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Research Article Research Article

Efficient, Baseline Separation of Pyrethrins by Centrifugal Partition Chromatography

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Abstract

Pyrethrins are natural pesticides present in the oil extracted from the flowers of *Chrysanthemum cinerariaefolium*. They are potent ion-channel neurotoxins which have higher selective toxicity for insects compared to mammals. These phytochemicals have an exceptionally safe environmental profile and are an attractive natural alternative to organophosphate insecticides currently used in agriculture. The oil extract contains six pyrethrins compounds, which are structurally related esters. Isolation of reference standards for pyrethrins and their derivatives in multi-gram quantities is a prerequisite for studying toxicity and soil degradation for these natural pyrethrins. Chromatography is difficult because of the high hydrophobicity and close structural similarity. The isolated pyrethrin compounds also have poor stability because they are photosensitive. To facilitate these studies, we have developed a two-step purification process, in which the two groups were first separated by normal phase liquid chromatography on silica gel. The separation of pyrethrin, cinerin, and jasmolin within each group was achieved by centrifugal partition chromatography (CPC). We explored and successfully optimized solvent systems that were subsequently applied to achieve multi-gram scale separation. The pyrethrins I group compounds were separated using a solvent system containing heptane-methanol-acetonitrile (6:1:2, v/v) in ascending mode. The pyrethrins II group compounds were separated using a solvent system containing heptane-terbutylmethyl ether-acetonitrile-water (8:1:5:1.5, v/v) also in ascending mode. In both cases, gram quantities of individual pyrethrins with purity exceeding 99% were produced.

Keywords: Pyrethrin; Jasmolin; Cinerin; Centrifugal partition chromatography; Countercurrent chromatography; Natural pesticides; Chrysanthemum

Highlights

Centrifugal partition chromatography was applied for the separation of six natural pesticidal pyrethrins. Solvent systems were optimized for the separation of pyrethrins I group and pyrethrins II group. Pyrethrin I, cinerin I, and jasmolin I were separated in gram quantity per single CPC run. Similarly, pyrethrin II, cinerin II, and jasmolin II were purified nearly base-line.

Introduction

Pyrethrins are natural pesticides obtained from the extraction of *Chrysanthemum cinerariaefolium* flowers, which are cultivated largely in Kenya. Pyrethrin derivatives have been developed for agriculture and household pesticide products. In recent years, the use of natural non-derivatized pyrethrins for agriculture is increasing in popularity. Pyrethrin compounds have a good environmental safety profile stemming from the fact that these esters are photosensitive and have a short half-life following application to crops. The basic issues related to production, chemistry, and toxicology were reviewed by Maciver [1], while issues related to extraction, refining and analysis were reviewed by Carlson [2]. The Pyrethrum oil contains six pyrethrins belonging to two groups. Pyrethrin I (**1**), cinerin I (**2**), and jasmolin I (**3**) are esters of *trans*-chrysanthemic acid (**7**) and constitute group I. The pyrethrins II group are esters of carbomethoxychrysanthemic acid (pyrethric acid) (**8**) and comprise of pyrethrin II (**4**), cinerin II (**5**), and jasmolin II (**6**). The pyrethrins, cinerins, and jasmolins differs by the alkyl chain present in the alcohol portion, which is called pyrethrolone (**9**), cinerolone (**10**), and jasmolone (**11**), respectively (Figure 1).

Pyrethrins are potent ion-channel neurotoxins with higher selective toxicity for insects compared to mammals, and compared to other insecticides used in agriculture they have less toxicity towards humans. To facilitate the studies of toxicity and the environmental fate of pyrethrins, we needed to purify gram quantities of these esters. The unmodified phytochemicals are also used for the semi-synthesis of their environmental degradation products. The gram quantity separation was achieved by developing appropriate solvent systems for centrifugal partition chromatography (CPC), which gave baseline separation of the natural esters in high purity. CPC, also called liquid-liquid partitioning, belongs to the family of countercurrent chromatography techniques – the separation takes place in a series of small mixing chambers. The mobile liquid phase interacts with an immiscible stationary liquid phase during passage, carrying the analyte through the system based on the partition coefficient. In the CPC instrument, the stationary phase is immobilized by centrifugal force and the mobile phase diffuses through [3]. Retention time is determined by the partition coefficients of the individual components in the solvent system [4]. One of the greatest advantages of countercurrent chromatography is an absence of chemical degradation often seen with solid phase chromatography, allowing full recovery of the constituents of the mixtures [3].

A comprehensive environmental study required pure pyrethrins for use as reference standards and, additionally, as the starting materials to produce a variety of derivatives originating during environmental degradation. To accomplish this goal, we estimated the required quantity for pyrethrins to be in the range of 10 - 20 g, and 2 - 3 g for each of the cinerins and jasmolins. These were significant and unprecedented quantities to isolate. In early approaches, these esters were obtained by a semi-synthetic methodology, reacting acid chlorides with pyrethrolone (**9**), cinerolone (**10**), and jasmolone (**11**)

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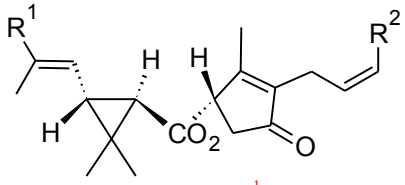
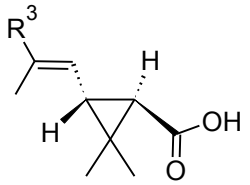
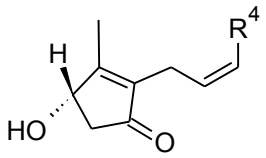


Pyrethrin I	(**1**):	$R^1 = CH_3$	$R^2 = CH{:}CH_2$
Cinerin I	(**2**):	$R^1 = CH_3$	$R^2 = CH_3$
Jasmolin I	(**3**):	$R^1 = CH_3$	$R^2 = CH_2CH_3$
Pyrethrin II	(**4**):	$R^1 = CO_2CH_3$	$R^2 = CH{:}CH_2$
Cinerin II	(**5**):	$R^1 = CO_2CH_3$	$R^2 = CH_3$
Jasmolin II	(**6**):	$R^1 = CO_2CH_3$	$R^2 = CH_2CH_3$



Chrysanthemic Acid	(**7**):	$R^3 = CH_3$
Pyrethric Acid	(**8**):	$R^3 = CO_2CH_3$



Pyrethrolone	(**9**):	$R^4 = CH{:}CH_2$
Cinerolone	(**10**):	$R^4 = CH_3$
Jasmolone	(**11**):	$R^4 = CH_2CH_3$

Figure 1: Structures of pyrethrins, with their constitutive acids and alcohols.

[5]. Until recently, purification of reference standards of pyrethrins directly from the extract have been described only a few times [6]. Rickett successfully used liquid-liquid partitioning on diatomaceous earth carrying nitromethane as the stationary phase, while hexane and carbon tetrachloride acted as the mobile phase, separating 270 mg of total pyrethrins in a 17-hour experiment [7]. In 2001, separation of pyrethrins was achieved by preparative HPLC using silica gel column [6]. The method was 100 min long, and after multiple injections, the collected amount varied between 11.4 mg for jasmolin II (**6**) (smallest component) and 93.4 mg for pyrethrin I (**1**) (largest component). The isolated pyrethrins were used for determining spectral properties and standardization of the pyrethrin oils. Scaling up any of the prior approaches to yield multi-gram quantity would be a daunting effort with a low chance of success. In addition, reference standards of these esters were often adulterated with stabilizing agents due to poor stability – the stabilizing agents will interfere with environmental degradation studies.

Pyrethrins are generally considered to be of strong hydrophobic character. The higher hydrophobicity of pyrethrins I group comes from the chrysanthemic acid (**7**) moiety, in comparison to the pyrethric acid (**8**) moiety of their group II counterparts. This difference in hydrophobicity is sufficient to separate the two ester groups by medium pressure chromatography on silica gel. Silica gel also induces partial separation within the group constituents, but under preparative conditions it is impractical to achieve a full separation. Analytical monitoring of the fractionation can be done by NP HPLC on Cyano columns or by RP HPLC on ODS columns. A review of chromatographic methods for pyrethrins was published by Gullick [8] and Chen [9].

Materials and Methods

Partition coefficients were measured by dissolving 5 mg of pyrethrin mixture in 2 mL of a bi-phasic solvent system. After equilibrating, even volumes (0.5 mL) of each phase were separated and evaporated to dryness under the stream of nitrogen. The dry residues were dissolved in 1.00 ml of MeOH and analyzed by high performance liquid chromatography coupled to a diode array detector (HPLC-DAD). A ratio of the peak areas from the stationary phase to the mobile phase constituted a partition coefficient for each compound.

Thin layer chromatography (TLC) analyses used silica gel plates developed in hexane-acetone (3:1), followed by spraying with cerium sulfate dissolved in 10% sulfuric acid and heated with a heat gun until the spots developed.

Pyrethrum oil sample (MGK Pyrocide 50%, code 224-14) was obtained from the McLaughlin Gormley King Co., Minneapolis, MN, USA. Medium pressure chromatography with silica gel (Premium Rf, 60 Å, 40 - 75 µm, Sorbent Technologies, Atlanta, GA) was performed on a glass column 4" ID × 47" (Spectrum Chromatography, Houston, TX), with a pump solvent delivery (model # 1212100 from Thomson Instrument Company). The HPLC analyzes were carried out on Agilent HPLC model 1100 (quaternary pump G1314, autosampler 1313A, DAD G1315A, Shimadzu degasser DGU-14). Normal phase HPLC analysis was done on a 4.6 × 100 mm Cyano column (YMC CN12S031046WTA), 3 µm, 120 Å, in a step gradient. Solvent A was hexane and solvent B was 10% THF in hexane. The flow rate was 2.0 mL/min: 0.0 min [1% B], 5.0 min [5.5% B], 5.1 min [12.0% B], 10.6 min [25% B], 13.0 min [25% B]. Reverse phase HPLC analysis was done on a XTerra MS C18, 5 µm, 2.1 × 100 mm; solvent A was 5% MeCN and 95% water with 0.1% TFA, solvent B was MeCN. The flow rate was 0.4 mL/min: 0.0 min [45% B], 12.0 min [75% B]. Centrifugal partition chromatography experiment uses the Kromaton FCPC-A (Rousselet Robatel, Annonay, France) with a 1 L rotor. Eluent was collected into test tubes of 16 or 18 mm × 20 cm using a fraction collector (Varian ProStar 701). The liquid phases were pumped into the FCPC instrument using a pump (Waters HPLC pump model 590). Solvents were purchased from Pharmco-AAPER, Brookfield, CT.

Results

In the initial fractionation, 300 g of Pyrethrum oil concentrate was dissolved in 2.7 L of hexane and pumped into a glass medium pressure column filled with 4 kg of silica gel (40 - 75 µm) in hexane. The solvent was pumped at the rate of 60 mL/min. Initially, the column was fed with 6 L of hexane; for every 2 L after, acetone content in hexane was increased by 0.33% v/v. The back pressure remained below 30 psi. Fractions were collected in 2 L increments. The pyrethrins I group, eluted in the fractions 11 - 15, had a total mass of 117 g. The pyrethrins II group, in the fractions 19 - 25 has a total mass of 78 g. In both cases the total mass comprises pyrethrins as well as other constituents. The earlier fractions in each sequence were enriched into jasmolin and cinerin and the later fractions contained almost exclusively pyrethrins.

Experiments on finding an appropriate solvent system for CPC separation of pyrethrin I group led to the solvent system SS-02, containing Hept-MeOH-MeCN (6:1:2, v/v) (Table 1). SS-02 offered good spread of the three esters (Figure 2A) within a run time of 1.5 hours at flow rate of 33 mL/min. All non-pyrethrin impurities had partition coefficients different from **1** - **3** and eluted either with the front of the mobile phase or were retained beyond the band of pyrethrin I. Further experimentation led to a discovery of an alternative solvent system, SS-03, containing Hept-MTBE-MeCN (8:1:5, v/v), in which the partition coefficients for **1** and **3** are near identical as in SS-02, but dramatically different for **2**, resulting in its early elution ahead of **1** (Figure 2C).

In a representative CPC process, first, the rotor was filled at 200 rpm with stationary phase (lower layer). With the valve configured to ascending run and the rotational speed increased to 1500 rpm, a solution of 7 - 8 g of a mixture of pyrethrins I group dissolved in a mixture of 20 mL of upper and 50 mL lower phase was injected into the rotor. Upon completion of the injection, the mobile phase was introduced to the system at a flow rate of 33 mL/min and the eluent was collected into 16 mm × 20 cm test tubes at 20 mL per tube. Only minimal loss of the stationary phase occurred during the run. The positions of eluted bands were determined by normal phase (NP) HPLC analysis on a YMC Cyano column. Retention times were as follows: **3** [4.42 min], **2** [4.63 min], and **1** [4.89 min], **6** [7.17 min], **5** [7.30 min], and **4** [7.48 min]. In reverse phase (RP) HPLC the retention times were: **5** [7.24 min], **4** [7.48 min], **6** [8.13 min], **2** [9.89 min], **1** [10.05 min], and **3** [10.65 min]. After analysis, the test tubes with similar content were combined, evaporated to dryness on a rotary evaporator and transferred to a freezer for storage. Each collected fraction from the silica gel column contained varying ratios of pyrethrins. The results illustrated in Figure 2A were of a fraction containing representative proportions of the three components found in pyrethrin I group, of which we obtained **3** [517 mg], **2** [726 mg], and **1** [3393 mg].

The CPC purification of pyrethrins II group was performed on these fractions from the silica gel fractionation that had a slightly higher content ratio of jasmolin and cinerin to pyrethrin (fractions 19 - 22). The selected solvent system SS-12, containing Hept-MTBE-MeCN-H_2O (8:1:5:1.5, v/v) was used in ascending mode. The run time was approximately 2 hours with a flow rate of 33 mL/min, and fractions were collected in 25 mL increments using 18 mm × 20 cm test tubes. A representative spread of the eluted bands of the pyrethrins II group is shown in Figure 2B, from which we obtained **6** [507 mg], **5** [967 mg], and **4** [2316 mg].

Discussion

Countercurrent chromatography is one of the gentlest of all chromatographic approaches capable of exploiting miniscule structural differences [4], and it provides separations on a scale much larger than preparative HPLC. The separations are carried out using solvent systems comprising several solvents, which form two phases after mixing. In ascending mode, the upper layer is used as mobile phase, while in descending, the lower layer is the mobile phase. In our experiment design, ascending mode was preferred since the eluted organic phase is easier to evaporate than the stationary phase. The mobile phase elutes components of a mixture according to the increasing partition coefficient ($K=[A]_{stationary}/[A]_{mobile}$). Considering close structural similarity of pyrethrins, it was necessary to find a solvent system that will maximally differentiate the components (increase the selectivity) without unnecessarily lengthening the run time. Tables 1-3 provide the partition coefficients (K) for each of the esters in selected solvent

Solvent System	Hept	MTBE	MeOH	MeCN	H_2O	Cpd	K	$\alpha=K_n/K_1$
SS-01	1.5	--	1	--	0.03	1	0.43	1.00
						2	0.38	0.88
						3	0.30	0.70
SS-02	6	--	1	2	--	1	2.63	1.00
						2	2.08	0.79
						3	1.61	0.61
SS-03	8	1	--	5	--	1	2.64	1.00
						2	0.68	0.26
						3	1.64	0.62

Table 1: Partition coefficients (K) for the pyrethrins I group in three selected solvent systems. K_n is the partition coefficient of a given pyrethrin

systems (SS), and their selectivity ($\alpha=K_n/K_1$ or K_n/K_4) to pyrethrin I (**1**) or pyrethrin II (**4**). The solvent systems with the largest differences in the normalized partition coefficients offer the best practical separations. Desired partition coefficients do not guarantee expected CPC separation, and only an actual run will verify if hydrodynamic properties of such a solvent system will perform.

Purification of pyrethrins I group by CPC

Large-scale chromatographic fractionation of hydrophobic substances faces limitations caused by a smaller selection of suitable solvents as well as usable chromatographic media. This applies to the liquid-liquid partitioning of the pyrethrins groups by imposing limitations regarding what solvents could be utilized to create the necessary bi-phasic solvent system. Hexane and water are found in the majority of the solvent systems used in countercurrent chromatography. We found, however, that hexane was not protecting the phases against homogenization in the presence of a sample load higher than 5%. Water, on the other hand, adds strong polar character to the lower phase and effectively excluding the pyrethrins and significantly alter the partition coefficient (K) values. To ameliorate these effects, we substituted hexane with heptane, which prevented homogenization and the MeCN was selected as the main component of the lower phase in place of water (Table 1). A solvent system based only on heptane and MeCN was very retentive and required addition of solvents that would increase polarity

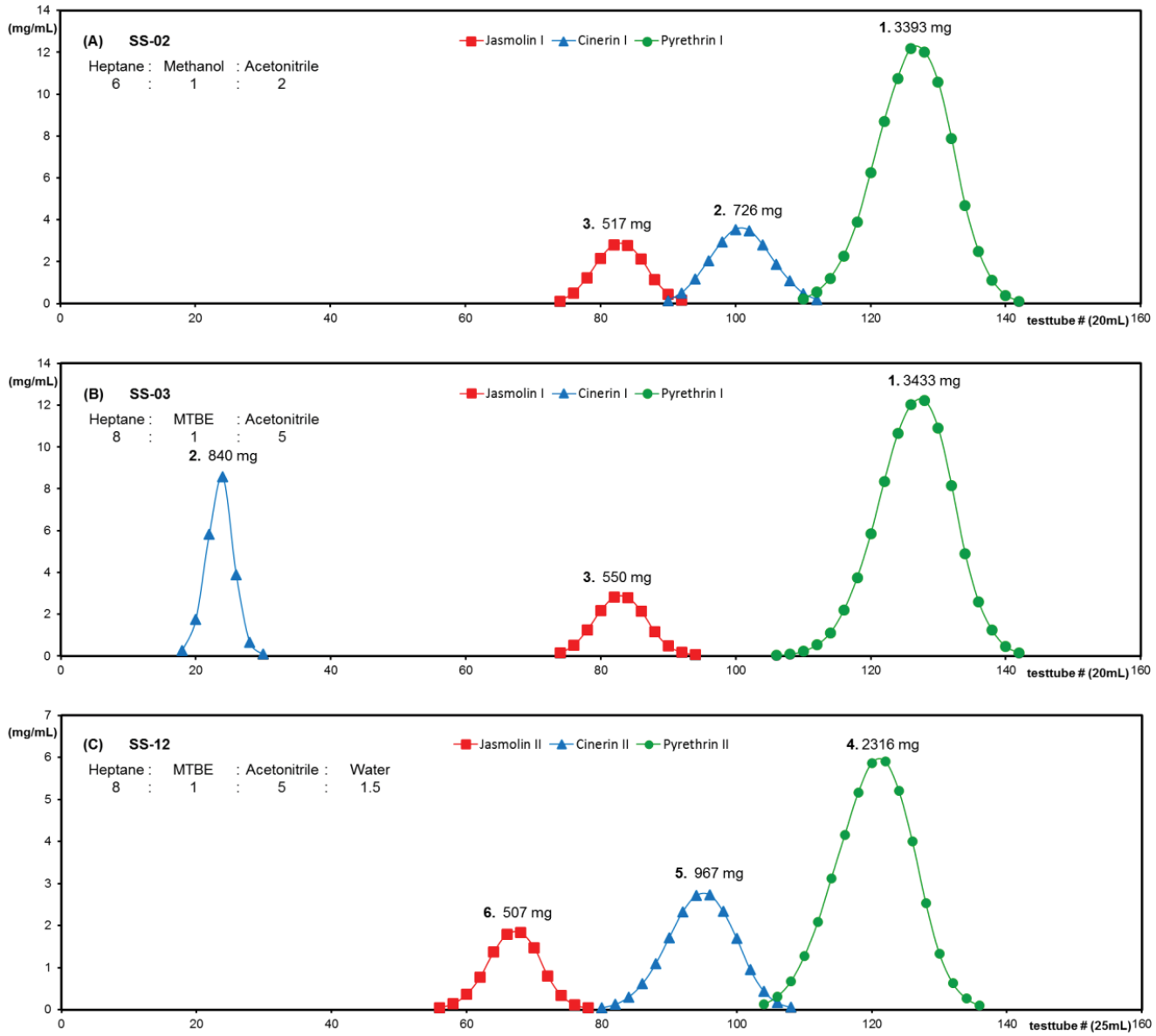


Figure 2: CPC chromatogram of pyrethrins I group in (A) SS-02, and (B) SS-03, and of pyrethrins II group in (C) SS-12. Concentrations of pyrethrin components in the collected fractions (20 mL for group I and 25 mL for group II) are measured using HPLC-DAD quantitation. Pyrethrin I (1), cinerin I (2), jasmolin I (3), pyrethrin II (4), cinerin II (5), and jasmolin II (6).

Solvent System	Hept	MTBE	MeOH	MeCN	H_2O	Cpd	K	$\alpha=K_n/K_4$
SS-04	5	--	2.5	0.5	--	4	6.25	1.00
						5	5.26	0.84
						6	4.76	0.76
SS-05	5	--	1.5	1.5	--	4	12.53	1.00
						5	9.53	0.76
						6	7.16	0.57
SS-06	5	--	1	2	--	4	8.82	1.00
						5	6.63	0.75
						6	4.85	0.55
SS-07	6	1	2	1	--	4	3.41	1.00
						5	3.10	0.91
						6	2.98	0.87
SS-08	6	0.5	2	1	0.5	4	2.94	1.00
						5	2.44	0.83
						6	1.79	0.61
SS-09	8	1	--	5	--	4	13.42	1.00
						5	9.87	0.74
						6	7.33	0.55
SS-10	8	2	--	4	--	4	7.84	1.00
						5	6.18	0.79
						6	4.90	0.63
SS-11	7	2	--	5	--	4	9.36	1.00
						5	4.17	0.76
						6	5.58	0.59
SS-12	8	1	--	5	1.5	4	4.54	1.00
						5	3.53	0.78
						6	2.26	0.50

Table 2: Partition coefficients (K) measured for the pyrethrins II group in selected solvent systems.

Solvent System	Hept	MTBE	MeOH	MeCN	H_2O	Cpd	K	$\alpha = K_n/K_{1,4,9}$
SS-02	6	--	1	2	--	1	2.63	1.00
						2	2.08	0.79
						3	1.61	0.61
						4	7.69	1.00
						5	6.67	0.87
						6	6.06	0.79
						9	136.0	1.00
						10	114.0	0.84
						11	79.11	0.58
SS-12	8	1	--	5	1.5	1	0.60	1.00
						2	0.14	0.24
						3	0.29	0.48
						4	4.54	1.00
						5	3.53	0.78
						6	2.26	0.50
						9	56.69	1.00
						10	48.40	0.85
						11	36.27	0.64

Table 3: Comparison of partition coefficients for the pyrethrins I group and pyrethrins II group and their respective alcohol moieties.

of the upper phase. This was accomplished by adding MeOH (SS-02). Experimentation with the pyrethrins I group was brief because SS-02 was simple in design and provided satisfactory CPC separation. It possessed the best combination of practical benefits: moderate run time and phase stability tolerating a sample load of 8 g (larger sample loads were not tested). This allowed isolation of up to 4 g of individual compounds per load, eluting in the order of jasmolin>cinerin>pyrethrin, with most of them at >99% purity (Figure 2A).

An alternative solvent system was found which incorporated MTBE (SS-03) instead of MeOH. The system also had good hydrodynamic properties, selectivity, and stability in the presence of a sample load. While the values of K for jasmolin I (**3**) and pyrethrin I (**1**) were similar to the values in SS-02 (K_3=1.61→1.64 and K_1=2.63→2.64), the elution of cinerin I (**2**) was significantly earlier (K_2=2.08→.68) (Figure 2B). The change in elution order was unexpected and we cannot offer a good hypothesis to explain this phenomenon. A disadvantage of SS-03 resulted from co-elution of cinerin I (**2**) with non-pyrethrin components in the early fractions. The different selectivity of the two solvent systems can be exploited if they are used in succession for achieving superior purity with increased sample load.

Purification of pyrethrins II group by CPC

During the development stage of a suitable solvent system (SS) for pyrethrins II group, we prepared and tested 33 SS (only the data from selected SS are shown in Table 2, measuring K for pyrethrin II (**4**), cinerin II (**5**), and jasmolin II (**6**) in order to assess their effectiveness for CPC separation. In addition, hydrodynamic behavior was evaluated by observation. The first stage in developing a solvent system involves searching for a combination of individual solvents that offer useful selectivity ($\Delta\alpha > 0.2$) for separation. Next, we fine-tuned K to an appropriate range by adjusting ratios of solvents; this is to maintain satisfactory separation while having moderate experiment time (2<K<3). The last step is to determine if the SS has suitable hydrodynamic properties for CPC use. In SS-04 to SS-06, the systems were generally excessively retentive (Table 2). Attempts to lessen retentivity by addition of EtOAc or CH_2Cl_2 failed due to loss of selectivity ($\Delta\alpha < 0.2$). In addition, the hydrodynamic properties of these solvents were poor, resulting in excessive loss of the stationary phase during CPC experimentation; with sample loads higher than 5%, phase homogenization was observed. By addition of MTBE to adjust K to an acceptable range, SS-07 had improved hydrodynamic properties and phase stability. However, the selectivity was poor, which would result in significant band overlapping. With additional fine-tuning (SS-08), the selectivity was improved, but baseline separation of **5** and **4** was not possible. The removal of MeOH from the SS resulted in significant improvements to selectivity (SS-09). Decrease of retentivity was achieved by multiple changes, involving an increase in the MTBE-MeCN ratio (SS-10), increase in the MTBE-Hept ratio (SS-11), or addition of water (SS-12). By adjusting the ratio of the Hept-MTBE-MeCN, K decreased, but with the tradeoff of decreased selectivity. With the addition of water, the partition coefficients can be brought into the practical range, while at the same time maintaining excellent selectivity. SS-12 provided the optimal efficiency with the ratio of Hept-MTBE-MeCN-H_2O at 8:1:5:1.5, v/v (Table 2).

Contributions of each moieties to K

To understand the contributions of the acid and alcohol moieties to the partition coefficients, we measured K of the esters **1**-**6** as well as their constitutional alcohols **9**, **10**, **11** in SS-02 and SS-12 (Table 3). K for the three alcohols are very high, as their polarity is higher than those of the esters. The more hydrophobic pyrethrins I group have lower partition coefficients than their group II counterparts. This reflects the dominant influence of the acid moiety with the hydrophobicity of these compounds. Because of this, chromatographic separation of the pyrethrin I and II groups is relatively easy on some chromatographic processes, including silica gel. Separation of the individual esters within each group is significantly more challenging, because of the subtle structural differences between them, laying with the alkyl chains of the constitutional alcohol moieties. K measured in SS-02, for the alcohols **9**, **10**, and **11**, as well as for both sets of the esters **1**-**6** show the same trend, eluting in the order of jasmolin>cinerin>pyrethrin. Thus, the structural differences in the alkyl chains are amplified in this particular solvent system (Table 3).

When the K values of the same nine compounds were measured in SS-12 (MTBE-based SS), we noticed an anomalous response only within the pyrethrins I group, expressed by a rearranged elution order of **2**>**1**>**3**. This anomalous effect of **2** was also observed in SS-03 (Table 1) This change in elution order is only observed with pyrethrin I group in MTBE-based SS; this effect was not observed with either the pyrethrin II group or the constitutional alcohols.

Conclusion

CPC is an effective method in separating structurally related compounds. The partition coefficients are dependent on the composition of the solvent system and its interactions with the analytes. Through extensive experimentation in manipulating partition coefficients, we can tailor solvent systems for specific compounds of interest. In this experiment, CPC coupled with optimized solvent systems allowed for baseline separation each natural pyrethrin esters; the minute difference is in the length of the alkyl chain or the degree of unsaturation. Using this method, gram quantities in high purity can be quickly obtained without the use of stabilizing agents, which would affect subsequent environmental studies.

By using a systematic approach to developing countercurrent solvent systems, it is possible to tailor an experiment to meet specific needs. The liquid-liquid systems developed for the lipophilic esters of pyrethrins can also be applied to the separation of other hydrophobic chemicals. A repository of solvent systems (with each providing unique selectivity) is necessary for countercurrent chromatography to be an effective and scalable alternative to solving challenging isolation problems.

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