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ELUTING TRACE COMPONENTS BEFORE MAJOR CONSTITUENTS

I. SENSITIVITY ENHANCEMENT IN ANALYTICAL DETERMINATIONS OF OPTICAL PURITY

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SUMMARY

High optical purity can be precisely determined by high-performance liquid chromatography if several conditions can be met. Derivatization, if required, must be non-chiral; the elution order of enantiomers must be reversible, to allow elution of the trace enantiomer before the major enantiomer; and the column must under large overload retain efficiency for the trace enantiomer. Also for enhancing sensitivity to traces, we have found that a given quantity of sample is better injected in larger-than-conventional volume and at less-than-conventional concentration. Combining these approaches allowed not only the determination of 99.9967% optical purity in one demonstrative example but also an apparent extensibility to considerably higher purities. The limits to that extensibility, not obvious, were not established.

INTRODUCTION

Trace analysis in high-performance liquid chromatography (HPLC) was extensively treated in 1974 in a paper by Karger *et al.*[1] and in 1979 in the second edition of Snyder and Kirkland[2]. In neither case was the order of trace elution suggested as a major variable.

Elution order as an electable variable came to our attention in direct chiral separations (*i.e.*, of enantiomers rather than diastereomers)[3,4]. In such direct separations, elution order is easily inverted (by changing from a D to an L column, or the inverse). A trace enantiomer can then be eluted as easily before as after the major enantiomer it contaminates. Elution order can be inverted in non-chiral separations by changing the phase relationship, *i.e.*, from reverse to normal, or the inverse. However, in this case decreased analyte solubility in the changed mobile phase may prevent the more favorable elution order, the trace being eluted first, from being fully exploited. Otherwise, the enhancement of detectability achievable if the trace components can be eluted first seems general.

In this study (in which only conventional equipment was used) we show that when traces are eluted before rather than after major components, trace detectability

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not only improves by 3–4 orders of magnitude but also can be made to reach surprisingly low trace concentrations.

EXPERIMENTAL

Materials

For this study, trifluoro-1-(9-anthryl)-ethanol was used. The chromatograms of the L enantiomer and the racemate, purchased from Aldrich, are shown in Fig. 1. The highly purified enantiomer shown in Fig. 4 was purified at Regis from a racemate that had been kindly supplied by Dr. William Pirkle of the University of Illinois (for details of the purification see ref. 5). HPLC-grade solvents were used throughout.

Columns

The analytical columns used in this study were laboratory-packed and are commercially available from Regis. The analytical columns were 25 cm × 4.6 mm

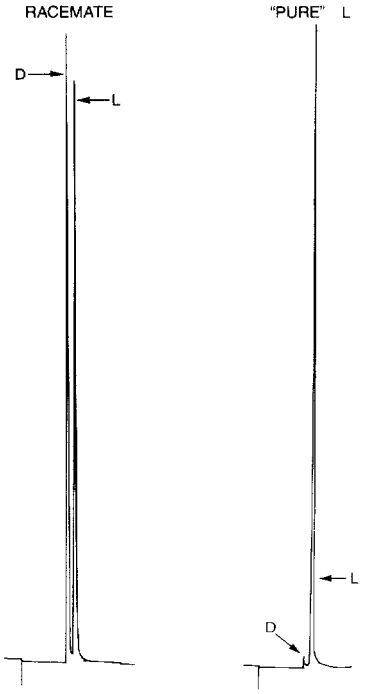

Fig. 1. By definition, a racemate contains equal proportions of each isomer (if there are only two, 50% of each). This fact allows a response factor to be calculated for each enantiomer in a given chromatogram of the racemate (here, see the left chromatogram: first peak, the D enantiomer; second peak, the L enantiomer). Used in a corresponding chromatogram of a "pure" enantiomer (here, the right chromatogram: first peak, the trace D enantiomer; second peak, the "pure" L enantiomer), that response factor allows calculation of the contamination of the major enantiomer by the trace enantiomer; the difference between 100% and this percent contamination is identically the optical purity of the major enantiomer. For details of the calculation, see Results and discussion. Stationary phase, L-phenylglycine; relative retention, 1.10; mobile phase, isopropanol–hexane (30:70, v/v); flow-rate, 0.6 ml/min; injected, 0.1 μg of L-fluoroalcohol dissolved in the mobile phase; 10 μl volume.

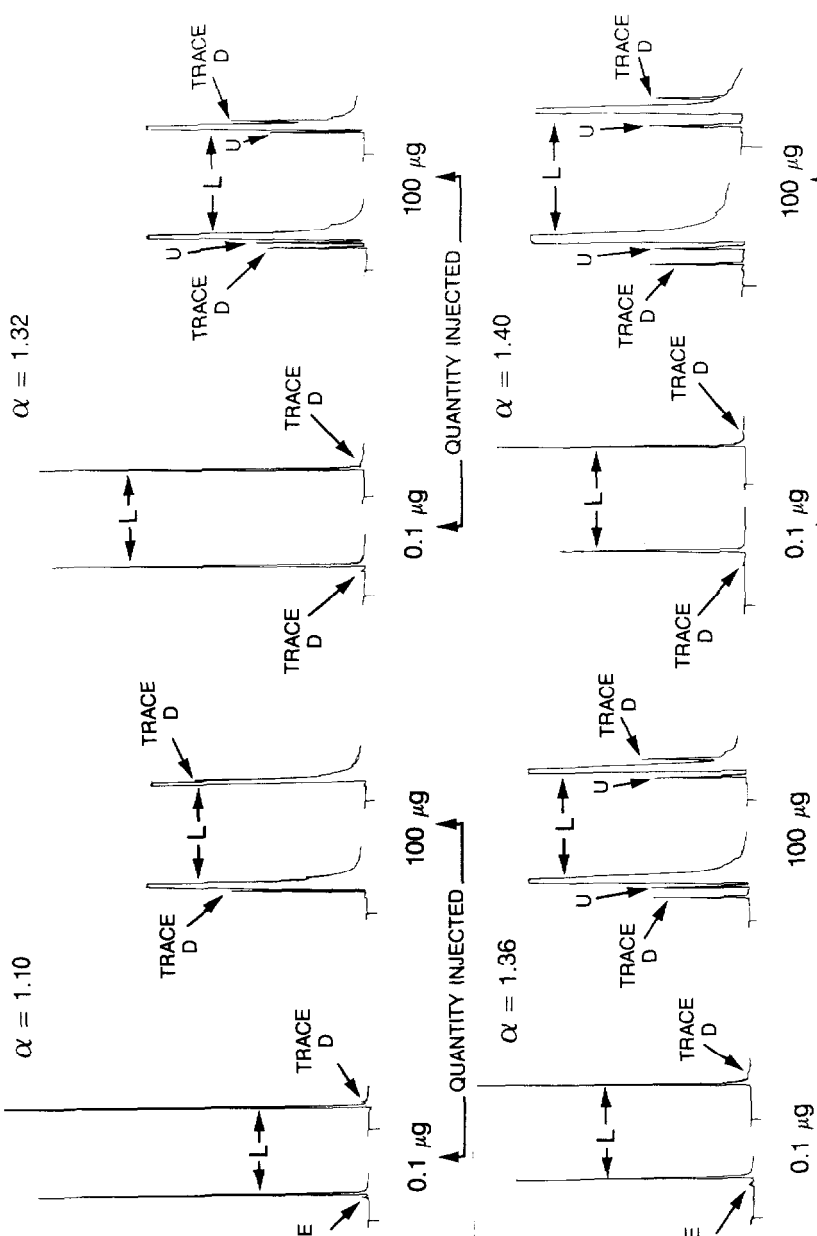

Fig. 2. In each pair of chromatograms, the first chromatogram was made with the L-phenylglycine stationary phase; the second, with the D-phenylglycine. Eluting the contaminating trace enantiomer first usually allows much larger quantities to be injected without causing the trace peak to overlap the major peak. In consequence, sensitivity is correspondingly enhanced. Note that the efficiency shown by each peak reflects the amount of component injected rather than the total amount of the sample; the trace peaks continue to reflect full column test efficiency even though the total amount injected may have been increased 1000 times. (The 100-μg chromatograms of relative retentions 1.32, 1.36 and 1.40 show a peak U: usually an unidentified impurity.) Column test efficiency: about 55 000 plates per meter, not corrected for dead volume; injected volume, 10 μl throughout; injection solvent, mobile phase (see Experimental, Procedures, first sentence); mobile phase flow-rate, 0.6 ml/min.

I.D., packed with spherical silica to which the chiral stationary phase was covalently bonded. The spherical silica particles were 5 μm in diameter and has pores that before bonding were 8 nm in diameter. The chiral stationary phases were the dinitrobenzoyl derivatives of D- and L-phenylglycine.

Equipment

The columns were packed with Haskel Model MCP-110 constant-pressure pumps. The HPLC analytical experiments were conducted with Rheodyne Model 7010 injection valves and Beckman Model 110A and 153 pumps and detectors, respectively.

Procedures

Enhancing sensitivity to the D *trace in the* L *enantiomer.* Four relative retentions from 1.10 to 1.40 were obtained by varying the percent isopropanol in the isopropanol–hexane mobile phase: 1.10, 30%; 1.32, 20%; 1.36, 15%; and 1.40, 10%.

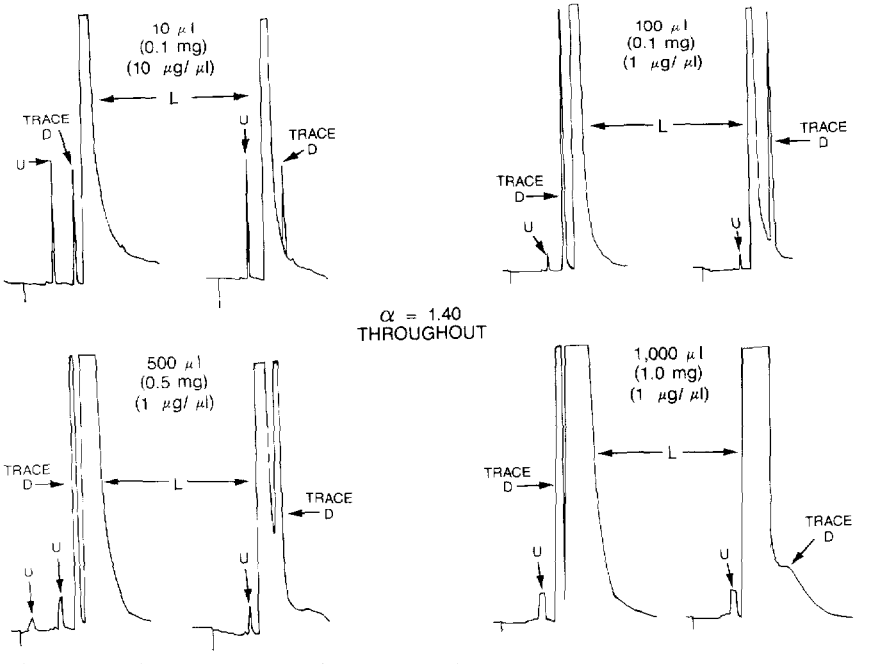

Fig. 3. In each chromatogram, the sample is the purifed fluoroalcohol L-enantiomer. In each pair of chromatograms, the first chromatogram was made with the L-phenylglycine stationary phase; the second, with D-phenylglycine. In the top row, the weight of solute injected was held constant. However, for the right chromatogram, the solute concentration was decreased 10-fold while the volume injected was increased 10-fold. In consequence, as can be seen, the height of the trace D peak more than doubled. In the top right and both lower pairs of chromatograms, the concentration was held constant but the injection volume was increased from 100 to 1000 µl. As can be seen, if the trace was eluted first the trace peak height and area continued to increase. Clearly, injecting these larger volumes at these lower concentrations dramatically increased the detectability of the trace. Injection solvent, mobile phase; mobile phase, isopropanol–hexane (10:90); flow-rate, 0.6 ml/min; "U" peaks, usually, unidentified impurities.

By applying the following regime first to the D-phenylglycine column and then to the L-phenylglycine column, 24 chromatograms were produced:

At each of the four relative retentions, three 10-μl solutions of the fluoroalcohol in the mobile phase were injected. Two of these injections contained 0.1 μg of fluoroalcohol: one, the racemate; the other, the L enantiomer. The chromatograms from these two injections are shown in Fig. 1; they were both recorded at the same sensitivity, which was set to keep all peaks on scale. The third injection contained 1000 times as much of the L-fluoroalcohol: 100 μg. The sensitivity for this chromatogram was set to hold the trace peak on scale.

The pairs of chromatograms in Fig. 2 show the effect on trace peak detectability of two parameters: elution order reversal at sample loadings 0.1 and 100 μg, and relative retention: 1.10, 1.32, 1.36, and 1.40.

In Fig. 3, the relative retention is held at 1.40 throughout, and the effect on trace detectability of reversing elution order continues to be shown in each pair of chromatograms. However, in Fig. 3 for the first time in this study the effect on trace detectability of injecting increasingly larger volumes at a lower concentration is also shown. In the top row in Fig. 3, the weight of enantiomer injected is held constant, but the concentration is decreased from 10 to 1 μg/μl from the left pair of chromatograms to the right pair. The top right pair and both lower pairs of chromato-

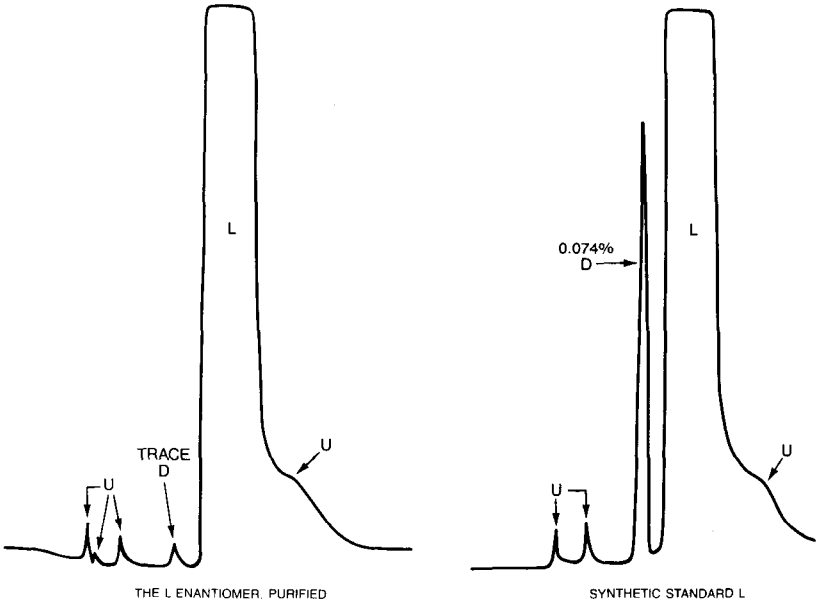

Fig. 4. Combining trace-first elution, larger injected quantity, and larger-than-conventional injected volume allows the trace peak to be brought out as adequately as desired and quantified as precisely as desired. The purified L enantiomer (left chromatogram), found to contain 0.0033% of the D enantiomer, thus shows 99.9967% optical purity. The material purity was somewhat less: the enantiomer also was determined to contain 0.0066% of the ketone precursor, shown in one of the preceding trace "U" peaks; and it also contained an undetermined proportion of some other impurity, revealed as a shoulder. ("U" peaks: usually, unidentified.) Stationary phase, L-phenylglycine; relative retention, 1.40; sample, purified L enantiomer (left), synthetic containing 0.074% D enantiomer in purified L (right); mobile phase, isopropanol–hexane (10:90); mobile phase flow-rate, 0.6 ml/min.

grams in Fig. 3 were all made with 1 $\mu g/\mu l$ sample concentration, but with successively larger volumes injected.

Quantifying an enantiomer of very high optical purity. Chromatograms of purified L enantiomers are shown in Fig. 4. The right chromatogram of the standard was made from solutions of purified D and L enantiomer. The solution of the standard was prepared by gravimetric and volumetric means. Throughout, the ultimate means of purification was preparative LC.

RESULTS AND DISCUSSION

Overall, the study was carried out in two parts. In the first part, experimental procedures to answer two questions about enhancing sensitivity were explored. In the second part, the results of the exploration were applied to determine the optical purity of a highly purified isomer.

First: exploring sensitivity enhancement

The exploratory work was carried out to answer two questions. We state the questions, then take up the corresponding experimental explorations.

(1) Concentration increase at constant injection volume. Consider a series of successively more sensitive determinations of a trace contaminant. Let the lowest initially determinable trace of percent X concentration give rise to a recorder pen deflection of one division. What lower percentage Y can be attained by increase of sample concentration, while the injection volume is held constant? What is the available sensitivity enhancement X/Y? Also, does that enhancement reflect whether the trace contaminant is eluted before or after the major component?

(2) Further increases in the both injection volume and sample concentration. What is the effect of that approach, and can the minimum detectable trace percentage be driven still lower?

(1) Concentration increase at constant injection volume. In Fig. 1, identically-made chromatograms of the fluoroalcohol racemate and of the L enantiomer are shown. This racemate is known to contain 50% of each enantiomer. From this and the chromatograms, the percent of the trace D enantiomer in the nearly pure L enantiomer can be established. Within the precision desired for this calculation, peak area can be taken as proportional to quantity injected and as given by the product of retention time and peak height[6–8]. Given these assumptions, we can use the L enantiomer peak area to monitor the weights of L enantiomer injected for the chromatograms of Fig. 1. With the identical retentions obtained, the areas are proportional to the peak heights: 69.0 with the racemate, 76.5 with the enantiomer. The enantiomer chromatogram was made with (76.5:69.0) or 1.11 more L enantiomer injected than was the racemate chromatogram. In the racemate chromatogram, the D enantiomer peak shows 74.8 divisions, thus 74.8 divisions/50%, or 0.668% D/division. In the chromatogram of the L enantiomer, the trace D peak is one division high, but 1.11 more L enantiomer was injected. The L enantiomer contains about 0.668%/1.11, or 0.60% D. (From the similar pairs of chromatograms made for this study, the percents D determined ranged as a function of relative retention from 0.3 to 1.0.) So we take 0.6% as the trace sensitivity to be enhanced. The object is to obtain a lower percent/division. In Fig. 2 can be seen the effects of changes in con-

centration injected, relative retention, elution order, and sensitivity setting. Increasing by a factor of 1000 the concentration injected increases the height of the trace peak (held on scale by recording at minimum sensitivity), and decreases the percent per division. A typical example: With the L column (trace-first elution), relative retention 1.10, a sensitivity setting of 0.64, and 0.1 μg injected, the trace D peak was 1.5 divisions high: a percent/division sensitivity of 0.67. With the same column and relative retention, but with a sensitivity setting of 2.64 and 100 μg injected, the trace peak height was 31.0 divisions: a percent/division sensitivity of 0.0326 at 2.64, or 0.00815 at the reference 0.64 sensitivity. The sensitivity enhancement was 0.60/0.00815, or 74. The factors of sensitivity enhancement shown in Fig. 2 and calculated for injecting a 1000-fold more concentrated sample ranged from 75 to 486. The expectable factor is probably about 100. That the available sensitivity enhancement is a function of elution order is not obvious from Fig. 2. The chromatograms in Fig. 2 do not reflect the problem of determining a peak height of just one division. For that to be feasible, the trace peak cannot ride on a massive and rapidly falling shoulder, but must stand free. The trace must be eluted before the major enantiomer, must be eluted first.

We then tested injecting 100 μg at one tenth the concentration but 10 times the volume. We found that this more than doubled the height of the trace peak. Accordingly, this approach was investigated further.

(2) Increase in both sample volume and concentration. The chromatograms shown in Fig. 3 show the striking sensitivity enhancements obtained by injecting larger volumes. These could not be kept on scale, therefore can be only qualitatively indicated. Eventually, increase in injection volume produces merely a broader peak with a flat top. At what increase this happened here cannot be seen in Fig. 3. Certainly, however, the peak height continues to increase at volumes far above conventional; this technique for enhancing sensitivity is rarely fully exploited. To illustrate sensitivity enhancement further, a sample with a smaller and known trace content had to be employed. The description of this work follows.

Second: example of actual sensitivity enhancement

The chromatograms shown in Fig. 4 were made with enantiomers that had been purified by preparative LC, a work to be described in the companion paper to this one (see ref. 5).

To determine the trace D in the purified L enantiomer, a solution of known trace D enantiomer content was added to a solution of known content of the purified L enantiomer. The peak area of the trace was then subtracted from the increased peak area of the trace from the synthetic, and the trace content of the purified material deduced: 0.0033% D. For this determination, the sensitivity could apparently have been further increased, if that had been necessary. We stopped at this point because the purification itself, only demonstrative, was discontinued. As will be pointed out in the companion paper[5], that purification could easily have gone on had there been reason.

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