Method Development and Validation of a High-Performance Liquid Chromatographic Method for Pyrethrum Extract

Ken Essig* and **Zhouming Zhao**

Aventis Environmental Science, 95 Chestnut Ridge Road, Montvale, NJ 07645

Abstract

A robust analytical methodology is developed for the quantitative determination of all six insecticidal components of pyrethrum extract: jasmolin I, cinerin I, pyrethrin I, jasmolin II, cinerin II, and pyrethrin II. This method, based on the separation technique normal-phase high-performance liquid chromatography, offers selectivity, accuracy, precision, linearity, range, ruggedness, and robustness as well as efficiency and ease.

Introduction

Pyrethrum extract is one of the most important natural insecticides extracted from plants. The extract is composed of three closely related insecticidal esters of chrysanthemic acid (cinerin I, jasmolin I, and pyrethrin I) and three closely related insecticidal esters of pyrethric acid (cinerin II, jasmolin II, and pyrethrin II). The three chrysanthemic acid esters are commonly identified as Pyrethrins 1, and the three pyrethric acid esters are identified as Pyrethrins 2 (Table I and Figure 1). Collectively, the esters are commonly known as "rethrins". Depending on the source of the extract, the concentration of each compound may vary from sample to sample. The pyrethrum extracts that are commercially available in the U.S. usually contain 20% or 50% total pyrethrins. These extracts are commonly used to formulate end-use insecticide products as well as over-the-counter head-lice control products that are regulated by the Food and Drug Administration.

Currently, a variety of analytical methods (1–15) based on chemical, spectrophotometric, and chromatographic procedures are used for the assay of pyrethrum extract. Because of the separation difficulty and lack of absolute standards for each compound, the routine quantitation of pyrethrum extract is usually reported as either "total pyrethrins" or as "total Pyrethrins 1" and "total Pyrethrins 2". It has been a long-term aim for many laboratories to find a reliable analytical method that can simultaneously determine the six rethrins in pyrethrum extract.

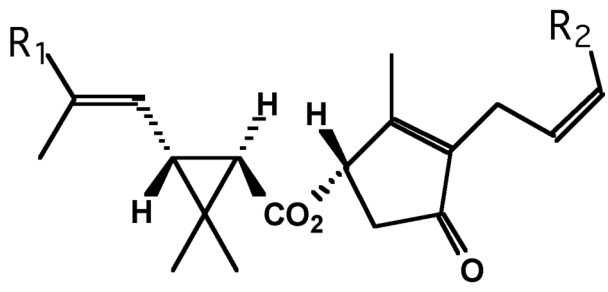
* Author to whom correspondence should be addressed: email ken.essig@aventis.com.

Some promising work has been done with gas chromatography (GC) methods for pyrethrins (1,2,15). However, after an initial evaluation, we did not use these methods because of the unavoidable thermal isomerization of pyrethrin I and II (15–18). In addition, the Pyrethrins 2 peaks were observed to be strongly retained and difficult to integrate consistently.

With the constraints of ease of use, short run time, thermal sta-

Table I. Six Individual Esters of Pyrethrum Extract

Common name	CAS number	Molecular formula	Molecular weight
Pyrethrins 1			
Jasmolin I	4466-14-2	$C_{21}H_{30}O_3$	330.4
Cinerin I	25402-06-6	$C_{20}H_{28}O_3$	316.4
Pyrethrin I	121-21-1	$C_{21}H_{28}O_3$	328.4
Pyrethrins 2			
Jasmolin II	1172-63-0	$C_{22}H_{30}O_5$	374.4
Cinerin II	121-20-0	$C_{21}H_{28}O_5$	360.4
Pyrethrin II	121-29-9	$C_{22}H_{28}O_5$	372.4



cinerin I : R1 = CH3 , R2 = CH3
jasmolin I : R1 = CH3 , R2 = C2H5
pyrethrin I : R1 = CH3 . R2 = -CH:CH2
cinerin II : R1 = CO2CH3 , R2 = CH3
jasmolin II : R1 = CO2CH3 , R2 = C2H5
pyrethrin II : R1 = CO2CH3 , R2 = -CH:CH2

Figure 1. Chemical structures of the six individual esters in pyrethrum extract.

bility, and accuracy, our attention became focused on high-performance liquid chromatographic (HPLC) methods of analysis. The current U.S. Pharmacopoeia (USP) analytical method for pyrethrum extract involves a reversed-phase HPLC analysis on a C_{18} ODS column with a 80:20 methanol–water mobile phase (11). In the past, this method has proved to be reliable, but it does not separate all six esters of pyrethrum extract. The cinerin and jasmolin peaks of both groups are usually merged. Our initial strategy for new method development was to adapt the reverse-phase procedure to allow for the separation of all six esters. Reversed-phase separation of all six esters was achieved rather easily, but a closer examination of the chromatograms revealed problems. Ultraviolet diode-array peak-purity evaluations consistently pinpointed small interfering components eluting with the analytes. The most perplexing of the interferences were from unidentified extract components that could not be resolved from jasmolin I and II. We undertook a lengthy investigation of many different types of reversed-phase columns with varying mobile phase compositions, flow rates, and temperatures. However, we failed to find a reversed-phase method that could separate the six esters without the minor exotic interferences.

After exhausting our reversed-phase options, we decided to pursue normal-phase HPLC methodology. As an alternative approach, normal-phase HPLC methods are regularly used in industry outside of the U.S. to analyze pyrethrins. Several previously published methods (3,5,9,10) formed the basis for our investigation. These methods demonstrated that it is possible to separate all six pyrethrin esters, but there were shortcomings such as long run time, lack of baseline separation of all esters, and mobile phases that were difficult to prepare. Because none of the published methods provided the speed and resolution we desired, we initiated the development of our own methodology. Finally, after a series of trial injections and screenings, we developed our own normal-phase HPLC method.

The purpose of this study was to provide a validated analytical method that can be used to simultaneously quantitate each of the six esters in pyrethrum extract. This method validation has been carried out according to the chromatographic method criteria regulated by USP in anticipation that it will be considered as a candidate method for the USP pyrethrum monograph (11). The method may eventually be used as a basis for the isolation of analytical standards for each of the six esters (Table II). These standards could potentially be used to standardize a fully characterized reference standard of pyrethrum extract.

Table II. Identification and Composition of the Analytical Standard

Pyrethrum extract*	%wt/wt
Jasmolin I	2.487
Cinerin I	5.053
Pyrethrin I	19.906
Jasmolin II	2.408
Cinerin II	5.331
Pyrethrin II	15.286
Total pyrethrins	50.47

* Lot # 96/11.2, R97-254.

Experimental

Materials

Reagents

HPLC-grade hexane, tetrahydrofuran (THF), 2-propanol, and triethanolamine were purchased from Fisher Scientific (Fairlawn, NJ). Octadecane and butylated hydroxytoluene (BHT) were bought from Sigma-Aldrich (Allentown, PA). Isopar M fluid (an isoparaffinic hydrocarbon used as a diluent for pyrethrum extracts) was obtained from Exxon Chemical Company (Houston, TX).

Pyrethrum extracts

All of the pyrethrum extracts used in this study were obtained from the Pyrethrum Board of Kenya (Nakuru, Kenya). These samples spanned five years of production with the expected variability in ester composition (20). The samples had all been previously assayed by the Association of Official Analytical Chemists (AOAC) Mercury Reduction Method 936.05 (4). Twenty of these samples (as listed in Tables III–VI) were used for the purpose of recovery validation. For the precision and range validation, a 60% pyrethrum extract, pyrethrum pale concentrate (Lot R98-183), and its three dilutions with pyrethrin contents of 50%, 20%, and 10% were used as indicated in Tables VII, IX, and X.

Analytical standard

Because no true analytical standards exist for pyrethrum extract or the individual esters, the standard chosen in this study was a sample of pyrethrum extract that had been analyzed by the AOAC Mercury Reduction Method 936.05 with subsequent normal-phase HPLC diode-array analysis. For this study, the AOAC reported values for the Pyrethrins 1 and Pyrethrins 2 group were assumed to be the true values. In order to make assignments of the individual ester purities, we did a calculation based on Beer's law. The percentage of each ester was calculated using normal-phase HPLC peak-area measurements of each ester at its maximum wavelength (λ_{max}) and by using the previously reported extinction coefficients for each of the esters (12).

Instrument and operating conditions

HPLC

HPLC assays were performed according to our developed normal-phase method using an HP1100 HPLC system with a diode-array detector (Hewlett-Packard, Wilmington, DE). A Spherex Cyano column (Phenomenex, Torrance, CA) (25-cm × 4.6-mm i.d., 5-µm particle) was used. The mobile phase was 97.75:2.25 hexane–THF with a flow rate of 1.5 mL/min. The column oven was set to 25°C, and the detector wavelength was set at 240 nm. The pyrethrum sample or standard concentration was set at 500 µg total pyrethrins per milliliter using hexane as the dilution solvent. Ten-microliter injections were made.

Capillary GC

GC characterization was carried out using an HP5890 Series II GC system (Hewlett-Packard) equipped with a DB-1 capillary column (30-m × 0.32-mm i.d., 0.25-µm film thickness) (J&W Scientific, Folsom, CA). The injection volume was 1 µL at a carrier (He) flow of 2 mL/min and a split vent flow rate of 42 mL/min. The oven temperature for each run was programmed to

be held at 180°C for 11 min, raised to 200°C at 10°C/min and held for 8 min, raised to 210°C at 10°C/min and held for 18 min, and then raised to 240°C at 30°C/min and held at 240°C for 4 min. The injector temperature was 250°C, and the detector (a flame ionization detector) temperature was 300°C. The sample preparation for the pyrethrum extract was the same as that for HPLC, except that 2-propanol containing 0.06% (w/v) octadecane (the internal standard) was used as the solvent.

Results and Discussion

Method development

During a series of trial injections for the normal-phase method development in addition to runs using different types of silica and cyano columns, the mobile phase varied in solvent type and composition, the temperature ranged from 25°C to 35°C, and the flow rate changed from 1.2 to 1.5 mL/min. Peak purity readings from the diode-array detector were used as a judge for separation in order to avoid coelution of different components, as was experienced during reversed-phase analysis. After screening, a normal-phase method was finally crystallized (listed in Figure 2). The method provided an optimal separation of each pyrethrin ester with no major interferences from the sample matrix. Ultimately, the detector wavelength selection (240 nm) was chosen based on the wavelength that provided the least chance of interference from any of the minor interferences in the sample matrix. Using these method parameters, we overcame the interference of the unidentified peaks conflicting with the jasmolins during the reversed-phase procedures.

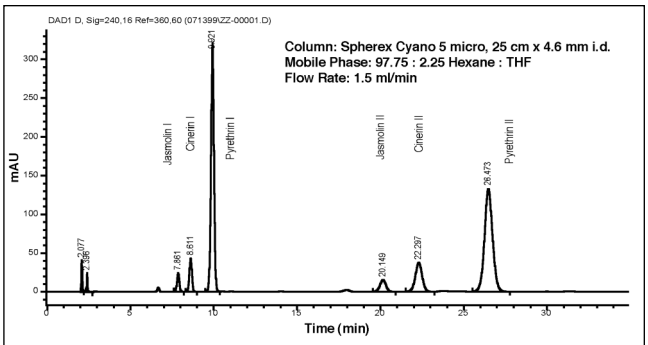


Figure 2. Chromatogram of separating pyrethrum extract using the developed normal-phase HPLC method.

The identity of the eluted peaks was determined by combining several approaches. The first and the most direct was to compare the determined elution order and λ_{max} with previously published literature (3,10,12). The second was to collect fractions of each peak as they eluted, followed by subsequent analysis using Fourier transform infrared spectrometry (FTIR) and GC–mass spectrometry (MS). The FTIR and mass spectral results, included in another part of our work (13), fully confirmed the identity of each of the eluted peaks as those assigned from the literature (2,14). The purity of each collected fraction was also checked by using packed-column GC, the HPLC normal-phase method, and the total ion chromatograms of GC–MS. Only one major single peak was found for each checked fraction.

Table III. Results of Pyrethrum Extract Samples Assayed for Accuracy

Sample	Identification (lot #)	AOAC pyrethrum content (%)	Current HPLC assay (%)	%Recovery
1	98/9.1	50.77	52.74	103.9
2	98/8.1	53.47	52.78	98.7
3	98/7.5	50.88	52.70	103.6
4	98/6.1	47.55	49.88	104.9
5	98/3.9	53.20	53.97	101.4
6	98/2.2	50.45	50.96	101.0
7	98/1.5	54.36	52.81	97.1
8	97/9.1	54.33	54.43	100.2
9	97/7.1	51.92	51.23	98.7
10	97/5.5	49.02	48.98	99.9
11	97/5.3	55.22	55.94	101.3
12	97/4.5	50.43	49.40	98.0
13	97/3.1	49.54	50.47	101.9
14	97/2.6	51.99	52.13	100.3
15	96/4.3	49.72	47.97	96.5
16	96/10.1	49.52	49.52	98.9
17	95/8.2	50.47	49.15	97.4
18	95/3.2	50.49	48.47	96.0
19	963	55.25	52.64	95.3
20	996	52.02	50.22	96.5
Average				99.6
Standard deviation				2.7
RSD				2.7

Table IV. Results of Pyrethrum Extract Samples Assayed for Accuracy as Pyrethrums 1 Group Recoveries

Sample	Identification (lot #)	AOAC Pyrethrums 1 content (%)	Current HPLC Pyrethrums 1 assay (%)	%Recovery
1	98/9.1	26.42	27.91	105.6
2	98/8.1	29.38	29.78	101.4
3	98/7.5	25.23	26.52	105.1
4	98/6.1	25.34	27.19	107.3
5	98/3.9	29.29	29.69	101.4
6	98/2.2	27.79	28.51	102.6
7	98/1.5	37.34	37.80	101.2
8	97/9.1	29.43	30.40	103.3
9	97/7.1	35.26	36.45	103.4
10	97/5.5	28.48	28.77	101.0
11	97/5.3	33.49	34.30	102.4
12	97/4.5	33.20	33.59	101.2
13	97/3.1	28.66	29.08	101.5
14	97/2.6	29.19	28.76	98.5
15	96/4.3	27.98	26.69	95.4
16	96/10.1	26.68	26.35	98.8
17	95/8.2	27.67	27.04	97.7
18	95/3.2	27.31	26.54	97.2
19	963	30.75	30.09	97.9
20	996	28.12	27.90	99.2
Average				101.1
Standard deviation				2.9
RSD				2.9

Method validation

Validation of the developed method was carried out to assess what the performance characteristics of the method were for the following parameters: accuracy, precision, linearity, range, selectivity, ruggedness, and robustness.

Table V. Results of Pyrethrum Extract Samples Assayed for Accuracy as Pyrethrum 2 Group Recoveries

Sample	Identification (lot #)	AOAC Pyrethrum 2 content (%)	Current HPLC Pyrethrum 2 assay (%)	%Recovery
1	98/9.1	24.23	24.83	102.5
2	98/8.1	24.09	23.01	95.5
3	98/7.5	25.65	26.17	102.0
4	98/6.1	22.21	22.69	102.2
5	98/3.9	23.91	24.29	101.6
6	98/2.2	22.66	22.45	99.1
7	98/1.5	17.02	15.01	88.2
8	97/9.1	29.43	26.03	88.4
9	97/7.1	16.66	14.78	88.7
10	97/5.5	20.54	20.21	98.4
11	97/5.3	21.73	21.64	99.6
12	97/4.5	17.23	15.81	91.8
13	97/3.1	20.88	21.39	102.4
14	97/2.6	22.80	23.37	102.5
15	96/4.3	21.74	21.28	97.9
16	96/10.1	23.40	23.17	99.0
17	95/8.2	22.80	22.11	97.0
18	95/3.2	23.18	21.93	94.6
19	963	24.50	22.55	92.0
20	996	23.90	22.32	93.4
Average				96.8
Standard deviation				4.9
RSD				5.0

Accuracy

For the 20 analyzed pyrethrum extracts, the percent recoveries were determined by assuming the AOAC analysis as the "true" level of pyrethrins in the samples. Table III shows that in all cases the recoveries were within 5% of the AOAC-reported assay values based on chemical analysis. The average recovery of 20 assayed samples was 99.6% with a relative standard deviation (RSD) of 2.7%. These results strongly indicated that the method was accurate. These data were also evaluated for recoveries of the Pyrethrins 1 and Pyrethrins 2 group versus the AOAC analysis results. From Tables IV and V, it can be seen that the average recoveries for both the Pyrethrins 1 and Pyrethrins 2 group of the 20 assayed samples were within 5% of the AOAC-reported assay values based on chemical analysis. This further supported that the new method was accurate. There was a slight difference in the average recovery of the Pyrethrins 2 group versus the Pyrethrins 1 group (101.1% versus 96.8%, respectively). This difference may be caused by the standard that was utilized. The Pyrethrins 1 and 2 assay totals for this standard were dependent on the AOAC wet chemical analysis and could have been slightly skewed because of the variability of this method. Further investigations are planned after a true standard is developed. In order to demonstrate the variation in purity of the individual esters in the pyrethrum extract, the analysis of the 20 batches is presented in Table VI with the determined purity for each of the esters. As anticipated, the data confirmed that the amount of each of the esters in the pyrethrum extract did indeed vary from batch to batch.

Precision

The 20% and 50% samples of pyrethrum diluted from the 60% pyrethrum extract were employed for the validation of the method's precision or repeatability. Six samples were prepared for each of these extracts. Each sample was run with an independently prepared standard. The analytical results are summarized

Table VI. Purity of Each of the Six Esters of Pyrethrum Extract in 20 Batches

Sample	Identification (lot #)	% Jasmolin I	% Cinerin I	% Pyrethrin I	% Jasmolin II	% Cinerin II	% Pyrethrin II
1	98/9.1	2.46	4.62	20.83	2.52	5.17	17.14
2	98/8.1	2.66	6.06	21.05	2.52	5.11	15.38
3	98/7.5	2.57	4.10	19.86	3.00	5.27	17.91
4	98/6.1	2.67	3.77	20.75	2.65	4.12	15.92
5	98/3.9	2.84	4.76	22.10	2.74	5.04	16.50
6	98/2.2	2.43	5.27	20.81	2.43	5.04	14.98
7	98/1.5	2.36	9.48	25.96	1.40	3.76	9.85
8	97/9.1	2.48	5.10	22.82	2.55	5.63	17.85
9	97/7.1	2.57	8.24	25.64	1.48	3.45	9.85
10	97/5.5	2.66	4.12	21.99	2.31	3.78	14.12
11	97/5.3	3.09	5.36	25.84	2.50	4.00	15.15
12	97/4.5	2.59	7.02	23.98	1.67	3.35	10.79
13	97/3.1	2.93	4.08	22.07	2.61	4.07	14.71
14	97/2.6	2.83	4.22	21.71	2.70	4.46	16.21
15	96/4.3	2.86	4.32	19.51	2.67	4.42	14.19
16	96/10.1	2.36	4.87	19.12	2.37	5.30	15.50
17	95/8.2	2.94	4.76	19.34	2.83	4.97	14.31
18	95/3.2	2.80	4.01	19.73	2.65	4.24	15.04
19	963	3.23	4.73	22.13	2.86	4.65	15.04
20	996	2.78	4.39	20.73	2.60	4.57	15.15

Table VII. Results of Six Replicate Analyses of Pyrethrum Extracts and Individual Esters for Precision

Species identification	20.69% Pyrethrum extract (dilute 2) Average content (%)	Standard deviation	RSD (%)	50.07% Pyrethrum extract (dilute 1) Average content (%)	Standard deviation	RSD (%)
Jasmolin I	0.98	0.0066	0.67	2.38	0.01	0.57
Cinerin I	1.99	0.01	0.70	4.82	0.03	0.57
Pyrethrin I	8.64	0.06	0.66	20.96	0.12	0.56
Jasmolin II	1.00	0.01	1.21	2.44	0.01	0.54
Cinerin II	2.16	0.02	0.86	5.31	0.04	0.85
Pyrethrin II	6.65	0.05	0.70	16.33	0.10	0.64
Total pyrethrins	21.43	0.15	0.70	52.24	0.32	0.61

Table VIII. Linear Regression Results

Compound	Regression analysis equation	Correlation coefficient
Jasmolin I	$y = 0.4408x + 0.6017$	0.999983
Cinerin I	$y = 0.8509x + 2.1909$	0.999978
Pyrethrin I	$y = 7.5606x + 31.8678$	0.999940
Jasmolin II	$y = 0.7499x - 0.7393$	0.999527
Cinerin II	$y = 2.0427x + 0.6272$	0.999856
Pyrethrin II	$y = 8.5528x + 23.4517$	0.999933

Table IX. High and Low End Range Results

Replicate	60.53% Pyrethrum extract (lot R98-183) Assay (%)	%Recovery	9.88% Pyrethrum extract (dilute 3) Assay (%)	%Recovery
1	63.08	104.2	10.25	103.7
2	62.31	102.9	10.38	105.1
3	63.05	104.2	10.21	103.3
4	62.26	102.9	10.23	103.5
5	63.26	104.5	10.20	103.2
6	62.62	103.5	10.13	102.5
Average	62.76	103.7	10.23	103.5
Standard deviation	0.43		0.084	
RSD	0.69		0.82	

Table X. RSDs of the Individual Pyrethrum Esters for Six Replicate Analyses

	60.53% Pyrethrum extract (lot R98-183) Average content (%)	Standard deviation	RSD (%)	9.88% Pyrethrum extract (dilute 3) Average content (%)	Standard deviation	RSD (%)
Jasmolin I	2.86	0.02	0.78	0.47	0.0045	0.96
Cinerin I	5.79	0.04	0.73	0.95	0.0078	0.82
Pyrethrin I	25.18	0.17	0.67	4.13	0.04	0.87
Jasmolin II	2.92	0.03	0.92	0.48	0.0022	0.46
Cinerin II	6.38	0.04	0.67	1.04	0.0087	0.83
Pyrethrin II	19.63	0.13	0.65	3.17	0.03	0.93

in Table VII. The RSD of the six runs for each analyzed extract was within 2%. The precision of the six individual esters in each analyzed extract was also determined from the reported results of the six runs. Table VII shows that the RSD of each ester for the six runs was also within 2%.

Linearity

Linearity was tested by injecting a group of five pyrethrum extract standard solutions over the range of 50% to 150% of the expected analyte concentration of the developed method (i.e., 500 µg/mL of total pyrethrins). Linear relationships were set up between the elution peak areas and the concentrations for each of the esters. Correlation coefficients were then calculated by linear regression analysis as listed in Table VIII. We found that all six correlation coefficients were higher than 0.999, which therefore confirmed the linear relationships for the six components within the tested concentration range.

Range

With the confirmation of linearity within the interval between the upper (150%) and lower levels (50%) of the analytes, the high and low range of the expected pyrethrum extract purity was also checked. In order to encompass typical pyrethrum extracts of 20% and 50% pyrethrins, the high end of the range was determined by analyzing the extract containing approximately 60% pyrethrins, which was the highest concentration available. The low end of the method's expected range was determined by analyzing the diluted extract containing approximately 10% pyrethrins. The precision and accuracy of these determinations are summarized in Table IX. The precision and accuracy of both high and low range samples were closely comparable to the precision and accuracy values determined with the 20% and 50% extracts previously reported. These results indicated that the method is suitable for use with pyrethrum extract products over the range of 10% to 60% pyrethrins. The precision of the six individual esters was also determined from the reported results of the six runs of each analyzed extract. The RSD of each ester for the six runs was within 2% and met the reproducibility requirement. These results are presented in Table X.

Selectivity

Before the quantitation of each ester in the pyrethrum extract can take place, a separation of each of the esters from each other and from components of the sample matrix must be achieved. With the conditions outlined in the method, all six esters in the pyrethrum extract were baseline-separated. No interference from the sample matrix was evident in the chromatogram.

Diode-array peak-purity analysis

A more quantitative assessment of selectivity was achieved using peak purity readings from the diode-array detector (19). Ten commercial batches of 50% pyrethrum extract comprised of the previous five years of production were randomly selected. Typically, when using the HP ChemStation diode-array software, a component with a peak-purity value higher than 990 would be considered most likely to be a pure substance and the separation would be acceptable. All peak-purity assessments were determined over a wavelength range of 215 to 390 nm using nine

spectra per peak. We used the HP "All Spectra with Similarity Curves—Automatic Mode" to generate the peak-purity factors. The peak-purity results for the ten lots are summarized in Table XI. Most of the peak purities were over 990, indicating a lack of significant interfering components. Jasmolin I peak purities were slightly below 990 for three of the ten lots of pyrethrum extract checked, indicating a potential for a slight interference. This potential interference, however, was considered minor, because all three peak purities were above 980 and it is given that jasmolin I is a relatively minor component of pyrethrum extract having a small peak area. Examination of the individual replicates of the suspect jasmolin I peaks also confirmed this notion. In all three cases, one injection gave a peak purity over 990 and the second injection was less than 990. This indicated that there was variation in the derived peak purities for the small jasmolin I peaks. There was also one pyrethrin I peak purity below 990. This was also considered minor because it was above 980 and occurred in only one of ten batches. Given the complexity of this natural extract and the number of different batches examined, these peak-purity results were considered adequate.

Table XI. Diode-Array Peak-Purity Assessments of Ten Lots of 50% Pyrethrum Extract

	Average peak purity of duplicate injections					
Lot	Jasmolin I	Cinerin I	Pyrethrin I	Jasmolin II	Cinerin II	Pyrethrin II
98/1.5	989.2	995.7	999.5	998.6	999.6	999.8
98/2.2	998.9	998.4	1000.0	996.8	999.2	998.0
97/2.6	999.4	999.7	999.6	999.8	999.3	998.1
97/5.5	998.8	999.7	998.7	999.7	999.4	994.0
96/4.3	980.1	999.0	999.1	997.7	999.3	999.2
96/11.2	999.0	999.0	999.1	999.7	999.1	995.8
95/3.2	997.0	997.7	980.5	998.1	997.2	997.0
95/8.2	998.7	999.7	999.1	997.5	999.4	994.2
963	986.8	997.9	997.4	990.9	998.2	992.8
996	999.5	999.5	998.7	998.4	999.2	997.3

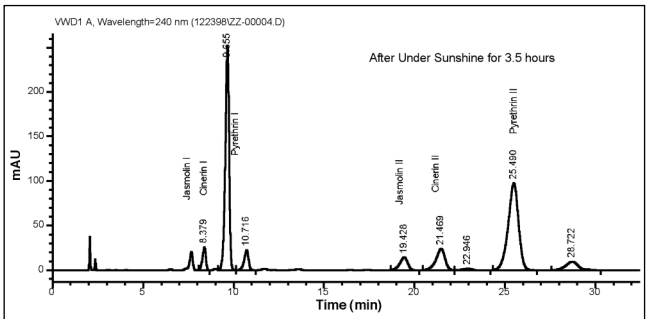


Figure 3. Chromatogram of pyrethrum extract after 3.5 h exposure to sunlight.

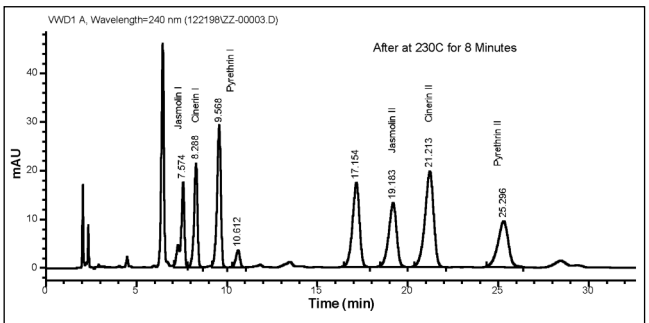


Figure 4. Chromatogram of pyrethrum extract after 8 min exposure to 230°C.

Inert ingredient screen

Because no placebo of pyrethrum extract was available, we were able to assess the potential for interference of only a few deliberate additives to pyrethrum extract. These were the solvent Isopar M fluid and BHT (stabilizer). No interfering component from either additive was present at the retention times (RTs) of the six esters of pyrethrum extract.

Forced degradation checks

Because there was no information readily available about the potential interference from the unidentified impurities or possible degradation products, this study also checked separation under conditions of forced degradation. The two most plausible modes for pyrethrin degradation were from exposure to light and high temperature. Additionally, an evaluation of forced degradation from exposure to alkaline conditions was made. Under each degradation case, a 100-mL volumetric flask containing 100 mg pyrethrum extract (50%) was put to test. The pyrethrum extract was spread over the flask bottom as a film. After the forced exposure, the flask was filled with hexane to the mark. This solution was then assayed to check the remaining pyrethrum extract and the degradation products.

An examination of the HPLC chromatograms showed that no significant degradation was evident when the sample was exposed to a regular light bulb overnight. However, when under mild sunshine in winter (December, Montvale, NJ) for 3.5 h, approximately

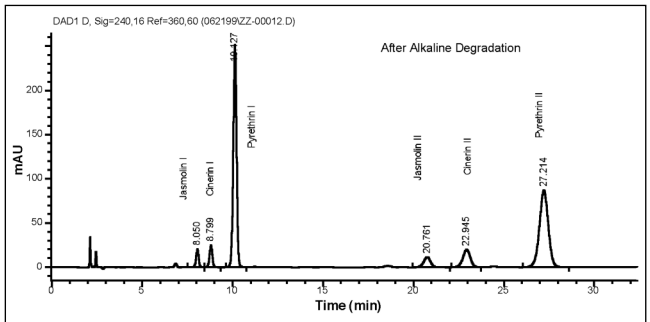


Figure 5. Chromatogram of pyrethrum extract subjected to alkaline conditions.

Table XII. Results for the HPLC Method Versus the AOAC Capillary GC Method

Replicate	GC method results Assay (%)	HPLC method results Assay (%)
1	52.81	52.40
2	53.25	52.63
3	53.70	52.22
4	53.56	52.23
5	52.77	52.28
6	53.32	51.67
Average	53.23	52.24
Standard deviation	0.38	0.32
RSD	0.72	0.61

6% degradation was found, as tested by the HPLC assay. Extra component peaks appeared in the chromatogram (Figure 3), and some of the six ingredients decreased in peak area. None of the extra peaks appeared to adversely interfere with the elution of each of the esters.

In order to check for forced heat degradation interferences, pyrethrum extract was exposed to temperatures greater than 230°C. When the pyrethrum extract was subjected to a temperature of 230°C for less than 8 min, approximately 34% of the pyrethrum extract had degraded. The color of the extract turned into charcoal black. The resulting chromatogram (Figure 4) showed the addition of two prominent and several minor degradation peaks. The two prominent peaks before jasmolin I and jasmolin II were believed to be the isomerization products of pyrethrin I and II called isopyrethrin I and II (15–18). Basically, no interference was found at the RTs of the six ingredients, except for a minor interference before jasmolin I. This interference can be partially separated from jasmolin I. Because jasmolin I was a minor ingredient compared with the other five, the minor interference to the jasmolin I should not significantly affect the quantitation of the total pyrethrin content.

The effect of alkalinity on the stability of pyrethrum extract was checked by mixing an organic base (triethanolamine) with pyrethrum extract (50:50, w/w). The color of the mixture turned reddish immediately. The HPLC assay of this mixture indicated that 33% of the pyrethrins degraded, presumably by ester cleavage. No peaks from degradation products were found in the chromatogram (Figure 5).

Verification using a secondary method

As a further check for assay bias, an assay was performed on the 50% dilution from pyrethrum pale concentrate using the developed HPLC method and a recently published AOAC capillary GC method as specified in the Experimental section (1). The same analytical standard was used for both procedures. Six samples of the extract were prepared. Each sample was run with an independently prepared standard. The analytical results are summarized in Table XII. The averaged result of 52.24% was obtained by the HPLC method and 53.23% by the AOAC GC method. Basically, the results from two different analytical approaches were in agreement with each other, further indicating that there was no bias in the selected HPLC procedure.

Ruggedness

In order to check the ruggedness of the method, samples of the same pyrethrum (20.69%) and pyrethrum (50.07%) extracts as analyzed previously in the Precision section were analyzed by a different analyst on different days using a different HPLC system

Table XIII. Ruggedness Results

	20.65% Pyrethrum extract		50.07% Pyrethrum extract	
Replicate	Chemist I Assay (%)	Chemist II* Assay (%)	Chemist I Assay (%)	Chemist II* Assay (%)
1	21.35	21.87	52.40	53.01
2	21.53	21.91	52.63	51.78
3	21.17	21.09	52.22	53.58
4	21.46	22.18	52.23	53.50
5	21.58	21.79	52.28	52.89
6	21.47	–†	51.67	53.83
Average	21.43	21.77	52.24	53.10
Standard deviation	0.15	0.41	0.32	0.74
RSD	0.70	1.87	0.61	1.39

* Chemist II was a novice and had no analytical experience.
† There was not enough sample available for run #6.

Table XIV. RT and Resolution of Pyrethrum Extracts Using a Mobile Phase of 97.75:2.25 (v/v) Hexane–THF

Pyrethrum ingredients	RT* (min)	RSD (%)	ΔRT† (min)	Resolution‡ (USP)
Jasmolin I	7.919	0.42	1.198	4.878
Cinerin I	8.667	0.38	0.748	2.715
Pyrethrin I	9.985	0.37	1.318	4.264
Jasmolin II	20.362	0.57	10.377	3.267
Cinerin II	22.515	0.53	2.153	2.989
Pyrethrin II	26.725	0.51	4.210	2.196

* RT and resolution were based on the average of two injections of the sample and four injections of the standard bracketing the sample.
† RT difference from the preceding ester (or impurity for Jasmolin I).
‡ Resolution for each ingredient was calculated between its preceding peak (impurity or active ingredient).

Table XV. RT and Resolution of Pyrethrum Extracts Using Mobile Phase of 98:2 (v/v) Hexane–THF

Pyrethrum ingredients	RT* (min)	RSD (%)	ΔRT† (min)	Resolution‡ (USP)
Jasmolin I	8.636	0.46	1.395	5.170
Cinerin I	9.471	0.46	0.835	2.761
Pyrethrin I	10.937	0.47	1.466	4.322
Jasmolin II	23.265	0.55	12.808	3.589
Cinerin II	25.748	0.56	2.483	3.000
Pyrethrin II	30.590	0.57	4.842	1.620

* RT and resolution were based on the average of two injections of the sample and four injections of the standard bracketing the sample.
† RT difference from the preceding ester (or impurity for Jasmolin I).
‡ Resolution for each ingredient was calculated between its preceding peak (impurity or active ingredient).

Table XVI. RT and Resolution of Pyrethrum Extracts Using Mobile Phase of 97.5:2.5 (v/v) Hexane–THF

Pyrethrum ingredients	RT* (min)	RSD (%)	ΔRT† (min)	Resolution‡ (USP)
Jasmolin I	7.371	0.32	1.047	4.486
Cinerin I	8.049	0.31	0.678	2.654
Pyrethrin I	9.243	0.32	1.194	4.172
Jasmolin II	18.252	0.30	9.009	2.221
Cinerin II	20.156	0.30	1.904	2.925
Pyrethrin II	23.864	0.35	3.708	2.914

* RT and resolution were based on the average of two injections of the sample and four injections of the standard bracketing the sample.
† RT difference from the preceding ester (or impurity for Jasmolin I).
‡ Resolution for each ingredient was calculated between its preceding peak (impurity or active ingredient).

and serial-number column. The results are expressed in Table XIII. Based on the average results obtained by chemists I and II in Table XIII, the RSDs were further calculated as 1.57% for the 20.65% pyrethrum extract and 1.63% for the 50.07% pyrethrum extract, which were both below 2%.

Robustness

The robustness of the method was examined by changing the composition of the mobile phase by ± 10% (i.e., 98:2 hexane–THF and 97.5:2.5 hexane–THF compared with the default 97.75:2.25). Samples of the 50.07% pyrethrum extract were run at each mobile phase respectively to check the effect of the mobile phase variation on the RT, resolution, and assay result for each pyrethrum active. The results are listed in Tables XIV through XVII. As expected, there were small changes in the RTs with varying mobile phase content. However, these RT shifts did not significantly affect the resolution of each pyrethrum ester with its preceding peak (impurity or pyrethrum ester). The resolution (USP) values were all greater than 1.5, which strongly supported the full separation of the six pyrethrum ingredients and any of the closely eluting impurities. The assay results in Table XVII provided further indication of the method tolerance for slight changes in a mobile phase. The assay results using each mobile phase composition were very close, with an RSD of only 0.23% between the three results. Also, under each of the mobile phase cases, it can be seen that the RTs for each pyrethrum ingredient (based on the six injections from the sample and corresponding standard) were all very consistent with an RSD less than 2%. As anticipated, the exact values of the RT and resolution changed with the variation of mobile phase composition, but neither the elution sequence nor the quantitative result were affected. This proves the method's robustness with varying mobile phase composition.

Conclusion

The normal-phase HPLC method described in this report has been developed and validated in terms of selectivity, accuracy, precision, linearity, range, ruggedness, and robustness. All of the criteria have been satisfied. The method is readily adaptable for use by the pyrethrum industry as a routine analytical procedure for the rapid and reliable monitoring of the blending, extraction, and refining process leading to marketable pyrethrum products. Furthermore, the method may be suitable as a basis for isolating the pure pyrethrin esters.

Table XVII. Assay Results Obtained Under Three Mobile Phase Compositions

Hexane–THF (v/v)	Formulated content (%)	Assayed content (%)
97.75:2.25	50.07	53.02
98.00:2.00	50.07	52.72
97.50:2.50	50.07	52.83
Average	–	52.83
Standard deviation	–	0.12
RSD	–	0.23

Acknowledgments

The authors of this study wish to thank Robin Slatter from Aventis Environmental Science and Donald Maciver for their help and suggestions during the completion of this work. The authors also wish to thank the Pyrethrum Board of Kenya for providing samples of the pyrethrum extracts.

References

1. K.T. Nguyen, R. Moorman, and V. Kuykendall. Determination of n-octyl bicycloheptene dicarboximide, pyrethrins, and butylcarbityl 6-propylpiperonyl ether in technical materials, concentrates, and finished products by capillary gas chromatography: collaborative study. *J. AOAC. Int.* **81:** 503–12 (1998).
2. R. Moorman and K.T. Nguyen. Identification and quantitation of the six active compounds in a pyrethrin standard. *J. AOAC Int.* **80:** 966–73 (1997).
3. A.M. McEldowney and R.C. Menary. Analysis of pyrethrins in pyrethrum extracts by high performance liquid chromatography. *J. Chromatogr.* **447:** 239–43 (1998).
4. *Official Methods of Analysis,* 15th ed. AOAC, Arlington, VA, 1990, pp. 170–73.
5. D.A. Otieno, I.J. Jondiko, P.G. McDowell, and F.J. Kezdy. Quantitative analysis of the pyrethrins by HPLC. *J. Chromatogr. Sci.* **20:** 566–70 (1982).
6. I.-H. Wang, V. Subramanian, R. Moorman, J. Burleson, J. Ko, and D. Johson. A validated reversed-phase HPLC method for analyzing pyrethrins, piperonyl butoxide, and (s)-methoprene in pesticide formulations. *LC-GC* **17:** 260–75 (1999).
7. C. Tomlin. *The Pesticide Manual, A World Compendium,* 10th ed. British Crop Protection Council, Surrey, U.K., and The Royal Society of Chemistry, Cambridge, U.K., 1994.
8. H.P. Their and H. Zeumer, *Manual of Pesticide Residue Analysis.* VCH, Weinheim, Germany, Volume 1, 1987.
9. R.J. Bushway. Normal phase liquid chromatographic determination of pyrethrins in formulations. *J. Assoc. Off. Anal. Chem.* **68:** 1134–36 (1985).
10. C.M. Dickinson. Stability of individual natural pyrethrins in solution after separation by preparative high performance liquid chromatography. *Pyrethrum Post* **16:** 105–10 (1987).
11. *U.S. Pharmacopeia 24/ National Formulary 19.* U.S. Pharmacopeia Convention, Rockville, MD, 2000, pp. 1445–46.
12. J.E. Cassida. *Pyrethrum, The Natural Insecticide.* Academic Press, New York, NY, 1973.
13. K. Essig and Z. Zhao. Preparation and characterization of pyrethrum extract standard. *LC-GC* **19:** 722–30 (2001).
14. *Wiley 138K Mass Spectra Database.* John Wiley and Sons, New York, NY, 1990.
15. *Capillary GC Separation of Thermally Labile Natural Pyrethrins.* Scientific Glass Engineering, Austin, TX, 1995.
16. N.C. Brown, R.F. Phipers, and M.C. Wood. The analysis of pyrethrins, part III: a correlation of various analytical methods with bioassays. *Pyrethrum Post* **4:** 24–29 (1956).
17. N.C. Brown, D.T. Hollinshead, R.F. Phipers, and M.C. Wood. New isomers of the pyrethrins formed by the action of heat. *Pyrethrum Post* **4:** 13–19 (1957).
18. L.W. Levy and D. Geller. A rate on the detection of the "isopyrethrins". *Pyrethrum Post* **5:** 12 (1959).
19. "HP ChemStation for LC 3D Systems—Installing and Understanding Your Spectra Module". Hewlett Packard, Waldbronn, Germany, 1995.
20. D.R. Maciver. "Constitutes of Pyrethrum Extract". In *Pyrethrum Flowers Production, Chemistry, Toxicology and Uses.* J.E. Casida and G.B. Quistad, Eds. Oxford University Press, Oxford, U.K., 1995, p 109.

Manuscript accepted September 5, 2001.