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Oxidation Products of α-Farnesene Associated with Superficial Scald Development in d'Anjou Pear Fruits Are Conjugated Trienols

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Conjugated triene (CT) oxidation products of the acyclic sesquiterpene (*E*,*E*)-α-farnesene are thought to induce development of the physiological storage disorder superficial scald in apple and pear fruits of susceptible cultivars. CTs that accumulate in peel tissues of Granny Smith and Delicious apples after several weeks of cold storage are known to be conjugated trienols (CTols) rather than the corresponding hydroperoxides produced by in vitro autoxidation of α-farnesene. Here, it is shown that CTols are also the predominant in vivo oxidation products of α-farnesene in cold-stored pear fruit of the highly scald-susceptible d'Anjou cultivar. Analysis by octadecylsilane reversed phase high-performance liquid chromatography with diode array detection, gas chromatography with flame ionization detection, gas chromatography−mass spectrometry, and proton nuclear magnetic resonance spectroscopy identified 3,7,11-trimethyldodeca-1,3(*E*),5(*E*),10-tetraen-7-ol as the major CT in hexane extracts of peel tissue from d'Anjou pears stored for 3−5 months in air at −1 °C. The possible origins of CTols in apples and pears and the hypothesized role of these oxidation products of α-farnesene in the induction of scald are discussed.

KEYWORDS: Pear fruit; *Pyrus communis*; α-farnesene oxidation; conjugated trienols; superficial scald; storage disorder

INTRODUCTION

Fruit of d'Anjou pear are highly susceptible to superficial scald, a physiological storage disorder that occurs in only certain cultivars of both apples and pears. Scald symptoms are manifested as brown or black patches on the fruit skin and generally worsen after removal from cold storage. Necrosis of the hypodermal cortical tissue is observed during scald development (*1*) and is thought to be induced by oxidation products of the sesquiterpene (*E*,*E*)-α-farnesene (*2*−*5*). Scald-susceptible apples and pears typically exhibit a high rate of ethylene-dependent α-farnesene synthesis a few weeks after placement in low-temperature storage (*6*−*8*). The sesquiterpene accumulates in the skin to high concentrations during the first 2−3 months and subsequently declines as its conjugated triene (CT) oxidation products increase to a maximum at about 4−6 months (*2*−*4*, *8*−*11*).

The predominant CT oxidation products of α-farnesene that accumulate in apple epicuticular wax and peel tissue during cold storage have been identified as conjugated trienols (CTols), 3(*E*) and 3(*Z*) isomers of 3,7,11-trimethyldodeca-1,3,5(*E*),10-tetraen-7-ol (**Figure 1**, structures **1a**,**b**, respectively) (*10*, *12*). In vitro autoxidation of α-farnesene, on the other hand, yields mainly the corresponding 3(*E*) and 3(*Z*) isomeric 7-hydroperoxides

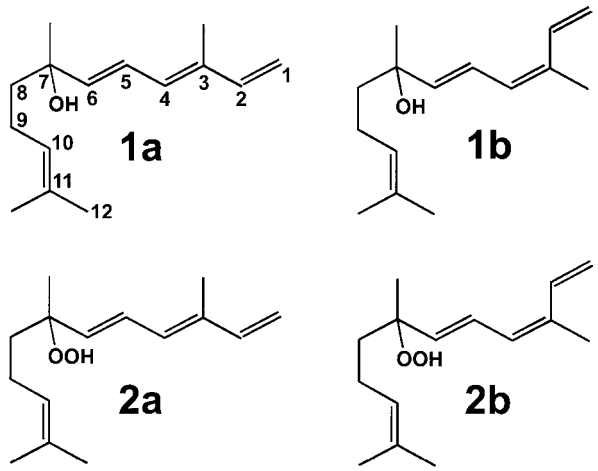

Figure 1. Structures **1a** and **1b**, respectively, are the major and minor CTol isomers 3,7,11-trimethyldodeca-1,3(*E*),5(*E*),10-tetraen-7-ol and 3,7,-11-trimethyldodeca-1,3(*E*),5(*Z*),10-tetraen-7-ol, which accumulate in the skin of cold-stored apple fruit. Structures **2a** and **2b**, respectively, are the 3(*E*) and 3(*Z*) CT 7-hydroperoxide analogues of **1a**,**b**, which are primary in vitro autoxidation products of (*E*,*E*)-α-farnesene.

(**Figure 1**, structures **2a**,**b**, respectively) (*12*, *13*), and it is not known how the CTols are produced in vivo in apple skin (*14*). When either synthetic CTols or their hydroperoxide analogues

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were applied to apple fruit prior to storage, they induced symptoms indistinguishable from naturally occurring superficial scald (*5*). Prestorage treatment of apples and pears with the antioxidants diphenylamine and ethoxyquin, respectively, inhibits oxidation of α-farnesene to CT products and largely prevents development of scald (*2*, *4*, *11*). Although the CTs in hexane-dip extracts of cold-stored apples and pears have identical UV absorbance spectra, this does not distinguish CTols from CT hydroperoxides, both of which exhibit three prominent absorbance maxima at 259, 269, and 281 nm (*12*, *13*, *15*). To date, the CTs from skin of scald-susceptible pear fruit have not been structurally elucidated. In the present study, CT oxidation products of α-farnesene were isolated from hexane extracts of peel tissue from d'Anjou pears stored for 3−5 months at −1 °C by normal phase column chromatography and reverse phase high-performance liquid chromatography with diode array detection (C_{18}-HPLC-DAD). The major CT species was analyzed by gas chromatography with flame ionization detection (GC-FID), gas chromatography−mass spectrometry (GC-MS), and proton nuclear magnetic resonance spectroscopy (^{1}H NMR) to determine whether it is identical to the predominant CT isolated from Granny Smith, Delicious, and Law Rome apples, namely, 3,7,11-trimethyldodeca-1,3(*E*),5(*E*),10-tetraen-7-ol (**Figure 1**, structure **1a**).

MATERIALS AND METHODS

Plant Material, Fruit Storage, Peel Tissue Sampling, and Scald Evaluation. d'Anjou pear (*Pyrus communis* L.) fruit were harvested from an orchard at the Mid-Columbia Agricultural Research and Extension Center in Hood River, OR, on September 15, 2004 (2 weeks after commercial maturity). Pears from three orchard blocks were segregated as three replicate lots, and defect-free fruit from each lot were packed in eight 20 kg wooden boxes (90 fruit per box) fitted with perforated polyethylene liners. The eight boxes of fruit from each replicate were immediately stored in air at −1 °C and >95% relative humidity. Fruit were sampled after 9, 33, 63, 94, 123, and 157 days in storage. Peel tissue, including the epidermis and 2−3 mm of hypodermal cortex, was excised with a stainless steel fruit peeler from the equatorial region of 10 randomized fruit (four + three + three fruit from the three replicate boxes) and immediately frozen in liquid N_2. Pooled 30 g samples were stored at −72 °C in Ziplock bags until shipped packed in dry ice to the U.S. Department of Agriculture, Agricultural Research Service Produce Quality and Safety Laboratory by overnight courier. Upon arrival, the peel tissue samples were stored at −80 °C until used for extraction and analysis of α-farnesene and its CT oxidation products.

Superficial scald was assessed visually in 60−70 fruit from each replicate 7 days after transfer from cold storage to 20 °C. Scald incidence was defined as the percentage of pears exhibiting slight to severe scald, that is, ≥0.6 cm^2 of the fruit surface exhibiting symptoms (*4*).

Extraction and Isolation of CT Oxidation Products of α-Farnesene. Pooled peel tissue samples (200 g) from fruit stored for 3−5 months (94, 123, and 157 days) at −1 °C, when CT concentrations were highest (**Figure 2**), were immersed in liquid N_2 for 2 min, sieved, and transferred to a 2 L Erlenmeyer flask containing 600 mL of hexane. The flask was flushed with N_2 gas, sealed with aluminum foil and Parafilm (American National Can, Greenwich, CT), and agitated on a rotary shaker at 125 rpm for 6 h at 4 °C in the dark. After it was decanted, the hexane extract was vacuum filtered through a sintered glass funnel fitted with a glass fiber disk, dried over anhydrous sodium sulfate, and reduced in volume to about 80 mL on a rotary evaporator at 30 °C. The remaining solvent was removed by evaporation under a gentle stream of N_2 without heating. Residue from the extract was dissolved in 5 mL of 2,2,4-trimethylpentane and transferred to a 15 mL screw cap tube, which was flushed with N_2, capped, and stored at −20 °C overnight to precipitate much of the epicuticular wax. The decanted supernatant was warmed to 20 °C and applied to a 0.8 cm i.d. glass column containing a 12 cm bed of 60−100 mesh Florisil (Fisher Scientific, Fair Lawn, NJ) slurried in 2,2,4-trimethylpentane. The column was eluted sequentially with 12 mL volumes of (i) hexane; (ii) hexane/isopropanol, 60:1; (iii) hexane/isopropanol, 20:1; (iv) hexane/ethanol, 5:1; and (v) hexane/ethanol, 2:1. UV absorbance spectra recorded on a Shimadzu UV160U spectrophotometer indicated that essentially all of the CT oxidation products of α-farnesene (absorbance maxima at 259, 269, and 281 nm; see **Figure 3**) eluted in column fractions 3 and 4. These two fractions were pooled, the solvent was removed by N_2 evaporation, and the residue was dissolved in 2 mL of methanol. One milliliter portions of the methanolic solution were transferred to two 2 mL amber HPLC vials, which were flushed with N_2, sealed, and stored at −20 °C until analyzed and fractionated by C_{18}-HPLC-DAD. Hexane extracts of peel tissue from cold-stored Law Rome apple fruit obtained during a prior study (*7*) and subsequently stored under N_2 atmosphere at −80 °C were used to prepare an analogous CT-enriched Florisil column fraction for analytical comparison with the d'Anjou pear sample.

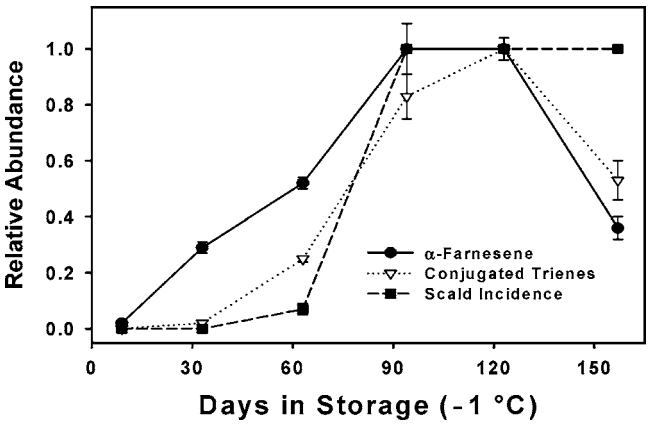

Figure 2. Changes in the relative levels of α-farnesene, CTs, and scald incidence in peel tissue of d'Anjou pears over the course of 157 days of storage at –1 °C in air. Separation and quantification of α-farnesene and CTs were performed by C_{18}-HPLC with UV monitoring at 232 and 269 nm, using HPLC-purified apple fruit α-farnesene and CTol samples of known concentrations as external standards (*8*). Data points were normalized relative to the highest value determined for each constituent, which was assigned a value of 1.0. Vertical bars indicate ± SD (n = 3).

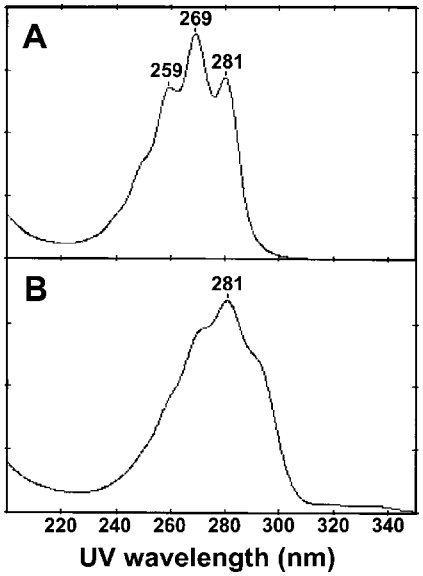

Figure 3. UV absorbance spectra of HPLC-purified CT1 (**A**) and CT2 (**B**) oxidation products of α-farnesene extracted from peel tissue of d'Anjou pears stored at –1 °C in air for 3–5 months. The pooled, thrice-purified CT1 and CT2 samples were dissolved in hexane.

HPLC of the CT-enriched pear and apple samples was performed with a Hewlett-Packard Series 1100 HPLC system (Agilent Technologies) including a quaternary pump, autosampler, and DAD. Initially, 80 μL aliquots were injected onto a Luna 5 μm particle size C18(2) column (250 mm long, 4.6 mm i.d.) from Phenomenex (Torrance, CA) and eluted with a gradient of methanol (A) and water/acetonitrile, 1:1 (B) as follows: 0−4 min, 75A:25B at 0.8 mL/min; 4−16 min, linear increase to 80% A at 0.8 mL/min; 16−18 min, linear increase to 100% A at 1.0 mL/min; 18−20 min, linear decrease to 80% A at 1.0 mL/min; and 20−22 min, linear decrease to 75% A at 0.8 mL/min. Using this gradient, major (CT1) and minor (CT2) constituents in a ratio of about 12:1 eluted at 10.5 and 11.0 min, respectively. The primary UV absorbance maximum of CT1 was at 269 nm, whereas that of CT2 was at 281 nm (**Figure 3**). Separate CT1 and CT2 eluates were collected from a series of 24 HPLC runs, the volume of each was reduced to 2 mL by N_2 evaporation followed by addition of 1 mL of deionized water, and the CT1 and CT2 constituents were recovered by extracting twice with 3 mL of hexane. After N_2 evaporation of the hexane, the CT1 and CT2 HPLC fractions were dissolved in 1.0 and 0.5 mL, respectively, of methanol/water, 4:1, and further purified by a second round of HPLC separations using the following modified gradient: 0−8 min, 60A:40B at 1.0 mL/min; 8−18 min, linear increase to 75% A at 1.0 mL/min; 18−20 min, linear increase to 100% A and 1.2 mL/min; 20−22 min, hold at 100% A with a decrease to1.0 mL/min; 22−25 min, linear decrease to 60% A at 1.0 mL/min; and 25−28 min, hold at 60% A and 1.0 mL/min. CT1 and CT2 eluted at 14.3 and 14.8 min, respectively, and the pooled eluates were again concentrated by N_2 evaporation and extracted with hexane to recover the CT oxidation products. A third series of HPLC separations was then performed using the modified mobile phase gradient to yield the final HPLC-purified CT1 and CT2 fractions.

GC-FID, GC-MS, and ^{1}H NMR Analyses of CT1 and CT2 HPLC Fractions. Aliquots of the d'Anjou and Law Rome CT1 and CT2 HPLC fractions dissolved in 2,2,4-trimethylpentane were initially analyzed by GC-FID using a Hewlett-Packard 5890 Series II gas chromatograph fitted with a 15 m × 0.25 mm i.d. HP-1 capillary column (film thickness, 0.25 μm). Injector and detector temperatures were 200 and 240 °C, respectively, and the oven was isothermal at 125 °C. Helium was the carrier gas with a flow rate of 1.1 mL/min and a linear velocity of 36.6 cm/s. Data were recorded and processed by an HP 3396 Series II integrator. GC-MS analyses (70 eV electron impact ionization; EI) were subsequently performed using an Agilent Technologies 6890N GC system equipped with a 50 m × 0.2 mm i.d., 0.33 μm film thickness HP Ultra-2 capillary column and a 5973N mass selective detector. Operation was in the splitless mode, with an MS interface temperature of 200 °C and with helium as the carrier gas at a flow rate of 0.9 mL/min and a linear velocity of 26.6 cm/s. The column oven was temperature programmed, holding at 140 °C for 6 min, increasing to 180 °C at 2 °C/min, and then holding for 30 min at 180 °C. Data were acquired and processed using ChemStation software (Agilent Technologies G1701DA version D01.00) loaded in a Pentium 4 PC.

A ^{1}H NMR spectrum of HPLC-purified d'Anjou CT1 dissolved in 0.8 mL of benzene-d_6 was acquired after deuterium lock at 25 °C, using a Bruker QE 300 MHz NMR spectrometer. Chemical shift values were assigned relative to the frequencies of residual nondeuterated benzene and water, which were externally referenced to tetramethylsilane.

RESULTS AND DISCUSSION

The d'Anjou pears that provided the peel tissue used in this study accumulated high levels of CTs after 2 months of −1 °C storage in air, reaching a maximum concentration of about 90 μg/g frozen peel at 4 months (**Figure 2**) (*8*). The increase in CTs was preceded by a parallel increase in α-farnesene and was closely correlated with the incidence of superficial scald (determined 7 days after transfer of fruit from −1 to 20 °C), which was 100% after 3 months of storage (**Figure 2**).

CT-enriched Florisil column fractions derived from hexane extracts of d'Anjou pear and Law Rome apple peel tissue included major constituents (CT1 and CT2) with identical C_{18}-HPLC elution times in chromatograms obtained using two different mobile phase gradients. Moreover, the CT1:CT2 ratio was roughly the same for the pear and apple extracts (about 12:1), and the UV absorbance spectra for CT1 and CT2 from pear (**Figure 3**) were identical to those for CT1 and CT2 from apple. GC-FID analysis of the HPLC-purified CT1 fractions from pear and apple showed the same pair of peaks with retention times of 12.51 and 13.34 min. The ratio of peak 1 to peak 2 was 8:92 for the pear CT1 sample and 9:91 for apple CT1. These data are consistent with the relative GC retention times and 1:9 ratio previously reported for the 3(*Z*) and 3(*E*) isomers of 3,7,11-trimethyldodeca-1,3,5(*E*),10-tetraen-7-ol isolated from peel tissue extracts of Delicious apples (*10*). The CT2 HPLC fraction from pear did not produce any major peaks on the GC-FID chromatogram, possibly due to thermal degradation in the injector, and showed only traces of the apparent CTol isomers at 12.51 and 13.34 min.

GC-MS analysis of pear and apple CT1 gave a single major peak (retention 28.6 min), suggesting that the 3(*Z*) and 3(*E*) CTol isomers were not separated on the HP Ultra-2 capillary column. The major CT peak in the GC-MS chromatogram of the HPLC purified CT1 fraction from d'Anjou pear peel tissue produced the 70 eV EI mass spectrum: *m*/*z* (rel. int. %) 220 (M^+, <1), 202 ($M^+ - H_2O$, 10), 187 (5), 177 (4), 162 (60), 159 (40), 147 (19), 137 (33), 133 (20), 119 (41), 107 (31), 105 (48), 95 (63), 93 (70), 91 (56), 79 (35), 69 (46), 55 (50), 43 (100), 41 (56). The corresponding GC-MS peak from the HPLC-purified CT1 fraction from Law Rome apple peel tissue gave a nearly identical EI mass spectrum: *m*/*z* (rel. int. %) 220 (M^+, <1), 202 ($M^+ - H_2O$, 9), 187 (4), 177 (3), 162 (52), 159 (36), 147 (17), 137 (30), 133 (18), 119 (38), 107 (29), 105 (45), 95 (58), 93 (65), 91 (53), 79 (33), 69 (44), 55 (50), 43 (100), 41 (58). Thus, the predominant peak from CT1 of the two pome fruits yielded matching EI mass spectra indicating a molecular ion of mass 220 (required for $C_{15}H_{24}O$) and ready loss of water to give *m*/*z* 202. These mass spectra are in good agreement with those previously reported for 3,7,11-trimethyldodeca-1,3(*E*),5-(*E*),10-tetraen-7-ol (*10*, *12*, *15*).

The ^{1}H NMR spectrum of HPLC-purified d'Anjou CT1 dissolved in benzene-d_6 was as follows: δ ^{1}H 1.14 (3H, s, 7-CH_3), 1.49 (2H, m, H-8), 1.53 (3H, s, 11-CH_3), 1.64 (3H, s, H-12), 1.76 (3H, s, 3-CH_3), 2.10 (2H, m, H-9), 5.00 (1H, d, J = 11 Hz, H-1b), 5.15 (1H, d, J = 18 Hz, H-1a), 5.18 (1H, m, H-10), 5.63 (1H, d, J = 15 Hz, H-6), 6.09 (1H, d, J = 12.4 Hz, H-4), 6.46 (1H, dd, J = 17, 11 Hz, H-2), 6.70 (1H, dd, J = 15.2, 11.2 Hz, H-5). After taking into account the slightly different chemical shifts attributed to the use of benzene-d_6 rather than $CDCl_3$ as the solvent, comparison of the d'Anjou CT1 ^{1}H NMR spectrum with published spectra for CTols (*12*, *16*, *17*) indicates that the major constituent in CT1 is the 3(*E*),5-(*E*) CTol isomer. In particular, the doublet of doublets at 6.46 ppm representing the single proton on C2 distinguishes the 3(*E*) from the 3(*Z*) isomer since the latter shows a 0.57 ppm downfield shift in this signal (*12*).

In contrast with the results of GC-FID analysis, the GC-MS chromatogram of the HPLC purified CT2 fraction from d'Anjou pear peel tissue showed two prominent, well-separated peaks. Their retention times were 43.5 and 47.9 min in a ratio of 1:3, and they yielded nearly identical 70 eV EI mass spectra, as follows: Peak 1, *m*/*z* (rel. int. %) 220 (M^+, 36), 202 ($M^+ - H_2O$, 20), 189 (4), 187 (4), 159 (28), 151 (30), 133 (55), 123 (32), 121 (39), 107 (77), 105 (100), 93 (56), 91 (89), 81 (46), 79 (40), 69 (61), 55 (36), 43 (25), 41 (97); Peak 2, *m*/*z* (rel. int. %) 220 (M^+, 37), 202 ($M^+ - H_2O$, 21), 189 (4), 187 (4), 159

(24), 151 (31), 133 (55), 123 (29), 121 (33), 107 (75), 105 (100), 93 (52), 91 (87), 81 (41), 79 (36), 69 (57), 55 (33), 43 (21), 41 (93). The close similarity of the EI mass spectra indicates that the two major compounds in fraction CT2 are most likely isomers, the second being three-fold more abundant than the first. The molecular mass of the CT2 isomers is 220, the same as that of the CTol isomers and consistent with the formula $C_{15}H_{24}O$. However, the molecular ion is much more abundant than that of the CTols, indicating a reduced tendency to lose a molecule of water. The UV absorbance spectrum of the constituents in CT2 is quite different from that of the CTols in CT1 (**Figure 3**), with a single maximum at 281 nm and shoulders on either side rather than additional, well-defined maxima. Among the acyclic sesquiterpenes with the formula $C_{15}H_{24}O$ described in the literature, it appears that the EI mass spectrum of 3,7,11-trimethyldodeca-2*Z*,4*E*,6*E*,10-tetraen-1-ol reported by Fielder et al. (*18*) most closely resembles the mass spectra of the two CT2 constituents. However, identification of these unknowns will require purification of sufficient material for ^{1}H NMR analysis and/or synthesis of suitable model compounds.

It was long assumed that the CT oxidation products of α-farnesene in the skin of cold-stored apple fruit are the same as those generated during in vitro autoxidation (*13*) until Rowen et al. (*12*) showed them to be 3(*E*) and 3(*Z*) isomers of 3,7,-11-trimethyldodeca-1,3,5(*E*),10-tetraen-7-ol (CTols) rather than the corresponding 7-hydroperoxy analogues (**Figure 1**). Whitaker et al. (*10*) later corroborated this finding using fruit of a different scald-susceptible apple cultivar. Like Granny Smith and Law Rome apples, d'Anjou pears are highly susceptible to superficial scald, and the incidence and severity of the disorder are closely correlated with accumulation of CT oxidation products of α-farnesene during low-temperature storage (**Figure 2**) (*2*, *4*, *6*−*8*, *10*, *11*). The results of the present study show that d'Anjou pears accumulate the same CTol isomers as those previously isolated from scald-susceptible apples.

Using the synthetic compounds, Rowan et al. (*5*) demonstrated that application of either CTols or their 7-hydroperoxy analogues to apple fruit prior to storage promoted development of scald symptoms. Although the alcohols are considerably more stable than the hydroperoxides (*5*, *13*), it was found that in vitro at 20 °C CTols isolated from apple skin are prone to rapid, free radical-mediated autoxidation (*19*). In vitro autoxidation of both CTols and α-farnesene yields the volatile 6-methyl-5-hepten-2-one (MHO) as a major product, probably via decomposition of a 7-alkoxy radical intermediate (*19*, *20*). It has been suggested that MHO plays a role in the induction of scald (*21*, *22*), but it appears more likely that this volatile is merely a byproduct of the free radical reactions that cause cell damage and eventual necrosis (*5*, *14*, *20*).

An important unanswered question is how CTols are generated in peel tissue of apple and pear fruits. During slow decomposition in pentane at −20 °C, about 10% conversion of CT hydroperoxides to the corresponding alcohols was observed (*12*). It is possible that a similar process occurs in the waxy coating of cold-stored pome fruits. However, it was recently reported that strains of three species of *Ascomycete* fungi are capable of enzymatically converting α-farnesene to the 3(*E*) and 3(*Z*) CTol isomers (*16*), suggesting that production of CTols in apples and pears could also be enzymatic. Members of two classes of plant enzymes, glutathione peroxidases (GPXs) and glutathione transferases (GSTs), can reduce alkyl hydroperoxides to the corresponding alcohols as part of the antioxidative defense system (*23*, *24*). Thus, either a GPX or a GST may convert the autoxidation products of α-farnesene to CTols in the skin of apples and pears.

It appears that the two minor constituents in the HPLC-purified CT2 fraction from d'Anjou pears, with UV absorbance maxima at 281 nm (**Figure 3**), have not been reported previously. However, Du and Bramlage (*25*) found a correlation between a high A_{281nm} to A_{258nm} ratio in hexane-dip extracts of cold-stored apples and the severity of scald after storage. This suggests that a particular CT_{281nm} species might trigger or intensify the free radical reactions leading to scald development. Additional analytical data are required to identify the two most abundant compounds in the CT2 HPLC fraction. The possibility that these are oxidation products of α-farnesene with a key role in scald induction warrants further investigation.

ABBREVIATIONS USED

C_{18}-HPLC-DAD, octadecylsilane reversed phase high-performance liquid chromatography with diode array detection; CT(s), conjugated triene(s); CTol(s), conjugated trienol(s); EI, electron impact ionization; GC-FID, gas chromatography with flame ionization detection; GC-MS, gas chromatography−mass spectrometry; GPX(s), glutathione peroxidase(s); GST(s), glutathione transferase(s); ^{1}H NMR, proton nuclear magnetic resonance spectroscopy; MHO, 6-methyl-5-hepten-2-one.

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