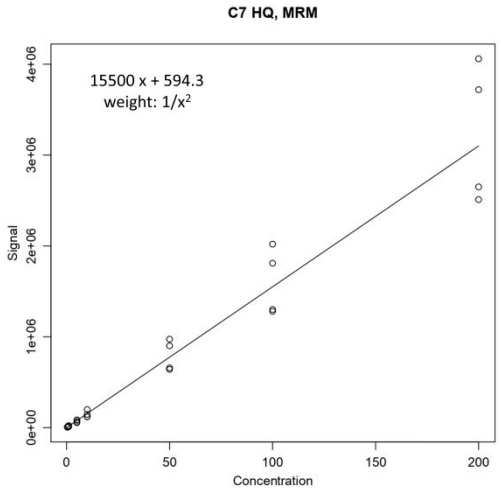
For Peer Review

-PI

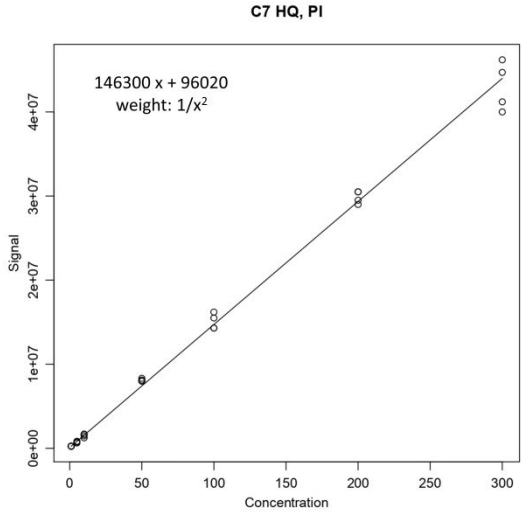
	AUC T_0	AUC $T_{freeze-thaw}$	Conc. (µg/L) T_0	Conc. (µg/L) $T_{freeze-thaw}$	STAB%	AVAREGE STAB%
LLOQ (1 µg/L)	2.98E+05	2.65E+05	1.38	1.16	83.7	90.2
	2.12E+05	1.99E+05	0.79	0.70	88.8	
	3.01E+05	2.97E+05	1.40	1.37	98.1	
ULOQ (300 µg/L)	4.23E+07	3.99E+07	289	272	94.3	96.2
	4.98E+07	4.59E+07	340	313	92.1	
	4.03E+07	4.12E+07	275	281	102	

2.3.8. Figure of average of four calibration curves

-MRM



-PI



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3. Tables

Table S1 Multiple Reaction Monitoring (MRM) scan parameters and selected transition for AHLs and HQs analysis (bolded transitions were used as quantitative ones). DP: Declustering Potential; EP: Entrance Potential; CE: Collision Energy; CXP: Collision Cell Exit Potential

Analyte	Precursor ion $[M+H]^+$	Product Ion $[M+H]^+$	DP (Volts)	EP (Volts)	CE (Volts)	CXP (Volts)
3-oxo-C12-AHL	298.2	102.2	109	10.0	26.9	12.0
	298.2	197.2	109	10	20.9	20
C4-AHL	172.1	102.2	49.0	10.0	12.0	13.0
	172.1	71.1	49.0	10.0	12.0	13.0
C7 HQ	260.0	188.0	290.0	14.0	42.1	10.0
	260.0	147.0	290.0	14.0	49.2	13.0
ND3	203.2	102.1	65.0	6.0	23.0	6.0
	203.2	74.1	65.0	6.0	20.0	7.0

Table S2 Neutral Loss (NL) and Product Ion (PI) scan methods parameters for AHL and HQ signalling molecules analysis. DP: Declustering potential; EP: Entrance Potential; CE: Collision Energy; CXP: Collision Cell Exit potential

Molecules family	MS Mode	Δm (Da)	DP (Volts)	EP (Volts)	CE start (Volts)	CE stop (Volts)	CXP start (Volts)	CXP stop (Volts)
AHL	NL	101.0	109.0	10.0	15.0	25.0	9.0	13.0
	PI	102.0	110.0	9.0	15.0	25.0	9.0	12.0
HQ	PI	175.0	110.0	9.0	35.0	45.0	9.0	11.0

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Table S3 Chemical formula, *m/z* ratio ($[M+H]^+$) and proposed structural protonated formula of detected AHL compounds with untargeted approach

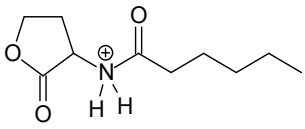
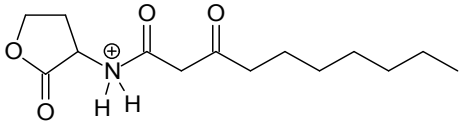
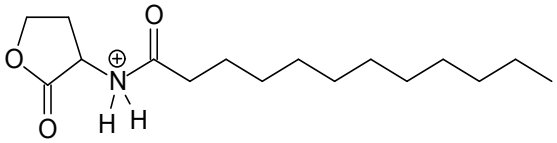
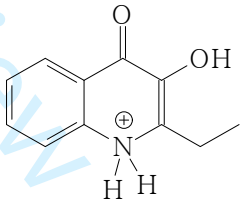
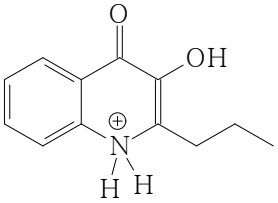
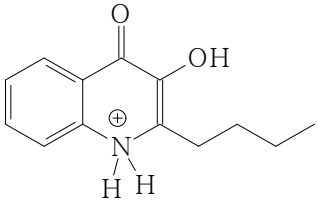
N° of carbon atoms	Compound	Chemical formula	$[M+H]^+$	Proposed chemical protonated structure
6	C_6-AHL	$C_{10}H_{17}NO_3$	200.1287	
10	3-oxo-C_{10}-AHL	$C_{14}H_{23}NO_4$;	270.1705	
12	C_{12}-AHL	$C_{16}H_{29}NO_3$;	284.2226	

Table S4 Chemical formula, *m/z* ratio ($[M+H]^+$) and proposed structural protonated formula of detected HQ compounds with untargeted approach. The structure of the species with an unsaturation on the acyl-chain (such as C6:1-HQ), due to the uncertainty of the position of the double bond along the chain, are not reported

N° of carbon atoms	Compound	Chemical formula	$[M+H]^+$	Proposed chemical structure
2	C_2-HQ	$C_{11}H_{11}NO_2$	190.0868	
3	C_3-HQ	$C_{12}H_{13}NO_2$	204.1024	
4	C_4-HQ	$C_{13}H_{15}NO_2$	218.1181	

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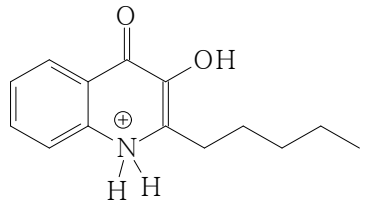
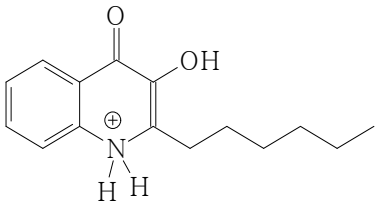
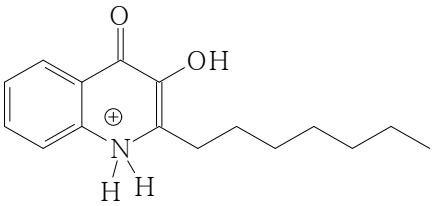
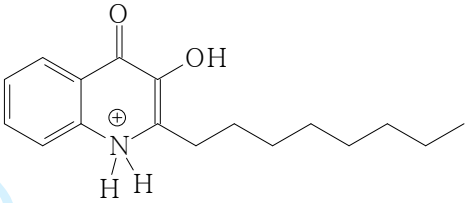
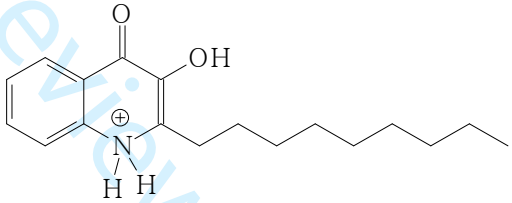
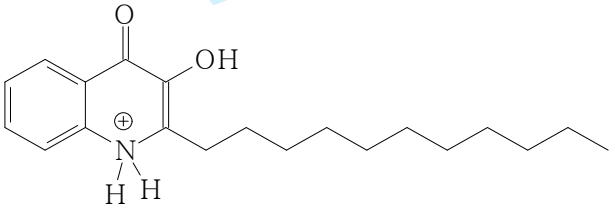
5	C_5-HQ	$C_{14}H_{17}NO_2$	232.1337	
6	C_6-HQ	$C_{15}H_{19}NO_2$	246.1494	
	$C_{6:1}$-HQ	$C_{15}H_{17}NO_2$	244.1337	-
7	C_7-HQ	$C_{16}H_{21}NO_2$	260.1650	
8	C_8-HQ	$C_{17}H_{23}NO_2$	274.1807	
	$C_{8:1}$-HQ	$C_{17}H_{21}NO_2$	272.1650	-
9	C_9-HQ	$C_{18}H_{25}NO_2$	288.1936	
	$C_{9:1}$-HQ	$C_{18}H_{23}NO_2$	286.1807	-
11	C_{11}-HQ	$C_{20}H_{29}NO_2$	316.2276	
	$C_{11:1}$-HQ	$C_{20}H_{27}NO_2$	314.2120	-

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Table S5 Results obtained from the study of the calibration models. The p-values obtained for the study of heteroscedasticity (F-test and Levene test), of the quadraticity (Partial F-test), and of the standardized residuals were considered significant if lower than 0.05 and reported in bold in the Table. The weights and the equations of the calibration models were obtained using an R routine

		Study of heteroscedasticity					
Analyte	Calibration range (µg/L)	F-test (p-value)[a]	Levene test (p-value)[a]	Weight	Partial F-test for quadraticity (p-value)[a]	Equation of the calibration curve	Normality of standardized residuals (p-value)[a]
3-oxo-C12-AHL, MRM	0.4-400	**1.2×10^{-9}**	**3.0×10^{-2}**	$1/x^2$	0.73	141900 x – 2609	0.99
3-oxo-C12-AHL, NL	1 – 300	**1.4×10^{-8}**	**2.5×10^{-2}**	$1/x^2$	0.68	490900 x + 152400	0.98
3-oxo-C12-AHL, PI	5 – 400	**7.3×10^{-8}**	**8.7×10^{-4}**	$1/x^2$	0.34	709400 x + 93520	0.71
C4-AHL, MRM	0.4 – 400	**2.7×10^{-9}**	**4.3×10^{-7}**	$1/x^2$	0.52	15520 x + 783.5	0.83
C4-AHL, NL	5 – 400	**6.0×10^{-6}**	7.2×10^{-1}	$1/x^2$	0.96	16740 x + 20230	0.99
C4-AHL, PI	1 – 300	**3.0×10^{-8}**	1.9×10^{-1}	$1/x^2$	0.28	151400 x + 2447	0.95
C7 HQ, MRM	0.4 – 200	**2.4×10^{-8}**	**8.5×10^{-11}**	$1/x^2$	0.53	15500 x + 594.3	0.29
C7 HQ, PI	1 – 300	**5.8×10^{-7}**	**1.7×10^{-8}**	$1/x^2$	0.80	146300 x + 96020	0.99

[a] 95% level of significance (p-value < 0.05)

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Table S6 Back calculation results

	Deviation (%)								
Analyte ↓| Concentration (ppb) →	**0.4**	**1**	**5**	**10**	**50**	**100**	**200**	**300**	**400**
3-oxo-C12-AHL, MRM	2	-5	0	2	11	8	0	-1	-2
3-oxo-C12-AHL, NL	\	\	4	-9	8	-5	1	-4	4
3-oxo-C12-AHL, PI	\	2	-10	-6	3	3	3	5	\
C4-AHL, MRM	-1	0	7	1	6	-4	-8	-1	0
C4-AHL, NL	\	\	3	-6	2	3	1	-4	1
C4-AHL, PI	\	-3	17	3	-4	-1	-5	-7	\
C7 HQ, MRM	-2	8	-10	-6	2	3	4	\	\
C7 HQ, PI	\	2	-10	-3	10	2	2	-2	\

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Analytical and Bioanalytical Chemistry

Electronic Supplementary Material

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

Federica Dal Bello, Michael Zorzi, Riccardo Aigotti, Davide Medica, Vito Fanelli, Vincenzo Cantaluppi, Eleonora Amante, Viviana Teresa Orlandi, Claudio Medana

For Peer Review

1. Figures

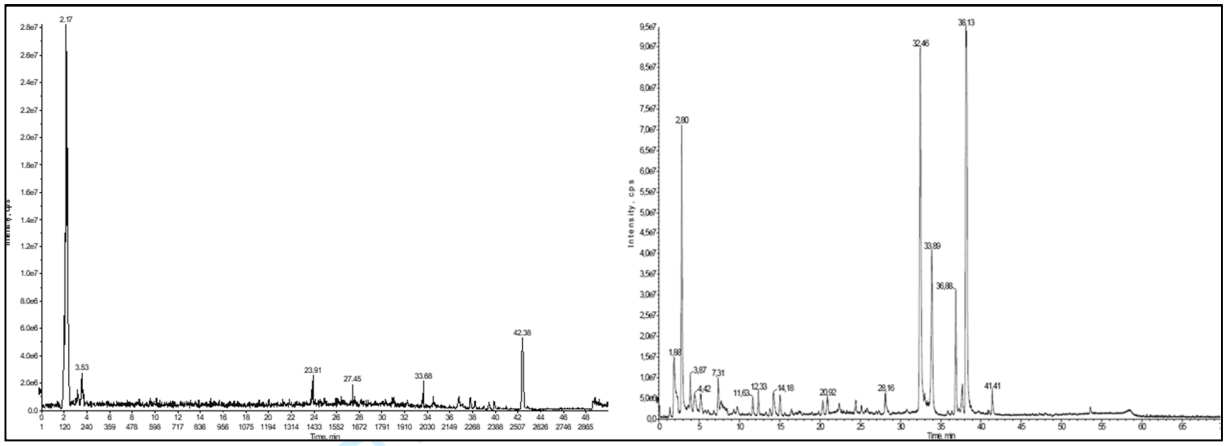

Fig. S1 Chromatograms acquired with NL MS approach for AHLs signalling molecules analysis. On the left it was presented the separation of few AHL compounds in a sample of patient plasma. As discussed in the main manuscript, since the sample was poor in AHL detection, the elution gradient was of 48 minutes and no overlapping peaks were observed. On the contrary, the right panel shows the NL AHLs separation in a sample of *Pseudomonas aeruginosa* wild type grown in Luria Bertani (LB) broth. Here, many AHL molecules were detected and the gradient separation was slower compared with plasma sample in order to obtain a satisfactory separation of peaks

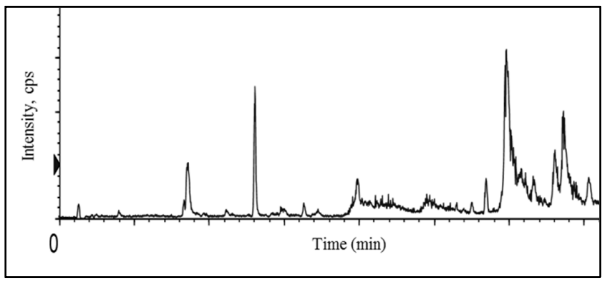

Fig. S2 Chromatogram acquired with PI MS approach for HQs signalling molecules analysis using 2-picolinic acid as aqueous mobile phase

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2. Validation tables and results

*The acronym AUC means "area under the curve".

2.1. 3-oxo-C12-AHL (N-(3-oxododecanoyl)-L-homoserine lactone)

2.1.1. Calibration curves AUC and equation

-MRM

Conc. (µg/L)	AUC cal. curve 1	AUC cal. curve 2	AUC cal. curve 3	AUC cal. curve 4
0.4	4.91E+04	4.81E+04	6.58E+04	5.75E+04
1	1.12E+05	1.19E+05	1.60E+05	1.40E+05
5	6.21E+05	7.30E+05	9.11E+05	5.80E+05
10	1.27E+06	1.48E+06	1.88E+06	1.12E+06
50	6.46E+06	7.94E+06	1.00E+07	7.06E+06
100	1.07E+07	1.30E+07	1.62E+07	1.25E+07
200	2.30E+07	2.84E+07	3.52E+07	2.67E+07
300	3.56E+07	4.25E+07	5.04E+07	40117103
400	4.56E+07	5.57E+07	6.81E+07	5.41E+07

Linear regressive analysis using a weighting factor of $1/x^2$: y= 141900x-2609

-NL

Conc. (µg/L)	AUC cal. curve 1	AUC cal. curve 2	AUC cal. curve 3	AUC cal. curve 4
5	2.67E+06	2.72E+06	2.69E+06	2.72E+06
10	4.12E+06	4.52E+06	4.88E+06	4.99E+06
50	2.31E+07	2.65E+07	2.80E+07	2.95E+07
100	4.03E+07	5.12E+07	4.83E+07	4.79E+07
200	9.90E+07	9.17E+07	9.99E+07	1.08E+08
300	1.41E+08	1.40E+08	1.41E+08	1.45E+08
400	1.90E+08	1.98E+08	2.14E+08	2.14E+08

Linear regressive analysis using a weighting factor of $1/x^2$: y= 490900x + 152400

-PI

Conc. (µg/L)	AUC cal. curve 1	AUC cal. curve 2	AUC cal. curve 3	AUC cal. curve 4
1	8.40E+05	8.57E+05	9.19E+05	6.66E+05
5	3.55E+06	7.69E+05	4.71E+06	4.13E+06
10	7.33E+06	5.67E+06	7.45E+06	6.54E+06
50	3.47E+07	3.59E+07	40318826	3.59E+07
100	7.27E+07	6.98E+07	78447022	7.07E+07
200	1.41E+08	1.37E+08	1.54E+08	1.50E+08
300	1.83E+08	2.30E+08	2.58E+08	2.25E+08

Linear regressive analysis using a weighting factor of $1/x^2$: y= 709400x + 93520

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2.1.2. Selectivity (SEL%)

- MRM

Sample	AUC	Average AUC	SEL%	8.12
QS-free_matrix_001	3.45E+03			
QS-free_matrix_002	4.67E+03	4.41E+03		
QS-free_matrix_003	5.12E+03			
STD_400 ppt_001	4.91E+04			
STD_400 ppt_002	4.81E+04	5.43E+04		
STD_400 ppt_003	6.58E+04			

-NL

Sample	AUC	Average AUC	SEL%	0.50
QS-free_matrix_001	1.14E+04			
QS-free_matrix_002	1.54E+04	1.34E+04		
QS-free_matrix_003	1.33E+04			
STD_5 ppb_001	2.67E+06			
STD_5 ppb_002	2.72E+06	2.69E+06		
STD_5 ppb_003	2.69E+06			

-PI

Sample	AUC	Average AUC	SEL%	1.10
QS-free_matrix_001	9.94E+03			
QS-free_matrix_002	8.77E+03	9.58E+03		
QS-free_matrix_003	1.00E+04			
STD_1 ppb_001	8.40E+05			
STD_1 ppb_002	8.57E+05	8.72E+05		
STD_1 ppb_003	9.19E+05			

2.1.3. Recovery (REC%)

- MRM

Sample	AUC	Average AUC
STD_0.4ppb_solv_01	1.26E+05	1.09E+05
STD_0.4ppb_solv_02	9.87E+04	
STD_0.4ppb_solv_03	1.02E+05	
STD_400ppb_solv_01	1.02E+08	9.87E+07
STD_400ppb_solv_02	9.74E+07	
STD_400ppb_solv_03	9.63E+07	
STD_0.4ppb_pls_01	5.00E+04	4.95E+04
STD_0.4ppb_pls_02	5.00E+04	

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STD_0.4ppb_pls_03	4.86E+04	
STD_400ppb_pls_01	5.34E+07	5.30E+07
STD_400ppb_pls_02	5.54E+07	
STD_400ppb_pls_03	5.03E+07	
REC%	**LLOQ**	**45.5**
	ULOQ	**53.7**

2.1.4. Carry-over (CO%)

	Sample	**AUC**		
MRM	STD_400 ppb	4.56E+07	**CO%**	**7.10**
	QS-free_matrix	4.67E+03		
	STD_400 ppt	6.58E+04		
NL	STD_400 ppb	1.90E+08	**CO%**	**0.57**
	QS-free_matrix	1.54E+04		
	STD_5 ppb	2.69E+06		
PI	STD_300 ppb	1.83E+08	**CO%**	**1.29**
	QS-free_matrix	9.94E+03		
	STD_1 ppb	7.69E+05		

2.1.5. Intra-day accuracy (BIAS%) and precision (CV%)

-MRM

Nominal conc. (µg/L)	Real conc. (back-calculated, µg/L)				CV%	BIAS%
	Cal. curve 1	Cal. curve 2	Cal. curve 3	Cal. curve 4		
0.4	0.36	0.36	0.48	0.42	12.4	1.65
1	0.81	0.85	1.15	1.00	14.0	4.92
5	4.39	5.16	6.44	4.11	18.0	0.50
10	9.00	10.5	13.3	7.88	20.0	1.51
50	45.5	56.0	70.6	49.8	17.1	9.88
100	75.5	91.8	114	88.2	15.1	8.16
200	162	200	248	189	15.6	0.15
300	251	300	355	283	12.8	0.96
400	322	392	480	381	14.3	1.61

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-NL

Nominal conc. (µg/L)	Real conc. (back-calculated, µg/L)				CV%	BIAS%
	Cal. curve 1	Cal. curve 2	Cal. curve 3	Cal. curve 4		
5	5.13	5.23	5.17	5.24	0.87	3.66
10	8.07	8.90	9.63	9.85	7.64	9.72
50	46.8	53.6	56.6	59.8	8.87	7.77
100	81.8	104	98.0	97.2	8.60	4.99
200	201	187	203	221	5.96	1.45
300	287	284	287	296	1.53	4.00
400	387	404	435	435	4.98	3.63

-PI

Nominal conc. (µg/L)	Real conc. (back-calculated, µg/L)				CV%	BIAS%
	Cal. curve 1	Cal. curve 2	Cal. curve 3	Cal. curve 4		
1	1.05	1.08	1.16	0.81	12.9	2.40
5	4.88	0.95	6.51	5.69	47.3	10.9
10	10.2	7.86	10.4	9.09	10.8	6.63
50	48.7	50.5	56.7	50.4	5.91	3.05
100	102	98.3	111	99.5	4.62	2.56
200	199	193	217	211	4.62	2.51
300	258	324	364	317	12.0	4.97

2.1.6. LOD and LOQ

-MRM

Sample	**AUC**		
QS-free_matrix_001	3.45E+03		
QS-free_matrix_002	4.67E+03		
QS-free_matrix_003	5.12E+03		
QS-free_matrix_004	5.02E+03		
QS-free_matrix_005	4.11E+03	Slope	1.42E+05
QS-free_matrix_006	4.53E+03	Intercept	-2.61E+03
Average AUC	4.48E+03		
Std. dev QS-free matrix	5.69E+02		**Conc. (µg/L)**
10std. Dev. + Average AUC	1.02E+04	**LOQ**	**0.090**

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-NL

Sample	AUC		
QS-free_matrix_001	4.58E+03		
QS-free_matrix_002	4.69E+03		
QS-free_matrix_003	4.82E+03		
QS-free_matrix_004	3.52E+03		
QS-free_matrix_005	4.11E+03	Slope	4.91E+05
QS-free_matrix_006	4.53E+03	Intercept	1.52E+05
Average AUC	4.37E+03		
Std. dev QS-free matrix	4.42E+02		**Conc. (µg/L)**
10std. Dev. + Average AUC	8.80E+03	**LOQ**	**0.293**

-PI

Sample	AUC		
QS-free_matrix_001	3.45E+03		
QS-free_matrix_002	4.67E+03		
QS-free_matrix_003	5.12E+03		
QS-free_matrix_004	5.02E+03		
QS-free_matrix_005	4.11E+03	Slope	7.09E+05
QS-free_matrix_006	4.53E+03	Intercept	9.35E+04
Average AUC	4.48E+03		
Std. dev QS-free matrix	5.69E+02		**Conc. (µg/L)**
10std. Dev. + Average AUC	1.02E+04	**LOQ**	**0.117**

2.1.7. Freeze-thaw stability (STAB%)

-MRM

	AUC T_0	AUC $T_{freeze\text{-}thaw}$	Conc. (µg/L) T_0	Conc. (µg/L) $T_{freeze\text{-}thaw}$	STAB%	AVAREGE STAB%
LLOQ (0.4 µg/L)	1.23E+05	1.23E+05	0.88	0.88	100	106
	5.54E+04	4.81E+04	0.41	0.36	115	
	5.43E+04	5.57E+04	0.40	0.41	102	
ULOQ (400 µg/L)	5.54E+07	5.63E+07	390	397	102	101
	5.55E+07	5.58E+07	392	393	100	
	5.60E+07	5.68E+07	394	400	101	

For Peer Review

-NL

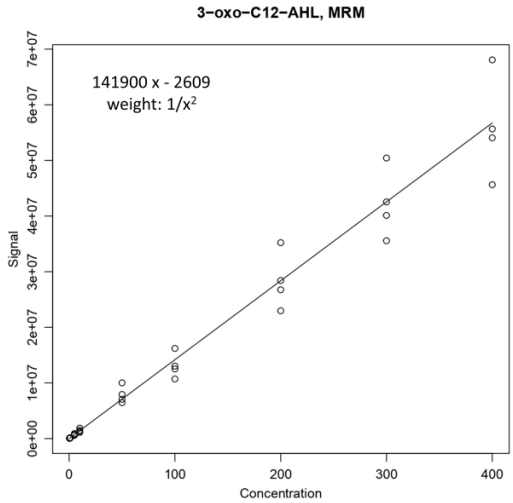
	AUC T_0	AUC $T_{freeze-thaw}$	Conc. (µg/L) T_0	Conc. (µg/L) $T_{freeze-thaw}$	STAB%	AVAREGE STAB%
LLOQ (5 µg/L)	2.78E+06	2.41E+06	5.35	4.60	86.0	89.5
	2.61E+06	2.59E+06	5.01	4.96	98.9	
	2.61E+06	2.21E+06	5.01	4.18	83.5	
ULOQ (400 µg/L)	1.98E+08	1.80E+08	403	367	91.1	88.6
	2.05E+08	1.82E+08	417	371	88.9	
	2.06E+08	1.77E+08	420	360	85.8	

-PI

	AUC T_0	AUC $T_{freeze-thaw}$	Conc. (µg/L) T_0	Conc. (µg/L) $T_{freeze-thaw}$	STAB%	AVAREGE STAB%
LLOQ (1 µg/L)	9.88E+05	7.20E+05	1.26	0.88	70.0	86.0
	6.59E+05	6.61E+05	0.80	0.80	100	
	8.14E+05	7.24E+05	1.02	0.89	87.6	
ULOQ (300 µg/L)	2.23E+08	2.13E+08	315	300	95.2	90.2
	1.99E+08	1.86E+08	281	261	93.2	
	1.87E+08	1.54E+08	264	217	82.2	

2.1.8. Figure of average of four calibration curves

-MRM



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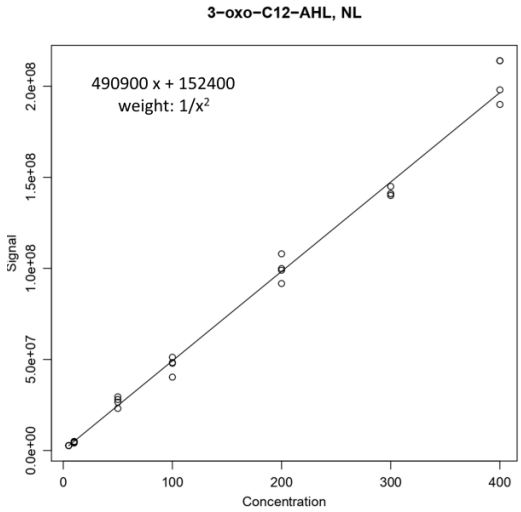
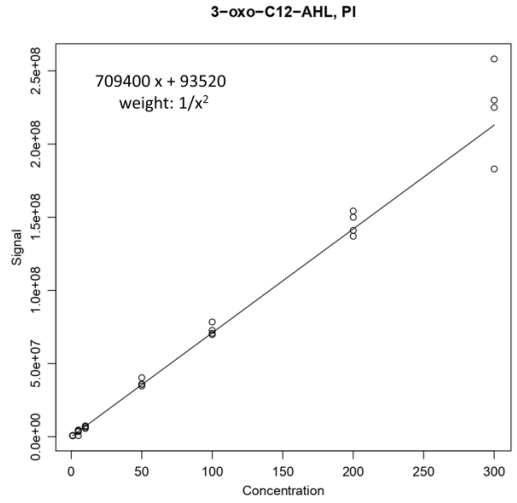
-NL



-PI



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2.2. C4-AHL (N-butanoyl-L-homoserine lactone)

2.2.1. Calibration curves AUC and equation

-MRM

Conc. (µg/L)	AUC cal. curve 1	AUC cal. curve 2	AUC cal. curve 3	AUC cal. curve 4
0.4	5.78E+03	6.18E+03	8.73E+03	7.13E+03
1	1.28E+04	1.40E+04	2.08E+04	1.75E+04
5	9.27E+04	1.00E+05	6.08E+04	8.16E+04
10	1.42E+05	1.48E+05	1.79E+05	1.61E+05
50	7.10E+05	7.58E+05	8.93E+05	9.20E+05
100	1.29E+06	1.44E+06	1.64E+06	1.59E+06
200	2.43E+06	2.89E+06	3.54E+06	2.58E+06
300	3.77E+06	4.18E+06	5.53E+06	5.03E+06
400	4.89E+06	5.68E+06	7.38E+06	6.83E+06

Linear regressive analysis using a weighting factor of $1/x^2$: y= 15520x + 783.5

-NL

Conc. (µg/L)	AUC cal. curve 1	AUC cal. curve 2	AUC cal. curve 3	AUC cal. curve 4
5	1.00E+05	7.95E+04	1.39E+05	1.06E+05
10	1.52E+05	1.52E+05	2.28E+05	1.77E+05
50	7.58E+05	7.22E+05	1.14E+06	8.73E+05
100	1.44E+06	1.40E+06	2.42E+06	1.75E+06
200	2.89E+06	2.91E+06	4.41E+06	3.40E+06
300	4.18E+06	4.11E+06	6.29E+06	4.86E+06
400	5.68E+06	5.55E+06	9.07E+06	6.77E+06

Linear regressive analysis using a weighting factor of $1/x^2$: y= 16740x + 20230

-PI

Conc. (µg/L)	AUC cal. curve 1	AUC cal. curve 2	AUC cal. curve 3	AUC cal. curve 4
1	1.68E+05	1.61E+05	1.16E+05	1.49E+05
5	8.05E+05	1.31E+06	5.64E+05	8.64E+05
10	1.34E+06	2.12E+06	1.24E+06	1.55E+06
50	7.62E+06	8.29E+06	5.21E+06	7.91E+06
100	1.50E+07	1.61E+07	1.11E+07	1.79E+07
200	3.02E+07	2.98E+07	2.22E+07	3.32E+07
300	4.47E+07	4.44E+07	2.99E+07	5.09E+07

Linear regressive analysis using a weighting factor of $1/x^2$: y= 151400x + 2447

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2.2.2. Selectivity (SEL%)

- MRM

Sample	AUC	Average AUC	SEL%	5.65
QS-free_matrix_001	4.82E+02			
QS-free_matrix_002	4.85E+02	7.44E+02		
QS-free_matrix_003	1.27E+03			
STD_400 ppt_001	1.88E+04			
STD_400 ppt_002	1.20E+04	1.32E+04		
STD_400 ppt_003	8.73E+03			

-NL

Sample	AUC	Average AUC	SEL%	1.63
QS-free_matrix_001	1.75E+03			
QS-free_matrix_002	1.65E+03	1.76E+03		
QS-free_matrix_003	1.88E+03			
STD_5 ppb_001	7.95E+04			
STD_5 ppb_002	1.39E+05	1.08E+05		
STD_5 ppb_003	1.06E+05			

-PI

Sample	AUC	Average AUC	SEL%	2.54
QS-free_matrix_001	5.94E+03			
QS-free_matrix_002	4.77E+03	5.58E+03		
QS-free_matrix_003	6.03E+03			
STD_1 ppb_001	7.64E+04			
STD_1 ppb_002	1.71E+05	2.20E+05		
STD_1 ppb_003	4.13E+05			

For Peer Review

2.2.3. Recovery (REC%)

Sample	AUC	Average AUC
STD_0.4ppb_solv_01	1.59E+04	1.42E+04
STD_0.4ppb_solv_02	1.42E+04	
STD_0.4ppb_solv_03	1.26E+04	
STD_400ppb_solv_01	1.59E+07	1.49E+07
STD_400ppb_solv_02	1.24E+07	
STD_400ppb_solv_03	1.63E+07	
STD_0.4ppb_pls_01	6.45E+03	7.26E+03
STD_0.4ppb_pls_02	7.86E+03	
STD_0.4ppb_pls_03	7.46E+03	
STD_400ppb_pls_01	1.03E+07	9.05E+06
STD_400ppb_pls_02	7.24E+06	
STD_400ppb_pls_03	9.65E+06	
REC%	**LLOQ**	**51.0**
	ULOQ	**60.9**

2.2.4. Carry-over (CO%)

	Sample	**AUC**		
MRM	STD_400 ppb	6.93E+06	**CO%**	**8.90**
	QS-free_matrix	4.82E+02		
	STD_400 ppt	5.41E+03		
NL	STD_400 ppb	6.77E+06	**CO%**	**1.65**
	QS-free_matrix	1.65E+03		
	STD_5 ppb	1.00E+05		
PI	STD_300 ppb	1.26E+05	**CO%**	**0.87**
	QS-free_matrix	1.27E+03		
	STD_1 ppb	1.46E+05		

For Peer Review

2.2.5. Intra-day accuracy (BIAS%) and precision (CV%)

-MRM

Nominal conc. (µg/L)	Real conc. (back-calculated, µg/L)				CV%	BIAS%
	Cal. curve 1	Cal. curve 2	Cal. curve 3	Cal. curve 4		
0.4	0.32	0.35	0.51	0.41	18.4	0.61
1	0.77	0.85	1.29	1.08	20.2	0.03
5	5.93	6.39	3.87	5.20	17.8	6.49
10	9.12	9.49	11.5	10.3	8.93	0.96
50	45.7	48.8	57.5	59.2	10.8	5.32
100	83.2	92.4	105	102	9.05	4.43
200	157	186	228	166	14.9	8.57
300	243	270	357	324	15.0	0.61
400	315	366	475	440	15.6	0.23

-NL

Nominal conc. (µg/L)	Real conc. (back-calculated, µg/L)				CV%	BIAS%
	Cal. curve 1	Cal. curve 2	Cal. curve 3	Cal. curve 4		
5	4.76	3.54	7.11	5.14	25.0	2.72
10	7.87	7.87	12.4	9.38	19.7	6.64
50	44.1	41.9	66.8	50.9	19.1	1.84
100	84.5	82.1	143	103	23.7	3.19
200	171	173	262	202	18.2	1.03
300	249	245	375	289	18.1	3.67
400	338	331	541	403	20.9	0.80

-PI

Nominal conc. (µg/L)	Real conc. (back-calculated, µg/L)				CV%	BIAS%
	Cal. curve 1	Cal. curve 2	Cal. curve 3	Cal. curve 4		
1	1.10	1.05	0.75	0.97	13.7	3.59
5	5.30	8.63	3.71	5.69	30.5	14.3
10	8.83	14.0	8.19	10.2	21.8	2.97
50	50.3	54.8	34.4	52.3	16.6	4.32
100	99.3	106	73.4	118	16.6	0.67
200	199	197	147	220	14.0	4.97
300	295	293	198	336	18.1	6.98

For Peer Review

2.2.6. LOD and LOQ

-MRM

Sample	AUC		
QS-free_matrix_001	4.82E+02		
QS-free_matrix_002	4.85E+02		
QS-free_matrix_003	1.27E+03		
QS-free_matrix_004	4.72E+02		
QS-free_matrix_005	1.27E+03	Slope	1.55E+04
QS-free_matrix_006	1.36E+03	Intercept	7.84E+02
Average AUC	8.88E+02		
Std. dev QS-free matrix	4.10E+02		**Conc. (µg/L)**
10std. Dev. + Average AUC	4.99E+03	**LOQ**	**0.271**

-NL

Sample	AUC		
QS-free_matrix_001	4.82E+02		
QS-free_matrix_002	4.85E+02		
QS-free_matrix_003	1.27E+03		
QS-free_matrix_004	1.36E+03		
QS-free_matrix_005	3.65E+03	Slope	1.67E+04
QS-free_matrix_006	4.02E+02	Intercept	2.02E+04
Average AUC	1.27E+03		
Std. dev QS-free matrix	1.13E+03		**Conc. (µg/L)**
10std. Dev. + Average AUC	1.26E+04	**LOQ**	**0.457**

-PI

Sample	AUC		
QS-free_matrix_001	1.27E+03		
QS-free_matrix_002	1.36E+03		
QS-free_matrix_003	3.65E+03		
QS-free_matrix_004	4.02E+02		
QS-free_matrix_005	2.47E+02	Slope	1.51E+05
QS-free_matrix_006	1.25E+03	Intercept	2.45E+03
Average AUC	1.36E+03		
Std. dev QS-free matrix	1.11E+03		**Conc. (µg/L)**
10std. Dev. + Average AUC	1.25E+04	**LOQ**	**0.066**

For Peer Review

2.2.7. Freeze-thaw stability (STAB%)

-MRM

	AUC T_0	AUC $T_{freeze-thaw}$	Conc. (µg/L) T_0	Conc. (µg/L) $T_{freeze-thaw}$	STAB%	AVAREGE STAB%
LLOQ (0.4 µg/L)	7.24E+03	6.59E+03	0.42	0.37	90.0	97.3
	6.72E+03	6.95E+03	0.38	0.40	104	
	7.27E+03	7.13E+03	0.42	0.41	97.9	
ULOQ (400 µg/L)	6.09E+06	6.32E+06	392	407	104	102
	6.16E+06	6.19E+06	37	399	100	
	6.18E+06	6.23E+06	398	401	101	

-NL

	AUC T_0	AUC $T_{freeze-thaw}$	Conc. (µg/L) T_0	Conc. (µg/L) $T_{freeze-thaw}$	STAB%	AVAREGE STAB%
LLOQ (5 µg/L)	9.10E+04	8.02E+04	4.23	3.58	84.8	91.1
	8.93E+04	9.30E+04	4.13	4.34	105	
	9.22E+04	8.01E+04	4.30	3.58	83.2	
ULOQ (400 µg/L)	6.84E+06	5.91E+06	407	352	86.4	86.9
	6.79E+06	5.91E+06	404	352	87.0	
	6.83E+06	5.95E+06	407	354	87.1	

-PI

	AUC T_0	AUC $T_{freeze-thaw}$	Conc. (µg/L) T_0	Conc. (µg/L) $T_{freeze-thaw}$	STAB%	AVAREGE STAB%
LLOQ (1 µg/L)	1.56E+05	1.41E+05	1.01	0.92	90.3	88.9
	1.61E+05	1.40E+05	1.04	0.91	86.9	
	1.60E+05	1.44E+05	1.04	0.93	89.6	
ULOQ (300 µg/L)	4.27E+07	4.07E+07	282	269	95.4	95.5
	4.26E+07	3.99E+07	281	263	93.6	
	4.22E+07	4.11E+07	278	272	97.6	

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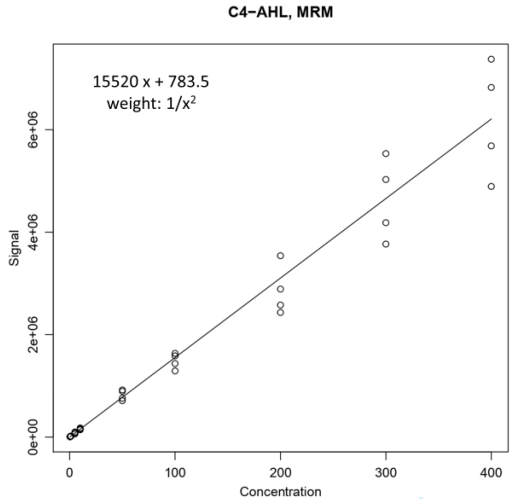
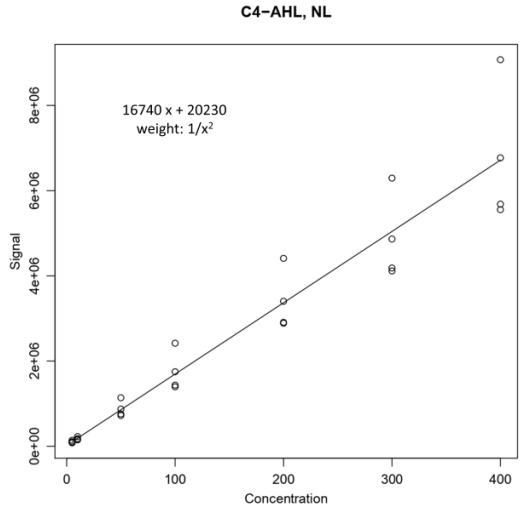
2.2.8. Figure of average of four calibration curves

-MRM

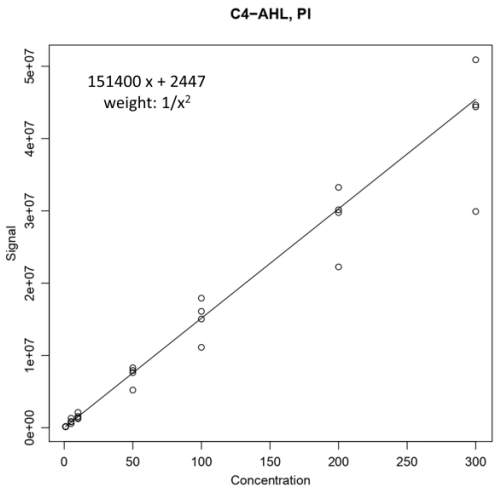


-NL



Peer Review

-PI



2.3. C7 HQ (2-heptyl-4-hydroxyquinoline)

2.3.1. Calibration curves AUC and equation

-MRM

Conc. (µg/L)	AUC cal. curve 1	AUC cal. curve 2	AUC cal. curve 3	AUC cal. curve 4
0.4	8.02E+03	5.06E+03	5.07E+03	8.51E+03
1	1.83E+04	1.63E+04	1.14E+04	2.31E+04
5	8.53E+04	5.54E+04	6.20E+04	8.01E+04
10	1.99E+05	1.19E+05	1.21E+05	1.46E+05
50	9.01E+05	6.42E+05	6.58E+05	9.74E+05
100	1.81E+06	1.30E+06	1.28E+06	2.02E+06
200	3.72E+06	2.65E+06	2.51E+06	4.06E+06

Linear regressive analysis using a weighting factor of $1/x^2$: y= 15500x + 594.3

-PI

Conc. (µg/L)	AUC cal. curve 1	AUC cal. curve 2	AUC cal. curve 3	AUC cal. curve 4
1	2.56E+05	2.27E+05	2.69E+05	2.30E+05
5	8.24E+05	7.36E+05	6.35E+05	8.15E+05
10	1.69E+06	1.24E+06	1.64E+06	1.47E+06
50	8.32E+06	8.00E+06	7.98E+06	8.12E+06
100	1.43E+07	1.62E+07	1.55E+07	1.43E+07
200	2.95E+07	3.05E+07	3.05E+07	2.90E+07
300	4.47E+07	4.62E+07	4.12E+07	4.00E+07

Linear regressive analysis using a weighting factor of $1/x^2$: y= 146300 x + 96020

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2.3.2. Selectivity (SEL%)

- MRM

Sample	AUC	Average AUC	SEL%	16.5
QS-free_matrix_001	1.01E+03			
QS-free_matrix_002	1.37E+03	1.19E+03		
QS-free_matrix_003	1.19E+03			
STD_400 ppt_001	8.02E+03			
STD_400 ppt_002	5.07E+03	7.20E+04		
STD_400 ppt_003	8.51E+03			

-PI

Sample	AUC	Average AUC	SEL%	4.73
QS-free_matrix_001	9.14E+03			
QS-free_matrix_002	1.13E+04	1.08E+04		
QS-free_matrix_003	1.20E+04			
STD_1 ppb_001	1.98E+05			
STD_1 ppb_002	2.57E+05	2.29E+05		
STD_1 ppb_003	2.31E+05			

2.3.3. Recovery (REC%)

Sample	AUC	Average AUC
STD_0.4ppb_solv_01	1.52E+04	1.26E+04
STD_0.4ppb_solv_02	1.13E+04	
STD_0.4ppb_solv_03	1.12E+04	
STD_200ppb_solv_01	1.12E+07	1.03E+07
STD_200ppb_solv_02	9.86E+06	
STD_200ppb_solv_03	9.74E+06	
STD_0.4ppb_pls_01	5.98E+03	6.28E+03
STD_0.4ppb_pls_02	6.85E+03	
STD_0.4ppb_pls_03	6.01E+03	
STD_200ppb_pls_01	3.65E+06	3.52E+06
STD_200ppb_pls_02	2.95E+06	
STD_200ppb_pls_03	3.95E+06	
REC%	**LLOQ**	**49.9**
	ULOQ	**34.3**

For Peer Review

2.3.4. Carry-over (CO%)

	Sample	AUC		
MRM	STD_200 ppb	2.59E+06	**CO%**	**16.1**
	QS-free_matrix	1.37E+03		
	STD_400 ppt	8.51E+03		
PI	STD_300 ppb	4.35E+07	**CO%**	**2.98**
	QS-free_matrix	7.04E+03		
	STD_1 ppb	2.36E+05		

2.3.5. Intra-day accuracy (BIAS%) and precision (CV%)

-MRM

Nominal conc. (µg/L)	Real conc. (back-calculated, µg/L)				CV%	BIAS%
	Cal. curve 1	Cal. curve 2	Cal. curve 3	Cal. curve 4		
0.4	0.48	0.29	0.29	0.51	26.5	2.11
1	1.14	1.01	0.70	1.45	25.3	7.09
5	5.46	3.54	3.96	5.13	17.6	10.5
10	12.8	7.61	7.80	9.36	22.3	6.41
50	58.1	41.4	42.4	62.8	18.4	2.27
100	117	83.8	82.6	130	20.1	3.21
200	240	171	162	262	20.7	4.14

-PI

Nominal conc. (µg/L)	Real conc. (back-calculated, µg/L)				CV%	BIAS%
	Cal. curve 1	Cal. curve 2	Cal. curve 3	Cal. curve 4		
1	1.10	0.89	1.19	0.92	11.9	2.28
5	4.97	4.38	3.68	4.91	11.6	11.5
10	10.9	7.80	10.6	9.36	12.6	3.55
50	56.2	54.0	53.9	54.9	1.72	8.68
100	97.1	110	105	97.3	5.34	2.34
200	201	208	208	197	2.19	1.68
300	305	315	281	273	5.84	2.27

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2.3.6. LOD and LOQ

-MRM

Sample	AUC		
QS-free_matrix_001	1.43E+03		
QS-free_matrix_002	1.01E+03		
QS-free_matrix_003	9.14E+02		
QS-free_matrix_004	1.13E+03		
QS-free_matrix_005	1.20E+03	Slope	1.55E+04
QS-free_matrix_006	9.03E+02	Intercept	5.94E+02
Average AUC	1.10E+03		
Std. dev QS-free matrix	1.83E+02		**Conc. (µg/L)**
10std. Dev. + Average AUC	2.93E+03	**LOQ**	**0.151**

-PI

Sample	AUC		
QS-free_matrix_001	1.20E+04		
QS-free_matrix_002	7.04E+03		
QS-free_matrix_003	1.37E+04		
QS-free_matrix_004	1.51E+04		
QS-free_matrix_005	1.46E+04	Slope	1.46E+05
QS-free_matrix_006	1.38E+04	Intercept	9.60E+04
Average AUC	1.27E+04		
Std. dev QS-free matrix	2.71E+03		**Conc. (µg/L)**
10std. Dev. + Average AUC	3.98E+04	**LOQ**	**0.385**

2.3.7. Freeze-thaw stability (STAB%)

-MRM

	AUC T_0	AUC $T_{freeze\text{-}thaw}$	Conc. (µg/L) T_0	Conc. (µg/L) $T_{freeze\text{-}thaw}$	STAB%	AVAREGE STAB%
LLOQ (0.4 µg/L)	8.02E+03	8.51E+03	0.48	0.51	107	115
	5.07E+03	6.67E+03	0.29	0.39	136	
	7.06E+03	7.25E+03	0.42	0.43	103	
ULOQ (200 µg/L)	3.72E+06	4.06E+06	240	262	109	118
	2.51E+06	3.23E+06	161	209	129	
	2.65E+06	3.04E+06	171	196	115	

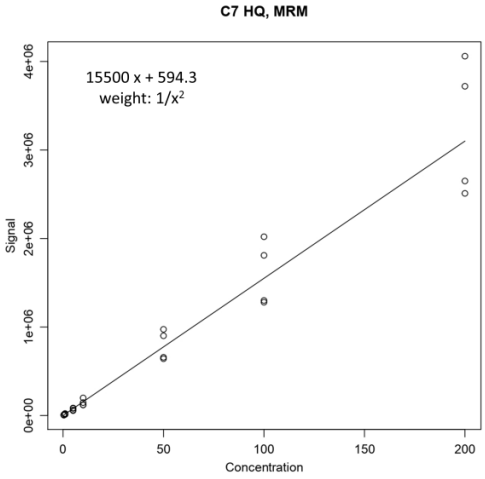
For Peer Review

-PI

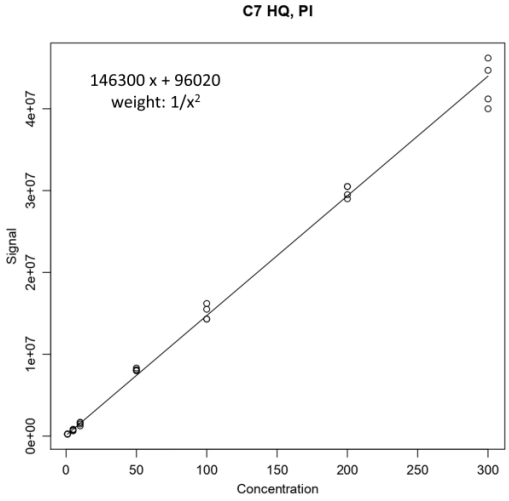
	AUC T_0	AUC $T_{freeze-thaw}$	Conc. (µg/L) T_0	Conc. (µg/L) $T_{freeze-thaw}$	STAB%	AVAREGE STAB%
LLOQ (1 µg/L)	2.98E+05	2.65E+05	1.38	1.16	83.7	90.2
	2.12E+05	1.99E+05	0.79	0.70	88.8	
	3.01E+05	2.97E+05	1.40	1.37	98.1	
ULOQ (300 µg/L)	4.23E+07	3.99E+07	289	272	94.3	96.2
	4.98E+07	4.59E+07	340	313	92.1	
	4.03E+07	4.12E+07	275	281	102	

2.3.8. Figure of average of four calibration curves

-MRM



-PI



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3. Tables

Table S1 Multiple Reaction Monitoring (MRM) scan parameters and selected transition for AHLs and HQs analysis (bolded transitions were used as quantitative ones). DP: Declustering Potential; EP: Entrance Potential; CE: Collision Energy; CXP: Collision Cell Exit Potential

Analyte	Precursor ion $[M+H]^+$	Product Ion $[M+H]^+$	DP (Volts)	EP (Volts)	CE (Volts)	CXP (Volts)
3-oxo-C12-AHL	298.2	102.2	109	10.0	26.9	12.0
	298.2	197.2	109	10	20.9	20
C4-AHL	172.1	102.2	49.0	10.0	12.0	13.0
	172.1	71.1	49.0	10.0	12.0	13.0
C7 HQ	260.0	188.0	290.0	14.0	42.1	10.0
	260.0	147.0	290.0	14.0	49.2	13.0
ND3	203.2	102.1	65.0	6.0	23.0	6.0
	203.2	74.1	65.0	6.0	20.0	7.0

Table S2 Neutral Loss (NL) and Product Ion (PI) scan methods parameters for AHL and HQ signalling molecules analysis. DP: Declustering potential; EP: Entrance Potential; CE: Collision Energy; CXP: Collision Cell Exit potential

Molecules family	MS Mode	Δm (Da)	DP (Volts)	EP (Volts)	CE start (Volts)	CE stop (Volts)	CXP start (Volts)	CXP stop (Volts)
AHL	NL	101.0	109.0	10.0	15.0	25.0	9.0	13.0
	PI	102.0	110.0	9.0	15.0	25.0	9.0	12.0
HQ	PI	175.0	110.0	9.0	35.0	45.0	9.0	11.0

For Peer Review

Table S3 Chemical formula, *m/z* ratio ($[M+H]^+$) and proposed structural protonated formula of detected AHL compounds with untargeted approach

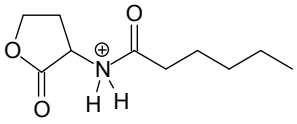
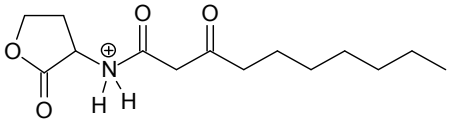
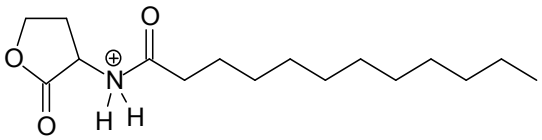
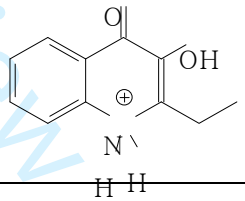
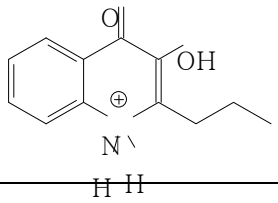
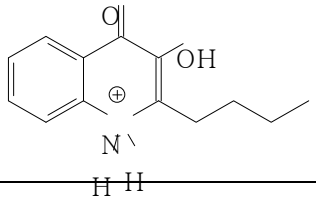
N° of carbon atoms	Compound	Chemical formula	$[M+H]^+$	Proposed chemical protonated structure
6	C_6-AHL	$C_{10}H_{17}NO_3$	200.1287	
10	3-oxo-C_{10}-AHL	$C_{14}H_{23}NO_4$;	270.1705	
12	C_{12}-AHL	$C_{16}H_{29}NO_3$;	284.2226	

Table S4 Chemical formula, *m/z* ratio ($[M+H]^+$) and proposed structural protonated formula of detected HQ compounds with untargeted approach. The structure of the species with an unsaturation on the acyl-chain (such as C6:1-HQ), due to the uncertainty of the position of the double bond along the chain, are not reported

N° of carbon atoms	Compound	Chemical formula	$[M+H]^+$	Proposed chemical structure
2	C_2-HQ	$C_{11}H_{11}NO_2$	190.0868	
3	C_3-HQ	$C_{12}H_{13}NO_2$	204.1024	
4	C_4-HQ	$C_{13}H_{15}NO_2$	218.1181	

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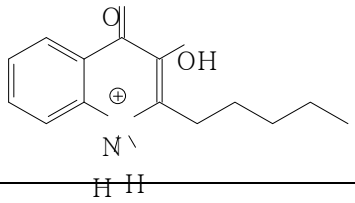
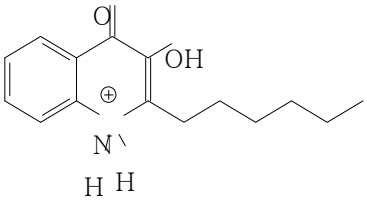
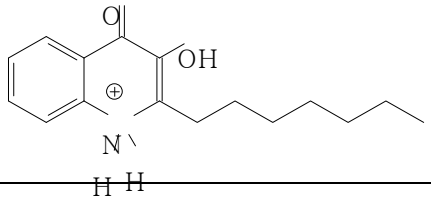
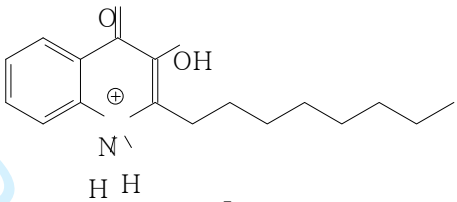
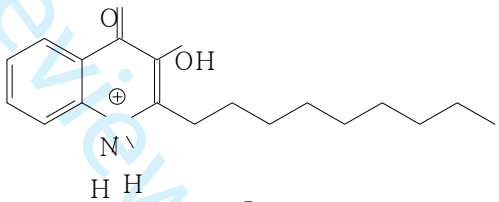
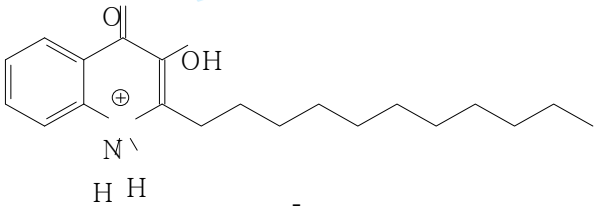
5	C_5-HQ	$C_{14}H_{17}NO_2$	232.1337	
6	C_6-HQ	$C_{15}H_{19}NO_2$	246.1494	
	$C_{6:1}$-HQ	$C_{15}H_{17}NO_2$	244.1337	-
7	C_7-HQ	$C_{16}H_{21}NO_2$	260.1650	
8	C_8-HQ	$C_{17}H_{23}NO_2$	274.1807	
	$C_{8:1}$-HQ	$C_{17}H_{21}NO_2$	272.1650	-
9	C_9-HQ	$C_{18}H_{25}NO_2$	288.1936	
	$C_{9:1}$-HQ	$C_{18}H_{23}NO_2$	286.1807	-
11	C_{11}-HQ	$C_{20}H_{29}NO_2$	316.2276	
	$C_{11:1}$-HQ	$C_{20}H_{27}NO_2$	314.2120	-

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Table S5 Results obtained from the study of the calibration models. The p-values obtained for the study of heteroscedasticity (F-test and Levene test), of the quadraticity (Partial F-test), and of the standardized residuals were considered significant if lower than 0.05 and reported in bold in the Table. The weights and the equations of the calibration models were obtained using an R routine

		Study of heteroscedasticity					
Analyte	Calibration range (µg/L)	F-test (p-value)[a]	Levene test (p-value)[a]	Weight	Partial F-test for quadraticity (p-value)[a]	Equation of the calibration curve	Normality of standardized residuals (p-value)[a]
3-oxo-C12-AHL, MRM	0.4-400	**1.2×10^{-9}**	**3.0×10^{-2}**	$1/x^2$	0.73	141900 x – 2609	0.99
3-oxo-C12-AHL, NL	1 – 300	**1.4×10^{-8}**	**2.5×10^{-2}**	$1/x^2$	0.68	490900 x + 152400	0.98
3-oxo-C12-AHL, PI	5 – 400	**7.3×10^{-8}**	**8.7×10^{-4}**	$1/x^2$	0.34	709400 x + 93520	0.71
C4-AHL, MRM	0.4 – 400	**2.7×10^{-9}**	**4.3×10^{-7}**	$1/x^2$	0.52	15520 x + 783.5	0.83
C4-AHL, NL	5 – 400	**6.0×10^{-6}**	7.2×10^{-1}	$1/x^2$	0.96	16740 x + 20230	0.99
C4-AHL, PI	1 – 300	**3.0×10^{-8}**	1.9×10^{-1}	$1/x^2$	0.28	151400 x + 2447	0.95
C7 HQ, MRM	0.4 – 200	**2.4×10^{-8}**	**8.5×10^{-11}**	$1/x^2$	0.53	15500 x + 594.3	0.29
C7 HQ, PI	1 – 300	**5.8×10^{-7}**	**1.7×10^{-8}**	$1/x^2$	0.80	146300 x + 96020	0.99

[a] 95% level of significance (p-value < 0.05)

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Table S6 Back calculation results

	Deviation (%)								
Analyte ↓| Concentration (ppb) →	**0.4**	**1**	**5**	**10**	**50**	**100**	**200**	**300**	**400**
3-oxo-C12-AHL, MRM	2	-5	0	2	11	8	0	-1	-2
3-oxo-C12-AHL, NL	\	\	4	-9	8	-5	1	-4	4
3-oxo-C12-AHL, PI	\	2	-10	-6	3	3	3	5	\
C4-AHL, MRM	-1	0	7	1	6	-4	-8	-1	0
C4-AHL, NL	\	\	3	-6	2	3	1	-4	1
C4-AHL, PI	\	-3	17	3	-4	-1	-5	-7	\
C7 HQ, MRM	-2	8	-10	-6	2	3	4	\	\
C7 HQ, PI	\	2	-10	-3	10	2	2	-2	\

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