Available online at www.sciencedirect.com
ScienceDirect
Phytochemistry 68 (2007) 1017–1025
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Composition of the cuticle of developing sweet cherry fruit

Stefanie Peschel [a], Rochus Franke [b], Lukas Schreiber [b], Moritz Knoche [a,*]

[a] Institute for Agronomy and Crop Science, Department of Horticulture, Martin-Luther-University of Halle-Wittenberg, D-06099 Halle (Saale), Germany
[b] Institute of Cellular and Molecular Botany, University of Bonn, Kirschallee 1, D-53115 Bonn, Germany

Received 4 December 2006; received in revised form 15 January 2007
Available online 27 February 2007

Abstract

The composition of wax and cutin from developing sweet cherry (*Prunus avium*) fruit was studied by GC–MS between 22 and 85 days after full bloom (DAFB). In this and our previous study, fruit mass and surface area increased in a sigmoidal pattern with time, but mass of the cuticular membrane (CM) per unit fruit surface area decreased. On a whole fruit basis, mass of CM increased up to 36 DAFB and remained constant thereafter. At maturity, triterpenes, alkanes and alcohols accounted for 75.6%, 19.1% and 1.2% of total wax, respectively. The most abundant constituents were the triterpenes ursolic (60.0%) and oleanolic acid (7.5%), the alkanes nonacosane (13.0%) and heptacosane (3.0%), and the secondary alcohol nonacosan-10-ol (1.1%). In developing fruit triterpenes per unit area decreased, but alkanes and alcohols remained essentially constant. The cutin fraction of mature fruit consisted of mostly C16 (69.5%) and, to a lower extent, C18 monomers (19.4%) comprising alkanoic, ω-hydroxyacids, α,ω-dicarboxylic and midchain hydroxylated acids. The most abundant constituents were 9(10),16-dihydroxy-hexadecanoic acid (53.6%) and 9,10,18-trihydroxy-octadecanoic acid (7.8%). Amounts of C16 and C18 monomers per unit area decreased in developing fruit, but remained approximately constant on a whole fruit basis. Within both classes of monomers, opposing changes occurred. Amounts of hexadecandioic, 16-hydroxy-hexadecanoic, 9(10)-hydroxy-hexadecane-1,16-dioic and 9,10-epoxy-octadecane-1,18-dioic acids increased, but 9,10,18-trihydroxy-octadecanoic and 9,10,18-trihydroxy-octadecenoic acids decreased. There were no qualitative and minor quantitative differences in wax and cutin composition between cultivars at maturity. Our data indicate that deposition of some constituents of wax and cutin ceased during early fruit development.

Keywords: *Prunus avium*; Rosaceae; Sweet cherry fruit; GC–MS; Cuticle; Wax; Cutin; Triterpenes

1. Introduction

The cuticular membrane (CM) covers all above-ground organs of primary origin of terrestrial plants and forms the interface between the plant and its environment. The CM serves as a protective barrier against water loss, nutrient leaching, mechanical damage, and invasion by pathogens (Martin and Juniper, 1970; Jeffree, 1996; Post-Beittenmiller, 1996). Maintaining these functions requires an intact CM during growth and development. This is a particular challenge for CM of fruit that often are characterized by rapid surface expansion until late in development. In the developing sweet cherry fruit, CM mass per unit fruit surface area decreases indicating that CM deposition, which is the net-effect of biosynthesis, transport and turnover (Jetter and Schäffer, 2001), does not keep pace with surface expansion (Knoche et al., 2001, 2004). Furthermore, the strain that develops as a consequence causes formation of microscopic cracks in the CM (Peschel and Knoche, 2005). These cracks impair the barrier function and predispose fruit to subsequent rain-cracking, a serious problem of sweet cherry production worldwide (Christensen, 1996).

The cuticle is composed of a cutin polymer matrix consisting mainly of esterified hydroxy- and epoxyhydroxy fatty acids with a chain length of 16 and 18 C-atoms and embedded cuticular and surface-deposited epicuticular

* Corresponding author. Present address: Institute of Biological Production Systems, Fruit Science Division, University of Hannover, Herrenhäuser Straße 2, 30419 Hannover, Germany. Tel.: +49 511 7629020; fax: +49 511 76219308.
E-mail address: moritz.knoche@obst.uni-hannover.de (M. Knoche).

0031-9422/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2007.01.008

waxes. In some species, cutan, a non-hydrolyzable part of the polymer matrix (Jeffree, 1996), polysaccharides and proteins (Martin and Juniper, 1970; Schönherr and Bukovac, 1973) may also be present in the CM. Cuticular and epicuticular wax comprise mixtures of long chain aliphatic hydrocarbons and their oxygenated derivatives including fatty acids and esters, alkanes, alcohols, aldehydes, ketones and cyclic compounds like triterpenes (Kollatukudy, 1996). Structure and composition of the CM varies between species, organ and developmental stage (Holloway, 1982; Jeffree, 1996; Kollatukudy, 1996).

Little information is available on the composition of the CM of developing sweet cherry fruit (Markley and Sando, 1937; Knoche et al., 2001) and at present it is not known, whether the decrease in CM mass per unit area during development affects all CM constituents uniformly. This would be expected if surface expansion merely distributed a constant amount of CM on an enlarging surface. Deeper insights in compositional changes of CM during fruit development will help to (i) assess consequences for mechanical properties and water permeability and (ii) develop strategies that help to "synchronize" fruit surface expansion and CM development and, ultimately, manipulate CM composition.

The objectives of the present study therefore were to (1) analyze the composition of wax and cutin of the sweet cherry fruit CM, (2) characterize developmental changes in CM composition and (3) establish potential differences in CM composition between selected cultivars.

2. Results

2.1. Fruit growth and CM development

The increase in fruit mass and calculated surface area between 22 and 85 days after full bloom (DAFB) followed a sigmoidal pattern that is typical for stone fruit development (Fig. 1a). This pattern is characterized by an initial lag phase indicative for development of the stony endocarp and seed (stage II) followed by a phase of rapid increase in fruit mass that approaches an asymptote towards maturity (stage III; final swell). Between 22 and 85 DAFB fruit mass and surface area increased from 1.4 to 12.0 g and from 6.0 to 24.7 cm^2, respectively. The mass of CM per unit fruit surface area increased in early stage II, but decreased throughout stage III (Fig. 1b). On a whole fruit basis, mass of CM increased by 50% between 22 and 85 DAFB, while fruit surface area increased by about 311%.

2.2. Main constituents of wax in CM of developing fruit

More than 95% by weight of the wax extract was identified by GC–MS. Triterpenes (75.6%) were the most abundant class of wax constituents of mature sweet cherry fruit, followed by alkanes (19.1%) and alcohols (1.2%; Table 1). Within triterpenes, ursolic (60.0%) and oleanolic acid

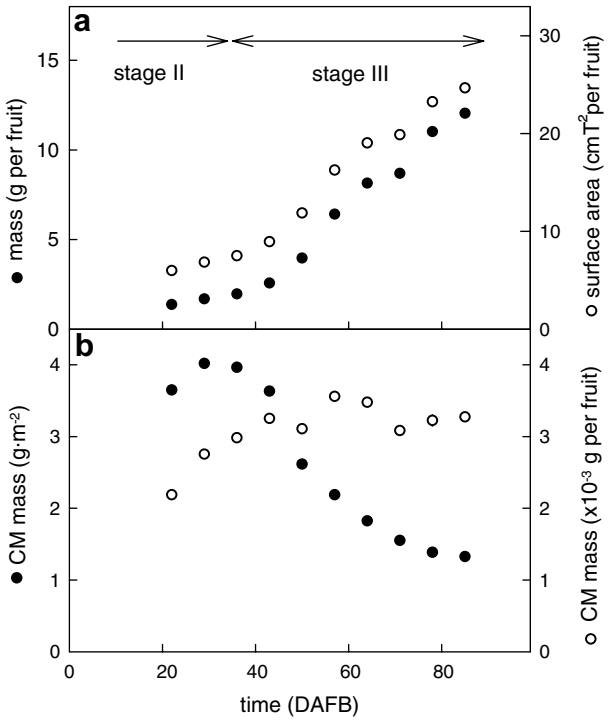

Fig. 1. Time course of change in (a) fruit mass and surface area and (b) mass of the enzymatically isolated cuticular membrane (CM) on a unit fruit surface area and a whole fruit basis. *X*-axis scale in days after full bloom (DAFB).

(7.5%) dominated. Triterpene alcohols (α-amyrine 0.4%, uvaol 0.8%) were present in lower amounts. Other triterpenes resembling hederagenin and its isomers as well as some triterpene esters were recognized by ion fragments and retention times typical for triterpenes. These are referred to as unidentified triterpenes in Table 1 and accounted for <7% of wax of mature fruit.

The alkane fraction (19.1%) comprised a homologous series of *n*-alkanes (C26–C31) with odd-numbered *n*-alkanes dominating above even numbered. In mature fruit the most abundant alkane was nonacosane (13.0%) followed by heptacosane (3.0%, Table 1). The alcohol fraction (1.2%) consisted primarily of the secondary alcohol nonacosan-10-ol (1.1%). Sweet cherry fruit wax did not contain long chain aldehydes, acids or ketones.

The wax composition changed in the course of fruit development (Fig. 2). While triterpenes decreased from 92.6% to 75.6%, between 22 and 85 DAFB, alkane and alcohol content increased about fourfold (from 5.7% to 19.1% and 0.4% to 1.2% for alkanes and alcohols, respectively; Table 1, Fig. 2b).

On a unit surface area basis, triterpenes increased in stage II, but decreased throughout stage III (Fig. 2b). Mass of alkanes and alcohols per unit surface area remained essentially constant (Fig. 2b).

Calculating amounts of wax constituents on a whole fruit basis revealed that total triterpenes per fruit remained about constant during development, but that of alkanes

Table 1
Constituents of wax of cv. Kordia sweet cherry fruit cuticles isolated at 22 and 85 days after full bloom (DAFB)

Constituents	Developmental stage					
	22 DAFB			85 DAFB		
	Mass			Mass		
	(% w/w)	($\times10^{-2}$ g m^{-2})	($\times10^{-3}$ g)[a]	(% w/w)	($\times10^{-2}$ g m^{-2})	($\times10^{-3}$ g)[a]
Triterpenes	*92.57 (0.41)*	*55.30 (3.70)*	*33.15 (2.22)*	*75.63 (0.68)*	*15.21 (0.69)*	*37.50 (1.70)*
Ursolic acid	72.55 (1.10)	43.28 (2.51)	25.94 (1.51)	60.03 (1.11)	12.08 (0.65)	29.78 (1.60)
Oleanolic acid	9.69 (0.16)	5.78 (0.32)	3.46 (0.19)	7.51 (0.08)	1.51 (0.07)	3.73 (0.18)
Uvaol	0.80 (0.07)	0.48 (0.06)	0.29 (0.04)	0.82 (0.06)	0.16 (0.01)	0.40 (0.02)
α-Amyrine	0.23 (0.04)	0.14 (0.03)	0.09 (0.02)	0.38 (0.06)	0.08 (0.01)	0.19 (0.04)
Unidentified triterpenes	9.30 (0.78)	5.62 (0.82)	3.37 (0.49)	6.89 (0.71)	1.38 (0.12)	3.40 (0.30)
Alkanes	*5.65 (0.48)*	*3.42 (0.51)*	*2.05 (0.30)*	*19.06 (0.41)*	*3.82 (0.05)*	*9.43 (0.13)*
Nonacosane (C29)	4.02 (0.37)	2.43 (0.39)	1.46 (0.23)	12.95 (0.34)	2.60 (0.03)	6.40 (0.07)
Hentriacontane (C31)	0.64 (0.04)	0.39 (0.05)	0.23 (0.03)	1.28 (0.04)	0.26 (0.01)	0.63 (0.02)
Heptacosane (C27)	0.45 (0.05)	0.27 (0.04)	0.16 (0.02)	2.95 (0.14)	0.59 (0.02)	1.46 (0.05)
Octacosane (C28)	0.26 (0.04)	0.16 (0.03)	0.09 (0.02)	1.00 (0.01)	0.20 (0.01)	0.50 (0.02)
Hexacosane (C26)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.11 (0.06)	0.02 (0.01)	0.06 (0.03)
Triacontane (C30)	0.28 (0.02)	0.17 (0.02)	0.10 (0.02)	0.77 (0.02)	0.15 (0.01)	0.38 (0.02)
Alcoholes	*0.36 (0.04)*	*0.21 (0.02)*	*0.13 (0.01)*	*1.16 (0.06)*	*0.23 (0.01)*	*0.57 (0.04)*
Nonacosane-10-ol (C29)	0.30 (0.04)	0.18 (0.02)	0.11 (0.01)	1.14 (0.08)	0.23 (0.02)	0.56 (0.05)
Hexacosanol (C26)	0.06 (0.00)	0.03 (0.00)	0.02 (0.00)	0.02 (0.02)	0.00 (0.00)	0.01 (0.00)
Unidentified[b]	*1.42 (0.08)*	*0.84 (0.05)*	*0.50 (0.03)*	*4.15 (0.28)*	*0.83 (0.03)*	*2.05 (0.07)*
Total	100	59.77 (4.23)	35.83 (2.54)	100	20.09(0.72)	49.56 (1.79)

Data are given as means and standard errors of means (±SE). At 85 DAFB fruit were at the fully mature stage.
[a] The amounts per fruit were calculated from mass per unit surface area multiplied by the surface area of the fruit.
[b] The unidentified wax constituents represented mostly long-chain-aliphatics based on their EIMS-spectra; structural identification was technically impossible because of their low abundance.

and alcohols increased to more than 400% of their respective amounts at 22 DAFB (Fig. 2c and d).

Interestingly, within these classes, shifts in composition occurred. For example, the amount of heptacosane per fruit increased about 10-fold, that of hentriacontane approximately tripled. The alcohol nonacosan-10-ol increased about 5-fold.

2.3. Main constituents of cutin in CM of developing fruit

About 90% of the cutin monomers released by depolymerization of the dewaxed CM from mature fruit were identified by GC–MS. The most abundant constituents of sweet cherry fruit cutin were C16 monomers; C18 monomers were present in lower amounts (Table 2). Monomers comprised alkanoic acids, ω-hydroxyacids, α,ω-dicarboxylic acids and midchain hydroxylated acids, the latter being most abundant (Table 2). Within the C16 and C 18 monomers the main constituents were 9(10),16-dihydroxy-hexadecanoic acid and 9,10,18-trihydroxy-octadecanoic acid, respectively. Mass of C16 and C18 monomers per unit area decreased in developing fruit (Fig. 3b). The decrease in both monomer classes approximately mirrored the increase in surface area. On a whole fruit basis, C16 monomers somewhat increased (+28%) between 22 and 85 DAFB, while C18 monomers decreased (–23%; Fig. 3c and d) resulting in a gradual increase in the ratio of C16/C18 monomers from 2.2 to 3.5 (Table 2). As for wax constituents, shifts in composition occurred within both classes of cutin monomers during development. The slight increase in C16 monomers was accounted for by a doubling of amounts of hexadecandioic and 16-hydroxy-hexadecanoic acids, that of 9(10)-hydroxy-hexadecane-1,16-dioic acid even tripled (Table 2). The decrease in mass of C18 monomers per fruit was the net effect of a decrease in 9,10,18-trihydroxy-octadecanoic (−55%) and 9,10,18-trihydroxy-octadecenoic acid (−33%) and an increase in 9,10-epoxy-octadecane-1,18-dioic acid (+359%; Table 2). Total dioic acids (C16 and C18) increased from 7.13% to 19.18% between 22 and 85 DAFB (Table 2).

2.4. Cultivars

No qualitative differences in composition of wax or cutin were detected between the four cultivars investigated (Table S1). Quantitatively, wax of Hedelfinger was lower in triterpenes, and higher in alkanes, that of Sam fruit contained significantly fewer alcohols. Composition of the cutin fraction was similar between cultivars.

3. Discussion

3.1. Composition of the CM of mature sweet cherry fruit relative to other species

The amount of CM of developing sweet cherry fruit was similar to data obtained in other cultivars, growing seasons

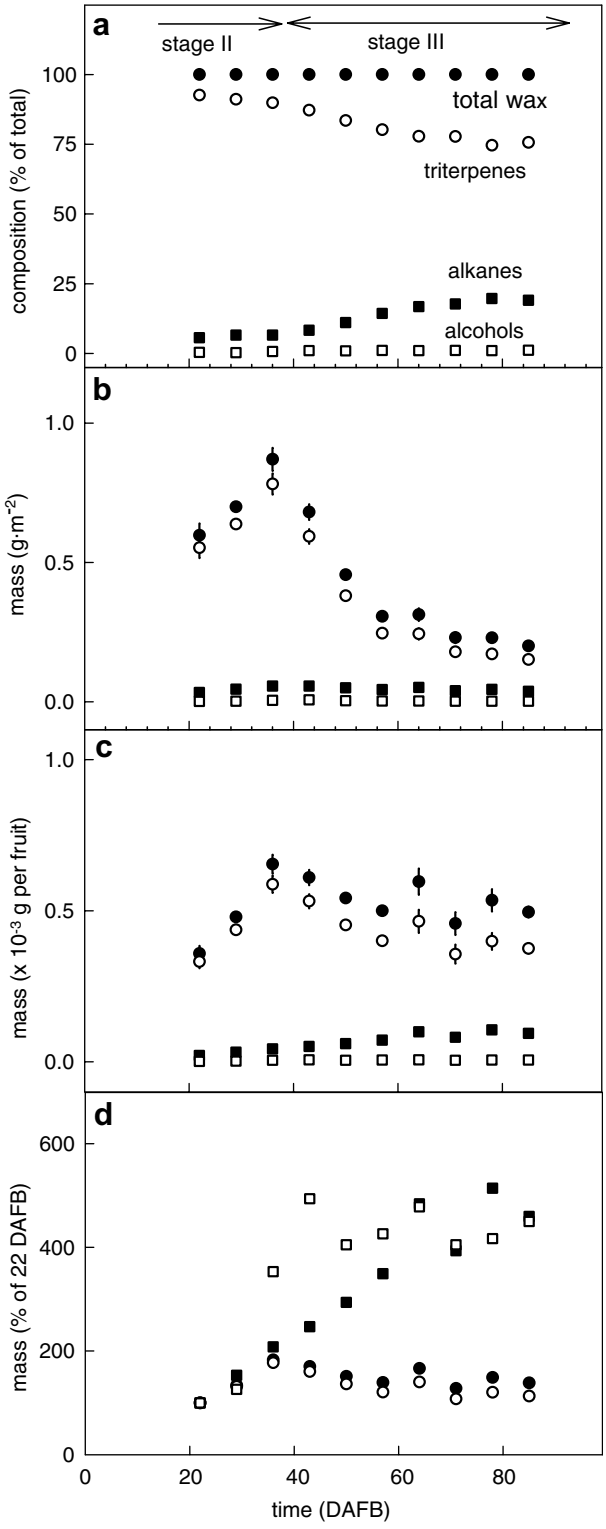

Fig. 2. Time course of change in classes of wax constituents of the cuticle of developing sweet cherry fruit. (a) Relative composition. (b) Mass per unit fruit surface area. (c) Mass on a whole fruit basis. (d) Mass per fruit as percentage of mass at 22 days after full bloom (DAFB).

or locations (Knoche et al., 2001, 2004). However, the wax and cutin yields calculated from our GC data were lower than those data generated using gravimetric assays. For the batch of mature Kordia fruit used in the present study, this discrepancy was smaller for wax (200.9 ± 7.2 vs 257.7 ± 8.3 mg m^{-2} equivalent to $15.1 \pm 0.6\%$ and $19.4 \pm 0.6\%$ for GC vs gravimetric quantification, respectively), but significantly larger for the cutin fraction. Methanolysis of the DCM yielded 351.2 ± 41.0 mg m^{-2} which is equivalent to a calculated cutin content of the CM of $26.5 \pm 3.1\%$. The cutin content thus obtained corresponds to about half of gravimetrically determined data after dewaxing and extraction of carbohydrates and proteins using 6N HCl (Schmidt and Schönherr, 1982). These differences can be attributed to (1) the lack of specific response factors for the quantification of secondary oxygenated acids (Graca and Pereira, 2000) in cutin or triterpenes as dominating wax components, (2) limited volatility of some depolymerization products under the GC conditions used (Graca et al., 2002), and (3) non-esterified cutin that is not depolymerised by methanolysis (Nip et al., 1986).

Composition of wax and cutin fractions of mature Kordia fruit was representative for sweet cherry fruit from other cultivars (Table S1; Markley and Sando, 1937). The wax represented a typical "fruit wax". This classification is based on several observations. First, wax of fruit is often high in triterpenes (Holloway, 1994). Ursolic acid (60.0%), the most abundant constituent in sweet cherry fruit wax is also found in apple (Belding et al., 2000), cranberry (Croteau and Fagerson, 1971) and blueberry (Freeman et al., 1979). Oleanolic acid, the second major triterpene in sweet cherry fruit wax (7.5%) is the most abundant constituent of grape berry wax (Radler and Horn, 1965). In contrast, amyrin that was detected as a minor constituent of sweet cherry fruit wax represented the dominating triterpene in tomato (Baker et al., 1982) and cranberry (Croteau and Fagerson, 1971). Second, like in apple (Belding et al., 2000) and cranberry (Croteau and Fagerson, 1971) nonacosane (C29) is the most abundant odd-numbered alkane in sweet cherry fruit wax. Third, the occurrence of secondary alcohols with nonacosane-10-ol being most abundant is characteristic for fruit of the Rosaceae family (Wollrab, 1969). Unlike Markley and Sando (1937) we detected only trace amounts of sitosterol and no palmitic, stearic, linoleic and oleic acid. These compounds are characteristic for membrane lipids. Their presence in the study by Markley and Sando (1937) was most likely due to the use of dried sweet cherry "press cakes" rather than isolated CM as a source of wax.

The cutin of sweet cherry fruit is of a mixed C16/C18-type according to the classification by Holloway (1982). Mixed type cutins were also reported for leaves of *Malus pumila*, *Prunus laurocerasus* and *Vitis vinifera* and fruit of *Solanum melongena*, *Ribes nigrum* (Holloway, 1982 and references therein), peach and pear (Walton and Kollatukudy, 1972). Like cutin from fruit of apple (Holloway, 1973), peach, pear (Walton and Kollatukudy, 1972), black currants, cranberry, buckthorn, lingonberry and bilberry (Kallio et al., 2006) the sweet cherry fruit cutin is primarily composed of midchain oxygenated hydroxyacids such as 9(10),16-dihydroxy-hexadecanioc acid and 9,10,18-trihy-

Table 2
Cutin constituents obtained by methanolysis of cv. Kordia sweet cherry fruit cuticles isolated at 22 and 85 days after full bloom (DAFB)

Constituents	Developmental stage					
	22 DAFB			85 DAFB		
	Mass			Mass		
	(% w/w)	($\times 10^{-2}$ g m^{-2})	($\times 10^{-3}$ g per fruit)[a]	(% w/w)	($\times 10^{-2}$ g m^{-2})	($\times 10^{-3}$ g per fruit)[a]
C16	63.14 (1.38)	78.50 (2.80)	47.06 (1.68)	69.52 (0.91)	24.48 (3.16)	60.37 (7.79)
C18	28.88 (1.70)	35.96 (2.61)	21.55 (1.56)	19.43 (1.27)	6.74 (0.44)	16.62 (1.10)
Alkan-1-ioc acids	*0.75* (*0.10*)	*0.93* (*0.16*)	*0.56* (*0.09*)	*0.96* (*0.17*)	*0.3* (*0.04*)	*0.81* (*0.11*)
Hexadecanoic acid (C16)	0.50 (0.04)	0.62 (0.07)	0.37 (0.04)	0.56 (0.09)	0.19 (0.02)	0.47 (0.05)
Octadecanoic acid (C18)	0.25 (0.06)	0.31 (0.09)	0.19 (0.06)	0.40 (0.09)	0.14 (0.02)	0.34 (0.06)
α,ω-Dicarboxylic acids	*1.03* (*0.52*)	*1.30* (*0.65*)	*0.78* (*0.39*)	*1.96* (*0.16*)	*0.68* (*0.02*)	*1.67* (*0.06*)
Hexadecane-1,16-dioic acid (C16)	1.03 (0.52)	1.30 (0.65)	0.78 (0.39)	1.96 (0.16)	0.68 (0.02)	1.67 (0.06)
ω-Hydroxyacids	*3.17* (*0.06*)	*3.94* (*0.21*)	*2.36* (*0.13*)	*3.52* (*0.08*)	*1.23* (*0.11*)	*3.03* (*0.28*)
16-Hydroxy-hexadecanoic acid (C16)	1.03 (0.00)	1.27 (0.03)	0.76 (0.02)	1.75 (0.06)	0.62 (0.09)	1.52 (0.21)
18-Hydroxy-octadecadienioc acid (C18(2))	2.14 (0.07)	2.67 (0.18)	1.60 (0.11)	1.77 (0.13)	0.61 (0.03)	1.51 (0.08)
Midchain oxygenated hydroxyacids	*87.07* (*0.87*)	*108.29* (*3.52*)	*64.91* (*2.11*)	*82.51* (*0.08*)	*28.98* (*3.41*)	*71.49* (*8.40*)
9(10)-Hydroxy-hexadecane-1,16-dioic acid (C16)	4.72 (0.33)	5.85 (0.27)	3.51 (0.17)	11.66 (0.45)	4.12 (0.62)	10.17 (1.53)
9(10),16-Dihydroxy-hexadecanoic acid (C16)	55.86 (0.66)	69.46 (2.21)	41.64 (1.33)	53.59 (0.71)	18.87 (2.45)	46.55 (6.04)
9(10),18-Dihydroxy-octadecanoic acid (C18)	1.47 (0.04)	1.84 (0.12)	1.10 (0.07)	1.51 (0.07)	0.54 (0.08)	1.32 (0.20)
9,10,18-Trihydroxy-octadecanoic acid (C18)	19.97 (1.71)	24.87 (2.44)	14.91 (1.46)	7.80 (0.88)	2.70 (0.27)	6.66 (0.67)
9,10,18-Trihydroxy-octadecenoic acid (C18(1))	2.61 (0.21)	3.24 (0.16)	1.94 (0.09)	1.50 (0.06)	0.52 (0.05)	1.29 (0.12)
9,10-Epoxy-octadecane-1,18-dioic acid (C18)	1.37 (0.07)	1.70 (0.03)	1.02 (0.02)	5.56 (0.63)	1.90 (0.04)	4.69 (0.11)
9,10-Epoxy-18-hydroxy-octadecanoic acid (C18)	1.07 (0.04)	1.33 (0.07)	0.80 (0.04)	0.89 (0.20)	0.33 (0.10)	0.81 (0.26)
Coumaric acid	0.77 (0.02)	0.96 (0.00)	0.57 (0.00)	0.30 (0.04)	0.11 (0.03)	0.27 (0.07)
Unidentified midchain hydroxy-acids	4.08 (0.32)	5.10 (0.57)	3.06 (0.34)	3.77 (0.26)	1.35 (0.26)	3.32 (0.63)
Unidentified aliphatics	3.13 (040)	3.87 (0.39)	2.32 (0.23)	6.98 (0.15)	2.45 (0.28)	6.04 (0.70)
Total	100	124.38 (4.23)	74.56 (2.53)	100	35.13 (4.10)	86.63 (10.10)

Data are given as means and standard errors of means (±SE). At 85 DAFB fruit were at the fully mature stage.

[a] The amounts per fruit were calculated from mass per unit surface area multiplied by the surface area of the fruit.

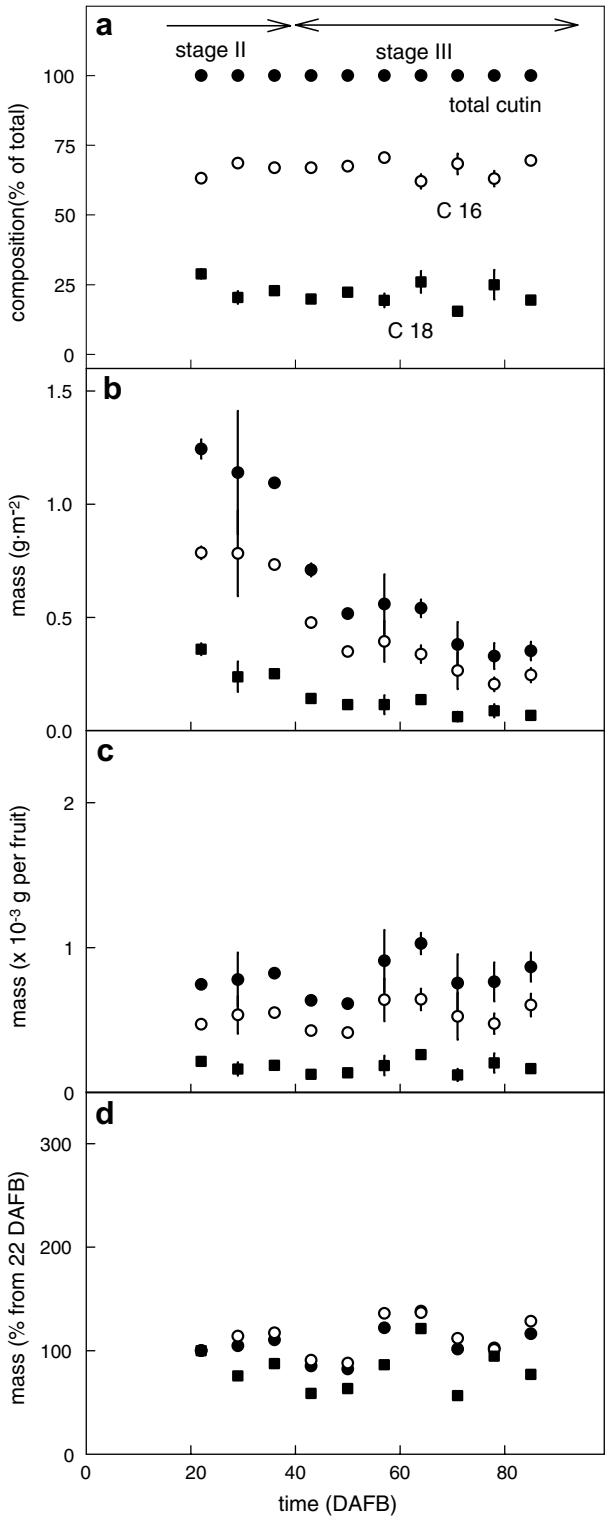

Fig. 3. Time course of change in C16 and C18 cutin monomers of the cuticle of developing sweet cherry fruit. (a) Relative composition. (b) Mass per unit fruit surface area. (c) Mass on a whole fruit basis. (d) Mass per fruit as percentage of mass at 22 days after full bloom (DAFB).

droxy-octadecanoic acid. Unlike apple fruit (Holloway, 1973) C16 is the dominating chain length of monomers in sweet cherry. Also, abundance of unsaturated (C18) monomers (3.3%) and epoxides (6.5%) is markedly lower in sweet cherry than in apple (Holloway, 1973). Interestingly, α,ω-diacid structures, i.e., 9(10)-hydroxy-hexadecane-1,16-dioic acid, 9,10-epoxy-octadecane-1,18-dioic acid and hexadecandioic acid, make up a considerable portion (19.2%) of the sweet cherry fruit cutin. These monomers have been suggested to be important for cross-linking of the cutin polymer (Bonaventure et al., 2004; Franke et al., 2005). The aromatic *p*-coumaric and ferulic acids are characteristic constituents of suberin (Riley and Kollatukudy, 1975) and *p*-coumaric acid is also found as a minor component in cutin of leaves and fruit of other species (Riley and Kollatukudy, 1975; Graca et al., 2002).

3.2. Wax and cutin composition of CM of developing fruit

Our data demonstrate that mass of wax and cutin per unit fruit surface area markedly decreased during stage III of sweet cherry fruit development (Figs. 2 and 3). This decrease mirrored the increase in fruit surface area indicating that, on a whole fruit basis, mass of wax and cutin must have remained essentially constant relative to the marked increase in surface area. The CM of sweet cherry fruit differs in this respect from CM of other species such as apple (Knoche, unpublished data), tomato fruit (Baker et al., 1982) or leaves of *Clivia* (Riederer and Schönherr, 1988) or *Hedera* (Hauke and Schreiber, 1998) which continued to increase in thickness throughout development. Compositional analysis of wax and cutin fractions in sweet cherry revealed that this decrease was not a mere dilution effect caused by surface area expansion, but resulted from specific changes of individual constituents (Tables 1 and 2; Figs. 2 and 3).

The decrease in wax mass per unit fruit surface area was accounted for by a decrease in triterpenes, while alkanes and alcohols on a unit area basis remained approximately constant throughout stage III. Thus, deposition of alkanes and alcohols kept pace with surface expansion, but not that of triterpenes. These opposing changes resulted in a constant percentage of wax of the developing CM (Knoche et al., 2001).

The decrease in triterpenes in developing sweet cherry fruit was similar to that reported for leaves of other *Prunus* species (peach, Bukovac et al., 1979; sour cherry, Baker and Hunt, 1981; *P. laurocerasus*, Jetter and Schäffer, 2001) and apple (Hellmann and Stösser, 1992). They differed from those for leaves and fruit of blueberry (Freeman et al., 1979), where triterpenes increased throughout development. In developing tomato fruit, the increase in triterpenes even overcompensated the decrease in long chain aliphatics (Baker et al., 1982).

The decrease in mass of cutin per unit surface area during stage III development is accounted for by a low or lacking deposition of the midchain oxygenated hydroxyacids 9(10),16-dihydroxy-hexadecanoic acid and 9,10,18-trihydroxy-octadecanoic acid that represent the main constituents of sweet cherry fruit cutin. Some deposition of alkan-1-ioc acids, α,ω-dicarboxylic acids, ω-hydroxy acids

and some of the minor midchain oxygenated hydroxyacids occurred during development, but this was not sufficient to compensate for the 311% increase in fruit surface area. Similarly, for grape berries, Commenil et al. (1997) reported a 2.5-fold decrease in cutin mass per unit surface area. But for CM of *Clivia* leaves (Riederer and Schönherr, 1988), apple (Knoche unpublished) and tomato fruit (Baker et al., 1982) cutin deposition outnumbered surface expansion resulting in an increase in CM thickness during development.

3.3. Potential consequences for fruit development

The change in cuticle composition may affect one or several of the following CM properties:

(i) transport barrier
(ii) wetting characteristics and
(iii) mechanical properties of the CM.

According to current models of the CM as a *transport barrier*, the crystalline fraction of embedded cuticular wax represents the primary barrier within the CM. Amorphous regions and defects in crystal structure are considered as permeable domains within the wax, but crystalline wax is inaccessible (Riederer and Schneider, 1990; Reynhardt and Riederer, 1994). Based on this model, a close relationship between wax composition and transport properties of CM is unlikely (Riederer and Schneider, 1990; Vogg et al., 2004). However, some general trends have been identified. Fruit CM are rich in triterpenes and often more permeable than leaf CM (Becker et al., 1986; Schreiber and Riederer 1996). Also, triterpenes were shown to form a less effective barrier to water transport than long chain hydrocarbons (Grncarevic and Radler, 1967). The latter constituents are considered as primary determinants of transport properties of wax (Vogg et al., 2004).

Wetting characteristics of (plant) surfaces depend on the chemistry of the functional groups exposed on the surface of epicuticular wax and its physical fine structure (Holloway, 1969, 1970). Both factors are interrelated (Jeffree et al., 1975; Jetter and Riederer, 1994). Wax containing large amounts of long chain alkanes often has a distinct fine structure, which is difficult to wet. The sweet cherry fruit CM, however, contains mostly triterpenes, has little fine structure and a fairly easy-to-wet surface (Peschel et al., 2003). This would be expected since triterpenes represent a more polar class of wax constituents. In *P. laurocerasus* triterpenes are a major constituent of intracuticular wax (Jetter and Schäffer, 2001), but their location in the sweet cherry fruit wax is unknown. This would be needed to elucidate causal relationships.

Little information is available on the contribution of individual constituents of the CM to its *mechanical properties*. Rheological studies using isolated CM established that the dewaxed CM determines primarily mechanical properties and wax only acts as a supporting filler (Petracek and Bukovac, 1995). For synthetic polymers mechanical properties are affected by the degree of cross-linking that, in turn, depends on the functional groups of the monomers. Analysis of cutin monomers in sweet cherry fruit revealed a continuous decrease in mass per unit area of the major cutin constituents. However, this effect is partially offset by increasing amounts of midchain hydroxylated acids and α,ω-dicarboxylic acids, compounds with two or more functional groups that could be important in cross-linking of the cutin matrix as suggested previously (Kollatukudy, 1996; Bonaventure et al., 2004; Franke et al., 2005). The mismatch between deposition and surface expansion, however, remains as an overriding factor that results in strain of the CM (Knoche et al., 2004), formation of microcracks (Peschel and Knoche, 2005) and – together with the change in wax constituents – impaired barrier properties of the CM (Knoche et al., 2001). Since these consequences are economically important, there is considerable interest in developing strategies to understand and ultimately manipulate synthesis, turnover and deposition of cuticle and its constituents. The analysis of the composition of wax and cutin fractions presented herein provides the basis therefore.

4. Experimental

4.1. Plant material

Sweet cherry fruit (*Prunus avium* L., cv. 'Hedelfinger', 'Kordia', 'Sam', 'Van'; grafted on *P. avium* 'Alkavo' rootstocks) were sampled in 2000 and 2001 from a commercial orchard located near Eisleben, Germany (latitude 51°31′N and long. 11°44′E). Fruit were harvested at commercial maturity except for 'Kordia' fruit that was collected at weekly intervals between 22 and 85 days after full bloom (DAFB). Fruit were selected for uniformity based on color and size and freedom from visual defects, processed fresh or held at 5 °C for no longer than 3 days.

4.2. Cuticle isolation

Exocarp segments were excised from the cheek region using a cork borer (i.d. 8.9 mm) except for 'Sam', where segments from cheek, suture, stylar end and pedicel cavity were used. Only one segment per fruit was excised. CM were isolated enzymatically by incubating in pectinase (90 ml l^{-1}; Panzym Extra; Novo Nordisk Ferment Ltd., Dittingen, Switzerland) and cellulase (5 g l^{-1}; *Aspergillus niger*, Sigma Chemical Co., St. Louis, MO, USA) prepared in 50 mM citric acid buffer at pH 4.0. NaN_3 was added at a final concentration of 1 mM to prevent microbial growth. Enzyme solutions were replaced periodically. After separation from the tissue CM were desorbed in borax buffer (0.02 M; pH 9.18) and deionized water (Schönherr and Riederer, 1986). Subsequently CM were dried and stored until used. CM mass was determined gravimetrically. Mass of CM on a whole

fruit basis was calculated by multiplying CM mass per unit area by the surface area of the fruit. The latter was estimated from fruit mass assuming a spherical shape and a density of 1000 kg m^{-3} as first approximations.

4.3. Wax extraction and preparation

Samples (0.75 mg per sample) of CM were dewaxed with 700 µl $CHCl_3$ for 16 h at 25 °C in Teflon-sealed reaction vials. After adding 2 µg tetracosane as an internal standard the extract was evaporated in a stream of nitrogen at 40 °C and derivatized. Free hydroxyl and carboxyl groups were converted into their trimethylsilyl (TMSi) ethers and esters using bis-(*N*,*N*-trimethylsilyl)-tri-fluoroacetamide (Machery-Nagel) in pyridine for 30 min at 70 °C prior GC–MS analysis. Dewaxed CM (DCM) were dried and weighed for determining mass per unit area.

4.4. Cutin depolymerization and extraction

DCM were depolymerised using the procedure described by Franke et al. (2005). Briefly, DCM were transesterified with 1N MeOH/HCl for 2 h at 80 °C. After cooling, 2 µg dotriacontane was added as an internal standard. One milliliter saturated NaCl was added to the methanolysate. The cutin monomers were extracted 3× with 1 ml hexane, the extracts combined, the solvent evaporated and derivatized as described above.

4.5. Qualitative and quantitative analysis of cuticular components

Wax components and cutin monomers released after transesterification were analyzed as TMSi-derivatives and methylesters by GC and GC–MS. Constituents of wax and cutin fractions were identified using a GC (GC6890 N, Agilent) coupled to a quadrupole mass selective detector (5973N, Agilent). One microliter-samples were injected on-column (capillary column DB-1, 30 m × 0.32 mm, 0.1 µm; J&W). H_2 (2 ml min^{-1}) was used as carrier gas. For separation of wax and cutin constituents the following temperature programs were used: wax, 2 min at 50 °C, 40 °C min^{-1} to 200 °C, 2 min at 200 °C, 3 °C min^{-1} to 310 °C, 30 min at 310 °C; for cutin, 2 min at 50 °C, 10 °C min^{-1} to 150 °C, 1 min at 150 °C, 3 °C min^{-1} to 310 °C, 15 min at 310 °C. Individual constituents were identified based on their EIMS spectra (70 eV, *m*/*z* 50–700), published data (Holloway, 1982) and commercial libraries.

Constituents of wax and cutin were quantified with an identical GC system equipped with flame ionisation detector using the same separation conditions as described above. Mass of wax and cutin was calculated using the internal standards. Content of individual constituents was expressed (i) as percentage of total wax and cutin respectively, (ii) on a unit surface area basis by dividing the amount of constituents by the cumulative CM surface area in the respective sample or (iii) on a whole fruit basis by multiplying amounts of constituents per unit fruit surface area by the surface area of the fruit. The latter calculation implies that wax and cutin composition of the cheek region is representative for that of the entire fruit surface. This was confirmed in preliminary experiments where CM composition in cheek, suture, stem cavity and stylar region of Sam sweet cherry fruit was compared. In general, only minor differences were detected between regions. The only exception formed alcohols that ranged from 0.1 ± 0.0% in the cheek to 0.7 ± 0.0% in the pedicel region of Sam sweet cherry fruit.

4.6. Data analysis and presentation

GC analyses were carried out with three replications. Data in figures and tables are presented as means ± standard error of means (SE). Where not shown, error bars were smaller than data symbols. Analysis of variance and mean separation were carried out using the Statistical Analysis System software package (version 8.02; SAS Institute Inc., Cary, NC).

Acknowledgements

We thank Isabel Briesen for her assistance in sample processing and MS analysis and Ruth Richter for preparation of cuticles. The work was supported in part by grants from the GiSeLa-foundation and the Deutsche Forschungsgemeinschaft.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2007.01.008.

References

Baker, E.A., Hunt, G.M., 1981. Developmental changes in leaf epicuticular waxes in relation to foliar penetration. New Phytol. 88, 731–747.

Baker, E.A., Bukovac, M.J., Hunt, G.M., 1982. Composition of tomato fruit cuticle as related to fruit growth and development. In: Cutler, D.F., Alvin, K.L., Price, C.E. (Eds.), The Plant Cuticle, . In: Linnean Society Symposium Series No. 10. Academic Press, New York, pp. 33–44.

Becker, M., Kerstiens, G., Schönherr, J., 1986. Water permeability of plant cuticles: permeance, diffusion and partition coefficients. Trees 1, 54–60.

Belding, R.D., Sutton, T.B., Blankenship, S.M., Young, E., 2000. Relationship between apple fruit epicuticular wax and growth of *Peltaster fructicola* and *Leptodontidium elatius*, two fungi that cause sooty blotch disease. Plant Dis. 84, 767–772.

Bonaventure, G., Beisson, F., Ohlrogge, J., Pollard, M., 2004. Analysis of the aliphatic monomer composition of polyesters associated with *Arabidopsis* epidermis: occurrence of octadeca-*cis*-6,*cis*-9-diene-1,18-dioate as the major component. Plant J. 40, 920–930.

Bukovac, M.J., Flore, J.A., Baker, E.A., 1979. Peach leaf surfaces: wettability, retention, cuticular permeability, and epicuticular wax chemistry during expansion with special reference to spray application. J. Am. Soc. Hort. Sci. 104, 611–617.

Christensen, J.V., 1996. Rain-induced cracking of sweet cherries: its causes and prevention. In: Webster, A.D., Looney, N.E. (Eds.), Cherries. CAB International, Wallingford, United Kingdom, pp. 297–327.

Commenil, P., Brunet, L., Audran, J.C., 1997. The development of the grape berry cuticle in relation to bunch rot disease. J. Exp. Bot. 48, 1599–1607.

Croteau, R., Fagerson, I.S., 1971. The chemical composition of the cuticular wax of cranberry. Phytochemistry 10, 3239–3245.

Franke, R., Briesen, I., Wojciechowski, T., Faust, A., Yephremov, A., Nawrath, C., Schreiber, L., 2005. Apoplastic polyesters in *Arabidopsis* surface tissues – a typical suberin and a particular cutin. Phytochemistry 66, 2643–2658.

Freeman, B., Albrigo, L.G., Biggs, R.H., 1979. Cuticular waxes of developing leaves and fruit of blueberry, *Vaccinium ashei* Reade cv. Bluegem. J. Am. Soc. Hort. Sci. 104, 398–403.

Graca, J., Pereira, H., 2000. Methanolysis of bark suberins: analysis of glycerol and acid monomers. Phytochem. Anal. 11, 45–51.

Graca, J., Schreiber, L., Rodrigues, J., Pereira, H., 2002. Glycerol and glyceryl esters of x-hydroxyacids in cutins. Phytochemistry 61, 205–215.

Grncarevic, M., Radler, F., 1967. The effect of wax components on cuticular transpiration – model experiments. Planta 75, 23–27.

Hauke, V., Schreiber, L., 1998. Ontogenetic and seasonal development of wax composition and cuticular transpiration of ivy (*Hedera helix* L.) sun and shade leaves. Planta 207, 67–75.

Hellmann, M., Stösser, R., 1992. Saisonale, ontogenetische und sortentypische Veränderungen des Oberflächenwachses von Apfelblättern. Angew. Bot. 66, 109–114.

Holloway, P.J., 1969. Chemistry of leaf waxes in relation to wetting. J. Sci. Food Agric. 20, 124–128.

Holloway, P.J., 1970. Surface factors affecting the wetting of leaves. Pest. Sci. 1, 156–163.

Holloway, P.J., 1973. Cutins of *Malus pumila* fruits and leaves. Phytochemistry 12, 2913–2920.

Holloway, P.J., 1982. The chemical constitution of plant cutins. In: Cutler, D.F., Alvin, K.L., Price, C.E. (Eds.), The Plant Cuticle, . In: Linnean Society Symposium Series No. 10. Academic Press, New York, pp. 45–85.

Holloway, P.J., 1994. Plant cuticles: physicochemical characteristics and biosynthesis. In: Percy, K.E., Cape, J.N., Jagels, R., Simpson, C.J. (Eds.), Air Pollutants and the Leaf Cuticle. Springer, Berlin Heidelberg, pp. 1–13.

Jeffree, C.E., 1996. Structure and ontogeny of plant cuticles. In: Kerstiens, G. (Ed.), Plant Cuticles. BIOS Scientific Publishing Ltd., Oxford, pp. 33–82.

Jeffree, C.E., Baker, E.A., Holloway, P.J., 1975. Ultrastructure and recrystallization of plant epicuticular waxes. New Phytol. 75, 539–549.

Jetter, R., Riederer, M., 1994. Epicuticular crystals of nonacosan-10-ol: in-vitro reconstitution and factors influencing crystal habits. Planta 195, 257–270.

Jetter, R., Schäffer, S., 2001. Chemical composition of the *Prunus laurocerasus* leaf surface. Dynamic changes of the epicuticular wax film during leaf development. Plant Physiol. 126, 1725–1737.

Kallio, H., Nieminen, R., Tuomasjukka, S., Hakala, M., 2006. Cutin composition of five finnish berries. J. Agric. Food Chem. 54, 457–462.

Knoche, M., Peschel, S., Hinz, M., Bukovac, M.J., 2001. Studies on water transport through the sweet cherry fruit surface: II. Conductance of the cuticle in relation to fruit development. Planta 213, 927–936.

Knoche, M., Beyer, M., Peschel, S., Oparlakov, B., Bukovac, M.J., 2004. Changes in strain and deposition of cuticle in developing sweet cherry fruit. Physiol. Plant. 120, 667–677.

Kollatukudy, P.E., 1996. Biosynthetic pathways of cutin and waxes, and their sensitivity to environmental stresses. In: Kerstiens, G. (Ed.), Plant Cuticles. BIOS Scientific Publishing Ltd., Oxford, pp. 83–108.

Markley, K.S., Sando, C.E., 1937. The wax-like constituents of the cuticle of the cherry, *Prunus avium* L. J. Biol. Chem. 119, 641–645.

Martin, J.T., Juniper, B.E., 1970. The Cuticle of Plants. Edward Arnolds, London.

Nip, M., Tegelaar, E.W., de Leeuw, J.W., Schenck, P.A., Holloway, P.J., 1986. A new non-saponifiable highly aliphatic and resistant biopolymer in plant cuticles – evidence from pyrolysis and ^{13}C NMR analysis of present day and fossil plants. Naturwissenschaften 73, 579–585.

Peschel, S., Knoche, M., 2005. Characterization of microcracks in the cuticle of developing sweet cherry fruit. J. Am. Soc. Hort. Sci. 130, 487–495.

Peschel, S., Beyer, M., Knoche, M., 2003. Surface characteristics of sweet cherry fruit: stomata-number, distribution, functionality and surface wetting. Sci. Hort. 97, 265–278.

Petracek, P.D., Bukovac, M.J., 1995. Rheological properties of enzymatically isolated tomato fruit cuticle. Plant Physiol. 109, 675–679.

Post-Beittenmiller, D., 1996. Biochemistry and molecular biology of wax production in plants. Ann. Rev. Plant Physiol. Plant Mol. Biol. 47, 405–430.

Radler, F., Horn, D.H.S., 1965. The composition of grape cuticle wax. Austr. J. Chem. 18, 1059–1069.

Reynhardt, E.C., Riederer, M., 1994. Structures and molecular dynamics of plant waxes. II. Cuticular waxes from leaves of *Fagus sylvatica* L. and *Hordeum vulgare* L. Eur. Biophys. J. 23, 59–70.

Riederer, M., Schönherr, J., 1988. Development of plant cuticles: fine structure and cutin composition of *Clivia miniata* Reg. leaves. Planta 174, 127–138.

Riederer, M., Schneider, G., 1990. The effect of the environment on the permeability and composition of Citrus leaf cuticles II. Composition of soluble cuticular lipids and correlation with transport properties. Planta 180, 154–165.

Riley, R.G., Kollatukudy, P.E., 1975. Evidence for covalently attached *p*-coumaric acid and ferulic acid in cutins and suberins. Plant Physiol. 56, 650–654.

Schmidt, H.W., Schönherr, J., 1982. Development of plant cuticles – occurrence and role of non-ester bonds in cutin of *Clivia miniata* Reg. leaves. Planta 156, 380–384.

Schönherr, J., Bukovac, M.J., 1973. Ion exchange properties of isolated tomato fruit cuticular membrane: exchange capacity, nature of fixed charges and cation selectivity. Planta 109, 73–93.

Schönherr, J., Riederer, M., 1986. Plant cuticles sorb lipophilic compounds during enzymatic isolation. Plant Cell Environ. 9, 459–466.

Schreiber, L., Riederer, M., 1996. Ecophysiology of cuticular transpiration: comparative investigation of cuticular water permeability of plant species from different habitats. Oecologia 107, 426–432.

Vogg, G., Fischer, S., Leide, J., Eyal, E., Jetter, R., Levy, A., Riederer, M., 2004. Tomato fruit cuticular waxes and their effects on transpiration barrier properties: functional characterization of a mutant deficient in a very-long-chain fatty acid beta-ketoacyl-CoA synthase. J. Exp. Bot. 55, 1401–1410.

Walton, T.J., Kollatukudy, P.E., 1972. Determination of the structures of cutin monomers by a novel depolymerization procedure and combined gas chromatography and mass spectrometry. Biochemistry 11, 1885–1897.

Wollrab, V., 1969. Secondary alcohols and paraffins in the plant waxes of the family of Rosaceae. Phytochemistry 8, 623–627.