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Preharvest Calcium Sprays Improve Volatile Emission at Commercial Harvest of 'Fuji Kiku-8' Apples

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Apple (*Malus* × *domestica* Borkh.) fruit intended for long-term storage are frequently harvested commercially before becoming fully ripe, often resulting in poor aroma development. Since postharvest calcium dips have proved effective for the enhancement of flavor-related volatile esters after cold storage of apples, this study was undertaken in order to assess whether preharvest calcium sprays (7 weekly applications at 1.6%, w/v, 81−123 days after full bloom) could also aid in improving this important attribute at harvest. This procedure significantly increased calcium content in treated fruit. The emission of aroma-related volatile esters by untreated and calcium-treated 'Fuji' apples was then monitored during maturation and ripening over two months prior to commercial harvest. Results indicate that most of the compounds contributing to overall flavor in ripe fruit were enhanced in response to preharvest calcium applications, suggesting that this procedure may be suitable for the improvement of fruit aroma at harvest. The emission of acetate esters was particularly favored, consistent with higher acetaldehyde contents in treated fruit. These effects arose apparently from increased pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) activities, possibly leading to a better supply of alcohols and acyl CoAs for ester biosynthesis.

KEYWORDS: Alcohol *o*-acyltransferase; alcohol dehydrogenase; apple; aroma; preharvest calcium sprays; pyruvate decarboxylase; volatile esters

INTRODUCTION

Because most apple (*Malus* × *domestica* Borkh.) production is aimed for mid- or long-term storage, it is a common practice to harvest fruit before full ripeness with the purpose of improving storage potential and resistance to postharvest handling procedures. Yet, this practice is not free from drawbacks, as the volatile profile emitted by apple fruit changes continuously throughout maturation and ripening (*1*, *2*), and the emission of flavor-contributing volatile compounds during the postharvest period is dependent upon the developmental stage at harvest. Consequently, fruit often fails to develop full flavor after harvest if it is picked before optimal maturity (*3*−*5*). Since flavor is a key attribute for sensory quality and consumer acceptance of apple fruit (*6*), disregard of these aspects often causes unsatisfactory eating quality in spite of benefits in terms of firmness and external appearance. Therefore, the improvement of aroma-related volatile production has become an important challenge for the fruit industry.

Calcium treatment of apples is a widely used practice, which has been demonstrated to be useful for delaying or reducing softening rates (*7*), physiological disorders (*8*), and fungi-caused decay (*9*). In contrast, little information has been reported to date on the effects of calcium treatments on fruit flavor. Interestingly, recent work has shown that postharvest calcium chloride ($CaCl_2$) applications in 'Fuji' (*10*) and 'Golden Reinders' (*11*) apple fruit improve the emission of aroma volatile compounds after mid-term cold storage under either air or controlled atmosphere, particularly of those compounds having the most impact on overall flavor. Therefore, the question arises whether preharvest calcium treatments might be a feasible procedure to avoid undesirable effects on aroma quality when apple fruit is harvested before full ripeness, at a maturity stage suitable for long-term storage. Here we report the production of aroma volatile compounds by untreated and calcium-treated 'Fuji' apples during maturation and ripening over two months prior to commercial harvest.

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MATERIALS AND METHODS

Plant Material, Calcium Treatment, and Standard Quality Analysis. 'Fuji' apple (*Malus* × *domestica* Borkh.) fruit, growing on 7-year-old trees grafted on M-9 EMLA rootstocks at an experimental orchard in Mollerussa (NE Spain), were sprayed weekly with $CaCl_2$ (1.6%, w/v). Treatment period was 23 June to 4 August 2008, corresponding to 81 and 123 days after full bloom (dafb), respectively. Uniform and defect-free fruit samples from treated and untreated trees were then picked weekly over two months (11 August to 22 October), covering a dafb range of 130−202. Samples were coded H1−H10, corresponding to successive picking dates. Commercial harvest at the producing area took place at 195 dafb (H9 stage). At each sampling date, 15 apples per treatment were assessed individually for standard quality parameters. Firmness (*N*) was measured on two opposite sides of each fruit, using an Effegi penetrometer equipped with an 11-mm diameter convex tip. Soluble solids content (SSC)

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and titratable acidity (TA) were measured in juice pressed from the whole fruit. SSC was determined with a hand-held refractometer (Atago, Tokyo, Japan), and results were expressed as % sucrose in an equivalent solution. TA was determined by titrating 10 mL of juice with 0.1 N NaOH to pH 8.1 using 1% (v/v) phenolphthalein; results were given as g of malic acid L^{-1}. Starch hydrolysis (SI) was rated visually using a 1-10 EUROFRU (CTIFL, France) scale (1, full starch; 10, no starch), after dipping cross-sectional fruit halves in 0.6% (w/v) I_2−1.5% (w/v) KI solution for 30 s.

Chemicals. The chemicals used were of the highest quality available, supplied by Sigma-Aldrich (Steinheim, Germany) unless otherwise indicated. Ethyl acetate, *tert*-butyl propanoate, propyl acetate, 1-propanol, ethyl butanoate, ethyl 2-methylbutanoate, butyl acetate, 1-butanol, pentyl acetate, 2-methyl-1-butanol, hexyl acetate, 1-hexanol, and hexyl 2-methylbutanoate were obtained from Fluka (Buchs, Switzerland). Ethanol was purchased from Panreac Química, S.A. (Castellar del Vallès, Spain). 2-Methylpropyl acetate was obtained from Avocado Research Chemicals Ltd. (Madrid, Spain).

Determination of Calcium Content. Lyophilized tissue (1 g) was ashed in a muffle furnace at 500 °C for 2 h. Ashes were digested thereafter with 4 mL of HCl/distilled water (1:1, v/v) and heated at 70 °C until complete sample dehydration as described in a previous work (*10*). Dried material was then resuspended in 2 mL of HCl/distilled water (1:1, v/v) for 15 min, filtered through 'Whatman 40 Ashless', and the filtrate was diluted to 50 mL in distilled water. Samples were then analyzed by inductively coupled plasma emission spectroscopy (ICP-OES) in a 'Horiba Jobin Yvon ACTIVA' spectrometer, and results were expressed as mg 100 g FW^{-1}.

Analysis of Volatile Compounds. Eight kilograms of intact apples (2 kg/replicate × 4 replicates) were taken for the extraction of volatile compounds according to the method of dynamic headspace as described previously (*10*). Briefly, intact fruit from each treatment were placed into an 8-L Pyrex container, and an air-stream (900 mL min^{-1}) was passed through for 4 h. The effluent was then recovered in an adsorption tube (ORBO-32; SUPELCO, Bellefonte, PA, USA) filled with 100 mg of activated charcoal (20/40 mesh), from which volatile compounds were desorbed by agitation for 40 min with 0.5 mL of diethyl ether. Identification and quantification of volatile compounds were achieved on a Hewlett-Packard 5890 series II gas chromatograph equipped with a flame ionization detector and a cross-linked free fatty acid phase (FFAP; 50 m × 0.2 mm i.d. × 0.33 μm) capillary column. The injection volume was 1 μL from each extract in all the analyses. The oven program was set at 70 °C (1 min) and the temperature was initially raised by 3 °C min^{-1} to 142 °C and then by 5 °C min^{-1} to 225 °C. It was then kept constant for 10 min at this final temperature. Helium was used as the carrier gas at a flow rate of 0.8 mL min^{-1} (42 cm s^{-1}), with a split ratio of 40:1, in the presence of air (400 mL min^{-1}) and H_2 (32 mL min^{-1}). The injector and detector were held at 220 and 240 °C, respectively. A second capillary column (SGE, Milton Keynes, UK) with 5% phenyl polysilphenylene-siloxane as the stationary phase (BPX5, 30 m × 0.25 mm i.d. × 0.25 μm) was also used for compound identification under the same operating conditions as described above. Volatile compounds were identified by comparing retention indexes with those of standards and by enriching apple extract with authentic samples. The quantification was made using butylbenzene (assay > 99.5%, Fluka) as the internal standard, run with each added standard aside from the matrix to develop standard curves for each volatile analyzed. A GC-MS system (Agilent Technologies 6890N − 5973N) was used for compound confirmation, in which the same capillary column was used as in the GC analyses. Mass spectra were obtained by electron impact ionization at 70 eV. Helium was used as the carrier gas (42 cm s^{-1}), according to the same temperature gradient program as described above. Spectrometric data were recorded (MSD Chemstation D.03.00.611) and compared with those from the NIST NBS75A original library mass spectra. The concentration of each volatile compound was expressed as μg per kg of fruit.

Analysis of Acetaldehyde Concentration. Juice samples (5 mL) obtained individually from 15 fruit were introduced in 10-mL test tubes and incubated 1 h at 65 °C for the analysis of acetaldehyde content as described elsewhere (*12*). A 1-mL headspace gas sample was taken and injected into a Hewlett-Packard 5890 Series II gas chromatograph, equipped with a column containing Carbowax (5%) on Carbopack (60/80, 2 m × 2 mm i.d.) as the stationary phase, and a flame ionization detector. Nitrogen was used as the carrier gas (24 cm s^{-1}), and operating conditions were as follows: oven temperature 80 °C, injector temperature 180 °C, detector temperature 220 °C. Acetaldehyde was identified and quantified by comparison with external standards (Merck, Darmstadt, Germany), and the results were expressed as μL L^{-1}.

Table 1. Standard Quality Parameters and Calcium Content of 'Fuji Kiku-8' Apples around the Commercial Harvest[a]

parameter		H8	H9	H10
firmness (*N*)	control	68.93 a	63.96 b	62.29 b
(LSD = 4.70)	calcium	73.44 a	69.95 a	68.77 a
SSC (%)	control	14.72 a	15.21 a	15.47 a
(LSD = 1.93)	calcium	14.25 a	15.07 a	15.29 a
TA (g L^{-1})	control	2.94 b	2.85 b	2.61 b
(LSD = 0.52)	calcium	3.51 a	3.56 a	3.43 a
SI (1−10)[b]	control	6.7 a	8.1 a	9.0 a
(LSD = 0.91)	calcium	6.5 a	7.1 b	7.8 b
calcium content (mg 100 g^{-1})	control	3.27 b	3.10 b	2.92 b
(LSD = 0.42)	calcium	4.16 a	3.92 a	3.89 a

[a] Values represent means of 15 (standard quality) or three (calcium content) replicates. Means followed by different letters within the same column for a given parameter are significantly different at $P \leq 0.05$ (LSD test). [b] EUROFRU 1−10 scale (1, full starch; 10, no starch).

Extraction and Assay of Aroma Volatile-Related Enzyme Activities. Samples of skin and flesh tissues were taken separately at each picking date (2 apples/replicate × 3 replicates), frozen in liquid nitrogen, freeze-dried, powdered, and kept at −80 °C until processing. One hundred milligrams of lyophilized powdered tissue was used for each determination. Extraction and assay of lipoxygenase (LOX; EC 1.13.11.12), pyruvate decarboxylase (PDC; EC 4.1.1.1), alcohol dehydrogenase (ADH; EC 1.1.1.1), and alcohol *o*-acyltransferase (AAT; EC 2.3.1.84) activities on crude enzyme extracts were performed as described elsewhere (*13*). Hydroperoxyde lyase (HPL; EC 4.1.2.-) activity was extracted and assayed according to ref (*14*). Total protein content in the enzyme extract was determined with the Bradford method (*15*), using BSA as a standard. In all cases, one activity unit (U) was defined as the variation in one unit of absorbance per minute. Each determination was done in triplicate, and results were expressed as specific activity (U mg $protein^{-1}$).

Statistical Analysis. A multifactorial design with calcium treatment and sampling time as factors was used to statistically analyze the results. All data were tested by analysis of variance (GLM-ANOVA procedure) with the SAS System 9.0 program package (SAS Institute, Cary, NC, 2002). Means were separated by the Fisher's LSD test at $P \leq 0.05$.

Partial least-squares regression (PLSR) was used additionally as a predictive method to relate a matrix of dependent variables (Y) to a set of explanatory variables (X) in a single estimation procedure, with full cross-validation as a validation procedure. The Unscrambler 6.11a software package (CAMO ASA, 1997) was used for developing these models.

RESULTS AND DISCUSSION

Significant increases in calcium content were found in the flesh of treated fruit around the commercial harvest date (**Table 1**), showing that exogenous calcium was actually incorporated and that it penetrated into the inner tissues. SSC and TA levels of H8, H9, and H10 fruit (**Table 1**) were suitable for storage according to local recommendations (TA < 4 g L^{-1}, SSC > 13%), but only H9 samples showed SI values within the optimal range (7−8), and untreated H9 and H10 fruit were slightly less firm than recommended (68.5−78.5 N). SSC was apparently unaffected by calcium applications. Contrarily, treated fruit showed lower starch index at harvest, together with higher firmness and acidity levels, indicative of delayed ripening (**Table 1**). This delay in the ripening process may be favorable for storage of produce; however, it may also have exacerbated the lack of aroma development often encountered when apple fruit are picked before being fully ripe. Therefore, we focused on this important attribute for the eating quality of fruit.

Preharvest Calcium Sprays Enhanced the Emission of Key Volatile Esters by Ripe Fruit. Volatile esters are reportedly the most important contributors to apple aroma (reviewed in ref *16*),

Table 2. Emission (μg kg^{-1}) of Straight- (**A**) and Branched-Chain (**B**) Esters by 'Fuji Kiku-8' Apples during On-Tree Maturation[a]

A

compound	RI[b]	RI[c]	OTH[d]		H1	H2	H3	H4	H5	H6	H7	H8	H9	H10
methyl acetate	854	–	8300	control	19.0 a	19.4 a	31.7 a	25.1 a	26.8 a	20.7 a	21.6 a	27.6 a	26.5 a	25.4 a
(LSD = 3.7)				calcium	16.7 a	21.9 a	30.2 a	30.2 a	24.8 a	19.7 a	23.4 a	27.7 a	25.7 a	25.7 a
ethyl acetate	882	609	5000	control	28.1 a	43.2 b	84.8 b	82.6 b	87.1 b	79.4 b	113.7 b	117.7 b	73.4 a	83.3 b
(LSD = 7.1)				calcium	34.8 a	50.3 a	104.9 a	110.3 a	119.9 a	119.4 a	132.3 a	130.2 a	70.7 a	71.6 a
propyl acetate	945	649	2000	control	–	–	–	–	–	1.0	1.2 a	1.5 a	4.2 a	17.4 b
(LSD = 3.3)				calcium	–	–	–	–	–	–	2.1 a	2.6 a	6.8 a	25.2 a
methyl butanoate	955	656	5	control	–	–	–	–	–	2.1 a	2.6 a	2.1 a	4.7 a	6.8 a
(LSD = 1.5)				calcium	–	–	–	–	–	2.0 a	2.1 a	2.4 a	4.5 a	7.1 a
ethyl butanoate	1002	803	1	control	1.3 a	1.7 a	1.6 a	0.6 a	2.5 a	1.2 a	5.1 a	5.0 a	4.5 a	4.7 a
(LSD = 1.0)				calcium	1.1 a	1.7 a	1.4 a	1.0 a	2.4 a	1.5 a	5.0 a	5.1 a	4.7 a	5.4 a
propyl propanoate	1008	809	57	control	–	–	–	–	–	–	–	–	2.2 a	12.0 a
(LSD = 1.9)				calcium	–	–	–	–	–	–	–	–	2.7 a	9.8 a
butyl acetate	1040	813	10	control	1.5	2.2	3.8	1.6	4.2 a	5.6 a	7.5 a	14.8 b	67.8 b	110.7 b
(LSD = 12.3)				calcium	–	–	–	–	0.5 a	4.7 a	7.1 a	27.8 a	91.6 a	133.1 a
butyl propanoate	1123	910	25	control	–	–	–	–	–	–	–	3.6 a	19.8 a	53.0 a
(LSD = 3.6)				calcium	–	–	–	–	–	–	–	4.7 a	16.8 a	47.8 b
pentyl acetate	1161	914	5	control	18.1 a	14.7 a	14.2 a	9.0 a	7.0 a	5.5 a	6.6 a	8.2 a	14.2 a	17.8 a
(LSD = 4.0)				calcium	10.2 b	7.6 b	6.6 b	6.8 a	6.4 a	6.2 a	5.5 a	9.4 a	13.2 a	18.9 a
butyl butanoate	1218	1000	100	control	–	–	–	–	–	–	–	3.1 a	17.6 a	26.7 a
(LSD = 3.1)				calcium	–	–	–	–	–	–	–	3.9 a	16.2 a	25.3 a
hexyl acetate	1292	1015	2	control	15.8 a	14.2 a	15.5 a	10.0 a	9.3 a	5.2 a	6.3 a	10.6 b	35.6 b	60.4 b
(LSD = 3.2)				calcium	6.5 b	6.9 b	6.1 b	5.5 b	5.1 b	3.4 a	5.2 a	14.5 a	45.9 a	81.8 a
propyl hexanoate	1360	1099	–	–	–	–	–	–	–	–	–	–	3.3 a	13.6 b
(LSD = 1.7)				calcium	–	–	–	–	–	–	–	–	2.0 a	15.5 a
hexyl propanoate	1379	1109	8	control	6.5 a	6.6 a	9.4 a	10.2 a	11.7 a	12.9 a	12.6 a	15.8 b	20.1 b	31.2 b
(LSD = 3.2)				calcium	1.4 b	2.1 b	1.7 b	2.4 b	2.7 b	12.6 a	13.7 a	20.7 a	29.0 a	47.3 a
butyl hexanoate	1473	1196	250	control	11.4 b	12.2 b	12.4 b	13.6 b	17.8 b	20.4 b	20.2 b	21.9 b	41.9 b	54.4 b
(LSD = 4.4)				calcium	16.3 a	16.8 a	17.0 a	24.2 a	23.6 a	25.7 a	26.3 a	30.5 a	51.4 a	67.5 a
hexyl butanoate	1477	1197	250	control	21.3 b	20.4 b	21.9 b	17.9 a	16.2 a	7.8 a	6.0 a	8.5 b	20.4 b	29.2 b
(LSD = 5.2)				calcium	27.4 a	28.0 a	29.4 a	21.9 a	19.5 a	9.5 a	6.6 a	14.4 a	30.7 a	40.5 a
ethyl octanoate	1502	1201	92	control	8.5 b	4.8 b	4.2 b	3.8 b	1.9 b	1.5 b	0.7 a	–	–	–
(LSD = 3.1)				calcium	17.3 a	16.6 a	14.0 a	12.5 a	11.4 a	5.7 a	3.4 a	–	–	–
pentyl hexanoate	1590	1293	–	control	–	–	–	–	–	–	–	–	6.6 a	7.6 a
(LSD = 1.3)				calcium	–	–	–	–	–	–	–	–	6.7 a	7.9 a
hexyl hexanoate	1687	1392	6400	control	23.5 a	18.7 b	14.4 b	9.2 b	11.5 b	9.3 b	8.4 b	11.5 b	31.2 a	31.4 a
(LSD = 4.1)				calcium	25.9 a	26.2 a	25.3 a	27.3 a	30.2 a	26.4 a	22.7 a	22.8 a	26.0 b	27.2 b
butyl octanoate	1690	1394	–	control	–	–	–	–	–	–	–	0.8 a	5.3 a	4.4 a
(LSD = 2.0)				calcium	–	–	–	–	–	–	–	2.7 a	5.6 a	4.7 a

B

compound	RI[b]	RI[c]	OTH[d]		H1	H2	H3	H4	H5	H6	H7	H8	H9	H10
tert-butyl propanoate	928	717	19	control	15.4 a	14.3 b	16.2 a	16.3 a	19.7 a	15.7 a	11.6 a	11.4 a	11.3 a	9.3 a
(LSD = 4.5)				calcium	14.3 a	20.7 a	20.1 a	17.0 a	19.3 a	14.2 a	12.3 a	12.4 a	10.2 a	10.4 a
2-methylpropyl acetate	976	691	5	control	1.0	4.5	8.4	8.7	10.4	4.5	5.7 a	5.5 a	6.2 b	8.7 b
(LSD = 2.2)				calcium	–	–	–	–	–	–	–	3.6 a	9.5 a	13.0 a
ethyl 2-methylbutanoate	1015	845	0.006	control	8.1	3.2	4.5	3.3	1.2	<0.5	–	–	–	–
(LSD = 2.1)				calcium	–	–	–	–	–	–	–	–	–	–
2-methylpropyl propanoate	1046	865	–	control	–	–	–	–	–	–	–	–	3.2 a	4.1 a
(LSD = 1.4)				calcium	–	–	–	–	–	–	–	–	2.9 a	4.2 a
2-methylbutyl acetate	1096	876	5	control	2.2 a	4.8 a	14.1 a	17.2 a	45.7 a	44.0 a	92.8 a	165.7 b	287.8 b	401.6 b
(LSD = 29.8)				calcium	0.6 a	3.3 a	7.7 a	8.9 a	37.2 a	44.8 a	90.1 a	214.6 a	395.1 a	531.4 a
butyl 2-methylpropanoate	1129	1009	80	control	–	–	–	–	–	–	–	–	–	0.9 a
(LSD = 0.2)				calcium	–	–	–	–	–	–	–	–	–	0.7 a
2-methylpropyl butanoate	1138	954	–	control	4.4 a	4.3 a	5.3 a	4.2 a	4.0 a	0.9 a	<0.5	–	–	–
(LSD = 1.5)				calcium	5.4 a	4.2 a	5.9 a	3.1 a	3.5 a	1.0 a	0.6	–	–	–
2-methylbutyl propanoate	1180	950	19	control	4.5 a	4.7 a	4.6 a	4.4 a	4.2 b	4.0 b	4.9 b	6.3 b	9.1 b	19.9 b
(LSD = 3.8)				calcium	4.1 a	4.8 a	4.2 a	5.2 a	10.4 a	9.0 a	13.1 a	17.6 a	18.1 a	25.4 a
2-methylbutyl 2-methylpropanoate	1190	1016	–	control	17.0 a	12.9 a	5.8 a	4.5 a	1.9 a	2.5 a	–	–	–	–
(LSD = 2.1)				calcium	17.8 a	12.6 a	6.2 a	4.6 a	2.1 a	1.6 a	–	–	–	–
butyl 2-methylbutanoate	1235	1042	17	control	4.1 a	6.4 a	11.0 a	10.1 a	10.4 a	11.3 a	10.9 a	12.2 a	24.7 a	43.9 a
(LSD = 4.9)				calcium	0.8 a	1.0 b	0.8 b	1.9 b	1.6 b	3.7 b	3.2 b	7.8 a	16.7 b	34.8 b
2-methylbutyl 2-methylbutanoate	1324	1106	–	control	36.3 a	37.7 a	35.2 a	29.4 a	11.1 a	4.9 a	2.2 a	2.5 a	3.1 a	12.1 a
(LSD = 6.6)				calcium	11.8 b	11.1 b	8.8 b	7.8 b	3.1 b	1.6 a	0.8 a	1.2 a	4.2 a	9.1 a

Table 2. Continued

					B									
compound	RI[b]	RI[c]	OTH[d]		H1	H2	H3	H4	H5	H6	H7	H8	H9	H10
hexyl 2-methylbutanoate	1488	1239	6	control	22.1 b	18.4 b	20.5 b	20.3 b	27.3 b	26.9 b	30.2 b	32.8 b	37.2 b	61.0 b
(LSD = 6.0)				calcium	29.1 a	29.6 a	27.3 a	27.8 a	36.2 a	39.6 a	39.0 a	41.0 a	46.5 a	74.6 a

[a] Values are the means of four samples obtained each from 2 kg of apples after 4h of collection (−: non-detected). For a given ester, means within the same column followed by different letters are significantly different at $P \leq 0.05$ (LSD test). [b] Kovats retention index in a cross-linked FFAP column. [c] Kovats retention index in a BPX5 column (−: eluted with the solvent). [d] Odor thresholds (μg kg^{-1}) in water as reviewed in ref *11* (−: not found).

both in quantitative and qualitative terms. Nineteen straight-chain and 12 branched-chain esters were identified in the volatile fraction emitted by fruit during the experimental time, although not all of them were detected at all sampling dates considered (**Table 2**). Some of these volatile esters were apparently unaffected by treatment, while significant differences between treated and untreated fruit were observed in other instances. In some cases, treatment effects were dependent upon the maturity stage of samples (**Table 2**). Since an objective of this work was to assess whether preharvest calcium sprays might be useful for the improvement of aroma quality of fruit at harvest, particular attention was placed on the latter phases of fruit maturation. The emission of eight straight-chain esters (ethyl acetate, propyl acetate, butyl acetate, propyl hexanoate, hexyl acetate, hexyl propanoate, butyl hexanoate, and hexyl butanoate) and of four branched-chain esters (2-methylpropyl acetate, 2-methylbutyl acetate, 2-methylbutyl propanoate, and hexyl 2-methylbutanoate) was increased significantly in treated fruit around the commercial harvest date (**Table 2**). In contrast, the production of butyl propanoate, hexyl hexanoate, and butyl 2-methylbutanoate decreased in response to treatment.

The question arose whether the alterations in ester production observed in response to treatment were relevant for the aroma profile of fruit at harvest. Therefore, ester production must be considered not only in quantitative, but also in qualitative terms. Twelve out of the 31 volatile esters identified during the experimental period were found to have log odor units (OU) > 0 by the time of commercial harvest (**Table 3**) and thus deemed as likely to have an impact on overall flavor (*17*). Most of these contributing compounds, with the exception of 2-methylpropyl and pentyl acetates, have been shown to be also important for the aroma of 'Fuji' apples after cold storage under air or ULO conditions (*10*), some of them (ethyl butanoate, 2-methylbutyl acetate, hexyl acetate) reportedly providing fruity odors to apple aroma (*18*). Interestingly, many of these compounds, particularly those showing the highest log OU values and thus putatively having the most impact on fruit aroma, were enhanced in treated samples, suggesting that preharvest calcium applications have a potential to improve this attribute at harvest.

The impact of treatment was dependent upon the chemical nature of each ester. Butanoate esters were apparently unaffected, while log OU values of acetate esters were higher in treated fruit, with the exception of pentyl acetate (**Table 3**). This is consistent with the observation of increased acetaldehyde content in calcium-treated samples (**Figure 1A**), as acetaldehyde can be used by plant tissues as a precursor for the biosynthesis of acetyl CoA (*19*), one of the substrates required for the biosynthesis of acetate esters by AAT action, and indeed a good correlation was found between acetaldehyde content and the emission of acetate esters (**Figure 1B**). An alcohol moiety is the second substrate necessary for AAT-catalyzed ester production, and data show that the emission of 1-butanol, 2-methyl-1-butanol, and 1-hexanol was higher in treated fruit, while that of 1-pentanol was unaffected (**Table 4**), which might explain why pentyl acetate was not modified in response to treatment and illustrates the relevance of alcohol supply for ester production. Accordingly, the emission and thus the log OU value at harvest of hexyl and 2-methylbutyl propanoates, as well as of hexyl 2-methylbutanoate, were also enhanced in response to treatment. However, results also show that additional factors may play an important role in ester production: for instance, the log OU values for butyl propanoate and butyl 2-methylbutanoate were lower in calcium-treated fruit in spite of the higher availability of 1-butanol (**Table 4**) and contrarily to the observations for butyl acetate (**Table 3**), which suggests that acetyl CoA was the preferred acyl CoA substrate for the AAT isoforms present in the tissues.

Table 3. Log_{10} of Odor Unit Value (Concentration/Odor Threshold) of Volatile Esters Contributing to Overall Flavor of 'Fuji Kiku-8' Apples around the Commercial Harvest[a]

compound		H8	H9	H10
methyl butanoate	control	<0	<0	0.14 a
	calcium	<0	<0	0.15 a
2-methylpropyl acetate	control	0.04	0.09 b	0.24 b
	calcium	<0	0.28 a	0.41 a
ethyl butanoate	control	0.70 a	0.66 a	0.67 a
	calcium	0.71 a	0.67 a	0.73 a
butyl acetate	control	0.17 b	0.83 b	1.04 b
	calcium	0.44 a	0.96 a	1.12 a
2-methylbutyl acetate	control	1.52 b	1.76 b	1.90 b
	calcium	1.63 a	1.90 a	2.03 a
pentyl acetate	control	0.21 a	0.45 a	0.55 a
	calcium	0.27 a	0.42 a	0.58 a
butyl propanoate	control	<0	<0	0.33 a
	calcium	<0	<0	0.28 b
2-methylbutyl propanoate	control	<0	<0	0.02 b
	calcium	<0	<0	0.13 a
butyl 2-methylbutanoate	control	<0	0.16	0.41 a
	calcium	<0	<0	0.31 b
hexyl acetate	control	0.72 b	1.25 b	1.48 b
	calcium	0.86 a	1.36 a	1.61 a
hexyl propanoate	control	0.29 b	0.42 b	0.59 b
	calcium	0.41 a	0.56 a	0.77 a
hexyl 2-methylbutanoate	control	0.74 b	0.79 b	1.01 b
	calcium	0.83 a	0.89 a	1.09 a

[a] Values are the means of four samples obtained each from 2 kg of apples after 4 h of collection. For a given ester, means within the same column followed by different letters are significantly different at $P \leq 0.05$ (LSD test).

Preharvest Calcium Sprays Increased the Availability of Specific Precursors for Ester Biosynthesis. AAT activity is necessary for ester production (*20*), and detectable levels were found throughout the experimental time (**Table 5**). However, the emission of volatile esters (**Table 2**) did not appear to parallel AAT dynamics. The highest AAT activity levels in the flesh were found at the H7 stage, the only sampling point for which significant differences were observed between treated and untreated samples (**Table 5**). Contrarily, AAT activity in the skin tissue was altered significantly in response to treatment throughout the experimental period. Untreated fruit displayed a maximum at the H8 stage,

one week before commercial harvest, which in treated samples was more moderate and advanced by approximately one month.

These results agree with previous observations for 'Fuji' apples (*10*, *21*) suggesting that, provided a minimum level of AAT activity is present in the tissues, an adequate supply of precursors is the actual key factor accounting for ester biosynthesis. In agreement with those reports, a partial least-squares regression (PLSR) model developed for flavor-contributing esters (*Y* variables) revealed a strong relationship to acetaldehyde and alcohol precursors (*X* variables), this model explaining up to 80% of total variability in ester emission during the whole two-month period considered (data not shown). If only advanced maturity stages were considered in the model (H6−H10), 87% of variability could be accounted for, calcium-treated fruit displaying higher levels of important precursors such as 1-butanol, 2-methyl-1-butanol, 1-hexanol, and acetaldehyde.

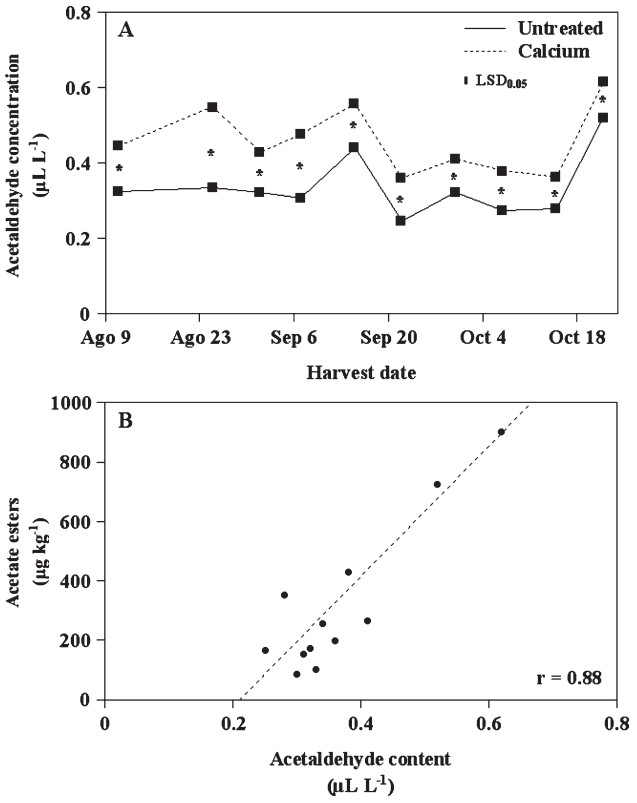

Figure 1. Acetaldehyde content (**A**) and correlation to the emission of acetate esters (**B**) by 'Fuji Kiku-8' apples during on-tree maturation. In panel **A**, asterisks indicate significant differences between treated and untreated fruit at $P \leq 0.05$ (LSD test). Vertical bar indicates LSD. Points represent means of 15 replicates.

This observation highlights the relevance of upstream enzymes providing these intermediates for ester biosynthesis, and therefore an additional PLSR model was developed in order to have an overview of the possible involvement of different volatile-related enzyme activities in improved availability of these substrates in mature (H6−H10) fruit. The loadings plot for this model (**Figure 2**) showed that the production of most alcohols, with the exception of ethanol, was related to PDC and ADH activities, which suggests that these activities were relevant for the observed increase in the emission of volatile esters. Indeed, higher PDC and ADH activities were found for calcium-treated fruit during the last stages of fruit maturation both in the skin and in the flesh (**Figure 3**). In the case of PDC activity, higher activity levels in the skin of treated samples were observed throughout the whole experimental period. This is consistent with previous reports for 'Fuji' fruit, indicating that postharvest $CaCl_2$ treatments enhanced the biosynthesis of some impact compounds after mid-term storage through an increase in PDC and ADH activities associated with better supply of acetaldehyde and alcohol precursors (*10*). The calcium-related increase in these enzyme activities has been attributed to increased O_2 gradients across apple tissues in response to the treatment, due to higher difficulty for O_2 diffusion (*22*) and to augmented internal CO_2 levels (*23*−*25*), causing hypoxia-like induction of PDC and ADH.

PDC uses a 2-oxoacid to render CO_2 and an aldehyde, which is metabolized further to either the corresponding alcohol by ADH-catalyzed reduction, or to an acyl-CoA by aldehyde dehydrogenase (ALDH, EC 1.2.1.5) (*26*). Both alcohols and acyl-CoA moieties are the required substrates for AAT-mediated ester formation. The observation that ethanol was apparently unrelated to ADH activity suggests that acetaldehyde was being diverted preferentially to the synthesis of acetyl-CoA, necessary for the production of acetate esters, and agrees with the general increase in the emission of acetate esters by treated fruit (**Tables 2** and **3**). Therefore, ADH may have used aldehydes other than acetaldehyde for obtaining the required alcohols. This is interesting in the light of results showing increased HPL activity in the

Table 4. Emission of Alcohols (μg kg^{-1}) by 'Fuji Kiku-8' Apples during On-Tree Maturation[a]

compound	RI[b]	RI[c]		H1	H2	H3	H4	H5	H6	H7	H8	H9	H10
ethanol	912	−	control	24.7 b	30.3 b	58.1 b	56.4 b	56.0 b	59.0 a	65.0 a	62.1 a	55.3 a	50.8 a
(LSD = 4.6)			calcium	30.5 a	35.0 a	68.5 a	66.8 a	66.5 a	38.1 b	54.7 b	55.9 b	42.5 b	40.7 b
1-propanol	992	−	control	−	−	−	−	−	−	−	−	3.3 a	11.3 b
(LSD = 3.5)			calcium	−	−	−	−	−	−	−	−	5.1 a	15.0 a
1-butanol	1119	626	control	−	−	<0.5	−	0.8	1.1 a	2.5 a	5.1 a	19.3 b	23.9 b
(LSD = 3.8)			calcium	−	−	0.5	0.5	−	2.2 a	3.1 a	8.4 a	24.8 a	28.3 a
2-methyl-1-butanol	1199	667	control	−	−	−	−	4.6 a	3.9 a	8.7 a	14.5 b	32.2 b	45.7 b
(LSD = 5.2)			calcium	−	−	−	−	5.4 a	4.7 a	12.2 a	20.6 a	46.5 a	59.5 a
1-pentanol	1262	688	control	1.6 a	1.8	−	−	−	−	−	−	−	−
(LSD = 0.3)			calcium	1.8 a	−	−	−	−	−	−	−	−	−
1-hexanol	1392	869	control	−	−	−	−	−	−	−	1.8 a	4.3 b	5.3 b
(LSD = 2.5)			calcium	−	−	−	−	−	−	−	3.9 a	11.1 a	14.8 a
2-ethyl-1-hexanol	1565	1031	control	46.1 b	35.4 b	21.1 b	21.5 b	16.8 b	11.8 b	7.8 b	10.0 a	6.8 a	3.7 a
(LSD = 10.6)			calcium	70.2 a	67.8 a	57.7 a	50.3 a	43.1 a	30.2 a	22.1 a	14.7 a	11.6 a	10.4 a

[a] Values are the means of four samples obtained each from 2 kg of apples after 4 h of collection (−: non-detected). For a given alcohol, means within the same column followed by different letters are significantly different at $P \leq 0.05$ (LSD test). [b] Kovats retention index in a cross-linked FFAP column. [c] Kovats retention index in a BPX5 column (−: eluted with the solvent).

Table 5. Flavor-Related Enzyme Activities (U mg protein^{-1}) in 'Fuji Kiku-8' Apples during On-Tree Maturation[a]

tissue	activity		H1	H2	H3	H4	H5	H6	H7	H8	H9	H10
skin	LOX	control	109.13 a	128.85 a	63.04 a	72.23 a	85.03 a	84.05 a	102.32 a	84.83 a	84.59 a	74.11 a
	(LSD = 16.1)	calcium	99.34 b	104.93 b	54.83 a	54.60 b	67.91 b	67.09 b	76.95 b	67.89 b	73.75 a	59.81 a
	HPL	control	61.05 a	65.41 b	43.78 b	39.70 b	57.25 b	57.55 b	54.77 a	49.55 a	52.12 a	62.50 a
	(LSD = 7.5)	calcium	65.95 a	101.92 a	59.67 a	57.43 a	76.00 a	73.01 a	51.52 a	41.75 b	42.57 b	52.94 b
	AAT[b]	control	54.56 b	58.21 b	81.24 b	90.26 b	90.85 b	89.74 a	104.78 a	128.54 a	62.25 a	51.79 a
	(LSD=14.2)	calcium	92.22 a	90.78 a	107.05 a	106.98 a	107.80 a	75.34 b	81.61 b	71.02 b	41.98 b	41.19 a
flesh	LOX	control	9.59 a	6.44 a	4.85 a	4.77 a	12.25 a	11.65 a	46.05 a	34.25 a	31.21 a	35.67 a
	(LSD = 5.5)	calcium	7.22 a	11.30 a	4.21 a	6.14 a	11.18 a	10.80 a	36.17 b	30.09 a	28.39 a	28.50 b
	HPL	control	39.47 a	37.93 a	19.86 a	18.02 a	11.56 a	20.76 a	15.93 a	14.91 a	15.45 a	19.05 a
	(LSD = 5.9)	calcium	35.76 a	31.45 b	18.41 a	17.88 a	14.11 a	17.39 a	13.11 a	11.70 a	10.55 a	13.66 a
	AAT[b]	control	16.72 a	17.82 a	15.25 a	12.69 a	17.35 a	22.50 a	26.56 a	21.02 a	18.48 a	17.07 a
	(LSD = 3.7)	calcium	14.67 a	17.68 a	14.51 a	12.14 a	14.96 a	21.37 a	21.81 b	20.18 a	18.10 a	14.04 a

[a] Values are the means of three replicates. Different letters within the same column for a given enzyme activity indicate significant differences at $P \leq 0.05$ (LSD test). [b] AAT activity data are given as mU mg protein^{-1}.

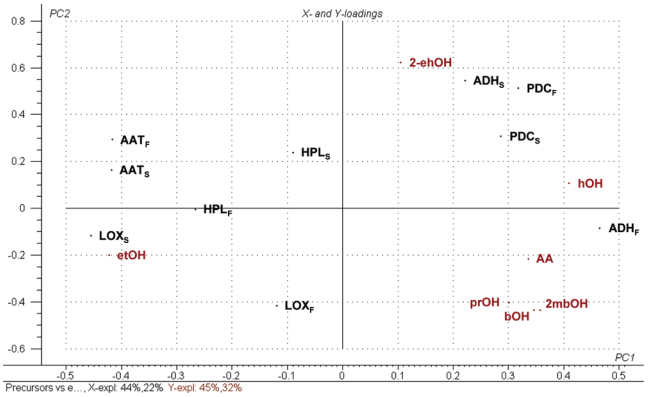

Figure 2. Loadings plot of PC1 versus PC2 corresponding to a PLSR model for emission of alcohols and acetaldehyde content (*Y* variables) vs volatile-related enzyme activities (*X* variables) in mature (H6 to H10) 'Fuji Kiku-8' apples (AA, acetaldehyde; etOH, ethanol; prOH, 1-propanol; bOH, 1-butanol; 2mbOH, 2-methyl-1-butanol; hOH, 1-hexanol; 2ehOH, 2-ethyl-1-hexanol). For enzyme labels, the suffix 'S' or 'F' refers to the activity in the skin or the flesh, respectively.

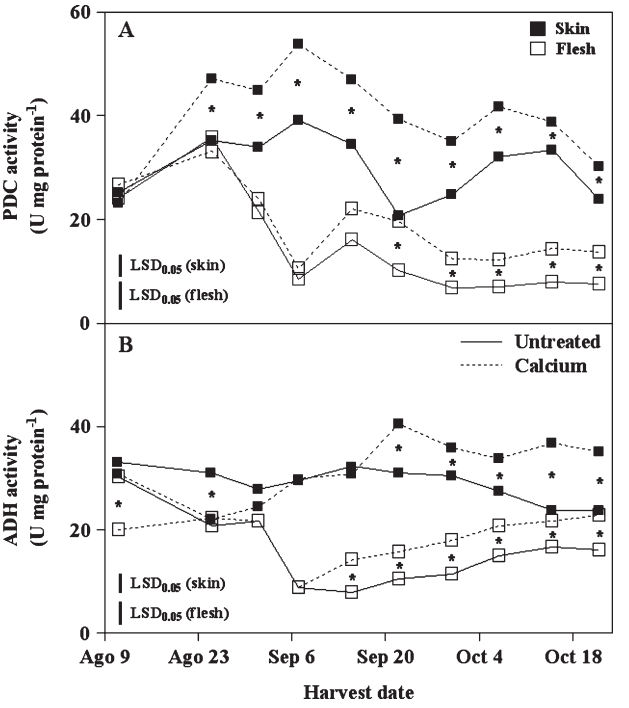

Figure 3. Pyruvate decarboxylase (**A**) and alcohol dehydrogenase (**B**) in the skin and flesh tissues of 'Fuji Kiku-8' apples during on-tree maturation. Asterisks indicate significant differences between treated and untreated fruit at $P \leq 0.05$ (LSD test). Vertical bars indicate LSD. Points represent means of three replicates.

skin tissue of calcium-treated samples up to H6 maturity stage (**Table 5**). HPLs catalyze the cleavage of fatty acid hydroperoxides generated by LOX action to aldehydes and oxoacids, the aliphatic aldehydes hexanal and 3-hexenal being major products of its action on 13-hydroperoxy linoleic or linolenic acids, respectively (*27*). In this work, 1-hexanol was the alcohol showing the highest dependence on ADH activity (**Figure 2**), and it has been reported that hexanal and hexyl acetate are produced mainly in the skin of apple fruit (*28*). LOX activity in the skin was generally lower in treated than in untreated samples, consistent with the protective role exerted by calcium on structural integrity of membranes (*29*). Although this may seem in contradiction with enhanced production of straight-chain esters, generally considered to arise from lipid metabolism through the LOX pathway (*21*), it has been hypothesized that the protective role of calcium on membranes might allow better regulation of LOX activity and hence higher straight-chain ester emission in spite of lower LOX activity levels (*10*). In contrast, no significant differences in LOX or HPL activities between treated and untreated samples were observed in general for the flesh tissue (**Table 5**).

Data reported herein are thus suggestive that preharvest calcium sprays, a simple and economical procedure, would allow harvesting 'Fuji' apples at a maturity stage suitable for long-term storage, while attenuating or overcoming the detrimental effects on aroma development often encountered when fruit are picked too early.

ABBREVIATIONS USED

AA, acetaldehyde; AAT, alcohol *o*-acyltransferase; ADH, alcohol dehydrogenase; HPL, hydroperoxide lyase; ICP-OES, inductively coupled plasma emission spectroscopy; LOX, lipoxygenase; OTH, odor threshold; OU, odor unit; PDC, pyruvate decarboxylase; PLSR, partial least-squares regression; SI, starch index; SSC, soluble solids content; TA, titratable acidity.

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