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Molecular Markers for Pyrethrin Autoxidation in Stored Pyrethrum Crop: Analysis and Structure Determination

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Supporting Information

ABSTRACT: Pyrethrum is a natural insecticide extracted from *Tanacetum cinerariifolium*. Six esters, the pyrethrins, are responsible for the extract's insecticidal activity. The oxidative degradation of pyrethrins through contact with aerial oxygen is a potential cause of pyrethrin losses during pyrethrum manufacture. Described here is the first investigation of the autoxidation chemistry of the six pyrethrin esters isolated from pyrethrum. It was found that pyrethrins I and II, the major pyrethrin esters present in pyrethrum, undergo autoxidation more readily than the minor pyrethrin esters, the jasmolins and cinerins. Chromatographic analysis of pyrethrin I and II autoxidation mixtures showed some correlation with a similar analysis performed on extracts from *T. cinerariifolium* crop, which had been stored for 12 weeks without added antioxidants. Two pyrethrin II autoxidation products were isolated, characterized, and shown to be present in extracts of stored *T. cinerariifolium* crop, confirming that autoxidation of pyrethrin esters does occur during crop storage.

KEYWORDS: *Tanacetum cinerariifolium, pyrethrum, pyrethrins, autoxidation, degradation*

INTRODUCTION

"Pyrethrum" is the term used to describe the insecticide that is extracted and refined from dried pyrethrum daisies (*Tanacetum cinerariifolium*). These daisies are grown commercially in temperate regions or highland areas of the tropics. The potent insecticidal properties of pyrethrum and its fast action, low toxicity to plants and mammals, and rapid degradation in the environment combine to make it ideal for many insecticidal applications. In particular, it is most suited for "sensitive" uses such as in food warehouses, homes, pet shampoo, and organic agriculture and for human health. Pyrethrum contains six insecticidal esters known collectively as "pyrethrins".[1] These bioactive compounds occur in two groups (Figure 1), pyrethrins I (pyrethrin I,**1**; cinerin I, **2**; and jasmolin I, **3**) and pyrethrins II (pyrethrin II, **4**; cinerin II, **5**; and jasmolin II, **6**). Chemically, these compounds are esters, formed between chrysanthemic acid or chrysanthemic diacid and three different hydroxylated cyclopentenones,[2] the latter known collectively as the "rethrolones". Pyrethrins I and II are the major components of pyrethrum with the ratio of pyrethrins I to pyrethrins II varying depending on plant variety, geography, and timing of harvest.

The susceptibility of pyrethrins to degrade upon exposure to light,[1,3,4] oxygen, and heat[5] is a desirable attribute from an environmental perspective. Care, however, must be taken to minimize pyrethrin losses during manufacture of the pyrethrum product. To this end, the crop is protected from exposure to light and high temperatures after harvesting and is amended with the antioxidant butylated hydroxytoluene (BHT).

Limited information has been published on the chemical transformations of pyrethrins that occur during postharvest processing of pyrethrum, but one such process most likely involves oxidative attack. To date, information about the oxidative degradation of pyrethrins has been largely derived from studies related to photoxidation and oxidative mammalian metabolism. Chen and Casida[6] studied the photoxidation of pyrethrin I and used ^{14}C-labeled pyrethrin I and 2D thin layer chromatography to show that pyrethrins decompose to give many products when exposed to a sun lamp in the air. Although the exact identity of the degradation products was not determined, many appeared to result from oxidation of the chrysanthemic acid portion of the pyrethrins. These workers also indicated that the "alcohol moiety", presumably the rethrolone fragment, of pyrethrin I is "most susceptible to photodecomposition".

Extensive studies of the oxidative mammalian metabolism of pyrethrins have been undertaken by Casida and co-workers,[1,7] who identified processes that led to the formation of side-chain epoxides and diols, as well as oxidative processes that occur on the chrysanthemic acid portion of the pyrethrins. Preliminary work by Williams[8] led to the suggestion that the diols **7**−**9** (Figure 1), oxidation products of pyrethrin II and previously identified by Casida as pyrethrin metabolic products, were produced upon heating ground pyrethrum seed.

Another potential route of oxidative degradation of pyrethrins is via microbial activity,[1] although products formed by this mode

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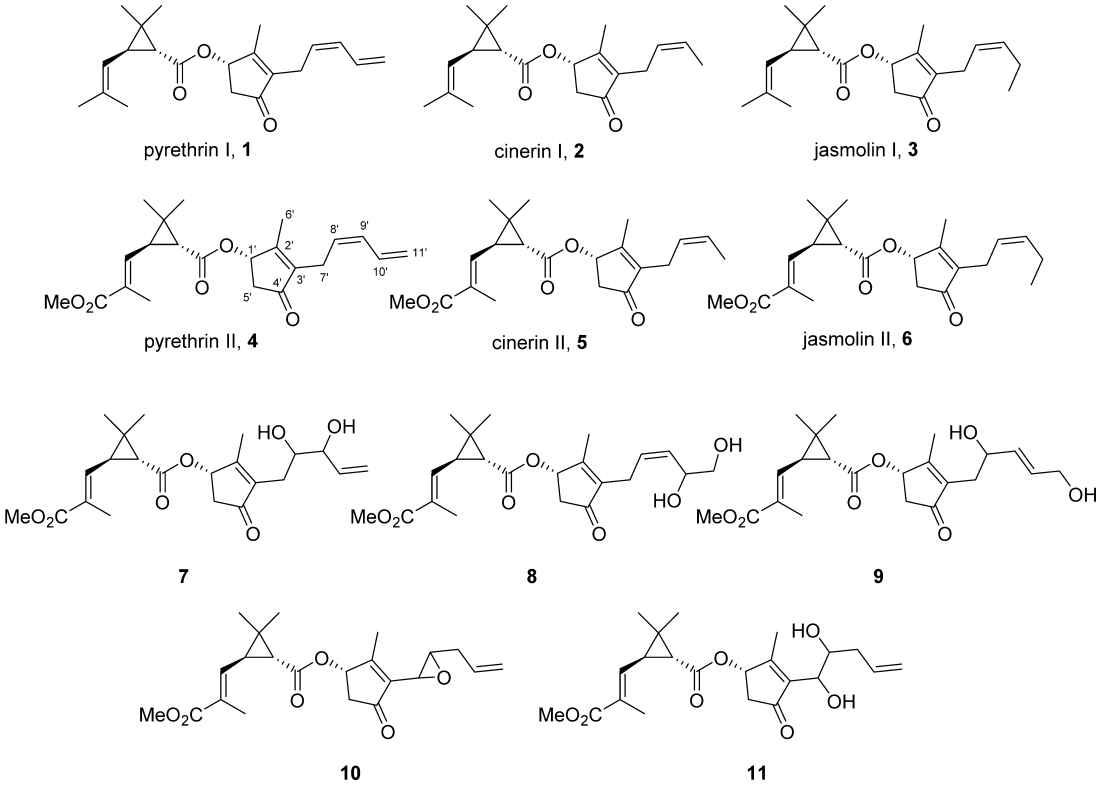

Figure 1. Six insecticidal esters of pyrethrum (**1**−**6**) and side-chain oxidation products of pyrethrin II (**7**−**11**).

of degradation have not been reported. To date, no evidence has been found to suggest that microorganisms play a role in the metabolism of pyrethrins under the conditions in which the crop is stored.[5]

A study of the electrochemical oxidation of synthetic pyrethroids and pyrethrins I and II was published by Bond and co-workers.[9] They found that pyrethrins could be irreversibly oxidized under conditions of cyclic voltammetry. No attempt was made to identify the oxidation products; however, the authors indicated that a wide range of compounds were formed, and the overall reaction pathways for oxidation of the pyrethrins are complex.

To date, the autoxidation chemistry, that is, free radical chain reactions mediated by molecular oxygen, of pyrethrins has not been subjected to close investigation. The structural similarity of the "doubly allylic center" (i.e., methylene centers flanked by two double bonds) in the rethrolone side chain of the pyrethrins to unsaturated fatty acids such as linoleate,[10] however, enables parallels to be drawn between the autoxidation of lipids and potential autoxidation reactions of pyrethrins. The chain oxidation process that is thought to apply in the autoxidation of lipids is as follows:[11]

Initiation:

$$\mathrm{In{-}In} \rightarrow 2\mathrm{In}^{\bullet} \quad (1)$$

$$\mathrm{In}^{\bullet} + \mathrm{L{-}H} \rightarrow \mathrm{In{-}H} + \mathrm{L}^{\bullet} \quad (2)$$

Propogation:

$$\mathrm{L}^{\bullet} + \mathrm{O_2} \rightarrow \mathrm{L{-}OO}^{\bullet} \quad (3)$$

$$\mathrm{L{-}OO}^{\bullet} + \mathrm{LH} \rightarrow \mathrm{L{-}OOH} + \mathrm{L}^{\bullet} \quad (4)$$

Termination:

$$2\mathrm{L{-}OO}^{\bullet} \rightarrow [\mathrm{L{-}OO{-}OO{-}L}] \quad (5)$$

$$[\mathrm{L{-}OO{-}OO{-}L}] \rightarrow \text{nonradical products} + \mathrm{O_2} \quad (6)$$

In−In represents a radical initiator, and LH represents a lipid.

The major observable product expected from this process is a hydroperoxide, L−OOH, where L represents the lipid chain. Hydroperoxides may be unstable and undergo a number of nonradical decomposition reactions. The rate of formation of L−OOH (eq 4) is concentration-dependent and also depends on the C−H bond dissociation energy of L−H. Through experimental observations and theoretical calculations, it has been established that the doubly allylic positions are most susceptible to autoxidation.[10]

In the study described herein, extracts of stored pyrethrum crop were analyzed for the presence of pyrethrin degradation products in the hope of gaining insights into the main causes of pyrethrin losses during storage and into potential ways of reducing these losses in the future. Evidence for the presence of molecular markers of metabolic oxidation and autoxidation of pyrethrin esters was sought, and the results were rationalized in terms of the autoxidation processes known to occur with unsaturated lipids. Included in this work is the first reported investigation of the autoxidation chemistry of pyrethrins, jasmolins, and cinerins.

MATERIALS AND METHODS

Chemicals and Reagents. *m*-Chloroperbenzoic acid (*m*-CPBA), azobis(isobutyronitrile) (AIBN), and BHT were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC solvents were purchased from Fisher Scientific (Loughborough, UK), and all other solvents were obtained from Merck (Darmstadt, Germany). Water for HPLC and

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UPLC was purified through a Milli-Q system (Millipore, Billerica, MA, USA).

General Experimental Procedures. All NMR spectra were recorded using $CDCl_3$ as the solvent. Proton NMR spectra were recorded on either an Av400 H spectrometer (Bruker, Billerica, MA, USA) operating at 400.13 MHz or a BioSpin Av500 spectrometer (Bruker) operating at 500.13 MHz. Carbon NMR spectra were recorded either at 100.62 MHz on an Av400 H spectrometer or on a BioSpin Av200 spectrometer (Bruker) operating at 50.33 MHz. Two-dimensional COSY and HSQC spectra were recorded on an Av400 H spectrometer, and two-dimensional HMBC were recorded on a BioSpin Av500 spectrometer. Low- and high-resolution mass spectrometric experiments (electron impact) were performed on a MAT 95XL mass spectrometer (ThermoQuest, San Jose, CA, USA). Analysis by LC-MS used a Q Exactive high-resolution FT mass spectrometer (Thermo Scientific, Waltham, MA, USA), employing atmospheric pressure chemical ionization (resolution, 70,000; scan range, m/z 100−1000; capillary temperature, 320 °C; sheath gas, 25 (arbitrary units); auxiliary gas, 10 (arbitrary units); discharge voltage, 2.8 kV; discharge current, 5.0 μA; vaporizer temperature, 350 °C). The column used was an Acquity 50 mm × 2.1 mm i.d., 1.7 μm, BEH RP-8 (Waters, Milford, MA, USA). Solvent A was H_2O with 5% CH_3CN, and solvent B was CH_3CN with 5% H_2O. HPLC analysis was programmed as follows: 100% solvent A for 20 min, followed by a linear gradient from 100% A to 100% B in 10 min, then 100% B for 2 min, then a linear gradient from 100% B to 100% A in 1 min, and finally a hold at 100% A for 5 min. Flow injection analysis was performed with the same instrument and settings as described above with the exception that the vaporizer temperature was 450 °C and the mass range was m/z 100−1500. The sample was not passed through the HPLC column; instead, it was introduced with a 0.2 mL/min flow of methanol, injected directly into the mass spectrometer.

UPLC analyses were performed on an Acquity UPLC system (Waters) using a 50 mm × 2.1 mm i.d., 1.7 μm, Acquity BEH Shield RP-18 column (Waters), with a flow rate of 0.4 mL/min and detection by photodiode array (PDA) scanning in the range from 190 to 400 nm. For the analysis of the autoxidation of individual pyrethrins, solvent A was H_2O with 50% CH_3CN and solvent B was CH_3CN with 5% H_2O. UPLC analysis was programmed as follows: 100% solvent A at 0 min followed by a linear gradient from 100% A to 99.9% B in 4 min, then a linear gradient from 99.9% B to 100% A in 0.5 min, and finally a hold at 100% A for 2 min. For the analysis of crop extracts, the same solvent regimen and program as used for LC-MS analysis, described above, was employed. Data acquisition and processing were performed using Empower software (Waters), and representative chromatograms were extracted at 223 nm for all analyses except those for the analytical scale autoxidation experiments. In that case, the following wavelengths were used; **1**, 223 nm; **2**, 228 nm; **3**, 221 nm; **4**, 229 nm; **5**, 221 nm; **6**, 229 nm.

Semipreparative HPLC separations were performed on a HPLC system (Waters) using a 250 mm × 22 mm i.d., 5 μm, Altima RP-18 column with detection by a 2487 dual-wavelength absorbance detector (Waters) set at 223 nm. Preparative HPLC was performed on a HPLC system (Waters) using a 300 mm × 40 mm i.d., 15 μm, Deltaprep RP-18 column, a mobile phase of 40% acetonitrile/water, and a flow rate of 80 mL/min and with detection by a 490E programmable multiwavelength detector (Waters) set at 223 nm. Radial chromatography was performed with silica gel (60 PF_{254}) coated (4 mm) glass rotor plates using a Chromatotron 7924T (Harrison Research, Palo Alto, CA, USA).

Crop Material. Samples of crop extracted included *T. cinerariifolium* crop harvested from a pyrethrum site, which had not been stored (i.e., "unstored crop"), and crop from the same site that had been stored for 12 weeks at 22 °C (i.e., "stored crop"). These samples did not contain BHT.

Extraction of Crop Material. A known amount of sample (~500 mg) was weighed into a 50 mL centrifuge tube. Ethyl acetate (25 mL) was added, and the sample was mixed at room temperature for 2 h on a rotisserie shaker. The mixture was centrifuged (4 °C, 2000 rpm, 10 min) and the liquor decanted and concentrated under vacuum. The residue was taken up in acetonitrile (2 mL), and a portion (200 μL) was taken and further diluted up to 1.5 mL with acetonitrile. The diluted sample was analyzed by UPLC (3 μL injection).

Purified Pyrethrin Esters. The six individual pyrethrin esters (Figure 1) were obtained in >99.5% purity from refined pyrethrum concentrate through a combination of dry column vacuum chromatography and careful preparative HPLC using a 900 mm × 100 mm i.d. glass column packed with 100 μm Chromatorex RP-18 packing material (Fuji Silysia Chemical Ltd., Kasugai, Japan) using an acetonitrile/water gradient pumped at 350 mL/min by a micropump. Detection was by Waters 481 LC spectrophotometer at 223 nm.

Preparation of 8′,9′ and 10′,11′ Epoxides and Diols from Pyrethrin II. *Synthesis and Isolation of Pyrethrin II 8′,9′ and 10′,11′ Epoxides.* To a solution of pyrethrin II (195 mg, 0.52 mmol) in dichloromethane (3 mL) was added *m*-CPBA (0.79 mmol) portionwise over 5 min. The reaction was monitored by TLC developed with EtOAc/petroleum spirits (1:4, v/v) until determined to be complete (1.5 h). The solids were removed by filtration through glass fiber paper, and the filtrate was concentrated to give a white sticky solid. The solid was purified by radial chromatography (elution with 1:4 to 3:7 EtOAc/petroleum spirits) providing two bands. Further purification of each band by semipreparative HPLC (eluent: 50% CH_3CN/H_2O, 8 mL/min) afforded the 8′,9′-epoxide **1** (39 mg, 19%) and the 10′,11′-epoxide **2** (14 mg, 7%) as clear colorless oils (both compounds were isolated as a ~1:1 mixture of diastereomers).

8′,9′-Epoxypyrethrin II ***12***:[12] δ_H (400 MHz, $CDCl_3$) 6.46 (1H, m), 5.83 (1H, m), 5.68 (1H, m), 5.50 (1H, m), 5.42 (1H, m), 3.73 (3H, s), 3.44 (1H, m), 3.24 (1H, m), 2.91 (1H, ddd, J = 2.2, 6.4, 18.7 Hz), 2.54 (1H, dt, J = 4.8, 14.1 Hz), 2.40 (1H, dd, J = 7.3, 14.3 Hz), 2.30−2.20 (2H, m), 2.07 (3H, s), 1.95 (3H, d, J = 1.4 Hz), 1.74 (1H, t, J = 5.4 Hz), 1.31 (3H, s), 1.24 (3H, d, J = 2.3 Hz) (all diastereomeric ^{1}H signals overlap); δ_C (100 MHz, $CDCl_3$) 204.0, 203.9, 171.3, 171.3, 168.2, 168.2, 167.0, 166.9, 140.3, 140.3, 139.1, 132.1, 132.0, 129.9, 120.8, 73.6, 73.5, 57.4, 57.3, 56.8, 56.8, 52.0, 42.2, 42.1, 35.9, 35.9, 33.1, 33.0, 30.7, 22.5, 22.4, 22.3, 20.6, 14.5, 14.5, 13.0 (some diastereomeric ^{13}C signals overlap); m/z (EI, 70 eV) found $M^{\bullet+}$ 388.1875, $C_{22}H_{28}O_6$ requires 388.1880.

10′,11′-Epoxypyrethrin II ***13***:[12] δ_H (400 MHz, $CDCl_3$) 6.46 (1H, m), 5.69−5.58 (2H, m), 5.09 (1H, m), 3.76 (1H, m), 3.73 (3H, s), 3.25−3.11 (2H, m), 3.02 (1H, m), 2.89 (1H, dd, J = 6.5, 18.5 Hz), 2.66 (1H, m), 2.27−2.19 (2H, m), 2.07 (3H, s), 1.94 (3H, d, J = 1.5 Hz), 1.74 (1H, d, J = 5.2 Hz), 1.30 (3H, s), 1.23 (3H, s) (all diastereomeric ^{1}H signals overlap); δ_C (100 MHz, $CDCl_3$) 203.5, 203.4, 171.2, 168.1, 165.2, 165.1, 141.8, 138.9, 131.2, 131.1, 129.8, 128.9, 128.8, 73.4, 73.4, 51.8, 48.6, 47.8, 42.0, 42.0, 35.8, 33.0, 30.6, 29.7, 22.3, 21.9, 21.8, 20.4, 14.4, 12.9 (some diastereomeric ^{13}C signals overlap); m/z (EI, 70 eV) found $M^{+\bullet}$ 388.1854, $C_{22}H_{28}O_6$ requires 388.1880.

Synthesis and Isolation of Diols from 8′,9′-Epoxypyrethrin II. 8′,9′-Epoxypyrethrin II **12** (39 mg, 0.10 mmol) was dissolved in methanol/water (2:1, 600 μL), and one drop of 0.5 M H_2SO_4 was added. The reaction was stirred for 1 h at room temperature and analyzed by TLC, developed with EtOAc/petroleum spirits (2:3, v/v), which showed no starting material present. A 5% $NaHCO_3$ solution was added to neutralize the reaction, and then the mixture was concentrated to remove the methanol. The aqueous phase was extracted with EtOAc (four times), and the combined organic extracts were dried ($MgSO_4$), filtered, and concentrated. Purification by semipreparative HPLC (eluent: 45% CH_3CN/H_2O, 10 mL/min) afforded 8′,9′-dihydroxy pyrethrin II **7**[11] (20 mg, 49%) as a clear colorless oil (mixture of diastereomers). δ_H (400 MHz, $CDCl_3$) 6.45 (1H, m), 5.88 (1H, ddd, J = 6.0, 10.5, 16.9 Hz), 5.68 (1H, brt, J = 5.1 Hz), 5.37 (1H, m), 5.27 (1H, m), 3.91 (1H, m), 3.73 (3H, s), 3.62 (1H, m), 3.22 (1H, brs), 2.93 (1H, dd, J = 6.3, 18.9 Hz), 2.81 (1H, brs), 2.58−2.40 (2H, m), 2.28 (1H, ddd (obscured), J = 2.0, 3.9, 19.0 Hz), 2.23 (1H, dd (obscured), J = 5.2, 9.7 Hz), 2.06 (3H, s), 1.94 (3H, d, J = 1.3 Hz), 1.74 (1H, dd, J = 2.2, 5.2 Hz), 1.30 (3H, s), 1.24 (3H, s) (all diastereomeric ^{1}H signals overlap); δ_C (100 MHz, $CDCl_3$) 206.5, 171.4, 171.4, 168.3, 168.3, 168.3, 141.0, 139.1, 137.4, 137.4, 130.1, 117.8, 117.8, 75.4, 75.4, 73.7, 73.6, 72.9, 72.8, 52.0, 42.2, 42.1, 35.9, 35.9, 33.2, 33.2, 30.9, 30.9, 28.1, 28.1, 22.5, 20.6, 20.6, 14.6, 14.5, 13.1 (some diastereomeric ^{13}C signals overlap); m/z (EI, 70 eV) found $M^{\bullet+}$ 406.1981, $C_{22}H_{30}O_7$ requires 406.1986.

Synthesis and Isolation of Diols from 10′,11′-Epoxypyrethrin II. 10′,11′-Epoxypyrethrin II **13** (10 mg, 0.025 mmol) was dissolved in

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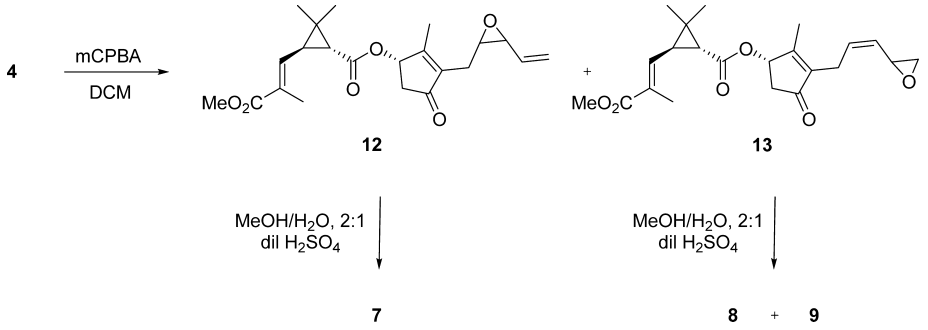

Figure 2. Synthesis of compounds **7**–**9** identified as pyrethrin II mammalian metabolic products by Casida.[2]

methanol/water (2:1, 900 μL), and one drop of 0.5 M H_2SO_4 was added. The reaction was stirred for 1 h at room temperature and analyzed by TLC, developed with EtOAc/petroleum spirits (2:3, v/v), which showed no starting material present. A 5% $NaHCO_3$ solution was added to neutralize the reaction, and the mixture was concentrated to remove the methanol. The aqueous phase was extracted with EtOAc (four times), and the combined organic extracts were dried ($MgSO_4$), filtered, and concentrated. Purification by semipreparative HPLC (eluent: 40% CH_3CN/H_2O, 3 mL/min) afforded 10′,11′-dihydroxy pyrethrin II **8** (6 mg, 59%) and 8′,11′-dihydroxy pyrethrin II **9** (3 mg, 30%), respectively, as clear colorless oils (both diols were obtained as mixtures of diastereomers).

10′,11′-Dihydroxypyrethrin II ***8****:*[12] δ_H (400 MHz, $CDCl_3$) 6.46 (1H, m), 5.67 (1H, d, J = 5.9 Hz), 5.55 (1H, m), 5.39 (1H, m), 4.73 (1H, m), 3.74 (3H, s), 3.71–3.49 (3H, m), 3.24 (1H, m), 2.99 (1H, m), 2.90 (1H, dd, J = 6.3, 18.8 Hz), 2.28–2.18 (3H, m), 2.09 (3H, s), 1.95 (3H, d, J = 1.4 Hz), 1.74 (1H, dd, J = 1.5, 5.2 Hz), 1.31 (3H, s), 1.24 (3H, s) (all diastereomeric ^{1}H signals overlap); δ_C (100 MHz, $CDCl_3$) 205.3, 205.2, 171.4, 168.3, 166.4, 141.4, 139.1, 130.8, 130.7, 130.0, 128.6, 128.5, 67.9, 67.8, 66.3, 52.1, 42.3, 35.9, 33.2, 30.9, 22.7, 22.5, 20.6, 14.4, 13.1 (some diastereomeric ^{13}C signals overlap).

8′,11′-Dihydroxypyrethrin II ***9****:*[12] δ_H (400 MHz, $CDCl_3$) 6.46 (1H, m), 5.92–5.83 (1H, m), 5.78–5.65 (2H, m), 4.33 (1H, m), 4.15 (2H, t, J = 4.9 Hz), 3.74 (3H, s), 2.92 (1H, dd, J = 6.3, 18.8 Hz), 2.67 (1H, dd, J = 4.5, 11.4 Hz), 2.55 (1H, dt, J = 3.9, 14.0 Hz), 2.47 (1H, dd, J = 7.8, 14.1 Hz), 2.32–2.21 (2H, m), 2.06 (3H, s), 1.95 (3H, d, J = 1.2 Hz), 1.75 (1H, dd, J = 2.3, 5.2 Hz), 1.38 (1H, t, J = 5.5 Hz), 1.31 (3H, s), 1.25 (3H, s) (all diastereomeric ^{1}H signals overlap); δ_C (100 MHz, $CDCl_3$) 206.0, 206.0, 171.4, 171.4, 168.4, 168.1, 140.9, 139.1, 133.4, 130.1, 130.0, 73.8, 73.7, 70.8, 70.7, 63.1, 63.1, 52.1, 42.2, 35.9, 33.2, 33.2, 32.0, 30.9, 30.9, 22.5, 20.6, 14.6, 13.1 (some diastereomeric ^{13}C signals overlap).

Radical Stability Predictions. Radical stabilities were calculated using the MOPAC semiempirical molecular orbital program (Stewart Computational Chemistry, Colorado Springs, CO, USA) using the AM1 parametrization. The radical stabilities were calculated by removing the relevant hydrogen atom from the geometry-optimized structure of pyrethrin I and using the UHF method to calculate the stability of the resulting radical. The geometry optimization was carried out using the Tripos force field[13] and Gasteiger–Huckel charges in the Sybylx1.1 modeling package (Certera, Princeton, NJ, USA).

Autoxidation Experiments Performed with Pyrethrin I and II, Cinerin I and II and Jasmolin I and II. Stock solutions of the six pyrethrins **1**–**6** (>99.5% purity, 1.0 M) and a stock solution of AIBN (0.1 M) were made up in cyclohexane. A stock solution of BHT (1.0 M) was made up in petroleum ether.

An autoxidation experiment was first performed with pyrethrin I: Stock solutions of pyrethrin I (10 μL) and AIBN (10 μL) were added to a series of 1.9 mL UPLC vials and then diluted with cyclohexane (80 μL) to give final pyrethrin I and AIBN concentrations of 0.1 and 0.01 M, respectively. The vials were sealed and placed in an oven set at 64 °C. Vials were removed periodically and quenched with BHT solution (50 μL). For the zero time experiment, BHT (50 μL) was first added to the stock solution of pyrethrin I (10 μL) and cyclohexane (80 μL), followed by AIBN (10 μL). After each vial was removed from the oven, the solvent was removed under a stream of nitrogen and the vial was stored under nitrogen at −8 °C until analysis by UPLC. Immediately prior to analysis, the samples were diluted with acetonitrile. The extent of the disappearance of pyrethrin I over time was measured relative to the BHT peak. This procedure was reproduced with pyrethrin II, cinerins I and II, and jasmolins I and II. The results are shown graphically in Figure 4. Curves were fit until convergence using OriginPro 9.1 software (OriginLab Corp., Northampton, MA, USA) employing the Rational Nelder Model and the Levenberg–Marquardt iteration algorithm and according to the following equation:

$$y = (x + a)/(b_0 + b_1{*}(x + a) + b_2{*}(x + a) \wedge 2) \quad (7)$$

Preparative Autoxidation Experiment Performed with Pyrethrins II. A freshly prepared solution of pyrethrins II, consisting of 80% pyrethrin II, 12.9% cinerin II, and 2.7% jasmolin II (1.08 g, 0.1 M), and AIBN (0.01 M) in benzene (29 mL) was placed in a 100 mL round-bottom flask fitted with an air condenser and heated at 64 °C in an oil bath for 24 h. The solvent was then removed under reduced pressure. The pyrethrins II autoxidation mixture (~1 g) was processed using preparative HPLC, and peaks of interest were collected and analyzed by UPLC and LC-MS. The compound eluting at 26.25 min from the UPLC column, compound **A**, was obtained but was contaminated with another component, compound **B**, eluting at 24.75 min in the UPLC. Compound **A** was passed through the HPLC two more times; however, it remained contaminated, whereas compound **B** could be isolated in pure form. Compound **A** (13 mg) was obtained as a yellow oil, which darkened over time. Compound **B** (14 mg) was a colorless oil. Compound **A** was identified as 7′,8′-epoxypyrethrin II (**10**) and compound **B** as 7′,8′-dihyroxypyrethrin II (**11**).

7′,8′-Epoxypyrethrin II ***10****:* δ_H (400 MHz, $CDCl_3$) 6.44 (1H, d, J = 9.5 Hz), 5.66 (1H, m), 5.57–5.39 (2H, m), 5.23 (1H, dd, J = 1.1, 9.7 Hz) 3.72 (3H, s), 3.15–3.10 (1H, m), 2.98–2.84 (3H, m), 2.67–2.58 (1H, m), 2.43 (1H, dd, J = 6.0, 14.2 Hz), 2.30 (3H, m), 2.05 (3H, brs), 1.93 (3H, brs), 1.76–1.70 (1H, m), 1.30 (3H, s), 1.22 (3H, s) (all diastereomeric ^{1}H signals overlap); δ_C (50 MHz, $CDCl_3$) 204.0, 203.9, 171.4, 168.3, 167.7, 167.6, 139.6, 139.5, 139.1, 135.1, 130.0, 119.8, 73.6, 73.5, 58.8, 58.3, 52.0, 42.2, 42.1, 36.0, 33.2, 33.1, 30.8, 26.1, 22.5, 20.6, 14.6, 13.1 (some diastereomeric ^{13}C signals overlap); IR (cm^{-1}) 2952 (br), 1711 (st), 1655, 1435, 1385, 1262, 1222, 1175, 1149, 1112, 994, 926, 831, 761; HRMS (APCI) m/z calcd for $C_{22}H_{29}O_6$ $[M + H]^+$ 389.1959, found 389.1934.

7′,8′-Dihyroxypyrethrin II ***11****:* δ_H (400 MHz, $CDCl_3$) 6.45 (1H, d, J = 9.5 Hz), 5.98–5.86 (1H, m), 5.70–5.63 (1H, m), 5.35 (1H, dd, J = 1.3, 17.3 Hz), 5.27 (1H, d, J = 10.6 Hz), 4.08–4.00 (1H, m), 3.72 (4H, brs), 3.10 (1H, brdd, 3.5, 17.8 Hz), 2.91 (1H, dt, J = 6.6, 18.9 Hz), 2.63 (1H, brd, J = 37.4 Hz), 2.54–2.38 (2H, m), 2.32–2.19 (2H, m), 2.06 (3H, s), 1.92 (3H, s), 1.73 (1H, d, J = 5.2 Hz), 1.29 (3H, s), 1.23 (3H, s) (all diastereomeric ^{1}H signals overlap); δ_C (50 MHz, $CDCl_3$) 206.9, 171.4, 168.7, 168.5, 168.3, 141.4, 141.3, 139.1, 136.7, 130.1, 117.5, 75.5, 75.5, 73.8, 73.7, 73.4, 73.2, 52.1, 42.1, 42.2, 35.9, 33.2, 30.9, 26.5, 26.4, 22.5, 20.6, 14.6, 14.6, 13.1 (some diastereomeric ^{13}C signals overlap); IR (cm^{-1}) 3435 (br), 2952 (br), 1708 (st), 1645, 1435, 1386, 1266, 1222, 1176, 1150, 1112, 994, 923, 830, 761, 734; HRMS (APCI) m/z calcd for $C_{22}H_{29}O_6$ $[M - H_2O + H]^+$ 389.1959, found 389.1959; calcd for $C_{22}H_{31}O_7$ $[M + H]^+$ 407.2064, found 407.2063; calcd for $C_{22}H_{30}O_7Na$ $[M + Na]^+$ 429.1884, found 429.1882.

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RESULTS AND DISCUSSION

Analysis of Crop Material for Molecular Markers of Oxidative Metabolism. Pyrethrum crop was examined for the presence of diols **7**−**9** (Figure 1), identified by Casida[7] as resulting from biological metabolism of pyrethrin esters and proposed by Williams[8] to be present in ground pyrethrum seed after heating at 100 °C for 10 h. These compounds were synthesized using methods slightly modified from those previously reported,[12] as outlined in Figure 2, and used as analytical standards for the analysis of extracts prepared from crop.

Crop was extracted with a range of organic solvents of various polarities, and the extracts were carefully analyzed by reverse phase UPLC. Not surprisingly, the extracts contained a complex mixture of compounds. Careful analysis revealed no evidence for the presence of the three diols **7**−**9** in any of the extracts studied.

Autoxidation Studies of Pyrethrin Esters. Oxidative metabolism that occurs in higher living systems typically employs P_{450} enzymes.[14] Whereas free radicals have been implicated as intermediates in these processes, the involvement of oxo-heme species distinguishes oxidative metabolism from autoxidation. It follows, therefore, that oxidative metabolism of pyrethrins is likely to produce a different product distribution from that produced from the autoxidation of pyrethrins in the air.

As indicated above, the pentadienyl substituent of the pyrethrins is somewhat reminiscent of the structures found in unsaturated lipids, the autoxidation chemistry of which has been extensively investigated. Figure 3 shows that a key step in the

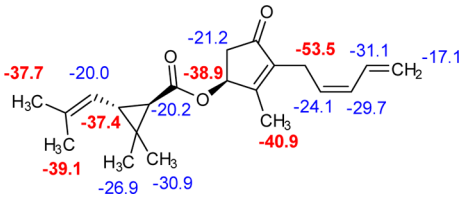

Figure 3. Calculated stabilities (kJ/mol) of radicals produced by hydrogen abstraction from various positions on the pyrethrin I molecule.

autoxidation chain is the abstraction of hydrogen from the substrate to generate a substrate radical (eq 2). The investigation of the autoxidation chemistry of pyrethrins described here began with semiempirical calculations of the relative stabilities of the possible free radicals generated by hydrogen abstraction from pyrethrin I. The MOPAC semiempirical molecular orbital program using the AM1 parametrization was employed, and relative radical stabilities were calculated via the UHF method. The results are displayed in Figure 4 and predict that hydrogen abstraction from the 7′-position of pyrethrin I would produce the most stable free radical. This is not unexpected, given that such a radical would be highly delocalized.

Autoxidation experiments were carried out with the six pyrethrin esters (Figure 1) following a procedure that was modified from that reported for the autoxidation of fatty acids.[10] Cyclohexane solutions of the isolated pyrethrin esters (0.1 M) containing AIBN (0.01 M) were heated under air at 64 °C in sealed vials. Vials were allowed to cool and quenched with BHT at various time points and their contents analyzed by UPLC. The extent of the disappearance of the pyrethrin esters over time was measured relative to the BHT peak. The analysis of the AIBN-initiated reaction mixtures showed substantial degradation of

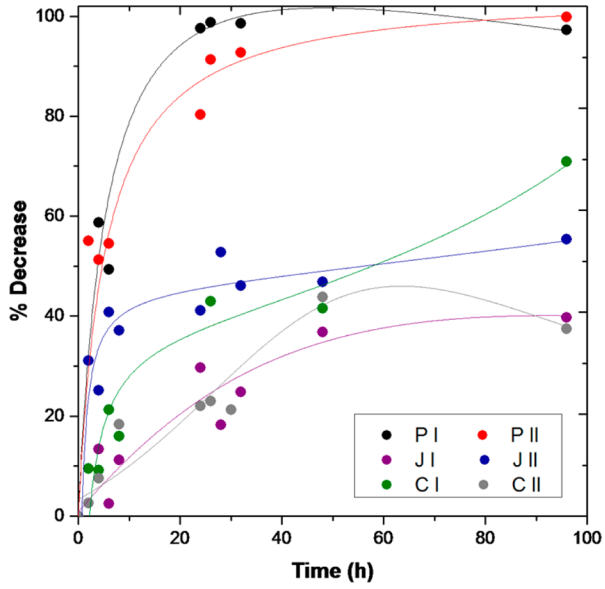

Figure 4. Plots of the percent decrease of pyrethrin esters versus time in AIBN-promoted autoxidation reactions. PI, **1**; CI, **2**; JI, **3**; PII, **4**; CII, **5**; JII, **6**.

pyrethrins I and II but less for the cinerins and jasmolins (Figure 4). It was found that at least 80% of pyrethrins I and II had degraded after 24 h and very little of either of these pyrethrins remained after 96 h. The control reactions in which AIBN was not included led to only 10−11% degradation of these compounds. The fact that the cinerins and jasmolins are much less susceptible to autoxidation than pyrethrins I and II is consistent with the above arguments about the ease of hydrogen abstraction from the 7′-position, because abstraction of a hydrogen from this position in the cinerins and jasmolins would give a less highly conjugated and hence less stable free radical.

Identification of Autoxidation Products in Pyrethrum Crop Extract. UPLC traces of the reaction mixtures from the AIBN-initiated autoxidation experiments showed a significant number of new peaks, which mainly eluted before the pyrethrins and increased in intensity with time. An attempt was made to find evidence for the autoxidation of pyrethrins in the *T. cinerariifolium* crop by correlating UPLC peaks that appeared in the AIBN-initiated autoxidation reaction mixtures, which also increased in intensity as the reaction proceeded, with peaks found in the crop extracts. Between 10 and 20 peaks showed such a correlation, based on retention time (Figure 5).

The strongest correlation between the number of peaks in crop extracts coincident with apparent autoxidation products, from the AIBN-initiated reactions, occurred with the extracts of stored crop samples, which had been stored in the absence of antioxidants. The three most intense peaks that grew over time in each of the pyrethrin I and II autoxidation reaction mixtures, and were found to be coincident with peaks in the stored crop extracts, were identified and their UV profiles extracted and compared (Figure 5, peaks 2−7). UPLC peaks 2 and 7 in the autoxidation mixture were both found to have UV profiles quite different from those of the coincident peaks in stored crop extract. Conversely, the UV profiles of UPLC peaks 3−6 in the autoxidation mixtures appeared to be similar to the UV profiles of coincident peaks in stored crop extract. The latter results provide strong circumstantial evidence that pyrethrin autoxidation occurs

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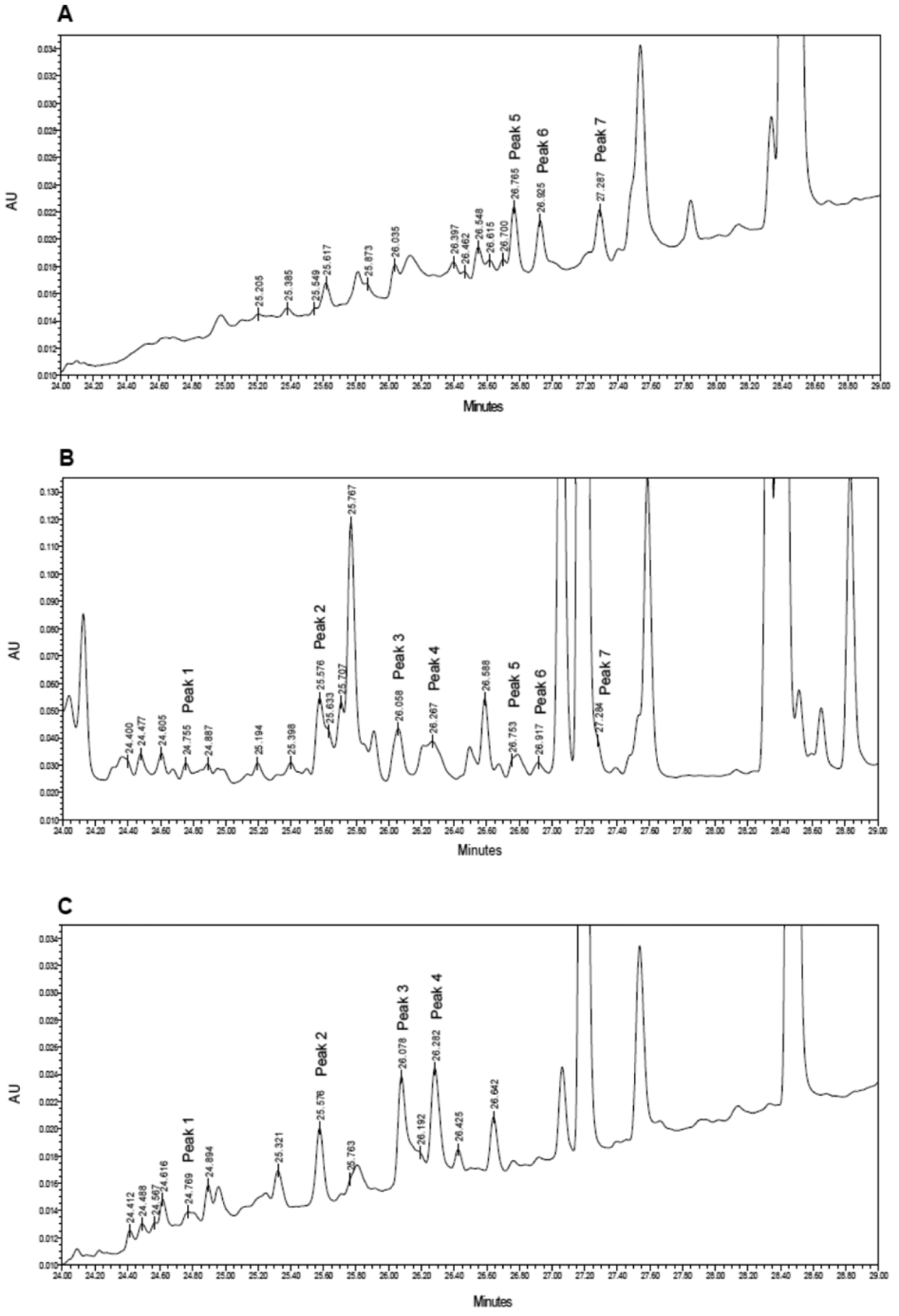

Figure 5. UPLC trace of the extract of *T. cinerariifolium* crop that had been stored for 12 weeks without BHT (B), compared with UPLC traces of pyrethrin I (A) and II (C) autoxidation mixtures.

when crop material is stored for extended periods in the absence of antioxidants.

The contention that autoxidation is a contributory cause of pyrethrin losses during crop storage would be further supported if some of the autoxidation products generated in laboratory experiments could be isolated, identified, and shown unambiguously to be present in stored crop material. The autoxidation reactions were therefore repeated with pyrethrin I and II on a gram scale, using a 24 h reaction time with the hope of generating enough of the autoxidation products to allow isolation and characterization. These samples had UPLC profiles very similar to those produced on a smaller scale, although a more significant peak due to residual cinerin II was observed in the pyrethrin II mixture as the pyrethrin II used had some cinerin II contamination. As found with the autoxidation experiments with pure pyrethrins, pyrethrin II was found to degrade more quickly than cinerin II in this latter autoxidation experiment. Stored crop was freshly extracted, and all three samples, the two resulting from autoxidation reactions and the crop extract, were analyzed by LC-MS (Figure 6).

Of the components of the pyrethrin I autoxidation mixture, identified as potentially important on the basis of the arguments given above, peaks 5 and 6, the compound responsible for peak 6 could not be readily identified in the LC-MS trace of stored crop

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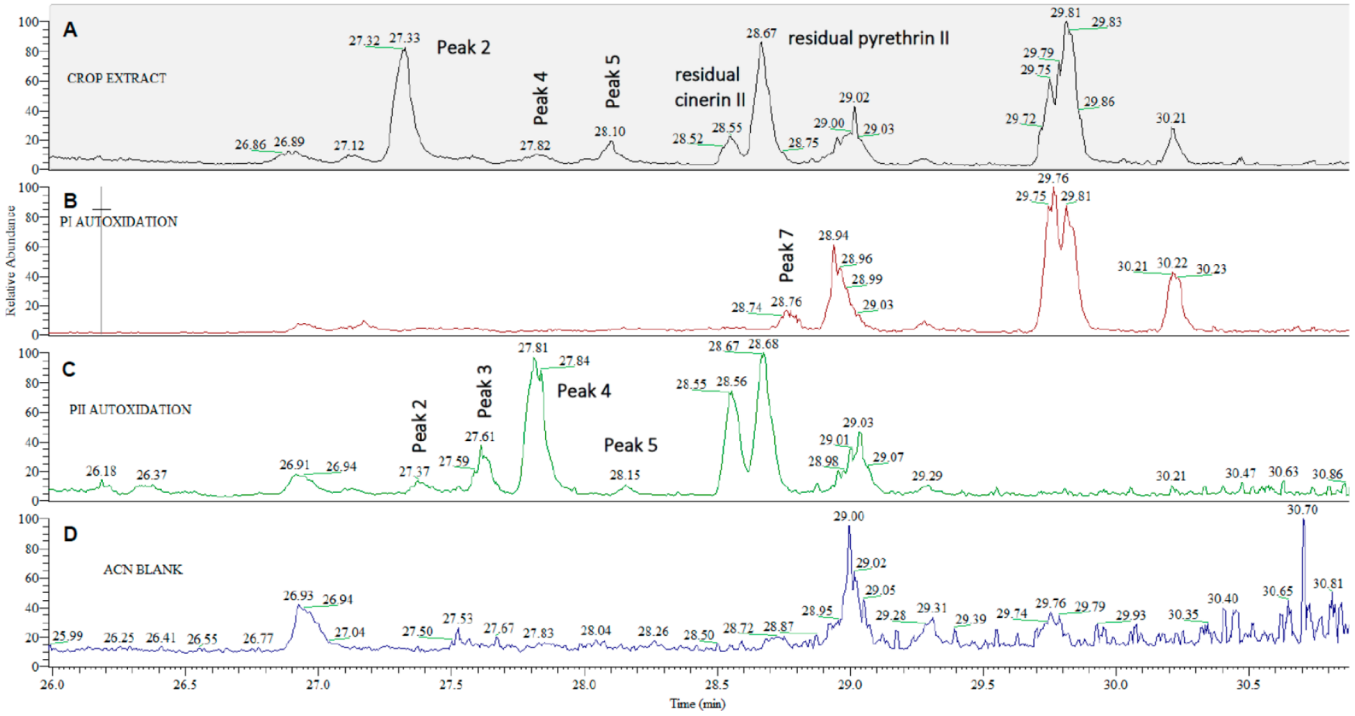

Figure 6. Relevant sections of LC-MS trace of the extract of *T. cinerariifolium* crop that had been stored for 12 weeks without BHT (A), compared with LC-MS traces of pyrethrin I (B) and II (C) autoxidation mixtures and a CH_3CN blank (D). Peak detection was via ion current in the mass range m/z 250−600.

extract as this peak was obscured by large peaks due to pyrethrin II and cinerin II in the crop extract. Peak 5 in the LC-MS trace of stored crop extract appeared to represent two separate compounds, one of which matched a compound in the pyrethrin I autoxidation mixture, whereas the other matched a compound in the pyrethrin II autoxidation mixture.

The chosen components of the pyrethrin II autoxidation mixture, peaks 3 and 4, could be observed as relatively small peaks in the LC-MS trace of stored crop extract. It was found that the larger of the two peaks, peak 4, had the same dominant molecular ion (m/z 389.1959/389.1960, corresponding to a molecular formula of $C_{22}H_{29}O_6$ for this M + H^+ peak) in both mixtures. The smaller peak, peak 3, was found to have at least two matching components (m/z 389.1958 and 411.1775) as well as matching components from the pyrethrin I autoxidation mixture (m/z 309.1695 and 343.1903).

As the LC-MS results suggested that peak 4 resulted from a single component, it was chosen for purification and characterization. The whole of the remaining pyrethrin II autoxidation reaction mixture (~1 g) was passed through a preparative scale C_{18}-reversed phase HPLC column using aqueous acetonitrile as the eluent, and the compound of interest was obtained as a colorless oil (compound **A**). LC-MS analysis of this compound was consistent with that previously obtained in crop and autoxidation mixtures; however, UPLC analysis showed it to be impure. HPLC purification was repeated two more times, but the compound remained contaminated. It became apparent that compound **A** was unstable in the aqueous acetonitrile eluent and consistently degraded to a component that eluted much earlier, with a UPLC retention time of 24.8 min (peak 1). This latter compound, compound **B**, was obtained in high purity, and mass spectral analysis showed it to have molecular ions at m/z 407.2063, corresponding to a molecular formula of $C_{22}H_{31}O_7$ for the M + H^+ peak, and m/z 429.1882, corresponding to a molecular formula of $C_{22}H_{30}O_7Na$ for the M + Na^+ peak. The strongest peak in the spectrum had m/z 389.1959, corresponding to (M − H_2O) + H^+ and had a mass identical to that of compound **A**, from which it is apparently formed. This mass spectrometric evidence strongly suggested that there was a component, compound **A**, formed in the autoxidation reaction of pyrethrin II that was also present in stored crop extract, and that this compound was hydrolytically unstable, hydrolyzing to compound **B**.

Single-ion LC-MS analysis of stored crop extract was used to further confirm that compound **A** was present in stored crop. A similar analysis was used to show that compound **B** was also present in stored crop. The UPLC retention time for this compound was also coincident with a peak in stored crop extract. A small peak at the same retention time was observed in pyrethrin II autoxidation mixtures. It is logical that compound **B** was formed in only small amounts in the autoxidation experiments as these were performed in dried solvent.

Compound **B** showed a strong infrared band at 3435 cm^{-1}, consistent with the presence of an alcohol functionality. Conversely, compound **A** did not show a strong IR band in the same region. Compound **A** has a molecular formula of $C_{22}H_{28}O_6$, which is related to the molecular formula of pyrethrin II by the addition of a single oxygen. At this point, suspicions arose that compound **A** could be an epoxide and compound **B** a 1,2-diol, formed by hydrolytic opening of the epoxide. This is consistent with previous observations of the behavior of the pyrethrin epoxides shown in Figure 2.

Compounds **A** and **B** were extensively investigated using one- and two-dimensional NMR spectroscopy. It was soon established that compounds **A** and **B** were structurally related to pyrethrin II, with both possessing the entire chrysanthemic diacid and cyclopentenone part of pyrethrin II. The unsaturated side chains of compounds **A** and **B**, however, were different from

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pyrethrin II. Two-dimensional COSY, HSQC, and HMBC spectra were used to establish that the structures of compounds **A** and **B** are the diastereomeric epoxide **10** and the diastereomeric diol **11** (Figure 1). These compounds are very closely related to the structures of the 8′,9′-epoxide **12** and 8′,9′-diol **7** derived from pyrethrin II. In fact, the NMR spectra of compounds **A** and **B** are very similar to those of the previously prepared compounds, although they are sufficiently different to be certain that they are not the same compounds. In addition, the UPLC retention times for compounds A and B are slightly different from those of **7** and **12**. It has been shown that epoxides are formed during autoxidation reactions of lipids.[11] The mechanism for the formation of compound **A** (**6**) has not been determined but is very likely derived from the free radical formed by hydrogen abstraction from the 7′-position of pyrethrin II.

In summary, the oxidation chemistry of the pyrethrins was investigated in an attempt to identify the causes of pyrethrin losses during crop storage. Compounds previously identified as metabolic oxidation products of pyrethrin II were prepared and used as analytical standards to investigate pyrethrum crop extracts; however, they were not detected in these extracts. It is thought, therefore, that biological degradation processes are unlikely to be the cause of pyrethrin losses during crop storage.

Autoxidation experiments were performed with isolated pyrethrins for the first time. It was found that the pyrethrins bearing the butadiene side chain, pyrethrin I and II, degrade significantly more quickly than the jasmolins and cinerins. The autoxidation of pyrethrin I and II was studied in more detail and found to yield a complex mixture of products, a number of which appeared to be also present in crop extracts. In particular, the 7′,8′-epoxide and 7′,8′-diol of pyrethrin II were isolated from autoxidation mixtures and confirmed to be present in extracts of crop material that had been stored for an extended period without BHT. These results provide strong evidence that pyrethrin autoxidation occurs in pyrethrum crop on standing and hence may contribute to pyrethrin losses during crop storage. It is expected that the pyrethrin autoxidation products identified here will be suitable analytical standards useful in gauging the effectiveness of amelioration measures aimed at minimizing pyrethrin losses during postharvest processing.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.6b02959.

Representative UPLC traces of pyrethrin autoxidation mixtures; data from AIBN-promoted autoxidation experiments; LC-MS analysis of individual peaks in autoxidation mixtures and crop extracts; figures showing UPLC, LC-MS, IR, and NMR characterization of compounds **A** and **B** (PDF).

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Notes

The authors declare no competing financial interest.

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