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Sodium Chlorite Delignification of Soil in the Investigation of Soil Polysaccharides

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The sodium chlorite-dilute acetic acid reagent was tested as a means of removing soil humic substances to facilitate soil polysaccharide analysis. The soil sample was an ether and cold water pre-extracted pine forest soil from Western Norway. The results of the polysaccharide determinations are compared with results obtained by a direct, fractionated acid hydrolysis method not involving chlorite. It turned out that more than 40 % of the total carbohydrates were lost in the chlorite procedure, mainly in a dialysis step. The method thus cannot be used for total soil polysaccharide analysis unless modified to recover dialysable carbohydrates.

The chlorite cellulose fraction, which closely corresponds to the common plant cellulose definition, consisted entirely of glucose units, while the "cellulose" obtained in the direct hydrolysis procedure was impure. On this basis is concluded that the chlorite method probably gives more reliable values than the latter method for "true cellulose" in soil.

According to the direct hydrolysis method the total carbohydrates constituted 22.4 % of the humus, estimated by circular paper chromatography and calculated as anhydro-sugars. Chlorite cellulose amounted to 2.5–2.8 %.

The investigation of soil polysaccharides is rendered difficult by their diversity and by the interference from and interaction with other soil constituents, in particular the humic substances. The occurrence of more or less firm bonds between soil polysaccharides and humic substances seems established;[1] the presence of chemical bonds is moreover to be expected from the continuous humification process which takes place in soil. In addition to the usual loss from decomposition, incomplete hydrolysis, *etc.*, an unavoidable loss of carbohydrates from condensation with the omni-present humic substances must be taken into account in soil polysaccharide analysis. Obviously this last type of loss would be minimised if the humic matter were removed from the soil sample, or made unreactive, prior to polysaccharide analysis. Various extraction methods have proved ineffective in separating soil poly-

saccharides from humic matter,[1] however, and little effort seems to have been made at finding other methods.

A possible approach would be by oxidation of the humic matter while leaving the soil polysaccharides unattacked as a "holocellulose" residue, on the model of plant material delignification. Sodium chlorite delignification [2-4] has previously been suggested in peat polysaccharide analysis.[5]

It was found of interest to test this method on a sample of highly organic Norwegian soil. In a preliminary work [6] the sodium chlorite reagent was applied to the soil in order to examine the applicability of paper chromatographical polysaccharide analysis to soil chlorite holocellulose. This work has now been supplemented by similar analyses of fractions obtained by a more drastic chlorite treatment as well as by a direct acid hydrolysis of the soil not involving chlorite. In this paper are discussed the results obtained with the three methods, which throughout are termed I (direct, fractionated acid hydrolysis), II (ordinary chlorite), and III (drastic chlorite treatment).

EXPERIMENTAL

Soil sample. The soil sample was an aliquot from the sample described by Grov.[7] It consisted of the combined, undistinguishable F-layers of a pine forest soil from Western Norway, was of dark brown colour, and contained fibres of incompletely decomposed plant materials. Before use it was extracted with ether, dried, and extracted with cold water for an investigation of the cold water soluble carbohydrates.[8] The air-dry residue is termed extracted soil.

Moisture and ash (Table 1). Moisture contents were determined by drying in an oven at 105°C, and ash was obtained by direct ignition.

Elementary analyses (Table 1). The carbon and hydrogen contents of extracted soil and holocellulose II were determined according to Reihlen.[9]

Acid lignin (Table 1). The acid lignin determinations were carried out according to the 72 % sulfuric acid method of Ritter, Seborg, and Mitchell.[10]

Fractionated acid hydrolysis of soil (Method I). The soil was heated in a boiling water bath for 7.5 h with 0.5 N sulfuric acid, filtered, and washed. The residue was further hydrolysed with 2 N acid for 4 h, filtered, washed, and dried with alcohol and ether. The powdered cake was digested with 72 % sulfuric acid for 2 h at room temperature, diluted to 1 % and boiled for 15 h, and filtered. — After removal of sulfate all solutions were concentrated *in vacuo* at max. 40°C to small volume, weighed, and conserved with thymol.

Chlorite treatments (Methods II and III). The treatments were carried out as described by Wise, Murphy, and D'Addieco [2-4] on 10 g samples of air-dry, extracted soil; the ordinary treatment (4 h) employing the specified amount of sodium chlorite, the drastic

Table 1. Analyses of extracted soil and holocelluloses.

	Extracted soil	Holocellulose II	Holocellulose III	Expressed as percentage of
Moisture	11.5	3.4	4.9	air dry subst.
Ash	22.2	52.2	63.2	105°C dry subst.
Carbon	55.1	48.2	—	org. matter
Hydrogen	5.50	6.55	—	org. matter
Acid lignin	49.6	13.1	—	org. matter

one (14 h) with three times this amount. In both instances the holocellulose was collected and washed in the centrifuge at 3000 rpm until free from Cl^-, and dried with alcohol and ether. The dry weights of the very voluminous, fibrous, light grey preparations were 4.0 g and 3.43 g, respectively.

In both treatments the chlorite mother liquor contained black, colloidal substances, and further amounts went into colloidal solution during the washing of the holocelluloses. The II colloids were separated by membrane filtration, but were not investigated. Colloids III were isolated by centrifugation for 1 h at 13 000 rpm. After washing with water, alcohol, and ether the preparation (about 400 mg) was obtained as a black, amorphous powder containing 16.3 % ash. Iron content, 9.9 % of the ash.

The chlorite mother liquors and washings were dialysed in cellophane bags against tap water until free from Cl^-, and evaporated to dryness *in vacuo* at 40°C. The tough, horny, dark brown masses weighed 2.26 g (II) and 3.03 g (III). Ash content of III, 18.9 % to a large part consisting of sodium carbonate. Iron, 0.85 % of the ash.

Fractionation of holocelluloses with alkali. Holocellulose II was extracted with 5 % and 24 % aqueous potassium hydroxide according to standard procedures [2,3] to give alkali-soluble fractions termed hemicellulose A (22 % yield) and B (6 %), respectively, as dense, grey powders. The fibrous, very voluminous, light grey, mineral-containing residue was termed α-cellulose II.

Holocellulose III was extracted with 24 % alkali only, which dissolved 14.1 % substance (hemicellulose III, light grey powder), leaving a light grey residue of α-cellulose III.

Hydrolysis of chlorite preparations. The mother liquor dry substances II and III were hydrolysed with 0.5 N sulfuric acid in a boiling water bath for 4 and 7.5 h, respectively. Hemicelluloses A and B of series II were heated with 2 N acid for 5 h, the hemicellulose III for 7.5 h. The α-cellulose preparations II and III were pre-hydrolysed with 2 N acid for 4 to 5 h, and the alcohol and ether dried residues were digested with 72 % acid for 2 h. After dilution to 3 % and 1 %, respectively, the mixtures were further hydrolysed for 4 to 5 h in a boiling water bath. — All hydrolysates were processed as described for method I.

The bulk of the mother liquor dry substances consisted of water-soluble, non-carbohydrate material of caramel-like appearance, probably degraded humic substances. Part of this material coagulated during the hydrolysis and was removed, but most of it remained in solution, and caused trouble in the chromatography by blocking up the paper.

Chromatography. The hydrolysates were analysed for monomeric sugars by circular paper chromatography employing a slight modification of the multisector, multiple development technique described by Giri and Nigam.[11] The papers (Whatman No. 1; 24 and 30 cm diameter circles) were run in specially designed, small volume cabinets of 18 mm internal height.[12] The micropipets were calibrated against each hydrolysate by weighing to eliminate errors arising from different densities of the hydrolysates.

Solvent systems employed were (a) butanol:pyridine:water (6:4:3),[11] (b) ethyl acetate:-acetic acid:water (3:1:3, upper layer),[13] (c) ethyl acetate:pyridine-water (40:11:6),[14] (d) ethyl acetate:pyridine:water:acetic acid (5:5:3:1).[15]

Colour reagents: (e) Aniline hydrogen phthalate,[16] (f) diphenylamine aniline phosphoric acid,[17] (g) α-naphthylamine phosphoric acid,[11] (h) *p*-anisidine phosphoric acid,[18] and ninhydrin.[19]

Quantitative determinations with triphenyl tetrazolium bromide were performed with slight modifications according to Giri and Nigam.[11] The formazan colour was eluted with 96 % alcohol and measured against paper blanks in Pulfrich Stufenphotometer with filter 4900Å (series II [6]), or in Beckman DB spectrophotometer at 480 mμ (series I and III), in at least triplicates. Rhamnose was determined on separate chromatograms in duplicate. The accuracy of the sugar determinations was estimated to be within ± 10 %. The results are compiled in Table 2, expressed as percentage of extracted soil organic matter, which is taken as the loss on ignition (Alvsaker [20]).

The sugars galactose, glucose, mannose, arabinose, xylose, and rhamnose were completely separated in system (c). The absence of ribose in all hydrolysates was demonstrated in system (c), and of fucose in system (b). Rapid-moving, methylated sugars could not be detected. None of the chromatograms gave response with the ketose reagents (g) and (h). In series II weak bands giving uronic acid colour reactions moved in system (d) identically with galacturonic and glucuronic acids. In series I and III residues on the

starting line giving uronic acid reactions were observed in system (c), but were not further investigated. All hydrolysates gave weak bands moving slower than galactose and giving pentose or hexose reactions; they were interpreted as oligosaccharides arising from incomplete hydrolysis, but were not closer examined. Hexosamines could not be detected, but most of the hydrolysates in series I and III gave several ninhydrin-positive spots of mobilities in the range of amino acids in system (c).

DISCUSSION

Total polysaccharides. The total amount of polysaccharides found in the extracted soil by method I is seen to be almost twice as great as when determined by any of the chlorite methods II or III (Table 2). It is thus evident that the direct acid hydrolysis of soil is superior to the chlorite methods for the determination of water-insoluble polysaccharides in soils of this type. However, the losses of carbohydrates which are inherent in polysaccharide analysis, due to incomplete hydrolysis, a possible condensation with humic matter during hydrolysis, acid-catalysed decomposition of sugars, loss in the chromatography, *etc.*, all contribute in lowering the results. Consequently even the value found by method I, 22.4 % of extracted soil organic matter, should be taken as a minimum value for the content of water-insoluble, acid hydrolysable polysaccharides in this soil.

Cellulose. The polysaccharides left in the chlorite II and III cellulose fractions after treatment with alkali and subsequent pre-hydrolysis with dilute acid were found to be composed entirely of glucose units (Table 2). Disregarding the refractory humic matter still present, these polysaccharides undoubtedly represent an extensively purified cellulose, as defined by the same procedures as employed for defining plant chlorite cellulose.[2-4] It may be argued that the combination of unreactivity and insolubility which makes these glucose residues appear in this fraction may be displayed not only by "true cellulose" but also by refractory compounds between humic matter and shorter-chain carbohydrates. However, the complete absence of other sugars than glucose in the fraction does not seem to support this argument, since the ability to be incorporated into humic matter compounds certainly is not specific for glucans alone.

Even if the existence in soil of cellulose-producing bacteria implies that part of the soil chlorite cellulose may be of microbial origin, the term cellulose seems appropriate, because microbial and plant cellulose are identical in every respect.[21]

Judging from the close similarity, within the limits of error, between the cellulose contents of the two chlorite preparations, the intensity of the chlorite treatment appears to be of little importance for the amount of cellulose determined. This is in accordance with statements [2-4] that the chlorite reagent has but little degrading effect on polysaccharides. The slight degradation observed has been ascribed to an oxidation of 1,4-bridges to form ester linkages which are easily cleaved by acid or alkali; at the same time the reducing end groups are oxidised to glyconic acid groups.[22] These factors may in part explain the lower cellulose values of II and III as compared with I, since acidic polysaccharide fragments may be extracted in the alkali treatment of the holocellulose. It is not likely, however, that such degradation and loss of cellulose

Table 2. Individual anhydrosugars (glycans) and total polysaccharides, expressed as percentage of humus.

Method	Soil fraction	Galactan	Glucan	Mannan	Araban	Xylan	Rhamnan	Total polysac.
I	½N acid	2.4	2.5	1.6	2.5	3.5	1.1	13.6
	2 N acid	trace	1.0	0.24	trace	0.16	—	1.4
	72 % acid	—	7.2	0.24	—	—	—	7.4
	Sum	2.4	10.7	2.1	2.5	3.7	1.1	22.4
II	Moth.liq.	1.2	0.72	0.89	0.89	0.56	0.07	4.2
	Hemi A	0.56	1.9	0.38	0.39	1.0	0.13	4.4
	Hemi B	0.14	0.32	0.22	0.05	0.19	trace	0.93
	α-Cellul. Prehydr.	0.14	0.29	0.18	0.05	0.06	—	0.72
	α-Cellul.	—	2.8[a]	—	—	—	—	2.8[a]
	Sum	2.0	6.0	1.7	1.4	1.8	0.20	13.1
III	Moth.liq.	0.50	0.20	0.18	0.74	0.58	trace	2.2
	Colloids	0.13	0.65	0.10	0.05	0.08	0.03	1.0
	Hemicell.	0.40	1.6	0.20	0.32	1.3	—	3.8
	α-Cellul. Prehydr.	0.54	1.0	0.79	—	0.33	—	2.7
	α-Cellul.	—	2.5[a]	—	—	—	—	2.5[a]
	Sum	1.6	6.0	1.3	1.1	2.3	0.03	12.2

[a] Cellulose.

should occur here to any larger extent than in ordinary plant holocellulose treatment.

On the other hand, the cellulose preparation obtained in I by treating the soil with dilute acids is seen to contain some mannose (Table 2). Since the more extensively purified preparations II and III contain glucose only, this mannose probably originates from hemicelluloses which still accompany the cellulose and which are not removed by acid treatments only. The presence of gluco-hemicelluloses as well may explain the large content of glucose in I relative to the II and III cellulose fractions.

It may be concluded, therefore, that the cellulose value I most probably is too high, while the chlorite method seems to give a more reliable cellulose determination, at least in this type of poorly decomposed, highly organic soils.

Hemicelluloses. The holocelluloses II and III contained considerable and nearly equal amounts of hemicelluloses along with the cellulose (Table 2). The two successive extractions with 5 % and 24 % potassium hydroxide in procedure II dissolved more hemicelluloses than did the single treatment with 24 % alkali in III. The subsequent pre-hydrolysis of the alkali-extraction residues seemed to dissolve all residual hemicelluloses in both cases, however, presumably because the swelling action of the strong alkali upon the cellulose matrix makes the hemicelluloses readily accessible to the dilute acid. The main difference between the hemicellulose A + B fraction of series II and

that of series III thus seems to be due merely to the difference in methods employed: If the hemicellulose content of the chlorite holocellulose is defined as the content of non-cellulosic polysaccharides it is not significantly influenced by variations in the chlorite or alkali treatments.

The hemicelluloses determined by method I are less clearly defined. It is interesting to note that the sum carbohydrates in the last two fractions of I amounts very closely to the holocellulose carbohydrates of II and III (Table 2). The mutual identity of these three groups of polysaccharides is not real, however, because their compositions differ. This fact makes doubtful also the otherwise obvious assumption that the most easily hydrolysable polysaccharide fraction of I (0.5 N acid step) be identical with the fraction which dissolved in the chlorite treatment and of which only the non-dialysable part is recovered.

Colloids. The iron content of as much as 1.6 % of the colloids may suggest that a relatively large proportion of their organic matter is made up of iron complexes of humic acids. The colloidal character points to very large structures, and it would seem likely that the colloids in their original form occur in soil as part of insoluble, super-molecular humic matter aggregates, which partially are broken down by the action of the chlorite. The observation that the cold water pre-extraction of the soil does not dissolve any significant amounts of colloidal matter, while the colloids are easily washed out after the chlorite treatment by the cold water washings, may support this. The great stability of the colloids against the chlorite reagent suggests that more reactive locations on the original humic aggregates have been oxidised, leaving the fragments more stable to further attack.

The colloid fraction polysaccharides seem to be exceptional in their high proportion of glucan, which places them intermediate between cellulose and the other soil fractions (Table 2). It is not improbable that the colloids contain some cellulose; this would be made clear by a sub-fractionation with alkali.

Carbohydrate loss. The losses of carbohydrates in series II and III amount to 42 and 45 %, respectively, relative to the series I value. The loss may have occurred by dialysis of polysaccharide fragments, or by irreversible condensation of carbohydrates with humic substances. However, such condensation would probably be acid-base catalysed and therefore should occur to a lesser extent in the weakly acidic chlorite solution than in the 0.5 N acid step of method I. It seems, therefore, that the main loss of carbohydrates in the chlorite procedures occurs in the dialysis step. The requirement of dialysability then necessarily restricts the size of the carbohydrates being lost to a few monosaccharide units.

As glycosidic bonds and polysaccharide ring units in general suffer little degradation by the chlorite reagent,[2-4,22] it may be assumed that such short-chain saccharides occurring in the reaction mixture, to a small extent only are produced by the splitting of longer chains. Since the soil beforehand was extracted with water it is not likely that these carbohydrates are present in the free state in the extracted soil, but are rather set free from complexes or compounds between short-chain saccharides and humic matter by oxidative degradation of the humic part of the compounds, or even by the slight hydrolytic action of the chlorite solution. The bonds involved in such complexes

may thus in part be rather labile, as is also indicated by observations that polysaccharide fractions prepared by mild extractive procedures may contain up to 90 % of dialysable matter.[1]

Acid lignin. In plant analysis [2-4] the residual lignin left in chlorite holocellulose is usually determined as the 72 % sulfuric acid insoluble organic matter.[10] When applied to soil holocellulose II it turned out, however, that the material determinable as acid lignin (Table 1) comprised only 19.5 % of the non-carbohydrate organic matter, against 60 % in the extracted soil. Clearly the non-carbohydrate part of the organic matter remaining in the holocellulose has been subjected to alterations resulting in increased acid solubility, and the conclusion lends itself that this acid lignin method is inapplicable to soil studies.

Ash balance. While only 59 % of the organic matter was recovered in procedure III the sum of the ignition residues of the soil fractions corresponded to a recovery of 143 % of the original ash. This surprising result may be explained only by an uptake of sodium ions from the chlorite mixture, *e.g.*, through carboxylic or other acidic groups formed by partial oxidation of humic matter. The combined ignition residues of the holocellulose and the colloids amounts to 112.3 % of that of the extracted soil. The rest of the uptake is to be ascribed to the mother liquor dry substances, which give as much as 18.9 % ignition residue, to a major part composed of sodium carbonate. The sodium content roughly corresponds to an equivalent weight of 240 for these substances. Considering their water-solubility this low value is not improbable. At the same time their non-dialysability suggests a molecular weight of several times this value. Thus it may be assumed that the major part of the recovered mother liquor dry matter probably consists of sodium salts of complex, polybasic acids derived from humic matter by partial oxidation. Being relatively stable to chlorite they are only slowly further degraded to dialysable products, and accumulate in the mother liquor.

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