

Development and application of a method for the analysis of *N*-acylhomoserine lactones by solid-phase extraction and ultra high pressure liquid chromatography

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Abstract

A robust method based on solid-phase extraction (SPE) followed by ultra high pressure liquid chromatography (with trade name of Ultra Performance Liquid Chromatography: UPLC; Waters, Milford, MA, USA) is proposed for the determination of five derivatives of *N*-acylhomoserine lactones (AHLs) that play a biological role as signal molecules of several gram-negative bacteria. Different commercial SPE cartridges were tested for sample extraction, clean-up and preconcentration. Since the sample matrix was a complex growth media, careful optimization of the SPE with respect to washing procedure, elution solvent and sample solvent was necessary. No sample loss was observed when up to 100 mL spiked full media was added onto the cartridge. Applying UPLC for the determination of AHLs, the performance characteristics of the method showed good separation efficiency and high speed. In order to demonstrate the applicability of the method, supernatants with the known AHL producer *Burkholderia cepacia* LA3 grown in different media were investigated. Additionally, the method was successfully used for the degradation/uptake study of AHLs from a liquid matrix in which barley was grown under controlled condition.

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1. Introduction

Bacterial cell–cell communication, also called *quorum sensing* or autoinduction, is mediated by small molecules (autoinducers) amongst which the most frequently studied group are *N*-acylhomoserine lactones (AHL) [1]. AHLs play a central role in regulation of virulence genes in many plant and

animal pathogens and control diverse physiological processes including bioluminescence, swarming, antibiotic biosynthesis, plasmid conjugal transfer and biofilm development among gram-negative bacteria [2]. AHL-dependent autoinduction in *Yersinia pseudotuberculosis*, for example, is involved in the control of cell aggregation and swimming motility [2]. Moreover, these signal molecules might prove valuable for diagnostic purposes, since they could be used as indicators for different diseases. The *N*-decanoyl-homoserine lactone and derivatives of oxo-homoserine lactones were found in mucropurulent respiratory secretions from cystic fibrosis patients at nM range [3].

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Because these autoinducers are mostly found in trace amounts, a sensitive method is required for their determination. The detection of AHLs has been greatly facilitated by bacterial whole-cell biosensors, which can be analysed by cytometry [4–6] or microscopic techniques. With these methods the homoserine lactones are detected indirectly with bacterial strains containing sensor constructs, which respond to the presence of the AHLs by expressing fluorogenic or luminogenic gene products. Another way for their determination is the application of separation techniques. For the extraction of AHLs, mostly liquid–liquid extraction (LLE) has been used with dichloromethane, ethyl acetate or chloroform as solvents. For purification of the extracts, thin-layer chromatography (TLC) has been frequently used [7–13]. TLC was also used for the characterization of the detectable autoinducers since the active spot can thus, be scraped off and analysed further by other analytical techniques, e.g. mostly mass spectrometry (MS) [7,8,12,13]. Another purification technique is semi-preparative or preparative high-performance liquid chromatography (HPLC) [9], however, for these techniques the amount of bacterial supernatant that need to achieve the required sensitivity is too high. Separation techniques like gas chromatography (GC) [11,14], classical HPLC, nano-LC [9,10,15–17], capillary electrophoresis (CE) in zone electrophoretic [18] and micellar mode [19], coupled mostly to MS have been used for the target analysis of homoserine lactones. Mass spectrometry is frequently used for the identification of AHL derivatives [7,8,12,13] without coupling to a separation technique as well. The collision induced fragmentation of AHLs was examined in detail [14,16,20]. In electrospray, the $[M + H]^+$ ions may be protonated: (i) at the secondary amino group; (ii) at the carbonyl function of the acyl chain or; (iii) at the ethoxy function of the lactone ring. Fragmentation (i) leads to the $[M + H - C_4H_7NO_2]^+$ ion with a mass of $[M + H - 101]^+$ via neutral loss of the homoserine and to an m/z 102 ion by protonation of the homoserine, fragmentation of (ii) and (iii), respectively, yields the $[M + H - H_2O]^+$ and the $[M + H - CO]^+$ ions. The very characteristic m/z 102 fragments in this context are suitable for screening unknown samples for the presence of AHLs.

We herein describe the optimization of a solid-phase extraction (SPE) protocol for the purpose of extraction and purification

as an alternative to LLE prior to liquid chromatographic analysis performed by ultra high pressure liquid chromatography (trade name UPLC, Waters Corporation, Milford, MA, USA). During the optimization process the type of the sorbent material and the condition of the sample treatment was investigated. Representative real samples from running projects on bacterial-plant molecular interactions and metabolomics in the rhizosphere were chosen to validate the applicability of the method.

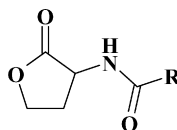
2. Experimental

2.1. Chemicals

The selected *N*-acylhomoserine lactones (C_4 -, C_6 -, C_7 -, C_8 -, C_{10} -, C_{12} -, C_{14} -AHL) (see Fig. 1), *N*-(β -ketocaproyl)-homoserine lactone (3-oxo- C_6 -AHL) and *N*-(3-oxooctanoyl)-homoserine lactone (3-oxo- C_8 -AHL) were obtained from Sigma–Aldrich (Steinheim, Germany). Stock solutions of the analytes were prepared by dissolving the substances in acetonitrile (ACN) at concentrations of 4500 μ M. The stock solutions were kept at -20°C and could be stored over a four-weeks period without observable losses. Standard solutions were prepared by diluting the stock solutions with a water-ACN mixture at a volume ratio of 70/30 (v/v). Hydrolyzed AHLs (C_4 -, C_6 -, C_7 -, C_8 -, C_{10} -, C_{12} -, C_{14} -HL) were prepared by the method as published before [18]. Syringe Filter (13 mm, w/0.2 μ m, PTFE Membrane) was purchased from VWR (West Chester, PA, USA). ACN at “hypergrade” quality for the UPLC analysis, methanol and isopropanol (iPrOH) were purchased from Merck (Darmstadt, Germany), and hexane from Riedel-de Haen (Seelze, Germany). Water was purified by a Milli-Q system (Millipore, Billerica, MA, USA). All chemicals used in the experiment were at least of analytical grade.

2.2. Real samples

The first sample was selected from the study of AHL degradation during plant growth. Barley seeds (*Hordeum vulgare*, cv. “Barke”) were surface sterilized using 1% Tween 20, ethanol,



R	Name	Abbreviation	LogP
$-C_2H_4-CH_3$	N-butanoyl-homoserine lactone	C_4 -AHL	0.03
$-C_4H_8-CH_3$	N-hexanoyl-homoserine lactone	C_6 -AHL	1.02
$-C_5H_{10}-CH_3$	N-heptanoyl-homoserine lactone	C_7 -AHL	1.94
$-C_6H_{12}-CH_3$	N-octanoyl-homoserine lactone	C_8 -AHL	1.97
$-C_8H_{16}-CH_3$	N-decanoyl-homoserine lactone	C_{10} -AHL	2.96
$-C_{10}H_{20}-CH_3$	N-dodecanoyl-homoserine lactone	C_{12} -AHL	4.02
$-C_{12}H_{24}-CH_3$	N-tetradecanoyl-homoserine lactone	C_{14} -AHL	5.09

Fig. 1. Chemical structure of AHLs. Abbreviation: log *P* is *n*-octanol/water partition coefficient expressing the analyte's hydrophobicity.

12% sodium hypochlorite solution and sterile water. Seeds were grown in the dark on nutrient broth (NB no. 4, Fluka, Buchs, Switzerland) for 72 h and then transferred into a sterile system containing mineral media (Murashige-Skoog media, Duchefa, Haarlem, The Netherlands) or modified Hoagland [21]. C₈-AHL and C₁₀-AHL were dissolved in ethanol and were added separately to a final concentration of 10 μM. Control plants were given an equal volume of ethanol without AHL. The plants were grown in a Heraeus-Vötsch (Hanau, Germany) chamber at 14 h light per day (150 μE) and harvested 21 days after surface sterilization, which means 17 days exposure of the plants to AHL. Plants were removed carefully to avoid damage; mineral media was separated from glass beads and stored at -20 °C.

The second sample involved pure cultures of *Burkholderia cepacia* LA3 was isolated from the rhizosphere of rice [19]. To obtain AHLs, 10–50 mL of nutrient broth, Luria Bertani media (LB, containing 10 g/L tryptone, 5 g/L yeast extract, 4 g/L NaCl), King's B media (20 g/L protease peptone, 1.5 g/L MgSO₄, 1.5 g/L K₂HPO₄, 10 mL/L glycerol) or M9 media [22] with glucose as C-source were inoculated and grown at 30 °C and 175 rpm overnight. To obtain cell free supernatants, cultures were centrifuged at 4 °C and 5000 rpm for 5 min in a Hettich Universal 32R centrifuge equipped with a 1620A rotor (Hettich, Tuttlingen, Germany).

2.3. Analysis

For the sample preparation, SPE cartridges, namely Bond Elut LRC C18-OH, Mega Bond Elut C18, Bond Elut PPL, Bond Elut PRS and Bond Elut SCX were purchased from Varian (Darmstadt, Germany); Bakerbond C18, Octadecyl polar plus, Bakerbond phenyl, Bakerbond Silica Gel, Bakerbond Florisil, Bakerbond Diol, Bakerbond WP CBX, Bakerbond Cation Exchange were from Baker (Griesheim, Germany); Strata-X Cation Exchange was from Phenomenex (Aschaffenburg, Germany); Oasis MAX from Waters (Waters, Milford, MA, USA); Chromabond HR-P was from Macherey (Duren,

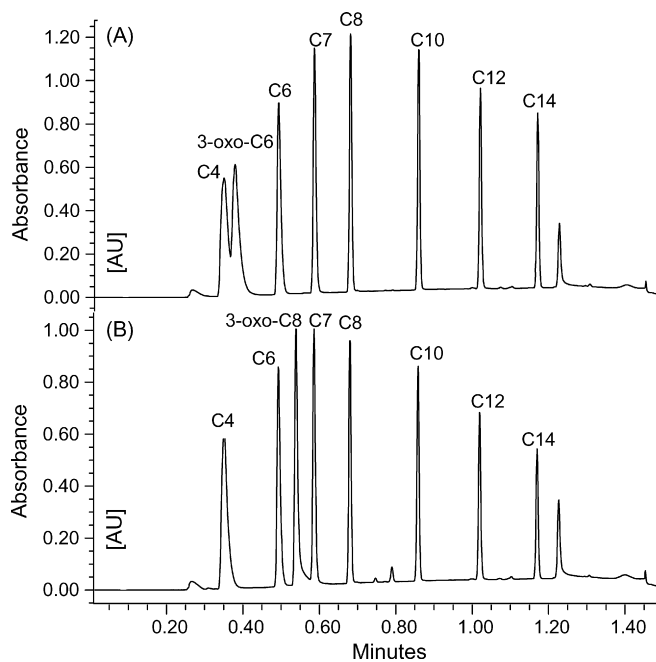


Fig. 2. Chromatogram of a standard solution of AHLs with 3-oxo-C6-AHL (A), or 3-oxo-C8-AHL (B). Separation conditions: gradient from 30% ACN to 100% ACN in 1.0 min period. Column: C₁₈, 2.1 mm × 100 mm, 1.7 μm. Column temperature: 60 °C. Flow rate: 0.9 mL/min. Peak identification: C₄ = C₄-AHL, C₆ = C₆-AHL, C₇ = C₇-AHL, C₈ = C₈-AHL, C₁₀ = C₁₀-AHL, C₁₂ = C₁₂-AHL, C₁₄ = C₁₄-AHL, 3-oxo-C6 = *N*-(β-ketocaproyl)-homoserine lactone and 3-oxo-C8 = *N*-(3-oxooctanoyl)-homoserine lactone. Concentration of the standard solution was 67.5 μM.

Germany); and Adsorbex NH₂ was purchased from Merck (Darmstadt, Germany).

The analysis was performed on a UPLC Waters Acquity System (Waters, Milford, MA, USA) equipped with a 2996 PDA detector. The column had dimensions of 2.1 × 100 mm, filled with BEH C₁₈ packing material with 1.7 μm particle size. The column was thermostated at 60 °C, the sample system at 27 °C. Twenty microliters of sample was injected via full loop injection. The system was run with a linear gradient starting with water

Table 1
Performance characteristics of the UPLC method

	C ₄ -AHL	C ₆ -AHL	C ₇ -AHL	C ₈ -AHL	C ₁₀ -AHL	C ₁₂ -AHL	C ₁₄ -AHL
<i>t_R</i> (min)	0.354	0.496	0.589	0.684	0.865	1.031	1.182
<i>t_R</i> within day RSD	0.15%	0.23%	0.19%	0.22%	0.17%	0.14%	0.12%
<i>t_R</i> day-to-day RSD	1.18%	2.89%	3.46%	4.20%	4.60%	5.27%	5.55%
Retention factor ^a	0.57	1.19	1.59	2.00	2.79	3.52	4.20
Selectivity factor	2.09	1.34	1.26	1.40	1.26	1.20	
Resolution	6.33	6.97	8.93	19.18	17.57	16.30	
Asymmetry	1.51	1.41	1.22	1.06	1.04	1.02	1.02
Number of plates ^a	2353	17,785	43,342	79,650	138,759	174,293	248,420
Working range	0.1–20.0 mg/L (0.4–90.0 μM)						
<i>R</i> ²	0.9993	0.9999	0.9999	0.9998	0.9999	0.9999	0.9992
RSD of peak area	0.26%	0.14%	0.09%	0.07%	0.09%	0.54%	3.87%
LOQ (μM)	6.6	5.5	5.0	4.6	4.1	3.7	3.2
Recovery	97%	96%	98%	97%	94%	96%	96%

Abbreviations: *t_R*, retention time; RSD, relative standard deviation; *R*², regression coefficient; LOQ, limit of quantification.

^a Apparent.

containing 30% (v/v) ACN to 100% (v/v) ACN in 1.0 min. The flow rate was set to 0.9 mL/min, which results in a maximum system pressure of 950 bar. Detection was performed at 195 nm at a scan rate of 20 Hz and the peak areas were calculated using Waters Empower software. Each injection was repeated three times and the mean value was taken into account. The *n*-octanol water partition coefficient ($\log P$) values of the analytes were calculated with Pallas 3.1. (CompuDrug International, Budapest, Hungary).

3. Results and discussion

3.1. UPLC analysis

The selected AHLs summarized in Fig. 1 were expected to be separable by a gradient using increasing amounts of water miscible organic solvents, since they highly differ in their polarity ($\log P$ differences are higher than 2). Applying the optimized conditions described in Section 2.3, baseline separation of the AHLs was achieved within 1.5 min. A representative chromatogram is shown in Fig. 2.

Separation of other AHL derivatives, which may be additionally produced by microorganisms, might also be taken into account. AHLs are sensitive to alkaline lactonolysis [18], thus, their hydrolysis products, namely *N*-acylhomoserines, were detected with the method developed for determination of AHLs. No *N*-acylhomoserines peaks were observed within the elution window of the targets, but broad peaks were detected at the dead time, which suggested that the *N*-acylhomoserines had no retention on the column (data not shown). The commercially available oxo-AHLs, namely 3-oxo-C6-AHL and 3-oxo-C8-AHL were eluted with the target components as well and separation from the targets was achieved (see Fig. 2). A system peak at 1.28 min was always observed in the chromatograms even when high purity water was injected into the UPLC.

To validate the applicability of the separation, the performance characteristics of the method were determined (see Table 1). Retention time (t_R) had to be used for the identification purpose since the spectra of the analytes are not very characteristic; therefore, high precision of the t_R is substantial. Repeating the separation five times at different concentrations, the RSDs determined within one day were between 0.12% and 0.23%. The day-to-day RSDs determined from a three-day period were below 6%. The determined resolutions were above 6 showing good separation of the analytes from each other. Small tailings of C4-AHL and C6-AHL were observed (symmetry factor was 1.5 and 1.4, respectively), but the other peaks had symmetric shapes.

The quantitative ability of the method for the determination of the selected AHLs was evaluated with seven different amounts of analytes over the range of 0.4–90.0 μM . The linear least-squares standard calibration graphs were linear with correlation coefficients $r^2 > 0.9992$. The limit of detection (LOD) was determined to be 0.4–1.0 μM . By running five replicates of the standard solutions at concentration of 45 μM , each analyte showed high reproducibility in terms of peak area integrated from the chromatogram. The RSDs of peak areas were below 0.5% except for

Table 2
Properties of selected reversed phase SPE and detected analyte amounts in the wash-out after loading the sample

SPE	Name	Functional group	Supplier	Sorbent mass (mg)	Reservoir volume (mL)	Pore size (Å)	Particle diameter (μm)	Surface area ($\mu\text{g}/\text{m}^2$)	C ₄ -HSL in wash-out (%)	C ₆ -C ₁₄ -HSL in wash-out (%)
1	Bakerbond C18	Octadecyl	Baker	100	1	60	55	321	86	0–73
2	Chromabond HR-P	End-capped octadecyl	Macherey	200	3	60	45	500	47	0
3	Octadecyl polar plus	Octadecyl	Baker	2000	6	–	52	315	0	0
4	Bond Elut LRC C18-OH	Octadecyl	Varian	500	10	150	120	300	62	0
5	Mega Bond Elut C18	End-capped octadecyl	Varian	1000	6	78	57	462	35	0
6	Bakerbond phenyl	Phenyl	Baker	100	1	60	54	217	17	0–12
7	Bond Elut PPL	Modified PSDVB	Varian	200	3	150	125	600	7	0

The cartridge was loaded with 2 mL of a 45 μM standard solution of the seven selected AHLs dissolved in 30/70 (v/v) ACN/water after conditioning with 2 mL methanol and 2 mL water. The wash-out was examined and the recoveries were determined.

C₁₄-AHL (3.8%). The recovery values were calculated at different concentrations and were independent; they were determined to be between 94% and 98%.

3.2. Development of solid-phase extraction

Direct injection of the sample such as full media may block the column (e.g., due to the presence of proteins) or may adversely affect the separation. SPE has been proved to be an effective tool for removing the sample constituents, enabling higher sensitivity and more robust analysis. Different types of sorbent materials are currently available and may lead to different selectivity, thus, sorbent materials with different trademarks and chemistry were studied. However, the focus of the interest was mainly the use of reversed phase materials because of the wide polarity range of the targets. SPE cartridges with type of ion-exchange or H-bridge activity were tested as well to investigate the possibility of the discrimination of AHLs from matrix constituent. Properties of the selected commercial available SPEs and recoveries of the AHLs in the wash-out are shown in Table 2, among the solid phases small differences in the recoveries in the wash-out were observed. C₄-AHL was difficult to adsorb onto the sorbent and therefore, the results are shown separately from the recovery values of the other target compounds. Among the reversed phase SPE columns, four (SPE nos. 2, 3, 5, 7) were selected for further detailed examination. The recoveries in the wash-out applying silica gel based solid phases (bakerbond silica gel and bakerbond florisil) were 0–23% reflecting the polar nature of the homoserine ring. Since a dependence on the recoveries of the carbon number in the alkyl chain and the loaded amount was observed, the normal phase materials were not suitable for our purpose. Solid phases with ion exchange ability (Strata-X Cation Exchange, Bond Elut PRS, Bakerbond WP CBX, Bond Elut SCX, Oasis MAX and Adsorbex NH₂) could not be used for discriminating the AHLs since a fraction of the loaded amount always retained (15%–77%).

The elution properties of the AHLs were studied applying the four selected SPEs (SPE nos. 2, 3, 5, 7). After adding 2 mL of standard solution at a concentration of 45 μM, onto the solid phase, they were separately eluted with ACN, methanol and iPrOH. Best recoveries were determined when iPrOH was applied (Fig. 3), thus, it was used for further examination. With increase of the alkanoyl side chain length of the AHLs, the elu-

tion ability decreased except for SPE 2 and SPE 5, which also gave the best recoveries of the adsorption (wash-out contained least amount of C₄-AHL) and elution (the recoveries were in the range of 65–95%). Since less disturbing peaks were observed at the detection wavelength in the chromatogram when SPE 5 was used compared with those in SPE 2, SPE 5 was chosen for further studies. In order to increase the recoveries of long side chain AHLs (C₁₂- and C₁₄-AHL) and to maintain the recoveries of shorter side chain AHLs, the polarity of the elution solvent was changed. Hexane, toluene and tetrahydrofuran (THF) were added to iPrOH at volume ratios of 25%, 50%, 75% and 100% (v/v), respectively and the best results were obtained with a solvent containing hexane (data not shown). By stepwise increasing the hexane content in the elution solvent a maximum in the recovery of long side chain AHLs was found at 25% (v/v). The recovery of C₁₂-AHL increased to a value larger than 95% but for C₁₄-AHL it remained low (75%).

For washing the sample-loaded solid phases, water and a water-methanol mixture were tested and no significant decrease in the recoveries of the selected AHLs were determined until the methanol content was lower to 15% (v/v). Applying methanol to the washing solvent was useful to remove the highly polar solutes present in liquid media extracts, especially in the full media like LB or NB (Fig. 4).

ACN was added to the spiked samples (LB media) at volume ratios between 10% and 40% (v/v) to reduce the matrix effect and to continuously condition the reversed phase sorbent when high sample volumes were applied. As a result, greatest elimination of matrix effect was obtained when 25% (v/v) ACN was added to the sample. Because of the bactericidal effect of ACN, it was helpful to transport and store the real samples in the refrigerator as well. The determined recoveries of AHLs in the spiked LB media were higher than 90% for AHLs with alkyl side chains from hexanoyl (C₆-AHL) to dodecanoyl (C₁₂-AHL), thus, no observable loss was recorded when adding organic solvent in the abovementioned concentrations.

The optimized SPE method could be used for quantitative determination of AHLs with side chains from hexanoyl to dodecanoyl, since the recoveries with standard solution and spiked full media (LB) were higher than 90% while C₄-AHL and C₁₄-AHL can be determined semiquantitatively. The final protocol of the sample preparation is the following: the sample (with 25%, v/v ACN) was applied onto the MegaBond Elute car-

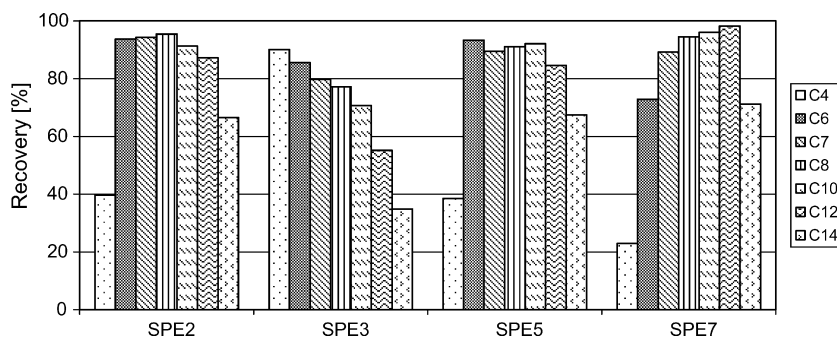


Fig. 3. Recoveries of AHLs after extraction with four selected SPE cartridges. SPE2: Chromabond HR-P, SPE3: octadecyl polar plus, SPE5: Mega Bond Elut C18, SPE7: Bond Elut PPL. Elution: 2 mL of 100% iPrOH. Loading with 2 mL of a 45 μM, standard solution. Abbreviations as above.

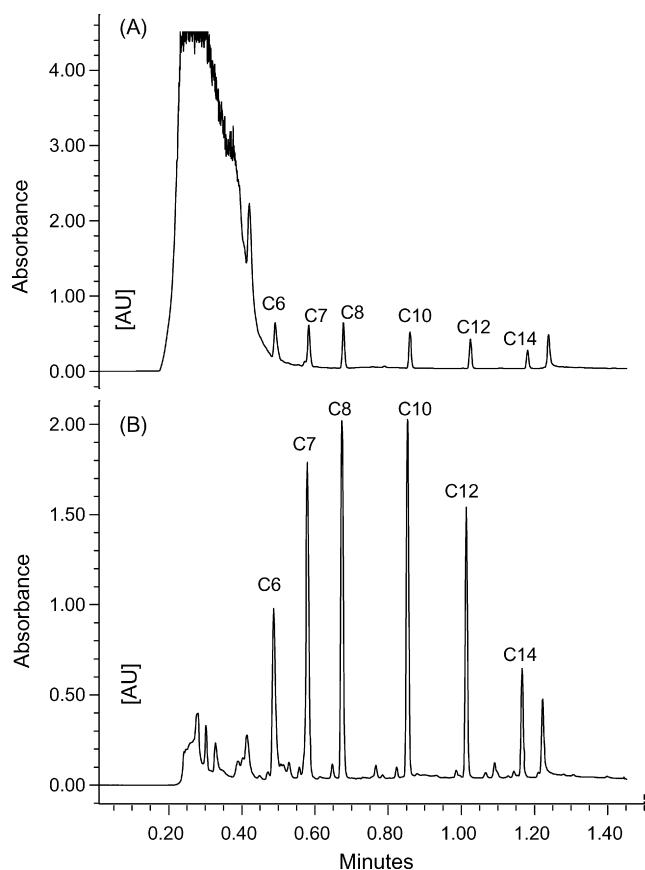


Fig. 4. Chromatogram of an extract of spiked LB media (A) without washing the loaded SPE cartridge and without addition of ACN to the sample; and (B) with washing the loaded SPE cartridge with 15% (v/v) methanol in water and an ACN content of 25% (v/v) in the sample. Separation conditions as in Fig. 2.

tridge, which was formerly conditioned by 2 mL water and 2 mL methanol sequentially. The loaded column was washed with 15/85 (v/v) methanol/water and the analytes were eluted with 25/75 (v/v) hexane/iPrOH. The elute was dried by nitrogen in room temperature by 30% (v/v) ACN/water resolved solution was sequentially filtered by PTFE Syringe Filter into UPLC, via no breakthrough was observed when 2 mL of a 10 mg/L standard solution was diluted with LB media to 10 mL, 25 mL, 50 mL and 100 mL (data not shown). Thus, applying 10 mL sample volume for SPE was safe and no losses of analytes is to be expected. Repeating the whole analysis three times with spiked LB media, the RSDs were determined to be between 1% and 4%. The sensitivity of the whole analysis process when 10 mL of the sample was applied was in the range of 0.05 μM . Furthermore, other sample matrices like NB and mineral media (e.g., Hoagland and Murashige-Skoog) were also studied with the above mentioned method, explicit elimination of the sample constituents and satisfied recoveries were obtained, thus, the method can be applied for other media as well.

3.3. Degradation study with barley plants

The optimized method was used for studying the uptake or degradation of AHLs from mineral media in which barley was

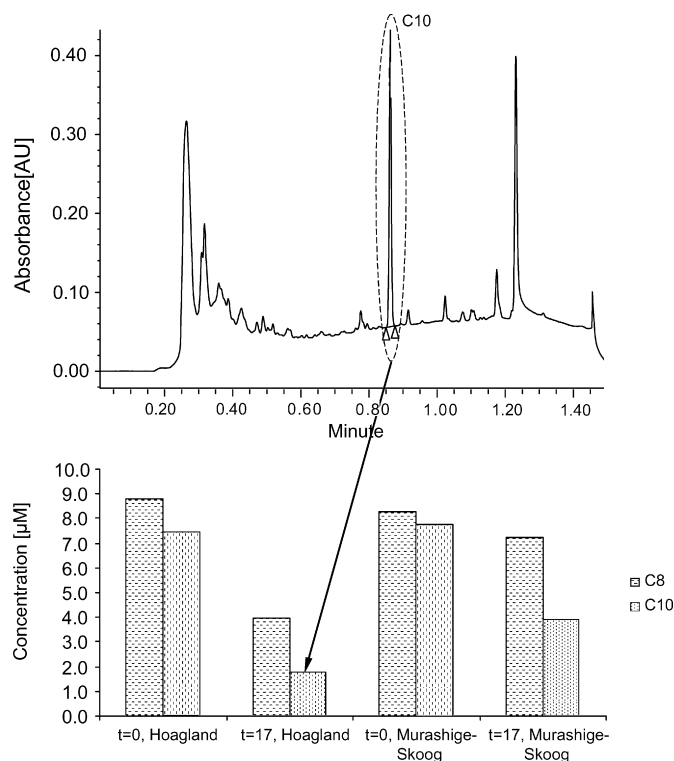


Fig. 5. Uptake/degradation study of C₈-AHL and C₁₀-AHL by barley, cv. “Barke” growing in modified Hoagland media and Murashige-Skoog media. A chromatogram is shown for modified Hoagland media spiked with C₁₀-AHL in which barley was grown for 17 days. Recoveries are shown before and after incubation in two different media. Abbreviation: t=0, Hoagland: samples were not incubated in modified Hoagland media; t=17, samples were incubated for 17 days and barley was grown in modified Hoagland media; t=0, Murashige-Skoog: samples were not incubated in Murashige-Skoog media; t=17, Murashige-Skoog: samples were incubated for 17 days and barley was grown in Murashige-Skoog media (see Section 2.2). SPE of 10 mL sample under optimized conditions (see text).

grown under controlled conditions. The initial concentration of C₈-AHL and C₁₀-AHL was 10 μM in 10 mL modified Hoagland or Murashige-Skoog media. The amount of AHL in samples taken at the onset of incubation (t=0) was determined to be between 75% and 89% of initial concentration for both media (Fig. 5). At the end of the incubation time (17/408 days/h) and after harvesting the plant, losses of AHL and increases of additional peaks in the chromatogram were observed. A loss of 13% for C₁₀-AHL and 50% for C₈-AHL in Murashige-Skoog media and of 56% for C₁₀-AHL and 77% for C₈-AHL in modified Hoagland media (Fig. 5) was observed. It can be concluded that the degradation or uptake of AHLs during the growing of the plant thus, correlates with the alkanoyl chain length of AHL and the media of choice.

3.4. Investigation of AHL production as an effect of the media

Supernatants of a *Burkholderia cepacia* LA3 culture grown in LB media were also analyzed applying the optimized method. The chromatogram shows more complex peak patterns (see Fig. 6B) than that of the spiked LB media (see Fig. 4B), which

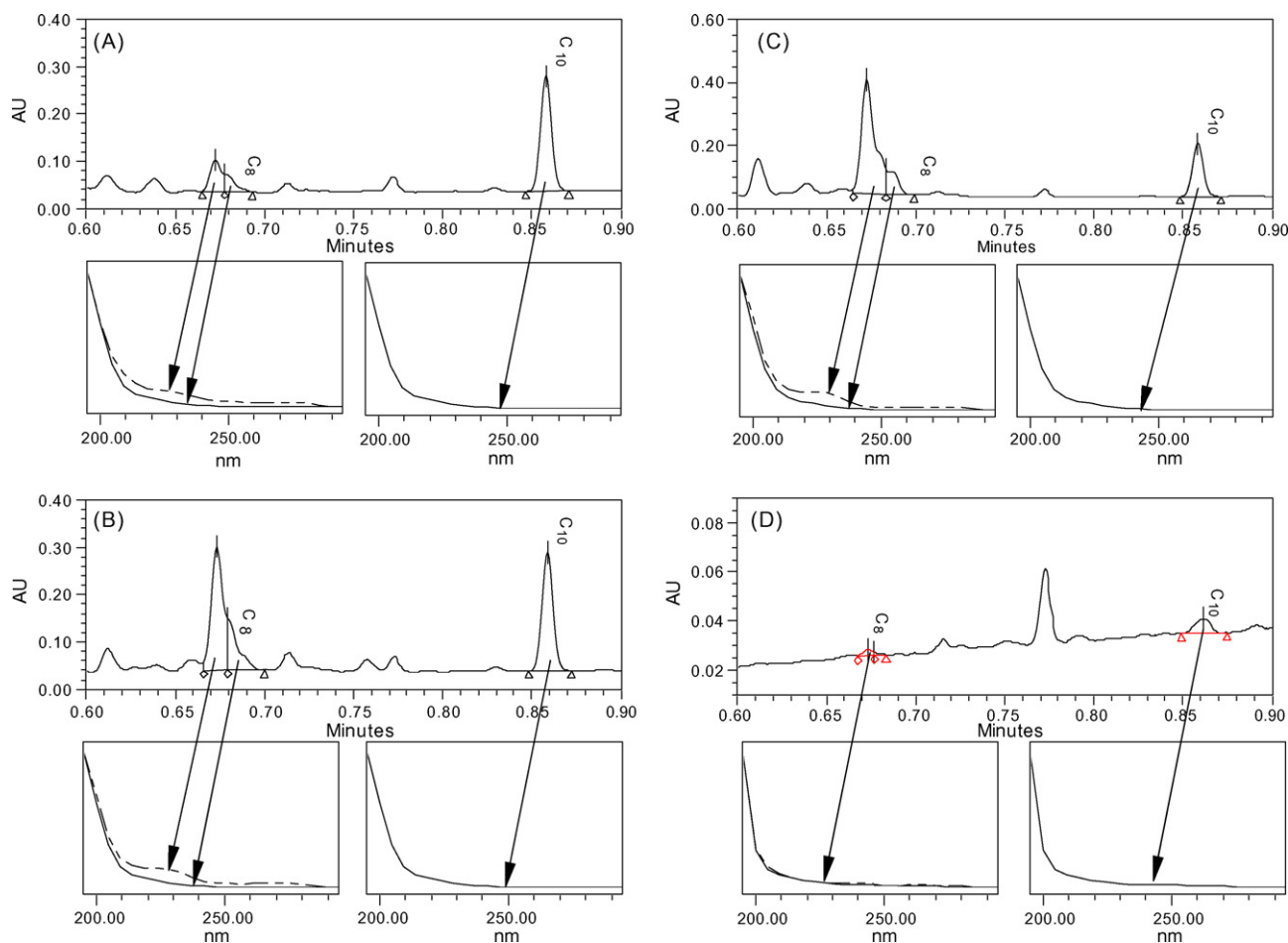


Fig. 6. Chromatograms and UV-vis spectrum of C_8 -AHL and C_{10} -AHL extracted from the supernatant of *Burkholderia cepacia* grown in NB media (A); LB media (B); King's B media (C); and M9 minimal media (D). Loading of the SPE with 10 mL sample after 25% (v/v) ACN was added and eluted with 2 mL iPrOH containing 25% (v/v) hexane after washing it with 4 mL water/methanol at 85/15 (v/v).

may be caused by the compounds produced by the bacteria. Co-elution of a matrix compound together with C_8 -AHL was observed from the shoulder peak with retention time at 0.672 min and 0.682 min. The former peak was identified as a sample constituent, because it has a maximum UV absorbance at 240 nm, different from the characteristic spectra of AHLs, which have no apparent absorbance, and because of its shifted retention time as well (see Fig. 6). C_{10} -AHL was successfully identified and quantified among the target compounds. To confirm these results, the same sample was investigated with Fourier Transform Ion Cyclotron Resonance Mass Spectrometry (FT-ICR-MS) that allows an ultrahigh resolution and accuracy of the masses and the previously identified C_8 -AHL and C_{10} -AHL showed up nicely (data not shown).

The effect of different media (LB, NB, King's B and M9) on the AHLs producer *Burkholderia cepacia* LA3 was investigated as well (Fig. 6). The interference peak of C_8 -AHL was observed when LB, NB and King's B full media were used but no co-elution was found when the bacteria were grown in M9 minimal media. The production of C_{10} -HSL was 40 times higher in LB media (1.69 μM) than minimal media (0.04 μM), 1.5 times less C_{10} -AHL was produced in King's B media (1.11 μM) and no differences were observed between LB and NB media (1.61 μM).

The same phenomenon was observed with FT-ICR-MS; the peak abundance in M9 minimal media was lower than in full media. Thus, the production of AHLs was correlated to the media composition approving also the fact that the bacteria grow slower in minimal media than in full media.

4. Conclusion

A method based on SPE-UPLC was developed to analyse AHLs in bacterial supernatants. The separation time of the analytes by UPLC was less than 1.5 min and the peaks could be observed at concentrations below 0.4 μM . The sensitivity of the current method can further be improved by increasing sample loading volume and decreasing the elute resolving volume during the SPE process which worked not only as pre-concentration step but also as clean-up. The method, being fully electrospray compatible, shows great potential for coupling with mass spectrometry, which is far more sensitive and selective compared to DAD detection. However, UPLC-DAD was a suitable tool for the optimization of the sample preparation method from complex matrix. The method was successfully applied to follow the degradation/uptake rate of AHLs in barley, cv. "Barke" making the method suitable for studies of the behaviour of AHLs

in the rhizosphere. Additionally, supernatants of *Burkholderia cepacia* cultures were examined. C₈-AHL was identified and C₁₀-AHL was determined in different growth media at different amounts. For the confirmation of the results, due to the lack of a selective on-line detector, offline FT-ICR-MS was applied and the same AHLs were identified. Further studies are ongoing to enable a direct analysis of AHL and their hydrolysis products in one UPLC run.

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