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A simple and robust set-up for on-column sample preconcentration – nano-liquid chromatography – electrospray ionization mass spectrometry for the analysis of *N*-acylhomoserine lactones

Received: 27 October 2003 / Accepted: 5 November 2003 / Published online: 11 December 2003

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Abstract A simple method for the simultaneous, rapid and sensitive determination of *N*-acylhomoserine lactone signaling molecules in bacterial isolates, without prior sample preconcentration and with minimal sample cleanup, is presented. The analysis relies on the combination of analyte preconcentration and separation on a single device: a relatively large sample volume (1–5 μ L) is directly loaded onto a laboratory-made, miniaturized (75 μ m i. d.) reverse phase nano-liquid chromatography column, connected on-line to a microelectrospray-ionization ion trap mass spectrometer. In a first step the analyte is adsorbed (and so concentrated) at the beginning of the column, and is eluted and selectively separated in a second step by the organic mobile phase. Sample preconcentration follows the mechanisms of solid phase extraction on a nano-scale, while separation takes place according to classical liquid chromatography separation principles. The columns can be manufactured easily, are simply connected, and used with minimal solvent amounts; this makes this method extremely robust and cost-effective. The analytical setup was found to be routinely quantitative down to a concentration of 10 ng/mL (corresponding to a total analyte amount of 10 pg or ca. 50 fmol). The limit of detection was reached

at 1 ng/mL (1 pg, ca. 5 fmol). Compared to the classical AHL analysis of bacterial cultures with biosensors, where selectivity and sensitivity is often limited, this rapid analytical technique is a substantial qualitative and quantitative improvement. Two unsubstituted *N*-acylhomoserine lactones could be identified and quantified from a *Burkholderia cepacia* culture supernatant in a chloroform extract.

Keywords Quorum sensing · AHL · Nano-HPLC · Sample preconcentration · Mass spectrometry

Introduction

Over the last few years, it has become more and more apparent that bacterial cells can sense their local population density through a communication process mediated by small diffusible molecules (also referred to as “autoinducers”). This mechanism, known as “quorum sensing” (QS), allows bacteria to initiate specific physiological reactions depending on the cell number and these can therefore be seen as a kind of multicellular behavior in prokaryotes. Physiological processes regulated by QS include bioluminescence, swarming, antibiotic biosynthesis, plasmid conjugal transfer, and the production of virulence determinants in animal and plant pathogens; for overviews see [1, 2, 3, 4, 5].

In Gram-negative bacteria, the best understood QS mechanism is mediated by *N*-acylhomoserine lactones (AHLs), which vary in the length and the saturation of the acyl side chain and its substitution (oxo or hydroxy functions at C-3). Special attention is focused on the AHL-type of interactions, as they can govern the development of virulence factors in human pathogenic bacteria [6]. The coordinated initiation of pathogenicity in the lungs of cystic fibrosis patients is, for example, regulated by AHLs in the biofilms of *Pseudomonas aeruginosa* [7, 8] and members of the *Burkholderia cepacia* complex [9, 10]. In bacterial colonies, different signals can be sent simultaneously, even resulting in inter-species signal recognition/

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processing patterns with complex physiological rules and interacting signal networks [11, 12]. AHL molecules play an essential role in the interaction between plants and bacteria [13], such as in the N_2 -fixing rhizobia/leguminosa symbiosis [14, 15, 16]. Effective colonizers of plant root surfaces such as *Serratia* sp. [17, 18] and *Pseudomonas* sp. [19] can activate the production of natural antibiotic and antifungal substances in an AHL-controlled regulatory mechanism. Specific stems of *Pseudomonas* sp. and *Burkholderia* sp. exhibit human pathogenicity and are colonizers of rice and tomato roots in the same manner [20]. The increased understanding of bacterial communication with other bacteria and with higher organisms finds more and more applications. New therapeutic drugs can be designed based on the interference of communications between bacteria [21, 22] inducing antibiotic activity without the risk of possible resistance [23].

These numerous examples show an increasing need for selective and sensitive analytical techniques; the overwhelming majority of articles published on QS, however, still rely on modifications of a thin-layer chromatography (TLC) assay originally established by Shaw et al. in 1997 [24]. The assay involves extraction of the samples with organic solvents [25] and covering of the TLC plates with genetically modified reporter strains exhibiting easily detectable physiological reactions, like light emission or pigment production, when exposed to elevated AHL concentrations.

Although there is no denying that the TLC assay may often be an excellent tool for analyzing AHLs from bacterial broths, as it is cost-effective, does not require special laboratory hardware and is in some cases highly sensitive, the constraints are obvious and already noted in the early papers. Genetically engineered reporter strains (which have to be produced or purchased) are often practically limited to a subgroup of molecules (only short-chained, only hydroxylated AHLs) and react to other types with reduced sensitivity or not at all; structural and quantitative information from TLC is poor.

Recently, we showed that selective analysis of AHLs in bacterial culture supernatants is possible using partial-filling micellar electrokinetic chromatography (PF-MEKC) coupled to electrospray ionization mass spectrometry [26]. The method was found to be suitable for the detection of AHLs in preconcentrated culture extracts, but due to sensitivity limitations, it could not be successfully applied to AHL detection in untreated samples. Additionally, routine quantitative analysis was found to be limited in PF-MEKC/MS.

Here, the development of a simple and robust on-column sample preconcentration system using an in-house made capillary column is presented. We show its direct coupling to micro-electrospray ionization – mass spectrometry combining concentration and chromatographic separation for the quantitative analysis of *N*-acylhomoserine lactones from bacterial culture supernatant extracts. The method relies on principles which have been successfully applied in capillary LC [27, 28] and in capillary electrochromatography (CEC) [29, 30]; corresponding applications in nano-

scale LC are at the moment almost exclusively limited to protein and peptide analysis in “Proteomics”.

Materials and methods

Chemicals, standards and real samples

All chemicals were of analytical grade and were purchased from Merck (Darmstadt, Germany). Water was provided in double-distilled form (Westdeutsche Quarzschmelze, Geesthacht, Germany). *N*-acyl-D/L-homoserine lactone standards (C_4 -HSL, C_6 -HSL, C_7 -HSL, C_8 -HSL, C_{10} -HSL, C_{12} -HSL, and C_{14} -HSL) were supplied by Sigma-Aldrich (Buchs, Switzerland). The standards were dissolved in methanol at 1 mg/mL and diluted with water to reach the desired concentration.

In-house glycerol stock cultures of *Burkholderia cepacia* strain JA-8 were plated on Luria-Bertani agar [31] containing 4 g/L NaCl instead of the normal 10 g/L, and were incubated at 30 °C overnight. Single colonies were picked and were grown in 150 ml of Luria-Bertani medium for 24 h. 1 mL aliquots of the cultures were centrifuged at 14,000 rpm for 10 min. A 500 μ L-portion of the cell-free supernatant was extracted one or three times with chloroform (continuous shaking for 5 min and centrifugation at 14,000 rpm for 3 min). As any heating, even to 70 °C, was found to be a reason for dramatic recovery losses, the combined organic phases were taken to dryness under a stream of nitrogen at ambient temperature. If necessary, the extracts were stored in dry form (–20 °C) and were rediluted immediately before assaying them with 500 μ L of water.

Columns

To produce packed columns, fused-silica capillaries (75 μ m inner diameter, 40 cm length; Polymicro Technologies, Phoenix, AZ, USA) were used. The filling protocol was compiled from many sources and heavily modified; an overview of the different concepts is given in [32]. For the filling procedure, an emptied HPLC pre-column (4.6 mm inner diameter, 50 mm length) was connected to a Gilson (Middleton, WI, USA) 307 pump. This column served as slurry reservoir; its outlet is referred to as the “filling port”.

The capillaries were flushed with acetone (1 mL/min, 5 min). One end was dipped repeatedly in bare (unmodified) silica (Merck, Darmstadt, Germany) and was heated over the flame of a flue to form a temporary frit. A slurry of ODS (5 μ m Hypersil; Grom, Herrenberg, Germany) was prepared in acetone (5%, w/v). To provide maximum homogeneity, the suspension was treated for 5 min in an ultrasonic bath. The slurry was emptied into the reservoir and was pumped into the capillary with high pressure (40 MPa) under vigorous shaking. The actual filling process was accomplished in less than one minute and was carefully monitored under a light microscope (Nr. 355190, Reichert, Vienna, Austria). The pump was switched off and the pressure was allowed to dissipate overnight. The capillary was disconnected from the filling port and all parts were washed carefully with water to remove any remaining slurry. After remounting, a compression step with water (40 MPa, 120 min; the final 30 min in an ultrasonic bath) was performed. This step results in the compression of the filling material by ca. 5%. With the capillary still under pressure, a first final frit was sintered with the help of a laboratory-constructed electrical filament heater. The capillary was dismounted, and after cutting off the temporary frit, a second compression step identical to the first one but from the opposite direction was performed. A second frit was fabricated as above.

The filling material needed for one capillary is in the lower mg range and the effective total working time for filling (of one or an array of several columns) does not exceed 30 min (except the waiting periods of several hours during the night). No column conditioning was necessary between runs. A single column could be used successively nearly 500 times without a loss in performance, making nano-LC columns very attractive.

Liquid chromatography

A Series 200 LC pump (Perkin-Elmer, Norwalk, CT, USA) was connected to a stainless steel filter (5 μm ; Upchurch Scientific, Oak Harbor, WA, USA) and to a splitting tee equipped with a pressure reduction column (100 cm/50 μm , fused silica). After optimization, excess liquid was recycled to the solvent reservoir. The separations were driven in isobar mode (in other words, the flow rate was adjusted to result in constant pressure – usually ± 0.3 MPa) and elution took place isocratically. The room temperature was maintained at 20 ± 1 °C to give reproducible solvent viscosities. For injections, a Vici (Schenkon, Switzerland) CN2 (for 1 μL injections) or a C3 valve (for 5 μL injections) was used. After a modification of the original valve position, the packed columns (with free fused-silica segments of 5 cm on both sides) could be connected directly to the valve's outlet port. The separation column ended directly in the ionization interface.

This setup is competitive in terms of feasibility and cost-effectiveness compared to classical nano-LC/MS, which makes it available for routine analytical laboratories.

Mass spectrometry

For mass spectrometry, a LCQ Duo (Thermoquest, San José, CA, USA) ion-trap instrument was used. Ionization was performed in electrospray mode using a laboratory-built sheath-liquid interface without sheath gas or auxiliary gas. The construction (see Fig. 1) consisted of a polymer tee (Upchurch Scientific, Oak Harbor, WA, USA; 0.020" borings) equipped with a 22-gauge syringe needle (Hamilton, Reno, NV, USA). The voltage was applied to the needle via a stainless steel nut. The separation column was guided through the tee and was positioned flush with the outlet of the needle. This positioning generally doesn't provide ideal conditions in terms of sensitivity, but was easily controlled to provide maximum reproducibility. A 5 mm section of the polyimide coating was removed from the end of the separation capillary by flame cleaning to provide better mixing characteristics and to minimize disturbing effects. The reverse side of the tee was caulked by an elastomer ferrule (Beckman, Waldbronn, Germany).

Sheath liquid (methanol/water/glacial acetic acid, 50/50/1, v/v/v) was delivered from the side port of the tee using a Series 1100 HPLC pump (Hewlett-Packard, Palo Alto, CA, USA) operated at a flow rate of 1 $\mu\text{L}/\text{min}$. The flow rate was measured and found to be constant. The sheath liquid was grounded at a distance of 15 cm from the ionization interface. An optimization procedure (details not shown here) resulted in a distance between ionization needle and heated capillary of 5.0 mm and an electrospray voltage of 4 kV. For overview chromatograms, full scans were acquired over a mass range $m/z=100\text{--}400$; when quantifying low amounts of analytes, single reaction monitoring (SRM) was used with $m/z=102$

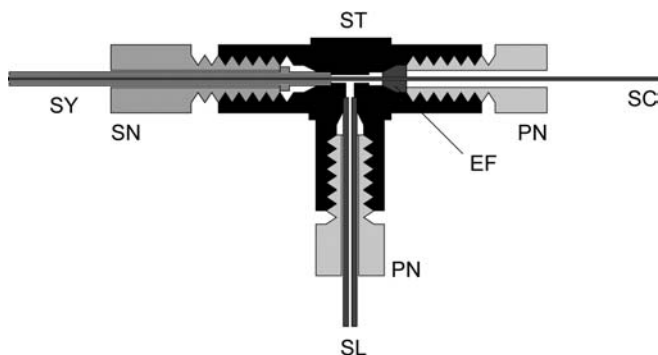


Fig. 1 Laboratory constructed microspray interface. ST, polymer tee; SY, syringe needle; SN, stainless steel nut; SL, sheath liquid channel; SC, separation capillary; PN, polymer nuts; EF, elastomer ferrule

as the target fragment. Data acquisition was performed with the Xcalibur (Thermoquest, San José, CA, USA) Software, v1.0. The data presented in the following sections were not smoothed or further processed.

Results and discussion

Optimization of MS parameters

Besides the molecular ion $[M+H]^+$, a prominent signal with a mass of $[M+18]^+$ and one with lesser intensity at $[M+74]^+$ were observed in the spectra of all examined AHLs (results not shown). The $[M+18]^+$ peak most probably represents a reversible hydration product that could exceed the intensity of the molecular ion by more than 10 times in some extreme heated capillary temperatures (C_{10} -HSL, 50 °C). Because the maximum intensity of the signals in electrospray ionization mass spectrometry is often a balancing act of thermal stability and adduct formation [26, 33], the temperature-dependant cluster formation and degradation behavior of the molecules was examined by taking chromatograms with temperatures of the MS heated capillary varying from 50 °C up to 300 °C (upper instrument limit) in steps of 25 °C. Figure 2 gives a representation of the measured peak areas of the mother peaks versus the temperature of the heated capillary. $[M+18]^+$ was nearly completely repressed at 200 °C (ratio molecular ion/hydration product ≥ 1); at this temperature, however, the abundance of the molecular ion dropped to values from 2.5% (C_4 -HSL) to 9.0% (C_{14} -HSL). The observed higher thermal stability of the long-chained AHLs can be explained by the free motility of the acyl side-chain, allowing more steric positions and decreasing rigidity of the molecule. In terms of thermal stability only, the lowest value (50 °C) would be the optimum analysis temperature for AHLs, but at low temperatures (<100 °C), even at very low flow rates the mass spectra showed a dramatic loss in resolution due to the incomplete water removal during the electrospray ionization process. Hence, for further analyses the heated capillary temperature was adjusted to 100 °C.

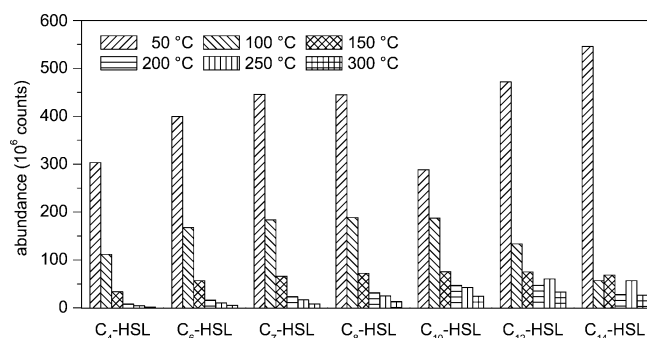


Fig. 2 Integrated peak areas of the seven AHLs versus the temperature of the heated capillary (chromatograms of 1 $\mu\text{g}/\text{mL}$ AHL standards). The intermediate temperature values (75, 125, 175, ... °C) were removed for clarity

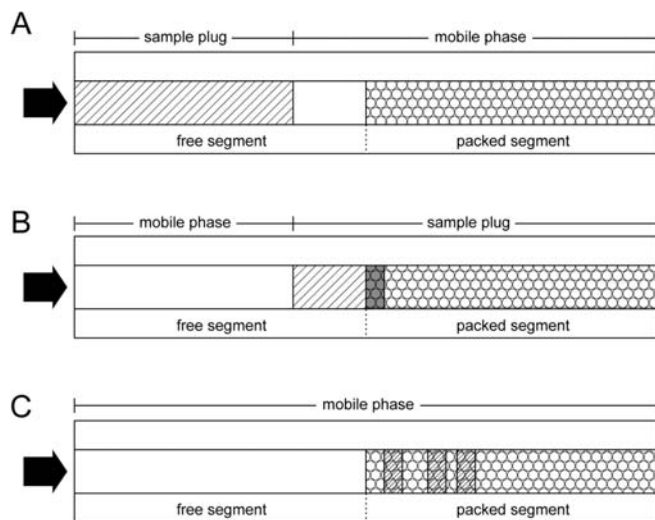


Fig. 3 Principles of the on-column pre-concentration-nano liquid chromatography (for a practical example of steps A to C, refer to Fig. 4). Step A, an aqueous sample plug is injected onto a reversed-phase (ODS) column; step B, partitioning of the analytes from the aqueous sample matrix towards the hydrophobic packing material, while bypassing the border between the free and the packed segment; step C, elution and separation of the pre-concentrated analytes with the methanolic mobile phase

Optimization of the separation parameters and column performance

The on-column concentration method applied in our experiments combines characteristics of solid phase extraction (SPE) and liquid chromatography (LC) on a single column. Similar principles were successfully applied in capillary HPLC [27, 28] and more recently in capillary electrochromatography [29, 30] and are described for LC in Fig. 3. The separation principles of this technique do not differ fundamentally from those in other types of reverse-phase separations. With the used experimental setup (refer to the *Liquid Chromatography* section), gradient elution was not possible due to the construction limitations of the flow splitter, and the mobile phase was optimized only with regard to the organic modifier content. Briefly, 80% of methanol was found optimal for the separation of the AHLs of interest (other organic additives did not yield better results); the addition of acetic or formic acid did not improve resolution. The pressure was adjusted to 15 MPa, yielding a flow-rate of ca. 300 nL/min. Higher pressures rapidly led to outgassing problems at the column outlet, even after degassing in a vacuum or with sparging gas (helium). At or below 15 MPa, this problem was not found to occur, and both the mobile phase and the sample did not require prior treatment.

The sample matrix composition was optimized with respect to the organic modifier content: at 30% methanol we observed a breakthrough of the concentration mechanism. The addition of acetic acid or formic acid to the sample slightly improved peak sharpness, but also caused unwanted degradation of the molecules. The injected volume could be increased to at least 5 μ L, but for the sam-

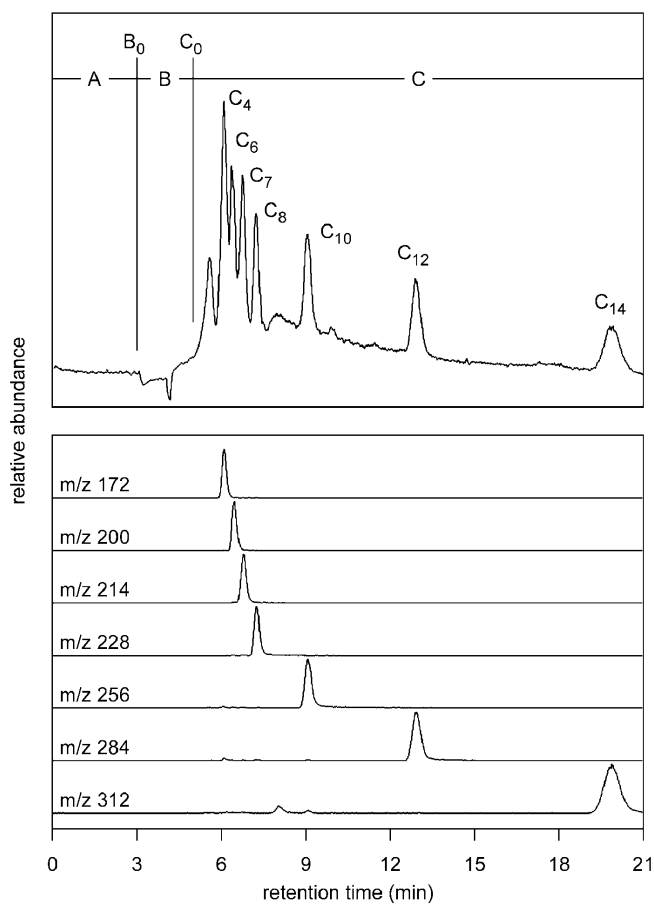


Fig. 4 Practical realization of the separation principles presented in Fig. 3 under optimized conditions. Lettering analogous to Fig. 3. Analyte, mix of seven AHL standards (C_4 -HSL, C_6 -HSL, C_7 -HSL, C_8 -HSL, C_{10} -HSL, C_{12} -HSL and C_{14} -HSL; 1 μ g/ml each). Top, total ion current of $m/z=100-400$. Step A, elution of the initial column contents (pre-run, methanolic); B_0 , onset of the sample matrix elution (aqueous); step B, sample matrix elution (aqueous, the different eluent composition causes disturbances in the total ion current); C_0 , onset of the sample elution (pre-concentrated during phase B, methanolic); step C, sample elution and separation (methanolic). Bottom, selected mass traces for the individual compounds

ples examined here, 1 μ L was sufficient. These optimization procedures led to chromatograms as presented in Fig. 4, and allowed a base-line separation of the peak pair C_8 -HSL/ C_{10} -HSL (substances of interest). For other analytical targets, the organic modifier content of the mobile phase has to be adjusted; base-line separation of all analytes (usually not necessary in LC-MS) can only be achieved in a reasonable time when using a setup that allows gradient elution.

Standards

Standard mixtures were analyzed in full scan MS, in full scan MS/MS, and in single reaction monitoring (SRM), in the latter cases exploiting a fragmentation principle common for at least all unsubstituted AHLs (Fig. 5). The predominant fragmentation reaction is the cleavage of the

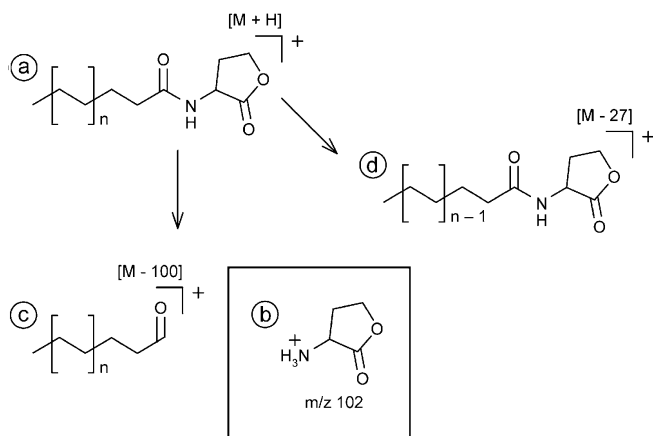


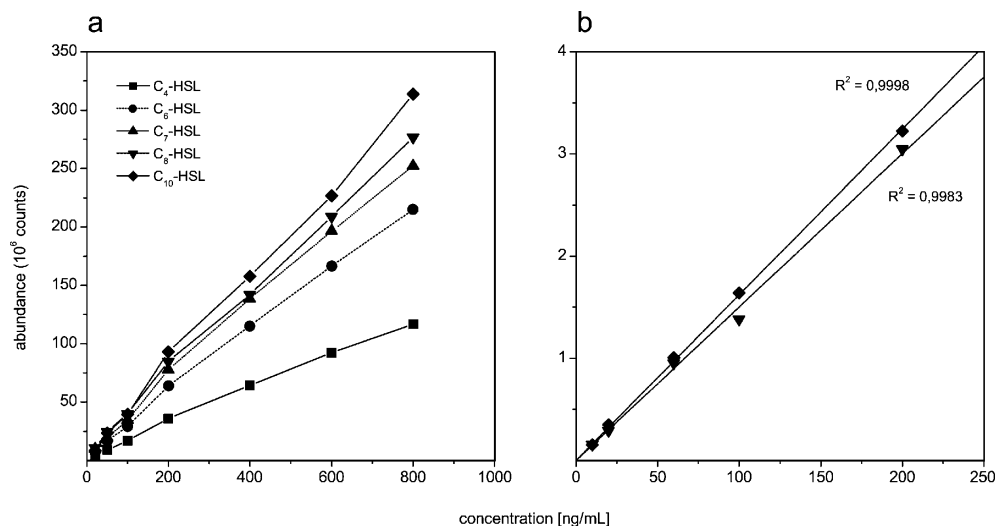
Fig. 5 Fragmentation patterns of AHL molecules in MS/MS. **a** molecular ion; **b** characteristic homoserine lactone fragment; **c** remaining acyl side chain; **d** cleavage of the acyl side chain by one C_2 unit. Fragmentation pattern was obtained with MassFrontier v1.0

AHL molecules to form unacylated homoserine lactone ($m/z=102$) and the corresponding side chain fragment ($[M-100]^+$); to a lesser extent a reduction of the acyl side chain by one C_2 subunit ($[M-27]^+$) and a subtraction of water ($[M-18]^+$) was observed. The fragment $m/z=102$ was already found to be useful for AHL identification in unknown samples [26, 34, 35, 36]. For structural characterization (where the presence of *all* fragmentation products has to be ensured) and for SRM quantification (where an optimum intensity of $m/z=102$ is desirable) the individual collision energies were optimized, and this resulted in the values presented in Table 1. Quantification was performed in full scan MS and in SRM (Fig. 6). The quantification was found to be difficult for C_{12} -HSL and C_{14} -HSL

Table 1 Optimum fragmentation energies for structural characterization (MS/MS) and SRM quantification

Compound	C_4 -HSL	C_6 -HSL	C_7 -HSL	C_8 -HSL	C_{10} -HSL	C_{12} -HSL	C_{14} -HSL
MS/MS (%)	20	20	20	22.5	22.5	25	25
SRM (%)	25	25	25	27.5	27.5	30	30

Fig. 6 AHL quantification under optimized conditions. **a** five AHL standards in full scan MS; **b** C_8 -HSL (bottom) and C_{10} -HSL (top) determined as the $m/z=102$ fragment in single reaction monitoring (SRM, 27.5% collision-induced dissociation)



due to the high retention time (peak broadening). The limits of quantification (LOQ) and of detection (LOD) in full scan MS, defined as $s/n \leq 10$ or $s/n \leq 3$ for *one* of the seven standards, were reached at 50 ng/mL or 10 ng/mL. For SRM, the values were found to be 10 ng/mL (LOQ) and 1 ng/mL (LOD), the latter corresponding to 3.9–5.8 nM (C_{10} -HSL – C_4 -HSL) or a total substance amount of 1 pg (3.9–5.8 fmol).

Real sample extracts

A primary aspect of this work was to establish an assay for analyzing AHLs from bacterial cultures which are most of the microbiologist's real samples. A major goal was to keep a minimal extraction procedure to enable rapid and reproducible sample preparation in any laboratory prior to sending them to chemical analysis. Because enzymatic activity may alter the AHL concentration in untreated cultures, a rapid cleanup may be reasonable. An extraction efficiency of 85% was found acceptable when extracting the sample with chloroform. Accessing in situ sampling and measurements of environmental or clinical samples will require further efforts in the near future (precise extraction evaluation, nano-volume handling).

Because of high background signals, we were not able to detect any of the target compounds in the raw real samples. The Luria-Bertani medium used to cultivate the bacteria has a high salt content (4 g/L NaCl) and a crude mix of protein and other macromolecules, sugars, amino acids, and so on (according to common protocols in total 15 g/L "bacto tryptone" and "bacto yeast extract"). This makes sample cleanup (extraction, purification) obligatory. Analysis of the extracts was performed in three steps: a) identification of possible candidates in full scan MS; b) selective fragmentation to exclude false-positive peaks (Fig. 7),

Fig. 7 Characterization: detection of C_8 -HSL in a *Burkholderia cepacia* culture supernatant extract. **a** selected mass trace of $m/z=228$ in full-scan MS; **b** selected mass traces of $m/z=228$ and $m/z=102$ in full scan MS/MS (22.5% collision-induced dissociation on $m/z=228$); **c** mass spectrum at the peak maximum of $m/z=102$ in subfigure **b**; **d** mass spectrum at the peak maximum of $m/z=102$ in a reference standard (100 ng/mL) under identical chromatographic and mass spectrometric conditions

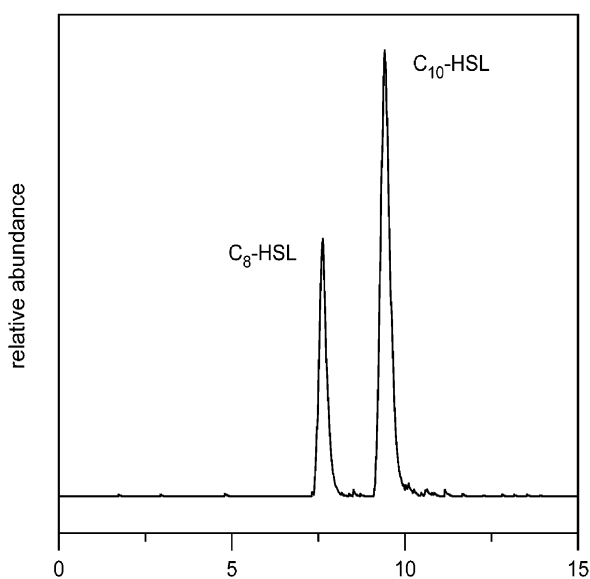
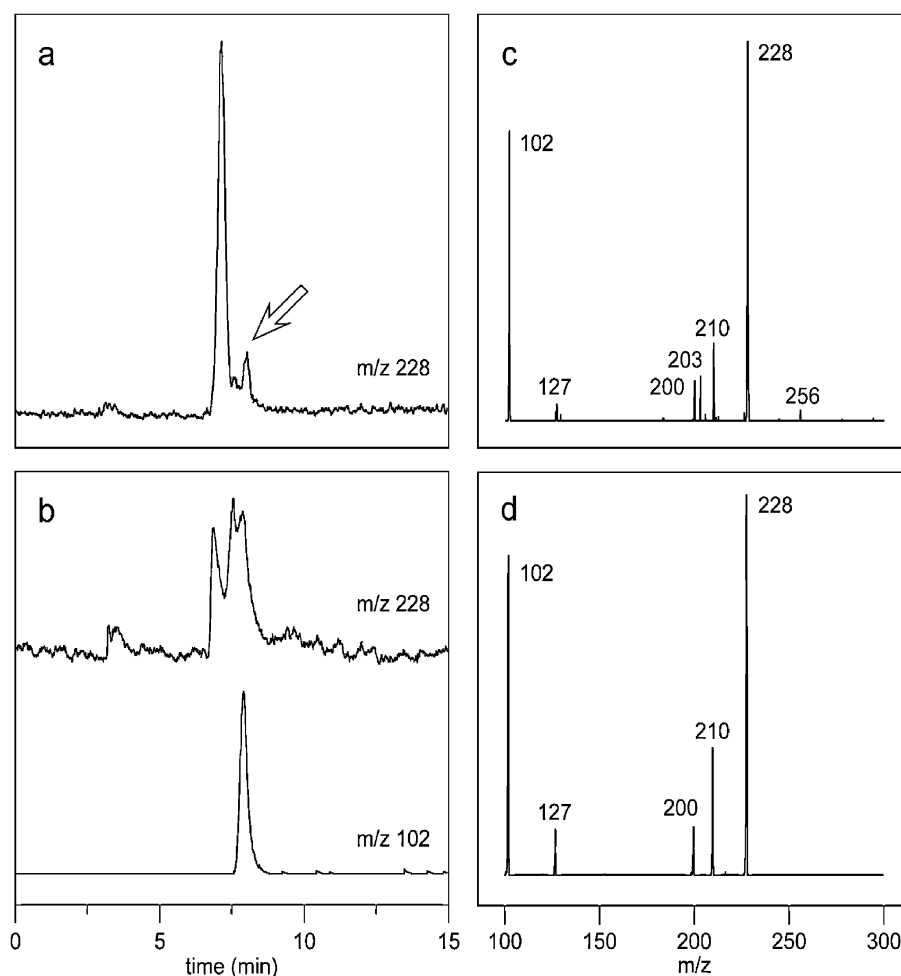


Fig. 8 Quantification: detection of C_8 -HSL and C_{10} -HSL as the $m/z=102$ fragment in a *Burkholderia cepacia* culture supernatant extract. Single reaction monitoring (SRM) with 27.5% collision-induced dissociation on $m/z=228$ (0–8.5 min) and on $m/z=256$ (8.5–15 min)

and; c) quantification in SRM. Knowledge about the exact retention time of peaks of interest allows a shortening of the analysis time by switching fragmentation parameters during one run (Fig. 8) and therefore being able to quantify two or more compounds in one run. Using this strategy, *Burkholderia cepacia* JA-8 was identified to produce 40 ng/mL or 180 nM (12% RSD, $n=4$) of C_8 -HSL and 70 ng/mL or 260 nM (9% RSD, $n=4$) of C_{10} -HSL.

Conclusions

In this paper we presented an easy and robust nanoscale approach for the separation and characterization of *N*-acyl-homoserine lactones in bacterial culture extracts. Coupling nano-LC to mass spectrometric detection allowed sensitive quantification, overcoming limitations caused by low UV-absorbance of AHLs. The sensitivity reached with this combination allowed the analysis of samples using a simple purification step avoiding matrix effects. Efforts are ongoing to critically evaluate the extraction yields of different cleanup procedures and the necessity for cleanup when analyzing real samples other than culture supernatants. The sample amount could be limited to a mini-

mum (1 μ L). Compared to the classically-used bioanalytical tools in quorum sensing analysis (TLC and biosensors to track AHLs), the chemical analysis proposed herein offers qualitative and quantitative advantages.

The high sensitivity, together with a small sample volume, should allow in-situ analysis in the rhizosphere of plants, in naturally occurring biofilms, and clinical samples in the near future.

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