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LEAF WAX OF *LACTUCA SATIVA* AND *PLANTAGO MAJOR*

MARTINE I. BAKKER,*† WIM J. BAAS,‡ DICK T. H. M. SIJM§¶ and CHRIS KOLLÖFFEL†

† Department of Plant Ecology and Evolutionary Biology, Transport Physiology Research Group; ‡ Ecophysiology Research Group; § Research Institute of Toxicology, Environmental Chemistry Group, Utrecht University, PO Box 80.084, 3508 TB Utrecht, The Netherlands

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Key Word Index—*Lactuca sativa*; Compositae; *Plantago major*; Plantaginaceae; leaf cuticular wax; extraction; composition; semivolatile organic compounds; hydrophobic organic chemicals.

Abstract—Wax layers of plants are able to accumulate semivolatile organic compounds (SOCs) from the atmosphere. In this study, the composition of the leaf cuticular waxes of lettuce (*Lactuca sativa*) and common plantain (*Plantago major*) was determined for future studies on the role of cuticular waxes in the uptake and bioaccumulation of SOCs. In addition, to find a suitable extraction solvent to be used in these studies, the extraction efficiency of several solvents for the cuticular wax of the plants was studied. Leaf wax of *L. sativa* consists mainly of long-chain linear alcohols and minor amounts of fatty acids, while the major components off leaf wax of *P. major* are the free polar triterpene acids, oleanolic and ursolic acid, and the linear alkanes $C_{27}H_{56}$–$C_{33}H_{58}$. The wax composition of both species only slightly changes with leaf developmental stage. This property makes them highly suitable as test plants in studies on uptake of SOCs. The waxes of both plant species are readily extractable with chloroform, toluene and dichloromethane. A mixture of chloroform and methanol 2 : 1 additionally extracted internal lipids and chlorophyll and, therefore, is not suitable. The apolar solvent, *n*-hexane, did not extract the triterpene acids of *P. major*. However, this solvent readily extracted the relatively apolar leaf wax of *L. sativa*. Since the extraction of SOCs (also from deeper embedded wax layers) can only be efficient if all the components of the cuticular wax are removed, we recommend to test the extraction efficiency of the solvent for each plant species beforehand.

INTRODUCTION

The aerial parts of all terrestrial plants are covered by a hydrophobic layer, the cuticle. The cuticular wax, which can be divided into surface (epicuticular) and embedded (intracuticular) wax, is found to accumulate semivolatile organic compounds (SOCs) from the ambient air, such as polychlorobiphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs) and polychlorodibenzo-*p*-dioxins (PCDDs) [e.g. 1–3]. The uptake rate and bioaccumulation of SOCs from the atmosphere are dependent on the properties of the plant, as well as the physicochemical properties of the compounds and the environmental characteristics [4]. Since composition, polarity, amount and structure of the leaf cuticular wax differ widely among plant species [5], the uptake of SOCs in the leaf wax (and thus by the plant) may be plant specific. In addition, these plant properties are dependent on leaf developmental stage and growth conditions [6, 7]. For studying uptake rates and bioaccumulation in plants, it is thus important to have information about the chemical and physical properties of the cuticular waxes.

In order to determine the amount of SOCs in the leaf cuticular wax of plants (epi- and intracuticular wax together as a single fraction), the wax has to be extracted, without extracting internal lipids from the cytoplasmic compartment. Since most cuticular waxes contain a mixture of both polar and non-polar constituents, solvents need to be carefully chosen if they are to dissolve all wax components [8]. Chloroform is most generally used as a solvent, since it is able to dissolve almost all compounds known to occur in leaf waxes [7–9]. Other frequently used solvents are petrol and mixtures of chloroform and methanol [10]. Since SOCs are hydrophobic chemicals, they are readily soluble in apolar organic solvents. For the extraction of SOCs in cuticular waxes, dichloromethane is widely used [e.g. 11–13], while *n*-hexane [e.g. 14–16] and toluene [17] have been used for the extraction of SOC in homogenized plant material. Chlorinated SOCs are often detected by electron capture detection. This method is very sensitive to chlorine atoms and there-

* Author to whom correspondence should be addressed.
¶ Present address: RIVM-CSR, P.O. Box 1, 3720 BA Bilthoven, The Netherlands.

Table 1. Composition (%) of chloroform extracts of leaves from *Lactuca sativa* from three developmental stages

Component	Young	Medium	Old
Linear alcohols	88	86	83
Fatty acids	8	6	7
Squalene			1
Unidentified	4	8	9

fore an extraction solvent without chlorine atoms would be preferable.

In the present study, we determined the cuticular wax composition and the extraction efficiency of several solvents for the waxes of the plants used in our studies on uptake and bioaccumulation of SOC, namely lettuce (*Lactuca sativa*), representing a food crop for man, and plantain (*Plantago major*), a common species of natural ecosystems. To limit the variation in wax composition between different plant "batches", due to changing environmental circumstances, plants were grown under controlled environmental conditions. To study the influence of leaf developmental stage on the wax composition, wax extracts from leaves of different developmental stages were analysed. Leaf cuticular wax of *P. major* is known to contain polar, free triterpenes [18], but its composition has never been presented in detail. For *L. sativa*, to the best of our knowledge, no data on the composition of the cuticular wax have been published before.

RESULTS AND DISCUSSION

Wax composition

The total amount of extracted wax of *L. sativa* changed considerably with developmental stage; the young, middle age and old leaves containing 5, 11 and 8 mg wax g^{-1} dry wt, respectively. In contrast, its composition is little affected by leaf developmental stage (Table 1). However, only in the wax of old leaves traces of the triterpenoid precursor, squalene, could be detected. The wax components of fully expanded, older lettuce leaves are listed in Table 2. It consists mainly of linear primary alcohols and, to a lesser extent, of fatty acids, their biochemical precursors. In old leaves, 9% of the components could not be identified.

For *P. major*, almost no influence of leaf developmental stage on the total amount of extracted wax was found; the young leaves contained 10 mg wax g^{-1} dry wt, while middle age and old leaves had 8 mg wax g^{-1} dry wt. Also, the composition of the leaf wax was relatively constant with leaf developmental stage (Table 3). Table 4 summarizes the components from the methylated extract of cuticular wax of old leaves of *P. major*. Main compounds are the polar, non-volatile, free triterpene acids, oleanolic and ursolic acid, which were detected by GC-FID as their volatile methyl ester derivatives. Besides these triterpene acids, linear alkanes were present as major compounds.

The constant composition of the extractable cuticular wax with leaf age of the plants, and the fact that the two species have a very different leaf wax composition, makes them highly suitable as test plants in research on foliar uptake of semivolatile organic contaminants.

Solvents

Leaf extracts from both species made with chloroform, dichloromethane, toluene or *n*-hexane were colourless. However, the extracts obtained from a mixture of chloroform and methanol were green. Spectrophotometric measurements confirmed the presence of 5 μg chlorophyll g^{-1} fr. wt in these extracts of *P. major*. For the other solvents, this was less than 0.1 μg g^{-1} fr. wt (extraction time 15 min). In contrast to this, Bewick and coworkers [19], already detected several μg g^{-1} fr. wt of chlorophyll in the extracts

Table 3. Composition (%) of chloroform extracts of leaves from *Plantago major* from three developmental stages

Component	Young	Medium	Old
Triterpenic acids	68	64	57
Linear hydrocarbons	18	13	19
Linear alcohols	1	1	1
Unidentified	13	22	23

Table 2. Components from methylated wax of old leaves of *Lactuca sativa*, extracted with chloroform.

Component	RT (min)	% total area	Component	RT (min)	% total area
Henicosanol	3.92	< 1	Pentacosanol	8.49	2
Docosanol	5.07	26	Squalene	9.04	< 1
Me docosanoate	5.35	< 1	Hexacosanol	10.39	24
Tricosanol	7.04	< 1	Me hexacosanoate	11.16	2
Tetracosanol	7.50	22	Heptacosanol	13.54	3
Me tetracosanoate	8.49	3	Octacosanol	16.07	5
			Unidentified		9

Table 4. Components from methylated wax of old leaves of *Plantago major*, extracted with chloroform.

Component	RT (min)	% total area	Component	RT (min)	% total area
Hexadecanol	2.57	< 1	Triacontane	13.52	< 1
Me heptadecanoate	2.95	< 1	Henitriacontane	16.87	10
Me nonadecanoate	3.32	< 1	Dotriacontane	21.20	< 1
Heptacosane	7.40	< 1	Tritriacontane	26.78	3
Hexacosanol	10.50	< 1	Me oleanolate	35.51	14
Nonacosane	10.93	3	Me ursolate	39.49	44
			Unidentified		23

of torpedograss and black nightshade after 2 s of immersing the leaves in chloroform. Apparently, the cuticles of these plants are penetrated by chloroform more rapidly compared with those of *P. major* and *L. sativa*.

Besides chlorophyll, internal cytoplasmic lipids (e.g. for *L. sativa*: the membrane sterols, β-sitosterol, campesterol and stigmasterol, and for *P. major*, tocopherol) were found in the extracts made with the chloroform–methanol mixture. The presence of internal compounds indicates that, in contrast to the other solvents used, this solvent penetrates into the interior of the leaf. This is due to methanol, which penetrates the cytoplasmic compartment relatively fast. For this reason, methanol or a combination of methanol with other solvents is not suitable for the extraction of leaf cuticular waxes of lettuce and plantain. In Fig. 1, the composition of extracts of *L. sativa* leaves, extracted with the various solvents, is shown. The wax compounds are grouped as linear alcohols, fatty acids, squalene and membrane sterols. The extract made with chloroform–methanol is the only one containing internal lipids. The distribution of the wax compounds over the groups is very similar for the extracts made with chloroform, dichloromethane, toluene and *n*-hexane (Fig. 1). This indicates that these solvents extract leaf wax of *L. sativa* equally well. However, this was not the case for the leaves of *P. major* (Fig. 2). The relative extracted amounts of the two dominant types of compounds of leaf wax of *P. major*, namely linear alkanes and triterpene acids (the sum of which is arbitrarily set to 100%) are plotted for the various solvents. The apolar solvent, *n*-hexane, extracts less triterpene acids relative to the linear alkanes than the other, more polar solvents.

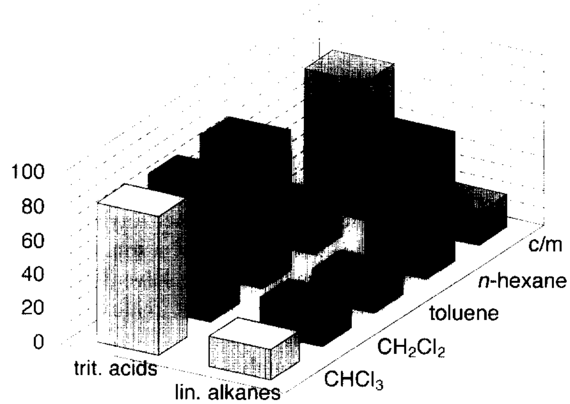

Fig. 2. Relative amounts (%) of triterpene acids (trit. acids) and lin. alkanes (lin. alkanes) in extracts of *P. major*, made with chloroform ($CHCl_3$), dichloromethane (CH_2Cl_2), toluene, *n*-hexane and chloroform–methanol (2 : 1) (c/m). The sum of extracted triterpenes and *n*-alkanes is set to 100%.

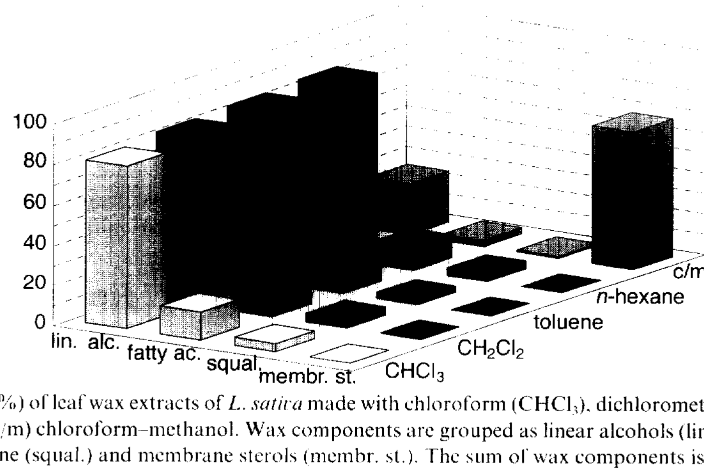

Fig. 1. Composition (%) of leaf wax extracts of *L. sativa* made with chloroform ($CHCl_3$), dichloromethane (CH_2Cl_2), toluene, *n*-hexane and (2 : 1) (c/m) chloroform–methanol. Wax components are grouped as linear alcohols (lin. alc.), fatty acids (fatty ac.), squalene (squal.) and membrane sterols (membr. st.). The sum of wax components is set to 100%.

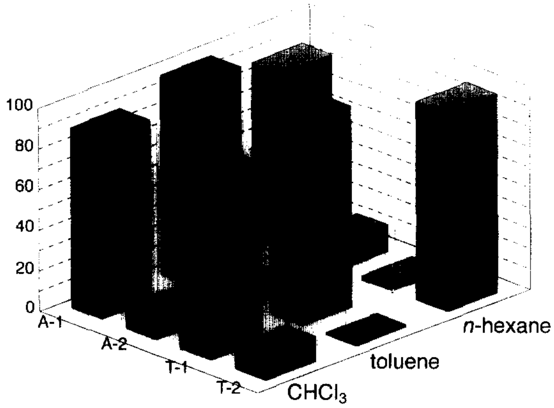

Fig. 3. Relative amounts (%) of linear alkanes (A) and triterpene acids (T) in initial (1) and *post-* (2) extracts of *P. major*. Initial extracts were made with chloroform ($CHCl_3$, extraction time 3 min), toluene (extraction time 60 min) and *n*-hexane (extraction time 60 min). *Post*-extraction was done with chloroform (extraction time 15 min). The sum of initial and *post*-extraction is set to 100%.

This finding is supported by an additional experiment, in which already extracted leaves were *post*-extracted with chloroform for 15 min (Fig. 3). Whereas *n*-hexane extracts the linear alkanes relatively well (only 20% of the total is present in the *post*-extract), the amount of triterpene acids present in the *post*-extract is 50 times higher than the initial *n*-hexane-extract. In contrast, the *post*-extracts of the two other solvents (toluene and chloroform) only contained a small amount of triterpene acids. Thus, the apolar solvent *n*-hexane is not suitable for extraction of the relatively polar leaf wax of *P. major*.

As a consequence, although *n*-hexane is widely used for extracting SOCs from plant leaves, this solvent thus cannot be used for *P. major*. The presence of polar triterpene acids may inhibit the penetration of the apolar solvent into the deeper embedded cuticular wax layers and, therefore, prevent the extraction of SOCs that have diffused into these layers. However, the three more polar solvents (chloroform, toluene and dichloromethane) can be used for the extraction of SOC from cuticular wax of *P. major*.

Since we are unaware of experiments in which authors checked the efficiency of *n*-hexane as an extraction solvent for SOCs, we recommend that the extraction efficiency of a solvent other than chloroform (or the comparable dichloromethane) is tested for each plant species beforehand.

EXPERIMENTAL

Plants

Lettuce (*Lactuca sativa* L. cv. Meikoningin, commercial seeds) and plantain (*Plantago major* L. wild origin) were grown under controlled conditions in water culture (Hoagland). A 12 h light period (21°) was followed by a 12 h dark period (15°). Relative humidity was kept at 70%. Photon flux density was 250 μmol m^{-2} s^{-1} at plant level.

Extraction procedures

Fully expanded leaves of old (ca 3 months) plantain and lettuce were weighed (typical sample wt 5 g) and extracted by immersing them in 50 ml of $CHCl_3$, *n*-hexane, toluene, $CHCl_3$–MeOH (2 : 1) or CH_2Cl_2 for 15 min at room temp. while shaking gently with a horizontal shaker. Extracts were evapd to dryness under a gentle stream of N_2. To study the influence of leaf developmental stage on wax composition, leaves of older lettuce and plantain plants were divided in three groups: young (not fully expanded), middle age (just fully expanded) and older leaves.

Spectrophotometrical measurements

To the wax extracts, 2.5 ml of 80% Me_2CO was added. The concentration of chlorophyll was determined spectrophotometrically [20].

Gravimetric measurements

Extracts of young, middle age and older leaves, made with chloroform, were put in methanol-washed, preweighed glass vials and evaporated under a stream of N_2 to constant wt.

Gas chromatography and identification

Wax extracts were analyzed by GC-FID before and after methylation with CH_2N_2 on a 25 m CP-SIL 5CB-column (ID 0.32 mm, film thickness 0.12 μm, carrier gas N_2 at 6 psi inlet pressure). Injector and detector temps were 280°. Oven temp. 250° (45 min) was quickly raised to 300° and kept at that temp. for 45 min. Peaks were identified by RR_t compared with the int. standard, 5α-cholestane. Additional identification of the wax compounds was done after separation of the leaf wax on a silica column with stepwise elution with a Et_2O–petrol gradient. Elution with 100% petrol gave the alkanes, whereas the esters are present in the 5% Et_2O fr. in petrol. The alcohols were eluted with 25% Et_2O in petrol and 100% Et_2O eluted the triterpene acids.

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