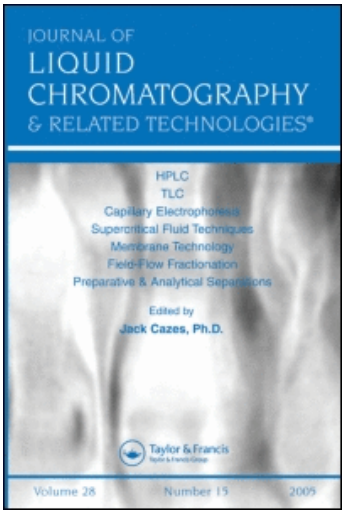

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APPLICATIONS OF ROTATION LOCULAR COUNTERCURRENT CHROMATOGRAPHY IN NATURAL PRODUCTS ISOLATION

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ABSTRACT

Purification of natural products and the fractionation of crude plant extracts are processes which traditionally involve adsorption and/or molecular exclusion chromatography. While countercurrent chromatography avoids the irreversible adsorption and decomposition frequently encountered in adsorption chromatography, the classical countercurrent distribution technique is both time- and solvent-consuming, consequently it is of limited value today. Rotation locular countercurrent chromatography (RLCC) is one of three new countercurrent techniques, the others being droplet contercurrent chromatography (DCCC)[1] and planet coil countercurrent chromatography (PCCCC)[2], which have revived the chromatographic application of liquid-liquid partition. We have employed a commercially available RLCC apparatus in the isolation of several natural products with a broad range of polarity demonstrating the versatility of the RLCC with regard to the functionality of the compounds to be isolated.

INTRODUCTION

Rotation locular countercurrent chromatography is a technique originally proposed by Signer et al.[3] and later developed by several groups.[4-9] As a countercurrent technique, RLCC has the advantages of complete recovery of all material (i.e., no irre-

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versible adsorption) and no danger of decomposition catalyzed by a solid support or compounds adsorbed on the solid support. Furthermore, RLCC requires only a biphasic solvent system for partitioning, difficulties such as emulsion formation or an inability to form droplets are avoided with this technique.

These characteristics render RLCC suitable for the fractionation of crude plant extracts and the purification of natural products. We have applied RLCC to the facile isolation of plant and bacterial glycosides as well as flavonoid aglycones whose isolations were extremely tedious using other methods. The fractionation of a crude plant extract (Daucus carota L.) possessing plant growth regulatory activity was routinely performed with no loss of material. Finally, resolution of racemic norephedrine as its ammonium salt with a lipophilic anion, hexafluorophosphate, was also achieved employing a partitioning between an aqueous phase and a lipophilic phase containing (R,R)-di-5-nonyltartrate.[10]

APPARATUS AND MECHANISM

The RLCC apparatus (Tokyo Rikakikai Co., Tokyo, Japan) consists of sixteen glass columns (50 cm X 11 mm i.d.) mounted cylindrically about a rotational axis. Each column is divided into 37 compartments or loculi by teflon disks with a hole in the center to allow solvent flow between the compartments. The columns are connected in series with teflon tubing (1 mm i.d.). Solvent is applied via a high pressure, constant flow pump. After selecting the solvent system and determining the mode (ascending or descending, see below), the solvent serving as the stationary phase is loaded into the machine, completely expelling all air bubbles. Upon loading of the stationary phase, the columns are inclined to a 20-40^{o} angle with the horizontal. When using the ascending mode, as in Figure 1 for the n-PrOH/n-BuOH/H_2O (1/2/3) system, the lighter mobile alcohol phase is

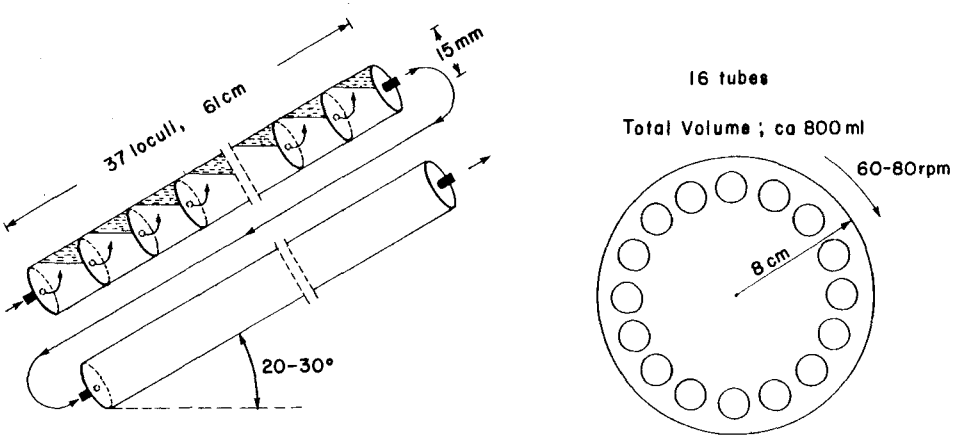


FIGURE 1

RLCC Ascending Mode. Example: n-PrOH/n-BuOH/H_2O (1/2/3); Stationary Phase: Lower Layer; Mobile Phase: Upper Layer.

applied to the bottom of the first column at a rate of 15-25 ml/hr while the columns rotate about the central axis at a speed of 60-80 rpm. Passing through the initial perforation, the alcohol phase rises to the uppermost corner of the first loculus, displacing stationary phase as its volume increases until it attains the level of the hole in the disk leading to the next loculus. The alcohol passes through this hole and enters the second loculus which is slightly elevated from the first. This process continues through all the loculi in the column until the mobile phase emerges from the uppermost loculus and is directed to the bottom of the next column in the series by the teflon tubing.

When the apparatus is charged with mobile phase, the sample, dissolved in the mobile phase, is loaded into a teflon sample loop (volume 4 ml) with syringe suction and subsequently transferred to the first column. Within each loculus the sample is partitioned between the mobile and stationary phases, and eluted

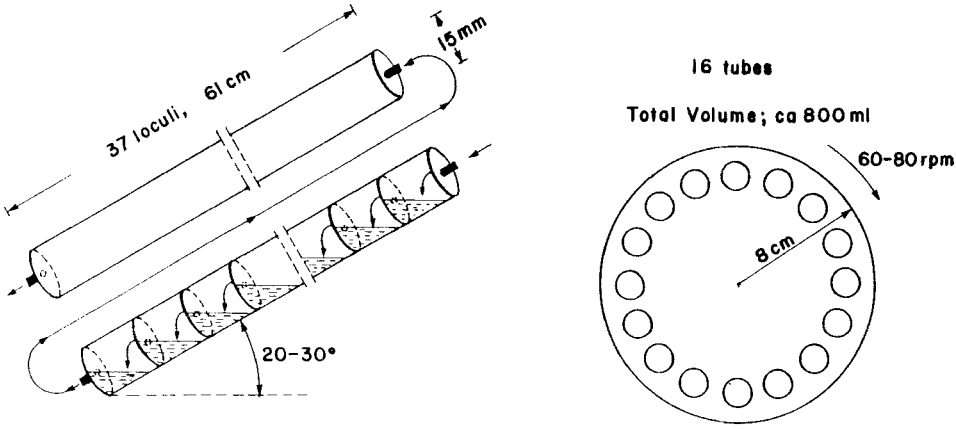


FIGURE 2

RLCC Descending Mode. Example: $CHCl_3/MeOH/H_2O$ (35/65/45); Stationary Phase: Upper Layer; Mobile Phase: Lower Layer.

through each of the columns. Any sample which remains in the stationary phase and not completely eluted from the apparatus can be displaced by flushing the columns with excess stationary phase.

The rotation of the columns serves to renew the contact surface between the two phases, therefore aiding in equilibration, though not allowing emulsions to form due to excessive agitation. The RLCC apparatus has 250-300 theoretical plates, thus the partitioning in each loculus does not represent a complete equlibration.

When the descending mode is selected for separation, as in Figure 2 for the $CHCl_3/CH_3OH/H_2O$ (35/65/45) system, the operation remains the same except that the heavier mobile phase is applied at the top of each column and descends through each loculus.

SELECTION OF THE SOLVENT SYSTEM

A preliminary screening of appropiate biphasic systems using silica gel TLC plates was employed. The TLC's of the sample

using each of the two phases were developed; those systems giving R_f values greater than 0.8 in one of the phases and between 0.2 and 0.4 in the other were potentially applicable systems.

Final selection was based on the distribution of 5 to 10 mg of sample between 5 to 10 ml of each of the the two phases. A system in which 15-25% of the sample was distributed in one of the phases is chosen. The phase which contained the higher percentage of sample functions as the stationary phase. Such systems usually enable the chromatography to be completed within 36 hrs prior to displacement of residual compounds dissolved in the stationary phase. If the equilibration gave a more equal distribution of the sample, the chromatography was completed in less time, but with less resolution. If the equilibration gave a less equal distribution (<15% in one of the phases), the time factor and hence the amount of solvent required increased significantly.

EXPERIMENTAL

All solvents were spectral grade or distilled prior to use. Biphasic solvent systems were prepared in a 2 l. separatory funnel and allowed to equlibrate overnight prior to separation of the phases. Following elution from the apparatus, the eluant was collected in ten ml fractions with an automatic fraction collector and analyzed by TLC or UV-spectrophotometry. Ultraviolet spectra were recorded on a UVIKON 810 or a JASCO UVIDEC-505 spectrometer. Circular dichroism were recorded on a Jobin-Yvon III Dichrograph.

Separation of flavonoid Aglycones

A mixture of approximately 30 mg each of hesperetin 1, kaempferol 2, and quercetine 3 was prepared and applied to the RLCC. Solvent system: $CHCl_3$/MeOH/H_2O (33/40/27); mobile phase: lower layer; flow rate: 48 ml/hr; detection: UV 280 nm. The results are shown in Figure 3.

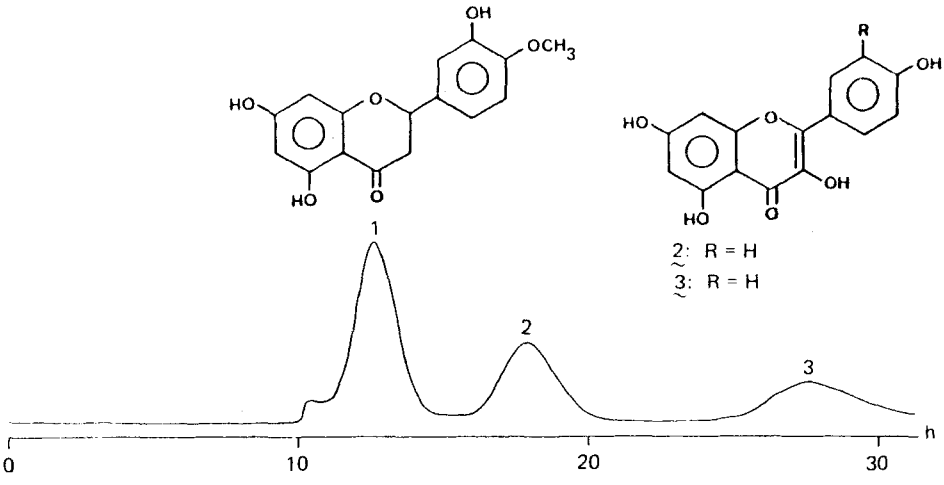


FIGURE 3

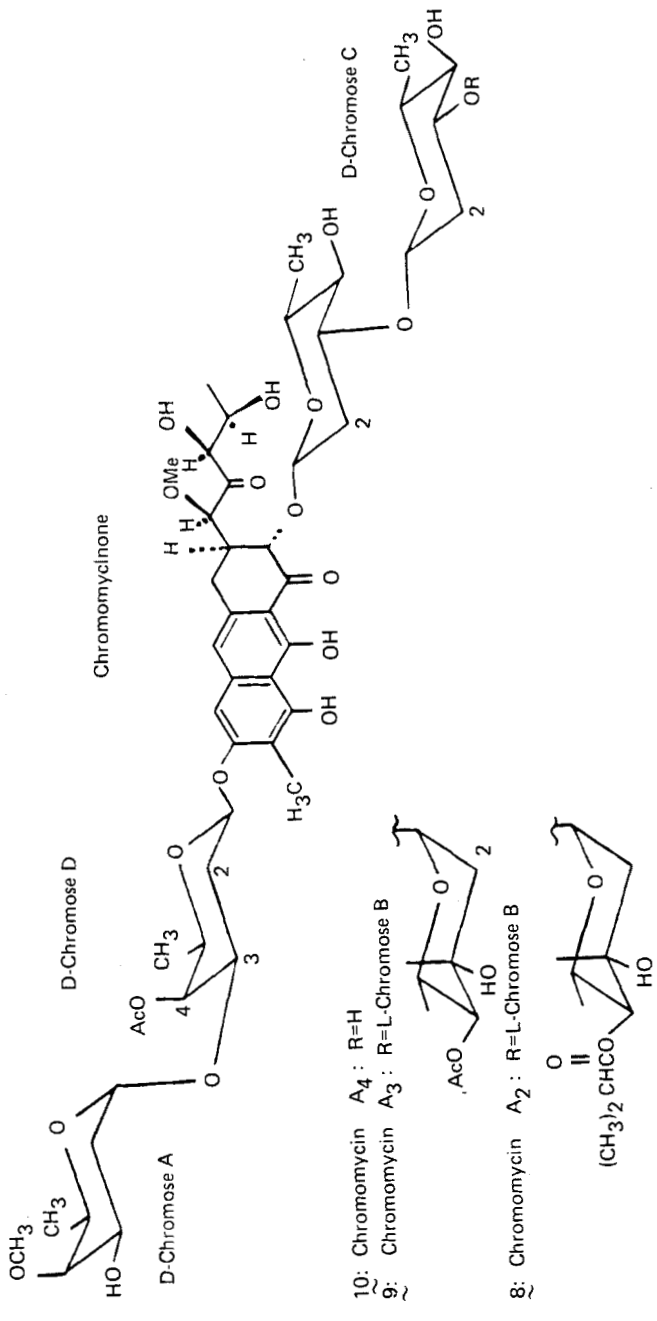
Separation of Flavonoid Aglycones. Solvent System: $CHCl_3$/MeOH/H_2O (33/40/27); Mobile Phase: Lower Layer.

Separation of Swertia perennis L. (Gentianaceae) Constituents

The crude methanol extract of the leaves and stems of Swertia perennis L. was chromatographed on a Sephadex LH-20 column (methanol).[11] The fraction (100 mg) containing three flavonoid C-glycosides 4, 5, and 6, and a xanthone 7, was applied to the RLCC. Solvent system: AcOEt/n-PrOH/H_2O (40/20/70); mobile phase: lower layer; flow rate: 48 ml/hr; detection: UV 280 nm. The results are shown in Figure 4.

Separation of Chromomycins A_2, A_3 and A_4, 8, 9 and 10, Respectively

The crude chromomycin extract[12] (700 mg) was separated using a $CHCl_3$/AcOEt/MeOH/H_2O (2/4/2/1) solvent system as shown in Figure 5. In this solvent system, 79% of the extract equilibrated in the lower phase, hence the ascending mode was employed. Mobile phase: upper phase; flow rate: 30 ml/hr; detection: TLC (silica gel, 1% oxalic acid in AcOEt). Increasing the charge to 5g of the extract gave the same results.



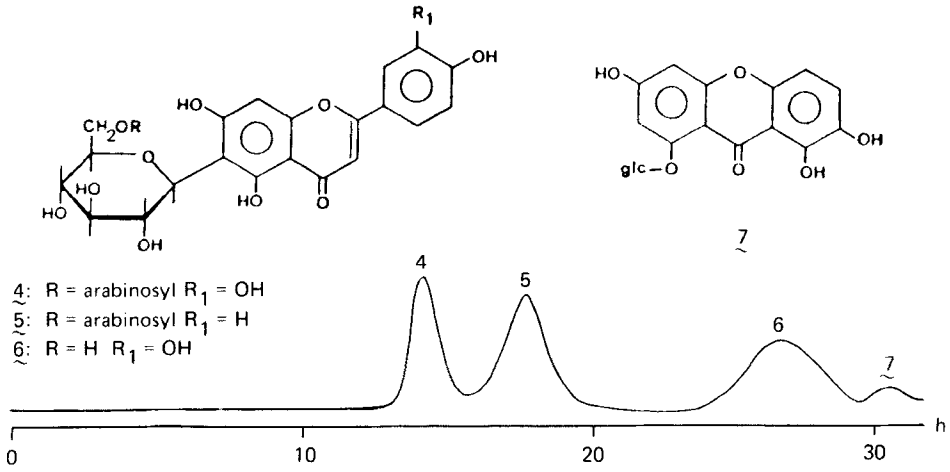


FIGURE 4

Separation of Swertia perennis L. (Gentianaceae) Constitutents. Solvent System: AcOEt/N-PrOH/H_2O (40/20/70); Mobile Phase: Lower Layer.

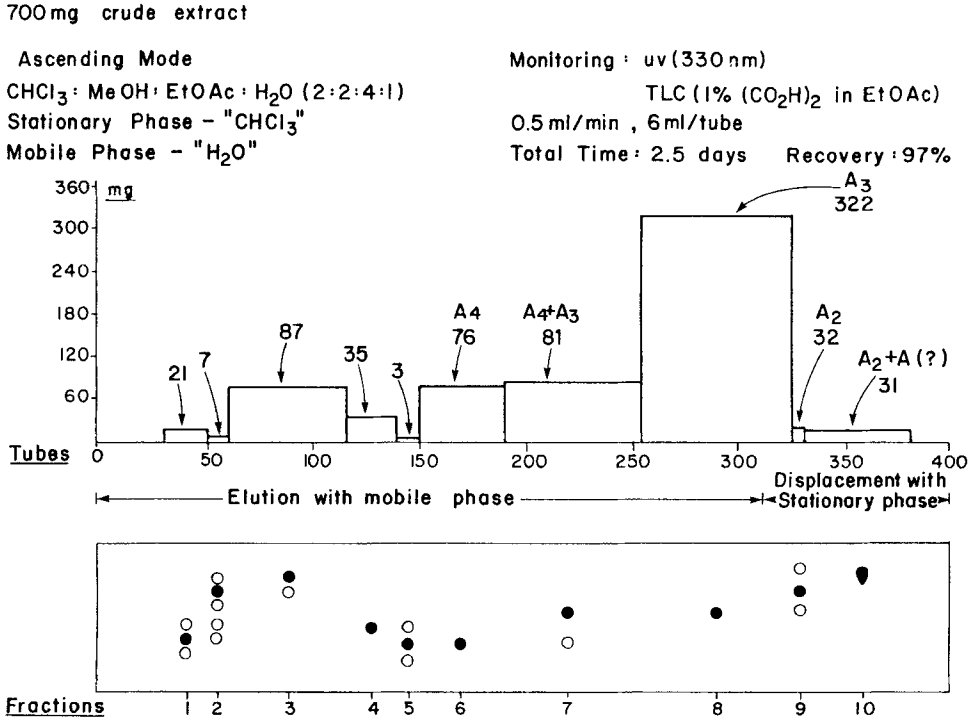


FIGURE 5

Separation of Chromomycins.

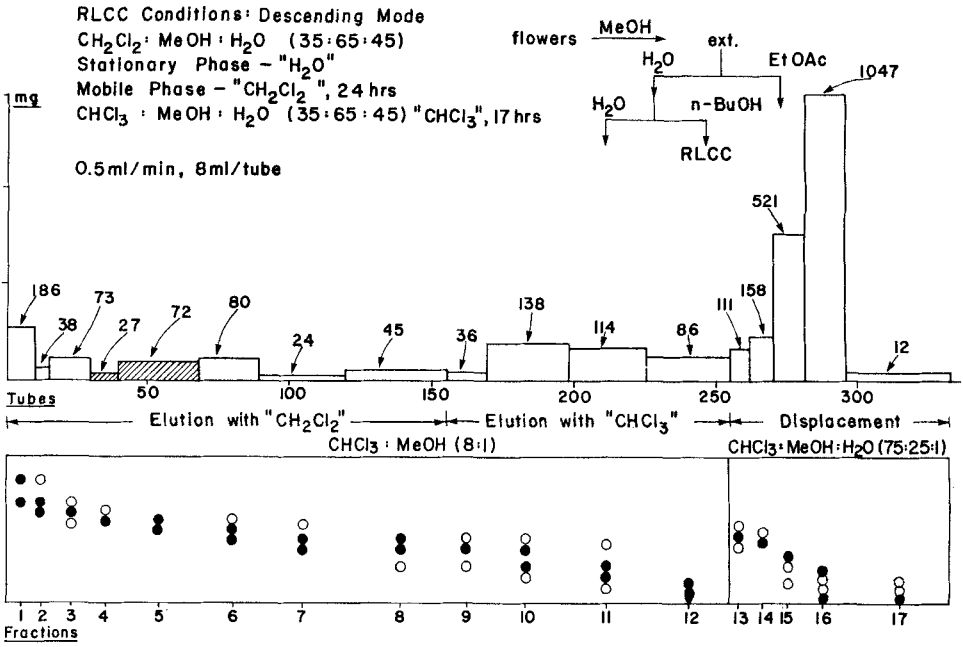


FIGURE 6

Isolation of Plant Growth Regulator from Daucus carota L., Shaded Areas are Active Fractions.

Fractionation of Extract of Daucus carota L. Flowers

The crude methanol extract of the fresh flowers of wild carrot or Queen Anne's lace, Daucus carota L., (Ammiaceae) which showed plant growth regulatory activity, was partitioned between water and ethyl acetate, and the residue from the active, aqueous layer was subsequently partitoned between water and n-butanol. The residue from the n-butanol fraction contained the biologically active compound and was applied to the RLCC as shown in Figure 6. Due to the large number of compounds in this fraction as shown by TLC, the elution of the crude fraction from the aqueous stationary phase was carried out with two separate organic phases using the descending mode. Initially a CH_2Cl_2/MeOH/H_2O

(35/65/45) was applied. In this system, the sample was only 10% partitioned into the lower layer, but TLC had shown that a major component would be quite mobile in this layer. The aqueous layer was eluted for 24 hrs with the lower layer of this system. Following this elution, the stationary phase was then eluted with the lower layer of a $CHCl_3$/MeOH/H_2O (35/65/45) system. In this second biphasic system, the sample was 20% partitioned into the lower layer. Elution with the lower phase of the chloroform containing system was continued for 17 hrs prior to displacement of residual compounds dissolved in the stationary phase using the aqueous layer of the second solvent system. These results are shown in Figure 6.

Resolution of Racemic Norephedrine

The resolution of racemic norephedrine employing the RLCC has been previously described.[10]

RESULTS AND DISCUSSION

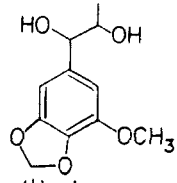
The separation of the flavonoid aglycones, Figure 3, and the Swertia perennis L. constituents, Figure 4, proved to be routine. In the case of the Swertia perennis L. constituents, the RLCC separation represents a notable improvement over a time-consuming polyamide column chromatography employing a water-methanol gradient originally used in the purification of these compounds.[11] Furthermore, the solvent system employed in this isolation could not be used on the DCCC as it would not form droplets.

The chromomycins, 8-10, are clinically used antitumor antibiotics isolated from Streptomyces griseus No. 7 culture broth; the structure of chromomycin A_1 is still undetermined.[13,14] While countercurrent chromatography appeared to be ideally suited for the purification of these compounds, the non-polar sugars of the chromomycins rendered the solvent systems employed on the DCCC ineffective. Moreover, a considerable amount of the extract

had to be separated in order to obtain the desired quantity of the chromomycins.

Using the solvent system as shown in Figure 5, the chromomycins were readily separated in gram quantities. This purification was a great simplification over the original isolation which used chromatography on silica gel employing a 1% oxalic acid solution in ethyl acetate.[12] The chromomycins were readily separated from early eluting impurities which had caused difficulties in the original isolation due to similar R_f values in adsorption chromatography. Impressively, 9 and 10 were obtained in pure states while 8, which differs from 9 only in the nature of the acid group esterified to the 4-OH group of the terminal chromose B sugar (8 is esterified with isobutyric acid while 9 is esterified with acetic acid), was obtained in a nearly pure state.

The crude methanol extract of the fresh flowers of wild carrot or Queen Anne's lace, Daucus carota L. (Ammiaceae), exhibited plant growth regulatory activity in the lettuce seed germination bioassay. This bioassay was then used as a guide for the isolation of the active compound. Final fractionation of the n-butanol fraction on the RLCC led to the isolation of two active fractions, Figure 6 fractions 4 and 5. Final purification of the active compound on C-18 reverse phase HPLC, MeOH/H_2O (25/75) lead to the phenylpropanoid 11.[15]

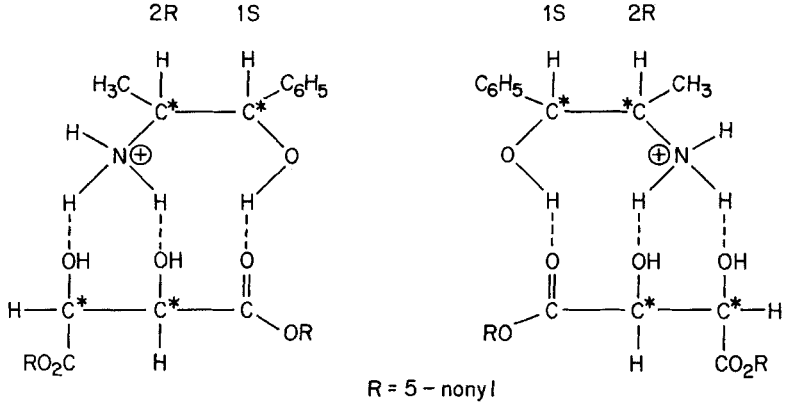


(±) - threo

11

This application of RLCC illustrates the significant promise for the fractionation of polar components of crude natural product extracts. These compounds are traditonally troublesome to fractionate via adsorption chromatography due to irreversible adsorption, decomposition, and severe tailing. The utilization of RLCC provides a stepping stone to ultimate purifications with HPLC or another countercurrent technique such as DCCC.

The final application of RLCC described in this work, the resolution of racemic norephedrine, is based on the preliminary, elegant work of Prelog et al.[16] who resolved racemic α-amino-alcohols as their salts with lipophilic anions by partition between an aqueous phase and a lipophilic phase, 1,2-dichloroethane, containing (R,R)-di-5-nonyltartrate. In this work, the stationary aqueous phase was adsorbed on a Kieselguhr support. For application of RLCC, the stationary phase employed was a 0.5M sodium hexafluorophosphate solution, pH adjusted to 4 with HCl, and the mobile phase was a 0.3M solution of (R,R)-di-5-nonyltartrate in 1,2-dichloroethane.[10] Prelog has suggested that the racemic norephedrine forms diastereotopic complexes 12 and 13 with the (R,R)-5-nonyltartrate esters which have different partitioning properties. While baseline separation of the enantiomers



12 13

was not achieved, practically pure enantiomers ($\geq$ 95%) were obtained.

CONCLUSIONS

Applications of countercurrent chromatography, especially with regard to polar extracts, can be much more suitable than column chromatography due to the high recovery of material. When elution with the mobile phase is completed, the material remaining in the stationary phase can be conveniently recovered by flushing with excess stationary phase. Moreover, countercurrent chromatography avoids the hazards of decomposition and severe tailing frequently encountered when using adsorption chromatography. Equally important is the advantage of partitioning behavior versus adsorption behavior. Thus, compounds which may not be separated via adsorption chromatography due to similar adsorption behavior may be routinely separated via partitioning, as in the case of the chromomycins.

Rotation locular countercurrent chromatography complements the recently developed DCCC and reverse phase HPLC. While RLCC has only 250-300 theoretical plates compared to approximately 1,000 for DCCC, RLCC is more practical for the fractionation of crude extracts as gram quantities can be loaded and separated with the selection of the solvent system restricted only by the requirement of being biphasic and not by the limitations of droplet formation.[17] Furthermore, application of gradient systems as well as a wider variety of non-aqueous systems are possible on RLCC.

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