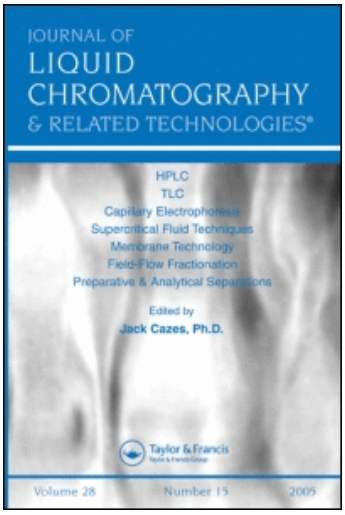

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SEPARATION OF CRUDE PLANT EXTRACTS WITH HIGH SPEED CCC FOR PRIMARY SCREENING IN DRUG DISCOVERY

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EXTRACTIONS AND PURIFICATIONS

SEPARATION OF CRUDE PLANT EXTRACTS WITH HIGH SPEED CCC FOR PRIMARY SCREENING IN DRUG DISCOVERY

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ABSTRACT

High speed countercurrent chromatography (HSCCC) was used in a pre-fractionation pilot study to improve the quality of crude plant samples for primary screening in drug discovery efforts. The methanol extracts of sixty-four plant samples were (i) defatted, (ii) treated with poly-N-vinylpyrrolidone (PVP) for polyphenolic removal, and (iii) fractionated with a multilayer coil planet centrifuge.

The ternary solvent system CH_2Cl_2:MeOH:H_2O (5:6:4, v/v/v) was used based upon elution of known plant natural product standards with ranging polarities. Elution was carried out until a partition coefficient (K) of 1, followed by column contents extrusion

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to exploit stationary phase separation and to increase the polarity range of compounds, fractionated.

Fractionation was found to be consistent for all separated extracts with respect to sample recovery, stationary phase fraction (S_f), and weight distribution by fraction number. Biological evaluation was conducted in 20 mechanism-based, *in-vitro* assays with an evaluation of biodata trends. Bioassay interfering agents such as polyphenolics and fatty acids were chromatographically localized and rapidly identified.

INTRODUCTION

Although natural products offer significant contributions to modern high-throughput drug discovery programs,[1-3] crude extracts present problems with current protocols.[4] For example, natural product extracts harbor compounds, such as tannins, that give false-positives in biological test systems.[5] It has, therefore, been proposed that an initial fractionation procedure can better adapt extracts for high-throughput screening methodologies.[6] Sample quality could theoretically be improved by separating bioactive constituents from interfering components that nonspecifically denature receptor-binding and enzyme assays.[5] A preliminary fractionation could also serve to enrich secondary metabolites found in trace quantities and could potentially accelerate the isolation process.[4-7]

This concept of pre-fractionation to engineer higher quality samples as compared to classical preparation methods, was tested with a pilot study using 64 methanol plant extracts. High speed countercurrent chromatography (HSCCC) was used for fractionation because it is a gentle form of chromatography based upon liquid/liquid partition that does not lead to decomposition, artifact formation, or compound loss due to irreversible solute adsorption onto a solid support.[8,9] Additionally, this form of chromatography readily accepts crude sample mixtures, has a high sample capacity, and results in excellent recovery.[8,9] The separation protocol was developed with the use of five pilot species and pure plant natural products serving as guides.

EXPERIMENTAL

Sample Selection and Preparation

A random set of 64 plant extracts was chosen from a sample collection that is part of a collaborative effort with the Instituto Nacional de Biodiversidad (INBIO), located in Heredia, Costa Rica[10] (Table 1). The plant samples were

Table 1. Plant Samples and Plant Part Fractionated. The Country of Origin for All Species Is Costa Rica

Plant Sample Number	Species	Plant Part
1	*Muntingia calabura* L.	leaves
2	*Alibertia edulis* (Rich.) A. Rich.	leaves
3	*Alibertia edulis* (Rich.) A. Rich.	twigs
4	*Erblichia odorata* Seem.	green stems
5	*Erblichia odorata* Seem.	twigs
6	*Enterolobium cyclocarpum* (Jacq.) Griseb.	twigs
7	*Pseudobombax septenatum* (Jacq.) Dugand	bark
8	*Pseudobombax septenatum* (Jacq.) Dugand	green stems
9	*Pseudobombax septenatum* (Jacq.) Dugand	leaves
10	*Lindenia rivalis* Benth.	leaves
11	*Mabea occidentalis* Benth.	leaves
12	*Mabea occidentalis* Benth.	roots
13	*Drimys granadensis* L. f.	green stems
14	*Drimys granadensis* L. f.	twigs
15	*Ateleia Herbert-Smithii* Pittier	leaves
16	*Calycophyllum candidissimum* (Vahl) DC.	leaves
17	*Hemiangium excelsum* (Kunth) A. C. Sm.	leaves
18	*Schoepfia schreberi* J. F. Gmel.	green stems
19	*Erblichia odorata* Seem.	leaves
20	*Enterolobium cyclocarpum* (Jacq.) Griseb.	leaves
21	*Tapura* sp.	green stems
22	*Vouarana guianensis* Aubl.	fruit
23	*Vouarana guianensis* Aubl.	green stems
24	*Vantanea barbourii* Standl.	fruit
25	*Ampelocera macrocarpa* Forero & A. H. Gentry	green stems
26	*Talisia nervosa* Radlk.	bark
27	*Talisia nervosa* Radlk.	green stems
28	*Buchenavia* sp.	fruit
29	*Buchenavia* sp.	green stems
30	*Olmedia aspera* Ruiz & Pavon	twigs
31	*Tocoyena pittieri* (Standl.) Standl.	green stems
32	*Tocoyena pittieri* (Standl.) Standl.	twigs
33	*Apeiba tibourbou* Aubl.	green stems
34	*Hampea appendiculata* (Donn.-Sm.) Standl.	green stems
35	*Hyeronima poasana* Standl.	green stems
36	*Escallonia myrtilloides* L. f.	twigs
37	*Sciadodendron excelsum* Griseb.	leaves
38	*Davilla kunthii* A. St.-Hil.	whole plant
39	*Trigonia rugosa* Benth.	whole plant

(continued)

Table 1. Continued

Plant Sample Number	Species	Plant Part
40	*Vouarana guianensis* Aubl.	leaves
41	*Vantanea barbourii* Standl.	leaves
42	*Olmedia aspera* Ruiz & Pavon	green stems
43	*Bernoullia flammea* Oliv.	twigs
44	*Alzatea verticillata* Ruiz & Pav.	green stems
45	*Wercklea insignis* Pittier & Standl. Ex Standl.	twigs
46	*Centropogon granulosus* C. Presl.	whole plant
47	*Heliocarpus appendiculatus* Turcz.	twigs
48	*Heliocarpus appendiculatus* Turcz.	leaves
49	*Cyclanthus bipartitus* Poit.	whole plant
50	*Conostegia xalapensis* (Bonpl.) D. Don	twigs
51	*Conostegia xalapensis* (Bonpl.) D. Don	green stems
52	*Hyeronima poasana* Standl.	twigs
53	*Escallonia myrtilloides* L. f.	leaves
54	*Tapura* sp.	bark
55	*Uribea tamarindoides* Dugand & Romero	leaves
56	*Uribea tamarindoides* Dugand & Romero	green stems
57	*Uribea tamarindoides* Dugand & Romero	twigs
58	*Olmedia aspera* Ruiz & Pavon	leaves
59	*Tocoyena pittieri* (Standl.) Standl.	leaves
60	*Apeiba tibourbou* Aubl.	leaves
61	*Apeiba tibourbou* Aubl.	twigs
62	*Bernoullia flammea* Oliv.	bark
63	*Alzatea verticillata* Ruiz & Pav.	leaves
64	*Wercklea insignis* Pittier & Standl. Ex Standl.	leaves

extracted with methanol and classically prepared for pharmaceutical screening with solvent partitioning between hexane, methylene chloride, and water.[11] The same methanol extract was prepared for HSCCC fractionation using an initial defatting step by partitioning the extract (1.5 g) between hexane (15 mL) and 90% aqueous methanol (15 mL.) The hexane layer was removed, concentrated to dryness, and weighed. The methanol layer was treated for polyphenolic removal with poly-N-vinylpyrrolidone (PVP) in the batch mode.[12,13] A PVP methanol slurry (30 mL, 3.0 g equivalent of dried PVP) was added to the methanol layer and placed on a shaker overnight. The solvent was removed from the PVP with a vacuum manifold, the resin washed with MeOH (10 mL), and the combined eluates concentrated to dryness.

Separation with High Speed CCC

Separations were carried out using a multilayer coil planet centrifuge obtained from P.C., Inc. (Potomac, Md., USA) containing a single coil of #14 teflon tubing (1.68 mm I.D.) with a volume of 300 mL and a β value ranging from 0.57 to 0.85. The ternary solvent system CH_2Cl_2:MeOH:H_2O (5:6:4, v/v/v) was used in normal- and dual-phase mode. The detanninated and defatted MeOH feeds were dissolved in up to 500 mg aliquots with equal portions of pre-saturated mobile and stationary phases (18 mL total volume). Greater sample solubility was achieved by using this mixture of the solvent phases, than could be achieved with either of the above single phases. Elution was carried out with one column volume of mobile phase, rotation stopped, and the column contents extruded in the forward direction with methanol. During elution with mobile phase, the column rotation was 800 rpm and the mobile phase had a flow rate of 3.0 mL/min.

Evaluation of Natural Product Standards

HSCCC retention for each of the natural product standards was determined with the partition coefficient, K. The K value was defined as C_S/C_M, with C_S and C_M representing solute concentrations in stationary and mobile phase, respectively.[14] The fraction of elution for each of the natural product standards was determined with Thin Layer Chromatography (TLC). Pre-coated silica gel 60 F_{254} 10 X 20 cm plates with a 0.25 mm thickness (E. Merck, Darmstadt, Germany), were spotted horizontally with sample eluant. The following solvent systems and spraying reagents were used (i) ethyl acetate/2-butanone/formic acid/water (5:3:1:1, v/v/v/v), 3% cesium sulfate in 3N H_2SO4, (ii) cyclohexane/diethylamine (3:1, v/v), Dragendorff's reagent, (iii) CH_2Cl_2/MeOH/H_2O (8:2:0.2, v/v/v) and *p*-anisaldehyde for the flavonoid and coumarin standards, the alkaloids, and the terpenoids, respectively.

Sample Collection and Assay Plate Preparation

The eluent for each sample fractionated was collected into 16 x 100 mm test tubes at 12 mL per tube for a total of 47 fractions. The void volume was discarded and the fractions were concentrated with a TurboVap LV evaporator (Zymark, Hopkinton, Massachusetts, USA) and weighed. The fractions were dissolved in DMSO to a maximum redissolution concentration of 10 mg/mL, with a MultiPROBE II liquid handling station (Packard, Meriden, Connecticut, USA). A higher end concentration was used if redissolution required greater than 10 mL

to achieve the 10 mg/mL target concentration. Low weight fractions were dissolved in a manner to be up-normalized approximately three-fold.

Experimental infrastructure mandated the use of 16 × 100 mm test tubes for fraction collection, which gave a greater number of fractions (47) than was desired for biological testing (19). Therefore, the following pooling protocol was implemented to limit the number of assay testing samples: Every two fractions were combined for the first eight HSCCC fractions, while every three fractions were combined for the next 27 HSCCC fractions, and every two fractions were combined for the remaining 12 HSCCC fractions. A greater number of fractions were combined for the middle fractions, since as can be seen in Figure 1, plant components elute over a greater fraction range for the middle fractions as compared to the end fractions. The twentieth testing sample for each plant extract was set aside for a feed sample with an assay concentration of 10 mg/mL.

In our 96-well format, 80 positions were occupied by testing samples with 16 wells left available for assay standards. Each plant species had 19 pooled HSCCC fractions and a feed sample, which allowed for four fractionated plant samples to be tested per assay plate.

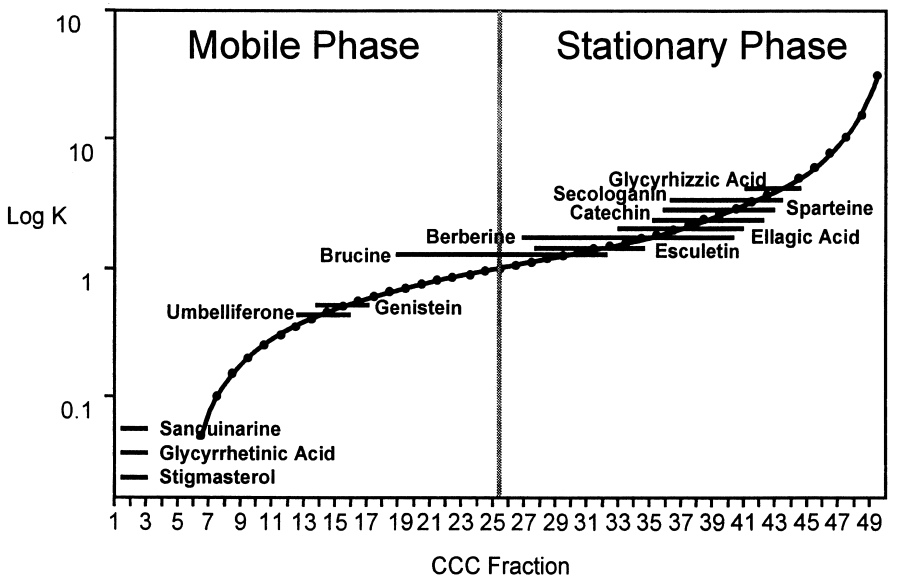

Figure 1. HSCCC fractionation of plant natural product standards. Solvent system: CH_2Cl_2:MeOH:H_2O (5:6:4, v/v/v); normal-phase mode. Natural products plotted by elution order and theoretical K value. Bars represent fractions of elution.

Biological Evaluation

The assay plates with the fractionated HSCCC samples were submitted to our natural product extract library. These assay plates were subsequently distributed to various biological departments for evaluation in biochemical, cell-based, and functional assays.[10] In total, the pre-fractionated samples were evaluated in twenty mechanism-based assays.

RESULTS AND DISCUSSION

Sample Preparation for HSCCC Fractionation

The average yield by weight from 1.5 g of crude plant extract was 241 mg for the hexane partition and 638 mg for the PVP-treated methanol partition. It is this methanol partition that has been classically used to make the CH_2Cl_2 and aqueous sub-fractions. However, experience has shown that although, the classically produced hexane and CH_2Cl_2 extracts can be generally screened, the aqueous extract often results in a significant amount of assay interferences. Therefore, a fractionation protocol was developed for the above methanol partition that would attempt to reproduce the classically derived CH_2Cl_2 extract, while optimizing for the resolution of interesting aqueous soluble constituents from the uninteresting tannin and sugar components in the aqueous sub-fraction.

Solvent System Selection

The solvent system, CH_2Cl_2:MeOH:H_2O, was chosen due to its long history for plant CCC fractionation and its excellent solubilizing properties for crude plant extracts. The solvent ratio for this ternary system was determined by qualitatively evaluating the compound distribution following CCC separation of five representative plant species. Additionally, retention of the following pure plant natural products was measured: (i) flavonoids (genistein, catechin), (ii) coumarins (esculetin, umbelliferone), (iii) alkaloids (berberine, brucine, sanguinarine, sparteine), and (iv) terpenes (18-β-glycyrrhetinic acid, glycyrrhizic acid, secologanin, and stigmasterol). Several glycosides were added as representatives of potentially interesting polar, non-CH_2Cl_2 extractable compounds, present in the aqueous extract. HSCCC separation of the standards using various solvent proportions, determined that a composition of 5:6:4, v/v/v provided the best distribution of standards. Compounds eluted as predicted by structural type with non-polar compounds eluting early (Figure 1).

The normal-phase mode was selected based on the following advantages over the reversed-phase mode: (i) stationary phase loss was less, (ii) stationary

phase fraction, S_f (see below) was more consistent, (iii) there was a better distribution of natural product standards, (iv) there were fewer interfering fractions with HTRF (homogeneous time resolved fluorescence) based assays, and (v) there was an increase in the number of theoretical plates when the "heavier" phase was used as the mobile phase. (Conway, unpublished observations, CentriChrom Inc.)

A modified, dual-mode HSCCC separation was employed for this study. The separation was developed with one column volume of mobile phase only, corresponding to elution of components with a K~1, as column rotation stopped. The column contents were then extruded and fractions collected to exploit the separation of polar components in the stationary phase. Column contents extrusion probably yielded less resolution of the stationary phase components than a true dual-mode operation, but this modified dual-mode method was more compatible with existing equipment. The net result was complete recovery of all injected solutes, with fractions corresponding to elution polarity.[15]

Stationary Phase Retention

Resolution and sample capacity is increased when a larger fraction of stationary phase is retained in the column at equilibrium. The stationary phase fraction (S_f) in HSCCC is defined as the ratio of volume of stationary phase at equilibrium, V_s, to total column volume, V_c. Decreases in S_f result from sample components, such as emulsifying agents, which disturb the liquid-liquid equilibrium. S_f is typically in the range of 0.5 to 0.9 with the CCC instruments used in this study.[16] In our pilot study, the average S_f was 0.69 and was consistent among all 64 samples separated (Figure 2).

Sample Consistency, Recovery, and Weight Distribution

This pilot study demonstrated excellent consistency for fractionation of crude plant extracts with respect to sample recovery and weight distribution. HSCCC fractionation proved to be a robust method, since it was not necessary to repeat any separation, and there was good sample recovery for all extracts. The average recovery in the fractions was 88% of the applied feed sample and was extremely consistent amongst all 64 separated samples.

The sample weight distribution for all samples was similar, and was characterized by high weight at both ends of the polarity range with a low weight profile in the middle fractions. These fractions were subsequently pooled into nineteen assay testing samples (Figure 3). Based upon compound content, the first two or three fractions resembled the classical CH_2Cl_2 extract and the last four-five fractions were similar to the aqueous extract after solvent partition.

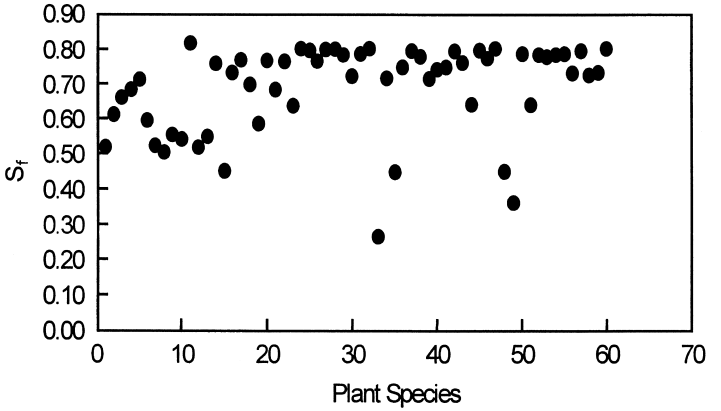

Figure 2. Stationary phase fraction, S_f, for each HSCCC separation. The 64 plant samples represented are referenced in Table 1.

Assay Sample Generation

The goals for assay sample generation were: (i) to increase the concentration of the highly-resolved, low-weight intermediate fractions in an attempt to identify bioactive constituents normally found in trace quantities, and (ii) to make the less-resolved, high-weight end fractions more equivalent to the corresponding crude CH_2Cl_2 and aqueous extracts. The end fractions were reconstituted to a

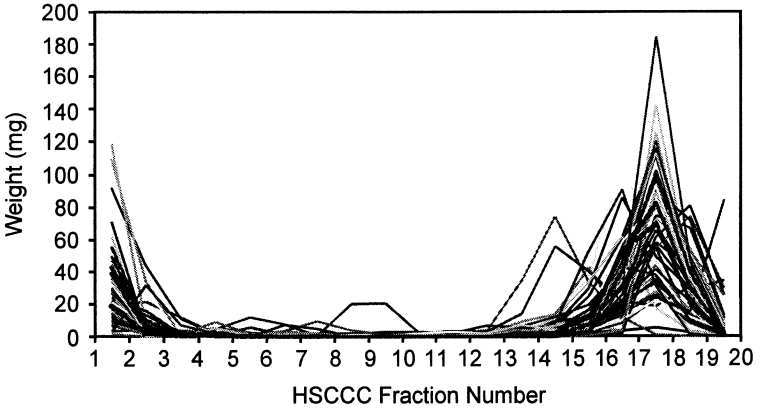

Figure 3. Fraction weight for the HSCCC assay samples for all 64 fractionated plant extracts. Weight distribution is skewed to either end of the polarity range (that is, the end fractions).

concentration similar to the crude plant extracts (10 mg/mL) and the middle fractions were increased approximately three-fold relative to their fraction weight. The fractionated samples were subjected to primary screening in various *in vitro* HTS assays, generally at a single concentration, followed by confirmatory assay(s).

Biological Results

In 20 high-throughput *in vitro* assays tested, each plant sample had three independent opportunities to yield positive results in our biological screens, since the extracts were tested with the following three preparations: (i) the classically partitioned samples (hexane, CH_2Cl_2 and aqueous), (ii) the HSCCC starting material, which was hexane extracted and PVP treated, and (iii) the HSCCC fractionated samples. The confirmed biological activity for these three preparations is summarized in Table 2. The classically partitioned samples, the HSCCC starting material, and the HSCCC fractionated samples identified 14, 18, and 42 plant species, respectively, as having biological activity in at least one of the 20 mechanism-based assays.

To understand the higher hit rate for the HSCCC fractionated samples, the distribution of the confirmed active fractions relative to plant species is demonstrated in Figure 4. Bubble diameter is directly proportional to the number of assays for which a given fraction was designated as active and ranges from 1 to 5 assays. Two major groupings of activity by fraction number were observed, with the largest group containing 133 actives in the polar fractions (fractions 14-19), and the second corresponding to 37 actives in the most non-polar fraction (fraction 1). This grouping of activity corresponded to the highest weight fractions and also to those that were the most likely to contain common interfering agents,

Table 2. Distribution of Confirmed Active Samples from 20 In Vitro Assays

	Classical Partition	HSCCC Starting Material	HSCCC Fractionation
Total Samples	19	6	121
Total Assay Points (~20 Assays/sample)	384	128	2432
Confirmed Active	37 (9.6%)	46 (36%)	193 (7.9%)
Number of the 64 Original Plant Samples Yielding Activity	14 (22%)	18 (28%)	42 (66%)

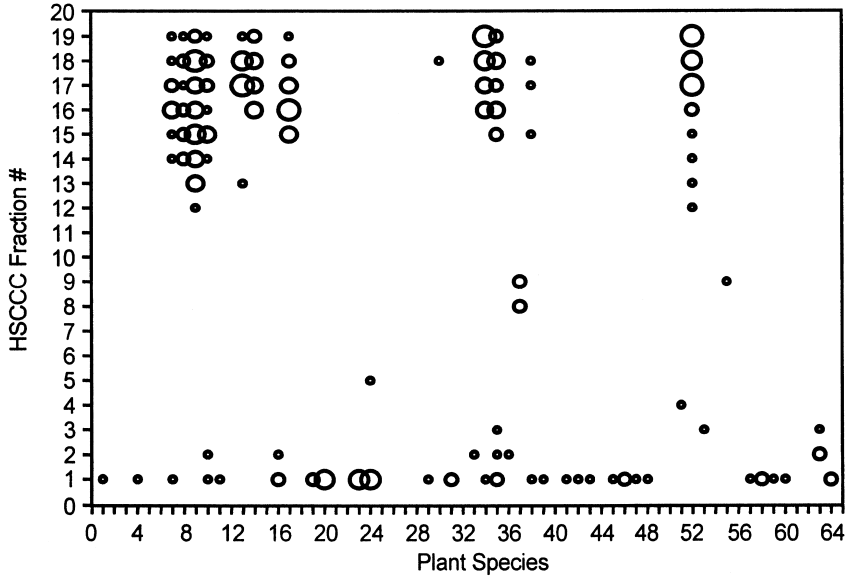

Figure 4. Bioactivity distribution for mechanism-based assays of the HSCCC separated plant samples. Bubble diameter is directly proportional to the number of assays for which a given fraction was designated as active and ranges from 1 to 5 assays. The 64 plant samples represented are referenced in Table 1.

such as polyphenolic compounds (fractions 14-19) and fatty acids (fraction 1). Thus, the simplest explanation for the higher hit rate for the fractions, relative to the other extracts, is that the HSCCC fractionation simply concentrated interfering compounds.

The two groups of active fractions were not uniformly distributed amongst the 64 plant samples. For example, only 10 of the 64 plant samples had more than one active fraction between fractions 14 and 19. There was also a significant difference in activity distribution for the 20 *in vitro* screening assays, since the assays used several different biochemical methodologies and varied in their susceptibility to common interfering agents. For example, only seven of the assays had a significant number of samples testing positive in the 14-19 fraction range; whereas one nuclear receptor assay, known to give false positives from fatty acids, had the majority of active samples from fraction one (20 of 37). With this solvent system and protocol, HSCCC concentrates the total extract fatty acids into the first fraction.

A grouping of activity among several plant samples suggests a common active component (possible interfering agent). For example, biodata for all fractions for a particular protease assay were analyzed by plotting the primary screen-

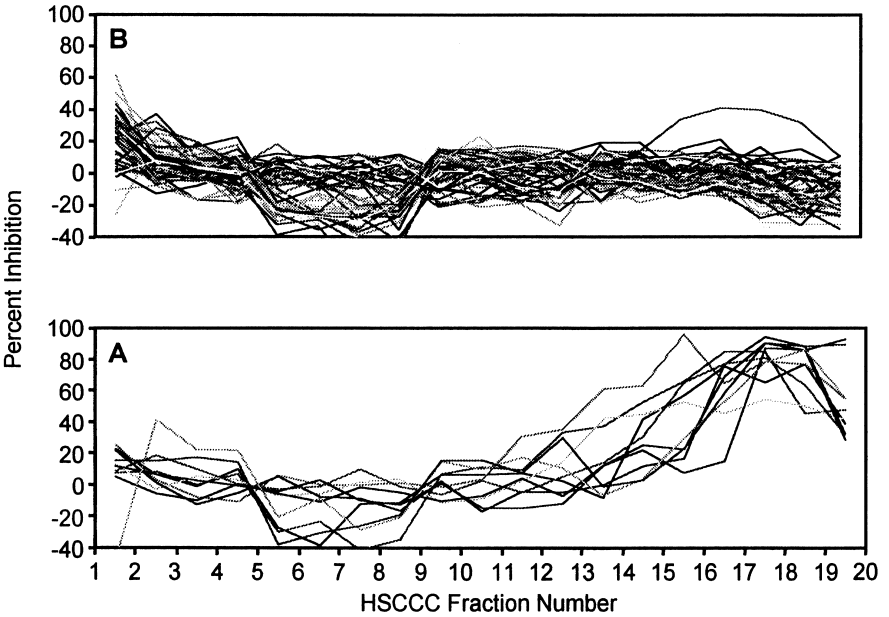

Figure 5. Percent inhibition data plotted against HSCCC fraction number for a protease screening assay. A) All active samples. B) All non-active samples. All active samples exhibit a broad peak of activity in the polar region of the separation; whereas, no activity is exhibited in the polar region for the non-active species.

ing data (% inhibition) versus fraction number for all plant samples, which had one or more active fractions (Figure 5A) and for those without an active fraction (Figure 5B). All 10 of the active plant samples exhibited the same broad peak of activity in fractions 14-19. Since this protease assay is susceptible to polyphenolic interferences, the samples with the same uniform distribution of activity in fractions 14-19 were disregarded for follow-up study and attributed to polyphenolics.

Potential interfering compounds were, however, removed from the intermediate fractions, 2 through 13, and these yielded a total of 23 (0.15%) actives. This group was distributed over 14 (22%) different plant samples, 8 different *in vitro* assays, and contained 19 unique fractions. Several of these fractions were from plant samples that did not show activity when extracted using the other two methods. This, of course, is a desirable outcome for a pre-fractionation protocol and evaluation of the active components in these samples is ongoing.

In summary, when bioactivity distribution is assessed for each assay, activity uniqueness or a lack thereof can be determined. Figure 6 illustrates examples of the active fraction distribution for three different types of assays: A) an assay

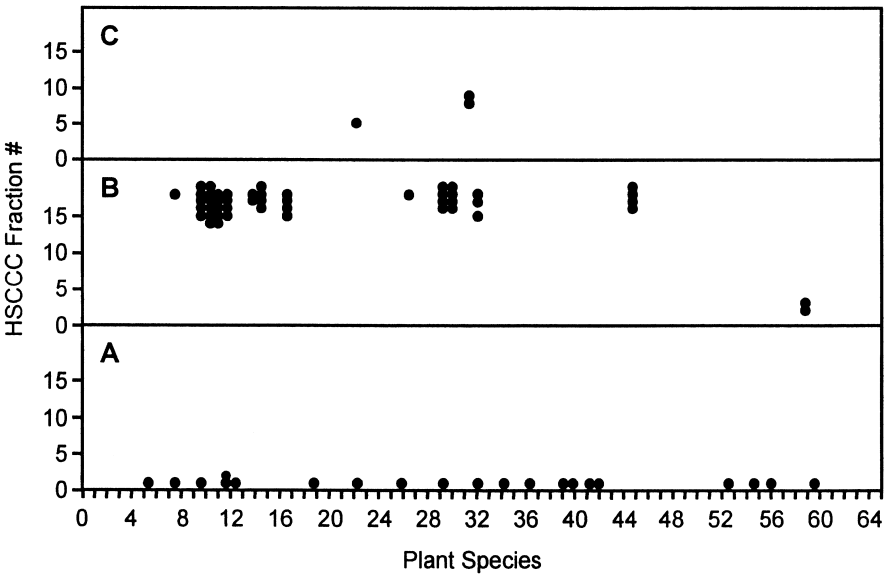

Figure 6. Bioactivity distribution for three in vitro assays. A) Assay susceptible to fatty acid interference. B) Assay susceptible to polyphenolic interference. C) Assay with no interference.

susceptible to interference from high concentrations of fatty acids; B) an assay susceptible to interference from polyphenols and tannins; and C) an assay with little or no known interferences. Interference from polyphenolic compounds (Figure 6B) was suggested by grouping of sequential polar fractions in several extracts with very little other activity. Fatty acid interference (Figure 6A) was suggested by the predominance of activity in fraction one. Finally, the ideal scenario is shown in Figure 6C; no interference and the identification of activity in the middle, low-weight/high-resolution portion of the fractionation.

CONCLUSIONS

This pilot study of pre-fractionation demonstrated the robustness and reproducibility of preliminary fractionation using HSCCC prior to high-throughput drug screening. Consistency with respect to weight distribution, percent recovery, and stationary phase fraction for extracts of disparate plant samples, proved that a standard fractionation protocol can be implemented. By analyzing data trends within the fractions of a single extract source and across many different samples, assay interferences were rapidly recognized, while assay outliers,

fractions that represented unique spikes of bioactivity, were identified. Assay interferences were chromatographically localized within the HSCCC fractionated samples and, in many cases, were more concentrated than the corresponding crude extract. By chromatographically concentrating areas of interferences, there is a possibility that an active could be masked by an interfering group, such as polyphenolics. However, in many assays, the entire fraction set was free from interferences, thus, no masking of activity was created.

Implementation of an extract pre-fractionation protocol for natural products extracts would require the separation of thousands of extracts. This type of "industrialization" would require a careful cost-benefit analysis to determine the rate of "unmasking" and discovery of previously unseen metabolites. Such fractionation is labor and resource intensive. Robust equipment and protocols would be necessary to ensure the reproducibility of the process.

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