Evolution of Strawberry Alcohol Acyltransferase Activity during Fruit Development and Storage

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Four strawberry (*Fragaria ananassa* × *Duch*.) varieties Oso Grande, Chandler, Tudla, and I-101) were studied in relation to their alcohol acyltransferase (AAT) activity. AAT activity profile during fruit development and ripening was determined for each variety. The highest AAT activity value corresponded to Oso Grande, which is also the variety showing the earliest maximum peak of AAT activity. The effect of passive modified atmosphere (MA) storage on strawberry AAT was also studied. An increase in AAT activity was found in strawberries stored under high CO_2 (>30%). This higher level of AAT activity could be attributed to a detoxifying function of AAT that might be activated to eliminate ethanol generated by fermentation. Data obtained show a good correlation between AAT activity and flavor quality in strawberries.

Keywords: *Strawberry; flavor; ester biogenesis; alcohol acyltransferase*

INTRODUCTION

Volatile esters formed by esterification of alcohols and carboxylic acids constitute one of the largest and main group of volatile compounds extensively studied in fruit aroma. Nevertheless, there are very few results on the biochemical aspects of ester formation in fruits. Esters are important contributors not only to the aroma of fruits but also to the flavor of foods and fermented beverages. The mechanism of ester formation is better known in microorganisms in which two different enzymes seem to be implicated: alcohol acyltransferase (AAT) and esterase (Yoshioka and Hashimoto, 1981; Yamauchi et al., 1989; Malcorps and Dufour, 1992; Mauricio et al., 1993). Both enzymes, AAT and esterase, have been described in fruits (Ueda and Ogata, 1976). Experiments using esterase inhibitors have demonstrated that esterification of alcohols and acids in fruits is a coenzyme A-dependent reaction and that esterase has only hydrolytic activity (Ueda and Ogata, 1977).

The first attempt for AAT purification in fruits was carried out by Harada et al. (1985). The enzyme was localized in the soluble fraction of banana pulp cells and described as an alcohol acetyltransferase. More recently, two more fruits have been studied in relation to their AAT activities: apple (Fellman et al., 1991) and strawberry (Pérez et al., 1993), although only the strawberry AAT enzyme has been purified and characterized.

In our previous paper (Pérez et al., 1993), protein with AAT activity was purified about 29-fold from Chandler strawberry fruits shown to have a pH optimum of 8.0, an optimum temperature of 35 °C, and an apparent molecular mass of 70 kDa. The enzyme was tested for its preference in using different acyl-CoAs and alcohols. Strawberry AAT was found to be active against acetyl-CoA (100%), butyl-CoA (70%), and propyl-CoA (20%), while hexanol was the preferred substrate among five alcohols tested (Pérez et al., 1993). In a further study, strawberry and banana AAT specificities were compared. Banana AAT proved to be a more selective enzyme forming acetate esters and only very low amounts of propionate and butyrate esters. In these experiments, clear differences were also found in relation to alcohol specificity (Olías et al., 1995). In both cases, the results showed a clear correlation between substrate specificity and volatile esters present in each fruit aroma, suggesting a determining role for AAT in flavor biogenesis.

Despite the importance of AAT as a key enzyme in aroma biochemistry, many aspects such as its action mechanism or physiological relevance are still not fully understood. In this sense, to the best of our knowledge, no study on AAT changes during fruit development has been published, and only preliminary studies on the effect of different storage conditions on AAT activity have been carried out (Fellman et al., 1993; Ke et al., 1994).

In this research, we studied the AAT activity profile during maturation of four strawberry varieties, the relationship between AAT and flavor quality at fruit harvest, and the changes in AAT activity during the postharvest life of strawberries.

EXPERIMENTAL PROCEDURES

Materials. Fruits from four strawberry (*Fragaria ananassa* × *Duch*.) varieties (Chandler, Oso Grande, Tudla, and I-101) were used in this study. Strawberry plants were greenhouse grown and marked on blooming days. Fruit maturity was expressed as days after blooming (dab). The study of AAT activity during storage was carried out with Oso Grande strawberries harvested at the usual commercial picking date. Fruits were placed in 0.5-kg baskets filmed with polypropylene (PP) and stored at two temperatures, 1 and 17 °C.

Preparation of Enzyme Crude Extract. Strawberries were cut symmetrically into four pieces. Four pieces of four different strawberries (25 g) were blended in a Waring blender with 4 g of polyvinyl polypyrrolidone (PVPP) and 33 mL of 0.5 M Tris-HCl pH 8.0 buffer containing 0.1% Triton X-100. The resulting homogenate was vacuum filtered through a Whatman No. 1 filter paper, and the residue was washed two times with 8 mL of the above described buffer. The extract was centrifuged at 27000*g* for 20 min, and the supernatant was used as the crude extract.

Assay of AAT activity. The standard assay mixture consisted of 0.85 mL of 0.5 M Tris-HCl pH 8.0 buffer containing 11.6 mM $MgCl_2$, 0.3 mM acetyl-CoA, 10 mM butanol, and 0.15 mL of the enzyme solution. Two different assay methods were tested: GLC analysis and spectrophotometric assay.

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GLC Analysis. The mixture was incubated at 35 °C for 15 min in a 11-mL sealed vial. The vial was then transferred into a automatic headspace sampler (Hewlett Packard 19395 A) where a 15-min equilibrium time at 80 °C was set to allow the produced ester to enter the gas phase. The reaction product, butyl acetate, was determined by GLC in a Hewlett Packard gas chromatograph (HP-5890), equipped with FID and a stainless steel FFAP (2 m × 2mm) column at 120 °C. The amount of ester was calculated from a calibration curve in the range of 3−750 nmol. One unit of AAT activity was defined as the enzyme forming 1 μmol of butyl acetate/min.

Spectrophotometric Assay. The assay method was modified from that of Fellman et al. (1991). The mixture was incubated at 35 °C for 15 min, and then 50 μL of 20 mM 5,5′-dithiobis(nitrobenzoic acid) (DTNB) was added and allowed to stand at room temperature for 10 min. The increase in absorbance at 412 nm over time due to a yellow thiophenol product formed by the reaction of DTNB with the free CoA-SH liberated during the catalytic reaction was meassured by means of a spectrophotometer.

Protein Determination. Protein was measured according to the method described by Bradford (1976), using the Pierce Coomasie protein assay reagent with crystalline BSA as the standard protein.

Evaluation of Quality Parameters during Storage. Strawberry fruits stored at two different temperatures, 17 and 1 °C, were evaluated for skin color and firmness. Thirty fruits from three baskets were evaluated on days 0, 2, 4, 7, and 9. Gas composition inside each basket was also analyzed during storage. Three replicates were used for each treatment.

Color. Strawberry skin color was evaluated using a Minolta CR-200 portable tristimulus colorimeter (Minolta, Ramsey, NY) and expresed as *L*, *a*, and *b* values.

Firmness. Firmness was measured as penetration force with a Zwick 3303 penetrometer, using a 5-mm plunger tip, and expresed as Nw/cm^2.

Atmosphere Composition. CO_2 and O_2 percentage were analyzed by a gas chromatograph equipped with a thermal conductivity detector on a stainless steel Carbosieve S-II (3 m × 3 mm i.d.) column and helium as carrier gas.

Analysis of Volatile Compounds. *Headspace Sampling.* Strawberries (75−100 g) were placed in a desiccator housed within a thermostated water bath (25 °C). Methyl octanoate was added to the sample as the internal standard. The vessel was continuosly flushed with nitrogen (99.9% pure), flow rate 115 mL/min. For sampling, a Tenax TA trap (150 mg) was attached to the outlet of the desiccator; sampling time 15 min.

Desorption and GLC Analysis. The desorption of volatiles trapped in the Tenax was carried out by using a Chrompack thermal desorption cold trap injector (TCT). Identification of compounds in the headspace was made by means of GLC-MS. Volatiles were analyzed using a GLC (HP-5890) equipped with a 60 m × 0.25 mm i.d. fused silica capillary column SPB-1, carrier gas was helium at a flow rate of 30 cm/s. The column was held for 15 min at 40 °C and then programmed at 2 °C/min to 160 °C. A MS-30/70-VG mass spectrometer was directly coupled to the gas chromatograph described above. Identification of volatile compounds was made by matching against the Wiley/NBS library and by GLC retention time against standards.

RESULTS AND DISCUSSION

In our previous work on strawberry AAT, the enzyme activity was assessed by direct meassurement of the ester formed. The reaction product, butyl acetate, was determined by headspace GLC analysis and quantified from a calibration curve in the range of 3−750 nmol. The method proved to be accurate and allowed us to identify the reactions products in experiments with a number of different substrates. However, two major disadvantages can be attributed to this method: first, the requirement for total absence of remaining esterase activity in crude extracts, and second, the long time required for GLC analysis. The spectrophotometric procedure reported in this paper was developed to avoid both limitations. Instead of measuring ester formed, a colorimetric reaction with DTNB was used to detect acyl-CoA hydrolysis. Interferences due to esterase activity were avoided by this method, and AAT extraction protocol could be simplified since pectinase treatment of fruit tissue used to get rid of esterase enzyme (Pérez et al., 1993) was no longer necessary. Spectrophotometric assays showed that the formation of butyl acetate was proportional to the amount of thiophenol generated in the reaction mixture. The increase in absorbance at 412 nm, due to this yellow tiophenol product, formed by reaction of DTNB with free coenzyme A, was stable in the time range of 10−30 min. Good correlation was found between activity values calculated by GLC and spectrophotometric methods in the range of activity from 0 to 180 mU (Figure 1). In the described experimental conditions, the spectrophotometric assay proved to be linear up to 300 mU.

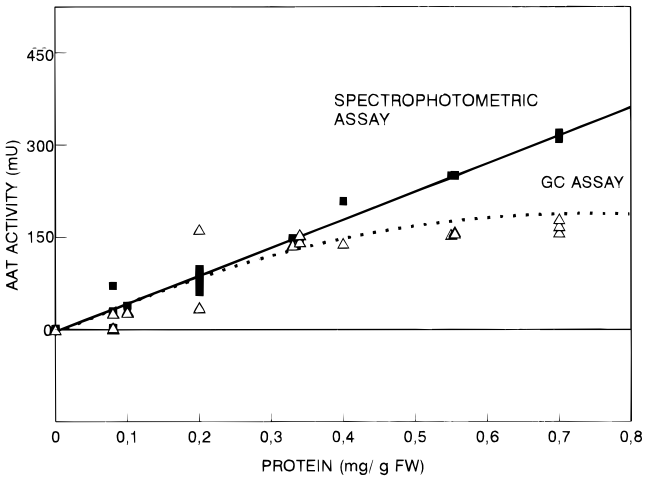

Figure 1. Correlation found between strawberry AAT activity values calculated by GLC and spectrophotometric methods, in the range of activity from 0 to 300 mU.

Using this spectrophotometric method, AAT activity was determined through strawberry maturation and storage. Crude extracts were prepared according to the experimental procedure described. PVPP and Triton X-100 were found to be essential for obtaining good activity levels. PVPP was employed to remove phenolics from crude homogenate. The absence of PVPP markedly increases the loss of AAT activity (75%). Triton X-100 was used to solubilize the enzyme. Previous data suggested that AAT could be a membrane-bound enzyme, in this paper, this possibility was examined with the criterion that membrane-associated activity should sediment at 150000*g*, 1 h (Razin, 1972). When strawberry crude extracts were centrifuged under these conditions, more than 70% of AAT activity was found in the pellet and an addition of 0.1% Triton X-100 resulted in total recovery of AAT activity in the supernatant.

Four strawberry varieties (Oso Grande, Chandler, Tudla, and I-101) were studied in relation to their AAT activities during maturation (Figure 2). Four fruit maturity stages were established according to external color: I (white), II (pinky), III (bright-red), and IV (dark-red). Only in Chandler fruits was AAT detected in maturity stage I. In the other three studied varieties, no AAT activity was detected at the early stages of maturity. All varieties under study showed an increase in AAT specific activity along maturation. Chandler and Oso Grande fruits, two Californian varieties, ex-

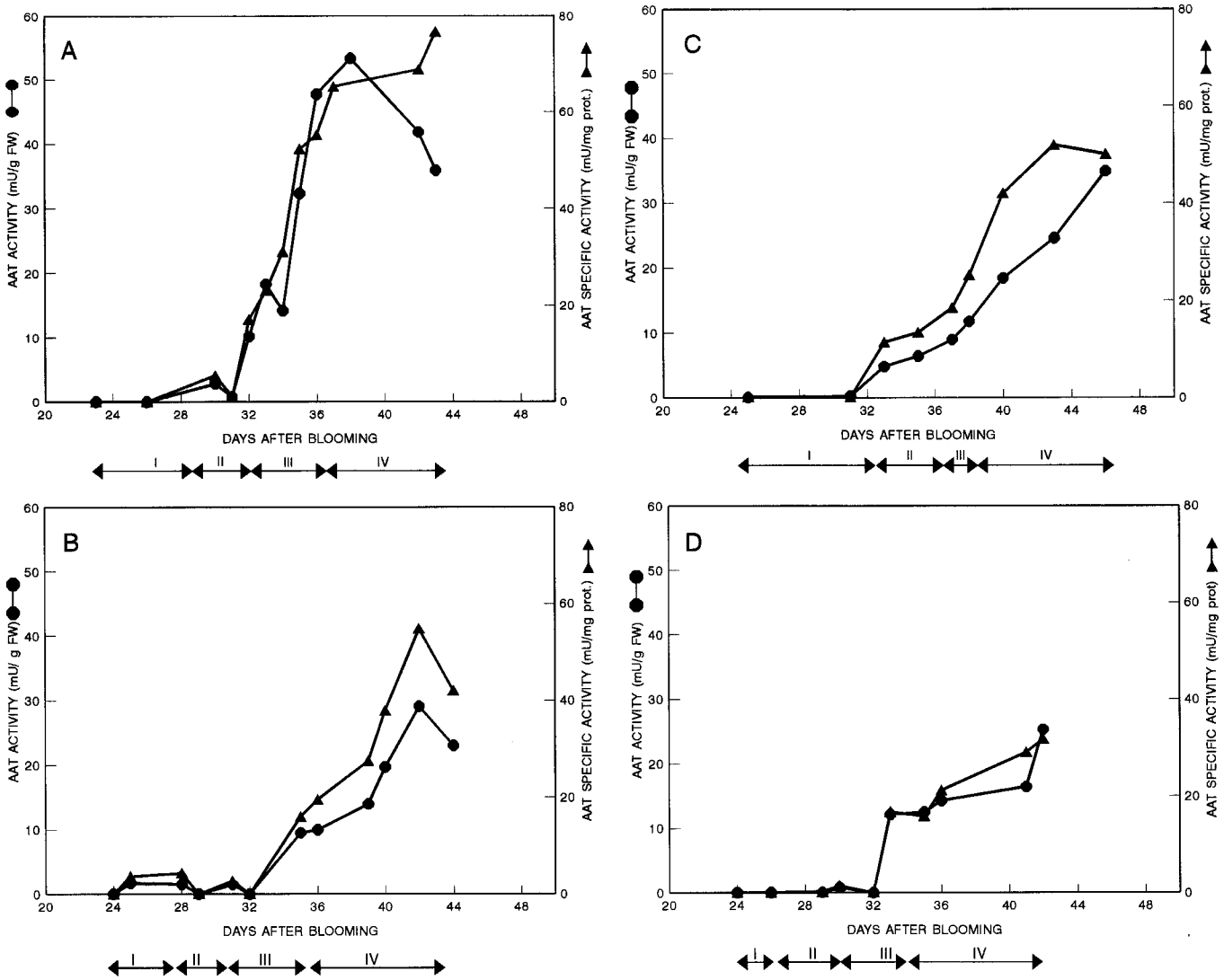

Figure 2. AAT activity and specific activity profiles during maturation of four strawberry varieties: (A) Oso Grande, (B) Chandler, (C) Tudla, and (D) I-101.

hibited similar AAT activity profiles. Both absolute and specific AAT activities reached a maximum and then a clear decrease at the overripe stage. Differences among varieties were found not only in relation to maximum AAT values read but also in the pattern of AAT activity during fruit maturation. The highest AAT activity value, 55 mU/g FW, corresponded to Oso Grande (stage IV, 38 dab), which is also the variety showing the earliest maximum peak of AAT activity. On the contrary, I-101 fruits presented the lowest AAT activity level among the four varieties under study. The low AAT specific activity of this strawberry cultivar could be responsible for its poorly flavored fruits (López-Aranda et al., 1995).

The importance of AAT as a key enzyme in aroma biogenesis in fruits has been pointed out by several authors (Harada et al., 1985; Fellman et al., 1993; Pérez et al., 1993), and a clear correlation has been found between AAT substrate preference and volatile esters composition in fruits such as banana and strawberry (Olías et al., 1995). High AAT activity should result in higher ester production and subsequently in fruits with enhanced aroma. In this sense, higher AAT activity of Chandler and Oso Grande varieties could well account for their superior organoleptic properties, and I-101 poor flavor could be due to its low AAT activity (López-Aranda et al., 1995).

As it can be seen at the bottom of each graph (Figure 2), the length of every maturity stage differs among strawberry varieties. Althoug strawberry growth and maturation is a continous developmental process, there is a definite point in growth when the physiology of the fruits is turned over to the maturation process. After this physiological change in the fruit, visual redness can be correlated with ripening and softening and used as criterion for the picking date (Knee et al., 1977; Given et al., 1988a,b; Manning, 1994). Ripening takes place between late stage III and early stage IV. Younger fruits lack of flavor, and older fruits rapidly become too soft. This fact makes Oso Grande an especially valuable strawberry cultivar because of the good flavor of stage III fruits, still firm, that could be due their high AAT activity.

Despite quantitative differences found among varieties, the study of AAT substrate specificty in these four cultivated strawberries showed similar results. As previously reported for Chandler AAT (Pérez et al., 1993), hexanol was the preferred substrate for this enzyme in the studied strawberry varieties. In the same way, AAT from these varieties showed decreasing esterification rates as compared to hexanol against butanol (50−60%), isoamyl alcohol (35−40%), propanol (30−40%) and ethanol (20−25%). Slight differences in

Table 1. Effect of Passive Modified Atmosphere Storage of Strawberries on CO_2/O_2 Composition, Color, and Firmness of Fruits

		atmosphere		color			
day	temp (°C)	% CO_2	% O_2	*L*	*a*	*b*	firmness (Nw/cm^2)
0		0.03 ± 0.01[a]	21.01 ± 1.22	35.76 ± 3.81[b]	30.96 ± 2.59	21.95 ± 6.07	17.50 ± 4.36[b]
2		3.47 ± 1.71	17.71 ± 1.55	34.67 ± 3.63	31.43 ± 3.51	23.83 ± 6.07	19.32 ± 4.24
4	17	26.23 ± 6.42	1.44 ± 0.66	35.42 ± 3.14	31.41 ± 2.81	20.61 ± 4.81	18.61 ± 3.97
1		5.92 ± 2.02	16.23 ± 1.48	37.95 ± 5.71	28.80 ± 5.35	22.81 ± 4.77	18.85 ± 4.01
7	17	47.79 ± 9.09	0.89 ± 0.23	33.38 ± 3.97	30.15 ± 3.41	19.11 ± 5.21	18.27 ± 2.81
1		7.81 ± 2.83	13.41 ± 0.61	36.21 ± 4.10	29.20 ± 3.36	22.37 ± 5.24	20.01 ± 3.83
9	17	57.64 ± 4.21	0.83 ± 0.14	32.67 ± 3.74	28.84 ± 3.05	16.07 ± 3.52	18.17 ± 4.41
1		8.94 ± 2.41	14.02 ± 0.81	35.38 ± 3.79	31.37 ± 3.96	22.28 ± 4.51	18.72 ± 5.32

[a] Average of three replicates. [b] Average of 30 determinations.

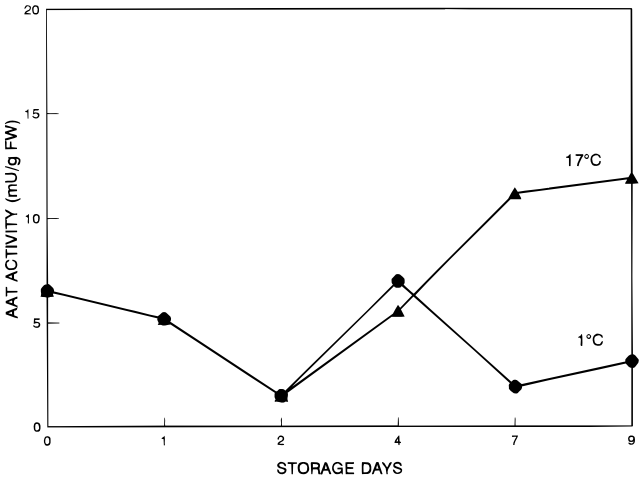

Figure 3. Changes in AAT activity (mU/g FW) during strawberry storage in polypropylene-filmed baskets kept at two temperatures, 17 and 1 °C.

alcohol specificity were found among these cultivated strawberries and the wild type *Fragaria vesca* (data not shown).

Fruits harvesting before they reach full ripening is a commom practice used to extend shelf-life that ussually causes a lack of organoleptic quality. In this sense, especially for short shelf-life fruits like strawberries, it is important to select varieties that had good flavor at early stages of ripening, when fruits are still firm and less susceptible to decay during transportation and storage. Oso Grande has proved to be an excellent strawberry variety, with firm and flavored fruits, being the major exported variety in southern Spain. As stated previously, high AAT activity found in immature Oso Grande strawberries could be a major factor involved in its precocious aroma and flavor biogenesis. Strawberry fruits of Oso Grande variety were used to study AAT activity during transportation and storage. Some studies on strawberry quality preservation have shown the benefits from films that produce passive modified atmospheres (MA), although off-flavor development caused by MAs has also been reported (Ke et al., 1991; Shamaila et al., 1992).

Strawberry baskets filmed with PP were stored at different temperatures in a system designed to mimic the commercial life of strawberries. Fruits were initially stored at 1 °C for 2 days to simulate refrigerated transport and then stored at 17 °C (with a control at 1 °C) for 7 more days to simulate the shelf-life period. AAT activity (Figure 3), O_2/CO_2 concentrations, color, and firmness of stored fruits (Table 1) were determined during the experience. Fruits were harvested at the usual commercial picking date, that is, an early stage III, around 32 dab. Insufficient degree of ripeness can be inferred from the low initial color values compared to those described for mature fruits of this variety (Sacks and Shaw, 1993). The slight decrease of *L**, *a**, and *b** values observed during storage can be related to habitual fruit darkening. AAT activity profile showed an initial decreased from day 0 to day 2, and then a steady increase for fruits stored at 17 °C was observed. On days 7 and 9, clear differences in AAT activity were found between 1 and 17 °C strawberries, with higher values for the latter (Figure 3). The effect of temperature on the atmosphere developed in the basket is clearly shown in Table 1. From day 2 to day 4, CO_2 content increased from 3.5 to 26.2% CO_2. This high level of CO_2 had beneficial effects on firmness and decay prevention (no decay fruits were found among stored strawberries) and did not cause any detrimental effect on flavor. On day 7, the CO_2 content is near 50%, while on day 9 is near 60%, and although no visual injury was observed, strawberries developed a strong off-flavor due to anaerobic metabolism. Three volatile compounds have been implicated in strawberry off-flavor: acetaldehyde, ethanol, and ethyl acetate (Li and Kader, 1989; Ke et al., 1991; Larsen and Watkins, 1995). Acetaldehyde is regarded as one of the most prevalent products of anaerobic respiration, but it may also be reduced to ethanol. Finally the most likely mode of eliminating these offending compounds involves their conversion to esters, mostly ethyl acetate. Pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) are responsible for acetaldehyde and ethanol production, respectively, and finally AAT would catalyze the formation of ethyl acetate. It has been reported that CA treatments enhanced PDC and ADH activities in strawberries, but slightly decreased AAT activity (Ke et al., 1994). Fellman and Mattheis (1995) found that AAT activity increased with the advanced maturity of apples but was supressed by exposure to 0.5 or 1% O_2. Lack of flavor of long-term CA stored apples could be atributed to an inhibition of AAT activity that causes a reduction of fruit ester content (Lidster et al., 1983; Mattheis et al., 1991; Fellman et al., 1993; Brackman et al., 1993). In this study, we found a slight increment in AAT activity on days 7 and 9, when strawberries are stored under high CO_2 (>50%) and low O_2 (<1%) atmospheres. This increase of strawberry AAT activity was related to an unusual aroma pattern, clearly different from that of fresh and control fruits kept at 1 °C (Table 2), in which methyl and ethyl acetate were the major compounds and only minor amounts of other esters were determined in the strawberries headspace. This higher level of AAT activity found in 17 °C fruits could be related to a possible detoxifying role by AAT, which might be activated to eliminate excess of ethanol. Control fruits kept at 1 °C, with 9% CO_2 and 14% O_2 on day 9, did not show this increment in AAT activity nor off-flavor

Table 2. Changes in Concentration of Volatile Esters in Oso Grande Strawberries Stored in Polypropylene-Filmed Baskets Kept at Two Temperatures (17 and 1 °C)

strawberry esters	day 0	day 9 (17 °C)	day 9 (control 1 °C)
methyl acetate	948.82[a]	3987.36	214.52
ethyl acetate	371.97	2968.72	142.71
methyl butanoate	1615.89	tz[b]	315.37
ethyl butanoate	181.99	22.04	72.95
isopropyl butanoate	38.85	tz	24.32
ethyl 2-methylbutanoate	32.04	tz	tz
butyl acetate	13.63	tz	tz
methyl hexanoate	175.85	tz	47.42
butyl butanoate	12.27	tz	tz
ethyl hexanoate	17.72	tz	37.40
hexyl acetate	17.71	tz	7.90
3-hexenyl acetate	58.62	tz	29.50
hexyl butanoate	23.17	tz	19.80

[a] Relative amounts are expressed as nanograms (gram of fresh weight)$^{-1}$ (1.7 L)$^{-1}$. Average of three determinations. [b] Values lower than 3 ng (g FW^{-1} (1.7 L)$^{-1}$.

development. Differences found between two treatments (1 and 17 °C) indicate the requirement for a strict temperature control when passive MA are used in strawberry storage.

Data obtained in this study on AAT activity, different AAT profiles found among strawberry varieties during ripening, and the relationship established between AAT activity and off-flavor development, confirm the main role of AAT in fruit aroma biogenesis.

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