# CUTICULAR AND SUBERIN POLYMERS OF EDIBLE PLANTS – ANALYSIS BY GAS CHROMATOGRAPHIC-MASS SPECTROMETRIC AND SOLID STATE SPECTROSCOPIC METHODS

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*To my dear Family*

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# ABSTRACT

Cutin and suberin are structural and protective polymers of plant surfaces. The epidermal cells of the aerial parts of plants are covered with an extracellular cuticular layer, which consists of polyester cutin, highly resistant cutan, cuticular waxes and polysaccharides which link the layer to the epidermal cells. A similar protective layer is formed by a polyaromatic-polyaliphatic biopolymer suberin, which is present particularly in the cell walls of the phellem layer of periderm of the underground parts of plants (e.g. roots and tubers) and the bark of trees. In addition, suberization is also a major factor in wound healing and wound periderm formation regardless of the plants' tissue. Knowledge of the composition and functions of cuticular and suberin polymers is important for understanding the physiological properties for the plants and for nutritional quality when these plants are consumed as foods.

The aims of the practical work were to assess the chemical composition of cuticular polymers of several northern berries and seeds and suberin of two varieties of potatoes. Cutin and suberin were studied as isolated polymers and further after depolymerization as soluble monomers and solid residues. Chemical and enzymatic depolymerization techniques were compared and a new chemical depolymerization method was developed. Gas chromatographic analysis with mass spectrometric detection (GC-MS) was used to assess the monomer compositions. Polymer investigations were conducted with solid state carbon-13 cross polarization magic angle spinning nuclear magnetic resonance spectroscopy ($^{13}$C CP-MAS NMR), Fourier transform infrared spectroscopy (FTIR) and microscopic analysis. Furthermore, the development of suberin over one year of post-harvest storage was investigated and the cuticular layers from berries grown in the North and South of Finland were compared.

The results show that the amounts of isolated cuticular layers and cutin monomers, as well as monomeric compositions vary greatly between the berries. The monomer composition of seeds was found to differ from the corresponding berry peel monomers. The berry cutin monomers were composed mostly of long-chain aliphatic ω-hydroxy acids, with various mid-chain functionalities (double-bonds, epoxy, hydroxy and keto groups). Substituted α,ω-diacids predominated over ω-hydroxy acids in potato suberin monomers and slight differences were found between the varieties. The newly-developed closed tube chemical method was found to be suitable for cutin and suberin analysis and preferred over the solvent-consuming and laborious reflux method. Enzymatic hydrolysis with cutinase was less effective than chemical methanolysis and showed specificity towards α,ω-diacid bonds. According to $^{13}$C CP-MAS NMR and FTIR, the depolymerization residues contained significant amounts of aromatic structures, polysaccharides and possible cutan-type aliphatic moieties. Cultivation location seems to have effect on cuticular composition.

The materials studied contained significant amounts of different types of biopolymers that could be utilized for several purposes with or without further processing. The importance of the so-called waste material from industrial processes of berries and potatoes as a source of either dietary fiber or specialty chemicals should be further investigated in detail. The evident impact of cuticular and suberin polymers, among other fiber components, on human health should be investigated in clinical trials. These by-product materials may be used as value-added fiber fractions in the food industry and as raw materials for specialty chemicals such as lubricants and emulsifiers, or as building blocks for novel polymers.

## LIST OF ABBREVIATIONS

| | |
|---|---|
| AFM | atomic force microscopy |
| ANOVA | analysis of variance |
| APCI | atmospheric pressure chemical ionization |
| CcCUT1 | *Coprinus cinereus* cutinase |
| CIMS | chemical ionization mass spectrometry |
| CLSM | confocal laser scanning microscopy |
| COSY | homonuclear correlation spectroscopy |
| CP-MAS | cross polarization magic angle spinning |
| DAD | diode array detector |
| DP-MAS | direct polarization magic angle spinning |
| EI | electron impact ionization |
| ELSD | evaporative light scattering detector |
| ESI | electrospray ionization |
| FID | flame ionization detector |
| FTIR | Fourier transform infrared spectroscopy |
| GC | gas chromatography |
| HMBC | heteronuclear multiple-bond correlation |
| HMQC | heteronuclear multiple quantum coherence |
| HPLC | high performance liquid chromatography |
| HR-MAS | high resolution magic angle spinning |
| HSQC | heteronuclear single quantum coherence |
| IR | infrared |
| LC | liquid chromatography |
| LM | light microscopy |
| LSD | light scattering detector |
| LSIMS | liquid secondary ion mass spectrometry |
| MS | mass spectrometry |
| MALDI | matrix-assisted laser desorption ionization |
| MS/MS | tandem mass spectrometry |
| NMR | nuclear magnetic resonance spectroscopy |
| NOESY | nuclear overhauser effect spectroscopy |
| SEC | size exclusion chromatography |
| TLC | thin layer chromatography |
| TOCSY | total correlation spectroscopy |
| ToF | time-of-flight |
| UV | ultraviolet |

# LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles referred to in the text by the Roman numerals I-V:

I Kallio, H., Nieminen, R., Tuomasjukka, S., Hakala, M. (2006) Cutin composition of five Finnish berries. *J. Agric. Food Chem.* 54; 457-462.

II Järvinen, R., Kaimainen, M., Kallio, H. (2010) Cutin composition of selected Northern berries and seeds. *Food Chem.* 122, 137-144.

III Järvinen, R., Silvestre, A.J.D., Holopainen U., Kaimainen, M., Nyyssölä, A., Gil, A.M., Pascoal Neto, C., Lehtinen, P., Buchert, J., Kallio, H. (2009) Suberin studies of potato; comparison of the effect of Cutinase CcCut1 hydrolysis with chemical depolymerization. *J. Agric. Food Chem.* 57, 9016-9027.

IV Järvinen, R., Silvestre, A.J.D., Gil, A.M., Kallio, H. Solid state $^{13}C$ CP-MAS NMR and FTIR spectroscopic analysis of cuticular fractions of berries and suberized membranes of potato. *JFCA* (In press).

V Järvinen, R., Rauhala, H., Holopainen, U., Kallio, H. Differences in suberin composition between two varieties of potatoes (*Solanum tuberosum*) and effect of post-harvest storage. Submitted to *LWT - Food Science and Technology*.

# 1 INTRODUCTION

Living organisms are wrapped in polymeric lipid-derived coverings. The functions and characteristics of the cuticular layer present in the epidermal part of aerial plants are strongly influenced by the insoluble cuticular polymers cutin and cutan, as well as by cuticular polysaccharides and soluble cuticular waxes (1). A similar protective layer is formed by a polyaromatic-polyaliphatic biopolymer suberin, present particularly in the outer cell walls of the periderm of the underground parts of plants (e.g. roots and tubers) and the bark of trees (2, 3).

Berry is a common term for fleshy, juicy fruits with one or typically more seeds, including grape (*Vitis vinifera*), gooseberry (*Ribes uva-crispa*), black currant (*Ribes nigrum*), cranberry (*Vaccinium macrocarbon*), sea buckthorn (*Hippophae rhamnoides*), tomato (*Solanum lycopersicum*), strawberry (*Fragaria ananassa*) and raspberry (*Rubus idaeus*) (4). Some are actually accessory fruits, even though they are generally considered berries. For example, strawberry is not a berry but a pseudocarp, the edible red pulp of which is a swollen receptacle of the flower. Stone fruits, raspberries and cloudberries are aggregate fruits with multiple drupes. The peel of a berry has a major influence on how berries are preserved in nature. The cuticular polymers, together with associated waxes, are thought to be the major contributors to morphology and preservation (1). Fruit surface influences the appearance of the whole fruit including color, glossiness, texture and uniformity, efficacy of post-harvest treatments, storage, transport and shelf life (5). Thus, knowledge regarding fruit surface characteristics is fundamental for the improvement of fruit quality.

The mature periderm of potato (*Solanum tuberosum*) forms an efficient protective layer on the tuber and consist of three layers; phellem, phellogen and phelloderm. Suberized cells, the major contributing factor of the protective effect, are located in the phellem i.e. in the skin of potato (6-8). An immature or wounded periderm is susceptible to infection and skinning (excoriation of the skin), making the potato crop vulnerable during harvest and post-harvest storage, potentially causing significant economical losses (6, 7).

Dietary fiber has a complex and highly variable composition. It is principally composed of the polysaccharides of cell walls, but many edible plants have cell types that contain hydrophobic polymers, for example, cutin, suberin and lignin. These biopolymers are also a significant part of the by-products of the food and wood processing industry (9-12). The lack of knowledge of these natural biopolymers hinders their utilization.

The original research of this thesis project revealed the composition of cutin monomers and gave insight into the structures of cuticular polymers of several berries. Potato aliphatic

suberin was compared of two varieties with different phenotypes during post-harvest storage. In addition, chemical and enzymatic cutinase depolymerization techniques were developed and compared in the analysis of cutin and suberin.

# 2 REVIEW OF THE LITERATURE

Plants need protection against many biotic and abiotic factors. Being sessile and not able to move away from unfavorable conditions or escape from impending attacks, plants have developed survival strategies by differentiating their cell wall structures. Re-modelling and reconstruction also takes place during growth and development, thus keeping the plant in a state of constant progress to ensure proper growth, reproduction and defense (13). In addition to changes in cell wall polysaccharides, plants have developed specialized structural and protective biopolymers, such as cutin, suberin and lignin. Suberin and lignin are polymers located inside the cell walls, while cutin is present in the outermost extracellular layer called cuticle.

Development of cuticle was a leading factor that enabled plants to move onto land from aquatic environments about 400 million years ago (1). The ubiquitous presence of suberin in root tissues controlling water and ion uptake also suggests that suberin played a major role in the adaptation of vascular plants to terrestrial life (14). These lipid-polyester layers are unique for plants, while the structural components of animals are composed of amino acid chains which form proteins or polysaccharides such as chitin (1, 15, 16).

The genetics underlying plant polyester biosynthesis is largely unknown. The analysis of genes affecting the composition of cutin and suberin monomers have recently performed on *Arabidopsis* (14, 17-19), tomato (5, 20) and cork oak (*Quercus suber*) (21). The use of *Arabidopsis* as model plant for cutin biosynthesis is questionable, as the polyester composition of *Arabidopsis* ultrathin cuticle resembles that of suberin, rather than cutin (22). Suberin present in the endodermis and periderm of the roots of *Arabidopsis* is very similar to other species, indicating that these can serve as a model for suberized tissues (18). In recent years, research regarding the influence of cutin and suberin polymers on physiological and mechanical properties as well as microbial susceptibility has increased with the possibility of studying different transgenic plants or mutants where genes involved in cutin/suberin biosynthesis have been altered (19, 20, 22, 23).

The purpose of this literature review is to focus on research findings on the chemical structure and composition of cuticular and suberin polymers, present especially in edible plants, and to introduce the methods used to analyze these polymers.

## 2.1 CUTICULAR POLYMERS ARE MAJOR CONSTITUENTS OF THE CUTICLE

The cuticle is composed of cuticular polymers, polyester cutin and highly resistant cutan, in addition to cuticular waxes embedded in the cuticle matrix. The structure and composition of

cuticle varies considerably among plants, organs and growth stages (1, 24). Differences in cutin composition might occur from different protection and nutrient needs, in addition to biochemical properties such as elasticity and hydration of the different parts of plants (23, 25).

Cuticle acts not only as a barrier, but also as a gateway between the plant and its environment. Plant cuticles have the ability to adjust to hydration, temperature and other stresses imposed by their environment (26, 27). Cutin, usually the largest constituent of cuticle, plays an important role in cuticle as a structural component, as a defense barrier against pathogens (1, 20, 28), as protection against the uncontrolled loss of water together with waxes (19), as well as in transporting substances across plant tissues (27, 29). Cutin can act as a chemical signaling site to alert the host of a pathogen attack and trigger a defense reaction, but also activate the invading pathogen (1, 28, 30). Cutin has a critical role in ensuring organ identity during development, preventing fusion of cell walls from adjacent organs (31). The importance of cutin is seen during the imbibition of seeds (32). Cuticular polysaccharides, in addition to the effects of the cutin matrix, are responsible for the elastic modulus and stiffness of the cuticle, providing differences in mechanical properties of various plant species (33). Cuticle is the first barrier for agrochemicals to pass through; thus, the composition of cuticle and cutin plays a major role in the effects of pesticides, herbicides and fertilizers, in addition to sorption of organic pollutants (34-38).

The biosynthesis of cutin, although not completely understood, is a specialized function of terminally differentiated epidermal cells (1, 24, 27). Direct hydroxylation of $C_{16}$-cutin monomers from hexadecanoic acid (i.e. palmitic acid) was shown *via* incorporating $^{14}C$-labeled monomer precursors to cutin polymer by Kolattukudy *et al*. in the early 1970s (39). The $C_{18}$-monomer biosynthesis pathway from octadecen-9-oic acid (i.e. oleic acid) and octadeca-9,12-dienoic acid (i.e. linoleic acid) includes ω-hydroxylation, peroxygenase catalyzed epoxidation of double bonds and finally hydroxylation (40, 41). Recent developments of cutin biosynthesis with gene-altered plants have lead to the identification of several genes and enzymes relevant to the process, but the exact intracellular location, organization of the enzymes and the polymerization reaction itself, including cross-linking, remains unclear (14). A new hypothesis for cutin synthesis has been suggested by Heredia *et al*. (42-44) which includes the self-assembly of monomers and transportation in *cutinsomes*, lipid vesicles formed by cutin monomers which serve as the putative building units of the polyester. This hypothesis implies that cutin polymer formation ends in a physico-chemical controlled process following genetically controlled sequences of fatty acid precursor modifications to cutin monomers (42-44).

While the monomer units of the cutin polyester are starting to be well known in various plants and their tissues, the structure of the cutin polymer itself and the presence and nature of highly resistant cutan have still not been comprehensively revealed.

The ultrastructure and morphology of cuticle have been investigated with different microscopic methods (24, 45, 46), including recent developments in 3-D imaging using confocal scanning laser microscopy (47). This thesis will concentrate on chemical composition, thus the ultrastructural characteristics of cuticle will be only briefly discussed related to the location (section 2.1.1) and development of cuticle (section 2.1.3.3).

### 2.1.1 Location and occurrence

The cuticle is present in the outermost layer of the aerial parts of vascular plants and is formed very early in epidermal cell development (24). Cuticle has been found in lower plants including mosses, lycopods, liverworts and ferns (48) in addition to higher plants, both angiosperm and gymnosperm species (1, 24, 49). Only the epidermal cells of aerial organs are known to be capable of synthesizing the constituents of the cuticle, and it is always absent in root epidermis (24). Cutin-containing layers are found on the surfaces of all the primary parts of aerial plants, such as stems, petioles, leaves, flower parts, fruits and some seed coats. In addition, cutin may be found on some internal parts of plants such as the juice-sacs of citrus fruits (46).

In a simplified picture of cuticle (Figure 1), the cutin-containing layer is attached to the epidermal cell walls *via* a pectin layer, but the boundaries between cuticular layers, cell wall polysaccharides and pectin are intermingled, thus the layers are not easily simplified (1). Modern ultrastructural studies have revealed at least six types of cuticles, whose fine structures vary predominantly from layers containing lamellae to reticulate type structures. Usually, cuticle contains an outermost layer formed of epicuticular waxes and a cuticle proper composed of cutin and/or cutan polymers (Figure 2). The cuticular layer underneath the cuticle proper consists of cutin-cutan polymers and possibly polysaccharides with embedded waxes. These layers which make up the cuticular membrane are connected to the epidermal cell wall *via* a pectinaceous layer. The fine structure of cuticle varies according to the stage of development and growth (24). It is still not clear how the fine structure of cuticles relates to the chemical composition of cutin and cutan polymers or the properties of cuticle (1, 14).

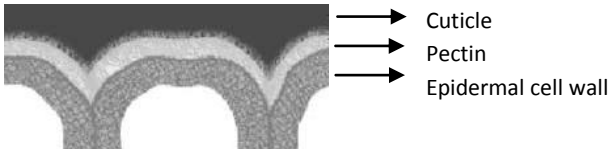



**Figure 1.** Schematic presentation of simplified cuticle (not to scale). Reproduced with permission (1, 15). 

Cuticular membrane occupies approximately 0.1-10 µm of the aerial plant surface (50). A major part of plant polyesters is located in the cuticle. The weight of an isolated cuticle ranges from 2000 µg/cm$^2$ (fruits) to 450-800 µg/cm$^2$ (leaves), of which usually 40-80% corresponds to cutin, thus it can be considered as major plant lipid polyester (27, 51, 52).

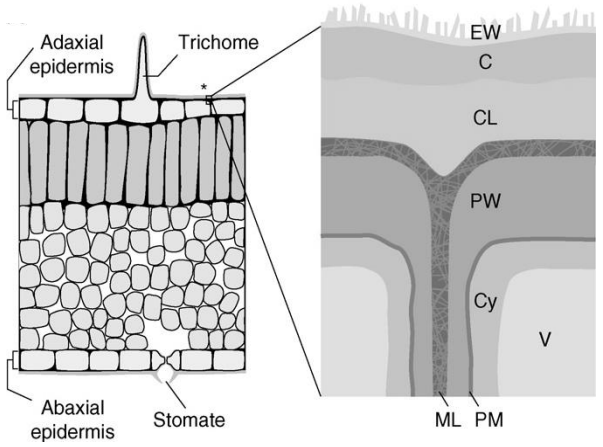



**Figure 2.** Schematic representation of cutin deposition in a leaf (not to scale). EW, epicuticular waxes; C, cuticle proper; CL, cuticular layer; ML, middle lamella; PW, primary cell wall; PM, plasma membrane; Cy, cytoplasm; V, vacuole. Reproduced with permission (14). Copyright 2008 Elsevier.

Investigations into the cuticular layers of seed coats, such as soy bean (*Glycine max*) (32, 53), have indicated that seeds contain two thin cuticular layers, one on the outer seed coat and another associated with the inner seed coat. Seeds from apples (*Malus pumila*) (54) have cuticular aliphatic components only in the inner seed coat, while seeds of grapefruit (*Citrus paradisi*) (46) exhibit lamellar suberin layers in the chalazal region in addition to an amorphous cuticular layer encircling the seed. Also, analyzes of *Arabidopsis thaliana* seed and rapeseed (*Brassica napus*) have indicated the presence of cutin and suberin in the inner and outer seed coat, respectively (55, 56). Three cutinaceous layers have been found from the husk of cereal such as barley (57).

Cuticular polymers are resistant to biological degradation, as these have been found in sewage sludge (58) and soil (59, 60). However, enzymes hydrolyzing cutin have been isolated from fungi, pollen and mammals (16). The composition of cuticle has been found to affect its biodegradability, with those containing cutan being more durable (36). Cutan is even durable during diagenesis as it has been found in fossilized plant cuticles (61, 62). Thus, cuticular analysis is regarded as an important standard technique in paleobotanical analysis.

### 2.1.2 Structure and composition of cutin

The monomeric composition of cutin has been widely studied in various plants, but the linkages between the monomers and the three-dimensional structure are still not fully understood. The main monomers of cutin were essentially identified in the 1970s by using chemical degradative techniques (39, 48, 49, 52, 54, 63-74), but evaluations of different plant materials have continued until today (18, 55, 56, 75-77). Plant-specific compounds have been found, even though cutin monomers were generally found to be composed of different mixtures of similar $C_{16}$- and $C_{18}$-hydroxy acids.

The insoluble nature of cuticular polymers has hampered the analysis of intact structure, and knowledge on the composition of these materials has been based on the monomers obtained after depolymerization reactions. The reasons behind the insolubility are unknown, but as listed by Pollard *et al.* (14), may be the covalent anchoring of cutin polymer to cell walls or cutan, undefined cross-linking within the aliphatic polymer or the very high molecular weight of the polymer molecules. According to recent studies, evidence of the covalent bonds between the cutin polymer and the cell wall polysaccharides has been found (78, 79).

Cutin monomers constitute a complex network by forming ester linkages through primary and secondary hydroxyl and carboxyl groups (1). Elucidation of the covalent bonds between monomers and the presence of free functional groups were previously based on the estimation of chemical reactivity (1, 16, 80). During the late 1990s and 2000s, the structure of cutin has been evaluated by using enzymatic and chemical methods producing oligomers (9, 81, 82) and by analyzing intact polymers by spectroscopic methods, atomic force microscopy (AFM) and X-ray diffraction (78, 83-89).

According to chemical reactivity, virtually all primary (terminal) hydroxyl groups are part of the linear bonds, while some of the secondary (mid-chain) hydroxyl groups form branches or cross-links with other polymer chains (1, 16, 80). There are only very few non-esterified carboxyl groups (27). When independently considering the monomers and linkages, the data suggest that a polyester dendrimer is likely to be a dominant structural element in ω-hydroxy acid-rich cutins, the size of which may be enlarged by glycerol (14).

Walton and Kolattukudy (66) and Croteau and Fagersom (74) in their work in the early 1970s proposed that cutin also consist of other bonds in addition to ester linkages. According to the current knowledge and definition of cutin, it only consists of ester-bound monomers (27), while cutan (or non-ester cutin) has other types of hydrolysis-resistant bonds, e.g. ether and peroxide linkages. Thus, in this thesis, cutin is defined as an ester-bonded biomacromolecule.

The structure is heavily dependent on the length of the monomers forming chains and different functional groups that may be involved in the cross-linking. For example, polymers

rich in epoxy-substituted monomers may not form cross-links and are essentially linear. On the contrary, polymers rich in dihydroxy substituents have a greater potential for cross-link formation (46, 65). The increased hydroxyl groups can also affect the hydrophilicity of the cutin by facilitating the impregnation of water (25). Absorbed water can act as a plasticizer by disrupting hydrogen bonded cross-links, which promotes molecular flexibility and decreases the elastic modulus (89). The mechanical properties of cuticles are influenced by the $C_{16}$-$C_{18}$-monomer ratio. A higher content of trihydroxy acid monomers and a near-equal $C_{16}$ to $C_{18}$ ratio characterizes the cutin of elastic tissues, while mostly $C_{16}$ containing cutins are more rigid (23, 25). Also, the amount of phenolic compounds is correlated with the rigidity of a cutin matrix (25, 33). All these functionalities affect the various properties of the polymer.

Work done by Graça *et al*. (51) has revealed that glycerol is an essential part of cutin. In addition to sole ω-hydroxy acid monomers, they found glycerol esterified to the monomers. These findings suggest that glycerol may act as an anchor for linear and cross-linked ω-hydroxy acids, thus increasing the complexity of the polymer. Conformation of the glycerol molecule by rotation around single bonds permits macromolecular expansion to several directions, bringing middle-chain substituents, such as epoxy, free hydroxy and keto group, close enough to establish hydrogen bonds capable of constituting an important structural factor. This might influence the organization behind many lamellar type cuticles, but structural models with glycerol have not yet been published. Lipids, which are composed of glycerol esterified to fatty acids and also play important roles, e.g. as part of biomembranes in the animal and plant kingdoms and as storage for lipids, are different from cutin which is formed by polymerized macromolecules attached *via* ester bonds.

So far, oligomer analysis of cutin has been published for mainly $C_{16}$-cutins, thus information on the $C_{18}$- and $C_{16}$-$C_{18}$ mixed-type cutins is lacking. Therefore, the proposed cutin structure (Figure 3) is mainly a model of a $C_{16}$-cutin. Linear dimer esters of 8,ω-, 9,ω- and 10,ω-dihydroxyhexadecanoic acids were among the first oligomeric structures identified from tomato cutin *via* partial chemical and enzymatic hydrolysis (90). Later, fragments up to pentameric oligomers were obtained by analysis of different high performance liquid chromatography mass spectrometric (HPLC-MS) methods (9). Solution-state nuclear magnetic resonance (NMR), electron impact mass spectrometry (EI-MS) and liquid secondary ion mass spectrometry (LSIMS) analysis of oligomers of lime fruit cutin have proven directly the existence of secondary alcohol ester linkages in addition to various oligomers consisting of primary ester bonds (Figure 4) (78, 81, 82) together with a provisional glycoside-linkage in a hydroxy fatty acid tetramer (79). Recently, Tian *et al*. (79) have revealed that the monomers may be assembled in any possible order which increases the heterogeneity of the polymer. Also, strengthening of the polymeric network by hydrogen bonding is possible when a mixed-monomer architecture is synthesized (79).

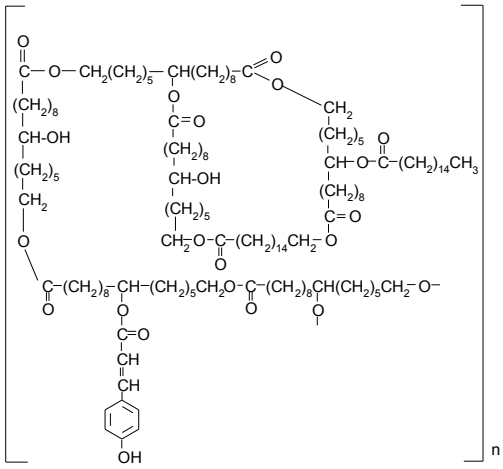


**Figure 3.** Proposed model for mainly $C_{16}$-cutin structure (1, 15, 16).

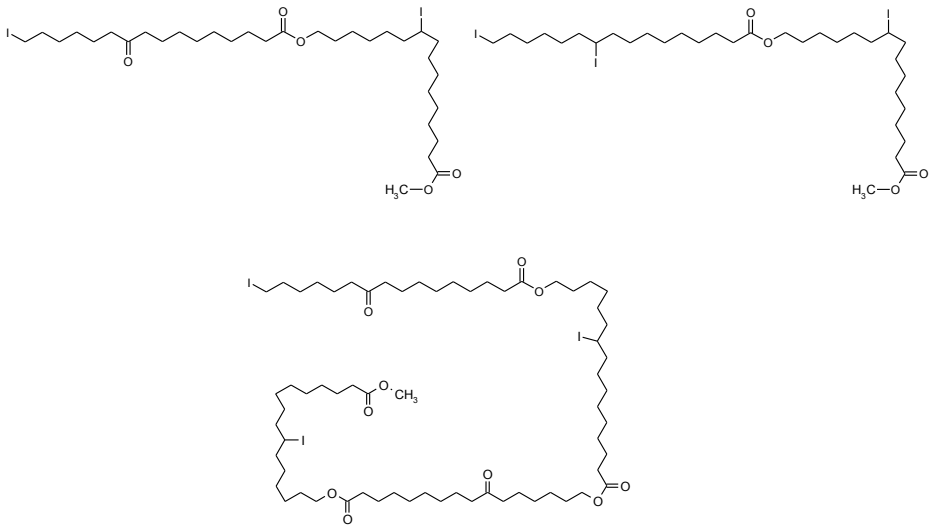


**Figure 4.** Oligomers from lime fruit cutin obtained with trimethylsilyl iodine cleavage (82).

More direct analysis of the cutin polymers has been achieved by using solid state and solvent-swelled NMR methods together with Fourier transform infrared spectroscopy (FTIR). These methods have produced complementary information as the depolymerization products do not give information on the polymer architecture. The polymer structure has been defined as a mostly aliphatic polyester with olefinic and aromatic structures. Functional groups present in cutin polymers, such as methylenes, methines, carboxyls, carbonyls and aromatic structures have been identified by using carbon-13 cross-polarization magic angle spinning ($^{13}$C CP-MAS) NMR and FTIR (82, 83, 85, 86, 91-94). Together with direct polarization methods (DP-MAS),

cutin was demonstrated to be a moderately elastic network, with motion-hindering positions possibly at the cross-link sites of the polymer (83, 91).

In NMR studies of lime (*Citrus aurantifolia*) fruit cutin, which contain a large amount of secondary keto-groups, over half of the methylenes were assigned to the rigid category. The authors concluded that in cutins, which contain more secondary hydroxyl groups capable of forming cross-links, an ever larger part of the methylenes may be part of the rigid category. Ester linkages between aliphatic and aromatic compounds, in addition to esters of primary and secondary alcohols, were confirmed (78, 83).

Cutin architectures involving α-branched monomers and their esters, pointing towards cross-linking, have been identified from solvent-swelled cuticular polymers from tomato and agave (*Agave americana*) by high resolution magic angle spinning (HR-MAS) NMR spectroscopy. Also, epoxy groups, free primary and secondary alcohol groups, as well as free carboxylic acid groups have been detected (85, 88).

#### 2.1.2.1 Cutin monomers

The basic monomeric constituents of cutin polyester are polymorphous hydroxy acids with skeletons typically of 16- or 18-carbons (Figure 5), although longer and shorter chains are also present in minor compounds. Hydroxyl groups or their derivatives, including keto and epoxy moieties, are located at typical double bond positions of fatty acids and in the ω-position at the end position of the chain. Usually, saturated compounds dominate unsaturated monomers. In addition to hydroxy acids, cutin contains ester-bound long chain fatty acids, diacids, alcohols, phenolic compounds and glycerol (1, 15, 16, 27, 51). The composition of cutin is unique, as for example keto-substituted fatty acids are only rarely found in the lipids of plant kingdom, except for some seed oils (64).

##### 2.1.2.1.1 ω-Hydroxy acids

The main monomers of the $C_{16}$-group are isomers of dihydroxyhexadecanoic acids and 16-hydroxyhexadecanoic acid. The presence of dihydroxyhexadecanoic acids has been suggested to be the only single indication that distinguishes cutin from suberin (95).

Dihydroxyhexadecanoic acid isomer composition is distinctive to certain plants and the most common isomers are 9,16- and 10,16-, although 7,16- and 8,16-positions have been detected (67, 96). Positional isomers of 16-hydroxy-oxo-hexadecanoic acid are important constituents of e.g. citrus fruits, cape gooseberry (*Physalis peruviana*) and black currant cutin (46, 64, 82, 97). Suberin-like monohydroxyhexadecan-1,16-dioic acid, 9,10,16-trihydroxyhexadecanoic acid together with ω-oxo- i.e. aldehyde and dihydroxy-oxo-derivatives of $C_{16}$-acids may be found as minor constituents of cutin polymer (51, 69, 72, 98, 99).

The main monomers of the $C_{18}$-group are 9,10-epoxy-18-hydroxyoctadecanoic acid and 9,10,18-trihydroxyoctadecanoic acid, together with their unsaturated derivatives. In addition to these, 18-hydroxyoctadecanoic acid and its mono- ($C_9$) and diunsaturated ($C_9$ and $C_{12}$) derivatives may be found (1, 27, 65, 68). Also, some dihydroxy-$C_{18}$-compounds, such as 9/10/11,18-dihydroxyoctadecenoic and 9/10,18-dihydroxyoctadecanoic acid (51) and ω-oxo-derivatives such as 9,10-epoxy-18-oxo-$C_{18}$-acids (71) have been found as minor constituents.

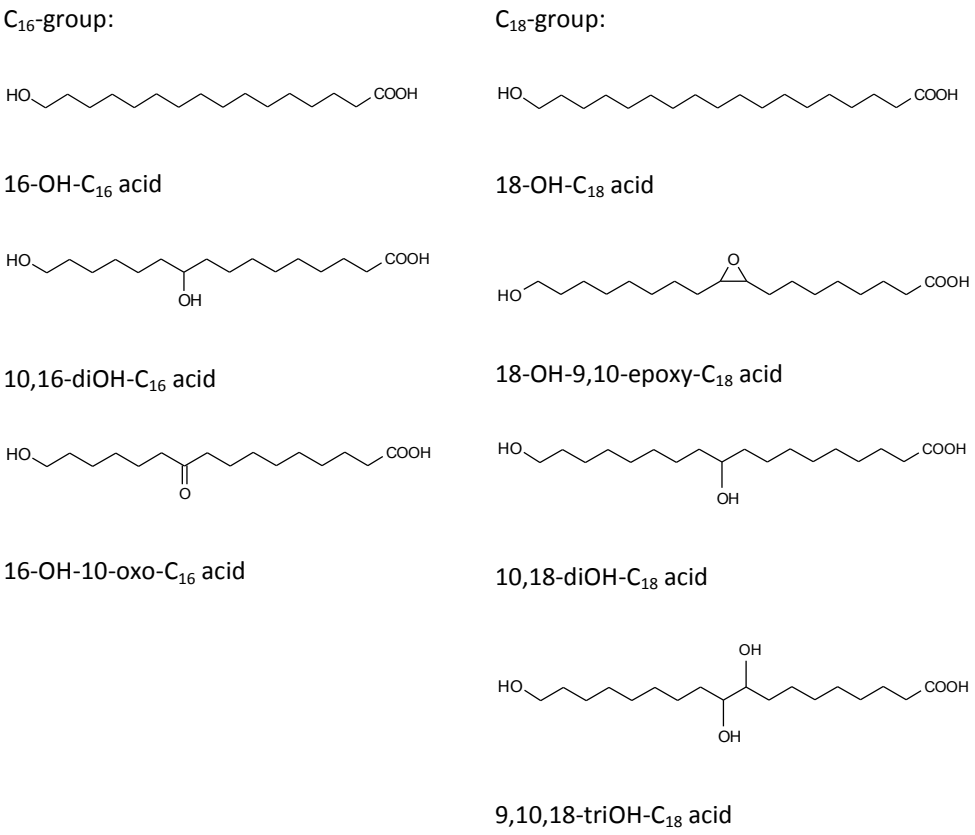



**Figure 5.** Examples of the major components of cutin.

In addition to $C_{16}$- and $C_{18}$-monomers, cutin may contain shorter and longer chain ω-hydroxy acids and hydroxy substituted α,ω-diacids (1). In many plants, these are usually of chain lengths $C_{20}$, $C_{22}$ and $C_{24}$. In addition, $C_{14}$-hydroxy acids has been found in coffee leaves (*Coffea arabica*) (69) and in the seed coats of rapeseed and *Arabidopsis* (55). Longer chain dihydroxy acids, such as 10,20-dihydroxyeicosanoic acid have been found from wood apple (*Limonia acidissima*) leaf cutin (100).

Most commonly, cutin monomers are composed of even-numbered carbon chains but odd-numbered chain lengths have also been detected. Graça *et al*. (51) have found 9/10,15-dihydroxypentadecanoic acid isomers from tomato cutin and Holloway and Deas (69) have isolated monohydroxy- and dihydroxypentadecanoic acid from the leaf cutin of the coffee plant. Rarely, α-branched hydroxy acids (85, 100) pentahydroxy-$C_{18}$-acids and epoxy-trihydroxy-$C_{18}$-acids (70) have been detected. The primitive cutin of lower plants (ferns, lycopods and mosses) may contain reduced $C_{16}$-monomers or fewer hydroxylated monomers (1). Caldicott and Eglinton (48) have reported ω-1 hydroxy acids as important monomers in two liverwort species (*Astarella lindenbergiana* and *Conocephalum conicum).*

### 2.1.2.1.2 Other compounds

Long chain fatty acids, diacids, alcohols and aromatic compounds have been found among the depolymerization products of cutin. Generally, the proportion of these monomers is small (<5%), although exceptions can be found e.g. in walnut leaf cutin (*Juglans regia*) with a content of 30% (51).

Dicarboxylic acids and very long chain (>$C_{18}$) fatty acids are major components of the suberin aliphatic domain and are usually only minor components in cutin (1, 95). Unusually, *Arabidopsis* leaves and stems have been found to contain octadeca-6,9-diene-1,18-dioic acid as the main monomer (52% of total monomers) (17). Minor amounts of fatty alcohols, diols and triols have been detected, for example 1,7,16-hexadecantriol from lime fruit cutin (81).

The most commonly-found aromatic compounds esterified to the cutin structure are cinnamic acid derivatives, such as hydroxycinnamic (i.e. coumaric), dihydroxycinnamic (i.e. caffeic) and hydroxymethoxycinnamic (i.e. ferulic) acid (Figure 6) (16, 55, 100-102). Das and Thakur (100) reported that wood apple leaf cutin contained approximately 5% of aromatic compounds including *m*- and *p*-coumaric acids (3- and 4-hydroxycinnamic acid), 4-hydroxybenzoic acid and 4-hydroxy-3-methoxybenzoic acid and concluded that aromatic compounds esterified to cutin polymer might play a protective role when released by the hydrolytic enzymes excreted by fungi.

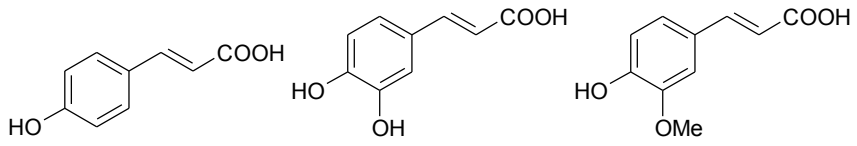



**Figure 6.** Structures of *p*-coumaric (4-hydroxycinnamic acid), caffeic (3,4-dihydroxycinnamic acid) and ferulic (4-hydroxy-3-methoxycinnamic acid) acid (103).

After recognition of the important role of glycerol in polyaliphatic suberins, its presence in cutin has been established by Graça *et al*. (51). Previously, it had not been detected among the depolymerization products as all water-soluble material was discarded due to the extraction methods employed after depolymerization reactions. In addition to monomeric glycerol (1 to 14% of monomers depending on plant species), 1- and 2-monoacylglyceryl esters of ω-hydroxy acids have been obtained *via* partial depolymerization reactions. Interestingly, glycerylesters of epoxy-substituted hydroxy acids have not been found (51).

### 2.1.3 Non-depolymerizable cutan

After removal of cuticular wax and ester-bound cutin monomers, a non-depolymerizable and insoluble fraction usually remains. Generally, this fraction is species-dependent and accounts for 10-55% of the weight of the isolated cuticular fraction (16, 61, 87). In most cases, the residue is mostly composed of polysaccharides that represent the portion of the epidermal cell wall to which the cuticular membrane was attached, but in some cases it contains a highly resistant polymethylenic biopolymer called cutan or non-ester cutin (24, 61, 62, 87, 104).

Many structural models of cutan have been proposed by applying different analysis methods including FTIR, $^{13}$C-NMR and X-ray diffraction, together with degradative methods like thermochemolysis, ozonolysis, oxidation and pyrolysis coupled to GC-MS (61, 62, 87, 105, 106). In most studies, agave and kaffir lily (*Clivia miniata*) cuticles have been used as the sample material. The results of these studies are controversial and the exact nature of cutan still remains to be unraveled. Certainly, chemical purification is a crucial step in the isolation of cutan and is inevitably linked to its definition.

Nip *et al*. (61) presented a non-saponifiable, highly aliphatic and resistant biopolymer releasing mainly hydrocarbons in addition to sugars derived from polysaccharides by pyrolysis. Tegelaar *et al*. (62) concluded that cutin and cutan can be present in any ratio, and differ in their relative abundance at different stages of development and the structure of polymethylenic chains covalently bound to the polysaccharide moiety. Studies by McKinney *et al*. (106) showed no evidence of polysaccharide units in cutan. These authors proposed the presence of very long chain fatty acids esterified to substituted aromatic-ring structures as part of the polymers. The presence of ester bonds of long chain fatty acids and aromatic-OHs in addition to ether bonds was supported by Schouten *et al*. (107). The presence of ether bonds was also demonstrated by Kolattukudy *et al*. (1) by observation that HI treatment released part of the insoluble core as a soluble material. Villena *et al*. (87) showed that polysaccharidic and aromatic moieties could be completely removed upon purification, thus suggesting that cutan is a highly aliphatic three-dimensional macromolecular network linked by ether bonds containing double bonds and free carboxylic acid functions. Direct HR-NMR studies by Deshmukh *et al*. (88) suggested that cutan contains long chain fatty acids and fatty alcohols

ester-linked to aryl-O systems, but did not find evidence for ether linkages. Since the cuticle is known to contain phenolic compounds and peroxidase enzymes, peroxidately-coupled phenolics might be present in cuticular polymers and especially in the non-ester bonded fraction (1). Based on these ambiguous results and hypotheses, highly aliphatic polymethylenic chains are the only consistent marker for cutan which remains after commonly-used ester breaking reactions.

Some cuticles appear to totally lack cutan structures, such as the cuticles of tomato fruit (9, 61) and lemon leaf (*Citrus limon*) (61), while it may be the principal component of some cuticles. The mixed-type cuticle containing both polymers may be very ubiquitous, as cutan has been detected from fossilized cuticles, terrestrial sediments and coals (61, 62, 87). Boom *et al*. (105) obtained alkene and alkane structures from pyrolysis of several succulent plants confirming the wide presence of cutan in plants. They concluded that all the plants investigated have thick cuticles and some degree of adaptation to drought, suggesting that cutan enhances the hydrophobic nature of these cuticles (105). So far, cutan-type structures have been found in only a few edible plants including sea beet (*Beta vulgaris ssp. maritime*) (61), apple fruit (62, 94) and pepper (*Capsicum annuum)* fruit (36, 94).

In studies of fossilized cuticle, cutan and cutin-type polymers that have remained non-hydrolyzed during diagenesis have been found, although ester-cutin has not survived (62). These studies have implicated that cutins which contain high amounts of epoxy groups could undergo secondary reactions forming non-hydrolyzable (ether) bonds, perhaps *via* a ring opening reaction. Thus, the preservation potential of angiosperm species with a large number of epoxy groups is higher because of the modification of cutin to cutin-derived material.

In addition to fossils, the amount of cross-links and complexity of the polymer has been found to increase during cutin ageing of some presently living species. Thus, the cutin composition may change while the cuticle develops during the life cycle of the plant and form more resistant structures (62, 104, 108). Cutanization of the cuticular layer therefore probably arises from the maturation process involving progressive modification of previously-deposited cutin and possibly embedded polysaccharides and waxes, rather than *de novo* synthesis and secretion of a new layer (24). However, nothing is known regarding the reactions involved in the modification of cutin to cutan (22).

### 2.1.4 Effects of genetic background, developmental stage and environment

The amount and structure of the cuticle and the composition of cutin vary greatly according to plant species, age, organ (fruit, leaf, etc.) and the stage of growth (1, 15, 25, 52, 62, 95). Evolutionary stage has also been suggested to affect the composition of cutin monomers. Hunneman and Eglinton (63) concluded that the higher the plant is in the evolutionary scale,

the higher the oxidation state of the cutin acids. In general, the chemical composition of cutin monomers may be predominantly similar with plants that belong to very different families or, on the contrary, be very different between plants of same family.

The monomeric composition of cutin is also dependent on the method of depolymerization and analysis. Changes in monomeric composition may happen during isolation and storage, especially in polyunsaturated components (52). Attention should be paid to the current definition of cutin (polyester structure) when comparing monomeric compositions of some older work. As an example, Croteau and Fagersom (74) used saponification together with 4% NaI in AcOH-*i*-PrOH and 1% HI in AcOH-*i*-PrOH, which reduces peroxide and ether linkages, respectively, thus releasing possible cutan components as well.

Monomeric analysis after degradative methods may give an incomplete picture of the polymer, as reported by many authors (50, 51, 76, 77, 109-112, 112-114). It is therefore reasonable to assume that more heavily cross-linked regions in the polymer are less accessible to the reagents used (79). Nevertheless, monomeric compositions of plants have revealed the wide variety of compounds present in cutin, but the influence of polymorphic monomer composition on the polymeric structure and function is still very poorly understood.

#### 2.1.4.1 Cutin in different edible plants

Fruit cuticles can be considered as one of the most important targets when studying factors affecting shelf life and fruit quality e.g. fruit cracking, which commonly affect, for example, cherry tomatoes (115) and susceptibility to fungal diseases, often related to grape berries (116, 117). The most studied plant cutins are representatives of economically important food crops such as apple (49, 52), tomato (20, 23, 33, 47, 85, 86, 90, 115) and citrus fruits (46, 64, 81, 98). Only recently, studies concerning cuticle composition of the world's most important oilseeds, such as soy bean seeds (32) and rapeseeds, have been conducted. The plants for which information is available (Table 1) form a miscellaneous subset of species and still, the cuticles of important crop plants such as rice, most grains and sugarcane are either unknown or poorly understood.

Fruit peel contains usually more cutin per surface area than the leaf of the same plant. Grapefruit (var. Macfed) (46) fruit peel contained cutin 39 $\mu g/cm^2$ whereas leaves only contained 17 $\mu g/cm^2$. The lowest (0.2 $\mu g/cm^2$) and highest (342 $\mu g/cm^2$) amounts of aliphatic components were obtained from the juice-sac and chalazal region of the inner seed coat of the same grapefruits, respectively. The isolated cuticular membranes of spinach (*Spinacia oleoracia*) leaves have been found to contain 7-10 $\mu g/cm^2$ cutin, representing about 20% of the weight of solvent-extracted membranes (118). Apple fruit peel contains 7-15 times more

cutin per area than the surfaces of leaves and the amount is variety-dependent. Cutin content of the adaxial (upper) leaf membranes was higher than those of the abaxial (down) side (52).

In five cultivars of rose (*Rosa*) leaves, the total cutin monomer content was very similar between the adaxial and abaxial sides of the leaves, but the proportion (%) of the most abundant monomer, 10,16-dihydroxyhexadecanoic acid was slightly more abundant on the adaxial side compared with the abaxial side (75).

Analysis of the leaf, fruit peel, juice-sac and inner seed coat of grapefruit (var. Macfed) (46) revealed great differences in the relative proportions of the most abundant monomers, x,ω-dihydroxyhexadecanoic (90%, 32%, 30% and 2%, respectively) and ω-hydroxy-x-oxohexadecanoic (0%, 61%, 26% and 0%, respectively), in addition to differences in the positional isomeric distribution of these monomers. The seed coat was found to contain suberin-type polymer in the chalazal region (43% of α,ω-acids), but the rest of the seed coat was more similar to juice-sac monomer composition and fruit peel ultrastructure, and was thus classified as cutin (46).

The hydroxy acid composition of apple cutin was different in various anatomical parts (52, 54). The fruit peel, leaves, flower petals, stigma and inner seed coat contained 73%, 35%, 14%, 12% and 80% of hydroxy and hydroxyepoxy-$C_{18}$-acids, respectively. The corresponding values for dihydroxyhexadecanoic acid content were 21%, 58%, 77%, 77% and 16%, respectively. The inner seed coat cutin was most similar to the fruit peel cutin in its monomer composition, the only difference being a smaller amount of 18-hydroxyoctadecen-9-enoic acid (54). Minor compositional variation was found between different apple cultivars (52), but not between sweet cherry cultivars (77).

The cutin composition of the inner and outer seed coats of soybean (var. Merr) has been found to differ from each other and from the leaves and pods of the corresponding plant (32). Seed cuticles contained unusual monomeric components predominated by 2-hydroxy and ω-hydroxy fatty acids, with an absence of mid-chain hydroxylated monomers.

In general, the cutins of angiosperm plants may be roughly divided to three categories: $C_{16}$, $C_{18}$ and $C_{16}$-$C_{18}$ mixture type compositions. Peel cutins of citrus fruits (46, 97, 98), sweet cherry (*Prunus avium*) (77) and papaya (*Carica papaya*) (66, 99) consist mainly of only $C_{16}$-monomers, while the cutin from spinach leaves has almost entirely $C_{18}$-monomers (118). Cutins of pear (*Pyrus communis*) and peach (*Prunus persica*) fruits (66) and apple (52) leaves and the juice-sac of grapefruit (46) contain almost equal amounts of monomers from both groups. Gymnosperm and primitive plants, such as moss and liverwort, usually contain very little of $C_{18}$-acids (48, 63).

Epoxy hydroxy acids are general monomers of angiosperm plant cutin, consisting of 9,10-epoxy-18-hydroxyoctadecanoic and its monounsaturated counterpart. Epoxy acids are not

rare in seed oil triglycerides either, and are formed by epoxidation of one of the double bonds of oleic, linoleic or linolenic acids (65). High amounts of epoxy cutin acids are present in spinach (73%) (118), barley (29%) (119) and rye leaves (*Secale cereale*) (73%) (120). In addition to the most common epoxy hydroxy monomers, 9,10-epoxy-octadecan-1,18-dioic acid and 9,10-epoxy-12,18-dihydroxy-$C_{18}$ acids have been found in spinach leaves (118).

The sorptive characteristics of cuticles are influenced by their composition (35, 38). Cuticles containing a high amount of epoxy acids covalently bind chemical compounds containing a carboxyl function, thus increasing the persistency of such compounds in nature (35). Those also influence on the structural architecture by limiting cross-linking (65). Lequeu *et al.* (41) found that reducing the synthesis of epoxy and hydroxy fatty acids in maize leaves by the inhibition of peroxygenase led to a restricted accumulation of the whole cuticle, seen by a dramatic decrease in cuticle thickness. Also the protective effect against pesticides and fungal infection was influenced, which proved the evident importance of these compounds in various functions of the cuticle (41).

Usually oxo- and epoxy-derivatives of hydroxy-$C_{16}$- and -$C_{18}$-acids, respectively, are not present in same cutin polymer, except for grapefruit juice-sacs, which contain almost equal amounts of hydroxy-oxo-$C_{16}$- (25.8%) and epoxy-hydroxy-$C_{18}$-acids (22.6%) (46). As another exception, spinach leaves have been found to contain a small amount of 18-hydroxy-oxo-$C_{18}$-acid in addition to their very high epoxy acid content (118).

Cutin from the peels of mature citrus fruits, such as grapefruit, lemon and orange (*Citrus sinensis*), contain high amounts of ω-hydroxy-oxo-$C_{16}$-fatty acids, typically 50-60% depending on the species (46, 64, 98). Citrus leaf cutin usually does not contain these monomers, except for a minor amount in bitter orange (*Citrus aurantium*) leaf. In addition to citrus fruits, hydroxy-oxo-$C_{16}$-acids have been found in cape gooseberry (23%) and black currant (14%) cutin.

In addition to a large amount of ω-hydroxy-oxo-$C_{16}$-fatty acids, Espelie *et al*. (98) have reported the presence of 9,16-dihydroxy-10-oxohexadecanoic acid in several citrus fruit peel cutins. As citrus cutins contain substantial amounts of mid-chain keto fatty acids, which limits the potential for cross-links and branching, the presence of additionally mid-chain hydroxylated keto acid further increases the potential for a cross-linked matrix.

Monomers which only have a secondary hydroxyl group (not ω-OH) have been found from some cutins. For example, lime fruit cutin contains 10-hydroxyhexadecanoic acid (97, 99). In some cases, especially in lower plants, reduced $C_{16}$-compounds such as 1,8,16-hexadecantriol may be major monomers (1), although they can also be found in fruit cutins such as in lime (1,7,16-hexadecantriol) (81).

Also, dihydroxy acid monomers, other than those of hexadecanoic acid, are present in plant cutins. Dihydroxy-substituted monomers of $C_{18}$-acid have been detected as minor components of the fruit peels of pepper (51) and cranberry (74). Leaf cutin of wood apple contains both $C_{18}$- and $C_{20}$ -dihydroxy acids in addition to dihydroxy-$C_{16}$ acids (100). Minor amounts of odd chain dihydroxy acid monomers have been detected in e.g. tomato fruit cutin (51) (9/10,15-dihydroxypentadecanoic acid) and in coffee leaf cutin (69) (monohydroxy- and dihydroxypentadecanoic acid).

Trihydroxy acid monomers present in cutins are usually either $C_{16}$- or $C_{18}$-compounds, with those of $C_{18}$ dominating. Unsaturated 9,10,18-trihydroxy-$C_{18}$ acids prevail in addition to the saturated counterpart. Trihydroxyhexadecanoic acids have been found in citrus fruit cutins (<2%) (98) and from the peels of papaya (5.8%) (99). Tomato fruit cutin contains both of the common trihydroxy acids; 0.7% of trihydroxyhexadecanoic acid and 1.9% of trihydroxyoctadecanoic acid (51).

Monohydroxy dioic acids are usually present in cutins in low amounts. The unusual composition of wood apple leaf cutin contains over 20% of these suberin-like monomers (100). Also, sweet cherry fruit contains almost 20% dioic, hydroxy dioic and epoxy dioic acids (77). Most commonly, the monohydroxy dioic acids are 7/8-positional isomers of hydroxyhexadecan-1,16-dioic acid (for example wood apple leaves 15% (100) and coffee leaf 6% (69)), but also $C_{14}$- and $C_{15}$-chain lengths have been detected.

Monomers shorter than normal cutin monomers (< $C_{16}$) and branched chain hydroxy acid monomers may be detected in some cuticles. For example, wood apple leaf cutin contains about 17% $C_3$-$C_{15}$-compounds and 11% branched chain compounds (100). Recently, maize (*Zea mays*) leaf cutin has been found to contain α-hydroxy carboxylic acids after saponification by chromatographic analysis (76). Shao *et al*. (32) and Ranathunge *et al*. (53) have characterized unusual 2-hydroxy-$C_{22}$ – $C_{26}$ acids as major components of soy bean seed cutin layers. Rapeseed coat was found to contain branched-chain alcan-1-ols, but not branched-chain hydroxy acid monomers (55). α-Branched hydroxy acid monomers were detected in tomato cutin by NMR (85). These types of monomers may be more resistant to general degradation methods and may remain as oligomeric fragments, and thus, could be ignored in GC analysis because of their insufficient volatility.

**Table 1.** Composition of cutin from edible plants.

| Plant (*Latin*) | Organ | Depolymerization method | mono-mers % | mono-mers µg/cm$^2$ | ω-OH-$C_{16}$ | ω-OH-$C_{18}$ | Other mono-OH [a] | diOH-$C_{16}$ | diOH-$C_{18}$ | triOH-$C_{16}$ | triOH-$C_{18}$ | ω-OH-oxo-$C_{16}$ | ω-OH-epoxy-$C_{18}$ | Total $C_{16}$ | Total $C_{18}$ | Unsaturated | Aromatics | Others [b] | Unidentified | Ref. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| apple *(Malus pumila* var. Jonathan) | fruit peel | 1.3 M NaOMe in MeOH | 55 | 1050 | 2.8 | 16.9 | 0.4 | 23.4 | 0.4 | - | 15.1 | - | 34.8 | 26.2 | 67.2 | 42 | - | 3.1 | 3.5 | 52 |
| apple *(M.pumila)* | fruit peel | $LiAlH_4/LiAlD_4$ in THF | 87-94 | - | 6.6 [k] | 9.8 | - | 23.1 | [l] | - | 10.9 | - | 27.7 | 29.7 | 68.5 | 28 | - | 1.7 | - | 66 |
| apple *(M.pumila)* | fruit peel | 3% KOH in MeOH | 75 | - | 8 | 18 | 5 | 24 | (14) [n] | - | 27 | - | [o] | 32 | 45 | 21 | - | 2.2 | 5.3 | 68 |
| apple (*M.pumila* var. Dove) | fruit peel | 3% KOH in EtOH | - | | 3 | | | 18.9 | | | 6.5 | | | | | | | 1.4 | 39.5 [r] | 49 |
| apple *(M.pumila)* | inner seed coat | $LiAlD_4$ in THF | - | | 0.6 | 31.1 | 1.4 | 16 | - | - | 20.3 | - | 29 | 16.6 | 80 | 32.6 | - | 1.7 | - | 54 |
| apple (*M.pumila* var. Dove) | leaf | 3% KOH in EtOH | - | | 6.5 | | | 36.8 | | | 7 | | | | | | | 3.8 | 27.7 [r] | 49 |
| apple (*M.pumila* var. Jonathan) | leaf(adaxial) | 1.3 M NaOMe in MeOH | 80 | 130 | 2.7 | 0.8 | - | 37.1 | - | - | 8.2 | - | 33.2 | 39.8 | 42.2 | 6.3 | - | [f] | 18 [f] | 52 |
| beet (*Beta vulgaris)* | leaf | 3% KOH in EtOH | - | | - | | | - | | | - | | | | | | | 38.2 | 21 [r] | 49 |
| bitter orange (*Citrus aurantium)* | leaf | 50 mM NaOMe in MeOH | 53 | 68 | 2.6 | - | - | 69.9 | - | 1.1 | - | 1.4 | - | 75 | - | - | 0.1 | 20.2 [c] | 4.7 | 51 |
| broad bean (*Vicia faba*) | leaf | $LiAlH_4/LiAlD_4$ in THF | 60-70 | - | 7.1 | - | - | 84.9 | - | - | - | - | - | 92 | - | - | - | 6.6 | 1.4 | 66 |
| cherry (*Prunus avium* cv. Kordia) | fruit peel | 1 N MeOH/HCl | 26.5 | 35.1 SD 4 | 1.8 | 1.8 | | 53.6 | 1.5 | | 9.3 | | 6.5 [u] | 69.5 | 19.4 | 3.3 | 0.3 | 14.6 | 10.8 | 77 |
| coffee *(Coffea arabica)* | leaf | 4% KOH in EtOH | > 60 | 600 | 2.7 | - | 9.9 | 66.2 [e] | - | - | 1 | - | - | 67.2 | 1 | - | - | 9.3 | 10.9 | 69 |
| crabapple (*Malus* cv. "Golden hornet") | fruit | 3% KOH in EtOH | - | | 1.6 | | | 20.5 | | | 26.5 | | | | | | | 0.7 | 29.7 [r] | 49 |
| crabapple (*Malus* cv. "Golden hornet") | leaf | 3% KOH in EtOH | - | | 6.1 | | | 38.2 | | | 5.4 | | | | | | | 5.3 | 37.6 [r] | 49 |
| cranberry (*Vaccinium macrocarpon*) | fruit | 3% KOH in MeOH, 5%NaI + 1% HI in HOAc-isoPrOH | 90 [s] | | 2.3 | 12.1 | 0.3 | 16.7 | 2.5 | - | 44.3 | - | 10.3 | 19 | 68.6 | 14.4 | | 6.4 [t] | 5.2 | 74 |
| grape *(Vitis vinifera)* | fruit peel | $LiAlH_4/LiAlD_4$ in THF | > 80 | - | 1.3 [k] | 6.4 | - | 12.7 | [l] | - | 13.8 | - | 62.1 | 14 | 82.3 | 35.7 | - | 1 | - | 66 |
| grape (*V. vinifera* cv. Siebel) | fruit peel | 3% KOH in EtOH | - | | - | | | 16.7 | | | 7.5 | | | | | | | 2.2 | 40.3 [r] | 49 |

| Plant (*Latin*) | Organ | Depolymerization method | mono-mers % | mono-mers µg/cm² | ω-OH-$C_{16}$ | ω-OH-$C_{18}$ | Other mono-OH [a] | diOH-$C_{16}$ | diOH-$C_{18}$ | triOH-$C_{16}$ | triOH-$C_{18}$ | ω-OH-oxo-$C_{16}$ | ω-OH-epoxy-$C_{18}$ | Total $C_{16}$ | Total $C_{18}$ | Unsaturated | Aromatics | Others [b] | Unidentified | Ref. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| grape (*V. vinifera* cv. Riesling sylvaner) | fruit peel | 3% KOH in EtOH | | - | | - | | 17.9 | | | 14 | | | | | | | 3.3 | 43.0 [r] | 49 |
| grape vine (*V. vinifera* cv. Riesling sylvaner) | leaf | 3% KOH in EtOH | | - | | 4.4 | | 21.1 | | | 3.6 | | | | | | | 5.5 | 40.7 [r] | 49 |
| grape vine (*V. vinifera* cv. Siebel) | leaf | 3% KOH in EtOH | | - | | - | | 15.4 | | | 3.4 | | | | | | | 35.4 | 23.7 [r] | 49 |
| grapefruit *(Citrus paradisi)* | fruit peel | $LiAlH_4$/$LiAlD_4$ in THF | | - | | 1.3 | - | - | 29.7 | - | 1.9 | - | 61.8 [p] | - | 93.8 | - | - | - | 2.5 | 3.8 | 98 |
| grapefruit *(C. paradisi)* | fruit peel | $LiAlH_4$/$LiAlD_4$ in THF | - | | 39 | 2.3 | - | - | 31.9 | - | - | - | 61.4 | - | 95.6 | - | - | [i] | 3.5 | - | 46 |
| grapefruit (*C. paradisi*) | juice-sac | $LiAlH_4$/$LiAlD_4$ in THF | - | | 0.2 | 1.2 | - | - | 30.3 | - | - | 12.3 | 25.8 | 22.6 | 57.3 | 34.9 | 26.2 | [i] | 3.6 | - | 46 |
| grapefruit *(C. paradisi)* | leaf | $LiAlH_4$/$LiAlD_4$ in THF | - | | 13 | 1.4 | - | - | 89.5 | - | - | 2.2 | - | - | 91 | 2.2 | - | [i] | 6.6 | - | 46 |
| grapefruit *(C. paradisi)* | inner seed coat | $LiAlH_4$/$LiAlD_4$ in THF | | | 17 | 1.1 | 5.6 | 4.2 | 2.0 | - | - | 34.0 | - | 43.1 | 3.1 | 82.7 | 31.2 | [i] | 10.0 | - | 46 |
| grapefruit *(C. paradisi)* | chalazal region of seed | $LiAlH_4$/$LiAlD_4$ in THF | | | 342 | 7.8 | 19.6 | 18.5 | 0.0 | - | - | 1.0 | - | 10.2 | 7.8 | 30.8 | 27.2 | [i] | 42.9 | - | 46 |
| lemon *(C. limon)* | fruit peel | $LiAlH_4$/$LiAlD_4$ in THF | | - | | 2.1 | - | - | 29.1 | - | 0.8 | - | 66.1 [p] | - | 98.1 | - | - | - | 1.3 | 0.6 | 98 |
| lettuce (*Lactuca sativa)* | leaf | 3% KOH in EtOH | | - | | - | | | 4.2 | | | - | | | | | | | 59.6 | 11.1 [r] | 49 |
| lime *(Citrus aurantifolia)* | fruit peel | TMAH 250°C | | - | | 12.8 | - | 3.8 | 37 | - | - | - | 3.9 | [m] | 57.5 | - | - | 5.8 | 20.8 | 15.5 | 99 |
| lime *(C. aurantifolia)* | fruit peel | $LiAlH_4$/$LiAlD_4$ in THF | | - | | 0.2 | - | - | 45.4 | - | 0.1 | - | 53.3 [p] | - | 99 | - | - | - | 0.1 | 0.9 | 98 |
| lime (*C. aurantifolia)* | leaf | 3% KOH in EtOH | | - | | - | | | 69.3 | | | - | | | | | | | 6.3 | 12.6 [r] | 49 |
| onion *(Allium cepa)* | leaf | 3% KOH in EtOH | | - | | - | | | 24.8 | | | 10.4 | | | | | | | 9.8 | 42.5 [r] | 49 |
| orange (*Citrus sinensis)* | fruit peel | $LiAlH_4$/$LiAlD_4$ in THF | | - | | 1 | - | - | 36.3 | - | 0.2 | - | 62.1 [p] | - | 99.6 | - | - | - | 0.4 | - | 98 |
| papaya *(Carica papaya)* | fruit peel | TMAH 250°C | | - | | 3.2 | - | - | 25.9 | - | 5.8 | - | - | [m] | 34.9 | - | - | 0 | 58.4 | 6.7 | 99 |
| papaya *(C. papaya)* | fruit peel | $LiAlH_4$/$LiAlD_4$ in THF | > 80 | - | | 4.2 [k] | - | - | 74.4 | [l] | - | - | - | - | 78.6 | - | - | - | 14.2 | 7.2 | 66 |

| Plant (*Latin*) | Organ | Depolymerization method | mono-mers % | mono-mers µg/cm$^2$ | ω-OH-$C_{16}$ | ω-OH-$C_{18}$ | Other mono-OH [a] | diOH-$C_{16}$ | diOH-$C_{18}$ | triOH-$C_{16}$ | triOH-$C_{18}$ | ω-OH-oxo-$C_{16}$ | ω-OH-epoxy-$C_{18}$ | Total $C_{16}$ | Total $C_{18}$ | Unsaturated | Aromatics | Others [b] | Unidentified | Ref. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| peach *(Prunus persica)* | fruit peel | $LiAlH_4/LiAlD_4$ in THF | 25 | - | 0.9 [k] | 6.9 | - | 33 | [l] | - | 17.8 | - | 39.6 | 33.9 | 64.2 | 20.4 | - | 1.9 | - | 66 |
| pear *(Pyrus communis)* | fruit peel | $LiAlH_4/LiAlD_4$ in THF | > 80 | - | 2.7 [k] | 12.8 | - | 44.8 | [l] | - | 7.7 | - | 31.4 | 47.5 | 52 | 37.1 | - | 0.6 | - | 66 |
| pepper *(Capsicum annuum)* | fruit peel | 50 mM NaOMe in MeOH | 83 | 1513 | 3.5 | 0.2 | - | 64.4 | 3.1 | - | 7.7 | 1 | 1 | 68.9 | 12 | 3.6 | 1.6 | 6.2 [c] | 11.3 | 51 |
| rosehip (*Rosa canina)* | fruit | 3% KOH in EtOH | | - | 1.9 | | | 65.3 | | | - | | | | | | | 1.1 | 14.8 [r] | 49 |
| spinach (*Spinacia oleracea*) | leaf | 3% KOH in EtOH | | - | - | | | 2.8 | | | - | | | | | | | 26.9 | 5.7 [r] | 49 |
| spinach *(S. oleracea)* | leaf | 1.3 M NaOMe in MeOH | ~ 20 | 7-10 | 0.3 | 2.9 | - | 2.9 | - | - | 13.4 | 0.4 [f] | 73.0 [g] | 3.2 | 89.7 | 4.6 | - | 2.8 | 4.3 | 118 |
| tomato (*Solanum lycopersicum)* | fruit peel | 50 mM NaOMe in MeOH | 85 | 717 | 4.2 | 0.5 | - | 82.5 [j] | 1 | 0.7 | 1.9 | 2 | - | 88.4 | 3.4 | 0.7 | 0.2 | 4.3 [c] | 2.7 | 51 |
| tomato (*S. lycopersicum)* | fruit peel | 3% KOH in EtOH | | - | 5.2 | | | 71.1 | | | 2.6 | | | | | | | 1.2 | 12.4 [r] | 49 |
| tomato (*S. lycopersicum)* | leaf | 3% KOH in EtOH | | - | - | | | 5.5 | | | tr | | | | | | | 38.6 | 14.9 [r] | 49 |
| white deadnettle (*Lamium album)* | leaf | 3% KOH in EtOH | | - | - | | | 36.8 | | | - | | | | | | | 6.9 | 14.6 [r] | 49 |
| wood apple *(Limonia acidissima)* | leaf | 3% KOH in EtOH | | - | 0.9 | - | 14.1 | 27.9 | 12.0 [d] | - | - | - | - | 50 | 1.5 | - | 5 | 25.6 | 14.3 | 100 |

a) Includes (ω-1)-OH-, 2-OH-, 10-OH-$C_{16}$-acids, branched and < $C_{16}$ and >$C_{18}$ ω-OH-acids. b) Includes fatty acids, alcohols, OH-diacids, diacids. c) Includes also glycerol. d) Includes 10.5% 10,20-di-OH-$C_{20}$-acid. e) Includes 1.7% di-OH-$C_{15}$-acid. f) 18-OH-oxo-octadecanoic acid. g) Includes 7.1% of 9,10-epoxyoctadecan-1,18-dioic acid and 1.6% 9,10-epoxy-12,18-di-OH $C_{18}$-acid. h) Others included in unknowns if present. i) Only aliphatic compounds detected. j) Includes 1.0% di-OH-$C_{15}$-acid. k) May include 1,16-$C_{16}$-diacid due to the method used. l) diOH-$C_{18}$ acids not separated from e.g. epoxy-OH-$C_{18}$-acids due to the method used. m) Epoxy acids not identified due to the method used. n) Unidentified diOH-acid. o) Not separated from triOH-$C_{18}$-acids due to the methods used. p) Contain minor (0.1–4.2%) amounts of diOH-oxo-$C_{16}$-acids. q) 10,15-di-OH-$C_{16}$-acid. r) Other OH-acids not identified (included in unknowns). s) Also peroxide and ether linkages cleaved. t) 0.3% 8,9-diOH-1,17-dioic and 9,10-diOH-1,18-dioic acids. u) Includes 5.6% of 9,10-epoxy-octadecan-1,18-dioic acid.

### 2.1.4.2 Isomer composition of cutin monomers

The position of functional groups in the cutin monomers is suggested to have an important relationship to the biosynthetic pathways of cutin formation. The isomeric composition of dihydroxyhexadecanoic acid differs between plants, and between organs in the same plant, in addition to developmental stage. The most common isomers are usually the 10,16- and 9,16-, but 7,16- and 8,16-isomers have also been detected (Table 2). Cucumber (*Cucumis sativus*) cutin has been reported to contain 8,16-dihydroxyhexadecanoic acid as the major monomer (>70%) (96). The positional monomers and their relative composition may be deduced from their mass spectral fragmentation by the α-cleavage of the carbon atoms bearing the secondary hydroxyl group (67).

Another cutin monomer for which the positional isomeric distribution has been evaluated is 16-hydroxy-x-oxohexadecanoic acid. It has been found to be very similar between various citrus fruits and parts of the same fruit (Table 3). Similarly to dihydroxyhexadecanoic acid isomeric distribution, the keto group is also mainly located at position C-10. The main isomers in cape gooseberry have been found to be 9- and 10-oxo, together with minor amounts of 8-isomers, while black currant cutin 16-hydroxy-x-oxohexadecanoic acid isomerism has been found to be close to that of lemon (64). Deas *et al*. (64) has proposed that these compounds are derived from the oxidation of dihydroxy acids, as the isomeric composition was very similar between these monomers in the same cutin. The isomer composition of ω,x-hydroxyhexadecanoic acid and ω,x-hydroxypentadecanoic acid obtained from coffee leaf cutin are somewhat identical (69).

**Table 2.** Positional distribution of x,ω-diOH-$C_{16}$ acid isomers in different plants.

| Plant (*Latin*) | Organ | % of ω,*x*-diOH-$C_{16}$ acid | | | | Ref. |
|---|---|---|---|---|---|---|
| | | $C_7$ | $C_8$ | $C_9$ | $c_{10}$ | |
| apple (*Malus pumila*) | inner seed coat | - | 6 | 16 | 78 | 54 |
| apple (*Malus pumila*) | fruit peel | - | 6 | 18 | 76 | 52 |
| apple (*Malus pumila*) | fruit peel | - | 5 | 16 | 79 | 99 |
| coffee (*Coffea arabica*) | leaf | 3 | 11 | 56 | 30 | 67 |
| coffee (*Coffea arabica*) | leaf | 3 | 12 | 55 | 30 | 69 |
| crap apple (*Malus zumi*) | fruit peel | 2 | 8 | 21 | 69 | 67 |
| crap apple (*Malus zumi*) | leaf | 2 | 8 | 24 | 66 | 67 |
| grapefruit (*Citrus paradisi*) | leaf | 1 | 5 | 33 | 61 | 46 |
| grapefruit (*Citrus paradisi*) | fruit peel | - | 4 | 18 | 78 | 46 |
| grapefruit (*Citrus paradisi)* | fruit peel | - | 4 | 18 | 78 | 98 |
| grapefruit (*Citrus paradisi*) | juice-sac | 2 | 5 | 25 | 68 | 46 |
| grapefruit (*Citrus paradisi*) | inner seed coat | 0 | 5 | 16 | 79 | 46 |
| lemon (*Citrus limon*) | fruit peel | - | 6 | 15 | 79 | 98 |
| lime *(Citrus aurantifolia)* | fruit peel | - | - | 9 | 91 | 97 |
| lime *(Citrus aurantifolia)* | fruit peel | - | 4 | 16 | 80 | 98 |
| lime *(Citrus aurantifolia)* | fruit peel | - | 3 | 13 | 84 | 99 |
| lime *(Citrus aurantifolia)* | leaf | 2 | 5 | 30 | 63 | 67 |
| maize (*Zea mays*) | leaf | 9 | 13 | 71 | 7 | 67 |
| oat (*Avena sativa*) | leaf | 12 | 38 | 36 | 14 | 67 |
| orange (*Citrus sinensis*) | fruit peel | - | 5 | 21 | 74 | 98 |
| papaya (*Carica papaya*) | fruit peel | - | 1 | 98 | 1 | 99 |
| rosehip (*Rosa canina*) | fruit peel | 1 | 3 | 20 | 76 | 67 |
| spinach (*Spinacia oleracea*) | leaf | 13 | 31 | 25 | 41 | 118 |
| tomato (*Solanum lycopersicum)* | fruit peel | 2 | 9 | 12 | 77 | 67 |
| tomato (*Solanum lycopersicum)* | fruit peel | - | 6 | 8 | 86 | 99 |

**Table 3.** Positional distribution of ω-OH-x-oxo-$C_{16}$ acid isomers in different citrus fruits.

| Plant (*Latin*) | Organ | % of ωOH-*x*-oxo-$C_{16}$ acids | | | | Ref. |
|---|---|---|---|---|---|---|
| | | $C_7$ | $C_8$ | $C_9$ | $c_{10}$ | |
| grapefruit (*Citrus paradisi*) | fruit peel | - | 4 | 13 | 83 | 46 |
| grapefruit (*Citrus paradisi*) | juice-sac | - | 9 | 19 | 72 | 46 |
| grapefruit (*Citrus paradisi)* | fruit peel | - | 4 | 13 | 83 | 98 |
| lemon (*Citrus limon*) | fruit peel | 2 | 9 | 16 | 73 | 64 |
| lemon (*Citrus limon*) | fruit peel | - | 6 | 13 | 81 | 98 |
| lime *(Citrus aurantifolia)* | fruit peel | - | 5 | 17 | 78 | 98 |
| orange (*Citrus sinensis*) | fruit peel | - | 6 | 13 | 81 | 98 |

### 2.1.4.3 Cuticle development and cutin composition during different developmental stages

A limited amount of research has been performed related to cuticle and cutin development. The plant cuticle undergoes very large changes in area as cells expand during growth. Some organs which undergo rapid expansion have a very loose and wrinkled cuticle, which allows for increases in the surface area (24, 45).

Riederer and Schönherr (108) showed that kaffir lily leaf cuticle undergoes significant morphological changes during cell expansion, which continues long after the expansion has ceased. The cuticle was found to be very thin (73 nm) at the base i.e. the youngest part of the leaf, but rapidly increased in thickness (up to 140 nm) at maturity i.e. with distance from the leaf base. With increasing age, the cuticular layer becomes more resistant to ester-breaking reactions, thus changing from ester-bound cutin to non-ester cutin (108), which was discussed in detail in Chapter 2.1.2.

Domínguez *et al*. (115) studied cuticle formation microscopically, in addition to thickness measurements and compositional analysis of four genotypes of cherry tomatoes over 55 days from anthesis. They found a positive correlation of fruit growth (measured by fruit diameter) with the increase in cuticle thickness and the amount of cuticle and its cutin and polysaccharide components. The amount of cuticle, cutin and polysaccharides per unit surface area ($\mu g/cm^2$) reached its maximum at day 15 after anthesis and remained more or less constant until ripening (115). Kosma *et al*. (23) reported a continued increase in cutin content until red ripening in tomato genotypes with extended storage longevity.

Casado and Heredia (45) performed morphological and ultrastructural studies using different microscopic techniques and revealed that grape cuticle (var. Palomino fino) was already present at the early stages of growth, changed in appearance during rapid growth and formed a smooth, continuous and homogenous cuticle at a thickness of 3 µm at full maturity of the berry (45).

The content of grape berry (var. Pinot noir) cutin per unit surface area was found to decrease by a factor of 2.5 between young and mature berries, making the berry susceptible to fungal infection (116). The authors saw a correlation between the changes in cuticular microstructure, particularly in thinning of the primary cuticle at harvest. Kretschmer *et al*. (117) observed that Riesling-cultivar skin thinning was more pronounced resulting in berries that were more susceptible to fungal infection when compared with Pinot noir berries.

Peschel *et al*. (77) and Knoche *et al.* (29) investigated the composition of developing sweet cherry fruits. They reported a rapid increase in the amount of cuticle during the first stages of

development, followed by reduced cuticle synthesis during the rest of the growing period, resulting in impaired barrier properties of the thinned cuticular membrane.

These studies indicate differences in cuticle and cutin formation between plants and genotypes of the same plants. Thus based on these studies, no general conclusions can be made about cuticle development, although different mechanisms seem to occur between non-climacteric (such as cherry and grape) and climacteric (such as tomato) fruits. However, thick cuticles of certain monomer types with a nearly equal $C_{16}$ to $C_{18}$ ratio seem to provide better mechanical stability and fungal resistance. In addition to different ripening pathways, it is possible that environmental effects (light, precipitation, temperature, humidity) may be key factors in the faster or slower development of the cuticle.

Developmental changes occur in the monomeric composition during plant growth and ageing. Kolattukudy and coworkers (1, 16, 66) have suggested, after careful analysis of cutin compositions, that fast-growing plants and plant parts tend to have a higher content of $C_{16}$-group monomers. Leaf cutin from broad bean (*Vicia faba*) leaves contained up to 92% $C_{16}$-monomers (39). Conversely, cutin covering slow-growing winter rye was found to have a greater proportion of $C_{18}$- than $C_{16}$-monomers compared with faster-growing summer rye (120). This generalization can be argued, however, as $C_{18}$-monomers dominated over $C_{16}$-monomers in growing leaves of barley (*Hordeum vulgare*) (119).

The cutin of kaffir lily leaves contained 26 monomers in the young part, but only 13 monomers could be found in the older part of the leaf, although the major monomers were the same during ageing. With increasing age, the $C_{18}$-monomers became more important. The relationship between $C_{16}$- and $C_{18}$-monomers was 1:1.7 in the young parts and 1:3.5 in the mature parts of leaves. Furthermore, the number of secondary functional groups (possible sites of cross-linking) increased with age (108). On the contrary, growing barley leaves kept their cutin composition rather stable (study of 4 chosen monomers), although an increase in the total amount was detected between 10 and 80 mm from the leaf insertion (119).

The decrease found in cutin content of developing sweet cherry cutin was pointed out to be due to specific changes in cuticular wax and cutin monomer composition, in addition to an increase in fruit surface area (29, 77). The low or lacking deposition of midchain oxygenated hydroxy acids, 9/10,16-dihydroxyhexadecanoic acid and 9,10,18-trihydroxyoctadecanoic acid, caused changes in cutin monomer composition during development. A gradual increase in the ratio of $C_{16}$- to $C_{18}$-monomers from 2.2 to 3.5 was also observed (77).

Young parts of cutin may contain ω-oxo-derivatives as their major monomers, but the amount of these aldehydes diminished remarkably during plant development, and their detection might become impossible at a mature state (16). 9,10-epoxy-18-oxo-octadecanoic acid has

been found from very young apple fruit cutin (71) and 9/10-hydroxy-16-oxo-hexadecanoic acid was the major monomer of in cutin from the embryonic shoots of germinating broad bean and its developing leaves (72). In young tissues, terminal aldehyde-groups may act as an anchor between cutin polyester and proteins (with the formation of a Schiff's base with an amino group) or polysaccharides (forming an acetal with the hydroxy group) in the early development of the cuticle (72).

No significant differences were found in the qualitative composition of the cutins of young and mature coffee leaves, although some small quantitative differences were found. Also, the cutin monomer content released by hydrolysis of young membranes was slightly higher than from membranes isolated from mature leaves (69).

Das and Thakur (100) stated that the high abundance of dihydroxy acids (40.4%) and the hydroxy diacids (20.4%) and the absence of fatty acids together with very low levels of 16-hydroxyhexadecanoic acid could be taken as proof of a well-developed cutin structure.

Benítez *et al*. (86) compared young and ripe tomato cuticles by using FTIR, $^{13}C$ CP-MAS NMR and AFM methods. Using the signal ratios of methylenes (asymmetric and symmetric vibrations near 2900 $cm^{-1}$) and ester carbonyl C-O stretching (1730 $cm^{-1}$), they concluded that the ratio was lower (0.72) in ripe tomato cuticles than in young fruit cuticles (0.92), indicating higher cross-linking in ripe, mature cuticles. The weight per area was 1528 and 994 $\mu g/cm^2$ for ripe and young cuticles, respectively, again confirming the more dense structure in mature tomato cuticles. Spin relaxation times, as an indirect measure of chemical mobility, also suggested a more dynamically restricted environment in ripe cutin. Increased aromatic signals in both NMR and FTIR spectra were observed for ripe tomatoes when compared with young cuticles (86).

Cutin-bound phenolic compounds have been found to change during the maturation of tomato fruits (23, 101). The main phenolic monomer, coumaric acid, was found to increase from 2 $\mu g/cm^2$ to 24 $\mu g/cm^2$, which accounted for <1% and >2% of the total weight of the isolated membrane, respectively (101). Accumulation of phenolic acids continued during the climacteric phase when cuticular thickness remained fairly constant. Flavonoids, present in epicuticular waxes and cutin polymer, were absent in unripe fruits before the climacteric phase. Cutin-bound flavonoids, mainly naringenin and chalconaringenin, accounted for 30-43 $\mu g/cm^2$ (5%) of the total weight in ripe fruit cuticles, and the varieties were found to differ from each other (101). López-Casado *et al*. (33) concluded that the amount of phenolics in the cutin network of tomato fruit cuticle were the main candidates to explain the increased rigidity from mature green to red ripe cutin.

Shao *et al*. (32) concluded that a relatively high level of hydroxylated fatty acids in the outer cuticle of soy bean (var. Merr) seed correlated with a lack of cuticular cracks during development. Soy bean seed crackability, rather than permeation, was found to be the main factor behind soy bean seed softness and imbibition. Ranathunge *et al*. (53) reported a steady deposition of cutin monomers for six weeks post anthesis, after which cutin biosynthesis ceased while the seed kept expanding. Throughout seed development, the main cutin monomers remained the same. The ability of the seed to resist cracking was thus dependent on the strength of the cuticle with embedded polysaccharides without new cutin synthesis. This was supposed to result from the differences in cutin monomer-cell wall polysaccharide interactions and the elasticity of cuticle during the last stages of soy bean seed expansion. Microarray analysis showed that gene expression patterns changed massively during development, which could also be impacted on by environmental effects (53).

Molina *et al*. (55, 56) found that the deposition of rapeseed polyesters was not coupled with cell expansion and occurred fairly late in the seed maturation. Furthermore, compositional changes occurred right up to seed maturity. In particular, the deposition of 16-hydroxyhexadecanoic and 10,16-dihydroxyhexadecanoic acid was a very late event, contrary to the rapid deposition of 1,18-dioic acids.

#### 2.1.4.4 Environmental factors affecting composition

There are no studies which have focused specifically on the role of temperature or light on the synthesis of the cuticle related either to the cutin matrix or polysaccharides. Domínguez *et al*. (115) noted, while studying cherry tomato fruit cuticle development, that fruits at the same stage of growth (15 days from anthesis) harvested in late April 2007 contained a lower amount of cuticle (805.2 µg/cm$^2$) than fruits collected in May 2007 (1307.35 µg/cm$^2$), which was very similar to fruits from 2006 (harvested in early June). The explanation for this was found to be increased mean temperature (18°C to 21°C) and photosynthetic radiation (23 to 32 mol/m$^2$ per day). No differences very found in humidity (74% to 73%) between the investigated time points (April-May) (115).

Franich and Wolkman (121) suggested differentiation in the stem cutin monomers of Monterey pine (*Pinus radiate*) seedlings grown under winter and summer environmental conditions. The cutin grown under winter temperatures and light radiation contained twice the amount of 9,16-dihydroxyhexadecanoic acid (40%) than the corresponding summer cutin (20%). The authors concluded that the slow-growing winter cutin was significantly more cross-linked than summer cutin, which was composed of monomers capable of forming more linear polyester (121).

The cuticular membrane and monomer compositions have been studied for rye leaves grown at 5°C and 20°C. Lower temperatures caused an increase in lipid polymer, which could be seen as a thicker cuticle and two to four times more aliphatic polymeric material. Also, the proportions of the major monomers differed significantly. The relative amount of 18-hydroxy-9,10-epoxyoctadecanoic acid increased during the growth from 47% at 20°C to 73% at 5°C, while the amount of dihydroxyhexadecanoic acid decreased from 29% at 20°C to 8% at 5°C (120). Possibly, these changes in the polymer are an important factor in the cold-tolerance of the plant, both in terms of structural support and moisture control (1, 122, 123). Hunt and Baker (101) found that light stimulated the production of phenolic acids and flavonoids in tomato fruit cuticles, and that the effect was most profound for fruits receiving red light compared with red-deficient light and darkness.

## 2.2 SUBERIZED MEMBRANES

Even the concept of suberin is contradictory in the scientific forum. Some groups define suberin as a two-domain structure, containing an ester-bound mainly polyaliphatic domain and a non-ester polyaromatic domain (2, 8, 15, 124-135), while some restrict the term suberin only for the ester-bound domain composed mainly of aliphatic compounds with some aromatics (110, 136-140).

Suberin is essentially deposited in specific cell wall locations in external and internal tissues during plant development, such as the periderm of bark and tubers, but suberization also occurs in plants wherever and whenever a plant needs to form an effective diffusion barrier, such as after wounding (3, 6, 7, 14, 125, 129, 132, 135, 141, 141-143).

The periderm is formed during secondary growth to replace the epidermis in stems, branches and roots. The periderm is a three-layered system composed of phellem, phellogen and phelloderm. The outer phellem and inner phelloderm are formed due to the activity of the phellogen in between. The phellem cells are characterized by the formation of suberin, which is deposited internally to the cell wall. In most tree stems, one periderm layer is functional only during a limited period and it is substituted by a new functional periderm located on the inner side. In a single growth period, the number of layers of phellem cells that is produced varies in different species due to the activity of the phellogen, which also depends on environmental conditions such as light and temperature (142).

In tubers, suberization occurs in the phellem cells of the peel, the outer layer of the periderm. The phellem cells are produced by a single layer of phellogen cells which, upon maturation of tuber skin, become inactive (6, 7, 141, 143).

Suberin plays an important role in protection against environmental aggressions and pathogens, and in controlling temperature and water loss (137, 144, 145). Heavily suberized phellem minimizes mechanical freezing injury to grapevines by maintaining a low, constant moisture content in tree trunks through the winter (122, 123). The thickness and water permeability of suberized phellem has been found to correlate (143), as well as suberization with the elasticity of cotton fibers (*Gossypium* sp.) (146). The distinct polyaliphatic and polyaromatic domains of suberin have separate roles in the development of resistance to bacterial and fungal infections during suberization (145). The ester-bound hydroxycinnamic acids of suberin may be released by esterase activity of an attacking micro-organism, thus creating an antimicrobial environment (134).

Despite the physiological importance of suberin, its biosynthesis and deposition remain poorly understood. As suberin deposition occurs in specific tissues, and induced by wounding and stress caused by environmental factors, it must be the result of a complex regulation process. Some parts of the biosynthetic pathway are common for cutin and suberin. Fatty acid elongation and ω-oxidation represent two characteristic processes of suberin biosynthesis, as diagnostic suberin monomers contain very long chain derivatives ($>C_{18}$-$C_{30}$) and α,ω-diacids. Large variations in suberin monomer composition between species require different pathways, leading to mid-chain oxidation or unsaturation (15, 147). The biosynthesis of suberin aromatic compounds is even less understood, but apparently derives from phenylalanine, which is the precursor for nearly all phenylpropanoids (135).

Wound-induced suberization of potato disks has served as a useful model for suberin biosynthesis (125, 129, 132). The rate of suberization is influenced by genotype, age and environmental conditions, such as temperature and humidity (141). Wound-induced suberization of potato discs has been found to reach a constant level in 7 to 11 days at +20°C (144). Alkyl ferulates have been found to start accumulating in healing potato periderm 3-5 days after wounding (125). Yan and Stark (131) reported accumulation of both hydroxycinnamic acids and monolignols at day 1 after wounding, which stabilized after reaching a maximum in approximately 5 days at +25°C. Suberin-associated waxes are deposited with a similar time course to suberin aliphatic compounds (148).

In addition to aliphatic polyester and polyaromatic structures, suberized tissues contain large amounts of non-polymeric extractable material called suberin waxes (136). These compounds play crucial role in waterproofing the periderm together with the suberin matrix (8, 148, 149). Tegelaar *et al*. (150) have also suggested the presence of non-hydrolyzable aliphatic biomacromolecules called suberan in some angiosperm barks to explain finding suberinite material in fossils. Suberin, in addition to cutin, has been found in soil (59, 60), although cutin-hydrolyzing enzymes found in nature also hydrolyze suberin structures (16). The rate of degradation has been found to differ from approximately one year for leaf cutin to six years

for root suberin in studies of maize litter (151). This may be due to differences in the macromolecular structures and composition of polymers impacting on microbial access.

### 2.2.1 Location and occurrence

Suberin is located in the phellem cells of the periderm of plants, such as the bark of trees and the peel of tubers (Figure 7) (2, 8, 128, 137, 142). Suberized cells are also found in some internal tissues of plants, such as the endodermis and hypodermis (Casparian strip) of roots (Figure 8) (127, 134, 152), the chalazal region of seeds (46) and the secondary seed coat and fibers of cotton (146, 153, 154).

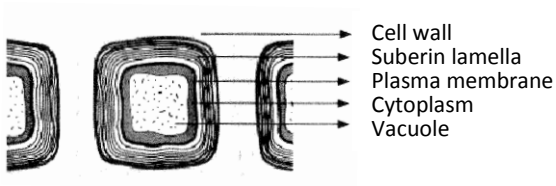



**Figure 7.** Schematic representation of simplified suberized phellem cells. Reproduced with permission (15). Copyright 2001 Wiley-VCH Verlag GmbH & Co. KGaA.

Suberin is additionally formed as a result of wounding to seal tissues, regardless of the plant part (1, 3, 8, 141). As an example, the wounding of grapefruit peel did not induce resynthesis of cutin as protective barrier; instead, suberin was found, according to NMR studies (155). Suberin has been found in cavities after an internal injury of potato tubers called hollow heart disorder (156). Suberization may be induced by stress, as has been shown in the cell walls of intercellular adhesion-strengthened potato tuber parenchyma (157) and grapevine trunks during winter (122, 123).

Generally, the phellem consists of 5-8 heavily suberized cell layers (158). In birch bark, in addition to heavily suberized cells, other layers of only slightly suberized cells have been found (158). Suberized cell walls are generally less than 1 µm thick (137). Although the number of cork cell layers has been found to be quite equal within plants, the thickness of the phellem has been found to vary between 50 µm and 330 µm due to large differences in the thickness of the individual cork cell layers between species and varieties. Thickening also happens as a result of post-harvest storage of tubers and during ageing of tree phellems (143). The number of cell layers in the phellem has usually been found to be greater in native periderm than in wound-induced periderm (7, 8). The thickness of suberin was estimated to be 50 nm in potato periderm 7 days after wounding (126). Potato has served as a popular substrate for isolation of suberized tissues, as it is easily available in large quantities. Almost everything that is known about wound-induced suberin has been obtained from incubated potato slices (8, 125, 129, 159).

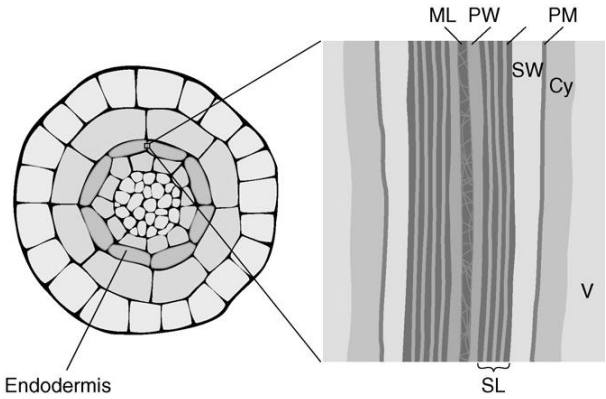



**Figure 8.** Schematic representation of suberin deposition in the endodermis of roots. ML, middle lamella; PW, primary cell wall; SL, suberin lamella; SW, secondary cell wall; PM, plasma membrane; Cy, cytoplasm; V, vacuole. Reproduced with permission (14). Copyright 2008 Elsevier.

The amount of suberin in the bark of trees differs greatly. The outer bark of the cork oak tree is an exceptionally suberin-rich tissue, known as commercial cork (140). The cork layer can grow up to several centimeters thick and is harvested as a renewable resource every 9 or 10 years (160). It has also been commercially exploited for hundreds of years, and is thus perhaps the most widely studied source of native suberin.

In a study of 15 different tree species, the amount of suberin varied between 8 and 62% of the extractive-free cork material, the highest being from golden chain (*Laburnum anaqyroides*) and birch (*Betula pendula*) and the lowest being from winged spindle (*Euonymus alatus*). Most of the tree barks contained 40 to 50% suberin, such as beech (*Fagus sylvatica*), cork oak, chestnut (*Castanea sativa*), English oak (*Quercus robur*) and aspen (*Populus tremula*) (139). In cork oak, the suberized cell walls contained polyaliphatic suberin (50%), polyaromatic compounds (30%) and polysaccharides (20%) as structural components (137, 142), but the proportions varied in different geographical locations (161). The suberin content of the stem and roots of apple tree (*M. pumila*) ranged between 15 to 35% of isolated cork layers, depending on cultivar and age (162).

In solid state NMR studies, suberin has been found to be attached to the cell wall discretely through both its aliphatic and aromatic domains (163). Suberin has been found to be spatially separated from polysaccharides and lignin (164). Gil *et al*. (165) found that most suberin aliphatics are mobile and close to aromatic compounds, while more hindered suberin is closer to the polysaccharide fraction of cork bark. Evidence on covalent bonding through the aromatic rings of suberin and the glycosides of cell walls have been found (131).

The suberin ultrastructure has demonstrated alternating electron-dense opaque and translucent lamellas by transmission electron microscopy (TEM) (137, 146, 154). The lighter

bands are thought to consist of suberin aliphatics and associated waxes, while the darker lamellas are composed of the polyaromatic domain (132, 148, 154). Microscopic studies have shown that the secondary wall accounts for most of the thickness and contains aliphatic suberin and some of the polyaromatics (Figure 9) (137).

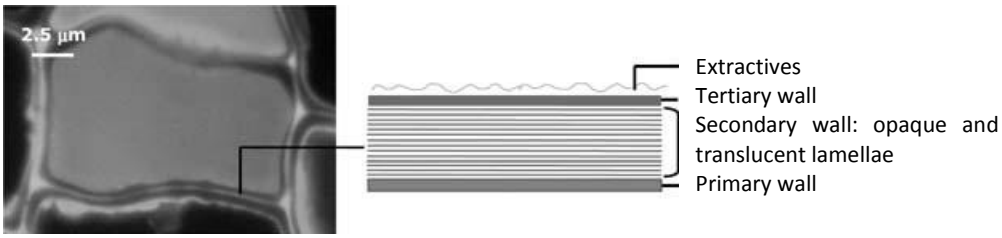



**Figure 9.** Cross-section of suberized cork cell (UV fluorescence microscopy). Reproduced with permission (137), Copyright 2007 Wiley-VCH Verlag GmbH & Co. KGaA.

### 2.2.2 Structure and composition

The macromolecular assembly of suberin is poorly understood (135, 137). In situ, suberin is an insoluble polymer which may be depolymerized by ester-breaking reactions (the aliphatic domain) and other degradative methods (the polyaromatic domain). Some structural pieces have been obtained after partial depolymerization reactions producing oligomers (110, 140, 159, 160, 166) and solid state NMR studies of the intact polymers (124, 126, 131-133, 149, 157, 161, 163-165, 167, 168). Recent development in methods utilizing ionic liquids may open new opportunities for studies on suberin, as it has been found to be soluble after suitable derivatization in an imidatzolium-based ionic solvent (169).

The variability in monomer composition affects the structure, although a common pattern of monomers have been identified from different plant sources (137). An excess of hydroxyl groups compared with carboxylic acid groups has been found in cork suberin depolymerizates (170). The aliphatic domain of suberin containing high amounts of mid-chain hydroxylated or epoxy groups, such as cork suberin, may establish relatively strong hydrogen bonding at mid-chain, in addition to covalent cross-links (137). Again, suberins with a small amount of mid chain polar substituents, like potato suberin, build up a different structure. NMR studies have shown that suberin organization in cork is distinct from that of suberin in potato tissue where higher motional restriction is found (164).

No free carboxyl groups have been detected in cork suberin (113, 168), unlike in cutin (80). Therefore, fatty acids with only one functional group must be esterified to the polymer in the terminal positions and all α,ω-diacids are esterified from both of their end groups. These

diacids may act as bridges between different glycerol molecules and other multifunctional monomers, allowing for cross-linking of the polymer (113).

Partial depolymerization reactions with methanolysis using calcium oxide or calcium hydroxide as catalysts have revealed several types of oligomers (Figure 10): linear dimeric esters of α,ω-dicarboxylic acids and ω-hydroxy acids and their monoacylglyceryl esters; monoacylglyceryl esters of α,ω-diacids, ω-hydroxy acids and fatty acids; diglycerol diesters of α,ω-diacids and diacylglyceryl esters of α,ω-diacids (110, 140, 160, 166). Acylglyceryl esters of almost all the aliphatic acids of potato suberin have been detected and generally occur at both the primary and secondary positions of glycerol (110). Glycerol, with its three esterifying positions, can continue the polymer in several planes and in opposite directions (166).

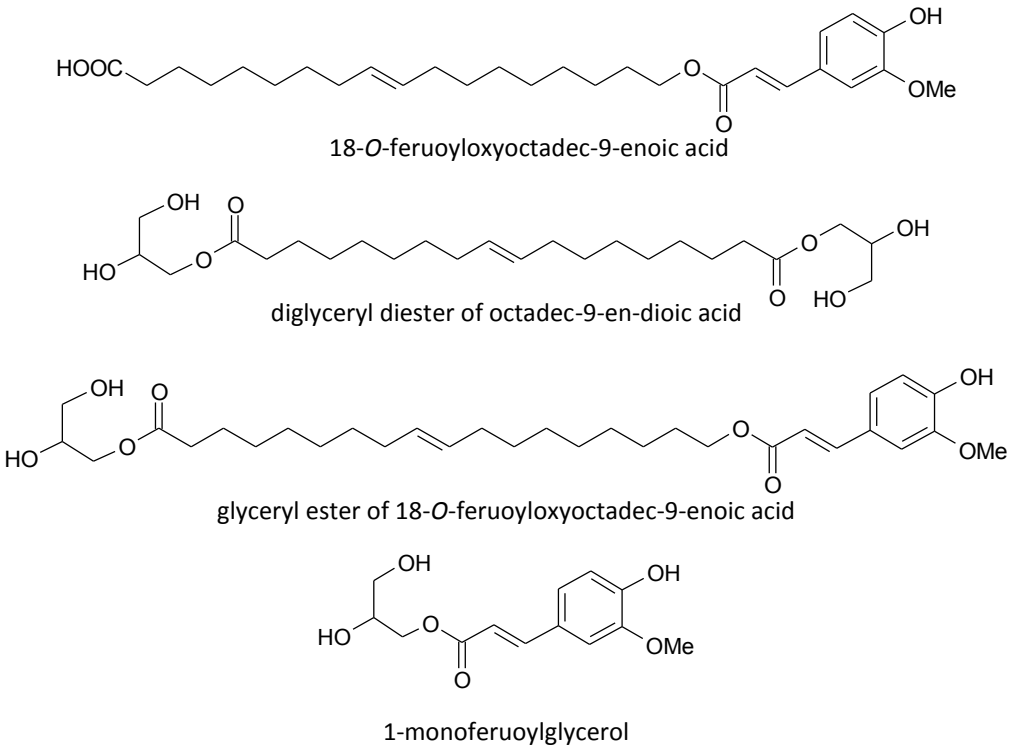



**Figure 10.** Examples of the oligomeric fragments of suberin.

Hydroxycinnamates are thought to play an important role in suberin structure and its linkages to the other components of suberized cell walls (136). Partial depolymerization using mild conditions have revealed feruloyl esters of ω-hydroxy acids and glycerol (110), in addition to dimeric hydroxycinnamates and dimeric structures of ferulic acid linked to hydroxycinnamic amines (136). As these compounds are directly bound to the aliphatic polyester of suberin, they are suggested to act as cross-linkers between the aliphatic domain and the aromatic

domain (110, 137). The polyaromatic domain consists of hydroxycinnamic acids and monolignol monomers in a highly cross-linked network (131).

Wang *et al*. (159) have reported triacylglycerol structures obtained via partial hydrolysis in methanolic KOH of wound-induced potato suberin, but not the above mentioned structures found by Graça *et al*. These triacylglycerols were of $C_{16}$- and $C_{18}$-homologs typical of potato suberin monomers, but also many $C_8$-$C_{12}$ species were found. However, these compounds do not possess any chemical moieties capable of covalent bonding with other parts of suberin, thus making their involvement in suberin structure inconsistent, but definitely interesting. In addition to triacylglycerols, Wang *et al*. (159) reported linear trimeric esters of fatty acids and ether-bound aromatic dimers solubilized after hydrolysis.

Solid state NMR studies have revealed important information about the structure and linkages of potato suberin. The ratio of rigid and mobile chain-methylene groups have been estimated to be in a ratio of 1:4 (126), and aromatic and unsaturated linkages outnumbered aliphatic methylenes in a ratio of 2:1 (124).

Bernards has proposed a two domain structure (Figure 11) for potato suberin in his review (135). This model is a thin two dimensional (2D) slice of a supposed 3D network which separates the macromolecule spatially and chemically into aliphatic ester-bound suberin and aromatic suberin domains which are covalently bound. The polyaromatic domain (where intermolecular linkages remain tenuous) in this model is located inside the primary cell wall and is covalently bound to cell wall polysaccharides (C). The aliphatic part also containing ester-bound phenolics accounts for the lamellate appearance of suberin in microscopic studies (135).

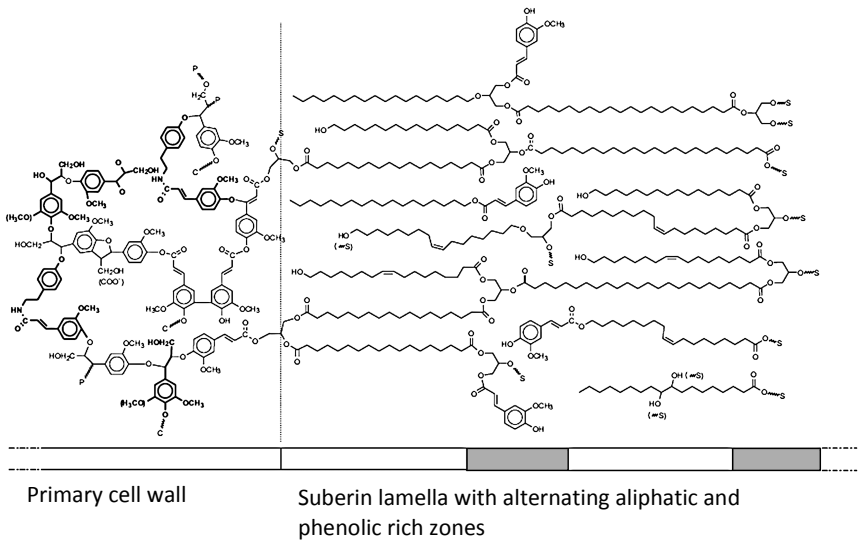



**Figure 11.** Bernards' model of suberin. Reproduced with permission (135). Copyright 2008, NRC Canada.

However, according to Pereira (142), this model does not fit well with the monomeric composition of cork oak suberin, because it is based mainly on findings from potato suberin. The results of oligomeric, monomeric and intact polymer analysis have lead Graça *et al*. to propose a slightly different model, mainly for the aliphatic ester-bound suberin structure, taking into account the monomeric composition of cork suberin (Figure 12). In this model, a monolayer of α,ω-acid monomers anchored by glycerol on both sides presents the basis of the structure. This glycerol-aliphatic structure is surrounded by polyaromatic structures, with ferulic acid being the linkage molecule between the domains. The translucent aliphatic lamellas and opaque lamellas of aromatics are connected by linear chains made of ω-hydroxy acids, which cross through the polyaromatic layers connecting the repeating lamellas of the suberin structure. In addition, intra- and intermolecular bonds may exist at the mid-chain positions of oxygen-containing substituted groups (137).

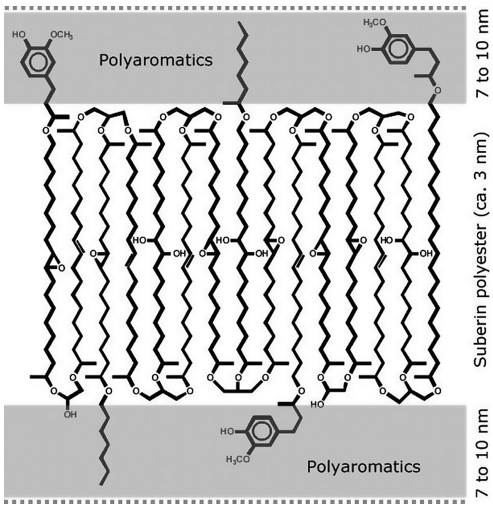



**Figure 12.** Proposed poly(acylglycerol) polyester model for suberin. Reproduced with permission (137), Copyright 2007 Wiley-VCH Verlag GmbH & Co. KGaA.

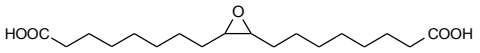
### 2.2.2.1 Aliphatic domain of suberin

The aliphatic domain of suberin consists of a complex network of esterified and usually substituted long chain α,ω-dicarboxylic acids, ω-hydroxy acids, fatty acids and alcohols. In addition, glycerol and hydroxycinnamic acid derivatives are an essential part of this structure, although it is called a polyaliphatic domain (135, 137, 142).

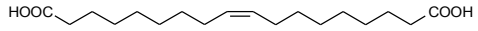
Suberin aliphatic acids have dominant chain lengths of 16, 18 and 22 carbons, of which the $C_{16}$ and $C_{22}$ are mostly saturated, while the $C_{18}$-compounds have mid-chain unsaturation, hydroxy or epoxy groups (128, 138, 160). Monomers up to $C_{32}$ have been found (128, 139). α,ω-

Dicarboxylic acids and ω-hydroxy acids are major components and other compounds are present in lower amounts. Generally, the monomers are very alike to cutin monomers, although longer chain lengths and greater proportions of α,ω-dicarboxylic acids have been found in suberin (Figure 13).

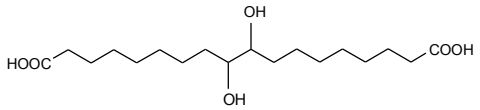
Some suberins have very low proportions of mid-chain oxidized monomers, such as in several tubers (128, 171) or *Arabidopsis* (18), while in some suberins the major components such as epoxy acids and diacids, constitute over 40% of the monomers, as seen in cork suberin aliphatics (113, 139) and birch outer bark (172). Of the unsaturated compounds, such as the major monomers of potato suberin, octadec-9-en-1,18-dioic and 18-hydroxyoctadecen-9-oic acids, both *cis*- and *trans*-isomers have been found (110).




9,10-epoxy-1,18-dioic-$C_{18}$ acid




1,18-dioic-$C_{18:1}$ acid




9,10-dihydroxy-1,18-dioic-$C_{18}$ acid

**Figure 13.** Examples of suberin aliphatic monomers.

Some unusual compounds can be essential components of some suberins such as *Ribes* species stem suberin which has been found to contain up to 10% of α,ω-diols (139, 173). 2-hydroxy fatty acids have been found as minor components of the endodermal cell wall suberin of the primary roots of maize (127) and Norway spruce (*Picea abies*) (95).

Glycerol is one of the main monomers and can account for up to 26% (Douglas fir, *Pseudotsuga menziesii*) of the total monomer mixture (109, 154), but is often left unanalyzed due to methodological choices (8, 112, 113, 128, 138, 139, 156, 162, 171, 173, 174). In cork oak, complete depolymerization of the aliphatic suberin domain gives about 14% glycerol (109), while the proportion in partial depolymerization can account up to 80% of the products, depending on conditions (140). In potato suberin, the content of glycerol is about 22% of the released monomers (110).

The hydroxycinnamates found after depolymerization of suberin polyester are commonly ferulic acid and coumaric acid. In addition, caffeic acid, vanillin, benzoic acid and hydroxycinnamic amines such as tyramine have been found (136). Depending on the method

of depolymerization, the amounts detected have been from <1-1.5% (111, 112) to 3.4%-7.6% (113, 174, 175) in cork. In tubers, covalently bound ferulic acid has been found in small proportions (<0.2%) (102). *Arabidopsis* suberin contains up to 5% ferulic and coumaric acids, although it is otherwise very similar to potato suberin (18).

In some studies, pentacyclic triterpenoids, such as cerin, friedelin, betulinic acid and betulin have been detected among the depolymerizates (112, 150), although they are not generally regarded as part of the polymer.

#### 2.2.2.2 Polyaromatic domain of suberin

After the removal of soluble extractives and ester-bound suberin polyaliphatics, a polyaromatic residue with residual cell wall polysaccharides remains from suberized tissues. In cork, this residue is about 40% of the extractive-free suberized cell walls and about 70% of potato natural periderm (136). A solid state NMR study of isolated potato suberin-enriched material showed that roughly 50% of the content was composed of polysaccharides while the rest was comprised of a 2:1 ratio of aromatic and aliphatic structures (124).

Structural studies of the polyaromatic domain of suberin have given slightly contradictory results depending on the methods used, similar to studies on cutan. The difficulty in performing structural studies is the fact that suberin itself cannot be isolated in its pure form using currently known methods (2).

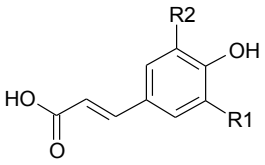
The suberin aromatic domain has been proposed to be a lignin-like structure, but even more cross-linked than usual wood lignins (130). A number of studies have shown that suberin polyaromatics have a low methoxyl content (136). The presence of fewer methoxyl groups may be the factor in enhancing the probability of extensive cross-linking (176).

The few degradative methods applied to the polyaromatic domain of suberin have been adopted from lignin research. Cottle and Kolattukudy (176) used nitrobenzene oxidation to release mostly vanillin and *p*-hydroxybenzaldehyde from wounded potato periderm. Vanillin is an aldehyde arising from the oxidation of 3-methoxyphenol phenylpropanoid units common to the ferulic acid and coniferyl alcohol of guaiacyl lignins, and *p*-hydroxybenzaldehyde arises from non-methoxylated phenylpropanoids (136). Lapierre *et al*. (130) utilized thioacidolysis in a study of the polyaromatic domain of natural and wound-induced suberin from potato, and found that the yield of aromatic monomers was about one tenth of that obtained from common wood lignins. Both *β-O*-4-linked guaiacyl (G) and syringyl (S) units were detected, and the amount in wound-induced suberin was twice as high as in the natural periderm samples. On the other hand, comparative analysis by nitrobenzene oxidation saw no differences between natural and wound-induced suberin (130). Negrel *et al*. (177) reported the release of

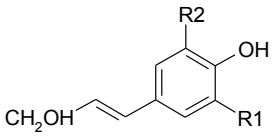
ether-bound ferulic acid amides by thioacidolysis from suberin-enriched samples of native and wound-induced potato periderm. Twice the amount of dicovalently linked ferulic acid amides were found from wound-induced potato periderm, indicating more efficient strengthening of the cell walls in wound periderm (177). In both thioacidolysis studies, the authors concluded that the localization of the released compounds was impossible to determine, thus the ferulic acid amides and phenylpropanoid units could originate from suberin itself or from other cell wall materials present in the suberin-enriched preparations (130, 177).

Solid state NMR studies after incorporating $^{14}$C-labelled precursors to wound-induced potato suberin have revealed the presence of both hydroxycinnamic acids and lignin-like monolignols that covalently cross-link in a dense network. However, differences in the amounts have been obtained as Bernards *et al*. (132) observed mainly hydroxycinnamic acids, while in studies by Yan and Stark (131) both components were found in roughly equal amounts. In the latter study (131), the authors found that the relative amounts of these aromatic building blocks depended on the details of the suberization protocol, including the concentrations of precursors, pH, oxygen concentration, timing and duration of the incubation and purification steps.

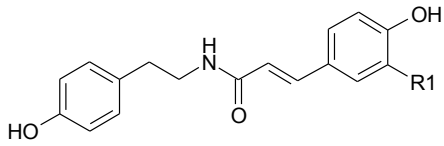
The insolubility of suberin and its polyphenolic domain has been overcome by using solubilization in imidatzole-based ionic liquids, such as 1-allyl-3-methylimidazolium chloride ([Amim]Cl). In this recent study (169), the polyaromatic fraction of suberin-enriched material from natural potato periderm was characterized in liquid form by phosphorus NMR ($^{31}$P NMR). The derivatized hydroxy groups of the solubilized material revealed the presence of *p*-hydroxyphenyl and guaiacyl structures in addition to the remaining polysaccharides. The phenolic group content of the residue was calculated to be 0.7 mmol/g (169).




R1 = R2 = H Coumaric acid
R1 = OH, R2 = H Caffeic acid
R1 = $OCH_3$, R2 = H Ferulic acid
R1 = R2 = $OCH_3$ Sinapic acid




R1 = R2 = H Coumaryl alcohol
R1 = OH, R2 = H Coniferyl alcohol
R1 = R2 = $OCH_3$ Sinapyl alcohol




R1 = H Coumaroyltyramine
R1 = $OCH_3$ Feruoylcoumarine

**Figure 14.** Phenolic precursors of suberized tissues.

This evidence points to the fact that suberized tissues contain a significant amount of hydroxycinnamic acids and a relatively small amount of monolignols compared to lignified tissues (129, 135). Graça and Santos (137) have recently proposed that suberized tissues contain two different populations of aromatic compounds: a domain closely related to

polyester suberin located in the secondary cell wall composed mostly of a non-lignin polyhydroxycinnamic acid polymer, and a second type of polyaromatic structure which is located in the primary cell wall and is thought to be a guaiacyl-type lignin, i.e. consisting of coniferyl alcohol monomers (137).

### 2.2.3 Variability of aliphatic suberin in different plants related to genetic background, age and environmental conditions

The relative proportions of each monomer group, the dominating chain lengths, together with the type and degree of substitution in the aliphatic chains are all quite variable among suberins from different plant origins. A large part of the studies on suberin have been made on periderm samples of different tree trunks, such as cork oak (111-113, 133, 138, 140, 161, 174, 175), birch (138, 160, 172), apple (162), *Ribes* (139, 173) and the roots of maize (127), apple tree (162) and kaffir lily (134). Only a few plant species with edible parts containing suberin, i.e. tubers (128), have been studied in addition to potato.

Complete depolymerization of aliphatic suberin from cork oak yields approximately 55-60% of the total material as soluble products (109, 160). The main chain lengths of α,ω-diacids and ω-hydroxy acids are $C_{18}$ and $C_{22}$, of which the $C_{18}$-monomers have mid-chain substitutions (unsaturation, dihydroxy, epoxy) while the $C_{22}$ monomers are saturated (140). Only small differences between virgin (25-30 year old first cork) and reproductive (collected every 9 years thereafter) cork suberin aliphatic composition has been observed, but variation between individual trees has been found to be more substantial (174). The suberin and polysaccharide content of cork was found to correlate with cork quality, but not with the geographic location of the cork trees (161).

Holloway (139) published a study on the suberin composition of 15 different tree barks and potato periderm, concluding that wide variation occurred in the relative proportions of different monomer classes. The investigated suberins could be roughly divided into two main chemical groups according to the relative amounts of more polar epoxy and dihydroxy $C_{18}$-monomers. In suberins containing a small amount of these polar monomers, the predominant monomers were simple ω-hydroxy acids and α,ω-dioic acids. Suberins with a higher polar $C_{18}$-monomer content had always less than 10% α,ω-dioic acids (139). The main differences between cork oak and birch suberin monomer composition have been reported to be the relative abundance of 9,10,18-trihydroxyoctadecanoic acid and 9,10-dihydroxyoctadecen-1,18-dioic acid, which accounted for 7.7%, 42.7% and 15.4%, 1.3% of cork and birch suberin monomers, respectively (138).

Holloway (173) studied the cork aliphatic suberin composition of six closely related *Ribes* species, including *R. americanum*, *R. nigrum*, *R. uva-crispa*, *R. houghtonianum*, *R. davidii* and *R.*

*futurum*. The aliphatic suberin content varied from 16% to 31% of dry weight and cork from young branches had the lowest suberin contents. The major constituents were α,ω-diacids and ω-hydroxy acids of $C_{16}$ to $C_{24}$ chain length, comprising about 80% of the total suberin acids. The composition was qualitatively common for all six species, but varied in quantitative composition. Qualitatively, the composition was similar to cork oak and birch bark suberin (138), but *Ribes* species showed a lesser degree of hydroxylation, a higher proportion of unsaturation and a shorter overall chain length (173).

Suberin content was found to decrease from 1-year-old to 3-year-old apple tree (var. Cox's Orange Pippin) stem and slight differences could be observed in monomer composition. The amount of epoxy-substituted monomers (9,10-epoxyoctadecan-1,18-dioic and 9,10-epoxy-18-hydroxyoctadecanoic acid) decreased from 19.8% to 14.5% and the corresponding hydroxylated monomers (9,10-dihydroxyoctadecan-1,18-dioic and 9,10,18-trihydroxyoctadecanoic acid) increased from 24.6% to 30.3%, respectively. There was also an increase in the relative amounts of hexacosanol, tetracosanoic, 22-hydroxydocosanoic and 24-hydroxytetracosanoic acids. Root suberin contained more epoxy compounds (40%) than stem suberin in same variety, but variety-dependent differences were also observed in monomer compositions. It was concluded that apple tree cork suberin most resembled those of cork oak and birch (162).

The endodermal cell wall suberin of the developing roots of maize has been found to change during maturation. The suberization increased and monomer composition changed in relation to root length. The young parts were characterized by higher amounts of carboxylic acids and 2-hydroxycarboxylic acids, while in mature parts, the typical suberin monomers ω-hydroxy acids and α,ω-diacids predominated. A shift towards longer chain lengths was also observed (127).

Ryser and Holloway (146) revealed that suberin is formed in secondary cell walls of cotton fibers of colored wild cottons, but not white cottons. Interestingly, over 65% of the monomers obtained from green lint cotton (*G. hirsutum* var. green lint) were 22-hydroxydocosanoic acid, followed by substantial amounts of 24-hydroxytetracosanoic acid and minor amounts of $C_{16}$- and $C_{18}$-monomers. The secondary seed coats of all the studied wild and cultivated cottons were found to contain suberin, and this was found to differ in composition from the leaf cutin and periderm suberin (146).

#### 2.2.3.1 Suberin in the edible parts of plants and its changes during storage

Potato suberin, both in the native (7, 8, 110, 139, 169, 171) and wounded form (7, 8, 132, 144, 159, 178) has been studied for suberization, aliphatic monomer composition and polyaromatic domain composition. Potato periderm suberin differs from the suberin of many of other

sources in that it contains very high amounts of unsaturated monomers, mainly octadec-9-enoic-1,18-diacid, and only very small amounts of hydroxylated polar monomers (139). Graça and Pereira (110) reported that potato suberin contains about 20% glycerol and less than 1% ester bound phenolics, including ferulic acid and coniferyl alcohol.

Wound-induced suberin, natural periderm suberin and suberin from the internal cavities of hollow heart disorder of potato tubers have been found to be ultrastructurally and chemically somewhat similar (144, 156). However, subsequent studies have established that the cell wall biochemistry differentiates these sources (7). Schreiber *et al*. (8) reported that isolated natural phellem consisted of 8±2 cell layers, which was independent of storage conditions, while wound phellem cell layers constantly increased during storage, up to 6±1 in 30 days. The aliphatic domain of suberin was found to be qualitatively similar between these suberin types, although present in smaller amounts in wound suberin (8).

The suberin composition of six tubers commonly used for food purposes (Table 4) were studied by Kolattukudy *et al*. (128). In all cases, ω-hydroxy acids and α,ω-diacids constituted the major components, similar to potato tubers (110, 171). Taxonomical relations of these tubers resembled their suberin compositions; carrot and parsnip (*Umbelliferae*) and rutabaga and turnip (*Cruciferae*) showed similar aliphatic suberin compositions. Red beet (*Chenapodiaceae*) appeared to have a distinct composition compared with the other tubers, although the ω-hydroxy acids and α,ω-diacids of this species were similar to turnip. Sweet potato (*Convolvulacea*) resembled the composition of closely-related potato (*Solanacea*e) (110, 171). Small portions of ferulic acid were found from the six tubers varying between 1.5 and 2.2 mg/g of suberin (102).

Post-harvest conditions affect the development of the phellem and suberin of potato tubers. According to Lendzian (143), the post-harvest storage of potatoes resulted in an increase in phellem thickness from 116 to 128 µm and in dry weight from 3.1 to 4.3 mg/cm$^2$ for the variety Combi N and from 85 to 98 µm and from 2.1 to 2.4 mg/cm$^2$ for the variety Erna, respectively, within two months. The changes occurred most clearly in high humidity with an ambient temperature of +20°C (143). Schreiber *et al*. (8) reported a 2-fold increase in the amount of suberin in isolated native phellem after one month's storage at room temperature. The wound suberin reached about 40% (75 µm/cm$^3$) of the amount of native suberin in the same time period. The mean chain length of the suberin aliphatic compounds was also found to increase during storage (8).

**Table 4.** Suberin monomer composition of the peels of edible tubers.

| Plant (*Latin*) | Depolymerization method | Suberin monomers % | $C_{16}$ dioic | $C_{18}$ dioic | diOH-$C_{18}$-dioic | epoxy-$C_{18}$-dioic | Other α,ω-dioic | ω-OH-$C_{16}$ | ω-OH-$C_{18}$ | ω-OH-epoxy-$C_{18}$ | triOH-$C_{18}$ | diOH-$C_{16}$ | Other ω-OH | Total $C_{16}$ | Total $C_{18}$ | Unsaturated | Aromatics | Other[f] | Alcohols and diols | Fatty acids | Unidentified | Ref. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| carrot *(Daucus carota)* | 14% $BF_3$ in MeOH 24 h | 36 | 6.3 | 16.7 | [b] | [b] | 1 | 7.1 | 23.7 | [b] | [b] | - | 4.9 | 13.4 | 40.4 | 29.7 | 9.0[c] | 3 | 3 | 14 | 25 | 128 |
| parsnip *(Pastinaca sativa)* | 14% $BF_3$ in MeOH 24 h | 34 | 6.4 | 16.7 | - | [b] | 3.6 | 9.5 | 25.5 | [b] | [b] | - | 1.8 | 15.9 | 42.2 | 28.2 | 7.0[c] | 4 | 4 | 14 | 21 | 128 |
| potato (*S.tuberosum* var. Russet Burbank) | 14% $BF_3$ in MeOH 48 h | 22-25 | 1 | 22.9 | - | [b] | - | tr | 15.3 | [b] | [b] | [b] | 0.7 | 1.1 | 38.2 | 38.2 | [e] | - | 6 | 8 | 46.0[d] | 171 |
| potato (*S. tuberosum*) | 0.012 M NaOMe in MeOH 3 h | 25 | 0.8 | 38.6 | 0.1 | - | 1.3 | 1.7 | 15.5 | - | tr | - | 1.8 | 2.5 | 54.1 | 54.4 | 0.7 | - | 3.2 | 10.8 | 24.9 | 110[a] |
| potato (*S. tuberosum* var. Whites) | 0.5 M NaOMe in MeOH | 12.1 | 0.4 | 32.2 | 1.5 | tr | 0.1 | 0.2 | 25.9 | 0.5 | tr | tr | 5.6 | 0.6 | 60.1 | 56.4 | 1.5 | - | 11.1 | 8.2 | 12.8 | 139 |
| red beet (*Beta vulgaris)* | 14% $BF_3$ in MeOH 24 h | 20 | 3.9 | 10 | - | [b] | 1.9 | 3 | 24.6 | [b] | - | [b] | 5.2 | 6.9 | 34.6 | 28.4 | 3.0[c] | 4 | 6 | 18 | 27 | 128 |
| rutabaga *(Brassica napobrassica)* | 14% $BF_3$ in MeOH 24 h | 49 | 9.4 | 10.4 | [b] | [b] | 2.3 | 14.9 | 20 | [b] | [b] | [b] | 5.9 | 24.3 | 30.4 | 23 | 8.0[c] | 3 | 5 | 4 | 30 | 128 |
| sweet potato (*Ipomoea batatas*) | 14% $BF_3$ in MeOH 24 h | 26 | 1.4 | 18.8 | - | [b] | 0.8 | 1.8 | 34.5 | [b] | [b] | - | 1.1 | 3.2 | 53.3 | 49.6 | 6.0[c] | 3 | 4 | 9 | 27 | 128 |
| turnip *(Brassica rapa)* | 14% $BF_3$ in MeOH 24 h | 30 | 4.7 | 15.2 | [b] | [b] | 2.2 | 10 | 36.4 | [b] | - | - | 6.6 | 14.7 | 51.6 | 37.7 | 5.0[c] | 3 | 3 | 8 | 21 | 128 |

a) original results mg/g converted to %. Glycerol (220.3 mg/g) was removed and proportions recalculated to correspond to other results. b) compound identified, but not quantified. c) whole phenolic fraction isolated from the soluble monomeric fraction after depolymerization. d) includes about 10% of polar hydroxy and epoxy acids and diacids which were not quantified. e) were not separated and are included in the unknowns. f) polar fraction including di- and trihydroxy and epoxy-substituted monomers.

## 2.3 METHODS OF ANALYSIS

The extracellular cuticular layer is attached to the epidermal cell walls *via* a pectin layer, and thus it can be isolated with adequate chemical and enzymatic treatments, although the boundaries between the layers can be intermingled. As suberin is attached to, and located inside of cell walls, only suberin-enriched fractions can be obtained for analysis and the residues always contain variable amounts of polysaccharides (1, 3). The suberin-enriched material, i.e. the suberized membrane, consists of the isolated phellem while other layers of the periderm (phellogen and phelloderm) are destroyed in the isolation process (8, 143). Seeds are complex organs and contain aliphatic polyesters, both cutin and suberin, in several locations of distinct cell layers (55, 179), thus making their isolation even more challenging.

### 2.3.1 Isolation of cuticular polymers and suberin-enriched membranes

Various chemical and enzymatic methods in addition to physical separation may be used to isolate the cuticular polymers and suberin from the remaining plant material (Figure 15).

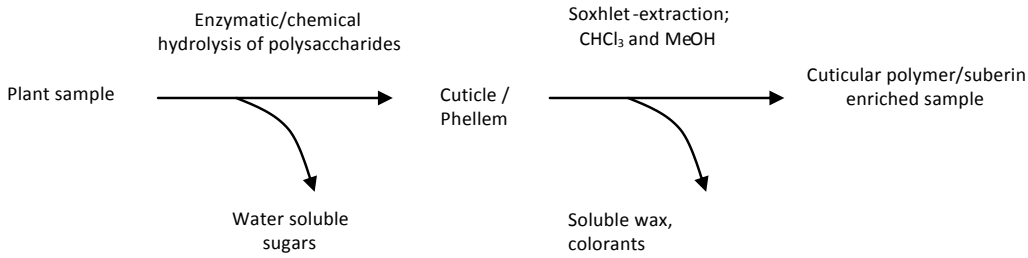



**Figure 15.** Schematic representation of the isolation of cuticular polymers and suberin.

Chemical methods for the removal of polysaccharides, without affecting the cutin or suberin, include treating the sample with ammonium oxalate-oxalic acid (1, 16, 66, 128), zinc chloride ($ZnCl_2$) together with hydrochloric acid (HCl) (63) or hydrofluoric acid (HF) (33, 87). In a study by Pacchiano *et al*. (92), oxalate was found to get trapped inside the polymer matrix, thus should be avoided. The use of HF has been found to be effective and does not affect the chemical nature of cutin, while the hydrolysis of polysaccharides with acids, such as HCl or $H_2SO_4$ in harsh conditions was found to cause microscopic holes and changes to the phenolic material of cutin (33).

Enzymatic methods include treating the plant material with pectinase, cellulase and hemicellulase in acetate or citrate buffer (1, 16, 66, 77, 171). The necessary incubation time and temperature are enzyme and sample dependent, and sodium azide ($NaN_3$) can be used to prevent microbial growth during incubation (33, 77).

Cuticular and suberin-associated waxes are removed by using extractions with different solvents. Frequently, a continuous Soxhlet-extraction vessel is used, and solvents include a repeating series or combinations of chloroform ($CHCl_3$), dichloromethane ($CH_2Cl_2$), ethanol (EtOH), methanol (MeOH) and water ($H_2O$) (1, 16, 51, 128).

Generally, several repeated isolation processes have been found to be more effective than one long treatment. Complete removal of cell wall materials from suberin samples depend on the methods used and have been reported to take up to 4 weeks (potato suberin) and even longer for the phellem layers of tree trunks (143). Before further analysis, the sample may be powdered and freeze-dried (1, 15, 16). The purity may be evaluated gravimetrically or using spectroscopic methods, such as solid state NMR and FTIR (33, 168).

More recently, *Arabidopsis* cutin has been analyzed from delipidated whole tissues without any isolation process (17). The authors were able to show that most polyester-derived products originated from the epidermis, but noticed a potential disadvantage in the presence of non-epicuticular polyester contaminants (e.g. suberin of the vascular system).

## 2.3.2 Depolymerization of cutin and aliphatic suberin

Cutin and aliphatic suberin polymers can be depolymerized using various methods to cleave ester bonds, yielding products that depend heavily on the methods used. Different chemical and enzymatic methods release monomers, oligomers or mixtures thereof, depending on the conditions used. The non-depolymerizable residue may contain highly resistant polymers, cutan and suberan, in addition to the polyaromatic domain of suberin and residual polysaccharides (1, 11, 15, 16). The most commonly used methods of cutin and suberin depolymerization are discussed in the following chapters.

Certain functional groups of cutin and suberin monomers (e.g. epoxy and aldehyde) may not be stable under some depolymerization conditions. Identification of these reactive groups may need additional derivatization before depolymerization e.g., transforming epoxy groups into their corresponding chlorohydrins (35, 108). These groups may also be derivatized *in situ* by using deuterated reagents. After such derivatization, the original position of the functional group in the intact polymer may be identified.

### 2.3.2.1 Chemical depolymerization methods

#### 2.3.2.1.1 Alkaline hydrolysis

Cutin polymer is effectively hydrolyzed by using alcoholic aqueous potassium hydroxide (KOH). A widely used alkaline depolymerization reaction includes hydrolysis with an excess of 3%

alcoholic aqueous potassium hydroxide (KOH), removal of alcohol, acidification with a mineral acid and extraction of the monomer acids (49, 68, 90, 100, 138).

Alkaline hydrolysis was extensively used in the early days of cutin analysis, but was largely substituted in the 1970s by the development of lithium aluminum hydride ($LiAlH_4$) reduction and methanolysis (65, 66) which better preserved the most labile functional groups, such as epoxides, in the reaction. In addition to $OH^-$-catalyzed hydrolysis, epoxy groups undergo acid-catalyzed reactions and the end products are dependent on the nature of the mineral acid and acidification conditions resulting in a mixture of products (65). Ekman (172) reported the preservation of epoxy acids when avoiding unnecessarily severe hydrolysis conditions and over-acidification. However, alkaline hydrolysis is best suited for analysis of polymers that do not contain epoxy groups such as tomato cutin, and has been used to produce both monomers and oligomers of tomato cutin by varying the conditions (90).

### 2.3.2.1.2 Reductive $LiAlH_4$ ($LiAlD_4$) hydrogenolysis

Reductive hydrogenolysis by $LiAlH_4$ in tetrahydrofuran (THF) to cleave ester bonds in cutin yields hydroxyl derivatives of the monomeric acids (alcohols, diols, triols, tetraols) which can be extracted from the reaction with diethylether (66, 144, 156, 171). By using deuterated $LiAlD_4$, the labile functional groups such as epoxides and carbonyls are D-labeled, thus are identifiable by mass spectrometry (Figure 16). Kolattukudy *et al*. developed this method in the early 1970s to establish the presence of epoxy- and oxo-derivatives among cutin monomers (66). However, this method does not permit the accurate quantitative determination of epoxy acids since the $C_{18}$-triol is formed from many other monomers as well (65).

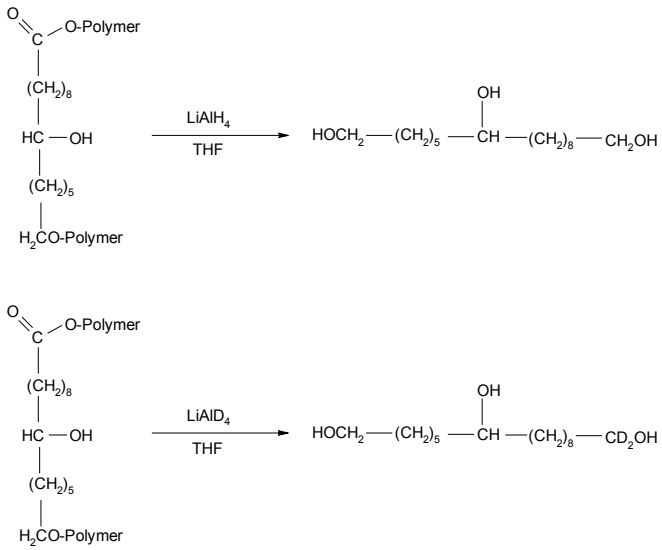



**Figure 16.** $LiAlH_4$/$LiAlD_4$ reduction of cutin polymer.

More recently, $LiAlH_4$ reduction has been used to reveal the cutin composition of *Arabidopsis*, but completed with data from transmethylation for identification purposes (17). Bonaventure *et al*. (17) asserted several advantages of using $LiAlH_4$/$LiAlD_4$ reduction, including the fact that polyunsaturated monomers remained unoxidized in reductive conditions, and obtaining simple mixture of products and D-labeling followed by GC-MS which allowed for the complete identification of monomers. However, other authors have also completed the results from hydrogenolysis with transesterification (144, 171).

Molina *et al*. (55) documented that one drawback of hydrogenolysis is due to the requirement of high resolution GC-MS to distinguish the degree of deuteration of fatty polyols in order to make assignments of their structures. For example, a 1,ω-diol may be derived from 1,ω-diol, ω-hydroxy fatty acid and/or 1,ω-dicarboxylic acid (55). $LiAlH_4$ reduction also cleaves peroxide bonds (66), thus it is not a suitable method according to the current definition of cutin.

#### 2.3.2.1.3 Transesterification

Frequently used transesterification employing either methanolic sodium methoxide (NaOMe) (51, 52, 65, 109, 110, 112, 113, 139), potassium methoxide (KOMe) (9, 159), boron trifluoride ($BF_3$) (5, 95, 108, 114, 128, 134, 144, 171) or methanolic HCl (20, 154) yield methyl esters of the monomers.

Using mild conditions and shorter reaction times, oligomeric components may be created (9, 51, 112, 113, 159). Bento *et al*. (113) compared several NaOMe concentrations (0.05% to 3%) and found out that a concentration of 1-3% NaOMe was sufficient for full cork suberin depolymerization, while 0.05% released only 80% of the monomers. Also, the monomer compositions were different, indicating different reactivity and/or accessibility of the monomers as alkanoic acids and α,ω-diacids which were more easily removed compared with ω-hydroxy acids (113). Those may also be located in sites which are less accessible to methanolysis, such as the core of the suberin polymer (112). Wang *et al*. (159) found that neither the overall or oligomer yield was related to KOMe concentration, but were affected by the duration of the reaction. In addition to the transesterification methods mentioned above, partial depolymerization of cork (140, 160) and potato suberin (110) has been achieved by using calcium oxide- (CaO) and calcium hydroxide- [$Ca(OH)_2$] catalyzed methanolysis.

After depolymerization, the monomers and oligomers formed can be extracted using organic solvent-water partitioning (112) or by precipitation (9). Using mild conditions (e.g. 50 mM NaOMe in MeOH), the monomer mixture may be analyzed without partitioning water-extraction, thus making possible the detection of water-soluble monomers e.g. glycerol simultaneously with aliphatic hydroxy acid monomers. However, mild conditions may result in incomplete depolymerization of cutin and suberin (51, 110).

Holloway and Deas (65) were among the first to describe a quantitative depolymerization method that revealed the abundant occurrence of epoxy acids in cutin and suberin depolymerizates. By using sodium methoxide (NaOMe) or sodium ethoxide (NaOEt) catalyzed transesterification, the epoxy monomers originally present in the polymer were successfully converted to their corresponding alkoxyhydrin alkyl ester isomers (Figure 17), providing a convenient method for their identification by GC-MS. With lower NaOMe concentrations, epoxy acids may survive unmodified (109).

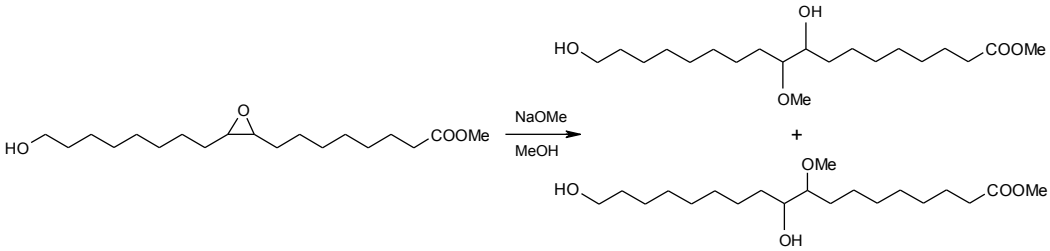



**Figure 17.** Opening of the epoxy ring in methanolysis.

One disadvantage of the methanolysis reaction is its vulnerability to moisture. Irreversible saponification competes with reversible transesterification when water is present in a methanolysis reaction. Saponification leads to the formation of free acids, although methylation is preferred in equilibrium. The formation of free acids may be prevented by using dried or anhydrous reagents, performing the reaction in a closed environment or under a nitrogen atmosphere (65, 97, 180). In some studies, methyl acetate has been used as a co-solvent to compete with saponification, with the disadvantage of producing acetylated monomers. This does not matter if full acetylation is used prior to gas chromatographic (GC) analysis (55), but becomes problematic if other derivatization protocols are being used.

Ray *et al*. (97) found in depolymerization studies of lime fruit cutin that transesterification (14% $BF_3$ in MeOH ($N_2$), 8 h) yielded fewer oxo-derivatives and more dihydroxy acids when compared with alkaline hydrolysis (1,5 M KOH in MeOH, 8 h). Mendez-Millan *et al*. (76) obtained only three cutin-specific monomers when using $BF_3$ in MeOH, compared to the eight monomers obtained with saponification (76). In both cases, the results were speculated to be due to the presence of these monomeric acids in the highly crosslinked areas of the biopolymer, which were not accessible to $BF_3$ acting as a catalyst and a Lewis acid (76, 97).

#### 2.3.2.1.4 Other chemical methods

Thermochemolysis with tetramethylammonium hydroxide (TMAH) has been used in the biopolymer analysis of cutin and suberin (10, 99, 174, 175). TMAH chemolysis is a one-step thermolysis and methylation, after which the monomeric products are immediately ready for gas chromatographic analysis, without any further derivatization. The hydroxylated sites

(COOH, OH) and ester bonds are converted by TMAH to methyl ethers and esters, thus producing methyl derivatives of the corresponding cutin and suberin aliphatic acids. The disadvantage of this method is that it converts epoxy compounds to the corresponding dimethoxy compounds, disabling the identification and separation of 9,10-epoxy-18-hydroxy-$C_{18}$-monomers from 9,10,18-trihydroxy-$C_{18}$-monomers (99), although some research has suggested that epoxides may survive intact (175). Methylation with TMAH does not always occur quantitatively for all free hydroxyl groups. In particular, primary alkanols are often not methylated unless they are in esterified form (175).

Ray *et al*. (82) used trimethylsilyl iodide (TMSI) to cleave the sterically-hindered ester bonds of secondary alcohols to yield oligomeric fragments (22%) of lime fruit cutin polymer. Trimethylsilyl iodide is a mild chemical reagent that can be used readily in an organic solvent under neutral conditions and at room temperature. In the method, iodine is introduced to the positions of secondary ester bonds, while leaving some of the primary bonds unattached. The monomers and oligomers identified contained all major monomers of lime fruit cutin, including ω-hydroxy-oxo-$C_{16}$ and 10,16-dihydroxyhexadecanoic acid (82).

Another chemical degradation method yielding oligomers is low-temperature hydrogen fluoride (HF) treatment. Fang *et al*. (78) used anhydrous HF at 0°C for 0.5 h to yield oligomers (23%) from lime fruit cutin, whose identifications were further resolved in a report by Tian *et al*., (79). HF is known to cleave sugars from glycoproteins (acetal bonds) and break glycosidic linkages of neutral sugars in polysaccharides. The oligomeric compounds from HF-treated lime fruit cutin were isolated as ethyl esters of carboxylic acids, due to workup with ethyl ether and free alcohol groups at the other end as a result of ester and acetal cleavage. This method allowed for the identification of a novel tetramer retaining an ester linkage between sugars from cell wall polysaccharides and cutin polyester, in addition to several dimer and trimer products (79).

#### 2.3.2.2 Enzymatic depolymerization

Esterases may be used to cleave cutin and suberin polyaliphatic ester polymers. Pancreatic lipases have been shown to hydrolyze cutin *in vitro*, releasing oligomers and monomers (1, 81, 181).

Brown and Kolattukudy (181) reported that pancreatic lipase was responsible for the hydrolysis of apple cutin, releasing oligomers and monomers. Ray and Stark (81) used porcine pancreatic lipase to release monomers and oligomers (2 w/w% of soluble product) from lime fruit cutin. This lipase was found to selectively cleave esters of primary alcohols, producing oligomers composed of monomers which were ester linked through secondary alcohol-primary carboxyl groups. A pentamer characterized by EI-MS, LC-MS and $^{1}$H NMR spectroscopy did not contain ω-hydroxy-n-oxo-$C_{16}$-monomers, although other major monomers of lime fruit

cutin were part of it. The authors concluded that the low yield of products and the absence of keto-monomers were due to steric hindrance of the cross-linked polyester structure and the lack of an aqueous environment which affected lipase activity (81).

Special polyester hydrolyzing enzymes called cutinases have been found to evolve in pollen and micro-organisms such as bacteria and fungi (1, 3, 16, 90, 182-189). A large number of publications concerning cutinase screening and characterization have been published, but are outside of the scope of this thesis. Cutinases are involved in microbial attacks, causing disruption of the cuticular and suberized barriers of the host plants during infection. The active site of cutinase contains serine, aspartate and histidine residues, which form a catalytic triad in a similar arrangement to those of serine esterases and several lipases (1, 183). In addition to cutin and suberin, cutinases are able to degrade a variety of synthetic polyesters and triacylglyserols (183, 186, 187). Biochemical characterization of bacterial and fungal cutinases have indicated similar substrate specificity and catalytic properties, but the thermostability, tolerance to detergents and organic solvents and optimum pH range of these enzymes are variable (187, 188).

Cutinases have been found to preferentially hydrolyze primary alcohol esters, making them suitable for producing oligomers that possibly contain cross-links of secondary alcohol ester bonds (1, 182). The first fungal cutinase was purified from *Fusarium solani pisi* (182). Fett *et al*. (184, 185) have screened large amounts of nonfilamentous (232 strains) and filamentous (45 strains) bacteria for cutinase activity by using apple or tomato cutin as the substrate. Only very few of these were found to be cutinase positive, thus demonstrating that cutinase activity is not widespread among bacteria. Osman *et al*. (90) reported on the treatment of tomato cutin with partially purified cutinase obtained from *Fusarium solani*. By HPLC-MS analysis, they only obtained ions in the mass area $M_r$ 100-1500 while the mass area $M_r$ 500-1500 lacked peaks, indicating that only monomers ($M_r$ 100-500) were produced (90). Kontkanen *et al*. (189) have purified and characterized a cutinase CcCUT1 from *Coprinus cinereus* that was able to depolymerize apple cutin and birch bark suberin, producing both oligomers and monomers. CcCUT1 cutinase was also used in the experimental part of this thesis work.

### 2.3.3 Cutan degrading methods

Virtually all cuticular preparations leave behind non-depolymerizable residues after exhaustive treatments with ester-breaking reagents. In some cases, this residue is mainly polysaccharides, but in certain species it may also contain non-ester cutin i.e., cutan. Some degradative methods have been used in the analysis of cutan in addition to spectroscopic and other non-invasive methods.

Pyrolysis-GC-MS has been used in studies of cutan and fossilized cuticles (61, 62, 105, 106). Flash pyrolysis achieves a thermal dissociation of macromolecular structures in an inert atmosphere

yielding smaller fragments. Pyrolysis coupled with GC for separation and MS for identification of low-molecular weight pyrolysis products provides a method of molecular characterization of insoluble high-molecular-weight material. Characteristic pyrolysis products of cutan are extended homologous series of $C_7$-$C_{35}$ n-alkanes, n-alkenes, n-α,ω-alkanedienes and long chain methyl ketones (62, 105). Nip *et al.* (61) also identified sugars derived from the polysaccharides of the cutan fractions of cuticles of agave, kaffir lily and sugar beet leaf (*Beta vulgaris*). Additionally, McKinney *et al.* (106) applied TMAH thermochemolysis to cutan analysis. TMAH thermochemolysis is highly selective for polar functionalities such as esters, phenols and acids. Thermochemolysis of agave cutan yielded mainly methyl esters of aliphatic and aromatic acids and methyl ethers of phenols. Methoxylated aromatic structures detected after TMAH thermochemolysis were not detected in pyrolysis-GC-MS, which was explained by the poor chromatographic behavior of unmethylated oxygen-substituted aromatic compounds (106).

Oxidative methods have been found to completely break down the cutan structure. Ruthenium tetroxide ($RuO_4$) oxidation has been found to cleave alkyl chains from aromatic moieties, double bonds and ether bonds. Schouten *et al*. (107) obtained very low yields of mono- and α,ω-dicarboxylic acids ($C_9$, $C_{26}$-$C_{34}$) from agave cutan. According to the authors, these products suggested a cutan structure of alkyl moieties bound to aromatic rings *via* ether or ester bonds. Complete solubilization of cutan isolated from leaf cuticles of agave and kaffir lily was obtained by ozonolysis (6 h at room temperature), which was followed by refluxing with 30% $H_2O_2$ and formic acid and GC analysis of trimethyl silylated products (87). Ozonolysis cleaves double bonds and ether bonds, forming the corresponding aldehydes and acids. Agave and kaffir lily leaf cutans yielded $C_4$-$C_{10}$ α,ω-dicarboxylic acids, which indicated the presence of unsaturation at critical positions in the aliphatic biopolymer in addition to ether bonds.

### 2.3.4 Polyaromatic suberin degrading methods

Classical lignin degradation methods have been used to depolymerize the polyaromatic domain of suberin. Cottle and Kolattukudy (176) used alkaline nitrobenzene oxidation followed by diethyl ether extraction to obtain aromatic aldehydes released from the polyaromatic domain. A disadvantage of this method is that the propyl chains of the phenylpropanoid monomeric units are broken, thus complicating identification of the released compounds (178).

Thioacidolysis, another common method of lignin degradation, releases the structures of labile alkyl-aryl ether bonds referred to as *β-O*-4-linkages (130, 134). The method releases thioethylated monomers of *p*-hydroxyphenyl (H), syringyl (S) and guaiacyl (G) units, which are all common to wood lignin. An advantage of thioacidolysis compared with nitrobenzene oxidation is its high selectivity towards *β-O*-4-linkages, thus releasing unambiguous lignin monomers. Although released, ester- or amide-linked hydroxycinnamic acids do not cause an

increase in the abovementioned thioethylated monomers, thus various phenolic structures do not interfere with this analysis (130). Negrel *et al*. (177) reported the release of thioetherified feruoyl amides by thioacidolysis, but the quantitative abilities of the method were questioned.

Pyrolysis coupled with GC-MS has been applied to wine cork samples. Thermal degradation at a temperature of 600°C in an inert atmosphere produced 75 identified products: phenolic compounds with guaiacyl, syringyl and p-hydroxyphenyl moieties originating from the suberin polyaromatic domain and lignin; hydrocarbons originating from the suberin aliphatic domain and associated waxes; and degradation products of polysaccharides (10).

### 2.3.5 Analysis and identification of monomers and oligomers

Cutin monomer composition has been analyzed using various chromatographic methods, including thin layer chromatography (TLC), GC and LC, of which GC combined with MS detection is the most commonly used method. These methods are often limited by the size and properties of the target molecules, such as restricted volatility in GC analysis even after derivatization. Identification methods based on NMR, HPLC coupled with different detectors and also direct MS methods, such as electrospray ionization tandem mass spectrometry (ESI MS/MS) have been developed with the purpose of analyzing oligomeric fragments (79, 81, 82, 140, 160). Usually, many different or combined methods must be used to add information in order to identify oligomeric fragments.

In this chapter, the three most frequently used techniques, GC-MS for monomers and HPLC and NMR for oligomers, are introduced. The main focus is on the GC-EI-MS method, which was used in the experimental part for monomer analyses, while the others are briefly discussed based on the literature.

#### 2.3.5.1 Gas chromatography – mass spectrometry

Gas chromatography (GC) is based on the separation of volatile compounds between the mobile phase (inert carrier gas) and the stationary phase of the column. The analytes are separated based on their interactions with the stationary phase, resulting in different elution times through the column to the detector where the compounds are detected at various retention times. Temperature program of the column oven affects the separation of the analytes. Detection yields a sample-specific chromatogram where the concentrations of the analytes in the sample are proportional to integrated peak areas (190).

Several types of detectors may be used in GC depending on the application. In the analysis of cutin and suberin monomers, a flame ionization detector (FID) and MS are generally used. In FID analysis, detection is based on ion formation when burning organic molecules in a hydrogen-air-flame, yielding a detector response for all compounds that are combustible.

Identification of the compounds is based on the retention times of reference compounds or retention indexes. In MS, the analytes are ionized to produce charged molecules or fragments and detected by their mass to charge (m/z) ratios. In addition to the total ion chromatogram, each peak yields a mass spectrum which can be used to identify the compound present in the sample. Ionization methods are variable such as chemical (CI) or electron impact (EI) ionization, of which the latter was used in the work contained in this thesis. In cutin and suberin monomer analyses, the use of MS detection is essential because many of the monomeric compounds are eluted with the same retention times due to their close similarity in structures (63). Thus, GC-MS is the preferable method for analyzing complex sample mixtures.

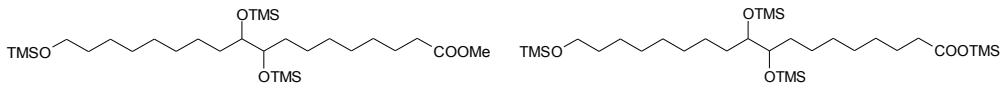
Cutin and suberin monomers have to be derivatized due to their polar functional groups to make them amenable to GC analysis (16, 68, 73, 191). The reduction in polarity may also improve the gas chromatographic properties of the compounds, yielding better peak shapes and more diagnostic fragmentation ions. Silylation is the most generally used derivatization procedure for GC-MS analyses (108, 191), but acetylation has also been used (55). Silyl derivatives are formed by displacement of the active proton in hydroxyl (-OH), thiol (-SH) or amine (-NH) groups by an alkylsilyl group in the silylation reagent to yield silyl ethers and/or esters (191).

The most commonly used trimethylsilylation (TMS) method is an easy derivatization step for which there are many commercial reagents available. Trimethylsilylated compounds are thermally stabile and highly volatile, thus having excellent chromatographic properties. TMS-groups [$(CH_3)_3Si$-] may also increase the total ion current, therefore increasing the sensitivity of detection (191).

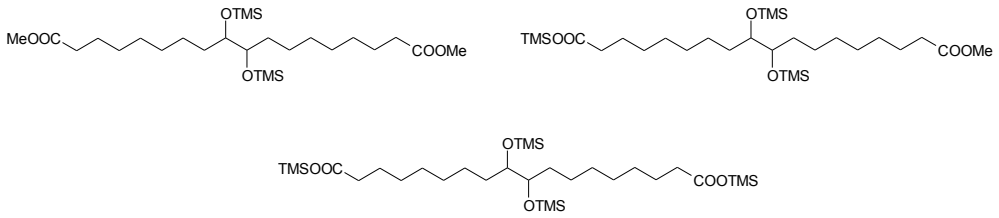
Identification of the locations of double bonds in the carbon chain is not unambiguous due to their migration in EI-MS. The location may be confirmed by chemical methods such as conversion of the double bond to the corresponding vicinal diol by osmium tetroxide ($OsO_4$) before analysis (68, 73, 128, 192).

#### 2.3.5.1.1 Typical fragmentation patterns of trimethylsilyl derivatives of ω-hydroxy acids and α,ω-dioic acids

Fragmentation patterns of aliphatic carbon chains containing functional groups such as carbonyl and hydroxyl groups are typically very specific for the compounds. The diagnostic ions enable identification of the monomers based on their mass spectra from even complex mixtures of compounds (16, 68, 73, 191). In addition, molecules with two functional groups may undergo rearrangements not found for those with either group alone (191), and added functionality leads to a variety of rearrangements (73).




9,10,18-hydroxyoctadecanoic acid methyl ester triTMS ether and TMS ester triTMS ether




9,10-hydroxyoctadecadioic acid dimethyl ester diTMS ether, methyl ester TMS ester diTMS ether and diTMS ester diTMS ether

**Figure 18.** Possible derivatives of dihydroxylated ω-hydroxy acids and α,ω-diacids after methanolysis and trimethylsilylation.

The following chapters concentrate on the typical fragmentation patterns of the major cutin and suberin monomers in GC-EI-MS after transesterification with NaOMe-catalyzed methanolysis and trimethylsilylation. Depending on the success of the methanolysis reaction (180), the monomeric compounds may be present in the product mixtures as methyl and/or TMS esters, while all the hydroxy groups are TMS etherified (Figure 18). During the early days of cutin analysis, the monomers obtained from depolymerization were methylated using diazomethane ($CH_2N_2$) (63, 68, 69, 138) and preliminarily fractionated by TLC before GC analysis (66, 68, 173) to simplify the challenging analysis of the mixtures of monomers.

*2.3.5.1.1.1 ω-hydroxy acids*

TMS-derivatives of aliphatic ω-hydroxy acids and their methyl esters give very clear diagnostic ions in EI-MS. The base peak is usually *m/z* 73 $[(CH_3)_3Si]^+$ or *m/z* 75 $[(CH_3)_2SiOH]^+$. Other common peaks, but of varying intensities are *m/z* 89 $[(CH_3)_3SiO]^+$, *m/z* 129 $[CH_2{=}CH{-}CH{=}OSi^+(CH_3)_3]$, *m/z* 103 $[CH_2{=}OSi^+(CH_3)_3]$ and *m/z* 117 $[(CH_3)_3SiO^+CH_2{=}CH_2]$. The molecular ion at the end of the spectrum is usually too weak, often remaining undetectable (73, 193).

TMS ethers of the ω-hydroxy acid methyl esters exhibit intense peaks at $[M\text{-}15]^+$ ($CH_3$ cleavage from the TMS-group) and $[M\text{-}47]^+$ ($CH_3OH$ cleavage from $[M\text{-}15]^+$) ions, in addition to a weaker $[M\text{-}31]^+$ ion (73, 191). In addition, $[M\text{-}90]^+$ ($[M\text{-}TMSiOH]^+$) and $[M\text{-}90\text{-}32]^+$ ($[M\text{-}TMSiOH\text{-}CH_3OH]^+$) are common (191). Increasing functionality of the compound, such as an additional epoxy group, reduces the intensity of the peaks at high end of the spectrum and changes the ratio of $[M\text{-}15]^+$, $[M\text{-}31]^+$ and $[M\text{-}47]^+$ ions (73).

Fragmentation of TMS ethers of the ω-hydroxy acid TMS esters yields typically intense $[M-15]^+$, $[M-31]^+$ and $[M-105]^+$ ($[M-TMSiOH-Me]^+$) ions (73, 191, 193).

Rearrangement of the TMS group in both TMS ethers of the ω-hydroxy acid methyl esters and TMS esters yield typical ions of *m/z* 146 $[(C_6H_{14}SiO_2]^\bullet$ and *m/z* 159 $[(C_7H_{15}SiO_2]^+$ for methyl esters and *m/z* 204 $[(C_8H_{20}Si_2O_2]^{\bullet+}$ and *m/z* 217 $[(C_9H_{21}Si_2O_2]^+$ for TMS esters, respectively (73, 191, 193). The TMS migration ions are formed through five- or eight-membered cyclic transition states and the formation appears to be more efficient for compounds with a chain length higher than $C_{14}$. In the case of shorter chain lengths, the formation of the TMS transfer competes strongly with the formation of *m/z* 147 $[(CH_3)_3SiO^+=Si(CH_3)_2]$ (191), which is typical for all compounds with more than one TMS-group (73, 191, 193).

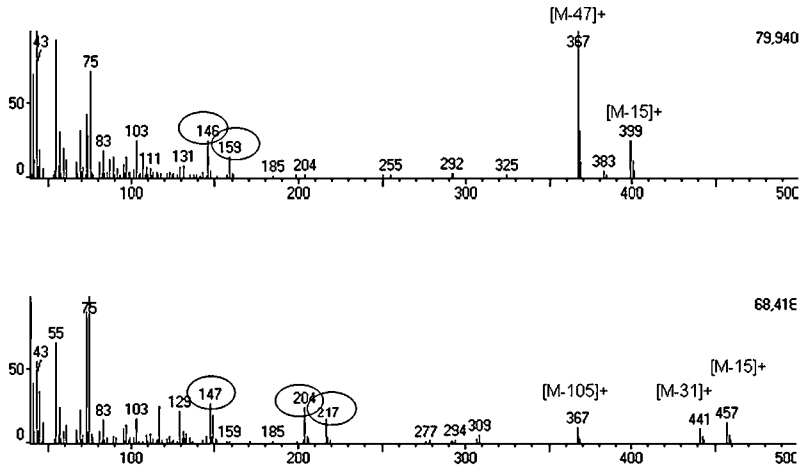



**Figure 19.** Examples of EI-MS spectra of the TMS ether of 20-hydroxyeicosanoic acid methyl ester (upper) and the TMS ester (down).

*2.3.5.1.1.2 α,ω-dicarboxylic acids*

Trimethylsilylation of the α,ω-dioic acids may result partly or fully TMS derivatized compounds, depending on the success of the methanolysis. Saturated and unsaturated methyl diesters of α,ω-dioic acids (no TMS groups) show differences in the mass spectra in the mass range of *m/z* 93-98. The *m/z* 98 peak is dominant in saturated diesters, while mono-unsaturated compounds have an additional strong peak at *m/z* 95 and di-unsaturated diesters display peaks at *m/z* 93, 94 and 95. The ions $[M-31]^+$ and $[M-73]^+$ are more intense for saturated compounds, while $[M-32]^+$, $[M-64]^+$ and $[M-74]^+$ are more intense for unsaturated compounds (68, 194).

In addition to the classical fragmentation patterns of trimethylsilylated long chain acids ($[M-15]^+$, *m/z* 73, 75, 103, 117, 129), a fragmentation ion at $[M-131]^+$ is produced from TMS

diesters. Migration of the TMS group yields rearrangement ions of *m/z* 204 and 217 for TMS diesters and *m/z* 146 and 159 for methyl ester TMS esters, just as in the case of TMS derivatives of ω-hydroxy acids. The transfer appears to be more intense between two TMS ester groups than with TMS ester and ether groups (191, 193).

*2.3.5.1.1.3 Middle chain substituents*

Long chain ω-hydroxy acids and α,ω-dioic acids that have middle chain substituents, such as derivatized hydroxyl groups, undergo α-cleavage on the either side of the carbon bearing the TMSO group (Figure 20). These cleavage ions enable identification of the location of the middle chain substituents. The cleavage ion containing the TMSO and carboxyl group of the molecule is usually more stable than the ion from the other cleavage (67, 73).

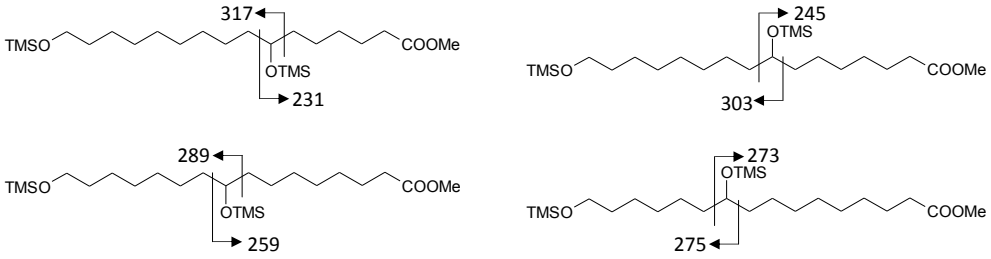



**Figure 20.** Examples of the α-cleavage of 7,16-, 8,16-, 9,16- and 10,16-dihydroxyhexadecanoic acid methyl ester bisTMS isomers.

In vicinal hydroxyl groups, the most preferable cleavage happens between the carbons bearing the TMSO groups (Figure 21). The two fragments from this cleavage form the most intense peaks at the end of the spectrum. The sum of formed cleavage ions will give the molecular weight of the compound. Usually in cutin and suberin monomers, the vicinal hydroxyl groups are in positions of 9 and 10 of octadecanoic acid, which yields a rearrangement ion of *m/z* 332 for methyl ester TMS ether (73) and of *m/z* 390 for TMS ester TMS ether.

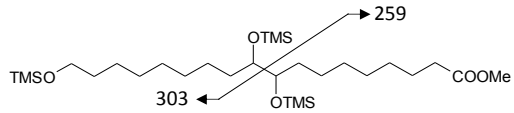



**Figure 21.** Example of the α-cleavage of 9,10,18-trihydroxyoctadecanoic acid methyl ester trisTMS ether.

Fragmentation of compounds with middle chain oxo groups (C=O) is preferred at both α- and β-cleavage with respect to the keto-group on either side of the carbon (Figure 22). The cleavage ions containing the methyl ester and TMS group readily give away MeOH and TMSOH, respectively (64).

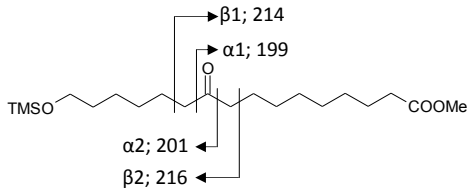



**Figure 22.** EI-MS fragmentation of 16-hydroxy-10-oxohexadecanoic acid methyl ester TMS ether.

**Table 5.** Diagnostic ions of long-chain aliphatic cutin and suberin monomer derivatives.

| Original compound | retention time and mass spectral cleavage of each derivative | | |
|---|---|---|---|
| **ω-hydroxy acids** | **Me ester, TMS ether m/z** | **TMS ester, TMS ether m/z** | **Ref.** |
| 16-hydroxyhexadecanoic acid | 343[M-15]$^+$, 327[M-31]$^+$, 311[M-47]$^+$, 159, 146, 103, 89, 75, 73, 55 | 401[M-15]$^+$, 385[M-31]$^+$, 311[M-105]$^+$, 217, 204, 147, 117, 103, 75, 73, 55 | 68, 73 |
| 16-hydroxy-10-oxohexadecanoic acid | 357[M-15]$^+$, 341[M-31]$^+$, 325[M-47]$^+$, 216, 214, 201, 199, 126, 111, 83, 75, 73, 55 | 415[M-15]$^+$, 272, 251, 215, 201, 126, 111, 83, 75, 73, 55 | 64 |
| 8/9/10, 16-dihydroxyhexadecanoic acid | 431[M-15]$^+$, 415[M-31]$^+$, 399[M-47]$^+$, 317, 303, 289, 275, 273, 259, 245, 159, 146, 147, 129, 103, 95, 75, 73, 55 | 489[M-15]$^+$, 399[M-90-15]$^+$, 383[M-105]$^+$, 331, 317, 303, 289, 275, 217&204*, 147, 129, 103, 95, 75, 73, 55 | 67, 73 |
| 18-hydroxyoctadecadienoic acid | 367[M-15]$^+$, 335[M-47]$^+$, 149, 135, 129, 107, 95, 75, 73, 67, 55 | 440[M]$^+$, 425[M-15]$^+$, 409[M-31]$^+$, 335[M-105]$^+$, 217, 204, 147, 135, 129, 121, 117, 109, 95, 75, 73, 55 | 68, 73 |
| 18-hydroxyoctadecenoic acid | 384[M]$^+$, 369[M-15]$^+$, 353[M-31]$^+$, 337[M-47]$^+$, 159, 146, 129, 109, 95, 75, 73, 55 | 442[M]$^+$, 427[M-15]$^+$, 411[M-31]$^+$, 337[M-105]$^+$, 383, 217, 204, 147, 135, 129, 117, 109, 95, 75, 73, 55 | 73 |
| 18-hydroxyoctadecanoic acid | 339[M-47]$^+$, 159, 146, 129, 107, 95, 75, 73, 55 | 444[M]$^+$, 429[M-15]$^+$, 413[M-31]$^+$, 339[M-105]$^+$, 217, 204, 147, 129, 117, 103, 75, 73, 55 | |
| 9, 10-epoxy-18-hydroxyoctadecenoic acid [a] | 487[M-15]$^+$, 471[M-31]$^+$, 383, 303, 271, 259, 243, 213, 191, 155, 129, 121, 109, 103, 89, 75, 73, 55 | 545[M-15]$^+$, 441, 423, 361, 329, 317, 301, 271, 259, 191, 149, 129, 121, 109, 103, 75, 73, 67 | 65 |
| 9, 10-epoxy-18-hydroxyoctadecanoic acid [a] | 489[M-15]$^+$, 473[M-31]$^+$, 457[M-47]$^+$, 385, 303, 274, 259, 201, 155, 129, 121, 109, 103, 81, 75, 73, 55 | 547[M-15]$^+$, 443, 425, 332, 317, 303, 243, 147, -129, 121, 109, 103, 75, 73, 67 | 65 |
| 9, 10, 18-trihydroxyoctadecanoic acid | 545[M-15]$^+$, 361, 317, 271, 259, 155, 147, 129, 109, 103, 75, 73 | 513[M-105]$^+$, 419, 329, 317, 301, 217, 204, 191, 147, 129, 109, 103, 75, 73 | 68, 73 |
| 9, 10, 18-trihydroxyoctadecanoic acid | 547[M-15]$^+$, 332, 303, 259, 243, 212, 155, 147, 129, 109, 103, 81, 75, 73 | 515[M-105]$^+$, 390, 317, 303, 217, 204, 147, 129, 109, 103, 81, 75, 73 | 68, 73 |
| 20-hydroxyeicosanoic acid | 399[M-15]$^+$, 383[M-31]$^+$, 367[M-47]$^+$, 159, 146, 103, 89, 75, 73, 55 | 457[M-15]$^+$, 441[M-31]$^+$, 367[M-105]$^+$, 217, 204, 147, 129, 117, 103, 75, 73, 55 | |
| 22-hydroxydocosanoic acid | 442[M]$^+$, 427[M-15]$^+$, 411[M-31]$^+$, 395[M-47]$^+$, 159, 146, 129, 103, 83, 75, 73, 55 | 485[M-15]$^+$, 469[M-31]$^+$, 395[M-105]$^+$, 217, 204, 147, 129, 117, 103, 75, 73, 55 | 73, 191 |
| 24-hydroxytetracosanoic acid | 470[M]$^+$, 455[M-15]$^+$, 439[M-31]$^+$, 423[M-47]$^+$, 159, 146, 129, 103, 87, 75, 73, 55 | 513[M-15]$^+$, 497[M-31]$^+$, 423[M-105]$^+$, 217, 204, 147, 129, 117, 103, 75, 73, 55 | |
| 26-hydroxyhexacosanoic acid | 483[M-15]$^+$, 467[M-31]$^+$, 451[M-47]$^+$, 159, 146, 129, 103, 87, 75, 73, 55 | 541[M-15]$^+$, 525[M-31]$^+$, 451[M-105]$^+$, 217, 204, 147, 129, 117, 103, 75, 73, 55 | |
| 28-hydroxyoctacosanoic acid | 511 [M-15]$^+$, 495[M-31]$^+$, 479[M-47]$^+$, 159, 146, 129, 103, 87, 75, 73, 55 | 569[M-15]$^+$, 553[M-31]$^+$, 479[M-105]$^+$, 217, 204, 147, 129, 117, 103, 75, 73, 55 | |

[a] identified as corresponding methoxyhydrin compounds.

| Compound - derivative | mass spectral cleavage of each derivative | | | |
|---|---|---|---|---|
| **α, ω-dioic acids** | **Di Me ester, TMS ether m/z** | **Me ester TMS ester, TMS ether m/z** | **Di TMS ester, TMS ether m/z** | **Ref.** |
| hexadecan-1,16-dioic acid | 283[M-31]$^+$, 250[M-64]$^+$, 241, 209, 191, 168, 154, 135, 125, 112, 98, 84, 74, 69, 55 | 357[M-15]$^+$, 307, 269, 159, 146, 129, 117, 98, 75, 73, 69, 55 | 430[M]$^+$, 415[M-15]$^+$,299 [M-131]$^+$, 217, 204, 170, 147, 129, 117, 75, 73, 55 | 100, 194 |
| 7/8-hydroxy hexadecan-1,16-dioic acid | 402[M]$^+$, 387[M-15]$^+$, 371[M-31]$^+$, 273, 259, 245, 231, 169, 155, 159, 146, 95, 73, 67, 55 | | 503[M-15]$^+$,331, 317, 303, 289, 147, 117, 73 | 63, 69, 100 |
| octadec-9-en-1,18-dioic acid | 340[M]$^+$, 309[M-31]$^+$, 308, 290, 276, 98, 95, 81, 67, 55 | 383[M-15]$^+$, 333, 295, 159, 129, 117, 98, 95, 81, 75, 73, 67, 55 | 456[M]$^+$, 441[M-15]$^+$, 325[M-131]$^+$ ,217, 204 170, 147, 129, 117, 75, 73, 55 | 68 |
| octadecan-1,18-dioic acid | 311[M-31]$^+$, 278[M-64]$^+$, 269, 237, 196, 154, 140, 126, 112, 98, 84, 74, 69, 55 | 385[M-15]$^+$, 335, 297, 159, 146, 129, 117, 98, 83, 75, 73, 67, 55 | 458[M]$^+$, 443[M-15]$^+$, 327 [M-131]$^+$, 311, 217, 204, 170, 147, 129, 117, 75, 73, 55 | 68, 193, 194 |
| 9, 10-dihydroxyoctadecan-1,18-dioic acid | 503[M-15]$^+$, 487[M-31]$^+$, 332, 259, 243, 155, 147, 129, 109, 73, 55 | 390, 317, 259, 217, 204, 155, 147, 129, 109, 73, 55 | 390, 317, 301, 227, 217, 204, 147, 129, 109, 73, 55 | 73 |
| 9, 10-epoxyoctadecan-1,18-dioic acid [a] | 445[M-15]$^+$, 429[M-31]$^+$,416[M-47]$^+$, 274, 259, 243, 227, 155, 137, 129, 109, 89, 73, 55 | 332, 317, 259, 217, 204, 155, 137, 129, 109, 73, 55 | 341, 329, 317, 309, 217, 204, 147, 129, 109, 75, 73, 55 | 65, 128 |
| eicosan-1,20-dioic acid | 339[M-31]$^+$, 306[M-64]$^+$, 297, 265, 154, 25, 112, 98, 84, 74, 55 | 413 [M-15]$^+$, 363, 325, 159, 146, 129, 117, 98, 83, 75, 73, 67, 55 | 486[M]$^+$, 471[M-15]$^+$, 355 [M-131]$^+$, 217, 204, 147, 129, 117, 75, 73, 55 | 194 |
| docosan-1,22-dioic acid | 367[M-31]$^+$, 334[M-64]$^+$, 325, 293, 154, 35, 125, 112, 98, 84, 74, 55 | 441[M-15]$^+$, 391, 353, 159, 146, 129, 117, 98, 83, 75, 73, 67, 55 | 514[M]$^+$, 499[M-15]$^+$, 383 [M-131]$^+$, 217, 204, 147, 129, 117, 75, 73, 55 | 191, 194 |
| tetracosan-1,24-dioic acid | 395[M-31]$^+$, 362[M-64]$^+$, 353, 321, 154, 35, 125, 112, 98, 84, 74, 55 | 469[M-15]$^+$, 419, 381, 159, 146, 129, 117, 98, 83, 75, 73, 67, 55 | 542[M]$^+$, 527[M-15]$^+$, 411 [M-131]$^+$, 217, 204, 170, 147, 129, 117, 75, 73, 55 | 194 |
| hexacosan-1,26-dioic acid | 423[M-31]$^+$, 381, 349, 154, 35, 125, 112, 98, 84, 74, 55 | 497[M-15]$^+$, 447, 409, 159, 146, 129, 117, 98, 83, 75, 73, 67, 55 | 570[M]$^+$, 555[M-15]$^+$, 439 [M-131]$^+$, 217, 204, 147, 129, 117, 75, 73, 55 | |
| octacosan-1,28-dioic acid | 451[M-31]$^+$, 409, 377, 154, 135, 112, 98, 84, 74, 55 | 525[M-15]$^+$, 475, 437, 159, 146, 129, 117, 98, 75, 73, 55 | 598[M]$^+$, 583[M-15]$^+$, 467 [M-131]$^+$, 217, 204, 147, 129, 117, 75, 73, 55 | |

[a] identified as corresponding methoxyhydrin compounds.

#### 2.3.5.1.2 Issues of monomer quantification

The quantification of cutin and suberin monomers using internal standards has been very difficult, as only a few reference compounds are commercially available. There is evidence that response factors for the secondary oxygenated acids are much higher than those for their non-substituted counterparts (109), thus using only one or a few compounds for external calibration may not be sufficient.

Graça *et al*. (51) used the response factor of 12-hydroxyoctadecanoic acid calibrated against 16-hydroxyhexadecanoic acid to quantify all ω-hydroxy acid monomers. The GC-detected and calculated portion of the total loss of mass (gravimetric) was 74% from potato suberin and rarely more than 50% from several cutins after methanolysis (51, 110). This was attributed to an underestimation of the compounds due to false response factors or, alternatively, to volatility issues of some of the depolymerization products i.e. soluble oligomers. Similar conclusions were drawn by Peschel *et al*. (77) and Cordeiro *et al*., (111) when they obtained about 50% and 40%, respectively, of the monomers by GC quantification compared with gravimetrical data. Graça and Pereira (109) detected about 65% of the mass released by methanolysis from cork suberin and 38% of Douglas fir suberin, while Lopes *et al*. (112) obtained less than 30% of cork suberin. Bento *et al*. (113) confirmed the presence of oligomeric fragments by size exclusion chromatography (SEC) and matrix-assisted laser desorption-ionization coupled with mass spectrometry (MALDI-MS), similar to Lopes *et al*. (112) who used different NMR techniques after 3% NaOMe catalyzed methanolysis corresponding to the complete removal of suberin.

Riederer and Schönherr (114) reported an underestimation of the actual amounts of cutin acid methyl esters by 6.2-21.3%, depending on the polarity of the monomers by using diethyl ether–water partitioning after methanolysis. They suggested that reductive depolymerization using $LiAlH_4$ yielding considerably more polar alcohols than methods producing methyl esters lead to even higher losses in the extraction of monomers.

Mendez-Millan *et al*. (76) used nonadecanoic acid as an internal standard calibrated against 16-hydroxyhexadecanoic acid and obtained lower amounts from transmethylation reactions than saponification. In addition to differences in depolymerization mechanisms, this might be due to different response factors of trimethylsilylated reaction products, as the products of transmethylation were methyl ester TMS ethers while saponification products were analyzed as TMS ester TMS ethers.

Many of the so-called quantitative results of cutin and suberin monomers have not been corrected for losses during work-up or for different responses of the GC system, thus they remain somewhat qualitative or semiquantitative although they are claimed to be quantitative. Also, GC analysis of the depolymerization extracts only accounts for monomers

and therefore is only a partial characterization of the solubilized products (112, 113), although also mono- and diacylglyceryl esters of aliphatic acids and ferulic acid have been detected by GC-MS (110). The non-detected, but soluble, higher molecular weight fractions may also be composed of cutan and suberan-type structures (11).

Nevertheless, qualitative descriptions of the monomer composition are enough to understand the differences between various plants and organs, but perhaps are not sufficient for deductions on the exact polymer composition.

#### 2.3.5.2 High performance liquid chromatography

High performance liquid chromatography (HPLC) is based on the separation of compounds between a liquid mobile phase (eluent) and the stationary phase of the column. The sample components are first dissolved in a solvent before injection, but the molecular weight, thermal stability or volatility does not restrict the analysis. Analytes are separated by their interaction with the stationary phase and eluent during the flow through the column at high pressure. The eluent solvent system may be manipulated during the flow to affect the separation of the analytes. HPLC may be coupled with versatile detectors such as spectroscopic (UV, fluorescence, IR, MS, NMR) or electrochemical (conductivity, potentiometric) detectors. Many detectors may also be combined together for the analysis of various compound mixtures from small non-polar products to very large polar molecules (190). In HPLC coupled with MS for the analysis of natural products, the most commonly used interfaces are atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI) (195).

Most of the studies of cutin and suberin have been conducted by GC and only very few investigations have been performed with HPLC methods. Gérard *et al*. (196) introduced a feasible HPLC method combined with an evaporative light scattering detector (ELSD) method for the quantitative analysis of underivatized mono-, di- and trihydroxy and epoxy acids and used it in cucumber cutin analysis (96). The HPLC-CIMS method has been used to analyze tomato cutin monomers and oligomers after partial hydrolysis (90). In addition to several monomers, a dimer of two dihydroxyhexadecanoic acids could be detected. Osman *et al*. (90) concluded that introduction of the cutin hydrolyzates *via* particle-beam separator limited the analysis of larger compounds, such as trimers. After re-examination of these oligomer mixtures after O-acetylation by HPLC-size exclusion chromatography (SEC) using a light scattering detector (LSD), fragments up to pentamers were observed (9).

Ray *et al*. (97) utilized HPLC combined with ELSD in studies of lime fruit cutin monomers produced with alkaline hydrolysis and transesterification. By using this method, twelve monomers were detected of which nine were identified by comparison with the retention times of known monomers (97). An oligomeric extract produced by hydrofluoric acid (HF) from

lime fruit cutin was purified by reverse-phase HPLC connected to UV and ELS detection followed by NMR spectroscopic analyses and mass spectrometry utilizing electrospray ionization (ESI-$MS^n$) and time-of-flight (ToF) -MS techniques (79).

Suberin oligomeric fragment mixtures have been purified using HPLC connected to UV, diode array detector (DAD) and ELS detections followed by identification with LC-APCI-MS and LC-ESI-$MS^n$ (159).

### 2.3.5.3 Nuclear magnetic resonance spectroscopic methods

Nuclear magnetic resonance (NMR) spectroscopy is based on the magnetic properties of certain atomic nuclei having unpaired spin (I=½), such as $^{1}H$, $^{13}C$, $^{19}F$ and $^{31}P$. In a very simplified description to measure a NMR spectrum, the sample is placed in a strong static magnetic field which makes the different spin states energetically different, is rapidly spun and nuclei are brought to resonance by the application of radiofrequency radiation. Acquisition of the signals emitted by the excited nuclei in the sample is carried out and then transformed into a spectrum. The frequency at which a given nucleus resonates, recorded as the chemical shift (ppm) from a reference compound, is an effect based on the local atomic environment in which the nucleus is situated. The integrated intensity of a signal is directly proportional to the number of the nuclei which contribute to the particular signal. The splitting of signals results from a magnetic interaction between neighboring atoms, known as spin-spin coupling, while dipole-dipole coupling involves magnetic interactions through space and not chemical bonds (197).

Generally, in proton ($^{1}H$) NMR, saturated hydrocarbons absorb approximately in the region of 1.0-4.0 ppm, alkoxy protons in ether, alcohol and ester groups at 3.0-4.5 ppm, olefinic protons at 5.0-6.5 ppm and aromatic protons between 7.0-8.5 ppm while aldehyde and acid protons absorb between 9.0-14.0 ppm relative to tetramethylsilane (TMS) used as internal standard. In carbon ($^{13}C$) NMR, the sensitivity is about 100 times less than in proton NMR due to the low natural abundance of the NMR active isotope. Generally, alkane carbons resonate between 0-80 ppm, alkyne carbons at 60-95 ppm, alkene carbons at 100-150 ppm and carbonyl carbons at 160-220 ppm (197). In most cases, quantitative information is not obtained from $^{13}C$ NMR spectra due to the specific limitations of signal acquisition, and the long relaxation times of $^{13}C$ nuclei (197).

One-dimensional (1D) $^{1}H$ NMR can be used to analyze the protons of monomers and oligomers, while 1D $^{13}C$ NMR reveals the types of carbons present in the same molecules (81). In the case of cutin and suberin, two-dimensional (2D) homo- and heteronuclear NMR may be used to obtain information for example on chemically bonded $^{1}H$ and $^{13}C$ nuclear spins within and between soluble monomers and oligomers (159).

Several different methods have been used in the structural analysis of cutin oligomers, including $^{1}H$-$^{1}H$ total correlation spectroscopy (TOCSY) to determine through-bond connectivities up to five bonds, $^{1}H$-$^{13}C$ gradient-assisted heteronuclear multiple quantum correlation (HMQC) to detect directly bonded carbon-proton pairs and $^{1}H$-$^{13}C$ gradient-assisted heteronuclear multiple-bond (long range) correlations (HMBC) to assess, for example, the presence of ester carbonyl groups (78, 79, 82). In addition to these methods, suberin oligomeric fragments have been analyzed with proton correlation spectroscopy (COSY) revealing pairs of through-bond coupled $^{1}H$ nuclei (159).

NMR analyses may be also performed after derivatization. Trichloroacetyl isocyanate (TAI)-derivatized hydroxyl and carboxyl groups of suberin depolymerization product mixtures have been analyzed by $^{1}H$ NMR to determine the ratio between the groups and between primary and secondary hydroxyls (170).

### 2.3.6 Spectroscopic methods in the analysis of intact polymers and depolymerization-resistant residues

The FTIR and solid state NMR spectroscopy are non-destructive methods which enable the characterization of solid materials such as the natural polymers of cutin and suberin. Solution state methods may be applied after solvent-swelling of the polymers. These methods have been widely used in the analysis of different functional groups and structure (83-85, 88, 91, 93, 94, 97, 124, 126, 131-133, 149, 152, 155, 157, 161, 161, 163, 164, 167, 168, 179).

#### 2.3.6.1 Fourier transform infrared spectroscopy

The infra-red (IR) spectroscopy gives information on the different functional groups and chemical bonds that are present in a material based on their specific absorbance of vibrational energy. Infrared radiation is passed through a sample and the absorbed energy is transformed into a spectrum, which shows signals at specific wavelengths (frequency of a vibration) that can be assigned to different types of bonds between the atoms in functional groups (198). IR vibration types are specified in Figure 23.

The FTIR spectrum of cuticular polymers and suberized membranes are complex because of the structures of various macromolecules present in the samples. Thus, signals often overlap (86, 93, 142). In addition, the complexity of an IR spectrum of large molecules arises from the coupling of vibrations over a large part or over the complete molecule. These are called skeletal vibrations and form fingerprint signals of the molecules rather than of a specific functional group (198). For example, a typical suberin fingerprint is seen at 1464, 1240 and 1175 $cm^{-1}$ (133).

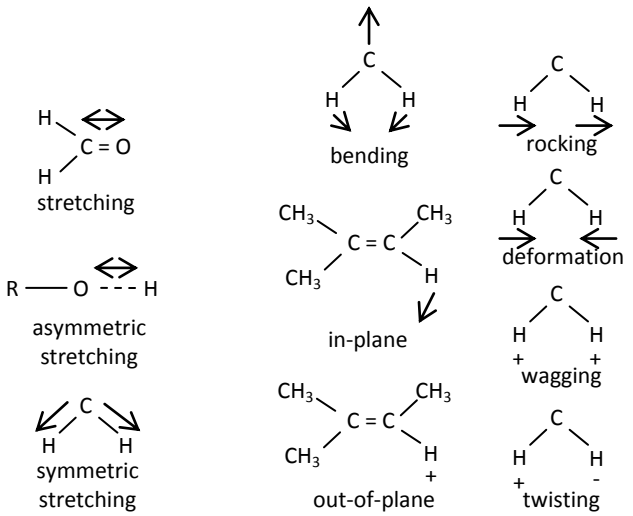



**Figure 23.** IR vibration types (198).

**Table 6.** Assignments of the most relevant signals in the FTIR spectra.

| FTIR /$cm^{-1}$ | assignment[a] |
|---|---|
| 3345-3355 | str (O-H----O) |
| 3040-3020 | str (aromatic C-H) |
| 2925 | str asymmetric ($CH_2$) |
| 2852 | str symmetric ($CH_2$) |
| 1473, 1463, 1313, 723 | bend ($CH_2$) |
| 1229 | str (C-C) chain |
| 1720-1750 | str (C=O) ester group (with a shoulder ~1700 str(C=O----H) H-bonded ester groups) |
| 1690-1710 | str (C=O) free acid group |
| 1167, 1103 | str asymmetric and symmetric (C-O-C) ester |
| 1624 | str (C=C phenolic acid) |
| 1606, 1588 | str (C-C aromatic) |
| 1551, 1515, 1440 | str (C-C aromatic conjugated with C=C) |
| 830 | bend oop (C-H and C-C) aromatic |
| 1247, 1063, 1030 | str (C-O) polysaccharides |

str, stretching; bend, bending; oop, out-of-plane. [a] Assignments after (83, 86, 92-94, 133, 152, 155, 164, 168).

The mid-infrared spectrum (4000-400 $cm^{-1}$) may be approximately divided into different regions which can be generally used to determine the nature of the group. The four regions are as follows: the X-H stretching (4000-2500 $cm^{-1}$), the triple bond (2500-2000 $cm^{-1}$), the double bond (2000-1500 $cm^{-1}$) and the fingerprint region (1500-600 $cm^{-1}$) (198). Table 6 introduces the main frequencies and assignments of components of the cuticular and suberized fractions.

### 2.3.6.2 Solid state nuclear magnetic resonance spectroscopy

The structural complexity, heterogeneity and amorphous nature of biopolymers limit the resolution of the rather broad spectral lines in solid state NMR (50, 88, 199). The line broadening arises from many possible orientation-dependent interactions such as anisotropic, chemical shielding and dipole-dipole coupling between the magnetic nuclei. In solution NMR, rapid random tumbling of the molecules averages each of these interactions to or near zero, resulting in sharp peaks with excellent resolution (199, 200). Suppression of these effects in the solid state may be achieved by using fast rotation of the sample, cross-polarization (CP) and magic angle spinning (MAS). Magic angle spinning, i.e., spinning the sample at a specific axis (54.7°) with respect to the external magnetic field ($B_0$) at high speed, suppresses the dipole-dipole coupling to zero while eliminating dipolar line broadening. The sensitivity of dilute spins like carbon-13 can be improved by using cross-polarization that enhances the signal-to-noise ratio (S/N) by transferring the polarization from abundant spins (like hydrogen-1) to dilute spins (199).

$^{13}C$ CP-MAS NMR has been widely used in the structural characterization of cutin and suberin in plants such as lime fruit (78, 82, 83, 92, 97), tomato, pepper, apple and olive leaf (94), wheat bran (201), cork (164, 168) and potato (92, 124, 126, 131, 132, 157, 163). Also, quantification of cork suberin and the variability of cork quality have been studied using $^{13}C$ CP-MAS NMR (133, 161). Solid state NMR studies of berry dietary fibers have been conducted before (202), but not on isolated cuticular fractions. The spectral assignments (Table 7) have been made by analogy with similar chemical compounds.

Determination of spin relaxation times may be used as an indirect measure of dynamics, i.e. the chemical mobility of the polymer chains (rigid and mobile carbons) and molecular organization including location in the polymer and separation from the different structures (83, 86, 163, 165). Relaxation behavior and differing cross-polarization (CP) efficiencies of the various structures make the exact quantification uncertain, as some signals may be attenuated in selected conditions (131, 201). CP contact time length has a great effect on the intensity of the signals (83, 131). For example, keto group signals of hydroxy-oxo fatty acids were enhanced by increasing the time from 0.7 ms to 5 ms (155). Direct polarization (DP) MAS techniques may be used in addition to CP-MAS for dynamic studies and quantification of specific functional groups in challenging biopolymers (91, 157, 163).

**Table 7.** Assignments of the most relevant signals in the $^{13}C$ solid state NMR spectra.

| $^{13}C$ CP-MAS NMR /ppm | assignment[a] |
|---|---|
| 26, 29, 33 | -$(CH_2)$n- |
| 40,42 | -$\underline{C}H_2$-$CH_2$-O-CO-R |
| 56 | Ar-$OCH_3$, -$OCH_3$, CH-O-CH- |
| 65 | -$CH_2$O-, -$CH_2$-O-CO-R, -$CH_2$OH (overlapping C6 polysaccharide) |
| 72 | >CHO-CO-R, >C-OH, -CHOH- (overlapping C2,3,5 polysaccharide) |
| 74 | C-OR-, Ar-CHOH-,-CHOH- (overlapping C2,3,5 polysaccharide) |
| 82 | -CHOH-, -CHOAr- (overlapping C4 polysaccharide) |
| 88 | -CH-O-, -CHOH-, C-OR, C-R (overlapping C4-polysaccharide) |
| 105, 115, 120-135, 148, 156 | Aromatic -CH-, -CH=CH-, Ar-C- (overlapping C1-polysaccharide) |
| 168 | -$CH_2$-O-$\underline{C}$=OR |
| 173 | >CH-O-$\underline{C}$=OR |
| 179 | -$CH_2$-$\underline{C}$OOH |
| 209 | C=O keto group |

[a] Assignments after (83, 92-94, 124, 126, 131, 133, 155, 161, 164, 165, 168, 201)

### 2.3.6.3 High resolution magic angle spinning nuclear magnetic resonance

The problems of solid state NMR can be overcome by using high-resolution magic angle spinning (HR-MAS), a hybrid NMR technique in which solid samples are swelled in organic solvents (such as dimethyl sulfoxide, DMSO) to enhance molecular mobility and to produce high-resolution spectra. The enhanced molecular mobility and application of MAS removes or minimizes the effects of various line-broadening effects (50, 84, 85, 88, 157).

In addition to $^{1}H$ NMR, several two-dimensional NMR methods may be used in HR-MAS analyses such as $^{1}H$-$^{13}C$ HMQC, heteronuclear single quantum coherence (HSQC) and HMBC, $^{1}H$-$^{1}H$ COSY, nuclear overhauser effect spectroscopy (NOESY) and TOCSY (84, 85, 88, 157). HR-MAS NMR provides a direct assessment of the molecular structure giving information of directly or remotely bound $^{1}H$–$^{13}C$ within a functional group, establishes covalent connectivities between monomers and differentiates which groupings are close to one another in space (50, 157).

## 2.4 UTILIZATION OF CUTIN AND SUBERIN CONTAINING MATERIALS

Processing of fruits, vegetables and forest products annually yields millions of tons of so-called by-products of little of no commercial value. These masses are partly used as animal feed, but are mainly disposed of as waste. These by-products contain, in addition to traditional dietary fiber, a large quantity of compounds such as cutin, suberin, associated waxes and secondary metabolites that could be of commercial value if the appropriate methods for their recovery and utilization were available (9, 11, 12).

As an example, tomato juice press residue contains cuticular waxes and cutin in addition to other hydrophobic compounds such as lycopene. During the year 1994, up to 550 million kilograms of tomato waste was generated, which contained approximately 5 million kilograms of 9,16- and 10,16-dihydroxyhexadecanoic acids, the main monomers of tomato cutin (9). Wine making produces approximately 30 w/w% of the by-product pomace, resulting in millions of tons of waste material annually (203). Wood processing, such as the paper, pulp, construction and furniture industries, produces thousands of tons of bark by-products, of which typically 20-50% represents suberin (11). According to Ekman (172) over 4000 tons/year of suberin monomers could be produced as by-product from birch kraft pulp milling. Renewable suberin-rich cork is used as cork stoppers to seal wine bottles (10, 11) and in technologically demanding applications such as in materials for thermal and acoustic insulation (11, 137), yielding high amounts of cork powders as by-products of limited use. Industrial potato and other vegetable peeling produces millions of tons of peel by-product annually (204). According to a study by Kolattukudy and Purdy (58), cutin constitutes approximately 12-28% of organic matter in domestic sewage sludge. Thus, even sewage sludge could be a source of hydroxy acids.

### 2.4.1 Cutin and suberin as part of cattle feed

In Western countries, cattle consume cutin and suberin in their feed, either derived directly from grass forage, straw, fruits, vegetables and seeds, but also as industrial cutin and suberin-rich by-products. Very little is known about the fate of cutin and suberin polymers in the intestinal tract of livestock, but apparently animals are able to degrade cutin and suberin polymers to some extent and utilize the released monomers. However, both short and long term studies on the effects and caloric value of these compounds are missing.

Brown and Kolattukudy (12) have performed a study in which they fed radioactive $^{14}C$-labeled apple cutin to rats. The label was found in all tissues and organs, including intestinal walls, liver, urine and blood. The polymer was hydrolyzed in the intestine and products were absorbed and metabolized in the liver. One enzyme involved in the hydrolysis was found to be a pancreatic lipase in the small intestine (12). Subsequent purification of a cutin-degrading enzyme from porcine pancreas confirmed the action of pancreatic lipase. Other hydrolases present may also participate in the hydrolysis of oligomers generated by pancreatic lipase (181). A high proportion of the label in the intestinal contents and feces was found in chloroform-soluble lipids and the remaining portion in insoluble materials representing the unhydrolyzed part of cutin. Thus, even though substantial amounts of apple cutin were hydrolyzed, a significant portion remained unabsorbed and also unhydrolyzed (12). Winemaking by-products, such as grape pomaces, were found to be even lower in their *in vitro* digestibility than standard feeds (grain and grasses) due to their higher lignin and cutin

content (205). It has been suggested that cutin, rather than lignin, plays an important role in protecting wheat bran from microbial degradation in the rumen and human gut (201).

### 2.4.2 Cutin and suberin as part of dietary fiber

In addition to their important features in plants, cutin and suberin may be important compounds among other fiber components in human nutrition affecting, for example, carcinogenesis, gastrointestinal health and the metabolism of sugars and fats. Although cuticular and suberized material composes only a very small fraction of dietary fiber, which is mainly composed of cell wall material, they may have important role in the prevention of colorectal cancer (201, 206, 207).

Dietary fiber is defined as the edible parts of plants resistant to digestion and absorption in the human small intestine with complete or partial fermentation in the large intestine. This definition includes polysaccharides, oligosaccharides, lignins and associated plant substances, to which cuticular polyesters together with suberin and waxes can be included (208). Dietary fibers vary in composition and properties, thus they might posses protective but also even enhancing effects in terms of carcinogenesis. The results of animal carcinogenesis studies are variable, but generally sources of insoluble fiber have been found to be more protective than soluble fiber. The hydrophobic suberin and cuticular polymers may protect plant cell walls from degradation in the colon and enhance the removal of harmful hydrophobic carcinogens by absorption from the colon. The aliphatic and aromatic components released from cutin and suberin by hydrolysis may affect the human body in different ways. However, the effects of the whole dietary fiber fraction cannot be calculated as a sum of the effects of each individual compound present and *vice versa* (206, 207, 209).

Hydrolysis of dietary cutin and suberin generates monomers, including potentially toxic epoxy acids. Since the pancreatic lipase from different animals shows similar substrate specificity, the probability of hydrolysis of such biopolymers taking place in the human intestine is high (16). Dietary hydroxy and epoxy fatty acids derived from fats (triglycerides) are absorbed in intestine of humans and have been implicated in the development of atherosclerosis and coronary heart disease. The effect of multiple oxidized substituents is not known, but diepoxy fatty acids seem to be absorbed less readily than monoepoxy fatty acids (210, 211). A microsomal epoxy hydrase, which catalyzes the hydration of epoxy acids of cutin, was found in the intestinal walls of rats and rabbits. The activity was found to be especially high in the upper portion of the intestine. This type of enzyme evidently plays an important role in detoxifying epoxy acids, which are major constituents of several fruit and vegetable cutins, such as apple and grape peels and spinach (16).

The polymorphous composition of cuticular material, including cutin and cutan, their amounts, monomeric compositions and different bonding types may all affect the processes of the human intestinal tract and metabolism. The effect of cuticular material as a part of dietary fiber and its composition in different edible and useful plants should be investigated more thoroughly to clearly establish the real significance of this material as a part of human nutrition. More studies should be conducted before adding various undefined by-product materials to produce functional high-fiber foods, although many of the fruit and vegetable processing by-products contain substantial amounts of dietary fiber-associated phenolic compounds with various beneficial effects (203, 212, 213).

### 2.4.3 Technological applications

Cutin and suberin monomers might be used to produce both soluble and solid polymers, to be utilized in various high-value applications. Such materials are also likely to be immunologically inert to allow their use in biomedical applications (1) and in cosmetics as smoothing components of skin formulas (214). Monomers and oligomers could be used in inks, paints and coatings as drying oils, plasticizers, wetting agents, viscosity modifiers, as substitutes to fats in oils (9, 11, 215) or as precursors to produce polyurethanes, polyesters and polyamides (11, 216).

The chemical industry is constantly seeking better, greener, more sustainable and renewable raw materials. Diacids and hydroxylated fatty acids have been noted as multifunctional, and thus sought-after compounds, but challenges in their prize-competitive and sustainable production hinders their usage in various applications (216, 217). These could be produced without difficult synthetic chemical modifications by depolymerizing variable industrial by-products containing suberized and cuticular materials.

The cutinase polyesterases have many potential commercial uses, such as acting as biocatalysts in laundry detergents, biodegradation of plastics, industrial waste water treatments or as adjuvants in agricultural chemical formulations and incorporating food additives (1, 186).

# 3 AIMS OF THE STUDIES

The overall aim of this thesis research project was to establish new knowledge on the composition of cuticular polymers of several Northern berries and suberized membranes of two varieties of potatoes with different phenotypes.

The first aim of the project was to create monomeric profiles of ester-bound berry cutin and potato suberin by using gas chromatography combined with mass spectrometry (GC-MS). In addition to the traditional reflux-method, a new small-scale depolymerization method was developed. Chemical methods were compared with enzymatic cutinase hydrolysis by a novel CcCut1 -cutinase (*Coprinus cinereus)* to obtain information on the mechanism of action of cutinase.

The second aim was to characterize the intact cutin and suberin biopolyesters, in addition to the remaining depolymerization-resistant residues obtained after cleavage of the ester-bound monomers. Solid state spectroscopic methods and microscopy were applied to the polymer investigations.

In addition, the effects of growth location for cuticular polymers of black currant berries and changes in potato suberin during post-harvest storage were investigated.

# 4 MATERIALS AND METHODS

## 4.1 MATERIALS

### 4.1.1 Berries (I, II, IV) and seeds (II)

Berries used in the present studies (Table 8) were either industrial powdered whole berries, press residue from juice processing, straining by-products or prepared by hydraulic juice pressing or manual peeling. Berries were collected at optimal ripeness for industrial berry processing. Isolated berry samples were throughout washed with water and the remaining seeds were manually separated after drying in an oven at 60°C.

**Table 8.** Berries used in the present studies.

| Berry | | Sample | Study |
|---|---|---|---|
| Bilberry | *Vaccinium myrtillus* | berry powder | I |
| Black chokeberry | *Aronia melanocarpa* | peel | II |
| Black currant | *Ribes nigrum* | press residue | I |
| Black currant | *Ribes nigrum* | crushed seed | II |
| Black currant[a] | *Ribes nigrum (var. Ola)* | peel[b] | IV |
| Cloudberry | *Rubus chamaemorus* | press residue | II |
| Cloudberry | *Rubus chamaemorus* | crushed seed | II |
| Cranberry | *Vaccinium oxycoccos* | press residue | I |
| Crowberry | *Empetrum nigrum* | straining residue | II |
| Lingonberry | *Vaccinium vitis-idaea* | berry powder | I |
| Raspberry | *Rubus idaeus* | press residue | II |
| Rosehip | *Rosa rugosa* | peel | II |
| Rowanberry | *Sorbus aucuparia* | straining residue | II |
| Sea buckthorn | *Hippophaë rhamnoides* ssp. *mongolica* | peel | I, IV |
| Sea buckthorn | *Hippophaë rhamnoides* | crushed seed | II |
| Strawberry | *Fragaria x ananassa* | press residue | II |

[a] Collected from Piikkiö, southern Finland (latitude 60°23' N, longitude 22°33' E, altitude 5-15 m) and from Apukka, northern Finland (latitude 66°34' N, longitude 26°01' E, altitude 100-105 m).

[b] peel samples were divided into soft, transparent peels and hard, dense receptacles based on a clear difference in appearance before depolymerization.

### 4.1.2 Potatoes (III, IV, V)

All potatoes were grown in Karijoki, Finland (Table 9). After harvest, the potato crop was kept at +12°C for 1 month to induce wound repair and the storage temperature was lowered by 1°C per week until +4°C, at which temperature the crop was stored. Potato peels were isolated manually.

**Table 9.** Potatoes used in the present studies.

| | | variety | Storage time | Study |
|---|---|---|---|---|
| Potato | *Solanum tuberosum* | Nikola | 2 months | III |
| | | Nikola | 0, 3, 6, 9, 12 months | V |
| | | Asterix | 0 months | IV |
| | | Asterix | 0, 3, 6, 9, 12 months | V |

[a] 0 month potatoes were obtained 2 weeks after harvest.

## 4.2 ISOLATION OF CUTICULAR AND SUBERIN POLYMERS

Cuticular and suberized membranes were isolated by a series of enzymatic treatments and extractions. Overnight enzymatic treatment of the dried berry samples/potato peel with cellulase (Econase CE, AB Enzymes, Darmstadt, Germany) and pectinase (Pectinex Ultra SP-L, Novozymes, Bagswaerd, Denmark) in acetate buffer was performed to hydrolyze the cell wall polysaccharides. Extraction of the soluble waxes was performed in a Soxhlet-apparatus with $CHCl_3$ and MeOH, with extraction times depending on the amount of soluble cuticular/suberin-associated waxes in each berry and potato sample. The isolation process was repeated to yield dried extractive-free cuticular (i.e. raw cutin) and suberin-enriched membranes (i.e. raw suberin). The crushed berry seeds were treated with the same combined enzymatic and extraction method once.

## 4.3 DEPOLYMERIZATION METHODS

### 4.3.1 Chemical depolymerization of cutin/suberin

Chemical depolymerization of the raw cutin/suberin was performed by transesterification with sodium methoxide (NaOMe)-catalyzed methanolysis yielding methyl esters of monomeric compounds. Three different conditions were used:

A) 1-2 M NaOMe reflux method (I, II, III)
B) 1 M NaOMe closed tube method (II, III, IV, V)
C) 0.05 M NaOMe closed tube method (III)

Both closed tube methods (B and C) were performed at ambient temperature with constant agitation. After application of methods A and B, the monomers were extracted with $CHCl_3$ to isolate lipophilic compounds, but after method C the $H_2O/CHCl_3$ partitioning was omitted. Cutin/suberin monomers were determined gravimetrically and stored in $CHCl_3$ in a freezer (-20°C) for further analysis. Resistant residues were freeze-dried after thoroughly washing with water (III, IV).

### 4.3.2 Enzymatic depolymerization of cutin/suberin by cutinase

The cutinase CcCUT1 produced and characterized previously by VTT Biotechnology (Espoo, Finland) (189) was used for the enzymatic hydrolysis of raw suberin (var. Nikola) and raw cutin of black currant peels. As a reference, the raw suberin and cutin were also treated with the Optimyze 525 esterase (Buckman Laboratories, Memphis, Tennessee). The hydrolyses were performed at 50°C, pH 8 for 24 hours in the presence of 500 nkat/mg of enzyme preparation with 0.1% hydrophobin II (HFBII) in phosphate buffer. Released compound mixtures were extracted with $CHCl_3$ and purified by filtration (0.45µm) to acquire samples comparable to chemical depolymerization. Hydrolysis-resistant residues were dried after thoroughly washing with water (III).

### 4.3.3 Cellulase and pectinase hydrolysis of depolymerization-resistant residues

The dried depolymerization-resistant residues from chemical and cutinase depolymerization were further treated with cellulase and pectinase, as an attempt to remove the residual cell wall polysaccharides (III, IV).

## 4.4 CHROMATOGRAPHIC AND MASS SPECTROMETRIC IDENTIFICATION OF MONOMERS

Monomeric components were trimethylsilylated before chromatographic analysis using the Tri-Sil® reagent (HMDS and TMCS in pyridine) (Pierce Chemicals Co., Rockford, IL).

The monomer composition was determined by GC-EI-MS with a Shimadzu GC-MS QP5000 instrument (Shimadzu, Kyoto, Japan) using a DB-1 MS column (30 m, id. 0.25 mm, $d_f$ 0.25 µm) (Agilent J&W, Folsom, CA). A mass range of *m/z* 45 – 550 was acquired. As a comparison of different detection methods, FID detection (Shimadzu GC 17A, Kyoto, Japan) was used in addition to MS analysis (II, III, V). All monomer proportions were calculated as the average peak area percentage of total peak areas from MS or FID chromatograms from at least triplicate analyzes.

Compounds were identified by comparing the EI-MS spectra of their TMS derivatives (methyl ester TMS ether or TMS ester TMS ether) with those of reference compounds and with published spectra with the aid of retention times (63-65, 67, 68, 68, 73, 100, 128, 191, 193, 194). For compounds lacking a reference, the chemical structures were deduced according to mass spectrometric fragmentation typical to long chain ω-hydroxy acids and α,ω-diacids

A TMS-derivative of cholesterol was used as an internal standard when determining the relative degree of depolymerization of the different methods in the case of potato suberin (III, V).

## 4.5 SOLID STATE NMR AND FTIR SPECTROSCOPY

Solid residues from depolymerization reactions and intact raw cutin/suberin were analyzed by FTIR (Spectrum BX FTIR, Perkin Elmer, Waltham, MA) and solid state $^{13}C$ CP-MAS NMR spectroscopy (Bruker AVANCE-400 (DRX), Bruker BioSpin, Karlsruhe, Germany) (III, IV).

## 4.6 MICROSCOPY

Black currant raw cutin, potato peels, raw suberin and solid residues of chemical depolymerization and enzymatic hydrolysis were analyzed by microscopic imaging.

Enzymatic modifications of raw suberin with CcCUT 1 and Optimyze 525 were visualized using confocal laser scanning microscopy (CLSM) equipment (Bio-Rad Radiance Plus confocal scanning system, Bio-Rad, Hemel Hempstead, Hertfordshire, UK; Nikon Eclipse E600 microscope, Nikon Corp., Tokyo, Japan). For imaging, samples were stained with Nile Blue (Gurr Products, Romford, Essex, UK). The final CLSM micrographs were reconstructed by the superimposition of a green-filtered emission image and a red-filtered emission image, in which suberin-containing cell walls appeared yellow (III, V).

Light microscopy (LM) was used for some samples due to their fine particle size, which made them unsuitable for confocal microscopy (57). The samples for LM analysis were stained with Oil Red O (BDH Chemicals, Poole, Dorset, UK). Cutin and suberin containing structures appeared as yellow-orange stained structures in the micrographs (III).

## 4.7 STATISTICAL ANALYSES

Statistical analyses were carried out using the statistical software SPSS for Windows (Version 14.0; SPSS Inc., Chicago, IL). All results were expressed as means and standard deviations (SD). Differences between the depolymerization methods A and B in the case of berry cutin (II) and between the suberin monomeric compositions of two varieties of potatoes (V) were evaluated by independent sample *t*-tests (normal distribution) and the Mann-Whitney U-test (abnormal distribution). One-way ANOVA (normal distribution) and Mann-Whitney U-test (abnormal distribution) were used for the analysis of differences between samples of the same variety during the post-harvest storage period of 0 to 12 months (V). Differences reaching a $p$-value $< 0.05$ were considered statistically significant.

# 5 RESULTS

## 5.1 BERRY CUTIN

### 5.1.1 Isolation of raw cutin

Berry raw materials used in the present studies varied from manually isolated peels to straining residues and commercial powders. Thus, the isolation yields cannot be directly compared between all the samples. However, the results obtained show the high variation in the extractive-free cuticular materials in different berries. The content of raw cutin ranged from approximately 14% in sea buckthorn peel (IV) to 51% in black chokeberry peel (II). In general, the proportion of raw cutin accounted for about 20-40% of the berry samples (II, IV).

The high amount of cuticular material (58-74%) obtained from seeds evidently contained not only cuticular components, but also other components remaining after the isolation process. Seeds are complex organs with polyesters in many distinct cell layers (55). Thus, the structure and composition of seeds, which deviates substantially from the peel, may have reduced the efficacy of enzymes and solvents, in addition to the fact that the process was not repeated (II).

The raw cutin obtained from black currant was further divided into two subgroups after the isolation of raw cutins according to their morphological differences; soft transparent peels and dense receptacle particles. Berries grown in the south yielded a higher proportion of receptacles compared to peels, while berries grown in the north had almost equal amounts of both (IV).

### 5.1.2 Depolymerization of cutin polymer

#### 5.1.2.1 Chemical depolymerization

Depolymerization of berry cutin was achieved with sodium methoxide-catalyzed methanolysis reaction, using either reflux conditions (method A) (I, II) or performing the reaction in a closed tube in an overnight reaction at ambient temperature (II, IV). The small-scale closed tube method (method B) (II) was developed because the traditional and laborious reflux method was greatly affected by moisture in the reaction and required large amounts of solvents.

The two methods provided comparable results. The slight differences achieved, depending on the sample (for rosehip and cloudberry $p<0.05$), may have resulted from the heterogeneous nature of the small aliquots of the cuticular samples. The newly-developed closed tube

method was found to be rapid, repeatable and reliable for providing information on the cuticular polymers and description of the cutin monomer composition (II).

The amount of depolymerized, $CHCl_3$-soluble cutin monomers, again, revealed differences between cuticular polymers in different berries (Figure 24). Strawberry, bilberry, and raspberry raw cutin contained only 2-7% of depolymerizable cutin. Conversely, 15-30% of black chokeberry, rowanberry, cranberry and lingonberry raw cutin was composed of ester-bound cutin. Cloudberry and sea buckthorn berry raw cutin released 38-48% of monomers. The highest yields of monomers (51-84%) were obtained from crowberry and rosehip raw cutin. Seed raw cutin released only very small amounts of $CHCl_3$-soluble monomers (1-3%) (II).

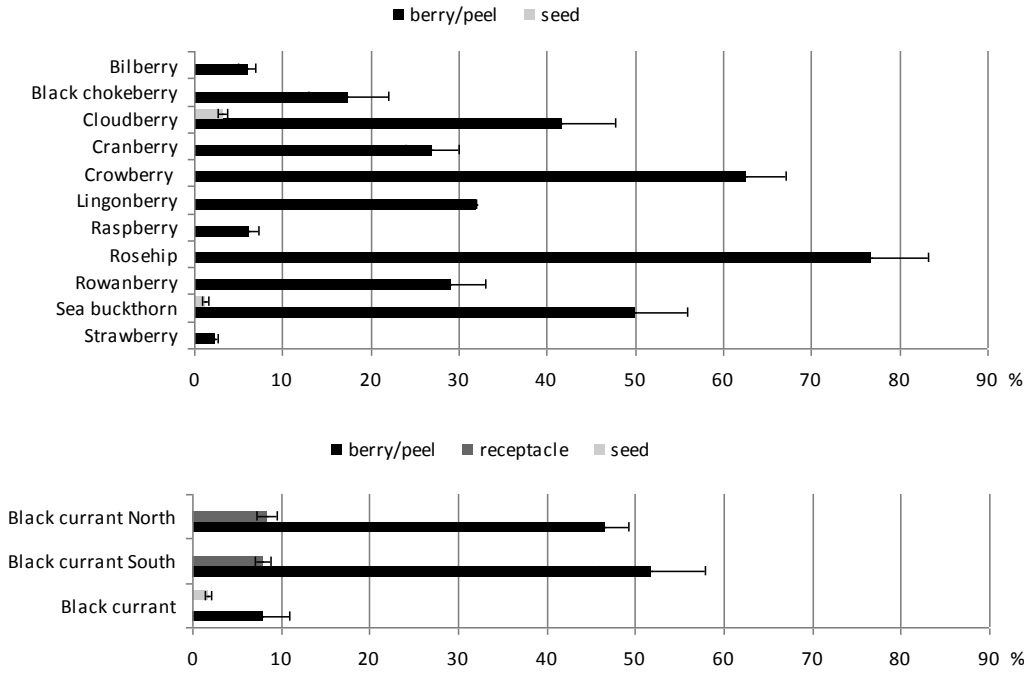



**Figure 24.** $CHCl_3$-soluble monomer fraction released from raw cutin (%).

Raw cutin isolated from commercial black currant powder yielded only 8% of the constituent monomers (I). When peel was analyzed separately from the receptacles, the yields were 46-52% and 8%, respectively (IV).

About 5% more cutin monomers were obtained from raw cutin isolated from black currant peel from berries grown in southern Finland when compared with berries from northern Finland (year 2006) (IV). Crowberries (growth locations not specified) collected in 2004 contained about 10% fewer ester-bound cutin monomers than those collected in 2005 (II).

These results were inadequate for further conclusions, but could propose that weather conditions and cultivation location might have important effects on the cuticular composition of berries.

The depolymerization-resistant solid residues obtained after methanolysis yielded 40-43% of raw cutin from peels of black currant (southern-northern Finland) and 34% from sea buckthorn berry peel. Black currant peel yields differed significantly from the yields obtained from receptacles, which were 77% (south) and 67% (north), respectively (IV). The remaining proportion besides residue and $CHCl_3$-soluble cutin monomers consisted of water-soluble components, e.g. glycerol (51), which were not further investigated.

#### 5.1.2.2 Cutinase hydrolysis of black currant cutin

The results showed that the black currant raw cutin is hydrolyzable by both CcCUT1 and Optimyze. Microscopic analysis (Figure 25) showed a clear alteration in the yellow-orange colored cutin structures in the enzyme-treated samples, but not in the reference sample. However, based on the micrographs, the mechanism of action of the hydrolyzing enzymes used seemed to be different: CcCUT1 clearly cut the cuticular layer, including the remaining cellular structures, while Optimyze partly broke down the cutin without affecting the other structures.

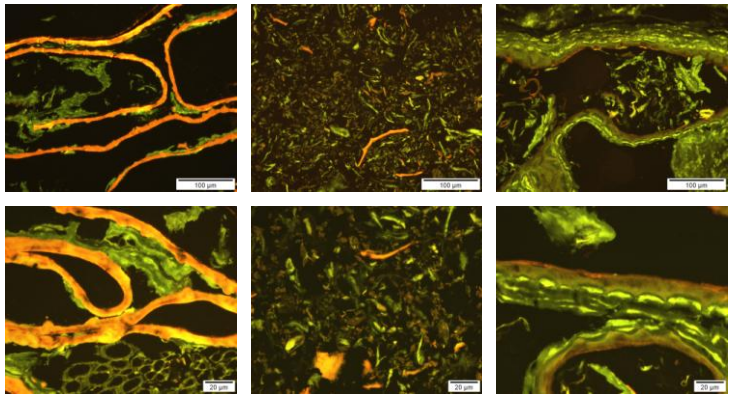


**Figure 25.** Microscopic analysis of black currant cutin after cutinase CcCut1 treatment (middle) and Optimyze 525 (left). Reference sample (right) was incubated without the enzyme.

### 5.1.3 Monomer compositions

The cutin monomers identified from berries comprised mainly of long-chain ω-hydroxy acids with mid-chain functionalities (Table 10), known to commonly exist in cutin polymers of different plant materials (46, 49, 51, 52, 54, 66, 68, 69, 74, 77, 98-100, 118).

Compounds with chain lengths $C_{16}$ and $C_{18}$ dominated in all berry cutins studied, but minor amounts of fatty acid derivatives with chain lengths $C_{15}$ and $C_{20}$ to $C_{24}$ were also found (I, II, IV). Interestingly, rosehip and cloudberry contained almost only $C_{16}$-monomers (85%, 77%) and in cranberry, lingonberry and sea buckthorn $C_{18}$-monomers predominated (86%, 74%, 78%), respectively. In studies II and IV, α,ω-diacids common in suberin and their mid-chain hydroxylated derivatives were also found. The other compound groups present were fatty alcohols, fatty acids, aromatics and unidentified compounds. Trace amounts of short-chain ($<C_{10}$) and branched-chain compounds were found in some cutins, but were included as unknown monomers because of their uncertain identifications (I, II, IV).

The major monomers in many berry cutins were dihydroxyhexadecanoic acid isomers (8,16-, 9,16- and 10,16-; which elute as a single peak). Epoxy acids ($C_{18}$) were the major monomers in black chokeberry, sea buckthorn, lingonberry and cranberry. Black currant contained 12% 16-hydroxy-(9/10)-oxo-hexadecanoic acid, which was present also in smaller proportions in rowanberry and rosehip, but not in any other berries (I, II). Only cranberry, lingonberry and bilberry contained over 20% trihydroxy acids (I).

Bilberry, strawberry and black currant cutin contained 4-6% ester-bonded aromatic compounds, while the other berries contained less than 1%, and rosehip and cloudberry lacked them totally. The aromatic compounds identified were mainly coumaric acids (*m*- and *p*-), with minor proportions of benzoic acid and its hydroxy- and/or methoxy-substituted derivatives, hydroxybenzaldehyde and ferulic acid (I, II).

Cloudberry seed cutin monomer profiles most resembled the corresponding berry cutin, whereas those of black currant and sea buckthorn seed differed markedly from their respective berry peel compounds. Sea buckthorn seed monomers evidently also contained fatty acids derived from seed oil (II).

The monomer profiles of black currant peel and receptacle differed significantly from each other, with receptacle monomer composition resembling a more suberin-like profile with 15-20% α,ω-diacids. The clear difference in appearance between the peels and receptacles was supported by differences in the amount of $CHCl_3$-soluble monomers and in the relative proportions of practically each monomer or monomer group. Slight differences in monomer proportions were seen between the black currants grown in southern and northern Finland, and similarly between crowberries from two harvest seasons (2004 and 2005). However, further conclusions regarding the effects of growing conditions (i.e. radiation, precipitation, temperature) without analyzing berries from several years are beyond the scope of this study (II, IV).

**Table 10.** Groups of $CHCl_3$-soluble cutin monomers from berries (area % of total chromatogram peak areas) (for individual compounds see references I,II,IV).

| | Billberry | Black chokeberry | Black currant powder | Black currant seed | Black currant peel | Black currant receptacle | Cloudberry | Cloudberry seed | Cranberry | Crowberry | Lingonberry | Raspberry | Rose hip | Rowanberry | Sea buckthorn | Sea buckthorn seed | Strawberry |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | S / N | S / N | | | | | | | | | | | |
| **ω-OH acids** | **76.5** | **78.6** | **70.9** | **80.8** | **84.2 / 82.8** | **62.2 / 65.4** | **86.4** | **69.5** | **96.6** | **92.3** | **95.0** | **79.2** | **88.9** | **78.9** | **94.0** | **21.8** | **59.5** |
| of which | | | | | | | | | | | | | | | | | |
| $C_{16}$ | 13.9 | 17.6 | 42.0 | 9.5 | 66.7 / 62.8 | 29.5 / 32.9 | 77.0 | 45.6 | 9.8 | 33.3 | 20.9 | 57.7 | 84.7 | 54.0 | 15.8 | 15.8 | 40.6 |
| $C_{18}$ | 61.8 | 60.6 | 23.0 | 69.7 | 17.3 / 18.7 | 22.6 / 23.7 | 9.4 | 18.8 | 86.2 | 58.8 | 73.9 | 20.4 | 3.8 | 24.3 | 77.6 | 3.4 | 17.8 |
| other | 0.6 | tr | 6.0 | 1.6 | tr / tr | 11.1 / 8.6 | - | 5.1 | 0.5 | tr | tr | 0.9 | tr | tr | 0.9 | 2.6 | 0.9 |
| of which additionally secondary substituted with | | | | | | | | | | | | | | | | | |
| epoxy | 26.3 | 44.0 | 8.7 | 46.9 [c] | 10.4 / 11.1 | 2.5 / 3.2 | tr | 1.6 | 59.9 | 33.7 | 39.2 | 0.5 | - | 9.2 | 70.8 | 0.9 | 7.9 |
| OH | 13.0 | 16.8 | 27.2 | 7.3 | 49.8 / 42.1 | 18.4 / 21.0 | 73.9 | 42.5 | 9.3 | 29.8 | 20.3 | 48.7 | 79.2 | 52.2 | 14.7 | 14.2 | 37.8 |
| diOH | 26.2 | 11.4 | 1.2 | 12.5 | 5.5 / 6.9 | tr / 1.1 | 5.6 | 4.4 | 20.6 | 2.1 | 29.2 | 1.9 | - | 13.4 | 4.5 | 1.1 | 3.4 |
| oxo | - | [b] | 12.0 | 1.5 | 15.5 / 19.3 [b] | 4.0 / 6.6 [b] | - | - | - | - | - | - | [b] | [b] | - | - | |
| OH-subst. α,ω-diacids | [a] | 0.7 | [a] | 1.2 | 1.0 / tr | tr / tr | 5.4 | 5.4 | [a] | 1.0 | [a] | 4.1 | 5.5 | 5.0 | [a] | 9.4 | 1.9 |
| Alkanols and diols | 2.5 | tr | 2.4 | 0.4 | - / 0.1 | 4.0 / 3.4 | - | 0.8 | - | - | - | 0.3 | - | tr | - | 0.6 | 0.7 |
| Fatty acids and diacids [d] | 6.5 | 3.6 | 7.0 | 6.0 | 1.9 / 2.5 | 21.8 / 19.0 | 3.6 | 14.4 | tr | 2.4 | 0.5 | 9.6 | 2.9 | 3.9 | 0.5 | 51.9 | 26.4 |
| Aromatics | 5.9 | 0.5 | 3.4 | 0.6 | 2.8 / 4.0 | 2.1 / 2.2 | - | 1.1 | tr | 0.6 | 1.4 | 0.6 | - | 0.9 | 0.7 | - | 6.0 |
| Hydrocarbons | - | - | - | tr | - | - | - | 1.2 | - | - | - | - | - | - | - | 0.8 | |
| Unidentified | 8.7 | 16.5 | 12.5 | 10.8 | 9.2 / 8.5 | 5.5 / 5.2 | 4.6 | 7.5 | 2.7 | 3.6 | 2.9 | 6.2 | 2.8 | 11.2 | 4.5 | 10.8 | 5.5 |
| **TOTAL** | **100** | **100** | **100** | **100** | **100 / 100** | **100 / 100** | **100** | **100** | **100** | **100** | **100** | **100** | **100** | **100** | **100** | **100** | **100** |

S, southern Finland (Piikkiö); N, northern Finland (Apukka); - not detected; tr < 0.5%; [a] not analyzed; [b] not separated from 7/8/9/10-OH-hexadecan-1,16-dioic acid, [c] includes 3% epoxy-1,18-diacid; [d] saturated and unsaturated fatty acids and α,ω-diacids.

### 5.1.4 Solid state spectroscopic analysis of raw cutin and depolymerization-resistant residues

The NMR technique utilized in the present study proved to be a useful technique for analyzing carbon functionalities, although signals overlapped in complex biopolymers. However, together with FTIR spectroscopy, the overall composition of cuticular fractions could be elucidated.

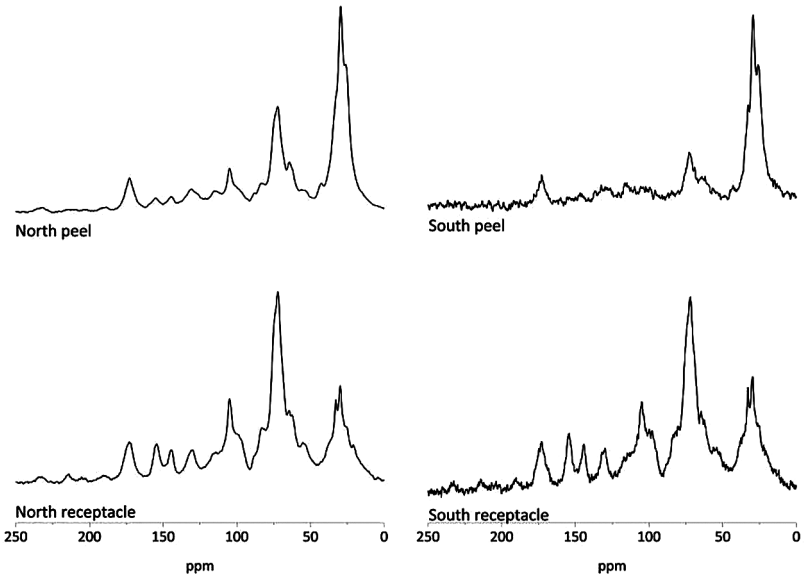



**Figure 26.** $^{13}C$ CP-MAS NMR spectra of black currant peel and receptacle raw cutin from the north and the south of Finland.

Solid state spectroscopic analysis of black currant peel and receptacle cutin further confirmed the difference between these two biomaterials. Peel cutin was essentially of an ester-bonded aliphatic nature, while receptacle raw cutin exhibited significant signals for aromatic carbons, indicating the presence of lignin-like aromatic moieties. Berries grown in the north of Finland tended to have more intense aromatic signals when compared with berries grown in the south of Finland. Figure 26 shows the $^{13}C$ CP-MAS NMR spectra comparison of raw cutin fractions from the peel and receptacle of berries grown in the south and the north of Finland. NMR spectra of sea buckthorn peel raw cutin was found to be very similar to the black currant peel spectra, but clear signal differences were seen in FTIR arising from the differences in monomer composition (Figure 27).

The analysis of the depolymerization-resistant residue revealed the efficacy of the depolymerization reaction in the removal of ester-bound cutin monomers, since the signals corresponding to ester functionalities were absent. The evident resonances of polysaccharides

present in the samples of the depolymerization-resistant residues were partly removed by subsequent enzymatic treatments, further clearly revealing the presence of the remaining aromatic and aliphatic structures. In addition to aromatic lignin-like structures, the results indicated the presence of cutan-type aliphatic polymers in berry cuticle residues.

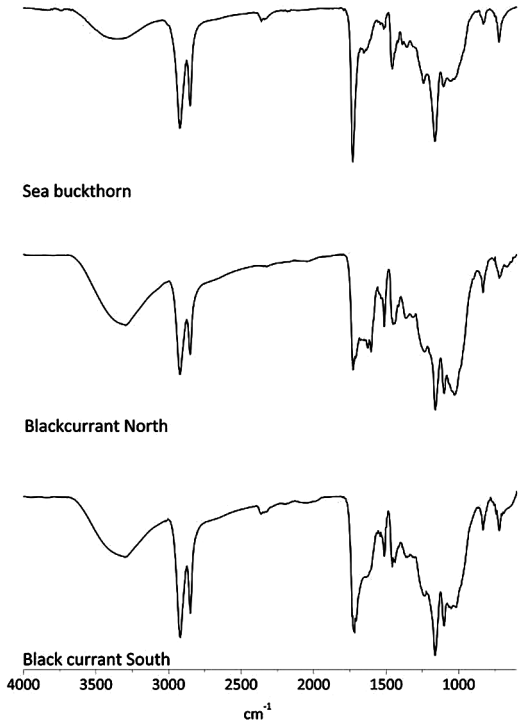



**Figure 27.** FTIR spectra of sea buckthorn and black currant (north and south) peel raw cutin.

## 5.2 POTATO SUBERIN

### 5.2.1 Peel and raw suberin isolation from potatoes

In study III, the amount of peel and raw suberin per average surface area was measured from Nikola potatoes after 2 months' storage as being 1.8 mg/cm$^2$ and 1.0 mg/cm$^2$, respectively (III).

In study V, the potato peel accounted for 2.3 g/kg and 3.5 g/kg of fresh potatoes in Nikola and Asterix, respectively, at the time of harvest (2008). The raw suberin fraction in Nikola was 280 mg/g and in Asterix was 201 mg/g of peel, also at the same time point (V).

### 5.2.2 Profiles of suberin monomers in potatoes

The most abundant aliphatic compound groups in potato suberin were α,ω-diacids and ω-hydroxy acids, accounting together for over 70% of monomers, with small proportions of fatty acids ($C_{16}$-$C_{28}$), alcohols ($C_{16}$-$C_{28}$) and aromatic compounds. Overall, Asterix contained more α,ω-diacids, fatty acids and aromatics, but fewer ω-hydroxy acids and fatty alcohols than Nikola (each $p<0.05$) (III, IV, V).

The three most abundant compounds were octadec-9-ene-1,18-dioic acid, 18-hydroxyoctadec-9-enoic acid and ferulic acid. They accounted for 38.5%, 26.6% and 4.1% of total monomers in Nikola and 39.8%, 25.1% and 4.3% of total monomers in Asterix at the time of harvest, respectively. All other individual compounds accounted for less than 2% of total monomers. Differences between varieties in the proportion of octadec-9-ene-1,18-dioic acid and 18-hydroxyoctadec-9-enoic acid were found to be statistically significant (each $p<0.05$) (V). These monomers have also been reported by others to be the most abundant compounds in potato suberin (110, 171, 218).

### 5.2.3 Comparison of depolymerization methods

Different depolymerization techniques were compared with potato suberin. To discover, in general, how effectively raw suberin was depolymerized by the various methods, the yields of the released soluble fractions were determined gravimetrically. Method A (1 M reflux) and B (1 M closed tube) both yielded 27% (SD 3%) of $CHCl_3$-soluble compounds, leaving 56% (SD 2%) of solid depolymerization-resistant residue. Method C (0.05 M closed tube) yielded 21% (SD 2%) of $CHCl_3$-soluble compounds, and was thus less effective than A or B (III).

Enzymatic hydrolysis with cutinase CcCUT 1 yielded 15% (SD 2%) of $CHCl_3$-soluble product. The solid depolymerization-resistant residue accounted for 72% (SD 1%). It was subjected to further chemical depolymerization by method B, which yielded an additional 11% of $CHCl_3$-soluble monomers. The most effective depolymerization took place with 1.0 M NaOMe-catalyzed methanolysis, yielding almost twice the amount of $CHCl_3$ -soluble compounds produced by cutinase hydrolysis (III).

The derivatized $CHCl_3$-soluble monomer fractions were further analyzed by GC using cholesterol as an internal standard, to reveal which part of the fraction was volatile enough to be detectable by GC. Compound-specific correction factors for the monomer derivatives were not determined due to the lack of reference compounds, but the internal standard was used to obtain information about the relative proportions of monomers vs. oligomers produced by the different methods (III, V). The proportions of monomers detected from methods A, B, C and cutinase hydrolysis were 35%, 40%, 20% and 50%, respectively (III). These results revealed that all the methods left, in addition to the polymeric fraction, soluble remnant compounds

that were not detected in GC analysis, which is in accordance with earlier published results (110, 112). Interestingly, these results showed that enzymatic hydrolysis produced relatively more of the compounds analyzable with GC even though the total degree of depolymerization was clearly lower.

#### 5.2.3.1 Monomeric composition of suberin

Method B (closed tube) yielded a similar monomeric profile as method A (reflux), but method C (mild closed tube) resulted in a substantially different monomer mixture, e.g. the amount of ω-hydroxy acids was lower and epoxy-acids were absent. Similar results have previously been reported for cork suberin depolymerization with several different NaOMe concentrations (112, 113).

In method C, the water-chloroform partitioning after methanolysis could be omitted and the amount of glycerol determined (about 20% of total peak areas in GC-MS and FID chromatograms) was consistent with earlier results from potato suberin hydrolysis (variety not known) (110).

Cutinase CcCUT1 showed a clear predominance in the release of α,ω-diacids over ω-hydroxy acids when compared with the chemical methods. The monomers released by cutinase were the same as the monomers released by chemical depolymerization, but the ratio of ω-hydroxy acids to α,ω-diacids was significantly lower in enzymatic hydrolysis. Epoxy-substituted monomers were absent in cutinase hydrolysis products, just as they were in milder NaOMe-methanolysis (method C) (III).

#### 5.2.3.2 Solid state spectroscopic analysis of depolymerization-resistant residues

Solid state $^{13}C$ CP-MAS NMR and FTIR analysis of the solid depolymerization-resistant residues were used to gain additional information on the mechanism of action of the cutinase CcCUT1 compared with chemical depolymerization. The $^{13}C$ CP-MAS NMR and FTIR spectra of these residues confirmed the results obtained from the other analyses and from previous observations on similar residues (124, 164). The residue from chemical depolymerization showed a clear predominantly cellulose profile overlapping with resonances from lignin-like aromatic structures, whereas the cutinase hydrolysis residue still showed clear signals derived from suberin ester-bonded aliphatic compounds (III).

The predominance of polysaccharides in the residues could be due either to their incomplete hydrolysis during the isolation procedure or to the shielding effect of suberin polyaliphatic layers, which hindered cellulase hydrolysis in the isolation of raw suberin. After cellulase treatment of the depolymerization-resistant residues, the $^{13}C$ CP-MAS NMR and FTIR analysis

revealed that the polysaccharides were partly removed, leaving broad signals of the remaining components (aromatics, carbonyls and aliphatics). The cutinase residue was further chemically depolymerized before cellulase treatment, which apparently caused a total collapse of the cellular system as seen in spectra of this residue (III).

### 5.2.3.3 Microscopic imaging of depolymerization-resistant residues

According to confocal microscopic analysis (Figure 28), both the enzymes CcCUT1-cutinase (B and E) and Optimyze 525-esterase (C and F) effectively hydrolyzed suberin in the suberized cell walls in the raw suberin samples (III).

In all samples, suberin appeared as yellow lamellar structures and the cellular network of potato periderm was apparent. In the reference raw suberin samples (A and D), suberin was seen as a continuous lamellar component surrounding the cork cells. After enzyme treatment with the cutinase CcCUT1 (B and E), suberin layers were only weakly visible when imaged from the top of the sample. In the cross section, suberin lamellae still showed the cellular network in the middle of the sample, but the outer layers of the sample had reduced fluorescence for suberin when compared to the reference sample. Fairly similar effects were obtained in the sample treated with the Optimyze 525 enzyme preparation (C and F). However, suberin seemed to be more efficiently hydrolyzed with Optimyze 525, as some cell walls did not show any fluorescence at all but some were still brightly fluorescent. Unlike in the case of cutin, CcCut1 did not break down the cellular structures of suberin.

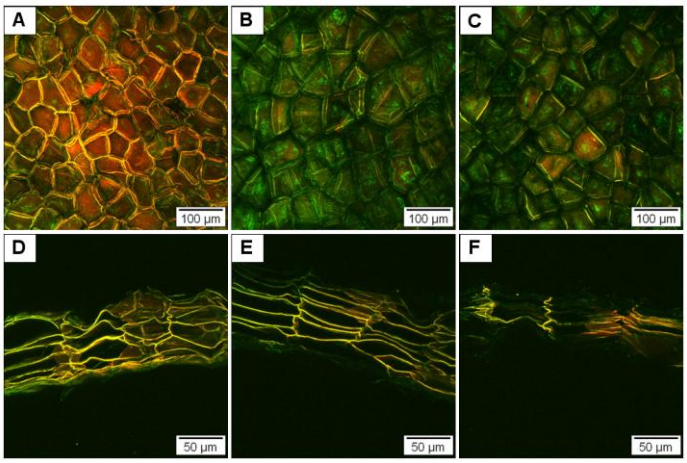



**Figure 28.** Microstructure of raw suberin (A-C from top, D-F cross-section) after cutinase CcCUT1 (B, E) and Optimyze (C, F) hydrolysis. Reference sample (A, D) was incubated without the enzyme.

The residues from chemical depolymerization (A) and cutinase CcCUT1 hydrolysis (after further chemical depolymerization) were visualized by light microscopy due to their fine

particle size which was not suitable for confocal microscopy (Figure 29). Neither of these residues contained any structures showing the bright orange fluorescence typical of suberin-containing particles stained with Oil Red O, although the slightly brownish lamellar structures observed may be the remnants of suberized cell walls.

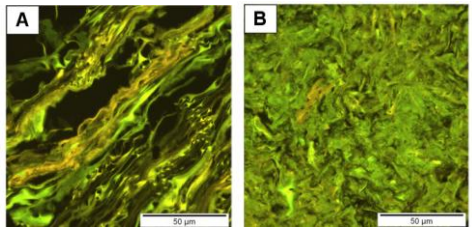



**Figure 29.** Microstructure of modified potato suberin fractions stained with Oil Red O and imaged using epifluorescence microscopy. Potato suberin residue after chemical depolymerization (A) residue after cutinase hydrolysis and chemical depolymerization (B).

Based on the microscopic data combined with the information obtained from chromatographic and spectroscopic analysis, it seems that chemical depolymerization results in more effective depolymerization of suberin than the enzymatic procedures with CcCut1 and Optimyze (III).

## 5.2.4 Changes in potato suberin during 12 months of post-harvest storage

### 5.2.4.1 Peel, raw suberin and $CHCl_3$-soluble monomer fractions

The total amount of potato peel obtained from the potatoes increased during the 12-month storage period from 2.3 g/kg to 4.8 g/kg (an increase of 106%) in the yellow-skinned Nikola and from 3.5 g/kg to 5.5 g/kg (an increase of 58%) in the red-skinned Asterix. The difference between the varieties at each time point was about 0.1% and remained relatively constant during the storage months (V).

Microscopic images of the peel from variety Nikola showed that both phellem and phellogen were identified in the peel samples representing different time points, and no significant differences were observed in the microstructure of the phellem layers (Figure 30). However, the 0 month sample was notably lacking the phelloderm layer, which was visible in the peel of Nikola after nine months. Adhesion of the phelloderm layer during maturation can be considered as one of the explanations for the increase in peel weight (V).

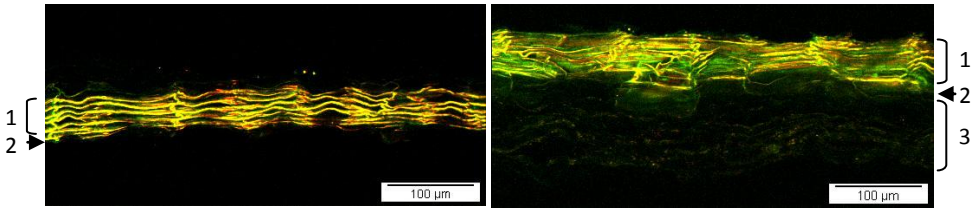



**Figure 30.** Microscopic images of Nikola peel at the 0 month (left) and 9 month (right) time points; phellem (1), phellogen (2) and phelloderm (3).

The amount of raw suberin isolated from the peel varied between 20% and 29%, being on average in Nikola 258.1±16.6 mg/g and in Asterix 250.7±36.3 mg/g during the 12-month storage period. If the 0 month results are not considered (due to the lack of phelloderm layer in peel), the amount of raw suberin was highest at the 6 month time point (Nikola 270.2 mg/g and Asterix 290.2 mg/g of peel). Microscopic images (Figure 31) of the Nikola raw suberin fraction at the 0 and 9 month time points show that the suberized cell walls and the phellem layers were both of equal thickness and no differences in microstructures were observed (V).

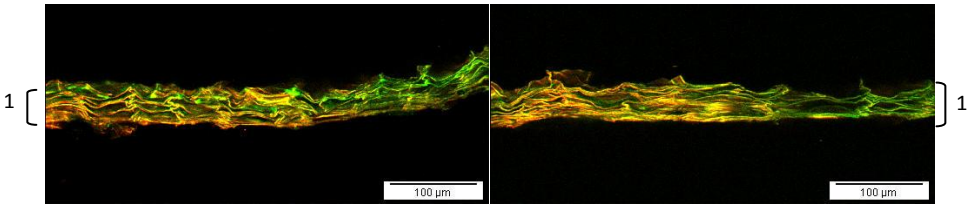



**Figure 31.** Microscopic images of Nikola peel raw suberin at the 0 month (right) and 9 month (left) time points show only the phellem layer.

Overall, the weight of the raw suberin fraction in Nikola was higher than in Asterix until 3 months of storage. After 12 months, the fractions were of equal amounts in both varieties (V).

The $CHCl_3$-soluble monomer fraction obtained by NaOMe-catalyzed methanolysis followed by extraction accounted for 19-22% of raw suberin and about 4.5-6% of peel from both varieties. There were no statistical differences in the amounts of the ester-bound monomeric fraction from raw suberin during storage, although a decreasing trend was observed in Asterix (V).

These variations indicate distinct rates in the developmental changes of the different components in peel fractions (polysaccharides, wax and suberin), which also possibly affect the effectiveness of the isolation process. The relative proportions of the aliphatic and aromatic domains of suberin did not change significantly during storage, but there was an increase in suberin up until 6 months of storage. Thereafter, the increase in peel weight was mostly due to the other components present (V).

### 5.2.4.2 Monomer profiles

Proportions of the most abundant monomer groups, the α,ω-diacids and ω-hydroxy acids, were quite constant during storage (Figure 32). Both decreased by about 1% during the storage period in both varieties, but the change from 0 months to 12 months was significant only for ω-hydroxy acids in Asterix (p= 0.000).

At the level of individual compounds, the amount of 18-hydroxyoctadec-9-enoic acid was found to change in both varieties during the storage period (0 months – 12 months p=0.047 Nikola, p=0.000 Asterix). In Nikola, changes during the storage period (0 months – 12 months) were found in the proportions of 9,10-dihydroxyoctadecan-1,18-dioic acid (p=0.020), docosan-1,22-dioic acid (p=0.006) and 9,10-epoxy-18-hydroxyoctadecanoic acid (p=0.002). In Asterix, there were no differences in the individual compounds of these groups during the storage period (V).

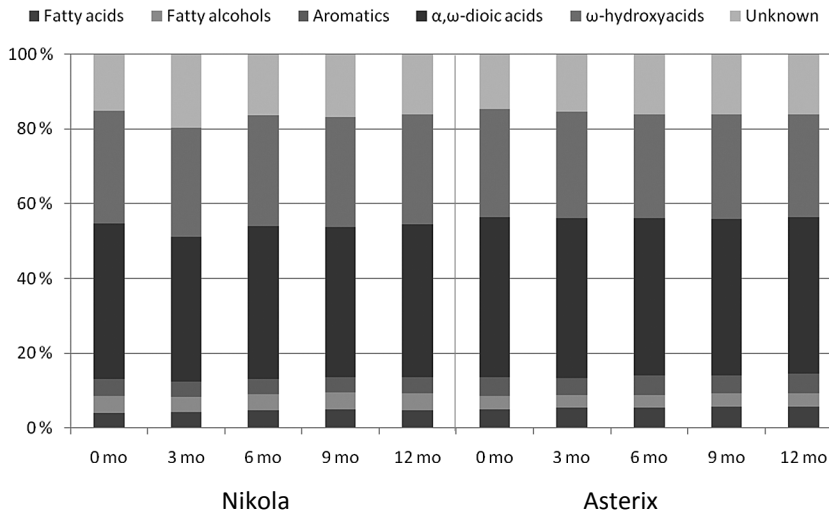



**Figure 32.** Changes in the monomer groups of suberin in Nikola and Asterix over 12 months of storage.

The proportions of the other compound groups (fatty acids, fatty alcohols and aromatics) were also found to remain stable during storage, although a slight increase in the amount of fatty acids (0 months – 12 months Nikola p=0.000, Asterix p=0.001 ) and a decrease in alcohols (0 months – 12 months Nikola p=0.038) were found. The fatty acid composition was found to differ during the 12-month post-harvest storage period in both varieties. In Nikola, an increase from 0 to 12 months was found in the relative proportions of octadecenoic acid (18:1, p=0.002), tetracosanoic acid (24:0, p=0.000) and pentacosanoic acid (25:0, p=0.039). In Asterix, there was a decrease in the proportion of octadecadienoic acid (18:2, p=0.046) and an increase in the proportions of octadecanoic acid (18:0, p=0.003) and octacosanoic acid (28:0,

p=0.016). Between the 0 month and 12 month time points for the same variety, of the individual fatty alcohols, only the amounts of nonadecanol (19-OL, p=0.014) in Nikola and eicosanol (20-OL, p=0.003) in Asterix were found to differ. Of individual aromatic compounds, only the proportion of coumaric acid in Asterix was found to increase (p=0.005), while the other aromatic compounds (ferulic acid, 4-hydroxy-3-methoxybenzoic acid and 4-hydroxy-3-methoxybenzaldehyde) in both varieties remained constant (V).

# 6 CONCLUSIONS

The method of depolymerization has a great influence on the resulting monomer composition. The results of the newly-developed closed tube method (B) for methanolysis were found to be comparable with the results obtained from the heavily solvent-consuming and moisture-vulnerable traditional reflux method (A). The closed tube method resulted in very repeatable results, without the elaborate mixtures of various derivatives caused by competing saponification. It also allowed for the screening of multiple samples simultaneously and was thus preferred in both cutin and suberin monomer analysis (II, III, IV, V).

Mild chemical depolymerization was clearly less effective compared with stronger methanolysis in the case of potato suberin (III). Cutinase CcCut1 degraded both cutin and suberin as revealed by microscopic imaging. Cutinase treatment seemed to be a more selective method for releasing monomeric compounds from suberin than the chemical methods, indicating the potential for specific industrial purposes. The results indicated that cutinase had a higher specificity for the hydrolysis of α,ω-diacids, rather than ω-hydroxy acids. This may have been because the former are mainly involved in more labile glyceryl-ester bonds, whereas the latter are in the form of less reactive wax-type ester bonds. In addition, the prevalence of α,ω-diacids over ω-hydroxy acids could also be the result of the varying organization of the units in the polymer and the varying amount of cross-linkages (III).

Berries differ greatly in their cuticular composition, both in the amount of isolated extractive-free cutin (raw cutin) and in the proportion of ester-bound cutin monomers, in addition to their highly variable monomer composition. According to the results obtained in these studies, there was no evident correlation between the amounts of cuticular material and ester-bound cutin in berry cuticles (I, II, IV). Differences in the extent of ester-linked cutin versus more resistant cutan, variable cutin monomers affecting the cross-linking and structure of cutin polymers and the composition of cutan in berries all have a strong influence on the various functions and physiological properties of cuticles in berries, and also for their nutritional qualities when consumed as food and as a part of dietary fiber.

As cutin is known to be a protective barrier against water loss together with cuticular waxes, in addition to genetic background, growth conditions and climate also provide significant explanations for the differences between berries. The amount of the cuticular material, the cutin/cutan ratio and also the monomeric units together with the cuticular waxes all have a crucial influence on the preservation of different berries in nature and when collected for food. The composition and structure of the surface tissues ultimately define the characteristic preserving properties of berries against drying, chemical attacks, mechanical injuries and microbial infections. For example, crowberries and lingonberries maintain their basic

morphology and composition for almost a year in nature, whereas strawberries, raspberries and cloudberries are typically destroyed by drying or infections in early autumn.

Polymers mainly composed of unsubstituted ω-hydroxy acids and α,ω-diacids or mid-chain substituted monomers with epoxy- or keto-functionalities are essentially linear, as there are not mid-chain groups present that could be part of interesterification forming cross-linkages. Thus, a polymer structure made up mostly of epoxy monomers differs greatly from di- or trihydroxy monomer-based polymers, which can form extensively cross-linked and perhaps more resistant polymers. A higher content of trihydroxy acid monomers and a near-equal $C_{16}$ to $C_{18}$ ratio has been found to characterize the cutin of elastic tissues, while mostly $C_{16}$ containing cutins are more rigid (23, 25). The role of glycerol in the three-dimensional structure of berry cutin cannot be addressed on due to the fact that it was not analyzed in the present study (I, II, IV).

The presence of aromatic acids esterified to the polyesters is an important feature in the cutin polymer and might play a protective role when released by the hydrolytic enzymes excreted by fungi (100). Also, the amount of phenolic compounds is correlated with the rigidity and maturation of cutin matrix (25, 33).

Residues investigated in this study suggest that cutan and lignin-like polymers are present in berry cuticular fractions. The composition of these residual cuticular polymers needs to be investigated more extensively to make solid conclusions about their structures. Black currants contained, in addition to peel cutin, a part derived from the receptacle which more resembled suberin, rather than cutin (IV). Previously, only very few edible plants had been investigated for their cutan content (36, 61, 62, 94, 94).

The slight differences in monomer proportions and polymer structures seen between the berries grown in southern and northern Finland give an insight about the effects of cultivation location, but further conclusions about the effects of growing conditions (i.e. radiation, precipitation, temperature) without analyzing berries from several years are impossible to make (IV). In previous studies, the effects of temperature and the amount of radiation have been observed to affect the cuticular composition (101, 115, 120, 121).

Suberin from two commercially important varieties of potatoes, red-skin Asterix and yellow-skin Nikola, was found to differ. The amount of peel was found to increase in both potato varieties during post-harvest storage, with a higher amount in Asterix compared to Nikola. The raw suberin fraction accounted for 20-29% of the peel, being highest at 6 months of storage. In addition to an increase in suberin up to 6 months of storage, the major contribution to the increased mass of peel was due to other components of periderm such as wax and polysaccharides. The ratio of the polyaliphatic and polyaromatic domains in suberin remained

quite constant over the storage period. Changes in the monomer compositions during the storage period were small between the post-harvest time points within a variety, but differences between the varieties were found. The α,ω-diacids, fatty acids and aromatic compounds were more abundant in Asterix, while ω-hydroxy acids and fatty alcohols were more abundant in Nikola (V).

As all plant-based foods contain cuticular and suberin polymers, they should evidently be included in non-soluble dietary fiber, although this has not been clearly stated in common definitions. A significant part of the berry fiber has been found to be insoluble, rather than soluble (202, 219), corresponding also to the substantive amount of cuticular material. Thus, knowledge of these plant substances is evidently important for human nutrition. Further studies on the structures of these polymers and on their effects in human nutrition are required for a more comprehensive understanding of their evident significance. Clinical studies focused on the role of cutin and suberin among other fiber components should be conducted related to, for example carcinogenesis, gastrointestinal health and the metabolism of sugars and fats.

The importance of so-called waste material from industrial potato and berry processes as a source of either dietary fiber or specialty chemicals should be investigated. The studied materials contained significant amounts of different types of biopolymers that could be utilized for several purposes before or after processing. These materials could be used as value-added fiber fractions in the food industry. Sandell et al. (220) evaluated the sensory properties of a similar residual fraction (ethanol and carbon dioxide-extracted juice press residue) and found it to be practically tasteless, which could be beneficial when used as a natural fiber supplements. The possible technological applications of cutin and suberin derived-products are extensive (11).

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