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ORIGINAL PAPER

Single drop microextraction of homoserine lactones based quorum sensing signal molecules, and the separation of their enantiomers using gas chromatography mass spectrometry in the presence of biological matrices

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Abstract N-acylated homoserine lactones (AHLs) are produced by Gram-negative bacteria as communication signals and are frequently studied as mediators of the "quorum sensing" response of bacterial communities. AHLs are optically active components and the L-form is known to have activity in the quorum sensing. However, the knowledge regarding the stereospecific production of the AHLs in bacterial cultures is limited; therefore, there is a need for a fast and easy method for their chiral analysis. A method was developed for the preconcentration of the AHLs using single drop microextraction or liquid-liquid microphase extraction in toluene for their analysis using GC-MS. The performance of the method were determined and discussed for the chiral separation of these autoinducers using a capillary column coated with heptakis-(2,3-di-O-acetyl-6-O-t-butyldimethyl-silyl)-β-cyclodextrin. The salient feature of this study is the demonstration, that *Burkholderia cepacia* LA3 produced D-decanoyl-homoserine lactone beside L-decanoyl- and L-octaonyl- enantiomers.

Keywords Chiral separation · *N*-acyl-homoserine lactones · Quorum sensing · GC-MS · Single drop micro-extraction

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Introduction

Bacteria are able to coordinate their certain behaviour in local cell density dependant manner that termed as quorum sensing or cell-to-cell communication [1]. Autoinducers (AI) are the vehicle of the quorum sensing that are produced and released to the environment by the microorganism. Different component classes of AIs have been identified like oligopeptides, (2S,4S)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran borate also called as Autoinducer 2 [2] and hydroxyl-alkyl-quinolones [3–5] but the most known AI produced by several Gram-negative bacteria are the derivatives of N-acyl-homoserine lactone (AHLs). AHLs consist of a furen-2-on group with an acyl chain consisting of 4 to 14 (less frequently up to 18) carbons and thus differs in hydrophobicity; the octanol-water coefficient (log P) were between 0.03 for butanoyl-homoserine lactone (C4-HSL) to 5.09 for tetradecanoyl-homoserine lactone (C14-HSL). AHLs can be substituted at the β-carbon by a keto- or hydroxyl-function and the fatty acid side chain may be unsaturated that shows high diversity of the produced AHL based bacterial signals. The AHL biosynthesis derives from the fatty acid biosynthesis pathway. This is the reason why several AHLs with an even

numbered C-chain are found in nature [6]. The amino acid substrate of the AHL biosynthesis is S-adenosyl-L-methi-nonine [7] reflecting the formation of L-enantiomers (see Fig. 1b).

Several methods have been developed for the direct analysis of AHLs based on bioassay, chromatography, electrophoresis coupled to different detection techniques like mass spectrometry and reviewed elsewhere [8–11]. Less methods have been developed for their chiral discrimination although the differentiation of the optical isomers became more and more important since it was showed that e.g., only L-AHL derivatives had comparative immune modulatory activity [12] or the uptake rate of AHLs by barley and yam bean was enantiomer dependant [13]. Chiral stationary phase high performance liquid chromatography (HPLC) were applied to confirm the optical purity of the synthesized material [14] but no real sample was applied for the method. Published methods developed for enantiomer separation of AHLs from bacterial supernatant are based on nuclear magnetic resonance spectrometry (NMR) after fractionation by reversed phase HPLC [15] and gas chromatography (GC) [13, 16–18]. The chiral stationary phase used for GC containing cyclodextrin derivative as a selector that are directly bonded to a polysiloxane to prevent the migration of the chiral selector to different locations in the surface film resulting in better and more robust resolution between the isomers. Pomini et al. applied flame ionisation detection and reported retention time of 56.72 min for R-N-hexanoyl homoserine lactone and 56.89 min for S-N-hexanoyl homoserine lactone (S-C6-AHL) that reflects quite low resolution of the enantiomers [17]. Thus confirming the results on the production of the pure S-C6-AHL by *Pantoea* species, circular dichroism—a conventional method for determination of the asymmetric centre of a molecule—was applied [18]. For the determination of the AHL enantiomers by GC, a pre-concentration was added by liquid-liquid extraction (LLE) applying ethyl acetate as an organic solvent. Relatively high volume of the cell-free supernatant (1L and 8L) and thus high volume organic solvent (3× 500 mL) were required for the treatment before the GC analysis. In our earlier work [13], similar chiral column was used for the GC analysis for studying the stereo selective uptake of AHLs in spiked mineral mediums in which barley and yam bean was grown. A high resolution (higher than 1.5) and lower retention times were achieved. Moreover, MS detection was applied to improve the selectivity of the method although the method lacked on sensitivity.

The main aim of the present work was to develop a sensitive, selective and rapid sample treatment using GC-MS for the simultaneous chiral resolution of the AHL derivatives that differs highly in the hydrophobicity in complex mixtures such as bacterial cultures. For the preconcentration of AHLs single drop microextraction (SDME) and liquid phase microextraction (LPME) were tested to decrease the detection limits and the organic solvent consumption that is needed for their extraction. SDME uses a single drop of an organic solvent of a few microliters volume which combines the sampling and preconcentration in the same step and whole of the extracted analyte(s) is used in determination in a simple manner [19–30]. The performance characteristics of the method were determined and applied for the analysis of the isomer ratios of the produced AHLs in the bacterial extracts of the *Burkholderia cepacia* LA3.

Experimental

Chemicals

N-Hexanoyl- (C6-HSL), N-octanoyl- (C8-HSL) and N-decanoyl- (C10-HSL) and N-dodecanoyl- (C12-HSL) homoserine lactone were obtained from Sigma-Aldrich (http://www.sigmaaldrich.com/germany.html, Steinheim, Germany) and kept at −20 °C. Stock solutions for standards were prepared by dissolving these substances in acetonitrile at a concentration of 1000 mg L^{-1}. The stock solutions were kept at −20 °C and could be stored over a 4-week period. Standard solutions were prepared by diluting the stock solutions with acetonitrile. Toluene, hexane, ethyl acetate, chloroform and methanol were purchased from Merck (http://de.vwr.com, Darmstadt, Germany). Acetoni-trile was purchased from Biosolve (http://www.biosolve.nl, Valkenswaard, the Netherlands). Water was provided by a Milli-Q system (Millipore Corporation, Billerica, USA). All other chemicals and reagents used in the present study were at least of analytical grade.

Equipment

AHLs were separated on a gas chromatograph (http://www.varianinc.com, Varian GC 3900 series, Varian Chromatog-raphy Systems, Middleburg, the Netherlands) coupled with a mass spectrometer (http://www.varianinc.com, Varian

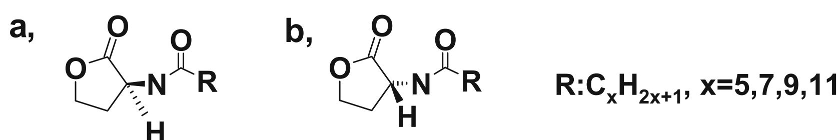

Fig. 1 Structure of AHLs as D- (**a**) and L- enantiomer (**b**)

Saturn 2100T, Varian Chromatography Systems, Walnut Creek, CA, 94598, USA). A 2 µL aliquot of the sample was injected in the splitless mode (2 min) using helium (ultrahigh purity) with a column flow of 1.3 mL min^{-1} and pulse mode injection (pulse pressure 15.0 psi, pulse duration 0.25 min). The injection port (CP 1177) contained an unpacked gooseneck injection port liner with a tapered lower section (4-mm internal diameter, deactivated) at 210 °C. The separation capillary column was a fused silica with an inner diameter of 0.25 mm and 25 m length covered with heptakis-(2,3-di-O-acetyl-6-O-t-butyldimethyl-silyl)-β-cyclodextrin with film thickness of 0.2 µm (http://www.macherey-nagel.com, Macherey- Nagel, Düren, Germany). The capillary was connected directly from injection port to a mass spectrometer via the interface region (280 °C). The oven temperature was programmed to increase from 50 °C at 30 °C min^{-1} to 220 °C (21 min). Higher temperature were avoided to limit fast blooding and loosing of the non covalently bonded film used as stationary phase and the destruction of the phase. Mass spectrometry conditions were as follows: electron ionization source set to 70 eV, emission current 500 mA at mass spectrometer trap of 190 °C and manifold of 80 °C.

Single-drop liquid-phase microextraction (SDME) and liquid-phase microextraction LPME)

For SDME, one 5 µL microsyringe with a bevel needle tip (http://www.hamiltoncompany.com, Hamilton, Reno, NV, USA) was used for introducing of the organic drop in 4 mL glass vial containing the 2 mL sample. For single-drop microextraction the sample solution was agitated with a magnetic stirrer by means of a 10×3 mm stir bar at room temperature. The microsyringe was rinsed with the organic solvent for several times to ensure that no air bubbles were left in the barrel and the needle. For extraction purpose, a specified volume of organic solvent (2 µL) was drawn into the syringe. The needle tip was put into the solution at about 1cm below the meniscus of the solution. The plunger of the syringe was depressed to make a drop at the tip of the needle. After stirring the solution for 30 min at a rate of 200 rpm, the drop was retracted into the syringe and the organic solvent was immediately injected into the GC injection port for the analysis.

For LPME, a 100 µL portion of toluene was added to 2 mL of the sample and shaken vigorously for about 1 min in a sample vial. The sample vial was kept undisturbed for the toluene droplets to coalesce and settle as a separate layer on the top of the aqueous phase. A 2 µl portion of organic phase was carefully withdrawn by a 5 µL Hamilton syringe and injected into the GC.

Real sample

To obtain AHLs produced by pure cultures of *Burkholderia cepacia* LA3 grown in 50 mL M9 minimal medium [10] with glucose as carbon source and in 50 ml nutrient broth (NB no. 4, Fluka, Buchs, Switzerland) full medium were inoculated and grown at 30 °C and 175 rpm overnight. Bacteria were harvested at 3488×g and 4 °C for 5 min in a Hettich Universal 32R centrifuge equipped with a 1620A rotor (http://www.hettich-zentrifugen.de, Hettich, Tuttlingen, Germany) and the supernatant was used for AHL extraction.

Results and discussion

GC-MS analysis

As previously reported [13], enantiomer separation of C6-, C8 and C10-HSL was achieved on heptakis-(2,3-di-O-acetyl-6-O-t-butyldimethyl-silyl)-β-cyclodextrin film by GC within one run that was extended with C12-HSL. When optically pure target was injected at different concentration the D-form showed higher complex affinity to the selector and no spontaneous racemisation was observed at the separation condition. As expected, the retention times of the L and D-enantiomers of the analytes (see Table 1) logarithmically increased with the number of the carbon of the alkyl side chain giving the possibility to confirm the identity of an another AHL derivatives as the targets using the method of Kovats-retention index. The resolution factors (R_s) of the enantiomers were between 2.7 and 3.8 (Table 1) reflecting the baseline chiral separation and were independent on the length of the alkyl side chain. Small tailing of the peaks were observed that increased with the retention strength of the target components that could be caused by the occurrence of stronger secondary interaction (e.g., H-bond). Another effect due to the relative polar nature of the AHLs was the relatively high detection limit determined for the separation thus a concentration step before the analysis was negligible for sample analysis. The fragmentation of the AHLs in the electron impact ionisation was identical as previously reported [31]. Although the main fragment is the ion 143, the m/z of the molecule ion was always observable giving more help in the identification.

Extraction method

Generally in SDME 1–3 µL of the organic solvent is employed for the extraction and it combines both the aspects of sampling and preconcentration of the AHLs. The main significant advantage of the method is that whole of

Table 1 Performance characteristics of a method

			L-C6-HSL	D-C6-HSL	L-C8-HSL	D-C8-HSL	L-C10-HSL	D-C10-HSL	L-C12-HSL	D-C12-HSL
retention	retention time		8.78	9.08	12.57	13.14	19.99	21.02	34.25	36.28
	RSD of the retention time [%]		0.19	0.22	0.19	0.17	0.23	0.39	0.17	0.11
	Resolution factor		3.8		3.7		2.7		3.6	
SDME	repeatibility [%]		2.76	4.37	5.00	6.36	7.61	7.34	7.71	7.94
	used conc range [mg/L]		0.5–10							
	limit of detection [mg/L]		0.5		0.1		0.1		0.3	
	slope of the calibration curve	standard solution	0.07	0.07	1.18	1.18	5.07	4.92	3.07	3.03
	regression coefficient		0.904	0.904	0.986	0.986	0.993	0.992	0.9352	0.931
	slope of the calibration curve	spiked growing medium	0.08	0.08	1.64	1.64	8.18	7.77	3.45	3.63
	regression coefficient		0.999	0.999	0.978	0.978	0.999	0.995	0.944	0.954
LPME	repeatability [%]		4.80	6.03	4.29	3.13	2.48	1.18	0.50	4.26
	used conc range [mg/L]		0.1–15							
	limit of detection		0.05		0.01		0.01		0.05	
	slope of the calibration curve	standard solution	0.43	0.42	0.41	0.43	1.27	1.27	1.57	1.54
	regression coefficient		0.988	0.994	0.906	0.903	0.934	0.934	0.985	0.981
	slope of the calibration curve	spiked growing medium	0.22	0.23	0.63	0.63	1.45	1.45	1.45	1.36
	regression coefficient		0.996	0.990	0.963	0.956	0.98	0.98	0.973	0.963

the target components extracted is directly transferred to the GC-MS, due to which the sensitivity of the method is increased many fold. Parameters which might influence the extraction of the AHLs in the organic solvent were investigated.

Since the AHLs are hydrolysed to homoserine lactones under the alkaline conditions [32], the extraction of the AHLs was carried out under the neutral conditions. Various organic solvents like, chloroform, ethyl acetate, hexane and toluene were tested for the extraction of these AHLs from the aqueous medium. Chloroform and ethyl acetate showed low extraction effectivity (recovery was less than 50%). The recoveries of the hexane extraction were dependant on the target hydrophobicity. When toluene was applied the extraction rate was higher than 80% and it was less dependent on the polarity of the AHLs. A stirring time of 30 min was selected from the studied range between 1 and 30 min since peak area of the analytes showed saturation after 30 min. It was also observed that the extraction efficiency increased from 80% to 95% with the increase in the drop volume of toluene 1–4 μL. However, a 2 μL of toluene was selected since the drop was stable during the extraction from the bacterial cultures and still gave sufficient recoveries for the extraction. It was found that stirring of the stirring of the solution at 200 rpm was sufficient as stirring at higher speed causes drop to fall down. Figure 2 shows a typical chromatogram of AHLs at a concentration of 2 mg·L^{-1} spiked in growing medium.

Liquid-liquid extraction of the AHLs from cell-free supernatant was often used, therefore SDME was compared to LPME applying the optimized condition although the volume of the organic solvent had to be increased. A 100 μL of toluene was used for the extraction of these AHLs. It was observed that LPME is another alternative for the extraction of these analytes into toluene (see Table 1).

Performance characteristics of a method

The performance characteristics of the method are summarized in Table 1. The method was applied for the chiral separation and determination of these AHLs in the standard solution and in the spiked growing medium using SDME and LPME The matrix effect was studied using two different growing medium; at first, a so-called rich medium that is extract of the yeast (NB medium) in which all elements present for fast and effective growing of the bacteria and secondly, a defined minimum medium (M9) that contains only the necessary substrates (inorganic salts and sugar as C-source) for the growth. No difference in the extraction efficiency was observed compared the two

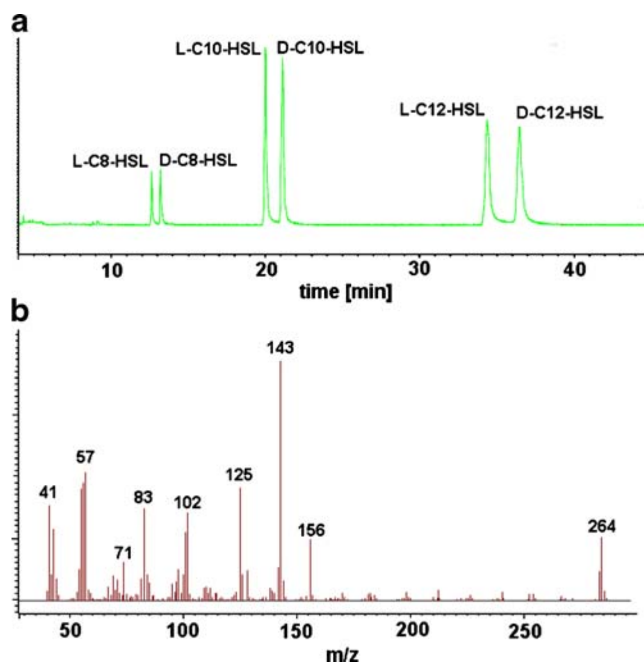

Fig. 2 Chromatogram of standard solution of D and L enantiomer of C8-, C10- and C12-HSL at concentration of 1 mg•L-1 L **a** and mass spectrum of C12-HSL at 34.2 min **b** in the case of SDME treated medium spiked with HSLs derivatives at concentration of 2 mg•L-1 L

medium types thus the characteristics were determined from one medium (M9) since this is most frequently used in for purpose of chemical analysis. Extraction from blank growing medium was applied and no interference was observed.

The separation showed a robust character since the injection-to-injection relative standard deviation (RSD) of the retention times were between 0.1–0.4% and the day-to-day was less than 3%. The repeatability of the method applying both treatment techniques was determined between 0.5–8%.

The used concentration range were between 0.1 mg/L and 10 mg/L, as saturation in the curve was observed with higher concentrations than 10 mg/L reflecting the overloading of the drop by the analytes. The regression coefficients (R^2) were between 0.956 and 0.999 that can be improved with application of internal standard. The most applied internal standard for AHL analysis [33] is the heptanoyl-homoserine lactone although its production as autoinducers have been identified [17]. Small increase in the slope values of the calibration curve was observed when standard solution and spiked M9 medium was used that might be due to the difference in the ionic strength although the difference was not synergistic. The mathematical limit of detection (LOD) was determined as signal-to-noise ratio 3 and were confirmed by injection of he determined concentration. LPME was more sensitive than SDME, but as a small organic volume is used in SDME and whole of which can be transferred into GC-MS makes it preferable over the LPME method.

The enantiomer ratio of the commercially available AHLs was found to be constant (0.98–1.04) in the range studied with an RSD of less than 4.5% showing the possibility of accurate determination of the enantiomer ratio. The ratio showed also non-dependency on the type of the extraction method and the day when the solutions were prepared reflecting the negligible racemisation of the chiral centre as was also published elsewhere [34].

Sample analysis

The method was applied for the analysis of the AHLs in a known producer, *Burkolderia cepacia* LA3 bacterial culture samples. This species produce mainly C10-HSL among C8-HSL at published concentration of 1.61 μM [35] that was comparable to the recent determined concentration (2.5 μM) for that preconcentration by solvent evaporation (factor of ten) before analysis was applied. L-C8-HSL and, surprisingly, both optical isomer of C10-HSL were determined from the cell-free superna-

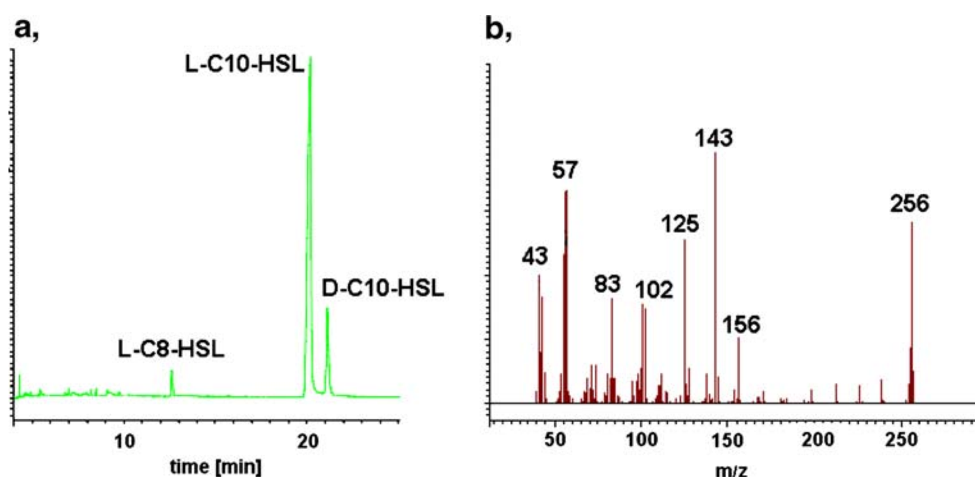

Fig. 3 GC-MS chromatogram of *Burkholderia cepacia* LA3 supernatant (**a**) with corresponding EI-MS spectra of the C10-HSL at 20.0 min (**b**)

tant (see Fig. 3). The D/L isomer ratio of the target component was 0.2 thus five times more L-C10-HSL was determined. Confirming the results, growing medium was extracted directly and similar results were determined; no presence of D-C8-HSL and D/L ratio of C10-HSL of 0.29. The production of the D-C10-HSL could be derived from its biosynthesis since it forms form amino acid derivatives and form the bacteria were shown to be able to produce D-methionine derivatives [36]. It is well known that also higher organisms show the production of D-amino acids and especially in the neural central system these were studied as building blocks with L-amino acids of neuropeptides signaling molecules [37–39] having thus a much higher structural diversity than when constituted from L-amino acids only. We speculate that in bacterial communication this strategy could exist giving these organisms to have an evolutionary communicative advantage.

Conclusion

A primary aspect of this work was to establish an assay for analyzing the AHL enantiomers from bacterial cultures. A major goal was to keep a minimal extraction procedure to enable rapid, reproducible and GC-compatible sample preparation. Two methods, SDME and LPME were studied and compared. Since the performances characteristics of the two treatment methods did not differ each other, SDME make the extraction and sample preconcentration in a single step therefore, it was highly advantageous. Applying the method, no matrix effect was observed reflecting the sufficient clean up. No drying of the solution before the injection to the GC was needed decreasing the number of the steps and thus the possibilities of failures. The AHL enantiomers differs highly in solubility were separated on a chiral column coated containing nonpolar β-cyclodextrin derivative within one run that makes the identification of the optical isomers from different strains since the produced AHLs mostly differs in the length and substitution alkyl side chain producers. D-decanoyl-homoserine lactone was found beside L-decanoyl- and L-octaonyl- derivatives to be produced by the *Burkholderia cepacia* LA3 stain showing that bacteria also may produce D-configured signalling molecules and showing the need of analytical technologies to differentiate enantiomers that may have different biological activity.

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