

# Biosynthesis of 2-Methylbutyl, 2-Methyl-2-butenyl, and 2-Methylbutanoate Esters in Red Delicious and Granny Smith Apples Using Deuterium-Labeled Substrates

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2-Methylbutanoate esters, especially ethyl 2-methylbutanoate, are key contributors to fruit aroma. The biosynthetic origins and interconversions of 2-methylbutyl and 2-methylbutanoate esters in Red Delicious and Granny Smith apples were determined by feeding deuterium-labeled substrates with GC–MS identification of the deuterated aroma volatiles produced. Deuterium-labeled isoleucine was fed to apple peel, and 2-methylbutanoic- $d_3$  acid, 2-methylbutanol- $d_3$ , and ethyl 2-methylbutanoate- $d_3$  were fed as vapor to whole apples. An array of labeled 2-methylbutyl and 2-methylbutanoate esters was produced from each substrate with significant differences in products and product distributions between the two apple cultivars. Novel 2-methyl-(2*E*)-butenyl esters were identified as biosynthetic products in the aroma of Red Delicious but not Granny Smith apples.

**Keywords:** *Biosynthesis; apple; aroma volatiles; 2-methylbutyl; 2-methylbutanoate; 2-methyl-2-butenyl; deuterium*

## INTRODUCTION

2-Methylbutanoate esters occur widely in the aroma volatiles of fruit and other foodstuffs where they contribute to aroma and flavour (Paillard, 1990) and may also function as insect attractants (Lugemwa *et al.*, 1989; Aluja *et al.*, 1993) and allelochemicals (Connick *et al.*, 1989). Ethyl 2-methylbutanoate (EMB) has an aroma threshold of 0.1 ppb (v/v) with an intense odor characterized as apple-like, green, fruity, and “giving an impression of ripeness” (Paillard, 1990). EMB has been identified as a key aroma impact compound of several fruits including apples (Flath *et al.*, 1969; Paillard, 1990; Kollmannsberger and Berger, 1992), pineapples (Rettinger *et al.*, 1991), and Asian pear (Takeoka *et al.*, 1992) and in synthetic apple juice odor (Dürr and Röthlin, 1981) and apple essences (Paillard, 1990). Methyl 2-methylbutanoate was identified as a primary aroma compound of muskmelon (Schieberle *et al.*, 1990) and was an important odor note of fresh dill herb (Blank and Grosch, 1991). Propyl 2-methylbutanoate is important in apple aroma (Kollmannsberger and Berger, 1992), as is hexyl 2-methylbutanoate which has a typical apple-like aroma and is reported to improve the quality of Golden Delicious apples (Paillard, 1990).

2-Methylbutyl esters (*e.g.*, 2-methylbutyl acetate, aroma threshold 5 ppb; Flath *et al.*, 1967) contribute to the aroma of Royal Gala apples (Young *et al.*, 1995) and were the major ester components in the flesh of Rome-type apples (Fellman *et al.*, 1993). In contrast, 2-methylbutan-1-ol was described as possessing a butter, strawlike, and fusel-oil-like odor and was considered an undesirable constituent in commercial apple essences (Paillard, 1990). 2-Methylbutanol also occurs in apple juice as glycosidic conjugates (Schwab and Schreier, 1990) which may function as aroma precursors.

The aroma quality of stored apples is affected by storage conditions which may change the activity of

particular biosynthetic pathways and reduce the production of all or particular aroma volatiles. Anaerobic storage (0.05% O<sub>2</sub>/0.2% CO<sub>2</sub>) of Delicious apples for 30 days (Mattheis *et al.*, 1991) caused an increased concentration of ethyl 2-methylbutanoate along with increases in the concentrations of ethanol, acetaldehyde, and six other ethyl esters in the headspace volatiles. Controlled atmosphere (CA) storage at low ethylene concentrations (3% O<sub>2</sub>, 3% CO<sub>2</sub>, 1–6 ppm ethylene) for up to 8 months suppressed formation of butanoates, 2-methylbutanoates, pentanoates, and hexanoates during storage and of some volatiles during later ripening in air (Yahia *et al.*, 1991). Production of aroma volatiles was not enhanced by subsequent air, oxygen, ethylene, or light treatments (Yahia, 1989). Production of straight carbon (C) chain volatiles was suppressed by prolonged storage under ultralow oxygen conditions (1% O<sub>2</sub>), while production of branched C-chain aroma compounds (2-methylbutanol, 2-methylbutyl acetate) was suppressed by high (3%) CO<sub>2</sub> concentrations in Golden Delicious apples (Brackmann *et al.*, 1993).

Natural 2-methylbutanoates in apples have the *S* configuration and are understood to arise from *L*-isoleucine (Rettinger *et al.*, 1991; Mosandl, 1992). Incubation of ripe banana tissue disks with [U-<sup>14</sup>C]-*L*-leucine gave incorporation of the radiolabel into 3-methylbutanol and 3-methylbutyl acetate (Myers *et al.*, 1970), and this has been conceived as a general biosynthetic pathway for the production of branched chain volatiles by plants (Drawert, 1975). However in hazelnuts, 2-methylbutanoate esters and 2-methylbutanol were shown by deuterium labeling to be derived from 6-methyl-5-oxooctanoic acid which was in turn partially derived from 2-methylbutanoic acid (Silberzahn and Tressl, 1993). 2-Methylbutyl acetate was the sole product detected on incubation of cortical tissue slices of Cox's Orange Pippin apples with 2-methylbutanol (Knee and Hatfield, 1981).

In view of their importance to apple aroma and the complex and economically important changes which occur to their production during storage, we were

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interested in determining in more detail the biosynthetic interconversions of 2-methylbutanoates. This paper describes the use of deuterium-labeled substrates to define the biosynthetic origins and interconversions of 2-methylbutyl and 2-methylbutanoate esters in two apple cultivars: Red Delicious and Granny Smith. Differences between the cultivars in the processing of precursors and a novel biosynthetic product are described.

## MATERIALS AND METHODS

**General.** Apples (*Malus domestica* Borkh. var. Red Delicious) were obtained from the New Zealand Apple and Pear Marketing Board from CA storage and maintained at 1 °C until use. Cultivar Granny Smith apples were obtained from a local retailer. Red Delicious and Granny Smith apples were harvested in April and March 1994, respectively, and experiments were performed in the period from November 1994 to January 1995.

Unlabeled aroma precursors and reagents were obtained from the Aldrich Chemical Co., Inc. (Milwaukee, WI). Hexyl 2-methylbutanoate was synthesized by acid-catalyzed esterification of hexanol with 2-methylbutanoic acid. Aroma precursors were of >99% purity by GC. Dry THF and ether were prepared by distillation from sodium benzophenone blue. L-Isoleucine- $d_{10}$ , uniformly labeled to 98% isotopic purity, was obtained from Cambridge Isotope Laboratories, Inc., Woburn, MA. NMR spectra were recorded on a Bruker AC300 (300 MHz) spectrometer in  $CDCl_3$  and referenced to  $\delta$  7.24 for  $^1H$  and  $\delta$  77.0 for  $^{13}C$  NMR spectra.  $^{13}C$  multiplicities were determined using the DEPT-135 pulse sequence. Traps of Tenax TA (Alltech) were conditioned at 250 °C for 4 h under a stream of oxygen-free nitrogen (30–50 mL/min) before use.

**Synthesis of Ethyl 2-Methylbutanoate- $d_3$  (EMB- $d_3$ ).** *n*-Butyllithium (5.96 mL, 1.93 M in pentane) was added to a stirred solution of diisopropylamine (1.44 mL, 11.0 mmol) in dry THF (50 mL) at -78 °C (acetone/dry ice). The solution was stirred for 5 min, and ethyl butyrate (1.32 mL, 10.0 mmol) was added dropwise. After stirring for 30 min, iodomethane- $d_3$  (0.75 mL, 12.0 mmol) was added as a single aliquot, and the flask was allowed to warm to room temperature over 30 min. The flask contents were poured into a separating funnel containing 1.0 M HCl (50 mL) and ether (50 mL), and the aqueous layer was re-extracted with ether (50 mL). The combined organic phases were washed with water (50 mL) and then saturated brine (50 mL), dried ( $MgSO_4$ ), filtered, and concentrated *in vacuo* on a rotary evaporator with a water bath of 10 °C, to give a yellow oil (1.50 g). Short path distillation (Kugelrohr) of the fraction distilling at 130–140 °C (760 mmHg) gave the crude product (0.94 g, 70%). Preparative GC gave the title compound as a colorless oil: EIMS see Table 1;  $^1H$  NMR  $\delta$  0.90 (3H, t,  $J$  = 7.5 Hz, H-4), 1.25 (3H, t,  $J$  = 7.1 Hz,  $CH_3CH_2O$ ), 1.43 (1H, m, H-3), 1.67 (1H, m, H-3), 2.33 (1H, t,  $J$  = 6.8 Hz, H-2), 4.13 (2H, q,  $J$  = 7.1 Hz,  $CH_3CH_2O$ );  $^{13}C$  NMR  $\delta$  11.6 (C-4), 14.3 (C-2), 26.7 (C-3), 40.9 (C-2), 60.1 (C-1), 176.8 (C-1).

**Synthesis of 2-Methylbutanoic- $d_3$  Acid.** Ethyl 2-methylbutanoate- $d_3$  (96 mg, 0.72 mmol) was stirred at room temperature for 48 h in a 10% aqueous NaOH solution (5.0 mL). The reaction mixture was extracted with ether (2  $\times$  5 mL) and acidified with 1 N HCl (8 mL) and the aqueous phase re-extracted with dichloromethane (3  $\times$  10 mL). The combined dichloromethane extracts were dried ( $MgSO_4$ ), filtered, and concentrated *in vacuo* to give the title compound as a clear pungent oil (75.5 mg, quantitative):  $^1H$  NMR  $\delta$  0.95 (3H, t,  $J$  = 7.4 Hz, H-4), 1.51 (1H, m, H-3), 1.71 (1H, m, H-3), 2.39 (1H, t,  $J$  = 6.8 Hz, H-2), 9.10 (1H, bs, COOH);  $^{13}C$  NMR  $\delta$  11.5 (C-4), 26.5 (C-3), 40.7 (C-2), 183.2 (C-1).

**Synthesis of 2-Methylbutanol- $d_3$ .** Lithium aluminum hydride (153 mg, 4.02 mmol) was added in one portion to a stirred solution of 2-methylbutanoic- $d_3$  acid (0.212 g, 2.01 mmol) in dry ether (15 mL) at room temperature. After 4 h, the reaction was quenched with saturated aqueous  $NH_4Cl$  solution (2 mL) and the mixture extracted with ether (2  $\times$  20

mL). The combined ethereal extracts were dried ( $MgSO_4$ ), filtered, and concentrated *in vacuo* to give a crude product which was subjected to short path distillation (Kugelrohr), and the fraction distilling at 125–135 °C (760 mmHg) was collected (140 mg, 76%). Further purification by preparative GC gave 2-methylbutan-1-ol- $d_3$  as a colorless mobile oil: EIMS see Table 1;  $^1H$  NMR  $\delta$  0.91 (3H, t,  $J$  = 7.3 Hz, H-4), 1.15 (1H, m, H-3), 1.43 (1H, m, H-3), 1.51 (1H, m, H-2), 1.71 (1H, bs, OH), 3.51 (2H, m, C-1);  $^{13}C$  NMR  $\delta$  11.3 (C-4), 25.7 (C-3), 37.1 (C-2), 68.0 (C-1).

**Synthesis of 2-Methylbutyl- $d_3$  Acetate.** 2-Methylbutan-1-ol- $d_3$  (2  $\mu$ L) was treated with acetic anhydride (5  $\mu$ L) and pyridine (5  $\mu$ L) in a pear-shaped HPLC vial at room temperature overnight. Dilution with ether (0.8 mL) and successive washing with 10% HCl, saturated  $NaHCO_3$ , and brine followed by drying ( $MgSO_4$ ) gave an ethereal solution of the required compound for GC-MS analysis.

**Synthesis of 2-Methyl-2-Butenyl Acetate.** To a stirred solution of *trans*-2-methyl-2-butenol (0.87 g, 10 mmol) in dry ether (20 mL) was added portionwise lithium aluminum hydride (0.20 g, 2.0 equiv). After 90 min at room temperature, saturated  $NH_4Cl$  (6 drops) was cautiously added, and the solution was diluted with ether (20 mL) and dried ( $MgSO_4$ ) and the solvent removed under reduced pressure. Kugelrohr distillation gave 2-methyl-(2*E*)-butenol (0.48 g, 54%) as a clear oil: GC-MS gave EIMS  $m/z$  86 ( $M^+$ , 41), 71 (100), 68 (12), 67 (14), 57 (11), 55 (13), 53 (26), 43 (33), 41 (44), 39 (27);  $^1H$  NMR ( $CDCl_3$ )  $\delta$  1.52 (3H, dm,  $J$  = ~1, 5.6 Hz, H-4), 1.59 (3H, s, 2-Me), 2.18 (1H, bs, OH), 3.91 (2H, bs, H-2), 5.47 (1H, m, H-3);  $^{13}C$  NMR ( $CDCl_3$ )  $\delta$  12.9 (C-4), 13.1 (2-Me), 68.7 (C-1), 120.3 (C-3), 135.5 (C-2).

To 2-methyl-(2*E*)-butenol (0.30 g, 3.5 mmol) in pyridine (0.5 mL) and acetic anhydride (0.5 mL) was added a catalytic quantity of *p*-(dimethylamino)pyridine, and the solution was left at room temperature overnight. The reaction mixture was diluted with ether (60 mL) and the ethereal solution washed with 10% HCl (2  $\times$  10 mL), saturated  $NaHCO_3$  (2  $\times$  10 mL), and then brine (1  $\times$  10 mL) before drying ( $MgSO_4$ ) and evaporation of the solvent under reduced pressure. Kugelrohr distillation of the residual oil gave 2-methyl-(2*E*)-butenyl acetate (0.39 g, 87%) as a clear oil: GC-MS (Carbowax, RI 19.7 min) EIMS see Table 1;  $^1H$  NMR ( $CDCl_3$ )  $\delta$  1.56 (3H, dt,  $J$  = 1.0, ~5 Hz, H-4), 1.59 (3H, bs, 2-Me), 1.99 (3H, s, OAc), 4.39 (2H, bs, H-1), 5.48 (1H, qm,  $J$  = ~1.4, 5.5 Hz, H-3).  $^{13}C$  NMR ( $CDCl_3$ )  $\delta$  13.0 (C-4), 13.4 (2-Me), 20.7 (OAc), 70.1 (C-1), 123.9 (C-3), 130.8 (C-2), 170.7 (C=O). The 2*E* stereochemistry was confirmed by NOE.

**Synthesis of 3-Methyl-2-butenyl Acetate.** By acetylation of 3-methyl-2-buten-1-ol as above: GC-MS (Carbowax, RI 19.9 min) EIMS  $m/z$  128 ( $M^+$ , 1.6), 113 (0.4), 86 (16), 71 (22), 69 (29), 68 (81), 67 (48), 53 (25), 43 (100), 41 (54), 39 (20);  $^1H$  NMR ( $CDCl_3$ )  $\delta$  1.63 (3H, bs), 1.67 (3H, d,  $J$  = 0.8 Hz), 1.96 (3H, s, OAc), 4.49 (2H, d,  $J$  = 6.2 Hz, H-1), 5.24 (1H, bt,  $J$  = 6 Hz, H-2);  $^{13}C$  NMR ( $CDCl_3$ )  $\delta$  17.8 (3-Me), 20.7 (OAc), 25.5 (C-4), 61.2 (C-1), 118.6 (C-2), 138.7 (C-3), 170.8 (C=O).

**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 30 m  $\times$  0.25 mm i.d. Carbowax Econo-Cap capillary column (Alltech), film thickness 0.25  $\mu$ m, 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 of hydrogen. Deuterated aroma volatiles were partially or fully resolved on GC from nonlabeled analogues and were characterized as new peaks not present in controls or in apples treated with nonlabeled analogues and eluting some 4–30 s before nonlabeled material. Chemical identity was established by direct comparison with authentic compounds, from Kovat indices and by GC-MS identifications.

Preparative GC was carried out using a modified HP 5840A gas chromatograph fitted with a 2 m  $\times$  5 mm i.d. stainless steel column packed with 10% OV-101 on GasChromQ run isothermally at 150 °C with  $N_2$  carrier gas at 100 mL/min.

Samples were collected via a heated exit port directly into cooled pear-shaped sample vials.

**Capillary Gas Chromatography–Mass Spectroscopy (GC–MS).** Electron impact GC–MS were recorded on a VG70-250S double-focusing magnetic sector mass spectrometer with ionization potential of 70 eV, mass spectral interface 180 °C with 1 s scans and a 0.2 s delay with GC conditions as above, except a 2 psi of helium column head pressure was used.

Chemical ionization (CI) GC–MS was performed with ammonia gas with the gas pressure adjusted to give both  $MH^+$  and  $MNH_4^+$  ions and characteristic fragmentation ions for each peak. Aroma volatiles were identified by their retention times and mass spectra (Jennings and Shibamoto, 1980), by computer library matching of the mass spectra (NIST and Wiley databases), and, in some cases, by comparison with authentic materials. Deuterated metabolites (Table 1) were identified as new aroma constituents with characteristic 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 in the EI GC–MS (McLafferty, 1973), by the presence of higher molecular weight ions in the CI GC–MS, and, in some cases, by direct comparison with authentic labeled materials.

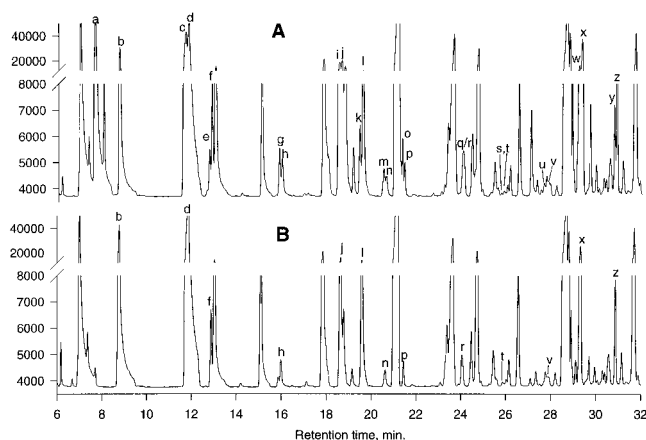
**Fresh Fruit Experiments.** Apples were removed from storage at 1 °C and allowed to warm to room temperature over 24 h. Two apples were placed in each of three 1.5 L glass jars (160 × 105 mm i.d.). Air, filtered through silica gel and then activated carbon, was drawn into the bottom of each jar by the vacuum from a water pump at a rate of 80 mL/min. Volatiles were entrained in the flow of air and absorbed onto Tenax TA (350 mg) packed into glass tubes (5 mm i.d.) attached to the top of each jar. Octyl acetate (10 μL/mL in ether) was used as an internal standard to correct for differences in the efficiency of the Tenax traps and in the recovery of the sample from the traps. Before each collection, 10 μL of the above solution was carefully added by syringe directly onto a glass fiber paper disk placed above the apple 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/jar, were added to the glass fiber filter paper held in the outlet of the jar above the apples and used for addition of the octyl acetate internal standard. 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 was then removed, and the apparatus was left open to the air for 1 h to remove precursor vapor. The glass fiber paper was replaced, octyl acetate internal standard was applied as above, and volatiles were trapped on Tenax as before for one or two successive 24 h periods. Volatile production by untreated apples was measured as a control.

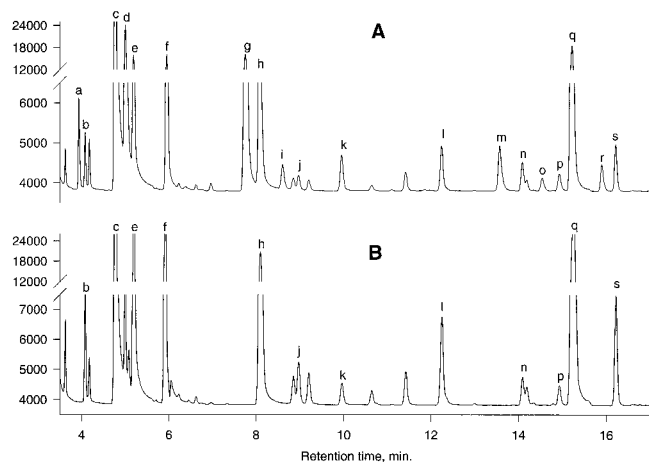
Volatiles were recovered from the Tenax traps by elution with freshly redistilled ether (2.5 mL) under slight positive pressure. Control experiments indicated that 2 mL of ether was sufficient to elute all volatiles from the traps. The eluent was collected into 3 mL screwcap vials, tightly capped, and stored at –20 °C. Immediately prior to GC analysis, 10 μL of a toluene solution (10 μL/mL in ether) was added as a second internal standard.

**Juice Experiments.** After the feeding experiments were completed, apples were juiced in a hydraulic press, and the juice was filtered through two layers of Miracloth (Calbiochem). Juice (30 mL) plus octyl acetate internal standard (1 μL of 10 μL/mL solution in ether) was sparged with nitrogen at 100 mL/min for 5 h in a 100 mL round bottomed flask. Volatiles were absorbed onto Tenax traps and analyzed as above except the ether eluent was concentrated to approximately 0.5 mL under a stream of nitrogen gas before GC and GC–MS analyses.

**Incorporation of Isoleucine into Apple Peel.** Apples were removed from storage and allowed to warm to room temperature over 24 h. Peel (80 g) was removed with a commercial peeler and rinsed (3 × 150 mL) in 0.011 M MES buffer, pH 6.45, 0.33 M in mannitol and containing chloram-

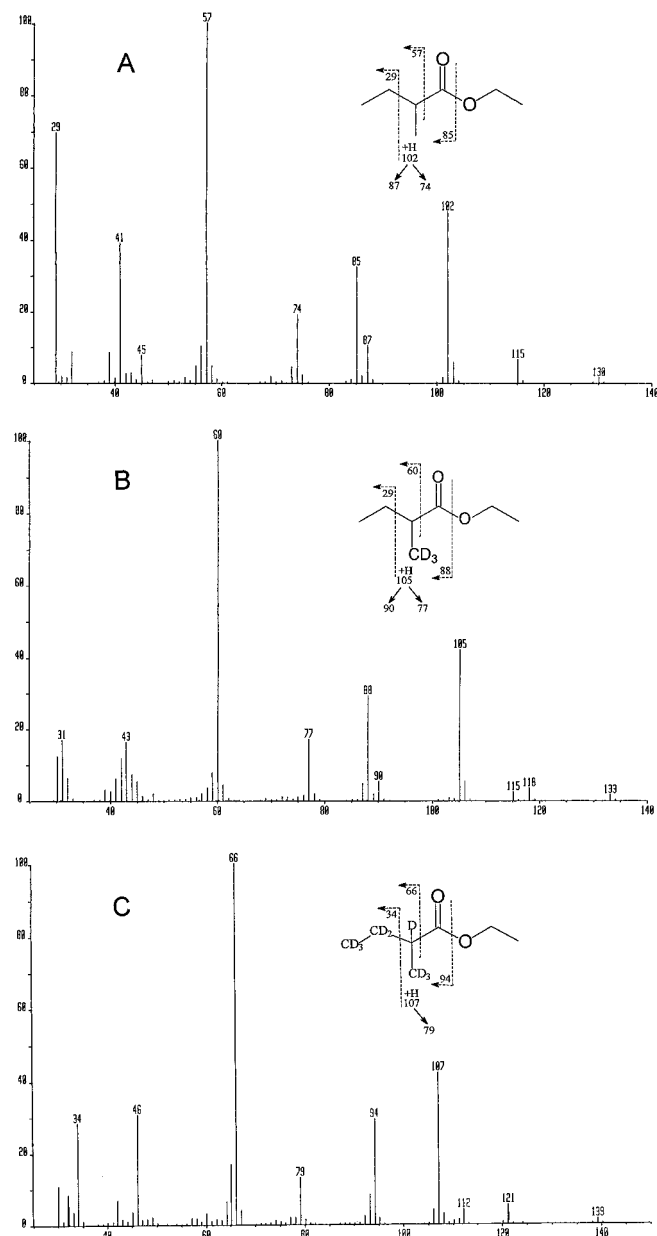


**Figure 1.** Capillary GC of aroma volatiles collected from (A) ethyl 2-methylbutanoate- $d_3$  treated- and (B) control Red Delicious apples: (a) ethyl 2-methylbutanoate- $d_3$ , (b) ethyl 2-methylbutanoate, (c) 2-methylbutyl- $d_3$  acetate, (d) 2-methylbutyl acetate, (e) propyl 2-methylbutanoate- $d_3$ , (f) propyl 2-methylbutanoate, (g) 2-methylbutyl- $d_3$  propionate, (h) 2-methylbutyl propionate, (i) butyl 2-methylbutanoate- $d_3$ , (j) butyl 2-methylbutanoate, (k) 2-methyl-2-butenyl- $d_3$  acetate, (l) 2-methyl-2-butenyl acetate, (m) 2-methylbutyl- $d_3$  2-methylpropionate, (n) 2-methylbutyl 2-methylpropionate, (o) 2-methylbutyl- $d_3$  2-methylbutanoate and 2-methylbutyl 2-methylbutanoate- $d_3$ , (p) 2-methylbutyl 2-methylbutanoate, (q/r) pentyl 2-methylbutanoate- $d_3$  and pentyl 2-methylbutanoate, (s) 2-methylbutyl- $d_3$  pentanoate, (t) 2-methylbutyl pentanoate, (u) 2-methyl-2-butenyl- $d_3$  2-methylbutanoate and 2-methyl-2-butenyl 2-methylbutanoate- $d_3$ , (v) 2-methyl-2-butenyl 2-methylbutanoate, (w) hexyl 2-methylbutanoate- $d_3$ , (x) hexyl 2-methylbutanoate, (y) 2-methylbutyl- $d_3$  hexanoate, and (z) 2-methylbutyl hexanoate.



**Figure 2.** Capillary GC of aroma volatiles collected from (A) isoleucine- $d_{10}$  treated- and (B) control Red Delicious apple skin slices: (a) methyl methylbutanoate- $d_9$ , (b) methyl 2-methylbutanoate, (c) toluene (internal standard), (d) ethyl 2-methylbutanoate- $d_9$ , (e) ethyl 2-methylbutanoate, (f) butyl acetate, (g) 2-methylbutyl- $d_9$  acetate, (h) 2-methylbutyl acetate, (i) propyl 2-methylbutanoate- $d_9$ , (j) propyl 2-methylbutanoate, (k) butanol, (l) methyl hexanoate, (m) 2-methylbutanol- $d_9$ , (n) 2-methylbutanol, (o) butyl 2-methylbutanoate- $d_9$ , (p) butyl 2-methylbutanoate, (q) ethyl hexanoate, (r) 2-methyl-2-butenyl- $d_7$  acetate, and (s) 2-methyl-2-butenyl acetate.

phenicol (0.68 mg/L) and cycloheximide (0.11 mg/L). Washed peel (ca. 35 g) was suspended in the above buffer (15 mL) in a 150 mL round bottomed flask and isoleucine- $d_{10}$  (10 mg in 0.5 mL of water) added. The flask contents were mixed by gentle swirling, maintained at 25 °C in a water bath, and sparged with air at 50 mL/min. Volatiles were collected onto Tenax for 5 h and then for a further 16 h and analyzed as above after concentration to approximately 0.5 mL under a stream of nitrogen gas.

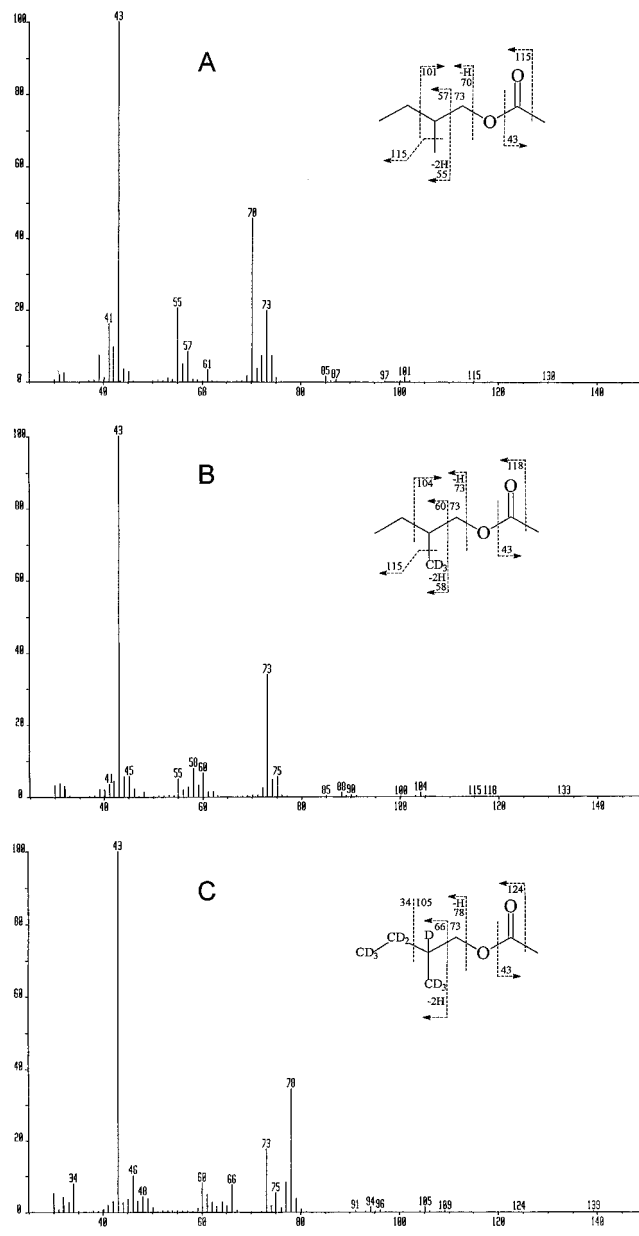


**Figure 3.** Mass spectra (GC-MS, 70 eV) of (A) ethyl 2-methylbutanoate, (B) ethyl 2-methylbutanoate- $d_3$ , and (C) ethyl 2-methylbutanoate- $d_9$ .

## RESULTS AND DISCUSSION

**Identification of Deuterated Aroma Constituents.** Exposure of intact apples to vapor of EMB- $d_3$ , 2-methylbutanoic- $d_3$  acid, or 2-methylbutanol- $d_3$  or incubation of apple peel with isoleucine- $d_{10}$  led to the production of deuterium-labeled aroma compounds which were trapped on Tenax and identified and quantified by GC and GC-MS. The chemical identity of unlabeled compounds was established on the basis of their GC retention times and by mass spectral comparison with authentic compounds and reference spectra. Deuterium-labeled aroma compounds were identified as new peaks eluting in the chromatogram some 4–30 s before their nondeuterated analogues and not appearing in head-space samples prepared from control (untreated) fruit or in fruit treated with unlabeled precursors (Figures 1 and 2).

The presence of deuterium in the aroma constituents was confirmed by EI and CI GC-MS. The EI mass spectra of 2-methylbutanoate esters show characteristic



**Figure 4.** Mass spectra (GC-MS, 70 eV) of (A) 2-methylbutyl acetate, (B) 2-methylbutyl- $d_3$  acetate, and (C) ethyl 2-methylbutanoate- $d_9$ .

fragments ions at  $m/z$  57, 85, and 103 (Jennings and Shibamoto, 1980) which are shifted to  $m/z$  60, 88, and 106 in the 2-methylbutanoates- $d_3$  and to  $m/z$  66, 94, and 112 in the 2-methylbutanoates- $d_9$  derived from isoleucine- $d_{10}$  (Figure 3). In ethyl 2-methylbutanoate (Figure 3A), an additional McLafferty rearrangement ion at  $m/z$  102 is shifted to  $m/z$  105 and 107 in the  $d_3$  and  $d_9$  metabolites, respectively (Figure 3B,C). 2-Methylbutyl esters were identified from the characteristic mass spectral fragment ion at  $m/z$  70 as exemplified by 2-methylbutyl acetate (Figure 4A), which was shifted to  $m/z$  73 in the  $d_3$  and to  $m/z$  78 in the  $d_9$  derivatives (Figure 4B,C).

A further minor series of labeled compounds (1.8–3.2% label) appeared to be the deuterated analogues of a previously uncharacterized series of natural aroma constituents. The most abundant of these deuterated compounds (to 2.1% label) appeared to be the  $d_3$  (or  $d_7$ ) analogue of a naturally occurring unsaturated C5 acetate ( $M^+$  128,  $m/z$  68 (46), 43 (100)). The natural compound was identified as 2-methyl-(2*E*)-butenyl ac-

**Table 1. Mass Spectra (GC–MS, 70 eV) of 2-Methylbutanoate, 2-Methylbutyl, and 2-Methyl-2-butenyl Esters Identified in the Aroma Volatiles of Red Delicious and Granny Smith Apples Fed Deuterated Aroma Precursors**

| compound  | retention index <sup>a</sup> | mass spectrum   |
|---|------------------------------|---|
| methyl 2-methylbutanoate- <i>d</i> <sub>9</sub>   | 1003                         | EIMS 125 (M <sup>+</sup> , 1.2), 107 (26), 94 (30), 93 (90), 92 (12), 74 (5), 66 (100), 65 (21), 64 (14), 59 (11), 46 (48), 42 (14), 34 (43), 30 (20); CIMS (NH <sub>3</sub> ) 126 (MH <sup>+</sup> )   |
| methyl 2-methylbutanoate  | 1010                         | EIMS 116 (M <sup>+</sup> , 1.8), 101 (26), 88 (96), 85 (32), 69 (9), 59 (14), 57 (100), 41 (54), 39 (18), 29 (49); CIMS (NH <sub>3</sub> ) 117 (MH <sup>+</sup> )   |
| ethyl 2-methylbutanoate- <i>d</i> <sub>9</sub>  | 1048                         | EIMS 139 (M <sup>+</sup> , 1.3), 121 (5), 112 (4), 107 (42), 94 (29), 79 (13), 66 (100), 46 (31), 34 (28); CIMS (NH <sub>3</sub> ) 140 (MH <sup>+</sup> )   |
| ethyl 2-methylbutanoate- <i>d</i> <sub>3</sub>  | 1053                         | EIMS 133 (M <sup>+</sup> , 3.3), 118 (8), 115 (6), 106 (11), 105 (100), 90 (6), 88 (29), 77 (18), 60 (100), 43 (17), 29 (42); CIMS (NH <sub>3</sub> ) 134 (MH <sup>+</sup> ), 151 (M + NH <sub>4</sub> <sup>+</sup> )   |
| ethyl 2-methylbutanoate   | 1055                         | EIMS 130 (M <sup>+</sup> , 1.5), 115 (7), 103 (6), 102 (48), 87 (10), 85 (32), 74 (19), 57 (100), 41 (39), 29 (70); CIMS (NH <sub>3</sub> ) 131 (MH <sup>+</sup> )  |
| 2-methylbutyl- <i>d</i> <sub>9</sub> acetate  | 1126                         | EIMS 139 (M <sup>+</sup> , 0.1), 124 (0.1), 105 (1), 94 (1), 78 (34), 77 (8), 73 (17), 66 (8), 60 (8), 46 (10), 43 (100), 34 (8); CIMS (NH <sub>3</sub> ) 140 (MH <sup>+</sup> ), 157 (M + NH <sub>4</sub> <sup>+</sup> )   |
| 2-methylbutyl- <i>d</i> <sub>3</sub> acetate  | 1131                         | EIMS 133 (M <sup>+</sup> , 0.1), 118 (0.1), 115 (0.1), 104 (1), 88 (1), 73 (34), 60 (7), 58 (8), 55 (5), 43 (100); CIMS (NH <sub>3</sub> ) 134 (MH <sup>+</sup> ), 151 (M + NH <sub>4</sub> <sup>+</sup> )  |
| 2-methylbutyl acetate   | 1134                         | EIMS 130 (M <sup>+</sup> , 0.1), 115 (0.1), 101 (1), 85 (1), 73 (14), 70 (33), 55 (16), 43 (100), 41 (12), 29 (11); CIMS (NH <sub>3</sub> ) 131 (MH <sup>+</sup> ), 148 (M + NH <sub>4</sub> <sup>+</sup> )   |
| propyl 2-methylbutanoate- <i>d</i> <sub>9</sub>   | 1144                         | EIMS 121 (9), 112 (71), 94 (61), 79 (13), 66 (100), 46 (26), 43 (25), 34 (25); CIMS (NH <sub>3</sub> ) 154 (MH <sup>+</sup> )   |
| propyl 2-methylbutanoate- <i>d</i> <sub>3</sub>   | 1148                         | EIMS 119 (10), 106 (60), 88 (67), 77 (21), 60 (100), 43 (53), 41 (23); CIMS (NH <sub>3</sub> ) 148 (MH <sup>+</sup> ), 165 (M + NH <sub>4</sub> <sup>+</sup> )  |
| propyl 2-methylbutanoate  | 1150                         | EIMS 129 (M <sup>+</sup> , 0.6), 116 (11), 103 (59), 87 (12), 85 (66), 74 (21), 60 (15), 57 (100), 43 (44), 41 (50), 39 (14); CIMS (NH <sub>3</sub> ) 145 (MH <sup>+</sup> ), 162 (M + NH <sub>4</sub> <sup>+</sup> )   |
| 2-methylpropyl 2-methylbutanoate- <i>d</i> <sub>3</sub>   | 1181                         | EIMS 106 (6), 88 (17), 60 (24); CIMS (NH <sub>3</sub> ) 162 (MH <sup>+</sup> )  |
| 2-methylpropyl 2-methylbutanoate  | 1183                         | EIMS 130 (4), 103 (23), 85 (58), 74 (9), 57 (100), 41 (38); CIMS (NH <sub>3</sub> ) 159 (MH <sup>+</sup> )  |
| 2-methylbutyl- <i>d</i> <sub>3</sub> propionate   | 1192                         | EIMS 118 (0.8), 99 (4), 87 (16), 74 (24), 73 (36), 57 (100), 45 (9), 43 (10); CIMS (NH <sub>3</sub> ) 148 (MH <sup>+</sup> ), 165 (M + NH <sub>4</sub> <sup>+</sup> )   |
| 2-methylbutyl propionate  | 1194                         | EIMS 115 (0.8), 87 (10), 70 (35), 57 (100), 55 (14), 43 (14); CIMS (NH <sub>3</sub> ) 145 (MH <sup>+</sup> ), 162 (M + NH <sub>4</sub> <sup>+</sup> )   |
| 2-methylbutanol- <i>d</i> <sub>9</sub>  | 1207                         | EIMS 78 (26), 66 (100), 64 (61), 60 (10), 46 (62), 34 (60), 30 (21)   |
| 2-methylbutanol- <i>d</i> <sub>3</sub>  | 1209                         | EIMS 73 (28), 60 (100), 59 (64), 43 (35), 31 (24); CIMS (NH <sub>3</sub> ) 131 (M <sup>+</sup> ), 132 (MH <sup>+</sup> ), 149 (M + NH <sub>4</sub> <sup>+</sup> )   |
| 2-methylbutanol   | 1211                         | EIMS 70 (37), 57 (100), 56 (84), 41 (83), 31 (32); CIMS (NH <sub>3</sub> ) 128 (M <sup>+</sup> ), 129 (MH <sup>+</sup> ), 146 (M + NH <sub>4</sub> <sup>+</sup> )   |
| butyl 2-methylbutanoate- <i>d</i> <sub>9</sub>  | 1232                         | EIMS 135 (6), 112 (85), 94 (68), 79 (15), 66 (100), 57 (22), 56 (38), 46 (20), 41 (15), 34 (19), 29 (20); CIMS (NH <sub>3</sub> ) 168 (MH <sup>+</sup> ), 185 (M + NH <sub>4</sub> <sup>+</sup> )   |
| butyl 2-methylbutanoate- <i>d</i> <sub>3</sub>  | 1237                         | EIMS 161 (M <sup>+</sup> , 0.1), 133 (6), 118 (1), 106 (71), 88 (66), 77 (20), 60 (100), 57 (34), 56 (38), 43 (13), 41 (32); CIMS (NH <sub>3</sub> ) 162 (MH <sup>+</sup> ), 179 (M + NH <sub>4</sub> <sup>+</sup> )  |
| butyl 2-methylbutanoate   | 1239                         | EIMS 158 (M <sup>+</sup> , 0.1), 130 (4), 115 (1), 103 (51), 85 (50), 74 (15), 57 (100), 56 (32), 41 (40); CIMS (NH <sub>3</sub> ) 159 (MH <sup>+</sup> ), 176 (M + NH <sub>4</sub> <sup>+</sup> )  |
| 2-methyl-2-butenyl- <i>d</i> <sub>7</sub> acetate   | 1253                         | EIMS 135 (M <sup>+</sup> , 5), 93 (23), 92 (25), 75 (22), 74 (30), 73 (38), 72 (23), 70 (24), 62 (6), 57 (42), 55 (11), 43 (100); CIMS 135 (M <sup>+</sup> )  |
| 2-methyl-2-butenyl- <i>d</i> <sub>3</sub> acetate   | 1256                         | EIMS 131 (7, M <sup>+</sup> ), 116 (0.6), 89 (44), 88 (10), 74 (17), 72 (19), 71 (39), 70 (42), 69 (17), 56 (9), 55 (9), 43 (100), 42 (16), 41 (13); CIMS (NH <sub>3</sub> ) 131 (M <sup>+</sup> ), 132 (MH <sup>+</sup> ), 149 (M + NH <sub>4</sub> <sup>+</sup> ) |
| 2-methyl-(2 <i>E</i> )-butenyl acetate  | 1258                         | EIMS 128 (3, M <sup>+</sup> ), 113 (0.3), 86 (28), 71 (18), 69 (14), 68 (46), 67 (35), 61 (3), 53 (18), 43 (100), 41 (32), 39 (14), 29 (8); CIMS (NH <sub>3</sub> ) 128 (M <sup>+</sup> ), 129 (MH <sup>+</sup> ), 146 (M + NH <sub>4</sub> <sup>+</sup> )          |
| 2-methylbutyl- <i>d</i> <sub>3</sub> butanoate  | 1270                         | EIMS 132 (0.7), 131 (0.7), 118 (1), 101 (5), 88 (5), 74 (11), 73 (46), 71 (100), 58 (8), 43 (46), 41 (12); CIMS (NH <sub>3</sub> ) 162 (MH <sup>+</sup> ), 179 (M + NH <sub>4</sub> <sup>+</sup> )  |
| 2-methylbutyl butanoate   | 1272                         | EIMS 129 (0.6), 128 (1), 115 (0.9), 101 (6), 89 (7), 85 (3), 71 (100), 70 (43), 55 (14), 43 (51), 41 (18); CIMS (NH <sub>3</sub> ) 159 (MH <sup>+</sup> ), 176 (M + NH <sub>4</sub> <sup>+</sup> )  |
| 2-methylbutyl- <i>d</i> <sub>3</sub> 2-methylbutanoate- <i>d</i> <sub>3</sub>                                     | 1282                         | EIMS 150 (3), 118 (2), 107 (5), 106 (8), 88 (85), 74 (23), 73 (68), 60 (100), 45 (22); CIMS (NH <sub>3</sub> ) 179 (MH <sup>+</sup> ), 196 (M + NH <sub>4</sub> <sup>+</sup> )  |
| 2-methylbutyl- <i>d</i> <sub>3</sub> 2-methylbutanoate and 2-methylbutyl 2-methylbutanoate- <i>d</i> <sub>3</sub> | 1283                         | EIMS 147 (4), 118 (2), 115 (3), 106 (4), 104 (6), 103 (7), 88 (34), 85 (88), 74 (33), 73 (73), 70 (26), 60 (42), 57 (100), 45 (24), 43 (23), 41 (31); CIMS (NH <sub>3</sub> ) 176 (MH <sup>+</sup> ), 193 (M + NH <sub>4</sub> <sup>+</sup> )                       |
| 2-methylbutyl 2-methylbutanoate   | 1284                         | EIMS 144 (3), 115 (3), 103 (13), 85 (97), 70 (70), 57 (100), 55 (21), 43 (35), 41 (30); CIMS (NH <sub>3</sub> ) 173 (MH <sup>+</sup> ), 190 (M + NH <sub>4</sub> <sup>+</sup> )   |
| pentyl 2-methylbutanoate- <i>d</i> <sub>3</sub>   | 1330                         | EIMS 147 (6), 115 (4), 106 (84), 88 (68), 77 (19), 70 (48), 60 (100), 55 (19), 42 (24); CIMS (NH <sub>3</sub> ) 176 (MH <sup>+</sup> ), 193 (M + NH <sub>4</sub> <sup>+</sup> )   |
| pentyl 2-methylbutanoate  | 1332                         | EIMS 144 (4), 115 (4), 103 (81), 85 (67), 74 (18), 70 (44), 57 (100), 55 (22), 43 (67), 41 (45); CIMS (NH <sub>3</sub> ) 173 (MH <sup>+</sup> ), 190 (M + NH <sub>4</sub> <sup>+</sup> )  |
| 2-methylbutyl pentanoate- <i>d</i> <sub>3</sub>   | 1365                         | EIMS 85 (100), 73 (50), 56 (84), 43 (94), 41 (56); CIMS (NH <sub>3</sub> ) 176 (MH <sup>+</sup> )   |

**Table 1 (Continued)**

| compound  | retention index <sup>a</sup> | mass spectrum  |
|---|------------------------------|--|
| 2-methylbutyl pentanoate  | 1367                         | EIMS 85 (100), 70 (39), 57 (43), 43 (35), 41 (38); CIMS (NH <sub>3</sub> ) 173 (MH <sup>+</sup> ), 190 (M + NH <sub>4</sub> <sup>+</sup> )   |
| 2-methyl-2-butenyl- <i>d</i> <sub>3</sub> 2-methylbutanoate and 2-methyl-2-butenyl 2-methylbutanoate- <i>d</i> <sub>3</sub> | 1398                         | EIMS 173 (M <sup>+</sup> , 5.1), 106 (14), 89 (6), 88 (35), 72 (36), 71 (17), 70 (15), 69 (15), 68 (12), 60 (100), 55 (13), 43 (81); CIMS (NH <sub>3</sub> ) 174 (MH <sup>+</sup> )  |
| 2-methyl-2-butenyl 2-methylbutanoate  | 1400                         | EIMS 170 (M <sup>+</sup> , 3.1), 113 (1), 103 (15), 86 (12), 85 (55), 68 (54), 67 (11), 57 (100), 43 (8), 41 (17); CIMS (NH <sub>3</sub> ) 171 (MH <sup>+</sup> )  |
| hexyl 2-methylbutanoate- <i>d</i> <sub>3</sub>  | 1420                         | EIMS 131 (20), 112 (93), 94 (54), 84 (43), 66 (100), 56 (53), 55 (28), 46 (20), 43 (71), 41 (30), 34 (24), 29 (19); CIMS (NH <sub>3</sub> ) 196 (MH <sup>+</sup> )   |
| hexyl 2-methylbutanoate- <i>d</i> <sub>3</sub>  | 1423                         | EIMS 189 (M <sup>+</sup> , 0.1), 161 (2), 146 (0.3), 129 (2), 118 (1), 106 (87), 88 (55), 84 (35), 77 (18), 69 (16), 60 (100), 56 (43), 55 (22), 43 (88), 41 (32); CIMS (NH <sub>3</sub> ) 190 (MH <sup>+</sup> ), 207 (M + NH <sub>4</sub> <sup>+</sup> ) |
| hexyl 2-methylbutanoate   | 1425                         | EIMS 158 (0.03), 143 (0.6), 129 (4), 115 (1), 103 (100), 85 (54), 84 (33), 74 (15), 69 (12), 57 (63), 56 (29), 55 (14), 43 (36), 41 (23); CIMS (NH <sub>3</sub> ) 187 (MH <sup>+</sup> ), 204 (M + NH <sub>4</sub> <sup>+</sup> )                          |
| 2-methylbutyl- <i>d</i> <sub>3</sub> hexanoate  | 1456                         | EIMS 118 (5), 117 (5), 99 (100), 74 (28), 73 (80), 71 (28), 60 (11), 58 (8), 55 (11), 43 (51); CIMS (NH <sub>3</sub> ) 190 (MH <sup>+</sup> ), 207 (M + NH <sub>4</sub> <sup>+</sup> )   |
| 2-methylbutyl hexanoate   | 1458                         | EIMS 117 (9), 99 (100), 71 (51), 70 (78), 60 (13), 55 (24), 43 (63), 41 (30); CIMS (NH <sub>3</sub> ) 187 (MH <sup>+</sup> ), 204 (M + NH <sub>4</sub> <sup>+</sup> )  |
| heptyl 2-methylbutanoate- <i>d</i> <sub>3</sub>   | 1513                         | EIMS 175 (2), 143 (1), 110 (8), 106 (36), 98 (12), 95 (6), 88 (19), 82 (28), 67 (27), 60 (34), 57 (23), 43 (100), 41 (31); CIMS (NH <sub>3</sub> ) 204 (MH <sup>+</sup> ), 221 (M + NH <sub>4</sub> <sup>+</sup> )   |
| heptyl 2-methylbutanoate  | 1516                         | EIMS 172 (1), 103 (32), 98 (10), 85 (17), 74 (10), 70 (12), 61 (13), 57 (50), 45 (66), 43 (100); CIMS (NH <sub>3</sub> ) 201 (MH <sup>+</sup> ), 218 (M + NH <sub>4</sub> <sup>+</sup> )   |
| 2-methylbutyl- <i>d</i> <sub>3</sub> heptanoate   | 1554                         | EIMS 131 (6), 113 (100), 110 (18), 95 (36), 87 (50), 85 (20), 73 (94), 69 (63), 55 (34), 43 (72), 41 (66); CIMS (NH <sub>3</sub> ) 204 (MH <sup>+</sup> )  |
| 2-methylbutyl heptanoate  | 1556                         | EIMS 143 (1), 131 (10), 113 (100), 95 (15), 87 (9), 85 (19), 71 (38), 70 (97), 55 (27), 43 (86), 41 (35); CIMS (NH <sub>3</sub> ) 201 (MH <sup>+</sup> ), 218 (M + NH <sub>4</sub> <sup>+</sup> )  |
| 2-methyl-2-butenyl- <i>d</i> <sub>3</sub> hexanoate   | 1578                         | EIMS 187 (M <sup>+</sup> , 4), 118 (12), 117 (2), 99 (74), 89 (27), 72 (52), 71 (100), 70 (38), 55 (14), 43 (86)   |
| 2-methyl-2-butenyl hexanoate  | 1581                         | EIMS 184 (M <sup>+</sup> , 5), 155 (1), 141 (1), 128 (1), 117 (17), 99 (85), 86 (32), 71 (67), 69 (62), 68 (100), 67 (46), 55 (12), 53 (19), 43 (86), 41 (73); CIMS (NH <sub>3</sub> ) 185 (MH <sup>+</sup> ), 202 (M + NH <sub>4</sub> <sup>+</sup> )     |
| octyl 2-methylbutanoate- <i>d</i> <sub>3</sub>  | 1622                         | EIMS 112 (33), 106 (100), 88 (53), 84 (27), 83 (32), 70 (46), 60 (94), 57 (58), 43 (63), 41 (56), 29 (29); CIMS (NH <sub>3</sub> ) 218 (MH <sup>+</sup> )  |
| octyl 2-methylbutanoate   | 1623                         | EIMS 112 (19), 103 (77), 85 (38), 84 (19), 83 (18), 70 (31), 57 (100), 43 (35), 41 (46), 29 (33); CIMS (NH <sub>3</sub> ) 215 (MH <sup>+</sup> ), 232 (M + NH <sub>4</sub> <sup>+</sup> )  |
| 2-methylbutyl- <i>d</i> <sub>3</sub> octanoate  | 1655                         | EIMS 127 (78), 73 (100), 43 (64); CIMS (NH <sub>3</sub> ) 218 (MH <sup>+</sup> ), 232 (M + NH <sub>4</sub> <sup>+</sup> )  |
| 2-methylbutyl octanoate   | 1656                         | EIMS 145 (10), 127 (77), 71 (38), 70 (100), 57 (56), 55 (27), 43 (54), 42 (32); CIMS (NH <sub>3</sub> ) 215 (MH <sup>+</sup> ), 232 (M + NH <sub>4</sub> <sup>+</sup> )  |
| 2-methyl-2-butenyl- <i>d</i> <sub>3</sub> octanoate   | 1767                         | EIMS 127 (66), 89 (20), 72 (42), 71 (40), 70 (39), 57 (100), 55 (36), 43 (46), 41 (27); CIMS (NH <sub>3</sub> ) 216 (MH <sup>+</sup> )   |
| 2-methyl-2-butenyl octanoate  | 1767                         | EIMS 212 (M <sup>+</sup> , 4), 145 (15), 127 (53), 86 (24), 69 (47), 68 (73), 67 (29), 57 (100), 43 (26), 41 (54); CIMS (NH <sub>3</sub> ) 213 (MH <sup>+</sup> )  |

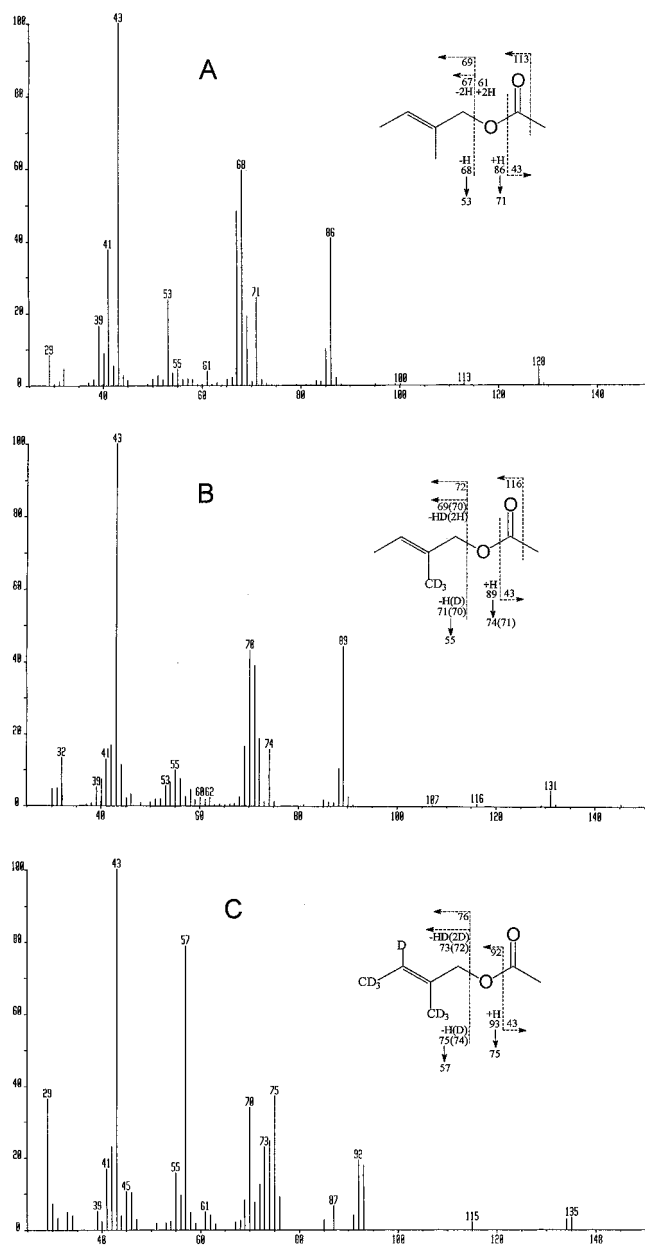
<sup>a</sup> Retention indices calculated for Carbowax capillary column (Jennings and Shibamoto, 1980).

etate by GC–MS comparison with synthetic samples of 2-methyl-(2*E*)- and 3-methyl-2-butenyl acetates. The 2-methylbutanoate and hexanoate analogues were likewise identified on the basis of their deuterium labeling, characteristic mass spectral ions, and chromatographic retention times (Table 1). 2-Methyl-2-butenyl esters are not listed as apple constituents in the review by Paillard (1990), although 2-methylbut-2-enal is reported in the cultivar Golden Delicious.

Deuterated 2-methyl-2-butenyl esters were identified among the aroma volatiles from the characteristic cluster of fragment ions at *m/z* 67, 68, and 69 (Figure 5A) shifted to appropriate higher mass ranges on deuteration (Figure 5B,C). In all, deuterium-labeled forms of 11 2-methylbutanoate esters, 8 2-methylbutyl esters, 3 2-methyl-2-butenyl esters, and 2-methylbutanol were variously identified in the two apple varieties examined. Kovats indices and mass spectral data for these compounds and their natural nondeuterated analogues are listed in Table 1.

**Metabolism of Labeled Precursors.** Some 0.54–3.7 mean mol % (calculated as EMB equivalents) of the deuterium label was collected during the first 24 h collection period after intact apples were exposed to the

vapor from precursors added at 10  $\mu$ L of neat liquid/apple. Differences in incorporations between varieties or precursors were not generally significant (unpublished data). When apple peel was feed labeled isoleucine, 0.38 and 0.25 mean mol %, respectively, of the deuterium label was recovered in the first 5 h collection of volatiles. The proportions of deuterated metabolites reflected those of unlabeled material in the aroma profile of each apple variety (Figures 1 and 2). 2-Methylbutyl compounds did not appear to be particularly good aroma precursors in these apple varieties as significantly more label was incorporated into the aroma volatiles on feeding *trans*-2-hexenol-6,6,6-*d*<sub>3</sub> (16.5% recovery as *trans*-2-hexenyl acetate equivalents). Release of labeled volatiles from whole apples continued for at least 2–3 days after exposure to the precursor, and labeled aroma compounds were recovered by sparging the juice pressed at this time. Deuterated ethyl, propyl, butyl, and hexyl 2-methylbutanoates, 2-methylbutyl acetate, 2-methylbutanol, and 2-methyl-2-butenyl acetate were identified by GC–MS in juice from Red Delicious apples which had been incubated with EMB-*d*<sub>3</sub> as whole fruit. Deuterated methyl 2-methylbutanoate was identified in the headspace from juice and



**Figure 5.** Mass spectra (GC-MS, 70 eV) of (A) 2-methyl-2-butenyl acetate, (B) 2-methyl-2-butenyl- $d_3$  acetate, and (C) 2-methyl-2-butenyl- $d_7$  acetate.

peel fed labeled isoleucine but was not found in the aroma from intact fruit.

Esters were the major biosynthetic products produced from all substrates by both apple varieties. The mean percent distributions (mean  $\pm$  standard error of the mean, SEM) of deuterium-labeled compounds found in the aroma volatiles of Red Delicious and Granny Smith apples fed the various precursors are presented in Tables 2–5. Exposure of Red Delicious apples to EMB- $d_3$  resulted in both transesterified (39% of recovered labeled volatiles), into principally the butyl (18%) and hexyl (18%) esters, and reduction to alcohol (0.1%) and further esterified (57%) to 2-methylbutyl esters, principally to 2-methylbutyl acetate (49%). Further oxidation also gave a series of 2-methyl-2-butenyl esters which constituted 3% of the volatile biosynthetic products. With Granny Smith apples, transesterification of EMB to principally the butyl (49%) and hexyl (27%) esters was the major (91%) biosynthetic pathway. Only a minor amount of reduction (3%) to 2-methylbutyl

**Table 2. Deuterated Products (Mean Relative Percent  $\pm$  SEM) Determined in Headspace of Whole Apples after Exposure to Vapor of Deuterated Ethyl 2-Methylbutanoate**

| biosynthetic product                             | apple variety   |                 |
|--|-----------------|-----------------|
|  | Red Delicious   | Granny Smith    |
| ethyl 2-methylbutanoate- $d_3$                   | <i>a</i>        | <i>a</i>        |
| propyl 2-methylbutanoate- $d_3$                  | 1.50 $\pm$ 0.10 | 5.3 $\pm$ 2.2   |
| 2-methylpropyl 2-methylbutanoate- $d_3$          | nd <sup>b</sup> | 2.77 $\pm$ 0.56 |
| butyl 2-methylbutanoate- $d_3$                   | 17.9 $\pm$ 0.71 | 49.4 $\pm$ 12.3 |
| 2-methylbutyl- $d_3$ 2-methylbutanoate- $d_3$    | 0.68 $\pm$ 0.09 | 1.97 $\pm$ 0.25 |
| 2-methylbutyl 2-methylbutanoate- $d_3$           | 1.24 $\pm$ 0.21 | 3.8 $\pm$ 1.4   |
| pentyl 2-methylbutanoate- $d_3$                  | 0.66 $\pm$ 0.05 | 1.46 $\pm$ 0.28 |
| hexyl 2-methylbutanoate- $d_3$                   | 17.5 $\pm$ 3.5  | 27.0 $\pm$ 8.9  |
| heptyl 2-methylbutanoate- $d_3$                  | 0.37 $\pm$ 0.20 | 1.07 $\pm$ 0.24 |
| octyl 2-methylbutanoate- $d_3$                   | 0.22 $\pm$ 0.05 | nd              |
| total % 2-methylbutanoate- $d_3$ esters          | 38.8 $\pm$ 4.5  | 90.5 $\pm$ 1.7  |
| 2-methylbutyl- $d_3$ acetate                     | 48.9 $\pm$ 4.5  | 0.62 $\pm$ 0.03 |
| 2-methylbutyl- $d_3$ propionate                  | 1.53 $\pm$ 0.21 | tr <sup>b</sup> |
| 2-methylbutyl- $d_3$ butanoate                   | 1.10 $\pm$ 0.08 | 1.11 $\pm$ 0.47 |
| 2-methylbutyl- $d_3$ pentanoate                  | 0.06 $\pm$ 0.01 | nd              |
| 2-methylbutyl- $d_3$ hexanoate                   | 3.02 $\pm$ 0.29 | tr              |
| 2-methylbutyl- $d_3$ heptanoate                  | 0.26 $\pm$ 0.03 | tr              |
| 2-methylbutyl- $d_3$ octanoate                   | 1.90 $\pm$ 0.40 | nd              |
| total % 2-methylbutyl- $d_3$ esters <sup>c</sup> | 58.0 $\pm$ 4.2  | 3.61 $\pm$ 0.71 |
| 2-methyl-2-butenyl- $d_3$ acetate                | 2.14 $\pm$ 0.33 | nd              |
| 2-methyl-2-butenyl- $d_3$ 2-methylbutanoate      | 0.48 $\pm$ 0.07 | nd              |
| 2-methyl-2-butenyl- $d_3$ hexanoate              | 0.50 $\pm$ 0.03 | nd              |
| total % 2-methyl-2-butenyl- $d_3$ esters         | 3.13 $\pm$ 0.37 | 0.0             |
| 2-methylbutanol- $d_3$                           | 0.13 $\pm$ 0.01 | 6.0 $\pm$ 1.1   |

<sup>a</sup> Recovered precursor constituted 24.5  $\pm$  1.1% and 93.4  $\pm$  1.2% of the deuterated volatiles collected from Red Delicious and Granny Smith apples, respectively. <sup>b</sup> nd, not detected; tr, trace only. <sup>c</sup> Includes contribution from 2-methylbutyl- $d_3$  2-methylbutanoate- $d_3$ .

esters occurred with an increased proportion of the label (6%) being recovered as free 2-methylbutanol. No 2-methyl-2-butenyl esters were detected with this cultivar.

Exposure of apples to vapor of 2-methylbutanoic- $d_3$  acid produced a similar distribution of classes of metabolites as with EMB- $d_3$ . However for Red Delicious, incorporation into 2-methylbutanoates was increased relative to 2-methylbutyl derivatives, and less 2-methylbutanol was present. The distribution of individual metabolic products also differed between the two cultivars. While Red Delicious apples produced largely hexyl (42%) and butyl (10%) 2-methylbutanoates with little EMB (1.4%), Granny Smith gave 84% of the recovered label as EMB with less than 5% as hexyl 2-methylbutanoate.

Red Delicious apples converted 2-methylbutanol exclusively to the corresponding 2-methylbutyl esters, principally the acetate (72%) and hexanoate (14%). No deuterated 2-methylbutanoates or 2-methyl-2-butenyl esters were detected. Granny Smith apples also produced predominantly the 2-methylbutyl esters (94%), but limited oxidation to 2-methylbutanoates (7%) was also observed.

Incubation of apple skin with deuterated isoleucine produced a similar distribution of 2-methylbutanoates and 2-methylbutyl esters as was produced by exposing intact fruit to 2-methylbutanoic acid. Red Delicious apples converted deuterated isoleucine to deuterated

**Table 3. Deuterated Products (Mean Relative Percent  $\pm$  SEM) Determined in Headspace of Whole Apples after Exposure to Vapor of Deuterated Methylbutanoic Acid**

| biosynthetic product                             | apple variety    |                 |
|--|------------------|-----------------|
|  | Red Delicious    | Granny Smith    |
| ethyl 2-methylbutanoate- $d_3$                   | 1.39 $\pm$ 0.36  | 84.1 $\pm$ 3.5  |
| propyl 2-methylbutanoate- $d_3$                  | 1.40 $\pm$ 0.31  | 1.18 $\pm$ 0.36 |
| 2-methylpropyl 2-methylbutanoate- $d_3$          | tr <sup>a</sup>  | 0.32 $\pm$ 0.16 |
| butyl 2-methylbutanoate- $d_3$                   | 10.12 $\pm$ 0.46 | 0.73 $\pm$ 0.29 |
| 2-methylbutyl- $d_3$ 2-methylbutanoate- $d_3$    | 1.61 $\pm$ 0.27  | 0.67 $\pm$ 0.16 |
| 2-methylbutyl 2-methylbutanoate- $d_3$           | 0.83 $\pm$ 0.24  | 0.54 $\pm$ 0.06 |
| pentyl 2-methylbutanoate- $d_3$                  | 1.02 $\pm$ 0.14  | 0.25 $\pm$ 0.07 |
| hexyl 2-methylbutanoate- $d_3$                   | 41.9 $\pm$ 3.3   | 5.04 $\pm$ 2.65 |
| heptyl 2-methylbutanoate- $d_3$                  | 0.80 $\pm$ 0.17  | 0.09 $\pm$ 0.06 |
| octyl 2-methylbutanoate- $d_3$                   | 0.67 $\pm$ 0.21  | nd*             |
| total % 2-methylbutanoate- $d_3$ esters          | 58.8 $\pm$ 2.1   | 97.3 $\pm$ 0.6  |
| 2-methylbutyl- $d_3$ acetate                     | 26.1 $\pm$ 3.4   | 0.18 $\pm$ 0.10 |
| 2-methylbutyl- $d_3$ propionate                  | 1.37 $\pm$ 0.32  | tr              |
| 2-methylbutyl- $d_3$ butanoate                   | 0.92 $\pm$ 0.02  | 0.26 $\pm$ 0.08 |
| 2-methylbutyl- $d_3$ pentanoate                  | 0.08 $\pm$ 0.02  | nd              |
| 2-methylbutyl- $d_3$ hexanoate                   | 4.13 $\pm$ 1.21  | tr              |
| 2-methylbutyl- $d_3$ heptanoate                  | 0.51 $\pm$ 0.12  | tr              |
| 2-methylbutyl- $d_3$ octanoate                   | 3.85 $\pm$ 0.79  | nd              |
| total % 2-methylbutyl- $d_3$ esters <sup>b</sup> | 37.9 $\pm$ 1.6   | 1.11 $\pm$ 0.2  |
| 2-methyl-2-butenyl- $d_3$ acetate                | 1.60 $\pm$ 0.44  | nd              |
| 2-methyl-2-butenyl- $d_3$ 2-methylbutanoate      | 0.45 $\pm$ 0.09  | nd              |
| 2-methyl-2-butenyl- $d_3$ hexanoate              | 1.21 $\pm$ 0.34  | nd              |
| total % 2-methyl-2-butenyl- $d_3$ esters         | 3.21 $\pm$ 0.56  | 0.0             |
| 2-methylbutanol- $d_3$                           | 0.14 $\pm$ 0.01  | 1.64 $\pm$ 0.37 |

<sup>a</sup> nd, not detected; tr, trace only. <sup>b</sup> Includes contribution from 2-methylbutyl- $d_3$  2-methylbutanoate- $d_3$ .

**Table 4. Deuterated Products (Mean Relative Percent  $\pm$  SEM) Determined in Headspace of Whole Apples after Exposure to Vapor of Deuterated 2-Methylbutanol**

| biosynthetic product                    | apple variety   |                  |
|---|-----------------|------------------|
|   | Red Delicious   | Granny Smith     |
| ethyl 2-methylbutanoate- $d_3$          | nd <sup>a</sup> | 4.8 $\pm$ 2.7    |
| propyl 2-methylbutanoate- $d_3$         | nd              | tr <sup>a</sup>  |
| 2-methylpropyl 2-methylbutanoate- $d_3$ | nd              | tr               |
| butyl 2-methylbutanoate- $d_3$          | nd              | tr               |
| pentyl 2-methylbutanoate- $d_3$         | nd              | tr               |
| hexyl 2-methylbutanoate- $d_3$          | nd              | 1.64 $\pm$ 0.27  |
| total % 2-methylbutanoate- $d_3$ esters | 0.0             | 6.5 $\pm$ 3.0    |
| 2-methylbutyl- $d_3$ acetate            | 72.1 $\pm$ 9.6  | 24.3 $\pm$ 2.9   |
| 2-methylbutyl- $d_3$ propionate         | 3.07 $\pm$ 0.43 | 15.7 $\pm$ 1.2   |
| 2-methylbutyl- $d_3$ butanoate          | 3.07 $\pm$ 0.34 | 25.04 $\pm$ 0.63 |
| 2-methylbutyl- $d_3$ 2-methylbutanoate  | 2.56 $\pm$ 0.15 | unresolved       |
| 2-methylbutyl- $d_3$ pentanoate         | tr              | 2.18 $\pm$ 0.13  |
| 2-methylbutyl- $d_3$ hexanoate          | 14.2 $\pm$ 4.9  | 21.5 $\pm$ 1.2   |
| 2-methylbutyl- $d_3$ heptanoate         | 1.39 $\pm$ 0.62 | tr               |
| 2-methylbutyl- $d_3$ octanoate          | 5.2 $\pm$ 3.0   | 4.74 $\pm$ 0.03  |
| total % 2-methylbutyl- $d_3$ esters     | 100.0           | 93.5 $\pm$ 3.0   |
| 2-methylbutanol- $d_3$                  | <i>b</i>        | <i>b</i>         |

<sup>a</sup> nd, not detected; tr, trace only. <sup>b</sup> Recovered precursor constituted <0.4% and 49.0  $\pm$  4.3% of the deuterated volatiles collected from Red Delicious and Granny Smith apples, respectively.

2-methylbutanoates (61%), 2-methylbutyl esters (38%), and 2-methyl-2-butenyl esters (2%) (Table 5) in a ratio similar to that observed on feeding 2-methylbutanoic

**Table 5. Deuterated Products (Mean Relative Percent  $\pm$  SEM) Determined in Headspace of Apple Peel Treated with Isoleucine- $d_{10}$** 

| biosynthetic product                  | apple variety              |                  |
|---------------------------------------|----------------------------|------------------|
|                                       | Red Delicious <sup>a</sup> | Granny Smith     |
| methyl 2-methylbutanoate- $d_9$       | 4.22 $\pm$ 0.45            | 0.62 $\pm$ 0.09  |
| ethyl 2-methylbutanoate- $d_9$        | 50.7 $\pm$ 4.0             | 98.39 $\pm$ 0.13 |
| propyl 2-methylbutanoate- $d_9$       | 2.05 $\pm$ 0.41            | nd <sup>b</sup>  |
| butyl 2-methylbutanoate- $d_9$        | 1.27 $\pm$ 0.26            | nd               |
| hexyl 2-methylbutanoate- $d_9$        | 2.54 $\pm$ 0.23            | nd               |
| total 2-methylbutanoate- $d_9$ esters | 58.6 $\pm$ 2.0             | 99.02 $\pm$ 0.09 |
| 2-methylbutyl- $d_9$ acetate          | 34.4 $\pm$ 3.4             | nd               |
| 2-methyl-2-butenyl- $d_7$ acetate     | 1.70 $\pm$ 0.24            | nd               |
| 2-methylbutanol- $d_9$                | 3.10 $\pm$ 0.72            | 0.98 $\pm$ 0.08  |

<sup>a</sup> *n* = 2. <sup>b</sup> nd, not detected.

acid (or EMB). In contrast, Granny Smith apples gave 2-methylbutanoates (99%, 98% EMB) as the major biosynthetic product with 2-methylbutanol (1%) being the only other biosynthetic product detected.

**Biosynthesis of 2-Methylbutanoates in Red Delicious and Granny Smith Apples.** Isoleucine was considered the biosynthetic precursor of 2-methylbutanoic acid and its esters in apples (Paillard, 1990) by analogy with the biosynthesis of 2-methylpropyl esters from [<sup>14</sup>C]valine and 3-methylbutyl esters from [<sup>14</sup>C]leucine in bananas (Myers *et al.*, 1970; Tressl *et al.*, 1970), but, to our knowledge, this hypothesis has not previously been confirmed. The pattern of metabolic products observed here is consistent with a metabolic scheme where isoleucine is first metabolized to 2-methylbutanoic acid with subsequent reduction to 2-methylbutanol competing with direct esterification to 2-methylbutanoate esters. Formation of 2-methyl-2-butenyl esters in Red Delicious apples would seem to occur from 2-methylbutanoic acid, consistent with the known conversion of 2-methylbutyryl-CoA to 2-methyl-2-butenyl-CoA (tiglyl-CoA) which occurs during the  $\beta$ -oxidation of isoleucine to acetyl and propionyl-CoA (Ikeda and Tanaka, 1983).

Straight chain carboxylic acids (C2–C6) and their methyl esters (C4–C8) underwent transesterification or reduction to the corresponding alcohol (which was then esterified) when supplied as vapors to whole fruit or tissue slices of Cox's Orange Pippin and Golden Delicious apples (De Pooter *et al.*, 1981; Bartley *et al.*, 1985).  $\beta$ -Oxidation could also be a significant pathway leading to the production of chain-shortened esters (De Pooter *et al.*, 1983; Bartley *et al.*, 1985). Straight chain alcohols (C2–C8) were esterified to predominantly acetate (Knee and Hatfield, 1981; Bartley *et al.*, 1985) or butanoate esters (Paillard, 1979) or oxidized to aldehydes (Knee and Hatfield, 1981). Branched 2-methylbutanoates were similarly transesterified; however there were distinctive differences both in the products formed and in the biosynthetic capability of the two apple varieties examined. Formation of 2-methylbutyl and 2-methyl-2-butenyl esters, principally the acetates, was characteristic of Red Delicious apples. This predominance of 2-methylbutyl esters is perhaps a consequence of the irreversible reduction of 2-methylbutanoic acid to 2-methylbutanol which seems to occur in this variety. Production of 2-methylbutanoate esters and especially EMB appeared characteristic of Granny Smith apples; isoleucine produced almost exclusively EMB (98%), and 2-methylbutanoic acid gave 84% recovered label as EMB. With Red Delicious, 2-methylbutanoate esters were lesser contributors to the aroma volatiles and



occurred largely as the hexyl esters. Production of 2-methyl-2-butenyl esters by Granny Smith apples was not observed. Free alcohols and aldehydes (Bartley and Stevens, 1981) were not observed as major biosynthetic products in this study which may reflect the undamaged state of the apple tissue.

While unlabeled aroma precursors have been used to identify the biosynthetic origins and relationships between aroma constituents in apples (DePooter *et al.*, 1983; Bartley *et al.*, 1985; Paillard, 1990), the variability encountered in the measurement of the concentrations of the aroma volatiles limits the usefulness of this method for the identification of changes in the concentrations of major biosynthetic products. Use of deuterium-labeled precursors enables unambiguous identification of both major and minor biosynthetic products in complex matrices (Allen *et al.*, 1992; Silberzahn and Tressl, 1993). Chromatographic or mass spectrometric separation of labeled from endogenous aroma volatiles provides quantitative measures of the extent of conversion of label into metabolic products. In the present case, deuterium incorporation has confirmed isoleucine as the biosynthetic precursor of several important aroma constituents of apples and enabled the detection and identification of new and minor metabolic products (2-methyl-2-butenyl esters). Deuterium labeling should prove useful in the study of other biosynthetic pathways which lead to aroma formation and to elucidate the changes in biosynthetic capacity which leads to loss of aroma during long term or controlled atmosphere storage of fruit.

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