Figure 1. pH dependence of k' values of $1\text{-}^{16}O_2$ normalized by using the k' value of benzyl alcohol in each mobile phase, and the pH dependence of ^{18}O isotope effect on retention, $^{16}k'/^{18}k'$. The curves were drawn by using eq 2 by applying $k'_{AH} = 2.646$, $k'_{A^-} = 0.128$, $^{16}K_a = 3.631 \times 10^{-5}$, and $^{18}K_a = 3.561 \times 10^{-5}$, which were estimated from the results between pH 2.4 and 6.3.

Equation 2 indicates that the k' of a carboxylic acid is determined by the extent of ionization of the acid. The dissociation constant of benzoic acid containing ^{18}O is expected to be smaller than that of $1\text{-}^{16}O_2$.[5] This will result in the lesser extent of ionization of $1\text{-}^{18}O_2$ and $1\text{-}^{16}O\text{-}^{18}O$ compared to $1\text{-}^{16}O_2$ at a pH near the pK_a. Therefore, the retention of benzoic acids containing ^{18}O is expected to be greater than that of $1\text{-}^{16}O_2$ in such a pH region. This assumes that the secondary oxygen isotope effects on k'_{AH} and k'_{A^-} are small compared to the equilibrium oxygen isotope effects on acid dissociation constants K_a, which influence the retention of the acid according to eq 2.

The present chromatographic system consisted of an octadecylsilylated silica stationary phase[6] and a methanol–water mobile phase (20/80 v/v) containing acetic acid–sodium acetate buffer.[7]

$1\text{-}^{16}O_2$ and $1\text{-}^{18}O_2$ showed very similar k' values below pH 3 and above pH 6 (where the acids dissociate very little or dissociate almost completely). In the pH region near the pK_a, however, differences of up to 1.2% in k' values were found between $1\text{-}^{16}O_2$ and $1\text{-}^{18}O_2$. $1\text{-}^{18}O_2$ was actually retained longer than $1\text{-}^{16}O_2$. The maximum separation was achieved near pH 5, as shown in Figure 1.

From eq 2, where k'_{AH} and k'_{A^-} were found to be similar for the two isotopic acids, eq 3 can be derived, where $^{16}k'$ and $^{18}k'$

$$^{16}K_a/^{18}K_a = \frac{k'_{AH} - {}^{16}k'}{{}^{16}k' - k'_{A^-}} \bigg/ \frac{k'_{AH} - {}^{18}k'}{{}^{18}k' - k'_{A^-}} \qquad (3)$$

stand for the capacity factors of $1\text{-}^{16}O_2$ and $1\text{-}^{18}O_2$, respectively. This equation yields the isotope effects, $^{16}K_a/^{18}K_a$, based on the k' values in RPLC, k'_{AH} at low pH, k'_{A^-} at high pH, and $^{16}k'$ and $^{18}k'$ at intermediate pH.

$^{16}K_a/^{18}K_a$ was found to be 1.020 ± 0.002 on the basis of the results between pH 4 and 6 in 20% methanol.[8] The oxygen isotope effect on the dissociation of formic acid in water was reported
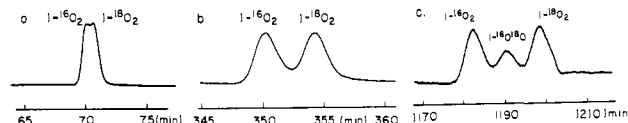

Figure 2. Separation of isotopic benzoic acids in recycle chromatography. Column: Cosmosil 5-C_{18}-P, 4.6-mm i.d., 15 cm × 4. Mobile phase: 20% methanol, 0.05 M acetic acid–sodium acetate buffer, pH 4.83. Flow rate: 0.8 mL/min. Temperature: 30 °C. (a) 1 cycle, (b) 5 cycles, (c) 17 cycles. Mass of samples: 0.5 μg each of $1\text{-}^{16}O_2$ and $1\text{-}^{18}O_2$ in (a) and (b); 0.25 μg of $1\text{-}^{16}O\text{-}^{18}O$ and 0.5 μg each of $1\text{-}^{16}O_2$ and $1\text{-}^{18}O_2$ in (c). A part of the tail of the $1\text{-}^{18}O_2$ peak was shaved off during the recycle operation in (c).

to be 1.022. Taking into account the presence of an organic solvent, the present results with benzoic acid agree well with the results for formic acid.[5a] Thornton also estimated the oxygen isotope effect on the acidity of H_3O^+ to be about 2%.[5b]

Column switching and solute recycling were utilized to resolve the three isotopic benzoic acids at pH 4.8. Two pairs of two 15-cm columns (60-cm total) were connected through a six-port valve.[1c,9] The chromatograms after 1, 5, and 17 recycles, giving the effective column lengths of 0.6, 3.0, and 10.2 m, respectively, are shown in Figure 2. The chromatogram in Figure 2c shows the resolution of the three isotopic benzoic acids with about 340 000 theoretical plates. Figure 2a shows that partial separation can be achieved without any solute recycling.

The results suggest the possibility of the enrichment of minor isotopes in various dissociable compounds from the natural abundance to near 100% by the ionization control technique in RPLC.

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Separation of Nitrogen Isotopic Compounds by Reversed-Phase Liquid Chromatography on the Basis of Nitrogen Isotope Effects on the Dissociation of Amines

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Isotope separation has been one of the most interesting subjects in separation science, and the importance of isotopic separation is increasing due to the wide use of isotopes in energy and life science oriented applications. We have reported the measurement of oxygen isotope effects on the dissociation constant of benzoic acid and the separation of the three isotopic benzoic acids, $C_6H_5C^{16}O^{16}OH$, $C_6H_5C^{16}O^{18}OH$, and $C_6H_5C^{18}O^{18}OH$ by ionization control in reversed-phase liquid chromatography (RPLC).[1] We wish to report here the first example of the separation of

(1) Tanaka, N.; Araki, M., J. Am. Chem. Soc., preceding paper in this issue.
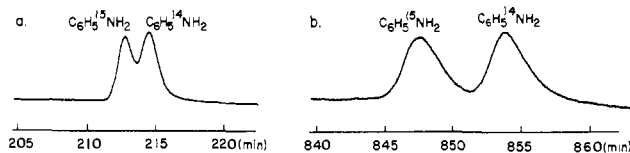
Figure 1. Separation of isotopic anilines in recycle chromatography. Column: Cosmosil 5-C_{18}-P, 4.6-mm i.d., 15 cm × 4, (a) 5 cycles, (b) 20 cycles. Mobile phase: 5% methanol, 0.05 M acetate, 0.01% triethylamine, pH 4.24. Flow rate: 1 mL/min. Temperature: 30 °C. Amount of sample: 1.4 μg each of 1-^{14}N and 1-^{15}N.

nitrogen isotopic compounds by RPLC and the measurement of the nitrogen isotope effect on the dissociation of aniline. Aniline-^{15}N (1-^{15}N) was completely separated from aniline-^{14}N (1-^{14}N) on the basis of the small difference in the dissociation constants of these isotopic anilines. The nitrogen isotope effect on the dissociation of **1**, $^{14}K_a/^{15}K_a$, was found to be 1.019 in 5% methanol.

Ionization control in RPLC[2] has enabled the measurement of oxygen isotope effects and the separation of oxygen isotopes based on the difference in the dissociation constants of carboxylic acids.[1] A similar difference in the dissociation constants due to the difference in mass of the nitrogen atom is expected with amines. Presumably amines containing ^{15}N are expected to be stronger bases and protonated more easily than amines containing ^{14}N. The measurement of the nitrogen isotope effect and the separation of nitrogen isotopic compounds were attempted with 1-^{14}N and 1-$^{15}N^3$ by RPLC.

The HPLC instrumentation employed in this study was similar to that described earlier.[1] Two pairs of two 15-cm columns (60-cm total) packed with octadecylsilylated silica gel[4] were used with a six-port valve in the solute recycle mode.[5] Methanol–water mixtures (5/95 v/v) containing 0.02 M phosphate at pH 2.37 and 7.24 and 0.05 M acetate buffer at pH 4.24 were used as mobile phases.[6] In order to eliminate slight peak tailing associated with basic compounds on the silica-based stationary phase, 0.01% triethylamine was added to the mobile phase.

The retention of amines in RPLC can be described by eq 2,[2]

$$RNH_3^+ + H_2O \rightleftharpoons RNH_2 + H_3O^+$$
$$K_a = [RNH_2][H_3O^+]/[RNH_3^+] \quad (1)$$
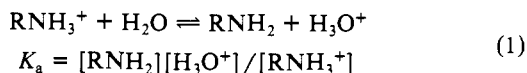
$$k' = \frac{k'_{BH^+}[RNH_3^+]}{[RNH_3^+] + [RNH_2]} + \frac{k'_B[RNH_2]}{[RNH_3^+] + [RNH_2]} = \frac{k'_{BH^+}}{1 + K_a/[H_3O^+]} + \frac{k'_B K_a/[H_3O^+]}{1 + K_a/[H_3O^+]} \quad (2)$$

where k'_{BH^+}, k'_B, and K_a stand for the k' of the protonated amine, the k' of the uncharged amine, and the acid dissociation constant, respectively. As uncharged species are retained much longer than charged species in RPLC, 1-^{14}N is expected to be retained longer than 1-^{15}N at a pH near the pK_a where a difference in the extent of protonation exists between the two isotopic anilines due to the nitrogen isotope effect.

No difference in retention was seen between 1-^{14}N and 1-^{15}N at pH 2.37 and 7.24, indicating that the nitrogen isotope effects on k'_{BH^+} and k'_B are negligible. At pH 4.24, 1-^{14}N gave a k' of 5.869 compared to a k' of 5.811 with 1-^{15}N ($^{14}k'/^{15}k'$ = 1.010). The nitrogen isotope effect on the dissociation of anilinium ion,

$^{14}K_a/^{15}K_a$, in 5% methanol was estimated to be 1.019 according to eq 3 derived from eq 2,[1,7] where $^{14}k'$ and $^{15}k'$ are the k' values

$$^{14}K_a/^{15}K_a = \frac{k'_{BH^+} - {}^{14}k'}{{}^{14}k' - k'_B} \Big/ \frac{k'_{BH^+} - {}^{15}k'}{{}^{15}k' - k'_B} \quad (3)$$

of 1-^{14}N and 1-^{15}N, respectively. The nitrogen isotope effect on the dissociation of anilinium ion is comparable with the case of the oxygen isotope effect which gave $^{16}K_a/^{18}K_a$ = 1.020 between benzoic acid-$^{16}O_2$ and benzoic acid-$^{18}O_2$. The results may include the effect of the difference in the amount of methanol in solution, 5% in the present case compared to 20% in the case of the oxygen isotope effect. (The isotope effect on the dissociation of formic acid was shown to decrease with the increase in acetone content.[8]) Further study is needed to obtain these isotope effects without added organic solvents in the chromatographic mobile phase.[9]

Figure 1 shows the chromatograms of the isotopic mixture of aniline after 5 and 20 recycles on the 60-cm column (effective column lengths of 3 and 12 m). Base-line separation was achieved in the latter case, suggesting the possibility of facile enrichment of ^{15}N isotope from the natural abundance of 0.37%. In this case the elution order is favorable for the recovery of ^{15}N-enriched material, because the tail of the major peak of ^{14}N compound does not decrease the isotopic purity of the enriched minor isotope, as might be the case with the oxygen isotope separation where the minor isotope eluted later.[1]

Although the separation factor obtainable by the present method based on the ionization control may be smaller than that in the conventional ion exchange process for the ammonium ion,[10] the wide availability of high-performance columns for RPLC can make this approach more attractive for the preparation of ^{15}N-enriched compounds in laboratory. Together with the results with the oxygen isotope separation described earlier,[1] the ionization control seems to be applicable for the isotopic separation of most weak acids and bases which undergo partial dissociation in aqueous media. Although most isotopic separations for oxygen and nitrogen have been carried out with simple compounds such as water, CO_2, NH_3, and urea,[11] one can get isotopic enrichment for the compounds of interest by the present approach, instead of synthesizing them from enriched reagents. Although the use of silica-based packing materials will limit the applicability of ionization control to compounds with a pK_a below 8, an organic polymer-based stationary phase will allow similar separations over a wider pH range. Such studies are currently in progress along with the measurements of heavy atom isotope effects in acid–base dissociation equilibria.

(2) Karger, B. L.; LePage, J. N.; Tanaka, N. "High Performance Liquid Chromatography"; Horváth, C., Ed.; Academic Press: New York, 1980; pp 113–206.

(3) 1-^{14}N stands for ordinary aniline without any isotopic enrichment, unless its separation from 1-^{15}N is specifically indicated. 1-^{15}N of 99% enrichment was purchased.

(4) Cosmosil 5-C_{18}-P (Nakarai Chem.) was used.

(5) Henry, R. A.; Byrne, S. H.; Hudson, D. R. *J. Chromatogr. Sci.* **1974**, *12*, 197–199.

(6) The apparent pH of mobile phase was measured with a pH meter. The pH values were not included in the subsequent calculation of the isotope effect according to eq 3.

(7) k' values were normalized by using the retention of hydroquinone in each mobile phase as a standard in order to eliminate the effect of the slight variation in methanol content in each mobile phase. k' values at pH 2.37 and 7.24 were used as k'_{BH^+} and k'_B, respectively, in eq 2.

(8) Ellison, S. L. R.; Robinson, M. J. T. *J Chem. Soc., Chem. Commun.* **1983**, 745–746.

(9) Elution without added methanol was accompanied by either prolonged retention times or poor peak shape with currently available packing materials. Nitrogen isotope effects on the dissociation of protonated amines including phenylalanine and pyridine are in a range of 1.016–1.021 in water (Hermes, J. D.; Weiss, P. M.; Cleland, W. W. *Biochemistry* **1985**, *24*, 2959–2967) and an unpublished observation by Kurz, J. L. See also: Ando, T.; Yamataka, H.; Wada, E. *Isr. J. Chem.*, in press.

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(11) Bairamov, V. T.; Gorshkov, V. I.; Mal'tseva, V. V.; Belousova, M. E. *Zh. Fiz. Khim.* **1984**, *58*, 1794–1795; *Chem. Abstr.* **1984**, *101*, 139203r.