Identification of Conjugated Triene Oxidation Products of α-Farnesene in Apple Skin

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Conjugated triene oxidation products of the sesquiterpene α-farnesene, implicated as causal agents of the storage disorder superficial scald, were extracted from apple skin wax and separated by GC–MS and normal and reversed phase HPLC. 2,6,10-Trimethyldodeca-2,7(*E*),9(*E*),11-tetraen-6-ol (**2b**) was the major triene (88–95% of total) present in the apple skin by HPLC but accounted for only 12–35% of the "triene" concentration of the apple skin washes as measured by UV spectroscopy. Concentrations of **2b** of up to 1.2 μg/cm^2 of surface area were measured on the five apple varieties examined. The 9*Z* isomer of **2b** constituted the remaining 5–11% of the triene content of most samples. 6-Hydroperoxy-2,6,10-trimethyldodeca-2,7(*E*),9(*E*),11-tetraene (**2a**) and its 9*Z* isomer were isolated from autoxidized samples of α-farnesene and identified as at most minor contributors (<5%) to the triene content of the skin. On standing, **2a** converted to **2b** and to a more polar triene of uncertain structure which was identical by HPLC with the remaining minor triene (0–4%) found in the apple skin.

Keywords: *Conjugated triene; α-farnesene; oxidation; apple; HPLC; GC–MS*

INTRODUCTION

α-Farnesene (**1**) (Figure 1), a sesquiterpene hydrocarbon found on the surface of apples and pears, has long been associated with the occurrence of superficial scald, a physiological disorder resulting in blackening of the skin after cold storage (Bain and Mercer, 1963; Meigh, 1970). α-Farnesene accumulates in the skin of stored apples, and its concentration in the skin has been shown in several studies (Meigh and Filmer, 1969; Huelin and Coggiola, 1970a,b; Meir and Bramlage, 1988) to correlate with the extent and severity of superficial scald. Autoxidation products of α-farnesene, including hydroperoxides or intermediary free radicals, have been suggested as the actual causal agents for scald (Anet, 1972a).

In an early study of α-farnesene autoxidation, Anet (1969) isolated and characterized the conjugated trienol **2b** and the endoperoxide **4b** after reduction of the primary autoxidation products with sodium borohydride. The characteristic UV absorption spectra of these conjugated trienes (λ_{max} 259, 269, and 281 nm) have subsequently been used to estimate "triene" concentrations in hexane washes from the skin of stored apples (Anet, 1972b), although the actual trienes present have not been identified [but see Gallerani and Pratella (1991)]. Conjugated trienes progressively accumulate on the surface of stored apples, and the concentrations of trienes, as measured by UV spectroscopy, correlate more closely with the occurrence and severity of superficial scald than does the concentration of α-farnesene (Huelin and Coggiola, 1970b; Meir and Bramlage, 1988; Chen *et al.*, 1990; Gallerani and Pratella, 1991). Natural antioxidants are also present on the surface of apples (Meir and Bramblage, 1988; Barden and Bramlage, 1994) and may retard the autoxidation of α-farnesene. A more complex hypothesis relating scald development to the levels of various UV absorptions in hexane extracts from the apple skin has recently been presented (Du and Bramblage, 1993).

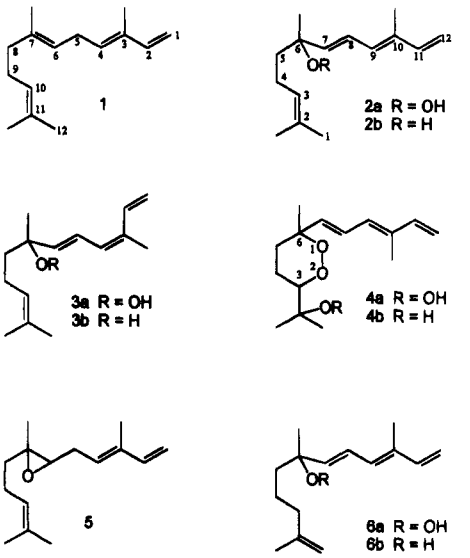

Figure 1. Chemical structures of α-farnesene (**1**) and some oxidation products.

While superficial scald can be controlled by postharvest treatment with the antioxidant diphenylamine (DPA) (Huelin and Coggiola, 1970a,b; Anet and Coggiola, 1974) or 6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline (ethoxyquin) (Chen *et al.*, 1990), alternative chemical (Blanpied, 1993; Klein and Lurie, 1994) and nonchemical control measures are increasingly being sought (Ingle and D'Souza, 1989; Lurie *et al.*, 1991;

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Blanpied and Creasy, 1993; Chen and Varga, 1993; Gallerani *et al.*, 1992). The rational development of alternative control strategies requires a better understanding of the role of α-farnesene in the initiation of superficial scald and of the significance of the α-farnesene oxidation products present or formed during the storage of apples. We recently revisited the chemical oxidation of α-farnesene (Spicer *et al.*, 1993) which has resulted in the first syntheses of Anet's trienol **2b** and endoperoxide **4b** (Brimble *et al.*, 1994). This paper describes the use of **2b**, **4b**, and the related triene hydroperoxides **2a** and **3a** to identify and quantitate conjugated triene oxidation products of α-farnesene present in the skin of fresh and stored apples.

MATERIALS AND METHODS

Materials. Apples, cv. Red Delicious, Fuji, Gala, Golden Delicious, and Granny Smith, were obtained from local retailers at 6–8 months after harvest. Additional Granny Smith apples were obtained directly from Hawkes Bay, New Zealand, stored at 1 °C, and sampled directly from cold storage. ^{1}H and ^{13}C NMR were obtained in $CDCl_3$ on a Bruker 300 or JEOL 270 spectrometer and are reported in parts per million relative to TMS. ^{13}C multiplicities were determined by DEPT-135. High-resolution EIMS from direct probe sample insertion were obtained on a VG-70S mass spectrometer. UV spectra were recorded on a Cary 1E spectrophotometer or using the scan function on a JASCO UV975 variable wavelength HPLC detector.

α-Farnesene [3,7,11-trimethyldodeca-1,3(*E*),6(*E*),10-tetraene] was prepared by the alkylation of 3-methylsulfolene with geranyl bromide (Fielder *et al.*, 1993). Trienol **2b** [2,6,10-trimethyldodeca-2,7(*E*),9(*E*),11-tetraen-6-ol] was obtained by base-catalyzed ring opening of 6,7-epoxy-3,7,11-trimethyldodeca-1,3(*E*),10-triene (**5**) synthesized in turn from α-farnesene by selective epoxidation (Spicer *et al.*, 1993; Brimble *et al.*, 1994). The 7*E*,9*E* stereochemistry of **2b** was confirmed by a $J_{7,8}$ coupling of 15 Hz (Anet, 1969) and by NOESY NMR experiments that showed the required interactions between the C-10 methyl and H-8 and between H-9 and H-11 (Brimble *et al.*, 1994). Endoperoxide **4b** was synthesized from 2,3-epoxy-2,6,10-trimethyldodeca-6(*E*),9(*E*),11-triene by dye-sensitized photoxidation and acid-catalyzed cyclization (Brimble *et al.*, 1994). All farnesene derivatives were unstable oils.

Extraction of Conjugated Trienes from Apple Wax. Individual apples were placed in glass beakers of slightly larger diameter, heptane (25 mL) was added, and the apple was gently washed with the solvent for 2 min (Huelin and Coggiola, 1968). For the determination of trienes by UV spectroscopy, the UV spectrum of the wash solution was measured and the triene concentration calculated as $A_{281} - A_{292}$ with $\zeta_{281-290} = 25\,000$ (Anet, 1972b).

For HPLC analysis, an aliquot of the heptane extract (2.0 mL) (supernatant of frozen samples or homogeneous solution after warming to room temperature) was applied under gravity to a 100 mg silica extraction column (Varian Bond Elut) conditioned with pentane (0.5 mL). The column was washed with pentane (0.5 mL) and then with ether/pentane (1:1, 1 mL) to elute the conjugated trienes. The solvent was removed under reduced pressure from a water aspirator and the residue dissolved in heptane (200 μL) and filtered through a 2 μm syringe filter (Upchurch Scientific) prior to analysis by HPLC. UV and HPLC analysis of the heptane and pentane fractions and of fractions obtained by subsequent elution of the extraction column with further pentane/ether (1:1) and with ether showed essentially all trienes (λ_{max} 269 nm) were recovered in the first pentane/ether fraction above.

For the detection of hydroperoxides, apples were taken directly from cold storage and immediately extracted with cold (4 °C) heptane. Extracts were processed and analyzed immediately as above.

Capillary Gas Chromatography (GC). Capillary GC analysis was carried out on a Hewlett-Packard Model 5890 Series II gas chromatograph equipped with a flame ionization detector (FID) and running under the control of Hewlett-Packard ChemStation software. Separations were achieved using a 30m × 0.25 mm i.d. HP5 column (0.25 μm film thickness) with a temperature program from 40 (3 min) to 150 °C at 8 °C/min and to 250 at 5 °C/min; the injector temperature was 160 °C and the detector temperature 280 °C. The column head pressure was 5 psi of hydrogen. Trienes **2b** and **6b** eluted at 27.6 and 27.3 min, respectively, between α-farnesene at 23.8 min and farnesol [3,7,11-trimethyl-2(*E*),6(*E*),10-dodecatrien-1-ol] at 28.7 min.

Capillary Gas Chromatography–Mass Spectroscopy (GC–MS). Electron impact GC–MS spectra were recorded on a VG70-250S double-focusing magnetic sector mass spectrometer with ionization potential of 70 eV, mass spectral interface 180 °C, 1 s scans, and a 0.2 s delay. GC conditions were as above except a 5 psi of helium column head pressure and a 180 °C injector temperature were used. Trienes **2b** and **6b** eluted at 25.5 and 25.3 min, respectively, between α-farnesene at 22.4 min and farnesol at 26.4 min.

HPLC Conditions. A JASCO PU 980 pump with low-pressure LG 980-02 ternary gradient unit and JASCO UV975 programmable variable wavelength detector was used to deliver solvent at 0.5 mL/min to a Brownlee Spheri-5 100 × 2.1 mm silica column with a 7 μm Newguard 15 × 3.2 mm silica precolumn. Typically 1 or 2 μL sample volumes were injected into the 10 μL injection loop of the Rheodyne injector. Detector output was digitalized and integrated on a Delta Junior Data System (Digital Solutions, Margate, Australia) operating on a 386 PC. UV spectra of eluting peaks were recorded using the "scan" option of the detector.

For analysis of apple skin washes, a gradient of solvent A (0.1% 2-propanol in heptane) and solvent B (10% 2-propanol in heptane) was run using 100% A for 5 min to 90% A at 17 min and then to 100% A at 19 min. This gradient was sufficient to elute all UV absorbing (269 nm) materials occurring in apple extracts or solutions of autoxidized α-farnesene. Retention times: **2a**, 5.1 min; **6a**, 5.7 min; **2b**, 6.4 min; **6b**, 7.7 min; unknown triene, 15.8 min. For resolution of the double-bond isomers **2a** and **2b**, the mobile phase was 0.02% 2-propanol in heptane. The retention times of **3b**, **2b**, and **6b** were 17.2, 18.6, and 22.1 min, respectively, under these conditions. For the routine analysis of apple skin washes, 0.15% 2-propanol in heptane was used (retention time of **2b** was 5.8 min). All HPLC peak identifications were confirmed by coinjection with authentic materials.

Isolation of Farnesyl Autoxidation Products. TLC examination (hexane/ethyl acetate 4:1) of a sample of α-farnesene, which had been stored as the neat oil at −20 °C for several months, showed two UV-active lower R_f autoxidation products: **2a** (R_f 0.70, blue/gray with vanillin/H_2SO_4 spray reagent) and a second autoxidation product at R_f 0.45 (purple/red with vanillin/H_2SO_4). Flash chromatography [Merck Kieselgel 60 (230–400 mesh), hexane/ethyl acetate 9:1] gave 6-hydroperoxy-2,6,10-trimethyldodeca-2,7(*E*),9(*E*),11-tetraene (**2a**) and 6-hydroperoxy-2,6,10-trimethyldodeca-2,7(*E*),9(*Z*),11-tetraene (**3a**) (9:1), 2mg as an oil: λ_{max} (hexane) 246.5 (sh), 261, 269, and 280 nm; **2a** δ ^{1}H 1.40 (3H, s, 6-CH_3), 1.61 (3H, s, 2-CH_3), 1.69 (3H, s, H-1), 1.69 (2H, m, H-5), 1.89 (3H, s, 10-CH_3), 2.01 (2H, m, H-4), 5.07 (1H, d, J = 10.6 Hz, H-12b cis), 5.12 (1H, t, J = 7.1 Hz, H-3), 5.24 (1H, d, J = 17.2 Hz, H-12a trans), 5.76 (1H, d, J = 15.8 Hz, H-7), 6.09 (1H, d, J = 11.4 Hz, H-9), 6.41 (1H, dd, J = 10.6, 17.2 Hz, H-11), 6.58 (1H, dd, J = 11.4, 15.8 Hz, H-8); **3a** δ ^{1}H 5.24 (1H, d, J = 17 Hz, H-12a trans), 5.70 (1H, d, J = 16 Hz, H-7), 6.01 (1H, d, J = 11 Hz, H-9), 6.72 (1H, dd, J = 11,16 Hz, H-8), 6.96 (1h, dd, J = 11,17 Hz, H-11); EIMS m/z 236.1769 (0.4%, M^+, $C_{15}H_{24}O_2$ requires 236.1776), 219 (4), 218 (3), 203 (6), 161 (12), 147 (10), 136 (10), 121 (23), 119 (20), 109 (26), 105 (23), 93 (42), 91 (29), 81 (38), 69 (100), 55 (45), 43 (91), 41 (78).

The second autoxidation product (R_f 0.45) was also isolated by flash chromatography but proved to be too unstable to characterize. Instead, purified material was immediately dissolved in ethyl acetate (10 mL) and treated with 0.1 M triphenylphosphine in ethyl acetate (0.1 mL) to give an oil (<1 mg): λ_{max} (hexane) 247 (sh), 261, 269, and 280 nm; δ ^{1}H 1.15

(3H, s), 1.20 (3H, s), 1.25 (s), 1.88 (3H, s, 10-CH_3), 3.90 (1H, m, H-3), 5.05 (1H, d, J = 10.6 Hz, H-12 cis), 5.22 (1H, d, J = 17.6 Hz, H-12 trans), 5.93 (1H, d, J = 15.8 Hz, H-7), 6.08 (1H, d, J = 11.7 Hz, H-9), 6.41 (1H, dd, J = 10.9, 17.2 Hz, H-11), 6.54 (1H, dd, J = 11.4, 15.8 Hz, H-8); EIMS m/z 252.1723 (11%, M^+, $C_{15}H_{24}O_3$ requires 252.1725), 194 (4), 147 (6), 143 (8), 137 (8), 133 (10), 121 (16), 119 (29), 105 (17), 94 (33), 93 (33), 91 (37), 85 (20), 81 (20), 79 (37), 77 (37), 59 (91), 55 (27), 43 (100), 41 (32).

Reduction of α-Farnesyl Hydroperoxides. To a solution of farnesyl hydroperoxides (**2a**:**6a** 58:33 by HPLC) in pentane (0.5 mL) at −20 °C was added 0.1 M triphenylphosphine in ethyl acetate (2 mL), and the mixture was left at room temperature for 40 min. The solvent was removed under a stream of nitrogen and the residue partially redissolved with gentle warming in pentane (0.5 mL). After cooling to −20 °C, the supernatant was recovered and returned to the freezer for a second crystallization. The resulting supernatant was used for subsequent analysis.

GC−MS (DB1 column as above) showed two major components at 33.9 and 34.2 min in a ratio of 1:2.6, respectively. The first component (RT 33.9 min) showed retention times (GC and HPLC) and mass spectra identical to those of **6b**, a minor component present in synthetic samples of **2b**. The second component (RT 34.2 min) showed m/z 220 (M^+, 2), 205 (3), 202 (12), 187 (4), 162 (47), 159 (30), 147 (10), 137 (33), 133 (17), 119 (25), 107 (18), 105 (29), 95 (34), 93 (45), 91 (30), 69 (34), 55 (35), 43 (100), 41 (48) and was identified as trienol **2b** by coinjection (GC and HPLC) and by comparison (GC−MS) with authentic material.

Isolation of 2,6,10-Trimethyldodeca-1,7(*E*),9(*E*),11-tetraen-6-ol (6b). Preparative centrifugal chromatography of a synthetic sample of trienol **2b** (30 mg) was performed using a Chromatotron (U.S. Patent 4139458) using a 1 mm thick silica gel (Merck GF-254, art. 7730) rotor impregnated with 5% w/w $AgNO_3$. Isomeric trienols were eluted with diethyl ether/30−40 petroleum ether 1:1 at a flow rate of 6 mL/min. Fractions were collected every 2 min and analyzed on 0.2 mm silica TLC plates that had been pretreated with 10% $AgNO_3$ in wet CH_3CN. TLC plates were developed in diethyl ether/petroleum ether as above and sprayed with vanillin/H_2SO_4. Chromatography gave trienol **2b** (10 mg, 33%) containing *ca.* 1% of the **3b** isomer by ^1H NMR and GC−MS and 2,6,10-trimethyldodeca-1,7(*E*),9(*E*),11-tetraen-6-ol **6b** (2 mg, 7%): δ ^1H 1.32 (3H, s, 6-CH3), 1.54 (4H, m, H-4,H-5), 1.70 (3H, s, 2-CH3), 1.88 (3H, s, 10-CH3), 2.01 (2H, t, J = 7 Hz, H-3), 4.67 (1H, s, H-1b), 4.70 (1H, s, H-1a), 5.04 (1H, d, J = 10.7 Hz, H-12b), 5.21 (1H, d, J = 17.3 Hz, H-12a), 5.81 (1H, d, J = 15.2 Hz, H-7), 6.07 (1H, d, J = 11.1 Hz, H-9), 6.40 (1H, dd, J = 10.7, 17.3 Hz, H-11), 6.58 (1H, dd, J = 11.1, 15.2 Hz, H-8); δ ^{13}C 12.1 (10-Me), 22.0 (C-3), 22.3 (2-Me), 28.3 (6-Me), 38.0 (C-3), 42.4 (C-5), 73.2 (C-6), 110.1 (C-1), 112.6 (C-12), 123.7 (C-8), 130.8 (C-7), 135.2 (C-10), 141.2, 141.4 (C-7, C-11), 145.7 (C-2); EIMS m/z 220 (M^+, 2), 205 (3), 202 (13), 187 (8), 159 (8), 147 (13), 137 (46), 119 (37), 109 (31), 107 (27), 105 (29), 95 (29), 94 (34), 93 (55), 91 (46), 69 (42), 55 (46), 43 (100), 41 (47).

RESULTS AND DISCUSSION

For the examination of the triene component of apple skin wax, a number of authentic α-farnesene oxidation products including the trienol **2b** and the endoperoxide **4b** were available by synthesis (Brimble *et al.*, 1994). To provide additional reference materials, attempts were made to isolate the hydroperoxides **2a** and **3a** and endoperoxide **4a** from autoxidized samples of α-farnesene as reported by Anet (1969). The autoxidation of synthetic α-farnesene on glass proved to be difficult to initiate and control. Better results were obtained using samples of α-farnesene stored as the neat oils on glass at −20 °C for several months.

Farnesyl Hydroperoxides 2a and 3a. 6-Hydroperoxy-2,6,10-trimethyldodeca-2,7(*E*),9(*E*),11-tetraene (**2a**) was isolated from aged samples of α-farnesene by flash chromatography and identified as a conjugated triene by its characteristic UV spectrum (λ_{max} 259, 269, and 281 nm) and the close similarity of the ^1H NMR spectrum to that of synthetic trienol **2b**. A methyl singlet at δ 1.40, assigned to the C-6 methyl of **2a**, was shifted 0.08 ppm downfield from its position in **2b** and was the only significant difference from the ^1H NMR of **2b**. The mass spectrum showed a molecular ion at m/z 236.1769 $C_{15}H_{24}O_2$ with significant fragments at m/z 219 (M^+ − OH) and 203 (M^+ − OOH).

Detailed analysis of the ^1H NMR of **2a** showed the presence of a minor component comprising some 10% of the sample, which was identified as the isomeric hydroperoxide **3a**. In particular, a low-field doublet of doublets (δ 6.96) was assigned as H-11, shifted downfield by 0.55 ppm, characteristic of the 3*Z* isomers of 3-methyl-1,2,5-hexatrienes (Brouwer *et al.*, 1992) and α-farnesenes (Anet, 1970; Gamba-Invernizzi *et al.*, 1993). Olefinic resonances at δ 5.70, 6.01, and 6.72, assigned as H-7, H-9, and H-8, respectively, were shifted −0.06, −0.08, and +0.14 ppm relative to their positions in **2a**, consistent with the above reference compounds and the assignments given by Anet (1969) for the 9(*Z*)-trienol **3b**.

The identity of hydroperoxides **2a** and **3a** was confirmed by reduction of the mixture with triphenylphosphine to give trienols **2b** and **3b** in a ratio of 8.3:1.0 by HPLC. Trienol **2b** was identified by direct comparison (UV, normal and reversed phase HPLC, GC−MS) with authentic synthetic material (Brimble *et al.*, 1994). Trienol **3b** was identical by UV and HPLC with the minor trienol isomer occurring in synthetic samples of **2b**. Detailed examination of the ^1H NMR of synthetic samples of **2b** identified this minor component as the 9(*Z*)-trienol isomer **3b**. In particular, a low-field doublet of doublets (δ 6.98) was assigned to H-11, shifted downfield by 0.57 ppm, and characteristic of the 3*Z* isomers of 3-methyl-1,2,5-hexatrienes (Brouwer *et al.*, 1992) and α-farnesenes (Anet, 1970; Gamba-Invernizzi *et al.*, 1993).

Samples of hydroperoxides **2a** and **3a** also contained variable amounts (0−25%) of a third hydroperoxide, which was subsequently identified as **6a** on the basis of the characteristic H-1 singlets at δ 4.66 and 4.70 in the ^1H NMR spectrum and by the formation of **6b** (Figure 2A) on reduction of the sample with triphenylphosphine.

Hydroperoxides **2a** and **3a** were proposed by Anet (1969) as early autoxidation products of α-farnesene after the isolation of a mixture of **2b** and **3b** from autoxidized and then $NaBH_4$-reduced samples of α-farnesene. Subsequently, hydroperoxides **2a** and **3a** have been presumed to be constituents of the triene component of apple skin wax (Gallerani and Pratella, 1991; Gallerani *et al.*, 1992) but have not previously been isolated or characterized.

Stability of Farnesyl Hydroperoxides 2a and 3a. Mass spectral analysis of a sample of farnesene hydroperoxides **2a** and **3a** showed the presence of a further oxidation product [M^+ 268.1680 (0.3%), $C_{15}H_{24}O_4$ requires 268.1675] which had not been observed during the analysis of this sample by ^1H NMR. HPLC analysis (Figure 2A) showed the presence of three major peaks [retention times (area ratios) 5.1 min (6.4), 5.7 min (3.6), 15.8 min (1.0)], each of which was identified as containing a conjugated triene chromophore from UV scans. The two early eluting peaks were identified as **2a** and **6a**, respectively, from their relative intensities in the

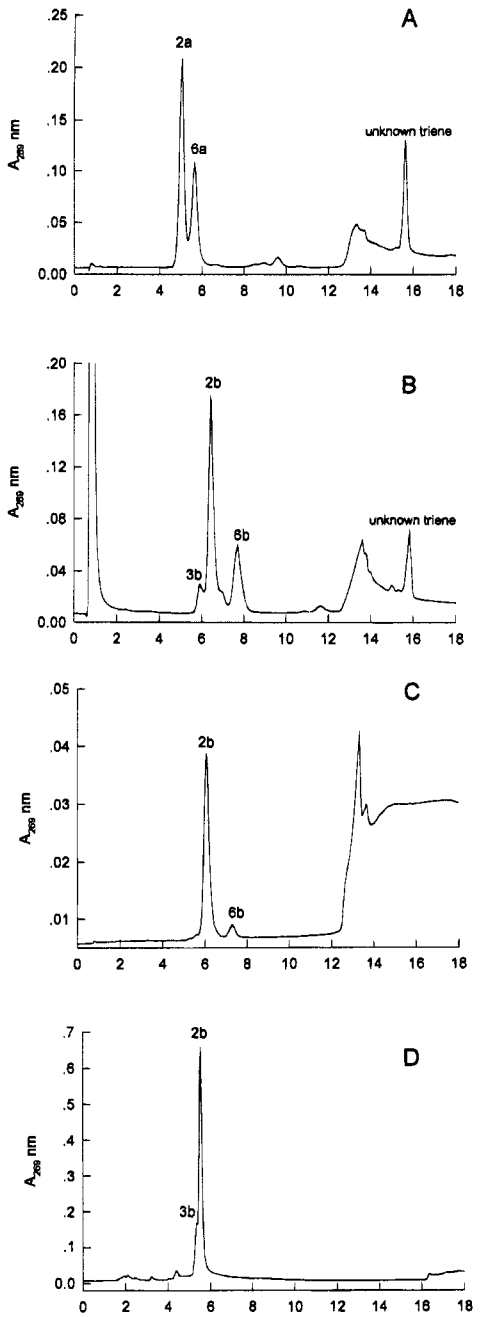

Figure 2. Normal phase HPLC profiles (gradient of 0.1−1% 2-propanol in heptane) of (A) a sample of farnesyl hydroperoxides, (B) above sample reduced with triphenylphosphine, (C) synthetic trienol **2b**, and (D) apple skin extract. Identification of peaks follows the structural formulas in Figure 1.

^{1}H NMR spectrum, UV (λ_{max} 269 nm), shorter retention times than trienol **2b,** and the formation of **2b** and **6b** (HPLC and GC−MS) on reduction of a portion of the sample with triphenylphosphine (Figure 2B).

The remainder of the sample was stored in pentane at −20 °C and the decomposition of the hydroperoxides followed by HPLC. For the major hydroperoxide **2a,** 53% decomposition had occurred after 6 days at −20 °C. Both isomeric hydroperoxides decomposed at approximately the same rate with partial conversion (21% at 6 days) to the third late eluting triene with a retention time of 15.8 min (Figure 2A). This late eluting triene coeluted, under a variety of conditions of normal and reversed phase HPLC, with the second component (R_f 0.45) isolated from the autoxidation of α-farnesene and is discussed further below. The retention time and UV spectrum of this third late eluting peak were not changed after reduction with triphenylphosphine. Trienol **2b** (*ca.* 10%) was also detected in the sample after 18 days when the bulk (69%) of the hydroperoxide had disappeared.

The Second α-Farnesene Autoxidation Product. The second compound (R_f 0.45) isolated from the autoxidation of α-farnesene proved to be too unstable to characterize by ^{1}H NMR. Consequently, after flash chromatography, this compound was immediately reduced with triphenylphosphine. High-resolution mass spectroscopy established $C_{15}H_{24}O_3$ as the molecular formula, and on the basis of previous work (Anet, 1969) structure **4b** was expected. The ^{1}H NMR and UV spectra showed absorptions characteristic of a conjugated triene. The olefin region of the ^{1}H NMR showed the presence of *ca.* 30% of a second triene system with the signal for H-7 (numbering as for **2b**) showing the greatest separation of resonances (δ 5.69 and 5.93, 0.3:0.7 H, respectively), suggesting the presence of isomers at C-6. Analysis of this material by reversed and normal phase HPLC confirmed the presence of two triene components (C_{18} ODS 75% methanol/water; 9.5 and 10.8 min, 2:1, respectively). In addition, the ^{1}H NMR showed the loss of the H-3 olefinic proton of **2a** and of the C-1 and 2-Me allylic methyl signals with the appearance of a new carbinol absorption at δ 3.9 (1H, m). The ^{1}H NMR, particulary the high-field position of the methyl singlets (δ 1.15, 1.20, and 1.25), was in general agreement with that reported by Anet (1969) for endoperoxide **4b**; however, the absence of a C-6 methyl resonance in the region δ 1.3−1.4 was inconsistent with data reported for synthetic endoperoxide **4b** by Brimble *et al.* (1994).

Conjugated Triene Components of Apple Skin. The conjugated triene constituents of the apple skin wax were extracted with heptane and purified by solid phase extraction onto silica prior to analysis by normal and reversed phase HPLC and GC−MS. No other triene components were obtained on repeated extraction of the apple skin with heptane or using 10% diethyl ether or dichloromethane in heptane. Solid phase cleanup on silica concentrated all of the conjugated trienes in the extract into a single fraction as determined by UV spectroscopy and HPLC. The triene-containing fraction was examined by normal phase HPLC using initially a 0−10% gradient of 2-propanol in heptane to examine all UV absorbing components. Conjugated triene components of the extract were determined by scanning the UV spectra of eluting peaks, and the gradient was refined to 0−1% 2-propanol/heptane for routine analysis. Isocratic conditions (0.02 and 0.1% 2-propanol/heptane) were then used for the separation of double-bond isomers and routine analysis of trienols **2b** and **3b**. Peak identities were confirmed by coinjection with authentic standards using both normal and C_{18} reversed phase HPLC (70% methanol/water). Analysis by C_{18} reversed phase HPLC did not reveal any additional highly polar trienes in the extracts.

Generally only two components with the characteristic UV absorptions of a conjugated triene were detected in the HPLC traces (Figures 2D and 3). The major conjugated triene in the apple skin wax was identified as trienol **2b** by normal and reversed phase HPLC, by coinjection on GC, and by GC−MS against authentic material obtained by synthesis (Brimble *et al.*, 1994). Trienol **2b** constituted some 89−95% of the conjugated trienes present in the apple extracts of the five apple varieties examined. The identification of

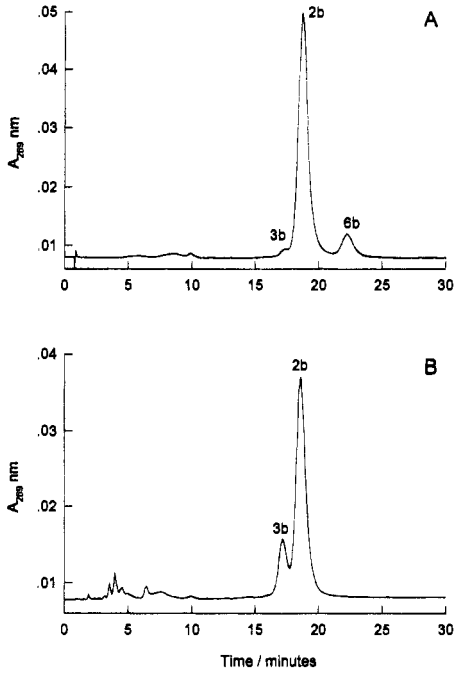

Figure 3. Normal phase HPLC (0.02% 2-propanol/heptane) of (A) synthetic trienol **2b** and (B) a typical apple skin extract. Identification of peaks follows the structural formulas in Figure 1.

trienol **2b** as the major conjugated triene present in the apple skin wax conflicts with the report of Gallerani and Pratella (1991), who identified hydroperoxide **2a** as the only conjugated triene in Granny Smith apples. The identification of **2a** by Gallerani and Pratella (1991) rests upon the observation of a significant increase in HPLC retention time of the triene peak upon reduction of the sample with sodium borohydride and on the isolation of trienol **2b** after borohydride treatment. We have found synthetic samples of hydroperoxide **2a** to be very unstable and have not observed HPLC peaks that would correspond to this compound in the majority of the apple samples we have examined.

The second triene component of the apple extracts was identified as trienol **3b** by HPLC comparison with the minor component in synthetic samples of **2b** and in the reduction of hydroperoxides **2a** and **3a**. Trienol **3b** constituted some 5–11% of the conjugated triene component of the apple skin wax and, together with **2b**, generally comprised the only conjugated trienes detected in most samples.

A third more polar conjugated triene was also found in some apple skin extracts where it comprised some 0–4% of the conjugated trienes present. This minor triene coeluted with the polar autoxidation product of α-farnesene and of hydroperoxides **2a** and **3a** (Figure 2), consistent with its being the endoperoxide **4b**. However, as discussed above, the ^{1}H NMR of a sample produced by autoxidation of α-farnesene did not match that of synthetic **4b**. The identity of this minor triene remains to be determined.

Hydroperoxide **2a** was the final conjugated triene observed in the apple skin washes. While this compound was not observed on routine analysis of fresh or stored fruit that had been warmed to room temperature, careful HPLC analysis of Granny Smith apples taken directly from cold storage and extracted with cold solvent showed the presence of small amounts of a triene eluting before **3b** and presumed to be **2a**. This peak constituted some 3–4% of the amount of trienol **2b** present in the samples analyzed and was presumed to be hydroperoxide **2a** on the basis of its disappearence on standing of the sample at room temperature, appropriate retention time (eluting before **3b**), UV spectrum, and disappearance on treatment of the sample with triphenylphosphine.

Triene Isomer in Synthetic 2b. Comparison of the isomeric trienol components of apple extracts with synthetic standards by normal phase HPLC (0.02% 2-propanol/heptane) showed the presence of an additional conjugated triene (RT 22.1 min, 3–10%) in the synthetic standard which was not present in apple skin extracts (Figure 3). This additional triene was isolated from a synthetic sample of **2b** using $AgNO_3$ centrifugal TLC and identified as **6b** by ^{1}H, COSY, and ^{13}C NMR experiments. The ^{1}H NMR showed the presence of a conjugated trienol system identical to that of **2b**. However, only one other vinylic methyl (δ 1.70) was present, while the H-3 olefinic proton was clearly absent. Two one-proton singlets (δ 4.67 and 4.70) were assigned to a C-1 methylene group with the two allylic H-3 protons occurring as a triplet at δ 2.01. The presence of a Δ^1 double bond was confirmed by the ^{13}C NMR which showed C-1 and C-2 as a triplet and singlet at δ 110.1 and 145.7, respectively. Re-examination of the ^{1}H NMR of samples of **2b** and of intermediates in its synthesis revealed that the concentration of the Δ^1 isomer was enriched from *ca.* 2 to 3–10% during the last step of the synthesis of **2b** when the strong mixed base potassium *tert*-butoxide/lithium diisopropylamine was used to open epoxide **5** (Brimble *et al.*, 1994).

Quantitation of Trienols 2b and 3b by HPLC. As **2b** and **3b** together comprised nearly all of the conjugated trienes present in the samples, HPLC conditions were adjusted so these compounds would coelute, and the combined peak was quantified against standards of synthetic **2b**. Recoveries of trienes after solid phase extraction on silica were estimated by standard addition experiments as approximately 58%. Five apple varieties and "early harvest" Granny Smith apples stored under high-humidity/low-ventilation conditions to promote superficial scald were sampled to obtain a range of typical triene concentrations. None of the apples examined showed symptoms of superficial scald. While triene concentrations of up to 1.2 μg of triene/cm^2 of apple surface (175 μg of triene per apple) were found, values of 3–20 ng of triene/cm^2 of apple surface (0.5–3 μg of triene per apple) were typical. Concentrations of trienes in apple skin washes have also been reported by Gallerani and Pratella (1991) using a similar HPLC system (diisopropyl ether/hexane) but as nanomole equivalents of diphenylamine per cm^2 of apple surface. We have measured the relative response of trienol **2b** to diphenylamine as 1:0.23 (w/w), respectively, in our HPLC system, which would place the triene values we have measured within the previously reported range.

The concentrations of conjugated trienes in the apple skin washes were also measured directly by UV spectroscopy [$A_{281} - A_{290}$ (Anet, 1972b)] and compared with values measured by HPLC. Concentrations of trienes by HPLC were only some 12–35% of the triene concentration as measured by UV spectroscopy, indicating that direct UV spectroscopy of apple skin washes can seriously overestimate triene concentrations. The measurement of trienes by HPLC should provide a more accurate method for the determination of triene concentrations in the apple skin, especially at the lower triene concentrations which occur early after harvest

when induction of superficial scald of the apple occurs. Further work to develop a robust quantitative HPLC method for the determination of conjugated trienes in the apple skin wax is in progress.

LITERATURE CITED

Anet, E. F. L. J. Autoxidation of α-farnesene. *Aust. J. Chem.* **1969**, *22*, 2403–2410.

Anet, E. F. L. J. Synthesis of (E,Z)-α-, (Z,Z)-α-, and (Z)-β-farnesene. *Aust. J. Chem.* **1970**, *23*, 2101–2108.

Anet, E. F. L. J. Superficial scald, a functional disorder of stored apples VIII. Volatile products from the autoxidation of α-farnesene. *J. Sci. Food Agric.* **1972a**, *23*, 605–608.

Anet, E. F. L. J. Superficial scald, a functional disorder of stored apples IX. Effect of maturity and ventilation. *J. Sci. Food Agric.* **1972b**, *23*, 763–769.

Anet, E. F. L. J.; Coggiola, I. M. Superficial scald, a functional disorder of stored apples X. Control of α-farnesene autoxidation. *J. Sci. Food Agric.* **1974**, *25*, 293–298.

Bain, J. M.; Mercer, F. V. The submicroscopic cytology of superficial scald, a physiological disease of apples. *Aust. J. Biol. Sci.* **1963**, *16*, 442–449.

Barden, C. L.; Bramlage, W. J. Accumulation of antioxidants in apple peel as related to preharvest factors and superficial scald susceptibility of the fruit. *J. Am. Soc. Hortic. Sci.* **1994**, *119*, 264–269.

Blanpied, G. D. Effect of repeated postharvest applications of butylated hydroxytoluene (BHT) on storage scald of apples. In *CA '93 Proceedings of the 6th International Controlled Atmosphere Research Conference*; Northeast Regional Agricultural Engineering Service Cooperative Extension: Ithaca, NY, 1993; Vol. 2, pp 466–469.

Blanpied, G. D.; Creasy, L. L. Concentrations of farnesene and conjugated trienes in the skin of Cortland apples stored in 2% and 4% oxygen with 1%, 3%, and 5% carbon dioxide at 2.2C. In *CA '93 Proceedings of the 6th International Controlled Atmosphere Research Conference*; Northeast Regional Agricultural Engineering Service Cooperative Extension: Ithaca, NY, 1993; Vol. 2, pp 481–486.

Brimble, M. A.; Rowan, D. D.; Spicer, J. A. Synthesis of conjugated trienes and related oxidation products of α-farnesene. *Aust. J. Chem.* **1994**, *47*, 1979–1988.

Brouwer, A. M.; Bezemer, L.; Jacobs, H. J. C. Steric perturbation of the conjugated triene chromophore. Conformational analysis of (E)- and (Z)-3-methyl-1,3,5-hexatriene and (Z)-3-*tert*-butyl-1,2,3-hexatriene. *Recl. Trav. Chim. Pays-Bas* **1992**, *111*, 138–143.

Chen, P. M.; Varga, D. M. Efficacy of step-wise low O_2, low O_2, and initial high CO_2 treatments on the control of superficial scald on d'Anjou pears. In *CA '93 Proceedings of the 6th International Controlled Atmosphere Research Conference*; Northeast Regional Agricultural Engineering Service Cooperative Extension: Ithaca, NY, 1993; Vol. 2, pp 453–465.

Chen, P. M.; Varga, D. M.; Mielke, E. A.; Facteau, T. J.; Drake, S. R. Control of superficial scald on d'Anjou pears by ethoxyquin: oxidation of α-farnesene and its inhibition. *J. Food Sci.* **1990**, *55*, 171–180.

Du, Z.; Bramlage, W. J. A modified hypothesis on the role of conjugated trienes in superficial scald development on stored apples. *J. Am. Soc. Hortic.* **1993**, *118*, 807–813.

Fielder, S.; Rowan, D. D.; Reay, P. F. The synthesis of 4-^{2}H-α-farnesene and 1-^{2}H-α-farnesene, *J. Labelled Compd. Radiopharm.* **1993**, *33*, 965–975.

Gallerani, G.; Pratella, G. C. Upgrading peroxide detection to prevent superficial scald of apples by HPLC. *Adv. Hortic. Sci.* **1991**, *5*, 139–143.

Gallerani, G.; Folchi, A.; Pratella, G. C.; Cazzola, P. Superficial scald and change in concentration of hydroperoxides in apples stored under ultra-low oxygen at varying CO_2 rates. *Ital. J. Food Sci.* **1992**, *4*, 39–45.

Gamba-Invernizzi, A.; Garlaschelli, L.; Rossi, A.; Vidari, G.; Vita-Finzi, P. New farnesane sesquiterpenes from *Lactarius porninsis*. *J. Nat. Prod.* **1993**, *56*, 1948–1953.

Huelin, F. E.; Coggiola, I. M. Superficial scald, a functional disorder of stored apples IV. Effect of variety, maturity, oiled wraps and diphenylamine on the concentration of α-farnesene in the fruit. *J. Sci. Food Agric.* **1968**, *19*, 297–301.

Huelin, F. E.; Coggiola, I. M. Superficial scald, a functional disorder of stored apples V. Oxidation of α-farnesene and its inhibition by diphenylamine *J. Sci. Food Agric.* **1970a**, *21*, 44–48.

Huelin, F. E.; Coggiola, I. M. Superficial scald, a functional disorder of stored apples VII. Effect of applied α-farnesene, temperature and diphenylamine on scald and the concentration and oxidation of α-farnesene in the fruit. *J. Sci. Food Agric.* **1970b**, *21*, 584–589.

Ingle, M.; D'Souza, M. C. Physiology and control of superficial scald of apples: a review. *HortScience* **1989**, *24*, 28–31.

Klein, J. D.; Lurie, S. Time, temperature, and calcium interact in scald reduction and firmness retention in heated apples. *HortScience* **1994**, *29*,194–195.

Lurie, S.; Klein, J. D.; Arie, R. B. Prestorage heat treatment delays development of superficial scald on Granny Smith apples. *HortScience* **1991**, *26*, 166–167.

Meigh, D. F. Apple scald. In *The Biochemistry of Fruits and Their Products*; Hulme, A. C., Ed.; Academic: New York, 1970; Vol. 1, Chapter 19.

Meigh, D. F.; Filmer, A. A. E. Natural skin coating of the apple and its influence on scald in storage III. α-Farnesene. *J. Sci. Food Agric.* **1969**, *20*, 139–143.

Meir, S.; Bramlage, W. J. Antioxidant activity in Cortland apple peel and susceptibility to superficial scald after storage. *J. Am. Soc. Hortic. Sci.* **1988**, *113*, 412–418.

Spicer, J. A.; Brimble, M. A.; Rowan, D. D. Oxidation of α-farnesene. *Aust. J. Chem.* **1993**, *46*, 1929–1939.

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