Biosynthesis of Straight-Chain Ester Volatiles in Red Delicious and Granny Smith Apples Using Deuterium-Labeled Precursors

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Biosynthesis of straight-chain ester volatiles by Granny Smith and Red Delicious apples was investigated using deuterium-labeled fatty acids, C-6 aldehydes, and alcohols. Perdeuterated saturated and monounsaturated fatty acids were metabolized to hexyl-d_{11}, hexanoate-d_{11}, heptanoate-d_{13}, and octanoate-d_{15} esters, whereas perdeuterated linoleic acid produced only hexyl-d_{11} and hexanoate-d_{11} esters. Exposure of fruit to vapors of deuterated 3*Z*-hexenal, 2*E*-hexenal, and hexanal identified the following biosynthetic processes: (1) isomerization between 3*E*, 3*Z*, and 2*E*-hexenals; (2) reduction to 3*E*, 3*Z*, and 2*E*-hexenyl esters; (3) reduction to hexanol and hexyl esters; (4) oxidation to hexanoic acid and formation of hexanoate esters; (5) β-oxidation of hexanoic acid leading to butyl and butanoate esters; and (6) α-oxidation of hexanoic acid leading to pentyl and pentanoate esters. Unsaturated straight-chain ester volatiles appear to arise only by the lipoxygenase pathway and may be useful indicators of lipoxygenase activity in fruit.

Keywords: *Biosynthesis; volatiles; apple; esters; fatty acids; hexenal; deuterium*

INTRODUCTION

Flavor is an important index of fruit and apple quality and is related to the concentrations of the complex mixture of low molecular weight esters, alcohols, aldehydes, and hydrocarbons found in the vapor and tissues of the fruit. In apples, the ethyl, butyl, and hexyl esters of acetic, butanoic, and hexanoic acids are often the major constituents of the headspace vapor and are important contributors to the flavor of ripe fruit (Cunningham et al., 1986; Paillard, 1990; Kollmannsberger and Berger, 1992; Young et al., 1996). In unripe fruit and juices, C-6 aldehydes and alcohols make additional contributions to the flavor (Paillard, 1990). Hexanal and 2*E*-hexenal are the major components, accompanied by lesser amounts of the corresponding alcohols and by 3*Z*-hexenal and 3*Z*-hexenol (Paillard, 1990). Some of the above flavor volatiles also show antimicrobial and insect attractant and deterrent properties (Berger, 1995) and are of interest as residueless, natural fumigants that might additionally be used to enhance aroma levels in treated fruit (Berger, 1990; Hamilton-Kemp et al., 1996; Song et al., 1996) with consequent changes to fruit quality.

Aroma production and biosynthesis are affected by the variety and age of the fruit and by storage conditions (Dirinck et al., 1989; Mattheis et al., 1991). Controlled-atmosphere (CA) storage reduces aroma production when fruit are ripened after storage (Dirinck et al., 1989; Willaert et al., 1983) with differential affects on the various aroma constituents. Prolonged storage may completely suppress the principal headspace components and block their subsequent regeneration on storage at room temperature (Lidster et al., 1983). Under low-ethylene CA storage of McIntosh and Cortland apples (Yahia et al., 1991), butanoate, 2-methylbutanoate, pentanoate, and hexanoate esters were suppressed, whereas aldehydes and acetates were unaffected. Under ultralow-oxygen CA storage of Golden Delicious apples (Brackman et al., 1993) the suppression of aroma production was restricted to straight-chain esters and appeared to be related to a decreased availability of the corresponding alcohol precursors (Knee and Hatfield, 1981). Production of branched-chain aroma compounds by Cox's Orange Pippin apples was suppressed by high carbon dioxide CA conditions (Knee and Hatfield, 1981), which may also reduce aroma production by interfering with carboxylic acid metabolism and alcohol dehydrogenase activity (De Pooter et al., 1987; Knee and Hatfield, 1981).

The straight-chain ester constituents of apple aroma are believed to be synthesized via β-oxidation of fatty acids to give acetic, butanoic, and hexanoic acids, which may then be reduced to the corresponding alcohols before transesterification (Paillard, 1990). Branched-chain alcohols and acids are produced from amino acid precursors (Rowan et al., 1996, 1997), whereas hexanal, 2*E*-hexenal, and 3*Z*-hexenal originate from the hydroperoxides of the unsaturated fatty acids (lipoxygenase pathway) (Hatanaka et al., 1987; Hatanaka, 1993). Hexanal may subsequently be oxidized to hexanoic acid or reduced to give hexanol. These C-6 substrates are then available for interesterification with intermediates from other pathways (Paillard, 1990). The operation of these pathways is supported by feeding experiments in which the addition of intermediates to stored whole apples, apple tissue disks, or homogenates gave enhanced levels of specific aroma compounds. Addition of ethyl through octyl alcohols to stored apples or apple tissue disks gave the corresponding acetates as the major products (Knee and Hatfield, 1981; Berger and Drawert, 1984; Bartley et al., 1985). Oxidation of alcohols to the corresponding aldehydes (C-2−C-4) was

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also reported (Knee and Hatfield, 1981). Addition of carboxylic acid (C-2−C-6) vapors to Golden Delicious apples (DePooter et al., 1983), of carboxylic methyl esters (C-4−C-8) to Cox's Orange Pippin apples (Bartley et al., 1985), or of the C-4−C-18 acid salts to tissue disks (Paillard, 1979) gave esters bearing shortened alkyl and alkanoate groups (C_{n-2}, C_{n-4}), confirming the presence of a β-oxidation pathway to aroma compounds. In similar experiments, addition of aldehydes (C-3−C-6) gave increased concentrations of the corresponding alkyl or alkanoate esters (De Pooter et al., 1983). The carboxylic ester hydrolases and alcohol dehydrogenase of apple have been partially characterized (Bartley and Hindley, 1980; Bartley and Stevens, 1981).

Although unlabeled flavor precursors can be used to infer biosynthetic relationships, their use is limited both by the natural variability of volatile production and by the increase in volatiles due to ripening that occurs during the experiment. These two factors make the unambiguous identification of minor increases in peak areas difficult. Radiolabeled (^{14}C) flavor precursors have been used to study volatile biosynthesis by banana tissue slices by Tressl and Drawert (1973), who showed conversion of ^{14}C-labeled C-2−C-10 fatty acids into the corresponding alcohols, esters, and ketones. ^{14}C-Linoleic and ^{14}C-linolenic acids were transformed into hexanal and 2*E*-hexenal by ripening bananas (Hatanaka, 1993; Jadhav et al., 1972) and into 2-nonenal and 2,6-nonadienal by unripe fruit, whereas ^{14}C-leucine and ^{14}C-valine gave methyl-branched esters, alcohols, and acids on feeding to ripe banana tissue slices (Myers et al., 1970; Tressl et al.,1970). Apple segments supplied with radioactive acetate incorporated label into virtually all compounds including the acetate and butyrate esters and ethanol, propanol, and butanol (Paillard, 1969). Feeding experiments with [2-^{14}C]propanoic acid have shown transformation into ethylene leading to accelerated ripening (De Pooter et al., 1984). Injection of ^{14}C-labeled hexanoic, octanoic, and linoleic acids was used by Beuerle and Schwab (1997) to define the biosynthesis of octane-1,3-diol and 3-hydroxy-octyl-β-D-glucopyranoside in fruit of cv. Peau de Chien apples.

Deuterium-labeled substrates have been less widely used to study aroma biosynthesis but have the advantage that deuterated biosynthetic products are normally separable from their nondeuterated analogues by gas chromatography and may be readily identified by GC/MS. In previous work, deuterium labeling allowed elucidation of previously unobserved biosynthetic transformations and of differences in amino acid metabolism between apple cultivars (Rowan et al., 1996, 1997). Berger and Kollmansberger (1985) used deuterated sorbic acid to model the oxidation of 2,4-unsaturated fatty acids by pineapple tissue. Engel et al. (1989, 1990) have used 3-hydroxyhexanoic acid-d_1 and 4*Z*-octenoic acid-*2,2*-d_2 to identify two pathways for the biosynthesis of γ- and δ-lactones in pineapple. Silberzahn and Tressl (1993) have also used deuterated flavor precursors to study isoleucine metabolism in hazelnuts. For apples, aroma biosynthesis from deuterated butanoic acid (Allen et al.,1992) and of branched-chain esters from isoleucine, leucine, valine, and 2-methylbutanoates (Rowan et al., 1996, 1997) has been reported. The straight-chain ester components of the aroma volatiles of fruit, however, may arise either from β-oxidation of fatty acids or from lipid hydroperoxides via the corresponding saturated and unsaturated aldehydes (Hildebrand et al., 1988). Saturated esters predominate in the headspace volatiles above whole apples, whereas unsaturated alcohols and aldehydes are more abundant in the headspace or extracts of juice or pulped tissue. However, aldehydes such as hexanal are rapidly metabolized by fruit (De Pooter et al., 1983, 1987; Song et al., 1996) so the precise biosynthetic origins of straight-chain esters are uncertain. Here we report the use of deuterium-labeled saturated and unsaturated fatty acids, C-6 aldehydes, and alcohols to study the biosynthesis of straight-chain aroma volatiles in Granny Smith and Red Delicious apples.

MATERIALS AND METHODS

General. Apples (*Malus domesticus* Borkh. cv. Red Delicious and Granny Smith) were obtained from local retailers and stored at 1 °C until required. Tenax traps (100 × 5 mm i.d. glass tubes) containing ~350 mg of Tenax were conditioned at 250 °C for 4 h under a stream of oxygen-free nitrogen (20−25 mL/min.).

Chemicals. Nondeuterated chemicals were obtained from the Aldrich Chemical Co. Hexanol-*6,6,6*-d_3, 2*E*-hexenol-*6,6,6*-d_3, and 3*Z*-hexenol-*6,6,6*-d_3 were obtained by synthesis (Brenstrum et al., 1993). 2*E*-Hexenal-*6,6,6*-d_3 was synthesized by alkylation of the trimethylsilyl derivative of malondialdehyde with propyl-*3,3,3*-d_3 magnesium bromide and was >98% the 2*E* isomer by GC (Fielder and Rowan, 1993). 3*Z*-Hexenal-*6,6,6*-d_3 was obtained by the oxidation of 3*Z*-hexenol-*6,6,6*-d_3 with the Des-Martin periodinane and 3*Z*-hexenal-*3,4*-d_2 from 3-hexynol by Lindlar reduction as described (Fielder and Rowan, 1995). Both deuterated 3*Z*-hexenals were >95% pure (major impurity 2*E*-hexenal) as determined by GC immediately before use. Deuterated hexanal was prepared by hydrogenation of 3-hexynol with deuterium gas using the Wilkinson catalyst [$RdCl_2(PPh_3)_2$ 10 mol %, benzene, 1 atm] followed by oxidation to the aldehyde with the Des-Martin periodinane (Fielder and Rowan, 1995). Hexanoic-*6,6,6*-d_3 acid and perdeuterated fatty acid methyl esters (98% D) were obtained from Cambridge Isotope Laboratories, Andover, MA. GC/MS analysis identified the deuterated fatty acid methyl esters as a mixture of C14:0, 1%; C16:0, 19.5%; C16:1, 2.9%; C18:0, 6.3%; C18:1, 17.1%; C18:2, 46.3%; and C18:3, 3.6%.

Perdeuterated methyl esters were separated into saturated, monounsaturated, and diunsaturated fatty acid classes by argentation chromatography on sulfonic acid derivatized silica (silver salt) (Christie, 1989). Typically, the deuterated esters (1 g) in CH_2Cl_2/pentane (1:4) were applied to a column of SCX silica (60 g, Varian Analytichem Bondesil, 40 μm) pretreated with $AgNO_3$ (6 g) as described. Elution with CH_2Cl_2/pentane (1:4), CH_2Cl_2, and acetone gave pure fractions of saturated [260 mg, C18:0 (70%), C16:0 (22%), C14:0 (8%)], monounsaturated (195 mg), and diunsaturated methyl esters (107 mg), respectively, as determined by GC. The position of the double bonds in each methyl ester fraction was determined by conversion of the double bonds to the diols (Khuddus et al., 1973) and GC/MS analysis of the corresponding trimethylsilyl ethers (Capella and Zorzut, 1968). The monounsaturated fraction consisted of C18:1 Δ^9 (methyl oleate-d_{33}, 88%), C16:1 Δ^7 and Δ^9 (8%), and traces of C17:1 Δ^7 and C18:1 Δ^{11}. The diunsaturated fraction consisted of pure C18:2 $\Delta^{9,12}$ (methyl linoleate-d_{31}). Perdeuterated free fatty acids were prepared from the esters by hydrolysis with methanol and aqueous potassium hydroxide immediately before use.

Gas Chromatography (GC). Capillary GC analysis was carried out using a 30 m × 0.25 mm i.d. Carbowax capillary column, film thickness = 0.25 μm, with a temperature program from 40 (10 min) to 100 °C at 3 °C/min and to 220 °C at 5 °C/min; injector temperature was 220 °C and detector temperature, 250 °C. The column head pressure was 5 psi hydrogen.

Capillary Gas Chromatography/Mass Spectroscopy (GC/MS). Electron impact GC/MS (EIMS) spectra were recorded on a VG70S mass spectrometer at 70 eV ionization

Table 1. Mass Spectra (GC/MS, 70 eV) of Deuterated Esters Identified in the Aroma Volatiles of Red Delicious Apples Fed Deuterated Fatty Acids

compound	retention index[a]	mass spectrum
ethyl hexanoate-d_{11}	1226	EIMS 128 (8), 121 (7), 110 (65), 105 (24), 91 (100), 82 (31), 77 (16), 63 (40), 50 (70), 46 (33), 34 (24), 30 (27); CIMS 156 (MH^+), 173 ($M + NH_4^+$)
hexyl-d_{11} acetate	1266	EIMS 94 (17), 76 (9), 73 (8), 64 (20), 62 (26), 50 (14), 43 (100); CIMS 156 (MH^+), 173 ($M + NH_4^+$)
propyl hexanoate-d_{11}	1308	EIMS 128 (78), 110 (100), 105 (12), 82 (30), 77 (15), 63 (36), 50 (56), 46 (23), 43 (36), 34 (18), 30 (18); CIMS 170 (MH^+), 187($M + NH_4^+$)
butyl hexanoate-d_{11}	1402	EIMS 128 (77), 110 (100), 95 (17), 82 (29), 63 (21), 56 (94), 50 (56), 41 (36); CIMS 184 (MH^+), 201 ($M + NH_4^+$)
ethyl octanoate-d_{15}	1423	EIMS 187 (2), 160 (2), 142 (30), 137 (8), 121 (5), 105 (32), 91 (100), 77 (18), 66 (38), 63 (28), 50 (20), 46 (30), 34 (18), 30 (15); CIMS 188 (MH^+), 205 ($M + NH_4^+$)
pentyl hexanoate-d_{11}	1493	EIMS 128 (82), 110 (48), 70 (100), 50 (46)
butyl heptanoate-d_{13}	1498	EIMS 144 (94), 126 (100), 77 (27), 66 (15), 64 (20), 63 (21), 56(95), 50 (75), 43 (59), 32 (53); CIMS 200 (MH^+), 217 ($M + NH_4^+$)
propyl octanoate-d_{15}	1506	EIMS 160 (88), 142 (100), 105 (27), 77 (29), 66 (78), 64 (73), 50 (36), 46 (46); CIMS 202 (MH^+), 219 ($M + NH_4^+$)
hexyl hexanoate-d_{11}	1600	EIMS 128 (100), 110 (86), 84 (76), 56 (72), 50 (58), 43 (73); CIMS 212 (MH^+), 229 ($M + NH_4^+$)
butyl octanoate-d_{15}	1596	EIMS 160 (55), 142 (57), 77 (16), 66 (41), 56 (100), 50 (30), 46 (27), 41 (30); CIMS 216 (MH^+), 233 ($M + NH_4^+$)

[a] Retention indices calculated for Carbowax capillary column (Jennings and Shibamoto, 1980).

Table 2. Deuterated Products (Mean Relative Percent ± SEM) Determined in Headspace of Red Delicious Apples Fed Perdeuterated Saturated and Monounsaturated Fatty Acids and Perdeuterated Linoleic Acid

	biosynthetic precursor		
biosynthetic product	saturated fatty acids	mono-unsaturated fatty acids	linoleic acid
hexyl-d_{11} acetate	10.2 ± 3.4	20 ± 13[a]	15.5 ± 7.4[a]
ethyl hexanoate-d_{11}	16.2 ± 4.4	17.0 ± 7.0	47 ± 15
propyl hexanoate-d_{11}	1.84 ± 0.72	tr[b]	19.8 ± 3.2
butyl hexanoate-d_{11}	11.0 ± 2.0	10.6 ± 5.0[a]	11.3 ± 3.1[a]
pentyl hexanoate-d_{11}	nd[b]	nd	tr
hexyl hexanoate-d_{11}	9.1 ± 1.4[c]	5.3 ± 4.3[a]	6.8 ± 1.9[a]
total % hexanoate-d_{11}	**38.2 ± 1.6**	**32.8 ± 3.4**	**84.5 ± 7.4**
butyl heptanoate-d_{13}	1.25 ± 0.22	nd	nd
ethyl octanoate-d_{15}	38.7 ± 4.4	33 ± 19	nd
propyl octanoate-d_{15}	11.7 ± 2.3	4.0 ± 0.1[a]	
butyl octanoate-d_{15}	*c*	9.9 ± 5.1[a]	nd
total % octanoate-d_{15}	**50.4 ± 4.4**	**47 ± 15**	nd

[a] Areas calculated using SIM-GC-MS. [b] nd, not detected; tr, trace only. [c] Not resolved (from butyl octanoate-d_{15}).

potential and a helium column head pressure of 5 psi (Rowan et al., 1996). Chemical ionization GC/MS (CIMS) was performed with ammonia with the gas pressure adjusted to give both MH^+ and $M + NH_4^+$ ions and characteristic fragmentation ions for each peak. Volatiles were identified from their retention times and mass spectra (Jennings and Shibamoto, 1980; NIST and Wiley databases) and, in some cases, by comparison with authentic materials. Deuterated metabolites (Tables 1 and 3) were identified as new peaks with GC retention times just before those of the unlabeled analogues. The presence of deuterium was confirmed by the shift to higher mass of characteristic fragment ions (McLafferty, 1973) and by the presence of higher molecular weight ions in the CIMS. In a few cases where peaks could not be resolved by GC, GC/MS with selected ion monitoring (SIM-GC/MS) was used to ascribe proportions of unresolved GC peaks to individual constituents. The following ions were used: hexyl, 84.094; hexyl-d_{11}, 94.157; hexanoate, 99.081/117.092; hexanoate-d_{11}, 110.150/128.160; octanoate, 127.112/145.123; octanoate-d_{15}, 142.206/160.216.

Biosynthesis Experiments. Apples were placed in 1.5 L glass jars (160 × 105 mm i.d.) as previously described (Rowan et al., 1996). Purified air (80 mL/min) was drawn into the bottom of each jar, and volatiles were adsorbed onto Tenax traps attached to the top of the jars. Before each volatile collection, 10 μL of an octyl acetate solution (10 μL/mL in ether) was carefully syringed onto a glass fiber paper disk held in the outlet of the jar above the apples and directly before the Tenax trap. The apparatus was immediately connected, and aroma volatiles were collected for 24 h. After 24 h, aroma precursors (labeled or unlabeled, typically 2−20 μL of neat C-6 alcohols or aldehydes per jar) were added to the glass fiber filter paper above the apples. The top inlet of the jar was sealed with Parafilm, the bottom air inlet opened to the atmosphere, and the vapor left to diffuse onto the apple for 2 h. The glass fiber filter disk and lid of each jar were then removed, and the apparatus was left open to the air for 1 h. After addition of aroma precursors, the glass fiber paper was replaced, octyl acetate internal standard was applied, and volatiles were trapped on Tenax as before for successive 24 h periods. Volatile production by untreated or squalane coated apples was measured as controls. Volatiles were recovered from the Tenax traps by elution with diethyl ether (2.5 mL) and stored at −20 °C prior to analysis.

Perdeuterated fatty acids or their methyl esters (3 mg) were dissolved in squalane (0.1 mL) and applied evenly over the surface of each of three apples with a soft artist's brush.

RESULTS AND DISCUSSION

Metabolism of Saturated and Unsaturated Fatty Acids. Perdeuterated fatty acid methyl esters were separated by argentation chromatography (Christie, 1989) into saturated, monounsaturated, and diunsaturated fatty acid classes and applied to the surface of Red Delicious apples in squalane. Aroma volatiles were collected on Tenax and analyzed by GC and GC/MS. Control experiments established that the squalane coating had no discernible effect on volatile production by the apples. Equivalent results were obtained using either the free acids or their methyl esters. Hexyl-d_{11}, hexanoate-d_{11}, heptanoate-d_{13}, and octanoate-d_{15} esters appeared as new (minor) volatile constituents eluting some 10 s before their nondeuterated analogues (Figures 1 and 2) and were identified from their GC retention times and shifts to higher mass of characteristic fragment (e.g., m/z 43 → 50 for C_3D_7; m/z 99 → 110 and 117 → 128 for hexanoate-d_{11} esters; m/z 127 → 142 and 145 → 160 for octanoate-d_{15} esters) and molecular ions in their EI and CIMS, respectively (Table 1). Perdeuterated saturated and monounsaturated fatty acids were converted to hexyl-d_{11} acetate (10−20% of total deuter-

Table 3. Mass Spectra (GC/MS, 70 eV) of Deuterated Metabolites Identified in the Aroma Volatiles of Red Delicious and Granny Smith Apples Fed Deuterated Hexenals

compound	retention index[a]	mass spectrum
ethyl butanoate-d_3 (partially resolved toluene)	1036	EIMS 92 (78), 91 (100), 89 (21), 74 (45), 46 (48); CIMS 120 (MH^+), 137 ($M + NH_4^+$)
butyl-d_3 acetate	1073	EIMS 73 (14), 59 (28), 43 (100); CIMS 120 (MH^+), 137 ($M + NH_4^+$)
propyl butanoate-d_3	1122	EIMS 92 (56), 74 (100), 46 (59), 43 (65); CIMS 134 (MH^+), 151 ($M + NH_4^+$)
ethyl pentanoate-d_2	1137	EIMS 89 (97), 87 (100), 61 (54), 59 (93), 43 (42), 42 (43); CIMS 133 (MH^+)
methylpropyl-d_3 butanoate-d_3	1160	EIMS 92 (13), 74 (100), 56 (38), 46 (38)
pentyl-d_2 acetate	1173	EIMS 72 (20), 61 (13), 56 (10), 43 (100); CIMS 133 (MH^+), 150 ($M + NH_4^+$)
pentyl-d_3 acetate	1173	EIMS 73 (41), 61 (14), 58 (12), 55 (9), 45 (17), 43 (100)
2*E*-hexenal-d_3	1202	EIMS 101 (39), 86 (10), 83 (78), 72 (48), 69 (35), 58 (55), 55 (100), 43 (78)
butyl-d_3 butanoate-d_3 and butyl-d_3 butanoate	1212	EIMS 92 (40), 74 (100), 59 (51), 46 (49)
butyl butanoate-d_3	1214	EIMS 92 (47), 74 (100), 56 (36), 46 (56); CIMS 148 (MH^+), 165 ($M + NH_4^+$)
butyl-d_3 2-methylbutanoate	1228	EIMS 103 (56), 85 (60), 74 (36), 59 (37), 57 (100), 42 (37)
hexyl-d_3 acetate	1270	EIMS 87 (15), 73 (9), 72 (6), 61 (15), 59 (30), 43 (100); CIMS 148 (MH^+), 165 ($M + NH_4^+$)
3*E*-hexenyl-d_3 acetate	1301	EIMS 85 (53), 70 (20), 67 (24), 43 (100); CIMS 146 (MH^+), 163 ($M + NH_4^+$)
3*Z*-hexenyl-d_3 acetate	1309	EIMS 85 (49), 70 (18), 67 (22), 43 (100); CIMS 146 (MH^+), 163 ($M + NH_4^+$)
propyl hexanoate-d_3	1312	EIMS 120 (15), 102 (28), 89 (46), 71 (82), 60 (19), 55 (25), 43 (100); CIMS 162 (MH^+), 179 ($M + NH_4^+$)
2*E*-hexenyl-d_3 acetate	1329	EIMS 145 (2), 105 (10), 103 (12), 85 (31), 70 (11), 67 (16), 43 (100); CIMS 146 (MH^+), 163 ($M + NH_4^+$)
hexanol-d_3	1355	EIMS 87 (7), 72 (9), 69 (9), 59 (100), 55 (26), 45 (26), 42 (27)
3*E*-hexenol-d_2	1367	EIMS 84 (39), 71 (70), 69 (50), 57 (55), 42 (100)
3*Z*-hexenol-d_2	1386	EIMS 84 (18), 71 (98), 69 (57), 57 (31), 43 (100)
2*E*-hexenol-d_3	1409	EIMS 103 (M^+, 2), 85 (14), 57 (100), 43 (13)
hexyl-d_3 proprionate	1334	EIMS 132 (2), 87 (42), 75 (44), 72 (11), 59 (37), 57 (100), 46 (20), 43 (32); CIMS 162 (MH^+), 179 ($M + NH_4^+$)
3*E*-hexenyl-d_2 proprionate	1376	EIMS 84 (65), 69 (57), 57 (100), 42 (18); CIMS 159 (MH^+), 176 ($M + NH_4^+$)
3*Z*-hexenyl-d_2 proprionate	1383	EIMS 84 (67), 69 (58), 57 (100), 42 (14)
2*E*-hexenyl-d_3 proprionate	1393	EIMS 85 (18), 69 (47), 57 (100), 41 (64); CIMS 160 (MH^+), 177 ($M + NH_4^+$)
butyl-d_3 hexanoate-d_3, hexyl-d_3 butanoate-d_3 and butyl hexanoate-d_3	1402	EIMS 120 (44), 102 (63), 92 (52), 87 (35) EIMS 74 (100), 59 (56), 56 (70), 46 (94); CIMS 179, 176 (MH^+), 196, 193 ($M + NH_4^+$)
hexyl-d_3 butanoate-d_3	1406	EIMS 92 (64), 87 (43), 74 (100), 59 (39), 46 (78), 43 (28); CIMS 179 (MH^+), 196 ($M + NH_4^+$)
hexyl-d_3 butanoate	1412	EIMS 89 (64), 87 (44), 71 (100), 59 (39), 55 (16), 46 (29), 43 (84), 41 (26); CIMS 176 (MH^+), 193 ($M + NH_4^+$)
hexyl-d_3 2-methylbutanoate	1422	EIMS 103 (100), 87 (46), 85 (59), 74 (19), 57 (100); CIMS 190 (MH^+), 207 ($M + NH_4^+$)
3*E*-hexenyl-d_3 butanoate-d_3	1447	EMIS 85 (100), 74 (76), 70 (27), 67 (33), 46 (64); CIMS 177 (MH^+), 194 ($M + NH_4^+$)
3*E*-hexenyl-d_3 butanoate	1450	EIMS 85 (100), 71 (78), 70 (35), 67 (34), 43 (73); CIMS 174 (MH^+), 191 ($M + NH_4^+$)
3*Z*-hexenyl-d_3 butanoate-d_3	1452	EIMS 85 (100), 74 (72), 70 (25), 67 (31), 46 (65); CIMS 177 (MH^+), 194 ($M + NH_4^+$)
3*Z*-hexenyl-d_3 butanoate	1455	EIMS 85 (100), 71 (71), 67 (34), 43 (65); CIMS 174 (MH^+), 191 ($M + NH_4^+$)
2*E*-hexenyl-d_3 butanoate-d_3	1469	EIMS 176 (2), 102 (3), 85 (31), 74 (100), 70 (9), 67 (13), 57 (28), 46 (50), 41 (17); CIMS 177 (MH^+), 194 ($M + NH_4^+$)
2*E*-hexenyl-d_3 butanoate (partially resolved)	1472	EIMS 172 (M^+, 3), 103 (4), 102 (3), 89 (8), 85 (100), 71 (43), 67 (27), 57 (72), 43 (47), 41 (26); CIMS 173 (MH^+), 190 ($M + NH_4^+$)
d_2-3*Z*-hexenyl 2-methylbutanoate	1467	EIMS 84 (95), 69 (49), 57 (100), 41 (27); CIMS 187 (MH^+), 204 ($M + NH_4^+$)
d_3-2*E*-hexenyl 2-methylbutanoate	1477	EIMS 85 (47), 70 (11), 57 (100), 41 (32)
hexyl-d_3 hexanoate-d_3	1601	EIMS 120 (100), 102 (100), 87 (87), 74 (40), 59 (68), 46 (66), 43 (44); CIMS 207 (MH^+), 224 ($M + NH_4^+$)
hexyl-d_3 hexanoate	1605	EIMS 117 (100), 99 (88), 87 (97), 71 (34), 59 (52), 46(33), 43 (96); CIMS 204 (MH^+), 221 ($M + NH_4^+$)
3*E*-hexenyl-d_3 hexanoate-d_3	1644	EIMS 147 (2), 120 (3), 102 (34), 85 (100), 74 (22), 70 (24), 67 (24), 57 (15), 41 (19); CIMS 205 (MH^+), 222 ($M + NH_4^+$)
3*E*-hexenyl-d_3 hexanoate	1646	EIMS 117 (2), 99 (31), 85 (100), 71(24), 67 (24), 43 (46); CIMS 202 (MH^+), 219 ($M + NH_4^+$)
3*Z*-hexenyl-d_3 hexanoate-d_3	1650	EIMS 102 (28), 85 (100), 74 (20), 70 (25), 67 (27), 57 (21), 41 (26); CIMS 205 (MH^+), 222 ($M + NH_4^+$)
3*Z*-hexenyl-d_3 hexanoate	1653	EIMS 117 (1), 99 (34), 85 (100), 71 (23), 67 (24), 43 (40); CIMS 202 (MH^+), 219 ($M + NH_4^+$)
2*E*-hexenyl-d_3 hexanoate-d_3	1665	EIMS 204 (5), 120 (4), 102 (100), 85 (29), 74 (48), 57 (17), 41 (28)
2*E*-hexenyl-d_3 hexanoate	1668	EIMS 201 (4), 117 (7), 99 (100), 85 (30), 71 (53), 43 (67), 41 (38)

[a] Retention indices calculated for Carbowax capillary column (Jennings and Shibamoto, 1980).

ated volatiles) and to hexanoate-d_{11} (33−38%), heptanoate-d_{13} (0−1%), and octanoate-d_{15} (47−50%) esters in similar proportions consistent with β-oxidation of both precursors (Table 2). However, a higher ratio of octanoate-d_{15} to hexanoate-d_{11} esters was produced than was found in the normal volatile profile of Red Delicious apples (Figure 1, ~1:1 octanoate/octanoate-d_{15}), suggesting early termination of the β-oxidation pathway. Exposure of fruit to perdeuterated linoleic acid (C18:2 $\Delta^{9,12}$) gave only hexyl-d_{11} acetate and hexanoate-d_{11}

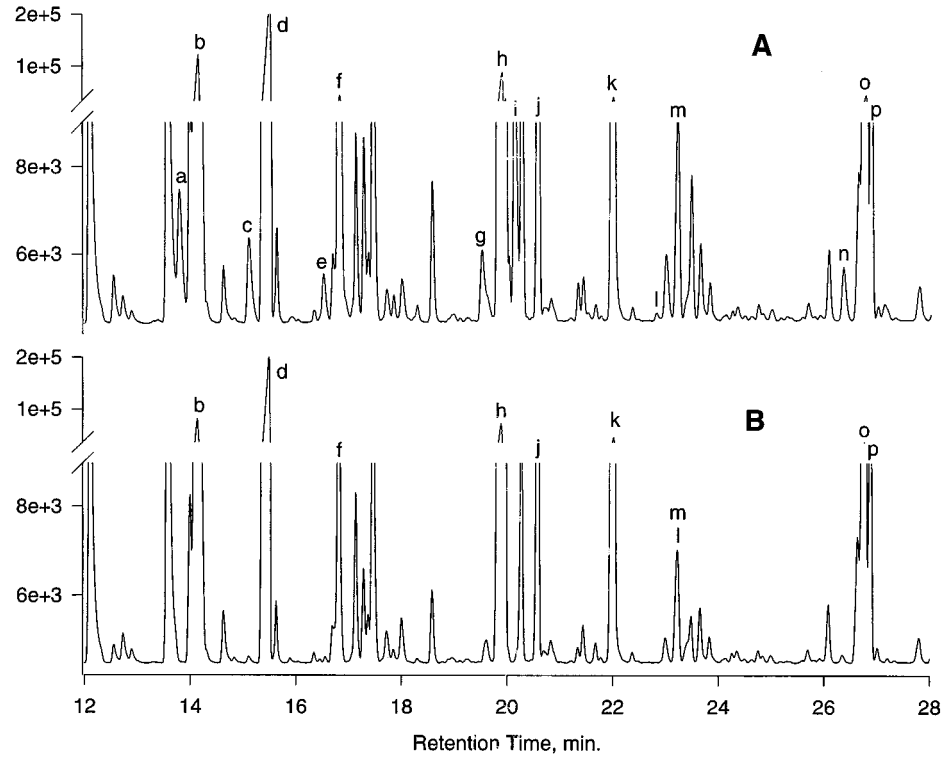

Figure 1. Capillary GC of aroma volatiles collected from Red Delicious apples treated with deuterated saturated fatty acids (A) and from a control fruit (B): (a) ethyl hexanoate-d_{11}; (b) ethyl hexanoate; (c) hexyl-d_{11} acetate; (d) hexyl acetate; (e) propyl hexanoate-d_{11}; (f) propyl hexanoate; (g) butyl hexanoate-d_{11}; (h) butyl hexanoate; (i) ethyl octanoate-d_{15}; (j) ethyl octanoate; (k) octyl acetate (internal standard); (l) butyl heptanoate-d_{13}; (m) butyl heptanoate; (n) hexyl hexanoate-d_{11} and butyl octanoate-d_{15}; (o) hexyl hexanoate; (p) butyl octanoate.

esters (Table 2). Analysis of the mass spectrum of the deuterated hexyl acetate confirmed in each case the presence of 11 deuterium atoms (MH^+ 156). In addition, the characteristic fragment ion of hexyl acetate, *m/z* 84 (C_6H_{12}), resulting from McLafferty rearrangement and elimination of acetic acid, now appeared at *m/z* 94 ($C_6H_2D_{10}$), confirming that two hydrogen atoms had been incorporated at C-1 ($C_5D_{11}CH_2OAc$). Subsequent losses of CD_3, $C_2H_2D_2$, and C_2D_4 account for the observed ions at *m/z* 76, 64, and 62, respectively. These deuterated metabolites therefore arose via a hexanoate-d_{11} intermediate rather than via hexanal-d_{12} and are derived from β-oxidation of the fatty acid precursor. These results confirm earlier observations of β-oxidation in apples when C-2−C-8 carboxylic acids or their methyl esters were fed to whole apples (De Pooter et al., 1983; Bartley et al., 1985; Beuerle and Schwab, 1997) and tissue disks were exposed to C-4−C-18 carboxylic acid salts (Paillard, 1979).

The intermediacy of hexanoic acid in the formation of hexyl acetate was confirmed by exposure of Red Delicious fruit to vapor of both hexanoic-d_3 acid and hexanol-d_3. Exposure to hexanoic-d_3 acid vapor gave not only production of hexyl-d_3 acetate and hexanol-d_3 but also β-oxidation, resulting in the production of butanol-d_3 and butyl-d_3 acetate (data not shown). Exposure of fruit to hexanol-d_3 gave conversion only to hexyl-d_3 esters (acetate to hexanoate); no labeled hexanoate esters were observed. These results indicate that the saturated straight-chain ester volatiles in the whole fruit of apples may be accounted for solely as products of fatty acid β-oxidation.

Metabolism of C-6 Aldehydes (3*Z*-Hexenal, 2*E*-Hexenal, and Hexanal). Straight-chain ester volatiles may also be produced by the action of lipoxygenase (Hildebrand et al., 1988) on unsaturated fatty acids through the intermediacy of the C-6 aldehydes, 3*Z*-hexenal, 2*E*-hexenal, and hexanal (Almosnino and Belin, 1991; Hatanaka et al., 1987; Tressl and Drawert, 1973). To examine the involvement of these aldehydes in fruit volatile metabolism, the deuterated analogues 3*Z*-hexenal-*6,6,6-d*$_3$, 3*Z*-hexenal-*3,4-d*$_2$, and 2*E*-hexenal-*6,6,6-d*$_3$ were synthesized (Fielder and Rowan, 1993, 1995). The d_3 analogues proved to be more useful for detection and identification of metabolites by GC/MS (Table 3), whereas the more conveniently prepared d_2 analogue was used to confirm identifications and for quantification (Table 4).

Exposure of Red Delicious and Granny Smith apples to vapors of deuterated 3*Z*-hexenal or 2*E*-hexenal resulted in the rapid and extensive uptake of these volatiles (Song et al., 1996) and the production of a complex mixture of labeled metabolites (Tables 3 and 4). Deuterated metabolites were identified as new peaks eluting some 5 s before their nondeuterated analogues (Figure 2) and showing shifts to higher mass of characteristic fragment ions (e.g., *m/z* 43 → 46 $C_3H_4D_3$, *m/z* 84 → 87 hexyl-d_3, *m/z* 82 → 85 hexenyl-d_3, *m/z* 56 → 59 butyl-d_3, *m/z* 71 → 74 butanoate-d_3 esters) and molecular ions (Table 3) in the GC/MS. Analysis of the metabolic products (Table 4) and additional feeding experiments using deuterated hexanal and isomeric hexenols (below) resulted in the identification of a number of biosynthetic processes:

(1) Double-bond isomerization between 3E-, 3Z-, and 2E-hexenal resulted in the formation of 3*E*-, 3*Z*-, and 2*E*-hexenyl esters from both 3*Z*- and 2*E*-hexenal. That isomerization occurs between the aldehydes themselves was shown by feeding experiments using 3*Z*-hexenol-d_3 and 2*E*-hexenol-d_3. Exposure of Red Delicious apples

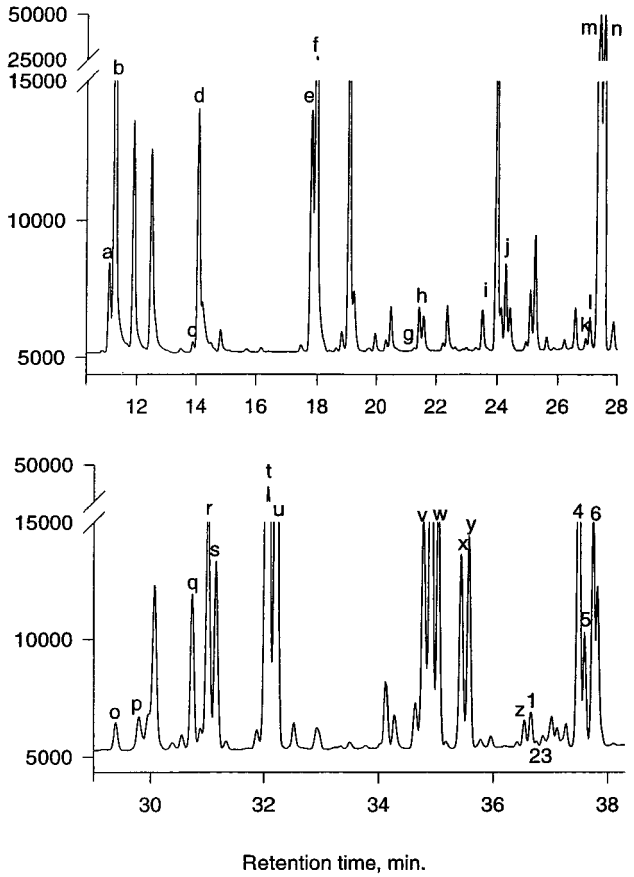

Figure 2. Capillary GC of aroma volatiles collected from a Granny Smith apple exposed to vapor of 2*E*-hexenal-d_3: (a) ethyl butanoate-d_3; (b) ethyl butanoate; (c) butyl-d_3 acetate; (d) butyl acetate; (e) propyl butanoate-d_3; (f) propyl butanoate; (g) pentyl-d_3 acetate; (h) pentyl acetate; (i) butyl butanoate-d_3; (j) butyl butanoate; (k) 2-methylbutyl butanoate-d_3; (l) 2-methylbutyl butanoate; (m) hexyl-d_3 acetate; (n) hexyl acetate; (o) 3*E*-hexenyl-d_3 acetate; (p) 3*Z*-hexenyl-d_3 acetate; (q) 2*E*-hexenyl-d_3 acetate; (r) hexyl-d_3 proprionate; (s) hexyl proprionate; (t) hexanol-d_3; (u) hexanol; (v) hexyl-d_3 butanoate-d_3; (w) hexyl butanoate; (x) hexyl-d_3 2-methylbutanoate; (y) hexyl 2-methylbutanoate; (z) 3*E*-hexenyl-d_3 butanoate-d_3; (1) 3*E*-hexenyl-d_3 butanoate; (2) 3*Z*-hexenyl-d_3 butanoate-d_3; (3) 3*Z*-hexenyl-d_3 butanoate; (4) 2*E*-hexenyl-d_3 butanoate-d_3; (5) 2*E*-hexenyl-d_3 butanoate; (6) octyl acetate (internal standard).

to vapor of 3*Z*-hexenol-d_3 gave greatly enhanced production of 3*Z*-hexenyl esters (acetate to hexanoate and 2-methylbutanoate), which were at most minor components of control fruit with no other labeled hexenyl, hexyl, or hexanoate esters being detected. Similarly, exposure of Granny Smith apples to 3*Z*-hexenol-d_3 vapor gave enhanced levels of both deuterated 3*Z*-hexenyl and hexyl esters (acetate to hexanoate and 2-methylbutanoate) but no isomeric hexenyl esters among the aroma volatiles. 2*E*-Hexenol-d_3 was converted exclusively to hexyl-d_3 esters (acetate to hexanoate and 2-methylbutanoate) by both cultivars.

A 3*Z*:2*E*-enal isomerase, which assists the isomerization of 3*Z*-hexenal and 3*Z*-nonenal to 2*E*-enals, has been partially purified from cucumber fruit by Phillips et al. (1979). Interestingly, neither 3*Z*-hexenol (as above) nor 3*E*-nonenal was a substrate for this enzyme. The presence of an analogous enzyme in apples is required to explain the extensive conversion of 2*E*-hexenal to the thermodynamically less stable 3*E*- and 3*Z*-hexenals, which are then trapped by conversion to their corresponding hexenyl esters (Table 4).

(2) Reduction to hexenols, resulting in the production of trace quantities of isomeric hexenols, was followed by esterification, leading to significant quantities (13−42% total) of 3*Z*-, 3*E*-, and 2*E*-hexenyl esters (Table 4). The order of these transformations was confirmed by feeding 3*Z*-hexenol-d_3 and 2*E*-hexenol-d_3 (above) and also 3*E*-hexenol. Exposure of fruit to vapor of 3*E*-hexenol gave significant quantities of only the 3*E*-hexenyl esters in the headspace volatiles (Table 5). Reduction of 3*E*-hexenol to hexyl esters was not a major pathway as judged by changes in peak areas but could not be excluded in the absence of labeled substrate.

3*Z* and 2*E*-hexenyl esters are normally minor components of the headspace volatiles of apples, whereas 3*E*-hexenyl esters do not appear to have been previously observed (Paillard, 1990). Presumably in whole fruit, low rates of lipid peroxidation and rapid conversion of 2*E*- and 3*Z*-hexenal to predominantly hexyl esters means that 3*E*-hexenyl esters are not formed in significant quantities.

(3) Reduction to hexanol, particularly with Granny Smith apples in which deuterated hexanol comprised 25−28% of the total deuterated volatiles, was followed by esterification to hexyl esters (acetate to hexanoate and 2-methylbutanoate). Similarly, exposure of Red Delicious and Granny Smith apples to vapor of hexanol-d_3 gave conversion to hexyl-d_3 esters (acetate to hexanoate and 2-methylbutanoate); no labeled hexanoates or butyl or butanoate esters were observed.

(4) Reduction to Hexanal with Subsequent Oxidation to Hexanoic Acid or Reduction to Hexanol. That oxidation to hexanoic acid occurs from hexanal was supported by the conversion of hexanal-d_4 to both hexyl-d_4 and hexanoate-d_4 esters by both Red Delicious and Granny Smith apples (Table 6) and by the absence of unsaturated hexenoate esters in the aroma volatiles. Only Granny Smith apples effected significant (>17% total labeled volatiles) oxidation of hexanal-d_4 to hexanoate-d_4 esters.

(5) Subsequent β-oxidation of hexanoic acid leading to d_3-butyl and d_3-butanoate esters (trace to 11% total labeled volatiles) was observed on feeding hexanoic acid-d_3 itself (above).

(6) Pentyl and Pentanoate Esters. Labeled pentyl acetate and ethyl pentanoate were observed as minor biosynthetic products with both apple varieties (Table 4). Their occurrence in feeding experiments involving 3*Z*-hexenal-d_2, 3*Z*-hexenal-d_3, and 2*E*-hexenal-d_3 precursors suggests that despite low percent conversions (0−0.9% of total labeled volatiles) these are genuine biosynthetic products. The retention of the deuterium label and mass spectral analysis of the d_2- and d_3-pentyl acetates (Figure 3) indicates loss of C-1 from the aldehyde precursor. Similarly, mass spectral analysis of ethyl pentanoate-d_2 (Figure 4) establishes the presence of deuterium at carbons 2 and 3.

Chain shortening of [2-^{14}C]hexanoic, [8-^{14}C]octanoic acid, and [10-^{14}C]decanoic acids by α-oxidation has been observed in banana tissue (Engel et al., 1990; Tressl and Drawert, 1973), whereas Paillard (1979) has reported the transformation of fatty acids into alcohols and methyl ketones with one fewer carbon atom in the apple cultivar Canada gris. α-Oxidation of aldehydes or carboxylic acids by Golden Delicious apples has been proposed by Dirinck et al. (1989) on the basis of feeding experiments with nonlabeled aldehydes and carboxylic acids (De Pooter et al., 1981, 1983); however, this

Table 4. Deuterated Products (Mean Relative Percent ± SEM) Determined in Headspace of Whole Apples after Exposure to Vapor of Deuterated 2*E*- and 3*Z*-Hexenal

	2*E*-hexenal-d_3		3*Z*-hexenal-d_2	
biosynthetic product[a]	Red Delicious apple	Granny Smith apple	Red Delicious apple	Granny Smith apple
ethyl butanoate-d_3	1.26 ± 0.24	3.7 ± 1.5	nd[b]	nd[b]
butyl-d_3 acetate	0.79 ± 0.05	0.23 ± 0.01	nd (0.11[c])	nd (0.09[c])
propyl butanoate-d_3	nd	5.89 ± 0.71	nd	nd (0.86)
2-methylpropyl butanoate-d_3	nd	0.17 ± 0.05	nd	nd
butyl butanoate-d_3	1.54 ± 0.16	0.90 ± 0.15	nd (0.18)	nd (0.32)
butyl-d_3 2-methylbutanoate	nd	0.15 ± 0.06	nd (0.05)	nd (0.18)
2-methylbutyl butanoate-d_3	nd	0.13 ± 0.03	nd (0.02)	nd (0.14)
total % deuterated C-4 esters	**3.59 ± 0.25**	**11.16 ± 0.54**	**nd (0.36)**	**nd (1.59)**
ethyl pentanoate-d_2	nd	nd	0.10 ± 0.03	nd (0.02)
pentyl-d_3 acetate	0.20 ± 0.01	0.06 ± 0.01	0.91 ± 0.31	nd (0.07)
total % deuterated C-5 esters	**0.20 ± 0.01**	**0.06 ± 0.01**	**0.67 ± 0.29**	**nd (0.09)**
2*E*-hexenal-d_2			**0.74 ± 0.24**	**7.71 ± 1.59**
hexyl-d_3 acetate	43.0 ± 5.0	28.5 ± 3.0	50.1 ± 6.4	17.2 ± 2.2
hexyl-d_3 propanoate	1.43 ± 0.07	3.42 ± 0.94	1.44 ± 0.04	4.46 ± 0.57
hexyl-d_3 butanoate[d]	2.39	5.40	1.55	8.03
hexyl-d_3 2-methylbutanoate	2.10 ± 0.93	2.41 ± 0.64	2.27 ± 0.03	3.96 ± 0.51
hexyl-d_3 hexanoate	4.42 ± 0.30	1.37 ± 0.32	unresolved	unresolved
propyl hexanoate-d_3	0.66 ± 0.07	0.49 ± 0.24	0.18 ± 0.05	0.25 ± 0.11
butyl hexanoate-d_3[d]	0.70	0.25	1.33	tr[b]
total % deuterated C-6 esters	**54.1 ± 5.7**	**41.8 ± 3.1**	**56.1 ± 5.9**	**33.9 ± 2.5**
3*E*-hexenyl-d_3 acetate	2.42 ± 0.15	0.63 ± 0.02	2.88 ± 0.20	0.42 ± 0.06
3*Z*-hexenyl-d_3 acetate	1.26 ± 0.07	0.89 ± 0.01	5.00 ± 0.52	3.39 ± 0.23
2*E*-hexenyl-d_3 acetate	22.2 ± 7.2	4.63 ± 0.72	22.6 ± 3.1	nd
3*E*-hexenyl-d_3 propanoate	tr	0.46 ± 0.11	tr	nd
3*Z*-hexenyl-d_3 propanoate	tr	0.26 ± 0.01	tr	0.40 ± 0.07
2*E*-hexenyl-d_3 propanoate	1.45 ± 0.30	1.07 ± 0.31	1.30 ± 0.16	2.22 ± 0.13
3*E*-hexenyl-d_3 butanoate	0.73 ± 0.16	0.49 ± 0.12	unresolved	2.99 ± 0.21
3*Z*-hexenyl-d_3 2-methylbutanoate	0.14 ± 0.03	0.15 ± 0.04	unresolved	3.28 ± 0.45
3*Z*-hexenyl-d_3 butanoate	nd	nd	nd	1.73 ± 0.06
2*E*-hexenyl-d_3 butanoate	2.83 ± 0.07	1.51 ± 0.37	3.91 ± 0.87	12.8 ± 2.2
2*E*-hexenyl-d_3 2-methylbutanoate	1.93 ± 0.53	1.76 ± 0.48	1.63 ± 0.26	2.52 ± 0.21
3*E*-hexenyl-d_3 hexanoate	0.28 ± 0.02	0.11 ± 0.05	0.53 ± 0.10	0.26 ± 0.03
3*Z*-hexenyl-d_3 hexanoate	tr	0.04 ± 0.01	0.80 ± 0.16	0.31 ± 0.05
2*E*-hexenyl-d_3 hexanoate	1.93 ± 0.25	0.91 ± 0.28	3.40 ± 0.66	unresolved
total % deuterated hexenyl esters	**35.2 ± 6.9**	**12.9 ± 2.0**	**42.6 ± 5.5**	**30.3 ± 2.7**
hexanol-d_3	3.6 ± 1.3	25.3 ± 2.8	0.39 ± 0.12	24.1 ± 1.1
3*E*-hexenol-d_3	nd	tr	nd	1.28 ± 0.17
3*Z*-hexenol-d_3	tr	tr	tr (0.09)	0.39 ± 0.06
2*E*-hexenol-d_2	nd	nd	tr	1.96 ± 0.18
total % alcohols	**3.6 ± 1.3**	**25.3 ± 2.8**	**0.39 ± 0.12**	**27.75 ± 0.95**
butyl-d_3 butanoate-d_3	tr	tr	nd	nd
hexyl-d_3 butanoate-d_3[d]	0.18	0.43	0.02	0.35
butyl-d_3 hexanoate-d_3	tr	nd	tr	nd
3*E*-hexenyl-d_3 butanoate-d_3	0.29	0.51 ± 0.13	nd	nd
3*Z*-hexenyl-d_3 butanoate-d_3	tr	tr	nd	nd
2*E*-hexenyl-d_3 butanoate-d_3	2.25 ± 0.23	7.51 ± 2.23	nd	nd
hexyl-d_3 hexanoate-d_3	0.61 ± 0.04	0.31 ± 0.05	unresolved	unresolved
3*E*-hexenyl-d_3 hexanoate-d_3	0.06 ± 0.01	tr	tr	nd
3*Z*-hexenyl-d_3 hexanoate-d_3	tr	tr	tr	nd
2*E*-hexenyl-d_3 hexanoate-d_3	nd	unresolved	tr	unresolved
total % doubly labeled esters	**3.38 ± 0.22**	**8.75 ± 2.38**	**0.02**	**0.35**

[a] Biosynthetic products contain two deuterium atoms when derived from 3*Z*-hexenal-d_2. [b] nd, not detected; tr, trace only. [c] d_3 metabolite, percent concentration on exposure to 3*Z*-hexenal-d_3. [d] SIM-GC/MS of unresolved GC peak using *m*/*z* butanoate 89.071, butanoate-d_3 92.089, and hexanoate-d_3 120.121.

appears to be the first demonstration of the occurrence of α-oxidation in volatile formation in apples using isotope labeling.

3*Z*- and 2*E*-hexenal are chemically labile, and labeled derivatives do not appear to have been used previously in biosynthetic studies with fruit. Transformation of these hexenals to hexanol, hexyl acetate, and the corresponding hexenyl acetates by strawberries (Hamilton-Kemp et al., 1996) and of the saturated aldehydes (C-3−C-6) to the corresponding alkyl and alkanoate esters by apples has been reported (De Pooter et al., 1983). A much greater range of metabolic products was

Table 5. Biosynthetic Products (Relative Percent) Determined in Headspace of Red Delicious and Granny Smith Apples after Exposure to Vapor of 3*E*-Hexenol, 4*E*/*Z*-Hexenol, and 5-Hexenol

hexenyl or hexenoate ester metabolite	precursor			
	3*E*-hexenol	3*E*-hexenol	4*E*/*Z*-hexenol	5-hexenol
	Red Delicious	Granny Smith	Red Delicious	Red Delicious
ethyl hexenoate	nd[a]	nd	1.8	0.8
propyl hexenoate	nd	nd	nd	0.1
hexenyl acetate	67.3	12.6	78.8	86.2
hexenyl proprionate	7.2	11.1	4.3	1.6
hexenyl 2-methylproprionate	0.2	1.4	nd	nd
hexenyl butanoate	7.1	24.6	5.2	2.3
hexenyl 2-methylbutanoate	10.6	40.8	2.6	1.3
hexenyl pentanoate	0.4	1.2	nd	nd
hexenyl hexanoate	7.2	8.4	7.2	6.9
hexenyl heptanoate	nd	nd	nd	0.3
hexenyl octanoate	nd	nd	nd	0.4

[a] nd, not detected.

Table 6. Deuterated Products (Mean Relative Percent ± SEM) Determined in Headspace of Red Delicious and Granny Smith Apples after Exposure to Vapor of Deuterated Hexanal

biosynthetic product	apple variety	
	Red Delicious[a]	Granny Smith[b]
hexyl-d_4 acetate	67.8 ± 2.0	20.4 ± 8.3
hexyl-d_4 proprionate	7.7 ± 1.4	6.7 ± 2.8
hexyl-d_4 2-methylbutanoate	7.6 ± 0.8	14.6 ± 6.3
hexanol-d_4	nd[c]	24.1 ± 11.5
propyl hexanoate-d_4	0.6 ± 0.1	0.3 ± 0.1
butyl hexanoate-d_4	nr[d]	16.1 ± 6.7
pentyl hexanoate-d_4	0.3 ± 0.1	0.6 ± 0.2
hexyl-d_4 hexanoate and hexyl hexanoate-d_4	15.8 ± 0.8	17.4 ± 7.2

[a] $n = 3$. [b] $n = 2$. [c] nd, not detected. [d] nr, not resolved from hexyl butanoate.

observed in the present instant and, given the complexity of the volatile profiles, not all metabolites may have been identified. Hexanal has been proposed as a residue-free fungicide that can be used to enhance apple flavor (Song et al., 1996). 2*E*-Hexenal and 2*E*-hexenol showed a greater inhibitory effect than their saturated analogues against *Alternaria alternata*, *Botrytis cinerea*, and *Pseudomonas syringae* pathovars in bioassay systems (Hamilton-Kemp et al., 1992; Deng et al., 1993); however, the potential of these volatiles and of 3*Z*-hexenal for flavor enhancement (Kollmannsberger and Berger, 1992) and postharvest disinfestation of fruit does not appear to have been explored.

Biosynthesis in Red Delicious and Granny Smith Apples. Overall, the cultivar Red Delicious showed a greater propensity toward the reduction of aldehyde substrates, giving a higher proportion of hexyl and hexenyl esters than Granny Smith (Table 4) but with suggestions also of a greater extent of α-oxidation. Granny Smith apples showed lower overall levels of volatile production (unpublished results), more evidence of β-oxidation, and the presence of significant quantities of hexanol among the aroma volatiles. Similar trends were observed in a study of 2-methylbutyl and 2-methylbutanoate ester metabolism by Red Delicious and Granny Smith apples (Rowan et al., 1996) and suggest a relative lack of the organic acid components (De Pooter et al., 1983) or of the enzymes required for ester formation in Granny Smith apples. This contrasts with the situation with Cox's Orange Pippin apples, in which low levels of ester production were considered to be due to low rates of alcohol synthesis (Knee and Hatfield, 1981).

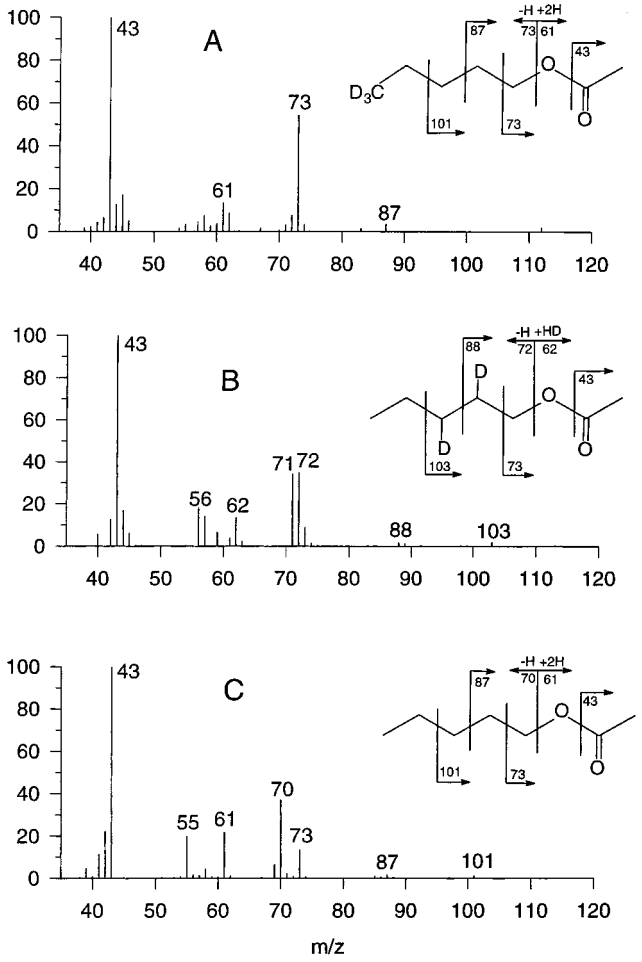

Figure 3. Mass spectra (GC/MS, 70 eV) of (A) pentyl-d_3 acetate, (B) pentyl-d_2 acetate, and (C) pentyl acetate.

Of interest is the inability of Red Delicious apples to reduce 3*Z*-hexenol to hexyl esters. To probe the selectivity of this process, three "unnatural" substrates (Paillard, 1990), 2*Z*-hexenol, a mixture of 4*E*/*Z*-hexenols, and 5-hexenol, were fed to Red Delicious apples. Exposure to vapor of 2*Z*-hexenol resulted in the formation of the corresponding 2*Z*-hexenyl esters. Feeding of *E*/*Z*-hexen-4-ol or 5-hexenol (Table 5) also gave the corresponding hexenyl esters together with small quantities (0.9−1.8%) of the corresponding hexenoate esters on the basis of their GC retention times and mass spectra. Reduction of the double bond could not be confirmed in the absence of labeled substrates but was not a major pathway as

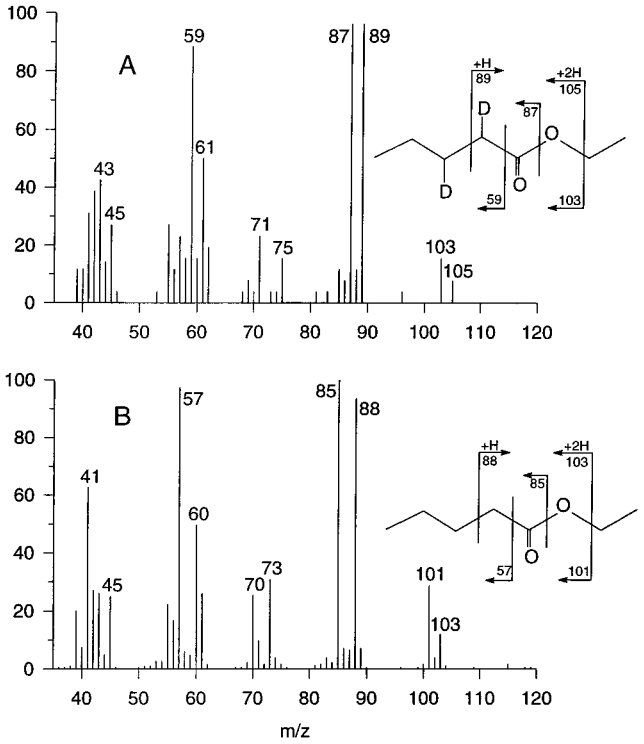

Figure 4. Mass spectra (GC/MS, 70 eV) of (A) ethyl pentanoate-d_2 and (B) ethyl pentanoate.

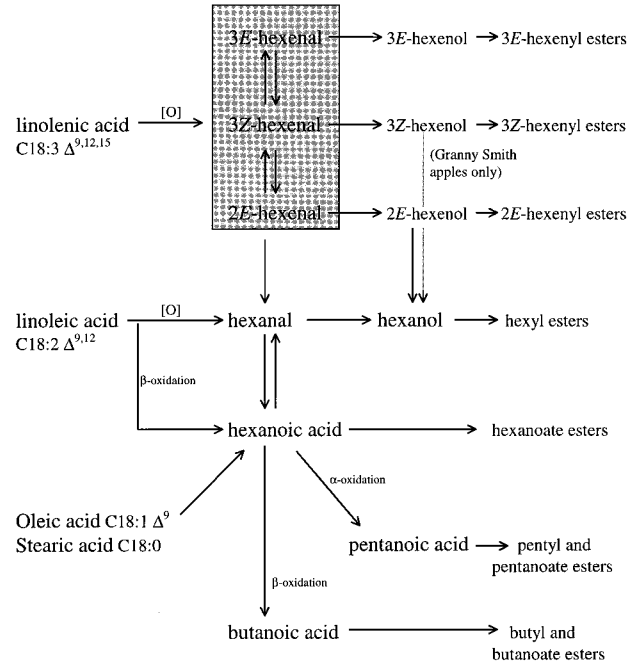

Figure 5. Biosynthetic pathways leading to straight-chain ester volatiles in Granny Smith and Red Delicious apples.

judged by relative peak areas of volatiles before and after exposure of fruit to the alcohols. For two of these nonnatural substrates, oxidation of the alcohol to the corresponding acid appears to be competitive with esterification. Although all of the isomeric hexenols may be esterified by Red Delicious apples, it appears that reduction to hexanol occurs only from 2*E*-hexenol.

Conclusion. Deuterium labeling has been used to identify the biosynthetic origins of the straight chain ester volatiles in Red Delicious and Granny Smith apples. Volatile biosynthesis from fatty acids, α- and β-oxidation processes, and the 2*E*/3*E*/3*Z* isomerization of isomeric hexenals has been demonstrated. These transformations are summarized in Figure 5 and extend our knowledge of the biosynthetic capabilities of apples. Addition of precursors allowed observation of normally hidden pathways such as the isomerzation of 2*E*-hexenal to 3*E*- and 3*Z*-hexenal with subsequent formation of novel volatiles, the 3*E*- and 3*Z*-hexenyl esters. The usefulness of deuterium labeling in biosynthetic studies was demonstrated by the observation of minor transformations such as the α-oxidation of hexanoic to pentanoic acid. Straight-chain ester volatiles in whole fruit arise predominantly by β-oxidation of fatty acids. Unsaturated straight-chain esters appear to arise only by the lipoxygenase pathway and may be useful indicators of lipoxygenase activity, particularly in cultivars such as Red Delicious that seem to be unable to convert 3*Z*-hexenol to hexyl esters.

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