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MANUAL FOR THE FRACTIONATION OF DISSOLVED ORGANIC MATTER IN NATURAL WATERS

by

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MANAGEMENT PERSPECTIVE

Environmental scientists study aquatic humics, or dissolved organic materials (DOM), because of their relationship to water quality. Most previous studies concentrated on the polymeric humic matter, since it is the largest component, often ignoring low molecular weight organics. The development of a procedure to isolate and quantify low molecular weight components in a manner that was compatible with previous studies was undertaken. Since they may be active species in problems like natural acidity, metal chelation, metal toxicity to fish, taste, and odour; these species should be studied.

This report represents the final product of development of a method for the fractionation of DOM based on the merging of traditional definitions and procedures used for many years by soil scientists, with new procedures utilized by aquatic chemists. This procedure separates DOM into seven fractions with little alteration of their chemical nature and no loss of organic carbon. It is applicable to a wide range of sample types and can accomodate large sample sizes. By isolating large quantities of fractions containing classes of similar components, further chemical characterization and biological testing is facilitated.

PERSPECTIVES DE GESTION

Les spécialistes de l'environnement étudient les matières aquatiques humiques, ou matières organiques dissoutes (MOD), à cause de leur rapport avec la qualité de l'eau. La plupart des études antérieures se concentraient sur les matières humiques polymères, étant donné que celles-ci sont les plus numbreuses, et ne tenaient pas compte des matières organiques à poids moléculaire peu élevé. On a donc entrepris de mettre au point une méthode qui permette d'isoler et de quantifier les composants à faible poids moléculaire de façon à obtenir des résultats compatibles avec ceux des études antérieures. Comme ces matières peuvent jouer le rôle d'agents actifs dans des phénomènes comme ceux de l'acidité naturelle, de la chélation du métal, de la toxicité des métaux pour les poissons, du goût et de l'odeur de l'eau, elles doivent être étudiées.

Ce rapport présente les derniers résultats de la mise au point d'une méthode de fractionnement des MOD basées sur la fusion des définitions classiques et des méthodes utilisées depuis de nombreuses années par les pédologues avec les nouvelles méthodes utilisées en chimie aquatique. Cette méthode permet de séparer les MOD en sept fractions sans altérer de façon significative leurs caractéristiques chimiques et sans perte de carbone organique. Elle peut s'appliquer à une vaste gamme de type d'échantillon et permet d'analyser des échantillons de grosse taille. Cette méthode, en isolant de grandes quantités de fractions contenant des classes de composantes similaires, facilite la tenue d'essais biologiques et la caractérisation chimique.

ABSTRACT

Environmental scientists study aquatic humics, or dissolved organic materials (DOM), because of their relationship to water quality. While polymeric material, usually called humic matter, makes up a large proportion of the DOM, other soluble components such as low molecular weight organic molecules, biological monomers, carbohydrates, etc., cannot be ignored.

Employing the same operational definitions of humic acid (HA) and fulvic acid (FA) from soil chemistry, total humic matter, as traditionally defined, is considered here identical to DOM, thus making maximum use of all the information it contains. FA is further fractionated by a method which is a modification of that developed by Leenheer and co-workers. Six subfractions of FA isolated are: hydrophobic acids, bases, and neutrals, and hydrophilic acids, bases, and neutrals.

This report details all of the steps involved in fractionation of DOM as utilized in this laboratory. Calculations of the distribution of DOM fractions and ancillary techniques are also included.

RÉSUMÉ

Les spécialistes de l'environnement étudient les matières humiques aquatiques ou matières organiques dissoutes (MOD) à cause de leur rapport à la qualité de l'eau. Bien que les matières polymères, habituellement appelées matières humiques, constituent une grosse proportion des MOD, d'autres composantes solubles, comme les molécules organiques à faibles poids moléculaires, les monomères biologiques, les hydrates de carbone, etc. ne peuvent être ignorées.

En se servant des même définitions opératoires de l'acide humique (AH) et de l'acide fulvique (AF) utilisées en chimie des sols, les matières humiques totales, selon la définition classique, sont considérées ici comme identiques aux MOD, ce qui permet d'utiliser au maximum toutes les informations qu'elles contiennent. L'AF est fractionné encore plus au moyen d'une méthode qui consiste en une modification de la méthode mise au point par Leenheer et ses collègues. Les six sous-fractions d'AF isolées sont les suivantes: acides, bases et composés neutres hydrophobes, et acides, bases et composés neutres hydrophiles.

Ce rapport décrit en détail toutes les étapes que comporte le fractionnement des MOD en laboratoire. Les calculs de la distribution des fractions de MOD et la description des techniques auxiliaires figurent également dans le rapport.

CONTENTS

DISCLAIMER

Throughout this document references are made to name brand products and model numbers. The inclusion of this information is intended for the purpose of identification only and to assist the reader in comparison of equivalent products. Such references in no way constitute an endorsement of such products by the authors or by Environment Canada.

INTRODUCTION

The study of humic matter extracted from soil has been ongoing for many years. It is generally accepted that humic matter is a heterogeneous mixture of amorphous macromolecular organic materials found in nearly all terrestrial and aquatic environments. Recently interest in humic substances has grown among researchers other than soil scientists. Environmental scientists have begun to study aquatic humics, or dissolved organic materials (DOM), because of their relationship to water quality. Questions concerned with the extent to which natural organic acids contribute to total acidity, and how they complex metals such as aluminum, can be addressed with more knowledge about the acidic nature and functionality of DOM. A better understanding of DOM character may be useful in predicting the occurrence of taste, colour, and odour problems, and the haloform reaction on drinking water quality.

The present research examines the total DOM found in water samples collected from the natural environment. While high molecular weight material, usually called humic matter, makes up a large proportion of the DOM, other soluble components such as low molecular weight organic molecules, biological monomers, carbohydrates, etc., cannot be ignored. Concerning issues such as acidity and metal toxicity, these components may be important active species in the overall water chemistry.

It is difficult to study the chemistry of DOM since the solutes usually occur in low concentrations. Isolation, identification, and quantification of individual compounds is an enormous task requiring a great deal of time and advanced technology. In the past much progress has been made in the isolation and purification of bulk amounts of humic matter. It has become common practice to separate base-soluble humic matter into two fractions: humic acid (HA), which is insoluble at low pH (pH <2) and fulvic acid (FA), which remains soluble under all pH

conditions (Stevenson, 1982). More recently, ion exchange resins and nonionic macroporous resins have been used in isolation procedures to better separate the organic components (Aiken, 1985; Leenheer, 1985).

Analogous to the isolation of humic matter by alkaline extraction of soils; natural waters are considered here to be extracts of "soil" obtained under ambient pH and temperature conditions, and to contain humic matter. The same operational definitions are employed for HA and FA as from soil chemistry discussed above. Under this framework total humic matter, as traditionally defined, is considered identical to DOM; thus retaining all the information contained in the DOM. These criteria provide a high degree of compatibility with the extensive body of research done on humic matter from the soil and aquatic environments.

The DOM fractionation scheme described here further reduces the heterogeniety of the system by using a sequence of resins to separate the FA fraction into six subfractions. The method is a modification of that developed by Leenheer and co-workers (Leenheer and Huffman, 1976; Leenheer, 1981; Leenheer and Noyes, 1983). The separation depends on factors such as the polarity of the solvent, the polarity and physical characteristics of the resin, and the ratio of resin volume to sample volume. The distinction made between hydrophobic and hydrophilic components is operational and not clear cut, but the breakthrough can be mathematically defined (Leenheer, 1981). Although the fractions are operationally defined, it is inherent in the procedure that species will be grouped together according to common chemical characteristics (e.g. aromaticity, solubility, molecular weight, acid functionality). A variety of analytical techniques have been used to determine many compound types found in each fraction (Leenheer et al., 1982; McKnight et al., 1985; Cronan and Aiken, 1985; Bourbonniere, 1986).

As a procedure to study the total DOM in a system, this fractionation scheme has many advantages:

1) Little alteration occurs to the chemical nature of the components isolated because the aqueous state is maintained throughout;
2) Inorganic compounds generally do not cause interferences;
3) All of the organic carbon is accounted for in the fraction definitions, none is discarded;
4) Use of ambient temperatures and slightly elevated pressures minimizes losses due to volatility;
5) The procedure is applicable to a broad range of sample types, from soil extracts to very dilute natural samples;
6) Large volumes of water can be processed and therefore significant amounts of the organic solutes can be isolated;
7) By isolating classes of similar components, further chemical or biological analysis is greatly facilitated.

The major disadvantage to the procedure is that it is time consuming and labour intensive.

SAMPLE COLLECTION

Water samples are collected in high density polyethylene containers. Conductivity (or salinity) and pH measurements are done on site. Samples are suction filtered (low vacuum) through pre-cleaned glass fibre filters (1.5 μm nominal retention size) to remove particulate matter. A one litre aliquot is filtered quantitatively using a 5.5 cm filter (for subsequent use in determining total particulates); the remainder is filtered through an 11 cm filter in a stainless steel support. The filters are frozen and later freeze dried. Subsamples of the filtrate are removed for major ion chemistry and other determinations, stored at 4°C, and labelled WHOLE FILTERED. The pH of the bulk sample is then lowered to pH <2 by the addition of concentrated HCl. The flocculant solids which precipitate (after standing at least 12 h) are defined as the HUMIC ACID fraction

(HA). At this point, the acidified bulk sample can be stored at room temperature, for up to several weeks, without further processing.

OVERVIEW OF FRACTIONATION PROCEDURE

For samples which contain high DOM contents isolation of HA from the whole filtered sample by refiltration 12 or more hours later is desirable. Isolation of HA is necessary to avoid its precipitation in subsequent low pH steps. The remaining DOM is equivalent to the "unpurified" FA fraction of soil scientists (Stevenson, 1982) and is subjected to further fractionation.

The Leenheer fractionation is used to separate the FA fraction into six distinct sub-fractions (Figure 1). Hydrophobic components of the DOM are defined by their ability to be adsorbed on Amberlite^{R} XAD-8, a macroporous methylmethacrylate copolymer. Hydrophobic base(s) (HPOB) and a portion of hydrophobic neutral(s) (HPON) are adsorbed when the sample is passed through the resin at pH 6. HPOB is eluted from the XAD-8 with HCl. Hydrophobic acid(s) (HPOA) and the remainder of the HPON are adsorbed when the sample, adjusted to pH 2, is passed through the same resin bed. HPOA is eluted from the XAD-8 with NaOH, and HPON remains adsorbed to the resin.

Hydrophilic components of the DOM are defined as those which are not adsorbed by the XAD-8 resin at either pH 6 or pH 2. Hydrophilic base(s) (HPIB) are bound to a strong acid cation exchange resin (in H^+ form) when the effluent from the XAD-8 is passed through at pH 2. Effluent from the cation exchange resin is passed through a strong base anion exchanger (in OH^- form) where hydrophilic acid(s) (HPIA) are bound. Finally, hydrophilic neutral(s) (HPIN) are defined as those components of DOM which pass through all (i.e. not retained on any) three resins. HPIB and HPIA are eluted from their respective resins with NaOH.

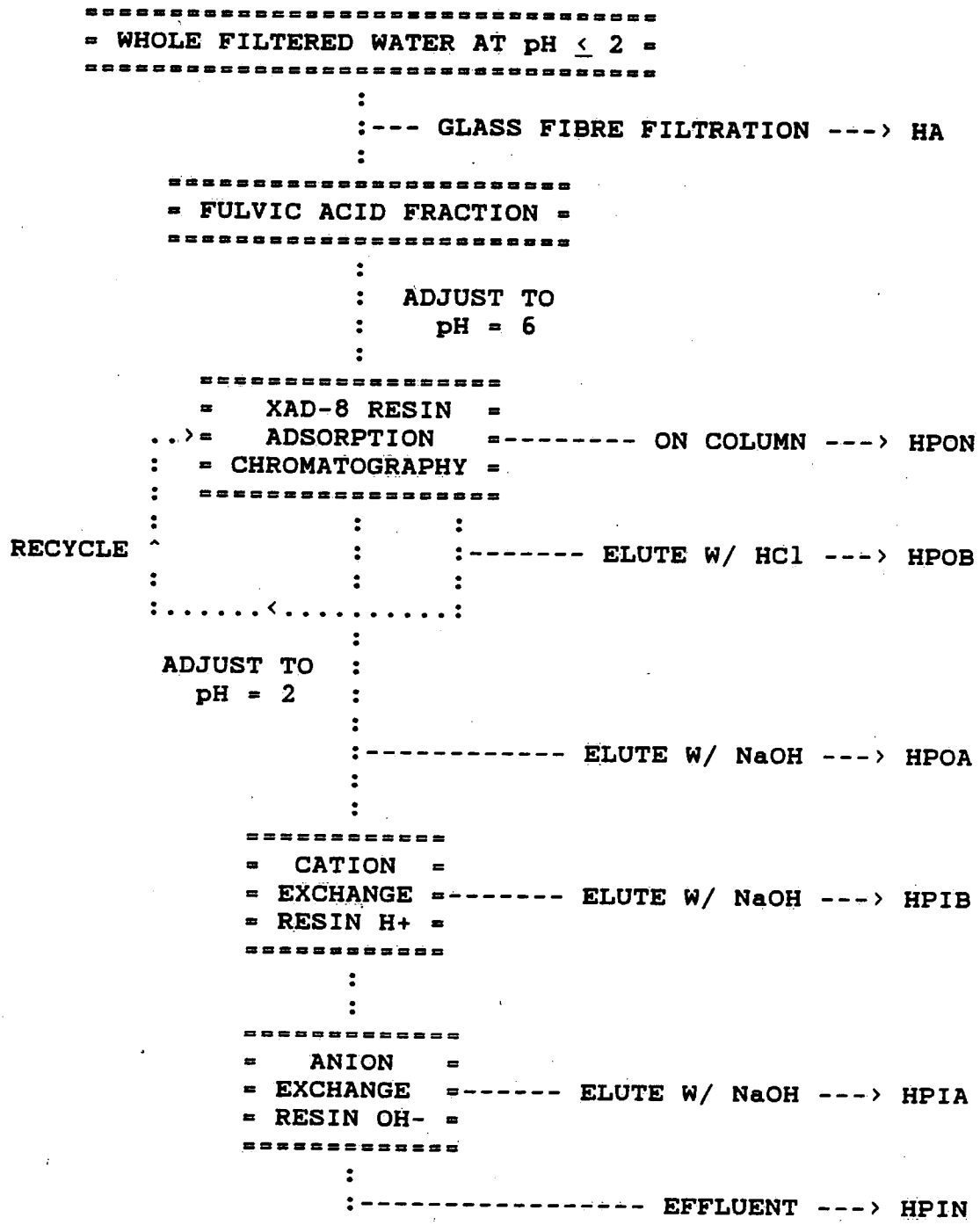

Figure 1. Schematic diagram of the procedure used to fractionate DOM from natural waters into seven subfractions. The chromatographic procedures applied to the fulvic acid fraction are adapted from the work of Leenheer and co-workers (see References).

The seven sub-fractions of DOM isolated can be distinguished by the following general characteristics:

- Humic acid (HA), a brown solid material, which itself is likely composed of a mixture of acidic, basic and neutral compounds, but which is considered here to be a weakly acidic mixture that is highly hydrophobic (i.e. insoluble) at low pH;
- Hydrophobic acid(s) (HPOA), components of the FA fraction which are often highly coloured and expected to be moderately acidic (e.g. fatty acids, phenols);
- Hydrophobic base(s) (HPOB), components of the FA fraction which are expected to be moderately basic (e.g. aromatic amines);
- Hydrophobic neutral(s) (HPON), components of the FA fraction which cannot be eluted from XAD-8 by acid or base (e.g. hydrocarbons);
- Hydrophilic acid(s) (HPIA), more soluble components of the FA fraction which are expected to be strongly acidic (e.g. short chain dicarboxylic acids);
- Hydrophilic base(s) (HPIB), more soluble components of the FA fraction which are expected to be strongly basic (e.g. aliphatic amines);
- Hydrophilic neutral(s) (HPIN), very soluble components of the FA fraction which are not sufficiently ionized at pH=2 to bind to the cation or anion resin (e.g. carbohydrates).

PRE-FRACTIONATION TREATMENT

The bulk sample is homogenized by vigorous shaking. Using the same filtration apparatus as above, HA is filtered off. An aliquot of filtrate is saved as representative of the FULVIC ACID fraction (FA). The filters are frozen and later freeze dried; and the remaining filtrate is the bulk FA sample, ready for further fractionation.

DETAILED STEPS FOR DOM FRACTIONATION

1) The pre-cleaned XAD resin column is rinsed with doubly distilled water (DDW) until the pH of the effluent rinse matches that of the influent DDW (a minimum of 1 L is used).
2) The pH of the sample (bulk FA fraction) is brought to within the range of 5.9 to 6.1 by slow addition of 10 M NaOH with stirring.
3) The sample is pumped downward through the prepared XAD resin bed at a rate of approximately 30 mL/min and the volume of sample processed is recorded. Any species which adsorb to the resin and can subsequently be desorbed with aqueous acid are defined as the HYDROPHOBIC BASE fraction (HPOB). Other species, called the HYDROPHOBIC NEUTRAL fraction (HPON), also adsorb to the resin at this pH, but cannot be desorbed by acid.
4) HPOB is desorbed from the resin by backflushing (upward flow) with 1 L of 0.1 M HCl. This eluate is stored in a glass bottle.
5) The pH of the bulk sample is brought to within the range 1.9 to 2.1 by slow addition of 12 M HCl with stirring.
6) Pump the sample downward through the same XAD column and record the volume processed. At the lower pH, the resin adsorbs the HYDROPHOBIC ACID fraction (HPOA) and more species which would be classified as HPON. Much of the original colour of the FA is generally removed at this step.
7) HPOA is desorbed from the resin by backflushing the column with 0.1 M NaOH (988 mL). Concentrated HCl (12 mL) is added to lower the pH to <2 for storage. The low pH prevents oxidation of the humic matter in the sample, minimizes microbial activity, and assists in removing carbonates prior to DOC analysis.
8) The HPON fraction contains those species which were not desorbed by either the aqueous base or acid. Any liquid remaining on the column at this point is pumped out and

discarded. After dismantling the column the resin is flushed out with methanol and stored in the same. (Extraction procedures to remove the HPON from the resin are given in Appendix III.)

9) The fully protonated cation exchange resin bed is rinsed with DDW until the effluent has a pH >4 (minimum of 1 L).

10) Pump the sample (bulk effluent from the XAD column at pH 2) downward through the cation exchange column using the same flow rate as before, recording the volume processed. Species which are adsorbed are defined as the HYDROPHILIC BASE fraction (HPIB).

11) HPIB is desorbed from the resin bed by backflushing with 1.0 M NaOH (920 mL). Concentrated HCl (80 mL) is added to acidify to pH <2 for storage.

12) Regenerate the cation exchange resin to its H^+ form by backflushing the column with 1 L of 1.0 M HCl, followed by backflushing with DDW until the effluent has a pH >4. The resin is then ready for step 9) for the next sample. Unlike the XAD resin, both the cation and anion exchange resins are reuseable without further cleaning.

13) The anion exchange resin bed is rinsed with DDW until the effluent has a pH <7. This can require a considerable amount of water (up to 10-15 L for 100 mL resin volume).

14) Pump the sample (bulk effluent from the cation exchange resin at pH 2) through the anion exchange resin using the same flow rate as earlier, recording the volume processed. Species which adsorb onto this column are defined as the HYDROPHILIC ACID fraction (HPIA). Any organic solutes still remaining in the bulk sample, having passed through all three resin beds, are defined as the HYDROPHILIC NEUTRAL fraction (HPIN). A subsample of this fraction is saved; the rest is discarded.

15) To remove HPIA from the anion exchange resin, the column is backflushed with 1.0 M NaOH (920 mL). Concentrated HCl (80 ml) is added to lower the pH to <2 for storage.

16) Regeneration of the anion resin is not necessary as the 1 M NaOH backflush also serves this purpose. The resin must be rinsed with DDW as in step 13), to be ready for the next sample. This should be carried out immediately after the HPIA is desorbed, because long-term standing at high pH is detrimental to the anion exchange resin.

MONITORING THE DOM FRACTIONATION BY DOC ANALYSES

At various points during the procedure 10 mL aliquots are removed and later analyzed for dissolved organic carbon (DOC). In this way the concentration of organic material in each fraction can be calculated. The following is a list of these aliquots indicating where in the procedure they are taken; numbered steps refer to the previous detailed section. The HPIA and HPIB fractions are not analyzed directly because their high salt content causes interferences in the DOC analysis.

VIAL #	ALIQUOT OF...
1	Whole filtered water
2	FA: taken from bulk sample filtrate (prior to step 2)
3	DDW rinse: effluent from XAD column just prior to application of the sample at pH=6 (end of step 1, serves as an XAD column bleed blank)
4	HPOB, 1 L of 0.1 M HCl backflush (homogenized, step 4)
5	Last few mL of 0.1 M HCl eluent AFTER backflushing of HPOB is completed (end of step 4, serves as a blank representing XAD, HCl eluent bleed)
6	HPOA, 988 mL 0.1 M NaOH + 12 mL conc HCl (homogenized, step 7)
7	Last few mL of 0.1 M NaOH after backflushing is completed (end of step 7, serves as a blank representing XAD, NaOH eluent bleed)

8	Bulk sample (homogenized), after XAD (twice) but before cation or anion exchange resins (after step 6 but before step 10, contains HPIB + HPIA + HPIN)
9	DDW rinse: effluent from cation column BEFORE sample (end of step 9, serves as a cation column bleed blank)
10	Bulk sample (homogenized) after cation resin but before anion resin column (after step 10 but before step 14, contains HPIA + HPIN)
11	DDW rinse: effluent from anion column BEFORE sample (end of step 13, serves as an anion column bleed blank)
12	Bulk sample (homogenized) after anion column (after step 14, contains HPIN)

DOC analyses of these samples should be done by whatever method is standard in the laboratory, but a few precautions should be noted. Vials #2, #3, #4, #5, #8, #9, #10, and #12 are samples which have been at pH 4 or less for a significant time and should contain very little or no dissolved inorganic carbon (DIC). Thus determination of DOC on these samples is direct. Vial #11 can contain some DIC because its pH is about neutral. This vial should be acidified with concentrated acid (1 drop) and purged with a fine stream of inert gas for about ten minutes to remove dissolved CO_2. Similarly vial #7 must be acidified and purged since it results from an NaOH elution; it could require several drops of concentrated acid. Vial #6 is already acidified in step 7, but it requires purging.

Note that acidification of high DOC samples can result in HA precipitation which can interfere with the precision of carbon determinations. Thus acidification should be avoided if possible and, if necessary (e.g. vials #7 and #11), should be done just prior to determination of carbon. The progress of HA precipitation will be minimized by such timing because of its slow kinetics. Care should be taken when storing high pH (>9)

samples. To minimize the possibility of oxidative polymerization, store DOC samples with little or no head space in well sealed vials for only a short time (1-2 days).

The necessity of acidifying the whole filtered sample (vial #1) to determine the original DOC of the sample depends on the sample pH. If it is pH 5 or less, 96% or more of the DIC will be present as dissolved CO_2 gas (cf. Wetzel, 1975) and subjected to equilibration with the atmosphere which results in negligible DIC values (cf. Riley and Chester, 1971). If the sample is pH 6 or more then a significant amount of the DIC may be in the form of HCO_3^- or CO_3^{2-} and therefore acidification and purging will be required to remove it. Clear water samples of whatever pH may best be acidified since even a small amount of DIC may be enough to cause a significant error in estimation of DOC from a measurement of total carbon. All of the cautions in this paragraph are unnecessary if the laboratory has the capability to reliably determine both total and inorganic carbon. In such a case the DOC can be estimated by difference.

CALCULATION OF FRACTION DISTRIBUTION

The formulas below are modified from those in Leenheer and Huffman (1976) and are keyed to the vial numbers representing the aliquots taken above. The value in each case (vial #X) is DOC in mg/L, and the results are expressed as Fraction-C in mg/L.

DOC = vial #1

HA = vial #1 - vial #2

$$\text{HPOA} = \frac{(\text{vial \#6} - \text{vial \#7}) \times (^{*}\text{dil fct}) \times {}^{**}\text{BFvol (L)}}{\text{vol (L) thru XAD at pH=2}}$$

$$\text{HPOB} = \frac{(\text{vial \#4} - \text{vial \#5}) \times {}^{**}\text{BFvol (L)}}{\text{vol (L) thru XAD at pH=6}}$$

HPON = vial #2 - (vial #8 - vial #3) - HPOA - HPOB

HPIA = (vial #10 - vial #9) - (vial #12 - vial #11)

HPIB = (vial #8 - vial #3) - (vial #10 - vial #9)

HPIN = vial #12 - vial #11

*dil fct = (1.000 / 0.988) IF backflush volume off the XAD is 1000 mL AFTER addition of 12 mL of conc. HCl

**BFvol = Total volume of backflush saved; in the case of HPOA this is AFTER addition of HCl. Normally this value is 1.0 L.

EQUIPMENT AND SUPPLIES

SAMPLE COLLECTION AND FILTERING:

- High density polyethylene (Nalgene^{R}) carboys.
- Glass fibre filters (Whatman 934-AH, nominally 1.5 um retention size) - 5.5 and 11 cm.
- Glass frit type filter support (Millipore XX10 047 22 and acces.) and filtering flask with aspirator connection (used for 5.5 cm filters).
- Stainless steel support (Sartorious SM16260) - for 11 cm filters.
- Low vacuum, hand pumped, bottle (VacuPump^{R}).
- pH meter, (Radiometer PHM 80), with combination electrode.

DOM FRACTIONATION:

- Cheminert^{R} LC-1 column (LDC/Milton Roy, Riviera Beach, FL) Column dimensions, tube sizes, etc. are specified in Fig. 2. Any well-sealing column that can withstand moderate fluid pressures, is capable of upward as well as downward flow, has inert contact surfaces, and convenient connections is appropriate.
- Peristaltic pumps, Masterflex^{R} (Cole-Parmer, Chicago, IL) or equivalent.
- RESINS:
  - Amberlite^{R} XAD-8 (Rohm & Haas) methylmethacrylate resin, 20-50 mesh, macroporous; 100 mL of wet resin has worked satisfactorily for up to 20 L of water with FA of 25 mgC/L.
  - Dowex^{R} MCS-1 (Dow Chemical) strong acid cation exchange resin, 20-50 mesh, macroporous sulfonated styrene divinylbenzene copolymer, wet capacity 1.5 meq/mL; 100 mL of wet resin has been used as above for XAD, but only for samples with conductivities of 5000 uS/cm or less (See Appendix IV).

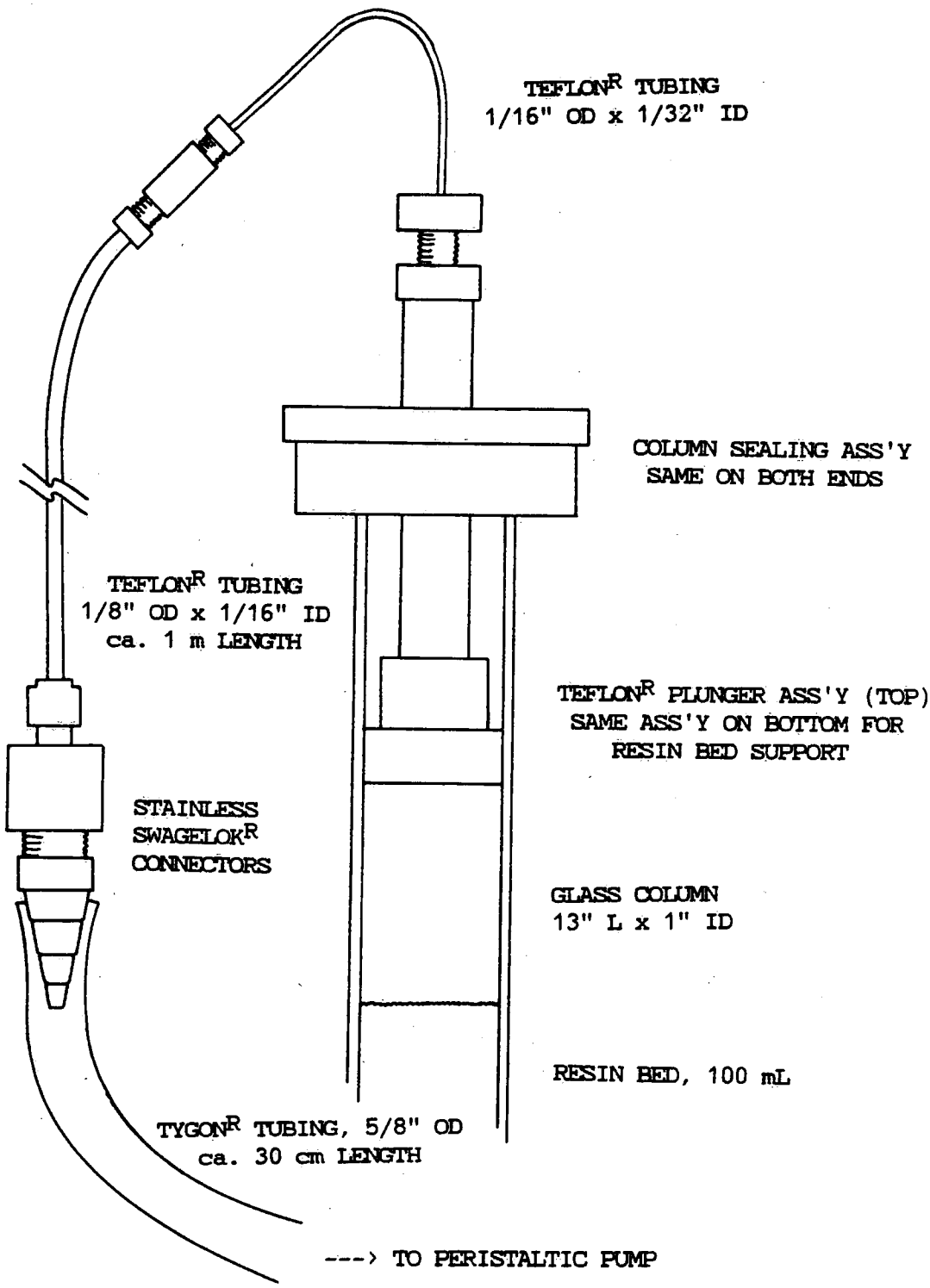

Figure 2. Schematic of one end of a Cheminert® LC-1 chromatography column and connections used for XAD-8, Cation Exchange and Anion Exchange portions of the procedure outlined in Figure 1. For more detailed description of the components see manufacturer's literature.

- Amberlite^{R} IRA 900 (Rohm & Haas) strong base anion exchange resin, 20-50 mesh, macroporous styrene divinylbenzene copolymer with quaternary ammonium functions attached, wet capacity 1 meq/mL, supplied in Cl^- form and must be converted to OH^- form before use; 100 mL of wet resin has been used as above for cation resin (See Appendix IV).
- Resin clean-up and conversion procedures are given in Appendix I.
- REAGENTS:
  - Reagent grade hydrochloric acid, concentrated, used to make 1 M and 0.1 M HCl solutions.
  - Reagent grade sodium hydroxide pellets, low in carbonate, used to make 10 M, 1 M, and 0.1 M NaOH solutions.
  - Doubly distilled water (DDW), distilled water from a common source tap, redistilled in an all-glass still.
- Procedures used for cleaning of glassware, filters, and polyethylene containers are given in Appendix II.

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APPENDIX I: RESIN CLEAN-UP METHODS

UNUSED XAD-8 RESIN:

1) Using a magnetic stirrer, mix together 1 volume resin: 2 volumes 0.1 M NaOH, stir ca. 1/2 h.
2) a) Let settle, decant off fines and discard, replenish the volume of wash with more 0.1 M NaOH.
   b) Stir 2 h.
3) Repeat step 2a) changing solution each night and morning for two days and stirring constantly.
4) Let settle, decant. Rinse 3X with 1-2 volumes of 0.1 M HCl, leaving resin to stir in last rinse for ca. 2 h.
5) Filter by suction to remove as much liquid as possible. Wash with doubly distilled water (DDW) to remove HCl. Wash with methanol to remove DDW.
6) Place resin in a glass Soxhlet thimble, directly onto the coarse (EC) glass frit. Extract 24 h with methanol.
7) Disassemble Soxhlet apparatus and place thimble of resin in a beaker. Stir with glass rod to "squeeze" out as much methanol as possible. Wash 5-6X with small amounts of hexane, attempting to remove as much residual methanol as possible. Extract 24 h with hexane.
8) Repeat the procedure of step 7), except the solvent change-over is now hexane to methanol. Extract 24 h with methanol.

NOTE: If resin is to be stored for long periods of time (e.g. several weeks or more) it should be kept in methanol (min. 25% v/v) and step 9) carried out just prior to use.

9) Wash the resin 3X with 0.1 M NaOH, 3X with 0.1 M HCl, and 3X with DDW. Final pH should be in the range of 5-6. Resin can be stored in DDW for 3-4 weeks (add methanol to at least 25% v/v for longer storage).

CATION EXCHANGE RESIN (H^+ form)

1) Place resin in a glass Soxhlet thimble, directly onto the coarse (EC) glass frit. Extract 24 h with methanol. If resin is to be stored leave in methanol.
2) Before use, rinse with 1.0 M NaOH, followed by a 1.0 M HCl rinse (5-6 volumes rinse : 1 volume resin), followed by DDW rinse.

ANION EXCHANGE RESIN

1) This resin is purchased in the Cl^- form (for stability reasons) and must be converted to the OH^- form. Place resin in a glass Soxhlet thimble, directly onto the coarse (EC) glass frit. Extract 24 h with methanol. Store in methanol.
2) To convert from Cl^- to OH^- rinse with 20 volumes of 1.0 M NaOH : 1 volume of resin.
3) Test for Cl^- in the effluent: acidify subsample of effluent with concentrated nitric acid, add 1% silver nitrate. A white precipitate indicates the presence of Cl^-. Continue rinsing with 1.0 M NaOH until Cl^- test negative.
4) Rinse with DDW until pH is 7 or below.

APPENDIX II: CLEANING OF LABORATORY EQUIPMENT

GLASSWARE

Any glass containers or apparatus used in the procedure must first be solvent cleaned as follows:

1) Wash with detergent and water, rinse with distilled water.
2) Rinse 2X with small amounts of methanol.
3) Rinse 2X with small amounts of dichloromethane.
4) Oven dry at 60° C.

GLASS FIBRE FILTERS

Place filters, a few at a time, in a Buchner funnel and wash sequentially with the following solvents:

1) 0.1 M HCl.
2) 1:1 mixture of methanol and toluene.
3) Dichloromethane.
4) Oven dry at 60° C.

HIGH DENSITY POLYETHYLENE CONTAINERS

1) Rinse container with ca. 2 M NaOH, swirling wash reagent around interior surfaces for several minutes. If possible, container can be filled with wash reagent and soaked overnight for more thorough leaching.
2) Rinse with ca. 2 M HCl, swirling as above or soaking overnight.
3) Rinse well with DDW.

APPENDIX III: ISOLATION OF HPON FRACTION

This serves the dual purpose of isolating the HPON fraction and regenerating the XAD resin so that it can be used again in the DOM fractionation.

PART I: EXTRACTION OF HPON FROM RESIN

1) XAD resin from the DOM fractionation should be stored in methanol. Place the resin in a glass Soxhlet thimble, directly onto the coarse (EC) glass frit, draining off and saving the original liquor in which the resin was stored. Using a glass stirring rod to mix resin, wash 3X with small amounts of methanol. Combine these washes with the original liquor.
2) Extract 24 h with methanol.
3) Disassemble Soxhlet apparatus and place thimble of resin in a beaker. Stir with glass rod to "squeeze" out as much methanol as possible. Wash 5-6X with small amounts of hexane, attempting to remove as much residual methanol as possible. These washes can be combined with the methanol from the 24 h extraction.
4) Extract 24 h with hexane.
5) Repeat the procedure of step 3), except the solvent change-over is now hexane to methanol. All solvent extractions and washes up to this point contain the HPON fraction and will be used in PART III.

PART II: FINAL CLEAN-UP OF XAD

(PART I should be completed before PART II)

1) Extract 24 h in methanol.
2) Continue with step 9) from the clean-up method for unused XAD resin in Appendix I.

PART III: CONCENTRATION OF THE HPON FRACTION

1) From PART I, there should be three "batches" of solvent: i) the methanol in which the the XAD was stored plus the first washes, ii) the first methanol extraction plus washes, and iii) the hexane extraction plus washes. A known amount of a suitable internal standard (we use n-hexatriacontane) is added to the hexane extract to assist in subsequent gas-liquid chromatographic (GLC) quantitation. All of the solvent "batches" are then combined in a separatory funnel.
2) Add acidified (ca. pH 1) half-saturated NaCl solution to the solvent mixture, 1 volume salt solution : 5-6 volumes solvent, and shake well.
3) Drain off the aqueous/methanol layer and discard. Transfer the hexane layer to a clean round bottom flask. (NOTE: The efficency of this single partitioning was tested. A second hexane extraction of the aqueous/methanol phase repeatedly showed neglible amounts of any organics by GLC, and therefore is considered unnecessary.)
4) The volume of the organic layer is reduced to near dryness by rotary evaporation (bath at 30° C). Dichloromethane (3X 1-2 ml) is used to transfer the sample to a 2 dram vial, the contents of which are also evaporated to near dryness, and can be stored at 4o C.
5) The sample can be analyzed by GLC after redissolving in an small volume of an appropriate solvent.

APPENDIX IV: RESIN CAPACITIES

The definitions of the fractions, especially the hydrophilic components, depend heavily on the capacities and volumes of the resins used. Definitions of the hydrophobic fractions also depend on the volume of sample passed through the XAD-8 column. In this appendix we will discuss some of the theoretical aspects of the definitions and the compromises that may occur if these are not considered. Also we present some of our experience with testing this procedure, and some general recommendations.

HYDROPHOBIC-HYDROPHILIC BREAK

Leenheer (1981) defines as the hydrophobic-hydrophilic break that point in the XAD-8 adsorption chromatography when 50% of an hypothetical solute is retained on an XAD-8 column and 50% is eluted. He describes this distribution mathematically as:

$$V_{0.5r} = 2V_0 \ (1 + k'_{0.5r})$$

where,

$V_{0.5r}$ is the volume of sample to be processed,
V_0 is the void volume of the XAD-8 column, and
$k'_{0.5r}$ is defined as the capacity factor at the hydrophobic-hydrophilic break.

In the procedure described in this manual a constant bed volume of XAD-8 (100 mL) is used. To maintain the same $k'_{0.5r}$ for all samples then the same volume of sample must be passed through. For XAD-8 the void volume is approximately 65% of the column bed volume (Leenheer, 1981). By substituting in the equation above the hydrophobic-hydrophilic break $k'_{0.5r}$ was calculated for various volumes of sample passed through a 100 mL bed, resulting in the values given in Table IV-1.

Table IV-1.

Sample Vol.(L)	$k'_{0.5r}$
5	38
10	76
15	114
20	152

For rigorous theoretically accurate comparisons all samples should be processed to attain the same $k'_{0.5r}$ values. This means that if the objectives of a particular study require that different volumes of sample must be processed (e.g. when a wide range of DOC concentration is encountered in a preparative study), then column volumes should be varied accordingly. Comparison of the $k'_{0.5r}$ values in Table IV-1 with the results of Thurman et al. (1978) indicate that many low molecular weight organic compounds have high k' values. Some exemplary values are listed in Table IV-2.

Table IV-2.

Compound	k'
Butanol	25
Butanoic acid	39
Pentanol	93
Pentanoic acid	125
Hexanol	266
Hexanoic acid	377

Source: Thurman et al. (1978)

The differences that would occur in the composition of the HPOA fraction if the hydrophobic-hydrophilic break were made between C4 and C5 acids and alcohols rather than between C5 and C6, should be of little concern if the bulk of HPOA consists of

higher molecular weight acids and polycondensates. Similar arguments can be made for HPOB and HPON using data from Thurman et al. (1978). According to these authors k' is inversely related to solubility in a way that is predictable using structural and functional group considerations.

Experience in this laboratory suggests that the researcher is afforded some latitude in comparison of samples fractionated with different $k'_{0.5r}$ values. We have consistently removed the bulk of the colour (and DOC) from fulvic acid (FA) fractions on 100 mL XAD-8 columns, passing through the entire range of volumes listed in Table IV-1. The volumes passed through were inversely related to sample DOC. Thus 5-10 L of FA was passed through for samples with DOC >25 mg/L and 15-20 L for samples with DOC <15 mg/L.

If the $k'_{0.5r}$ value was very important in determining the bulk character of the hydrophobic fractions, a relationship between HPIA/HPOA and total hydrophilics/total hydrophobics (THPI/THPO), for the FA fractions, with $k'_{0.5r}$ should be evident. Data for 159 samples taken from bogs or lakes and streams in organic watersheds from eastern Canada show no such relationship (see Fig. IV-1). If samples from a low DOC site and a high DOC site are selected from the total data set, no significant relationship between either of the ratios with $k'_{0.5r}$ is evident (see Fig. IV-2). The correlation coefficients are given for all of these comparisons in Table IV-3.

Table IV-3. Correlations (r) between $k'_{0.5r}$ and hydrophilic/hydrophobic ratios for various sample populations.

Samples Tested	N	HPIA/HPOA	THPI/THPO
All	159	0.001	-0.022
Low DOC Site	14	-0.384	-0.064
High DOC Site	15	0.156	-0.043

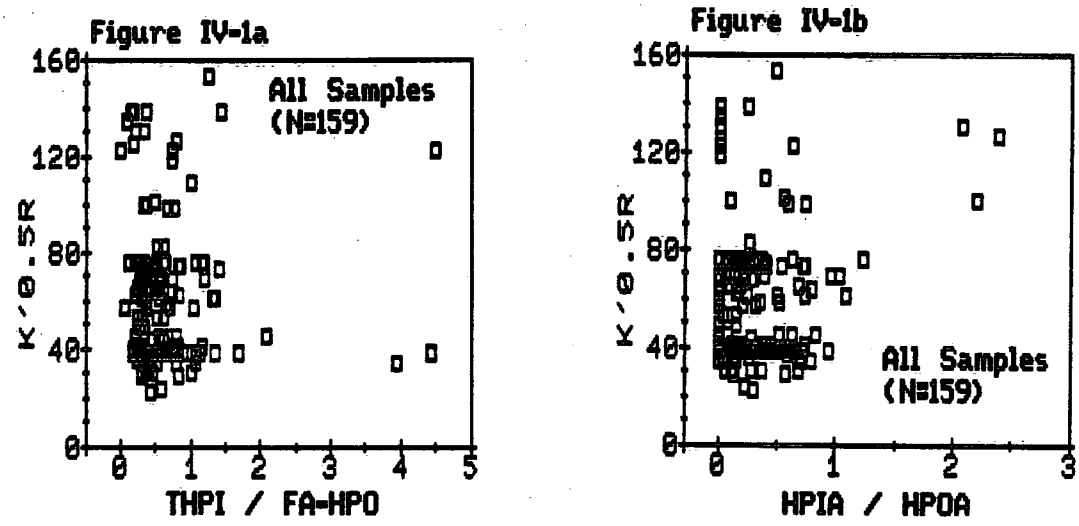

Figure IV-1. Scatter diagrams for hydrophilic/hydrophobic ratios compared with hydrophobic-hydrophilic $k'_{0.5r}$ used. N = 159 samples from eastern Canadian organic watersheds.

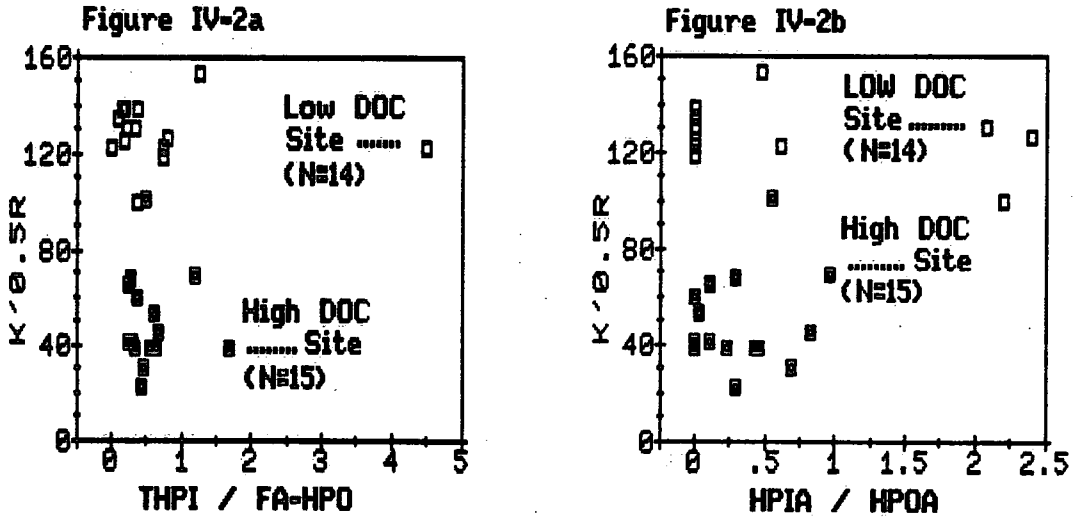

Figure IV-2. Scatter diagrams as above for a low DOC and a high DOC site selected from set represented above.

Data shown in Table IV-4 result from fractionations of replicate samples. The Mersey and Clyde river pairs were sampled sequentially from the same site over a 10 minute period. Data for the retort water pairs are taken from Leenheer (1981). For the retort water the ratios increased by only a factor of three even though the $k'_{0.5r}$ was varied by >10 times for the analytical vs. preparative fractionation.

Table IV-4. Relationship of hydrophobic-hydrophilic break tested on replicates of various samples. DOC in mg/L, Omega-9 retort water data from Leenheer (1981).

	DOC	$k'_{0.5r}$	THPI/THPO	HPIA/HPOA
Mersey R. 3/85	14.6	82	0.51	0.26
Mersey R. 3/85R	13.1	84	1.41	0.73
Mersey R. 8/85	16.8	41	1.16	0.73
Mersey R. 8/85R	18.5	41	0.41	0.31
Clyde R. 8/85	31.8	37	0.54	0.46
Clyde R. 8/85R	32.7	39	0.60	0.24
Omega-9 Anal.	977	45	1.04	1.53
Omega-9 Prep.	977	4	0.32	0.47

The poor correlations shown in Table IV-3, coupled with the variability of the ratios, at the same $k'_{0.5r}$, for the river sample replicates in Table IV-4 suggest that other factors override the importance of column capacity (in the range used here) in determining the hydrophobic-hydrophilic split for these types of samples. Since most of the ratios are <1, it appears that the bulk of the FA in these samples is characterized by high k' values on XAD-8. Comparisons of fraction compositions can probably be done for samples processed in the $k'_{0.5r}$ range of 40-150.

CAPACITIES OF CATION AND ANION EXCHANGE COLUMNS

The hydrophilic fractions are defined by their ability, or lack thereof, to be adsorbed on a strong acid cation exchange resin or a strong base anion exchanger, at pH=2. Original work by Leenheer and co-workers was done on low ionic strength samples and without isolation of an HA fraction. Thus the ionic strength of their samples applied to the cation and anion columns was determined primarily by the quantities of acid and base needed to adjust sample pH first to six (to isolate HPOB) and later to two (to isolate HPOA). The Na^+ and Cl^- which remains in the THPI solution will compete with the HPIB and HPIA for sites on the cation and anion resins respectively.

Testing of cation and anion columns in this laboratory with standard compounds, various column/elution conditions, and monitoring with HPLC, DOC analysis, and specific conductivity gave the following results:

1) For a high conductivity 20 L sample (salt equivalent of HA, HPOB and HPOA isolation), through 100 mL columns - Acetic, oxalic, citric and salycylic acids are not retained on the cation column; conductivity is increased after cation. None of the acetic, 20% of the oxalic, 70% of the citric and probably all of the salicylic acid added are retained on the anion column; conductivity is reduced about 40% by the anion column.
2) For a low conductivity (no salt) 4 L sample, all propanoic, oxalic, and citric acid (Tot. DOC = 112 mg/L) was retained on a 100 mL anion column in the OH^- form. Subsequent elution of this column with a volume of NaCl solution equal to the theoretical capacity of the column for Cl^- releases all of the propanoic acid but none of the other two acids. Further elution with NaCl releases more DOC and likely some of the citric and oxalic acids as suggested from 1) above.

3) Using the anion resin in the Cl^- form results in retention of salicylic acid only; propanoic, oxalic, and citric acids broke through, but were held up for some time. Salicylic acid also began to break through at the end of the experiment.
4) Experience with samples which were fractionated with minimized conductivity (no HPOB isolation) and greater anion column size (225-250 mL) indicates that HPIN conductivity can be reduced to zero if more anion resin is used.

General Recommendations Regarding Hydrophilics

The conclusion from these experiments is that the anion resin has the ability to retain HPIA in the OH^- form. The anion resin in the Cl^- form does not retain HPIA except for the aromatic acids. The presence of Cl^- in the sample up to the capacity of the resin will replace monocarboxylic acids, and in excess of the capacity will replace di- and tricarboxylic acids. Substantial amounts of hydrophilic acids can therefore appear in the HPIN fraction under these conditions.

To maximize the retention of HPIA it is advisable to increase the size of the anion exchange column and, if possible, refrain from isolating an HPOB fraction (thus reducing conductivity contributed by pH adjustments). Increasing the cation exchange column size should also help to reduce the conductivity brought over to the anion column, but this is untested. The disadvantage of larger exchange column bed volumes is the possibility for increasing the quantity of resin bleed contributed to the HPIB and HPIA backflushes, and the HPIN fraction. Storage of the anion column in its Cl^- form reduces the subsequent bleed compared to storage in the OH^- form.