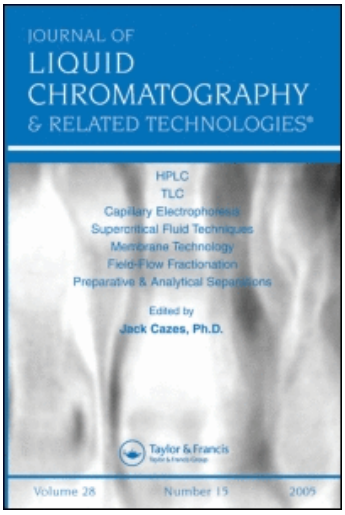

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Recent Examples of Natural Products Isolation by Countercurrent Chromatographic Methods

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RECENT EXAMPLES OF NATURAL PRODUCTS ISOLATION BY COUNTERCURRENT CHROMATOGRAPHIC METHODS

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ABSTRACT

The efficient isolation of two steroidal glycoalkaloids from the Andean potato "papa negra", three bitter quassinoids from the bark of *Castela tortuosa*, and several phytoecdysteroids from the root barks of *Vitex strickeri* was accomplished by countercurrent chromatographic methods.

INTRODUCTION

In general, when fractionation is guided by bioassay to isolate biologically active compounds from crude extracts, the first step is to partition the extracts between water and organic solvents such as *n*-hexane, diethyl ether (or methylene chloride or chloroform), ethyl acetate and *n*-butanol. This narrows the

spectrum of chemical constituents and concentrates the biological activity. If the biological activity is found in the polar fractions, such as the ethyl acetate and/or *n*-butanol fractions, countercurrent chromatographic methods[1,2] can be considered to be appropriate isolation techniques. Although the isolation of natural products usually involves a combination of chromatographic methods, some compounds have been isolated using only countercurrent chromatographic methods. In our continuing search for biologically active natural products, two countercurrent chromatographic methods, droplet countercurrent chromatography (DCCC) and rotation locular countercurrent chromatography (RLCC), have been demonstrated to be extremely useful, especially for the isolation of polar compounds[3-6].

I have previously described the isolation of various biologically active phytochemicals by RLCC and DCCC[5] in our laboratory, while illustrating the general principles of these techniques[3,7]. The aim of this paper is to report several new examples and describe appropriate methodologies[5]. Thus, efficient and simple methods for the isolation of various polar phytochemicals are described, among them: (1) two steroidal glycoalkaloids from an Andean potato, (2) three bitter quassinoids from the bark of *Castela tortuosa* (Simaroubaceae), a Mexican medicinal plant known as "chaparro amargo", and (3) several phytoecdysteroids from the root barks of *Vitex strickeri* (Verbenaceae).

MATERIALS AND METHODS

Apparatus. RLCC was performed on an Eyela RLCC-60 (Tokyo Rikakikai, Tokyo, Japan). DCCC was accomplished on an Eyela DCC-

G2 (Tokyo Rikakikai, Tokyo, Japan) with 300 glass columns (400 X 2 mm i.d.). Recycle HPLC (R-HPLC) was performed on a JAI-LC-09 (Japan Analytical Industry, Tokyo, Japan) using an Asahipack GS-320 column (50 X 2 cm i.d.).

Chemicals. Authentic α-chaconine and α-solanine were purchased from Sigma Chemical Co. (St. Louis, MO). Solvents were used reagent grade.

Plant Materials. The samples of papa negra were purchased at marketplaces in La Paz, Bolivia and their original scientific names were tentatively identified as either *Solanum* X *juzepczukii* or *S.* X *curtilobum* by Ms. I. O. Hinojosa. However, since all species of cultivated potatoes have been derived in some way through hybrization, the further taxonomic study is needed. "Chaparro amargo", or the bark of *Castela tortuosa* was purchased at marketplaces in Guadalajara, Mexico and identified by Prof. T. Ogura and Ms. R. E. Garcia. The root bark of *Vitex strickeri* was collected near Nairobi, Kenya and identified by Dr. G. M. Mungai.

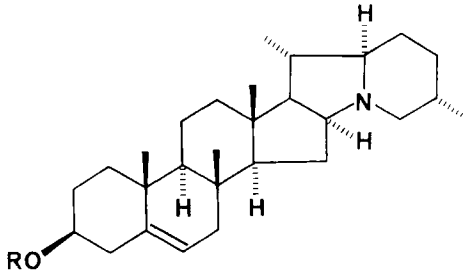
APPLICATIONS

Steroidal glycoalkaloids from potatoes.

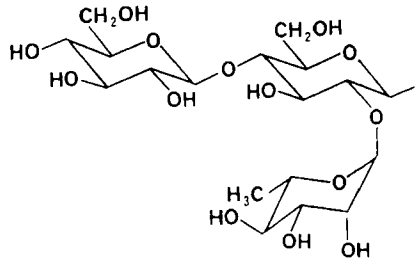
Throughout history, the potato - known as "papa" - has been one of the most important food crops for the inhabitants in the Andes. There are mainly two kinds of potatoes sold in the marketplaces, namely "papa negra" and "papa blanca". The main physical difference between the two is that the papa blanca is the same (or similar) in appearance to our cultivated potato, *Solanum tuberosum* (Solanaceae), while the papa negra has darker peelings. Although both potatoes belong to the genus *Solanum*, the

taxonomy of this genus is further complicated by varieties of *Solanum* hybrids. More than one hundred wild species of tuber-bearing *Solanum* from South America[8] have been identified. Moreover, all species of the cultivated potatoes have been derived in some way through hybridization. Based on their appearance, there are mainly two kinds of potatoes sold in the marketplaces, namely, papa negra and papa blanca. The potato has long been known to contain toxins. The presence of toxic steroidal glycoalkaloids such as solanine in potato is well documented[9]. In our preliminary TLC analysis, we found large amounts of alkaloids, consisting primarily of solanine and chaconine, which were positive to Dragendorff's reagent in the methanol extracts. These steroidal glycoalkaloids have previously been isolated by various chromatographic methods[10,11]. However, these methods have always required solid packing materials, which often cause irreversible absorption of unacceptably large amounts of polar steroidal glycoalkaloids. Therefore, an efficient isolation method of the steroidal glycoalkaloids, possibly without using any solid packing material, has been sought. In order to resolve this problem, countercurrent chromatography, based on two phase liquid-liquid partition chromatography, was applied. This attempt was based largely on our previous success in the efficient isolation of two similar steroidal glycoalkaloids, solasonine and solamargine from the fresh ripe fruit of an East African medicinal plant *Solanum incanum*. This was accomplished by a combination of RLCC and DCCC[12].

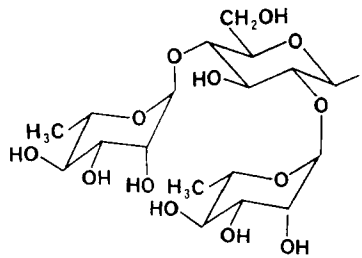
The two steroidal glycoalkaloids, α-chaconine (**1**) and α-solanine (**2**), were isolated from various potatoes obtained from the Andes area, by using RLCC. This was followed by removal of the extraction solvent (methanol), suspension of the residue in



1 R=



2 R=



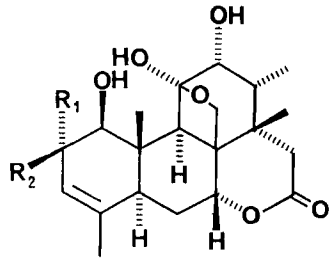
water, and partitioning with organic solvents. The aqueous layer was injected, without further purification, into the sample loop of the RLCC apparatus in the ascending mode. The column was filled with water as the stationary phase. After eluting water saturated ethyl acetate as the mobile phase to remove non-polar compounds, the solvent system was changed to the upper layer of ethyl acetate-*n*-butanol-water (4:1:1, v/v/v). This ratio was

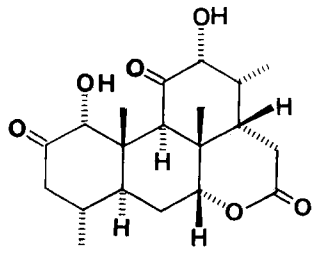
successively changed to 3:1:1 and 2:1:1 in order to increase the polarity of the mobile phase. The flow rate of the mobile phase was adjusted to 1 ml/min and 20 ml of the eluant was collected in test tubes. Each fraction was monitored by SiO_2-TLC developing by chloroform-methanol-ammonium hydroxide (2:4:1, v/v/v) with Dragendorff's spray reagent. This method was applied to the isolation of these steroidal glycoalkaloids from several Andean potatoes. For example, from 10 g of the freeze-dried aqueous portion of the papa negra purchased at a marketplace in La Paz, Bolivia, 59.5 mg of α-chaconine (**1**) and 57.9 mg of α-solanine (**2**) were isolated. The total quantity of glycoalkaloids in this papa negra was more than 1.17% of the dry weight. The biological significance of these findings will be reported in detail elsewhere.

Quassinoids from *Castela tortuosa* (Simaroubaceae).

The bark of *C. tortuosa*, a medicinal plant known as "chaparro amargo" in Mexico, was administered by the ancient Mexican people to treat liver diseases and is currently used to heal stomach aches and spasmodic pain. This extremely bitter tasting plant is commonly found in the subtropical regions of Mexico. In the course of our searches for insect control agents from plant sources, we have found that the methanol extract of chaparro amargo exhibited potent insect growth inhibitory activity in an artificial diet feeding bioassay against the lepidopteran pest insect *Heliothis virescens* (tobacco budworm). The quassinoids, which consist of the bitter constituents of the Simaroubaceae, have been shown to possess diverse biological activities, including insect growth inhibitory activity[13].

After concentration of the methanol extract under reduced pressure, the precipitate was removed by filtration. The precipitate was almost pure chaparrin (186 mg) (**3**). The filtrate was suspended in water and the suspension was successively partitioned into *n*-hexane, methylene chloride, ethyl acetate and water soluble fractions. Subsequent bioassay indicated the methylene chloride and ethyl acetate fractions to be active. The bioactive methylene chloride fraction was subjected to purification by DCCC. Based on TLC analysis[14], the solvent system, chloroform-methanol-water (7:13:8, v/v/v) was chosen for the descending method. The flow rate of the mobile phase was 12 ml/hr. Each 12 ml fraction was collected into test tubes and monitored by TLC, using vanillin-sulfuric acid spray as the development reagent. The sample injection could not exceed 1 g because of solubility limitations. Fractions 7 and 8 afforded chaparrin (**3**) (4.3 mg) and fractions 10 to 12 yielded chaparrinone (**4**) (34.0 mg). In addition, fractions 16 to 17 afforded a new minor compound (**5**) (0.8 mg). The repeated DCCC (X 9) led to the isolation of more of these three compounds, totaling **3** (46.7 mg), **4** (237.2 mg) and **5** (7.7 mg). Thus, fractionation guided by the bioassay led to the isolation of two known quassinoids, chaparrinone (**3**) and chaparrin (**4**), together with a new minor quassinoid. The new quassinoid was designated as "chaparramarin" and its structure was established as **5** by means of spectroscopic methods, in particular, the ^{1}H and ^{13}C NMR spectra. The significant structural characteristic of this new quassinoid, chaparramarin (**5**), is that it possesses an α-configuration of hydroxyl group at C-1. Although more than fifty quassinoids are known by now, among them, only castelanolide[15] was reported to possess an α-hydroxy group at C-1.





3 R_1=OH, R_2=H

4 R_1, R_2=O

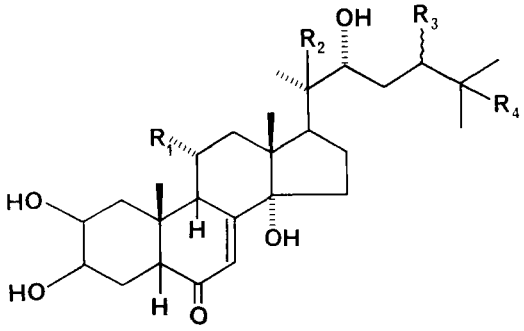
5

Phytoecdysteroids from *Vitex strickeri* (Verbenaceae).

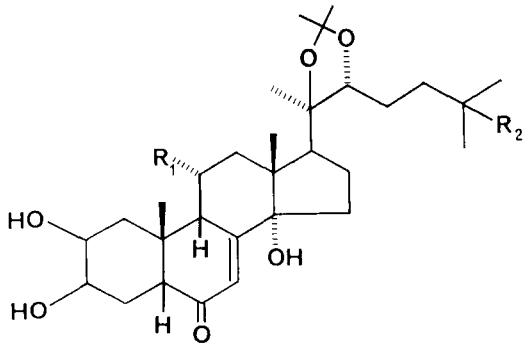
In our continuing search for insect control agents from tropical plants, large quantities of phytoecdysteroids, particularly 20-hydroxyecdysone, have been found in several African *Vitex* species[16] These plants have become an important source of ecdysteroids for our biochemical studies on insect molting[17]. Since the discovery of these resources, we have been studying ways to simplify their isolation. Among the separation methods attempted, countercurrent chromatographic methods, especially DCCC, have proved to be very efficient. They have high sample recovery rates and large loading capacities for isolating hydrophilic compounds directly from the plant crude extracts, and yet a typically low solvent consumption. However, there are some limitations on the use of DCCC. First, the choice of solvent system is restricted because the system must be favorable for the formation of droplets which are essential for separation. Second, the slow flow rate limits the speed of separation. Often, five to eight days are necessary for a whole separation, although some

modifications of the commercial apparatus have been made to increase flow rates[18]. In contrast to DCCC, RLCC does not require droplet formation, therefore, it is compatible with a wider range of solvent systems. More importantly, RLCC has a large capacity (10 times that of DCCC). Thus, with RLCC, one can easily extract and separate large quantities of crude sample by partitioning between two or more immiscible layers of components. The countercurrent chromatographic methods avoid the irreversible absorption of large amounts of polar compounds such as phytoecdysteroids, to the matrix, since they do not require any solid packing material. The separation of 20-hydroxyecdysone using RLCC alone has not been very successful, especially when a compound of similar polarity to 20-hydroxyecdysone, such as ajugasterone C, coexisted in the crude sample. As we have previously reported[12], combining RLCC with DCCC has been proven to be an efficient technique to separate these structurally similar phytoecdysteroids.

The methanol extract of the root bark of *V. strickeri* (1 g) was extracted with methanol (X 3) at ambient temperature. After removal the solvent, the extract was dissolved in 39 ml of a mixture of ethyl acetate-water (1:3, v/v) and injected directly into the RLCC apparatus. The column was filled with water as the stationary phase. After eluting water saturated ethyl acetate (400 ml) as the mobile phase in order to remove non-polar compounds, the solvent system was changed to the organic solvent layer of water-ethyl acetate-*n*-propyl alcohol (6:6:1). The flow rate of the mobile phase was adjusted to 0.5 ml/min. Each 20 ml fraction was collected into test tubes and monitored by TLC, using vanillin-sulfuric acid spray as the development reagent. From RLCC, 150 fractions were collected. Fractions 6 to 19



6 R_1=OH, R_2=OH, R_3=R_4=H
7 R_1=R_3=H, R_2=R_4=OH
10 R_1=H, R_2=R_3=R_4=OH
11 R_1=R_4=OH, R_2=R_3=H



8 R_1=H, R_2=OH
9 R_1=OH, R_2=H

afforded ajugasterone C (**6**) (33 mg) and fractions 30 to 45 yielded 20-hydroxyecdysone (**7**) (324 mg), respectively. Thus, we successfully obtained pure 20-hydroxyecdysone and ajugasterone C from the crude extract using a single RLCC injection. The other fractions from RLCC containing the ecdysteroid mixtures could not

be separated in this system and were subjected to further separation using recycle high performance liquid chromatography (R-HPLC)[19]. Thus, fractions 1 to 5 (54 mg) were a mixture of primarily two compounds which yielded 20-hydroxyecdysone-20,22-monoacetonide (**8**) (12 mg) and ajugasterone C-20,22-monoacetonide (**9**) (4 mg) after R-HPLC. Also, fractions 46 to 54 (44 mg) were a mixture and were further separated by R-HPLC to give 20-hydroxyecdysone (**7**) (21 mg), abutasterone (**10**) (10 mg) and 11α-hydroxyecdysone (**11**) (3 mg). As a result, 20-hydroxyecdysone (**7**) was the major component in the methanol extract of the root bark of *V. strickeri*. Among the ecdysteroids isolated, 11α-hydroxyecdysone (**11**) and ajugasterone C-20,22-monoacetonide (**9**) are unknown in literatures. The final separation might be accomplished by DCCC. However, R-HPLC was proven to be even superior technique for the separation of these phytoecdysteroids[20].

CONCLUSION

Based on our own experience using these countercurrent chromatographic techniques, RLCC and DCCC can be best utilized when the techniques are used in combination. Although RLCC alone has sometimes led to the isolation of pure compounds[6], it is advisable to use RLCC prior to DCCC. Thus, RLCC can be employed with a gradient elution for large scale separation of a crude extract into several coarse fractions prior to application of DCCC. The subsequent application of DCCC, with its higher resolution, gives pure compounds, still on a large scale[7]. This combined countercurrent chromatographic technique accomplishes separations with solvent-solvent partition chromatography without

any solid packing materials. Thus, irreversible absorption of compounds to absorbents can be avoided. This method, therefore, might be generally applicable for the isolation of polar and/or unstable natural products. In addition, the aforesaid initial partition between water and organic solvents can be achieved rapidly, at least in part (exception of *n*-butanol) by RLCC, utilizing a gradient elution. Noticeably, this avoids formation of troublesome emulsions that are frequently encountered in separatory funnel type solvent partitions.

REFERENCES

1. W. D. Conway, *Countercurrent Chromatography*, VCH, New York (1989).

2. N. Mandava and Y. Ito, *Countercurrent Chromatography*, Dekker, New York (1988).

3. I. Kubo, F. J. Hanke and G. T. Marshall, *J. Liq. Chromatogr.*, **11**, 173 (1988) and references therein.

4. F. J. Hanke and I. Kubo, *LC·GC*, **5**, 248 (1987).

5. I. Kubo, *J. Chromatogr.*, **538**, 187 (1991).

6. I. Kubo, P. C. Vieira and K. Fukuhara, *J. Liq. Chromatogr.*, **13**, 2441 (1990).

7. I. Kubo, M. T. Marshall and F. J. Hanke, *Countercurrent Chromatography* (N. B. Mandava and Y. Ito, eds.), Dekker, New York pp. 493-511 (1988) and references therein.

8. J. G. Hawkes, in *The Potato Crop* (P. M. Harris, ed.) Chapman and Hall, London, pp. 1-14 (1978).

9. J. M. Kingsbury, *Poisonous Plants of the United States and Canada*, Prentice-Hall, Englewood Cliffs, pp. 287-294 (1964).

10. S. B. Mahato, A. N. Ganguly and N. P. Sahu, *Phytochemistry*, **21**, 959 (1982).

11. A. Arnald and H. Georgina, *Toxicol Lett.*, **12**, 151 (1982).

12. K. Fukuhara and I. Kubo, *Phytochemistry*, **30**, 685 (1991).

13. Z. Lidert, K. Wing, J. Polonsky, Y. Imakura, M. Okano, S. Tani, M. Y. Lin, H. Kiyokawa and K. H. Lee, *J. Nat. Prod.*, **50**, 442 (1987).

14. K. Hostettmann, M. Hostettmann-Kaldas and O. Sticher, *J. Chromatogr.*, **186**, 529 (1979).

15. J. Polonsky and N. Bourguignon-Zylber, *Bull. Soc. Chim. France.*, 2793 (1965).

16. I. Kubo, Y. Asaka, M. J. Stout and T. Nakatsu, *J. Chem. Ecol.*, **16**, 2581 (1990) and references therein.

17. M. Zhang and I. Kubo, *Insect Biochem.*, in press.

18. F. J. Hanke and I. Kubo *J. Chromatogr.*, **329**, 395 (1985).

19. I. Kubo and T. Nakatsu, *LC·GC*, **8**, 933 (1990).

20. M. Zhang, M. J. Stout and I. Kubo, *Phytochemistry*, **31**, 247 (1992).