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Calcium Dips Enhance Volatile Emission of Cold-Stored 'Fuji Kiku-8' Apples

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Despite the relevance of volatile production for overall quality of apple (*Malus* × *domestica* Borkh.) fruit, only a few studies have focused on the effects of calcium treatments on this quality attribute. In this work, 'Fuji Kiku-8' apples were harvested at commercial maturity, dipped in calcium chloride (2%, w/v), stored at 1 °C and 92% relative humidity for 4 or 7 months under either air or ultralow oxygen (ULO; 1 kPa of O_2/2 kPa of CO_2), and placed subsequently at 20 °C. Ethylene production, standard quality parameters, emission of volatile compounds, and the activities of some related enzymes were assessed 7 days thereafter. Calcium concentration was higher in $CaCl_2$-treated than in untreated fruit, suggesting that the treatment was effective in introducing calcium into the tissues. Higher calcium contents were concomitant with higher flesh firmness and titratable acidity after storage. Furthermore, calcium treatment led to increased production of volatiles in middle-term stored apples, probably arising from enhanced supply of precursors for ester production as a consequence of increased pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) activities. After long-term storage, higher volatile emission might have arisen also from the enhancement of alcohol *o*-acyltransferase (AAT) activity, which was increased as a result of calcium treatment. In addition to storage period, the effects of calcium treatment were also partially dependent on storage atmosphere and more noticeable for fruit stored in air.

KEYWORDS: Alcohol *o*-acyltransferase; alcohol dehydrogenase; calcium applications; controlled atmosphere; pyruvate decarboxylase; volatile compounds

INTRODUCTION

Cold storage of apple fruit, under either air or hypoxic conditions, is a widespread technology used to delay many ripening-related modifications and, thus, to extend the commercial life of produce. In addition, calcium chloride ($CaCl_2$) has been widely used as a preservative and firming agent in the fruit and vegetable industry for whole as well as for fresh-cut produce. Prestorage calcium treatment of apples has been shown to reduce the incidence of physiological disorders (*1*), softening rates (*2*), and decay caused by fungi (*3*). Moreover, calcium treatment of fruit may have beneficial side effects on the nutritional quality of produce, as a bulk of findings link dietary calcium deficiency to some chronic diseases, including osteoporosis, hypertension, and colon cancer (*4*).

The ultimate objective of calcium applications, as of any other postharvest treatment, is to enhance consumer acceptance of the commodity and/or to maintain it for as long as possible. It has been found that calcium infiltration of apples significantly increased sensory hardness and overall acceptability scores (*5*). In addition to texture, also flavor is a key attribute determining consumer acceptance of apples (*6*). Thus, to better understand how consumer acceptance of fruit may be affected by calcium applications, more information is needed on the alterations in flavor induced by the treatments.

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Although fruit flavor depends upon taste and aroma, the latter is considered to play a dominant role (*7*). The aroma profile of a fruit is complex and depends on the combination of all volatile compounds emitted, in addition to the concentration and odor threshold of each individual emitted compound. Volatile production in fruits is a process under tight control, involving enzymes as well as substrates and energy supplied from many pathways. In particular, metabolism of fatty acids through both β-oxidation and the lipoxygenase (LOX; EC 1.13.11.12) pathway has been reported to be the principal source of precursors for the production of those volatile compounds responsible for the aroma of most fruits (*8*). β-Oxidation is generally considered to be the main metabolic pathway producing primary aroma in fruits, whereas the LOX system may account for the widest assortment of lipid-derived precursors of aroma compounds in disrupted plant tissues. Besides, cell walls and membranes become more permeable to different substrates in the course of ripening, and thus the role of the LOX pathway in the biosynthesis of volatiles becomes more significant (*8*).

Although the relationships between calcium treatments and aroma volatile production are of interest, only a few works on this subject have been published. Calcium has been shown to play an important role in maintaining structural integrity not only of cell walls but also of cell membranes, thus delaying lipid catabolism (*9*). Therefore, the production of lipid-derived precursors of volatiles, and hence of aroma-related volatile

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compounds, might be modified in response to calcium treatments. The purpose of this work was to investigate the suitability of calcium applications for preservation of the overall quality of apple fruit during the poststorage period, with special focus on the biosynthesis of volatile compounds through the LOX pathway. To simulate the usual commercial procedures for apples before marketing and to assess possible interactions, $CaCl_2$-treated and untreated samples were cold-stored under either air or ultralow oxygen (ULO) conditions.

MATERIALS AND METHODS

Plant Material, Calcium Treatment, and Storage Conditions. Apple (*Malus* × *domestica* Borkh. cv. Fuji Kiku-8) fruit were harvested in 2006 at commercial maturity (187 days after full bloom), from 5-year-old trees grafted on M-9 EMLA rootstocks at the IRTA-Experimental Station in Mollerussa, in the area of Lleida (northeastern Spain). Immediately after harvest, fruits were randomly divided into six lots, three of which were dipped in a 2% (w/v) $CaCl_2$ solution at ambient temperature for 5 min. After treatment, $CaCl_2$-treated and untreated apples were stored at 1 °C and 92% relative humidity (RH) in cold rooms under either air or ULO conditions (1 kPa of O_2/2 kPa of CO_2). The experimental chambers (20 m^3) at the UdL-IRTA research center were used for storage of fruit. The O_2 and CO_2 concentrations were monitored continuously and corrected automatically using N_2 from a tank and by scrubbing off excess CO_2 with a charcoal system. A humidifier was used to maintain RH to constant levels. Fruit samples were taken from each storage atmosphere after 4 or 7 months of storage and placed at 20 °C to simulate commercial shelf life and final quality of fruit that reach potential consumers. Unless stated otherwise, analyses were carried out after 7 days at 20 °C.

Chemicals. The chemicals obtained were of the highest quality available and were 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, 2-methyl-1-propanol, 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).

Analysis of Standard Quality Parameters. Fifteen apples per batch (storage period × atmosphere × calcium treatment) were used individually for the analysis of flesh firmness, soluble solids content (SSC), titratable acidity (TA), and skin color. Fruits were analyzed at harvest and 7 days after removal from cold storage as described above. Flesh firmness was measured on opposite sides of each fruit with a penetrometer (Effegi, Milan, Italy) equipped with an 11-mm diameter plunger tip; results were expressed in newtons. SSC and 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 percent of 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 grams of malic acid per liter. Fruit epidermis color was determined with a portable tristimulus colorimeter (Chroma Meter CR-200, Minolta Corp., Osaka, Japan) using CIE illuminant D_{65} and with an 8 mm measuring aperture diameter. Skin color was measured at two points on the equator of each fruit that were 180° apart: one on the side exposed to sunlight (ES) and the other on the shaded side (SS). Hue angle was measured on both the side exposed to the sun and the shaded side, and the resulting values were respectively used as measurements of superficial and background color.

Determination of Calcium Content. To determine calcium content, samples of pulp tissue were taken from five apples per batch, frozen in liquid nitrogen, freeze-dried, powdered, and kept at −80 °C until processing. Weight loss after lyophilization was consistently around 80%. One gram of lyophilized powdered tissue 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 dehydration of sample. Subsequently, dried material was resuspended in 2 mL of HCl/distilled water (1:1, v/v) for 15 min and passed through a Whatman 40 ashless filter. Finally, the filtrate was diluted to 50 mL in distilled water. Prepared samples were then analyzed by inductively coupled plasma emission spectroscopy (ICP-OES) in a Horiba Jobin Yvon ACTIVA spectrometer. Each determination was done in triplicate, and results were expressed as milligrams per 100 g of fresh weight (FW).

Determination of Ethylene Production. Ethylene production was measured at harvest date and 7 days after cold storage. Six apples per batch (storage period × atmosphere × calcium treatment) were weighed, placed into 3 L respiration jars, and continuously aerated with humidified air at a rate of 5 L h^{-1}. Gas samples of the effluent air from the respiration jars were taken with a 1 mL syringe and injected into a gas chromatograph (Agilent Technologies 6890N) equipped with a flame ionization detector and an alumina column (1.5 m × 3 mm). Gas analyses were conducted isothermically at 100 °C. N_2 carrier gas, air, and H_2 flows were 45, 400, and 45 mL min^{-1}, respectively. The injector and detector were held at 120 and 180 °C, respectively. Results were expressed as microliters of ethylene per kilogram and hour.

Analysis of Volatile Compounds. Eight kilograms of intact apples (2 kg/replicate × 4 replicates) were taken for extraction and analysis of volatile compounds. The extraction was performed according to the method of dynamic headspace as described in ref (*10*), with some modifications. Briefly, intact fruits 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) 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 of 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, U.K.) with 95% dimethyl-5% diphenylpolysiloxane 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 indices with those of standards and by enriching apple extract with authentic samples. 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, with the same capillary column as used 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 is expressed as micrograms per kilogram of fruit.

Analysis of Acetaldehyde Concentration. A 5 mL sample of juice obtained individually from 15 fruits per batch (storage period × atmosphere × calcium treatment) was introduced in a 10 mL test tube and frozen at −20 °C until analysis of acetaldehyde content as described previously (*11*). Test tubes closed with a rubber cap and containing frozen juice from each fruit were thawed and incubated at 65 °C for 1 h. Thereafter, a 1 mL headspace gas sample was taken with a syringe 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 microliters per liter.

Extraction and Assay of Aroma Volatile-Related Enzyme Activities. Samples of both peel and pulp tissue were taken separately from

Table 1. Meaning of *X*, *Y*, and *Z* Values for the Generic Sample Labels

	1	2
X^a	4	7
Y^b	air	1:2
Z^c	0	2

[a] Storage period at 1 °C (months) + 7 days at 20 °C. [b] Storage atmosphere conditions (O_2/CO_2). [c] Calcium treatment (% $CaCl_2$, w/v).

five apples per batch (storage period × atmosphere × calcium treatment), frozen in liquid nitrogen, lyophilized, powdered, and kept at −80 °C until processing. One hundred milligrams of lyophilized powdered tissue was used for each determination. Extraction and assay of LOX, 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 (*12*). Hydroperoxide lyase (HPL) activity was extracted and assayed according to the method in ref (*13*). Total protein content in the enzyme extract was determined with the Bradford method (*14*), 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^{-1} of protein).

Statistical Analysis. A multifactorial design with storage period, storage atmosphere, and calcium treatment as factors was used to statistically analyze the results. All data were tested by analysis of variance (GLM-ANOVA procedure) with the SAS program package (SAS Institute, Cary, NC, 1988) (*15*). Means were separated by the Fisher's LSD test at $P \leq 0.05$. Partial least-squares regression (PLSR) was used as a predictive method to relate a matrix of a dependent variable (*Y*) to a set of explanatory variables (*X*) in a single estimation procedure. Sample names were coded as *XYZ*, where *X*, *Y*, and *Z* refer to storage period, storage atmosphere, and calcium treatment, respectively, and take values of 1, 2, or 3 as indicated in **Table 1**. Unscrambler version 6.11a software (CAMO ASA, 1997) (*16*) was used for developing these models. As a pretreatment, data were centered and weighed by the inverse of the standard deviation of each variable to avoid dependence on measured units. Full cross-validation was run as a validation procedure.

RESULTS AND DISCUSSION

Standard Quality and Emission of Volatile Compounds at Harvest. Firmness of fruit at harvest averaged 71.2 N, the soluble solids content was 17.6%, and the titratable acidity was 3.5 g L^{-1}, thus indicating a suitable stage of maturity for long-term cold storage, according to recommendations for this cultivar (*17*). Thirty-six compounds were identified and quantified in the volatile fraction emitted by 'Fuji Kiku-8' apples at harvest, composed of 28 esters (9 acetates, 7 propanoates, 1 2-methylpropanoate, 4 butanoates, 3 2-methylbutanoates, and 4 hexanoates) and 8 alcohols (**Table 2**). Esters were quantitatively prominent among the volatiles produced, accounting for 73 and 89% of total volatiles emitted at harvest and after 1 week at 20 °C, respectively. Ethyl butanoate, ethyl 2-methylbutanoate, 2-methylbutyl acetate, butyl 2-methylbutanoate, pentyl propanoate, hexyl acetate, hexyl propanoate, and hexyl 2-methylbutanoate were found to have log odor units (OU) > 1 at harvest. Therefore, these molecules are suggested as having an impact on overall flavor (*18*). Ethyl butanoate, ethyl 2-methylbutanoate, 2-methylbutyl acetate, and hexyl acetate have been previously identified as compounds that contribute to 'Fuji' flavor at harvest (*19*, *20*), providing fresh-green and fruity odors (*10*, *21*). The number of putative flavor-contributing compounds increased in fruit kept at 20 °C for 7 days after harvest, as the log OU of four more esters (*tert*-butyl propanoate, butyl acetate, butyl propanoate, and 2-methylbutyl propanoate) also became positive, suggesting an enrichment of the aroma profile of apples in comparison to that observed immediately after harvest.

Did Calcium-Treated Fruit Actually Incorporate Calcium? To check the actual incorporation of calcium into the tissues after treatment and storage, flesh samples were analyzed by ICP-OES. Dipping of apples in 2% (w/v) $CaCl_2$ resulted in significantly increased concentrations of calcium in the pulp of fruit (**Table 3**). Range of increases observed was 16−39.6%, indicating that $CaCl_2$ treatment was efficient in incorporating calcium into fruit tissues. Calcium content was generally higher in fruit stored for 7 months than after 4 months, which is in agreement with earlier reports on calcium penetration rates into the flesh of apples from the epidermis. Calcium can penetrate the pulp through different feasible ways, including the lenticels as well as the microscopic fissures present in fruit cuticle and epidermis (*22*). Because fissures and other irregularities have been shown to become wider and deeper as storage duration increases (*23*), calcium ions could be taken up by fruit at higher rates. Thus, extending the storage period may allow easier and faster calcium incorporation into fruit pulp from the epidermis.

Standard Quality Parameters and Emission of Volatile Compounds after Cold Storage. Table 4 shows values for standard quality parameters in 'Fuji Kiku-8' apples after cold storage plus an additional period of 7 days at 20 °C, which simulated commercial life and final quality of the fruits that reach potential consumers. Similarly to previous reports (*2*), calcium treatment enhanced air-stored fruit quality by retarding flesh softening and reducing the rate of titratable acid decline. Moreover, air-stored, $CaCl_2$-treated fruit also had higher superficial red color. Superficial color is an important issue to take into account in the marketing of 'Fuji' apples, because insufficient red color development is generally associated with low visual consumer acceptability and has been reported as the most important instrumental quality parameter influencing apple purchasing patterns (*24*). Keeping fruit in ULO conditions also resulted in higher fruit firmness and TA content, regardless of calcium treatment. Furthermore, the combination of ULO storage and calcium treatment more effectively slowed the loss of fruit firmness and TA than either alone, suggesting that ULO and calcium treatment had an additive effect in slowing the loss of fruit firmness and TA.

Besides instrumental quality parameters, aroma is also a key attribute determining consumer acceptance of apples (*6*), and indeed the relevance of certain volatile compounds for consumer acceptability of 'Fuji' apples has been previously reported (*19*, *25*). Therefore, we were interested specifically in assessing the effects of a calcium treatment, alone or in combination with storage in ULO, on the emission of volatile compounds by 'Fuji Kiku-8' fruit during shelf life at 20 °C subsequent to cold storage. The emission of volatile compounds exhibiting positive log odor units (OU) either at harvest or after storage in air, and thus considered to have an impact on the overall flavor of fruit (*18*), is shown in **Table 5**. Butyl butanoate was also included due to its quantitative importance in the volatile fraction emitted by air-stored samples as well as ethyl acetate as an indicator of possible fermentative processes in stored fruit.

Results showed that ULO-stored apples exhibited reduced aroma volatile production, which is in accordance with previous reports in 'Fuji' apples (*19*, *25*). Although controlled atmosphere (CA) storage has many beneficial effects on fruit quality, it has been reported to cause a decrease in the production of volatiles due partially to a lack of lipid-derived precursors for ester biosynthesis (*20*, *26*). This reduction in the emission of volatile compounds by ULO-stored apples may decrease the value of produce. However, results reported herein suggest that prestorage $CaCl_2$ treatments caused enhanced emission of some impact compounds (**Table 5**). After 4 months of cold storage, the emissions of ethyl acetate, ethyl 2-methylbutanoate,

Table 2. Emission of Aroma Volatile Compounds (μg kg^{-1}) by 'Fuji Kiku-8' Apples 0 (H) and 7 (H+7) Days after Harvest

compound[a]	RI[b]	RI[c]	OTH[d]	H[e]	log OU[f]	H+7[e]	log OU[f]	code[g]
methyl acetate	854	–	8300 (a)	21.21 a	−2.59	48.25 a	−2.24	
ethyl acetate	882	609	13500 (b)	23.38 a	−2.76	32.93 a	−2.61	ea
ethanol	912	–	100000 (c)	204.34 a	−2.69	36.34 a	−3.44	etOH
tert-butyl propanoate	928	717	19 (a)	16.02 a	−0.07	35.46 a	0.27	tercbpr
propyl acetate	945	649	2000 (b)	13.88 b	−2.16	27.70 a	−1.86	
methyl butanoate	955	656	5 (d)	<0.5		0.57		mb
2-methylpropyl acetate	976	691	65 (e)	5.79 a	−1.05	9.79 a	−0.82	
1-propanol	992	–	9000 (c)	9.06 b	−3.00	22.66 a	−2.60	
ethyl butanoate	1002	803	1 (f)	4.46 b	0.65	11.16 a	1.05	eb
propyl propanoate	1008	809	57 (c)	12.39 b	−0.66	28.25 a	−0.30	
ethyl 2-methylbutanoate	1015	845	0.006 (e)	24.00 a	3.60	41.31 a	3.84	e2mb
butyl acetate	1040	813	66 (b)	45.36 b	−0.16	98.37 a	0.17	ba
2-methylpropyl propanoate	1046	865	–	<0.5		1.88		
2-methyl-1-propanol	1054	996	250 (g)	2.56 a	−1.99	4.83 a	−1.71	
2-methylbutyl acetate	1096	876	11 (e)	360.33 a	1.52	653.09 a	1.77	2mba
1-butanol	1119	626	500 (c)	18.23 b	−1.44	53.99 a	−0.97	bOH
butyl propanoate	1123	910	25 (c)	16.83 b	−0.17	42.70 a	0.23	bpr
butyl 2-methylpropanoate	1129	1009	80 (h)	12.90 b	−0.79	38.81 a	−0.31	
pentyl acetate	1161	914	43 (b)	11.54 a	−0.57	22.46 a	−0.28	
2-methylbutyl propanoate	1180	950	19 (a)	12.60 a	−0.18	45.10 a	0.38	2mbpr
2-methyl-1-butanol	1199	667	250 (f)	48.40 b	−0.71	135.81 a	−0.26	2mbOH
butyl butanoate	1218	1000	100 (h)	10.26 b	−0.99	32.47 a	−0.49	bb
butyl 2-methylbutanoate	1235	1042	17 (h)	21.31 b	0.10	70.37 a	0.62	b2mb
pentyl propanoate	1247	969	1 (e)	1.89 b	0.28	5.25 a	0.72	ppr
1-pentanol	1262	688	4000 (g)	1.47 b	−3.43	4.47 a	−2.95	pOH
hexyl acetate	1292	1015	2 (g)	42.78 b	1.33	98.35 a	1.69	ha
hexyl propanoate	1379	1109	8 (i)	15.72 b	0.29	58.28 a	0.86	hpr
1-hexanol	1392	869	500 (g)	1.69 b	−2.47	6.99 a	−1.85	hOH
2-methylpropyl hexanoate	1399	1153	–	1.65 b		23.90 a		
butyl hexanoate	1473	1196	700 (h)	23.62 b	−1.47	94.90 a	−0.87	
hexyl butanoate	1477	1197	250 (e)	9.01 b	−1.44	38.10 a	−0.82	
hexyl 2-methylbutanoate	1488	1239	6 (h)	58.67 b	0.99	169.53 a	1.45	h2mb
octyl acetate	1549	1215	12 (f)	1.20 a	−1.00	6.51 a	−0.27	
2-ethylhexanol	1565	1031	–	1.48 a		3.73 a		
pentyl hexanoate	1590	1293	–	3.39 a		7.98 a		
hexyl hexanoate	1687	1392	6400 (j)	8.99 b	−2.85	29.62 a	−2.33	

[a] Compounds identified on the basis of a comparison of mass spectrometric data and retention indices with authentic reference compounds. [b] Kovats retention index in cross-linked FFAP column. [c] Kovats retention index in BPX5 column; –, eluted with the solvent. [d] Odor threshold (μg kg^{-1}) in water reported in ref (a) (*34*), (b) (*35*), (c) (*36*), (d) (*37*), (e) (*38*), (f) (*39*), (g) (*18*), (h) (*40*), (i) (*41*), (j) (*42*); –: not found. [e] Values are the means of four samples obtained each from 2 kg of apples after 4 h of collection. Means within the same row followed by different lower case letters are significantly different at $P \leq 0.05$ (LSD test). [f] $\log_{10}$ of odor unit value = $\log_{10}$ (amount/OTH). [g] Codes used for multivariate analysis of data.

Table 3. Calcium Content (mg 100 g^{-1} FW) in the Flesh of 'Fuji Kiku-8' Apples after 7 Days at 20 °C following Cold Storage[a]

		storage period	
storage atmosphere	treatment	4 months	7 months
air	untreated	2.88 Bb	4.14 Ba
	$CaCl_2$	3.89 Ab	4.83 Aa
ULO	untreated	3.15 Bb	5.08 Ba
	$CaCl_2$	4.40 Ab	5.89 Aa

[a] Data represent means of three replicates. Means within the same column for a given storage atmosphere followed by different capital letters are significantly different at $P \leq 0.05$ (LSD test). Means in the same row followed by different lower case letters are significantly different at $P \leq 0.05$ (LSD test).

2-methylbutyl acetate, butyl propanoate, butyl butanoate, and pentyl propanoate were higher in $CaCl_2$-treated than in untreated apples, regardless of storage atmosphere, which suggests that calcium applications might help to improve aroma quality in this cultivar after middle-term storage. Indeed, sensory analysis by means of a consumer panel (data not shown) indicated higher acceptance scores for $CaCl_2$-treated fruit. For ULO-stored samples, calcium treatments enhanced the production of butyl acetate, butyl 2-methylbutanoate, hexyl acetate, and hexyl propanoate after 4 months of cold storage. Therefore, $CaCl_2$ applications prior to cold storage of apples under hypoxic conditions might also be useful as a means for partial regeneration of aroma quality during the commercial life of fruit, particularly after middle-term cold storage. These results are in agreement with earlier findings (*27*), when it was observed that calcium-treated 'Golden Delicious' apples produced the same or higher total flavor-associated volatile levels in comparison to untreated fruit when stored for at least 4 months.

After fruits were stored for 7 months, the effects of calcium treatment on the production of volatile compounds were different for the two storage atmospheres considered. The emission of some aroma volatile compounds, namely, ethyl acetate, *tert*-butyl propanoate, 2-methylbutyl acetate, 2-methylbutyl propanoate, butyl butanoate, butyl 2-methylbutanoate, and hexyl 2-methylbutanoate, was higher in $CaCl_2$-treated fruit stored in air than in untreated control fruit, whereas no such enhancement was found in apples stored under ULO (**Table 5**). Furthermore, decreased productions of butyl acetate, 2-methylbutyl acetate, butyl 2-methylbutanoate, and hexyl 2-methylbutanoate were observed in fruit stored in ULO, which partially agrees with previous work on 'Fuji' apples (*19*), when significantly reduced total aroma volatile emission was found after storage for 7 months in ULO. The reduction in total volatile emission after long-term storage of

Table 4. Maturity and Quality Parameters of 'Fuji Kiku-8' Apples at Harvest and after 7 Days at 20 °C following Cold Storage[a]

			storage period			
			4 months		7 months	
	at harvest	atmosphere	untreated	$CaCl_2$	untreated	$CaCl_2$
ethylene production (μL kg^{-1} h^{-1})	0.1	air	14.6 Aa	16.6 Aa	12.9 Ba	21.7 Aa
		ULO	0.5 Ab	0.5 Ab	1.3 Ab	1.4 Ab
acetaldehyde content (μL L^{-1})	0.3	air	0.9 Ba	1.4 Aa	1.0 Aa	0.7 Ba
		ULO	0.7 Bb	1.0 Ab	0.7 Ab	0.4 Ba
firmness (N)	71.2	air	64.3 Bb	70.2 Ab	55.3 Bb	63.1 Ab
		ULO	71.1 Ba	76.5 Aa	72.4 Aa	72.3 Aa
TA (g L^{-1})	3.5	air	1.5 Bb	2.1 Ab	0.9 Bb	1.2 Ab
		ULO	2.7 Aa	2.5 Aa	7.7 Ba	2.0 Aa
SSC (%)	17.6	air	16.8 Aa	16.9 Aa	16.1 Ba	16.6 Aa
		ULO	16.8 Aa	17.0 Aa	15.5 Bb	16.1 Ab
hue (SS)	101.9	air	89.1 Aa	91.3 Aa	90.4 Aa	75.1 Bb
		ULO	80.1 Aa	78.6 Ab	85.4 Aa	87.7 Aa
hue (ES)	44.8	air	54.3 Aa	44.5 Ba	58.0 Aa	45.4 Ba
		ULO	46.3 Ab	41.7 Aa	48.0 Ab	42.3 Aa

[a] Data represent means of 3 (ethylene production) and of 15 (acetaldehyde content and standard quality parameters) replicates. Means in the same row for a given storage period showing different capital letters are significantly different at $P \leq 0.05$ (LSD test). Means followed by different lower case letters within a column for a given parameter are significantly different at $P \leq 0.05$ (LSD test).

Table 5. Emission (μg kg^{-1}) of Volatile Esters Contributing to Overall Flavor of 'Fuji Kiku-8' Apples after 7 Days at 20 °C following Cold Storage[a]

		storage period			
		4 months		7 months	
	atmosphere	untreated	$CaCl_2$	untreated	$CaCl_2$
ethyl acetate	air	34.1 (−2.6) Ba	53.8 (−2.4) Aa	10.1 (−3.1) Ba	20.0 (−2.8) Aa
	ULO	16.4 (−2.9) Bb	35.5 (−2.5) Ab	13.9 (−2.9) Aa	16.4 (−2.9) Aa
tert-butyl propanoate	air	31.6 (0.2) Aa	33.4 (0.2) Aa	6.0 (−0.5) Ba	13.1 (−0.1) Aa
	ULO	5.9 (−0.5) Ab	9.4 (−0.3) Ab	4.0 (−0.7) Aa	4.5 (−0.6) Ab
methyl butanoate	air	19.9 (0.6) Aa	19.5 (0.6) Aa	9.9 (0.3) Aa	12.7 (0.4) Aa
	ULO	1.1 (−0.6) Bb	0.8 (−0.8) Bb	0.5 (−1.0) Ab	0.2 (−1.4) Ab
ethyl butanoate	air	44.5 (1.6) Aa	41.1 (1.6) Aa	14.2 (1.2) Aa	17.5 (1.2) Aa
	ULO	9.0 (1.0) Ab	14.7 (1.2) Ab	5.3 (0.7) Ab	1.0 (0.0) Ab
ethyl 2-methylbutanoate	air	151.5 (4.4) Ba	179.8 (5.5) Aa	48.0 (3.9) Aa	52.3 (3.9) Aa
	ULO	48.4 (3.9) Bb	82.7 (4.1) Ab	18.9 (3.5) Ab	11.6 (3.2) Ab
butyl acetate	air	203.2 (0.5) Aa	232.2 (0.5) Aa	96.6 (0.2) Aa	98.7 (0.2) Aa
	ULO	70.9 (0.0) Bb	142.8 (0.3) Ab	64.6 (0.0) Aa	18.7 (−0.5) Bb
2-methylbutyl acetate	air	536.6 (1.7) Ba	708.7 (1.8) Aa	247.6 (1.4) Bb	334.3 (1.5) Aa
	ULO	456.2 (1.6) Ba	706.8 (1.8) Aa	459.7 (1.6) Aa	167.9 (1.2) Bb
butyl propanoate	air	62.2 (0.4) Ba	96.5 (0.6) Aa	46.8 (0.3) Aa	50.0 (0.3) Aa
	ULO	22.1 (0.0) Bb	48.2 (0.3) Ab	21.4 (−0.1) Ab	9.8 (−0.4) Ab
2-methylbutyl propanoate	air	29.0 (0.2) Aa	46.3 (0.4) Aa	12.8 (−0.2) Ba	22.0 (0.1) Aa
	ULO	7.3 (−0.4) Ab	20.4 (0.0) Ab	12.7 (−0.2) Aa	6.9 (−0.4 Ab
butyl butanoate	air	161.9 (0.2) Ba	194.0 (0.3) Aa	79.6 (−0.1) Ba	124.8 (0.1) Aa
	ULO	35.1 (−0.5) Bb	64.4 (−0.2) Ab	30.8 (−0.5) Ab	17.9 (−0.7) Ab
butyl 2-methylbutanoate	air	324.5 (1.3) Aa	369.4 (1.3) Aa	107.6 (0.8) Ba	167.5 (1.0) Aa
	ULO	61.2 (0.6) Bb	134.0 (0.9) Ab	49.0 (0.5) Ab	15.9 (0.0) Bb
pentyl propanoate	air	4.9 (0.7) Ba	6.9 (0.8) Aa	1.8 (0.3) Aa	2.3 (0.4) Aa
	ULO	2.2 (0.3) Bb	4.6 (0.4) Ab	1.2 (0.1) Aa	0.8 (−0.1) Ab
hexyl acetate	air	245.0 (2.1) Aa	258.0 (2.1) Aa	113.7 (1.8) Aa	142.0 (1.9) Aa
	ULO	154.7 (1.9) Bb	204.5 (2.0) Ab	98.4 (1.7) Aa	49.6 (1.4) Ab
hexyl propanoate	air	154.6 (1.3) Aa	148.0 (1.3) Aa	47.7 (0.8) Ba	70.6 (0.9) Aa
	ULO	54.7 (0.8) Bb	87.9 (1.0) Ab	39.6 (0.7) Aa	25.7 (0.5) Ab
hexyl 2-methylbutanoate	air	966.5 (2.2) Aa	687.8 (2.0) Ba	255.1 (1.6) Ba	363.8 (1.8) Aa
	ULO	273.6 (1.6) Ab	359.2 (1.8) Ab	187.3 (1.5) Aa	85.0 (1.2) Bb

[a] Data represent means of four replicates obtained each from 2 kg of apples after 4 h of collection. Numbers between brackets stand for log OU, where OU = concentration/ odor threshold. Means in the same row for a given storage period showing different capital letters are significantly different at $P \leq 0.05$ (LSD test). Means followed by different small letters within a column for a given compound are significantly different at $P \leq 0.05$ (LSD test).

apples in CA has been suggested to arise from partial inhibition of some volatile-related enzyme activities (*20*, *26*). Therefore, the effects of calcium treatment and of storage conditions considered herein on several volatile-related enzyme activities were also analyzed.

Volatile-Related Enzyme Activities after Cold Storage. A PLSR model was developed for the emission of selected volatile esters, their alcohol precursors, and acetaldehyde content (Y variables), with LOX, HPL, PDC, ADH, and AAT activities as the X variables. The model accounted for 84% of total variability

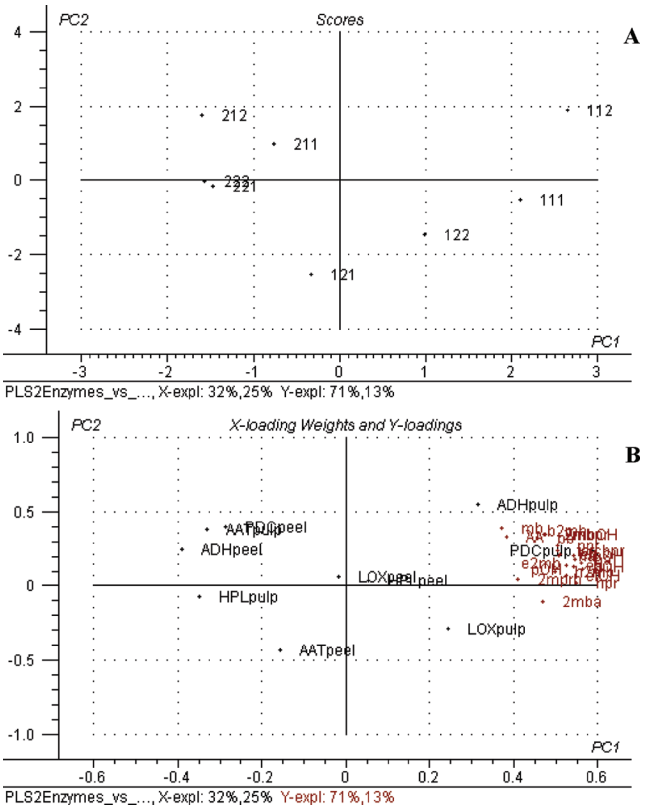

Figure 1. Scores (**A**) and loadings (**B**) plots of PC1 versus PC2 corresponding to a PLSR model for emission of volatile compounds (*Y* variables) versus volatile-related enzyme activities (*X* variables) in 'Fuji Kiku-8' apple fruit after cold storage under different conditions. Samples and volatile compounds are labeled as indicated in **Tables 1** and **2**, respectively (AA, acetaldehyde).

observed in the production of volatile compounds (**Figure 1**), which suggests a positive link to the enzyme activities included in the regression analysis. Samples distributed along the first principal component (PC1) of the corresponding scores plot according to storage period (**Figure 1A**). In turn, samples stored for 4 months separated according to storage atmosphere (PC1) and according to calcium treatment for each storage atmosphere (PC2). With regard to fruit stored for 7 months, no clear differentiation was observed between samples. The loadings plot (**Figure 1B**) showed that samples stored for 4 months were characterized by higher emission of all volatile compounds included in the model. Higher emissions were associated with higher PDC, ADH, and, to a lesser extent, LOX activities in the pulp tissue, particularly for fruit treated with $CaCl_2$ and stored in air. The plot also indicated higher volatile production for $CaCl_2$-treated fruit stored in ULO in comparison to untreated fruit, linked likewise to higher levels of LOX, PDC, and ADH activities. The model reflected the diminished capacity for biosynthesis of volatile compounds after long-term storage of fruit (**Table 5**), possibly arising from a lessening in these enzyme activity levels.

AAT activity is directly responsible for the production of volatile esters by linking an alcohol to an acyl-CoA (*8*). However, the regression model showed no apparent relationship between ester production and this enzyme activity (**Figure 1B**), suggesting the relevance of an adequate supply of substrates for the AAT-catalyzed reaction by enzymes located upstream in the biosynthetic pathway. This would explain the strong association observed between PDC and ADH activities in the pulp tissue and the emission of volatile esters after storage. PDC uses pyruvic acid

Table 6. Specific Activities (U mg^{-1} protein) of Volatile-Related Enzymes in the Pulp of 'Fuji Kiku-8' Apples after 7 Days at 20 °C following Cold Storage[a]

		storage period			
		4 months		7 months	
	atmosphere	untreated	$CaCl_2$	untreated	$CaCl_2$
LOX	air	5.519 Aa	4.381 Ba	3.875 Aa	2.925 Bb
	ULO	4.234 Aa	4.103 Aa	4.121 Aa	4.403 Aa
PDC	air	50.946 Ba	85.420 Aa	41.085 Aa	40.079 Aa
	ULO	29.304 Ba	55.962 Ab	25.079 Ab	24.815 Ab
ADH	air	34.961 Ba	56.833 Aa	42.488 Aa	42.257 Aa
	ULO	19.428 Bb	28.818 Ab	35.086 Ab	38.125 Aa
AAT	air	0.009 Aa	0.011 Aa	0.030 Ba	0.036 Aa
	ULO	0.011 Aa	0.013 Aa	0.022 Ab	0.023 Ab

[a] Data represent means of three replicates obtained each from 2 kg of apples after 4 h of collection. Means in the same row for a given storage period showing different capital letters are significantly different at $P \leq 0.05$ (LSD test). Means followed by different lower case letters within a column for a given enzyme activity are significantly different at $P \leq 0.05$ (LSD test).

to produce acetaldehyde, which can be processed subsequently by ADH or by aldehyde dehydrogenase (ALDH, EC 1.2.1.5) to render ethanol or acetyl-CoA, respectively. In turn, acetyl-CoA acts as the acylating agent for acetate ester formation (*28*). Although all of these products can accumulate during fruit ripening even under aerobic conditions, PDC and ADH are generally associated with anaerobic metabolism. Consequently, PDC and ADH activities can be induced by low O_2 levels in fruit tissues. Previous work (*29*) has shown that gradients in O_2 concentration across pulp tissue of apples were increased by vacuum infiltration of $CaCl_2$, due to decreased O_2 diffusivity in pulp tissues and increased skin resistance to gas diffusion. In addition, applications of calcium can also increase internal CO_2 levels in apples (*30*, *31*). Thus, dipping of fruit in a $CaCl_2$ solution could have lessened O_2 levels and caused CO_2 accumulation inside the fruit, which would explain enhanced PDC and ADH activities observed in $CaCl_2$-treated apples stored for 4 months (**Table 6**), possibly leading to improved availability of substrates for AAT-catalyzed ester production during the shelf life period. Indeed, hypoxia-induced acetaldehyde and ethanol accumulation has been shown to have the potential to increase the production of volatile esters in apple (*32*). Accordingly, the emission of alcohol precursors and acetaldehyde content in the samples were associated with the emission of volatile esters as shown by PLSR analysis (**Figure 1B**). Actually, an increase in the production of some alcohol precursors (ethanol, 1-butanol, 2-methyl-1-butanol, and 1-hexanol) due to $CaCl_2$ treatment was observed in apples stored for 4 months (**Table 7**). Acetaldehyde content in $CaCl_2$-treated fruit was also higher than in untreated apples (**Table 4**). This was possibly related to enhanced ADH and PDC activities, respectively, in the pulp of these apples (**Table 6**). In fact, good correlations were found between the emission of some specific ester families and their corresponding alcohol precursors. The plots of the selected ethyl and butyl esters emitted versus ethanol and 1-butanol are given as examples (panels **A** and **B**, respectively, of **Figure 2**), showing R^2 values of 0.74 and 0.94, correspondingly, suggesting the importance of substrate supply for AAT-catalyzed ester production.

LOX activity, which has been found to be essential for the recovery of the ability to synthesize volatile esters after cold storage of 'Fuji' apples (*26*), was inhibited by calcium treatment in air-stored fruit (**Table 6**). During fruit ripening, cell walls and membranes become more permeable to different substrates, increasing the possibility of a LOX-mediated cleavage of fatty acids, but this chance of reaction might be delayed in

Table 7. Emission (μg kg^{-1}) of Alcohol Precursors for Volatile Ester Biosynthesis by 'Fuji Kiku-8' Apples after 7 Days at 20 °C following Cold Storage[a]

		storage period			
		4 months		7 months	
	atmosphere	untreated	$CaCl_2$	untreated	$CaCl_2$
ethanol	air	24.2 Ba	41.3 Aa	11.7 Aa	13.9 Aa
	ULO	15.1 Ba	33.9 Aa	9.4 Aa	10.3 Aa
1-butanol	air	168.0 Ba	214.0 Aa	38.2 Ba	53.7 Aa
	ULO	37.4 Bb	69.7 Ab	23.7 Cb	3.2 Db
2-methyl-1-butanol	air	133.6 Ba	211.3 Aa	92.8 Aa	111.6 Aa
	ULO	68.3 Bb	124.5 Ab	109.6 Aa	59.7 Bb
1-pentanol	air	4.2 Aa	3.8 Aa	2.3 Aa	2.5 Aa
	ULO	1.9 Ab	2.4 Ab	1.7 Aa	1.6 Aa
1-hexanol	air	5.8 Ba	8.5 Aa	2.6 Ba	6.0 Aa
	ULO	4.8 Ba	8.1 Aa	4.7 Aa	1.8 Bb

[a] Data represent means of four replicates obtained each from 2 kg of apples after 4 h of collection. Means in the same row for a given storage period showing different capital letters are significantly different at $P \leq 0.05$ (LSD test). Means followed by different lower case letters within a column for a given compound are significantly different at $P \leq 0.05$ (LSD test).

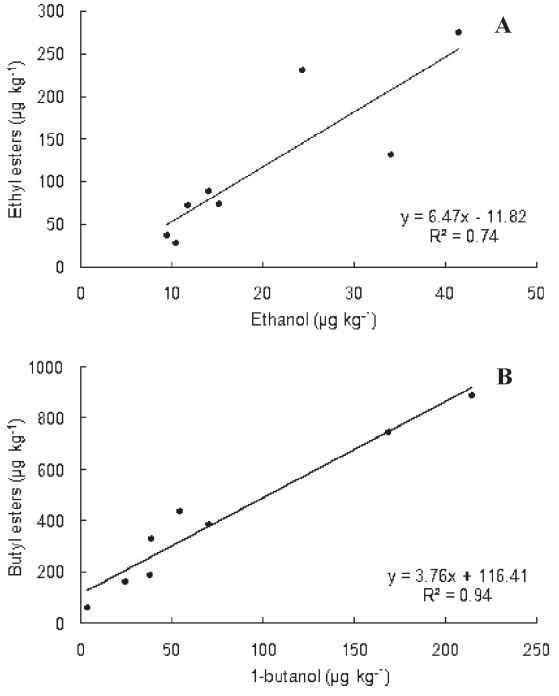

Figure 2. Correlation between the emissions of ethanol (**A**) and 1-butanol (**B**) and flavor-contributing ethyl and butyl esters, respectively, emitted by cold-stored 'Fuji Kiku-8' apples. Points represent the means of four replicates.

$CaCl_2$-treated fruit as a consequence of the beneficial role of this mineral in maintaining the structural integrity of membranes (*9*). The observation that the production of several straight-chain esters, generally considered to arise from lipid metabolism through the LOX pathway (*8*), was enhanced in $CaCl_2$-treated apples, particularly after 4 months of storage (**Table 5**), despite lower LOX activity levels (**Table 6**), suggests that applied calcium might be exerting a protective role on membranes, which would allow a better regulation of LOX activity.

With regard to fruit stored for 7 months, no significant influence of calcium treatment on PDC and ADH activities was found, although some differences in the volatile emission profile of these fruit were observed as a consequence of the factors considered (**Table 5**). Long-term-stored fruit had lower emission of most volatile esters selected than those stored for 4 months (**Table 5**). This may be related to lower levels of their alcohol precursors. Anyhow, air-stored samples dipped in $CaCl_2$ emitted higher levels of some volatile compounds than untreated fruit. After 7 months of cold storage, $CaCl_2$-treated apples stored in air were characterized by higher AAT activity in the pulp tissue (**Table 6**) and, indeed, showed enhanced production of some volatile esters (**Table 5**). These results indicate that, although not sufficient, AAT activity was necessary for the production of volatile esters after cold storage and that not only substrate supply but also ester-forming capacity itself may be compromised after long-term storage of 'Fuji Kiku-8' apples. Calcium dips prior to cold storage in air were thus shown to potentially have beneficial effects on the aroma quality of 'Fuji Kiku-8' apples, even after a long period of cold storage. The observed increase in AAT activity, which was concomitant with higher ethylene production (**Table 4**) in $CaCl_2$-treated fruit after long-term storage, may be in agreement with previous work reporting the activity of AAT to be ethylene-dependent (*33*). However, inhibition of AAT activity in ULO-stored samples (**Table 6**) was not reversed by calcium treatments, and indeed the beneficial effects of calcium applications on the emission of volatile esters after long-term storage were restricted to fruit stored in air (**Table 5**).

In summary, calcium treatment generally increased production of volatile esters after storage of 'Fuji Kiku-8' apples, probably as a consequence of enhanced PDC and ADH activities with concomitantly better supply of acetaldehyde and alcohol precursors in fruit stored for 4 months. Treatment also increased AAT activity in samples stored in cold air for a longer period, leading to higher emission of volatile esters. Inhibition of volatile ester emission after long-term storage in ULO was apparently not recoverable by calcium applications. Thus, postharvest calcium treatments have the potential to improve aroma quality of cold-stored 'Fuji Kiku-8' apples, with interesting implications from the commercial point of view: these treatments have no damaging effects on the environment, are considerably more economical and simple than CA technology, and may have beneficial side effects on the nutritional quality of produce.

ABBREVIATIONS USED

AA, acetaldehyde; AAT, alcohol *o*-acyltransferase; ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; CA, controlled atmosphere; ES, exposed side; HPL, hydroperoxide lyase; LOX, lipoxygenase; OTH, odor threshold; OU, odor unit; PC1, first principal component; PC2, second principal component; PDC, pyruvate decarboxylase; PLSR, partial least-squares regression; SS, shaded side; SSC, soluble solids content; TA, titratable acidity; ULO, ultralow oxygen atmosphere.

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