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Aroma Volatiles of Apples as Influenced by Ripening and Storage Procedures

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Abstract

Odour volatiles represent a major quality parameter for fresh produce. Consequently, improving the emission of volatiles in fruit has become an important challenge. A series of experiments were conducted in our laboratories in the last 20 years that aimed to elucidate the development of volatiles of various fruit types, but with an emphasis on apples. The major findings of these experiments are the following: 1) Early harvested apples had a poorer ability to produce volatiles, and that was coupled with lower respiration and less fatty acids (FA) levels. This may be related to the insensitivity of immature fruit to ethylene, since treatment with high concentrations of ethylene stimulated respiration, and increased the levels of FA and volatiles. 2) The biosynthesis of volatiles is highly reduced by apple fruit following ultra low oxygen (ULO) storage, and also after treatments with aminoethoxyvinyl glycine and 1-methylcyclopropene; the reduction became severe after an extended storage period. 3) Feeding apple fruit with volatile precursors (alcohols and aldehydes) stimulated the biosynthesis of the corresponding volatiles, mainly esters. Moreover, feeding AVG-treated fruit with precursors also led to a marked increase in the production of the corresponding volatiles. However, this effect was transitory with both ULO stored as well as with AVG-treated fruit.

INTRODUCTION

The aroma of fruit is a complex trait, due to the different biosynthetic pathways involved, and the complexity of the regulation of their biosynthesis (Fellman et al., 2000). The significance of odour volatiles to the quality of fruit is clear to the point that Pelayo et al. (2003) suggested that a new concept like "flavour life" needed to be defined, in addition to the concept of postharvest life. Flavour life was defined as 'the maximum period of storage during which fruit maintain a similar flavour profile to that present in freshly harvested fruit'. According to Schotsmans and Prange (2006) the main reason that aroma has not been included in standard descriptions of postharvest quality lies in the complexity of its measurements. It is worth to mention here that apple aroma is a mixture of substances; esters are the major group (78-92%) of these substances (Dixon and Hewett, 2000).

Concerning the effect of harvest time and storage conditions on qualitative and quantitative changes, a huge number of studies is available. Taking into account that apples are climacteric fruit, and that maturity stage is critical to the flavour development of fruit (Echeverría et al., 2004), it is expected that too early harvesting would result in a lack of full flavour development. Storage has also a pronounced effect on the flavour of fruit. Harb et al. (2000) showed that ULO-storage caused a marked reduction in the ability of fruit to produce volatiles. Differential impact of various controlled atmosphere

(CA) storage conditions, as well as treatments with AVG and 1-MCP, on the emission of odour volatiles has been also reported in numerous studies (Halder-Doll and Bangerth, 1987).

In this paper the results of our studies, that were conducted over the last 20 years, will be explored for the sake of better understanding of how harvest time, storage conditions, and postharvest treatments aimed to block ethylene synthesis and/or action influence the biosynthesis of odour volatiles. Moreover, changes at molecular level will be discussed since we have begun investigating differential gene expression under different conditions.

MATERIALS AND METHODS

Ripening Stages of Apple Fruit

Uniform apple fruit were collected from 18 year old 'Golden Delicious' trees on M9 rootstocks at the Experiment Station of the University of Hohenheim, Stuttgart, Germany. Stages of ripening of the fruit were characterized by their internal ethylene concentrations, starting with the 1st harvest on September 9th to the 6th harvest on October 20th. Internal ethylene concentration for the first harvest (immature) and the 5th harvest (mature) were 0.0009 µl L^{-1} and 0.50 µl L^{-1}, respectively. Analytical procedures to determine volatile emission, fatty acids, and ATP/ADP are described in more detail by Song and Bangerth (1996, 2003) and Tan and Bangerth (2001).

CA-Storage of Apples

'Golden Delicious' apples were obtained from the orchard of Kompetenzzentrum Obstbau Bodensee-Ravensburg, Germany. Fruit were stored under the following (kPa CO_2 + kPa O_2) conditions: (3+21, 3+3, 3+1, 1+21, 1+3, and 1+1). Volatile determination was conducted by two methods: the total emission adopting the method of volatile adsorption on small quantities of charcoal as described by Streif (1981), and individual volatiles by TENAX trapping method as reported by Harb et al. (2000).

Odour Volatiles Precursors

'Golden Delicious' apples were stored under ULO-condition (3 kPa CO_2 + 1 kPa O_2) at 1°C and 93% R.H. Directly after harvest and at two-month intervals during the subsequent storage period, the total amount of volatiles was determined as mentioned above. In another experiment 'Golden Delicious' trees were sprayed with aminoethoxy-vinyl glycine (AVG) at a concentration of 200 mg.L^{-1}. The trees were sprayed 4 times at weekly intervals starting one month before harvest. Fruit from AVG-treated trees were also stored under ULO-storage condition, and subjected to the same treatments and measurements as other fruit. At two periods (directly after harvest and after sixth months of storage) precursors were applied separately to the fruit as described earlier by Harb et al. (2000). The determination of volatiles was conducted as described by Song and Bangerth (1996) after seven days of conditioning at room temperature. For comparison, the r-factor was calculated based on the peak area of the standard; "r-factor" comprises the peak area of a particular odour volatile after the treatment divided by the peak area of the same odour volatile emitted by the control fruit.

1-Methylcyclopropene (1-MCP) Treatment

Apple (cv. 'Jonagold') and pear (cv. 'Conference') fruit were obtained from the orchards of the Experimental Station Bavendorf. Fruits were harvested at optimal ripening stage for long-term storage, cooled to 4°C within one day, and further treated with 625 ppb 1-MCP during 24 h according to the recommendations of AgroFresh Company. The subsequent storage of the 1-MCP treated and untreated apples were performed in experimental storage chambers kept under CA (3 kPa O_2 + 1 kPa O_2) or regular atmosphere (RA) conditions.

Statistical Analysis

Relevant results were subjected to analysis of variance (ANOVA) using the CoStat-software (CoHort Software, Monterey, CA, 1998), and mean separations were calculated by Student-Newman-Keuls range test at $P \leq 0.05$.

RESULTS

Influence of Ripening Stage on Respiration Rate, Emission of volatiles, and Fatty Acid Composition of 'Golden Delicious' Apples

Harvesting apple too early is of advantage for producers since the fruit are firmer, but it resulted in a poorer ability to produce volatiles (Fig. 1). This reduced ability to synthesize volatiles did not improve even over a conditioning period of seven days. These results were correlated with a lower concentration of total fatty acids in early harvested apples compared to late harvested fruit (Fig. 2). Furthermore, ripe apple fruit contained significantly higher amounts of both ATP and ADP (Fig. 3).

Influence of CA-Storage Conditions on Emission of Volatiles of 'Golden Delicious' Apples

The influence of various CA-storage conditions on the total amount of odour volatiles of 'Golden Delicious' is shown in Figure 4, whereas the influence on selected odour volatiles is shown on Table 1. It is clear that storing both cultivars under ULO-storage conditions (3 kPa CO_2 + 1 kPa O_2) resulted in dramatic and significant reduction on the biosynthesis of volatiles. Decreasing the O_2-level down to 3 kPa, without any increase in CO_2-level (1 kPa CO_2 + 3 kPa O_2), did not impair the capacity of fruit to synthesize the volatiles. However, increasing CO_2-level at the same O_2-level (3 kPa CO_2 + 3 kPa O_2) resulted in significant reduction of volatile levels. It is clear that decreasing O_2 down to 1kPa and/or increasing CO_2-level up to 3 kPa resulted in significant reduction in the emission of volatiles. Investigating changes of individual odour volatiles clearly revealed that the emission of straight-chain esters, in particular butyl acetate, pentyl acetate, and to lesser extent hexyl acetate, is influenced negatively by various CA-conditions, in particular ULO-storage conditions. On the other hand, the emission of branched esters, namely 3-methyl butyl acetate and hexyl 2-methyl butyrate, was higher by CA- and ULO-stored fruit than both air-stored and freshly harvested fruit. Concerning alcohols, the same trend can be seen, in which the emission of straight-chain alcohols was reduced, but the emission of branched alcohols was promoted upon CA- and ULO-storage.

Influence of Feeding with Precursors on the Emission of Volatiles of CA-Stored 'Golden Delicious' Apples

Feeding trials were conducted with 'Golden Delicious' apples, directly after harvest and after five months storage period under ULO storage (Tables 2 and 3). Feeding fruit with butanol resulted in dramatic increase in the emission of the major butyl- and -butyrate compounds: both straight-chain and branched compounds. Interestingly, the emission of both 2-propyl acetate and propyl acetate was also enhanced, while the emission of 2-methylpropyl acetate, pentyl acetate and hexyl acetate was generally retarded. Feeding fruit with 2-methylpropanol resulted also in the enhancement of the emission of 2-methylpropyl acetate (on all dates), and butyl 2-methylbutyrate and pentyl acetate (after ULO-storage). The emission of other odour volatiles was slightly affected.

Influence of AVG Treatment and Feeding with Precursors on the Emission of Volatiles of 'Golden Delicious' Apples

The effect of AVG treatment is shown in Table 4. The emission of volatiles, either straight-chain (propyl acetate, butyl acetate, pentyl acetate, butyl butyrate, and hexyl acetate) or branched (2-methylpropyl acetate, butyl 2-methylbutyrate, and hexyl 2-methyl butyrate) was diminished by this treatment. Moreover, the emission of the major alcohols

(1-propanol, butanol, hexanol, and 3-methyl 1-butanol) was also decreased. Feeding AVG-treated fruit with precursors resulted in a short-term stimulation in the emission of corresponding volatiles as shown in Table 5. Feeding pentanol to AVG-treated fruit resulted in more emission of pentyl acetate for all dates, and of pentyl butyrate directly after treatment; the stimulatory effect diminished quickly for this compound. However, this stimulation was at the expense of other volatiles, in particular butyl acetate and hexyl acetate with fruit treated directly after harvest; the emission of these volatiles was stimulated upon feeding of AVG-treated and ULO-stored fruit.

Influence of 1-MCP Treatment on the Emission of Volatiles (Total Amount) of 'Jonagold' Apples

Treatment of fruit with 1-MCP resulted in a significant reduction of the emission of odour volatiles, which persisted for months (Fig. 5). However, a partial recovery was registered after five months storage period for apples treated with 1-MCP but stored in air (21 kPa O_2); a corresponding recovery for 1-MCP treated but CA-stored (low O_2) apples is absent. Moreover, it is obvious that CA-storage alone led also to strong and significant reduction in the emission of odour volatiles.

DISCUSSION

The impact of early harvest on the reduced capacity of fruit to synthesize volatiles is well documented, and is coupled according to Song and Bangerth (1996) with lower respiration and fatty acid (FA) concentration. Furthermore, Tan and Bangerth (2001) and Saquet et al. (2003) traced back this phenomenon to impaired adenine and pyridine nucleotide levels which may limit FA biosynthesis. Moreover, the increase in the levels of volatiles may be coupled with other ripening related processes, such as changes in cell wall composition, which might deliver intermediates to ester biogenesis. In tomato, evidence was provided that activity of pectin methyl-esterase regulates levels of methanol in the fruit (Frenkel et al., 1998); methanol may then be utilized to generate methyl esters in ripe fruit. However, the major reason for the reduced capacity to synthesize volatiles has to be related to ethylene. Ethylene plays a paramount role in volatile production at harvest as well as during/after storage, which is particularly exemplified after AVG, but also after long term storage (Bangerth, 1984; Halder-Doll and Bangerth, 1987; Bangerth and Streif, 1987; Defilippi et al., 2005).

As mentioned above, the esters are the main group of volatiles in apples, and the biosynthesis of esters is limited by the alcohol concentration (Berger and Drawert, 1984), which highlighted the significance of alcohol dehydrogenase (ADH) or lipoxygenase (LOX) enzymes (Echeverría et al., 2004). Beekwilder et al. (2004) stated that the bottle-neck for ester production was the availability of alcohols. Furthermore, various studies focused on the last step of ester formation, in which alcohol acyltransferase (AAT) enzymes have a key role (Fellman et al., 2000; Yahyaoui et al., 2002; Beekwilder et al., 2004). AAT enzymes catalyze the last step in ester formation by transacylation from an acyl-CoA to an alcohol, and combinations between different alcohols and acyl-CoAs result usually in the formation of a range of esters, in which the most likely precursors are lipids and amino acids. On the other hand, the activity of the enzymes alcohol dehydro-genase and alcohol-acetyl CoA transferase have also been proposed as an additional limiting factor in volatile production of strawberries (Perez et al., 1993), although the substrate specificity of the AATs toward alcohols and acyl-CoAs appeared to be broad (Wyllie and Fellman, 2000; Olias et al., 2002).

The impact of various storage conditions on odour volatiles was intensively investigated by our group, and various conclusions were formulated. A major conclusion from various studies is that: long-term storage combined with early harvesting of fruit led to detrimental effect on the capacity of fruit to synthesize volatiles. Bangerth hypothesized that insufficient supply of "high energy" compounds (e.g. adenine and/or pyridine nucleotides) might limit the biosynthesis and availability of volatile precursors such as fatty acids. Furthermore, Bangerth (1984) revealed that the depression in both the

respiration and the emission of volatiles could not be overcome by the addition of ethylene, applied either during storage or after that.

Although much is known about the possible reasons behind the reduced ability of immature apples and/or CA-stored (in particular ULO-stored) fruits to synthesize odour volatiles, much is also unknown, in particular at the molecular level. Various experiments show that the lack of precursors is a serious limiting factor for volatile production, while the enzymatic machinery needed for ester formation seems less limiting. Accordingly, the following findings and statements are of importance to highlight the complexity of this issue: 1) Low levels of isoamyl acetate were synthesized upon feeding of plants with isoamyl alcohol, which indicate that the putative endogenous acyltransferase hardly uses acetyl-CoA, but preferably uses methylbutyryl-CoA and benzoyl-CoA (Beekwilder et al., 2004). 2) Stimulating fatty acid metabolism by overexpression of lipoxygenase genes resulted in delivery of linear alcohols like octanol (Beekwilder et al., 2004), whereas terpene alcohols (e.g. geraniol) could be provided by introducing a geraniol synthase (Iijima et al., 2004). 3) The selectivity of enzymes preceding AATs in the biosynthesis pathway of aroma volatiles plays a key role in determining ester composition (Wyllie and Fellman, 2000). 4) The combinations (alcohol + X-CoA) seem to be also specific; in strawberries butanol is hardly used with acetyl-CoA, but readily accepted with butyryl-CoA and hexanoyl-CoA, which explain why both butylbutyrate and butylhexanoate are considered as major volatiles in strawberries (Zabetakis and Holden, 1997). 5) AAT enzymes from different plant species differ in their selectivity: Researchers found that Wild Strawberry Alcohol Acyl Transferase (VAAT) enzyme hardly accepts octanol, nonanol, or decanol as substrates in combination with acetyl-CoA, although geraniol is accepted, while VAAT is much more active on short alcohol substrates (C4 to C6, but also ethanol and methanol), which are not preferred by strawberry SAAT (Beekwilder et al., 2004). 6) The ethylene silenced apples synthesize much lower amounts of both the hexyl and butyl esters, whereas aldehyde and alcohol precursors were inhibited by only 12-38 kPa; the significant change in the ratio of hexanal/(2E)- hexenal suggested that ethylene may regulate either lipoxygenase or hydroperoxide (Dandekar et al., 2004). 7) Villatoro et al. (2007) found no large variations in the activity of AAT with 'Pink Lady®' apple throughout the experimental period, although ester emission increased substantially, and LOX activity was increased at later stages of fruit development; researchers suggested that the enhancement of ester production at ripening arises mainly from greater availability of substrates.

Our future work will focus on elucidation of changes at the molecular level, with particular emphasis on fatty acid metabolism, since our previous results clearly indicated that the enzymatic machinery for esterification is functional even after very long ULO-storage.

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Tables

Table 1. Composition of 'Golden Delicious' apples odour volatiles directly after harvest and after 7 months storage under ULO-conditions (3 kPa CO_2 + 1 kPa O_2).

	October		March	
Aroma volatile	1st day	7th day	1st day	7th day
Ethyl acetate	240	297	250	253
2-Propyl acetate	335	349	386	296
Ethyl propionate	111	127	0	0
Propyl acetate	358	1333	0	328
2-Methylpropyl acetate	347	243	324	332
1-Propanol	290	398	0	156
Ethyl butyrate	0	0	51	59
Ethyl 2-methyl butyrate	0	0	158	101
Butyl acetate	21318	31469	2412	5604
Butanol	2133	3041	412	543
Pentyl acetate	906	1327	277	637
3-Methyl-1-butanol	376	500	338	463
Butyl butyrate	4063	2863	858	1155
Butyl 2-methylbutyrate	1851	1497	736	1586
Pentanol	24	21	0	0
Hexyl acetate	11171	16454	8612	8305
Pentyl butyrate	196	346	334	670
Hexyl propionate	856	1142	821	995
Hexanol	603	810	555	342
Hexyl 2-methylbutyrate	3019	2310	3604	5609
Hexyl hexanoate	52	319	970	1191

Table 2. Influence of feeding freshly harvested (October) and ULO-stored (March) 'Golden Delicious' apples with butanol on the emission of selected odour volatiles. r-factor is the ratio of peak area of treated to untreated peak area for each aroma volatile.

	r-Factor			
	October		March	
Aroma volatile	1st day	7th day	1st day	7th day
2-Propyl acetate	1.47	1.55	1.3	1.08
Ethyl propionate	0.32	0.52	0	0
Propyl acetate	3.21	1.31	113.48	1.52
2-Methylpropyl acetate	0.55	0.94	1.01	1.19
Ethyl butyrate	41.77	19.58	2.71	1.47
Ethyl 2-methyl butyrate	24.33	0	1.1	1.45
Butyl acetate	2.32	1.01	13.24	1.29
Propyl butyrate	0.61	0.92	1.16	1.33
Butanol	3.46	1.15	11.2	1.5
Pentyl acetate	0.71	0.64	1.53	0.89
3-Methyl-1-butanol	0.78	0.99	0.97	1.25
Butyl butyrate	3.39	1.33	10.2	1.26
Butyl 2-methylbutyrate	2.01	1.23	6.76	1.66
Hexyl acetate	0.77	1.06	1.23	0.86
Pentyl butyrate	0.64	0.71	0.97	1.39
Hexanol	1.11	1.31	1.32	1.02
Hexyl 2-methylbutyrate	0.42	1.03	1.2	0.93
Hexyl hexanoate	1.81	0.51	1.15	0.62

Table 3. Influence of feeding freshly harvested (October) and ULO-stored (March) 'Golden Delicious' apples with 2-methylpropanol on the emission of selected odour volatiles. r-factor represents the ratio of peak area of treated to the peak area of untreated fruit for each aroma volatile.

	r-Factor			
	October		March	
Aroma volatile	1st day	7th day	1st day	7th day
2-Propyl acetate	1.19	1.22	1.08	1.40
Propyl acetate	0.72	0.74	-	1.34
2-Methylpropyl acetate	110.2	93	61.3	36.8
Butyl acetate	0.74	0.75	1.51	1.19
Pentyl acetate	1.77	0.90	6.15	2.85
Butyl butyrate	0.69	0.73	0.81	1.04
Butyl 2-methylbutyrate	0.64	0.94	1.39	1.34
Hexyl acetate	1.1	0.90	1.01	0.98
Pentyl butyrate	0.46	0.49	0.49	1.28

Table 4. Influence of AVG-treatment combined with ULO-storage on the composition of odour volatiles of 'Golden Delicious' apples compared to freshly harvested and air stored fruit.

	Peak Area Units			
	October		March	
	AVG-treated	harvest time	AVG-ULO[1]	ULO[2]
Ethyl acetate	260	243	214	253
2-Propyl acetate	520	303	152	186
Propyl acetate	77	1647	0	165
2-Methylpropyl acetate	0	207	0	245
Butyl acetate	811	40219	113	9218
Propyl butyrate	464	12429	166	9637
Butanol	157	2882	0	491
Pentyl acetate	207	1470	23	631
3-Methyl 1-butanol	0	638	25	473
Butyl butyrate	157	4775	0	1192
Butyl 2-methylbutyrate	71	4216	0	229
Hexyl acetate	1867	47016	701	8885
Pentyl butyrate	0	531	0	153
Hexanol	222	1561	96	427
Hexyl 2-methyl butyrate	1529	16374	1160	4596
n-Hexyl hexanoate	310	1327	250	540
TOTAL	6652	135838	2900	39382

AVG-ULO[1] ULO-stored fruits treated with AVG;
ULO[2] ULO-stored not treated with AVG.

Table 5. Influence of feeding AVG-treated fruits with pentanol, directly after harvest and after five-months ULO-storage on the emission of selected odour volatiles. r-factor represents the ratio of peak area of treated fruits to the peak area of untreated fruit for each aroma.

	October				March			
	1st day		7th day		1st day		7th day	
	r1	k1	r2	k2	r3	k3	r4	k4
Ethyl acetate	0.84	309	0.69	260	0.98	214	0.85	214
2-Propyl acetate	1.37	922	0.96	520	1.11	199	0.93	152
Butyl acetate	0.04	1649	0.30	811	1.65	231	1.85	113
Pentyl acetate	18.26	1122	9.63	207	14649	1	51.8	23
1-Pentanol	43.44	123	497.3	1	4426	1	261	1
Hexyl acetate	0.27	3752	0.83	1867	1.6	1445	1.14	701
Pentyl butyrate	156.3	148	-	-	129	1	-	-
Hexanol	0.48	620	0.25	222	1.63	259	1.19	96

K means the peak area units at the designated sampling dates.

Figures

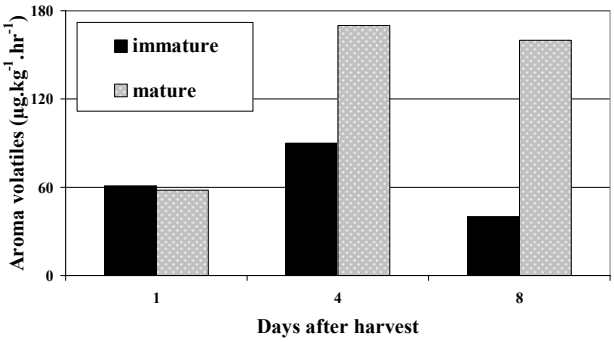

Fig. 1. Odour volatiles emission of `Golden Delicious´ apples at two ripening stages. Volatiles were trapped by the Tenax method in the outgoing air of the storage vessel during 7 d at 20°C.

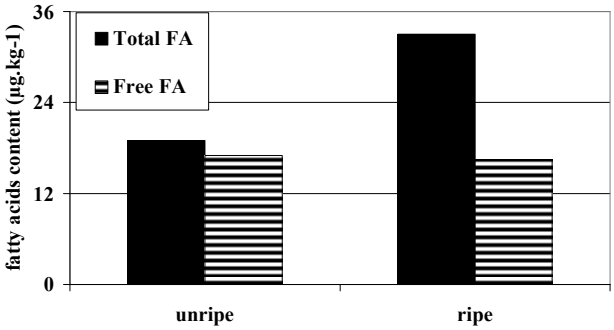

Fig. 2. Comparison between the concentrations of total and free fatty acids of apple fruit harvested at unripe and ripe stages. (total fatty acids in 100 g; free fatty acids in 1000 g).

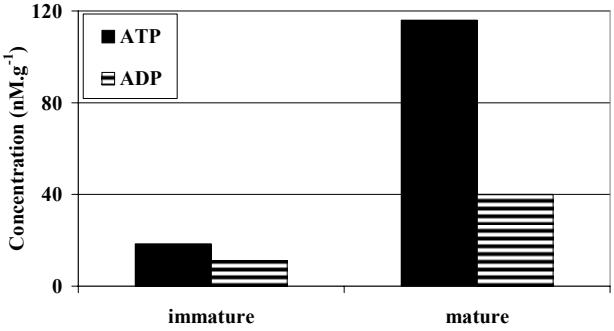

Fig. 3. Comparison between the concentrations of ATP and ADP of apple (peel) at two different ripening stages.

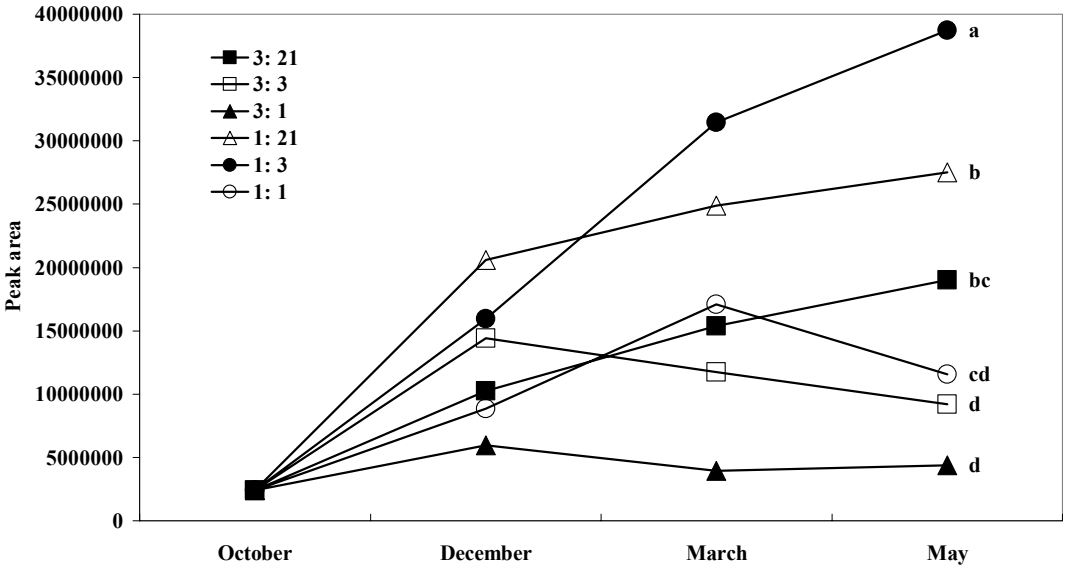

Fig. 4. Influence of CA-storage conditions on the emission of odour volatiles (total amount) of 'Golden delicious' apples after a conditioning period of 7 d at 20°C.

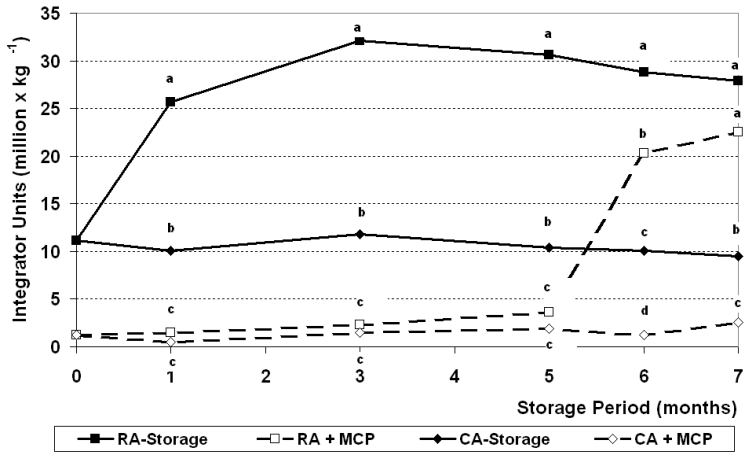

Fig. 5. Influence of '1-MCP' treatment and CA-storage on the emission of odour volatiles (total amount) of 'Jonagold' apples conditioned for 7 d at 20°C.