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Biogenesis of Volatile Methyl Esters in Snake Fruit (*Salacca edulis*, Reinw) cv. *Pondoh*

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The methyl esters of carboxylic acids are characteristic olfactory volatile compounds for the sweet aroma of snake fruit, (*Salacca edulis*, Reinw) cv. *Pondoh*. Although methanol was not detected as a volatile constituent, the crude enzymes showed activity to synthesize the methyl esters in the presence of acyl-CoA and methanol. Therefore, the biosynthetic origin of methanol was investigated, resulting in the detection of pectin methyl transferase activity in the flesh. This pectin methyl transferase activity increased during fruit maturation, in parallel with the level of methanol originating from hand-squeezed juice and with the methyl esters extracted from flesh of the fruit. Based on these results, the origin of methanol was confirmed to be the methyl esters of pectins. The crude enzyme also catalyzed the formation of methyl hexanoate, one of the esters of the fruit, in the presence of methyl pectins and hexanoyl-CoA that were used as precursors for a model reaction.

Key words: snake fruit; *Salacca edulis*, Reinw, cultivar *Pondoh*; pectin methyl-esterase; methyl ester biogenesis

We have previously reported that the methyl esters of short-chain carboxylic acids were the major volatile compounds in snake fruit (*Salacca edulis*, Reinw) cv. *Pondoh*.[1)] These esters are responsible for the sweet and fruity character of the fruit, and their olfactory contribution is about 68% of the total. Esters of carboxylic acids have been reported to be the major aromatic compounds in such fruits as bananas,[2)] strawberries,[3)] melons,[4)] pineapples,[5)] soursop,[6)] and guava.[7)] The major esters were acetates of ethanol, 2-butanol and isoamyl alcohol for banana, butanoates of methanol, ethanol and ethyl hexanoate for strawberry, acetates of hexanoyl alcohol, isoamyl alcohol, octyl alcohol and benzyl alcohol for melon, and ethyl acetate and ethyl 3-(methylthio)propanoate for pineapple. Ester formation in fruit has been intensively studied. In numerous fruits such as banana, strawberry, and melon, ester biosynthesis has been studied in relation to the alcohol acyltransferase (AAT) activity.[2–4)] The AAT activity in unripe fruits was low, but steadily increased during ripening. Methyl esters have also been observed as the major volatiles (>70%) in soursop, a tropical fruit, although there was no information on their methyl ester biogenesis.[6)] The flesh of snake fruit has been found to contain short chain-carboxylic acids, but methanol was not detected by the solvent assisted flavour evaporation (SAFE) method when the flesh was extracted in the presence of $CaCl_2$.[1)] This was the primary reason for investigating the biosynthesis of methyl esters in this fruit. We focused on investigating the origin of methanol in the fruit flesh in order to clarify the mechanism for ester formation. We describe here the detection of methanol in the fruit after pectin a methyl esterase (PME) treatment, and a possible mechanism for volatile compound formation.

Materials and Methods

Chemicals and biochemicals. All the reagents were of analytical grade and were purchased from Sigma, except for anhydrous sodium sulfate (Kanto Chemicals, Japan).

Plant materials. Snake fruits (*Salacca edulis*, Reinw), cultivar *Pondoh*, were obtained from Indonesia, and were harvested at various stages,

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defined as already reported:[1] stage 1 (3.5 months after pollination); stage 2 (4.0 months after pollination); stage 3 (4.5 months after pollination); stage 4 (5 months after pollination); stage 5 (5.5 months after pollination); and stage 6 (6.0 months after pollination). The samples were sent to Japan by air and stored at −80°C until being used.

Methanol determination. Hand-squeezed fruit juice (10 g of flesh at each maturation stage; 5 ml) was kept for 30 min at ambient temperature. Ethyl decanoate (0.15 μmol in 2000 μl of diethyl ether) was added as an internal standard. The juice was extracted with diethyl ether (1:3, 3 times), dried over anhydrous sodium sulfate, and concentrated to 100 μl. A sample volume of 1 μl was injected into a Hitachi G-3000 GC instrument with a split ratio of 1:40. A TC-Wax column (df 0.25 μm, 0.25 mm i.d. × 30 m) was used under the following temperature program: 5 min isothermal at 40°C and then increased incrementally to 150°C at rate 3°C/min. The amount of methanol was calculated from the regression curve ($y = 0.069x + 0.0032$; y = methanol in μmol and x = methanol/ethyl decanoate; $R^2 = 0.98$) that had been obtained by GC analyses of the mixtures of methanol and ethyl decanoate.

Crude enzyme preparation. The crude enzyme was prepared according to the method of Barnavon *et al.*[8] Briefly, ground snake fruit (5 g each) was homogenized in 10 ml of 0.5 M Tris-HCl buffer at pH 8.5 containing 1% Triton X-100, 20% glycerol, 5% polyvinyl polypyrrolidone (PVPP), 2 M NaCl, and 20 mM β-mercaptoethanol, and then stirred for 15 h at 4°C. The homogenate was centrifuged at 17,000 *g* for 30 min and the pellet re-homogenized in the same manner. The resulting supernatants were pooled, and ammonium sulfate was slowly added up to a concentration of 80%, the resulting suspension being stirred for 2 h and left to stand overnight. The precipitate was removed and dissolved in a 0.1 M phosphate buffer (pH 7.5), and the solution dialyzed and centrifuged to give the crude enzyme extract. The protein content was not evaluated.

Measurement of the pectin methyl esterase activity. PME activity was detected according to the method of Barnavon *et al.*[8] by following the release of methanol from esterified pectin (89% methylated citrus pectin, designated as methyl pectins, Sigma). The methanol generated from the pectins was oxidized, and the resulting formaldehyde was spectrophotometrically quantified as described by Klavon and Bennet.[9]

Ester formation assays. In experiment 1, the crude enzyme (100 μl) already mentioned was mixed with 6 mM methanol and 0.36 mM hexanoyl-CoA in an assay buffer (0.1 M phosphate at pH 7.5)[4] in a 5-ml sealed tube for 15 h at 35°C. The final volume of the reaction mixture was 1000 μl. In experiment 2, the crude enzymes (100 μl), hexanoyl-CoA (0.36 mM), and methyl pectins (1.5%) in the buffer (1000 μl final volume) were incubated for 15 h at 35°C. In experiment 3, the crude enzyme (100 μl) and methyl hexanoate (0.36 mM) in the buffer (1000 μl final volume) were incubated for 15 h at 35°C. Pentane (2 ml) was added to each reaction mixture, which was then vigorously vortexed for 30 s. The combined pentane layer (extracted 3 times) containing methyl hexanoate (for exps. 1 and 2), and hexanoic acid (for exp. 3) was separated by centrifugation (0°C, 3000 *g*), transferred to a glass tube, and dried over anhydrous sodium sulfate. Methyl hexanoate was detected either by GC-MS (for exp. 1) or GC-SIM (for exp. 2), and hexanoic acid by GC-SIM (for exp. 3), after concentrating the combined pentane layer to 300 μl under a nitrogen stream and injecting 1 μl for GC-MS. To detect methyl hexanoate, the characteristic ions at *m/z* 99, *m/z* 87, *m/z* 74, and *m/z* 59 were monitored. The ions at *m/z* 87, *m/z* 73, *m/z* 60 and *m/z* 41 were monitored for hexanoic acid. The conditions for GC-MS and GC-SIM are respectively shown in the legends to Figs. 2 and 3.

Results and Discussion

Methanol detection in hand-squeezed fruit juice and PME activity during fruit maturation

In our previous experiment, the methanol concentration was 0.01% of the total volatile compounds of snake fruit extracted by the SAFE[1,10] method in the absence of $CaCl_2$. However only a trace amount of methanol was detected when the flesh was extracted by the same method in the presence of $CaCl_2$,[1] which is known as an enzyme inhibitor. Therefore, we expected the presence of endogenous enzymes, which may hydrolyze methyl esters of the volatile compounds or large-molecule methyl esters, to result in the release of methanol. When the fruit juice prepared in the absence of $CaCl_2$ was kept at ambient temperature for 30 min, methanol was released. As shown in Fig. 1, the concentration of methanol increased as the fruit matured, reaching a maximum level in the juice obtained from the fruit at stage 6 (0.08 μmol/g of flesh). PME is a ubiquitous cell wall-bound enzyme that catalyzes the hydrolysis of methyl-ester groups from galacturonosyl residues.[8,11] Frenkel *et al.* have reported that the methanol content increased during the development of wild-type tomato, and that PME was the key enzyme for methanol production.[12] This observation was suggested to coincide with changes in the water-soluble pectin properties. Water-soluble pectin of tomato increased during maturation, while the degree of methylation decreased.[13] Methanol possibly pro-

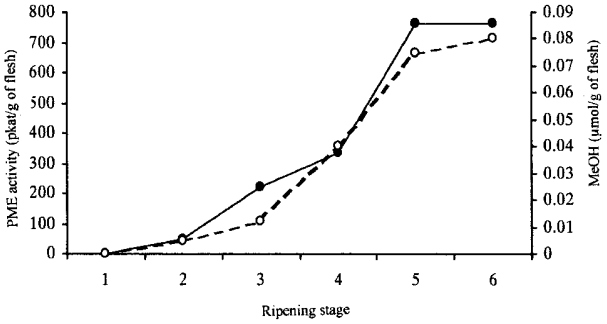

Fig. 1. Changes in Methanol Concentration in Snake Fruit Juice and Pectin Methyl Esterase: PME Activity during the Maturation of Snake Fruit.

○, Methanol concentration; ●, PME activity. Maturation stages are as described in the text.

duced by the action of PME has been reported as one of the undesirable contaminants in wine.[14] The presence of methanol has also been reported in fresh grapefruit juice and unpasteurized orange juice due to the action of naturally occurring PME in the fruits.[15,16] These observations and the results of previously reported work strongly suggest that methanol would be released from water-soluble pectin by the action of endogenous enzymes such as PME during the storage of snake fruit juice at ambient temperature.

As shown in Fig. 1, the PME activity of snake fruit increased with maturation stage, reaching a maximum level (about 760 pkt/g of flesh) at stages 5 and 6. This observation is similar to those previously reported during tomato and avocado ripening.[17,18] The PME activity during fruit maturation coincided with the concentration of methyl esters. The ester levels of pentane extracts resulting from microwave extraction were 0.09–3.07 μg/g flesh until stage 3, then rapidly increasing after stage 4 to exceed the level of carboxylic acids. The ratio of esters/carboxylic acids was 0.02–0.83 until stage 4, reaching 2.45 and 2.03 at stages 5 and 6, respectively.[1] Thus, the esters, an important contributor to the snake fruit aroma, were mainly biosynthesized between stages 4 and 6 to reach the highest level (21.7, 32.0 μg/g flesh at stages 5 and 6, respectively). Similar phenomena have been observed with guava,[7] peach,[19,20] strawberry,[21] and grape.[22]

Methyl ester formation by the crude enzyme (exps. 1, 2 and 3)

The major methyl esters found in snake fruit were methyl 3-methylpentanoate (13.3%) and methyl 3-methyl-2-pentenoate (35.2%). Unfortunately, acyl-CoAs corresponding to those methyl esters could not be used as model precursors for a methyl ester formation study, because they are not commercially available. Therefore, the formation of methyl hexanoate, which accounted for 4.7% of the total volatile compounds, was examined in this study instead of the dominant methyl esters. We confirmed the formation of methyl hexanoate from the reaction mixtures by comparing either the mass fragmentation patterns of MS spectra, MS chromatographic profiles, and/or SIM profiles of the sample solutions with those of authentic methyl hexanoate. To detect methyl hexanoate, the characteristic fragment ions of m/z 99 $(M\text{-}OCH_3)^+$, m/z 87 $(CH_2CH_2COOCH_3)^+$, m/z 74 $(CH_3OC(CH_2)=OH)^+$, and m/z 59 $(COOCH_3)^+$ were traced as shown in Figs. 2 and 3. In exp. 1, the GC-MS chromatographic analysis of the pentane extract clearly revealed the formation of methyl hexanoate, as shown in Figs. 2 and 3. A peak was detected at t_R 7.38 min (Fig. 2A), which was identical to that of authentic methyl hexanoate (Fig. 2E), on the chromatogram of the extract of the reaction mixture. This peak was not detected in the extract (Fig. 2C) containing the crude enzyme alone, nor in the reaction mixture (Fig. 2B) containing methanol and hexanoyl-CoA in the absence of the crude enzyme. The MS spectrum of the peak at t_R 7.38 min in Fig. 2A was identical to that of authentic methyl hexanoate (Fig. 2E). We therefore confirmed the formation of methyl hexanoate in the reaction mixture. The yield of methyl hexanoate detected in the reaction mixture was 1.0 mol% of hexanoyl-CoA, and this is likely to have been the result of low alcohol acyltransferase activity of the crude enzyme.

We also confirmed the formation of methyl hexanoate in exp. 2 (Fig. 3). GC-SIM traces showed peaks at t_R 6.67 min in the ratio of 13/29/100/33 = m/z 99/m/z 87/m/z 74/m/z 59 (Fig. 3A) that coincided with those (t_R 6.67 min; a ratio of 13/25/100/28 for m/z 99/m/z 87/m/z 74/m/z 59) for authentic methyl hexanoate (Fig. 3C). The yield of methyl hexanoate was 0.04 mol% under the conditions used. Although peaks were detected at around the t_R value for methyl hexanoate in the absence of methyl pectins (Fig. 3B), the ion intensity at m/z 74 was less than 10% that in Fig. 3A, and very small peak was detected at t_R 6.67 on the trace of m/z 59. Therefore, methyl hexanoate was produced in a trace amount by the crude enzyme in the absence of methyl pectins. It is suggested that PME was responsible for releasing methanol from methyl pectin. We tried to ascertain whether the crude enzyme would hydrolyze methyl hexanoate or not (Exp. 3). No detectable amount of hexanoic acid (at t_R of 33.41 min) was released in the reaction mixture consisting of methyl hexanoate and the crude enzyme, and indicating that the crude enzyme showed hydrolytic activity only toward methyl pectins and not toward methyl hexanoate. Therefore, the methyl esters of carboxylic acid were generated from acyl-CoAs and methanol essentially formed by the action of the crude enzymes in the presence of methyl pectins. Methyl ester formation using SAM (*S*-adenosyl-L-methionine) as the methyl donor[23] was

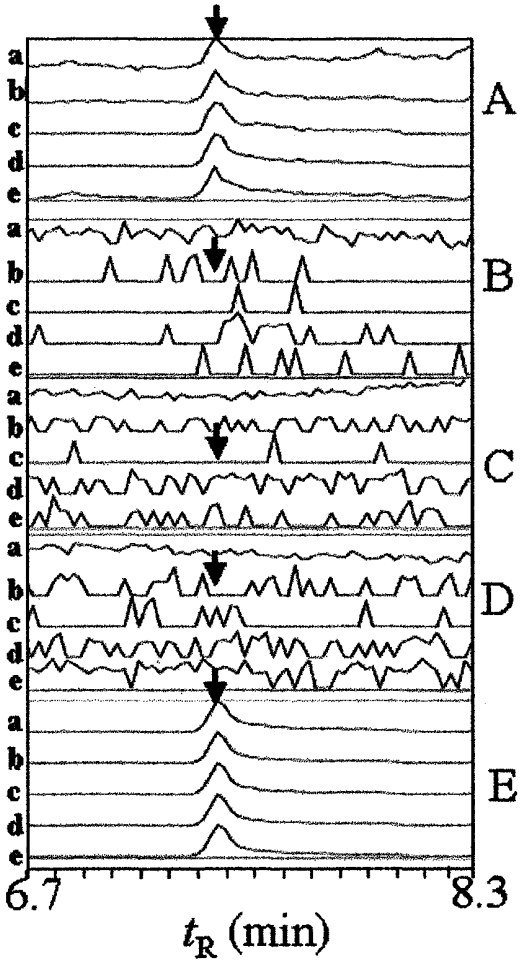

Fig. 2. MS Chromatograms of Enzymatic Reaction Mixtures and Methyl Hexanoate Standards.

A, crude enzyme + hexanoyl-CoA, and MeOH, a: TIC = 47.57, b: m/z 99 = 5.15, c: m/z 87 = 5.15, d: m/z 74 = 5.11, e: m/z 59 = 5.11; B, hexanoyl-CoA + MeOH, a: TIC = 12.5, b: m/z 99 = 0.11, c: m/z 87 = 0.11, d: m/z 74 = 0.10, e: m/z 59 = 0.11; C, crude enzyme, a: TIC = 38.46, b: m/z 99 = 0.14, c: m/z 87 = 0.14, d: m/z 74 = 0.13, e: m/z 59 = 0.13; D, crude enzyme + hexanoyl-CoA, a: TIC = 45.37, b: m/z 99 = 0.11, c: m/z 87 = 50.11, d: m/z 74 = 0.11, e: m/z 59 = 0.11; and E, methyl hexanoate authentic standard, a: TIC = 1765.13, b: m/z 99 = 46.6, c: m/z 87 = 46.2, d: m/z 74 = 46.7, e: m/z 59 = 46.9. GC was conducted with Hewlett Packard 5890 instrument equipped with a TC-Wax column (0.25 mm ID, 30 m length and 0.25 μm film thickness) under a program 5 min isothermal at 50°C, raised to 150°C at a rate 3°C/min, and then ramped at 10°C to 200°C. Splitless injection was used. GC/MS: Jeol JMS-DX 302 (m/z 30–300, 70 eV).

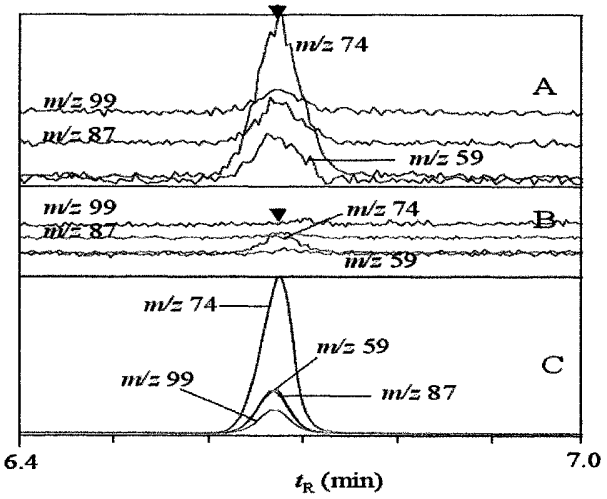

Fig. 3. GC-SIM Trace of Methyl Hexanoate and Extracts from the Reaction Mixtures.

A, crude enzyme + hexanoyl-CoA + methyl pectins, a: m/z 99 = 150, b: m/z 87 = 392, c: m/z 74 = 1310, d: m/z 59 = 430; B, crude enzyme + hexanoyl-CoA, a: m/z 99 = 1, b: m/z 87 = 8, c: m/z 74 = 121, d: m/z 59 = 1; and C, methyl hexanoate standard, a: m/z 99 = 572,500, b: m/z 87 = 139,540, c: m/z 74 = 558,140, d: m/z 59 = 156,260). GC: Shimadzu GC-17 A; GC/MS Shimadzu-QP 5050 (m/z 30–300, 70 eV). The column type and temperature program same as those described in Fig. 2, except for the injection split ratio being 1:25.

also examined. However, we could not confirm the formation of methyl hexanoate either by GC or GC-MS.

Based on these results, we propose the possible *in vitro* biosynthetic pathway for methyl ester formation of snake fruit as methanol released from methyl pectins by the action of PME being transferred to acyl-CoA by the action of an ester synthase.

In conclusion, the major olfactory contributors, the methyl esters of carboxylic acids, were confirmed to be enzymatically synthesized from corresponding acyl-CoAs in the presence of methyl pectins and crude enzymes such as PME. This is the first report on the biogenesis of methyl esters of short chain-carboxylic acids in fruits.

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References

1) Supriyadi, Suhardi, Suzuki, M., Yoshida, K., Muto, T., Fujita, A., and Watanabe, N., Changes in the volatile compounds and in the chemical and physical properties of snake fruit (*Salacca edulis*, Reinw) cv. *Pondoh* during maturation. *J. Agric. Food Chem.*, **50**, 7627–7633 (2002).
2) Ueda, Y., Tsuda, A., Ba, J. H., Fujishita, N., and Chachin, K., Characteristic pattern of aroma ester formation from banana, melon, and strawberry with reference to the substrate specificity of ester synthase and alcohol contents in pulp. *Nippon Shokuhin Kogyo Gakkaishi*, **39**, 183–189 (1992).
3) Perez, A. G., Sanz, C., Olias, R., Rios, J. J.,

and Olias, J. M., Evolution of tsrawberry alcohol acyltransferase activity during fruit development and storage. *J. Agric. Food Chem.*, **39**, 3286–3290 (1996).
4) Shalit, M., Katzir, N., Tadmor, Y., Larkov, O., Burger, Y., Shalekhet, F., Lastochin, E., Ravid, U., Amar, O., Edelstein, M., Karchi, Z., and Lewinshon, E., Acetyl-CoA: alcohol acetyltransferase activity and aroma formation in ripening melon fruits. *J. Agric. Food Chem.*, **49**, 794–799 (2001).
5) Umano, K., Hagi, Y., Nakahara, K., Shoji, A., and Shibamoto, T., Volatile constituents of green and ripened pineapple (*Ananas comosus* [L.] Merr.). *J. Agric. Food Chem.*, **40**, 599–603 (1992).
6) MacLeod, A. J., and Pieris, N. M., Volatile flavor components of soursop (*Annona muricata*). *J. Agric. Food Chem.*, **29**, 488–490 (1981).
7) Chyau, C. C., Chen, S. Y., and Wu, C. M., Differences of volatile and nonvolatile constituents between mature and ripe guava (*Psidium guajava* Linn) fruits. *J. Agric. Food Chem.*, **40**, 846–849 (1992).
8) Barnavon, L., Doco, T., Terrier, M., Ageorges, A., Romieu, C., and Pellerin, P., Involvement of pectin methyl-esterase during the ripening of grape berries: partial cDNA isolation, transcript expression and changes in the degree of methyl-esterification of cell wall pectins. *Phytochem.*, **58**, 693–701 (2001).
9) Klavon, J. A., and Bennett, R. D., Determination of methanol using alcohol oxidase and its application to methyl ester content of pectins. *J. Agric. Food Chem.*, **34**, 597–599 (1986).
10) Engel, W., Bahr, B., and Schieberle, P., Solvent assisted flavor evaporation—a new and versatile technique for the careful and direct isolation of aroma compounds from complex food matrices. *Eur. Food Res. Technol.*, **209**, 237–241 (1999).
11) Denes, J. M., Baron, A., and Drilleau, J. F., Purification, properties and heat inactivation of pectin methylesterase from apple (cv. Golden Delicious). *J. Sci. Food Agric.*, **80**, 1503–1509 (2000).
12) Frenkel, C., Peters, J. S., Tieman, D. M., Tiznado, M. E., and Handa, A. K., Pectin methylesterase regulates methanol and ethanol accumulation in ripening tomato (*Lycopersicon esculentum*) fruit. *J. Biol. Chem.*, **273**, 4293–4295 (1998).
13) Inari, T., Yamauchi, R., Kato, K., and Takeuchi, T., Changes in pectic polysaccharides during the ripening of cherry tomato fruits. *Food Sci. Technol. Res.*, **8**, 55–58 (2002).
14) Damberg, R. G., Kambouris, A., Francis, I. L., and Gishen, M., Rapid analysis of methanol in grape-derived distillation products using near-infrared transmission spectroscopy. *J. Agric. Food Chem.*, **50**, 3038–3084 (2002).
15) Cadwallader, K. R., and Xu, Y., Analysis of volatile components in fresh grapefruit juice by purge and trap/gas chromatography. *J. Agric. Food Chem.*, **42**, 782–784 (1994).
16) Moshonas, M. G., and Shaw, P. E., Quantitative determination of 46 volatile constituents of fresh and unpasteurized orange juices using dynamic head space gas chromatography. *J. Agric. Food Chem.*, **42**, 1525–1528 (1994).
17) Gaffe, P., Tieman, D. M., and Handa, A. K., Pectin methylesterase isoforms in tomato (*Lycopersicon esculentum*) tissues. *Plant Physiol.*, **105**, 199–203 (1994).
18) Sakurai, N., and Nevins, D. J., Relationship between fruit softening and wall polysaccharides in avocado (*Persea Americana* mill) mesocarp tissues. *Plant Cell Physiol.*, **38**, 603–610 (1997).
19) Chapman, G. W., Horvart, R. J., and Forbus, W. R., Physical and chemical changes during the maturation of peaches (cv. Majestic). *J. Agric. Food Chem.*, **39**, 867–870 (1991).
20) Robertson, J. A., Meredith, F. I., Horvart, R. J., and Senter, D. S., Effect of cold storage and maturity on the physical and chemical characteristics and volatile constituents of peaches (cv. Cresthaven). *J. Agric. Food Chem.*, **38**, 620–624 (1990).
21) Perez, A. G., Rios, J. J., and Olias, J. M., Aroma components and free amino acids in strawberry variety chandler during ripening. *J. Agric. Food Chem.*, **40**, 2232–2235 (1992).
22) Shure, K. B., and Acree, T. E., Changes in the odor-active compounds in *Vitis labruscana* cv. concord during growth and development. *J. Agric. Food Chem.*, **42**, 350–353 (1994).
23) Dudareva, N., Raguso, R. A., Wang, J., Ross, J. R., and Pichersky, E., Floral scent production in clarkia brewery. III. Biosynthesis and emission of benzenoic esters. *Plant Physiol.*, **116**, 599–604 (1998).