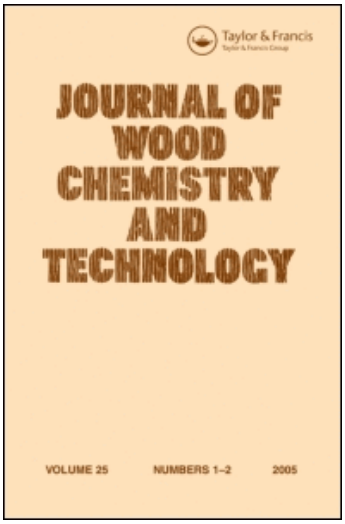

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Duane F. Zinkel[a]
[a] U.S. Department of Agriculture, Forest Products Laboratory, Forest Service, Madison, Wisconsin

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QUANTITATIVE SEPARATION OF ETHER-SOLUBLE ACIDIC AND NEUTRAL MATERIALS

Duane F. Zinkel

Forest Products Laboratory,[1] Forest Service
U.S. Department of Agriculture
Madison, Wisconsin 53705

ABSTRACT

The separation of neutral from acidic components of lipophilic materials (e.g., ether extractives, rosins, tall oil) can be accomplished rapidly and quantitatively without chemical alteration of the components using DEAE-Sephadex. Details and applications of the methodology are presented.

INTRODUCTION

Lipophilic extractives, such as the diethyl ether extractives of wood, usually are complex mixtures. In order to analyze an extraction mixture or to isolate components for further identification, some fractionation is a prerequisite. One of the first steps in the classical approach is to effect class separation based on the acidity of the components. Thus, the separation of neutrals from acids involves partition of the neutral components into a water-immiscible solvent, and the acids as salts (usually sodium salts) into an aqueous phase. This classical approach is not adaptable, however, for quantitative separation of extractives such as from conifers

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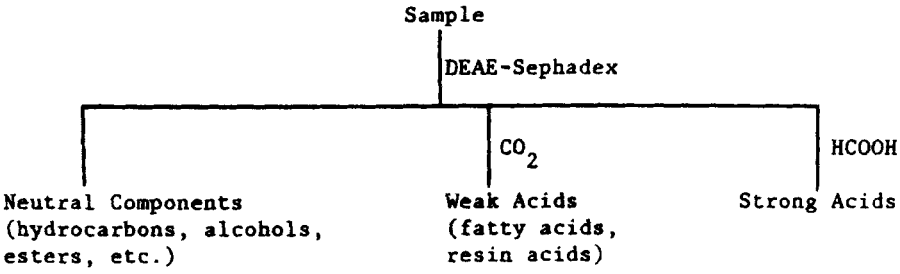

FIGURE 1 General scheme for the separation of acids and neutrals.

because of problems associated with the characteristics of soap (sodium salts of water-insoluble fatty and resin acids) solutions. To avoid such problems, a method was developed[2] that provides a rapid and quantitative separation of ether-soluble acidic and neutral materials (Fig. 1). In summary, the method consists of (1) the preparation of a column of DEAE-Sephadex (diethylaminoethyl-Sephadex) as the free base using an organic solvent system, (2) addition of a sample to the column, (3) elution of the neutral components, (4) elution of weak acids such as fatty and resin acids with carbon dioxide-saturated solvent, and (5) elution of strong acids with formic acid. The method has become the cornerstone of an analytical scheme for tall oil precursors[3] used at the Forest Products Laboratory and in many other laboratories. This report reviews laboratory experience with the DEAE-Sephadex separation method that has been acquired since publication of the brief note on the method in 1964.

MATERIALS AND METHODS

General Concepts

Ion exchange chromatography is an obvious approach to effect the separation of neutral from acidic materials. Ion exchange resins, however, are unsatisfactory in nonaqueous systems because

of possible resin bleeding into the organic solvent and resultant contamination of the fractionated solutes. Although DEAE-cellulose can be used to separate neutrals from acids, ion exchange capacity is limited and there is difficulty in packing columns with the filamentous DEAE-cellulose. These difficulties are greatly diminished when the cross-linked dextran derivative, DEAE-Sephadex, is used as packing material.

DEAE-Sephadex is used in the free base form with a solvent system of diethyl ether:methanol:water in the ratio of 89:10:1. The diethyl ether is the primary solvent for the lipophilic materials, and the methanol is necessary to effect ion exchange. The small amount of water represses the transesterification of esters[2] with methanol that would otherwise occur in an anhydrous diethyl ether:methanol solvent system.

An important feature of the method is the ease and selectivity in recovery of fatty and resin acids from the column by the simple expedient of elution with carbon dioxide-saturated solvent. Thus, a fatty acid/resin acid fraction is obtained without being contaminated by a nonvolatile eluting reagent. This is particularly important with diterpene resin acids, some of which (e.g., levopimaric acid) are very susceptible to acid-catalyzed isomerization.

Preparation of Column Packing and Columns

In our original publication,[2] preparation of the base form of the DEAE-Sephadex was described on a 1-g scale. Our current practice is to prepare the packing in 25-g batches. Storage for several months (in a refrigerator) does not adversely affect the efficiency of the packing.

A slurry of 25 g DEAE-Sephadex A-25 (Pharmacia Fine Chemicals, Piscataway, N.J.) in water is poured into a Buchner funnel with fritted disk (90-mm diameter disk; 600-mL capacity).

The DEAE-Sephadex is washed in the funnel at water aspirator pressure by slurrying sequentially with 200 mL 1N hydrochloric acid, 400 mL water, 200 mL 1N potassium or sodium hydroxide, and 400 mL water. This sequence is repeated twice. The final caustic wash, which leaves the DEAE-Sephadex in the base form, requires thorough washing with distilled water until the filtrate is neutral. Exchange from water to the organic solvent system is accomplished by slurrying the DEAE-Sephadex first with 400 mL methanol then with 200 mL diethyl ether, each in several portions, with gravity filtration between each addition. The packing is transferred to a 500-mL stoppered Erlenmeyer flask and 200 mL of 89:10:1 diethyl ether:methanol:water are added for storage in a refrigerator.

Simple glass columns were fabricated for dedication to DEAE Sephadex separations (Fig. 2). Column modifications include the addition of a water jacket for cooling the column in warm weather, and replacement of the simple capillary tubing with a capillary glass luer-fitting for ease in packing the column. The capacity of the 0.5- by 15-cm column is about 1.5-g DEAE-Sephadex (dry basis).

A small wad of fine glass wool is placed in the column to support the Sephadex packing. The column is closed at the bottom[4] and several milliliters of the solvent added. A small amount of the DEAE-Sephadex slurry is poured into the column. After allowing this packing to settle and form the initial bed, the column is opened at the bottom and additional slurry added with light external vibration of the column to obtain the desired height of the packed bed.

Performing the Neutrals/Acids Separation

Sample size.--The size of sample that can be applied to a given DEAE-Sephadex column will depend on the amount and nature

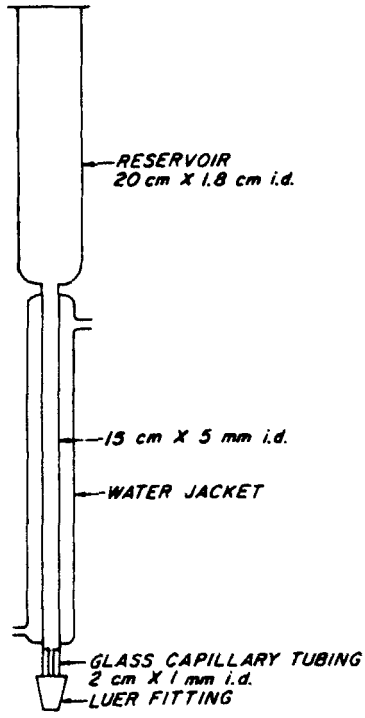

FIGURE 2 Column with waterjacket for DEAE-Sephadex separations.

of the acidic components in the sample. The ion-exchange capacity of the DEAE-Sephadex is listed by the manufacturer at 3.5 $\pm$ 0.5 meq/g. The practical capacity, however, is limited by the accessibility of the ion exchange sites and dissociation of the DEAE-salts after formation. The breakthrough capacity for pure diterpene resin acids and higher fatty acids (MW ~300) is about 150 mg/g DEAE-Sephadex, dry basis, and has been found to be consistent for several lots from the manufacturer. This breakthrough capacity will be reduced by the presence of any inorganic or low molecular weight organic acids. The breakthrough capacity is higher for "strong" acids such as cinnamic and benzoic acids.[2]

Sample preparation and introduction.--When samples are essentially dry, such as with rosins and tall oil, sample preparation consists merely of dissolving a weighed portion of the sample in an appropriate amount of the ether-methanol-water solvent. However, the sample often is an ether extract of green wood or other tissue and, thus, contains a considerable amount of water. Introduction of such solutions to the column will cause serious swelling of the DEAE-Sephadex with subsequent pressure drop and lack of flow through the column. This can be avoided by distilling nearly all the ether under reduced pressure and physically removing the water layer with a pipette. The ether solution of extractives can be transferred to an appropriate volumetric flask, the requisite amount of methanol added, and the flask then brought to volume with anhydrous ether. An aliquot of this solution is then applied to the DEAE-Sephadex column. The water contained in the concentrated ether solution suffices to avoid transesterification.

For typical analytical separations, 1 to 1-1/2 g of DEAE-Sephadex, dry basis, are used (1.5 g dry DEAE-Sephadex is equivalent to a 15-cm length of 5-mm-diameter column bed). Since the separation is not chromatographic in the usual sense, the sample need not be dissolved in a minimum volume of solvent; solutions containing a range of 0.1 to 2% total solutes result in no differences in breakthrough capacity. A typical sample aliquot contains 125 to 150 mg of rosin or tall oil, or 150 to 250 mg of pine wood extractives.

Column elution.--A 500-mL round-bottomed flask with ℨ joint is used to collect the neutral fraction from the column. The sample solution is added to the top of the column and allowed to flow into the DEAE-Sephadex bed. Elution of the neutrals is completed with an additional 100 to 150 mL of the ether-methanol-water solvent. In this fraction are found the usual neutrals: hydrocarbons, aldehydes, ketones, alconols, and esters. Babkin

and coworkers[5] examined the total acidics of black liquor and found that a variety of phenolics having $pk_a > 9$ are also found in the neutral fraction. Such phenolics constitute only a very small portion of the neutrals from pine sapwood extractives, rosin, or the tall oil as isolated in the Saltsman-Kuiken procedure.[6] Other phenolics can elute in the acidic fractions or remain on the column, depending upon the acidity and number of phenolic functionality.

If the acidic materials transferred to the column exceed the breakthrough capacity, some of them will obviously be found in the neutral fraction. Thus, it is desirable to have a simple test to monitor for such breakthrough. Fortuitously, nearly all conifer extracts, rosins, and tall oil samples contain small amounts of colored material that immediately precede the movement of the weak acidics (fatty and resin acids) through the column. This yellow to orange-brown band (see Applications and Observations section for further discussion) is sharpened and made more visible by the moving fatty acid/resin acid front. As long as this narrow band remains on the column, the breakthrough capacity is not exceeded. Examination of the shape of the band will also reveal any channeling in the column which can lower effective capacity. The occurrence of small voids during preparation and use of the column is usual, particularly during CO_2 elution (below), and will not unduly affect the separation. Void formation can be minimized by using a water-cooled jacketed column.

After the neutrals are eluted, the column tip is rinsed with solvent. The flask containing the neutrals is removed and replaced with another 500-mL flask. Elution of weak (resin and fatty acid) acidics is accomplished with carbon dioxide saturated ether-methanol-water solvent. A slight nitrogen pressure in the range of 3 to 20 KPa gauge (0.5-3 psig) minimizes column disruption and maintains a satisfactory flow rate during elution.

(A series of columns can be operated simultaneously from a common nitrogen tank using several low-pressure regulators such as the Matheson model 70.) The solvent can be saturated with carbon dioxide by shaking the solvent in a long-necked flask with carbon dioxide from a gas cylinder. However, we have found it more convenient to generate pure dry ice (to avoid potential contamination of the sample by using commercial dry ice) with a siphon cylinder of carbon dioxide and a generator such as the Redi-ice model 302 (Metalurgical Supply Company, Houston, Tex.), and saturating the solvent by adding dry ice. Elution of weak acids is complete after 200 to 300 mL of carbon dioxide-saturated solvent is passed through the column. It is recommended that the larger volume of the carbon dioxide-saturated solvent be used when α,β-unsaturated resin acids such as anticopalic acid and its derivatives or dicarboxylic resin acids such as pinifolic acid and dihydroagathic acid are present in the sample. The colored band noted above elutes with the weak acids. However, gas chromatographic analysis using internal standards shows that the contribution from this band is insignificant.

Elution of the column with 30 mL of the solvent containing several drops (*ca.* 0.1 mL) glacial acetic acid provides a routine confirmation of the efficacy of the carbon dioxide elution of weak acids. The acetic acid eluate is collected in a tared, 100-mL round-bottomed flask; the solvent is removed, and the total solute determined by weight. Strong acids can be removed with 30-50 mL of solvent containing several drops of formic acid. For most work with pine extractives, tall oil, and rosin, the strong acid fraction is not significant.[7] It is of note that the major proportion of the dark-colored material present in tall oil and some extractives remains at the top of the DEAE-Sephadex column after strong acid elution; the color usually represents less than 1% of tne total material.

The bulk of the solvent from both the neutral and weak acid fractions is removed on a flash evaporator. The fractions are then quantitatively transferred, with washing, using long Pasteur pipettes to tared 25- or 50-mL round-bottomed flasks (such as Corning 4280 or Kimble 25200). The remaining solvent is removed from the tared flasks by carefully heating the flasks on a steam bath under a stream of nitrogen, followed by drying under reduced pressure (e.g., water aspirator vacuum). After weighing the flasks and contents, the fractions are then available for further analysis.

APPLICATIONS AND OBSERVATIONS

The DEAE-Sephadex procedure has been successfully used with a wide variety of samples including oleoresins, rosins and derived products, tall oil, and extracts of wood, barks, and needles from many species. After separation of acids from neutrals by the procedure, the neutrals can be saponified and the resulting acids separated from nonsaponifiables[3] (the nonsaponifiables would include lipophilic alcohol products of saponification). Although the DEAE-Sephadex procedure was developed primarily to quantitatively separate resin acids and fatty acids from neutrals, it has been useful for other acid materials. *p*-Tolylvaleric acid in Douglas-fir extractives elutes in the weak acid fraction[8] as does todomatuic acid (the methyl ester is the insect juvenile hormone, juvabione), also found in Douglas-fir.[9] In other work, we have observed that furoic acid can be exchanged onto DEAE-Sephadex and eluted quantitatively with formic acid.

The DEAE-Sephadex procedure has been used for separation of pine needle epicuticular wax,[10] but chloroform-methanol-water was used as solvent because of the insolubility of the wax mixture in the ether-methanol-water solvent. Complete elution of the acidic

components was accomplished with chloroform-methanol-water-formic acid, but the separation was complicated by significant on-column formation of formate esters of ω-hydroxy fatty acids as artifacts.

In the separation of pine needle extractives, an intense green to yellow-green color obscures the colored band during sample introduction and initial washing. Most of the chlorophyll and carotene components responsible for the color elute into the neutral fraction, after which the colored band preceding the weak acids is visible.

As noted in the Materials and Methods section, the formation of narrow colored bands during separation of conifer extracts and rosins has been useful in monitoring the column capacity for a sample. The chemical nature of these colored bands has been an elusive problem. Recently, however, it was noted that yellow oleoresins of ponderosa pine gave a bright yellow band on DEAE-Sephadex.[11] The color characteristics of the band gave promise that a single component was responsible. The band material was isolated and identified as a mixture of monoterpene alcohol esters of ferulic acid (4-hydroxy-3-methoxycinnamic acid). Thus, the yellow components of the oleoresin were not responsible for the band, but rather, the band was due to on-column bathochromic shift of the absorption band of the DEAE-phenolate salt of colorless ferulic acid esters. Any esters of ferulic acid or isoferulic acid (3-hydroxy-4-methoxycinnamic acid) will show the same behavior. Thus, colorless methyl ferulate (addition of *ca.* 0.5 mg) can serve as an internal indicator for monitoring breakthrough capacity of DEAE-Sephadex columns for resin and fatty acids. Although methyl caffeate (methyl 3,4-dihydroxycinnamic acid) and scopoletin (6-methoxy-7-hydroxycoumarin) are yellow on the DEAE-Sephadex (scopoletin also has an intense fluorescence), these polar esters have greater interaction with the DEAE-Sephadex and are not moved in a frontal elution by fatty and resin acids

to form narrow bands. Acidic polyhydroxylic compounds such as the flavonoids myricetin, apigenin, genistein, and quercitin are seen at the top of the column and are difficult to elute even with strong acids.

The presence of large amounts of phenolic materials in extracts can cause complications in this procedure. The elution of phenolics with the neutral fraction,[5] as noted above, was also observed with extractives of Douglas-fir.[9] The phenolics remained in the water phase after ether extraction of the saponified Douglas-fir sapwood neutrals. In addition, the Douglas-fir heartwood extractives contained a large proportion of colored materials, probably phenolic polymers, which are seen on a substantial portion of the column yet are not eluted by additions of carbon dioxide or formic acid. The deep-purple pigment found in eastern hemlock bark extracts is also retained on the DEAE-Sephadex.[12] The DEAE-Sephadex procedure provides a convenient way to remove materials like the Douglas-fir phenolic polymers and the colored materials from hemlock extracts, but column capacity for the weak (fatty and resin) acids is decreased. The large amount of phenolics in Araucaria extractives resulted in incomplete separation of neutrals from acidics.[13]

Although the DEAE-Sephadex method has been used primarily in analytical work, application to preparative-scale separations is a logical extension. Acid-free neutrals can be readily isolated for characterization studies such as was done in a detailed investigation of the components in tall oil neutrals.[14] Conversely, gram quantities of neutral-free acidics can be obtained for further isolation and identification of components. With preparative-scale separations, it is particularly important to avoid a tightly-packed bed of DEAE-Sephadex as additional water in the sample, and the acids themselves cause swelling of the ion-exchanger and consequent plugging of the column. Often it is more convenient to use several analytical-scale separations to

avoid the plugging that can occur with a single preparative step. Examination of sequential portions of resin acids eluted from DEAE-Sephadex columns has not shown the technique to have potential for isolation of individual resin acids.

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The use of trade, firm, or corporation names in this publication is for the information and convenience of the reader. Such use does not constitute an official endorsement or approval by the U.S. Department of Agriculture of any product or service to the exclusion of others which may be suitable.

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