ENVIRONMENT CANADA

Forestry Service

PROJECT REPORT 11-3

ISOLATION OF TOXIC CONSTITUENTS OF KRAFT PULP MILL EFFLUENTS.

B.C. Research

Progress Report to March 31, 1972

PULP AND PAPER POLLUTION ABATEMENT

A research program sponsored by the
Department of the Environment
in cooperation with the
Canadian Pulp and Paper Industry.

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Distributed by:

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FOREWORD

This report covers work done in the fiscal year 1971/72 on a project sponsored by the Cooperative Pollution Abatement Research program of the federal government and the Canadian pulp and paper industry.

The program was first announced in August, 1970 by the Minister of Fisheries and Forestry and the Minister of Energy, Mines and Resources, to provide for the funding of research contracts aimed at reducing water pollution from Canadian pulp and paper operations. Various elements of these Departments were combined with the formation in 1971 of the Department of the Environment, which assumed the responsibility for the operation and funding of the program. In November, 1971 the Minister of the Environment announced that the program would be expanded in the next fiscal year to include pulp and paper air pollution abatement research.

The program administration and Secretariat are provided by the Canadian Forestry Service, Department of the Environment. Development and guidance of the program are the responsibility of a joint government-industry committee known as the Committee on Pollution Abatement Research (CPAR). The members represent the Department of the Environment, the Department of Industry, Trade and Commerce, the Canadian Pulp and Paper Association, the Pulp and Paper Research Institute of Canada and the pulp and paper companies in eastern and western Canada.

The Committee plans the program, assesses priorities, reviews progress and advises on the allocation of funds and awarding of contracts for research proposals from pulp and paper companies and any other recognized research institutions. Based on the Committee's recommendations, the federal government enters into contract agreements with the organizations concerned for the conduct of approved projects.

In the fiscal year 1970/71 funds in an amount up to $500,000 were authorized to finance approved water pollution abatement projects. From 1971/72 through 1975/76 up to $1,000,000 will be available each year for water pollution research provided the pulp and paper industry's own annual expenditures for this type of work are increased by a like amount over the 1970 expenditures. For the fiscal year 1972/73 funds in an amount up to $200,000 will be available on a similar basis for the support of approved pulp and paper air pollution abatement research projects. The same amount may be made available annually thereafter, through the fiscal year 1975/76.

CPAR PROJECT No. 11

ISOLATION OF TOXIC CONSTITUENTS OF KRAFT PULP MILL EFFLUENTS.

by

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Work Supported by the
Cooperative Pollution Abatement Research Program
of the
Department of the Environment
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NOTE:

This CPAR Project Report consists of three progress reports identified by B.C. Research as Nos. 3, 4 and 5, as follows:

No. 3: 10 pages, covering the period May 12 to July 9, 1971.
No. 4: 13 pages, covering the period July 10 to November 30, 1971.
No. 5: 22 pages, covering the period December 1, 1971 to March 31, 1972.

B.C. Research

Progress Report No. 3

May 12 to July 9, 1971.

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August 24, 1971

PROJECT REPORT NO. 3

Subject: ISOLATION OF TOXIC FRACTIONS IN UNBLEACHED WHITEWATER

A. OBJECT

To summarise the progress made in the above programme during the period May 12 to July 9, 1971.

B. BACKGROUND

A review was presented in Project Reports No. 1 and 2 on the limited work which has been done in various laboratories to isolate and identify the chemical toxicants in unbleached whitewater. A procedure developed at B.C. Research was outlined involving acid precipitation of toxic factors followed, after freeze-drying the precipitate, by petroleum ether extraction. Problems were encountered in consistently striking a toxicity balance after precipitation and during subsequent stages. The presence of acid-labile toxic components and degradation by aerial oxidation were suggested as factors responsible for toxicity losses.

This report describes further studies using the acid precipitation procedure and also results of tests to evaluate alternate methods for concentrating the chemical constituents responsible for the toxicity in unbleached whitewater.

C. PRESENT WORK - EXPERIMENTAL

1. Acid Precipitation Technique

Unbleached whitewater (1215 Imp. gal.), pH 9.8, was collected at a West Coast kraft pulp mill and acidified on-site to pH 1.9 with concentrated sulphuric acid (8.8 litres). After allowing the precipitate which was produced to settle, supernatant liquid was pumped off to leave a sludge (185 litres).

Three days after commencing collection of the samples bioassays were performed at several dilutions using 11 juvenile coho salmon in 3-1. vessels. Tests were made on a variety of fractions as follows:

a) A composite sample of nitrogen-stripped (pH 5.5) unbleached whitewater collected over two days of sampling at the mill.

b) The acid precipitate at a concentration equivalent to that in the original unbleached whitewater, after re-dissolving at pH 11.

c) The supernatant liquid from the precipitation step.

d) Recombined acid precipitate and supernatant liquid.

2. Lime Treatment of Unbleached Whitewater

The major advantage of the acid precipitation method is that it has allowed large volumes of effluent to be processed at a mill to give a small volume of toxic sludge. However, inconsistent results have sometimes been obtained. It was envisaged that a similar concentration of toxic material on-site might be achieved by other techniques. Precipitation with lime was considered as a preliminary treatment since the alkaline medium could eliminate potential acid hydrolysis reactions of the toxic components.

Calcium hydroxide was added to 3 1. samples of unbleached whitewater in amounts of 1, 3, 5 and 10 g/1, respectively. The mixtures were stirred briefly and the precipitates allowed to settle. Supernatant liquid was siphoned off and adjusted with carbon dioxide to pH 9.0 (point of minimum solubility of $CaCO_3$). The calcium carbonate was removed by filtration and the solutions adjusted to pH 7.0 by continued treatment with CO_2 prior to bioassays.

3. Nitrogen Stripping of Unbleached Whitewater

A nitrogen stream was passed for 24 hr. through two 3-litre samples of unbleached whitewater adjusted to pH 7.5 and 5.5, respectively. The foam which was produced was collected. Bioassays were performed on the mother liquors and on the recombined foam and mother liquor.

4. Freeze-Concentration of Dissolved Material in Unbleached Whitewater (1)

1-litre of unbleached whitewater was rotated in a round bottom flask in a bath held at $-2^{\circ}C$. In this manner a sheath of ice was produced on the walls of the flask and the dissolved components of the unbleached whitewater were concentrated in solution. Eventually, only a small volume of a very dark-coloured liquid remained. It was removed and the ice washed with a few ml. of cold water. After melting, the ice was tested by bioassay for toxicity towards juvenile coho salmon.

5. Use of Polymeric Absorbent Resins

Amberlite XAD-2 resin (Rohm and Haas Co.) was examined as an agent for concentration of toxic components in unbleached whitewater. Before use the resin was Soxhlet extracted with methanol to remove low molecular weight material, then placed in a 6 x 60 cm glass column to give a bed volume of 550 ml. Neutralised, nitrogen-stripped unbleached whitewater (30 l.) was passed down the column and accumulated portions of the eluate subjected to bioassay.

A series of solvents was then used to elute the toxic components from the column. First, 0.05N HCL (3 l.) was passed down the column. The second wash was with 0.05N sodium carbonate (9 l.) and gave a pale yellow solution. Then the resin was treated with 0.05N sodium hydroxide (6 l.) which also produced a yellow eluate. Finally, methanol (4 l.) was passed through the resin, resulting in a very dark brown solution. After removing the solvent at room temperature under reduced pressure, the residue was diluted and subjected to bioassay.

Unbleached whitewater at its original pH 9.8 was also passed through a column of XAD-2 resin. The absorbed material was eluted using 1% sodium hydroxide solution (7 l.) followed by methanol (4 l.). After treatment as before the solutions were tested by bioassay.

Preliminary examination of another Amberlite resin, XAD-7, has been made. Desorption of toxic material from the column is currently under investigation.

D. RESULTS AND DISCUSSION

1. Acid Precipitation

Figure 1 illustrates the results obtained from acid precipitation of the toxic constituents of UWW in a plot of log concentration versus log MST. Despite rapid processing of the precipitate formed on acidification of unbleached whitewater, a toxicity balance was not maintained. At 100% concentration the UWW has a median survival time (MST) of 45 min., whereas the precipitate diluted to its equivalent concentration in the original UWW had an MST of 168 mins. By inspection of Figure 1 it is evident that this is only 50% of the UWW toxicity. In contrast to previous samples treated by the acid precipitation technique, the supernatant liquid exhibited some toxicity, having an MST of 1480 min. at 100% concentration. Bioassay data in Figure 1 do not cover the complete range, but extrapolation of the log-log line for UWW to this MST value would suggest that approximately 17% of the original toxicity remained in the supernatant. That is, about 67% of the original toxicity of the UWW is accounted for by the sum of the toxicities of the acid precipitate and the supernatant. Recombination of the precipitate and the supernatant liquid in the correct proportions produced a solution having 76% of the UWW toxicity at 100% concentration. Moreover, the log-log curves for the UWW and the recombined acid precipitate and supernatant are non-parallel, suggesting the differential loss of one or more components. For example, at 60% of the original concentration, the toxicity of the recombined materials is only 70% of that of the UWW. It appears, therefore, that the process of precipitation in acid medium, at least in some instances, leads to irretrievable losses of toxic components. Furthermore, a repeat bioassay of the precipitate at 100% concentration after four days gave an MST value of 314 min., an additional loss of 11% of the original toxicity. These data imply that the toxic constituents are unstable in their free acid form.

2. Lime Precipitation of Toxicants

Lime is currently in the commercial demonstration phase at a few mills as an agent for removing colour from the UWW and from bleach plant alkali effluent. In view of the suspected lability of the toxic constituents of UWW in an acid medium, the prospects of using lime to precipitate toxic chemicals were explored.

From Table 1 increasing amounts of toxicants are removed as the lime loading is increased. The results suggest that massive lime treatment of pulp mill wastes for the purposes of colour removal may also be effective in precipitating toxic constituents. On the other hand, stoichiometric lime addition removes only a portion of the toxicity. At the level of lime addition (10 g/l) which was required to produce a non-toxic (MST > 3120 min.) supernatant liquid, recovery of the adsorbed and precipitated matter from the lime sludge proved troublesome. Since other, more promising avenues were concurrently being examined, further study of the program was shelved.

3. Nitrogen Stripping of UWW

The results of nitrogen stripping of UWW are listed in Table 2. Although there was some reduction in the toxicity of the UWW after stripping, concentration of the toxic factors in the foam from this sample is far from complete. It is interesting to note the consistently lower toxicities of samples acidified originally to pH 5.5. Though the effect is small, it is a further indication that acidification permanently destroys some toxic factors.

Two silicone-based defoaming agents for UWW tested during the course of acid precipitation studies were shown to be non-toxic. However, a practical corollary to the above observations is that addition of defoamers in pulp mill operations may indirectly contribute to effluent toxicity by redispersing toxic compounds. Where possible, it may be advantageous to skim off foam.

4. Freeze-Concentration of Dissolved Material in UWW (1)

The ice fraction from the freeze-concentration procedure, on thawing, showed some toxicity towards fish. It is likely that the rate of cooling was too rapid, causing entrainment of toxic organic materials in the ice phase. It seems improbable that this method could usefully be employed to concentrate the toxic factors from large volumes of effluent.

5. Use of Polymeric Absorbent Resins

Amberlite XAD-2 and XAD-7 resins are composed of cross-linked polystyrene; XAD-2 type resin is non-ionic, and XAD-7 resin is of intermediate polarity. They selectively absorb organic compounds from aqueous solutions and have been suggested for use as a "polishing" step in colour removal from pulp mill effluents.(2) Desorption of

material from the resins may be accomplished by use of aqueous solutions of suitable pH or with organic solvents. These properties suggested that the resins might be suitable for removal and fractionation of toxic components in UWW.

When 60 bed-volumes of UWW were passed down a column of XAD-2 resin the dark brown eluate was non-toxic. Similarly, constituents desorbed from the resin on elution with HCL, Na_2CO_3 and NaOH also exhibited no toxicity. However, when methanol was passed through the column, large amounts of coloured material were eluted, which, after removal of solvent and dissolution in an aqueous medium,had an MST of 300 min., compared with 225 min. for the original UWW. When dilutions of 80% and 60% were used, the gap between MST values for the methanol extract and the UWW widened. A decrease in toxicity was observed when the methanol fraction was combined with the three aqueous fractions in proportions corresponding to UWW. In this case, no mortalities occurred after 12 hr. Nonetheless, in all solutions containing the methanol eluate a violent "cough" response was produced in the test fish, together with a degree of distress and disorientation which would usually be expected to result in death within a short period of time. A possible explanation could be that a portion of the toxicity, which may act synergistically with the toxic components in the methanol extract, is lost or destroyed during the fractionation procedure. Evidence supporting this hypothesis was obtained from a bioassay on a sample composed of 50% UWW and a quantity of the methanol extract equivalent to 50% of its concentration in the original UWW. The MST was 234 min. compared with 225 min. for 100% UWW. Future tests will be made with the non-toxic UWW eluate in combination with the methanol fraction. Previous indications of the effects of acidification on the stability of toxic materials suggest that the nitrogen stripping of the UWW at pH 5.5 prior to absorption on the resin, and the use of HCL as the first eluant, are potential causes of toxicity loss.

Passage of UWW at pH 9.8 through XAD-2 resin also produced a dark coloured non-toxic eluate. The sodium hydroxide solution used as first eluant produced a fraction containing some toxicity but by far the bulk of the toxic matter was eluted using methanol. The methanol fraction at a concentration equivalent to the original UWW had an MST of 130 mins. compared with 144 min. for the UWW. Bioassay of the combined sodium hydroxide and methanol fractions gave an MST of 30 min. At present no explanation can be offered for the apparent increase in toxicity.

E. FUTURE WORK

It is proposed to establish conditions for optimum recovery of toxic constituents from resins XAD-2 and XAD-7 and to determine resin capacities for absorption of toxicants. Low temperature and a nitrogen atmosphere will be employed wherever desirable. If acceptable toxicity balances are obtained in the laboratory, a large-scale column will be used at a kraft mill to extract the toxic components from the UWW stream. The column will be returned to the laboratory for elution of absorbed material, bioassays and further fractionation of toxic constituents, probably by column chromatographic techniques.

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REFERENCES

1. Baker, R.A., Freeze Concentration of Microorganisms in Water, ASTM STP 448 (1968), 65-77.

2. Rohm and Haas Company Bulletin - Amberlite Polymeric Resins.

TABLE 1

MST FOR JUVENILE COHO SALMON IN UWW AFTER TREATMENT WITH LIME

Lime Loading (g/l)	1	3	5	10
MST (min)	1485	1320	1709	no mortalities after 3120

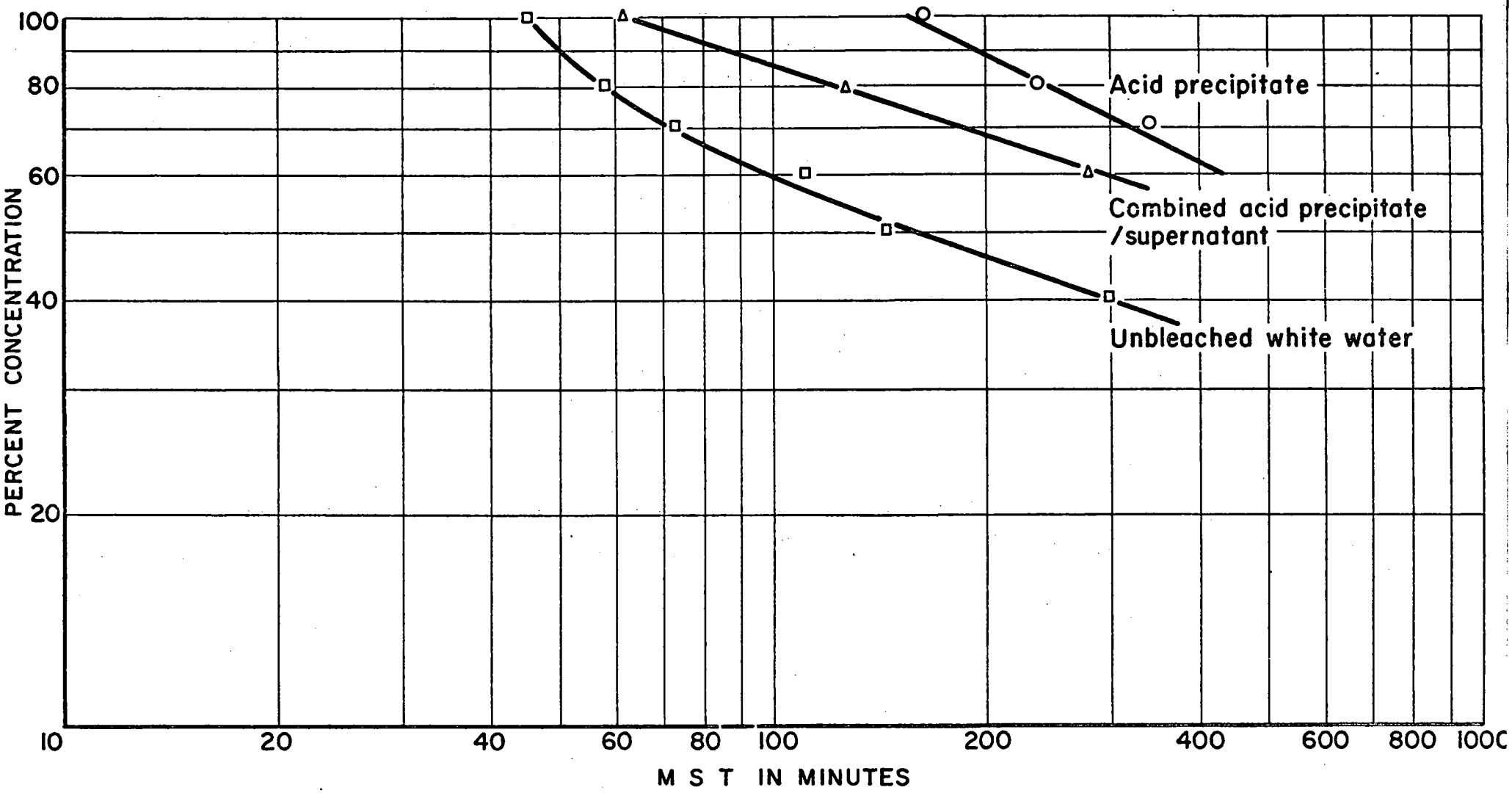
TABLE 2

MST FOR JUVENILE COHO SALMON IN UWW FOAM STRIPPED AT DIFFERENT pH VALUES

Sample	Foam Stripped UWW (pH 5.5)	Recombined Foam & Stripped UWW (pH 5.5)	Foam Stripped UWW (pH 7.5)	Recombined Foam & Stripped UWW (pH 7.5)
MST (min)	268	127	151	97

MST of original UWW, 85 min.

M S T AS A FUNCTION OF CONCENTRATION



B.C. Research

Progress Report No. 4

July 10 to November 30, 1971.

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December 31, 1971

PROJECT REPORT NO. 4

Subject: ISOLATION OF TOXIC FRACTIONS IN UNBLEACHED WHITEWATER

A. OBJECT

To summarise progress made in the above program during the period July 10 to November 30, 1971.

B. BACKGROUND

A number of techniques which had been evaluated for quantitative concentration of toxic factors in unbleached whitewater (UWW) were described in Progress Report No. 3. The most promising approach appeared to be selective absorption of material onto macroreticular polymeric resins, followed by desorption in an organic solvent. Preliminary results indicated a closer approximation to complete recovery of toxicity by this method than had hitherto been achieved on a regular basis by other means. The present report contains details of further tests to optimise absorption and recovery of toxic constituents, and the results when the technique was used on a large scale.

C. SUMMARY

A non-polar absorbent resin, Rohm and Haas "Amberlite XAD2 resin", was most effective in absorbing toxic materials from UWW. When 450 gallons UWW were passed through approximately 10 l. of the resin, the highly coloured eluate was not toxic to juvenile coho salmon. The absorbed

constituents could be removed by washing with methanol and showed an overall toxicity loss of 17% compared with the original UWW at 100% concentration. The degree of concentration of material derived from UWW was 46,000-fold. The methanol extract appears to be amenable to additional fractionation without further toxicity loss.

D. PRESENT WORK - EXPERIMENTAL

1. General

Amberlite XAD2 and XAD7 absorbent resins were exhaustively extracted with methanol in a Soxhlet apparatus before use in order to remove material of low molecular weight. A bioassay test was conducted on a portion of the methanol extract to ensure that no interference in toxicity measurements would result from any subsequent leaching of the resin during effluent processing. All operations involving absorption and desorption of effluent constituents were done in a walk-in refrigerator at 2°C. Nitrogen was used to air-strip the UWW feed to columns of the resin. Eluates were collected under a nitrogen atmosphere and all subsequent handling of UWW fractions was done as far as possible with exclusion of air. During solvent evaporation minimum temperatures commensurate with adequate distillation rates were maintained.

2. Evaluation of XAD2 Resin

A glass chromatography column was filled with a slurry of XAD2 resin (500 cc) in water, and UWW (30 l.), pH 9.5, fed through the resin at a rate of approximately 1 l/hr. Bioassays using 11 juvenile coho salmon were performed on the first 3 l. and final 3 l. of UWW eluate from the column. Material absorbed on the resin was eluted by successive passage of 0.05N sodium carbonate (12 l), 0.05N sodium hydroxide (12 l) and methanol (4 l.). Solvent was removed from the final fraction by rotary evaporation at 35-40°C under a water pump vacuum (ca. 15 mm) and each fraction then tested for toxicity by bioassay at various concentrations relative to the original UWW. Bioassays were also done on mixtures of the various eluates for comparison with the original UWW.

The absorption capacity of a column of the resin (100 cc) was tested by regular 3 l. bioassays of the eluate from passage of UWW (30 l.).

3. Evaluation of XAD7 Resin

Using conditions identical with those outlined above, XAD7 resin (500 ml) was treated with UWW (30 l.) and the absorbed material eluted with sodium carbonate solution, sodium hydroxide solution and methanol. Bioassay tests were run as before.

4. Large-Scale Fractionation of UWW Using XAD2 Resin

(a) Absorption of toxic constituents

Four glass columns, 4 ft long x 2½ in. diameter were packed with a total of 9810 cc methanol-extracted XAD2 resin. The resin columns were classified according to particle size by backwashing with water and set up at 2°C as shown in Figure 1.

UWW was collected from a West Coast kraft mill in 45 gal drums at approximately two week intervals and, after standing at 2°C for two days to permit fibre settlement, subjected to bioassay tests at a series of concentrations between 100% and 40%. The UWW effluent was then pumped into the columns and passed through the resin at a rate not exceeding 3 l./hr/column. A rapid stream of nitrogen, bubbled through the UWW feedstock above each resin bed, ensured anaerobic conditions within the column. Eluate was collected in four, calibrated Nalgene aspirator bottles of 20 l. capacity. At intervals of 80 l., i.e., when all four bottles were full, eluate (750 ml) was removed from each and combined for a 3 l. bioassay. A further aliquot (2 l.) was collected from each bottle and stored as a composite sample for future testing. The remaining UWW eluate was discarded. Overnight, when effluent was not being passed through the columns, they were sealed with Parafilm and a nitrogen blanket was maintained over the resin surface.

During the course of the experiment, the following chemical characteristics of the UWW feed and eluate were measured. Total organic and inorganic carbon were analysed using a Beckman Model 915 TOC analyser; "resin acid" concentrations were determined using the colourimetric procedure described in NCASI Technical Bulletin No. 182 (May 1965); specific conductance and pH were measured conventionally.

Four separate batches of UWW, totalling 450 gal (1700 l.) were passed through the resin. Each batch was tested for toxicity by bioassay at a number of concentrations. An aliquot from each batch was retained and stored at 2°C as a composite sample for later comparative bioassay tests. Of the eluate from the columns, 10% (170 l.) was retained as a composite sample.

Because the resin beds did not permit identical flow rates, different volumes of UWW were processed through each column. In order to produce a homogeneous reservoir of resin, the contents of all columns, after passage of UWW, were combined, mixed well and stored

as a slurry in UWW under nitrogen at 2°C. Aliquots of known volume were removed for subsequent fractionation of the absorbed components.

(b) Desorption of UWW toxic principles from resin

A glass column was set up containing an aliquot of the resin (250 cc; containing material from 43.32 l. UWW). The UWW used to slurry the resin was displaced by distilled water (2 l.) and then methanol (2 l.) was passed through the column. After removal of solvent from the methanol extract, the dark brown aqueous residue was diluted to 250 ml with 5% NaOH, and subjected to bioassays at various dilutions relative to the original UWW. Since 43.32 l. UWW gave rise to 250 ml concentrate, a volume of

$$\frac{3}{43.32} \times 250 \text{ ml concentrate } (= 17.3 \text{ ml})$$

was diluted to 3 l. with dechlorinated water to produce a solution equivalent to 100% UWW. A parallel series of dilutions of the composite UWW sample was set up for comparison. Bioassays were also performed on the methanol residue using UWW eluate as the diluent rather than dechlorinated water.

The UWW was allowed to drain from a second batch of resin (400 cc; ≡ 69.32 l. UWW), eliminating the water rinse used on the first batch. After elution of the absorbed constituents with methanol (5 l.) and removal of solvent from the eluate, bioassays were performed as before.

A further aliquot of resin (200 cc; ≡ 34.66 l. UWW) was used to ascertain the amount of material absorbed per unit volume of UWW. Methanol (4 l.) was again used to elute the absorbate from the resin. The aqueous residue produced by distillation of the methanol under reduced pressure was dried to constant weight under a partial vacuum over potassium hydroxide pellets. A bioassay was carried out on the dried, gummy residue (0.75 g). Another batch of resin (100 cc) was treated in the same manner, except that final removal of water was accomplished by freeze drying to leave a powdery residue (0.28 g).

E. RESULTS AND DISCUSSION

Results reported in Progress Report No. 3 showed that the toxic constituents of UWW are quantitatively absorbed onto Amerlite XAD2 and XAD7 resins. However, less than optimal toxicity balances were obtained on removal of the absorbed material from the resins. It was proposed that some acid-labile toxicants were lost during elution with dilute HCl. A similar suggestion had earlier been made to explain inconsistencies in toxicity balances obtained during acid precipitation of UWW components. Subsequent processing of toxic materials absorbed on the resins has been done using alkaline or organic media in which toxicant stability appears to be enhanced.

The efficiency of the two resins for quantitative fractionation of toxic material in UWW was compared in small-scale separations. Though very little colour appeared to be removed when 30 l. UWW were passed through 100 ml XAD2 or 100 ml XAD7 resin, the eluates were not toxic to juvenile coho salmon. The material absorbed on the resins was removed by washing with sodium carbonate and sodium hydroxide - each of which gave a pale yellow solution - followed by passage of methanol, which produced a dark brown eluate. Results of bioassay tests carried out on the various fractions are presented in Table 1. The presence of some toxicity in the sodium carbonate washes is evident inasmuch as fish mortalities occurred. However, a MST could not be established, presumably due to detoxification of the solution during the bioassays. In the case of the sodium hydroxide eluate, although no fish were killed, a pronounced and persistent "cough" response was evoked which may be symptomatic of toxic components causing sublethal effects. Notwithstanding these observations, the bulk of the toxicity was shown to reside in the methanol eluate. Good agreement was found between the MST of fish in the methanol fraction from each resin and the MST in the original UWW. A greater dependence of toxicity upon dilution to 80% and 50% of the equivalent UWW concentration was observed in the case of the methanol eluate from XAD2 resin than that from XAD7. Comparative values for UWW are unfortunately not available.

When UWW eluate was used instead of dechlorinated water for dissolving the methanol residue, a reduction in the overall toxicity was noted, becoming more pronounced with increasing dilution, especially in fractions from XAD7 resin. On combining the methanol fraction with the sodium carbonate and sodium hydroxide eluated from XAD7 resin, the MST increased relative to that for the methanol residue in water. For combinations of XAD2 eluates MST was virtually unchanged.

In tests to establish the capacity of the resins to absorb toxic material, both were found to be capable of detoxifying more than 300 ml UWW per cc resin.

The chemical structure of the resins was briefly discussed in the previous Progress Report. XAD2 resin is composed of a non-polar styrene polymer, crosslinked via divinyl benzene bridges. XAD7 resin is of intermediate polarity by virtue of ester groupings of the polymer chain.

The mechanism of absorption onto both XAD2 and XAD7 resins may be through the hydrophobic portion of a molecule involving Van der Waals attraction to the hydrophobic resin. Such bonds are generally weak. XAD7 resin additionally may absorb compounds via dipole-dipole interactions or hydrogen bonding involving polar groups on the absorbed molecule and the ester groups of the resin. Consequently, XAD7 should absorb the same materials as XAD2 plus certain more highly polar compounds which do not have the correct molecular configuration for absorption on non-polar XAD2. In other words, a narrower range of chemical types will be taken up on XAD2 resin. It is to be expected that materials absorbed through hydrophobic bonding will be more readily desorbed than those held to the resin by polar interaction. Evidence for this may be found in the presence of detectable toxicity in the sodium carbonate wash, particularly of XAD2 resin.

Thus, for the ensuing large-scale separation of toxic materials from UWW, XAD2 resin was used. The resin was chosen over XAD7 because of better agreement between MST values obtained with eluates from XAD2 and the original UWW during preliminary tests, and also because of the less complex mixture of components anticipated on XAD2 and the ease of desorption of these components from the resin. A scale of separation using XAD2 was chosen that was large enough to permit evaluation of a number of subsequent fractionation schemes. The known instability of the toxic constituents of UWW will undoubtedly give rise to imperfect toxicity balances during the period of time required to establish a successful separation procedure. However, when a viable fractionation scheme has been delineated, rapid processing of a fresh batch of effluent will permit an accurate assessment of the relative contributions of various fractions to the overall toxicity.

A total of 1700 l. (450 gal) UWW, collected in four separate batches, was passed through columns containing 9810 cc of XAD2 resin. The results of bioassays performed on each batch of UWW are shown graphically in Figure 2. It is evident from the shape of the graphs that

even after a relatively short period (approximately 125 min) mortality rate of batches C and D dropped drastically. Some fish mortalities were noted at 40% concentration of batches A, B, C, but median survival times were never attained for the groups of 11 fish. These observations can be explained in terms of detoxification or a difference in the characteristic toxicity-concentration relationship of these batches. Bioassay data for a composited sample of all the effluent batches bore no relationship to the results for any of the individual batches either in MST's or in the slope of the log concentration vs. log MST graph. Because of the volume of composite UWW sample (50 l.) and the storage time from first to final batch (8 weeks), it was impossible to exclude air from the sample. These factors may have contributed to the modification and loss of toxic principles which occurred, invalidating the measurement of acute toxicity.

Results of measurements made on the UWW feed to and eluate from the XAD2 resin are shown in Table 2. Specific conductance was unchanged, reflecting the lack of absorbance of strongly ionic materials from solution. The pH value of the eluate was slightly less than that of the feed, suggesting possible removal of salts of weak acids. Indeed, the Liebermann-Burchard reaction, which was until recently proposed as a quantitative test for resin acid soaps (1) showed a difference between feed and eluate of 16 mg/l resin acid soaps (calculated as abietic acid). However, the test lacks specificity; some resin acids, such as abietic and laevopimaric, give a positive reaction, whilst others, such as dehydroabietic acid, do not (2). Moreover, fatty acids, such as linoleic (2), sterols (3) and other extractives alo produce the characteristic violet-pink colour under the same reaction conditions as resin acids. Thus, it is unlikely that the 16 mg/l. absorbed material is composed exclusively of resin acid soaps.

As expected on the basis of specific conductance measurements, no inorganic carbon was absorbed from the UWW but removal of organically bound carbon was found to be 10 mg/l. UWW. For 16 mg/l. resin acid soaps, the corresponding organic carbon figure should be 12 mg/l. This is further indication that constituents other than resin acid soaps are included in the result of the Liebermann-Burchard test.

After passage of UWW was complete, the resin from all four columns was combined, mixed well and aliquots taken to assess toxicity and material balances. Since, in the exploratory work, sodium carbonate and sodium hydroxide washes were shown to remove a small proportion of the toxic material from the resin, they were elimated in the large-scale separation so that toxic components could be concentrated in a single methanol fraction. In the first aliquot of resin processed, the

UWW used to slurry the resin was removed by a distilled water rinse. The ensuing methanol washings were found to contain less than 60% of the toxicity in the original UWW feed. However, when UWW used to slurry the resin was drained and a methanol wash applied directly, the bioassay data presented in Figure 2 were obtained for the residue after solvent removal.

By comparison with graphs obtained for UWW batches A and C it can be seen that a 28% loss of toxicity has occurred. However, only 8% difference in toxicity levels exists between the methanol extract and UWW batch B.. When the volumes of each batch of UWW are taken into consideration, an overall toxicity loss of 17% is found at an effluent concentration of 100%. It should be pointed out that the bioassay data reported for the separate UWW samples were obtained two days after collection of each batch, when fibre settlement had taken place. Subsequently, bioassays were performed at 100% concentration during passage of each UWW batch through the XAD2 resin. As expected, MST's increased with time of standing. For instance, MST in batch D reached a maximum of 126 min, cf plotted value of 79 min, whilst for batch A the MST rose from 74 min to 97 min during the 8 days required to pass this effluent through the resin. In comparison with toxic factors applied to the resin columns, therefore, the toxicity loss is undoubtedly much less than 17%. When a satisfactory fractionation scheme has been established, more rapid effluent processing will drastically reduce this discrepancy and an accurate assessment of the contributions of each fraction to overall toxicity should be possible.

The methanol fraction diluted in UWW eluate showed a greater level of toxicity when subjected to bioassay than the same fraction diluted in water. However, little reliance can be placed on the values, inasmuch as the UWW eluate itself produced a strong cough response in the test fish. Evidently, components in the initially non-toxic UWW eluate have been transformed, on standing, to materials which cause toxic symptoms. Only rigorous exclusion of air will permit replicable bioassays such as those obtained for methanol extracts of XAD2-absorbed materials.

In quantifying the material concentrated onto the resin, and its toxicity, the following relationships pertain.

(a) The constituents from 1 l. UWW are absorbed on 5.77 cc resin.

(b) 100 cc resin contains materials from 17.34 l. UWW.

The residue from drying the methanol eluate of 200 cc resin over potassium hydroxide pellets weighed 0.75 g. This is equivalent to 21.6 mg absorbed

material per litre UWW, i.e., a 46,000-fold w/v concentration factor. When the methanol eluate from another batch of resin (100 cc) was freeze dried, the residue weighed 0.28 (≡ 17 mg/l. UWW). However, a bioassay showed it to be non-toxic, presumably due to loss of volatile toxic constituents: the KOH-dried material exhibited greater than 40% toxicity loss. From these observations, it would appear that volatile components, rather than resin acid soaps, are responsible for much of the toxicity of UWW.

F. FUTURE WORK

Preliminary studies indicate that toxic constituents in the residue obtained after removing solvent from the methanol eluate of XAD2 can be quantitatively extracted into pentane and/or diethyl ether. Thin layer chromatographic (TLC) examination of the extracts reveals only 8 - 12 components.

The next stage of the work will entail the establishment of correct combinations of solvent and stationary supporting media for TLC separation of the constituents. If possible, the conditions will be translated for use in preparative scale column chromatography. The use of analytical and preparative scale vapour phase chromatography will also be explored, since the evidence so far suggests the presence of readily volatilisable toxic materials.

G. REFERENCES

1. Colourimetric Measurement of Resin Acid Soaps in Pulp and Paper Mill Wastes, NCASI Technical Bulletin No. 182, (1965).

2. The Spectrophotometric Determination of Abietic-Type Dienoic Resin Acids. R.L. Stevens and R.V. Lawrence, Anal. Chem., 34, 199, (1962).

3. Organic Chemistry, an Advanced Treatise, Vol. II, p. 1390, H. Gilman.

4. The Origin of Toxic Material in Bleached Kraft Mill Effluent, CCWPAR Project Report No. 2 et. seq., (1971), A. Bruynesteyn and C.C. Walden.

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Table 1

MST of Juvenile Coho Salmon in UWW Fractions Eluted from Amberlite Resins
(11 fish in 3 l. bioassay test)

MST in original UWW 134 min.

Fraction	CONCENTRATION OF ORIGINAL UWW					
	100%		80%		50%	
	XAD2	XAD7	XAD2	XAD7	XAD2	XAD7
Na_2CO_3	4 dead 48 hr	1 dead 48 hr	-	-	-	-
NaOH	None dead 72 hr	None dead 72 hr	-	-	-	-
Methanol residue in water	132	118	196	144	None dead 48 hr	371
Methanol residue in UWW eluate	158	148	206	278	5 dead 48 hr	4 dead 48 hr
Methanol residue in Na_2CO_3 wash	139	265	-	-	-	-
Methanol residue in NaOH wash	102	146	-	-	-	-
Methanol residue in 50% NaOH + 50% Na_2CO_3 washes	138	208	-	-	-	-

Table 2

Chemical Characteristics of UWW Feed and Eluate

Parameter	UWW Feed	UWW Eluate
Specific conductance (micromhos/cm)	280	280
pH	10.0	9.7
Liebermann-active substances (mg/l)	19	3
Inorganic carbon (mg/l)	7	7
Organic carbon (mg/l)	89	79

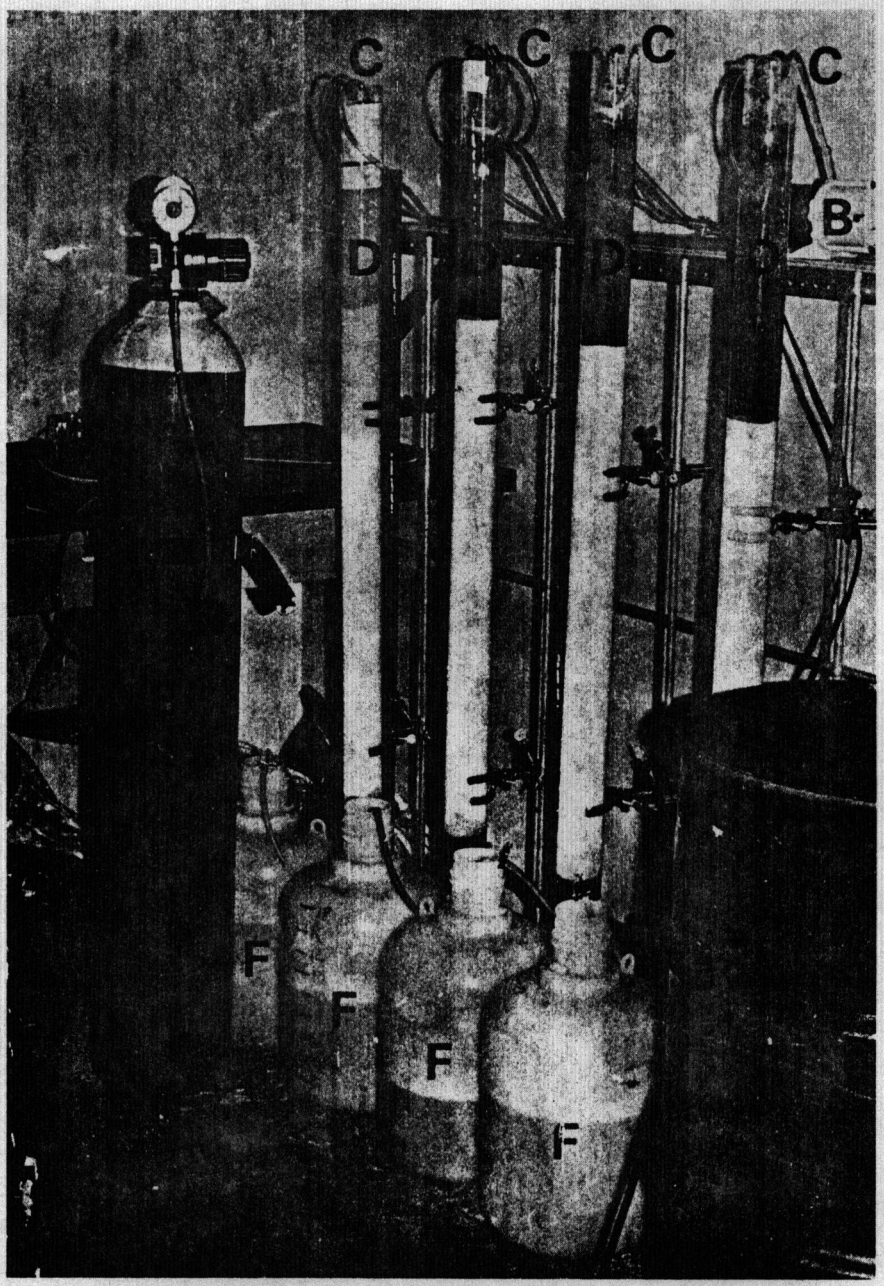


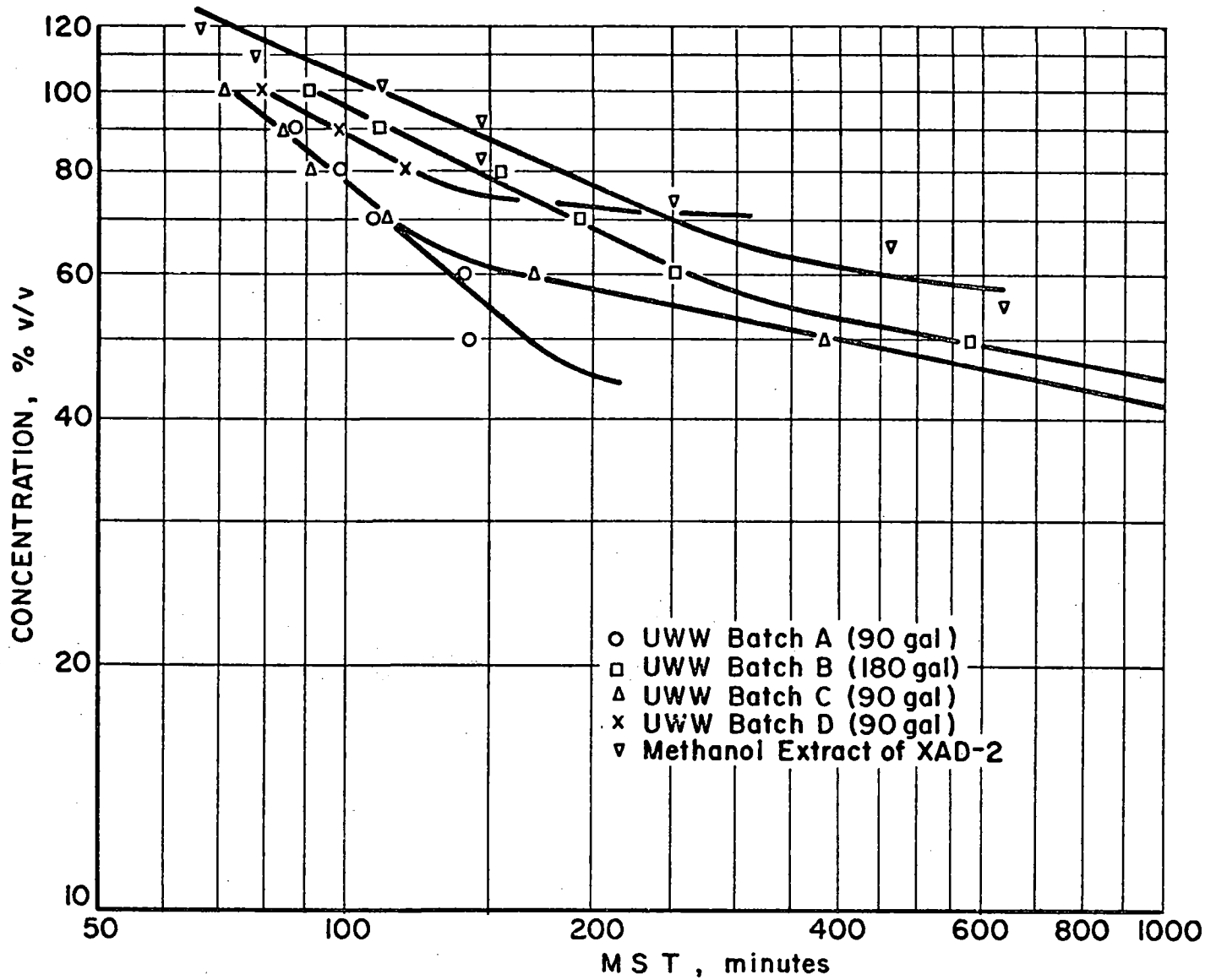
Figure 1

Key:

A - 45-gallon drum, UWW feedstock
B - Pump
C - UWW feed lines to columns of resin
D - Nitrogen sparge into UWW
E - Nitrogen cylinder
F - 20-litre aspirator bottles containing UWW eluate

Figure 2

BIOASSAY DATA FOR UNBLEACHED WHITEWATER FEEDSTOCK TO XAD-2 RESIN AND RECOVERED METHANOL EXTRACT



B.C. Research

Progress Report No. 5

December 1, 1971 to March 31, 1972.

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April 20, 1972

PROJECT REPORT NO. 5

To: Canadian Forestry Service
Department of the Environment

Subject: PROJECT NO. 11 - ISOLATION OF TOXIC MATERIALS IN BLEACHED KRAFT EFFLUENTS

A. OBJECT

The objective of the first phase of the project is to isolate and identify all the toxic constituents in unbleached whitewater, and to measure the contribution of each constituent to the overall toxicity of this effluent stream.

B. BACKGROUND

A technique for selective absorption of the toxic materials from unbleached whitewater (UWW) using Amberlite XAD-2 macroreticular resin was described in Project Report No. 4.

450 gallons of UWW from a West Coast kraft mill were processed through columns containing 9810 cc of the resin. The eluate from 100% concentration of UWW was not toxic towards juvenile coho salmon in conventional bioassay tests. Toxic constituents were removed from the resin by elution with methanol and, after evaporation of solvent, the residue was subjected to bioassay. Analysis of results from a plot of log concentration vs. log median survival time (MST) showed that 83% of the toxicity of the original UWW was recovered in the methanol extract.

Further stages in the fractionation of the toxic constituents of UWW and identification of a number of constituents are described in the present report which covers the period December 1, 1971 to March 31, 1972.

C. SUMMARY

A fractionation scheme, outlined in Chart 1, has been developed to isolate all materials responsible for the toxicity of UWW.

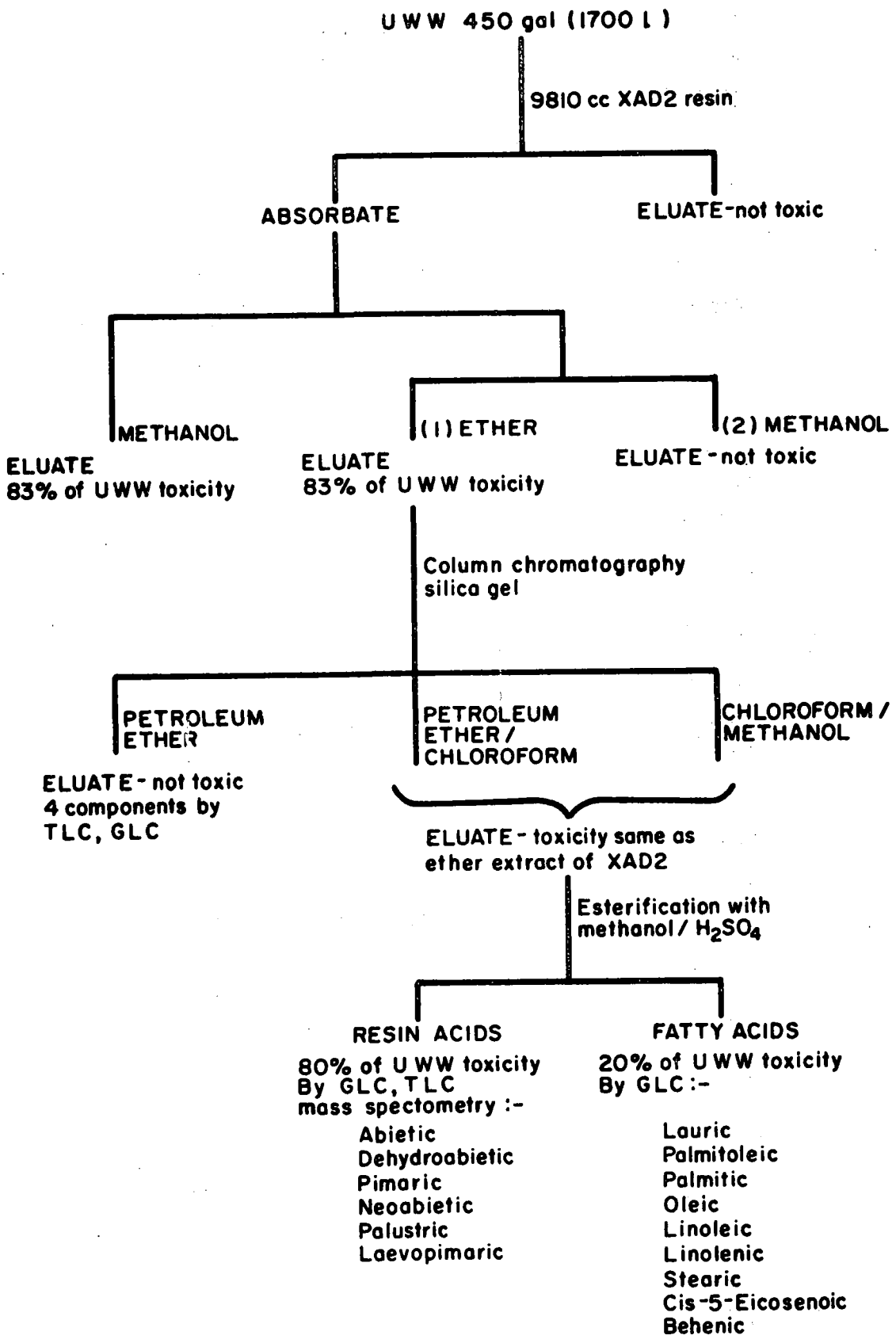
Overall, 83% of the toxicity of a batch of UWW was accounted for. Toxicity losses occurred only during the initial time-consuming stage when 450 gallons UWW were processed.

A total of six resin acids were identified, which together account for approximately 80% of the isolated toxic material. The other 20% of toxicity is contained in a fraction consisting largely of fatty acids, nine of which have been identified by gas chromatography.

It is expected that rapid processing of a fresh batch of UWW using the established fractionation scheme will achieve a toxicity balance close to 100%.

Chart 1

FRACTIONATION SCHEME FOR TOXIC CONSTITUENTS OF UWW



D. EXPERIMENTAL

1. Apparatus

Gas chromatographic analyses (GLC) were carried out using a Perkin-Elmer Model 881 temperature-programmed instrument. Two columns were used. Column A, 12 ft x 1/8 in. stainless steel, was packed with 15% silicone oil SE-30 on Chromosorb W (60/80). Column B, 5 ft x 1/8 in., contained 20% DEGS on Chromosorb W. Eluent vapours were monitored by a flame-ionisation detector connected to a Leeds and Northrup moving chart recorder.

Column chromatographic separations were made with the aid of a Gilson Model VL automatic fraction collector.

Infrared (IR) and ultraviolet (UV) spectra were obtained using a Perkin-Elmer Model 521 grating instrument and a Unicam SP 800 spectrophotometer, respectively. Nuclear magnetic resonance (NMR) spectra were measured at 60 Mc./sec with a Varian instrument on samples dissolved in carbon tetrachloride or in deuteriochloroform.

2. Solvent Extraction of Residue from Methanol Eluate of XAD-2 Resin

Amberlite XAD-2 resin (500 cc) containing the toxic constituents from 86.5 l. UWW, was washed with methanol (8 l.) under a nitrogen atmosphere at 2°C. The aqueous residue remaining after evaporation of methanol at 40°C under reduced pressure was made up to 500 ml with dechlorinated water and labelled "Solution A". Thus, 1 ml. Solution A contains the toxic constituents derived from 173 ml UWW. Bioassays were done using 11 juvenile coho salmon in aliquots of Solution A made up to 6 l. volume.

Solution A (100 ml.) was successively extracted under nitrogen with 200 ml. portions of pentane, diethyl ether, benzene and chloroform. After removal of solvent from each extract, the separate residues were dissolved in 5% sodium hydroxide solution and made up to standard volumes. Aliquots were taken for bioassay. A bioassay was also carried out on the aqueous residue remaining after extraction with the four solvents.

The extracts were examined by thin layer chromatography (TLC) using Silica Gel GF-coated plates and two-way solvent development with (a) chloroform, (b) 80/20 benzene:ethyl acetate. Spots were developed by spraying with antimony pentachloride solution, and the plates were examined under UV light.

A second portion of Solution A (100 ml.) was repeatedly extracted with pentane (10 x 50 ml.) under nitrogen, followed by extraction with diethyl ether. Solvents were removed from each fraction and bioassays performed on the combined pentane extracts, the ether extract, the mixed pentane and ether extracts and on the residue from Solution A after extraction.

Another aliquot of Solution A was extracted solely with diethyl ether under nitrogen. Both the ether extract after evaporation of solvent, and the residual Solution A were subjected to bioassay.

3. Desorption of Toxic Constituents of UWW from XAD-2 Resin Using Diethyl Ether

The toxic materials from 173 l. UWW, absorbed on Amberlite XAD-2 resin (1000 cc.), were eluted under nitrogen at 2°C with ether (2 l.), followed by methanol (10 l.). Solvents were removed in the usual way and the methanol residue made up to 500 ml. volume as before. The residue from evaporation of ether was bulked to 500 ml. with fresh ether.

The methanol extract and the ether extract after removal of solvent, separately and in combination, were tested by bioassays at several concentrations. The weight of material extracted was measured by evaporating solvent from an aliquot (34.6 ml. ≡ 12 l. UWW) and drying the residue under reduced pressure over phosphoric oxide. Bioassays were also done on the dried residue after dissolution in 5% sodium hydroxide and subsequent neutralisation. A further fraction of the ether extract (120 ml. ≡ 41.6 l. UWW) was dried over anhydrous magnesium sulphate, filtered and after removal of solvent, weighed and subjected to bioassay.

The ether extract was examined by GLC (Column A, programmed from 50-150°C at 12°C/min; flow-rate 30 cc N_2/min.), by TLC (Silica Gel GF; 90/10, chloroform:methanol), and by IR and NMR spectroscopy. Colourimetric tests for resin acids[1], using the Liebermann Bürchard reaction, were carried out on both ether and methanol extracts.

4. GLC Analysis of Methylated Ether Extract

Solvent was removed from a separate aliquot of the ether extract containing toxic components previously absorbed on XAD2 resin. The yellow oily residue (102 mg.) obtained was stirred for 18 hr. with diazomethane in ether (prepared by reaction of ethanolic

potassium hydroxide with N-nitroso-N-methyl urea). The ethereal solution was washed with 10% aqueous sodium hydroxide, separated and dried over anhydrous $MgSO_4$. After removal of solvent, the methylated residue was examined by GLC (Column A, programmed from 95-250°C at 6°C/min; flow-rate 214 cc N_2/min. Column B, programmed from 80-200°C at 12°C/min; flow-rate 30 cc N_2/min.). Retention volumes were compared with standard solutions of pure fatty acid and resin acid methyl esters.

5. Fractionation Procedure for Ether Extract of XAD-2 Resin

(a) Column chromatography using alumina

Solvent was removed from an aliquot (35 ml.) of the ether extract. The yellow oily residue (319 mg.) was chromatographed on a column of basic alumina (70 cc.) using gradient elution by petroleum ether, chloroform and methanol. Fractions were collected at 10 ml. intervals and each fraction was monitored by TLC. Materials eluting in petroleum ether were combined, solvent evaporated and the residue (84 mg.) subjected to examination by GLC and IR spectroscopy. Fractions eluting in petroleum ether-chloroform mixtures, in pure chloroform, in chloroform-methanol mixtures, and in pure methanol were combined and treated in the same fashion to give a residue (97 mg.).

(b) Semi-preparative scale TLC separation using Silica Gel

The oily residue (180 mg.) from evaporation of an aliquot of the ether extract was chromatographed by two successive developments with petroleum ether using glass plates coated with Silica Gel GF (2 mm thickness). The component bands were detected under UV light, removed and the constituents extracted with methanol. After evaporation of solvent, the residue was weighed (36 mg.).

(c) Column chromatography using Silica Gel

Ether was evaporated from a further sample of the extract of XAD-2 resin to leave a residue (228 mg.), which was chromatographed on Silica Gel (25 g; 80-200 mesh) using the solvent series, petroleum ether, chloroform, methanol. 70 aliquots (10 ml.) were collected and, after analysis by TLC, combined to give seven discrete fractions. Materials eluting in petroleum ether alone (54 mg.) were designated "non-polar". Components soluble in mixtures of petroleum ether-chloroform, chloroform alone or in chloroform-methanol (total wt. 173 mg.) were termed "polar".

Fractions were examined by TLC using three consecutive developing solvents; (a) petroleum ether, (b) chloroform, (c) methanol. Rf values were compared with samples of abietic and dehydroabietic acids which had previously been purified by column chromatography.

A second batch of the residue (368 mg.) remaining after removal of ether from the XAD-2 extract was chromatographed on Silica Gel (300 g.) using the same solvent system as before. A total of thirty-five, 50 ml. fractions were collected. After comparison of TLC analyses they were combined to give 8 major fractions. Two of the fractions contained non-polar constituents (64 mg.), the other six consisted of materials eluted in polar solvents (304 mg.).

Bioassay tests were performed at several concentrations on the polar and non-polar fractions separately and in combination. IR and UV spectra were obtained for the non-polar fraction.

6. Separation of Polar Fraction Obtained by Silica Gel Chromatography into Resin and Fatty Acids

(a) Cyclohexylamine precipitation of resin acids[2]

A synthetic mixture of dehydroabietic acid (93 mg.), abietic acid (287 mg.) and linoleic acid (37 mg.) was dissolved in acetone (10 ml.), and cyclohexylamine (1.5 ml.) added. The mixture was kept at 2°C for 24 hr., petroleum ether added and the precipitated cyclohexylamine salts of the resin acids removed by filtration. The salts were hydrolysed by reaction with dilute HCl (10 ml.), and the free acids extracted into ether. After separation of the ether layer, solvent was removed to give the recovered resin acids (180 mg.).

(b) Selective methylation of fatty acids[3]

The polar fraction (100 mg.) derived by silica gel chromatography of an aliquot of ether extract containing UWW toxicants was stirred for 18 hr. at room temperature in methanol (10 ml.) containing 25% sulphuric acid (0.2 ml.). Methanol was evaporated, the residue dissolved in ether (30 ml.) and the ethereal solution extracted with 10% sodium hydroxide solution (4 x 10 ml.). The ether layer was separated, dried over anhydrous $MgSO_4$, filtered and the solvent removed to leave a residue of fatty acid methyl esters (57 mg.), which were subjected to TLC (Silica Gel GF; 95/5, chloroform:methanol) and GLC (Columns A and B; conditions as before). The fatty acid esters were stirred for 16 hr. with 25% sodium hydroxide solution (10 ml.), the solution acidified by addition of hydrochloric acid and extracted with ether (4 x 10 ml.). Free fatty acids (55 mg.) were recovered by normal procedures.

The sodium hydroxide extract of the ether layer containing unesterified resin acids was acidified and treated in the usual manner to give free resin acids (43 mg.).

Bioassays were performed at several concentrations on the fatty acid and resin acid fractions separately and in combination. Authentic samples of commercially obtained fatty acids and resin acids were also subjected to bioassay tests.

7. Chromatography of Resin Acid Fraction on Silica Gel Impregnated with Silver Nitrate[4]

Silica Gel (30 g., 80-200 mesh) was stirred with silver nitrate (7 g.) in distilled water (90 ml.). Solvent was removed at 120°C and the support used for column chromatography of a resin acid fraction (321 mg.) derived as in Section 4 above. A total of 33 aliquots (10 ml.) were collected by elution with chloroform, and combined on the basis of analytical TLC (25% $AgNO_3$ on silica gel; developed with 90/10, chloroform:methanol) to give 8 fractions.

8. Analysis of Resin Acids by Combination GLC-Mass Spectrometry

A sample of the resin acid fraction was injected into a Varian Associates Model 1600 Aerograph gas chromatography instrument containing a DEGS column. The separated eluants passed directly into a Hitachi Perkin-Elmer Model RMU-6E mass spectrometer operating under the following conditions: inlet temperature 200°C, ionisation voltage 80 ev, ionising current 10 μA, scanning voltage 1800 v, magnetic current scanning 0-300 mu in 15 sec. Fragmentation patterns were obtained for four gas chromatographic peaks.

E. RESULTS AND DISCUSSION

1. Liquid-Liquid Extraction of Methanol Eluate of XAD-2 Resin

In Progress Report No. 4 it was stated that the toxic constituents of UWW, after absorption onto XAD-2 resin, could be desorbed by washing the resin with methanol. Subsequent work verified that 83% of the toxicity of the original UWW is recovered in the methanol extract.

Further fractionation of the toxic components of UWW was accomplished on the basis of their solubility characteristics. Thus, the residue obtained by removing solvent from the methanol extract of XAD-2 resin was dissolved in water and extracted successively with pentane, ether, chloroform, benzene.

Results of bioassay tests on the extracts are listed in Table 1. Toxic materials were dissolved only by the pentane and ether washes. When these were combined, the MST value at a concentration equivalent to 100% UWW was closely similar to that obtained for the original methanol extract. In bioassay tests on the material dissolved by benzene and chloroform, no fish were killed after 96 hr. at a concentration equivalent to twice that present in the original UWW. The aqueous residue after extraction by the four solvents was also not toxic. When all the extracts were recombined in the original proportions, the MST of fish was virtually identical with that observed for the methanol extract. TLC examination of both the pentane and ether fractions showed a similar pattern of eight spots, though relative quantities differed.

Since the same constituents appeared to be present in both toxic fractions, attempts were made to extract all the toxicants into pentane. The methanol extract of toxic material from XAD-2 resin, dissolved in water, was repeatedly washed with pentane, and then with ether. Bioassay tests, however, indicated that the fraction extracted by ether was still toxic. Consequently, ether was used directly to wash the aqueous methanol extract and was shown by bioassay to remove the toxic components quantitatively. Though accurate material weights were not obtained at this stage, it was apparent that most of the coloured components eluted from XAD-2 resin by methanol were not dissolved by ether. Ether was therefore used subsequently to obtain more selective desorption of the toxic factors of UWW from XAD-2 resin.

2. Extraction of UWW Toxic Materials from XAD-2 Resin with Ether

Bioassay data are presented in Figure 1 for materials extracted from two separate 1 litre batches of XAD-2 resin, each of which contained the toxic constituents from 173 l. UWW. The graphs for ether and methanol extracts were almost the same, with only minor differences which may be ascribed to normal deviations in bioassay results. Following the ether wash, exhaustive extraction of the XAD-2 resin with methanol removed all the coloured components, but did not produce additional toxic constituents. The weight of material extracted by ether was found, after rigorous drying over phosphoric oxide under reduced pressure, to be equivalent to 26.5 mg/litre UWW. However, bioassay tests showed that loss of some volatile or labile toxic components had occurred during the drying process. Milder drying conditions, using anhydrous magnesium sulphate, gave a yellow oil, equivalent of 33.4 mg/l. UWW.

The toxicity-concentration graph for the oil was identical with that of the ether extract from which it was derived.

3. Identification of Constituents in the Ether Extract

The NCASI standard colourimetric test for resin acids[1] indicated the presence in the ether extract of 17 mg./l. UWW of constituents giving a positive Liebermann-Bürchard reaction (expressed as abietic acid). In the methanol extract there was less than 1 mg. "resin acids" per litre UWW. TLC of the ether fraction suggested the presence of at least 12 constituents, two of which had Rf values identical with abietic acid and dehydroabietic acid. The NMR spectrum displayed absorptions at comparable chemical shifts to those observed for a synthetic mixture of abietic and dehydroabietic acids. There were, in addition, numerous signals of lesser intensity which could not be attributed to these acids.

Gas chromatographic analysis of the ether extract revealed the presence of 3 major and a number of minor components. However, under the column conditions used, free acids would not be expected to elute. Thus, the hydroxylated materials in the ether extract were converted to their more volatile methyl esters and ethers by treatment with diazomethane. The GLC trace obtained for the methylated extract is shown in Figure 2.

Resin acids listed in Table 2 below were identified in the ether extract by comparison of GLC retention volumes on both polar and non-polar stationary phases with synthesised methyl esters of the pure compounds. It was suspected that peaks of shorter GLC retention than resin acid esters were probably due to fatty acid esters. Reinforcement of these peaks using additions of the pure methyl esters of fatty acids indicated the presence of the fatty acids listed in Table 2.

Resin Acids	Fatty Acids	Carbon No.	Sites of Unsaturation
(For structural formulae see Figure 3)			
Dehydroabietic	Lauric	C12	Saturated
Abietic	Palmitoleic	C16	C-9
Laevopimaric	Palmitic	C16	Saturated
Palustric	Oleic	C18	C-9
Pimaric	Linoleic	C18	C-9,12
Neoabietic	Linolenic	C18	C-9,12,15
	Stearic	C18	Saturated
	Cis-5-Eicosenoic	C20	C-5
	Behenic	C22	Saturated

TABLE 2 - Components Indicated by GLC in an Ether Solution Containing the Toxic Constituents of UWW

At this stage in the fractionation procedure, two large GLC peaks of short retention and several other minor constituents remained unidentified.

4. Fractionation of Ether Extract by Silica Gel Chromatography

An initial attempt was made to separate the toxic UWW constituents contained in the ether extract by column chromatography using basic alumina. Only 57% of the material could be recovered from the column reflecting the high carboxylic acid content of the extract. When a semi-preparative thick layer chromatographic separation was attempted using pre-coated silica gel plates, good separation of components was observed. Unfortunately, only 20% of the starting material could be extracted from the absorbent support.

An excellent material balance was obtained when the ether extract was chromatographed on a column of silica gel using a solvent series consisting of petroleum ether, chloroform, methanol. The various fractions collected and their weights are given in Table 3. TLC revealed the presence of four components in Fractions 1 and 2. None of these components were detected in succeeding fractions. An additional four spots were shown by TLC to be distributed throughout Fractions 3-8. Because of their chemical similarity, the petroleum ether fractions 1 and 2 were combined and labelled "non-polar". The material in Fractions 3-8 was also pooled and designated "polar" fraction. By extrapolation from the weights of the separated fractions it can be calculated that the non-polar fraction constitutes 5.8 mg./l. UWW and the polar fraction 27.6 mg./l. UWW.

The non-polar material was not toxic towards salmon when tested at a concentration four times that found in UWW. GLC analysis showed that the three large peaks of short retention volume (see Figure 2) are due to constituents present in the non-polar fraction. IR spectroscopy showed absorptions ascribable to aromatic nuclei and also the complete absence of any functional groups. The UV spectrum of the mixture was indistinguishable from that of naphthalene, implying that the non-polar materials have a chemical skeleton based on the naphthalene nucleus. Further identification of these non-polar constituents has been shelved at the present time since they do not contribute to the toxicity of UWW.

The toxicity of the polar fraction from silica gel chromatography of the ether extract was tested by bioassay. Results are presented in Figure 1. Graphs for the ether extract and the polar fraction are essentially superimposable, having the same slope and concentration vs. toxicity characteristics. When the non-polar and polar

fractions were recombined in their original proportions the toxicity graph was again identical with that of the ether extract. Clearly, the toxic materials are completely retained during this chromatographic separation.

5. Separation of Polar Fraction into Resin Acids and Fatty Acids

The insolubility of amine salts of resin acids has been utilized[2,6,7,8] to effect separation of the free acids from other wood extractives. With cyclohexylamine, Riffer et al[2] obtained 86% recovery of resin acids from heartwood. In the present study, however, when a synthetic mixture of dehydroabietic acid, abietic acid and linoleic acid was treated with cyclohexylamine, only 47% of the resin acids were recovered by precipitation.

A far more effective procedure for separation of resin acids from fatty acids in the polar fraction involved selective esterification of the fatty acids with methanol and dilute acid[3]. Under the mild conditions employed, steric hindrance prevents methylation of the resin acids which can thus be readily extracted by base from the fatty acid methyl esters. When 100 mg. of the polar fraction, derived by chromatography of the ether extract containing UWW toxicants, was esterified, 55 mg. fatty acids and 43 mg. resin acids were isolated. GLC analysis showed that virtually complete separation of the two acid groups had been achieved. Bioassays of the two fractions separately and after recombination produced the graphs depicted in Figure 4, which is shown together with the graph for the ether extract. Approximately 95% of the original toxicity has been retained throughout the fractionation procedure. In fact, the discrepancy could be due entirely to the experimental error inherent in bioassay tests.

The data in Figure 4 indicate that the resin acid fraction, which comprises 12 mg./l. UWW, is responsible for 80% of the toxicity isolated in the ether extract. Fish in 100% concentration of the fatty acid fraction were alive after 1500 min. exposure, though in severe distress. By extrapolation it is evident that the fatty acids contribute the remaining 20% of UWW toxicity. Their concentration in the UWW is 15 mg./l.

In order to assess the contribution of individual acids to overall toxicity, column chromatography using silica gel impregnated with silver nitrate was employed. The technique permits the separation of unsaturated compounds from one another and from saturated constituents by the formation of weak coordination complexes between carbon-carbon double bonds in the organic molecule and the silver

ions in the chromatographic support[4]. When the resin acid fraction was chromatographed in this way a partial separation of the constituents was accomplished, as found from TLC and GLC examination of the various fractions. Pure samples of dehydroabietic and pimaric acids were obtained, though not quantitatively, since overlap occurred between these acids and the other resin acids present i.e., abietic, necabietic, laevopimaric and palustric.

Confirmation of the structural assignments of three of the resin acids was gained by using a mass spectrometer connected to a gas chromatograph. The resin acid mixture was injected in the conventional way onto the GLC column. As each separate constituent emerged from the instrument, the eluent vapour passed directly into the mass spectrometer and a fragmentation pattern characteristic of the compound was produced. In this way, by comparison with data on authentic samples, pimaric[9], dehydroabietic[10] and abietic[10] acids were identified. Further work is required to interpret the mass spectrum of the peak suspected to be due to laevopimaric and palustric acids, since these compounds are extremely difficult to separate by GLC. Mass spectral data do not rule out the possibility that isopimaric acid is also present in small quantity. Authentic samples of all of the resin acids, except isopimaric, have been obtained and used to confirm GLC assignments.

In order to illustrate the toxic characteristics of some of the components implicated by the present study, bioassays were performed at several concentrations using the following acids: oleic, linoleic, pure abietic, technical abietic, technical dehydroabietic. The results are presented in Figure 5. Clearly, the inherent toxicities of the acids differ greatly, and also the effects of dilution upon toxicity vary markedly between acids.

F. FUTURE PLANS

1. Additional bioassay tests will be run at various concentrations in order to confirm the contributions of resin and fatty acids to UWW toxicity.

2. Separation of individual resin acids and fatty acids using silver nitrate-impregnated silica gel and other techniques will be pursued.

3. Further use will be made of the GLC-mass spectrometer facility, made available by Simon Fraser University, in order to characterise individual compounds.

4. When the complete fractionation scheme has been evolved (within 6 weeks), rapid processing of a fresh batch of UWW through XAD-2 resin during a 48 hr. period will be done. In this way toxicity losses will be kept to an absolute minimum. The established fractionation procedure will be followed through in the shortest possible time so that a complete statement can be made of the nature and actual contribution of all toxic constituents in UWW.

5. Once the toxic materials are identified and their concentrations known, it will be a simple matter to repeat the separation procedure for comparison of UWW toxic components within and between mills.

6. A start will be made upon identifying toxic constituents of bleachery effluents.

G. REFERENCES

1. Colourimetric Measurement of Resin Acid Soaps in Pulp and Paper Mill Wastes, NCASI Technical Bulletin No. 182, (1965).

2. Composition of Turpentine and Resin Acid in Benguet Pine Stump Heartwood, R. Riffer, A.B. Anderson, E.V. Cabotage, A. Ynalvez. TAPPI, 49, 415 (1966).

3. Rapid Mixed Indicator Method for the Determination of Rosin Acids in Tall Oil. E.H. Sheers and L.S. Stevenson, TAPPI, 43, 190, (1960); Testing of Tall Oil, ASTM Standard D-803, (1970).

4. Thinlayer, Column, and Gas Liquid Chromatography of Resin Acid Esters and Related Terpenes. T. Norin and L. Westfelt, Acta. Chem. Scand. 17, 1828, (1963).

5. Reagents for Organic Syntheses, L.F. Fieser and M. Fieser, pp 1118-1121, (1967), Wiley.

6. An Improved Method for Isolation of Resin Acids, G.C. Harris and T.F. Sanderson, J. Amer. Chem. Soc., 70, 334, (1948).

7. Thermal Isomerisation of Laevopimaric Acid, V.M. Loeblich, D.E. Baldwin, R.T. O'Connor and R.V. Lawrence, ibid., 77,6311, (1955).

8. Organic Syntheses Collective Vol. 4, 1, (1963).

9. Structural Elucidation of Natural Products by Mass Spectrometry, H. Budzikiewicz, C. Djerassi, D.H. Williams, Vol 2, 155, (1964), Holden-Day Inc.

10. Resin Acid Analysis by Mass Spectrometer, C.A. Genge, Anal. Chem., 31, 1750, (1959).

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TABLE 1

Results of Bioassays on UWW Fractions
Extracted from XAD-2 Resin in Various Solvents

	MST (Mins)							
Concentration Relative to Original UWW	Methanol Extract of XAD-2	Pentane	Diethylether	Pentane and Diethylether	Benzene	Chloroform	Aqueous Residue After Solvent Extraction	Pentane, Ether, Benzene, Chloroform, and Aqueous Residue
200.1		74	2 dead after 96 hr.		No Mortalities	No Mortalities	No Mortalities	
150.1		94	No Mortalities					
100.1	115	2 dead after 96 hr.		119				120
80.1	220	No Mortalities						
70.1	325							
60.1	510							

TABLE 3

Fractions Eluted During Column Chromatography of Ether Extract of XAD-2 Resin on Silica Gel

Fraction Number	Weight (mg.)	Solvent
Extract applied to column	368	
1	57	Petroleum ether
2	7	Petroleum ether
3	3	10% chloroform, 90% pet. ether
4	3	25% chloroform, 75% pet. ether
5	14	50% chloroform, 50% pet. ether
6	11	50% chloroform, 50% pet. ether
7	76	50% chloroform, 50% pet. ether
8	197	Methanol
Total recovered	368	

Figure 1

BIOASSAY DATA FOR ETHER AND METHANOL EXTRACTS OF U W W TOXICANTS FROM XAD-2 RESIN AND FOR POLAR FRACTION OF ETHER EXTRACT

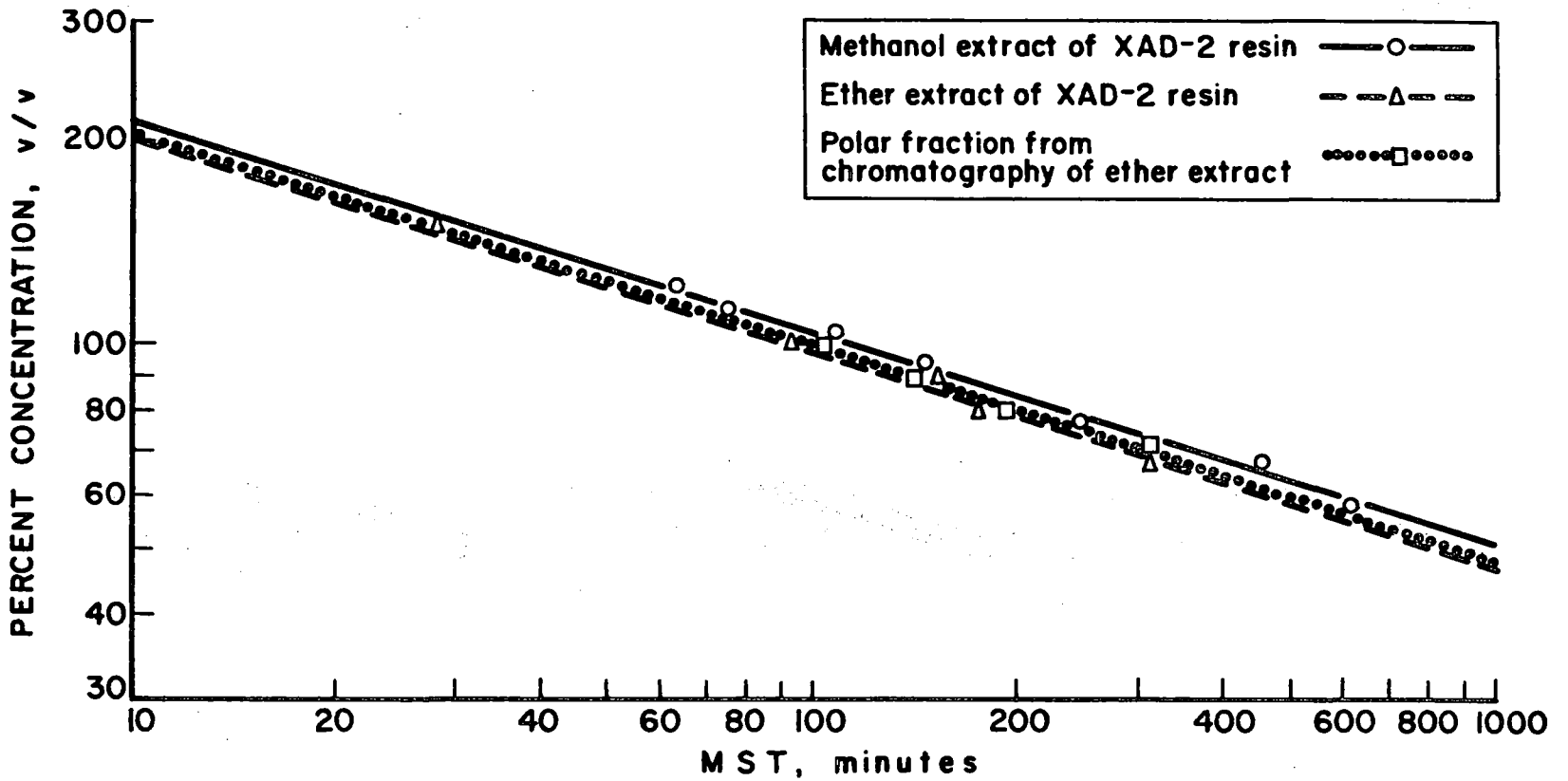


Figure 2

GAS CHROMATOGRAM OF ESTERIFIED ETHER EXTRACT

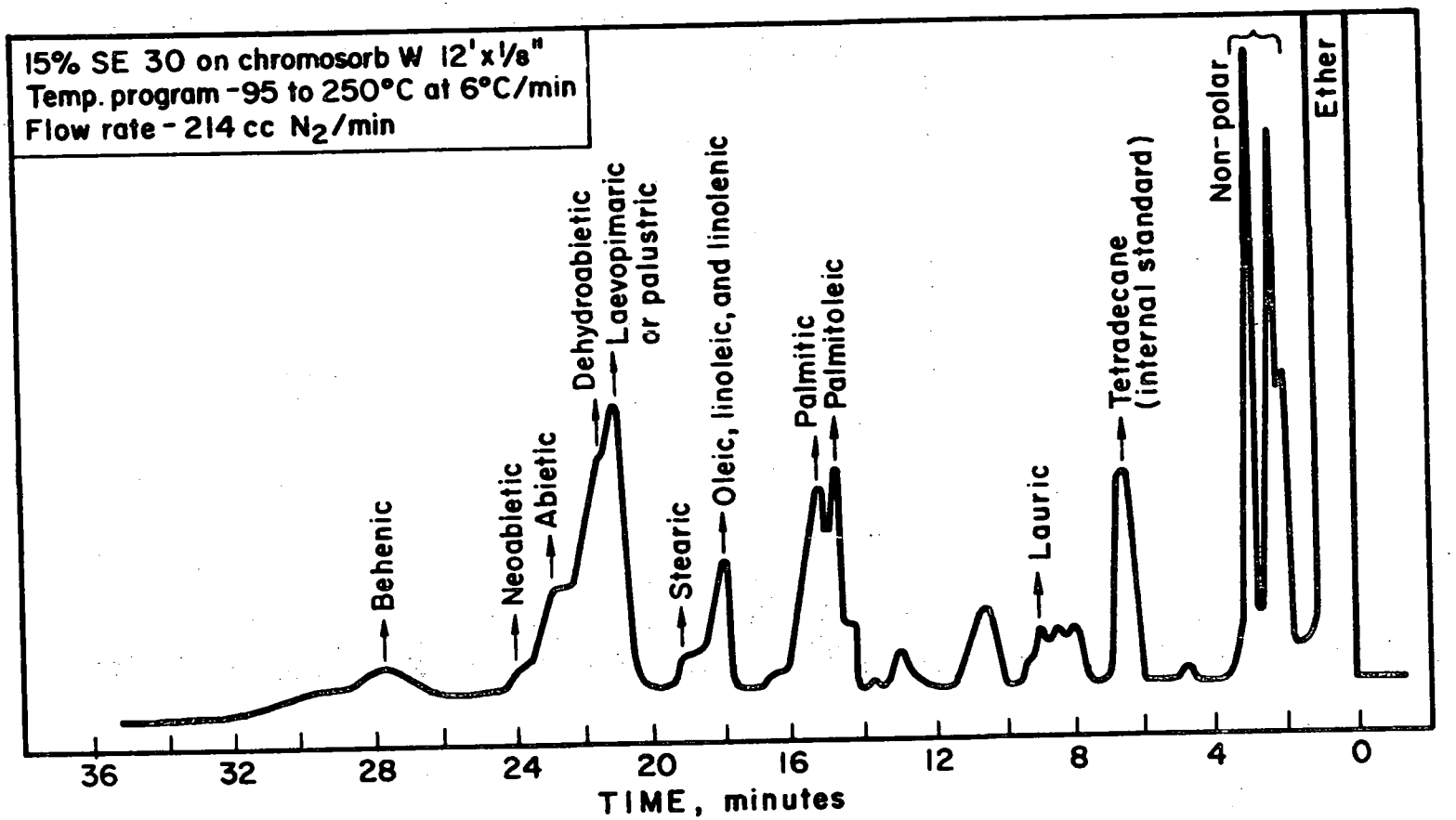
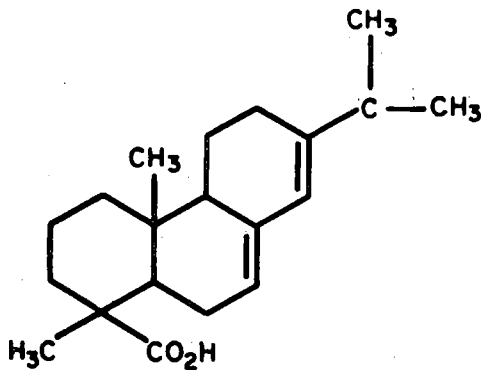
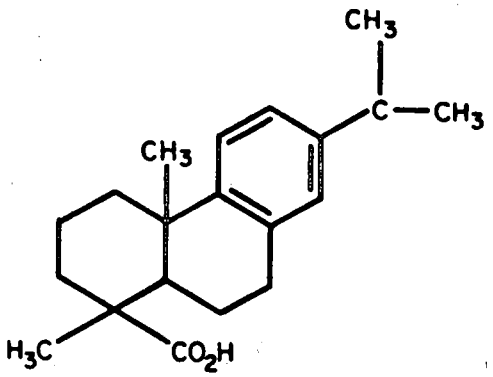


Figure 3

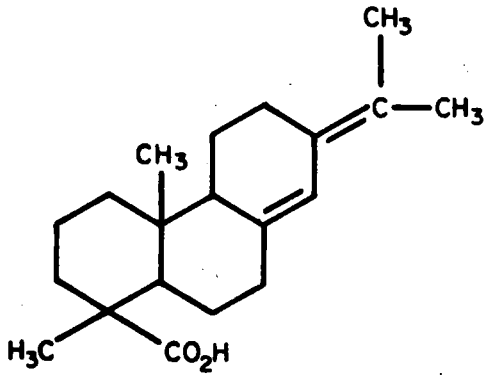
STRUCTURAL FORMULAE OF RESIN ACIDS FOUND IN ETHER EXTRACT OF XAD 2 RESIN



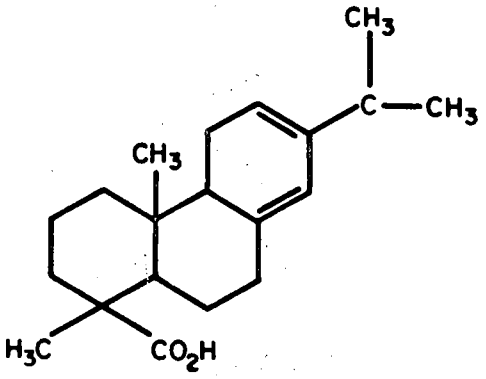
ABIETIC ACID



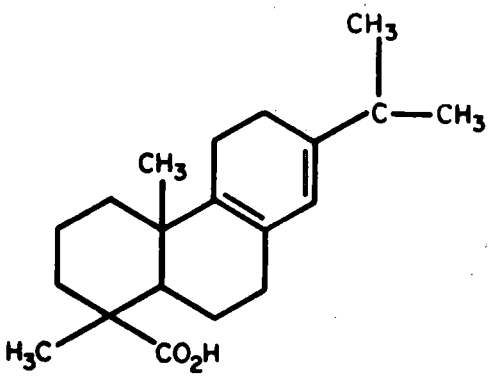
DEHYDROABIETIC ACID



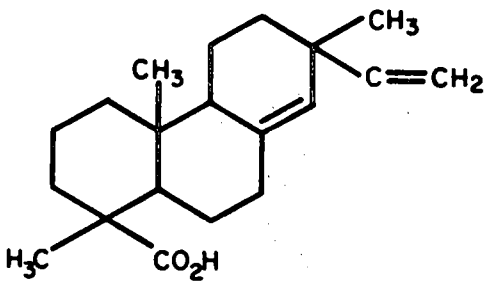
NEOABIETIC ACID



LAEVOPIMARIC ACID



PALUSTRIC ACID



PIMARIC ACID

Figure 4

BIOASSAY DATA FOR RESIN ACID AND FATTY ACID FRACTIONS OF U W W

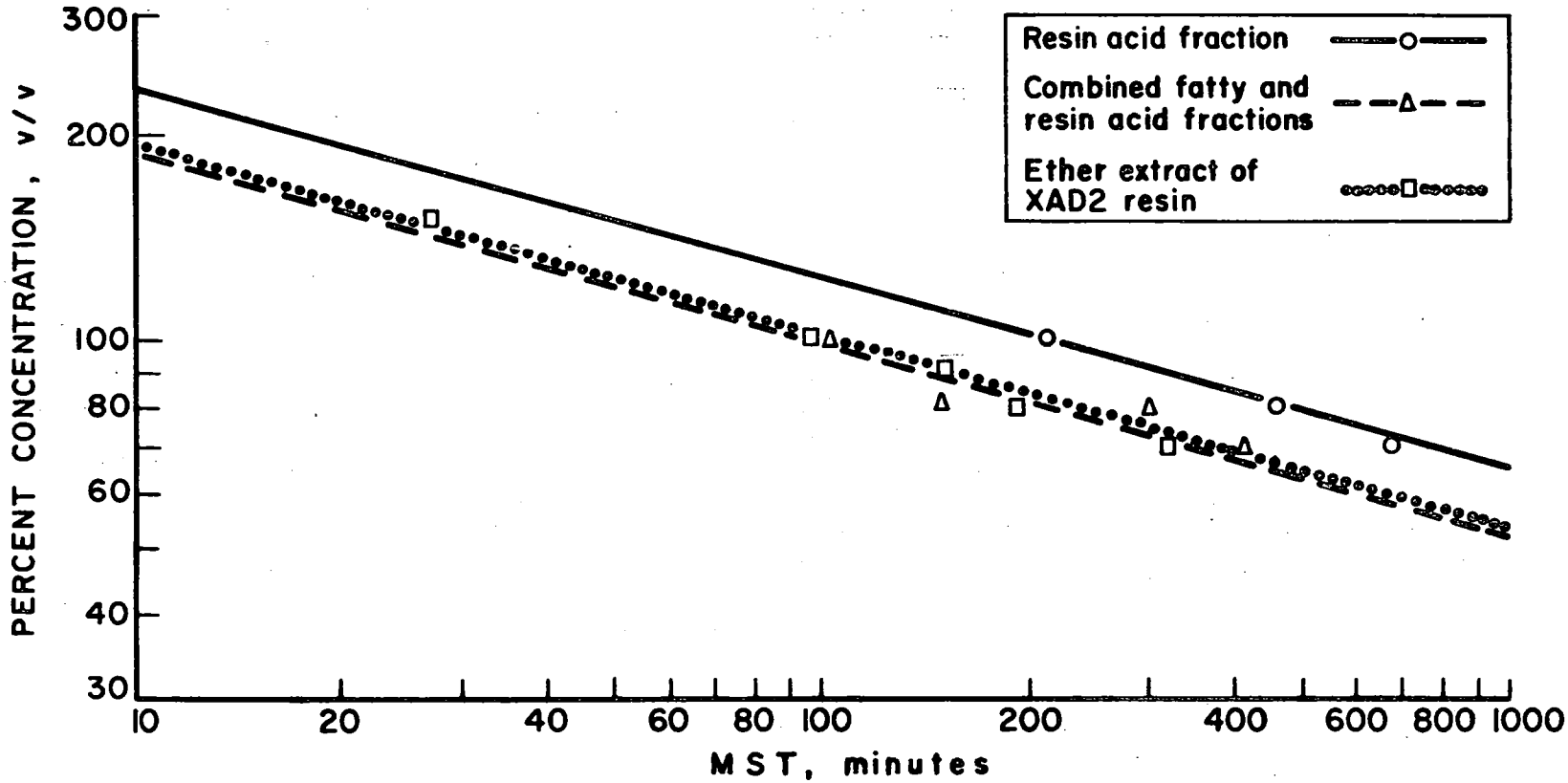


Figure 5

BIOASSAY DATA FOR SOME RESIN AND FATTY ACIDS