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Occurrence of *N*-Acyl Homoserine Lactones in Extracts of Bacterial Strain of *Pseudomonas aeruginosa* and in Sputum Sample Evaluated by Gas Chromatography—Mass Spectrometry

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Abstract

This study presents a fast, accurate and sensitive technique using gas chromatography-mass spectrometry (GC-MS) for the identification and quantification of *N*-acyl homoserine lactones (AHLs) in the extracts of bacterial strain of *Pseudomonas aeruginosa* and sputum sample of a cystic fibrosis patient. This method involves direct separation and determination of AHLs by using GC-MS as simultaneous separation and characterization of AHLs were possible without any prior derivatization. Electron ionization resulted in a common fragmentation pattern with the most common fragment ion at m/z 143 and other minor peaks at 73, 57 and 43. The limit of detection for N-butanoyl, N-hexanoyl, N-octanoyl, N-decanoyl, N-dodecanoyl and N-tetradecanoyl homoserine lactones was 2.14, 3.59, 2.71, 2.10, 2.45 and 2.34 µg/L, respectively. The presence of AHLs in the culture of *P. aeruginosa* strain and sputum of a cystic fibrosis patient was achieved in selected ion monitoring (SIM) mode by using the prominent fragment at m/z 143.

Keywords: Gas Chromatography—Mass Spectrometry, N-Acyl Homoserine Lactone (N-Butanoyl, N-Hexanoyl, N-Octanoyl, N-Decanoyl, N-Dodecanoyl and N-Tetradecanoyl) Homoserine Lactone, Sputum Sample, Bacterial Strain

1. Introduction

N-acyl homoserine lactones (AHLs) are important intercellular signaling molecules used by many bacteria to monitor their population density and control a variety of physiological functions in a cell-density-dependent manner by the process called quorum sensing. Quorum sensing involves synthesis and detection of extra cellular signals termed as auto inducers. Many Gram-negative bacteria like *Pseudomonas aeruginosa*, *Vibrio fischeri*, *Agrobacterium tumefaciens* etc., use acyl homoserine lactones (AHLs) as cell-cell communication molecules. When a threshold bacterial density (and corresponding AHL concentration) is reached, AHLs interact with transcriptional activators to trigger the expression of target genes. Members of the LuxI family of proteins synthesize these signals. These signal molecules diffuse from bacterial cells and accumulate in the medium as a function of cell growth.

Diverse gram-negative bacterial cells communicate with each other by using N-acyl homoserine lactones (AHLs) as signal molecules to coordinate gene expression with cell population density during invasion and colonization of higher organisms [1-3]. These AHL signal molecules are commonly referred as quorum sensing because the system enables a given bacterial species to sense when a critical population density has been reached in the host and in response activate expression of target genes required for succession [4,5]. All AHLs are characterized by a homoserine moiety and a fatty acyl group that differ in having various lengths, ranging from 4 to 18 carbons. They also have 3-oxo, 3-hydroxyl, or fully reduced methylene group at C-3 position or have an unsaturated bond within the side chain [6,7]. The acyl

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group of all natural AHLs of bacterial strain has even number of carbon atoms.

Nowadays, the determination of AHLs is of great interest, and development of reliable and sensitive analytical methods for their structural characterization can provide the information to elucidate their biological significance and activities. A wide variety of analytical procedures for the analysis of N-acyl homoserine lactones was developed and summarized in the literature [8-14, 20-25]. Screening for AHLs production from bacterial strains is based on bacteriological monitoring system, alone [8-10] or in simultaneous combination of these monitoring systems [11]. Using high performance liquid chromatography with tandem mass spectrometry in conjunction with chemical synthesis, Throup *et al.* [12] identified two signal molecules and showed them to be N-hexanoyl-L- homoserine lactone (HHL) and N-3-oxo-hexanoyl-L- homoserine lactone (OHHL). The production of OHHL was confirmed by Jacobi *et al.* [13] by thin layer chromatography in combination with an *Escherichia coli* AHL biosensor. A robust method based on solid-phase extraction (SPE) followed by ultra high-pressure liquid chromatography is proposed for the determination of five derivatives of *N*-acyl homoserine lactones [14]. *Pseudomonas aeruginosa* is a highly relevant opportunistic pathogen and is the most common gram-negative bacterium found in nosocomial, complicated and life threatening infections of immunosuppressed patients [15]. Patients with cystic fibriosis are especially disposed to *P. aeruginosa* infections and for these persons; the bacterium is responsible for the high rates of morbidity and mortality [16,17]. It can also colonize implanted devices, catheters, heart valves or dental implants [18]. Using different assays, a broad range of AHLs was detected in cell-free supernatants of bacterial cultures of *P. aeruginosa* [19-22]. Shaw *et al.* [23] reported the occurrence and separation of 3-hydroxy forms of N-hexanoyl, N-octanoyl, and N-decanoyl-L-homose rine lactones in the supernatant of *A. tumefaciens* cultures with thin layer chromatography. AHLs are the difficult compounds analyzed by the UV detectors as they have absorbance maxima at low wavelength and, coupled with the high background of commonly used solvents [24]. Moreover, these molecules are produced at very low concentrations, so the conventional techniques cannot be used. For this reason, Charlton *et al.* reported a GC/MS method for the quantification of 3-oxo AHLs based on their derivatization with pentafluorobenzyloxime derivatives [25]. The capillary separation techniques were also developed in conjunction with mass spectrometric detection for the analysis of AHLs from small sample volumes in *Burk- holderia cepacia* [27-29]. Analyses of N-acyl-L-homoserine lactone in some gramnegative bacteria were also reported using GC/MS method [30,31].

The main aim of the present work is to develop a procedure for the direct separation and detection of AHLs by GC/EI-MS. The method was applied for the analysis of *Pseudomonas aeruginosa* extracts and sputum sample of a Cystic fibrosis patient when a prominent fragment ion at m/z 143 was selected as a marker in selected ion monitoring (SIM) mode of mass detection.

2. Experimental

2.1. Chemicals and Reagents

N-Acyl homoserine lactone standards (N-butanoyl, N-hexanoyl, N-octanoyl, N-decanoyl, N-dodecanoyl and N-tetradecanoyl homoserine lactones) were obtained from Sigma-Aldrich (Steinheim, Germany) and kept at –4˚C. Stock solutions for standards were prepared by dissolving these samples in acetonitrile at a concentration of 1000 mg/L. The stock solutions were kept at – 4˚C and could be stored over a four week period. Acetonitrile was purchased from Merck (Mumbai, India). Standard solutions were prepared by diluting the stock solutions with acetonitrile. For GC-MS analyses, the stock solutions were mixed and diluted with acetonitrile to the desired concentration. Pure helium gas was delivered to the GC- MS system as a carrier gas.

2.2. Microorganism's Growth Conditions and Sample Extraction

The bacterial strain of *P. aeruginosa* Microbial Type Culture was grown in growth media. The composition used for the growth media was beef extract (1 g/L), yeast extract (2 g/L), peptone (5 g/L) and sodium chloride (5 g/L). Cultures were grown in an Erlenmeyer flask containing 50 ml growth media at a temperature of 37˚C for 48 hours. Liquid medium were inoculated with the strain and were sub-cultured every week. Cell suspension was centrifuged at 4000 rpm for 20 min and 10 ml of cell-free supernatant harvested during the stationary growth phase was extracted using the procedure described previously [30]. In brief, 10 ml of cell-free supernatant solution was extracted three times with an equal amount of chloroform. The combined organic phases were washed with 2 mL of water and taken to dryness in the oven kept at 20˚C. The residue was redissolved in 2 mL of acetonitrile. This culture solution was stored at freezing temperature until further use.

2.3. Sputum Sample Extraction

Sputum sample was collected from a cystic fibrosis pa-

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tient from a local clinic. The sample was centrifuged 1000 rpm for the 10 minutes. The supernatant was collected and stored at –4˚C. Extraction was done with 4 ml of chloroform using a separating funnel and then the organic layer was washed with equal amount of water to remove any matrix impurity and was taken to dryness at room temperature. The residue redissolved in 2 mL of methanol. This sample was further extracted with solid phase extraction using 7020-01 (100 mg/mL) monomer octadecyl cartridge (J. T. Baker, USA) and SPE process was conducted on Visiprep SPE vacuum manifold system Supelco (Bellefonte, PA, USA). For optimal procedure of RP-C_{18} SPE, cartridge was pre-conditioned with 2 ml of methanol, 2 mL of acetonitrile on an SPE manifold. Then, sample aliquots of 1 mL were passed through the cartridge at a flow rate of 1 mL/min. After that, the cartridges were dried by vacuum for 3 min and finally eluted with 2 mL of acetonitrile.

2.4. GC/MS Instrumentation and Conditions

Gas chromatographic mass spectrometric (GC-MS) system with model GC-MS-QP2010 Plus (Shimadzu Corporation, Kyoto, Japan) was used for the analysis. The capillary column used in the GC was Rxi-1ms (30 m × 0.25mm ID × 0.25μm) supplied by Restek U.S. (Bellefonte, PA, U.S.A.). Chromatographic data were collected and recorded by GC-MS Real Time Analysis software. Sample injection was done in split mode (split ratio 10:1). Helium was used as the carrier gas at a flow rate of 1 mL/min. The GC injector temperature was set at 270˚C. The column oven temperature was optimized to hold at 100˚C for 1 minute and then to increase by 10˚C/min up to 200˚C, then increase by 15˚C/min up to 260˚C and then by 30˚C /min up to 300˚C. Mass spectrometry conditions were as follows: electron ionization source set at 70 eV, MS source temperature 200˚C and solvent cut time was 3.5 minutes. The mass spectrometer was run in full scan mode (m/z 20-500) and in (SIM) mode at 143 m/z. The quantitation of samples was done by using the SIM mode.

3. Results and Discussion

3.1. Optimization of GC-MS Conditions

Standard solutions of AHL were assayed by GC/MS without derivatization using full scan acquisition mode. Using different oven temperature programs, the conditions were optimized. The best results in terms of selectivity and analysis time were obtained using an oven temperature gradient starting from 100˚C to 300˚C. Detection was performed in selected ion monitoring (SIM) mode by using fragment at m/z 143. A chromatogram of a mixture of six AHLs separated under the optimized experimental conditions is shown in **Figure 1**. As it can be seen, a very good separation of all AHLs was achieved in analysis time of 19 minutes (**Table 1**). All peaks of AHLs in the standard solution were detected and identified from the respective mass spectra (**Figure 2**.). The mass spectrum of each compound shows a molecular ion $[M]^{+\cdot}$ that was characteristic of each homoserine lactone (**Table 1**). A common fragmentation abundant ion was observed for all AHLs at m/z 143 and other minor peaks at 73, 57 and 43. A similar mass spectrum was reported by Pearson *et al.* [32] for the N-butyryl homoserine lactone (mol. wt. = 171) purified from *P. aeruginosa*.

As shown in **Scheme 1**, the fragment ion at 143 m/z is the most likely due to McLafferty rearrangement, which is a typical of carbonyl groups having a hydrogen atom in the γ-position. This rearrangement gives rise to an enolic fragment and an olefin. Two major fragmentation

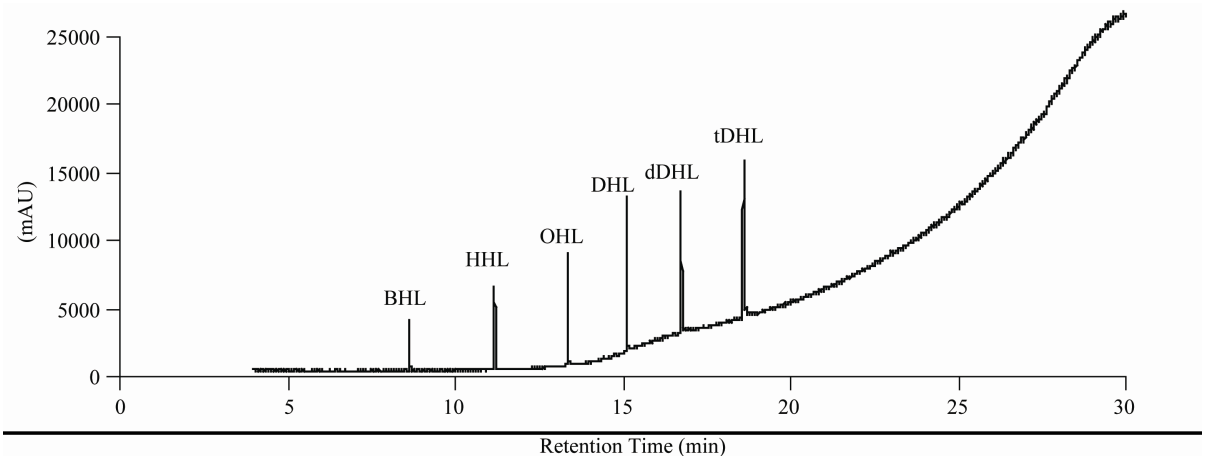

Figure 1. Typical GC/MS chromatogram of a mixture of BHL, HHL, OHL, DHL, dDHL and tDHL in acetonitrile at a concentration of 1 mg/L.

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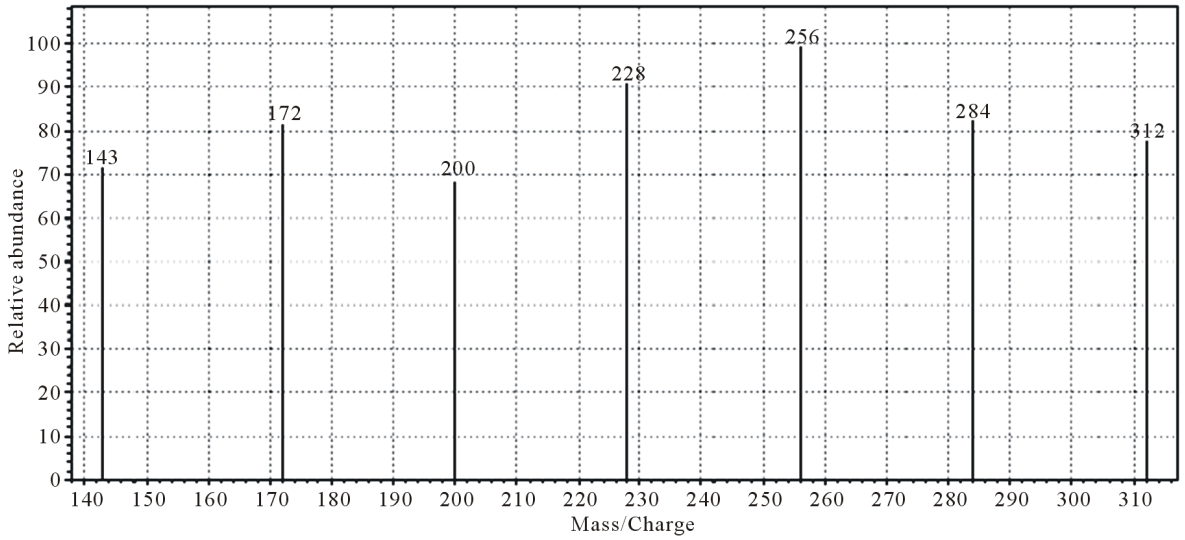
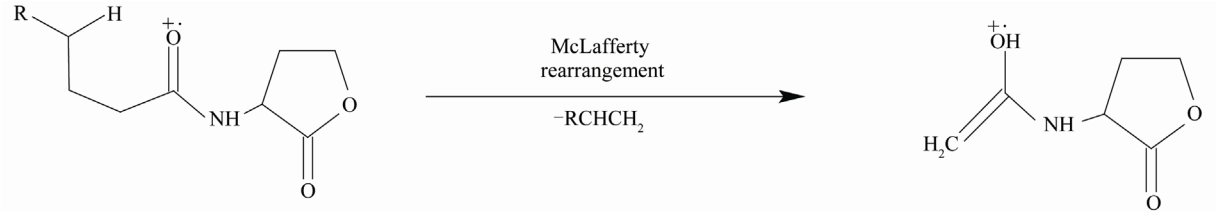


Figure 2. Representative GC/EI-MS spectrum recorded for N-Acyl homoserine lactone.

Table 1. Data of the N-acyl homoserine lactones investigated.

Homoserine lactones (HL)	Acronym	Melting point (˚C)	Molecular ion $[M]^+$	Retention time (min)
N-butanoyl HL	BHL	89.8	171	8.629
N-hexanoyl HL	HHL	102.6	199	11.178
N-octanoyl HL	OHL	105.8	227	13.351
N-decanoyl HL	DHL	112.5	255	15.108
N-dodecanoyl HL	dDHL	115.3	283	16.740
N-tetradecanoyl HL	tDHL	NA	311	18.602



Scheme 1. Electron impact formation of ion at m/z 143 due to cleavage of an acyl homoserine lactone by a McLafferty rearrangement.

processes involving an inductive effect and α-cleavage produce the other fragment ions at 73, 57 and 43.

3.2. Method Validation

Calibration curves of bacterial culture and sputum sample spiked with AHLs standards were performed in the range 1 - 500 µg/L on GC-MS with six concentration levels. The calibration curves were described by the linear regression equation:

$$y = mx + c$$

where y is peak area, x is the concentration, m is the slope and c is the intercept. The correlation coefficient was in the range of 0.987 - 0.999. The limit of detection (LOD) was set at the concentration when the signal/noise ratio was equal to 3:1 and found in the range of 2.1 - 3.6 µg/L (**Table 2**). The quality control (QC) samples were prepared with the concentration of 100 µg/L for AHLs. The accuracy and precision were calculated for the QC samples, both within and between days. The experiments were done six times during six different days. The RSDs for quality control samples were less than 6.24%. The extraction recoveries of the AHLs were calculated by comparing the peak areas of extracted QC samples from

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Table 2. GC-MS characteristics of N-acyl homoserine lactones in standard solutions.

N-AHL	BHL	HHL	OHL	DHL	dDHL	tDHL
R^2	0.9876	0.9979	0.9992	0.9889	0.9925	0.9957
Regression Equation	Y=7.7479x + 30.007	Y = 11.91x + 133.44	Y = 16.068x − 100.71	Y=20.988x − 281.12	Y = 22.627x − 489.4	Y = 33.898x − 1443.6
Slope (m)	7.7479	11.91	16.068	20.988	22.627	33.898
calibration range (µg/L)	10 - 1000	10 - 1000	10 - 1000	10 - 1000	10 - 1000	10 - 1000
LOD* = 3*S/N (10 µg/L)	2.14	3.59	2.71	2.10	2.45	2.34
Intra-day R.S.D*. (%)	2.66	1.56	1.82	1.91	2.43	1.58
Inter-day R.S.D*. (%)	2.98	1.57	2.13	2.27	2.57	1.94
Retention Factor	1.86	2.72	3.45	4.03	4.58	5.20
Selectivity factor	1.45	1.26	1.16	1.13	1.13	
Resolution	26.27	17.52	15.48	16.07	12.01	

*Each value is a mean of three measurements.

the bacterial culture and sputum sample to the peak areas of analyte standard solutions.The recovery of all AHLs in the sputum sample was found in the range of 94% - 99% and in the *Pseudomonas aeruginosa* was 93% - 98%. The statistical data (**Table 3**) reveals that the proposed method is acceptable for the quantification of AHLs in the real samples.

3.3. Real Sample Analysis

Using the optimized experimental conditions, the analysis of *P. aeruginosa* extract was performed. Three supernatant samples were extracted and injected into the GC-MS system. Chromatogram for the extracts of *P. aeruginosa* in full scan mode and corresponding SIM mode for m/z 143 has been shown in **Figure 3(a)** and **(b)**, respectively. The chromatograms recorded in full scan mode shows the interference in the identification of AHLs by intense signals of matrix. Analysis of bacterial extract in SIM mode provides signals for five AHLs efficiently. From the retention time and the characteristic fragment ion at m/z 143, the occurrence of BHL, OHL, DHL, dDHL and tDHL was confirmed and the respective concentrations of all five AHLs were obtained from real samples and RSD values were less than 6.5% (**Table 4**). These values of concentrations obtained were below the other reported methods. Therefore, the developed method is highly sensitive and is suitable for the determination and quantification of AHLs highly expressed by the bacteria.

AHLs were extracted from the sputum sample with the same procedure as mentioned above. The chromatograms in full scan mode (**Figure 4(a)**) and SIM mode (**Figure 4(b)**) were obtained for the sputum extract and the same common ion peak was obtained at m/z 143. In the sputum extract, only two AHLs *i.e.* OHL and DHL were obtained and the amount recovered were 3.47 and 3.44 µg/L, respectively (**Table 4**).

Significant differences in the AHL recovery profile were observed in this study and already published data for the same bacterial culture (**Table 5**). Most studies to detect *P. aeruginosa in vivo* have focused on detection of 3-oxo-C_{12} AHL and C_{14} AHL using reporters that are not completely specific for these AHLs. Middleton *et al*. [21] reported that the predominant AHL present in one sputum sample was C_6 AHL. Erickson *et al.* [26] found

Table 3. Results for determination of N-acyl homoserine lactones from spiked Sputum samples and extracts of bacterial strain of *Pseudomonas aeruginosa*.

Sample	Nominal (µg/L)	Amount of BHL found (µg/L)	Amount of HHL found (µg/L)	Amount of OHL found (µg/L)	Amount of DHL found (µg/L)	Amount of dDHL found (µg/L)	Amount of tDHL found (µg/L)
Sputum sample	100	95.59	97.60	96.57	98.75	94.23	95.38
Pseudomonas aeruginosa culture	100	93.78	95.32	97.89	97.34	96.45	96.74
RSD%		6.23	5.67	5.98	4.67	5.91	5.54

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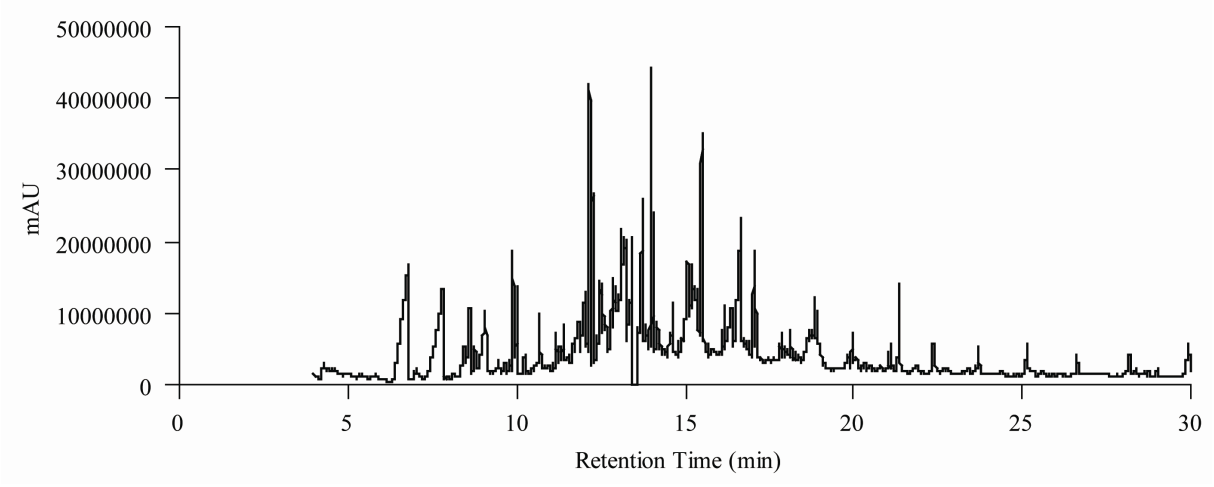
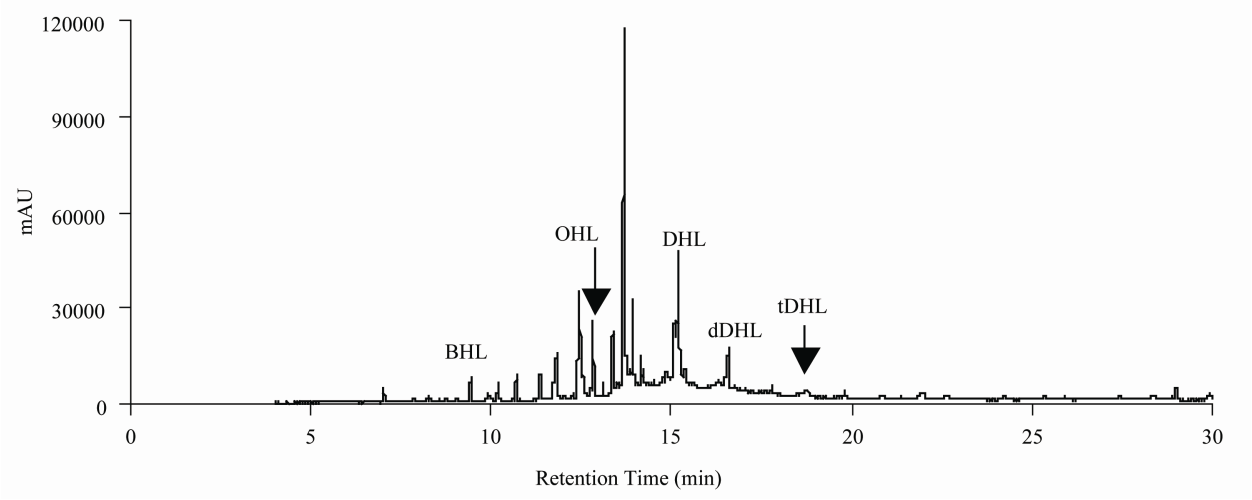


(a)



(b)

Figure 3. GC-MS chromatogram of an extract of cell-free supernatant of *Pseudomonas aeruginosa* in the TIC mode (a) and in SIM mode at m/z 143 (b).

Table 4. Results for determination of N-acyl homoserine lactones in extracts of bacterial strain of *Pseudomonas aeruginosa* and Sputum samples.

Sample	Amount of BHL found (µg/L)	Amount of HHL found (µg/L)	Amount of OHL found (µg/L)	Amount of DHL found (µg/L)	Amount of dDHL found (µg/L)	Amount of tDHL found (µg/L)
Sputum sample	_	_	3.47	3.44	_	_
Pseudomonas aeruginosa culture	2.78	_	4.89	2.34	2.75	3.51
RSD %	6.45		5.86	5.62	5.88	5.39

the significant amounts of 3-oxo-C_8 AHL and 3-oxo-C_{12} AHL in one of the sputum sample. All these studies suggest that it is important to identify specific AHLs present in clinical specimens and in a bacterial species that often produce more than one AHL, thus, the use of reporters to detect the AHLs produced in the bacterial culture is very complicated and problematic. Thus, definitive identification of AHLs would require mass spectrometry analysis. M. Frommberger *et al*. [28] detected N-acyl homoserine lactones using mass spectrometry along with capillary zone electrophoresis by studying the alkaline hydrolytic product of the same. In our study, six

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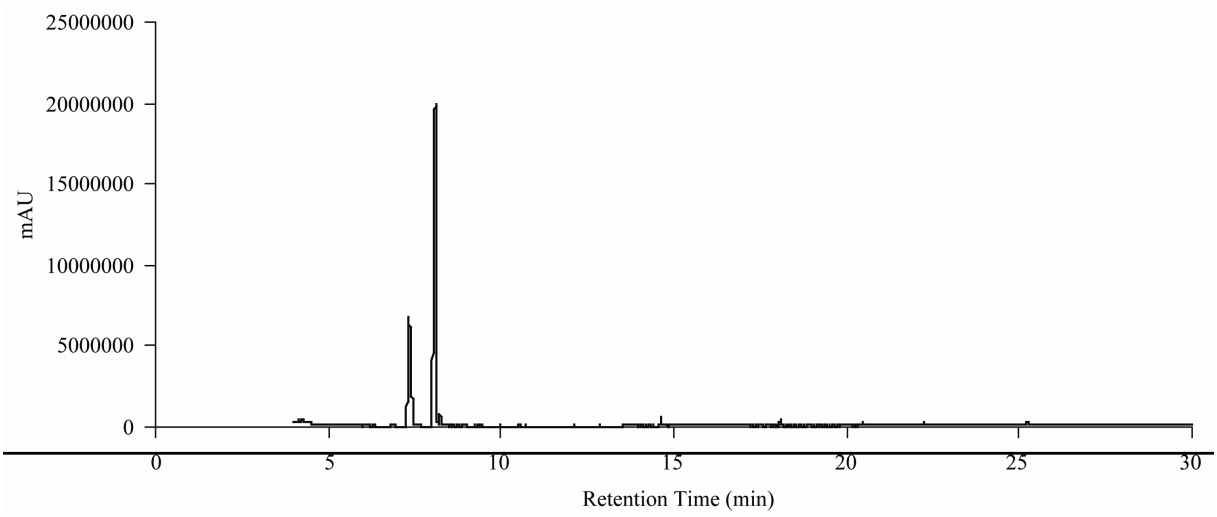
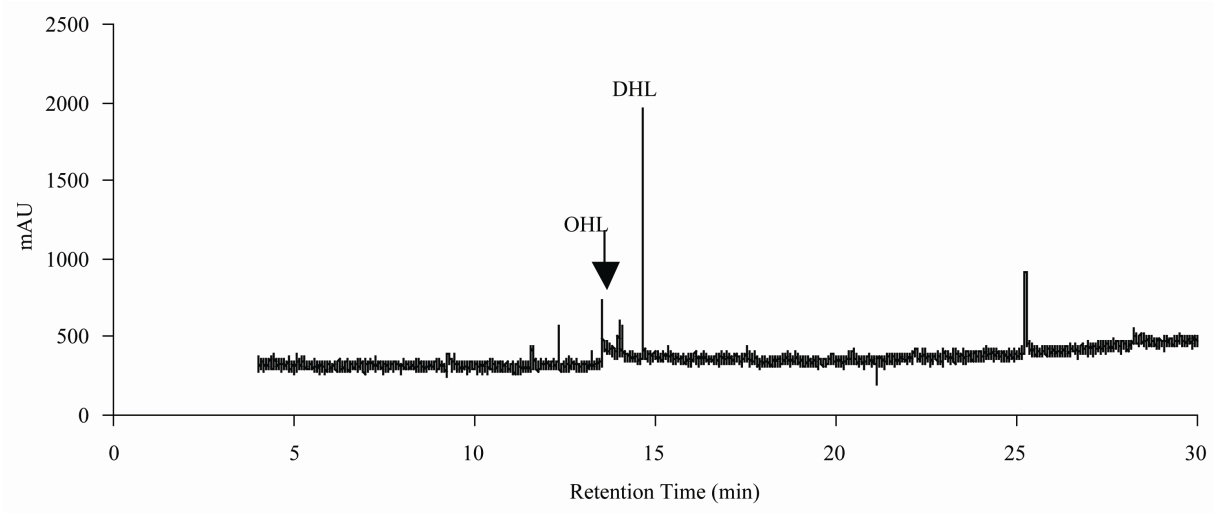


(a)



(b)

Figure 4. GC-MS chromatogram of the sputum sample of a cystic fibrosis patient in the TIC mode (a) and in SIM mode (b) at m/z 143.

Table 5. Comparison of AHLs recovered in developed method with literature data.

Sample	In our optimized method	In Published data	Reference
Sputum sample of Cystic fibriotic patient	OHL, DHL	HHL, OHL, DHL, ODHL,	[20][a]
Pseudomonas aeruginosa	BHL, OHL, DHL, dDHL, tDHL	BHL, HHL, OHHL, OHL, ODHL, OdDHL	[19][a]
		OdDHL, ODHL, OHL	[20][b]
		HHL, OdHL	[21][c]
		OtDHL, ODHL, OOHL	[25][d]
		BHL, DHL, d-DHL	[31][e]

Method of analysis employed: [a]biosensor strain *Escherichia coli*; [b]LC-tral-luxCDABE-based reporter; [c]LC-MS TLC; [d]GC-MS with derivatization; [e]Direct analysis on GC-MS.

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different AHLs were separated and identified in the spiked samples by GC-MS without any chemical modification. Five of these AHLs were identified in real sample of *P. aeruginosa* culture and two AHLs were found in the sputum sample of cystic fibrosis patient. Cataldi *et al*. [31] found the three-homoserine lactones *i.e*. N-butanoyl, N-decanoyl and N-dodecanoyl homoserine lactones in the *P. aeruginosa* culture in the range between 0.92-1.07 mg/L. In our study, AHLs are determined at lower concentration levels than the other reported me- thod [31]. Therefore, the developed GC-MS method is relatively rapid, reliable and almost 1,000 times more sensitive than the previous methods and could be used for assessing the patterns of AHLs production during growth of bacteria at different growth states.

4. Conclusions

A primary aspect of this work is to establish an assay for analyzing the AHLs from bacterial cultures and sputum samples. A major goal is to keep a minimal extraction procedure to enable rapid, reproducible and GC compatible sample preparation. SPE is used for the extraction of AHLs in the sputum sample. This is a reliable and convenient procedure for indicating the presence of AHL in real sample without using any chemical derivatization. It provides more comprehensive alternative to current bacteriological techniques for the detection of AHLs in biological extracts. The GC-MS technique can be used for characterizing AHLs on a routine basis, a necessary requirement for assessing potential health risks associated with microbial spoilage and to improve the understanding of AHLs behavior.

5. Acknowledgements

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