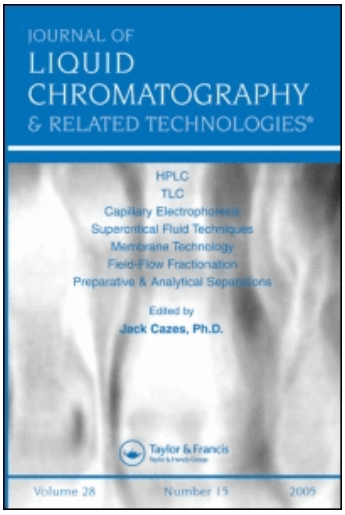

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Optimization for Minimum Analysis Time of the Separation of Benzene and Deuterated Benzene by Reverse Phase Microbore and Recycle HPLC, and the Utility of D_2O

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OPTIMIZATION FOR MINIMUM ANALYSIS TIME OF THE SEPARATION OF BENZENE AND DEUTERATED BENZENE BY REVERSE PHASE MICROBORE AND RECYCLE HPLC, AND THE UTILITY OF D_2O

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ABSTRACT

Optimization for minimum analysis time is demonstrated for HPLC separations of different degrees of difficulty. The separation of benzene and perdeuterobenzene can be performed in less than ten minutes, separation of benzene and monodeuterobenzene takes several hours.

The use of deuterium oxide and long microbore columns is not recommended since two-column recycle chromatography offers shorter analysis times than a long single-pass column.

INTRODUCTION

We are interested in demonstrating the advantages of microbore chromatography over standard HPLC procedures. The improved minimum detectable quantity of microbore chromatography when employing UV absorbance and fluorescence detection has been

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shown before.[1] The cost savings for expensive mobile phases such as deuterated solvents are obvious. It is well known that deuterated mobile phases can be necessary when NMR[2] or infrared[3] detection are to be used.

Perdeuterated analogs of the analyte(s) would be ideal internal standards for HPLC as they are in GC-MS (e.g. see [4]) but separation by HPLC of analyte and perdeuterated analyte appears not to be easy.[5,6] Deuteration may also be useful in the study of enzyme-mediated reactions and in the design of drugs with optimum therapeutic activity.[7]

A dramatic increase in selectivity has been reported for the separation of benzene and perdeuterobenzene when using long reverse phase microbore columns with deuterium oxide (D_2O) instead of water (H_2O) in the mobile phase.[8] This separation is notoriously difficult since no pK_a change occurs on hydrogen-deuterium substitution in this molecule, as in acids[5] or bases.[9] [Note that the isotopic effect of substitution of aliphatic hydrogens seems even smaller than that of aromatic ones.[5,7]]

The separation of benzene and perdeuterobenzene as reported in the literature takes several hours[8] even when using recycling.[10] By increasing the water content of the mobile phase it was predicted[5] and recently shown[11] that the separation time could be decreased to about an hour. Therefore an increase of selectivity by using deuterium oxide instead of water would result in substantial savings in separation time.

The objective of this study was to investigate the benefits of using deuterium oxide in difficult separations, specifically in the

separation of isotopically differing molecules. As an example the separation of benzene and (per)deuterobenzene was used. To assure that the improvement sought with D_2O could not be obtained otherwise [e.g. see [11]], a regular optimization of chromatographic conditions was performed using H_2O in the mobile phase.

EXPERIMENTAL

Separations were performed on a Varian LC 5560 liquid chromatograph equipped with a Model 7410 injection valve (Rheodyne, Cotati, CA., USA) and a UV 200 variable wavelength absorbance detector (Varian). Commercially available 15 cm x 4.6 mm Micropak SP C18-3 columns (Varian) were used except for the microbore experiment of Figure 5. In the microbore column experiment the LC 5560 was used in the split-flow mode[12] and an experimental 100 nl UV 200 flowcell was employed. In all other experiments the UV 200 detector contained the standard 0.5 ul microbore flowcell (Varian). The switching valve in the recycle experiment (Figure 6) was a Model C6U (Valco, Houston, TX, USA); the in-line filters (#7302, Rheodyne, Cotati, CA, USA) contained a 0.2 um Fluoripore® (Millipore, Bedford, MA, USA) insert[13].

Mobile phases consisted of deionized HPLC grade water, deuterium oxide (99.8% D, Sigma, St. Louis, MO, USA) and HPLC grade acetonitrile and methanol (Burdick and Jackson, Muskegon, MI, USA). Analytes were purchased from Sigma Chemical Co.

RESULTS

A. Optimization of Benzene/Perdeuterobenzene Separation

The minimum separation time of a mixture of two components, t_r, can be expresed in the chromatographic parameters as:

$$t_r = \left(\frac{R_{req}}{\alpha - 1}\right)^2 \cdot \left(\frac{1 + k_1'}{k_1'}\right)^2 \left(k'^2_2 + 1\right) \frac{H_1}{u} \quad (1)$$

in which:

k_1', k_2' = capacity ratio of compound one and two, respectively

α = selectivity factor of the two components = k'_2/k'_1

R_{req} = the required resolution between the compounds (expressed in standard deviations of the first peak)

H_1 = height of a theoretical plate for the first eluting compound

u = linear velocity of the mobile phase through the column.

As indicated in equation 1, the selectivity factor has a profound effect on the minimum analysis time for chromatographically very similar compounds like benzene and perdeuterobenzene. Several stationary phases were surveyed for their selectivity; Micropak SP C18 was chosen for optimization, giving good selectivity and high-efficiency columns. It is available in 3 um and 5 um particle size. Figure 1 shows the dependence of H/u on the pressure-drop per theoretical plate for Micropak SP C18 in the 3 um particle size with two mobile phases of difference viscosity. It is clear that the minimum value of H/u, corresponding to the fastest separation, can be reached for the highest available pressure-drop per number of theoretical plates required. Except at very high (>10^5) numbers of theoretical plates, the 3 um material gives faster separations than the 5 um material.

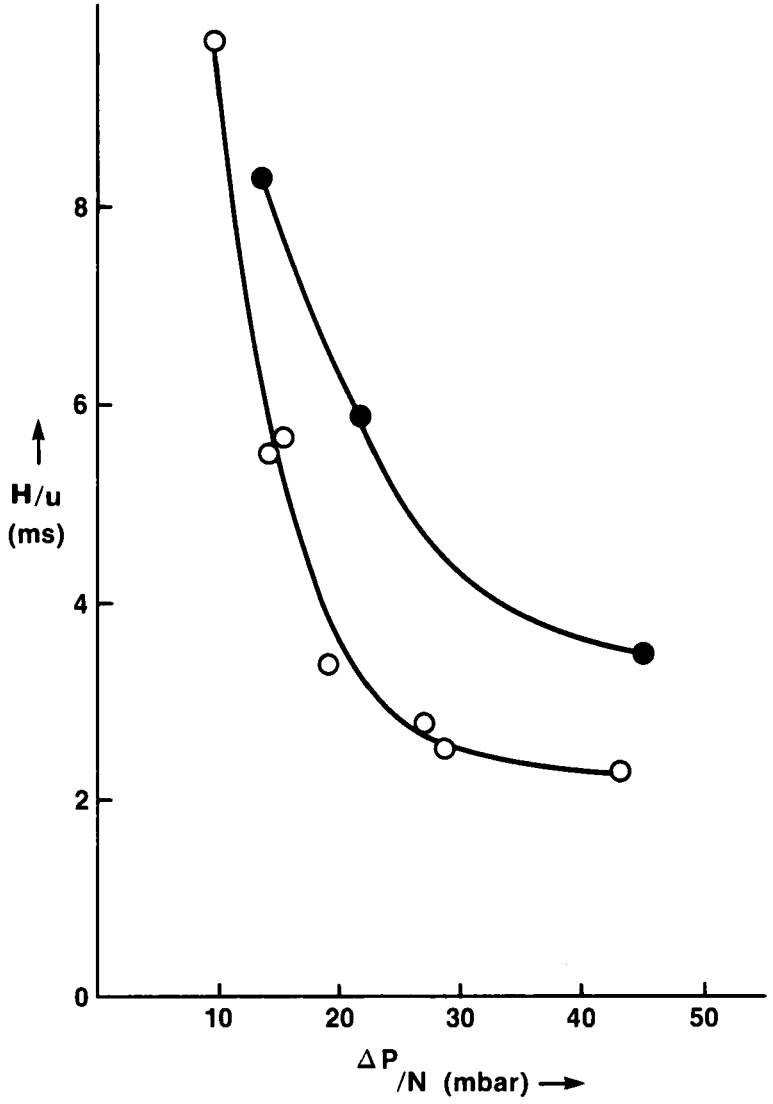

Figure 1 The dependence of H/u on P/N for Micropak SP C18-3. Closed symbols: 50% methanol, 50% water; open symbols: 35% acetonitrile, 65% water.

The number of theoretical plates required, N_{req}, is related to the other parameters by:

$$R_{req} = (\alpha - 1) \frac{k_1'}{k_1' + 1} \sqrt{N_{req}}$$

So, first the other parameters have to be set (R_{req}), or determined (α, k_1'). Excellent quantitation of compounds is possible for R_{req} = 4 and virtually complete "baseline" separation occurs at R_{req} = 6.

Since the effect of different organic modifiers was found to be generally minor, only the dependence of the selectivity on the capacity factor for methanol and acetonitrile as organic mobile phase modifiers is given in Figure 2 (Curve A and B). The minimum separation times for R_{req} = 6, calculated from equations 1 and 2 and Figure 1 and 2, are shown in Figure 3 as a function of mobile phase composition. The chromatogram of Figure 4 confirms that the perdeuterobenzene/benzene separation is not difficult at all after an optimization is applied. Thus, there is no incentive to use D_2O rather than H_2O for this separation.

B. Optimization of Benzene/Monodeuterobenzene Separation

A much lengthier separation time can be anticipated if the objective is the separation of benzene from monodeuterobenzene. An experimental 5 um reverse phase packing, based on a different silica, showed increased selectivity for this isotopic separation relative to Micropak SP C18-3 (c.f. Figure 2, experimental packing: curve C and D, vs Micropak: curve A and B). A 2250 x .3 mm fused silica column was packed with this material. The column appeared

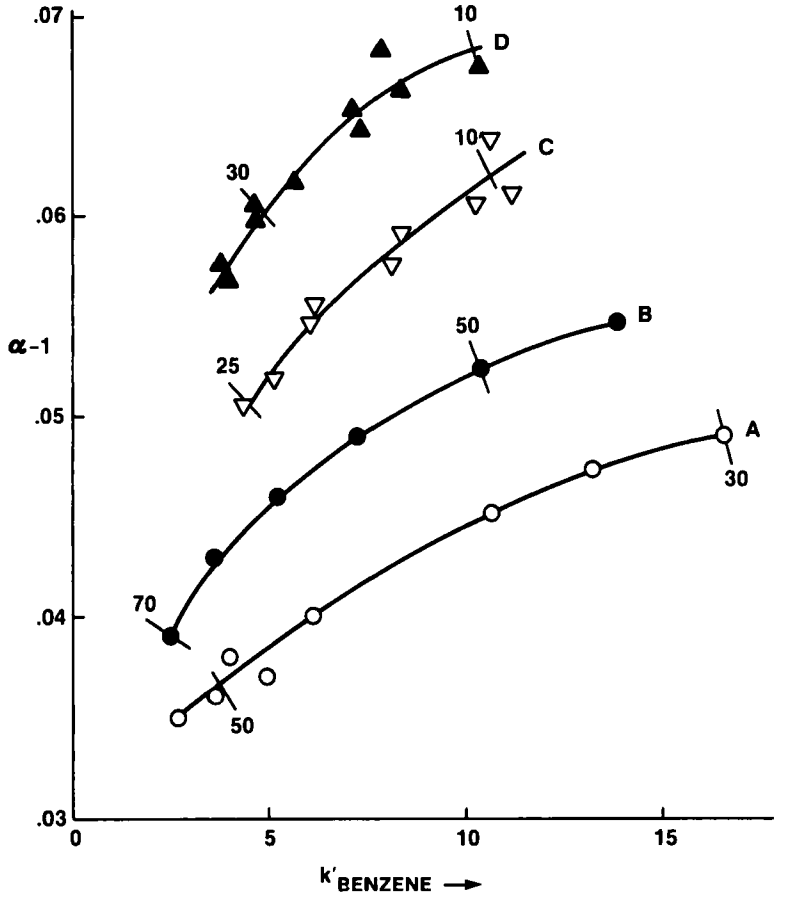

Figure 2 Dependence of (-1) on the capacity factor of benzene for it separation from perdeuterobenzene.
Curve A and B: Micropak SP C18-3; Curve C and D: experimental packing. Closed symbols: methanol modifier; open symbols: acetonitrile modifier; percentages of modifiers are indicated.

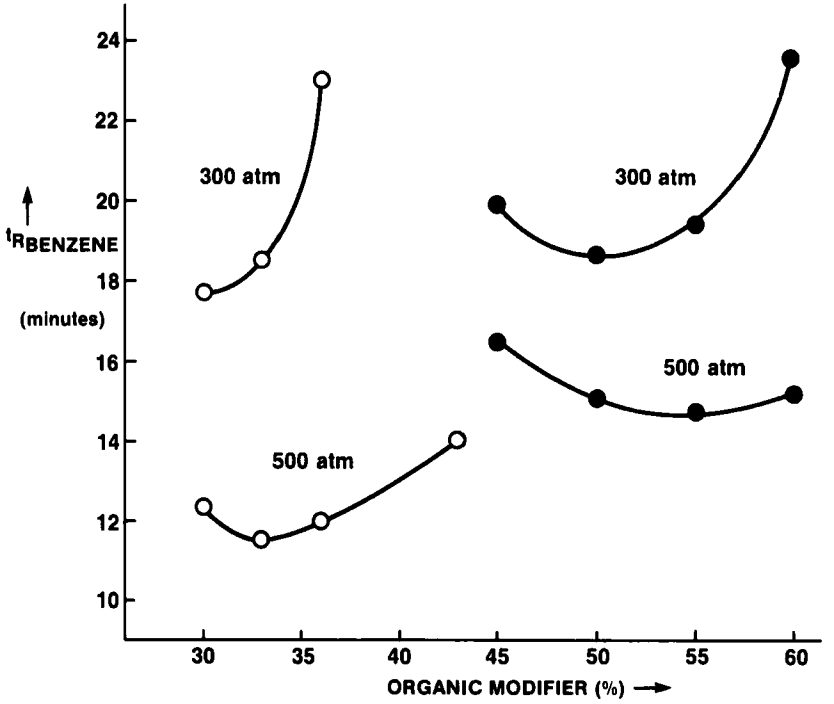

Figure 3 Minimum separation time of benzene and perdeuterobenzene as a function of modifier concentration in the mobile phase. Closed symbols: methanol modifier; open symbols: acetonitrile modifier.

not to be as efficient per unit length as the standard 150 x 4.6 mm Micropak SP C18 columns but its convenience of use (one, flexible column), reduced dilution of chromatographic peaks and superior selectivity (due to the different packing) might render it the column of choice over coupled standard columns for difficult isotopic separations. Figure 5 shows a representative result. Resolution was maximized at the expense of separation time. Complete separation will require larger particles or higher

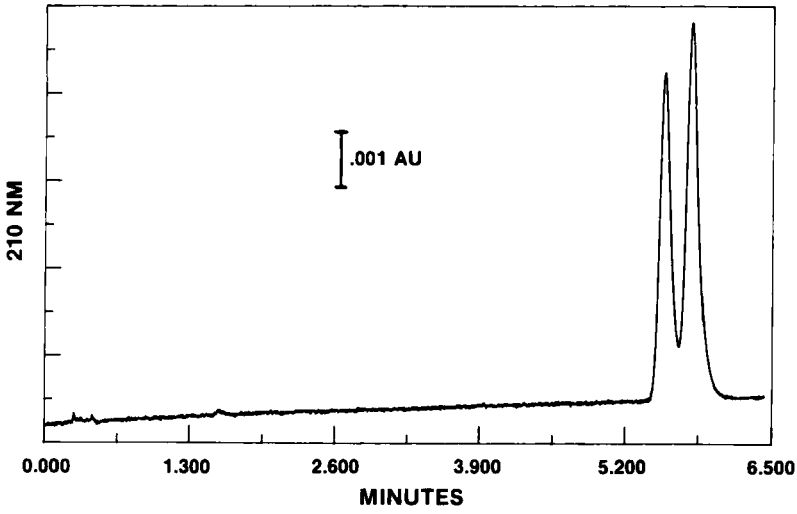

Figure 4 Separation of perdeuterobenzene and benzene.
Column: 150 x 4.6 mm Micropak SP C18-3
Mobile phase: 35% acetonitrile, 65% water
Pressure: 500 bar.

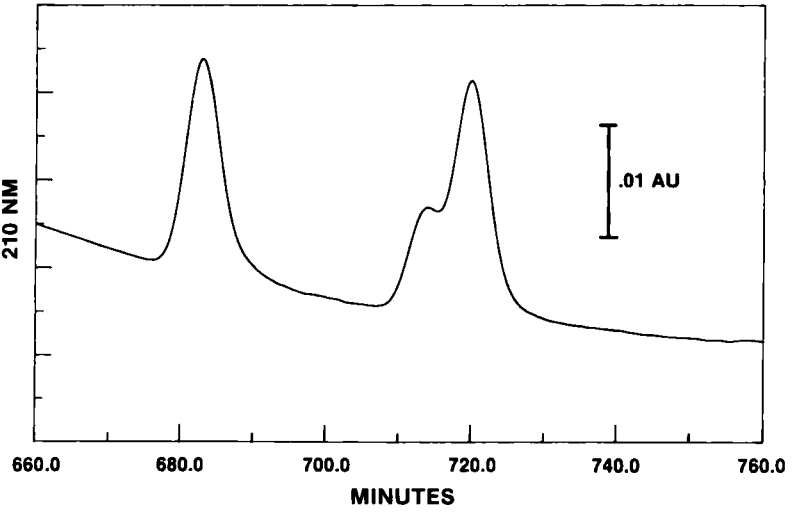

Figure 5 Separation of perdeuterobenzene, monodeuterobenzene and benzene.
Column: 2250 x .32 mm fused silica column with experimental packing.
Mobile phase: 15% acetonitrile, 85% water
Pressure: 400 bar.

pressure, a longer column and extremely long separation times. Figure 1 clarifies this: due to the maximum pressure allowed by the HPLC equipment and the large required number of theoretical plates, there is only a few millibar pressure available per required theoretical plate. In this region larger particles become preferable[14] but separations are extremely slow.

A means of reducing analysis time by removing the maximum operating pressure of the HPLC equipment as a severely limiting factor is to use two-column recycle chromatography.[6,10,15,16,17] A compromise has to be struck between higher speed operation with smaller capacity factors and shorter columns or higher peak capacity operation with larger retention and longer columns. The latter has the advantage of using fewer cycles and is less demanding on the accuracy of the timing of column switching (timing errors for column recycle are cumulative) on the performance of the columns (tailing) and on the equipment (extra-column bandbroadening). It was claimed that for high-performance recycle chromatography microbore columns are essential since 40% of the efficiency was lost during each coupling step on larger bore columns[10]. The multiplicative tailing effect[17] would not have shown up in only five cycles on long columns, however. It was therefore decided to take the more demanding high-speed approach using two standard 150 x 4.6 Micropak SP C18-3 columns and an UV-200 detector with an .5 ul microbore flowcell as an in-line detector (see Figure 6). The in-line detector appeared to be leak-free up to 400 atm. The extra-column bandbroadening of several

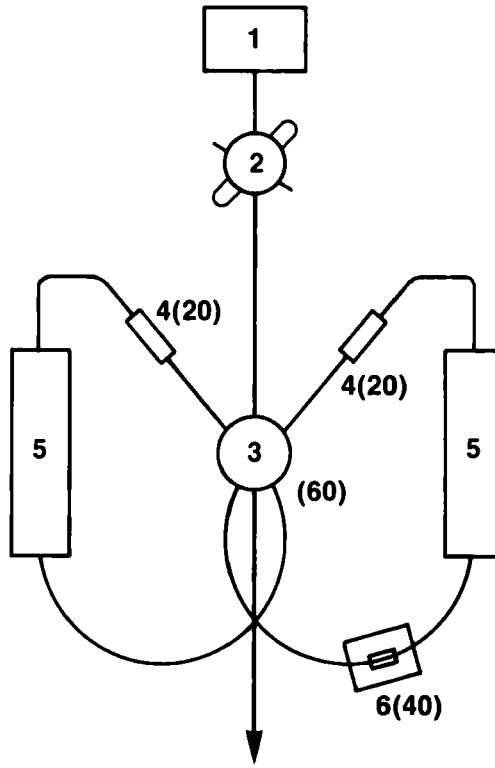

Figure 6 Schematic of high-speed column recycle equipment. Extra-column variance was measured analoguous to Ref. 18. The resulting variances are indicated in parentheses. 1 = HPLC pump, 2 = injection valve, 3 = switching valve, 4 = in-line filter, 5 = column, 6 = in-line detector.

components of the recycle system at 1.0 ml/min is also given in Figure 6. The in-line detector was used to guide the switching. A result obtained with this equipment after 19 cycles is shown in Figure 7: a distinct improvement over the separation on a long microbore column in separation time as well as resolution is observed.

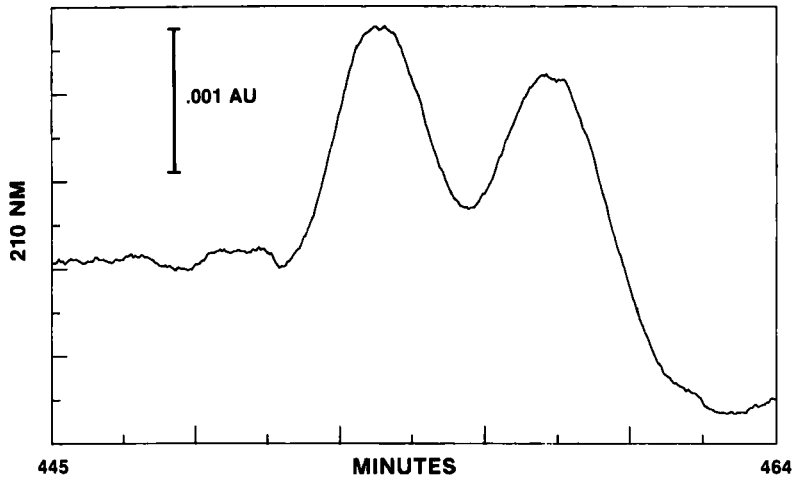

Figure 7 Recycle chromatogram #19 of monodeuterobenzene and benzene. System as in Figure 6. Mobile phase: 40% acetonitrile, 60% water. Flowrate: .85 ul/min, Pressure: 370 bar.

A definite conclusion on the trade-offs between microbore and standard column recycling could not be reached; further work elucidating the less-than-theoretical efficiency[16,17] requires novel hardware and is in progress in our laboratory.

C. The Use of D_2O

Jinno[8] observed a larger than threefold increase in selectivity when replacing H_2O with D_2O in the separation of benzene and perdeuterobenzene by reverse phase HPLC with a 30 - 40% methanol containing mobile phase. Unfortunately reproduction of these results in our laboratory was not successful.

D_2O is a slightly more polar solvent than H_2O and C_6D_6 a more polar analyte than C_6H_6[7]. The mechanism of isotopic separations

by reverse phase HPLC is thought to involve stronger Van der Waals' interaction of the stationary phase with C-H bonds than with C-D bonds[11], and Figure 3 shows a regular peakshape and efficiency for C_6D_6, giving no indication of a secondary equilibrium phenomenon.

D_2O is 23% more viscous than H_2O so longer separation times or higher pressures result. Moreover, the expense of D_2O will induce the use of microbore chromatography where the detrimental effect of extra-column bandbroadening is likely to wipe out any slight selectivity gain obtained by the use of D_2O.

It thus can be concluded that using D_2O in place of H_2O as a mobile phase constituent will not significantly improve the separation of isotopically different aromatic molecules.

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