Pergamon

PII: S0031-9422(96)00241-5

Phytochemistry, Vol. 43, No. 1, pp. 45–48, 1996

0031-9422/96 $15.00 + 0.00

LEAF EPICUTICULAR LIPIDS OF *PRUNUS LAUROCERASUS*: IMPORTANCE OF EXTRACTION METHODS

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(*Received in revised form* 29 *February* 1996)

Key Word Index—*Prunus laurocerasus*; Rosaceae; cherry laurel; epicuticular wax; alkanes; primary alcohols; free fatty acids; polar lipids; rinsing; dipping.

Abstract—Compounds of four chemical classes (alkanes, primary alcohols, free fatty acids and polar lipids) were isolated, identified and quantified for the first time from the epicuticular waxes of *Prunus laurocerasus* leaves. Alkanes were the major class and constituted 80% of the wax. Using chloroform, more wax was removed from leaves after dipping for 210 sec than by rinsing for the same length of time. Duration of solvent contact also had an effect on the composition of the soluble cuticular lipids extracted. Alkanes and primary alcohols were extracted earlier than fatty acids and, whatever the class of compounds, short chain-length homologues (C_{16}–C_{25}) needed more time to be extracted from the cuticle than longer ones (C_{26}–C_{35}). Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

The composition of leaf cuticular waxes is studied in different fields of research, such as chemotaxonomy [1], plant–insect relationships [2] and cuticular permeability [3]. Methods used to extract the waxes differ widely with regard to solvent, temperature and/or time of solvent contact [4]. In the present work, we compared two extraction methods for whole leaf surface lipids, viz. dipping and rinsing. These methods are interesting for *in situ* studies and, concerning rinsing, for isolation of waxes from the two leaf sides. Techniques and times of extraction were studied using *Prunus laurocerasus* as leaf material. Cherry laurel is widely used to study cuticular permeability, its leaves being hypostomatic and its cuticle being easy to remove [5, 6]. However, to date, no chemical analysis has been made on the soluble cuticular lipids. Compounds belonging to four chemical classes of soluble cuticular lipids were chosen to be studied, viz. alkanes, primary alcohols, free fatty acids and polar lipids (PL). This choice was done because of the wide range of polarity from the apolar alkanes to PL; such classes are representative components of epicuticular waxes.

RESULTS AND DISCUSSION

Comparison between the amounts of soluble cuticular lipids of cherry laurel extracted by dipping and rinsing the leaf surface with chloroform during 210 sec (Fig. 1) showed that dipping was twice as efficient for extracting the four wax classes than rinsing. This difference in the quantities extracted was valid for each of the four classes. Dipping extracted a slightly higher proportion of PL (Table 1). These lipids have no mobility on TLC plates and only their fatty acids were identified. Alkanes were the major class (Table 1) and free fatty acids (FFA) the minor one, irrespective of extraction methods. The relatively high proportion of alkanes in waxes of cherry laurel (80%) is not uncommon; for instance, waxes of *Solandra grandiflora*

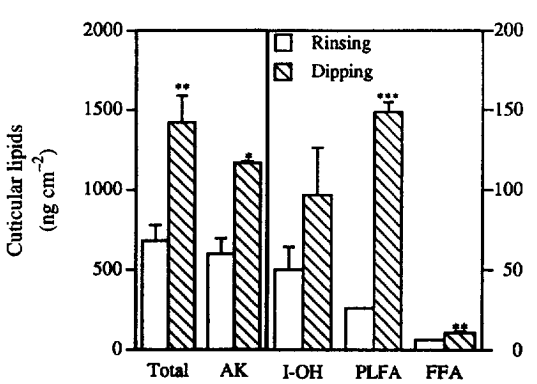

Fig. 1. Comparison between rinsing and dipping in $CHCl_3$ for 210 sec on removal of four chemical classes of epicuticular lipids. Total, sum of the four classes; AK, alkanes; I-OH, primary alcohols; PLFA, polar-lipid fatty acids; FFA, free fatty acids. Significant difference,* ($\alpha = 0.05$); highly significant,** ($\alpha = 0.01$); very highly significant,*** ($\alpha = 0.001$). Means of four and three samples for rinsing and dipping, respectively, ±s.e.

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Table 1. Relative percentages between the four classes of soluble cuticular lipids extracted by rinsing and dipping cherry laurel leaves during 210 sec of chloroform application

Classes	Rinsing	Dipping
Alkanes	88	82
Primary alcohols	7.4	6.8
Polar lipids	3.8	10.5
Free fatty acids	0.8	0.7

contain 92% of alkanes [7]. Plant waxes are often constituted by a dominant class of compounds, e.g. primary alcohols in leaves of *Pisum sativum* (abaxial surface) or ketones in *Allium* species [8].

A detailed examination of the homologues constituting the four classes (Table 2) showed that the chain length of alkanes extracted during 210 sec ranged from C_{18}–C_{35}, with a predominance of odd-numbered homologues. The major alkanes were C_{29} (50.8%) and C_{31} (20.2%). Primary alcohols in cherry laurel wax were constituted by homologues from C_{22}–C_{34}, with octacosanol (C_{28}) being the most abundant. FFA ranged from C_{16}–C_{30}. The C_{18} homologue in Table 2 represents the sum of stearic (18:0), oleic (18:1) and linoleic (18:2) acids; 18:1 and 18:2 constituted 24 and 15%, respectively, of C_{18}. Palmitic (16:0) was the major FFA. Thus, unlike primary alcohols and alkanes, FFA are mainly represented (96%) by short carbon chains. Fatty acids from polar lipids had even- and odd-numbered homologues with 16:0 dominant and C_{18} including 18:1 and 18:2. The range of carbon chain lengths described in each of these classes is common for soluble cuticular lipids [10].

Table 2. Homologue content (%) in each chemical class of soluble cuticular lipids extracted by dipping during 210 sec in chloroform

	Classes			
Carbons	AK	I-OH	FFA	PLFA
16	—	—	35.5	21.6
17	—	—	—	—
18	1	—	42.9	18.9
19	1.9	—	—	—
20	1.1	—	5.2	1.2
21	1.4	—	—	0.8
22	2.2	4.6	5.3	3.2
23	1.3	1.4	—	2.1
24	0.7	16.8	7	8.8
25	1.3	2.1	—	2.5
26	0.8	15.8	2.1	6.6
27	8.7	3	—	2.4
28	4	23.8	1.4	10.6
29	50.8	7.2	—	4.3
30	3	17.5	0.6	13.9
31	20.2	1.1	—	0.4
32	0.6	6	—	2.9
33	0.8	0.2	—	—
34	0.1	0.6	—	—
35	<0.1	—	—	—

AK = alkanes; I-OH = primary alcohols; FFA = free fatty acids; PLFA = fatty acids from polar lipids.

Homologues extracted by rinsing and dipping were compared for each class of compounds (Fig. 2). The dipping technique extracted 7.7 times more short chains ($<C_{25}$) and four times more long chains ($>C_{25}$) than the rinsing technique. This was particularly noticeable for alkanes and PL. For primary alcohols and FFA, these differences were much smaller or insignificant.

For the same time of chloroform contact, dipping was more efficient at extracting waxes, both quantitatively and qualitatively. In addition, the time of solvent contact also had an influence on extraction of the soluble cuticular lipids (Fig. 3). Thus, >50% of each class was extracted by dipping for 5 sec, rising to 80% after 30 sec (relative to the final time of 210 sec). Whatever the duration of the dipping, the order of compounds remained the same, with alkanes always being major components. They were quickly extracted, more than half before 5 sec and 90% within 50 sec (compared with 210 sec). However, long-chain compounds (C_{25}–C_{35}) were extracted more quickly than those with shorter chains (Fig. 4). Half of the former was obtained within 5 sec, whereas 40 sec was needed to reach the same proportion for the latter. Alkanes and primary alcohols were extracted very quickly, again

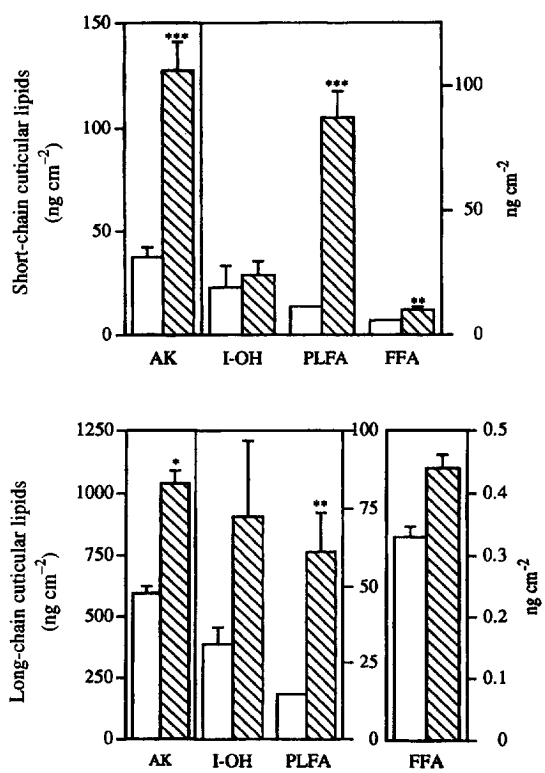

Fig. 2. Comparison between rinsing and dipping in $CHCl_3$ for 210 sec on removal of four chemical classes of epicuticular lipids in relation to carbon chain length [short chain, SC (C_{16}–C_{25}) and long chain, LC (C_{26}–C_{35})]. Abbreviations, statistical analysis and replication as Fig. 1.

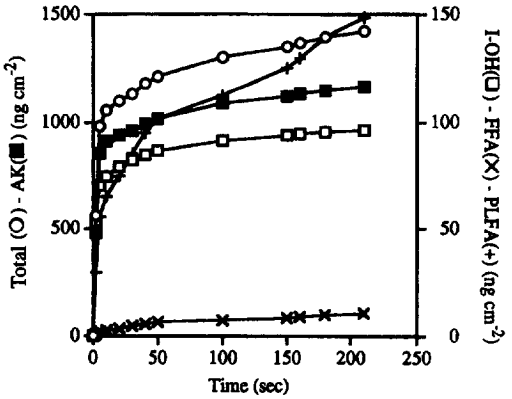

Fig. 3. Time-course of extraction of epicuticular lipids. Primary alcohols (I-OH), alkanes (AK), free fatty acids (FFA), polar lipids (PL) and their sum (Total). Points are means of three replicates of two leaves.

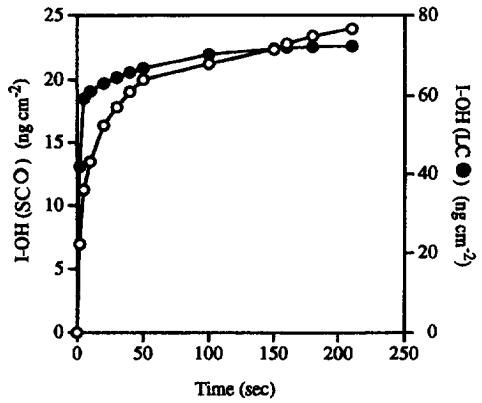

Fig. 5. Time-course of extraction of epicuticular alcohols (I-OH) in relation to chain length. Short chains (SC) from C_{22}–C_{25}, long chains (LC) from C_{26}–C_{34}. Points are means of three replicates of two leaves.

with their long-chain components before the short-chain ones (half of them in 2 sec) (Fig. 5). Polar compounds, such as PL and FFA, were extracted later than alkanes and primary alcohols; 20 and 40 sec were needed to obtain 50% of the PL and FFA, respectively (Figs 6 and 7). Long-chain homologues were always extracted before the short-chain ones, as observed for the other classes.

The fact that alkanes and primary alcohols were extracted before FFA and PL could be explained by difference in their solubility in chloroform. Solubility in this solvent for these two classes and for different chain lengths have been determined using commercial compounds [4]. For the same number of carbon atoms, alkanes are more soluble in chloroform than are primary alcohols, which in turn are more soluble than fatty acids; in all three classes of compound, short chains are more soluble in chloroform than long chains. Nevertheless, we found the contrary in our experiments. The extraction time-course showed no obvious relationships

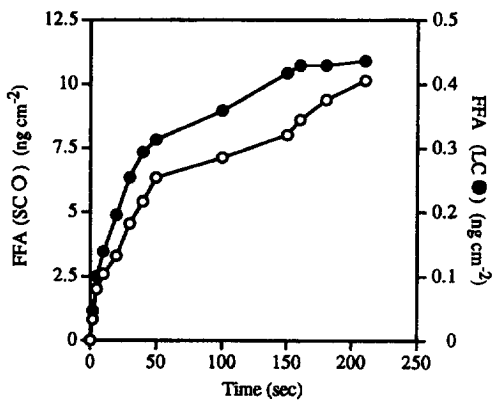

Fig. 6. Time-course of extraction of epicuticular free fatty acids (FFA) in relation to chain length. Short chains (SC) from C_{16}–C_{24}, long chains (LC) from C_{26}–C_{30}. Points are means of three replicates of two leaves.

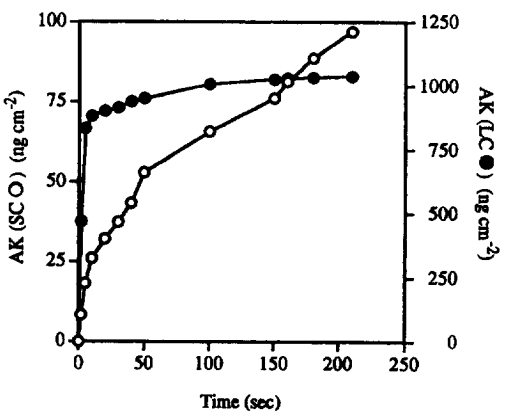

Fig. 4. Time-course of extraction of epicuticular alkanes (AK) in relation to chain length. Short chains (SC) from C_{18}–C_{25}, long chains (LC) from C_{26}–C_{35}. Points are means of three replicates of two leaves.

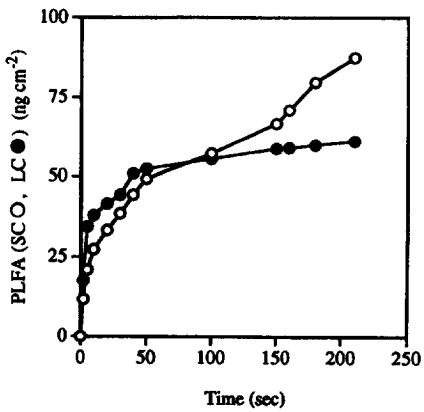

Fig. 7. Time-course of extraction of epicuticular polar-lipid fatty acids (PLFA) in relation to chain length. Short chains (SC) from C_{16}–C_{25}, long chains (LC) from C_{26}–C_{32}. Points are means of three replicates of two leaves.

with the chemical group and solvent affinities. This observation may provide circumstantial evidence for the superficial location of long-chain wax components in cherry laurel leaves. Besides the solubility of the substances in chloroform, the diffusion coefficients of compounds in the cutin matrix may also be involved. This coefficient is a function of the molar volume of the diffusing molecule; short-chain compounds diffuse quicker in the cutin matrix than those with long chains [4]. Thus, long-chain homologues could be more accessible to chloroform than the short-chain ones or localized differently within the cuticular waxes. Extraction times with chloroform for epicutical waxes range from 2 sec (*Zea mays* leaves) [10] to as long as 6 min (*Citrus halimii* leaves) [11]. Long exposure times to chloroform might reveal substances which do not occur at the surface of the cuticle.

EXPERIMENTAL

Plant materials. Fully expanded 1-year-old leaves of cherry laurel (*P. laurocerasus* L.) were obtained from trees grown under natural conditions (Arboretum of Amance, Champenoux, France). Soluble cuticular lipids were extracted either by rinsing both leaf sides with $CHCl_3$ during 210 sec or by dipping the leaves successively in 12 containers of 100 ml $CHCl_3$ with agitation for 2, 3, 5, 10, 10, 10, 10, 50, 50, 10, 20 and 30 sec, respectively. Individual extractives were evapd to dryness and kept at −80° until chemical analysis.

Separation of soluble cuticular lipids. Dry extracts were dissolved in 100 μl $CHCl_3$ and analysed by TLC on silica gel. Elution was carried out firstly with hexane followed by $CHCl_3$ after drying the plate. Bands were located with I_2 vapour. Bands corresponding to alkanes (R_f 0.95), primary alcohols (R_f 0.25), FFA (R_f 0.1) and PL (R_f 0) were removed from the plate, extracted from the gel and derivatized when necessary for GC-FID analysis.

Fatty acid methylation. Dried samples were reacted at 70° for 20 min with 1 ml BF_3–MeOH (7:50). After cooling, Me esters were extracted with hexane–H_2O (10:1). After centrifugation, the hexane layer was removed and evapd to dryness under N_2.

TMSi derivatives of primary alcohols. These were prepd by reaction with 200 μl pyridine and 70 μl BSTFA–TMCS (1:100) at 90° for 30 min.

GC-FID analysis. Dry samples were dissolved in 100 μl hexane and 5–10 μl were injected using the following chromatographic conditions according to class of compound. For alkanes and primary alcohols, temp. programme: 200° (1 min), then 200–300° at 9° min^{-1} and 300° for 9 min. Injector and detector temps were 250 and 310°, respectively. A BP1 capillary column (12 m × 0.33 mm) was used; carrier gas He, 0.3 bar for alkanes, 0.4 bar for alcohols. A Supelcowax 10 capillary column was used for fatty acid Me esters (30 m × 0.53 mm), He pressure 0.5 bar. Temp. programmes 200–235° at 2.5° min^{-1}, then 235–275° at 15° min^{-1} and 270° for 40 min. Peaks were identified by comparing their R_ts with those of standards. Quantification was achieved by adding to the sample before sepn on TLC, a known quantity of a homologue of each chemical class.

Leaf area. Foliar surfaces were measured with a Li 3000 planimeter (Li-Cor, U.S.A.).

Acknowledgements—This study was supported by a grant from INRA and the Lorraine Region.

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