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PURIFICATION OF NITROPHENYLVALERIC ACID REACTION MIXTURES BY COUNTER-CURRENT CHROMATOGRAPHY

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SUMMARY

Failure of purification of nitrated phenylvaleric acid reaction mixtures by conventional recrystallization and column chromatographic methods using silica gel, led to a trial of counter-current chromatography. Poor solubility of the nitration products precluded the use for aqueous systems and led to the examination of systems using ethylene glycol or formamide as the stationary phase, with chloroform and other solvents as mobile phases. Prospective solvent pairs were first screened by a micro-partitioning method and then by counter-current chromatography in an analytical micro counter-current chromatograph. Partition coefficients derived from these methods facilitated optimization of the solvent system and scale-up to a preparative counter-current chromatography range. Using the system chloroform–ethylene glycol, crude product was readily purified and then identified as 5-(2,4-dinitrophenyl)valeric acid. Purification using a 292-ml column provided high resolution and required 6 h. Purification with lower resolution on a 56-ml column required 2 h.

INTRODUCTION

Counter-current chromatography (CCC) has been used extensively for separation of water soluble natural products[1,2] and antibiotics[3,4] derived from fermentation. Application to lipophilic materials has been limited by their insolubility in water and lack of information on the physiochemical characteristics of various applicable nonaqueous solvent systems, which provide greater solubility.

The majority of non-aqueous solvent systems employed in CCC have been derived by mixing one or more additional solvents (such as methylene dichloride, ethyl acetate or nitromethane, with the immiscible pairs formed by alkyl hydrocarbons plus

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methanol or acetonitrile[5,6]. While applicable to essential oils and other very lipophilic materials, these provide insufficient solubility for preparative purification of substances with intermediate polarity, which class includes the majority of synthetic organic chemicals.

Many water-insoluble organic chemicals are quite soluble in ethylene glycol (EG) or formamide (FA), each of which forms two-phase systems with organic solvents ranging in polarity from hexane through ethyl acetate[7,8]. The present study applies these systems, particularly $CHCl_3$–EG, to the purification of the products obtained by nitration of phenylvaleric acid.

CCC symbols and nomenclature

In discussing non-aqueous solvent systems for CCC, the common, though not universal, practice of writing components of solvent systems from left to right, in order of increasing polarity (as in $CHCl_3$–CH_3OH–H_2O) will be followed. Thus the polar EG and FA components will be written to the right of the less polar CH_2Cl_2 or $CHCl_3$ as CH_2Cl_2–EG, $CHCl_3$–EG or $CHCl_3$–FA.

The retention volume, V_R, in CCC is given by the equation common to all forms of chromatography, $V_R = V_m + KV_s$, where V_m and V_s are the respective volumes of mobile and stationary phase and K is the partition coefficient defined as the ratio of solute concentrations in stationary and mobile phase, $K = C_s/C_m$. Either the normal-phase or reversed-phase mode can be employed in CCC. The generally accepted meanings of these terms, the normal-phase mode having the more polar phase stationary and the reversed-phase mode having the less polar phase stationary, can be equally applied to aqueous and nonaqueous solvent systems.

In surveying solvent systems for CCC, it is convenient to express solute partition coefficients as either $K_N = C_{\text{non-polar phase}}/C_{\text{polar phase}}$ or as $K_P = C_{\text{polar phase}}/C_{\text{non-polar phase}} = 1/K_N$. In aqueous systems the subscript N can be interpreted as either non-polar or non-aqueous, which are synomymous in aqueous systems. Since there is no adsorption in CCC, solute retention volumes, V_R, can be readily calculated from partition coefficients determined by non-chromatographic methods. One need only employ the appropriate partition coefficient as K in the above equation, $K = K_P$ for the normal-phase mode, or $K = K_N$ for the reversed-phase mode. Since only the normal-phase mode is employed in this paper, partition coefficients will be expressed as K_P.

Because of the resulting simplicity, it is always desirable to index counter-current chromatograms with the expected retention volumes, or the corresponding retention times, for solutes with $K = 0$, 1, 2, etc. Unretained solutes, $K = 0$, are eluted at the apparent solvent front, $V_R = V_m$. The retention volume for a solute with $K = 1$ is a focal point on the counter-current chromatogram and always occurs at one column volume, V_c, of eluent, $V_{R,K=1} = V_m + V_s = V_c$. Solutes with integrally higher K values are eluted with corrected retention volumes, $V'_R = V_R - V_m$, which are directly proportional to the solute partition coefficients, $V'_R = KV_s$.

To describe the counter-current chromatogram in terms of partition coefficients, it is convenient to rewrite the retention volume equation as $V_K = V_0 + K(V_1 - V_0)$ and the corresponding retention time equation as $t_K = t_0 + K(t_1 - t_0)$, in which V_K is the retention volume for a solute with partition coefficient K, V_0 is V_R for a solute with $K = 0$ and $V_1 = V_c + V_d$ is the column volume. As index points on a counter-current

chromatogram, V_0, V_1, V_2, etc., or the corresponding times t_0, t_1, t_2 etc., indicate the retention volumes, or times, for solutes with partition coefficients signified by the subscripts. The point of injection is indicated by V_i or t_i and any dead volume by V_d or t_d. The chromatograms in Figs. 1–3 are indexed in this way. Partition coefficients of chromatographic peaks can then be calculated by linear interpolation between these reference points.

MATERIALS AND METHODS

Solvents and chemicals employed were HPLC or reagent grade (Fisher Scientific, Rochester, NY, U.S.A.).

Nitration of phenylvaleric acid

To 1 g of a stirred mixture of 5-phenylvaleric acid (Alrich, Milwaukee, WI, U.S.A.) in 4 ml of conc. sulfuric acid, was added, dropwise, 4 ml of conc. nitric acid. The temperature was not allowed to rise above 60°C and was maintained at 45°C for 10 min following nitric acid addition. The reaction mixture was poured onto 25 g of crushed ice. The precipitate was collected by centrifugation and dried in air. The crude yield was 470 mg. The product was purified by CCC.

Partition coefficients

Partition coefficients were estimated for the crude nitrated phenylvaleric acid, in the solvent systems CH_2Cl_2–EG, $CHCl_3$–EG and $CHCl_3$–FA, by partitioning a small quantity of solute between 1 ml of each phase and determining the concentrations spectrophotometrically[9,10]. Results were expressed as K_P = concentration in polar phase/concentration in non-polar phase. This ratio corresponds to the K values employed in the CCC purification here, since the more polar EG phase was employed as the stationary phase. K_P values were 1.7, 2.8 and 3.1 for CH_2Cl_2–EG, $CHCl_3$–EG and $CHCl_3$–FA systems respectively.

Analytical counter-current chromatography

Preliminary examination of solvent systems was done using a micro multilayer coil planet centrifuge (P.C. Inc., Potomac, MD, U.S.A.). The orbital radius was 5 cm and the coil was a multilayer winding of 12.6 m of 0.86 mm I.D. PTFE tubing with a volume, V_c, of 7.8 ml. It was used with a Valco C6U injection valve, an SSI No. 350-60 chromatography pump, and an ISCO UA-5 detector with a Type 9 optical unit operated at 254 nm with a 2-mm preparative flow cell. Injected samples were 0.2 ml volume, containing 5 mg/ml of phenylvaleric acid or 1 mg/ml of crude nitrated product dissolved in mobile phase. The system dead volume sensed by the detector, V_d, was 0.6 ml, including half the injection volume. After saturation with the appropriate mobile phase (CH_2Cl_2 or $CHCl_3$), EG was loaded into the stationary CCC column manually, with a syringe. Rotation was then adjusted to 1500 rpm and mobile phase was pumped at 40 ml/h from a central head to a peripheral tail, (H) → T. After initial displacement of some stationary phase, the system remained stable for several hours, allowing repeated injection of samples.

The dead time, t_d, was calculated as $t_d = V_d/f$, where f = flow-rate in ml/min. The time, t_1, for emergence of a solute with $K = 1$, was calculated as $t_1 = (V_d + V_c)/f$.

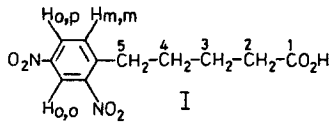
Emergence of an initial impurity peak was taken as an estimate of the time t_0, for emergence of the solute with $K = 0$. The fraction of column volume filled with stationary phase, S_F, was then estimated as $S_F = (t_1 - t_0)/(t_1 - t_d)$. The expected elution times, t_K, of solutes with higher partition coefficients were calculated as $t_K = t_0 + K(t_1 - t_0)$. All times are measured from the time of injection, t_i. The subscript 0 signifies a partition coefficient of zero. The partition coefficient is $K = K_P$ = solute concentration in stationary phase/solute concentration in mobile phase.

Preparative counter-current chromatography

Preparative CCC was done using an Ito multilayer coil separator extractor (P.C. Inc.) with a No. 14 (1.68 mm I.D.) coil, V_c 292 ml, a Milton Roy No. 196-31 pump and an ISCO V-4 monitor with a 2-mm preparative flow cell. Effluent was monitored at 270 nm. Samples were loaded using a Rheodyne No. 50410 four-way valve and a loop of No. 14 PTFE tubing. Fractions were changed manually. The dead volume, V_d, of 1.3 ml in the preparative system was neglected in calculating t_K values. Preparative CCC was also done using a No. 14 coil with a 56-ml volume, monitored at 254 nm using a 3-mm cell in a Glenco 5480 monitor.

After mutual saturation of $CHCl_3$–EG in a separatory funnel, the stationary column was filled with EG using the pump. The column was then rotated at 800 rpm and $CHCl_3$ pumped in at a rate of 120 ml/h in the (H) → T direction, where (H) signifies a central head. Pumping was continued for about 1 h, to establish a steady baseline, prior to injection of a 470 mg sample dissolved in 5 ml of the $CHCl_3$ phase. Fractions comprising the peak eluted at 4.8 h were combined and washed three times with one-tenth volumes of water to remove traces of EG. The $CHCl_3$ solution was dried with anhydrous Na_2SO_4 and evaporated to dryness to yield 123 mg of white crystals. Washing the product with 2 ml of water and drying over KOH, *in vacuo*, gave 117 mg white crystals, m.p. 86–89°C. Recrystallization of a 27-mg sample gave 26 mg of m.p. 88–89°C, which on analysis gave C, 49.37; H, 4.53; N, 10.40%. Theory for $C_{11}H_{12}N_2O_6$ is C, 49.26; H, 4.51; N, 10.44%.

A ^1H NMR spectrum was obtained on a Varian EM-390 spectrometer in C^2HCl_3 (1% tetramethylsilane) gave: δ 1.58–1.8 (m, four H, H_3 and H_4), 2.24–2.50 (m, two H, H_5), 2.80–3.14 (m, two H, H_2), 7.55 (d, one H, J = 9 Hz, Hm, m), 8.35 (dd, one H, J = 9.3 Hz, Ho, p), 8.73 (d, one H, J = 3 Hz, Ho, o) corresponding to 5-(2,4-dinitrophenyl)valeric aid (I).



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RESULTS AND DISCUSSION

Analytical CCC

Preliminary assessment of CCC for the purification of nitrated phenylvaleric acid using the P.C. Inc. micro multilayer CCC with EG as stationary phase and CH_2Cl_2 or $CHCl_3$ as mobile phases is summarized in Fig. 1. Each provided a stable

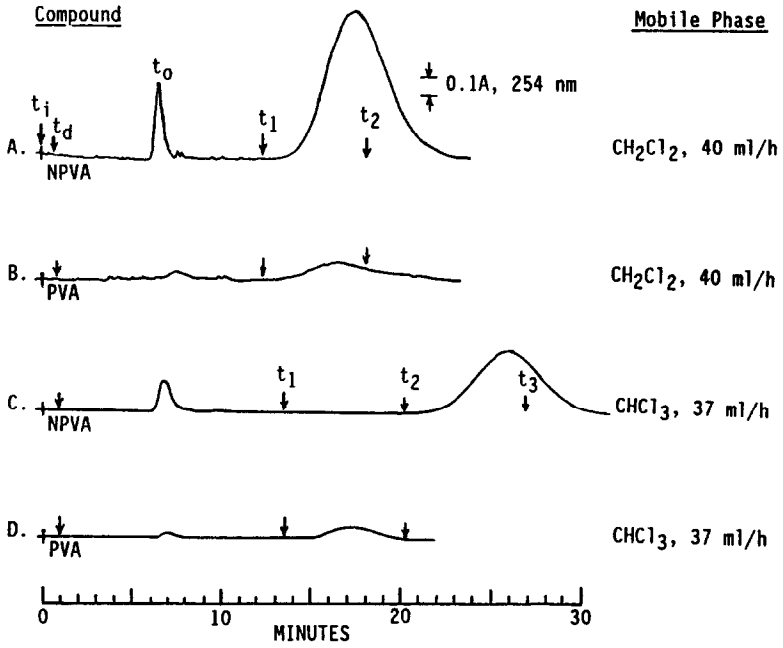

Fig. 1. Preliminary CCC of nitrated phenylvaleric acid (NPVA) and phenylvaleric acid (PVA) with P.C. Inc. micro CCC using a 12.6 m × 0.86 mm I.D. column at 1500 rpm. Stationary phase is ethylene glycol. A and B, mobile phase CH_2Cl_2 phase, 40 ml/h, (H) → T, S_F 0.50. C and D, mobile phase $CHCl_3$ phase, 37 ml/h, (H) → T, $S_F = 0.53$. Positions of column dead time, t_d and elution times, t_K, for $K = 1, 2$ etc., are indicated. The stationary phase fraction, S_F and t_K times were estimated by assuming that the first peak signified emergence of $K = 0$. Column volume, V_c, was 7.8 ml.

chromatographic system, retaining a stationary phase volume 50% or more of the column volume and permitted repetitive sample injection, with elution of solutes with partition coefficients up to 3 in less than 30 min. In both systems the synthetic product exhibited a small early impurity peak and a later major peak. Partition coefficients for the major chromatographic peaks are approximately 1.9 and 2.8 for the CH_2Cl_2 and $CHCl_3$ systems respectively, in good agreement with the values of 1.7 and 2.8 determined independently by a micropartitioning method. Comparison of the retention times for the starting material, phenylvaleric acid, indicates that a better separation would be obtained with the $CHCl_3$–EG system.

When subjected to CCC separation using the $CHCl_3$–FA system, in the microchromatograph, a retention time of 67 min was observed. This corresponds to a K_P value of about 8, compared to 3.1 estimated by micropartitioning. The reason for the discrepancy is not presently known but, because of the excessive retention time, the $CHCl_3$–FA system was not further investigated.

Preparative CCC

Preparative purification of a 470-mg sample of crude product, using a 292-ml column of 1.68 mm I.D. PTFE tubing, is summarized in Fig. 2. Evaporation of the $CHCl_3$ fractions comprising the major peak (after removal of EG by extraction with

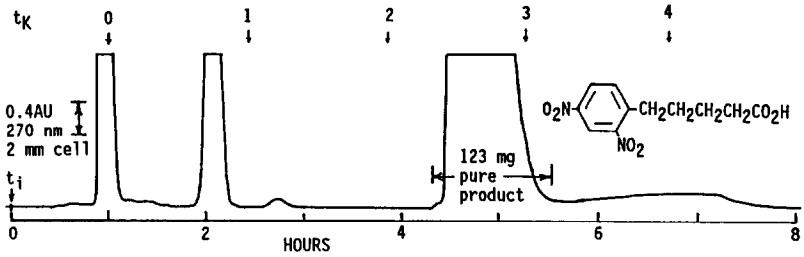

Fig. 2. Preparative purification of nitrated phenylvaleric acid in the P.C. Inc. Ito multilayer separator extractor. Conditions: 800 rpm, No. 14 (1.68 mm I.D.) coil, V_c = 292 ml, $CHCl_3$–EG, normal-phase mode, mobile phase $CHCl_3$, (H) → T, 120 ml/h. Sample was 470 mg crude product in 5 ml $CHCl_3$ phase. S_F = 0.52 from recovered column contents.

water) yielded 123 mg of crystalline product, which was shown by elemental analysis and NMR spectrometry to be 5-(2,4-dinitrophenyl)valeric acid. The partition coefficient of 2.8, calculated by interpolation of the t_K scale on the chromatogram, is in excellent agreement with values determined by micropartitioning and micro CCC methods. No crystalline material was recovered from the impurity peaks at 1.0, 2.1, 2.7 and 6.6 h. The crude product contained more polar materials which are retained in the ethylene glycol. A 26% yield of pure product was obtained from the heterogeneous reaction mixture.

CCC purification of crude material from a second synthesis is summarized in Fig. 3. Chromatographic conditions were identical except that a smaller column, with a volume of 56 ml was used. This permitted much faster chromatography, but with less than half the resolution of the 292-ml column. This 56-ml column has been shown to provide better resolution than can be achieved by "flash chromatography"[11]. Selection of a central cut of the major peak, between 40 and 60 min, washing the combined fractions with water and evaporation as above, yielded 66 mg of 5-(2,4-dinitrophenyl)valeric acid, identical to the product identified above. Note that the partition coefficient estimated from the chromatogram is also in good agreement with the values obtained in the other CCC runs and by micropartitioning. No crystalline material was recovered by processing fractions corresponding to the impurity peaks. Negligible additional material was also recovered by processing the

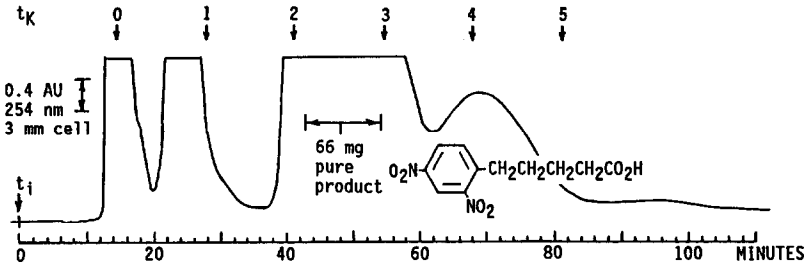

Fig. 3. Preparative purification of nitrated phenylvaleric acid by CCC. Conditions as in Fig. 2 except that a smaller volume No. 14 (1.68 mm I.D.) coil, V_c = 56 ml, was used. Sample was 180 mg crude product in 4 ml $CHCl_3$ phase. S_F = 0.48.

leading and trailing edges of the major peak. The chromatographic recovery of 37% of pure product from crude material again reflects degradation during the non-specific synthesis. The recovery of some *p*-nitrophenylbutyric acid from another comparable nitration of phenylvaleric acid is a further indication of the extent to which starting material is oxidized. The presence of more polar constituents in the crude material was also indicated by the observation of peaks eluting early in analytical CCC chromatograms using heptane–25% aqueous 2-propanol (1:1) in the reversed-phase mode.

CONCLUSIONS

Aqueous solvent systems, which are widely used in CCC, provide limited capacity to dissolve a wide range of compounds encountered in organic synthesis. The polar organic solvents, ethylene glycol and formamide, are excellent solvents for organic compounds, are immiscible with less polar solvents ranging from hexane to ethyl acetate and serve well as stationary phases for CCC purification of synthetic organic compounds.

REFERENCES

1 K. Hostettmann, M. Hostettmann and A. Marston, *Nat. Prod. Rep.*, 1 (1984) 471.
2 K. Hostettmann, M. Hostettmann and A. Marston, *Preparative Chromatography Techniques, Applications in Natural Product Isolation*, Springer, Berlin, 1986.
3 G. M. Brill, J. M. McAlpine and J. E. Hochlowski, *J. Liq. Chromatogr.*, 8 (1985) 2259.
4 D. B. Martin, C. Biles and R. E. Peltonen, *Am. Lab. (Fairfield, Conn.)*, 18, Oct. (1986) 21.
5 B. Domon, M. Hostettmann and K. Hostettmann, *J. Chromatogr.*, 246 (1982) 133.
6 K. Hostettmann, C. Appolonia, B. Domon and M. Hostettmann, *J. Liq. Chromatogr.*, 7 (1984) 231.
7 W. D. Conway and Y. Ito, *J. Liq. Chromatogr.*, 7 (1984) 291.
8 H. S. Freeman and C. S. Willard, *Dyes Pigments*, 7 (1986) 407.
9 W. D. Conway and Y. Ito, *J. Liq. Chromatogr.*, 7 (1984) 275.
10 W. D. Conway, *Countercurrent Chromatography*, VCH, New York, 1990, in press.
11 W. D. Conway, E. L. Bachert, A. M. Sarlo and C. W. Chan, *Northeast Regional Meeting of the American Chemical Society, Rochester, NY, November, 1987*, Abstract No. 111.