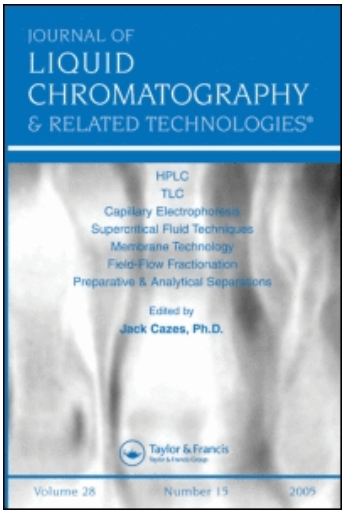

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Leitner, Tanja and Klampfl, Christian W.(2008) 'Development of a Simple Instrumental Setup for the Separation of Benzoic Acids by Comprehensive Liquid Chromatography with Microbore Columns and Monolithic Columns', Journal of Liquid Chromatography & Related Technologies, 31: 2, 169 — 178
To link to this Article: DOI: 10.1080/10826070701738738
URL: http://dx.doi.org/10.1080/10826070701738738

Journal of Liquid Chromatography & Related Technologies®, 31: 169–178, 2008

ISSN 1082-6076 print/1520-572X online
DOI: 10.1080/10826070701738738

Development of a Simple Instrumental Setup for the Separation of Benzoic Acids by Comprehensive Liquid Chromatography with Microbore Columns and Monolithic Columns

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Abstract: A simple instrumental setup for the separation of benzoic acid derivatives using comprehensive liquid chromatography is presented. The separation system consists of a 1 mm i.d. microbore anion exchange column coupled to a monolithic reversed phase column, using a simple interface realized by the combination of two conventional sixport valves. Thereby, the eluate from the first dimension could be transferred to the second chromatographic dimension throughout the whole run without any losses. Employing the combination of two different separation mechanisms namely ion exchange and reversed-phase chromatography allowed the resolution of all test analytes in less than 14 minutes.

Keywords: Benzoic acids, Liquid chromatography, Microbore columns

INTRODUCTION

The investigation of increasingly complex samples requires the use of more and more sophisticated analytical methods. A first step was the use of hyphenated methods such as the combination of high performance separation methods like gas chromatography (GC), high performance liquid chromatography (HPLC), or capillary electrophoresis (CE) with spectrometric

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techniques primarily mass spectrometry (MS).[1] These methods provide a substantial increase in information as chromatographic data (such as retention or migration times) are supplemented by spectroscopic information. Further improvement of the chromatographic separation can be achieved using multidimensional chromatographic approaches. By theory (if chromatographic techniques with complementary separation principles are employed), the use of two chromatographic dimensions can lead to a multiplication of their peak capacities, thereby, substantially increasing the resolving capability of the separation.[2,3] Although this theoretical enhancement can not be fully achieved in practice, a considerable increase in peak capacity can be observed, when two-dimensional chromatographic approaches are employed.

Focusing on the practical realization of this concept, much less obstacles are encountered in two-dimensional GC (GC × GC) compared to two-dimensional LC (LC × LC). This is also expressed in the large number of works reporting the use of GC × GC for the analysis of real world applications published within the last few years.[4–7] Whereas, the transfer of the eluate from the first to the second chromatographic dimension is relatively unproblematic in GC × GC,[4,5] it can be associated with a series of problems in LC × LC.[8] Therefore, a number of quite different approaches for LC × LC experiments, including off-line and on-line strategies, have been described in the recent literature. Focusing on on-line LC × LC the direct transfer of the eluate from dimension 1 to dimension 2 can be achieved via a single six-port valve. This quite simple setup is affected with the drawback, that while the six-port valve is in the injection mode (to inject the eluate into the 2nd column) the eluate from dimension one is lost.[9] This can be avoided by the so called "parking-mode", i.e., the flow in the first dimension is stopped during this time.[9] Although this strategy provides extra time for the separation in dimension 2, negative effects such as peak broadening due to diffusion phenomena and an increase in overall analysis time have to be taken into account. Another way to overcome the problem mentioned above is the use of a two six-port[9] or one ten-port[10] or even a twelve-port valve.[11] In this case, the separation time in the second dimension has to be adapted to the flow rate used in the first dimension and the size of the employed injection loops. A way to circumvent this dependency is the use of multiple parallel columns in the 2nd dimension, which are subsequently loaded with the eluate from the 1st dimension. Switching to the different types of columns (often also representing different chromatographic separation mechanisms), it can be seen that a variety of combinations have been described in the recent literature. These range from the use of two reversed-phase (RP) columns,[9,11–13] a RP column and a cyano or amino phase in the 2nd dimension,[11,14] C_{18} RP-phase with ion exchangers[15–17] (also loaded with Ag^+ ions,[18]), normal phase columns,[10] or other phases such as carbon clad zirkonia columns,[8,19] to the combination of quite different separation principles such as size exclusion chromatography and RP-chromatography.[20–22]

In the present paper, we describe a simple comprehensive LCxLC method for the separation of a set of substituted benzoic acids employing a combination of an anion exchange microbore column in the 1st and a monolithic RP column in the second dimension.

EXPERIMENTAL

Instrumentation

The instrumentation for comprehensive liquid chromatography consisted of an Agilent 1100 modular HPLC system (Agilent Technologies, Waldbronn, Germany), a Waters 486 tunable absorbance detector, a Waters 590 programmable HPLC pump (both Waters, Milford, MA, U.S.A.), and two Rheodyne sixport valves (Rheodyne, Berkeley, U.S.A.) equipped with 100 μL loops. For the first chromatographic dimension, a 140 mm × 1 mm i.d. column packed in house with Nucleosil 100-5-SB particles (Machery-Nagel, Düren, Germany) and for the second dimension a 100 mm × 4.6 mm i.d. Chromolith Performance RP-18e column (Merck, Darmstadt, Germany) was employed. Data acquisition was performed on an HP-Chemstation (Agilent Technologies, Waldbronn, Germany).

Reagents and Chemicals

All chemicals, were of analytical-reagent grade. Eluents were prepared from 18 MΩ high purity water obtained from a Milli-Q System (Millipore, Marlborough, MA, U.S.A.), acetonitrile, methanol, phosphoric acid (all J.T. Baker, Deventer, The Netherlands), and Na_2HPO_4 (Merck, Darmstadt, Germany). Molecular structures and pKa values of the benzoic acids used as test substances (Merck, Darmstadt, Germany, and Fluka AG, Buchs, Switzerland) are given in Figure 1.

RESULTS AND DISCUSSION

Choice of Column for the First and the Second Chromatographic Dimension

Theoretically, the approach for comprehensive chromatography promising the best results with respect to peak capacity, is the use of orthogonal stationary phases. Although this perfect condition cannot be fully reached in practice, the use of two quite different chromatographic separation principles, namely ion exchange in the first and a separation based on reversed-phase chromatographic principles in the second dimension, seemed the best choice for the

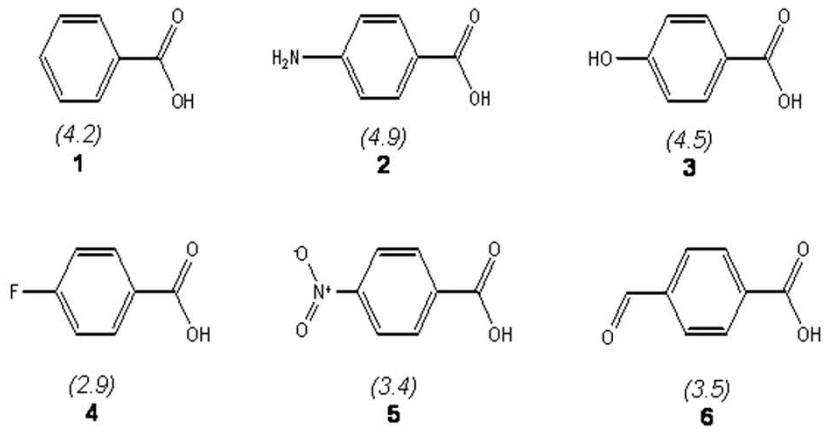

Figure 1. Molecular structures and pKa values of the benzoic acids used in this work.

investigations performed in the present work. A primary reason for the use of an anion exchange stationary phase for the separation in the first chromatographic dimension was the fact that all selected analytes included acidic functional groups, allowing the partial resolution of the mixture in the first dimension. A second benefit of this setup is that ion exchange separations are commonly performed using aqueous or almost aqueous mobile phases. Such conditions are a prerequisite to keep zone broadening a minimum when transferring the complete eluate from the first to the second dimension, in particular if the separation in the latter dimension is based on a reversed-phase mechanism. Besides the correct choice of the type of stationary phases employed, appropriate column dimensions also play an important role in comprehensive liquid chromatography. In principle, coupling capillary columns (in the first dimension) to larger diameter monolithic columns in the second dimension is a good choice with respect to several crucial parameters. First, the column of dimension 1 should be operated at low mobile phase flow rates, as the complete eluate from this column has to be transferred to the second dimension and injection volumes (in the second dimension) should be kept within reasonable limits. In addition to that, our setup (the flow rate of the first dimension column was kept constant for the full time of analysis) required the temporary storage of the eluate from the first column in an injection loop while the separation in the second dimension is performed. Therefore, assuming an analysis time of 75 seconds in the second dimension and a loop volume of 100 μL, the first dimension column had to be operated at a flow rate of maximum 0.08 mL, so the complete eluate accumulating during these 75 seconds could be temporarily stored. A sketch depicting the instrumental layout actually employed can be seen in Figure 2.

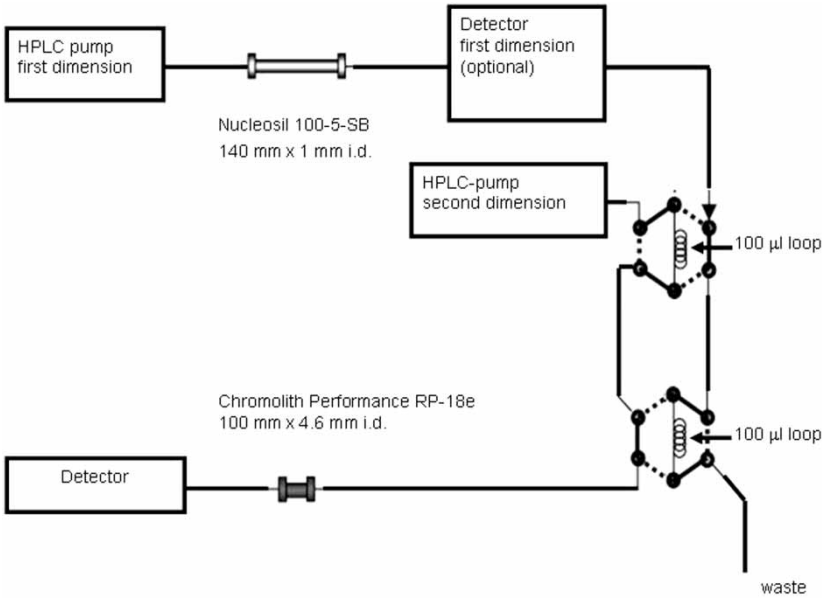

Figure 2. Instrumental setup used for two-dimensional comprehensive HPLC.

Optimization of Separation Parameters for the First Chromatographic Dimension

At the beginning of our investigations operational parameters for the first chromatographic dimension had to be optimized. Using the 140 mm × 1 mm i.d. Nucleosil 100-5-SB column, the following principal parameters were investigated: the eluent composition, the eluent flow rate, and the injection volume used. Starting with the eluent composition, eluents containing different amounts of organic modifier (acetonitrile) and buffers with different phosphate concentrations and pH were tested. Starting with the acetonitrile content, two facts had to be kept in mind: first, a certain amount of acetonitrile was needed to obtain acceptable peak shapes, whatever composition of the eluent with respect to the other ingredients was used; second, the amount of acetonitrile employed in the first dimension chromatographic separation should be kept as low as possible, to avoid excessive zone broadening when the eluate was finally transferred to the RP column. Focusing on the concentration and pH of the buffer employed, several different buffer compositions were investigated. Using an anion exchange column in the first dimension, separation could only be achieved when eluents with a pH where the selected benzoic acids are deprotonated were chosen. Regarding the pKa values depicted in Table 1, a slightly acidic to alkaline pH should provide the best performance of an anion exchange column. Therefore, phosphate buffers with

pH values ranging from 5 to 8 were tested. These experiments showed that buffers with higher pH values led to improved separations; because of the fact that silica based columns should not be operated at alkaline pH, the pH 7 phosphate buffer value was selected for the further experiments. In ion exchange chromatography the type and concentration of the eluting ion significantly influence the retention of the solutes. For this reason, eluents containing either phosphate or perchlorate as the eluting ion were compared. As the perchlorate eluent led to unfavorable analysis times, further investigations were performed with eluents with different phosphate concentrations. Thereby, an optimization with respect to the resolution of the selected solutes and the analysis time was performed. The separation finally obtained in the

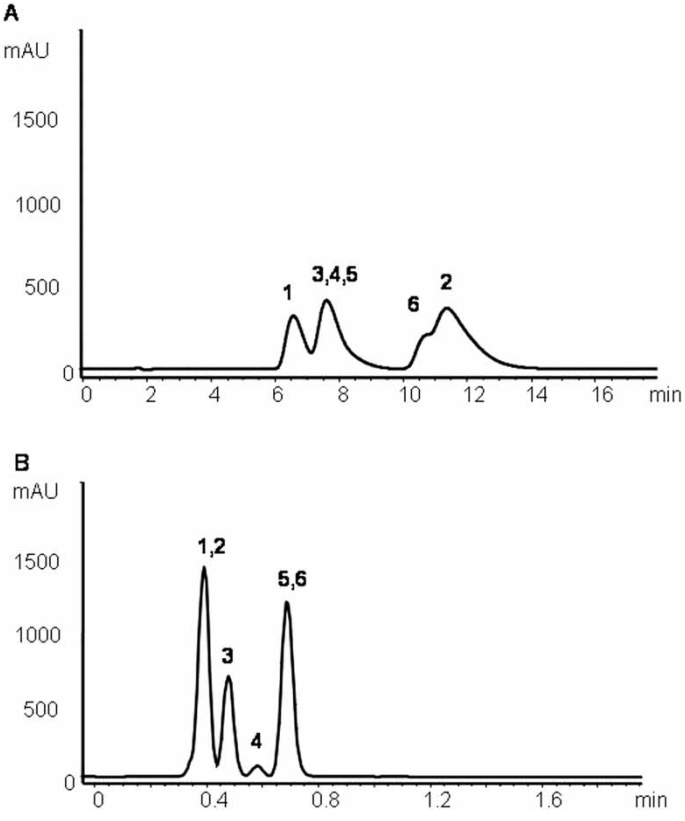

Figure 3. One dimensional separation of the test mixture using a 140/1 Nucleosil 100-5 SB anion-exchange column (A) and a chromolith performance RP-18e column (B). Chromatographic conditions: (A) mobile phase, water/acetonitrile/100 mM phospate buffer pH 7 (77/20/3); flow rate 0.08 mL min^{-1}; injection volume 5 μL; (B) mobile phase, water/acetonitrile/phosporic acid (65/35/0.2); flow rate, 3 mL min^{-1}; injection volume, 100 μL;. Peak assignement see Figure 1.

first dimension and the corresponding operational parameters can be seen in Figure 3A. As can be seen from this Figure, 4-formylbenzoic acid, benzoic acid, and 4-fluorobenzoic acid coeluted and 4-hydroxybenzoic acid and 4-nitrobenzoic acid were only marginally separated under these conditions.

Optimization of Separation Parameters for the Second Chromatographic Dimension

Crucial points in the optimization of the separation in the second chromatographic dimension were: i) substances coeluting in the first dimension have to be separated on this column; ii) conditions have to be adapted to the relatively large injection volume (100 μL); iii) the separation has to be fast, as the eluate from the first dimension has to be stored during this time. To meet the requirements arising from the first two points, a variety of eluent compositions including different amounts of acetonitrile, water, and phosphoric acid (to guarantee the protonation of the analytes required for their separation of the RP phase) were tested. The isocratic separation mode was preferred, as it allowed using the full time available for separation and no re-equilibration time was needed. An additional requirement was to move substance peaks from a small system peak (occurring at the start of each 2nd dimension chromatogram) originating from the operation of the Rheodyne valves used for the connection of the two chromatographic dimensions. Finally the last problem, namely the separation of the crucial analyte pairs in the time span available (less than one minute), had to be solved. A principle advantage of the monolithic column employed in this work is its ability to tolerate flow rates of 5 mL per minute and more without excessive backpressure and without loss in resolution. Figure 3B shows the separation of the selected test mixture at a flow rate of 3 mL min^{-1} using the optimized eluent composition.

Separations Employing the "Comprehensive Chromatography" Approach

The starting point for the following work can be understood by focusing on Figure 4. This figure shows a plot of retention times of the selected benzoic acids on the anion exchange column versus those obtained on the RP column. From these data, it can be concluded that the chromatographic resolution of all test substances should be possible employing the comprehensive chromatography approach. In the following analytical run, the two switching valves were operated manually in one minute intervals. To allow simultaneous operation of both valves, a fact that was essential to avoid excessive system peaks in the chromatogram, it was necessary to connect these valves by a simple metal clamp manufactured in the institute

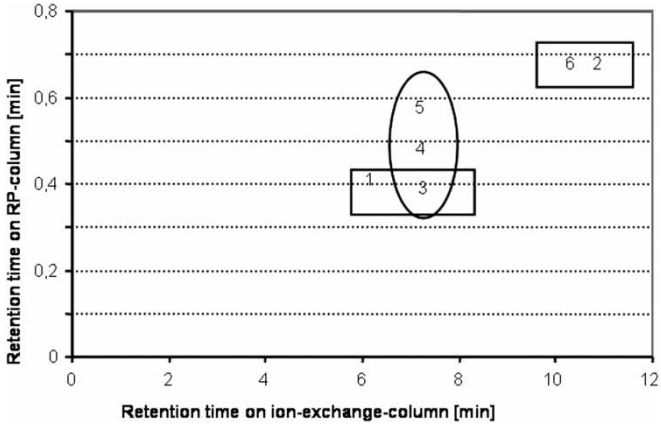

Figure 4. Comparison of retention times on 140/1 Nucleosil 100-5 SB anion exchange column and chromolith performance RP-18e column. Chromatographic conditions see text and Figure 3.

workshop. Figure 5 shows the separation of the test mixture employing the comprehensive chromatography approach. Valve switching points are indicated by arrows in the chromatogram. It can be seen that all selected benzoic acids could be resolved chromatographically. Focusing on the peak

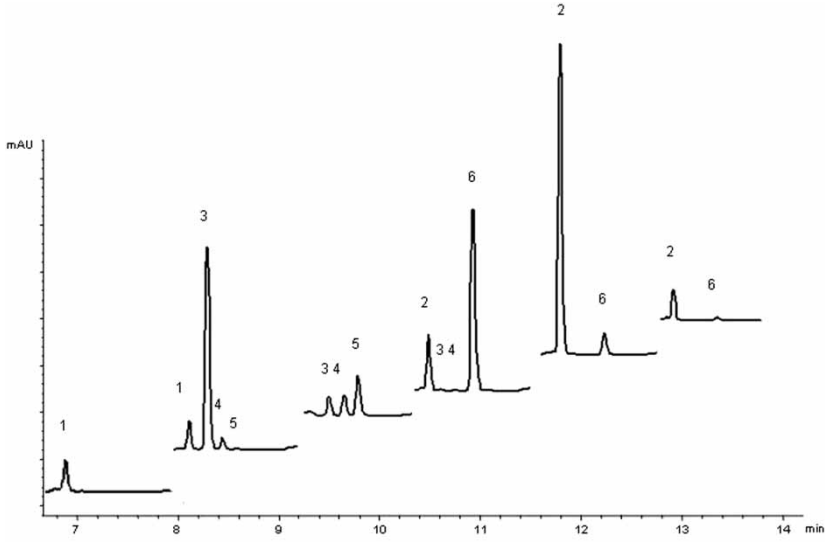

Figure 5. Two dimensional separation of the test mixture using a 140/1 Nucleosil 100-5 SB anion exchange column and a chromolith performance RP-18e column. Chromatographic conditions and peak assignement see text and Figure 3.

shapes obtained, no negative effect caused by the transfer of the eluate from the first to the second dimension could be observed.

CONCLUSIONS

Using a simple, lab made 2D-HPLC system based on the combination of an anion- exchange-and a reversed phase column, a series of benzoic acid derivatives could be separated within less than 14 minutes. It should be mentioned that the eluate from the first dimension was completely transferred to the second chromatographic dimension throughout the whole run. This could be achieved by combining a 1 mm i.d. column (1st dimension) with a monolithic column (2nd dimension). Adding more specific parts like automatic 10 port switching valves and specific 2-D chromatography software would allow a further improvement of the instrumentation presented in this paper, also facilitating its operation. Nevertheless, the major aim of this work was to demonstrate that comprehensive liquid chromatography can be performed employing basic chromatographic instrumentation available in almost every lab.

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Received June 22, 2007
Accepted July 16, 2007
Manuscript 6163