Table I. Limonoate Dehydrogenase Activity of *C. fascians* Grown on Various Carbon Sources[a]

substrates	cell yields, g	limonoate dehydrogenase	
		total activity, units	activity/ g of cells, units
limonoate	3.22	7392	2296
fructose	2.06	1546	750
galactose	2.20	2957	1344

[a] Growth media: 0.2% nutrient broth, 0.4% substrate of interest, and mineral salts in 500 mL.

monoate, galactose, or glucose, and the rates of growth on these carbon sources became very similar to that grown on fructose.

Table I shows the results of a typical experiment on analyses of limonoate dehydrogenase activity of cells grown on limonoate, galactose, or fructose. The activity was highest in cells grown on limonoate followed by galactose and fructose. An experiment similar to the above was done in triplicate with similar results in each case. The enzyme activity of *C. fascians* grown on limonoate was higher than that of either *A. globiformis* (Hasegawa et al., 1972b), *Bacterium* 342-152-1 (Hasegawa and Kim, 1975) or *Pseudomonas* 321-18 (Hasegawa et al., 1974a) when their cells were prepared under similar conditions. *C. fascians* grown on galactose produced limonoate dehydrogenase activity equaling that of *A. globiformis*, but higher than that of either Bacterium 342-152-1 or *Pseudomonas* 321.

The limonoate dehydrogenase of *C. fascians* was produced without the need for a limonoid inducer in the medium. This clearly showed that the organism produces constitutive limonoid-metabolizing enzymes. This is advantageous from a practical viewpoint because cells which possess limonoid-metabolizing enzymes can be produced conveniently and relatively cheaply by using inexpensive carbon sources. At present none of the citrus limonoids is commercially available.

ACKNOWLEDGMENT

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Objective Measurement of Aroma Quality of Golden Delicious Apples as a Function of Controlled-Atmosphere Storage Time

Guido A. Willaert, Patrick J. Dirinck,* Herman L. De Pooter, and Niceas N. Schamp

Flavor quality of Golden Delicious apples as a function of controlled-atmosphere storage time is studied by using aroma analysis as an objective criterium. For representative aroma isolation, a fast headspace concentration technique, which isolates the volatiles released during maceration of the fruits, is used. As flavor quality is a dynamic process, the evolution of volatiles as a function of ripening after removal from controlled atmosphere is followed for six periods during the storage season. Results objectively indicate an important decrease in aroma quality after long controlled-atmosphere storage (CA storage).

The volatile compounds of apples have been studied by several authors and over 250 volatile components have

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been identified (Van Strating and de Vrijer, 1973). The most important flavor compounds in apples are esters, alcohols, and aldehydes. According to Flath, the components directly associated with the characteristic Delicious apple-like aroma are ethyl 2-methylbutyrate, 1-hexanal, and *trans*-2-hexenal (Flath et al., 1967). Williams and co-workers established the importance of 4-methoxy-

allylbenzene for the spicy note of apple flavor (Williams et al., 1977).

Besides component identification and organoleptic evaluation, formation of apple volatiles by biogenesis and enzymatic reactions has also been extensively studied. The C-6 aldehydes hexanal and trans-2-hexenal are formed enzymatically by oxidative cleavage of linoleic and linolenic acid during disintegration of the fruits (Drawert et al., 1973). As to the biogenesis of the esters it is accepted that they are synthesised enzymatically by combining acid and alcohol moieties (Salunkhe and Do, 1976). The straight-chain fatty acid could be formed by β-oxidation of the straight-chain fatty acid moieties and the branched-chain acid moieties, such as 2-methylpropionates, 2-methylbutyrates, or 3-methylbutyrates, could be derived from amino acids. Until recently biosynthesis of carboxylic esters in fruits has been studied by incorporation experiments on simplified systems such as aged apple disks (Paillard, 1978, 1979) or cell suspensions (Ambid and Fallot, 1980). Recently also whole Golden Delicious apples have been treated with volatile precursors to study ester formation (De Pooter et al., 1981).

This study is part of a project on flavor quality evaluation of Golden Delicious apples, which represent 70% of the Belgian production, both by sensory analysis (taste panel) and instrumental analysis. It was suspected that flavor quality of Golden Delicious apples is influenced importantly by controlled-atmosphere storage time. Indeed, an important part of the Golden Delicious production is storage in a controlled atmosphere from October until June. In our opinion flavor quality evaluation by sensory analysis can only give reliable results when the samples are available at the same moment. Also, for this study an objective flavor quality evaluation technique was necessary and apple aroma was used as a criterion. For representative aroma isolation a headspace concentration technique, which isolates the volatiles released, when macerating the fruits, was used (Dirinck et al., 1977, 1981). The complete method consists of a combination of headspace enrichment on Tenax, high-resolution gas chromatography, mass spectrometric identification, and quantification of volatiles on a FID detector.

Although the relation of controlled-atmosphere storage with wastage due to rotting and physiological disorders has been intensively studied (Salunkke and Wu, 1974), only minor attention has been paid to the influence of controlled-atmosphere storage time on flavor quality. Drawert et al. (1969) followed the evolution of the main volatile components during storage for different varieties. However, as flavor quality is a dynamic process, it is important to follow aroma composition as a function of ripening in standard conditions, after removal from a controlled atmosphere. Guadagni et al. (1971) established that fruit from a commercial controlled-atmosphere store was defective in its ester production after transfer to air. Patterson et al. (1974) studied the influence of different combinations of temperature and concentrations of oxygen and carbon dioxide on the total amounts of Golden Delicious volatiles produced after storage. In our study we especially paid attention to the influence of storage time on flavor quality. Therefore, the evolution of aroma components of Golden Delicious apples ripened in standard conditions after removal from a controlled atmosphere at several times during the storage period was determined.

EXPERIMENTAL SECTION

Materials and Storage. Golden Delicious apples, harvested during the season 1978, were stored in a commercial controlled-atmosphere storehouse at 0.5 °C, 1–2%
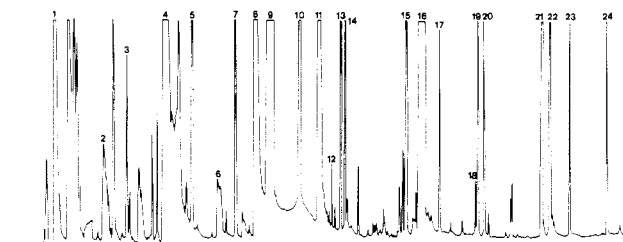

Figure 1. Typical gas chromatogram of Golden Delicious apples.

CO_2, and 93% relative humidity. During the storage period at different times, Golden Delicious apples were transferred from a controlled atmosphere to air for ripening in standard conditions (20 °C and 70% relative humidity). At regular intervals, depending on the degree of alteration, aroma analyses were performed during the following ripening periods: period I, from Dec 4, 1978, to Dec 15, 1978; period II, from Dec 18, 1978, to Jan 12, 1979; period III, from Jan 19, 1979, to Feb 2, 1979; period IV, from March 6, 1979, to April 5, 1979; period V, from April 9, 1979, to June 1, 1979; period VI, from May 9, 1979, to June 20, 1979.

Sampling Procedure and Isolation of Volatiles. For isolation of a fresh representative flavor mixture the volatiles, liberated while macerating 100 g of peeled apples in a commercial blender, were adsorbed on Tenax GC, 60–80 mesh, as described before (Dirinck et al., 1977). In order to minimize the differences in maturity of individual apples, the apple sample was provided by four different apples. The volatiles, liberated during disintegration of the material, were carried through a relatively large glass adsorption column (i.d. 1.6 cm; length 10 cm; 5 g of Tenax) by means of helium gas. For quantitative analysis helium passed through the adsorption column with a flow rate of 500 mL/min during 10 min. Loaded columns were closed and could be stored for a long period without loss of sample or changes in the composition. For sample injection the flavor components were desorbed at 220 °C in a helium stream and transferred to a cooling trap. Therefore, a Varian 3700 gas chromatograph was modified and equipped with a separate desorption oven and a thermostated two-position six-port, high-temperature injection valve (Valco Instruments Co, Houston, TX) as described before (Dirinck et al., 1981).

Gas Chromatography. The apple volatiles were separated on a 150 m × 0.5 mm i.d. glass column, coated with SE-52. Further operating conditions were as follows: linear temperature programming from 10 to 200 °C at 1 °C/min; carier gas He, 3 mL/min; injection valve temperature 250 °C; flame ionization detector temperature 250 °C.

Quantitative determination of apple volatiles was performed by electronic integration (Infotronics 204) and by relating the peak intensities of the different compounds to these of n-undecane and n-tridecane as internal standards. Therefore, a solution of the n-alkanes in carbon disulfide (1 μg of each hydrocarbon/μL of carbon disulfide) was injected on the adsorption column before desorption.

Gas Chromatography–Mass Spectrometry. Identification of the volatiles was performed by GS–MS, consisting of a Varian 2700 linked to a MAT 112 mass spectrometer, as described previously (Dirinck et al., 1981). In some cases identification was helped by gas chromatographic retention data of authentic materials synthesized in our laboratory.

RESULTS AND DISCUSSION

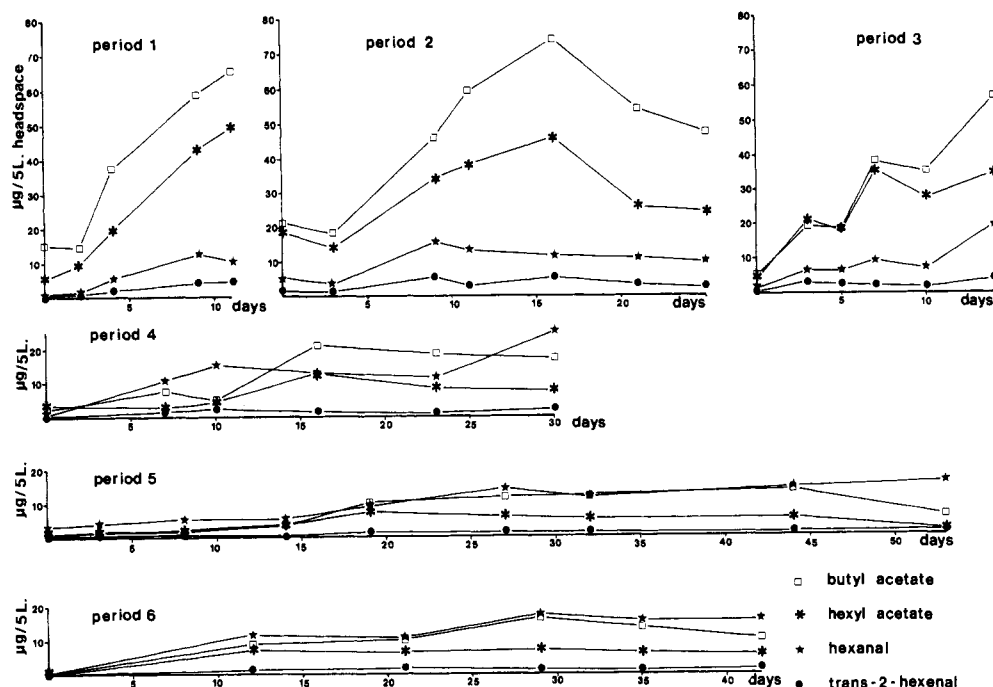A typical gas chromatogram of Golden Delicious apples is shown in Figure 1. The peak numbering is related to

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Figure 2. Evolution of the main esters, butyl acetate and hexyl acetate, and the main aldehydes, hexanal and *trans*-2-hexenal, for the different ripening periods (after removal from a controlled atmosphere).

Table I. Identified Compounds in Golden Delicious Apples

1, acetaldehyde	13, butyl propionate
2, propanol	14, pentyl acetate
3, ethyl acetate	15, butyl butyrate
4, butanol	16, hexyl acetate and
5, propyl acetate	*cis*-3-hexenyl acetate
6, 2- and 3-methylbutanol	17, butyl 2-methylbutyrate
7, 2-methylpropyl acetate	18, pentyl butyrate
8, hexanal	19, undecane (internal standard)
9, butyl acetate	20, hexyl propionate
10, *trans*-2-hexenal	21, hexyl butyrate and butyl
11, 3-methylbutyl acetate	hexanoate
12, propyl butyrate	22, estragole
	23, hexyl 2-methylbutyrate
	24, tridecane (internal standard)

the identifications in Table I, which show an important amount of volatile esters, some alcohols, some aldehydes, and estragole (4-methoxyallylbenzene). Undecane and tridecane are internal standards. The organoleptic important compounds are obviously the carboxylic esters and the aldehydes, which are responsible respectively for the fruity and green character of apples. These typical aroma compounds are only produced in higher amounts during ripening, related to the climacteric rise in respiration, and are only present in small concentrations during growth or at the time of harvest. This is shown in Table II, which represents relative amounts of flavor compounds in Golden Delicious immediately after harvesting as a function of picking date. The concentrations in Table II were obtained under analogous conditions as for the storage experiment and are expressed as micrograms per 5 L of headspace per 100 g of apples. Besides the fact that at harvest only minor concentrations of aroma compounds are present, from Table II it is also clear that hexanal and *trans*-2-hexenal are quantitatively important compounds in green immature apples, accounting for about 30% of the total volatile composition. These aldehydes are formed enzymatically from linoleic and linolenic acid in the headspace concentration during disintegration of the fruits and should also be responsible for the green flavor character of preclimacteric, immature apples. Other characteristics of apples at harvest are a higher concentration of hexyl acetate re-

Table II. Relative Amounts (Expressed as Micrograms per 5 L of Headspace per 100 g of Apple) of the Flavor Compounds in Golden Delicious Apples as a Function of the Data of Harvest

components	Sept 4, 1978	Sept 18, 1978	Sept 27, 1978	Oct 17, 1978
propyl acetate				
2-methylpropyl acetate				0.44
1-hexanal	1.18	1.10	1.57	1.05
butyl acetate	0.32	0.21	0.28	1.14
trans-2-hexenal	0.76	0.83	1.58	1.06
3-methylbutyl acetate	0.79	1.45	2.42	1.41
butyl propionate				
pentyl acetate	0.33	0.37	0.57	0.43
butyl butyrate				0.23
hexyl acetate	1.09	2.01	2.99	2.48
cis-3-hexenyl acetate	1.20	1.40	1.11	0.83
butyl 2-methylbutyrate				
pentyl butyrate				
hexyl propionate				0.24
4-methylpentyl butyrate				
hexyl butyrate				0.25
estragole				
hexyl 2-methylbutyrate				
sum	5.67	7.37	10.52	9.56

lated to butyl acetate and a high concentration of *cis*-3-hexenyl acetate.

As an illustration of the quantitative GC data in the storage experiment in Table III, the evolution of Golden Delicious volatiles (isolated by headspace concentration on Tenax during disintegration) as a function of ripening in standard conditions after removal from a controlled atmosphere is given for period II (ripening from Dec 18, 1978, to Jan 12, 1979). For the different ripening periods the evolution of the two principal esters, butyl acetate and hexyl acetate, and of the aldehydes, 1-hexanal and *trans*-2-hexenal, is given in Figure 2.

In ripening periods I–III (short CA storage) butyl actate and hexyl acetate are present in significantly larger amounts than the two aldehydes. On the other hand, from period IV hexanal becomes more important and it even is the main compound in periods V and VI, ressembling

Table III.   Relative Amounts (Expressed as Micrograms per 5 L of Headspace per 100 g of Apple) of the Flavor Compounds as a Function of the Days of Ripening for Period II: December 18, 1978, to January 12, 1979

| components	0	3	9	11	16	21	25
propyl acetate	0.35	0.61	2.48	2.57	3.39	3.65	3.86
2-methylpropyl acetate	1.96	2.38	2.93	2.29	1.55	1.22	1.18
1-hexanal	5.50	3.74	15.77	13.26	11.88	11.05	9.74
butyl acetate	21.56	18.38	46.10	59.28	73.99	54.14	47.49
trans-2-hexenal	1.79	1.60	5.43	3.98	5.22	3.28	2.45
3-methylbutyl acetate	4.06	4.54	6.17	9.92	11.00	10.33	11.93
butyl propionate	0.44	0.42	1.32	1.78	1.58	0.89	1.05
pentyl acetate	1.18	1.09	1.77	2.30	1.99	1.31	1.48
butyl butyrate	1.48	1.06	2.28	3.29	3.04	2.63	2.73
hexyl acetate	18.83	13.88	34.07	37.97	45.97	25.69	24.13
cis-3-hexenyl acetate	1.15	1.52	1.73	1.80	1.00	1.76	0.61
butyl 2-methylbutyrate	0.19	0.21	0.45	0.55	0.56	0.63	0.88
pentyl butyrate	0.06	0.08	0.17	0.11	0.10	0.15	0.11
hexyl propionate	0.24	0.24	0.64	0.71	0.59	0.38	0.55
4-methylpentyl butyrate	0.07	0.14	0.31	0.24	0.11	0.20	0.19
hexyl butyrate	2.16	1.52	3.45	4.76	4.28	3.40	3.64
estragole	0.21	0.22	1.93	2.79	1.86	1.12	0.98
hexyl 2-methylbutyrate	0.22	0.25	0.69	0.81	0.79	1.15	1.06
sum	61.45	51.88	127.66	148.41	168.90	122.98	114.06
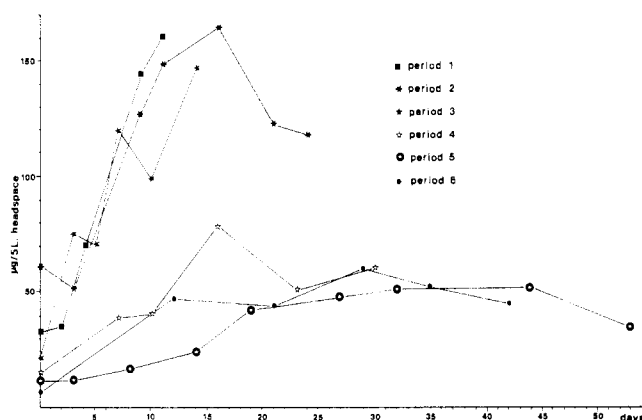
Figure 3.   Evolution of the sum of apple volatiles, isolated by headspace concentration on Tenax during disintegration, for the different ripening periods (as a function of CA storage time).
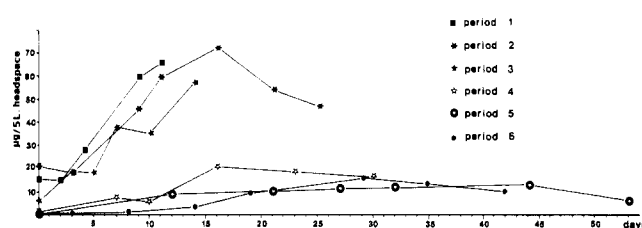

Figure 4. Evolution of butyl acetate for the different ripening periods (as a function of CA storage time).
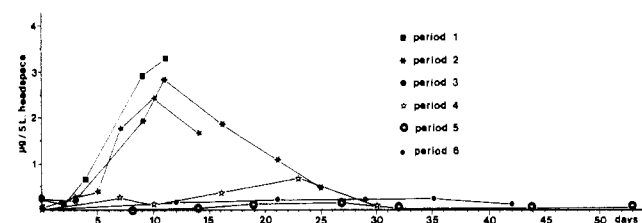

Figure 5.   Evolution of estragole for the different ripening periods (as a function of CA storage time).
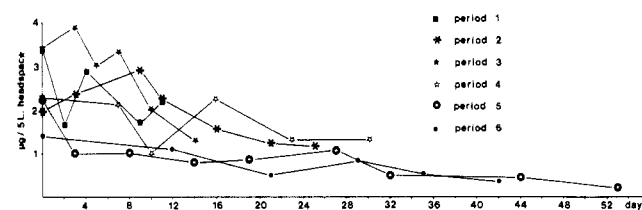

Figure 6.   Evolution of 2-methylpropyl acetate for the different ripening periods (as a function of CA storage time).

the situation of the green immature apples in Table II. Taking into account these evolutions, one can state that after long CA storage the green flavor character of Golden Delicious apples, due to aldehydes, is again becoming more important in relation to the fruity, estery character. This proposition is confirmed by the higher production of hexyl acetate relative to butyl acetate at the start of the ripening after long CA storage (periods III–V).

From the point of view of objective flavor quality measurement, the total sum of released volatiles during disintegration can be used as an aroma quality parameter. In Figure 3 the evolution of the sum of apple volatiles, isolated by headspace concentration on Tenax, while macerating the fruits, is given for the different ripening periods.   In relation to eating quality it is accepted that consumption is about 10 days after removal from a controlled atmosphere (distribution, shop, and home storage). After 10 days of ripening in standard conditions the total amounts of released volatiles, expressed as micrograms per 5 L of headspace per 100 g of apples, are respectively for period I, 152, for period II, 138, for period III, 98, for period IV, 40, for period V, 24, and for period VI, 40.   Examination of Figure 3 also clearly indicates that for shorter CA storage a faster and higher aroma production is observed and a maximum is reached within the normal consumption period.   Also, a clear distinction can be made between aroma evolution during ripening after short storage (until December and January) compared to that after long CA storage (March, April, May).   These results prove objec-

tively that although long-stored apples have excellent appearance and are free of disorders their flavor quality has decreased significantly.

A full discussion of the evolution of the individual aroma compounds is beyond the scope of this publication as it would need the presentation of the quantitative data for the different ripening periods.   Only some important aspects will be discussed.   Investigation of the quantitative GC data and graphic presentation showed that an important part of the volatile compounds, such as butyl acetate, hexyl acetate, and estragole, show an analogous evolution as the total sum of volatiles.   As an illustration the evolution of butyl acetate and estragol for the different ripening periods in function of CA storage time is illustrated in Figures 4 and 5.   After short CA storage a rapid

increase up to the maximum is observed for these compounds. After long CA storage slow formation of volatiles and a significantly lower maximum is obtained during ripening.

On the other hand, some compounds do not follow the evolution profile of the total sum of volatiles. An example is given in Figure 6, which shows a diminishing trend for 2-methylpropyl acetate for the different ripening periods. This can be explained by the fact that probably valine instead of a fatty acid is the precursor for the alcohol moiety of the carboxylic ester (Salunkke, 1976).

CONCLUSION

The work reported here shows that although CA storage prevents deterioration due to rotting and physiological disorder, it importantly influences the eating quality of the fruit. By means of a combination of a representative isolation of aroma compounds, quantitative high-resolution gas chromatography, and mass spectrometry, we have objectively proved that there is a significant flavor decrease for apples and a deficiency for ester production after long CA storage. If CA storage is used simply to extend storage life of a product of only average quality, apple consumption is unlikely to increase. In order to offer better quality, regulations for shorter storage periods should be established.

Registry No. Acetaldehyde, 75-07-0; 1-propanol, 71-23-8; ethyl acetate, 141-78-6; 1-butanol, 71-36-3; propyl acetate, 109-60-4; 2-methylbutanol, 137-32-6; 3-methylbutanol, 123-51-3; 2-methylpropyl acetate, 110-19-0; hexanal, 66-25-1; butyl acetate, 123-86-4; *trans*-2-hexenal, 6728-26-3; 3-methylbutyl acetate, 123-92-2; propyl butyrate, 105-66-8; butyl propionate, 590-01-2; pentyl acetate, 628-63-7; butyl butyrate, 109-21-7; hexyl acetate, 142-92-7; *cis*-3-hexenyl acetate, 3681-71-8; butyl 2-methylbutyrate, 15706-73-7; pentyl butyrate, 540-18-1; undecane, 1120-21-4; hexyl propionate, 2445-76-3; hexyl butyrate, 2639-63-6; butyl hexanoate, 626-82-4; estragole, 140-67-0; hexyl 2-methylbutyrate, 10032-15-2; tridecane, 629-50-5; 4-methylpentyl butyrate, 83471-19-6; CO_2, 124-38-9.

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Treatment of Golden Delicious Apples with Aldehydes and Carboxylic Acids: Effect on the Headspace Composition

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Application of vapors of C_3- to C_6-aldehydes or C_2- to C_6-carboxylic acids to intact Golden Delicious apples results in the formation of alcohols and esters. The aldehydes are either transformed into the corresponding alcohols and esterified with carboxylic acids present in the tissues or (to a small degree) oxidized into the acids, which combine with alcohols present as such, or derived from the aldehydes. Carboxylic acids yield either esters or alcohols (probably by way of the aldehydes) or smaller carboxylic acids by β-oxidation where possible, which are then in turn used as the substrate. While these additions have a great impact on the total composition of the headspace, as shown by GLC after enrichment on Tenax GC, no large or reproducible effect on the flavor of treated fruits could be demonstrated organoleptically.

Until recently there was a general trend to select high-yield apple varieties, with a good appearance, strong illness resistance, and good transport and storing properties but without paying too much attention to the flavor quality.

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With the improvement of storing technology, it is now possible to keep apples in an intact condition long after harvest. In general they suffer, however, from a loss of flavor, which depends moreover on the storing conditions (Shatat et al., 1978; Willaert et al., 1983). As part of a long-term project about the organoleptic quality of fruits and vegetables, a study was undertaken about the metabolic processes leading to the synthesis of aroma volatiles. The main substances present in the aroma of Golden