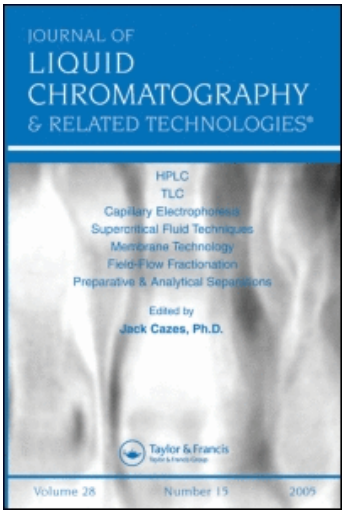


Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Optimization of Gradient Elution in UPLC: A Core Study on the Separation of Homoserine Lactones Produced by *Bukholderia Ubonensis* and Structure Confirmation with Ultra High Resolution Mass Spectrometry

Xiaojing Li[a]; Guonan Chen[a]; Jenoe Fekete[b]; Fang Yang[c]; Agnes Fekete[d]; Matthias Englmann[d]; Philippe Schmitt-Kopplin[d]

[a] Ministry of Education Key Laboratory of Analysis and Detection Technology for Food Safety, and Department of Chemistry, Fuzhou University, Fuzhou, Fujian, China [b] Department of Inorganic and Analytical Chemistry, Faculty of Chemical Engineering, Budapest University of Technology and Economics, Budapest, Hungary [c] FJCIQ-Fujian Entry-Exit Inspection and Quarantine Bureau, Technical Center, Fuzhou, Fujian, China [d] GSF-National Research Center for Environment and Health, Institute of Ecological Chemistry, Neuherberg, Germany

To cite this Article Li, Xiaojing , Chen, Guonan , Fekete, Jenoe , Yang, Fang , Fekete, Agnes , Englmann, Matthias and Schmitt-Kopplin, Philippe(2007) 'Optimization of Gradient Elution in UPLC: A Core Study on the Separation of Homoserine Lactones Produced by *Bukholderia Ubonensis* and Structure Confirmation with Ultra High Resolution Mass Spectrometry', Journal of Liquid Chromatography & Related Technologies, 30: 17, 2515 — 2531
To link to this Article: DOI: 10.1080/10826070701540084
URL: http://dx.doi.org/10.1080/10826070701540084

Journal of Liquid Chromatography & Related Technologies®, 30: 2515–2531, 2007

ISSN 1082-6076 print/1520-572X online
DOI: 10.1080/10826070701540084

Optimization of Gradient Elution in UPLC: A Core Study on the Separation of Homoserine Lactones Produced by *Bukholderia Ubonensis* and Structure Confirmation with Ultra High Resolution Mass Spectrometry

Xiaojing Li and Guonan Chen
Ministry of Education Key Laboratory of Analysis and Detection Technology for Food Safety, and Department of Chemistry, Fuzhou University, Fuzhou, Fujian, China

Jenoe Fekete
Faculty of Chemical Engineering, Budapest University of Technology and Economics, Department of Inorganic and Analytical Chemistry, Budapest, Hungary

Fang Yang
FJCIQ-Fujian Entry-Exit Inspection and Quarantine Bureau, Technical Center, Fuzhou, Fujian, China

Agnes Fekete, Matthias Englmann, and Philippe Schmitt-Kopplin
GSF-National Research Center for Environment and Health, Institute of Ecological Chemistry, Neuherberg, Germany

Abstract: Speed of analysis is a key factor in liquid chromatography. One possible way is to decrease the particle size and thus increase the pressure (ultra high pressure liquid chromatography, UHPLC). For the separation of a complex mixture, the application of gradient elution techniques is expedient. From a practical point of view, it has to be questioned how the high pressure influences the separation parameters at gradient mode. In

Address correspondence to Philippe Schmitt-Kopplin, GSF-National Research Center for Environment and Health, Institute of Ecological Chemistry, Ingolstädter Landstraβe 1 D-85764, Neuherberg, Germany. E-mail: schmitt-kopplin@gsf.de

our study we investigated the following chromatographic parameters: peak width versus temperature, peak width versus gradient time, retention time versus temperature, and retention factor versus flow rate. It was shown that even in sample volume overload conditions the gradient system worked in a reproducible way; the maximum possible sample volume (20 μL) was injected to get the lowest possible limit of detection. Increasing the flow rate, the peak width increased only moderately, and the same was found when the temperature was decreased. However, at 60°C the signal to noise ratio decreased in a small compass showing the thermal disequilibrium between the column outlet and the detector cell, which might have caused extra noise. Finally, a practical separation was presented in which the UHPLC can be applied for the determination of N-acylhomoserine lantones (AHLs), which have a relatively low UV absorption at low wavelength. The detection limit was low enough for practical applications because of the low dispersion due to a small diameter stationary phase and the low dead volume in the column. The UPLC method was applied as a first screening for AHLs and the results were confirmed by fourier transformation ion cyclotron resonance mass spectrometry (FTICR-MS), which has a high selectivity.

Keywords: Ultra high pressure liquid chromatography, Gradient elution, Overloading, Separation parameters, FTICR-MS

INTRODUCTION

Today, several approaches were developed to speed up the separation in liquid chromatography: (1) the use of monolithic columns (which have high permeability and the flow rate can be increased nearly by one order of magnitude),[1] (2) the application of short columns packed with particles of 3–5 μm, or in some cases, below 2 μm in a conventional pressure range, (3) the application of high temperature, and (4) the usage of particles below 2 μm and applying pressure above 400 bars. The last version of liquid chromatography is called, e.g., ultra high pressure chromatography (UHPLC) or ultra performance liquid chromatography (UPLC). The advantage of these systems lies in a lower analysis time without deterioration of the chromatographic performance characteristics. Moreover, some of them are even better.

There are several studies about advantages and disadvantages[2–6] using high pressure during the analysis. Several articles can be found of practical applications[7–12] emphasising advantages when mass spectrometry is used as a detector. Most of the theoretical studies were conducted with a constant mobile phase composition (isocratic elution). There was some indication about the heat gradient and some possible problem about thermal disequilibrium[13] but there is no detailed study on that. According to our best knowledge, the effect on separation and quantification parameters using gradient elution techniques was not jet studied in detail. In practical applications, there is a problem with sample volume overload when isocratic elution is applied. To reduce the heat gradient the column diameter is 2.1 mm and length is 5 or 10 cm. In UPLC the amount of stationary phase

applied compared to a conventional column (150 × 4.6 mm) is about one order of magnitude lower, and the same is true for the volume of the column and, therefore, the amount of injected sample must be in the same order of magnitude lower to get the same high efficiency (1–2 μL is the feasible injection volume). In this case, for a 20 μL injection the LOD will be at least ten times lower and the column overload will increase the peak width. To overcome this problem, the samples must be injected in a weak eluent according to the solvent strength of the mobile phase. To increase the column inlet concentration effect, gradient elution must be started from an eluent strength as low as possible, but complex mixtures where the polarity of compounds differs significantly from each other, will be an obstacle for applying a very weak solvent. In practical applications, this property of complex mixtures will also cause different apparent plate numbers for structurally related compounds, which differ in polarity and the calculated efficiency, and theoretically allow a column to differ widely. From a theoretical point of view, the second question will arise if the gradient elution is a basic need in the UPLC method, because the polarity of the compounds differs widely, or to avoid the extra peak dispersion: the plate number used in gradient elution has no physical meaning because the value depends on gradient time and other chromatographic parameters. Sander et al.[14] proposed an equation for calculating the plate number in the gradient elution technique, but the formula is quite complex and computer software used in LC and UPLC does not contain this option today. The peak width is a good indication for zone dispersion and UPLC was shown to be stable in terms of retention times and peak widths,[12,15–20] therefore, we use this parameter for the illustration of the system efficiency. Applying gradient elution, the last chromatographic peak will be concentrated at the column inlet and extra column dispersion will hardly affect the peak width. Theoretically, in a liner solvent strength (LSS) gradient elution, the width of all peaks will be found to be equal if no extra peak dispersion takes place. The sensitivity in detection of overloaded compounds and the peak capacity decreased by comparison with the theoretically predicted one, but this compromise had to be tolerated for the fast separation. The volume overload situation with practical consequences and some other parameters influencing the separation efficiency was studied.

In this approach, N-acylhomoserine lactons (AHLs) were used as reference compounds and a practical application is shown. The structure of AHLs varies in the length, the saturation of the acyl- side chain and its substitution (oxo or hydroxyl functions at C-3). They were recently found to serve as signalling molecules that mediate the communication between Gram-negative bacteria, as a part of bacterial communication systems.[21] It was reported that in more and more fields, AHLs were found, e.g, in human diseases,[22,23] animal and plant pathogens,[24,25] spoiled food,[26] and physiological processes including biloluminescence, swarming or plasmid conjugal transfer.[27] From an analytical point of view, the determination of AHLs is a

work full of challenges, since the concentrations of AHLs in real samples are in trace levels,[28] and AHLs do not have a strong chromophore group for spectrophotometric detection. So, to get reasonable limits of detection, volume overload has to be applied at least for the early eluting compounds. The UPLC method was applied as a first screening for these compounds and FTICR-MS was used for conformation of the identification. It is referred to in other papers focusing on this topic.[29,30]

EXPERIMENTAL

UPLC

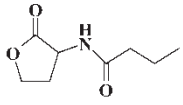
An Acquity UPLC System (Waters, Milford, MA, USA) equipped with a Model 2996 PDA detector was applied for the analysis. The 2.1 × 100 mm column was, filled with BEH C_{18} packing material with 1.7 μm particle size (Waters, Milford, MA, USA). The column oven was thermostated at different temperatures and practical application was run at 60°C, the sample system at 27°C. The sample (20 μL) was injected via full loop injection. The optimised system was run with a linear gradient starting with 30% (v/v) acetonitrile (ACN)/water to 100% ACN in 1.0 minute. The flow rate was set to be 0.8 mL/min, which results in a maximum system pressure of 950 bars. 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 at least three times and the mean value was taken into account.

FTICR-MS Analysis

Positive FTICR-MS spectra were acquired on a Bruker Daltonics (Bremen, Germany) Apex Qe 12 T system. Spectra were acquired in broadband mode and were calibrated externally on clusters of arginine (ca. 10 mg/L in 50% of methanol with 0.1% of formic acid) in the required mass range (m/z 175.11895, m/z 349.23062, m/z 523.34230, and m/z 697.45398). This external calibration was verified with the diketopiperazines *cyclo*-(Pro-Phe), *cyclo*-(Pro-Ile), and *cyclo*-(Pro-Trp), known to be present in the NB medium (verified by GC-MS and MS/MS, results not shown). Mass errors of these peaks were always less than 0.1 ppm (0.0001%).

Chemicals

The selected N-acylhomoserine lactones (C_4-, C_6-, C_7-, C_8-, C_{10}-, C_{12}-, C_{14}-AHL) (see Figure 1), were obtained from Sigma-Aldrich (Steinheim, Germany). Stock solutions of the analytes were prepared by dissolving the

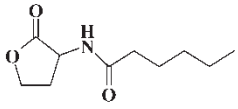


N-butanoyl-homoserine lactone

C_4-AHL

Mass protonated: 172.0968

Mass Na adduct: 194.0787

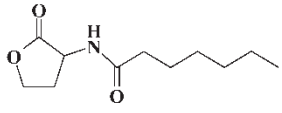


N-hexanoyl-homoserine lactone

C_6-AHL

Mass protonated: 200.1281

Mass Na adduct: 222.1100

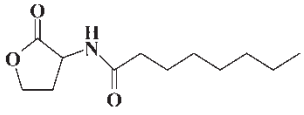


N-heptanoyl-homoserine lactone

C_7-AHL

Mass protonated: 172.0968

Mass Na adduct: 194.0787

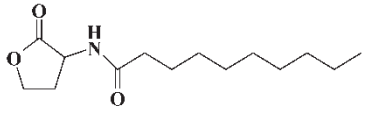


N-octanoyl-homoserine lactone

C_8-AHL

Mass protonated: 228.1594

Mass Na adduct: 250.1413

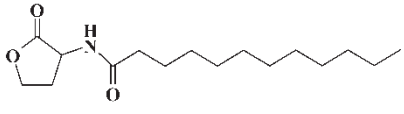


N-decanoyl-homoserine lactone

C_{10}-AHL

Mass protonated: 256.1907

Mass Na adduct: 278.1726

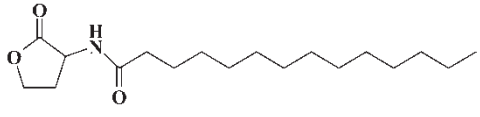


N-dodecanoyl-homoserine lactone

C_{12}-AHL

Mass protonated: 284.2220

Mass Na adduct: 306.2039



N-tetradecanoyl- homoserine lactone

C_{14}-AHL

Mass protonated: 312.2533

Mass Na adduct: 334.2352

Figure 1. Structure, name and exact mass of AHLs analysed as a target.

substances in ACN at concentrations of 1000 mg/L. The stock solutions were kept at -20°C and could be stored over a four week period without degradation. Standard solutions were prepared by diluting the stock solutions with a water-ACN mixture at a volume ratio of 7/3. ACN of "hypergrade" quality for the UPLC analysis, methanol, and isopropanol were purchased

from Merck (Darmstadt, Germany), and hexane from Riedel-de Haen (Seelze, Germany). Water was provided by a Milli-Q system (Millipore, Billerica, USA). All chemicals used in the experiment were at least of analytical grade.

Real Sample

As a real sample, a pure culture of *Burkholderia ubonensis, AB030584* was isolated from the rhizosphere of rice.[31] To obtain AHLs, 10–50 mL of nutrient broth (NB) medium was inoculated and grown at 30°C and 175 rpm overnight. To obtain cell free supernatants, the culture was centrifuged at 4°C and 5000 rpm for 5 min in a Hettich Universal 32R centrifuge equipped with a 1620A rotor (Hettich,Tuttlingen, Germany). The supernatant was purified according to a method published elsewhere.[29] Briefly, the sample (with 25% (v/v) ACN) was applied onto the MegaBond Elute cartridge (Varian, Darmsdtadt, Germany), which was previously conditioned by 2 mL water and 2 mL of 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/iso-propanol. The eluate was dried under a nitrogen stream at room temperature, resolved with 3/7 (v/v) ACN/water, and was filtered by a 13 mm PTFE Syringe Filter (VWR, Darmstadt, Germany).

RESULTS AND DISCUSSION

The effect of column temperature, gradient duration, and the flow rate of the mobile phase on peak width were taken into consideration to study the behaviour of the separation efficiency when high pressure is used for LSS. Under these conditions, the peak width turned out to be a better chromatographic parameter to show the separating efficiency because the plate number depends on the chromatographic condition.

To get the highest speed of analysis the temperature must be as high as possible. A difference of about 30°C in the temperature results in around 40% decrease in viscosity and the flow rate can be increased by the same rate. The instrument had no extra device to increase the temperature above the lower boiling points of the component in the mobile phase. In this situation, the highest allowed temperature must be around 20°C less than that of the lowest boiling point of the components. In the acetonitrile-water mixture, the boiling point of acetonitrile is 80°C and the one of water is 100°C, therefore, the highest allowed temperature in this separating system was set to 60°C. The effect of column temperature on peak width is shown in Figure 2. Generally, the peak width decreased by elevating the temperature. Nevertheless, at 60°C some increase can be observed (the last points on Figure 2), which indicates that there were some abnormalities when this

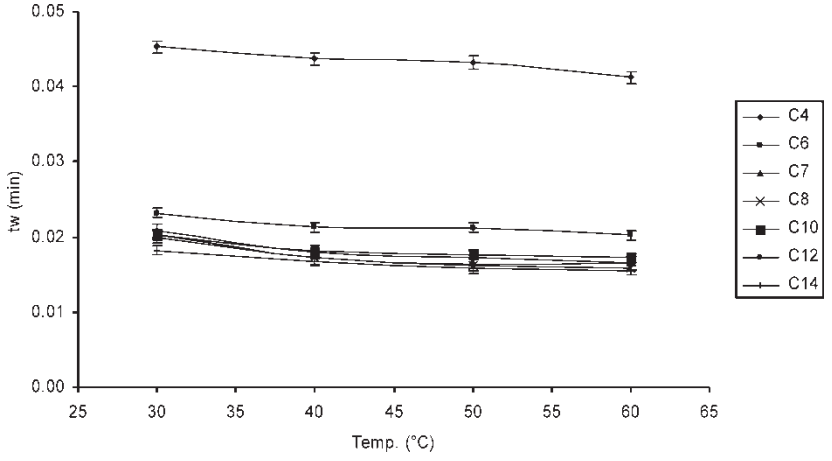


Figure 2. Dependency of the peak width of the column temperature. Separating conditions: gradient = 30% ACN/H_2O to 100% ACN/H_2O in 1.0 min, flow rate = 0.8 mL/min, concentration of C4 ~ C14-AHLs = 10 mg/L. Abbreviation: tw = peak width, temp. = temperature, Cn = Cn-AHL.

relatively high temperature was applied. These results will be referred to later when the temperature effect on the signal to noise ratio is discussed.

Influence of the temperature in the studied range ($\Delta T = 30^{\circ}C$) on retention time showed little variation. There is an empirical equation for temperature dependency on the retention time (t_R): $t_R = A + BT$,[32] where A and B are constant, T is the temperature in °C. The change of t_R in relation to temperature is given in Figure 3. Increasing the temperature within 30°C results in a maximum difference of the retention time of only 0.2 to 0.4 min. This small difference in retention time is unusual for compounds which have no ionisable group.

The peak width turned broader with the increase of gradient duration, especially when gradient times were more than 1.0 min (Figure 4). The advantage of using a short gradient was two fold: analysis time decreased and peak compression was higher, which led to a lower limit of detection. But, when the gradient time kept on decreasing, the influence of the isocratic part became more pronounced when combined with the resulting decreased peak compression; the separation efficiency was thus reduced. It was shown previously in the literature[33] and proven here that the fastest gradient can be run during the breakthrough curve (data not shown), but this is a special case when a sophisticated optimization procedure has to be applied. Technically, this is a stepwise gradient, but the change in solvent composition in the pores results in an S-shaped curve. Most of the gradient run can be conducted by LSS and the isocratic part is closely connected to the dwell time. Even in this low dispersion system, the application of a

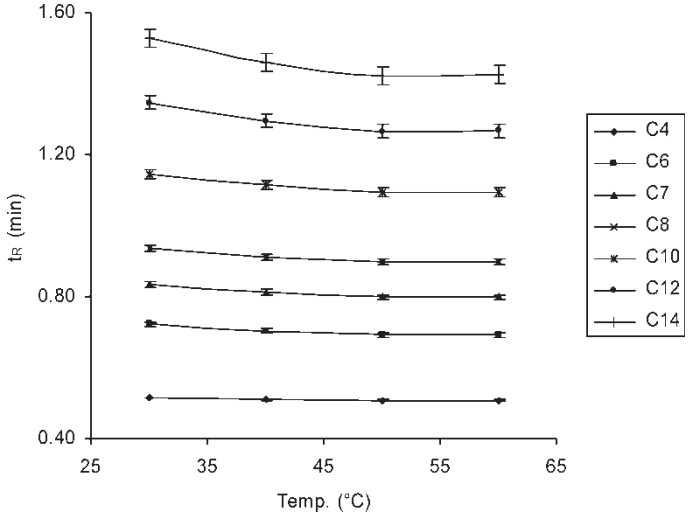


Figure 3. Dependency of the retention time of the column temperature. Separating conditions: gradient = 30% ACN/H_2O to 100% ACN/H_2O in 1.0 min, flow rate = 0.8 mL/min, concentration of C4 ~ C14-AHLs = 10 mg/L, respectively. Abbreviation: t_R = peak width, Temp. = temperature, Cn = Cn-AHL.

gradient elution results in the dwell volume being an obstacle to reduce the analysis time below dwell time and dead time of the column. More generally, the dwell volume and the dead volume are parameters that are more critical for a fast separation with UPLC.

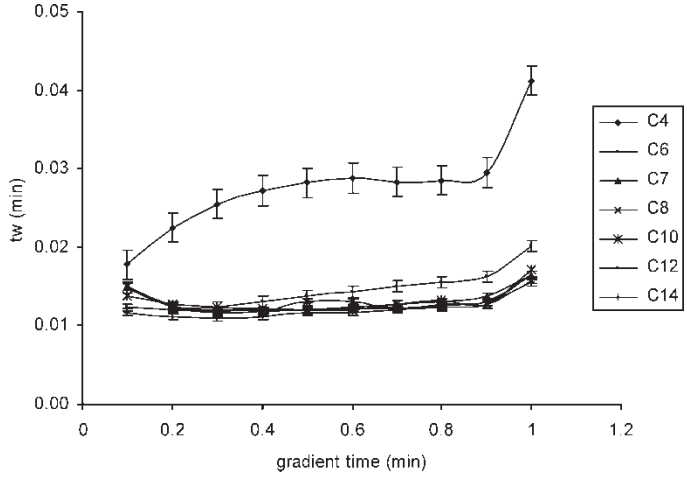


Figure 4. Dependency of the peak with of the gradient time. Separating conditions: column temperature = 60°C, flow rate = 0.8 mL/min, concentration of C4 ~ C14-AHLs = 10 mg/L, respectively. Abbreviation: tw = peak width, Cn = Cn-AHL.

The effect on the retention was observed by changing the flow rate (Figure 5). The pressure drop increased linearly with the flow rate. The results can be seen in Figure 6. In isocratic conditions, the equilibrium constantly depends on the change of the solute's partial molar volume change during the sorption. The dependence of the apparent retention (k^*) is given by the folowing equation: $\mathrm{Ln}\ k^*/p = -\nabla V/t_{R,}$ where p is the pressure, ∇V is the change of the partial molar volume of the solute, and t_R is the retention time. The inlet pressure during the gradient elution varies from time to time and from one to another length of the column. As it was shown in the literature,[34,35] the change of partial molar volume of the solute depends on the solute's size. For insulin, the partial molar volume change was in the order of -100 mL/mol at 350 bar and the retention factor was increased by a factor 2, for n-decane and n-eicosane the retention factor changed only 9% and 24%, respectively, when the pressure was increased from 100 to 350 bar. Tanaka et al.[36] showed another example and they found that the retention factor of nitrophenol was increased by 13% when increasing the pressure by 150 bar. This trend was observed below 400 bar, due to the linear retention behaviour of 4-methyl cathecol up to 680 bar. In our measurements, the separations were carried out above 400 bar. In the earlier examples, the pressure was bellow 400 bars where the mobile phase compressibility is negligible. Martin and Guiochon stated that above this pressure limit the dependency of the retention factor was more complex.[37] However, Sandra et al.[13] found there was only a slight decrease for small molecules, such as benzene and toluene.

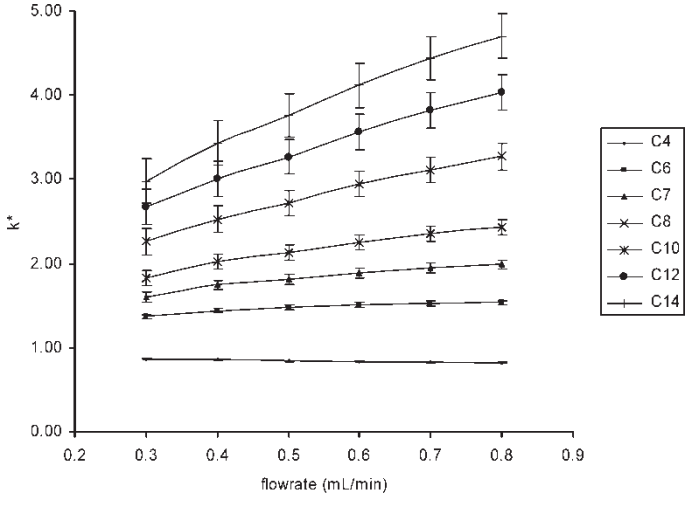


Figure 5. Dependency of the apparent retention factor of the flow rate of the mobile phase. Separating conditions: column temperature = 60°C, gradient = 30% ACN/H_2O to 100% ACN/H_2O in 1.0 min, concentration of C4 ~ C14-AHLs = 10 mg/L, respectively. Abbreviation: k^* = apparent retention factor, Cn = Cn-AHL.

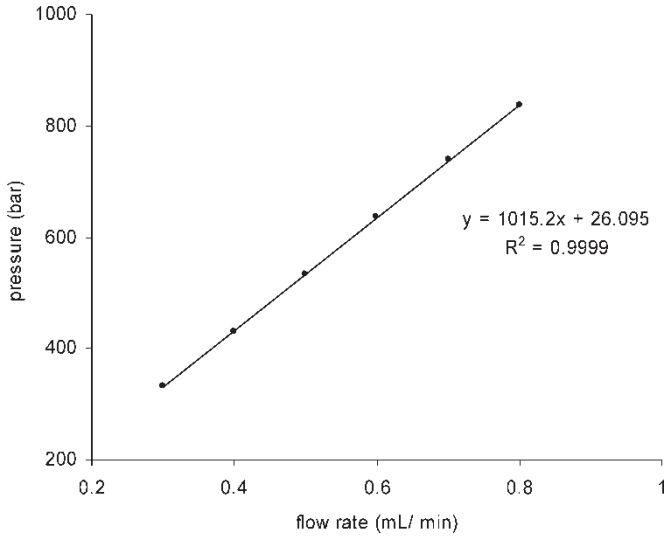


Figure 6. Relation of the column inlet pressure and the flow rate of the mobile phase. Separating conditions: column temperature = 60°C, gradient = 30% ACN/H_2O to 100% ACN/H_2O in 1.0 min, flow rate = 0.8 mL/min, concentration of C4 ~ C14-AHLs = 10 mg/L, respectively.

In our case, the AHLs can be considered as small molecules and, thus, the retention factors depended only on their molecular weight. The apparent retention factor increased with the flow rate (Figure 7); a possible explanation could be the applied higher pressure on the structure of AHLs. Namely, by elevating the flow rate, the column inlet pressure was rising, and, therefore, the k^* of AHLs with a higher molecular weight increased more than those with a smaller one. It is shown in Figure 7 that C4 was an exception where Δk^* was decreased with the increase of the inlet pressure. The main reason for this is its lower retention on the column. The relative ratio of the increase of k^* values was dependent on the molecular weight of the targets.

An optimized high speed separation was shown in Figure 8. The retention followed the Martine-rule.[38] Using a LSS, the retention time difference between two homologue solutes were almost equal (shown in Figure 8), namely, that the difference of t_R between C4 and C6, C6, and C8, C8, and C10, etc., were always similar. AHLs do not have a good chromophore group; therefore a detector with a lower limit of detection (LOD) was demanded when applying a conventional HPLC system. Alternatively, using a small diameter column with high pressure can help to reach a better LOD for the determination of a substance with poor chromophore groups. To get a low detection limit the column must be large and some loss in efficiency must be sacrificed. In the applied overload condition, the retention time and peak widths were repeatable since the relative standard deviations (RSDs)

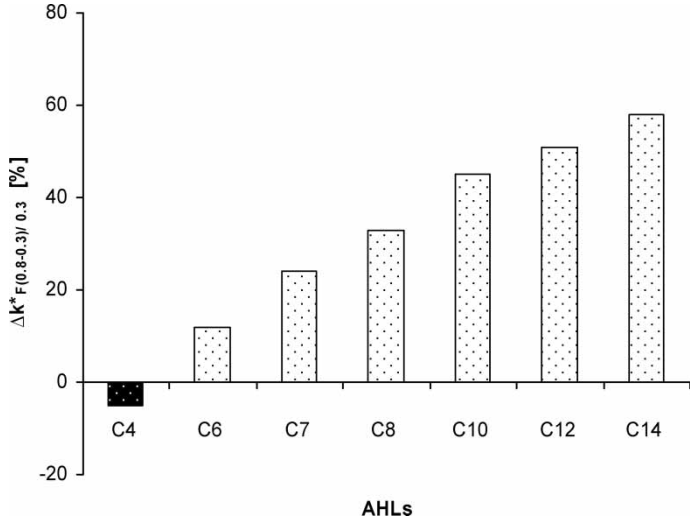


Figure 7. Influence of an increasing flow rate on the apparent retention factor. Separation column temperature = 60°C, gradient procedure = from 30% (v/v) ACN/H_20 to 100% (v/v) ACN/H_20 in 1.0 min, concentration of C4 ~ C14-AHLs = 10 mg/L, respectively, values for different bar = the change of k^* from Cn in flow rate 0.8 to 0.3 (mL/min)/k^* of Cn in flow rate 0.3 (mL/min), respectively. Abbreviation: k^* = apparent retention factor; Cn = Cn-AHL.

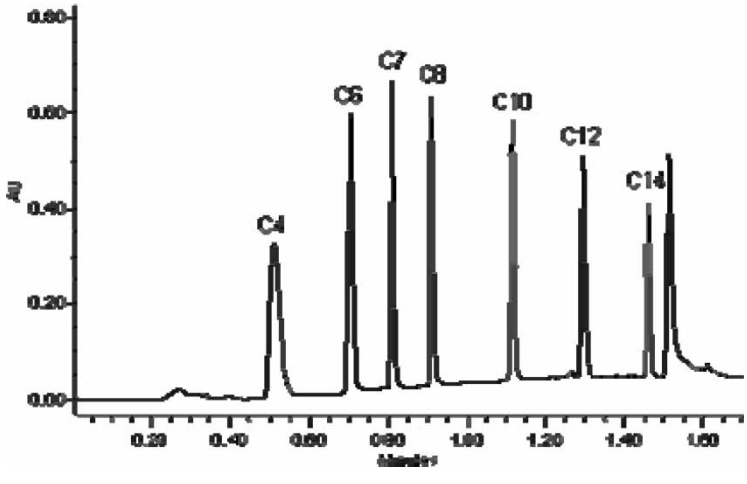


Figure 8. Chromatogram of optimized high speed separation. Separating conditions: Column temp. = 50°C, gradient = 30% ACN/H_2O to 100% ACN/H_2O in 1.0 min, flow rate = 0.8 mL/min, concentration of C4 ~ C14-AHLs = 10 mg/L, respectively. Abbreviation: Cn = Cn-AHL.

were less than 0.3%. For later eluting compounds the concentration and peak compression effects were higher, which could be explained by the dwell volume (V_d) which causes an isocratic part in the beginning of every gradient elution separation. In our system the dwell volume was 0.208 mL, and thus the dwell time was performed according to the following equation: $t_d = V_d/F$, where F is the flow rate in mL/min, t_d is the dwell time in min, and V_d is the dwell volume in mL which is 0.23 min when F is set to 0.9. By applying the gradient procedure the dwell time, which causes delay in the actual flow rate, also had to be taken into account. The LODs were in the 0.1 μg/L range and the technique was therefore attractive for the determination of AHLs in biological systems. Details of the sample preparation applied were given in other publications.[29,30]

From a practical point of view the signal to noise ratio is a basic parameter for the evaluation of analytical performance of a chromatographic system. At 60°C the signal to noise ratio decreased for all the compounds. It was discussed earlier by Sandra that a thermal[13] disequlibrium problem might exist in a UPLC system. The temperature of the mobile phase at its outlet of the column was 60°C and the detector cell was not thermostated; therefore some temperature gradient may exist causing extra noise. This effect might be more pronounced at low wavelength (around 190 nm). Every disturbance in liquid density will change the light scattering and cause some noise. This might be the same phenomenon observed for the flow rate by changing the signal to noise ratio.

In this case the highest flow rate was 0.9 mL/min and the pressure drop was more than 900 bars. After two weeks the pressure drop exceeded 1000 bars and the system stopped. Although the online filter was changed and the system was run at a lower flow rate without injecting any sample, the pressure drop increased again gradually. After that, a new column was used and the system worked perfectly. Two possible reasons were speculated to cause this phenomenon. The first one was that even though the mobile phase was filtered by a 0.2 μm membrane filter, some fines reached the column bed, which resulted in the blocking of the channels between column particles; the second one was that some of the particles in the column were destroyed under the high pressure run and caused the blockage in the channels in the column. Guiochon[39] proved earlier that during the packing process some particles were destroyed even though the column was packed for normal use in HPLC (400 bar). When pressure is applied, the injured particles would produce fines sooner or later.

Finally, one practical application is shown for the determination of AHLs from culture supernantant of *Burkholderia ubonensis*, *AB030584*, *a rhizosphere colonisating bacteria.* In Figure 9A, the chromatogram of the sample can be seen. In the growth medium of the bacteria a small amount of n-AHLs was found when focusing on the retention time. The result in this complex mixture was uncertain. Therefore, this was confirmed by FTICR-MS. In Figure 9B, the complex spectra in scan mode can be seen, the

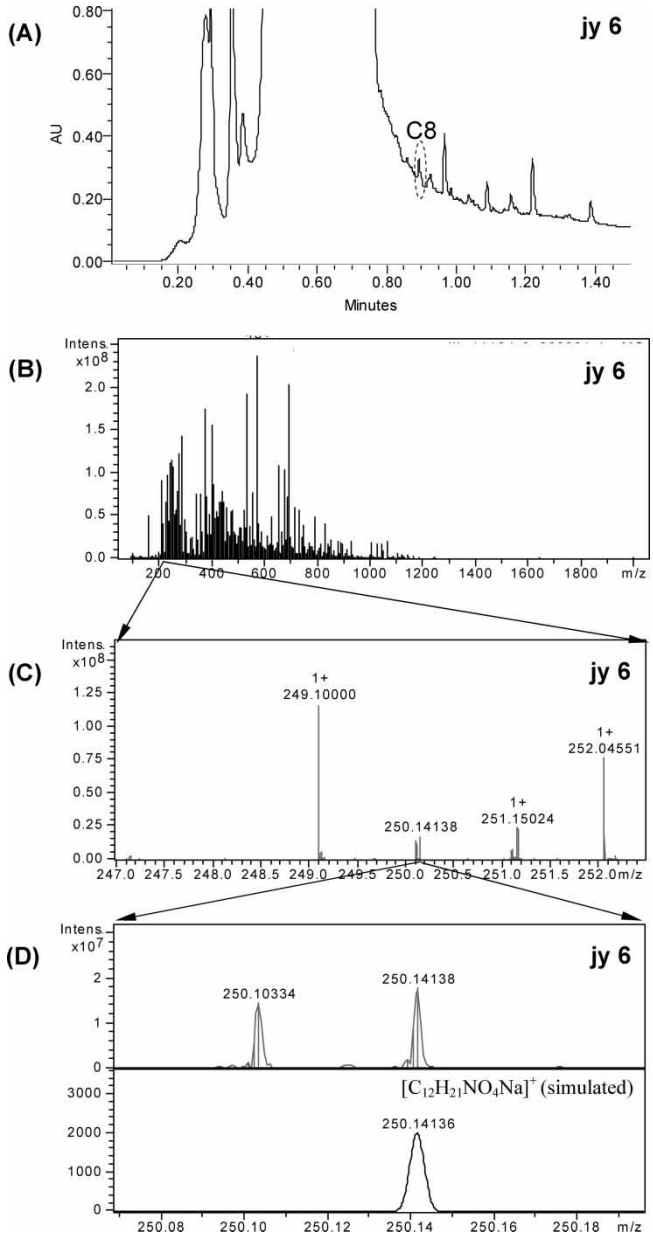


Figure 9. UPLC chromatogram (A) of 10 mL extracted culture supernatant of the *Burkholderia ubonensis*, AB030584 culture grown in NB medium, where C8 was found. The treatment of the sample based on solid phase extraction (see experimental part real sample section.). The extract was separated as in Figure 2. Overview scans (B), details of the FTICR-MS spectra (C), and the comparison with calculated spectrum (D) of the same extracted elution. C8 was thereby confirmed.

extracted spectra and the exact mass is displayed in Figure 8C, and the last step was the comparison of the theoretically calculated mass for C_8-AHL and the measured one. The difference between the two values (0.07 ppm) is negligible and practically the two masses can be considered to be equal. Based on the retention time in the UPLC experiment and the exact measured mass, the C_8-AHL was identified in the sample of a mixture of different species of bacteria.

CONCLUSIONS

UPLC is an appropriate method for the separation of poor UV light absorbing AHLs. Using gradient elution, 20 μL of samples can be injected because preconcentration at the inlet of the column took place, except for C4-AHL, which has a low retention. The retention factors were pressure dependent and the dead volume was also highly affected by changes in pressure. The peak width, as a parameter for characterizing the system efficiency, was hardly influenced by temperature. Surprisingly, a slight dependence of the retention times of the temperature was observed. It was shown that the effective dwell volume of this highly sophisticated system was 0.208 mL and must be taken into consideration when the gradient time is planned. In our case using the highest possible flow rate (0.9 mL/min), this effect caused a delay time of 0.231 min (0.208/0.9). Unfortunately, at this high flow rate the pressure drop was higher than 900 bar and a systematic increase in the pressure drop was observed, and after two weeks the pressure exceeded 1000 bar. There are two possible explanations: first, it is impossible to remove all fines from the samples and the solvents used for the mobile phases, the second one is that some of the particles in the column will be destroyed and block the channels. Setting the system pressure near to the upper limit may not be advisable. At lower flow rates the solvent dwell or delay time will be increased and must be taken into consideration by applying the gradient. By increasing the temperature, the signal to noise ratio decreased to some extent. In spite of this phenomenon, the possible maximum temperature (60°C) was suggested to run with the highest flow rate, and the fastest analysis can be reached.

ACKNOWLEDGMENTS

All work was done at the GSF National Research Center for Environment and Health with financial support of the Chinese Scholarship Council (CSC) of X. L. as guest scientist. The authors want to thank Dr. Michael Rothballer, ATP/GSF for preparing the *Burkholderia ubonensis* real samples. Special thanks to Brigitte Look and Heidi Neumeir for their precise technical assistance and for their always encouraging words.

REFERENCES

1. Ishizuka, N.; Minakuchi, H.; Nakanishi, K.; Hirao, K.; Tanaka, N. Chromatographic characterization of macroporous monolithic silica prepared via sol-gel process. Colloids Surf. A **2001**, *187*, 273–279.
2. Novakova, L.; Solichova, D.; Solich, P. Advantages of ultra performance liquid chromatography over high-performance liquid chromatography: Comparison of different analytical 2 approaches during analysis of diclofenac gel. J. Sep. Sci. **2006**, *29* (16), 2433–2443.
3. Nguyen, D.T.T.; Guillarme, D.; Rudaz, S.; Veuthey, J.L. Chromatographic behaviour and comparison of column packed with sub-2 μm stationary phases in liquid chromatography. J. Chromatogr. A **2006**, *1128* (1–2), 105–113.
4. Nguyen, D.T.T.; Guillarme, D.; Rudaz, S.; Veuthey, J.L. Fast analysis in liquid chromatography using small particle size and high pressure. J. Sep. Sci. **2006**, *29* (12), 1836–1848.
5. Murphy, B.J. UPLC outperforms HPLC in metabolite screening. Trac-Trends. Anal. Chem. **2006**, *25* (8), Vii–Viii.
6. Leandro, C.C.; Hancock, P.; Fussell, R.J.; Keely, B.J. Comparison of ultra-performance liquid chromatography and high-performance liquid chromatography for the determination of priority pesticides in baby foods by tandem quadrupole mass spectrometry. J. Chromatogr. A **2006**, *1103* (1), 94–101.
7. Wren, S.A.C.; Tchelitcheff, P. UPLC/MS for the identification of beta-blockers. J. Pharm. Biomed. Anal. **2006**, *40* (3), 571–580.
8. Nordstrom, A.; O'Maille, G.; Qin, C.; Siuzdak, G. Nonlinear data alignment for UPLC-MS and HPLC-MS based metabolomics: Quantitative analysis of endogenous and exogenous metabolites in human serum. Anal. Chem. **2006**, *78* (10), 3289–3295.
9. Mezcua, M.; Aguera, A.; Lliberia, J.L.; Cortes, M.A.; Bago, B.; Fernandez-Alba, A.R. Application of ultra performance liquid chromatography-tandem mass spectrometry to the analysis of priority pesticides in groundwater. J. Chromatogr. A **2006**, *1109* (2), 222–227.
10. Kovalczuk, T.; Jech, M.; Poustka, J.; Hajslova, J. Ultra-performance liquid chromatography-tandem mass spectrometry: A novel challenge in multiresidue pesticide analysis in food. Anal. Chem. Acta **2006**, *577* (1), 8–17.
11. Al-Dirbashi, O.Y.; Aboul-Enein, H.Y.; Jacob, M.; Al-Qahtani, K.; Rashed, M.S. UPLC-MS/MS determination of doxazosine in human plasma. Anal. Bioanal. Chem. **2006**, *385* (8), 1439–1443.
12. Swartz, M.E. Ultra performance liquid chromatography (UPLC): An introduction. LC-GC **2005**, 8–14.
13. de Villiers, A.; Lauer, H.; Szucs, R.; Goodall, S.; Sandra, P. Influence of frictional heating on temperature gradients in ultra-high-pressure liquid chromatography on 2.1 mm ID columns. J. Chromatogr. A **2006**, *1113* (1–2), 84–91.
14. *High-Performance Liquid Chromatography, Advances and Perspectives, 1*; Academic Press: New York, 1980, 207 pp.
15. Plumb, R.S.; Rainville, P.; Smith, B.W.; Johnson, K.A.; Castro-Perez, J.; Wilson, I.D.; Nicholson, J.K. Generation of Ultrahigh peak capacity LC separations via elevated temperatures and high linear mobile-phase velocities. Anal. Chem. **2006**, *78* (20), 7278–7283.
16. Yang, Y.; Hodges, C.C. Assay transfer from HPLC to UPLC for higher analysis throughput. LC-GC **2005**, 31–35.

17. King, S.; Stoffolano, P.J.; Robinson, E.; Eichhold, T.E.; Hoke, S.H.; Baker, T.R.; Richardson, E.C.; Wehmeyer, K.R. The evaluation and application of UPLC for the rapid analysis of dose formulations. LC-GC **2005**, 36–39.
18. Grumbach, E.S.; Wheat, T.E.; Mazzeo, J.R. Recent developments in columns for UPLC (TM). LC-GC **2005**, 48–49.
19. Chambers, E.; Diehl, D.; Mazzeo, J. Simple and fast SPE-UPLC (TM)-MS-MS method for an allergy tablet mixture of acids and bases. LC-GC **2005**, 74–75.
20. Waters ACQUITY UPLC system literature and presentation references. LC-GC **2005**, 45–46, [Anon].
21. Whitehead, N.A.; Barnard, A.M.L.; Slater, H.; Simpson, N.J.L.; Salmond, G.P.C. Quorum-sensing in gram-negative bacteria. Fems Microbiol. Rev. **2001**, *25* (4), 365–404.
22. Taminiau, B.; Daykin, M.; Swift, S.; Boschiroli, M.L.; Tibor, A.; Lestrate, P.; De Bolle, X.; O'Callaghan, D.; Williams, P.; Letesson, J.J. Identification of a quorum-sensing signal molecule in the facultative intracellular pathogen Brucella melitensis. Infec. Immu. **2002**, *70* (6), 3004–3011.
23. Khmel, I.A.; Metlitskaya, A.Z. Quorum sensing of genes expression—Perspective drug target against bacterial pathogenicity. Mol. Bio. **2006**, *40* (2), 195–210.
24. Pomini, A.M.; Manfio, G.P.; Araujo, W.L.; Marsaioli, A.J. Acyl-homoserine lactones from Erwinia psidii R. IBSBF 435(T), a guava phytopathogen (Psidium guajava L.). J. Agi. Food. Chem. **2005**, *53* (16), 6262–6265.
25. Singh, P.K.; Schaefer, A.L.; Parsek, M.R.; Moninger, T.O.; Welsh, M.J.; Greenberg, E.P. Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms. Nature **2000**, *407* (6805), 762–764.
26. Rasch, M.; Andersen, J.B.; Nielsen, K.F.; Flodgaard, L.R.; Christensen, H.; Givskov, M.; Gram, L. Involvement of bacterial quorum-sensing signals in spoilage of bean sprouts. Appl. Environ. Microbiol. **2005**, *71* (6), 3321–3330.
27. Frommberger, M.; Schmitt-Kopplin, P.; Ping, G.; Frisch, H.; Schmid, M.; Zhang, Y.; Hartmann, A.; Kettrup, A. 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. Anal. Bioanal. Chem. **2004**, *378* (4), 1014–1020.
28. Wagner-Dobler, I.; Thiel, V.; Eberl, L.; Allgaier, M.; Bodor, A.; Meyer, S.; Ebner, S.; Hennig, A.; Pukall, R.; Schulz, S. Discovery of complex mixtures of novel long-chain quorum sensing signals in free-living and host-associated marine alphaproteobacteria. Chembiochem. **2005**, *6* (12), 2195–2206.
29. Li, X.; Fekete, A.; Englmann, M.; Götz, C.; Rothballer, M.; Buddrus, K.; Cai, C.; Schröder, P.; Hartmann, A.; Chen, G.; Schmitt-Kopplin, P. Development of a solid phase extraction—ultra performance liquid chromatography method for the analysis of bacterial N-acylhomoserine lactones from complex samples. J. Chromatogr. A **2006**, *1135* (1), 101–108.
30. Fekete, A.; Frommberger, M.; Rothballer, M.; Li, X.J.; Englmann, M.; Fekete, J.; Hartmann, A.; Eberl, L.; Schmitt-Kopplin, P. Identification of bacterial N-acylhomoserine lactones (AHLs) with a combination of ultra-performance liquid chromatography (UPLC), ultra-high-resolution mass spectrometry, and in-situ biosensors. Anal. Bioanal. Chem. **2007**, *387* (2), 455–467.
31. Frommberger, M.; Schmitt-Kopplin, P.; Menzinger, F.; Albrecht, V.; Schmid, M.; Eberl, L.; Hartmann, A.; Kettrup, A. Analysis of N-acyl-L-homoserine lactones produced by Burkholderia cepacia with partial filling micellar electrokinetic chromatography-electrospray ionization-ion trap mass spectrometry. Electrophoresis **2003**, *24* (17), 3067–3074.

32. Dolan, J.W.; Snyder, L.R.; Wolcott, R.G.; Haber, P.; Baczek, T.; Kaliszan, R.; Sander, L.C. Reversed-phase liquid chromatographic separation of complex samples by optimizing temperature and gradient time III. Improving the accuracy of computer simulation. J. Chromatogr. A **1999**, *857* (1–2), 41–68.
33. Nagy, K.; Jakab, A.; Fekete, J.; Vekey, K. An HPLC-MS approach for analysis of very long chain fatty acids and other apolar compounds on octadecyl-silica phase using partly miscible solvents. Anal. Chem. **2004**, *76* (7), 1935–1941.
34. Liu, X.D.; Szabelski, P.; Kacmarski, K.; Zhou, D.M.; Guiochon, G. Influence of pressure on the chromatographic behavior of insulin variants under nonlinear conditions. J. Chromatogr. A **2003**, *988* (2), 205–218.
35. Liu, X.D.; Zhou, D.M.; Szabelski, P.; Guiochon, G. Influence of pressure on the retention and separation of insulin variants under linear conditions. Anal. Chem. **2003**, *75* (16), 3999–4009.
36. Tanaka, N.; Kobayashi, H.; Ishizuka, N.; Minakuchi, H.; Nakanishi, K.; Hosoya, K.; Ikegami, T. Monolithic silica columns for high-efficiency chromatographic separations. J. Chromatogr. A **2002**, *965* (1–2), 35–49.
37. Gritti, F.; Guiochon, G. Influence of the pressure on the properties of chromatographic columns-I. Measurement of the compressibility of methanol-water mixtures on a mesoporous silica adsorbent. J. Chromatogr. A **2005**, *1070* (1–2), 1–12.
38. Martin, A.J.P. Biochem. Soc. Symp. **1949**, 3–4.
39. Gritti, F.; Guiochon, G. Influence of the pressure on the properties of chromatographic columns III. Retention volume of thiourea, hold-up volume, and compressibility of the C-18-bonded layer. J. Chromatogr. A **2005**, *1075* (1–2), 117–126.

Received April 1, 2007
Accepted April 25, 2007
Manuscript 6099