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PHENOLIC CONSTITUENTS OF TOMATO FRUIT CUTICLES

GRACE M. HUNT and EDWARD A. BAKER

Long Ashton Research Station, University of Bristol, Bristol, BS18 9AF, U.K.

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Key Word Index—*Lycopersicon esculentum*; Solanaceae; tomato; fruit cuticle; flavonoids; chalconaringenin; naringenin; coumaric acids.

Abstract—Chalconaringenin, naringenin, naringenin-7-glucoside, and *m*- and *p*-coumaric acids have been identified in the fruit cuticles of three tomato cultivars. The phenolic content of the cuticles increased substantially during fruit development, those from immature green and mature ripe fruits of cv Ailsa Craig yielding respectively 2.8 and 61 μg/cm^2 (representing 1.4 and 6% of the total membrane wt). Coumaric acids, present only in the 'cutin-bound' phenolics, increased from 2 to 24 μg/cm^2 during fruit development. Flavonoids, synthesized mainly during the climacteric, occurred free in the epicuticular (0.3–7.2 μg/cm^2) and cuticular (0.7–5.7 μg/cm^2) phenolics but the major part of this class of constituents in ripe fruit cuticles was also 'bound' to the cutin matrix (30–43 μg/cm^2). The composition of the flavonoid fraction was controlled by the spectral quality of incident radiation, red light favouring the formation of chalconaringenin.

INTRODUCTION

Although other workers have identified phenolic compounds in extracts obtained from tomato fruit skins [1–4], the techniques used did not differentiate between those components associated with the cuticle and those originating from the adhering cellular tissues. Amongst the various phenolics identified in previous studies, naringenin and hydroxycinnamic acid derivatives have been most commonly reported [1, 2]. However, the yellow pigment responsible for the colour of ripe fruit cuticles has not been characterized. Quercitrin was isolated from the fruit skins of several cultivars [1, 4] but was not detected in a recent survey which utilized more selective analytical procedures [2]. In this paper we report the identity of the yellow pigment and describe changes in the distribution of cuticle phenolics during the development of the fruits of three tomato cultivars.

RESULTS

Surface phenolics

Yields of phenolics from tomato fruits at different stages of maturity, expressed per unit area of surface and as a proportion by weight of the epicuticular fraction, are shown in Table 1. Phenolics were minor constituents (<5%) of the surface extracts from green fruits, which consisted largely of hydrocarbons C_{25}–C_{34} (85%), triterpenols (α-amyrin 2%, β-amyrin 5%) and fatty acids (C_{16} 3%, C_{18} 4%) [5]. The dominant constituent of the phenolic fraction of these extracts appeared to be a simple hydroxy flavone (λ_{max}^{MeOH}nm: 310, $\lambda_{max}^{MeOH+NaOMe}$nm: 358; TLC, R_f system 1: 0.98, R_f system 2: 0.10) but repeated attempts to confirm this identification by ^{1}H NMR or MS proved unsuccessful due to difficulties in its isolation. In contrast, the surface extracts obtained from ripe fruits of the three cultivars contained much higher proportions of phenolics ranging from 7–14% at the onset of the climacteric to 21–26% at the post-climacteric stage. The qualitative composition of the epicuticular wax fractions from ripe fruits of the three cultivars differed markedly from those of green fruits, comprising larger proportions of triterpenols (α-amyrin 11%, β-amyrin 45%) and lesser proportions of hydrocarbons C_{29}–C_{31} (43%) [5]. Naringenin was recovered in trace amounts from all fruits sampled after the green mature stage but the marked increase in surface phenolics observed during the climacteric resulted mainly from the production of a yellow pigment λ_{max}^{MeOH}nm: 368, $\lambda_{max}^{MeOH+AlCl_3}$nm: 406, $\lambda_{max}^{MeOH+AlCl_3+HCl}$nm: 396, $\lambda_{max}^{MeOH+NaOMe}$nm: 420. This pigment, examined by TLC R_f system 1: 0.30, R_f system 2: 0.17, showed colour responses characteristic of a chalcone: yellow in UV light turning light brown with NH_3; pink colour with Fast Blue B. The TMSi ether underwent partial thermal ionization to the naringenin derivative *m/e* 488 (M^+) during GC–MS analysis but also showed the expected parent ion *m/e* 560 and cleavage fragments for chalconaringenin (see Experimental). This identification was confirmed by spectral and chromatographic comparison with an authentic marker, liberated by acid hydrolysis from chalconaringenin-2-glucoside. The latter compound was recovered by PLC from the methanol extract of *Salix purpurea* bark [6]. The amount of chalconaringenin in the surface extracts from mature ripe fruits was similar for all cultivars (6.1–7.1 μg/cm^2).

Cuticular phenolics

The cuticular extracts recovered by exhaustive

Table 1. Phenolic content of the epicuticular and cuticular extracts obtained from tomato fruits at various stages of maturity

Cultivar and development stage	Average fruit diameter (mm)	Epicuticular phenolics		Cuticular phenolics	
		$\mu g/cm^2$	% epicuticular fraction	$\mu g/cm^2$	% cuticular fraction
Ailsa Craig					
Immature green	23	0.3	1.5	0.9	0.9
Immature green	38	0.7	4.2	2.4	0.8
Immature green	46	0.6	3.5	3.3	0.7
Mature green	56	0.8	3.7	3.7	1.8
Onset-climacteric	56	1.5	6.8	5.5	1.9
Mid-climacteric	57	7.1	25.4	5.6	1.8
Post-climacteric	57	7.2	26.1	5.7	0.9
Alicante					
Onset-climacteric	41	2.5	9.5	3.4	2.4
Post-climacteric	41	6.4	21.4	3.7	1.4
Grower's Pride					
Onset-climacteric	42	3.0	13.6	4.2	3.5
Post-climacteric	47	6.2	20.4	3.5	2.1

methanol extraction of isolated fruit membranes consisted of a complex mixture of fatty acids (C_{16}; C_{18}), sugars (α- and β-glucose), triterpenols (α- and β-amyrin) and phenolics. Fatty acids (80–85%) were the dominant components of the cuticular extracts of green fruit membranes which also contained sugars (8–11%) and triterpenols (4–6%). In contrast, the extracts from ripe fruit membranes contained larger proportions of sugars (53–59%) and triterpenols (7–11%), although fatty acids were also present in significant amounts (27–35%). The amount of cuticular phenolics, composed entirely of flavonoids, increased steadily (0.9–5.7 $\mu g/cm^2$) during fruit development whereas the proportion of these constituents in the extracts remained low (0.7–3.5%) due to the steady production of sugars and fatty acids. TLC revealed that the phenolics from the smallest fruits of cv Ailsa Craig comprised a single component which yielded naringenin and glucose on acid hydrolysis. The slow rate of hydrolysis suggested that the flavonoid was substituted at the 7-position [7]. A pure sample of this glucoside isolated by PLC, co-chromatographed with an authentic specimen of naringenin-7-glucoside R_f system 1:0.61 and exhibited identical spectral properties λ_{max}^{MeOH}nm: 282, $\lambda_{max}^{MeOH+NaOMe}$nm: 355. The glucoside constituted the major component of the cuticular phenolics isolated from expanding fruits of this cultivar (0.9–3.3 $\mu g/cm^2$) but at the mature green stage the composition of the fraction changed abruptly. Naringenin, identified from spectral data (λ_{max}^{MeOH}nm: 288, $\lambda_{max}^{MeOH+NaOMe}$nm: 322, $\lambda_{max}^{MeOH+AlCl_3}$nm: 312), by TLC ($R_f$ system 1: 0.75, R_f system 2: 0.46) and by GC–MS (see Experimental), became the dominant cuticular phenolic and the quantities of this constituent increased steadily throughout the climacteric (3.7–5.7 $\mu g/cm^2$). In marked contrast to the surface phenolics, chalconaringenin occurred as a relatively minor component only in the extracts obtained from the membranes of fully ripened fruits.

Cutin-bound phenolics

Identification of phenolics bound within the cuticular membrane proved difficult due to side reactions occurring between the flavonoids and the alkaline reagents normally used for depolymerization. In particular, rapid cleavage of ripe fruit membranes using 3% NaOMe–MeOH yielded a mixture of flavone dimers: TLC system 1 R_f: 0.95; GLC of the TMSi ether derivative RR_t: 2.6–2.8; λ_{max}^{MeOH}nm: 385, $\lambda_{max}^{MeOH+NaOMe}$nm: 450. By comparison, phenolics were released unchanged together with cutin monomers using oxygen-free aqueous KOH.

Phenolic acids, identified by GC–MS, were the sole phenolic constituents of the cutins of green fruits of cv Ailsa Craig (Table 2). As the fruits expanded, the accumulation of these acids (1.0–8.2 $\mu g/cm^2$) paralleled the increase in cuticle thickness (200–1100 $\mu g/cm^2$) and consequently the proportion in the membrane remained constant (0.8%). However, during the climacteric, cuticle thickness increased only slightly whereas the phenolic acids continued to accumulate (11–24.6 $\mu g/cm^2$), finally exceeding 2% of the membrane weight. Although traces of ferulic and caffeic acids were detected in many of the hydrolysates, the phenolic acid fraction consisted largely of *m*- and *p*-coumaric acids present in a constant ratio (1.6:1). Flavonoids were barely detectable in the cutins of unripe fruits but they increased in amount steadily throughout the climacteric. The composition of the flavonoid fraction from fruit cutins of the three cultivars also changed markedly during ripening. Initially, naringenin was the major flavonoid but from the mid-climacteric stage the cutin matrix became progressively enriched with chalconaringenin. Flavonoids were notable constituents of the cutins from ripe fruits of cvs Alicante and Grower's Pride, representing more than 5% of the membrane weight.

The nature of the bond between the phenolics and the cutin complex and the uniformity of distribution of

Table 2. Distribution and composition of cutin-bound phenolics in the membranes of tomato fruits at various stages of maturity

	Cutin-bound phenolics		Major components (%)		
	$\mu g/cm^2$	% membrane wt	Coumaric acid	Naringenin	Chalconaringenin
Ailsa Craig					
Immature green	1.6	0.8	100	N.D.	N.D.
Immature green	4.7	0.8	100	N.D.	N.D.
Immature green	5.6	0.8	98	tr	tr
Mature green	8.2	0.8	98	2	tr
Onset-climacteric	12.5	1.1	88	12	tr
Mid-climacteric	23.0	2.1	60	25	15
Post-climacteric	48.0	4.2	51	19	29
Alicante					
Onset-climacteric	10	0.9	98	tr	tr
Post-climacteric	67	5.6	28	36	35
Grower's pride					
Onset-climacteric	10	0.9	97	2	1
Post-climacteric	65	5.5	22	41	37

the former within the membrane was examined in time-course experiments using cuticles isolated from mature green and fully ripened fruits of cvs Alicante and Grower's Pride. Membranes from ripe and unripe fruits hydrolysed at different rates; less than 20% of cutin monomers were released from the membranes of unripe fruits during the initial 4 hr treatment. After a similar period under reflux only 40% of the membranes from ripe fruits remained intact. Phenolic acids were found in each of the extracts recovered at different stages during the hydrolysis of membranes from unripe fruits (Fig. 1). GLC profiles showed that the ratio of coumaric to cutin acids in these extracts remained constant. The ratio of flavonoids, phenolic acids and cutin acids also remained constant throughout the hydrolysis of ripe fruit membranes.

Effect of light on the formation of tomato fruit cuticle phenolics

During the present investigations we noted variations in cuticle pigments amongst tomato fruits which visually appeared fully ripened. This observation prompted us to study the effect of light quality on the formation of tomato fruit cuticle phenolics. Accordingly, we compared the phenolics present in the membranes obtained from cv Ailsa Craig fruits ripened at 21°, 70% r.h., with the following light conditions: (a) radiant energy rate 100 W/m^2: fluorescent tubes with 30% supplement of tungsten filament lamps; (b) radiant energy rate 100 W/m^2: fluorescent tubes screened with a setocyanin filter (absorbs strongly in the region 580–675 nm [8]); (c) total darkness.

Light stimulated the production of phenolic acids and flavonoids in tomato fruit cuticles (Table 3). The effect was most pronounced for fruits receiving a supplement of red light; considerable amounts of phenolic acids and flavonoids binding to the cutin matrix whilst substantial quantities of chalconaringenin occurred free within the membrane. The effect of red light on phenolic acid and flavonoid synthesis was also evident from the reduced quantities of these constituents produced by fruits ripened under the setocyanin screen. In contrast, the colourless membranes of fruits ripened in total darkness contained much smaller quantities of phenolic acids and only trace amounts of flavonoids.

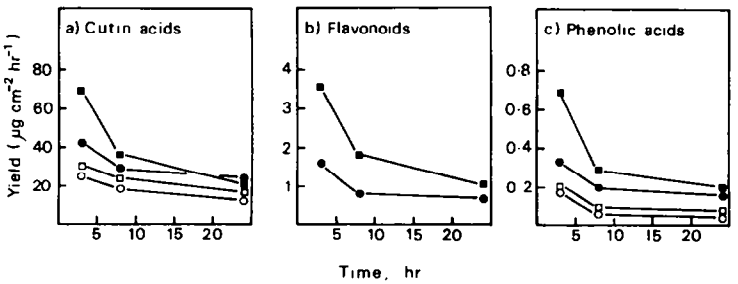

Fig. 1. Rates of release of cutin acids, flavonoids and phenolic acids from tomato fruit cuticles. ■, Grower's Pride ripe; □, Grower's Pride unripe; ●, Alicante ripe; ○, Alicante unripe.

Table 3. Effect of light quality on the composition of the cuticular and cutin-bound phenolics of tomato fruits cv Ailsa Craig

Ripening conditions	Major phenolics ($\mu g/cm^2$)		
	Coumaric acids	Naringenin	Chalconaringenin
Red supplemented light			
Cuticular phenolics	—	4	23
Cutin-bound phenolics	42	5	26
Red deficient light			
Cuticular phenolics	—	5	13
Cutin-bound phenolics	37	17	9
Darkness			
Cuticular phenolics	—	tr	tr
Cutin-bound phenolics	17	tr	tr

tr = trace.

DISCUSSION

The bulk of the tomato fruit cuticle phenolics remained firmly attached to the cuticular membrane despite repeated and prolonged treatment with hot organic solvents or mild alkali, e.g. aqueous $NaHCO_3$. Consequently these constituents could only be released following cleavage of the cutin monomers. Evidence obtained recently [9] indicates that the phenolic acids present in leaf and fruit cutins [9, 10] are covalently bonded to the polyestolide. The large amounts of phenolic acids and flavonoids retained by partially degraded membranes examined during the present investigations, gives support to this proposal and further suggests that the flavonoids are also bonded to the cutin matrix. The correlation observed during alkaline hydrolysis between the rate of release of cutin monomers and that of phenolic constituents indicates that the flavanones, chalcone and coumaric acids are not confined to a specific layer of the cuticle but are widely distributed within the membrane.

Naringenin-7-glucoside and chalconaringenin are reported for the first time as cuticular components. The latter compound clearly corresponds with the yellow pigment found previously in tomato fruit 'skins' by other workers [2, 3, 11]. Flavonoid glycosides occur extensively in the outer epidermis of leaves and fruits [12–14] but their precise location within this layer has not been clearly established. However, farina-bearing glandular hairs have been shown to be the source of flavones found in the dense surface coatings present on leaves of *Primula* spp. [15]. Neither quercitrin nor caffeic acid were identified as important components of the cuticles examined during the present investigations. We conclude, therefore, that the large quantities of these constituents isolated previously from tomato fruit skins [1, 2, 4] originated from the cellular tissues which adhere to the membrane if the cuticle is mechanically stripped from the fruit. The results of our experiments into the effect of red light on the production of phenolics in tomato fruit cuticles are in broad agreement with those reported previously by Piringer and Heinze [3] although these workers did not identify the yellow flavonoid. However, their studies also showed that the effect of red light on pigment formation could be rapidly reversed by immediate exposure to far-red radiation. From our limited studies it would seem likely that this regulation of pigment formation is achieved through an effect on the chalcone $\rightleftharpoons$ flavanone equilibrium.

EXPERIMENTAL

Plant material. The plants were raised from seed in pots maintained in a glasshouse at Long Ashton research station. Fruits of cv Ailsa Craig were sampled at 4 stages of growth prior to the climacteric and at 3 further stages during ripening, i.e onset-, mid- and post-climateric [2]. Fruits of cvs Alicante and Grower's Pride were taken at the onset- and post-climacteric stages.

Isolation of phenolics. Epicuticular phenolic constituents were removed from fruits by immersion (10 sec) in 3 successive portions (200 ml) of $CHCl_3$. Cuticular membranes were obtained from the stripped skins by incubation in pectinase soln [16], the epidermal remnants removed by careful brushing and absorbed phenolics isolated by refluxing in MeOH (3×100 ml). The mixture of hydroxy-fatty acids and phenolics released from cuticular membranes at intervals of 1, 3, 8 and 24 hr using either 3% NaOMe in MeOH [17] or 3% KOH were recovered in Et_2O after acidification [18].

Acid hydrolysis. Glycosides were hydrolysed by heating in a sealed tube with 5 ml 2 N HCl and the aglycones recovered from the acid soln using a 20×5 mm column of polyamide [19].

UV spectra. Quantitative estimations of phenolic extracts were achieved from UV spectra in MeOH [19], measured before and after the addition of (i) NaOMe, (ii) $AlCl_3$ and (iii) $AlCl_3 + HCl$.

Chromatography and spray reagents. Qualitative TLC was performed using either system 1: cellulose, $CHCl_3$–HOAc–H_2O (10:9:1) [20] or system 2: Si gel G, $CHCl_3$–EtOAc (7:3). Plates were examined in UV and after spraying with alkaline Fast Blue B [21] or $FeCl_3$–K ferricyanide [22]. Individual constituents fractionated by PLC on 0.75 mm layers of either cellulose or Si gel G were recovered from the isolated bands by refluxing in $CHCl_3$–MeOH (1:1).

GLC. Samples were methylated using excess CH_2N_2 [23]

and silylated with *N,O*-bis-trimethylsilylacetamide [16]. Constituents were identified from *RR*, data, determined on a 1 m × 3.5 mm stainless steel column packed with 1% Dexsil 300, using a dual FID instrument programmed 130–330° at 6°/min with tetracosane as int. standard. Quantitative assessments were obtained using response factors calculated from known reference compounds.

GC–MS. Analyses were performed at 70 eV using the Dexsil 300 column, temp. programmed as described above. Naringenin TMSi 70 eV *m/e* (rel. int. %): 488 (M^+ 3), 473 (16), 416 (38), 415 (38), 401 (14), 369 (23), 296 (10), 251 (13). 192 (45), 179 (83), 147 (10), 93 (38), 75 (100), 73 (50). Chalconaringenin TMSi 70 eV *m/e* (rel. int. %): 560 (M^+ 2) 545 (19), 532 (3), 369 (3), 342 (3), 296 (3), 281 (3), 267 (3), 269 (3), 193 (3), 179 (7), 149 (8), 147 (10), 145 (8), 132 (10), 117 (17), 97 (11), 93 (32), 75 (47), 73 (100).

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REFERENCES

1. Wu, M. and Burrell, R. C. (1958) *Arch. Biochem. Biophys.* **74,** 114.
2. Wardale, D. A. (1973) *Phytochemistry* **12,** 1523.
3. Piringer, A. A. and Heinze, P. H. (1954) *Plant Physiol.* **29,** 467.
4. Woldecke, M. and Herrmann, K. (1974) *Z. Lebensm. Unters. Forsch.* **155,** 216.
5. Baker, E. A., Hunt, G. M. and Bukovac, M. J. (1980) Manuscript in preparation.
6. Jarrett, J. M. and Williams, A. H. (1967) *Phytochemistry* **6,** 1585.
7. Harborne, J. B. (1965) *Phytochemistry* **4,** 107.
8. Gurr, E. (1971) *Synthetic Dyes in Biology, Medicine and Chemistry* p. 118. Academic Press, London.
9. Riley, R. G. and Kolattukudy, P. E. (1975) *Plant Physiol.* **56,** 650.
10. Caldicott, A. B. (1973) Ph.D. Thesis, University of Bristol.
11. Schonherr, J. and Bukovac, M. J. (1973) *Planta* **109,** 73.
12. Fisher, D. J. (1965) *Rep. Agric. Hortic. Res. Stn. Univ. Bristol* 255.
13. Wildanger, W. and Herrmann, K. (1973) *Z. Lebensm. Unters.-Forsch.* **151,** 103.
14. Starke, H. and Herrmann, K. (1976) *Z. Lebensm. Unters.-Forsch.* **161,** 137.
15. Blasdale, W. C. (1947) *J. Roy. Hortic. Soc.* **72,** 240.
16. Baker, E. A. and Procopiou, J. (1975) *J. Sci. Food Agric.* **26,** 1347.
17. Brieskorn, C. H. and Kabelitz, L. (1971) *Phytochemistry* **10,** 3195.
18. Huelin, F. E. and Gallop, R. A. (1951) *Aust. J. Sci. Res. Ser. B* **4,** 526.
19. Mabry, T. J., Markham, K. R. and Thomas, M. B. (1970) *The Systematic Identification of Flavonoids.* Springer, Berlin.
20. Eggers, K. (1961) *J. Chromatogr.* **5,** 74.
21. Betts, T. J. and Holloway, P. J. (1967) *J. Pharm. Pharmac.* **19,** 975 (suppl.).
22. Barton, G. M., Evans, R. S. and Gardner, J. A. F. (1952) *Nature* **170,** 249.
23. Schlenk, H. and Gellerman, L. (1960) *Analyt. Chem.* **32,** 1412.