Available online at www.sciencedirect.com
SCIENCE DIRECT®
ELSEVIER
Journal of Chromatography A, 1002 (2003) 79–92
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

On-line high-performance liquid chromatography–mass spectrometric detection and quantification of *N*-acylhomoserine lactones, quorum sensing signal molecules, in the presence of biological matrices

Danièle Morin*, Béatrice Grasland, Karine Vallée-Réhel, Chrystèle Dufau, Dominique Haras

Laboratoire de Biologie et Chimie Moléculaires, Université de Bretagne-Sud, B.P. 92116, F-56321 Lorient Cedex, France

Received 31 March 2003; received in revised form 22 April 2003; accepted 23 April 2003

Abstract

A protocol using reversed-phase liquid chromatography coupled with positive-ion electrospray ionization and ion trap mass spectrometry is described for the identification and quantification of *N*-acylhomoserine lactones (HSLs) in crude cell-free supernatants of bacterial cultures. The HSLs are produced by Gram-negative bacteria and act as intercellular signals inducing density-dependent gene expression. Compared with the multi-step procedures previously reported, which included chemical extraction, purification and the use of *Escherichia coli* HSL biosensors, this on-line LC–MS–MS method is fast and detects 11 HSLs. Its speed and robustness allow the analysis of a large number of samples without loss of performance (no signal variation for a control sample after 90 chromatographic injections). The selectivity is based on the MS–MS fragment ions of the molecular $[M+H]^+$ ions and on their relative intensities. For quantification, the m/z 102 ion, specific for the lactone ring and detected with a good signal-to-noise ratio, allows low detection limits even in complex matrix samples (0.28 up to 9.3 pmol). Moreover, this method allows the quantification of 11 HSLs whatever their chemical structure, substituted or not. The protocol was applied to *Vibrio vulnificus*, a marine bacterium. Six HSLs were detected and quantified with relative standard deviations for repeatability of $<10\%$.
© 2003 Elsevier Science B.V. All rights reserved.

Keywords: Quorum sensing; *Vibrio vulnificus*; Bacteria; Acylhomoserine lactones; Lactones

1. Introduction

Since the first report of the production of an *N*-acylhomoserine lactone (HSL), *N*-(3-oxohexanoyl)-L-homoserine lactone (3-oxo-C6-HSL), by the marine photobacterium *Vibrio fischeri* in 1981 [1], several other HSLs produced by a wide range of unrelated marine and terrestrial Gram-negative bacteria have been characterized [2,3]. HSLs are soluble and diffusible molecules, which act as intercellular signals inducing density-dependent gene expression, in a phenomenon termed quorum sensing [4]. During the growth of the bacteria, HSLs are synthesized by autoinducer synthases of the LuxI family and ac-

*Corresponding author. Tel.: +33-2-9787-4591; fax: +33-2-9787-4500.
E-mail address: daniele.morin@univ-ubs.fr (D. Morin).

0021-9673/03/$ – see front matter © 2003 Elsevier Science B.V. All rights reserved.
doi:10.1016/S0021-9673(03)00730-1

cumulated. When their concentrations reach a critical threshold, their interactions with the regulatory proteins of the LuxR family induce the expression of particular genes, operons or regulons (for a review, see Ref. [5]). Whatever the producing bacterium, all the HSLs are related in structure. They only differ by their acyl side-chain moiety, which can be of various length (four to 14 carbons), degree of saturation (presence or absence of a double bond) and can harbor various substituents at position 3 (no substituent, keto or hydroxy) [2,5,6]. Bacterial synthesis of HSLs has typically been reported using *E. coli* HSL biosensors to detect the presence of HSLs within cell-free supernatants of bacterial cultures.

The chemical characterization of the HSLs from cell-free supernatants of late exponential phase bacterial cultures requires a fastidious multi-step procedure (for a review, see Ref. [7]). Briefly, after organic solvent extraction and semipreparative HPLC, the presence of HSLs in each eluted fraction is tested using *E. coli* HSL biosensors. Structural elucidation and mass determination of the HSL present in the active fractions require ^{1}H-NMR and mass spectrometry (MS) analyses, respectively. Several ionization techniques have been used such as fast atom bombardment MS (FAB-MS) [8–11], electron impact MS (EI-MS) [12], chemical ionization MS (CI-MS) [13], and collision-induced dissociation MS (CID-MS) [14]. All the HSL structures identified today have been confirmed by spectral comparison with the corresponding chemically synthesized molecules. Indirect HSL detection by bacterial biosensors onto a thin-layer chromatography (TLC) plate [13], or in cell-free culture supernatants, can lead to artefactual results. The activation of a biosensor by an HSL-containing extract is dependent on the biosensor specificity, its sensitivity threshold for a particular HSL, the relative amount of each HSL produced by the studied bacterium, the nature of the solvent used for the supernatant extraction, the extraction yield, the medium and culture conditions used, and, finally, the presence of putative HSL-competitor molecules in the extract. Biosensor inactivity can thus reflect many potential artifacts and not only the inability of a bacterium to synthesize an HSL. Some of these drawbacks can be overcome by using a chemical approach as an alternative to bioassays. Using a chemical modification of the 3-oxo-HSLs to their pentafluorobenzyloxime derivatives, Charlton et al. [15] identified, by GC–MS, both in a biofilm of *P. aeruginosa* and in its effluent, the presence of *N*-(3-oxooctanoyl)-L-homoserine lactone (3-oxo-C8-HSL), *N*-(3-oxodecanoyl)-L-homoserine lactone (3-oxo-C10-HSL), 3-oxo-C12-HSL and *N*-(3-oxotetradecanoyl)-L-homoserine lactone (3-oxo-C14-HSL). The method described allows only the detection of the 3-oxohomoserine lactones. Here, we report a liquid chromatography–electrospray ionization ion trap MS–MS (LC–MS–MS) one-step method, which is useful to detect, characterize and quantify the *N*-acyl- and 3-oxo-acylhomoserine lactones directly in a crude cell-free supernatant of bacteria culture. The MS–MS analysis of the molecular ions allows HSL analysis in a single day. Our method was applied to the identification and quantification of HSLs produced by a marine bacterium, *Vibrio vulnificus*.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Pseudomonas aeruginosa PAO1 (wild type) and mutant strains, PAO-JP1 (Δ*lasI*::*tet*) and PDO100 (Δ*rhlI*::*Tn501-2*), were grown at 37 °C in a rich medium, Luria-Bertani (LB) (Bactotryptone 1%, yeast extract 0.5%, NaCl 1%) or in minimum mineral medium (M9-glucose) ($Na_2HPO_4 \cdot 7H_2O$ 1.28%, KH_2PO_4 0.3%, NaCl 0.05%, NH_4Cl 0.1%, glucose 0.4%). The marine bacterium *Vibrio vulnificus* (our collection) was grown on a rich medium, Marine Broth (Bacto Marine Broth 2216, Difco) at 20 °C without shaking. This medium (MB) contains: peptone 0.5%, yeast extract 0.1%, $FeC_6H_5O_7$ 0.01%, NaCl 1.945%, $MgCl_2$ 0.59%, Na_2SO_4 0.334%, $CaCl_2$ 0.18%, KCl 0.055%, Na_2CO_3 0.016%, KBr 0.008%, SrC $3.4 \cdot 10^{-3}$%, NaF $0.24 \cdot 10^{-3}$%, $Na_2Si_3O_7$ $3.4 \cdot 10^{-3}$%, H_3BO_3 $2.2 \cdot 10^{-3}$%, NH_4NO_3 $0.16 \cdot 10^{-3}$%, Na_2HPO_4 $0.8 \cdot 10^{-3}$%.

2.2. Extraction of HSLs

Extracts for analytical LC–MS were prepared from 50 mL of bacterial stationary phase liquid

cultures. Bacteria were removed by centrifugation at 3000 *g* for 20 min at 4 °C, and supernatants were extracted three times with half a volume of HPLC-grade dichloromethane (Carlo Erba, France). The dichloromethane extracts were dried over anhydrous magnesium sulfate (Sigma, France), filtered, and evaporated to dryness at 35 °C (rotavapor, Buchi, Switzerland). Residues were dissolved in 1 mL of HPLC-grade acetonitrile (Carlo Erba); the coefficient of concentration of the sample was thus 50. The extracts were stored frozen until analysis. Before LC injection, samples were filtered through a 0.45 μm PTFE syringe filter (Alltech, France).

2.3. The HSLs studied

Eleven HSLs were studied, six *N*-acyl- and five *N*-3-oxoacylhomoserine lactones. Their structures, names and abbreviations are listed in Fig. 1.

2.4. C_{18} Reversed-phase HPLC

An Agilent Technologies Series 1100 vacuum degasser, LC pump and autosampler (Hewlett-Packard, Germany) were used. Twenty microliters of acetonitrile solutions were applied onto an analytical C_{18} reversed-phase column (Hypersil ODS, 250×4.6 mm, particle size 5 μm). Smaller injected volumes were tried without improving the peak widths. The elution procedure consisted of an isocratic profile of methanol–water (50:50, v/v) for 10 min, followed by a linear gradient from 50 to 90% methanol in water over 15 min, and an isocratic profile over 25 min. The LC flow (0.4 mL/min) was split (1/12) using a micro-splitter valve (Upchurch Scientific, USA). The post-column additive, a mixture of 5 m*M* ammonium acetate and 0.05% trifluoroacetic acid (TFA) (Analysis grade, Carlo Erba) in methanol–water (50:50, v/v), was added using a Cole-Parmer (USA) syringe pump and a 2.5 mL SGE syringe at a flow-rate 150 μL/h. Detection was performed using mass spectrometry.

2.5. Mass spectrometric detection

The LC-separated compounds were detected by electrospray ionization ion trap mass spectrometry (ESI-MS) using a Bruker Esquire-LC spectrometer (Bruker Daltonic, Germany) under positive-ion conditions. For each compound, two ions were formed: the $[M+H]^+$ and the $[M+Na]^+$ ions. The $[M+H]^+$ ions were isolated for MS–MS fragmentation. The MS–MS chromatographic analysis is segmented for the isolation and fragmentation of the eluted $[M+H]^+$ ion. Some HSLs have similar retention times, consequently two injections were carried out: one for *N*-acyl-HSL analysis, and the second for the 3-oxo-HSLs (Table 1). The electrospray used nitrogen as nebulizing gas (pressure set to 15 p.s.i.; 1 p.s.i.= 6894.76 Pa) and drying gas (flow set to 7 mL/min). The drying temperature was 300 °C. The voltages of the capillary and end plate offset were −4500 and −400 V, respectively. Some parameters have an important effect upon sensitivity. Depending on the *m*/*z* values, they were tuned specifically for each segment. The conditions set are listed in Table 1. With the MS mode, the detection of all 11 HSLs is possible with one chromatographic injection (analysis time 50 min) and mean conditions. The others parameters were: octopole 2.4 V, octopole Δ 2.0 V, octopole RF 120.0 V, lens 1 −5.0 V and lens 2 −60.0 V. The detector voltages were: multiplier −1740 V and dynode −7.0 V. The helium pressure in the ion trap was $6 \cdot 10^{-6}$ mbar. Full-scan mode detection was used with a scan range from *m*/*z* 50 to 400. The software used was Bruker Esquire-LC NT version 6.08 and Agilent Technologies ChemStation May 1998.

2.6. Chemical synthesis of HSLs

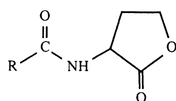
N-(3-Oxohexanoyl)-L-homoserine lactone (3-oxo-C6-HSL) was purchased from Sigma (France). The other HSLs were synthesized as described previously [16]. Structures were confirmed by MS and proton NMR spectroscopy.

3. Results

3.1. HPLC separation

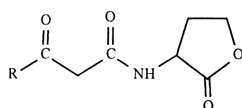
The molecular structures of the studied HSLs (Fig. 1) differ in the length of the acyl side-chain and the presence or absence of an oxo function on the third carbon. This leads to variations in the molecular

N-Acylhomoserine lactones



Structure	Chemical name	Abbreviation
R = $CH_3(CH_2)_2$	N-butanoyl-homoserine lactone	C_4-HSL
R = $CH_3(CH_2)_4$	N-hexanoyl-homoserine lactone	C_6-HSL
R = $CH_3(CH_2)_6$	N-octanoyl-homoserine lactone	C_8-HSL
R = $CH_3(CH_2)_8$	N-decanoyl-homoserine lactone	C_{10}-HSL
R = $CH_3(CH_2)_{10}$	N-dodecanoyl-homoserine lactone	C_{12}-HSL
R = $CH_3(CH_2)_{12}$	N-tetradecanoyl-homoserine lactone	C_{14}-HSL

N-3-Oxoacylhomoserine lactones



Structure	Chemical name	Abbreviation
R = $CH_3(CH_2)_2$	N-3-oxohexanoyl-homoserine lactone	3-oxo-C_6-HSL
R = $CH_3(CH_2)_4$	N-3-oxooctanoyl-homoserine lactone	3-oxo-C_8-HSL
R = $CH_3(CH_2)_6$	N-3-decanoyl-homoserine lactone	3-oxo-C_{10}-HSL
R = $CH_3(CH_2)_8$	N-3-dodecanoyl-homoserine lactone	3-oxo-C_{12}-HSL
R = $CH_3(CH_2)_{10}$	N-3-tetradecanoyl-homoserine lactone	3-oxo-C_{14}-HSL

Fig. 1. Structures, names and abbreviations of the 11 HSLs studied.

polarity, allowing the separation of HSLs by reversed-phase HPLC. However, because of the many interrelated experimental parameters involved in the HPLC–ESI-MS technique, it is difficult to apply a systematic approach to optimize the analytical method. The column, the mobile phase and the eluent gradient selected in our method are a compromise to achieve the best separation and the highest ESI-MS response. We used a C_{18} column with a mobile phase of methanol–water, which presents a low background signal with MS detection. The column size (I.D. 2 mm or less) generally used for LC–MS applications did not allow the separation of the 11 HSLs. We thus used a 250×4.6 mm I.D. column operated at 0.4 mL/min eluent flow-rate. To reduce contamination of the interface and mainly to improve the detector response, this flow-rate was divided (1/12) using a post-column split. Although acetoni-

Table 1
Segmentation of the chromatographic MS–MS analysis and the ESI-MS parameters tuned for the isolation of the $[M+H]^+$ ion and detection of the m/z 102 ion

Chromatographic segment (min)	$[M+H]^+$ ion monitored (m/z)	Skimmers (V)		Capillary exit offset (V)	Trap drive	Fragmentation	
		1	2			Amplitude	Cut off
N-Acylhomoserine lactones							
0–12	172	12.0	5.0	66.4	31.2	0.80	46
12–18	200	15.2	5.0	67.0	32.6	0.80	54
18–26	214	15.8	5.0	67.1	33.2	0.85	59
26–31	228	16.5	5.0	67.3	33.9	0.90	63
31–35	256	17.6	5.0	67.6	35.3	0.95	71
35–39	284	18.8	5.0	67.8	36.6	0.95	79
39–50	312	19.8	5.0	68.1	38.0	1.00	79
3-Oxoacylhomoserine lactones							
0–12.5	214	17.4	5.9	49.5	32.1	0.45	52
12.5–21.5	242	20.5	7.9	52.5	28.9	0.67	69
21.5–26.5	214	15.8	5.0	67.1	33.2	0.85	59
26.5–32	270	22.0	8.9	57.4	28.8	0.73	67
32–36.5	298	24.2	10.6	42.6	28.2	0.80	71
36.5–50	326	26.6	6.9	48.8	29.5	0.81	75

trile is often used in LC–MS analysis and in semi-preparative HPLC prior to bio-assays, we compared this eluent with methanol: with methanol, the earliest-eluted peaks were larger but better separated and the signal intensities were higher.

Using the experimental conditions described in the Materials and methods section, the analogues of a given type of structure (*N*-acyl- or 3-oxoacyl-HSLs) were well separated. Some *N*-acyl- and 3-oxoacyl-HSLs co-eluted (for example, C4-HSL and 3-oxo-C6-HSL co-eluted, C10 and C12-HSL nearly eluted with 3-oxo-C12- and 3-oxo-C14-HSL, respectively), but the specificity of MS detection allowed us to distinguish the co-eluted compounds by plotting specific ion $[M+H]^+$ chromatograms (Fig. 2).

3.2. MS and MS–MS detection of HSLs

A disadvantage of electrospray ionization is its low salt tolerance, resulting in cationization of the analyte. With MS detection, for each HSL, two ions, $[M+H]^+$ and $[M+Na]^+$, were effectively observed. The relative abundances of these ions were quite variable, but the sums of the $[M+H]^+$ and $[M+Na]^+$ ion intensities correlated with the concentration of the HSL in a linear relationship (coefficient of regression for *N*-acyl >0.999 for a range of tested concentrations from 0.25 to 5 mg/L). A rapid screening of the HSLs is thus possible with only one chromatographic injection (analysis time 50 min) using MS detection and plotting the specific $[M+H]^+$ or $([M+H]^+ + [M+Na]^+)$ chromatograms.

The bacterial culture media in which HSLs will be searched are complex matrices containing components of growth media and bacterial exoproducts, which could affect the specificity and sensitivity of MS detection. It is thus preferable to use MS–MS detection for identification and quantification. The adduct ion $[M+Na]^+$ is a stable cation which does not produce MS–MS fragment ions, therefore fragmentation was generated from the $[M+H]^+$ ion.

Three structures are possible for the $[M+H]^+$ ion, resulting from protonation of either the –NH group, or of the carbonyl function of the acyl chain, or of the ethoxy function of the lactone ring. The first leads, by two fragmentation mechanisms of the amide bond, to the $[M+H-C_4H_7NO_2]^+$ ion ($[M+H-101]^+$) by neutral loss of homoserine lactone, and to the m/z 102 ion by protonation of the homoserine lactone (nomenclature according to Refs. [17,18]). The two other protonations of HSLs lead, respectively, to the $[M+H-H_2O]^+$ and $[M+H-CO]^+$ ions. The proposed structures of the four MS–MS product ions from C12-HSL are shown in

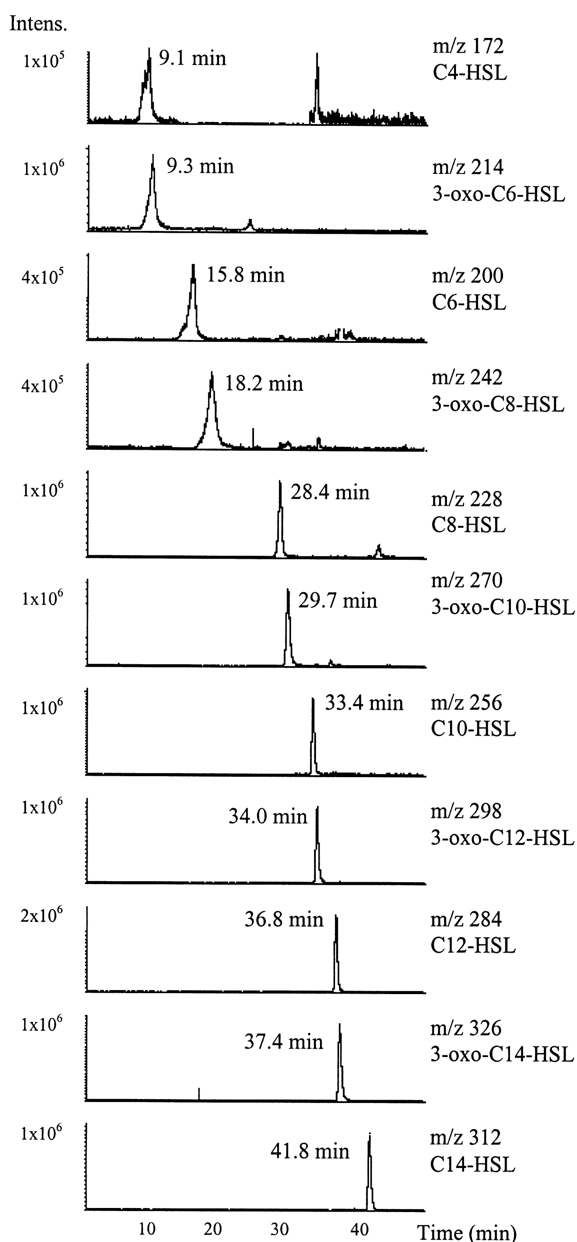


Fig. 2. Selected ion $[M+H]^+$ chromatograms for each HSL (1 mg/L). Chromatographic conditions are as described in the text.

Fig. 3. The relative abundances of the product ions are different for *N*-acyl- and 3-oxoacyl-HSLs (Table 2). For the first, the four ions were detected with abundances ranging from 15 to 70%, except for the $[M+H-101]^+$ ion of C4-HSL (<5%). For the second, only the ions m/z 102 and $[M+H-101]^+$ are major ions, the relative abundance of the other ions being under 5%. Fig. 4 illustrates these observations. It should be noted that the $[M+H-101]^+$ ion abundance increased with the acyl-chain length: small for small chains (about 5 and 10%, respectively, for C4-HSL and 3-oxo-C6-HSL), it reached 35% for C10-HSL and was large (100%) for 3-oxo-C12- and 3-oxo-C14-HSLs.

To increase the sensitivity, it was necessary to promote $[M+H]^+$ ion formation versus the $[M+Na]^+$ ion. This was achieved after we observed a positive conjugate action of ammonium acetate and TFA, itself known to depress analyte ion abundance in the ESI process. Mixtures of these two compounds enhanced the formation of $[M+H]^+$ and greatly reduced that of $[M+Na]^+$ (Fig. 5). Since the addition of these modifiers to the chromatographic eluents rapidly polluted the interface and required washing of the column, they were introduced by means of a post-column addition. A mixture of 5 m*M* ammonium acetate and 0.05% TFA in water–methanol (50:50), added with a syringe pump at 150 μL/h, allowed, for all the AHLs studied, a reduction in $[M+Na]^+$ ion production (<10%) without a suppression effect of the $[M+H]^+$ signal or an increase in the background signal.

The specific identification of the HSLs was based on the constant relative abundances of the four MS–MS product ions and on the chromatographic retention times of the standards.

3.3. Quantification

The difficulty in these analyses was the multiplicity of sample matrices, resulting from the growth media used, the studied bacteria (they synthesize more or less exoproducts), and the phase of bacterial growth. In order to increase the sensitivity and the accuracy of the method, the parameters involved in the quantification were examined: optimization of the detected signal, choice of internal standard, matrix effects, detection limits, and extraction yields. A quantification methodology was then proposed.

For quantification based on peak area measurements, MS–MS detection was used. While the summed intensities of the four ions could be used, only the ion at m/z 102 was selected because of its

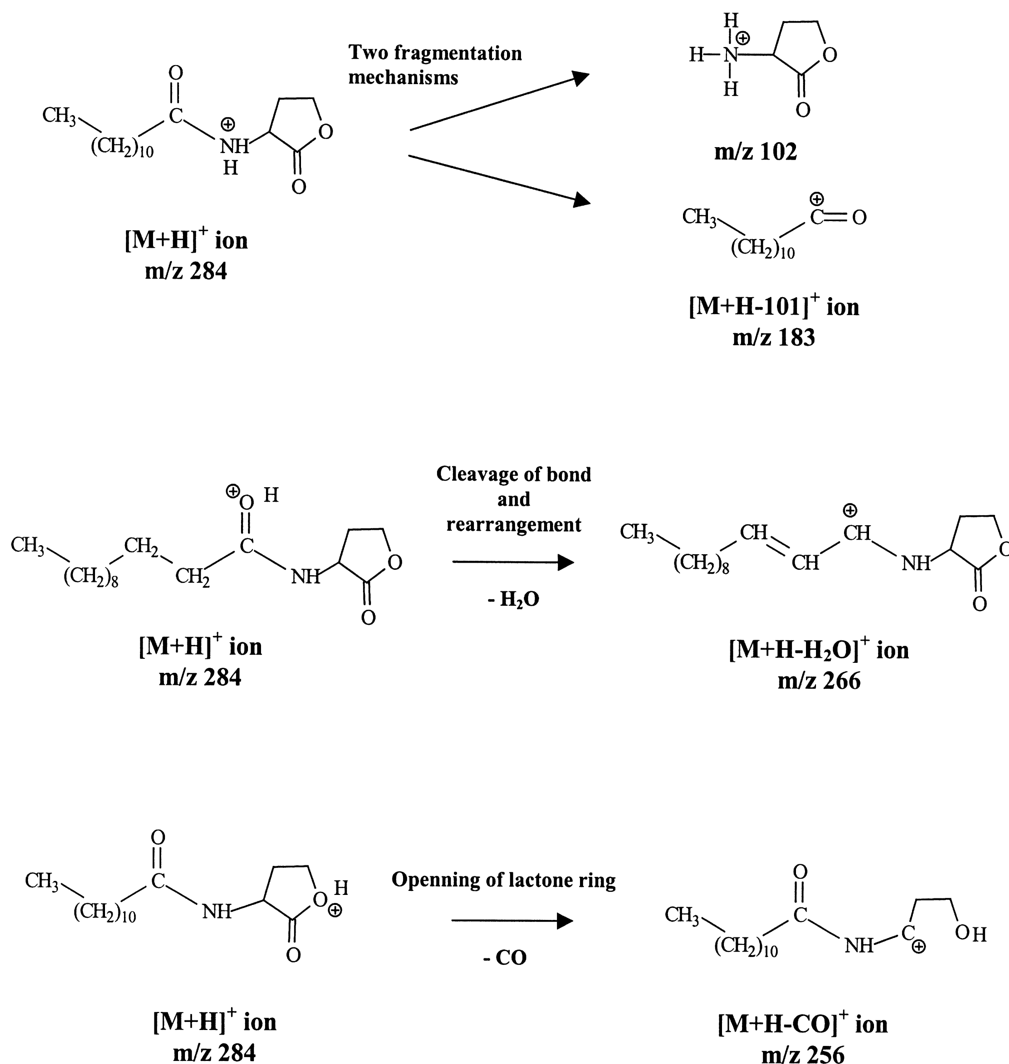


Fig. 3. MS–MS fragmentation of HSLs: proposed structures of the product ions obtained from the molecular ion $[M+H]^+$. Example of C_{12}-HSL.

specificity to the HSLs and its better signal-to-noise ratio.

As deuterated HSLs are not readily available, isotope dilution cannot be used to quantify HSLs. The internal standard chosen was *N*-heptanoyl-homoserine lactone, C7-HSL. C7-HSL, which has never been reported as being produced by bacteria, was never present in the unlabelled analytes and its signal was not affected by the matrix (same intensity whatever the matrix sample). Due of the large number of HSLs studied and of the possible matrix interferences affecting each HSL measurement, such an internal standard is not an all-purpose compound. The accuracy of its use was studied.

We extracted M9-glucose, LB and MB growth media with dichloromethane (coefficient of concentration 50) and plotted the calibration curves of the 11 HSLs dissolved in acetonitrile (ACN) and in the three growth media extracts. Before chromatographic injection, each sample was spiked with the same amount of internal standard. Calibration was based on measurements of the peak areas (*m*/*z* 102) of the

Table 2
MS–MS product ions of HSLs: m/z values and, in parentheses, the relative abundances (%)

HSL	Ions			
	m/z 102	$[M+H-101]^+$	$[M+H-H_2O]^+$	$[M+H-CO]^+$
C4-HSL	(100)	71 (≤5)	154 (50–70)	144 (15–20)
C6-HSL	(100)	99 (15)	182 (50–70)	172 (15–20)
C8-HSL	(100)	127 (30)	210 (50–70)	200 (15–20)
C10-HSL	(100)	155 (35)	238 (50–70)	228 (15–20)
C12-HSL	(100)	183 (35)	266 (50–70)	256 (15–20)
C14-HSL	(100)	211 (35)	294 (50–70)	284 (15–20)
3-Oxo-C6-HSL	(100)	113 (≈10)	196 (≤5)	186 (≤5)
3-Oxo-C8-HSL	(100)	141 (≈40)	224 (≤5)	214 (≤5)
3-Oxo-C10-HSL	(100)	169 (≈70)	252 (≤5)	242 (≤5)
3-Oxo-C12-HSL	(90)	197 (100)	280 (≤5)	270 (≤5)
3-Oxo-C14-HSL	(50)	225 (100)	308 (≤5)	298 (≤5)

internal standard and of four concentrations between 0.5 and 5.0 mg/L for each HSL. The calibration curves were plotted. For all AHLs, in the four matrices (ACN and M9, LB and MB extracts), the responses were linear functions of the concentration (coefficient of regression >0.996). A depressive matrix effect is observed for the earliest-eluting compounds: C4-, C6- and 3-oxo-C6-HSLs only in

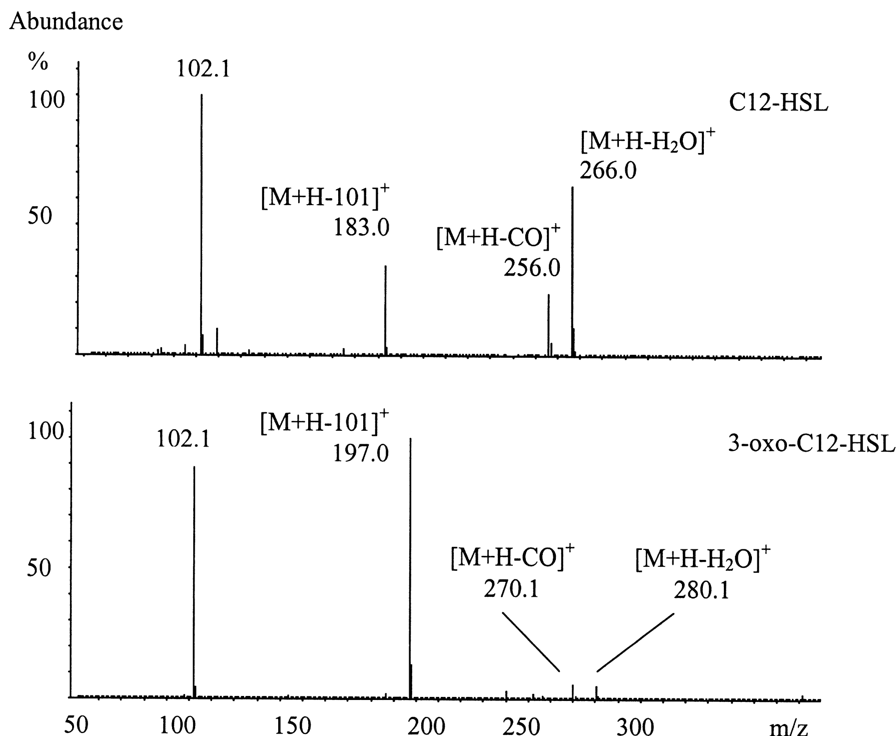


Fig. 4. Full-scan MS–MS spectra of an *N*-acylhomoserine lactone (C12-HSL) and of a 3-oxoacylhomoserine lactone (3-oxo-C12-HSL). Precursor ions: $[M+H]^+$.

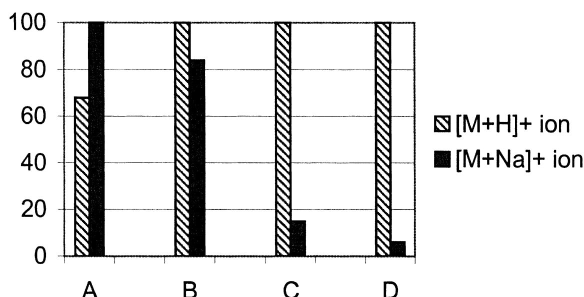


Fig. 5. Chromatography of C4-HSL: relative abundances of the $[M+H]^+$ and $[M+Na]^+$ ions according to the post-column addition of a modifier. (A) Without modifier. With modifier: (B) 5 m*M* ammonium acetate in methanol–water (50:50) at 100 μL/h; (C) 5 m*M* ammonium acetate and 0.05% TFA in methanol–water (50:50) at 100 μL/h; (D) 5 m*M* ammonium acetate and 0.05% TFA in methanol–water (50:50) at 150 μL/h.

LB extracts. Fig. 6 illustrates this effect for 3-oxo-C6- versus 3-oxo-C8-HSL, for which the responses are identical whatever the medium used.

In addition to this medium interference, matrix effects resulting from the exoproducts of the bacteria can depress the signals. We observed these effects on the late-eluting HSLs: the C12-, C14-, 3-oxo-C12- and 3-oxo-C14-HSLs when we spiked an extract of *P. aeruginosa* PAO1 wild type in LB medium with standard HSLs. Therefore, possible matrix effects have to be taken into account in the analytical methodology proposed below.

The detection limits were a variable function of the matrix complexity. To assess the sensitivity in a complex matrix, we used two *P. aeruginosa* mutants. The first, the *lasI* mutant *P. aeruginosa* PAO-JP1 strain, is unable to produce 3-oxo-HSLs and was therefore used to prepare a complex matrix lacking 3-oxo-HSLs. The second mutant, the *rhlI* mutant *P. aeruginosa* PDO-100, is deficient of C3-unsubstituted HSL synthesis and allows the generation of a matrix devoid of these HSLs [8,19]. We spiked corresponding LB medium extracts of PAO-JP1 and PDO100 strains with synthesized standards: 3-oxo-HSLs and C3-unsubstituted HSLs, respectively.

Decreasing concentrations of standards were tested until a signal-to-noise ratio of at least 3 was obtained on the selected m/z 102 chromatogram. We also ensured that the characteristic MS–MS product ions of each HSL appeared in the corresponding spectra

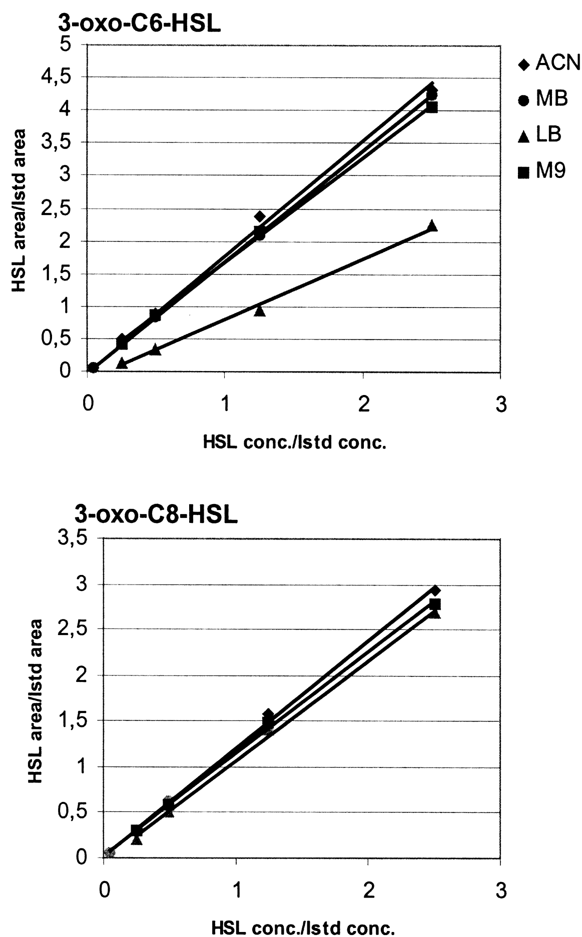


Fig. 6. Calibration curves of 3-oxo-C6- and 3-oxo-C8-HSLs dissolved in acetonitrile and extracts of MB, LB and M9-glucose growth media concentrated 50 times.

with a ratio close to those of the standards. Fig. 7A shows the MS–MS total ion chromatogram of the 3-oxo-HSLs at the detection limits from supernatants of PAO-JP1 cultures. We outlined the product ions of these HSLs on the full-scan spectra recorded at the retention times of 3-oxo-C6- and 3-oxo-C14-HSLs (9.1 and 37.4 min, respectively). For 3-oxo-C6-HSL, the background of the full-scan spectra was significant and it required a signal-to-noise ratio >3 on the selected m/z 102 chromatogram for unambiguous detection (Fig. 7B). The detection limits determined (molar amounts in the 20 μL injected LC–MS volume) and those published elsewhere are presented in Table 3. Our detection limits are between 0.3 and

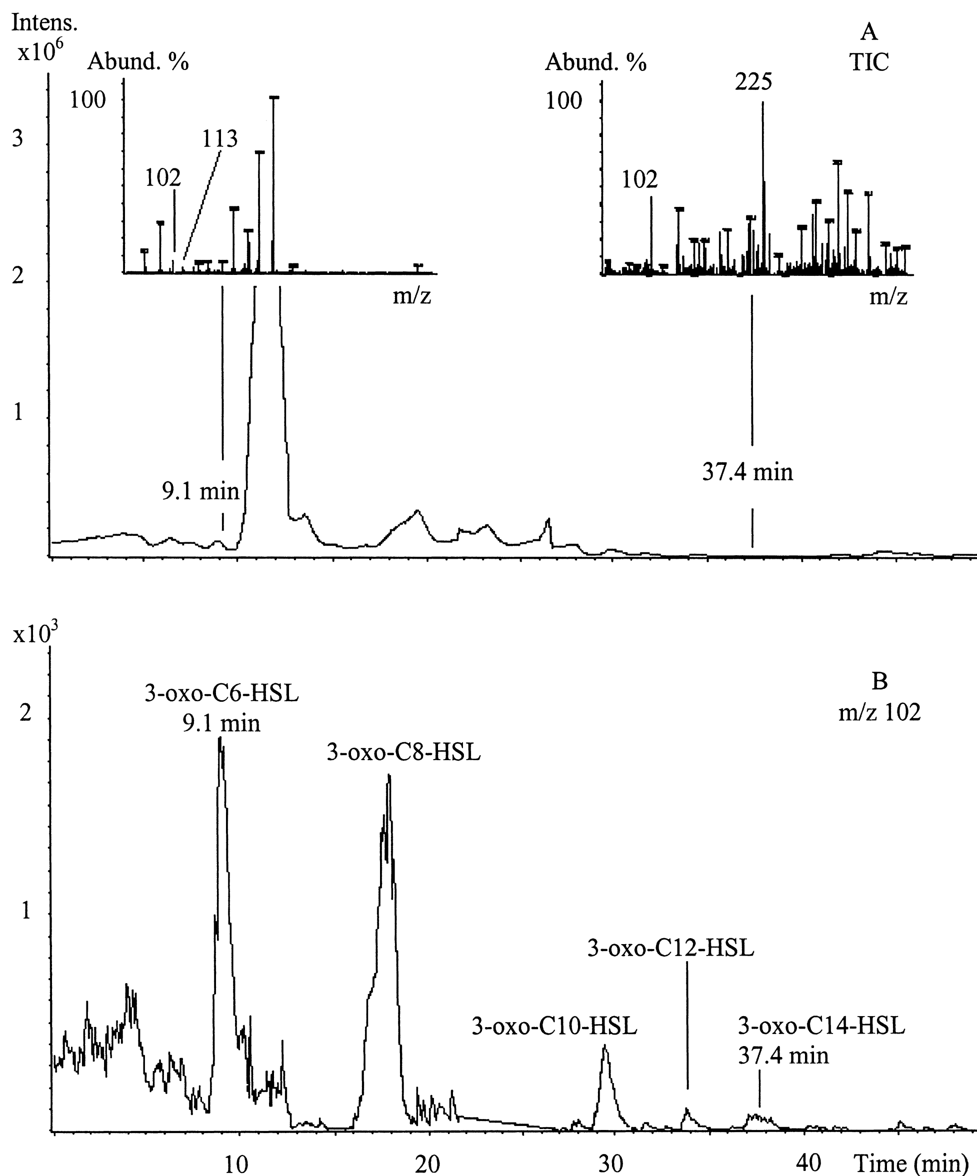


Fig. 7. MS–MS chromatograms of 3-oxoacyl-HSLs at the detection limits from supernatants of *P. aeruginosa* PAO-JP1 cultures. (A) Total ion chromatogram and full-scan MS–MS spectra at the retention times of 3-oxo-C6- and 3-oxo-C14-HSLs. (B) Selected *m/z* 102 chromatogram.

9.3 pmol. Compared with bioassays [13,20], realized with standard HSLs dissolved in pure acetonitrile, our limits are lower except for the 3-oxo-C6- and 3-oxo-C8-HSLs. The levels of detection by GC–MS [15] are lower. These differences can be explained by the instrumental technique and also by the sample matrix. The authors did not report the reproducibility of the measurements at their detection limits. In our case, the RSD values (<25%) show good precision of the HPLC–MS–MS signals for the detection limits in highly complex matrices.

Moreover, with the analytical conditions described

Table 3
Detection limits (amounts injected onto the chromatographic column for the LC–ESI-MS and GC–MS methods). First column: lowest amounts detected in a complex matrix (see description in the text) with our method (RSD values for five chromatographic injections). Second to fourth columns: bioassay limits. Fifth column: limits with a GC–MS method

HSL	LC–ESI-MS Our limits in a charged matrix (pmol) (RSD, %)	Bioassays			GC–MS [15] (fmol)
		[20] (nmol)	[13]		
			Minimum detection	Routine detection	
C4-	9.3 (16)	1.8	–	–	–
C6-	2.0 (16)	0.01	300 pmol	1200 pmol	–
C8-	0.88 (17)	0.44	2.4 pmol	38 pmol	–
C10-	0.63 (23)	2.4	100 pmol	400 pmol	–
C12-	0.28 (16)	110	–	–	–
C14-	0.51 (10)	–	–	–	–
3-Oxo-C6-	0.94 (11)	0.14	30 fmol	520 fmol	
3-Oxo-C8-	0.83 (25)	0.83	0.5 fmol	30 fmol	188
3-Oxo-C10-	0.30 (24)	40	190 fmol	3 pmol	to
3-Oxo-C12-	0.67 (20)	40	2 pmol	16 pmol	123
3-Oxo-C14-	1.25 (20)	130	–	–	

above, the method showed good performance characteristics over a long period of time. No signal variation for a control sample was observed after 90 chromatographic injections.

3.4. Extraction of HSLs

In a preliminary assay, we tested five solvents for extraction of HSLs: dichloromethane, chloroform, ethylacetate, ethylether and hexane. The first two solvents gave the best extractions with about the same yields. Therefore, dichloromethane was chosen for this study.

In order to examine the influence of the medium on the extraction, we added a standard mixture of HSLs to 50 mL of M9, LB and MB culture media. The HSL concentrations used were 20-fold the detection limits. Each sample was extracted three times with half a volume of dichloromethane. The first two extracts were pooled before chromatographic analyses, whereas the third was studied separately.

The extraction yield was not dependent on the medium used but, as expected, the yield decreased with the polarity of the extracted HSLs. Table 4 shows the extraction yields calculated from the three extractions. The results are the average values from six replicates for *N*-acyl-HSLs (two in each of the LB, MB and M9 media) and seven replicates for 3-oxo-HSLs (three in LB and MB, one in M9). For C4- and 3-oxo-C6-HSLs the yields were about 65%, and for the C6- and 3-oxo-C8-HSLs they were about 85%. The extractions were complete for the other HSLs. The third extraction was useful only for C4-HSL (10% extracted) and for 3-oxo-C6-HSL (6%).

Table 4
Extraction of HSL mixtures in M9, LB and MB growth media. Yields and relative standard deviations (RSDs). $n=6$ for the *N*-acyl-HSLs (two replicates in each medium), $n=7$ for the 3-oxoacyl-HSLs (three replicates in LB and MB media, one in M9)

N-Acyl-HSL	Extraction		3-Oxoacyl-HSL	Extraction	
	Yield (%)	RSD (%)		Yield (%)	RSD (%)
C4-HSL	65	9	3-Oxo-C6-HSL	63	9
C6-HSL	84	14	3-Oxo-C8-HSL	86	18
C8-HSL	96	12	3-Oxo-C10-HSL	116	20
C10-HSL	128	8	3-Oxo-C12-HSL	111	10
C12-HSL	122	15	3-Oxo-C14-HSL	102	11
C14-HSL	104	19			

For the other compounds, full recovery was obtained with two extractions. Extraction yields were above 100% for four HSLs. Given their relatively long retention times, this result may not be due to matrix effects. In the previous paragraph a depressive matrix effect was pointed out for the earliest-eluting HSLs when the LB medium was used. Moreover, to avoid this effect, standards for the chromatographic titration were dissolved in the same extract medium. For these experiments, which included extraction and LC–MS–MS analyses, the standard deviations were 9–20%.

3.5. Proposed quantification methodology

Our aim was to develop a rapid and accurate analytical method. Owing to the fact that the analyzed samples have various matrices, three methods are proposed. For each, the internal standard C7-HSL is added to the culture supernatant before extraction. The methods differ in the type of calibration: internal or external.

If the volume of the culture supernatant is sufficiently large to be divided, the best method is the spiked levels method: the supernatant is extracted without and with additions of standard HSLs.

A second method is to extract the supernatant and to analyze the extract without and with additions of standard HSLs. In this case, we have to take into account the incomplete extraction of some HSLs (proposed correction factor: 1/0.65 for C4- and 3-oxo-C6-HSLs and 1/0.85 for C6- and 3-oxo-C8-HSLs; see Table 4). This protocol can be applied, in default of the first method, to complex matrix samples, particularly when LB or rich growth media are used or when the samples contain many bacterial exoproducts.

The third method relies on an external calibration. The sample is analyzed and the signal intensities are compared with those of standard HSLs directly introduced into the LC–ESI-MS detector. We also take into account the correction factor for the extractions. This method can be applied when there is no or few matrix effects: for example, when minimum growth media are used or for samples at the beginning of the bacterial growth phase.

Standards were not added to the samples in the third method, which is thus less rigorous than the first two methods. We validated the third method by comparing it with the second method as follows.

3.6. Application

We studied a stationary phase culture of the marine bacterium *Vibrio vulnificus* in MB medium. Previous work using *E. coli* HSL biosensors revealed that this strain produces HSLs (data not shown).

The m/z 102 chromatograms showed that *V. vulnificus* synthesizes six HSLs (Fig. 8). Three HSLs, C4-, 3-oxo-C10- and 3-oxo-C12-HSLs, generated significant signals, whereas the other HSLs, C6-, 3-oxo-C8- and 3-oxo-C14-HSLs, gave weak peaks. The presence of the latter was confirmed by their fragment product ions in the full-scan spectra extracted from the TIC chromatogram at the retention times of the standard HSLs (respectively, 16.0, 18.0 and 37.1 min).

The HSLs were quantified using the second (internal calibration) and the third (external calibration) methods proposed. The results (Table 5) do not show significant differences with respect to the method used. Therefore, external calibration can be chosen in this case on the basis of its speed: it can be particularly useful if numerous samples have to be analyzed. The relative standard deviations are $<9\%$ for the major compounds and $<24\%$ for the others. For the latter, we quantified the HSLs contained in the 20 μL injected into the HPLC system. A comparison of the results (1.8, 0.58 and 0.04 pmol for C6-, 3-oxo-C8- and 3-oxo-C14-HSLs) with the limits presented in Table 3 (respectively, 2.0, 0.83 and 1.25 pmol for the same products) shows that the detection limits depend on the sample matrix.

4. Conclusion

The powerful selective LC–ESI-MS–MS method presented here enables the detection and quantification of *N*-acylhomoserine lactones in complex matrices without fastidious sample purification. Identification is based on three criteria: MS–MS fragmentation product ions, their relative intensities and HPLC retention times. For quantification, the m/z 102 ion is selected because of its specificity for the lactone

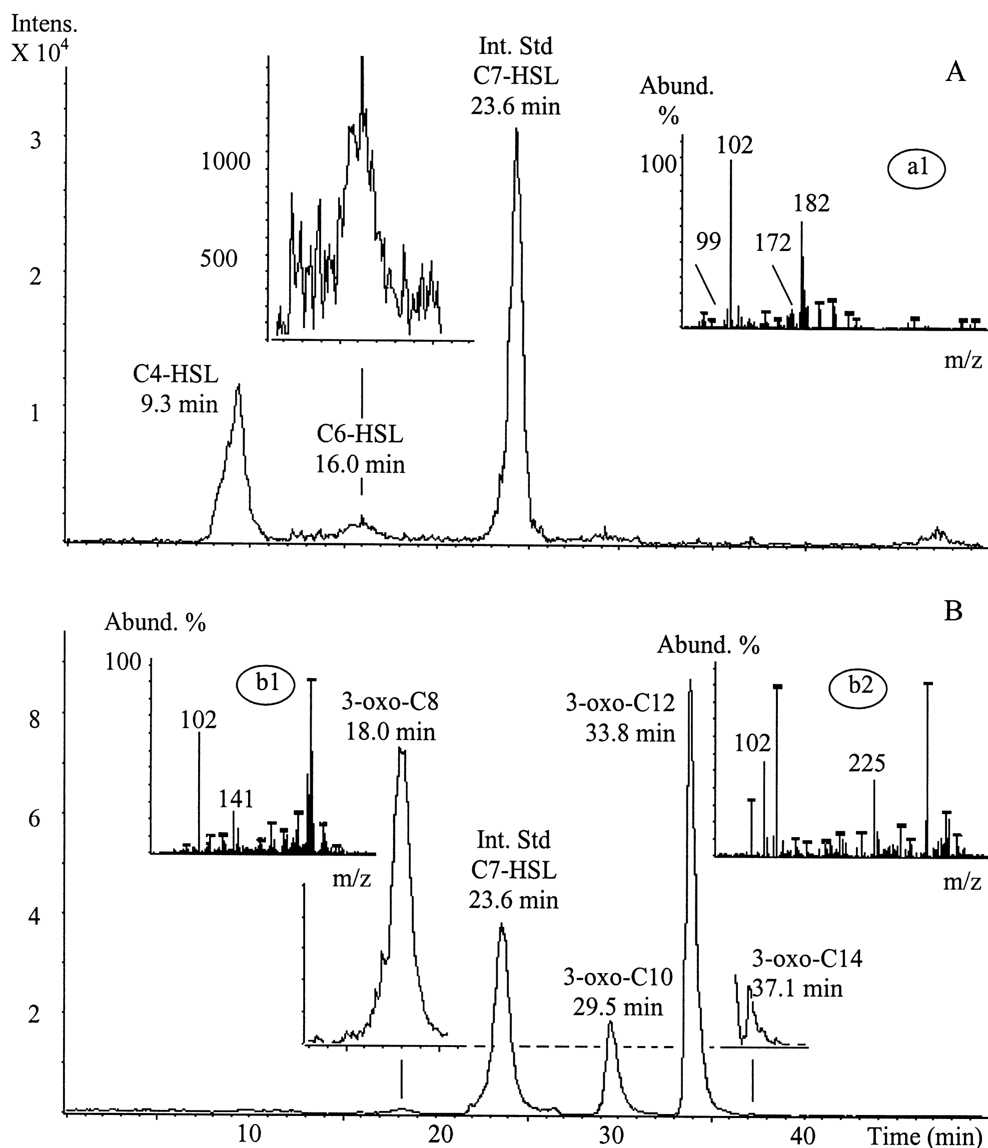


Fig. 8. *Vibrio vulnificus* culture supernatant analysis. Selected m/z 102 chromatograms of (A) *N*-acyl- and (B) 3-oxoacyl-HSLs. a1, b1 and b2 are the full-scan spectra at the retention times of the C6-, 3-oxo-C8- and 3-oxo-C14-HSLs, respectively.

ring. The corresponding chromatogram shows a good signal-to-noise ratio, which allows specific and sensitive detection. The method is robust and has good performance over a long period of time.

We applied this method to *V. vulnificus* and showed that this marine bacterium produces six *N*-acylhomoserine lactones: C4- and C6-HSL and 3-oxo-C8-, 3-oxo-C10-, 3-oxo-C12- and 3-oxo-C14-HSL. The deviation of the chromatographic analysis is $<10\%$.

The speed of the analytical protocol makes our method applicable to a large number of samples. Study of the variation of the communication network of bacteria as a function of their environmental conditions and way of life can thus readily be realized.

Table 5
HSL concentrations in extracted *Vibrio vulnificus* culture supernatant. One sample extracted. NQ, not quantified

	Internal calibration		External calibration	
	Conc. (μ*M*)	RSD (*n*=3) (%)	Conc. (μ*M*)	RSD (*n*=3) (%)
C4-HSL	3.5	8.9	3.9	4.6
C6-HSL	0.10	2.1	0.09	9.0
3-Oxo-C8-HSL	0.025	9.3	0.029	9.9
3-Oxo-C10-HSL	0.32	0.2	0.30	3.0
3-Oxo-C12-HSL	1.08	2.7	0.85	0.9
3-Oxo-C14-HSL	NQ	–	0.002	23.5

Acknowledgements

This work was supported by a research grant from the Region Bretagne, France (grant A2C288) and from the European Union (grant FEDER). We thank B. Iglewski and D. Ohmann for the gift of *P. aeruginosa* PAO-JP1 and PDO 100, A. Dufour for critically reading the manuscript and C. Marty for technical help.

References

[1] A. Eberhard, A.L. Burlingame, C. Eberhard, G.L. Kenyon, K.H. Nealson, N.J. Oppenheimer, Biochemistry 20 (1981) 2444.
[2] S. Swift, J.P. Throup, P. Williams, G.P.C. Salmond, G.S.A.B. Stewart, Trends Biochem. Sci. 21 (1996) 214.
[3] T.R. De Kievit, B.H. Iglewski, Infect. Immun. 68 (2000) 4839.
[4] C. Fuqua, S.C. Winans, E.P. Greenberg, J. Bacteriol. 176 (1994) 269.
[5] C. Fuqua, S.C. Winans, E.P. Greenberg, Annu. Rev. Microbiol. 50 (1996) 727.
[6] G.P.C. Salmond, B.W. Bycroft, G.S.A.B. Stewart, P. Williams, Mol. Microbiol. 16 (1995) 615.
[7] A.M. Schaefer, B.L. Hanzelka, M.R. Parsek, E.P. Greenberg, Methods Enzymol. 305 (2000) 288.
[8] M.K. Winson, M. Camara, A. Latifi, M. Foglino, S.R. Chhabra, M. Daykin, M. Bally, V. Chapon, G.P. Salmond, B.W. Bycroft, A. Ladzdunski, G.S.A.B. Stewart, P. Williams, Proc. Natl. Acad. Sci. USA 92 (1995) 9427.
[9] S. Swift, A.V. Karlyshev, A.V. Fish, E.L. Durant, M.K. Winson, S.R. Chhabra, P. Williams, S. Macintyre, G.S.A.B. Stewart, J. Bacteriol. 179 (1997) 527.
[10] L. Eberl, M.K. Winson, C. Sternberg, G.S.A.B. Stewart, G. Christiansen, S.R. Chhabra, B. Bycroft, P. Williams, S. Molin, M. Givskov, Mol. Microbiol. 20 (1996) 127.
[11] J.P. Throup, M. Camara, G.S. Briggs, M.K. Winson, S.R. Chhabra, B.W. Bycroft, P. Williams, G.S.A.B. Stewart, Mol. Microbiol. 17 (1995) 345.
[12] N.J. Bainton, P. Stead, S.R. Chhabra, B.W. Bycroft, G.P.C. Salmond, G.S.A.B. Stewart, P. Williams, Biochem. J. 288 (1992) 997.
[13] P.D. Shaw, G. Ping, S.L. Daly, C. Cha, J.E. Cronan, K.L. Rinehart, S.K. Farrand, Proc. Natl. Acad. Sci. USA 94 (1997) 6036.
[14] A. Kuo, N.V. Blough, P.V. Dunlap, J. Bacteriol. 176 (1994) 7558.
[15] T.S. Charlton, R. de Nys, A. Netting, N. Kumar, M. Hentzer, M. Givskov, S. Kjelleberg, Environ. Microbiol. 2 (1999) 530.
[16] S.R. Chhabra, P. Stead, N.J. Bainton, G.P.C. Salmond, G.S.A.B. Stewart, P. Williams, B.W. Bycroft, J. Antibiot. 46 (1993) 441.
[17] P. Roepstorff, J. Fohlman, Biomed. Mass Spectrom. 11 (1984) 601.
[18] K. Biemann, Methods Enzymol. 193 (1990) 886.
[19] J.P. Pearson, K.M. Gray, L. Passador, K.D. Tucker, A. Eberhard, B.H. Iglewski, E.P. Greenberg, Proc. Natl. Acad. Sci. USA 91 (1994) 197.
[20] K.H. McClean, M.K. Winson, L. Fish, A. Taylor, S.R. Chhabra, M. Camara, M. Daykin, J.H. Lamb, S. Swift, B.W. Bycroft, G.S.A.B. Stewart, P. Williams, Microbiology 143 (1997) 3703.