Journal of Chromatography, 352 (1986) 307–314
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 725

SEPARATION OF OXYGEN ISOTOPIC COMPOUNDS BY REVERSED-PHASE LIQUID CHROMATOGRAPHY

NOBUO TANAKA* and MIKIO ARAKI
Faculty of Textile Science, Kyoto Institute of Technology, Matsugasaki, Sakyo-ku, Kyoto 606 (Japan)
and
KAZUHIRO KIMATA
Nakarai Chem. Co., Kuze, Minami-ku, Kyoto 601 (Japan)

SUMMARY

The separation of oxygen isotopic compounds by reversed-phase liquid chromatography was attempted, based on the small differences in the dissociation constants of organic acids. In the case of benzoic acid, a separation factor, $^{18}k'/^{16}k'$, of maximum 1.012, and an oxygen isotope effect on the acid dissociation constants, $^{16}K_a/^{18}K_a$, of 1.020 were obtained. Three isotopic benzoic acids, $C_6H_5C^{16}O^{16}OH$, $C_6H_5C^{16}O^{18}OH$ and $C_6H_5C^{18}O^{18}OH$, were separated by ionization control at pH 4.8 in 20% methanol, using a C_{18} stationary phase and recycle chromatography. Similar separations were achieved with *p*-chlorobenzoic acid and *p*-nitrophenol, labelled with ^{18}O at the dissociating group.

INTRODUCTION

One of the major uses of high-performance liquid chromatography (HPLC) is the separation of compounds with structural similarity. In this respect, isotope separation represents one of the extremes. The importance of isotope separation is well recognized, and it has been studied extensively.

The most frequently employed chromatographic technique for isotope separation is ion-exchange chromatography, which enabled the separation of metal isotopes for the first time[1]. The separation of deuterium compounds from protium compounds by reversed-phase liquid chromatography (RPLC) was first reported in 1976[2,3]. Since then, several reports have appeared showing the separation of hydrogen isotopic compounds by RPLC, including the separation of deuterium and tritium[4–7]. Recently the separation of monodeuterobenzene from benzene was reported[7].

We report here the first examples of the separation of oxygen isotopic compounds by RPLC. A few acidic compounds labelled with ^{18}O were isotopically separated by ionization control in RPLC. The separation is based on the facts that the ^{18}O–H bond is harder to break than the ^{16}O–H bond, due to the isotope effect in acid dissociation, and that charged species show much smaller retention than uncharged species in RPLC. In other words, the difference in the extent of ionization seen with isotopic acids caused the difference in retention in RPLC.

0021-9673/86/$03.50

The ionization-control technique seems to be applicable to the isotopic separation of a wide range of acids and bases that undergo dissociation in aqueous media. This will enable one to utilize labelled compounds with complicated structures, because one can avoid the preparation of such molecules from enriched reagents of simple structure, such as $H_2{}^{18}O$ or $C^{18}O_2$, which are currently available. The present approach also enables the measurements of heavy atom isotope effects on acid–base dissociation equilibria[8].

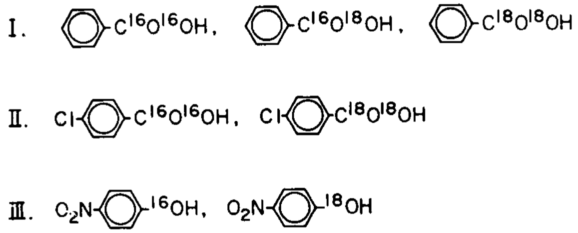
EXPERIMENTAL

Equipment

An HPLC system was assembled from an LC-3A pump (Shimadzu, Kyoto, Japan), 7125 valve injector (Rheodyne, Berkeley, CA, U.S.A.), M440 UV detector (Waters Assoc., Milford, MA, U.S.A.), 98.00 refractive index (RI) detector (Knauer, Berlin, F.R.G.) and 7000A data processor (System Instrument, Tokyo, Japan). A column recycle system consisted of two pairs of columns (each 15 cm × 4.6 mm I.D., Cosmosil 5-C_{18}-P; Nakarai Chem., Kyoto, Japan) and a 7010 six-port valve (Rheodyne). The valve operation was automated by using a Model 79 timer and air-drive unit (both from Gasukuro Kogyo, Tokyo, Japan).

Materials

Compounds labelled with ^{18}O shown in Scheme 1 were prepared from $H_2{}^{18}O$ of 97% enrichment (Centre d'Etudes Nucléaires de Saclay, France) by standard procedures. [$^{16}O_2$]Benzoic acid represents ordinary benzoic acid without any isotopic enrichment. All other chemicals were of the highest grade available, and used without further purification.



Scheme 1.

The mobile phase was made up by volume from LC-grade methanol, acetonitrile and distilled deionized water. This pH was maintained by a 0.05 *M* acetate buffer; 0.01 *M* phosphoric acid was added to 0.05 *M* acetic acid to obtain a mobile phase of pH 2.4. A pH meter (Horiba, Kyoto, Japan) was used to measure the pH of the mobile phase.

Chromatography

The column temperature was maintained at 30°C by a thermostatted water-bath. Chromatographic experiments were carried out in triplicate with a reproducibility better than ±0.2%. The k' values were normalized by using the k' of benzyl alcohol as standard in each mobile phase. The k' values of 2-phenylethanol showed a ± 1.5% variation without normalization, but its retention relative to benzyl alcohol showed a variation of less than ±0.25%, as shown in Fig. 1.

RESULTS AND DISCUSSION

The chromatographic method most frequently employed in the past for the separation of isotopes was ion-exchange chromatography, which utilizes the difference in the extent of coulombic interaction between isotopic ions. This mode of chromatography first enabled isotope separation and has extensively been used in the separation of metal isotopes as well as other ions, such as the ammonium ion[9–13].

The separation of hydrogen isotopic compounds by RPLC is based on the difference in the hydrophobic hydration between C–H and C–D of organic molecules in aqueous solvents[2,14]. This method is applicable to the separation of a variety of compounds, including monodeuterated substances[7].

The present approach of isotope separation by ionization control is based on the difference in the extent of ionization of dissociable organic compounds containing isotopes in their dissociating group.

Solute retention in RPLC is primarily determined by the hydrophobic properties of the solutes[14–16]. In the case of a carboxylic acid, a free acid (RCOOH) shows a much longer retention than the corresponding carboxylate ion ($RCOO^-$). The retention of a carboxylic acid in RPLC can be described by

$$RCOOH + H_2O \rightleftharpoons RCOO^- + H_3O^+$$

$$K_a = \frac{[RCOO^-][H_3O^+]}{[RCOOH]} \tag{1}$$

$$\begin{aligned} k' &= \frac{k'_{AH}[RCOOH]}{[RCOOH] + [RCOO^-]} + \frac{k'_{A^-}[RCOO^-]}{[RCOOH] + [RCOO^-]} \\ &= \frac{k'_{AH}}{1 + K_a/[H_3O^+]} + \frac{k'_{A^-}\ K_a/[H_3O^+]}{1 + K_a/[H_3O^+]} \end{aligned} \tag{2}$$

where k'_{AH} represents the capacity factor of the acid in its neutral form, k'_{A^-} that for carboxylate ion form and K_a is the acid dissociation constant[17]. Eqn. 2 indicates that k' for a carboxylic acid at a certain pH is determined by how much of the acid exists in the neutral form and how much in the carboxylate ion form. In other words, the extent of ionization controls the retention.

In the case of oxygen isotopic compounds, a carboxylic acid, labelled with ^{18}O, is expected to be a weaker acid than a carboxylic acid without labelling, due to the isotope effect on the dissociation equilibrium[18,19]. The ^{18}O–H bond is harder to break than the ^{16}O–H bond. Thus, more of the ^{18}O-labelled carboxylic acid exists in its neutral form compared to the carboxylic acid without labelling. Therefore, a greater retention is expected for the labelled carboxylic acid compared to the unlabelled compound, assuming that the secondary oxygen isotope effects on k'_{AH} and k'_{A^-} are small compared to the equilibrium oxygen isotope effects on the acid dissociation constants, K_a, which influences the retention of the acid according to eqn. 2.

In order to test the possibility of the separation of isotopic compounds by ionization control, the k' values of [$^{16}O_2$]benzoic acid (I-$^{16}O_2$) and [$^{18}O_2$]benzoic acid (I-$^{18}O_2$) were measured with two coupled 15-cm columns and 20% methanol, containing 0.05 *M* acetate buffer. The peak shape was poorer with phosphate buffer solutions. The results are shown in Table I. The k' values are shown as the values relative to the k' of benzyl alcohol at each pH in order to avoid the effect of the variation of k' due to the small variation in the methanol content of the mobile phase.

TABLE I

OXYGEN ISOTOPE EFFECTS ON RETENTION OF BENZOIC ACID

pH	$^{16}k'$*	$^{18}k'$*	$^{18}k'/^{16}k'$
	(2.646)**		
2.44	2.620	2.620	1.000
3.13	2.558	2.558	1.000
4.07	1.893	1.902	1.005
4.40	1.323	1.334	1.009
4.90	0.782	0.791	1.012
5.29	0.439	0.445	1.012
5.86	0.212	0.214	1.008
6.27	0.167	0.168	1.005
	(0.128)**		

* k' values relative to benzyl alcohol (k' = 10.08) are shown. Stationary phase: Cosmosil C_{18}-P × 2, 15 cm × 4.6 mm I.D. Mobile phase: methanol–0.05 *M* acetate (20:80). Temperature: 30°C.

** k'_{AH} and k'_{A^-} values estimated from the experimental results.

I-$^{16}O_2$ and I-$^{18}O_2$ showed very similar retention at low pH and at high pH where the acids dissociate very little or almost completely. However, in the pH region near the pK_a of these acids, very small but discernible differences of up to 1.2% in k' were found between I-$^{16}O_2$ and I-$^{18}O_2$. As expected, I-$^{18}O_2$ was retained longer than I-$^{16}O_2$.

In the case of I-$^{16}O_2$, the best fit of eqn. 2 to the experimental results was seen when a k'_{AH} of 2.646, k'_{A^-} of 0.128 and pK_a of 4.44 were assumed in eqn. 2, as shown in Fig. 1. These values were estimated from the results in Table I.

From eqn. 2, where the same k'_{AH} and k'_{A^-} can be used for both I-$^{16}O_2$ and I-$^{18}O_2$, eqn. 3 can be derived:

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

Eqn. 3 does not contain any pH-related term which might lead to ambiguity for the dissociation in mixed solvents. The oxygen isotope effects on acid dissociation can be directly obtained from the HPLC measurement, k'_{AH} at low pH, k'_{A^-} at high pH and k' of the two isotopic benzoic acids at intermediate pH values.

From the results obtained between pH 4 and 6, the oxygen isotope effect on the dissociation of benzoic acid, $^{16}K_a/^{18}K_a$, was calculated to be 1.020 ± 0.002. This corresponds to a difference in pK_a of 0.0084. The reported value for the oxygen isotope effect on the dissociation of formic acid is 1.022, or the difference in pK_a is 0.0095[18]. The agreement is excellent, taking into account the presence of organic solvent, which is known to reduce the isotope effect.

The oxygen isotope effect on the dissociation of an acid can provide the chromatographic separation factor between the two isotopic benzoic acids. As shown in Fig. 1, the agreement between the calculated curve and the experimental results is excellent for the separation factor. The separation factor, $^{18}k'/^{16}k'$, showed a maximum around pH 5, which is 0.5–0.6 pH units higher than the pK_a of these acids. The observed isotope effect of about 1.2% per atom was actually much larger than the isotope effects found in the retention of hydrogen isotopic compounds. This is due to the fact that the hydrogen isotope effect seen in RPLC was not associated with any bond cleavage.

In attempting to resolve isotopic benzoic acids at pH 4.8, recycle chromatography was utilized in order to generate a very large number of theoretical plates within the limits of our instrumental capability[20,21]. Two pairs of 15-cm columns

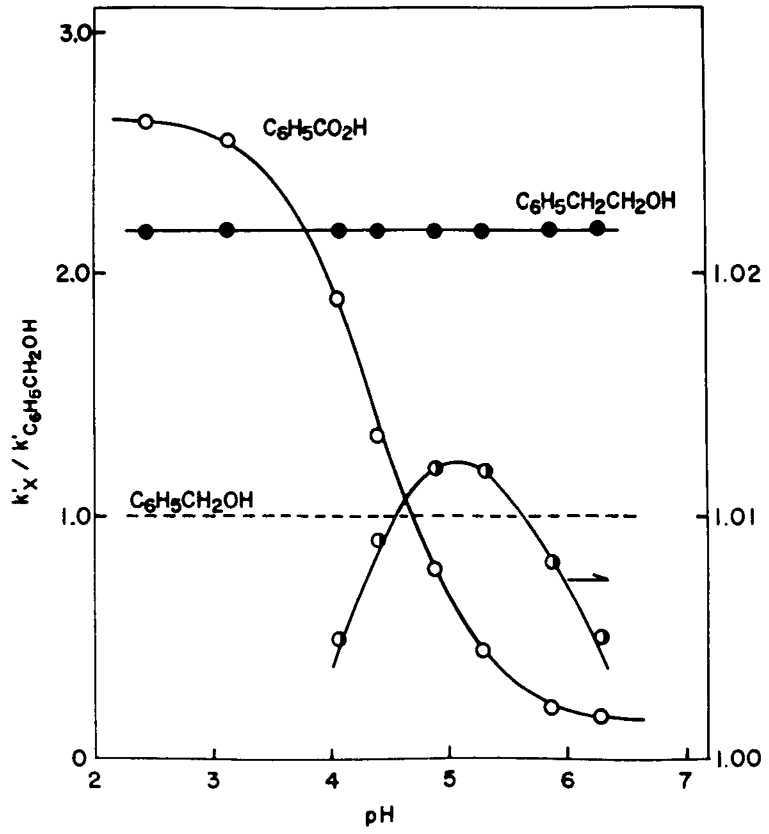


Fig. 1. The pH dependence of k' values for I-$^{16}O_2$, normalized by using the k' value of benzyl alcohol in each mobile phase, and the pH dependence of the separation factor, $^{18}k'/^{16}k'$. The curves were drawn using eqn. 2 by applying $k'_{AH} = 2.646$, $k'_{A^-} = 0.128$, $^{16}K_a = 3.631 \cdot 10^{-5}$ and $^{18}K_a = 3.561 \cdot 10^{-5}$.

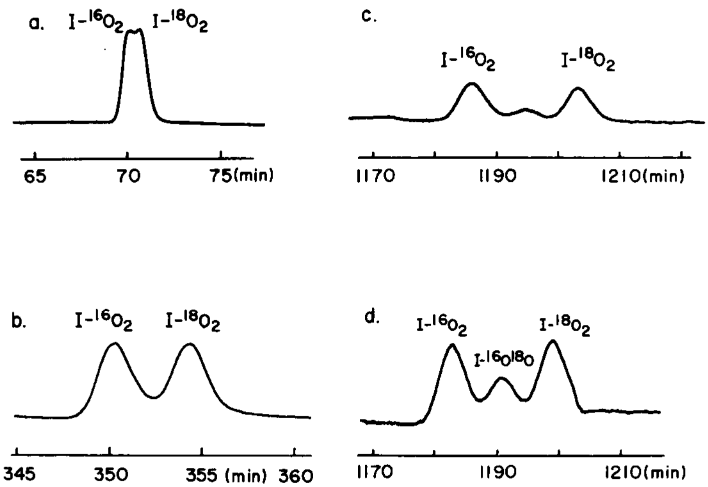


Fig. 2. Separation of isotopic benzoic acids in recycle chromatography. Column: Cosmosil 5-C_{18}-P, ×4, 15 cm × 4.6 mm I.D. Mobile phase: methanol–0.05 *M* acetate buffer (pH 4.83) (20:80). Flow-rate: 0.8 ml/min. Temperature: 30°C. Cycles: a, 1; b, 5; c, d, 17. 0.5 μg each of I-$^{16}O_2$ and I-$^{18}O_2$ (a–c); 0.25 μg of I-$^{16}O^{18}O$ and 0.5 μg each of I-$^{16}O_2$ and I-$^{18}O_2$ (d). A part of the tail of I-$^{18}O_2$ was shaved off during the recycle operation in d.

were used. The results obtained with the isotopic benzoic acids at 1, 5 and 17 cycles in the 60-cm column system are shown in Fig. 2.

I-$^{16}O_2$ and I-$^{18}O_2$ were partially separated with the 60-cm column, as shown in Fig. 2a. After five cycles, or with a column length of 3 m, baseline separation was obtained in about 6 h. As shown in Fig. 2c, after seventeen recycles or at an effective column length of 10.2 m, I-$^{16}O_2$ and I-$^{18}O_2$ were well separated from each other, and a small peak appeared between them. This peak is presumably caused by I-$^{16}O^{18}O$, produced during the preparation of I-$^{18}O_2$, because of the presence of at least 3% ^{16}O in ^{18}O-enriched water. This peak is also caused by I-$^{16}O^{18}O$, contained in the sample of I-$^{16}O_2$ at the natural abundance.

The resuls were confirmed by eluting the isotopic benzoic acids, as seen in Fig. 2d. This chromatogram suggests that the enrichment of naturally occurring I-$^{16}O^{18}O$ is possible, and that a similar separation of [$^{16}O^{17}O$]benzoic acid would be possible under similar chromatographic conditions, although it would need considerably longer columns.

Fig. 3 shows another example of the separation of oxygen isotopic compounds. *p*-Chloro[$^{16}O_2$]benzoic acid (II-$^{16}O_2$) and *p*-chloro[$^{18}O_2$]benzoic acid (II-$^{18}O_2$) were separated in 20% acetonitrile in the presence of 0.05 *M* acetate buffer at pH 4.8. Elution with 10% acetonitrile at pH 5.2 did not improve the separation, although the isotope effect is known to decrease with increasing organic solvent content[18]. In the separation of these isotopic substances the pH of the mobile phase seems to be of prime importance.

Phenols are another class of compounds that fit the present scheme of isotope separation, although the pK_a of phenolic compounds must be lowered to below 8 or so for chromatography on a silica-based stationary phase. *p*-Nitrophenol(III) has a pK_a of 7.15 in water.

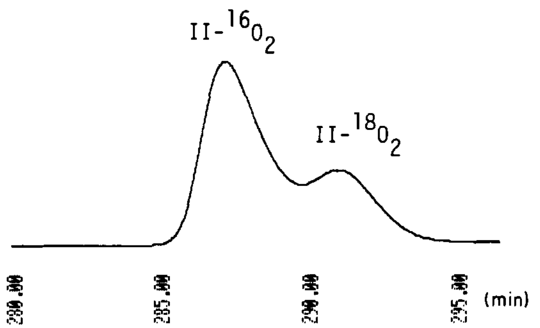


Fig. 3. Separation of isotopic *p*-chlorobenzoic acids in recycle chromatography after five cycles. Mobile phase: acetonitrile–0.05 *M* acetate buffer (pH 4.76) (20:80). Flow-rate: 1 ml/min. Samples: 5 μg or II-$^{16}O_2$ and 1.5 μg of II-$^{18}O_2$.

In the case of phenols, only one oxygen atom is involved in the dissociation process, compared to two oxygen atoms in the case of carboxylic acids. This is an advantage in the enrichment of naturally occurring ^{18}O, because the separation based on one ^{18}O atom would be much better with phenols than with carboxylic acids, assuming a similar extent of the isotope effect per atom on the dissociation. The single oxygen atom in phenols is involved in the dissociation process every time, while each oxygen atom in carboxylic acids is involved only half of the time.

The results shown in Fig. 4, however, are not as good as for the separation of isotopic benzoic acids. The separation factor between III-^{16}O and III-^{18}O was comparable to those in the separation of isotopic carboxylic acids, indicating a comparable isotope effect on dissociation. But the column performance was not as good as in the case of the carboxylic acids. While about 20 000 plates per cycle were obtained at 17 cycles for I, only about 10 000 plates per cycle were obtained for III at 10 cycles. Apparently some special band-broadening mechanism is operative; and the subject is currently under examination.

The ionization-control technique seems to be applicable to the isotopic separation of a wide range of organic acids and bases that undergo dissociation in aqueous media. RPLC systems, for which stable high-performance columns are readily available, will prove to be more easily adapted in the laboratory than other enrichment methods.

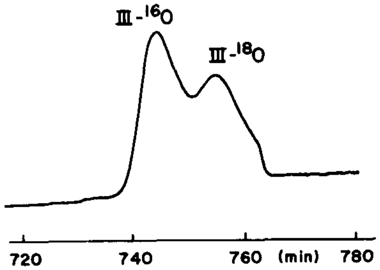


Fig. 4. Separation of isotopic *p*-nitrophenols in recycle chromatography after ten cycles. Mobile phase: methanol–0.02 *M* phosphate buffer (pH 7.7) (12:88). Flow-rate: 0.9 ml/min. Samples: 3.5 μg of III-^{16}O, 1.75 μg of II-^{18}O. A part of the peak of III-^{18}O was shaved off during the recycling operation.

Further studies on the separation of oxygen isotopic compounds including ^{17}O compounds, and isotope separation with other elements, are currently in progress in our laboratory[22].

CONCLUSION

Oxygen isotope separation was studied, utilizing the ionization-control technique in RPLC. Benzoic acid, *p*-chlorobenzoic acid and *p*-nitrophenol, labelled with ^{18}O in their dissociable groups, were isotopically separated on the basis of the slight differences in the dissociation constants caused by the oxygen isotope effects. In the case of benzoic acid, [$^{16}O_2$]benzoic acid, [$^{16}O^{18}O$]benzoic acid and [$^{18}O_2$]benzoic acid were successfully separated by recycle chromatography in about 20 h. The oxygen isotope effects of about 2% for the dissociation of benzoic acid, $^{16}K_a/^{18}K_a$, and about 1.2% for the retention of benzoic acid, $^{18}k'/^{16}k'$, were determined with 20% methanol.

REFERENCES

1 T. I. Taylor and H. C. Urey, *J. Chem. Phys.*, 6 (1938) 429.
2 N. Tanaka and E. R. Thornton, *J. Am. Chem. Soc.*, 98 (1976) 1617.
3 G. P. Cartoni and I. Ferretti, *J. Chromatogr.*, 122 (1976) 287.
4 P. Kucera and G. Manius, *J. Chromatogr.*, 216 (1981) 9.
5 J. D. Unadkat and M. Rowland, *J. Chromatogr.*, 261 (1983) 298.
6 P. K. F. Yeung, J. W. Hubbard, B. W. Baker, M. R. Looker and K. K. Midha, *J. Chromatogr.*, 303 (1984) 412.
7 S. Yamazaki and T. Tanimura, *Eighth International Symposium on Column Liquid Chromatography, New York, May, 1984, Abstract 1a-44.*
8 N. Tanaka and M. Araki, *J. Am. Chem. Soc.*, in press.
9 F. H. Spedding, J. E. Powell and H. J. Svec, *J. Am. Chem. Soc.*, 77 (1955) 6125.
10 T. Ishimori, *Bull. Chem. Soc. Jpn.*, 33 (1960) 516.
11 A. R. Gupta and S. K. Sarpal, *J. Phys. Chem.*, 71 (1967) 500.
12 M. Kotaka, T. Shono and H. Kakihana, *Chem. Lett.*, (1978) 315.
13 P. C. O. Trivelin, E. Matsui and E. Salati, *Energ. Nucl. Agric.*, 1 (1979) 1.
14 N. Tanaka and E. R. Thornton, *J. Am. Chem. Soc.*, 99 (1977) 7300.
15 Cs. Horváth, W. Melander and I. Molnár, *J. Chromatogr.*, 125 (1976) 129.
16 B. L. Karger, J. R. Gant, A. Hartkopf and P. H. Weiner, *J. Chromatogr.*,128 (1976) 65.
17 B. L. Karger, J. N. LePage and N. Tanaka, in Cs. Horváth (Editor), *High Performance Liquid Chromatography*, Academic Press, New York, 1980, pp. 113–206.
18 S. L. R. Ellison and M. J. T. Robinson, *J. Chem. Soc., Chem. Commun.*, (1983) 745.
19 E. R. Thornton, *J. Am. Chem. Soc.*, 84 (1962) 2474.
20 R. A. Henry, S. H. Byrne and D. R. Hudson, *J. Chromatogr. Sci.*, 12 (1974) 197.
21 P. Kucera and G. Manius, *J. Chromatogr.*, 219 (1981) 1.
22 N. Tanaka, A. Yamaguchi, M. Araki and K. Kimata, *J. Am. Chem. Soc.*, in press.