Preparation and Characterization of a Pyrethrum Extract Standard

The authors separated and collected milligram quantities of six pyrethrin esters in pyrethrum extract by semipreparative, normal-phase high performance liquid chromatography (HPLC). They structurally confirmed each ester by Fourier transform–infrared spectroscopy and gas chromatography–mass spectrometry. They also checked retention times based upon previously developed HPLC methodology. They determined the purity of each ester based upon the chromatographic area percentage. The authors then used the esters as individual standards to restandardize the content of pyrethrin esters in two selected pyrethrum extracts, which they previously had assayed using the Association of Official Analytical Chemists International titration method. They will submit one of the selected pyrethrum extracts to the *U.S. Pharmacopoeia* as a potential reference standard for additional applications.

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Pyrethrum extract comprises three closely related insecticidal esters of chrysanthemic acid — cinerin-I, jasmolin-I, pyrethrin-I — and three closely related insecticidal esters of pyrethric acid — cinerin-II, jasmolin-II, and pyrethrin-II. The three chrysanthemic acid esters commonly are identified as pyrethrins 1, and the three pyrethric acid esters commonly are identified as pyrethrins 2 (see Table I and Figure 1). Collectively, the esters are called *rethrins.* Because of the separation difficulty and lack of absolute standards for each component, the content of pyrethrum extract usually is not reported for the individual six rethrins but analyzed for total pyrethrins, total pyrethrins 1, or total pyrethrins 2.

Many laboratories have sought to develop a reliable analytical method that can simultaneously determine the content of each of the six components of pyrethrum extract (1–5). Although gas chromatography (GC) methods have been developed for this purpose, they cannot avoid sample thermodegradation because of the high temperatures used (1,2). In a previous study, we developed and fully validated normal-phase high performance liquid chromatography (HPLC) analytical methodology that meets the required validation criteria and has been used as a candidate for the forthcoming *U.S. Pharmacopoeia* (*USP*) pyrethrum extract monograph (6). This method provides the basis for quantitative analysis of each ingredient in pyrethrum extract. The remaining problem is the absence of true analytical standards or a reference standard for the quantitative determination of the six rethrins. The purpose of this study is to prepare and characterize milligram quantities of individual standards for each of the six esters in pyrethrum extract. These true standards then can be used to standardize and fully characterize reference standards of the pyrethrum extract.

Experimental

Materials: We obtained three pyrethrum extracts from the Pyrethrum Board of Kenya (Nakuru, Kenya). The extracts were pyrethrum pale concentrate bulk lot number 87/9-4 (AOAC assay 60.53%); pyrethrum extract lot number 96/11.2 (AOAC assay 50.47%); pyrethrum extract lot number 98/3.9 (AOAC assay 53.19%). Of these pyrethrum extracts, we used pyrethrum pale concentrate lot 87/9-4 for

preparing individual standards of the six ingredients and the other two extracts as candidates for reference standards. We purchased HPLC-grade hexane and tetrahydrofuran from Fisher Scientific (Fairlawn, New Jersey).

Instrument and running conditions: The HPLC assays were performed according to our previously developed normal-phase method using a standard Agilent 1100 HPLC system (Agilent Technologies, Wilmington, Delaware) with a diode-array detector (6). We used a 25 cm × 4.6 mm, 5-µm d_p Spherex cyano column (Phenomenex, Inc., Torrance, California). The mobile phase was 97.75:2.25 (v/v) hexane–tetrahydrofuran with a flow rate of 1.5 mL/min. The detector wavelength was set at 240 nm, and the sample injection size was 10 µL. Each run required 35 min. The instrument was calibrated with a single-point external standard calibration regime using the six individual collected esters as reference standards. Samples of each pyrethrum extract were bracketed by multiple injections of each reference standard ester. The peak area of each ester in the standard and sample solutions was within the linear range of the method.

GC–mass spectrometry (MS) characterization was performed using an Agilent 5890 II Plus series GC–MS system with an Agilent 5972 mass-selective detector (both from Agilent Technologies). The system was equipped with a 30 m × 0.25 mm, 0.25-µm d_f Agilent 5MS capillary column. The injection volume was 2 µL, the helium carrier-gas flow was 1.25 mL/min, and the split-vent flow rate was 20 mL/min. The oven temperature was programmed to start at 185 °C and hold for 30 min, increase to 220 °C at 10 °C/min, hold at 220 °C for 30 min, increase to 300 °C at 30 °C/min, and hold at 300 °C for 10 min. The injector temperature was 250 °C, and the MS transfer line temperature was 280 °C.

We performed packed-column GC analysis using an Agilent 5830A series packed-column GC system with a 3 ft × 2 mm column packed with 3% OV-101 on 80/120 Supelcoport (Supelco, Bellefonte, Pennsylvania). The on-column injection volume was 2 µL, and the run time was 30 min. We used nitrogen carrier gas with a flow rate of 40 mL/min. The isothermal oven temperatures were 160 °C for jasmolin I and cinerin I, 170 °C for pyrethrin I, and 180 °C for jasmolin II, cinerin II, and pyrethrin II. The temperature for the injector and flame ionization detector was 250 °C.

We performed Fourier transform–infrared (FTIR) spectroscopy analysis using a PerkinElmer 1600 series FTIR spectrophotometer (PerkinElmer Corp., Norwalk, Connecticut). The background was a potassium bromide crystal pallet in air, and the resolution was 4.0 cm^{-1}. The scan range was 440–4400 cm^{-1}, and the scan number was 16. We prepared samples for infrared (IR) spectroscopy by coating the pallets with the test solution and then air drying them. Then we measured the prepared sample on the spectrometer with the ratio calculated against the background. All the analyses were run at ambient temperature.

Standard preparation summary: We performed preparative separation of the standards using a standard Agilent 1100 HPLC system with a diode-array detector and a hexane solution that contained 20% pyrethrum pale concentrate, made for this purpose. The separation and collection were achieved based upon the extension of our previously developed normal-phase HPLC method for pyrethrum extracts (6). We used a 25 cm × 10 mm, 8-µm d_p semipreparative HS Hyper Prep 100 silica column (Alltech Associates, Inc., Deerfield, Illinois) instead of the cyano column and increased the injection volume to 50 µL. Because of the large injection volume and the semipreparative column, we adjusted the original mobile phase and flow rate to accommodate the proper separation and collection. Table II lists the gradient program we used to improve the separation efficiency. The total run time was 100 min. The detector wavelength was 240 nm.

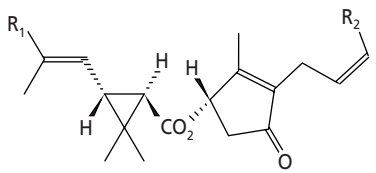
Each ester was collected manually after its elution. We dropped the starting and ending fractions to avoid any possible interfering components from neighbor species. We transferred the collected eluted analytes from each run into six preweighed flasks, one for each of the individual esters. We covered the flasks with foil to protect the analytes from

Table I: Six individual esters of pyrethrum extract

Common Name	Chemical Abstracts Service 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

Table II: Mobile-phase gradient program

Time (min)	Hexane–Tetrahydrofuran	Flow Rate (mL/min)
0	98.4:1.6	1.20
7.00	98.4:1.6	1.20
7.01	98.0:2.0	1.20
39.00	98.0:2.0	1.20
39.01	98.0:2.0	5.00
64.00	98.0:2.0	5.00
64.01	98.4:1.6	1.20
80.00	98.4:1.6	1.20
80.01	98.0:2.0	5.00
90.00	98.0:2.0	5.00



Cinerin-I : R_1 CH_3, R_2 CH_3
Jasmolin-I : R_1 CH_3, R_2 C_2H_5
Pyrethrin-I : R_1 CH_3, R_2 $CHCH_2$
Cinerin-II : R_1 CO_2CH_3, R_2 CH_3
Jasmolin-II : R_1 CO_2CH_3, R_2 C_2H_5
Pyrethrin-II : R_1 CO_2CH_3, R_2 $CHCH_2$

Figure 1: Structures of the six individual esters in pyrethrum extract.

light and allowed the flasks to vent at room temperature to promote solvent evaporation during the multiple-run collection process. The eluents were collected over a period of approximately two weeks, comprising approximately 45 injections. We dried the final eluted residues, after solvent evaporation, at room temperature under 30 in. Hg vacuum for 36 h in a precision vacuum oven. We ended vacuum drying when we observed no additional weight loss. The flasks were weighed to obtain the crude yield for each ester (see Table III). For analytical accuracy, we redissolved each ester with hexane and transferred it into a second carefully handled and preweighed vial. Those vials then underwent the same drying procedure to eliminate the solvent. After we weighed the flasks, we used the obtained weight (see Table III) for each ester in standard solution preparation. Because it would be very difficult to transfer such small quantities in milligrams, we pipetted solvent into each vial for the solution preparation. Based upon the weights obtained, we pipetted exactly 50 mL of hexane into each flask. We used these dilutions as master solutions of each extract. The master solutions then were further diluted by taking 6, 6, 5, 6, 6, and 5 mL of the master solution for jasmolin-I, cinerin-I, pyrethrin-I, jasmolin-II, cinerin-II and pyrethrin-II, respectively, and diluting them to 50 mL in hexane. We used these solutions as standard solutions for HPLC analysis.

Results and Discussion

Preparative separation: As Figure 2 shows, all six rethrin esters in pyrethrum extract were well separated with the following elution sequence: jasmolin-I (~34 min), cinerin-I (~38 min), pyrethrin-I (~43 min), jasmolin-II (~67 min), cinerin-II (~70 min), and pyrethrin-II (~78 min). The elution sequence actually was the same as the sequence we found previously using the normal-phase HPLC assay method. The elution could be clearly classified into two groups of jasmolin-I, cinerin-I, and pyrethrin-I and of jasmolin-II, cinerin-II, and pyrethrin-II; each grouping could be attributed to the chemical structure similarity. After drying, each ester was revealed as a clear gummy deposit. Table III lists the crude yields and refined weights.

Before we could use each of the isolated esters as standards for assay purposes, we needed to specify their structural identities and purities. In this study, we confirmed the structural identity by FTIR spectroscopy, GC–MS, and chromatographic elution behaviors. The purity was determined for each ester based upon the area abundance of the ester peak as a fraction of the total chromatographic area.

FTIR spectroscopy: We measured the FTIR spectra from each of the obtained esters using the master solutions coated on a potassium bromide plate. The spectra show that each ester exhibited enough of a fingerprint — in addition to the primary absorption bands caused by C=O (~1700 cm^{-1}), C=C (~1645 cm^{-1}), C–O (~1160 cm^{-1}), and C–H (~3000 cm^{-1}) — to permit differentiation. Based upon the C–O stretch bands between wave numbers of approximately 1100 and 1257 cm^{-1}, the spectra classify the six esters into two groups: pyrethrins 1, comprising jasmolin-I, cinerin-I, and pyrethrin-I, and pyrethrins 2, including jasmolin-II, cinerin-II, and pyrethrin-II. Pyrethrins 1 species are monoesters that exhibited bands at 1108, 1152, and 1257 cm^{-1}. Pyrethrins 2 species are diesters that exhibited bands at 1110, 1147, 1171, and 1257 cm^{-1}. The extra C–O split band at 1171 cm^{-1} was caused by the αβ-unsaturated carboxylate ester (R_1 position in Figure 1). For the same reason, the C=O band in the diesters shifted to the low frequency 1715 cm^{-1} because of the carbonyl conjugation, and the monoesters showed a C=O stretch at 1720 cm^{-1}.

The esters can be further distinguished within each group between jasmolin, cinerin, and pyrethrin. Pyrethrins — both pyrethrin-I and pyrethrin-II — can be recognized easily by the band at 910 cm^{-1}, which is attributed to the C–H out-of-plane deformation in the diene structure (R_2 position in Figure 1). Both jasmolins and cinerins showed C–H asymmetric and symmetric bands in the region of approximately 2860–2930 cm^{-1}. However, jasmolins could be identified simply by the higher band intensity caused by the ethyl group C_2H_5 (R_2) compared with those caused by the methyl group CH_3 (R_2) in cinerins.

GC–MS: We checked the master solutions of the collected esters using GC–MS to provide additional purity information and a confirmation of the structural identity. Based upon the total ion chromatograms, each ester displayed only one major peak in spite of minor impurity peaks that appeared in some samples. The retention-time profile for the esters matched those reported in the literature (1,2). As expected, we encountered extensive sample thermodegradation for the pyrethrin-I and pyrethrin-II samples. The thermal degradation was exhibited

Table III: Masses of the six esters obtained for the standard solutions

Ester Identification	Crude Separation Yield (mg)	Mass Used for Standard (mg)
Jasmolin-I	12.3	10.2
Cinerin-I	19.9	16.7
Pyrethrin-I	93.4	90.6
Jasmolin-II	11.4	7.8
Cinerin-II	24.7	21.0
Pyrethrin-II	86.9	80.1

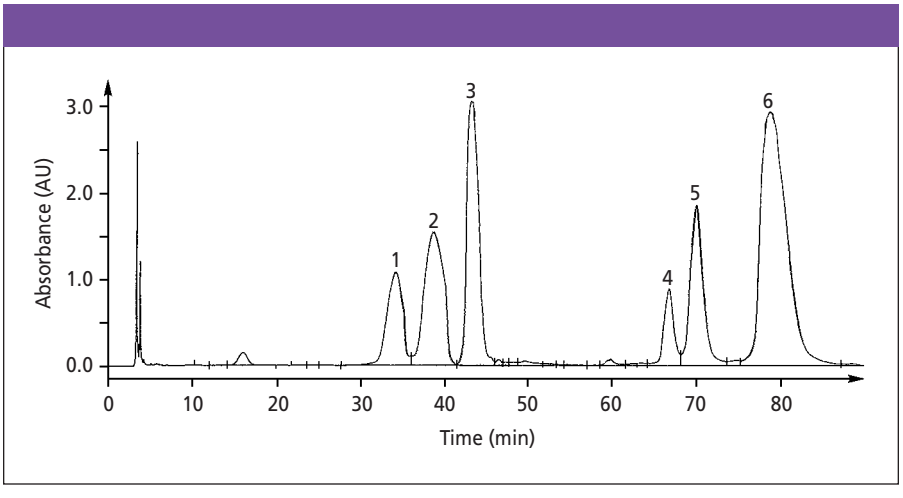


Figure 2: Chromatogram of preparative separation of pyrethrum extract. Peaks: 1 = jasmolin-I, 2 = cinerin-I, 3 = pyrethrin-I, 4 = jasmolin-II, 5 = cinerin-II, 6 = pyrethrin-II.

as a broad, tailing peak immediately after the main peak and as a small peak immediately preceding the main peak. As a further check, we found that adjusting the oven temperature indicated that the peaks caused by thermodegradation increased with temperature. For this reason, we considered the sample purities based upon the GC peak area abundance for pyrethrin-I and pyrethrin-II to be approximations.

We selectively surveyed electron ionization mass spectra for the eluted peaks summarized in Table IV. It was noticeable that the fragments *m/z* 123 and 168 (169) were characteristic of pyrethrins 1 species, and fragments *m/z* 168 (169) and 212 were characteristic of pyrethrins 2 species. However, the same *m/z* 168 (169) fragment actually represented different structure fragments for pyrethrins 1 and pyrethrins 2. Furthermore, fragment *m/z* 163 (164) was the fingerprint for jasmolins, fragment *m/z* 149 (150) was the fingerprint for cinerins, and fragment *m/z* 161 (162) was the fingerprint for pyrethrins. In addition, we also detected molecular ions for pyrethrins 1 species; that is, *m/z* 330 for jasmolin-I, *m/z* 316 for cinerin-I, and *m/z* 328 for pyrethrin-I. All the measured mass spectra contained the expected ion fragments and, therefore, strongly supported the structures of the obtained esters. The spectra also matched the mass spectra found in our mass spectra library (7).

Purity determined by chromatography: We assayed each master solution by HPLC using the normal-phase assay method that we developed earlier. We used diode-array ultraviolet (UV) detection for this purpose. In an effort to detect any non-UV absorbing species, we also used a refractive index detector for purity determination. The UV detector showed significantly better sensitivity compared with the refractive index detector; however, we encountered no significant additional impurities with the refractive index chromatograms. In the diode-array analysis, each ester displayed a main peak with a retention time that matched the times found in previous pyrethrum extract samples. All the main peaks showed high values for peak purity index, calculated as more than 990 by the instrument software (ChemStation, Agilent Technologies). The minor impurity peaks were well separated from the main peak, and this separation provided the basis for purity determination based upon peak area abundance.

In GC–MS characterization, we determined the purity for each obtained ester based upon the peak area abundance using total ion chromatograms. We used the master solutions to increase the detection level for any possible impurity. We also injected these solutions into a GC–flame ionization detection (FID) system using a short packed column. The short packed column was operated at a lower temperature with on-column injections to minimize thermal degradation. Thermal degradation appeared to be reduced with the packed column, but the method sensitivity and resolution of trace impurities was questionable.

Compared with GC, HPLC avoids the interfering traces in the pyrethrin-I and pyrethrin-II chromatograms that result from thermal degradation; therefore, HPLC is preferable and provides a more accurate determination of purity. The diode-array detector also provided UV spectra for each of the measured esters. Similar to FTIR and MS spectra, UV spectra of pyrethrins 1 species clearly differentiated from those of pyrethrins 2 species. Although the UV spectra represented the absorbance from combined chromophores, the difference between pyrethrins 1 and 2 was attributed mainly to the R_1 function group (see Figure 1); that is, CH_3 for pyrethrins 1 and CH_3OOC for pyrethrins 2. The HPLC area percentage results were chosen over the GC results because the HPLC procedure was the most sensitive and avoided the effects of thermal degradation. Despite the limitations of the GC characterization data, the GC results support those from HPLC. Table V lists purity results measured by HPLC (UV detection), GC–MS, and GC–FID. The lower assay value for the GC analysis of pyrethrin-II was caused by long-chain alkane contaminants found in the master solution. The identities of these contaminants were confirmed by GC–MS. We believe these contaminants were introduced after the collection and weighing of the pyrethrin-II fraction. Further substantiating

Table IV: Structure units detected from the mass spectra

Ester Identification	Common Fragments (*m/z*)	Characteristic Fragments (*m/z*)
Jasmolin-I	55, 107, 133	123, 164, 169, 330
Cinerin-I	55, 107, 133	123, 150, 168, 316
Pyrethrin-I	55, 107, 133	123, 162, 168, 328
Jasmolin-II	55, 107, 133	163, 168, 212
Cinerin-II	55, 107, 133	149, 169, 212
Pyrethrin-II	55, 107, 133	161, 169, 212

Table V: Purities of the six esters determined based upon chromatographic area abundance

Ester Identification	HPLC–UV Determination (%)	GC–MS Confirmation (%)	GC–FID Confirmation (%)
Jasmolin-I	97.67	99.93	100.0
Cinerin-I	98.23	99.88	100.0
Pyrethrin-I	96.96	99.86	99.00
Jasmolin-II	98.58	99.96	100.0
Cinerin-II	96.59	99.94	99.76
Pyrethrin-II	99.36	94.75	92.90

Table VI: Individual ester abundance as weight-to-weight percentage of the two pyrethrum extracts

	Pyrethrum Extract Lot Number 96/11.2		Pyrethrum Extract Lot Number 98/3.9	
Ester Identification	Calculated (%)	Current (%)	Calculated (%)	Current (%)
Jasmolin-I	2.487	2.69	2.333	3.01
Cinerin-I	5.053	4.88	3.880	4.54
Pyrethrin-I	19.906	20.09	23.076	22.13
Jasmolin-II	2.408	1.83	1.971	2.07
Cinerin-II	5.331	4.89	3.678	4.59
Pyrethrin-II	15.286	17.04	18.261	18.17

Table VII: Pyrethrins content as weight-to-weight percentage of the two pyrethrum extracts

	Pyrethrum Extract Lot Number 96/11.2			Pyrethrum Extract Lot Number 98/3.9		
Identification	AOAC Pasadena (%)	AOAC Kenya (%)	Current Assay (%)	AOAC Pasadena (%)	AOAC Kenya (%)	Current Assay (%)
Pyrethrins 1	27.44	27.46	27.66	29.30	29.27	29.68
Pyrethrins 2	23.03	22.78	23.77	24.98	22.84	24.83
Total pyrethrins	50.47	50.24	51.43	54.28	52.11	54.51

this notion is the fact that the contaminants were not identified in the starting pyrethrum pale concentrate when screened by GC–MS. In earlier work, we found similar contaminants and attributed them to rubber septa from autosampler vials. For these reasons, we are confident in the accuracy of the HPLC purity assignment.

Selection of the reference standard: Based on the six prepared esters used as absolute standards, the two selected pyrethrum extracts listed in Table 1 were assayed for individual ester content (see Table VI) using our developed normal phase HPLC methodology (6). We must point out that until now no other available approach could simultaneously provide the content for the six pyrethrin esters because of unavailability of the standards. For consideration, Table VI lists the calculated abundance of the six esters for both pyrethrum extracts based on the pyrethrins 1, 2, and total pyrethrins determined using the AOAC mercury reduction method 936.05 (8), the published spectral extinction coefficients for the six esters (9), and the HPLC peak area proportions at each lambda maximum wavelength. The Association of Official Analytical Chemists International (AOAC International) mercury reduction method 936.05 is the current industry-accepted technique for pyrethrins content determination that can provide pyrethrins 1, 2, and total pyrethrins. Due to the multistep nature and complexity of the AOAC International titration method, we found deviations between the results from different laboratories among the AOAC International reported values in our previous study (6).

Nevertheless, the only way for us to check the accuracy of our obtained results was to compare pyrethrins 1 and 2 and total pyrethrins with those determined using the AOAC International mercury reduction method. We added the determined ester abundances to provide pyrethrins content and compared these results with the AOAC International mercury reduction method results reported by the Aventis quality control department (Pasadena, Texas) and the Pyrethrum Board of Kenya, respectively. Table VII shows that the currently determined pyrethrins content — pyrethrins 1 and 2 and total pyrethrins — all were close to the results obtained by AOAC International method for both pyrethrum extracts. Pyrethrum extract lot number 98/3.9 was selected as the reference standard for further application because of its larger quantity.

Conclusion

This study explored the preparation of absolute standards for pyrethrum extracts. Based upon previously developed normal-phase HPLC methodology, we collected six insecticide esters in pyrethrum extracts in milligram quantities by semipreparative HPLC. We fully characterized the structural identity and purity of each. By using the obtained individual esters as absolute standards, we found the assay content of the two selected pyrethrum extracts to be close to that reported by the AOAC International titration method.

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OF INTEREST

Equipment grants available

Shimadzu Scientific Instruments, Inc. (Columbia, Maryland), has established an equipment grant program to assist academic and nonprofit organizations in acquiring analytical instruments for research and education. The grant program's goals are to encourage research into areas of mutual interest and to make analytical instruments available to laboratories. Shimadzu will award funds to universities, colleges, private foundations, and similar organizations that perform research aimed at the company's mission of contributing to society through science and technology.

Agilent and Advanced Chemistry Development sign agreement

Agilent Technologies Inc. (Palo Alto, California) has signed a co-marketing agreement with Advanced Chemistry Development Inc. (Toronto, Ontario, Canada), a supplier of software for chemistry, biochemistry, pharmacology, and related fields. The companies have agreed to share demonstration software, marketing materials, and sales leads for Agilent's mass spectrometry products and Advanced Chemistry Development's SpecManager software. The companies plan to investigate opportunities for closer integration of their products.