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Preparative separation of pyrrolizidine alkaloids by high-speed counter-current chromatography

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Abstract

We have applied a high-speed counter-current chromatography (CCC) technique to the separation and purification of pyrrolizidine alkaloids from *Amsinckia tessellata*, *Symphytum* spp., *Trichodesma incanum* (Boraginaceae), and *Senecio douglasii* var. *longilobus* (Asteraceae). Alkaloidal fractions were separated in a solvent system composed of a chloroform mobile phase and 0.2 *M* potassium phosphate buffer, of an optimum pH, as the stationary phase. Up to 800 mg of sample could be successfully separated in a single run, with excellent resolution of alkaloids. Lycopsamine and several of its acetylated derivatives were resolved from alkaloidal fractions of *Amsinckia* and *Symphytum*. However, diastereomeric pairs such as 7-acetyl-lycopsamine and 7-acetyl-intermedine, could not be separated. The presence of diastereoisomers was determined by gas chromatography–mass spectrometry. *Trichodesma* contained predominantly trichodesmine, which was resolved from a small quantity of incanine. We report the electron impact mass spectrum of incanine for the first time. Resolving power of CCC was sufficient to separate the closely related alkaloids senecionine and seneciphylline from *Senecio*, in addition to florosenine and retrorsine. Pyrrolizidine alkaloid compositions of the four species, determined by mass spectral techniques, were consistent with literature, except for the lack of riddelliine and the presence of the otonecine-based florosenine in *Senecio douglasii* var. *longilobus*.

Keywords: Preparative chromatography; Counter-current chromatography; Pyrrolizidine alkaloids; Alkaloids

1. Introduction

Counter-current chromatography (CCC) is a liquid–liquid partition chromatographic method that does not utilize a solid support matrix. With the development of new instruments for CCC, faster separations have become possible [1]. Ito et al. [2] observed that two immiscible liquids will become uniformly segmented in the coils of a helical column when the latter is rotated. The liquid designated as the stationary phase is retained by centrifugal force, while the mobile phase is pumped through the rotating column. Advantages of CCC include total sample recovery, good resolution (350–1000 plates) and high reproducibility. Since there is no solid support mechanism within the column, sample contamination and adsorption are normally eliminated [1]. A recently developed instrument for high-speed CCC, the multilayer coil planet centrifuge [3] used in these experiments is well suited for separation and purification of natural products on a preparative scale [1,4–6].

Pyrrolizidine alkaloids (PAs) are a large group of structurally similar compounds with wide geographi-

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cal and botanical distribution [7,8]. These compounds are hepatotoxic upon metabolism and represent a threat to both humans and livestock through accidental or intentional ingestion of pyrrolizidine-containing plants [7,9]. They are important in plant chemosystematics [10] and in the ecology of certain insects [11]. It is often necessary to isolate and purify large amounts of PAs for toxicological or other studies. Because PAs rarely occur in plants as a single alkaloid, there has been a need for convenient, rapid separation and purification methods for these compounds. Mixtures of PAs have been separated and purified with limited success using chromatography on various adsorptive media [7]. Earlier reports of PAs separated with counter-current techniques utilized the much slower droplet counter-current chromatography (DCCC) [12–15], or were performed on an analytical scale [16]. The separation of PAs on newer high-speed instruments has not been reported, and has been little used for alkaloids in general compared to other natural products of plant origin [6]. We demonstrate the preparative separation and purification of PAs (Fig. 1) isolated from *Amsinckia tessellata* A. Grey, *Symphytum* spp. (comfrey root), *Trichodesma incanum* Alph. DC., and *Senecio douglasii* DC. var. *longilobus* (Benth.) L. Benson, using high-speed CCC.

2. Experimental

2.1. Plant material

Amsinckia tessellata was collected near Tucson, Arizona, in the spring of 1994 and 1995. *Senecio douglasii* var. *longilobus* was collected in the spring of 1995 from Sonoita, Arizona. Seeds from *Trichodesma incanum* were collected from Uzbekistan in the summer of 1990. *Symphytum* spp., sold commercially as comfrey root, was purchased from a local supermarket. Whole-plant material from *Amsinckia* and *Senecio* was air-dried prior to extraction.

2.2. Alkaloid isolation

Prior to extraction, *Trichodesma* seeds and *Symphytum* root were finely ground in an electric coffee mill. Plant material was continuously extracted in a Soxhlet apparatus with EtOH for 24 h. Dried whole-plant material from *Senecio* and *Amsinckia* was ground in a commercial hammer mill through 3-mm mesh, followed by soaking in EtOH for four days in a commercial extractor. All extracts were then reduced under vacuum using a rotary evaporator. The residue was resuspended in H_2O and made acidic (pH 3–4) with citric acid and reduced with Zn dust overnight. The aqueous solution was filtered, and extracted with equal volumes of $CHCl_3$ until the organic phase was clear. The aqueous portion was then made basic (pH 8–9) with 30% NH_4OH and extracted three times with equal volumes of $CHCl_3$. Aqueous phases from *Amsinckia* and *Symphytum* extractions were saturated with NaCl to increase extraction of the more polar alkaloids lycopsamine and intermedine [17,18]. The organic portions were combined, dried over anhydrous Na_2SO_4, and evaporated to dryness yielding the alkaloid fraction. All alkaloid fractions were analyzed by GC–electron impact (EI) MS. Where EI-MS failed to produce a molecular ion, flow-injection atmospheric pressure chemical ionization MS was used.

2.3. Counter-current chromatography

An Ito multilayer coil counter-current chromatograph (P.C., Potomac, MD, USA) equipped with a P.C. #10 coil (PTFE tubing, 2.6 mm I.D., 380 ml capacity) was used in all separations. Potassium phosphate buffer, 0.2 *M*, at an appropriate pH was used as the stationary phase. The mobile phase was $CHCl_3$, driven by a metering pump (Milton-Roy, Riviera Beach, FL, USA), at a flow-rate of 210 ml h^{-1}, in a head-to-tail fashion. Prior to use, the two solvent phases were equilibrated by shaking together in a separatory funnel, and then allowed to separate. The column was initially filled with stationary phase, then rotated at 800 rpm while mobile phase was loaded. The column was considered in equilibrium when only $CHCl_3$ eluted from the tail end. At this point, alkaloid samples, either solid or non-solid, 200–800 mg, were dissolved in 3–5 ml of mobile phase and injected into the column. Mobile phase was collected by a fraction collector. Alkaloid remaining in column after separation was collected by purging the stationary phase with N_2, followed by

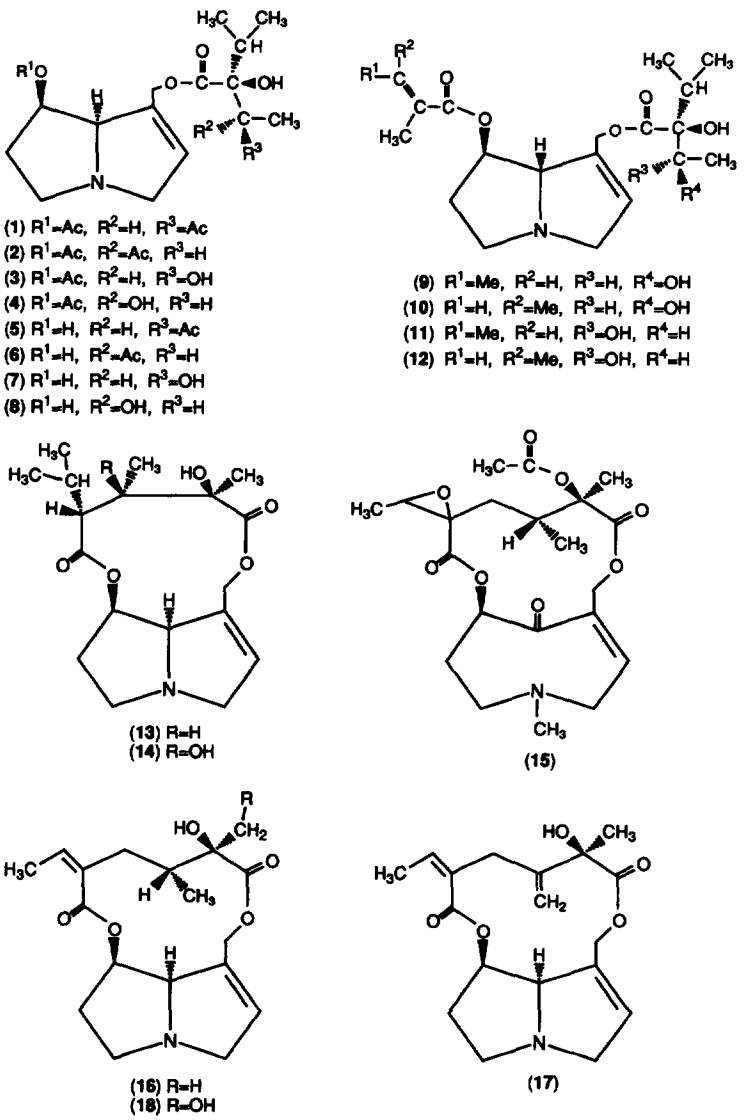

Fig. 1. Structures of pyrrolizidine alkaloids: (**1**) 3,7-diacetyl-lycopsamine; (**2**) 3,7-diacetyl-intermedine; (**3**) 7-acetyl-lycopsamine; (**4**) 7-acetyl-intermedine; (**5**) 3-acetyl-lycopsamine; (**6**) 3-acetyl-intermedine; (**7**) lycopsamine; (**8**) intermedine; (**9**) symphytine; (**10**) symlandine; (**11**) myoscorpine; (**12**) echiumine; (**13**) incanine; (**14**) trichodesmine; (**15**) florosenine; (**16**) senecionine; (**17**) seneciphylline; (**18**) retrorsine.

basifying and $CHCl_3$ extraction. Chromatograms were constructed by measuring relative PA content of each fraction colorimetrically with *p*-dimethylaminobenzaldehyde (Ehrlich's reagent) on a spectrophotometer (Beckman, Fullerton, CA, USA) at 562 nm [19]. Corrected retention time, t'_R, of solute peaks was found by subtracting time of peak maximum of the solvent front from time of peak maximum of the solute. Partition coefficients, K, defined as solute concentration in the mobile phase divided by solute concentration in the stationary phase, were estimated by the method of Zhang et al.

[5]. All peaks were analyzed on TLC and GC–MS or flow-injection atmospheric pressure chemical ionization (APCI) for identification and purity.

2.4. Thin-layer chromatography

Alkaloids were run on silica gel plates. *Amsinckia* and *Symphytum* alkaloids were run in $CHCl_3$–MeOH–NH_4OH (30%) (60:10:0.5, v/v). *Trichodesma* and *Senecio* alkaloids were run in the same solvents but in the ratio 85:14:1. Plates were developed by spraying with methanolic *o*-chloranil (0.5%, w/v), followed by modified Ehrlich's reagent (2% boron trifluoride etherate, v/v and 2% *p*-dimethylaminobenzaldehyde, w/v, in EtOH).

2.5. Gas chromatography–mass spectrometry

2.5.1. Method I

Performed by the Mass Spectrometry Facility, Department of Chemistry, University of Arizona, on a Hewlett-Packard 5890 system using an HP-5 fused-silica capillary column consisting of 5% phenyl–methyl silicone 25 m×0.2 mm with He carrier gas at 23 psig. Conditions: injector temperature, 250°C; starting temperature, programmed at 70°C, held for 1 min, to 300°C at 20°C min^{-1}, held for 7 min; split inj. ratio, 70:1, volume 1 μl (CH_2Cl_2). The column was connected directly to an HP 5970 mass selective detector. EI mass spectra were recorded at 70 eV.

2.5.2. Method II

Performed at the Southwest Environmental Health Sciences Center, University of Arizona, on a Fisons MD800 using a DB-5, 15 m×0.25 mm column with He carrier gas at 5 psig. Conditions: temperature program same as for method I; splitless injection volume, 1 μl (CH_2Cl_2). The column was connected directly to a mass selective detector. EI mass spectra were recorded at 70 eV.

2.6. Flow-injection atmospheric pressure chemical ionization mass spectrometry

Flow-injection APCI was conducted on a Finnigan TSQ triple quadropole mass spectrometer at the same location as GC–MS method II. Conditions: mobile phase, MeOH at 0.5 ml min^{-1}; samples introduced into the ion-source via direct injection; 50-μl injection volume (MeOH); 10 eV offset added to the region between sample introduction and mass analysis to increase fragmentation (in-source collision-induced decomposition). TSQ was equipped with an APCI source and was scanned from 100 to 700 amu at 1 scan s^{-1}.

3. Results and discussion

A biphasic solvent system was chosen for separation of PAs, consisting of a chloroform mobile phase and potassium phosphate buffer, 0.2 *M*, at various pH values (Table 1), as the stationary phase. The large density difference between chloroform and phosphate buffer promotes rapid phase separation and excellent stationary phase retention even at high mobile-phase flow-rates [6]. With an aqueous buffer stationary phase, good solute resolution can be obtained since structurally similar PAs differ in their pK_a values [7]. Additionally, the colored impurities often associated with PA isolations are unretained in an aqueous stationary phase, eluting at the solvent front. Resolution of PAs is determined by partitioning between the two liquid phases. Partition coefficients, *K*, defined as concentration of solute in the mobile phase divided by solute concentration in the stationary phase, ideally fall between 2 and 0.5 for the most efficient separations, but a wide range of *K* values may be acceptable depending on the amount and retention times of impurities (Table 1) [5]. Partitioning of PAs between solvent phases depends on the proportion of alkaloid in the ionized form, as well as its lipid solubility in the free base form. Partition coefficients and corrected retention time, t'_R, of PAs will therefore be profoundly affected by the stationary phase pH (Table 1). Because the stationary phase is a liquid, retained PAs are easily recovered, and their adsorptive loss to various solid chromatographic media as reported by Zalkow et al. [12], is prevented.

Amsinckia tessellata (Boraginaceae) is a member of a small genus of around fourteen species found in the U.S. and other countries [10]. Alkaloid content of *Amsinckia* is variable, but all contain PAs, dominated by the hepatotoxic diastereomers lycopsamine (**7**)

Table 1
Characteristics of separation of pyrrolizidine alkaloid fractions using counter-current chromatography

Species	Alkaloid	Stationary phase pH[a]	t'_R[b] (min)	K[c]
Amsinckia tessellata	3,7-Diacetyl-lycopsamine/-intermedine	7.4	2.6	27.4
	7-Acetyl-lycopsamine/-intermedine		13.1	6.4
	3-Acetyl-lycopsamine/-intermedine		74.0	1.3
	Lycopsamine/Intermedine[d]			
Symphytum spp.	Symphytine and/or isomers	5.6	14.5	6.4
	7-Acetyl-lycopsamine/-intermedine		113.1	0.9
	Lycopsamine/Intermedine[d]			
Trichodesma incanum	Incanine	6.0	7.7	11.3
	Trichodesmine		36.6	2.6
Senecio douglasii var. *longilobus*	Florosenine	5.0	0.9	52.0
	Senecionine		34.9	2.7
	Seneciphylline		70.6	1.4
	Retrorsine[d]			

[a] Solvent system composed of $CHCl_3$ mobile phase and phosphate buffer, 0.2 *M*, at indicated pH, as the stationary phase.
[b] Retention time, t'_R, defined as time of solute peak maximum minus time of solvent front.
[c] Partition coefficients, *K*, estimated according to Zhang et al. [5].
[d] Alkaloid(s) remained in column and were recovered as described in Section 2.

and intermedine (**8**), with smaller quantities of their acetylated derivatives and other uncommon PAs [10]. While human consumption is not known to be a problem with *Amsinckia*, they are a potential rangeland hazard for grazing animals. Roitman [17] used silica gel column chromatography for preparative separation of similar PAs from *A. menziesii*, while Culvenor and Smith [18] employed counter-current distribution to resolve PAs from several *Amsinckia* species. Both these techniques proved time-consuming since successive runs were necessary for complete resolution. *A. tessellata* collected near Tucson, AZ, contained 0.02% PA, per dry weight of plant. These mixed PAs were isolated as a brown gum, as reported previously [17,18]. Fig. 2A shows the resolution of the PAs using an aqueous phase pH of 7.4, from 710 mg of this gum. Three alkaloid peaks were obtained and additional PA remained in the column. Peaks I–III, plus the retained PA yielded a single spot on TLC, but GC–MS indicated each peak consisted of an isomeric pair, producing nearly identical mass spectra, but with slightly different retention times. Peak I, 3,7-diacetyl-lycopsamine/-intermedine (**1**, **2**) (5.0 mg) was retained sufficiently to elute after the colored impurities, and was well resolved from peak II, 7-acetyl-lycopsamine/-intermedine (**3**, **4**) (13.1 mg). Peak III, 3-acetyl-lycopsamine/-intermedine (**5**, **6**) (55.1 mg) completely eluted by 2 h. The peak exhibited small shoulders, possibly due to partial resolution of the two diastereomers. Lycopsamine and intermedine (70 mg) remained in the column and were recovered by purging. Mass spectra were consistent with literature data [10]. As reported by other researchers [17,18], the alkaloids, which were obtained as amber oils, could not be crystallized.

Comfrey, consisting of dried root or leaves of *Symphytum officinale* L. (common comfrey) or *S.* x *uplandicum* Nyman (Russian comfrey) (Boraginaceae), is widely used by humans as a herb, vegetable and food supplement [9]. *Symphytum* contains a mixture of PAs, consisting of open mono- and diesters of retronecine. They are mildly hepatotoxic and are carcinogenic in rats [20], and have been implicated in human poisonings [9,21]. Culvenor et al. [15] used counter-current distribution with a solvent system of chloroform and phosphate buffer, pH 7.5, to resolve 1.8 g of an alkaloid fraction from *S.* x *uplandicum*, yielding eight PAs. As with *Amsinckia*, the alkaloid of comfrey is isolated as a brown gum or tar. Several samples of commercial comfrey root were extracted, with alkaloid content ranging from 0.01 to 0.09% dry weight. Fig. 2B shows the resolution of 586 mg of

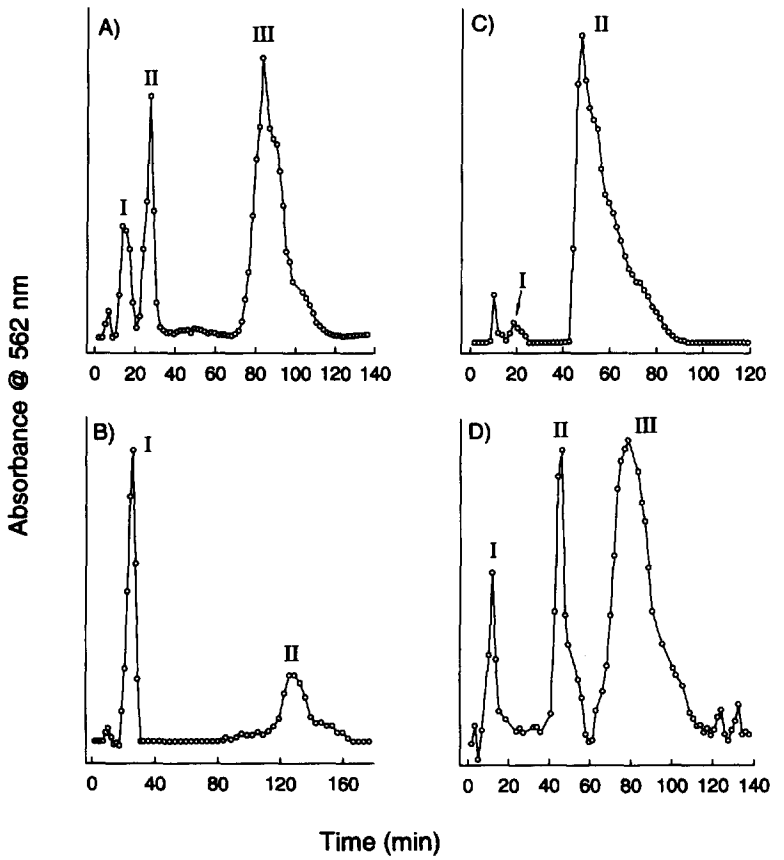

Fig. 2. Resolution of pyrrolizidine alkaloid fractions isolated from various plant sources using high-speed counter-current chromatography. Solvent system consisted of a $CHCl_3$ mobile phase and phosphate buffer, 0.2 *M*, at indicated pH, as the stationary phase. Alkaloid remaining in column was recovered by purging column contents with N_2. (A) *Amsinckia tessellata*; pH stationary phase=7.4; I=3,7-diacetyl-lycopsamine/-intermedine (**1**, **2**), II=7-acetyl-lycopsamine/-intermedine (**3**, **4**), III=3-acetyl-lycopsamine/-intermedine (**5**, **6**); lycopsamine/intermedine (**7**, **8**) remained in the column. (B) *Symphytum* spp.; pH stationary phase=5.6; I=symphytine and/or diastereoisomers (**9–12**), II=7-acetyl-lycopsamine/-intermedine; lycopsamine/intermedine remained in the column. (C) *Trichodesma incanum*; pH stationary phase=6.0; I=incanine (**13**), II=trichodesmine (**14**). (D) *Senecio douglasii* var. *longilobus*; pH stationary phase=5.0; I=florosenine (**15**), II=senecionine (**16**), III=seneciphylline (**17**); retrorsine (**18**) remained in the column.

alkaloidal fraction of comfrey, using a stationary phase pH of 5.6. Two well resolved peaks were obtained, with a third alkaloid remaining in the column. The colored impurities, unretained by the stationary phase, eluted with the solvent front. TLC indicated one spot for each eluted peak and the retained material, although GC–MS revealed the presence of isomers. Peak I (19.0 mg) consisted of 3 isomeric PAs, all producing nearly identical mass spectra, consistent with that reported for symphytine and its 3 diastereomers (**9–12**) [10]. Peak II, 7-acetyl-lycopsamine/-intermedine (13.6 mg), was retained until 113.1 min, due to the low pH of the stationary phase compared to the previous separation. Lycopsamine and intermedine (210.6 mg) remained in the column. Following chromatography, PAs were obtained as amber oils, but again could not be crystallized.

Native to Asia, *Trichodesma incanum* (Boraginaceae) has been responsible for outbreaks of human and livestock poisonings, reviewed in the Russian literature [22,23]. In addition to being hepatotoxic, causing an equine disease known as 'suiljuk', *T. incanum* is recognized as neurotoxic [9].

T. incanum has been shown to contain two PAs, incanine and trichodesmine [24]. Previously, trichodesmine isolated from various *Crotalaria* was separated from other PAs by column chromatography using Celite and Florisil, preparative TLC and DCCC [25–27]. Ethanolic extracts of seeds and surrounding capsule of *T. incanum* yielded 1.1% PA by weight. In agreement with the earlier account [24], the mixture of alkaloids consisted of two cyclic diesters of retronecine, incanine (**13**) and trichodesmine (**14**), as determined by GC–MS. Fig. 2C shows the chromatogram of a separation of 250 mg of the alkaloid fraction, using a stationary phase pH of 6.0. Two well resolved peaks were obtained: peak I, incanine (7.3 mg); and peak II, trichodesmine (238 mg). Separations were obtained with up to 800 mg of alkaloid, but in higher amounts the trichodesmine peak tailed considerably, although resolution was still satisfactory. Although peak II exhibited slight shoulders, no other alkaloids were detected from this peak by GC–MS. No stereoisomers of trichodesmine have been reported. The irregular peak shape is probably due to tailing. Due to the absence of colored material eluting at the solvent front, the high K value for incanine (Table 1) was acceptable. Lack of non-alkaloid contaminants allowed for rapid separation times (<2 h), since the PA did not have to be retained longer than impurities which elute at the solvent front. TLC and GC–MS analysis of each peak revealed the presence of a single PA, confirming complete resolution. The mass spectrum of incanine, which has not been reported, was (GC–MS method I): m/z 337 ($[M]^+$, 4), 294 (2), 250 (4), 222 (10), 206 (8), 155 (5), 136 (100), 120 (77), 119 (85), 94 (42), 93 (56), 80 (26), 53 (17), 43 (69).

The large genus *Senecio* (Asteraceae) has over 100 PA-containing species. *Senecio douglasii* DC. var. *longilobus* (Benth.) L. Benson, contains the hepatotoxic PAs riddelliine, senecionine (**16**), retrorsine (**18**) and seneciphylline (**17**) [28], all structurally similar cyclic diesters of retronecine. In the southwestern U.S., *S. douglasii* has been used extensively for ethno-medicinal purposes [9]. Infant poisonings and deaths have occurred in Arizona due to consumption of herbal preparations containing *S. douglasii* var. *longilobus* [29,30]. *Senecio* alkaloids have been separated by Zalkow et al. [12] using DCCC with a $CHCl_3$–C_6H_6–MeOH–H_2O solvent system. A 3-g mixture of ten alkaloids from *S. anonymus* was separated, including partial resolution of the diastereomers senecionine and integerrimine. This separation demonstrates the resolving power of counter-current techniques, although DCCC separations take longer, and choice of solvent systems are limited [1]. *S. douglasii* var. *longilobus* collected from Sonoita, AZ, contained ca. 0.03% PAs per dry weight of plant material, isolated as a clear-brown gum. GC–MS analysis of the alkaloidal fraction revealed the presence of senecionine, seneciphylline, retrorsine, and an otonecine-based PA, florosenine (**15**), not previously reported in *S. douglasii*. The PAs had a mass spectrum matching literature spectrum [31,32]. Riddelliine was not detected. Fig. 2D shows a chromatogram of the separation of 600 mg of this alkaloidal fraction. Excellent resolution was achieved at a stationary phase pH of 5.0. Peak I, florosenine (2.0 mg), was unretained at this or any other pH however, and eluted together with the colored material at the solvent front. Peak II, senecionine (23.0 mg), and peak III, seneciphylline (63.7 mg) were well resolved, while retrorsine (216.6 mg) remained in the column, and was easily recovered after the run. With the exception of florosenine, alkaloids were recrystallized from acetone. TLC and GC–MS analysis of peaks revealed single alkaloids. TLC failed to separate the structurally similar seneciphylline and senecionine [33]. Separation could be achieved on silica gel column chromatography only by the use of sequential runs [28]. Despite these earlier difficulties, they were resolved in the present CCC system. This separation demonstrates a major advantage of CCC in its ability to separate PAs from a large quantity of semi-solid material.

The use of high-speed CCC in the purification of natural products has become well established [6]. Our results show the value of this method for PAs. Plant compounds, including PAs, which are often isolated as a gum or tar, can be safely injected onto the column without fear of contaminating or fouling a solid matrix. Thus pre-separation clean-up steps are unnecessary. Further advantages demonstrated in this investigation include rapid separation times and low solvent consumption (<2 l per run). The simple chloroform–phosphate buffer solvent system used in these experiments was sufficient for the separations

performed, although the resolving power was insufficient to separate diastereomers. Similarly, attempts to resolve diastereomers by TLC were unsuccessful, regardless of the solvent system utilized. Preliminary experiments indicate that ternary solvent systems of $CHCl_3$–MeOH–H_2O may also be useful in separating PAs by CCC. More elaborate solvents and techniques will become necessary with increasingly complex PA mixtures. New techniques such as pH-zone-refining CCC described by Ma et al. [34], which utilizes a pH gradient, should prove especially useful in cases where PAs possess large differences in K values.

Acknowledgments

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