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Note

Recent applications of counter-current chromatography to the isolation of bioactive natural products

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In our continuing study of biologically active natural products, counter-current chromatographic (CCC) methods have been demonstrated to be useful, especially in the isolation of polar compounds. Thus, droplet counter-current chromatography (DCCC) and rotation locular counter-current chromatography (RLCCC), have an inherently large sample capacity, and yet a typically low solvent consumption. More importantly, CCC require no absorbent which often causes the irreversible absorption of polar compounds.

In general, when the fractionation is guided by bioassay to isolate active principles from crude extracts, the first step is to partition the extracts between water and organic solvents such as *n*-hexane, diethyl ether (or chloroform), ethyl acetate and *n*-butanol to narrow the spectrum of chemical constituents and concentrate the biological activity. If the biological activity is found in polar fractions such as the ethyl acetate and/or *n*-butanol fractions, CCC may be considered a practical application. Although the isolation of natural products usually involves a combination of various chromatographic methods, some compounds have been isolated using only CCC methods. We have previously reported on our isolation of various phytochemicals by DCCC and RLCCC [1–4]. The present paper is limited to a few additional phytochemicals recently reported in our laboratory. For example, efficient and simple methods for the isolation of various polar phytochemicals are described, such as (1) a flavone glycoside, rutin, from the leaves of *Esenbeckia pumila* (Ruraceae) by RLCCC, (2) two stilbene glycosides, rhaponticin and 4′-O-methylpiceid, from the roots of *Rheum palmatum* (Polygonaceae) by DCCC and (3) two antifungal steroidal glycoalkaloids, solasonine and solamargine, from the ripe fruits of *Solanum incanum* (Solanaceae) by a combination of RLCCC and DCCC.

APPLICATIONS

Flavone glycoside from Esenbeckia pumila

The dried powdered leaves of *E. pumila* (Rutaceae) (600 g), collected near Sãn Paulo, were extracted with dichloromethane followed by methanol at room tempera-

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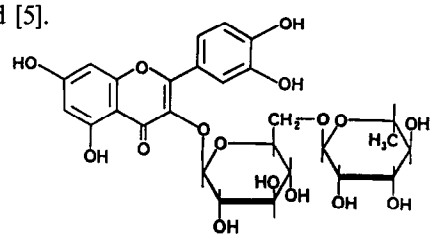
ture. Insect growth inhibitory activity against several lepidopterans was found in the methanol extract. The methanol extract was evaporated to dryness under 40°C to give a dark brown residue (15 g). A part of this residue (2.0 g) was suspended in a mixture of water and a small amount of methanol. The water suspension was partitioned with chloroform to remove non-polar components. The water layer was concentrated *in vacuo* to give a syrup (400 mg), which was dissolved in 20 ml of water. The water solution was injected into an RLCCC sample loop after filtration. A stationary phase of water, a mobile phase of water-saturated diethyl ether (500 ml) and water-saturated ethyl acetate (500 ml) and the upper layer of ethyl acetate–propanol–water (10:1:2 and 4:1:1, v/v, 1 l) proved to be a successful solvent system in the ascending method. The flow-rate of the mobile phase was adjusted to 1 ml/min, and the eluent was collected in 25-ml fractions. Each fraction was checked by thin-layer chromatography (TLC) with a vanillin–sulfuric acid spray reagent. Fractions 37–57 yielded 30 mg of an insect growth inhibitory yellow compound, which was then crystallized from methanol and water. The yellow needles were identified as rutin (**1**) by comparison of their spectroscopic data [UV, IR, fast atom bombardment mass spectrometry (FAB-MS), ^{1}H NMR and ^{13}C NMR] with those of an authentic sample. An efficient and simple method for the isolation of rutin by RLCCC using only a gradient elution as the mobile phase has been established [5].



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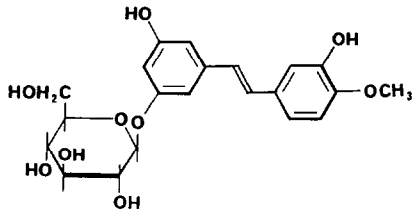

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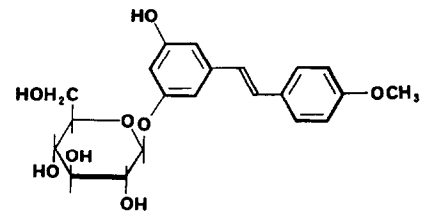

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The isolation of rutin has been reported from many plants by various methods. However, these reported methods use solid packing materials which often cause irreversible absorption (at least some if not all) of polar compounds such as rutin. In addition, all of these methods are time-consuming. In contrast, RLCCC is faster without using solid packing materials.

In addition, although an example is not provided in this paper, RLCCC can be used for the efficient initial fractionation using a gradient elution without forming troublesome emulsions that are frequently encountered in separatory funnel-type partitions.

Stilbene glycosides from Rheum palmatum

In Indonesia the roots of *R. palmatum* (Polygonaceae), known as "kelembak", are used to treat malaria and tropical cough. The roots of *R. palmatum* (397 g) were extracted with methanol at room temperature. This methanol extract inhibited β-glucosidase activity. Following suspension of a portion of the extract (4 g) in water, the water-insoluble portion was removed by filtration, and the suspension was partitioned into *n*-hexane, chloroform, ethyl acetate and water-soluble fractions, yielding 102, 147, 1108 and 1310 mg, respectively. The bioactive yellow precipitate was obtained from the ethyl acetate fraction after concentration of the volume to one twentieth, and a part of this (1 g) was dissolved in 14 ml of the stationary and mobile phase (1:1, v/v) for DCCC. The solvent system, chloroform–methanol–water (7:13:8, v/v) was chosen for DCCC separation in the descending method based on TLC analysis. The flow-rate of the mobile phase was adjusted to 21 ml/h. Each 21-ml fraction was collected into test tubes and monitored by TLC with use of a vanillin–sulfuric acid spray reagent. A total of 141 fractions were collected. Fractions 28–35 afforded 98.5 mg of compound **2** and fractions 87–141 yielded 184.5 mg of compound **3** (Fig. 2). Following crystallization from dichloromethane and ethanol, needles of compounds **2** and **3** were obtained. They were identified as rhaponticin, 3,5,3′-trihydroxy-4′-methoxystilbene 3β-D-glucopyranoside (**2**), and 4′-O-methylpiceid, 3,5-dihydroxy-4′-methoxystilbene 3β-D-glucopyranoside (**3**) [6], respectively, by spectroscopic data (UV, IR, FAB-MS, ^{1}H NMR and ^{13}C NMR) [7].

In contrast to RLCCC, typical DCCC separation is much more time-consuming since it operates at low flow-rates and pressures. The separation of 1 g of the above stilbene glycosides by DCCC required five to seven days, even though the flow limitation was reduced in part by a modification of the commercial instrument [8]. Another limitation of DCCC is selection of solvents which must form droplets [9–11]. Incidentally, this also limits the maximum elution flow-rate. Despite these limitations, DCCC is still very useful because of better resolution compared to RLCCC. An attempt to continuously inject the sample without washing the vertical glass columns of the DCCC system, to reduce the time between injections,failed due to the surfaces of the glass becoming wettable. Thus, washing the glass columns after each injection, at least in the case of the above separation, seems essential to maintain good resolution.

Steroidal glycoalkaloids from Solanum incanum

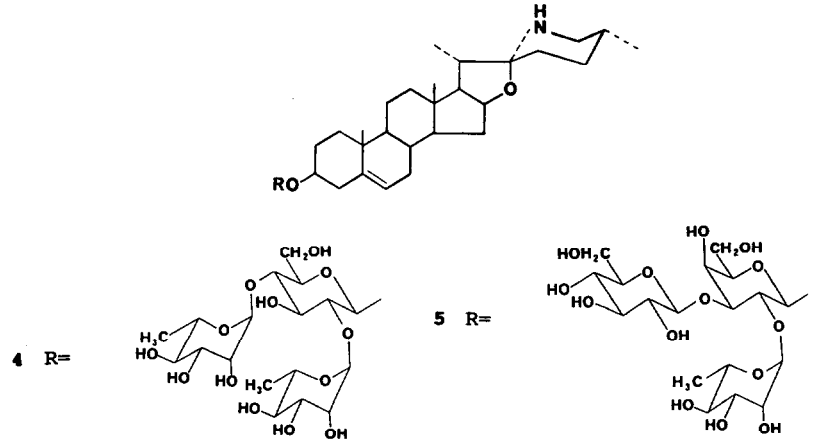
A shrub *S. incanum* (Solanaceae), known as the "sodom apple", is common in the tropics, especially in waste land. In East Africa the fruits of the shrub are used to treat skin diseases [12]. In our routine antimicrobial screening, the methanol extract of the ripe fruits of *S. incanum* exhibited antifungal activity.

Fresh ripe fruits of *S. incanum* (550 g) were extracted with methanol at room temperature. Following solvent partitions, the water-insoluble portion was removed, and the suspension was partitioned into *n*-hexane, chloroform, ethyl acetate and water-soluble fractions, yielding 0.7, 0.4, 0.5 and 16.6 g, respectively. The biological activity was found in the water-soluble fraction. The aqueous fraction was concentrated to 30 ml and injected directly into the sample loop of an RLCCC apparatus. For the successful RLCCC solvent system, water was used as a stationary phase, and a gradient elution of mobile phase was carried out in an ascending method. The first elution solvent, consisting of water-saturated ethyl acetate (1.5 l) was employed to

remove the non-polar components, as monitored by TLC with a vanillin–sulfuric acid spray reagent. The subsequent mobile phase, the upper layer of ethyl acetate–butanol–water (2:1:1, v/v), yielded crude antifungal alkaloid fractions consisting primarily of two components positive to Dragendorff's reagent.

A portion of the crude alkaloid fraction (400 mg) obtained from RLCCC was dissolved in 10 ml of the stationary and mobile phases (1:1, v/v) for DCCC. The solution was filtered and injected into a sample loop of a DCCC apparatus. The solvent system, chloroform–methanol–water–propanol–ammonium hydroxide (34:65:40:5:1, v/v), was chosen for DCCC separation in the ascending method based on TLC analysis of the fractionated alkaloids. The flow-rate of the mobile phase was adjusted to 3.0 ml/h. Each 9-ml fraction was collected into test tubes and monitored by TLC with vanillin–sulfuric acid and Dragendorff's spray reagents. A total of 95 fractions were collected. Fractions 41–51 afforded 84.3 mg of compound **5** and fractions 54–59 yielded 84.8 mg of compound **4**.

Following crystallization from methanol and water, fine needles of compounds **4** and **5** were identified as solamargine (**4**) and solasonine (**5**), respectively, by



comparison of their spectroscopic data (IR, FAB-MS, ^{1}H NMR and ^{13}C NMR) with those of authentic samples. Thus, the semi-preparative isolation of two bioactive steroidal glycoalkaloids, solamargine (**4**) and solasonine (**5**), was performed efficiently by the combination of RLCCC and DCCC [13].

Combining RLCCC with DCCC could exploit advantages of both techniques and, in our case, led to the isolation of the two steroidal glycoalkaloids from *S. incanum*. RLCCC was employed with a gradient elution for large-scale separation of a crude extract into several impure fractions prior to applying to DCCC. The subsequent application of DCCC, with its higher resolution, gave the pure steroidal glycoalkaloids on a large scale. This combined CCC technique accomplished the separation with solvent–solvent partition chromatography without solid packing materials. Thus, irreversible absorption of compounds to absorbents could be avoided. This method, therefore, might be generally applicable for the isolation of polar and/or unstable natural products.

CONCLUSION

The aforementioned, all-liquid separation techniques were completed without using any solid packing materials which might cause the irreversible absorption of large amounts of these polar compounds.

Although RLCCC is advisable to use prior to DCCC for initial fractionations to narrow the spectrum of chemical constituents for subsequent bioassay [1], RLCCC alone sometimes led to the isolation of pure compounds [1,3,14]. Usually, however, combining RLCCC with DCCC could maximize the advantages of both techniques.

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