

Studies on the Ether-extract from a Raw-humus

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The ether-extract from raw-humus has been shown to be an extremely complex mixture of a large number of different compounds by means of chromatographic separations. Infra-red spectra of the isolated fractions give absorption bands which can be attributed to hydroxyl, carboxyl, different carbonyls, aromatic compounds and aliphatic chains. The extract contains a series of n-alkanes ranging from C₁₆-C₃₃.

Relatively little attention has been paid to the wax-fractions of soils, isolated either as ether- or as benzene-extracts. Such fractions from soils are believed to contain similar components as may be found in plants, *i.e.* essential oils, fats, resins, and so on.¹ Gilliland and Howard² recently showed that the wax isolated from peat is an extremely complex mixture, in which they identified n-alkanes (C₂₇-C₃₃) and perylene. They also stated the existence of some non-identified polycyclic hydrocarbons, and a mixture of esters of high molecular weight. Morrison and Bick³ identified n-alkylmethyl ketones (C₁₇-C₃₇) in the wax-fraction from a mineral soil. The corresponding fraction from raw-humus has largely been neglected.

Chemical interest in the composition of this wax arises for the following reasons: a) Preliminary screening tests at this laboratory using gas chromatography, have shown that there is a significant difference between ether-extracts isolated from a raw-humus, and those from the same humus fertilized with urea. It is therefore possible that at least some of the compounds in the ether-extractable material react with urea or its products of hydrolysis, and give rise to some incorporation of the added nitrogen compound in the humus structure. b) The possibility that knowledge of the chemical structures in the extract can give more information on the humus structure.

In order to obtain information about the ether-extract from a typical raw-humus the following investigation has been carried out.

Direct gas chromatography of the ether-extract demonstrated the existence of a complex mixture and different methods of prior separation have been tried.

A steam distillation gave only 4 % yield of a product which on gas chromatography showed approximately 20 partly separated peaks. We did not

succeed in a complete separation, and it was concluded from the chromatogram that the mixture contained even more than the 20 compounds mentioned. A comparison with a sample of pine turpentine showed the latter to contain fewer and lower-boiling components; but some of the first eluted compounds from the steam distillate could be present in both samples according to retention data.

A vacuum distillation (at 10^{-2} mm Hg) was performed and two fractions with boiling ranges of 160–190°C and 190–215°C were collected with yields of 10 and 17 %, respectively. Both fractions were viscous syrups and showed a complicated and relatively similar separation picture of approximately 25 compounds in each fraction.

A classical alkaline extraction procedure for separating neutral materials from phenols and carboxylic acids did not prove satisfactory because of the formation of emulsions and insoluble materials. According to Zinkel and Rowe ⁴ the separation of ether-soluble acidic and neutral materials from pine is possible without structural changes when using DEAE-Sephadex in the free base form, and ether as solvent. An experiment with the ether-extract from humus gave a neutral fraction containing more than 50 components as found by gas chromatography. The yield was 13 %. The infra-red spectrum of the isolated fraction showed a relatively simple picture in spite of the number of compounds. There are absorption bands corresponding to absorption of hydroxyl, different kinds of carbonyl, and aliphatic chains of at least four methylene groups. The fraction has less absorption of aromatic character and the main part of it may therefore consist of alkanes, waxes, fat alcohols, and perhaps polycyclic ring systems. The fact that the infra-red spectrum is simple is not necessarily in conflict with the large number of compounds shown on the chromatogram. A more thorough treatment of the retention data indicates the existence of at least 3 homologous series.

Elution of the acidic compounds was done with ether-methanol saturated with carbon dioxide, and the total yield amounted to approximately 6 % divided over several fractions. Infra-red spectra of these fractions indicate mixtures containing the same functional groups as before, and, in addition, aromatics and perhaps fatty acids.

A further fraction was eluted with 4 % formic acid in ether and amounted to a yield of 10 %. The rest of the ether-extract added to the column, 70 %, could not be eluted with the solvent mixture, and even much stronger treatment was not sufficient to release the material. It looks as if humic compounds react with the Sephadex gel matrix, since this has also been observed in gel filtration with water solutions. The gel filtration method with DEAE-Sephadex does not give the same good results for the acidic components of humus as for pine extracts, but the method gives at least a very gentle isolation of the neutral compounds.

Isolations on activated aluminium oxide have been tried as a more specific procedure. An experiment was performed with neutral aluminium oxide, where petroleum ether, benzene, chloroform, and ethanol were used as eluting solvents, partly as pure solvents and partly as mixtures. A series of fractions was isolated which showed a rather complicated composition on gas chromatography.

The first fractions eluted with petroleum ether were identified as mixtures of n-alkanes extending from C₁₆ to C₃₃ as indicated by gas chromatography and the IR spectra. The other fractions showed the same functional groups in their IR spectra as has been mentioned earlier. The larger fractions obtained by the abovementioned methods were subjected to further attempts of separation using gel filtration (Sephadex LH-20) with chloroform as solvent. Although some of the above fractions were sparingly soluble in 2-propanol, this solvent was used in order to attempt better separations of aromatic substances.

The separations were for most of the fractions not completely successful; they did show some definite fractionation when monitored by UV absorption at 254 m μ and could be divided in 4–8 subfractions. In this connection it must be remembered that some groups of compounds which are supposed to be present do not absorb at 254 m μ . Gas chromatographic studies showed that the first eluted fractions, *i.e.* those with the highest molecular weight, did not elute at any of the temperatures used up to 275°C. The lower molecular weight fractions all showed a relatively complicated pattern of separation which, at least for some components, was found to be complete. Isolations by preparative gas chromatography were successful for some peaks where the compound was retrieved in an injection needle cooled by dry ice. The purity was checked by gas chromatography and found to be at least 90–95 %, but the total yield was too low for further investigations.

Most of the fractions isolated have also been investigated by thin-layer chromatography on activated silica-gel with developing solvents of different eluting power. For visualization, general reagents such as iodine vapour and chromic acid were used. Most of what has been said about the complexity of the different fractions is confirmed by the thin-layer chromatograms, and in most cases they also show a dark-coloured trail stretching over the greater part of the chromatogram.

EXPERIMENTAL

Material. A raw-humus described elsewhere⁵ was dried at 50°C and 12 mm Hg in a rotary evaporator for fast removal of most of the water, and thereafter dried to constant weight in a vacuum oven at 50° and 12 mm Hg. The dry humus (2.2 kg) was percolated with ether until a colourless eluate was obtained (30 litres for 10 days). The wax fraction was isolated as a dark brown resin after distilling off the ether. Yield 32.2 g (1.5 %).

Apparatus. Gas chromatographic analyses have been performed on a dual channel Aerograph 1520 B with flame detectors and nitrogen as carrier gas. The columns used were 1/8 in. stainless steel (s.s.) tubing of lengths 6 to 10 feet with stationary phases of 2–5 % SE-30, SE-52, Apiezon L, neopentyl glycol succinate, and Carbowax 20 M on acid-base washed and HMDS-treated Chromosorb W 60/80 mesh. At the higher temperatures, 10 feet of 1/8 in. s.s. column with 5 % SE-30 on the above mentioned support was mainly used.

For preparative gas chromatography, 10 feet of 1/4 in. s.s. column packed with 2 % Carbowax 20 M on acid-base washed Chromosorb W 60/80 mesh and a 20-foot 3/4 in. Al column packed with 3 % SE-30 on the same support were used.

Temperature programming in the range 125–275°C, and detector and injector temperatures of 300°C were necessary for most analyses.

A Hamilton fraction collector did not prove satisfactory at those high temperatures, because of condensation in the needle caused by bleeding (destruction products and column material). This gave mixed fractions and blocked the outlet needle. Better results were achieved when the samples were collected in needles cooled by dry ice-acetone

mixture according to Edwards and Fagerson.⁶ An additional oven had to be mounted on the gas chromatograph outlet.

Column chromatography. The eluate from the columns used was collected with an automatic fraction-collector and analyzed by continuous recording of the absorption at 254 m μ . The eluate was grouped in fractions according to its UV absorption and gas chromatogram.

Thin-layer chromatography. After different degrees of activation, the silica gel plates were developed with the solvents hexane, benzene, chloroform, and acetone either as pure solvents or as mixtures, and sometimes with the addition of acetic acid. For visualization the plate was placed upside-down as a lid on a container filled with iodine vapour and the spots marked directly on the glass plate. After the iodine had been removed by slightly warming, the plate was sprayed with a mixture of 5 g potassium dichromate in 100 ml 40 % sulphuric acid and warmed on a hot plate to 150°C. The two methods gave almost identical chromatograms but some spots were detected with iodine only. The potassium dichromate spray gave mostly brown-black spots, but some were coloured red, violet, or blue.

Infra-red spectra. The isolated fractions were recorded in potassium bromide pellets, as liquid films, and in Nujol mulls on a Unicam SP 200 spectrophotometer.

S e p a r a t i o n s

A. *Steam distillation.* The ether-extract (5 g) was steam-distilled for 25 h. The distillate was extracted with ether, and the product isolated from this as a viscous yellow oil, which turned brown under the influence of air. Yield 190 mg.

B. *Vacuum distillation.* The ether-extract (6.8 g) was dissolved in ethanol, a precipitate (1.1 g) filtered off, and the rest distilled at 10⁻² mm Hg, whereby two fractions were obtained.

Fraction B 1: B.p. 160–190°C yield 1.0 g

» B 2: » 190–215°C » 1.2 g

Both fractions were yellow viscous oils which gradually darkened on exposure to air. The chromatogram of fraction B1 was very similar to the steam-distillate, but contained more components. Fraction B 2 gave a slightly simpler chromatogram.

C. *Separations on DEAE-Sephadex.* DEAE-Sephadex in the free base form was, after swelling in the solvent mixture ether:methanol:water (89:10:1), packed in a column to give a bed. 2.7 × 40 cm, and equilibrated at a flow rate of 20 ml/h for 12 h. The ether-extract (6.8 g) was dissolved in 10 ml solvent mixture and a minor precipitate (0.9 g) filtered off. The rest was loaded on the column and eluted with: 1, 700 ml ether:methanol:water (89:10:1); 2, 1000 ml ether:methanol (90:10) saturated with carbon dioxide; 3, 4 % formic acid in ether until colourless eluate.

Table 1. Separation of the ether-extract from raw-humus on columns of DEAE-Sephadex.

Fraction	Solvent No.	Yield in g	Appearance of fraction
C 1	1	0.01	light yellow wax
C 2	»	0.90	»
C 3	2	0.05	light brown wax
C 4	»	0.02	»
C 5	»	0.05	brown waxy
C 6	»	0.10	red waxy
C 7	»	0.13	yellow waxy
C 8	»	0.05	»
C 9	»	0.05	light brown wax
C 10	»	0.09	»
C 11	3	0.91	dark brown tar

Before using solvent system 3, the column was washed with ether:methanol (90:10) in order to remove excess carbon dioxide. The fractions are given in Table 1, and are designated by the capital letter of the above heading.

D. *Separations on aluminium oxide.* Aluminium oxide, neutral M, was washed with distilled water until free of chloride ions, and activated at 180°C for 12 h. A slurry of the activated oxide in dry petroleum ether (60–80°C) was packed in a column giving a bed of 2.5×70 cm. The ether-extract (9.5 g) was boiled with 100 ml dry petroleum ether and filtered hot. A highly viscous black tar (0.9 g) remained undissolved. Cooling of the solution gave a white precipitate (0.8 g) which apparently consisted of fatty acids and fats or waxes according to the IR spectrum. The rest of the fraction (7.8 g) dissolved in 50 ml petroleum ether was loaded on the column and eluted, using the solvent schedule: 1, 600 ml petroleum ether; 2, 600 ml benzene; 3, 600 ml benzene:chloroform (2:1); 4, 600 ml benzene:chloroform (1:1); 5, 1000 ml chloroform; 6, 1400 ml chloroform:ethanol (1:1); 7, 500 ml 7.5 % acetic acid in chloroform:ethanol (1:1); 8, 500 ml 10 % acetic acid in chloroform:ethanol (1:1).

The eluates were worked up to give the main fractions listed in Table 2. The last fractions were contaminated with aluminium salts.

Table 2. Separation of the ether-extract from raw-humus on columns of aluminium oxide.

Fraction	Solvent No.	Yield in g	Appearance of fraction
D 1	1	0.03	white crystals
D 2	»	0.08	»
D 3	»	0.03	»
D 4	»	0.01	»
D 5	»	0.01	»
D 6	2	0.01	light yellow, wax
D 7	»	0.01	»
D 8	3	0.01	»
D 9	4	0.35	yellow wax
D 10	»	3.13	brown wax
D 11	5	0.38	dark green, waxy
D 12	»	0.16	»
D 13	6	0.25	brown waxy
D 14	»	0.12	»
D 15	»	0.17	light brown waxy
D 16	»	0.64	»
D 17	»	0.14	»
D 18	7		dark brown tar
D 19	»		»
D 20	8		»
D 21	»		»
D 22	»		»

The fraction D 2 was separated by gas chromatography on a 10-foot 1/8 in s.s. column packed with 5 % SE-30. Temperature-programming from 190–250°C with a 5°/min rate of increase, followed by isothermal operation at 250°C was used. The retention-times of the alternating high and low peaks suggest a homologous series some of which correspond to the pure reference compounds C₂₂, C₂₇, and C₂₈ n-alkanes; the infra-red spectrum of the fraction gives further confirmation that it is a mixture of n-alkanes of the composition given in Table 3. In addition there are trace amounts of the n-alkanes C₁₆-C₂₀. The molar distribution of the components was estimated on the assumption that the area under each peak was proportional to the molar concentration of each component.

Table 3. Gas chromatographic analysis of n-alkanes present in fraction D-2 (Table 2).

Carbon number	21	22	23	24	25	26	27	28	29	30	31	32	33
Mole per cent	1.8	1.8	2.2	1.6	4.7	3.5	13.3	9.7	25.6	5.7	26.8	1.4	1.6

Analogous results are found for the fractions D 1 and 3 and D 4–5, but the two latter most probably contain other hydrocarbons in addition to the n-alkanes. It has not been possible to make a satisfactory separation of the other fractions by gas chromatography. The aluminium ions in fractions D 18–22 cannot be removed by solvent extractions, and the fractions also harden with time and become more insoluble in the common organic solvents.

E. *Separation on Sephadex LH-20*. After swelling in chloroform, the Sephadex LH-20 was packed in a column to give a bed of 2.5×32 cm. Some separations have also been tried on a longer column with bed size 2.5×89 cm. An amount from 30 to 250 mg of the different fractions was dissolved in 1–2 ml of chloroform, loaded on the column, and the chromatogram then developed at a flow-rate of 20 ml chloroform/h. The smaller column was used for the fractions A, B1, B2, C2, D8-11, and the longer for the fractions D 8–11, D 13, and D 16. According to the UV analyzer the separation gave from 4 to 8 more or less separated peaks. It should be mentioned that at least some of the fractions contain compounds not absorbing at $254 \text{ m}\mu$, but no attention was paid to this when working up the eluate. As an example, the gel filtration of 207 mg D 10 in 2 ml chloroform on the short column may be cited (Fig. 1). The eluate was divided into sub-fractions at the minima of the recorder response curve, and the total recovery was 194 mg (95 %).

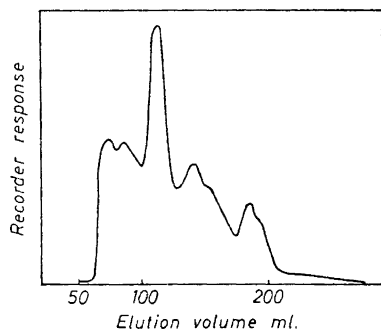


Fig. 1. Separation of fraction D 10 on Sephadex LH-20. Flow rate 20 ml chloroform per h.

The first fraction did not elute at all as far as could be determined on the gas chromatograph; all the other subfractions showed a more or less complicated chromatogram containing up to 25 single peaks. There is a certain overlapping between the fractions, but there is no doubt that there has been at least a qualitative separation by the gel filtration.

The longer column gave, in parallel runs, a better separation between the absorption peaks than the shorter column, although the peaks were at the same time somewhat broader. This could be caused by a higher degree of diffusion in the longer column, but also by better fractionation of compounds with nearly equal elution volumes.

Analogous gel filtration has been tried with 2-propanol as solvent in cases where the material placed on the column was sufficiently soluble, but the separations were generally not so good as with chloroform.

Acknowledgement. Thanks are due to the *Agricultural Research Council of Norway* for giving support to this work, which is a contribution from the Forest Soil Fertilization Research Group, Vollebekk, Norway.

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Received March 11, 1969.