Studies on Flavor Development in Strawberries. 4. Biosynthesis of Volatile Alcohol and Esters from Aldehyde during Ripening

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Strawberries were sampled at six different stages of maturity (5–45 days after blooming). A whole strawberry fruit at each stage was incubated in a flask with 5 μL of pentanal at 30 °C for 1 h. The headspace gas analysis by gas chromatography revealed the following facts: (1) the reduction of pentanal to 1-pentanol by strawberry fruit occurs at every stage of maturity, and the reduction rate is stimulated with progress of maturity; (2) the biosynthesis of 1-pentyl esters of volatile fatty acids via 1-pentanol formation from pentanal occurs not in immature fruit but in mature fruit. The generation of enzyme systems concerning alcohol and ester formation from aldehyde is discussed. It was confirmed that volatile free fatty acids in strawberries played an important role in ester formation. The method of determination of volatile fatty acid in strawberries by gas chromatography is also briefly discussed.

Development of flavor in fruits and vegetables is one of the most prominent changes that occurs during maturation before harvest and storage thereafter. Pitsarnitskii et al. (1970) reported the changes in volatile carbonyl compounds in strawberries during the maturization process. Tressl et al. (1969) isolated and identified 214 volatile compounds from ripe strawberries. Among them are esters as the major component, followed by alcohols and carbonyl compounds.

Recent evidence indicates that ester formation can be induced by addition of alcohol or aldehyde to ripe strawberry fruit (Yamashita et al., 1975, 1976a). The ester formation was located in the separated mesocarp cells and in the protoplasts of strawberry (Ueda et al., 1976).

In this paper, biosynthesis of volatile alcohol and esters from aldehyde in strawberries at different stages of maturity and relationship between ester formation and volatile fatty acid in strawberries are discussed.

MATERIALS AND METHODS

Strawberries. Strawberries (*Fragaria ananassa* Duchesne var. *Hoko*) used were grown at Chibaken Agricultural Experiment Station in 1976. Samples were collected at 5, 10, 20, 30, 40, and 45 days after blooming.

Reaction of Pentanal with Strawberries. A whole strawberry fruit was placed in a wide-mouthed, 100-mL Erlenmyer flask and 5 μL of pentanal was added onto the inside wall of the flask. Then the flask was sealed with a glass stopper equipped with a small silicone rubber septum and kept at 30 °C in a water bath in the dark for 1 h.

Control Test. A whole strawberry was incubated in a flask without addition of pentanal under the same conditions as mentioned above.

Analysis of Headspace Volatiles. Three milliliters of vapor sample in the flask was taken out through the silicone rubber septum by means of a gas-tight syringe and injected into a gas chromatograph equipped with a flame ionization detector. The two gas chromatographic systems (25% PEG 1000 and 30% Silicone DC 550 on Chromosorb W AW) were employed for the identification and determination of pentanal, 1-pentanol, and 1-pentyl esters as described elsewhere (Yamashita et al., 1976a). The concentration of volatile compounds was determined by a digital integrator and shown by the area counts. Pentanal, 1-pentanol, 1-pentyl acetate, 1-pentyl *n*-butyrate, and 1-pentyl isocaproate were identified by GC–MS recorded at 70 eV, comparing with their authentic samples.

Determination of Volatile Fatty Acids in Strawberries. The slurry was prepared by homogenizing 50 g of strawberry fruits in a Waring Blendor with 50 mL of cold water, and 4 N H_3PO_4 was added to adjust the pH to 2. The volatile fatty acids were distilled using a rotary evaporator into a flask containing 100 mL of water and 10 mL of 0.1 N NaOH. The volatile fatty acids in strawberry fruits were trapped as nonvolatile sodium salts. Then the distillate was concentrated and lyophilized in a small flask. Sodium salts of volatile fatty acids were esterified with sulfuric acid and 1-butanol (Yamashita et al., 1973) and determined by gas chromatography (Yamashita et al., 1974) using 4 m × 3 mm i.d. glass column of 30% Silicone DC 550 on Chromosorb W AW 60–80 mesh with programmed oven temperature from 90 to 190 °C at 2 °C/min.

Assay of Color Intensity of Strawberries. Twenty-five grams of strawberries were homogenized with 100 mL of ethanol containing 1% of HCl. The homogenate was filtered through the No. 2 filter paper, and then 5 mL of the filtrate was diluted with 5 mL of 80% ethanol containing 1% HCl. The color intensity was measured with spectrophotometer at 520 nm.

RESULTS AND DISCUSSION

Control Test. Tressl et al. (1969) found 91 esters including 1-pentyl acetate, 1-pentyl *n*-butyrate, and 1-pentyl *n*-caproate in the strawberry oil component which was prepared from 25 kg of strawberry fruit. On the contrary, only one strawberry fruit (less than 15 g) was used in the present investigation, and headspace gas analysis was carried out without any concentration procedure. Presumably for this reason, none of 1-pentyl esters were detected in the control samples. In fact, we reported the emanation of methyl acetate, methyl *n*-butyrate, ethyl acetate, and ethyl *n*-butyrate from a mature strawberry fruit during the incubation in the flask at 30 °C for 1 h. However, the esters of C_3–C_6 alcohol were not produced unless relatively high concentrations of these alcohols or corresponding aldehydes were incubated with strawberry fruit (Yamashita et al., 1975, 1976a).

As the similar results concerning the control tests were reported in the previous reports (Yamashita et al., 1975, 1976a), the gas chromatograms for the control samples were omitted in this paper.

Influence of Maturation on the Profiles of Volatile Compounds Produced by Incubation of Pentanal with Strawberries. Chromatograms from selected maturation levels illustrate the changes in the formation of alcohol and esters from aldehyde with increasing ma-

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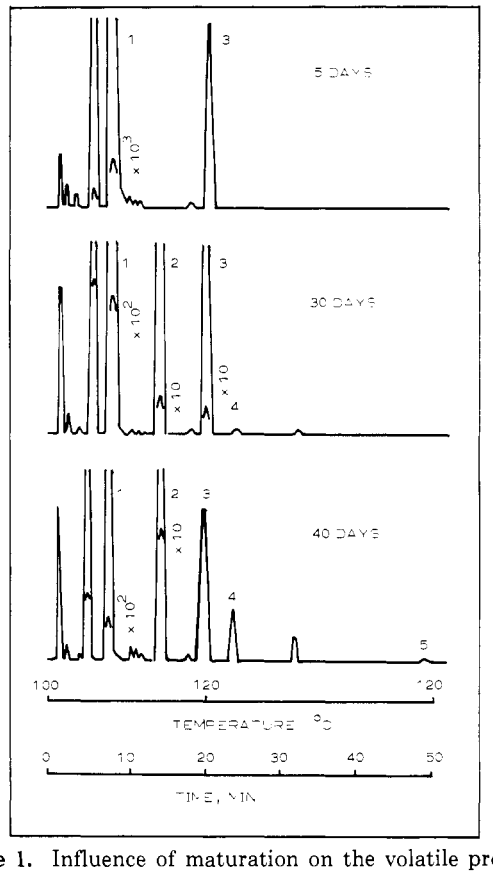

Figure 1. Influence of maturation on the volatile profile of strawberry reacted with pentanal: 1, pentanal; 2, 1-pentyl acetate; 3, 1-pentanol; 4, 1-pentyl *n*-butyrate; 5, 1-pentyl *n*-caproate; GC, 4 m × 3 mm i.d. glass column of 25% PEG 1000 on Chromosorb W AW 60–80 mesh; injection port and detector temperature, 150 °C; nitrogen, hydrogen, and air flow rate, 20, 20, 800 mL/min, respectively.

turity (Figure 1). An immature fruit at 5 days after blooming converted the added pentanal to 1-pentanol but no ester formation was observed. Esterification of 1-pentanol which was transformed from pentanal was found at 30-day and 40-day samples. 1-Pentyl acetate was dominant among the esters produced. 1-Pentyl *n*-butyrate and trace amount of 1-pentyl *n*-caproate was detected as it got ripened.

Formation of Alcohol from Aldehyde. The pattern of 1-pentanol formation in Figure 2 shows that the incubation of pentanal with a strawberry fruit causes the conversion of pentanal to 1-pentanol at every stage of maturity. The concentration of 1-pentanol reached its maximum at 20 days, and thereafter it continued to decrease until 45 days (over-maturation). However, considering the continuous decrease of residual pentanal from 5 to 45 days, the conversion of pentanal to 1-pentanol was intensified while ripening. The reason why the decrease of 1-pentanol concentration after 30 days should be due to the transformation of 1-pentanol to 1-pentyl esters, which was observed not during the initial stages but during the matured stages.

These results suggest that alcohol dehydrogenase (Yamashita et al., 1976b) activity per fruit continue to increase from the initial stage to the fully ripened stage.

Formation of Esters from Aldehyde Via Alcohol. In spite of the formation of 1-pentanol, production of 1-pentyl ester was not found before 30 days (Figure 2). Though it was in a small quantity, strawberry fruit at 30 days after blooming was able to esterify 1-pentanol with acetic acid and *n*-butyric acid. The amount of 1-pentyl acetate and

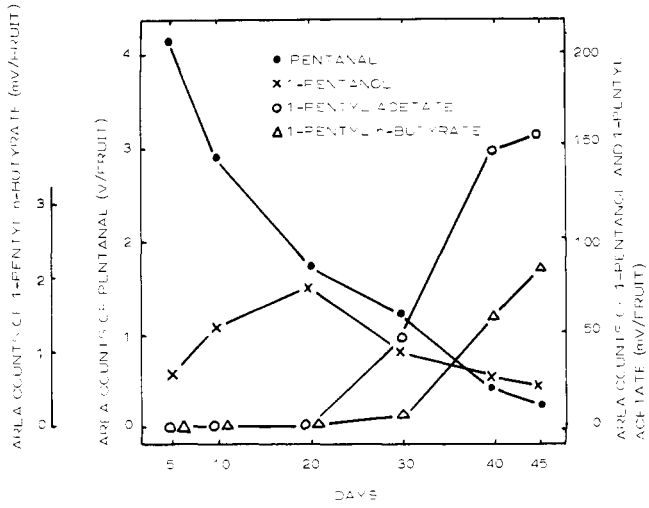

Figure 2. Influence of maturation on the formation of 1-pentanol and 1-pentyl esters from pentanal per fruit.

Table I. Change in Strawberry Characteristics during Maturation

Days after blooming	Weight of fruit, g	Color intensity absorbance, 520 nm	Sensory evaluation: Sweetness	Sensory evaluation: Flavor
5	0.37	0.038	–	No characteristic strawberry flavor
10	0.91	0.038	–	
20	2.50	0.040	–	
30	6.30	0.198	±	Faint strawberry flavor
40	15.00	0.540	+	Matured strawberry flavor
45	15.00	1.355	+	

1-pentyl *n*-butyrate was remarkably increased between 30 and 40 days. The change in the ester formation during the maturation paralleled well with the variation in strawberry characteristics such as weight and color intensity of fruit (Table I). Though 1-pentyl ester of *n*-caproic acid was also detected besides the esters of acetic acid and *n*-butyric acid, it was omitted from Figure 2 because of its low quantity.

In order to confirm these results, experiments were performed with 15 g of strawberries (leveled weight among the stages of maturity). Consequently the experiment did not give any new information concerning the ester formation (Figure 3). However, for the reduction of pentanal, Figure 3 shows that it is more rapid in immature fruit than mature fruit. In fact, no residual pentanal was detected at 5 and 10 days, and the concentration of 1-pentanol which was transformed from pentanal was maximum at the same days. It was, on the contrary, more rapid in mature fruit than immature fruit in the experiment per fruit (Figure 2).

Relationship between Ester Formation and Volatile Fatty Acid in Strawberries. The analytical method of volatile fatty acid described in this paper permits a good recovery of volatile fatty acids from aqueous solution (formic acid, 96.7%; acetic acid, 102%; propionic acid, 98.8%; isobutyric acid, 99.7%; *n*-butyric acid, 100.1%; isovaleric acid, 98.6%; *n*-valeric acid, 99.4%; isocaproic acid, 97.2%; *n*-caproic acid, 97.5%) and good esterification efficiency (formic acid, 97.7%; acetic acid, 90.3%; propionic acid, 94.7%; isobutyric acid, 101.4%; *n*-butyric acid, 97.1%; *n*-caproic acid, 96%). The resolution of C_1–C_6 normal and isovolatile fatty acid 1-butyl esters was complete, and the calibration curves of these 1-butyl derivatives of acids showed linearity.

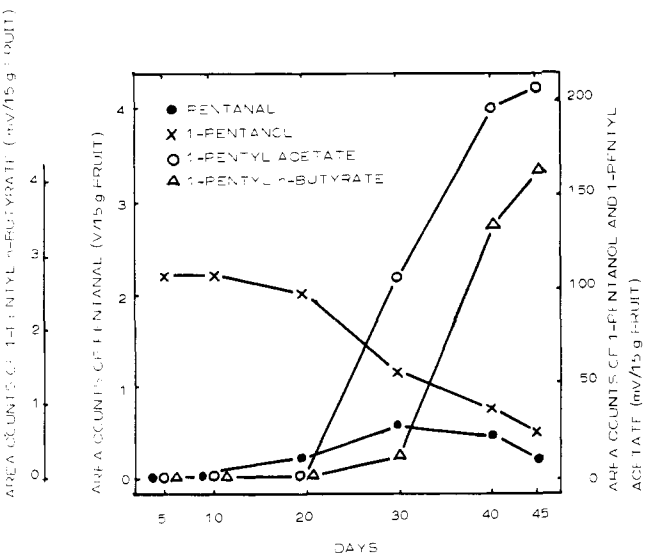

Figure 3. Influence of maturation on the formation of 1-pentanol and 1-pentyl esters from pentanal per 15 g of fruit.

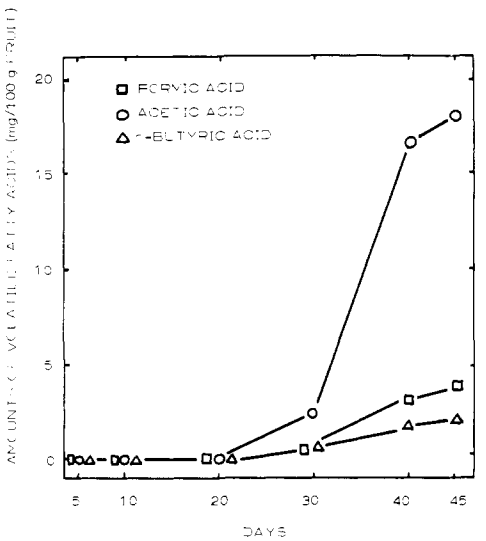

Figure 4. Changes in the volatile fatty acid content in strawberry during maturation.

The changes in volatile fatty acid content during maturation (Figure 4) are worthy of note. Small quantities of acetic acid and *n*-butyric acid (2.5 mg and 0.5 mg/100 g of fruit, respectively) were found at 30 days, and then their concentration increased rapidly until 45 days (18.2 mg and 2.2 mg/100 g of fruit), while no significant amount of acids were found from 5 to 20 days. The pattern of acetic acid and *n*-butyric acid is similar to those of ester production listed in Figures 2 and 3. These results suggest that there exists a clear correlation between ester formation and generation of volatile fatty acid in strawberry fruit.

Formic acid seems somewhat conflicting with the results obtained above. Formic acid was found at 30 days and increased with progress of maturity. In fact, the amount of formic acid at 40 and 45 days was around twofold of *n*-butyric acid; nevertheless, 1-pentyl formate was not detected through the experiments.

Some exogenous acids are known to be esterified as well as endogenous acids in strawberry fruit (Yamashita et al., 1975, 1976a). The simultaneous incubation of 1 μL of formic acid and 5 μL of 1-pentanol with a whole strawberry fruit of each stage of maturity from 5 to 45 days, however, did not result in the formation of 1-pentyl formate. On the other hand, added isocaproic acid was well esterified

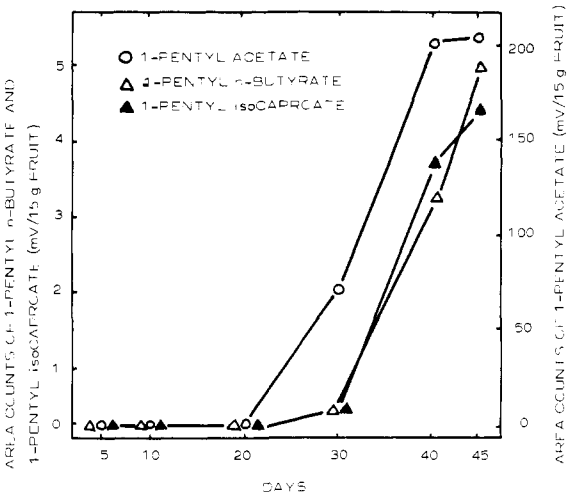

Figure 5. Influence of maturation on the formation of 1-pentyl esters by simultaneous incubation of 1-pentanol and isocaproic acid with 15 g of fruit.

under the same conditions (Figure 5). All results obtained here show that formic acid can not be esterified.

However, 3-methyl-1-butyl formate and 1-pentyl formate (McFadden et al., 1965), ethyl formate, 1-butyl formate, and 3-methyl-1-butyl formate (Tressl et al., 1969) are known to exist in strawberry oil components. Therefore, esters of formic acid are considered to be produced in strawberries. The most reasonable explanation for these facts is that biosynthesis of the ester of formic acid is too slow to be detected under the conditions employed in this experiments.

In Part 1 of this series, only one column was used in the experiment, and we concluded that some of the gas chromatographic peaks as esters of formic acid, which were later proved to be incorrect. Thus, from Part 2 on, we decided to employ two columns instead of one, namely polar and nonpolar columns, as described in the present paper, in order not to repeat the same mistake. Furthermore, identification with GC–MS was performed as long as the amount was enough for the analysis.

Relationship between Generation of Ester Producing Enzyme System and Fruit Maturity. To obtain further knowledge concerning the biosynthesis of esters during maturation, 5 μL of 1-pentanol and 1 μL of isocaproic acid were simultaneously incubated with 15 g of strawberries at each stage of maturity. As is illustrated in Figure 5, the pattern of the formation of 1-pentyl acetate and 1-pentyl *n*-butyrate is quite similar to those of Figure 2 and 3.

1-Pentyl isocaproate was not formed in spite of the addition of 1-pentanol and isocaproic acid to strawberry at initial stages of maturity. The formation of 1-pentyl isocaproate at 30 days means that the enzyme activity for ester formation generated 30 days after blooming, and the subsequent increase of 1-pentyl isocaproate represents the continuous increase of the activity from 30 to 45 days.

Consequently, it was found that lack of volatile fatty acid and ester producing enzymes were two factors influencing the absence of ester formation at immature stages.

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Olfaction and Molecular Shape. Chirality as a Requisite for Odor

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Although it has been established that chiral isomers can differ in odor, the number of cases reported in which the differences are small seems inconsistent with the logical assumption of optically active olfactory receptors. The observation that several terpenoid cyclohexanones in racemic form possess the urinous odor led to a program of synthesis of a group of these compounds in optically active form and the evaluation of their odors. At low concentrations all the compounds were either odorless or possessed the same characteristic urinous odor. However, the odor strength varied greatly with isomeric configuration although the high incidence of urinous anosmia known for *d*-androstenone and *cis*-(*tert*-butylcyclohexyl)isohexanone prevailed, suggesting the same olfactory receptor mechanism. Ratios of odor strength as high as 20:1 and one case of urine odorless vs. odorous between chiral isomers were observed. Liquid isomers were found to be stronger than solid isomers. At very high concentrations all the compounds had woody type odors with no observed cases of anosmia. An hypothesis for odor perception to account for all these observations is proposed.

Elucidation of the mechanism of olfaction remains for the future. Even the functioning of the olfactory cells, the first step in the perception of odors by animals, is still only a vaguely understood process. However, studies during the past two decades on the relationship of molecular shape to quality of odor have established useful criteria and led to a number of working hypotheses (Amoore, 1952, 1962, 1963; Beets, 1957; Davies, 1953; Theimer and Davies, 1967).

One particularly baffling aspect of structure–odor relationships has been difficulty in defining clearly the role of chirality. In contrast to large differences in other biological interactions between body tissues and dextro and levo forms of the same compounds, for example, in drug effectiveness and in taste perception, the differences between odors based solely on chirality have been so small that they have often been explained away as due to impurities only. This has been a roadblock on the way to a better understanding of the primary process of odor detection, for it has raised doubts about what would otherwise have been an a priori conclusion, namely, that the initial odor stimulus at the receptor cell results from an odor–molecule/receptor interaction on the olfactory epithelium. Since the epithelium, like all biological tissue, must have chirality, it should be able to differentiate between *d* and *l* isomers.

Fortunately, this ability to discriminate has now been established unequivocally by several discreet methods (Theimer and McDaniel, 1971; Friedman and Miller, 1971), but the relatively small differences in odor strength between chiral isomers has remained a disturbing element. The present study was undertaken to try to shed light on the reasons for the usually slight effect of chirality and to demonstrate that chirality can actually be dominant in determining odor strength as well as odor quality.

If we accept the existence of specialized receptors on the olfactory epithellium (Amoore, 1952, 1962, 1963; Beets, 1957; Davies, 1953; Theimer and Davies, 1967), we must conclude that an olfactory stimulus requires the entering molecules to have the proper shape in addition to other obvious properties such as volatility and suitable free-energy parameters. But the shape of a molecule as "sensed" by a receptor may depend not only upon its chemical structure but upon its particular conformation at the moment of contact and upon the attitude with which it presents itself to the receptor. Since intramolecular or spinning motion is rapid relative to intermolecular or translatory motion, the attitude of the molecule will not be important, since repositioning will permit effective interaction when this is at all possible. Similarly, the molecule will present itself in all its various conformations which can explain the similarity in odor between most chiral pairs studied to date.

Since chiral isomers are identical in all respects except spatial shape, they can, *if flexible*, assume conformations which are all but undistinguishable as far as the olfactory sensor is concerned. Such flexibility can be expected from acyclic molecules with many degrees of freedom. Molecular models show clearly the similarity in shape between, for example, the *d* and *l* forms of citronellol and of citronellal. The monocyclic carvones, more rigid, can be distinguished by odor quality, and their odor thresholds are also very different (Leitereg et al., 1971), as are also those of *d*-Nootkatone, the "Grapefruit ketone" and its enantiomer whose odor is far weaker and woody rather than fruity (Haring et al., 1972). On the other hand, *d*-

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