Cuticular Wax Composition of 'Celeste' and 'Somerset' Cherry Fruit

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Abstract

The cuticular layer plays an important role in protecting fruits against water loss and invasive microorganisms and insects, both during on-tree development and postharvest storage. There is also experimental evidence that cuticle composition and structure may be a relevant factor accounting for firmness and other textural attributes. Waxes are important cuticle components, together with the polymer matrices cutin and cutan. In this work, the chemical composition of waxes in the skin of 'Celeste' and 'Somerset' cherry (*Prunus avium* L.) fruit, which display quite different firmness levels, was investigated by means of gas chromatography-mass spectrometry (GC-MS). Fruit were picked at commercial maturity, and cuticles isolated enzimatically at harvest and after 3 days at 20 ºC. Total waxes were identified and quantified by means of GC-MS and GC-FID, respectively. In all cases, the most abundant constituent of cuticular waxes was the triterpene ursolic acid, which accounted for 49-56% ('Celeste') and 47% ('Somerset') of total wax composition. Important amounts of the alkanes nonacosane (6-10%, depending on cultivar and days at 20 ºC) and heptacosane (1-2%), as well as of the fatty acid linoleic acid (5-10%), were also found. Total alkane content was higher in 'Somerset' than in 'Celeste', but in contrast 'Celeste' cuticles were richer in triterpenes and fatty acids than those isolated from 'Somerset', thus showing some cultivar-related differences in the chemical composition of cuticular waxes.

INTRODUCTION

The surface of cherry fruit, like that of all aerial plant organs, is covered by a cuticle made of a mixture of the polyester cutin and a complex combination of cuticular waxes. Epicuticular and intracuticular waxes contain mixtures of long-chain fatty acids and their derivatives, esters, alkanes (Kolattukudy, 1996) and triterpenes (Belding et al., 1998; Verardo et al., 2003; Peschel et al., 2007). Alkanes originate from the decarboxylation of long-chain fatty acids or aldehydes. The wax layer plays a main role in protection against water loss (Riederer and Schreiber, 2001). Besides that, it has other physiological and ecological functions such as limiting surface permeability (Baur et al., 1996), reducing transpiration (Veraverbeke et al., 2003), and protecting against microorganisms and insects (Marcell and Beattie, 2002).

Wax production by plants is known to be greatly influenced by environmental factors such as precipitation/rain (Mayeux and Jordan, 1987; Rinallo and Mori, 1996), temperature (Lurie et al., 1996), breeze/wind (van Gardingen et al., 1991) and daylight (Letchamo and Gosselin, 1996). The composition of cuticular waxes is known to change during the development, ripening and shelf-life periods. Therefore, storage conditions

after harvest can affect the chemical composition and structure of waxes in fruits (Kolattukudy, 1984). A major function of cuticular wax during shelf-life is the restriction of water loss, hence limiting weight loss and influencing firmness. It has also been suggested that cuticular wax composition can be a biochemical marker for the identification of plant species (Spicer, 1989).

There is a lack of information on changes in the composition of cuticular waxes during the shelf life of fruit or in response to different postharvest procedures. However, given the relevance of cuticle for the preservation of particular fruit attributes, this information could be of interest as a possible indicator of storage potential of produce. In this work, we studied the composition of cuticular waxes of two cherry cultivars displaying different firmness levels, both at harvest and after 3 days at 20 ºC. The objectives of the present study were (a) to isolate, analyze and identify the chemical composition of extracted cuticular waxes, (b) to establish possible differences in cuticular wax composition between both cherry cultivars considered, and (c) to characterize changes in wax composition during the shelf life period at 20 ºC.

MATERIALS AND METHODS

Plant material

Cherry fruit (*Prunus avium* L. cv. 'Celeste' and 'Somerset') were sampled in 2011 from a commercial orchard located in Corbins, in the area of Lleida (NE Spain). Fruit were harvested at commercial harvest time (May 23rd and June 7th for 'Celeste' and 'Somerset', respectively), and transported immediately to the laboratory. Fruit were selected for uniformity of size, color, and freedom from disease, insect and visual defects. Average fruit weight at harvest was significantly higher for 'Somerset' than for 'Celeste' (10.49 *vs*. 8.99 g, respectively). Samples were analyzed immediately after harvest and after remaining 3 days at 20 ºC to simulate commercial shelf life. Weight (g) of 30 individual fruit was determined both at harvest and 3 days thereafter in order to determine weight loss regarding harvest date. Firmness was measured with an electronic durometer (Durofel DFT-100) equipped with a 5.64-mm tip, on two opposite faces on the cheek region of 30 fruit, and results were expressed as Durofel units (1-100) (Table 1).

Cuticle isolation

Exocarp segments (4 per fruit) were excised from the cheek region of 15 fruit using a cork borer (i.d. 13mm). Four skin disks were so obtained from each individual fruit. Cuticular membranes (CM) were isolated enzymatically by incubation at 37 ºC in 0.2% (w/v) cellulase and 100 U mL^{-1} pectinase in 50 mM citrate buffer (pH 4.0) for 10-15 days, until no more material was released. NaN_3 (1 mM) was added to the solution to prevent microbial growth. After isolation, CM were washed in citrate buffer (50 mM, pH 4.0) at 37°C, once or twice every day until no material was left in suspension, dried at 40°C, and kept in hermetically capped vials until used. CM mass was determined gravimetrically, and expressed as µg total wax per cm^2 of fruit surface area. The amount of CM isolated from 60 disks of fruit skin varied according to cultivar, yields obtained from 'Somerset' fruit being considerably higher than those from 'Celeste', particularly at harvest (Table 2).

Wax extraction and preparation

Waxes were recovered from the 60 dry CM disks (38.45 cm^2/sample) obtained in each case. CM samples were dewaxed by shaking in $CHCl_3$ (1 mg sample mL^{-1}) for 24 h

at 25 °C, followed by incubation (15 min) in an ultrasonic bath. After filtration, the chloroform extract was removed using a rotary evaporator at 50 °C, and the waxes transferred to a pre-weighed vial, dried under N_2 until complete dryness, and weighed with a microbalance for calculation of total wax yield. Free hydroxyl and carboxyl groups were converted respectively into their trimethylsilyl (TMSi) ethers and esters by derivatising with N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) in pyridine for 15 min at 100 °C. Samples were added eicosane (C_{20}) and dotriacontane (C_{32}) as internal standards, and injected to GC-MS and GC-FID for compound identification and quantitation.

GC-MS and GC-FID analysis of wax compounds

After derivatization, wax components were analyzed by GC-MS and GC-FID. Samples (1 µL) were injected into a gas chromatograph (GC) (Agilent 7890 A) equipped with a quadrupole mass selective detector (Agilent 5973N) and a capillary column (BPX5, 30m × 0.25 mm, 0.25µm; SGE). Wax compounds were identified from their EI-MS spectra (70eV, m/z 50-700) after capillary GC. The oven was set at 50 ºC (2 min), and the temperature was raised initially by 40 ºC min^{-1} to 200 ºC, kept at 200 ºC for 2 min, raised again by 3 ºC min^{-1} to 310 ºC, and then kept constant at 310 ºC for 30 min. H_2 was used as carrier gas (2 mL min^{-1}). Quantitative determination of wax compounds was carried out with an identical GC-system equipped with a flame ionization detector (FID) based on the injected wax compounds. All values shown in Tables 3 to 5 represent the average of two replicate samples.

Statistical analysis of data

All statistical analyses were performed using Minitab 15 (Minitab Inc., UK). To test changes in wax composition of fruit after harvest, as well as differences between cultivars, data were analyzed by one-way analysis of variance (ANOVA) and the means were compared using LSD test.

RESULTS

Recovery of cherry fruit waxes

The amounts of cuticle recovered from 60 skin disks of ‘Somerset’ fruit were higher than those recovered from ‘Celeste’. Furthermore, total wax (mg) and wax yields (µg cm^{-2}) were also higher for ‘Somerset’. However, the percentage of waxes identified in the extract was higher for ‘Celeste’ than for ‘Somerset’ samples (Table 2).

Wax components identified in cherry cuticles

Triterpenes were the most abundant class of wax components in both cherry cultivars considered. The second most important family of wax compounds in quantitative terms was alkanes in ‘Somerset’, and fatty acids together with alkanes in ‘Celeste’ (Table 3). Ursolic acid dominated within the triterpene wax compound type, in accordance with previous reports (Peschel et al., 2007) (Table 4). Among the alkanes, for both ‘Celeste’ and ‘Somerset’ fruit the most abundant compound was nonacosane (C_{29}) followed by heptacosane (C_{27}) (Table 5), which also agrees with results reported elsewhere on sweet cherry fruit (Peschel et al., 2007). However, ‘Somerset’ cuticles were richer in longer chain alkanes (C_{27} to C_{31}) than those obtained from ‘Celeste’.

Changes in wax composition during shelf life at 20 ºC

Additionally, total wax amount and wax yields showed some change after 3 days at 20 ºC in comparison with values at harvest (Table 2), with a remarkable increase for 'Celeste' fruit and an increasing trend for 'Somerset'. For both cultivars, total wax amount and wax yields were also higher after 3 days at 20 ºC than at harvest.

As to wax composition, the percentage of triterpenes increased notably during the shelf life of 'Celeste' samples (57.3 to 65.7% at days 0 and 3, respectively), whereas in 'Somerset' fruit triterpene levels were similar irrespective of period (Table 3). Within the triterpene-type wax compounds, the relative content of ursolic and oleanoic acids underwent noticeable increases in 'Celeste', but also not in 'Somerset' samples (Table 4). As to alkanes, the same trend was observed, with an augment during the shelf life of 'Celeste' and no significant change being found for 'Somerset'. The amount of fatty acids in cuticular waxes of 'Celeste' decreased along shelf life, while remained essentially unchanged in 'Somerset'. Generally speaking, changes in the total amounts of the main wax families after harvest were more intense for 'Celeste' than for 'Somerset'. Within the alkanes, however, the relative amount (%) of the important compounds heptacosane and nonacosane decreased in 'Celeste' fruit, opposite to the general trend for this wax compound family, whereas in 'Somerset' an important increase was found for nonacosane in spite of similar total alkane amounts both 0 and 3 days after harvest (Table 5).

DISCUSSION

The modifications commented in the previous section are indicative of intense alterations taking place in the biosynthesis of the cuticular waxes, and hence probably also in cuticle properties, after fruit harvest. The most abundant compound family was triterpenes, ursolic acid accounting for over 54% of waxes irrespective of cultivar and postharvest period. The most abundant alkanes were nonacosane (over 56%) and heptacosane (over 9%), which agrees with previous reports on other cherry cultivars (Peschel et al., 2007). The percentage of both alkanes and triterpenes in cuticular waxes of 'Celeste' increased during shelf life at 20 ºC, whereas fatty alcohols and fatty acids decreased during the same period in the same cultivar. This result suggests that alkanes arise from the decarboxylation of fatty acids and aldehydes (Hannoufa et al., 1993). No significant differences in the relative abundance of the different wax compound families were found for 'Somerset' after harvest.

More than 80% of wax components extracted from 'Celeste' and 'Somerset' cuticle samples were actually identified by GC. The highest wax yield (91.29 μg cm^{-2} was obtained from 'Somerset' fruit after being kept 3 days at 20 ºC (Table 2). Quantitatively, cuticular waxes from 'Celeste' were lower in alkanes and higher in triterpenes, whereas 'Somerset' samples were higher in fatty alcohols.

Data obtained in this work, together with those shown in previous reports, suggest that in spite of being comprised of similar chemical families, cuticular waxes are subject to cultivar-related differences in the specific composition and in the abundance of particular compounds. These differences may account for some of the quality or storability characteristics of each cultivar, including weight loss, firmness or susceptibility to infections or physiological disorders. For instance, firmness levels of 'Celeste' fruit decreased significantly after harvest, and samples dehydrated intensely as shown by important weight loss (Table 1), whereas firmness levels of 'Somerset' cherries were statistically similar both at harvest and 3 days thereafter, these fruit displaying also clearly lower transpirational water loss. These differences may have arisen partly from

dissimilarities in the chemical composition of ‘Somerset’ cuticles as compared with those obtained from ‘Celeste’ fruit. Further work on modifications in the relative amounts of the different cuticle component in response to internal and external factors will be required to shed light on the physiological consequences of these compositional changes, thus helping dissect the contribution of each particular compound family to the biological role of fruit cuticles.

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Tables

Table 1. Firmness and weight of 'Celeste' and 'Somerset' cherry fruit 0 and 3 days after harvest.

Parameter	'Celeste'		'Somerset'	
	0 d.	3 d.	0 d.	3 d.
Firmness (Durofel units)	72.10ab	58.50b	80.43a	77.23a
Weight loss (%)	-	9.57	-	5.24

Values represent means of 30 replicates. Mean values followed by a different lower-case letter within the same row are significantly different at $P \leq 0.05$ (LSD test). For weight loss assessment, the same 30 fruit were weighed jointly 0 and 3 days after harvest.

Table 2. Yields of cuticular membrane, total extracted wax, wax yield, and identified wax in 'Celeste' and 'Somerset' cherry fruit 0 and 3 days after harvest.

Parameter	'Celeste'		'Somerset'	
	0 d.	3 d.	0 d.	3 d.
Cuticular membrane (mg)	7.64	12.97	13.44	14.65
Total wax extract (mg)	2.49	2.82	3.10	3.51
Wax yield ($\mu g \cdot cm^{-2}$)	64.76	73.34	80.62	91.29
Identified wax (%)	83.25	88.59	82.20	81.42

Values represent means of 2 replicate determinations.

Table 3. Relative abundance (% area [z]) of different wax compound types in the chromatographically resolvable wax recovered from the cuticular membranes of 'Celeste' and 'Somerset' cherry fruit 0 and 3 days after harvest.

Compound family	'Celeste'		'Somerset'	
	0 d.	3 d.	0 d.	3 d.
Alkanes	10.29b	12.92a	15.34a	15.37a
Fatty Acids	13.03a	7.29ab	7.57ab	7.00b
Fatty Alcohols	0.87ab	0.54b	1.04a	0.84ab
Phytosterols	1.77b	2.11b	3.62a	3.01a
Triterpenes	57.28ab	65.73a	54.62b	55.19b
Unidentified	16.75ab	11.41b	17.80a	18.58a
Total	100	100	100	100

[z] Area (%) derived from GC-FID profile.

Values represent means of two replicates. Mean values followed by a different lower-case letter within the same row are significantly different at $P \leq 0.05$ (LSD test).

Table 4. Identified wax constituents in ‘Celeste’ and ‘Somerset’ cuticles 0 and 3 days after harvest.

Peak number	Compound	‘Celeste’				‘Somerset’			
		0 d.		3 d.		0 d.		3 d.	
		Area (%)[z]	**Relative content (%)[y]**	**Area (%)[z]**	**Relative content (%)[y]**	**Area (%)[z]**	**Relative content (%)[y]**	**Area (%)[z]**	**Relative content (%)[y]**
	Fatty Acids	**100**	**13.03a**	**100**	**7.29ab**	**100**	**7.57ab**	**100**	**7.00b**
1	C16:0 (Palmitic acid)	8.13	1.06a	6.36	0.46ab	5.40	0.41b	4.65	0.33b
2	C18:2 (*trans*-9,12)	0.25	0.03a	0.19	0.01b	0.00	0.00b	0.00	0.00b
3	C17:0 (Margaric acid)	0.68	0.09a	0.79	0.06b	0.77	0.06b	0.85	0.06b
4	C18:2 (Linoleic acid)	78.79	10.27a	77.58	5.66ab	74.77	5.66ab	74.99	5.25b
5	C18:1 (Oleic acid)	2.41	0.31a	2.90	0.21a	3.26	0.25a	3.43	0.24a
6	C18:0 (Stearic acid)	9.74	1.27a	12.18	0.89a	15.81	1.20a	16.08	1.13a
	Fatty Alcohols	**100**	**0.87ab**	**100**	**0.54b**	**100**	**1.04a**	**100**	**0.84ab**
1	C23-1-Tricosanol	39.01	0.34a	40.46	0.22a	28.21	0.29a	26.87	0.23a
2	C24-1-Tetracosanol (lignoceryl)	15.80	0.14a	7.59	0.04a	19.87	0.21a	6.19	0.05a
3	C26-1-Hexacosanol (ceryl)	15.86	0.14ab	10.13	0.05b	13.98	0.15a	20.53	0.17a
4	C28-1-Octacosanol	10.43	0.09b	13.18	0.07b	15.41	0.16a	18.00	0.15a
5	C30-1-Triacontanol	18.91	0.16b	28.63	0.15c	22.53	0.24a	28.40	0.24a
	Phytosterols	**100**	**1.77b**	**100**	**2.11b**	**100**	**3.62a**	**100**	**3.01a**
1	C28-Campesterol	46.67	0.83bc	33.62	0.71c	26.11	0.94ab	35.62	1.07a
2	C29-β-Sitosterol	53.33	0.95c	66.38	1.40bc	73.89	2.67a	64.38	1.94ab
	Triterpenes	**100**	**57.28ab**	**100**	**65.73a**	**100**	**54.62b**	**100**	**55.19b**
1	C30-Oleanolic acid	13.54	7.75ab	13.98	9.19a	13.68	7.47b	13.39	7.39b
2	C30-Ursolic acid	86.46	49.52ab	86.02	56.55a	86.32	47.15b	86.61	47.80b

[z] Area (%) derived from GC-FID profile. [y] Relative content (%) of total wax amount.

Values represent means of two replicates. Mean values followed by a different lower-case letter within the same row are significantly different at $P \leq 0.05$ (LSD test).

Table 5. Alkanes identified in cuticles recovered from ‘Celeste’ and ‘Somerset’ fruit 0 and 3 days after harvest.

Peak number	Alkanes	‘Celeste’				‘Somerset’			
		0 d.		3 d.		0 d.		3 d.	
		Area (%)[z]	**Relative content (%)[y]**	**Area (%)[z]**	**Relative content (%)[y]**	**Area (%)[z]**	**Relative content (%)[y]**	**Area (%)[z]**	**Relative content (%)[y]**
1	Decane n-C10	0.62	0.06ab	0.35	0.04b	0.43	0.07a	0.31	0.05ab
2	Undecane n-C11	0.29	0.03a	0.10	0.01a	0.19	0.03a	0.09	0.01a
3	Dodecane n-C12	0.90	0.09a	0.00	0.00b	0.00	0.00b	0.00	0.00b
4	Nonadecane nC19	0.32	0.03a	0.00	0.00b	0.00	0.00b	0.00	0.00b
5	Docosane n-C22	0.27	0.03b	14.23	1.84a	3.57	0.55ab	2.95	0.45ab
6	Tricosane n-C23	0.81	0.08a	0.26	0.03a	0.51	0.08a	0.38	0.06a
7	Tetracosane n-C24	2.13	0.22a	0.98	0.13ab	1.07	0.16ab	0.73	0.11b
8	Pentacosane n-C25	3.21	0.33a	1.38	0.18a	2.04	0.31a	1.38	0.21a
9	Hexacosane n-C26	3.20	0.33ab	1.45	0.19b	3.02	0.46a	1.92	0.30ab
10	Heptacosane n-C27	11.43	1.18b	9.43	1.22b	11.25	1.73a	11.36	1.75a
11	Octacosane n-C28	7.43	0.76b	5.59	0.72b	7.51	1.15a	6.39	0.98a
12	Nonacosane n-C29	57.96	5.96c	56.83	7.34bc	58.29	8.94ab	63.58	9.77a
13	Triacontane n-C30	5.04	0.52b	3.81	0.49b	5.27	0.81a	4.46	0.69a
14	Hentriacontane n-C31	6.41	0.66b	5.60	0.72b	6.83	1.05a	6.43	0.99a
	Total	**100**	**10.29b**	**100**	**12.92a**	**100**	**15.34a**	**100**	**15.37a**

[z] Area (%) derived from GC-FID profile. [y] Relative content (%) of total wax amount.

Values represent means of two replicates. Mean values followed by a different lower-case letter within the same row are significantly different at $P \leq 0.05$ (LSD test).