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On the determination of suberin and other structural components in cork from *Quercus suber* L.

by

A. VELEZ MARQUES
Dep. Quím. Org., Instituto Superior de Engenharia de Lisboa
and
HELENA PEREIRA
Dep. Eng. Florestal, Instituto Superior de Agronomia

RESUMO

Estudou-se a despolimerização da suberina na cortiça de *Quercus suber* L. em relação à influência da concentração de reagente e tempo de reacção, utilizando uma transesterificação com metóxido de sódio em metanol. A determinação da lenhina e dos polisacáridos foi feita em cortiça dessuberinizada.

A quantidade de suberina aumenta com a concentração de metóxido, ao contrário do que se passa para os outros componentes; o mesmo se observa com o aumento do tempo da reacção. A composição monomérica dos polisacáridos mantém-se sensivelmente constante para todas as condições de metanólise.

Propõe-se um método de despolimerização de suberina utilizando metóxido de sódio a 3% em metanol, em refluxo durante 3h, para uma amostra de 1,5g de cortiça livre de extractivos, de modo a maximizar a despolimerização da suberina mas com menores efeitos nas determinações subsequentes dos outros componentes estruturais.

SYNOPSIS

The depolymerization of suberin in cork from *Quercus suber* L. was studied in relation to the effect of reagent concentration and reaction time, using a transesterification with sodium methoxide in methanol. Lignin and carbohydrates were determined in the desuberinised cork samples.

The amount of suberin increases with the concentration of methoxide contrarily to the other componentes; the same effect is observed with an increase of reaction time. The monosaccharide relative composition remains fairly constant for all conditions of suberin depolymerization.

A method for suberin determination is proposed using 3% sodium methoxide in methanol for 3h in reflux for a sample of 1.5g extractive-free cork, which maximises the depolymerization of suberin with less effects on the subsequent determination of the other structural components.

1. INTRODUCTION

The cork of *Quercus suber* L. has a regular cellular structure of approximately prismatic cells with thin walls enclosing a comparatively large air-filled inside volume (Natividade 1950; Pereira et al. 1987). The chemical composition of cork cell walls is characterised by the presence of suberin, lignin, polysaccharides and extractives, which include mainly waxes and tannins (Ribas--Marques 1952; Guillemonat 1960; Pereira 1982). Cell walls have been reported as consisting of a thin primary wall of lignin and polysaccharides, of a thick secondary wall made up of alternating layers of suberin and waxes and of a thin polysaccharide containing tertiary wall (Sitte 1962).

The main chemical characteristic of cork tissues is the presence of suberin. The suberin content of corks depends on the species (Kolattukudy 1978; Hata et al. 1969); in the case of *Quercus suber* cork, suberin amounts to 30-50% of the cell wall and therefore constitutes its main chemical component (Pereira 1979).

The definition of suberin involves some controversy (Kolattukudy 1978). Initially, it was thought of as the anatomical entity observed in the microscope, the suberin complex, which builds up the secondary wall and includes a polymeric material and soluble

waxes. More recently, the term has been used in the chemical sense to designate only the polymeric material named suberin, and therefore excluding waxes and other components. It is in this chemical sense that the term suberin will be used in this study.

The structure of suberin is yet not fully elucidated. It is known that the molecule is a cross-linked polymer composed by a phenolic part, with some "lignin" character, and by an aliphatic part, both linked to one another by ester bonds. The components of the aliphatic part are mainly fatty acids, fatty alcohols, hydroxycarboxylic acids and dicarboxylic acids, with a number of carbon atoms between 16 and 28. A model for the suberin molecule has been proposed (Kolattukudy 1978) and is shown in Figure 1.

In *Quercus suber*, the aliphatic part of suberin is made up mostly of hydroxycarboxylic acids (Table 1) and the most important individual acids are the following: 22-hydroxydocosanoic acid (phelonic acid); 9, 10-dihydroxyoctadecanedioic acid (phloionic acid); docosanedioic acid (phelogenic acid); 9,10,18-trihydroxyoctadecanoic acid (phloionolic acid), 9-octandecendioic acid and 18--hydroxy-9-octadecenoic acid (Holloway 1972).

The presence of suberin in the cell wall complicates the procedure for the summative chemical analysis of cork since this analysis requires the separation and individual determination of the different components. A comparison of their chemical characteristics shows that suberin will be more easily depolymerised by hydrolysis of its ester bonds without major effects on lignin or polysaccharides. However, this depolymerization of suberin is likely to cause some attack on lignin and polysaccharides since they also contain hydrolysable ester and ether bonds, therefore affecting their subsequent quantification.

So far the methods used to depolymerise suberin are based on cleavage of ester bonds. Three methods have been used: hydrolysis with alcoholic alkali (Holloway 1972); transesterification with methanol containing a catalyst such as BF (Kolattukudy et al. 1975) or sodium methoxide (Holloway 1973; Pereira 1982); and hydrogenolysis with LiAlH (Kolattukudy 1978) (Figure 2).

The first method produces the free carboxylic acids, but has the disadvantage of oxidative reactions occuring during hydrolysis. The second method is frequently used and prefered by most authors for the analysis of the aliphatic components of suberin. The third one is more specific and it is used in the structural anal-

ysis of the phenolic part or for quick determination of the presence of suberin (Kolattukudy 1978).

FIGURE 1

A model proposed for suberin (adapted from Kolattukudy 1978)

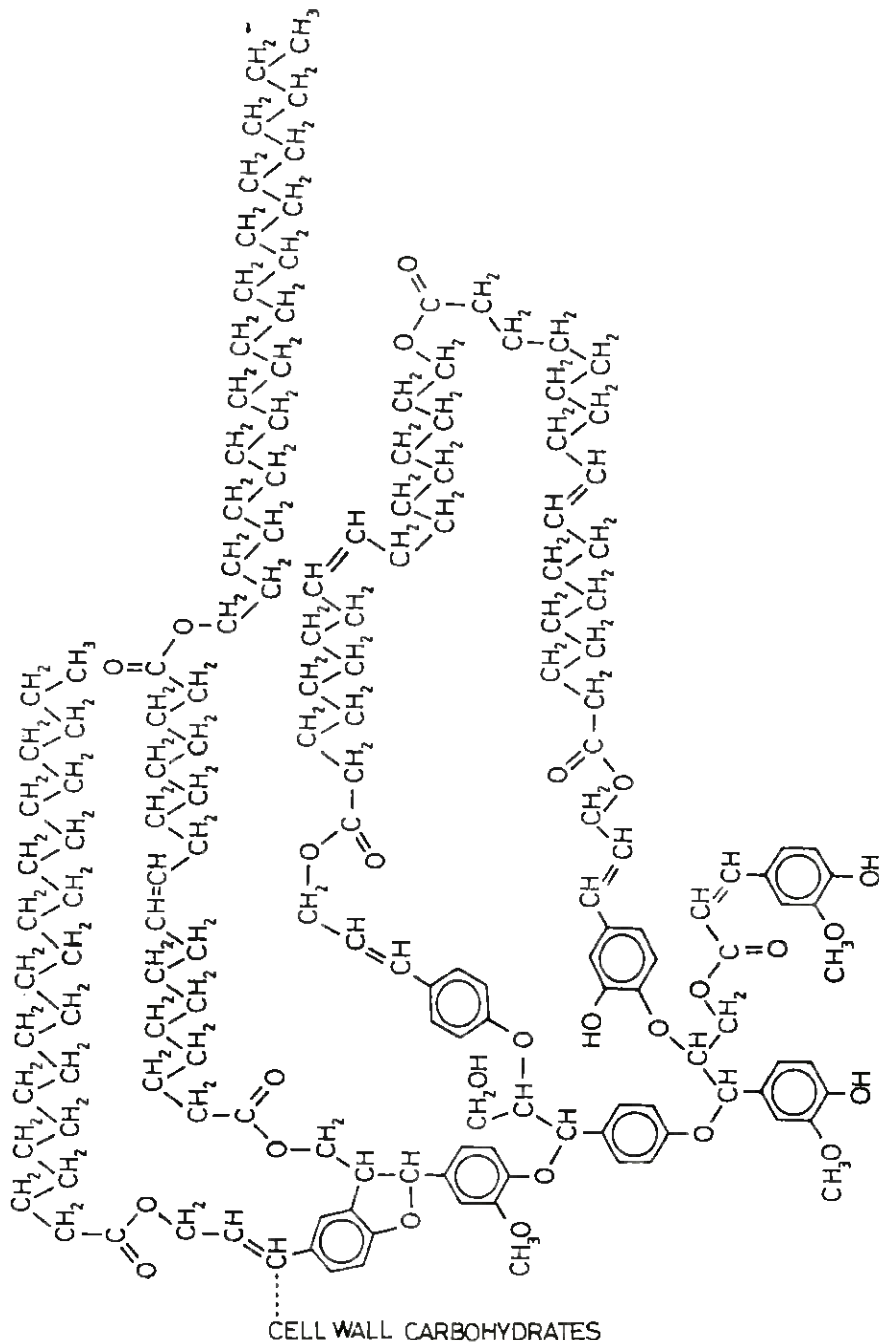


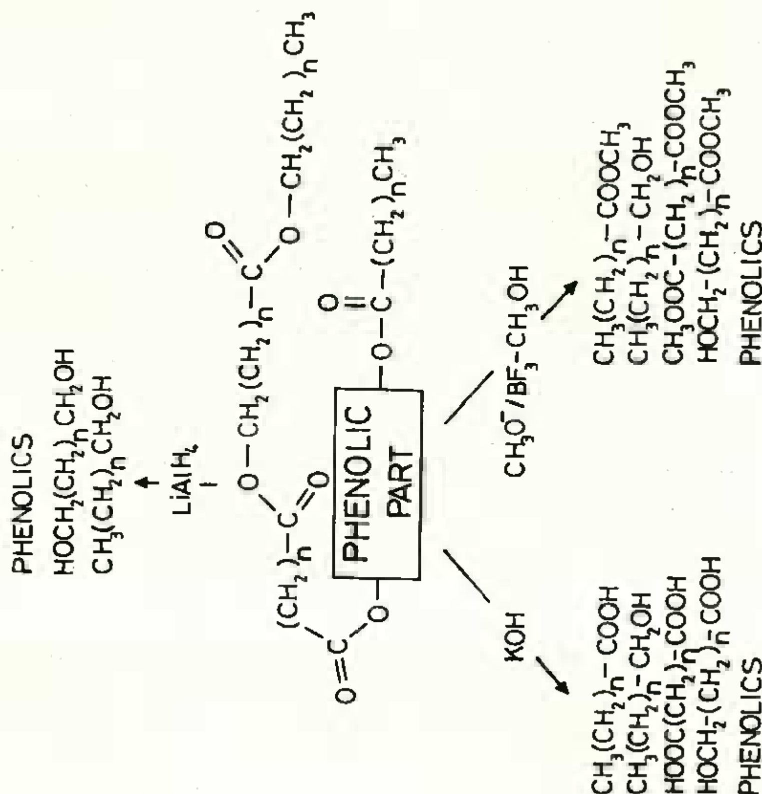
TABLE 1

Chemical composition of suberin from Quercus suber (Holloway 1972).

Component	%
Neutral	
1-Alcanols, C_{22}-C_{28}	2.7
Unidentified	3.4
Acids	
Monocarboxylic, C_{16}-C_{26}	1.9
α, w-Dicarboxylic, C_{16}-C_{26}	47.4
9,10-Dihydroxyoctadecanedioic	15.4
9,10,18-Trihydroxyodadecanoic	7.7
Unidentified	13.9

FIGURE 2

Schematic representation of possible methods for the depolymerization of suberin.



The implementation of a procedure for the summative chemical analysis of cork should take into account the effects of suberin depolymerization on the subsequent determination of lignin and polysaccharides. This paper reports results on suberin determination in cork of *Quercus suber* by using depolymerization with sodium methoxide and two reaction parameters: reaction time and methoxide concentration. The effect on the determination of monosaccharides and lignin content was studied for all cases.

2. MATERIAL AND METHODS

Samples of virgin cork of *Quercus suber* L. from Grândola (Herdade de Gouveia) were used. The samples were ground and the fraction 40-60 mesh used for analysis.

Extractive-free cork, to be used as the starting material for determination of suberin, was prepared by soxhlet extraction successively with dichloromethane, ethanol and water.

The depolymerization of suberin used sodium methoxide in methanol (Pereira 1982). 1.5 g of extractive-free cork were refluxed with 250 ml of sodium methoxide in methanol. Reaction time was varied from 0.5 to 5 hours and the concentration of sodium methoxide from 1 to 5% (w/w). The reaction products were filtered, the solid residue kept for subsequent analysis of lignin and carbohydrates, and the pH of the extracts adjusted to 6 with 2M H S0 in methanol. The methanolic extract was evaporated to dryness in a vacuum rotating apparatus and the residue suspended in 200 ml of water. The hydrolysis products were extracted with 200 ml chloroform three times. The extract was dried over anydrous Na_2 $S0_4$, filtered and evaporated. It was weighed and reported as suberin.

The solid residue remaining after suberin depolymerization was used for determination of lignin and carbohydrates by acid hydrolysis. The acid hydrolysis was done following the standard method TAPPI 249 pm-75, and used for determination of insoluble and soluble lignin and of monosacharides. Insoluble lignin was gravimetrically determined as the solid residue of hydrolysis and soluble lignin was estimated in the hydrolysis liquor by UV

absorption at 200-205 nm. The monosaccharides in the hydrolysis liquor were derivatized to alditol acetates and separated and quantitatively determined by gas-liquid chromatography, using myo-inositol as internal standard. A packed column of 3% ECNSS-M in Gas-Chrom Q 100-120 mesh, FID detector and integrator were used.

3. RESULTS AND DISCUSSION

The variation of reaction time and concentration of reagent for the methanolysis of suberin was made using three different samples of cork, A, B and C. Table 2 presents the corresponding results for the amount of suberin determinated with each set of reaction parameters, as well as the results for the subsequent determinations of lignin and carbohydrates.

These results show that the determination of each component is influenced, in greater or lesser degree, by the condition of reactions, namely by the concentration of $NaOCH_3$. The variation of the amount determined for suberin is positive with an increase of methoxide concentration, contrarily to the other components, for which this variation is negative. For instance, in sample A and with a reaction time of 1 h, the amount determined as suberin is 37.4% if 1% $NaOCH_3$ is used, but 44.8% if the concentration is increased to 5% $NaOCH_3$; on the contrary, the determination of insoluble lignin gives a value of 27.9% for the lower concentration and 23.4% for the higher concentration. A similar effect is seen in the determination of carbohydrates, with 17.2% for 1% $NaOCH_3$ and 13.0% for 5% $NaOCH_3$.

The variations in the determination of suberin, lignin and carbohydrates with the concentration of reagent rather counterbalance each other, since the total sum of components remains fairly constant for most cases.

The influence of reaction time is more difficult to be assessed because no direct comparison of the three samples can be made since they may have different chemical compositions. However, if suberin contents are calculated as percent of the maximum values obtained for each sample (respectively 45.9% for sample A, 42.1%

TABLE 2

Summative chemical analysis of three different samples of cork, which were desuberinised by methanolysis with varying reaction conditions.

Cork sample	Extractives (%)*	Conditions of methanolysis		Suberin (%)*	Lignin		Carbo-hydrates
		Time (h)	$NaOCH_3$ (%)		Insoluble (%)*	Soluble (%)*	(%)*
A	12.5	1	1	37.4	27.9	1.9	17.2
			2	43.8	24.9	1.1	13.1
			3	45.9	23.5	1.1	12.5
			4	43.6	n.d.	n.d.	13.1
			5	44.8	23.4	1.1	13.0
B	15.9	2	1	37.0	28.4	1.6	17.7
			2	39.2	22.9	0.7	18.9
			3	41.6	20.0	0.3	15.7
			4	42.1	22.1	0.9	n.d.
			5	39.5	21.5	1.0	16.5.
C	12.9	3	1	43.9	21.3	0.6	17.8
			2	45.7	20.9	0.9	16.1
			3	49.4	19.0	1.2	14.5
			4	47.0	19.8	0.9	15.2
			5	46.4	20.7	0.9	15.3

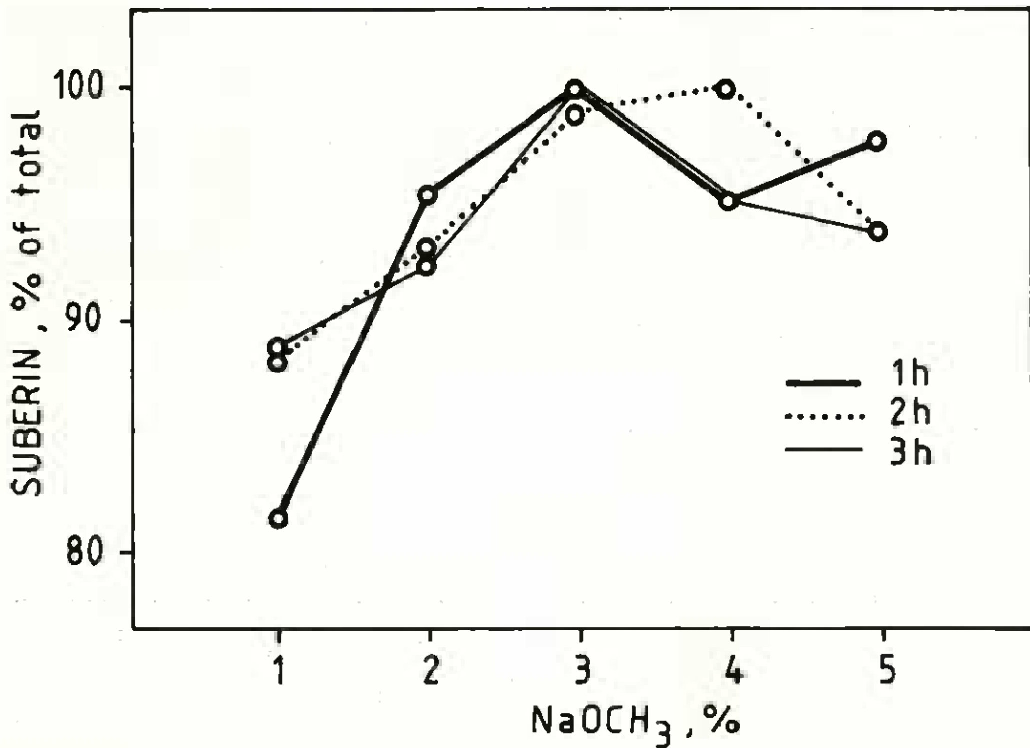
*percentage of oven-dry weight of cork
n.d. = not determined

for sample B, and 49.4% for sample C, which are then reported as 100%), the influence for reaction time in relation to methoxide concentration becomes clearer. This is shown by Figure 3.

The methoxide concentration is more important in the determination of suberin for shorter reaction times. With 1h reaction, approximately 20% more suberin is determined if the methoxide concentration is increased from 1% to 3%. For all cases, the amount of suberin determined increases to a maximum corresponding to 3-4% methoxide, and decreases somewhat with higher increases of methoxide concentration.

FIGURE 3

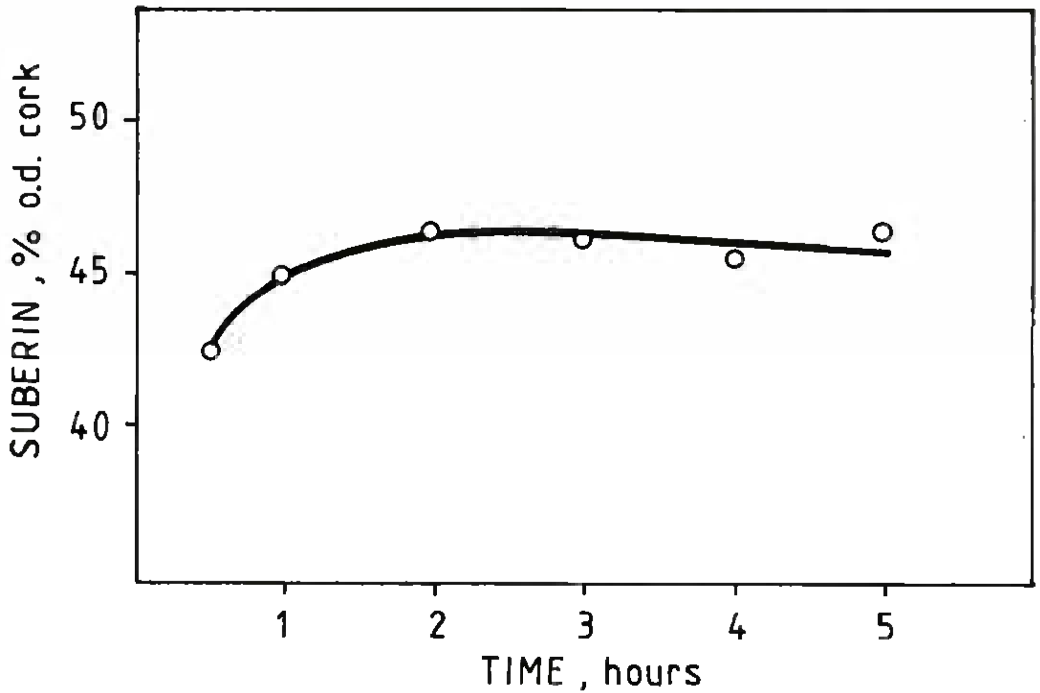
Determination of suberin using different concentrations of sodium methoxide and reaction times.
Values refer to percentage of maximum suberin determined for each reaction time.



The influence of reaction time on suberin determination for a methoxide concentration of 3% was further studied for another sample. The results are shown in Figure 4 and confirm that significant variations only occur with very short times.

FIGURE 4

Determination of suberin content using 3% sodium methoxide and different reaction times.
Values refer to percentage of suberin in relation to o.d. weight of cork.



The carbohydrate composition of the three samples after methanolysis in the different conditions is presented in Table 3.

TABLE 3

Carbohydrate composition of the three samples after methanolysis with different reaction conditions

Cork sample	Conditions of methanolysis		% of total monosaccharides					
	Time (h)	$NaOCH_3$ (%)	Rahmnose	Arabinose	Xilose	Mannose	Galactose	Glucose
A	1	1	1.6	10.3	32.0	3.4	6.4	46.5
		2	1.1	10.2	39.1	2.8	5.4	41.4
		3	1.0	11.2	30.5	3.9	6.8	46.6
		4	1.3	11.5	28.4	2.2	6.3	50.3
		5	1.4	11.1	29.0	3.5	6.9	48.2
B	2	1	1.8	6.3	34.5	2.7	3.3	51.3
		2	1.7	6.3	35.9	1.9	3.1	51.2
		3	2.2	6.3	33.5	2.2	3.2	52.3
		5	1.5	5.9	35.8	2.2	2.8	51.5
C	3	1	1.9	6.6	29.3	4.1	10.2	47.9
		2	1.9	7.2	29.4	3.0	8.6	49.9
		3	1.9	6.8	28.5	3.4	9.7	49.9
		4	1.1	7.4	28.0	–	9.7	47.5
		5	1.7	7.9	31.1	2.2	10.1	47.1

It can be observed that the monosaccharide composition slightly differs between samples. For instance, sample A has more arabinose than the other samples, or sample C more galactose. For each sample, however, the carbohydrate composition did not change appreciably with the different reaction conditions of the desuberinization, even if the total amount of carbohydrate material decreased with reaction time, as discussed previously.

The results so far obtained allow some discussion on the methanoysis of suberin. The attack on the ester bonds of suberin with the consequent depolymerization and solubilization is not completed with low concentrations of reagent or with short reaction time, thus giving rise to smaller values for the determination

of suberin. A similar effect was found when low ratios reagent: cork (volume of 3% methoxide: amount of cork) were used for the desuberinization (Pereira 1981).

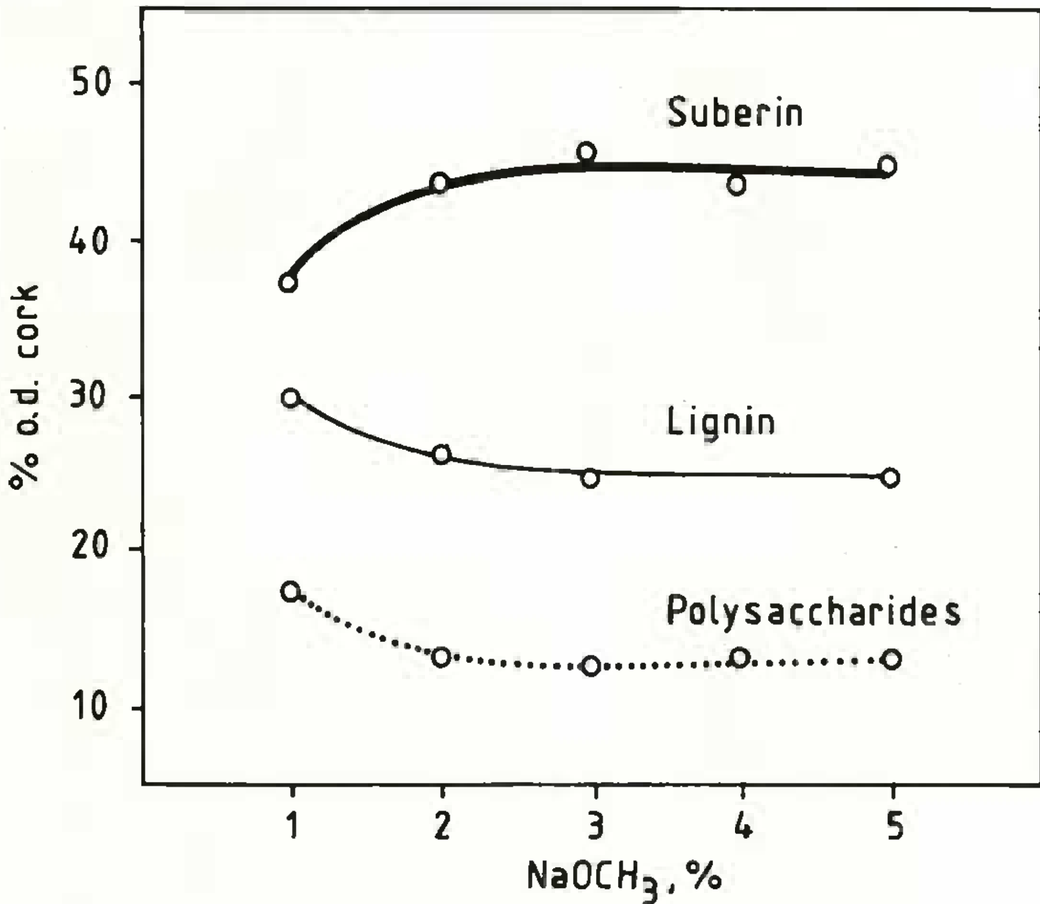
This does not apply to the carbohydrates which are not determined as a residue. For the case of carbohydrates, a decrease of the total amount is verified in relation to concentration of reagent: e.g. 17.2% for 1% NaOCH and 13.1% for 2% $NaOCH_3$ with a reaction time of 1h. It can be observed that the larger variations in the carbohydrate determination occur for the lower methoxide concentrations, 1% and 2%; for a methoxide concentration of 3% and above, the total carbohydrates remain fairly constant. This may indicate that carbohydrates are linked to the suberin molecule it as has been proposed in the model presented in Figure 1. For milder conditions of attack, e.g. low concentration of methoxide, part of the suberin is not depolymerized and remains in the solid residue of the reaction; any carbohydrates bound to this suberin also remain and therefore will be determined subsequently. In case of a stronger depolymerization, carbohydrates associated with suberin may to some extent be solubilized and lost for subsequent determinations.

A similar pattern of variation with methoxide concentrations is found for lignin, as seen in Figure 5, where the variation of suberin, lignin and carbohydrates with methoxide concentration is represented.

The increase of methoxide concentration and reation time leads to lower values of lignin and this fact could be considered as representing a stronger attack. However, the variation of lignin may result not from a different degree of attack by the methoxide but more from the fact that undepolymerised suberin will remain as solid residue after the acid hydrolysis and will be gravimetrically determined as "lignin". In this way, the remaining suberin increases the values determined for "lignin" when the acid hydrolysis is performed on cork desuberinised under the milder conditions. An extreme example might be found in the determination of lignin in cork which was not previously subjected to any desuberinization. In this case, the residue of the acid hydrolysis ("lignin") is approximately 60% (Pereira 1982), showing that most of the suberin is retained in this amount.

FIGURE 5

Determinations of suberin, lignin and carbohydrates with different $NaOCH_3$ concentrations (Sample A, 1h reaction time).



The conditions chosen for the depolymerization of suberin will therefore affect the other cell wall components. Variation of the results is smaller by use of longer reaction times, as should be expected: if 3 h is selected, then a methoxide concentration of 2-3% will achieve maximum suberin values, with small variations for lignin and carbohydrates determination.

4. CONCLUSIONS

In cork from *Quercus suber*, the use of methanolysis for the depolymerization of suberin followed by the quantitative determination of the chloroform-soluble components, as a step in its summative chemical analysis requires the selection of reaction conditions, e.g. reaction time and reagent concentration. Subsequent determinations of lignin and carbohydrates in the desuberinised cork material depend on the conditions of suberin depolymerization. A method using a concentration of 3% $NaOCH_3$ in methanol during 3 h in reflux is proposed for maximising suberin depolymerization with less effects on the determination of the other structural components.

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