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Analysis of *N*-acyl-L-homoserine lactones produced by *Burkholderia cepacia* with partial filling micellar electrokinetic chromatography – electrospray ionization-ion trap mass spectrometry

A method for the analysis of *N*-acyl-L-homoserine lactones (AHLs) with micellar electrokinetic chromatography coupled to electrospray ionization-ion trap mass spectrometry, combining the flexibility of capillary electrophoresis with the unmatched structural information provided by mass spectrometry is presented. Different surfactants were evaluated, with sodium dodecyl sulfate (SDS) yielding the best results considering sensitivity and flexibility. We examined the interaction of AHLs with the SDS micelles at different analysis conditions and applied the optimized method to the analysis of a real bacterial sample. Two AHLs from *Burkholderia cepacia* colonizing the rhizosphere of traditional Indian rice cultivars could be unambiguously determined in an ethyl acetate extract with high resolution flexibility.

Keywords: *Burkholderia cepacia* / Electrospray ionization – mass spectrometry / Micellar electrokinetic chromatography / *N*-Acyl-L-homoserine / Quorum sensing

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CE and CEC

1 Introduction

N-Acyl-L-homoserine lactones (AHLs) are a class of microbial signaling molecules that play an essential role in a regulatory process that is known as 'quorum sensing' (QS). For more detailed overviews on QS the reader is referred to [1–3]. QS is the ability of single cells to perceive the overall cell density in bacterial populations by sensing the concentration of small, diffusible molecules (also known as 'autoinducers'). QS was discovered more than 30 years ago with marine luminescent bacteria living in close symbiotic association to marine fish and squids [4, 5]. QS attracted broader attention mainly in the last decade, when a large and ever-growing number of Gram-negative bacteria became known to produce AHLs. AHLs are continuously produced by an AHL synthase catalyzing the lactonization of *S*-adenosyl-L-methionine and the transfer of a fatty acid side chain by acylated acyl carrier protein (acyl-ACP) to the lactonization product [6, 7]. Smaller AHLs are freely diffusible across the bacterial cell wall, and thus accumulate in the surrounding medium. When the AHL concentration reaches a critical threshold level, which correlates with a particular number of bacteria, *i.e.*, the 'quorum', the AHL molecule binds to its cognate receptor, which in turn activates or represses expression of target genes. Depending on the particular species, AHL molecules may vary in the length and the saturation of the hydrophobic side chain (C_4–C_{16}) and in the substitution at C_3 (oxo or hydroxy functions or no substitution) [8, 9]. Figure 1 gives an overview of the general structure of AHL molecules together with the substances used in this study. In this article, we follow the widely used naming scheme from [10].

Today, more than 50 Gram-negative species are known to communicate *via* AHL signals [11], among them a number of species of agricultural interest [12], and a number of important pathogens [13]. Members of the genus *Burkholderia* cover both fields because of their versatility: They occur in soil, water and air [14], root-associated *Burkholderia* spp. play a role as plant pathogens [15], but they are also capable of controlling other plant pathogenic microorganisms by producing antibiotics [16, 17]. Other species are able to degrade pollutants [18] or stimulate plant growth [19]. *B. cepacia* [20] and several members of the *B. cepacia* complex were isolated from the rhizosphere of rice, coffee, maize, and sorghum [21]. The medical importance of *B. cepacia* results from the fact that this microorganism is capable of infecting immundodeficient patients [22, 23], and has emerged as an important pathogen for patients suffering from the genetic disease cystic fibrosis [24].

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Abbreviations: AHL, *N*-acyl-L-homoserine lactone; **BBMA**, butyl acrylate/butyl methacrylate/methacrylic acid copolymer sodium salt; **FI**, flow injection; **PF**, partial filling

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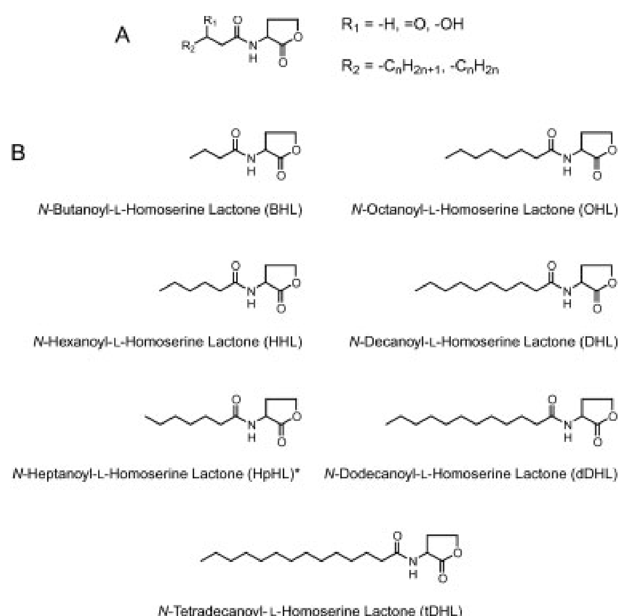

Figure 1. (A) General structure of AHL signaling molecules and (B) structures of the molecules examined in this paper. Naming scheme adopted from [10] (* analogous).

Taking into account the scientific relevance of bacterial communication, the bandwidth of analytic separation and quantification techniques is still limited: AHLs are classically purified from bacterial suspensions by liquid-liquid extraction with organic solvents, subsequentially subjected to thin-layer chromatography (TLC), and detected by covering the TLC plates with genetically modified AHL-sensitive reporter strains exhibiting easily detectible physiologic reactions like light emission or pigment production. Beneath other limitations (for discussion see [25]), especially the last step in classical AHL analysis is time-consuming and nonquantitative. Except for an approach with GC-MS [26], little profound data is present on systematic analysis of AHLs with modern chromatographic or electrophoretic techniques, presumably because the AHL molecules show a UV-absorbance maximum at or below 200 nm [27], which makes detection with UV difficult or requires large sample concentrations. MS detection, as a strictly concentration-dependent detection technique, overcomes this limitation, and, especially when run in selective fragmentation modes (MS/MS, MS^n), provides structural information unmatched by other detection techniques. The latter feature makes mass spectrometric detection preferable for analyzing heterogeneous and multicomponent matrices like represented by bacterial isolate suspensions. Electrospray ionization (ESI), as originally developed by Fenn and coworkers (see [28] for details), is today the most common ionization mode for liquid chromatography and capillary electrophoresis (CE). As a soft ionization technique, ESI allows the nondestructive detection of thermosensitive molecules.

CE is an attractive analytical technique due to its versatility. Coupling of CE with ESI-MS represents a powerful combination of high separation efficiency, high detection sensitivity and great capability for molecular characterization [29]. However, there are some constraints to be considered when CE and MS are coupled on-line; the most important is the buffer solution compatibility with the ESI process [30]. Due to the lack of a chargeable group (the secondary amine is stabilized by the neighboring keto function), AHLs can only be separated in micellar electrokinetic chromatography (MEKC), with the danger of sensitivity losses when contaminating the ion source with surfactant. Partial filling (PF)-MEKC tries to overcome this problem and involves filling the capillary with micellar buffer (10–90% of capillary volume) after filling with background electrolyte without surfactant [31]. The separation takes place in the micellar zone, the background electrolyte elutes into the MS and the micellar plug remains behind the capillary without (or with little) MS source contamination. However, with analytes exhibiting a strong affinity to the micelle (like long-chained AHLs), additional modifications may be necessary. One of these is the use of the reverse-migrating micelles, which is based on a mobility adjustment of the micelle and a slowdown of the EOF by conditioning the capillary with hydrochloric acid instead of sodium hydroxide and the use of buffers around pH 6 [32, 33]. Under these conditions, the micelles remain stationary in the capillary or even migrate towards the anode. Another strategy is the selection of MS-compatible agents like bile salts or high-molecular mass surfactants (reviewed in [34]). In this paper, we will give an overview of the ESI-MS behavior of some selected AHL molecules, the applicability of different surfactants in MEKC-ESI-MS and PF-MEKC-ESI-MS and discuss limitations and challenges of these methods for the analysis of AHLs in standard solutions and real samples.

2 Materials and methods

2.1 Chemicals

N-Acyl-D/L-homoserine lactones (BHL, HHL, HpHL, OHL, DHL, dDHL, tDHL; see Fig. 1), cholic acid and deoxycholic acid were purchased from Fluka (Buchs, Switzerland), analytical-grade ammonium acetate, methanol, hydrochloric acid, glacial acetic acid, dichlormethane, magnesium sulfate, sodium hydroxide, and ammonia from Merck (Darmstadt, Germany). Electrophoretic-grade SDS was purchased from Bio-Rad Laboratories (Hercules, CA, USA), butyl acrylate/butyl methacrylate/meth-

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acrylic acid copolymer sodium salt (BBMA) was a gift from Prof. S. Terabe (Himeji Institute of Technology, Hyogo, Japan). Ultrapure water (18.2 MΩ) was produced using a Milli-Q system from Millipore (Bedford, MA, USA).

2.2 Instrumentation

Analyses were performed using a Beckman (Waldbronn, Germany) P/ACE 5510 electrophoresis system with an on-line diode array detector and a high-voltage source delivering up to 30 kV. The CE system was coupled on-line to a Thermo Finnigan (San Jose, CA, USA) LCQ Duo ion-trap mass spectrometer using a self-modified Thermo Finnigan CE-MS (sheath liquid) interface which allowed accurate 3-D positioning of the CE outlet in respect to the MS orifice. The CE column outlet was set at the same height as the CE-ESI-MS probe to prevent siphoning. To maintain stable electrospray, a *ca.* 20 mm portion of the polyimide coating was removed from the outlet edge of the capillary. Formation of the electrospray and spray stability was monitored using a high-resolution CCD camera. MS data were acquired with the Xcalibur software, Version 1.0.

2.3 Capillary electrophoresis

Throughout all experiments, uncoated fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) with an inner diameter of 75 μm, an outer diameter of 360 μm and 80 cm total length (20 cm to the detector) were used. Proper conditioning proved to be essential for successful analyses, so prior to its first use and before/after each analysis period, the capillary was rinsed subsequently with 1 M hydrochloric acid (for SDS) or 0.1 M sodium hydroxide (for other surfactants) and the running buffer (30 min each). Between analyses, conditioning cycles were adjusted to 5 min each. CE routinely was operated at 30°C capillary temperature with +30 kV at the anode and +5 kV at the cathode (the electrospray interface), resulting in an effective voltage of 25 kV and a typical current of about 12 μA (10 mM ammonium acetate, pH 6.0). Micellar buffers and AHL samples were injected hydrodynamically with 0.5 psi. Plug lengths were calculated based on the Hagen-Poiseuille equation assuming a liquid viscosity of 1. As the substances examined are non-UV active, the only used detector for the peaks was the mass spectrometer.

2.4 Flow injections

Flow injections (FIs) of 100 μg/mL analyte solutions in 10 mM ammonium acetate, pH 6.0, were performed at 20 psi (20 s each) through a 75 μm inner diameter fused-silica capillary (80 cm length) followed by a rinsing step with buffer (10 mM ammonium acetate, pH 6.0, 4 min each). The resulting 20 s 'peaks' in the respective mass traces were integrated to characterize the AHL fragmentation behavior.

2.5 Mass spectrometry

FI-ESI-MS was performed with a nebulization gas (nitrogen) flow rate of 20 arbitrary units and an auxiliary gas (helium) flow rate of 5 arbitrary units. When coupled to CE, neither nebulization gas nor auxiliary gas was applied. Sheath liquid consisted of water, methanol and acetic acid (50/50/1 v/v/v) and was delivered by the use of a Hewlett-Packard (Palo Alto, CA, USA) 1100 HPLC pump with an on-line degasser operated at 1 $\mu L \cdot min^{-1}$. Ionization was performed in positive mode at 5 kV. For maximum spray stability, a distance of 5 mm between the electrospray needle and the surface of the heated capillary was found to be optimal. The temperature of the heated capillary was varied between 150 and 300°C. For screening and acquisition of fragmentation patterns, the mass spectrometer was run in full-scan mode, to perform routine analyses single-ion monitoring was used.

2.6 Standards and real samples

Stock solutions of 1 $mg \cdot mL^{-1}$ AHL standards in methanol were diluted in running buffer to the desired concentration. *B. cepacia* isolates from the rhizosphere of traditional Indian rice cultivars (B. Jha, L. N. Mithila University of Darbhanga, India) were grown in 250 mL modified Luria-Bertani (LB) medium [35] containing 4 $g \cdot L^{-1}$ NaCl instead of the normal 10 $g \cdot L^{-1}$ at 30°C and continous shaking at 150–200 (Innova 4200, Moncton, BC, Canada) up to a OD_{600} of 1 (CE 3021; Cecil, Cambridge, UK). Cell suspensions were centrifugated at 5000 rpm for 40 min (Varifuge 3.2 RS; Heraeus, Munich, Germany). The supernatant was shaken twice with 100 mL of dichlormethane. The combined organic phases were filtered after drying with magnesium sulfate (3MM Whatman paper; Whatman, Maidstone, UK) and taken to dryness with a rotation evaporator (Rotavapor R-114; Büchi, Flawil, Switzerland) at 42°C. The residues were redissolved with 250 μL of ethyl acetate and stored at −20°C. Prior to the analyses, the extracts were diluted 1:10 v/v with the respective running buffer.

3 Results and discussion

3.1 Ionization and fragmentation behavior of AHL molecules in FI-ESI-MS

All AHLs were subjected to FI-ESI-MS to understand their ionization and fragmentation behavior as a function of the experimental design prior to hyphenation to CE.

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Figure 2 illustrates the fragmentation patterns for (a) OHL (M_r = 227, *m/z* 228) at 300°C without and (b) OHL at 170°C with 25% collision-induced dissociation (CID, helium as collision partner). As a consequence of both the elevated temperature and the collision energy, the molecules fragment in a characteristic way. Similar fragmentation patterns were observed with all the substances examined (results not shown). Along with other fragmentation products, especially the fragment at *m/z* 102 and at *m/z* [M−27] is of importance. The former results from the cleavage of the *N*-acylic side chain from the homoserine lactone ring. This fragment was found to be common to all the compounds examined in this study, and can be used as a marker for scanning unknown mixtures for the presence of AHL signaling molecules. The fragment was also described previously by other authors using different ionization techniques [36–38]. In contrast, [M−27] most probably results from the shortening of the hydrophobic side chain by one C_2-subunit and causes signals identical to the next smaller AHL (HHL in case of OHL). This can be misleading when analyzing in FI without separation because one cannot be sure whether an observed signal at *m/z* 200 comes from the fragmentation of OHL or from unfragmented HHL. A number of characteristic adducts is formed during the electrospray process in a temperature-dependant manner.

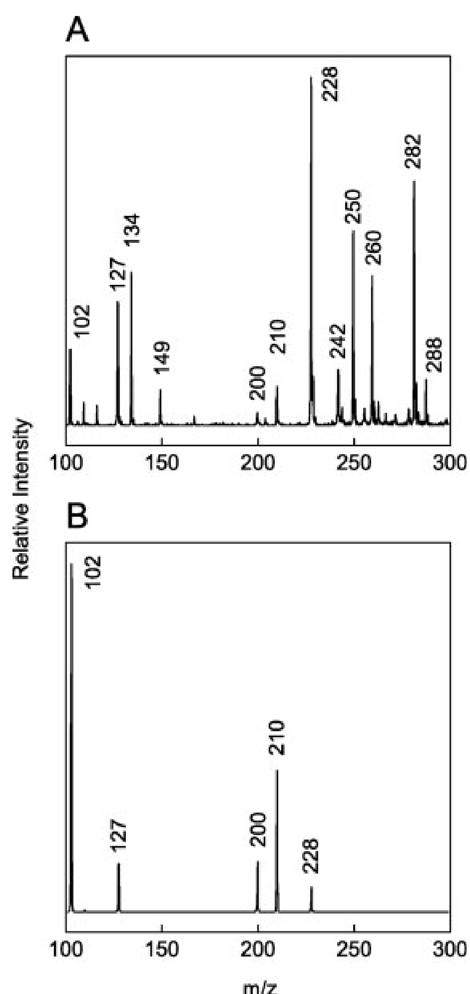

Figure 2. Ionization pattern of OHL (flow injections of 100 $\mu g \cdot mL^{-1}$ in ammonium acetate, pH 6, for additional information refer to Section 2). Heated capillary temperature (A) 300°C, no additional fragmentation, (B) 170°C, 25% CID.

In order to improve both the detection selectivity and the sensitivity, one has to ensure that the mother peak is favorably formed during the electrospray process, while the formation of adducts and the fragmentation of the molecule have to be limited. Optimization in respect to these demands can mainly be done by modifying the temperature of the MS orifice (the 'heated capillary'). Figure 3 visualizes the optimization results for OHL. From the large number of fragments and adducts (refer to Fig. 2a) only some characteristics are given, namely *m/z* 102, the mother peak, *m/z* 245, *m/z* 250, and *m/z* 260. The fragmentation increases for all AHL molecules when elevating the temperature. The mother peak's intensity, however, does not decrease reciprocal, as one might expect. This result is mainly due to the formation of adducts: the water adduct (*m/z* 245, [M+18]), decreases almost linearly when elevating the temperature. In contrast *m/z* 260, [M+33] shows an optimum at 200°C and then decreases. A third signal (*m/z* 250, [M+23]) is in contrast preferably formed at high temperatures. From these results, an optimum temperature for each molecule becomes hardly predictable. In Fig. 3b, individual curves for the temperature optima of the respective mother peaks of the AHLs are given. Correlating with the length of the hydrophobic side chain the temperature optimum, as a function of both thermostability and minimization of adduct formation, is shifted towards higher temperatures. This individual temperature optimum has to be kept in mind, when analyzing the different AHLs with optimum sensitivity. To simplify further steps and as a compromise, a temperature of 250°C was chosen for CE-ESI-MS analysis.

3.2 Suitability of different surfactants for direct MEKC-ESI-MS

When using deoxycholic acid and cholic acid (results not shown) as micelles, we observed a substantial contamination of the electrospray interface, leading to a domination of the mass spectrum with the surfactant and its respective ionization products. To minimize these disturbing effects, a PF technique with only 40% capillary filling was applied. The separation of the AHLs was pos-

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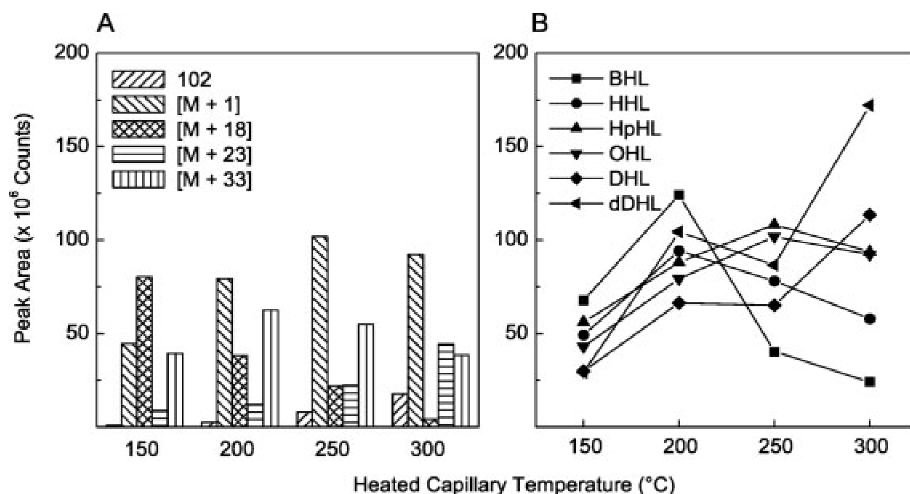

Figure 3. Electrospray behavior of AHL molecules. (A) Adduct formation and fragmentation of OHL in relation to the heated capillary temperature. Only selected masses are quantified. (B) Mother peak intensities of BHL-dDHL in relation to the heated capillary temperature.

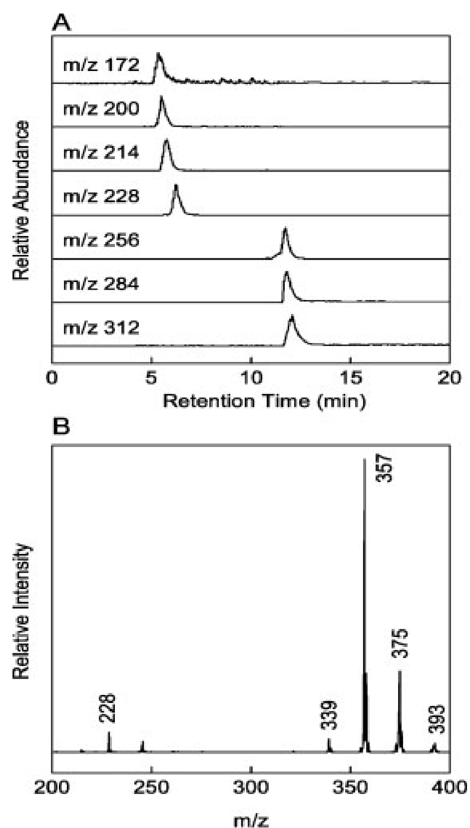

Figure 4. Separation of an AHL standard mix (BHL-tDHL, each 100 μg mL^{-1} in methanol, 10 s injection time) in PF MEKC using 20 mM deoxycholic acid in 20 mM ammonium acetate, pH 9.2 as surfactant (40% filling; BGE, 20 mM ammonium acetate, pH 9.2; for further details refer to Section 2). (A) reconstructed electropherogram of the seven standards BHL-tDHL. (B) Mass spectrum at the peak maximum of OHL after background subtraction over 1–5 and 7–11 min. A significant contamination with deoxycholic acid (M_r = 392) and its ionization products can be observed.

sible (Fig. 4a) after an optimization procedure including buffer acidity (pH 9.2–11), conductivity conditions, buffer molarity (5–20 mM), surfactant content (1–15 mM), organic buffer additives (5–20% methanol), and different PF degrees (5–60% of the total capillary volume). However, the contamination of the mass spectrum was not avoidable (Fig. 4b) because at least a small percentage of the surfactant migrated together with the EOF. The direct consequence was a dramatic loss of sensitivity because of the ionization concurrence at the electrospray. As a second limitation, we observed strong precipitation effects at the ESI interface both with acidic and basic sheath liquids, which can be considered as unhelpful for both sensitivity and reproducibility. As a third point, due to their solubility, the use of both bile salts was limited to a pH higher than 9, which did not provide enough flexibility for mobility adjustments. Polymeric surfactants like BBMA were evaluated, but did not yield satisfying results.

3.3 PF-MEKC-ESI-MS analysis of AHLs with SDS

The interaction of the studied AHL molecules with the micellar buffer was examined using different concentrations of SDS in the separation buffer and different plug lengths (PF) at constant concentration, respectively. Figure 5 shows the different migration times with a PF of 60% and a SDS concentration from 10 to 30 mM. Due to instabilities in the EOF times (ranging from 3.6 to 3.8 min after 20 cm), linearity of the different migration times is poor when taking raw data only (Fig. 5a). To improve linearity, raw data in MEKC is usually normalized and k' (the capacity factor) for neutral analytes is calculated according to Eq. (1) [39, 40].

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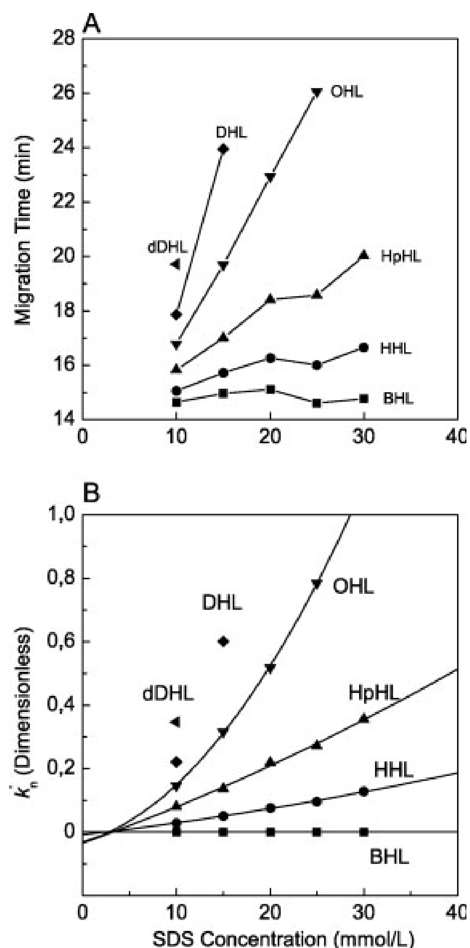

Figure 5. Migration time of six AHL standards (BHL-dDHL) in dependency of the SDS concentration at 60% PF in 10 mM ammonium acetate, pH 6.0. (A) Raw data, (B) transfer to k'_n (BHL-normalized capacity factors, refer to Section 3.3).

$$k' = \frac{t_R - t_{eo}}{t_{eo}\left(1 - \frac{t_R}{t_{mc}}\right)} \quad (1)$$

where t_R is the retention time of the substance under examination, t_{eo} is the time of the EOF and t_{mc} is the migration time of the micelle. In case of reverse migrating or stationary micelles, t_{mc} is infinity, and Eq. (1) can be expressed as

$$k' = \frac{t_R - t_{eo}}{t_{eo}} \quad (2)$$

The calculation of the capacity factors according to Eq. (2) still requires a detectible EOF, which, in our case, was not easily observable: The detection of the EOF after 20 cm (with diode array UV detection) was possible, but led to impreciseness when extrapolating to 80 cm capillary length.

As one can conclude from the results depicted in Fig. 5a, BHL, the smallest of the homoserine lactones examined, does interact only slightly with the SDS micelles. To describe the binding behavior of the other AHL molecules, respective, to the micelle, a new term was introduced:

$$k'_n = \frac{t_R - t_{BHL}}{t_{BHL}} \quad (3)$$

where k'_n is the BHL-normalized capacity factor of the particular AHL and t_{BHL} the migration time of BHL. The consequences of these considerations are summarized in Fig. 5b. The capacity factors, especially of the long-chained AHLs, did not increase linearly with an increasing concentration of SDS, which is somewhat unusual when compared to literature values (*e.g.*, [41]). An explanation for this behavior is, that because long-chained AHLs and SDS show structural similarities, it suggests a surfactant-like behavior of the analytes resulting in a more stable incorporation of the molecules to the micelle (formation of mixed micelles from high molar mass AHLs and SDS). According to these presumptions, high molar mass AHLs will not follow the regular partitioning mechanism of micellar interaction, but either affect partially the micelle formation/desintegration kinetics, which is in orders of magnitude slower than the former, or form metastable SDS adducts which may overlay the micellar separation.

The SDS concentration was kept as low as possible in order to maintain compatibility with mass spectrometric detection. A minimum concentration of SDS (10 mM) was used and the influence of different SDS plug lengths was examined. According to the SDS concentration studies above, this concentration was surely above the CMC. Theoretically, the analytical setup of reverse-migrating micelles should allow a complete filling of the capillary with SDS, but practically when filling the capillary with a plug exceeding 80% of the capillary length, a contamination of the mass spectrometer with SDS (*m/z* 310, presumably $[SDS-Na+NH_4+NH_3]$) was observed. This observation can be explained by the fact that not all of the SDS molecules are aggregated, but a small portion of them still remain in dispersed form. According to Eq. (3), the normalized mobility of the different substances was calculated. The results (Fig. 6) were again normalized to BHL (see above). The normalized capacity factors exhibit a strictly linear relation with the increasing SDS plug length and therefore allow flexible adjustment of the analysis conditions in relation to the resolution to achieve. Generally spoken, when applying mass spectrometric detection in comparison to other detection methods, resolution is often a minor issue but it has to be high enough to avoid pitfalls like mentioned in Section 3.1. No significant change in peak widths was observed

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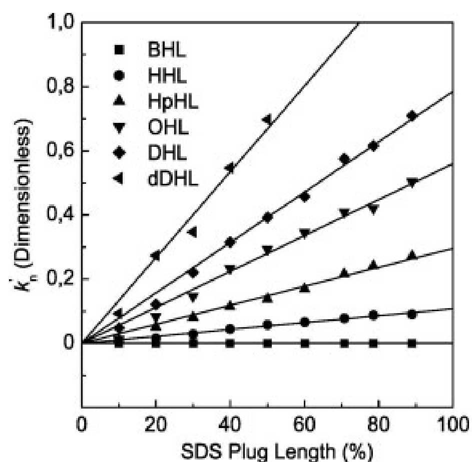

Figure 6. Values of k'_n (refer to Section 3.3) for six AHL standards (BHL-dDHL) in dependency of the SDS plug length (10 mM SDS in 10 mM ammonium acetate).

when varying the analysis conditions. This finding suggests that the peak broadness is less a consequence of the separation technique but of the electrospray interface. From 45 peaks in total an average peak width was determined (0.738 ± 0.102 min, all substances), and the resolution of adjacent peak pairs was calculated (Fig. 7). Depending the goal of the analysis (long-chained AHLs or short-chained AHLs), the optimum resolution can be easily adjusted by regulating the degree of PF.

Using the optimized procedure, consistent and reproducible qualitative data were obtained. Figure 8 shows the analysis of the AHL content in the culture of *B. cepacia* isolate LA3, purified by liquid-liquid extraction. Concluding from the peak time and from the characteristic fragment at *m/z* 102, the two peaks were ascribed to OHL

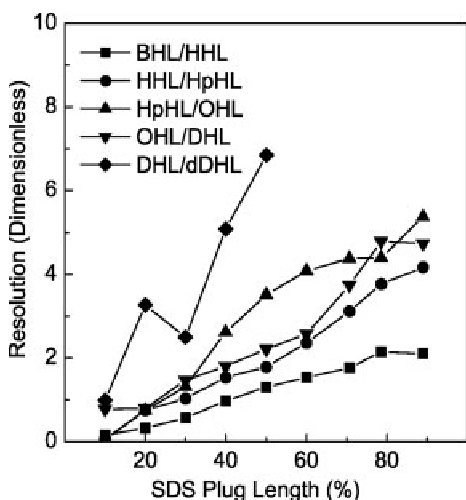

Figure 7. Resolution of adjacent peak pairs (BHL-dDHL) in relation to the SDS plug length (*cf.* Fig. 6).

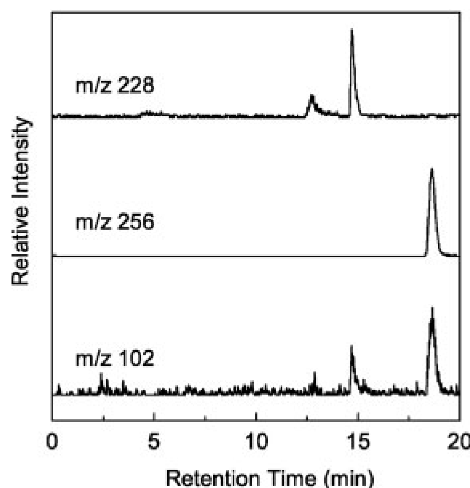

Figure 8. Analysis of a bacterial isolate extract (*B. cepacia*) in PF-MEKC-MS. Top lane, OHL (M_r = 227); middle, DHL (M_r = 255); bottom, AHL-characteristic fragment at *m/z* 102.

and DHL. However, quantification was found to be difficult when using PF-MEKC-MS and was not one of our first goals: we wanted to develop a method for the fast determination of AHLs in samples prepared in the 'classical' way, which is inherently not quantitative. However, a concentration down to *ca.* 1 μM was detectible without problems and with a reasonable sensitivity (S/N > 3). When measuring real samples from natural bacterial habitats, *i.e.*, without prior isolation and cultivation, methods will have to be refined all way from sampling to analysis to achieve improvements in terms of sensitivity and quantitative reproducibility.

4 Concluding remarks

AHL analysis by PF-MEKC with mass spectrometric detection proved to be a suitable tool for rapid detection of AHLs in a complex microbial extract sample. Two AHLs from *B. cepacia* could be unambiguously determined in a ethyl acetate extract with high resolution flexibility. Bearing in mind the classical way of AHL detection with sensitive reporter strains, the detection method presented here offers a significant shortening of the analysis time combined with an unmatched specificity. Moreover, this method also appears to be suitable for the identification of AHLs produced by bacteria in their natural habitats, *e.g.*, the rhizosphere of plants or tissue of infected animals. Quantification of AHLs, however, was found to be critical when using PF-MEKC-MS. Further analysis are ongoing using other hyphenated capillary separation techniques for the analysis of signaling molecules.

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