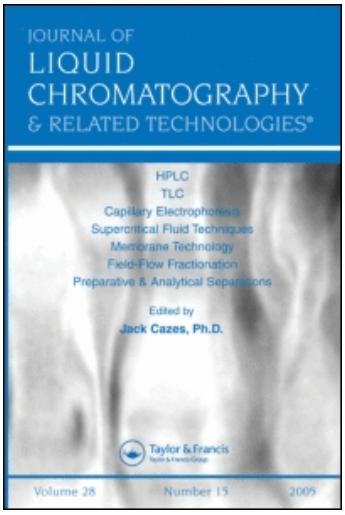

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SCREENING NATURAL PRODUCTS. BIOASSAY-DIRECTED ISOLATION OF ACTIVE COMPONENTS BY CENTRIFUGAL PARTITION CHROMATOGRAPHY

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ABSTRACT

A Centrifugal Partition Chromatograph (CPC), Model LLN by Sanki, was used on routine basis as a primary tool for over 200 assay-directed fractionations in search of active principles from extracts of natural products. Various extracts were found active in several receptor and enzymatic assays incorporated into a high capacity screening system targeting discovery of new antiinflammatory, immunomodulating and antiviral agents. Centrifugal partition chromatography is well suited for performing the assay-directed fractionations, since like other countercurrent techniques it does not involve solid phase adsorbents and thus is inherently less destructive. The instrument was found reliable, simple to operate and applicable to the entire range of polarity of natural products. It can handle milligram as well as multigram quantities.

This method alone was applied to a mixture of saponins from an extract of *Alysicarpus* sp. Isolation and subsequent identification of four new saponins, Alysicarpins A, B, C, and D allowed a necessary *in vitro* evaluation of their therapeutic potential. An extract of *Psychotria acuminata*, a tropical forest plant, gave inhibitory response in another assay. Two crucial fractionation steps carried out by the CPC increased the concentration of the active components

over 100 times. These steps greatly facilitated isolation of two plant pigments, Pheophorbide A and Pyropheophorbide A, each present in the dry plant material at a concentration of 0.5 ppm.

The high resolving power of the 1000E (prep.) cartridges was determined by a direct comparison with the 250W (analyt.) cartridges. The 1000E cartridges were applied to a scale up isolation of a minor (50 ppm) bioactive component.

INTRODUCTION

A Centrifugal Partition Chromatograph (CPC), Model LLN by Sanki, equipped with analytical 250W as well as 1000E preparative cartridges was used as a primary tool in assay-directed isolations of active principles from extracts of natural products. The activity of extracts was determined in several receptor-type and enzymatic assays incorporated into a high capacity screening system targeting discovery of new antiinflammatory, immunoregulating and antiviral agents. The assays were highly automated and capable of screening hundreds of solutions per week. During three years, 1987 - 90, the natural products program contributed to the overall screening effort with 7700 submitted extracts of plant and microbial origin. This included extracts derived from 2100 plant species.

Natural products are a rich source of chemical diversity with a great potential for discovery of new drugs. Screening of natural products, which may lead to these drugs, presents special problems not encountered with pure compounds. The enormous chemical complexity of extracts contributes non-specific responses and synergistic phenomena resulting in so called "false positives". For this reason, a bioassay-directed fractionation of an active extract does not always lead to the isolation of active principles. An apparent loss of activity on separation of synergistically acting components of low individual potency cannot be easily distinguished from the loss of activity resulting from chemical changes induced by a particular isolation technique. Use of the least destructive separation methods when performing the bioassay-directed isolation

of components of undefined *a priori* stability is therefore highly desirable. Centrifugal partition chromatography fulfills this condition. Its versatility is furthermore underlined by the speed of operation, straightforward scaling up and applicability toward the entire range of polarity of natural products.

RESULTS

During three years approximately 200 runs with CPC model LLN were performed in the pursuit of active components of various extracts. In the employed methodology the first fractionation step was frequently carried out on a specially designed fast flow silica column with a steep polarity gradient. The resulting fractions comprising components of similar polarity, were further fractionated routinely by the CPC. In the few cases, when isolation was succesfuly achived by CPC, other approaches based on solid-phase chromatography, resulted in a low recovery or even loss of activity.

The examples given below illustrate the essential role centrifugal partition chromatography played in accomplishing:

- Isolation of very minor plant pigments - pheophorbides. This example is also representative of demonstrating a general approach toward assay-directed fractionation.
- Separation of a mixture of saponins into individual components, including easily hydrolysable Alysicarpin D.
- Scale up isolation of a minor plant pigment.

Isolation of Pheophorbide A and Pyropheophorbide A. Activity of an extract of *Psychotria acuminata* (Rubiaceae), a tropical forest plant, was detected by a cellular receptor-type assay. Dry leaves, 730 grams, on extraction with two gallons of aqueous ethanol afforded a residue E1, 50.0 grams (Fig. 1).

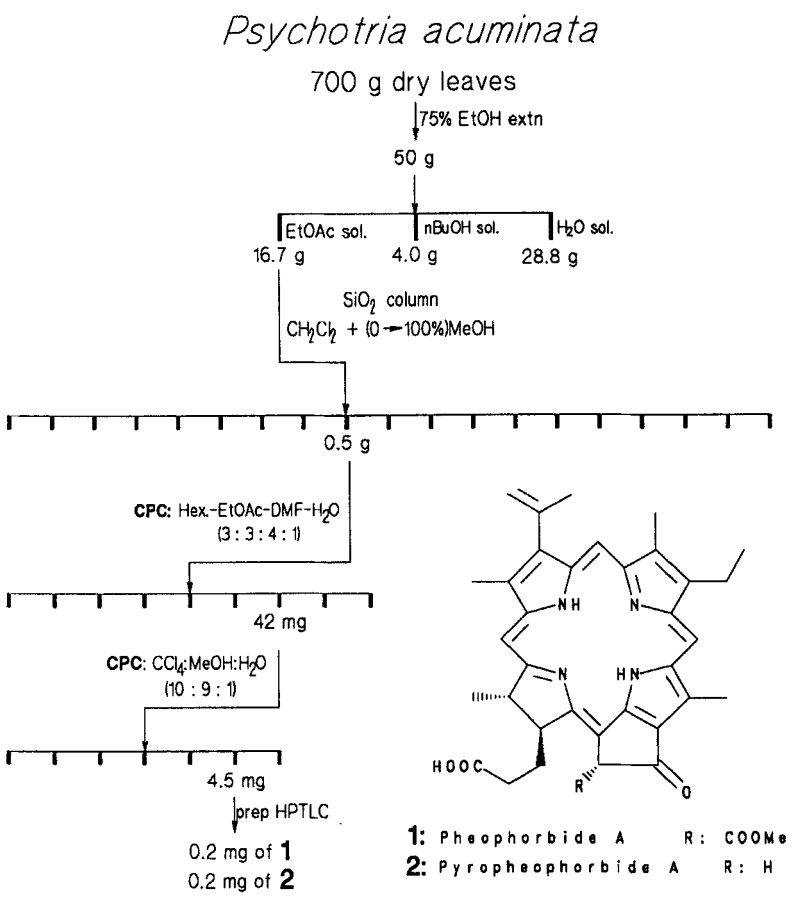


Figure 1: Bioassay-directed fractionation leading to the isolation of Pheophorbide A and Pyropheophorbide A.

This extract was partitioned between water and EtOAc to give 16.7 grams of a residue E2 from the organic layer. Out of this, 12.5 grams was fractionated on a column (Silica Gel 60, Merck 9385-9, 230-400 mesh, 100 grams) and eluted with CH_2Cl_2, 500 ml, containing 0, 1, 2, 4, 8, 16, 30% (v/v) of MeOH. Test tubes, each containing 25 ml of the eluent were combined according to similar (by TLC) content. The assay indicated activity in fractions F1 (test tubes 91-97,

inhib. 38%, 498 mg) and F2 (test tubes 98-101, inhib. 40%, 104 mg). The larger fraction F1, was chosen for subsequent countercurrent fractionation. Partitioning was carried out with 440 mg of F1 using 12 250W cartridges, ascending mode, C_6H_{14}-EtOAc-DMF-H_2O (3:3:4:1), 800 rpm, flow 1.3 ml/min, 7.0 ml/test tube. Test tubes containing eluent of similar composition (by TLC) were combined into 11 fractions. The activity was retained in fraction F3 (test tubes 66-80, inhib. 48%, 42 mg). A second countercurrent partitioning was performed on fraction F3, using 12 250W cartridges, ascending mode, CCl_4-MeOH-H_2O (10:9:1), 600 rpm, flow 0.5 ml/min, fraction vol. 7.0 ml. This time activity was detected in fractions F4 (test tubes 21-25, inhibition 66%, 2.3 mg) and F5 (test tubes 26-35, 51% inhibition, 4.4 mg). Both, F4 and F5 shared the same green pigments. Information gathered from the assay suggested that they were responsible for the biological activity. In order to avoid contaminating these few milligrams by the concentrated contaminants of the solvents it was decided to do the last purification step by TLC. A solution of F5 was applied horizontally to two 10 X 20 cm HPTLC silica plates (Merck 15552) and developed in CH_2Cl_2-MeOH (88:12) to give two bands of green pigments, at Rf 0.66 and 0.60, respectively. The upper band afforded Pheophorbide A **[1]** (0.2 mg, 52% inh. @ 25 μg/ml) and the lower Pyropheophorbide A **[2]** (0.2 mg, 46% inh. @ 25 μg/ml). Both pigments were identified by spectral methods (1).

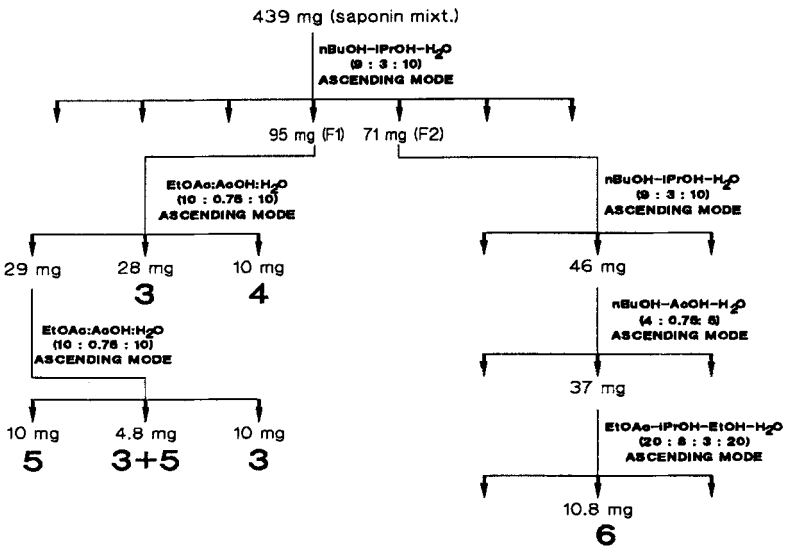
Pheophorbide A and Pyropheophorbide A are chlorophyll degradation products and they occur in *Chlorella* spp., abalone, salted (pickled) vegetables (2) and sea sediments (3) among others. Consumption of food products containing **1** and **2** is a matter of concern because it may induce photosensitivity dermatitis.

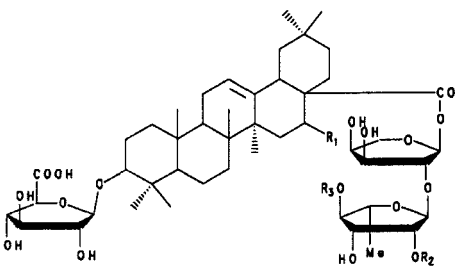
Isolation of Alysicarpins [A-D]. An aqueous-ethanolic extract of an African plant, *Alysicarpus* sp. (Leguminosae) showed activity in an assay monitoring cellular immune functions. The active fraction contained many

components, most being saponins, which were difficult to analyse by analytical HPLC. In order to conclude the research it was necessary to isolate, structurally characterize the components and evaluate their activity in pure state. Preliminary information indicated that the extract contained primarily pentacyclic triterpene saponins of the oleane type. Significant amounts of hydrolysis products appeared after each CPC operation indicating that at least some of the components had labile sugar linkages. Caution was also required because of the anticipated difficulties in obtaining additional supplies of the same plant material.

The entire saponin sample, 439 mg, was subjected to an initial countercurrent fractionation using 6 250A cartridges, nBuOH-iPrOH-H_2O (9:3:10), ascending mode, 1300 rpm, flow 2 ml/min, 5 ml/test tube. Analytical TLC was carried out using HPTLC silica plates (Merck 15552) developed in iPrOH-AcOH-H_2O (84:2:14). The activity was found in the pulled fractions F1, (test tubes 21-52, 95 mg) and, F2 (test tubes 53-69, 71 mg). Analysis of the composition either by C18-RP HPLC or HPTLC silica gel did not differentiate the components satisfactorily, but it was estimated that F1 contains two main components, while F2 - only one.

Fraction F1 was subjected to a CPC run (12 250A cartridges, ascending mode, EtOAc-AcOH-H_2O, 10:0.75:10, flow 2 ml/min, 800 rpm, 7 ml/test tube). Test tubes 59-74 afforded an echinocystic acid saponin named Alysicarpin A **[3]**, which is α-D-apiosyl-(1 →4)-α-L-rhamnopyranosyl-(1 →2)-α-L-arabinosyl ester of 3-(β-D-glucurono)-echinocystic acid, MW 1058, $[\alpha]_D^{20}$ -220° @ c=4.6 mg/mL of EtOH, 28 mg, >95% purity. The structure was rigorously characterized by numerous experiments with ^{13}C NMR, ^{1}H NMR and FAB-MS. The sugar linkage was established by extensive degradation and derivatisation experiments monitored by GC-MS. Test tubes 75-94 afforded Alysicarpin B **[4]**, MW 1072, 10 mg, >95% purity. The content of test tubes 29-43 considered initially to be a less pure Alysicarpin A was subjected to an additional run





	R_1	R_2	R_3
3:	OH	H	β-D-apiosyl
4:	OH	α-L-rhamnopyranosyl	H
5:	H	H	β-D-apiosyl
6:	OH	α-L-rhamnopyranosyl	β-D-apiosyl

Figure 2: Purification of Alysicarpins A (3), B (4), C(5) and D (6) by rotational countercurrent partitioning.

under similar conditions. A thorough correlation of the HPLC analysis with HPTLC led to an unexpected finding of Alysicarpin C [5], MW 1042, 10.0 mg, >95% purity. Alysicarpin C has sugar chains identical with those of Alysicarpin A but the aglycone is oleanolic acid rather than echinocystic. An additional 10.0 mg of **3** (>95% purity) was recovered from test tubes 52-65.

Isolation of the main component of fraction F2, estimated initially at approx. 60% purity turned out to be more troublesome and resulted in low final yield. First attempts indicated that this saponin, named Alysicarpin D [6] underwent cleavage of some of the sugar linkages on standing in solution and also during evaporation of its solutions. This hydrolysis-related instability added one extra CPC run to the purification process, which ended with 10.8 mg of **6** (>93% purity). The ^{13}C NMR and FAB MS data established that this saponin, MW 1204, contains echinocystic acid and five sugars. Its structure is parental to **3** (after loss of terminal rhamnose) and to **4** (after loss of apiose). All four Alysicarpins are novel natural products (Fig. 2). The isolated quantities were sufficient for structure determination as well as concluding their *in-vitro* evaluation (4).

Application of 1000E preparative cartridges for scale up isolation. Identification of an active component can be accomplished with only milligram quantities, but this is usually only the first step in the discovery process. For more comprehensive studies larger quantities are necessary. In one instance, an assay-directed isolation afforded 2 milligrams of a naphthoquinone. Collection of ten kilograms of a different plant material known to contain the same component was followed by an extraction, which afforded 280 grams of a residue. Among the explored solvent systems there were some which partitioned the naphthoquinone and some, which retained it mainly in one phase. The last solvent systems, being impractical for countercurrent chromatography, were applied to the crude extract, helping to concentrate the component. Thus, the entire extract was partitioned between 4 liters of hexane-

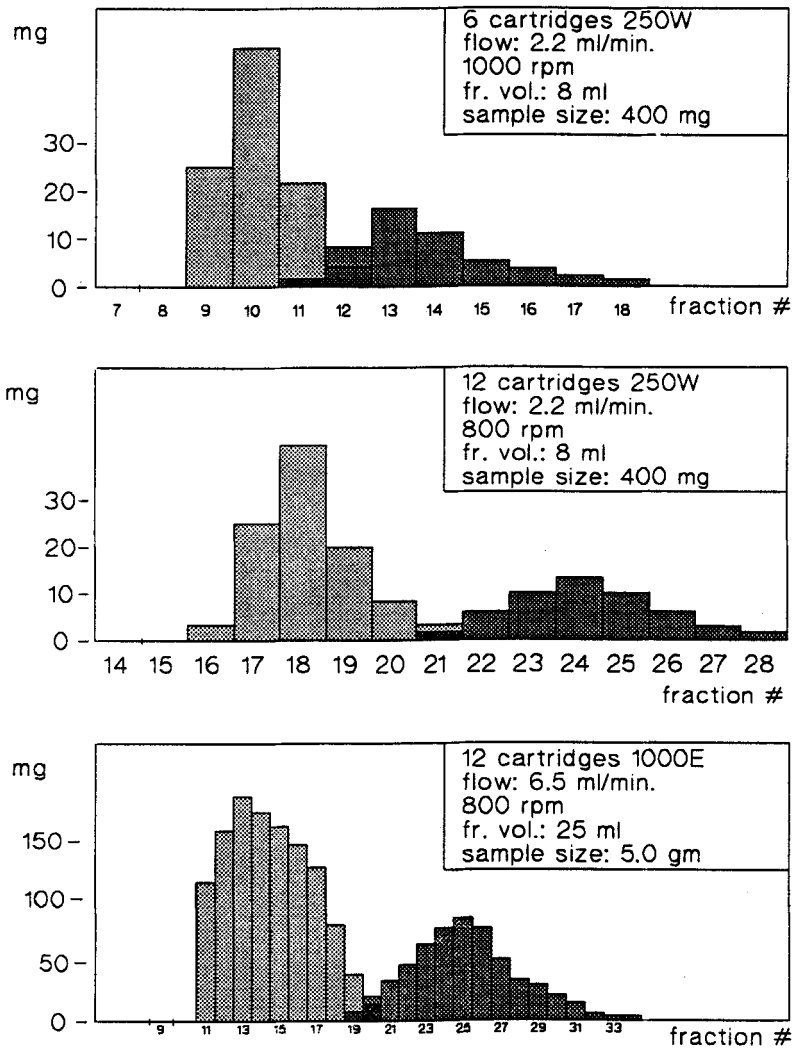


Figure 3: Separation of two alkaloids from *Voacanga africana*, using Countercurrent Partition Chromatograph Model LLN (Sanki) with three selected cartridge configurations. All runs performed in ascending mode in hexane-iPr_2O-MeOH-H_2O (5:5:8:2).

MeOH-H_2O (2:1:1). The upper layer containing over 95% of the component on evaporation gave 62 grams of a residue. This, in turn, was partitioned between hexane-acetonitrile. The lower layer containing again over 95% of the active component yielded 4.8 grams. At this stage the amount became easy to handle and was chromatographed using 12 1000E cartridges in hexane-EtOAc-DMF-H_2O (15:21:10:2), ascending mode, 800 rpm, flow 2.2 ml/min, 20 ml/test tube. The sample, in order to accommodate limited solubility of some components was dissolved in a mixture of both phases and loaded through a 100 ml sample chamber. Test tubes 43-52 provided 1.2 grams of the component of approximately 50% purity. At this stage, final purification of the naphthoquinone was easily achieved by crystallization.

Commonly, first steps of a fractionation involve multi-gram quantities of extracts. The ability to handle large quantities was examined on several occasions by using 1000E preparative cartridges, which gave satisfactory results even with loads of 20 grams or more. Specifically, a separation of two *Voacanga africana* (Apocynaceae) alkaloids by preparative cartridges was comparable to that with 250W analytical cartridges but done at a 12 times larger load, 5.0 grams (Fig. 3). On the other hand, comparison between 6 and 12 250W cartridges, both loaded with 400 mg, suggested that while a full set gives a somewhat better separation, the operational time and ease of controlling the parameters such as rotational speed and maximum flow rate are more favorable with 6 250W cartridges.

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