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DEVELOPMENTAL CHANGES IN THE PHENOL CONCENTRATIONS OF '*GOLDEN DELICIOUS*' APPLE FRUITS AND LEAVES

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Abstract—The phenolic content of apple fruit skin and leaves was determined at the developmental stage of each organ. Phenolic levels decreased on a dry weight basis during the seasonal development of fruits and leaves with respect to their ontogenesis but the single compounds did not behave uniformly. A shift in flavanol pools from monomeric to oligomeric structures during fruit growth indicated the biosynthetic tendency towards the formation of procyanidins at the end of the growing period. Among the procyanidins identified epicatechin-(4β,6)-epicatechin-(4β,6)-epicatechin has not been reported previously from apple.

INTRODUCTION

The occurrence of phenolic compounds in fruits and leaves of apple (*Malus domestica*) has been extensively reviewed by Macheix *et al.* [1] but quantitative data are still rare and mostly restricted to ripe fruits [2–11]. The only flavan-3-ols included in these analyses were catechin, epicatechin and procyanidins B2 and B5. Phenols have been suggested to play a role in plant resistance against many diseases [12], and in coincident response against apple scab (*Venturia inaequalis*) [13–15]. However, no clear conclusion could be drawn from the numerous findings until now because the localization of phenols within the fruit and seasonal and environmental influences on the concentration of individual compounds had not been studied.

In this paper we compare the concentrations of several characteristic phenolic constituents determined during the seasonal development of '*Golden Delicious*' apple fruits and leaves.

RESULTS AND DISCUSSION

Tissue-specific distribution within the young fruit

The concentrations of 15 phenolic constituents of skin, flesh and core of young apple fruits of the var. '*Golden Delicious*' sampled in mid-May and mid-June are listed in Table 1. The unequal distribution of chlorogenic acid and quercetin glycosides within the fruit (Table 1) agreed with the findings of Risch and Herrmann [5] and McRae *et al.* [7] for mature apples. It can also be seen that the levels of catechin and procyanidin B1 were highest in the flesh and decreased in skin and core at both developmental stages of the fruit. The levels of epicatechin and the procyanidins (epicatechin units B2, B5, C1 and E-B5) reached their highest values in the very young fruits in the core but also in the skin of older apples. The latter results agree in part with the observations of the above cited authors [5, 7] made on mature fruits where the skin was the main source of both catechin and epicatechin.

Phenolic content of the skin during fruit growth

During growth and maturation of the fruit the phenolic profile of the fruit skin changed markedly. Thus, chlorogenic acid exhibited its maximum value in spring followed by a constant decrease until September (Fig. 1). Similar curves are evidenced for the amounts of catechin and procyanidin B1 (Fig. 2). Thus, epicatechin and its oligomers reached a maximum in July. This holds true for both the 4,8- and the 4,6-linked procyanidins which comprise B2, C1 and B5, E-B5, respectively. The concentrations of phloridzin and the main flavonols, quercetin 3-galactoside and quercetin 3-arabinoside, are highest in spring and fell sharply during July. The remaining flavonols showed small fluctuations during the whole season.

With respect to biosynthesis it must be noted that the dry weight of apple fruits continued to increase until August [16]. The increasing flavanol contents calculated on a dry weight basis indicated high biosynthetic activity as found by Lister *et al.* [11], who estimated the phenolic content of fresh material. Regarding the co-ordination of synthesis it is obvious that the pool of hydroxycinnamic acids as represented by chlorogenic acid and the monomeric flavanols decreased in favour of flavonols and

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Table 1. Phenolic content of various tissues of young apple fruits cv 'Golden Delicious' (mid-May, mid-June) from South Tyrol, Italy*

		mg g^{-1} dry weight					
		Skin		Flesh		Core	
		Mean	SE	Mean	SE	Mean	SE
Chlorogenic acid	May	4.4	0.5	20.7	4.3	24.9	5.3
	June	1.9	0.6	5.7	0.6	10.54	0.8
Quercetin 3-Gal	May	20.2	1.7	2.4	0.5	6.0	0.9
	June	7.5	2.3	1.2	0.2	0.5	0.03
Quercetin 3-Glc	May	1.3	0.1	0.2	0.02	0.14	0.04
	June	0.4	0.1	0.1	0.03	0.04	0.01
Quercetin 3-Rha-Glc	May	4.5	0.2	0.1	0.02	0.10	0.01
	June	2.2	0.5	0.5	0.06	0.07	0.02
Quercetin 3-Rha	May	13.0	0.6	0.5	0.12	0.87	0.11
	June	3.5	0.8	0.8	0.1	0.1	0.02
Quercetin 3-Ara	May	19.6	0.6	0.5	0.12	0.41	0.15
	June	7.4	1.4	1.6	0.16	0.11	0.02
Phloretin 2'-Glc	May	114.1	8.6	40.6	5.2	134.6	21.0
	June	10.9	1.6	4.4	0.5	30.2	3.4
Phloretin 2'-Xyl-Glc	May	1.7	0.1	1.4	0.06	2.6	0.1
	June	1.1	0.1	1.3	0.17	2.4	0.3
Catechin	May	2.1	0.3	4.5	0.7	1.9	0.3
	June	0.4	0.1	0.8	0.1	0.2	0.03
Epicatechin	May	5.3	0.5	7.0	0.4	8.9	0.5
	June	6.9	0.3	5.8	0.4	2.2	0.4
Procyanidin B2	May	2.8	0.3	3.3	0.2	4.6	0.6
	June	4.0	0.2	3.8	0.2	1.9	0.3
Procyanidin B5	May	1.4	0.05	0.8	0.1	1.7	0.2
	June	1.0	0.1	0.5	0.02	0.4	0.08
Procyanidin B1	May	1.3	0.2	3.5	0.9	1.1	0.2
	June	0.6	0.1	1.3	0.1	0.1	0.05
Procyanidin E-B5	May	0.6	0.1	0.4	0.1	0.8	0.1
	June	0.6	0.1	0.3	0.03	0.2	0.04
Procyanidin C1	May	2.0	0.1	2.2	0.3	3.2	0.7
	June	2.6	0.3	2.4	0.1	1.2	0.2

Gal, Galactose; Glc, glucose; Ara, arabinose; Rha, rhamnose; Xyl, xylose.
*The mean values represent four replicates.

procyanidins. Plainly recognizable is the varying ratio epicatechin/procyanidin B2 which ranged from about 3 in spring to 1 in autumn. This observation could be interpreted as an enhanced oligomerization during the growing season. The last step of this process may be the formation of insoluble proanthocyanidins which is called 'the iceberg' by Haslam [17].

Leaf analysis

As compared with the fruit skin, the total phenolic content of apple leaves was much lower except for the flavonols and the dihydrochalcone phloridzin which was found in concentrations ranging from 200 to 400 μM g^{-1} dry weight. Phloridzin was excluded from routine analysis since the corresponding absorptions exceeded the detection range. Furthermore, the quercetin glycosides are quantitatively important compounds in young fruits. During leaf maturation the concentrations of the 3-galactoside and the 3-rhamnoside decline while the 3-arabinoside, the 3-rutinoside and the 3-glucoside remain on constant levels (Fig. 3). The influence of the developmental stage on the pools of the individual flavan-3-ols is obvious (Fig. 4). In the first fully expanded leaf the dimeric procyanidin B5 and the trimeric epicatechin-(4β,6)-epicatechin-(4β,6)-epicatechin (E-B5) have high concentrations. Thereafter they declined with increasing leaf age whereas epicatechin and procyanidin B2 exhibited a peak in older leaves. Trace amounts of catechin were found in all leaves and procyanidin C1 did not exceed 0.05 μM g^{-1} dry weight.

CONCLUSION

The results show that the phenolic content of apple tissues is not exclusively a function of the cultivar as some papers propose [7, 8] but is based also on the stage of maturity. This fact has to be considered when the physiological role of phenols or their contribution to plant resistance is considered. The diversity of phenolic com-

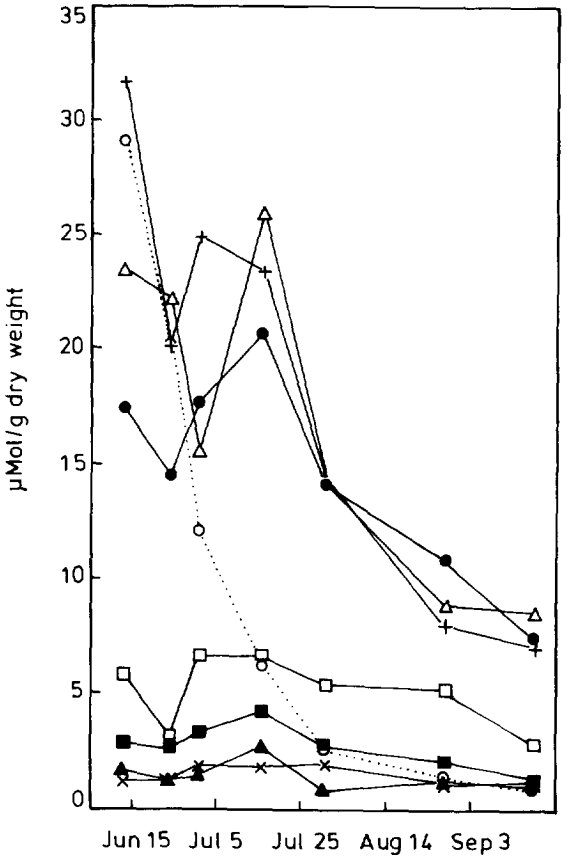

Fig. 1. Seasonal change of the phenol content in fruit skin of '*Golden Delicious*' apple. ○, Chlorogenic acid; ●, quercetin 3-arabinoside; △, quercetin 3-galactoside; ▲, quercetin 3-glucoside; ■, quercetin 3-rutinoside; □, quercetin 3-rhamnoside; +, phloretin 2'-glucoside; ×, phloretin 2'-xylosyl glucoside.

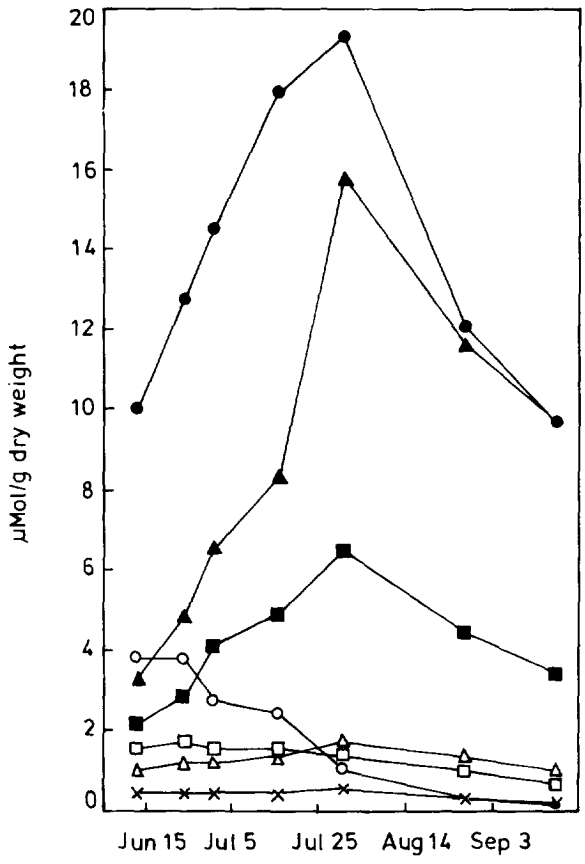

Fig. 2. Seasonal change of the flavanol content in fruit skin of '*Golden Delicious*' apple. ○, Catechin; ●, epicatechin; △, procyanidin B5; ▲, procyanidin B2; □, procyanidin B1; ■, procyanidin C1; ×, procyanidin E-B5.

pounds and their seasonal variability indicate an interrelationship more complex than linear.

EXPERIMENTAL

Plant material. Apple fruits of the cultivar '*Golden Delicious*' were grown in Freising-Weihenstephan and harvested on 13, 24 June, 1, 16, 31 July, 28 August, 18 September; for each analysis 4 fruits were collected, lyophilized, the fruit skin completely peeled off with a razor blade and the combined powder exhaustively extracted with 80% Me_2CO containing 6-methoxyflavone as internal standard for 30 min in a cooled water bath during sonication. The extract was filtered, the solvent evapd and the residue redissolved in small quantities of MeOH. This crude extract was centrifuged and directly injected for HPLC analysis.

For the analysis of the various tissues young fruits were harvested in mid-May and mid-June, lyophilized and the skin prepared as above. The core, including the young seeds, was cut out with a knife and ground in a ball-mill. The remaining fruit flesh was treated as the core and all fractions extracted and analysed separately.

For the study of the influence of the leaf age 5 comparable shoots were collected, the main veins discarded and the corresponding leaves combined for analysis.

High performance liquid chromatography. The phenolic compounds were separated on a column (250 × 4 mm i.d.) prepacked with Hypersil ODS, 3 µm particle size following a gradient as published elsewhere [18]. Chlorogenic acid, the flavonols and the dihydrochalcones were detected at 280 nm whereas the flavan-3-ols were estimated at 640 nm after post column derivatization with *p*-dimethylaminocinnamaldehyde [19].

Extraction, isolation and identification of compounds. Young, unripe apple fruits were divided, quickly frozen and lyophilized. From the dry material the fruit skin was peeled off with a razor blade. This fruit skin material (61 g) was exhaustively extracted with MeOH, diluted with H_2O (1:5) and defatted with petrol. The aq. phase was extracted with EtOAc and the extract concd to give a brown solid (928 mg), which was redissolved in a small amount of EtOH and subjected to CC on Sephadex LH-20 (50 × 3.5 cm) with EtOH as eluant. Fractions (each 15 ml) were collected and combined according to their TLC behaviour.

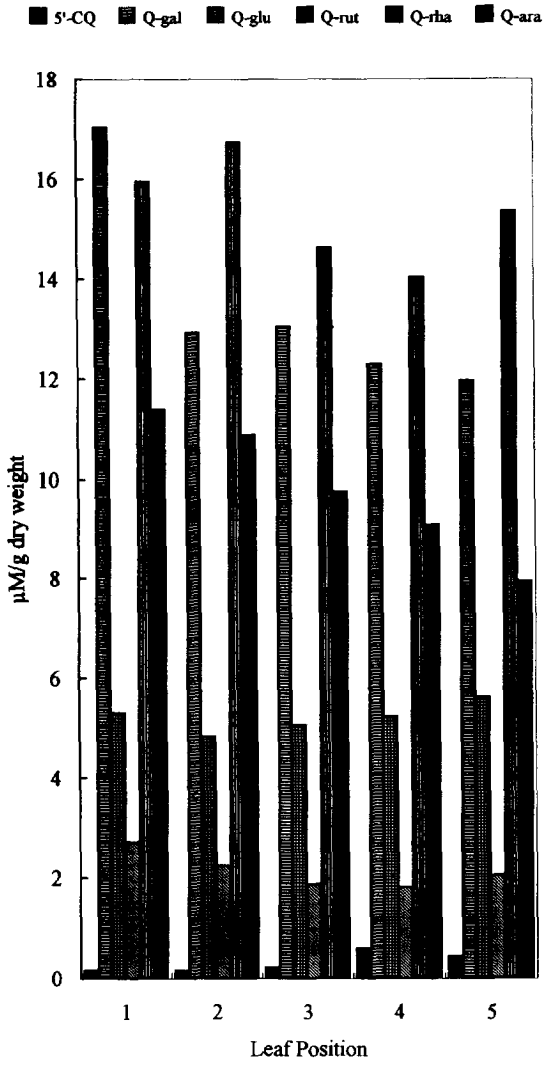

Fig. 3. The amount of chlorogenic acid (5'-CQ) and quercetin 3-O-glycosides (Q) in '*Golden Delicious*' apple leaves with respect to their position on the twig from the top (1st or youngest leaf) to the base (5th or oldest one).

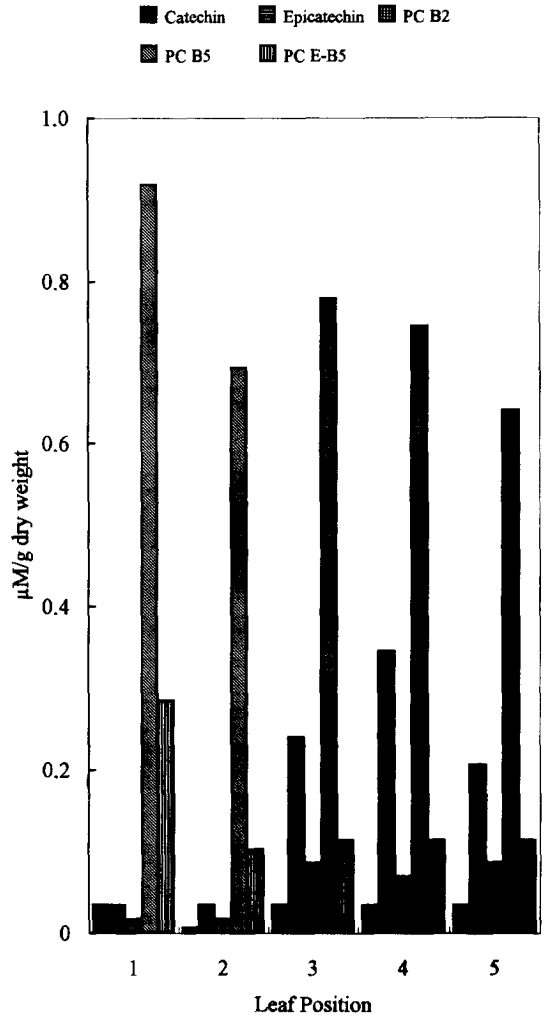

Fig. 4. The amount of monomeric flavan-3-ols (catechin, epicatechin) and procyanidins (PC) in leaves with respect to their position on the twig from the top (1st or youngest leaf) to the base (5th or oldest one).

Chlorogenic acid (R_t 45.1 min, λ_{max} 323 nm) and the monomeric flavan-3-ol catechin (R_t 33.5 min; CRD/UV ratio 12.4) were identified solely by their chromatographic behaviour (fraction A, tubes 8–14).

The procyanidins were characterized by TLC on silica gel with toluol–Me_2CO–HCO_2H (3:6:1) as the mobile phase [20], by HPLC and by their ratio CRD_{640}/UV_{280} exhibited after chemical reaction detection (CRD) with *p*-dimethylaminocinnamaldehyde in a system recently described but with a reaction time of 3 min [18]. Their structures were confirmed by their transformation to cyanidin and by hydrolytical cleavage in the presence of phloroglucinol. For chromatographic comparison standards were isolated from horse chestnut [procyanidins B2, B5, C1 and epicatechin-(4β,6)-epicatechin-(4β,6)-epicatechin (E-B5)] [21] and from grape seeds (procyanidin B1) [22].

The test tubes 15–22 (fraction B, 274 mg) contained epicatechin (R_t 60.4 min; CRD/UV ratio 18.9), phloretin derivatives and flavonols. The latter were identified as quercetin 3-galactoside (R_t 137.1 min, λ_{max} 354 nm), quercetin 3-glucoside (R_t 141.2 min, λ_{max} 354 nm), quercetin 3-rutinoside (R_t 144.5 min, λ_{max} 354 nm), and quercetin 3-rhamnoside (R_t 153.2 min, λ_{max} 346 nm), by co-chromatography with authentic samples on HPLC equipped with a photo diode array detector. The corresponding aglycone quercetin was confirmed by acid hydrolysis. An aliquot of fraction B was further fractionated by prep. HPLC on a Polygosil 5C18 (30 × 2 cm) column using a linear gradient from 5% HOAc to MeOH at a flow rate of 10 ml min^{-1} yielding phloretin 2'-xylosylglucoside (R_t 125.7 min, λ_{max} 284 nm), and quercetin 3-arabinoside (R_t 150.0 min, λ_{max} 350 nm). The structures were confirmed by chromatography of the aglycones (HPLC, TLC) and

the sugars (TLC) after acid hydrolysis in boiling 6N HCl under reflux. Quercetin 3-arabinoside was additionally characterized by spectrometric methods according to Mabry *et al.* [23]. Phloretin 2′-xylosylglucoside was also co-chromatographed with an authentic sample which was kindly provided by Dr F. A. Tomas-Barberan.

Tubes 31–40 (fraction D, 52 mg) contained procyanidin B2 (R_t 60.6 min; ratio CRD_{640}/UV_{280} 9.4). In tubes 41–66 (fractions E, F, 72 mg) the procyanidins B1 (R_t 29.3 min; CRD/UV ratio 8.7), B5 (R_t 120.8 min; CRD/UV ratio 10.2) and C1 (R_t 71.1 min; CRD/UV ratio 6.5) were found and further purified by prep. HPLC, as above. Tubes 67–78 (fraction G, 16 mg) contained procyanidin E-B5 [epicatechin-(4β,6)-epicatechin-(4β,6)-epicatechin; R_t 139.2 min; CRD/UV ratio 9.2], which was further purified by prep. HPLC. This compound was recently isolated from horse chestnut [21] and now for the first time found in apple tissue.

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