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DEVELOPMENT OF A RAPID COLOURIMETRIC ASSAY FOR RESIN AND FATTY ACIDS IN PULP AND PAPER MILL EFFLUENTS

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Master of Engineering

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Submitted October 1995

ISBN 0-612-07969-4

ABSTRACT

Researchers have linked resin and fatty acids (RFAs) to acute toxicity, especially in thermomechanical and chemithermomechanical pulp and paper mill effluents. Thus, the measurement of total RFAs may be a rapid, inexpensive alternative to standard methods of toxicity monitoring, such as 96-hour rainbow trout testing. Current procedures for the analysis of RFAs typically involve solvent extraction and concentration, derivatization, and analysis by GC, HPLC, or TLC. These procedures are far too expensive, complicated, and time-consuming for implementation at mill sites.

This thesis reports the development of a rapid, colourimetric assay based on the dye methylene blue (MB) for the quantification of resin and fatty acids in pulp and paper mill effluents. This MB assay uses the complexation of the cationic organic dye molecule to the carboxylic acid groups of RFAs to form a measurable chromophore. The electrically neutral, blue-coloured complexes are then extracted into a poorly polar organic solvent, dichloromethane. The measured absorbance at 655 nm is directly related to the total RFA concentration in the effluent sample.

The methylene blue assay is inexpensive and simple to use. It has a method detection limit of 0.589 mg/L total RFA. There are good correlations between the results obtained using the methylene blue assay and a well-established GC assay, and between RFA concentrations measured by the MB assay and acute toxicity measured by Microtox. The assay is sufficiently simple and rapid to be practical for routine in-mill monitoring.

RESUME

Une corrélation existe entre la toxicité et la teneur en acides gras et résiniques (ARG) des effluents de papetières utilisant les procédés thermomécaniques et chimicothermomécaniques. La mesure des ARG réprésente une alternative aux méthodes standards d'évaluation de la toxicité. Toutefois, les méthodes actuelles de quantification des ARG comprennent des étapes d'extraction, de concentration, de dérivation et d'analyse en chromatographie gazeuse, qui sont trop laborieuses et nécessitent trop d'instrumentation pour être facilement implantées en usine.

Une méthode colorimétrique rapide, employant le bleu méthylène, a été développée pour évaluer la concentration des ARG dans les effluents de pâtes et papiers. La méthode est basée sur la formation d'un complexe entre le colorant organique cationique et les groupements carboxyliques des ARG. Ce complexe chromophorique, électriquement neutre, peut être facilement extrait à l'aide de dichlorométhane, un solvant organique faiblement polaire. S'ensuit une mesure de densité optique à 655 nm, laquelle est directement proportionelle à la concentration des ARG dans l'échantillon.

La limite de détection pour la méthode est de 0.589 mg/L. Les résultats indiquent une bonne corrélation entre la méthode colorimétrique et, d'une part l'analyse conventionnelle par chromatographique gazeuse, et d'autre part avec des résultats d'analyse de toxicité aigue obtenus à l'aide du bioassai Microtox. L'analyse colorimétrique des ARG est suffisament économique, rapide et facile d'emploi pour être utilisée en usine.

ACKNOWLEDGEMENTS

The author would like to thank the following people and organizations for their contributions to this project: Bob Dawe (Dow Forestry Products) for his modifications to the original methylene blue assay for Dow's analyses of highly concentrated RFA solutions; Francis S. Young, Myriam Méthot, Francine Lafortune, Line Roy-Arcand (all from PAPRICAN), Génévière Harel (École Polytechnique), and Richard Blanchet (Université de Sherbrooke) for the provision of GC and Microtox data; Line Roy-Arcand for her translation of the abstract into French; Michael G. Paice, Bruce Sitholé, Gil Garnier, and Denys Leclerc (all from PAPRICAN) for consultation; Mike Landry (Fisher Scientific) and Steven Horvath (PAPRICAN) for technical support in the selection and development of equipment; Lyne Tremblay, P.Eng., and PAPRICAN member companies for the provision of mill effluent samples.

Special thanks are expressed to the Natural Sciences and Engineering Research Council (NSERC), McGill University Department of Chemical Engineering, Dr. Ronald J. Neufeld, and PAPRICAN for the funding of this project.

Finally this project could not have been completed without the countless contributions and guidance of Dr. Frederick S. Archibald (PAPRICAN), Dr. Line Roy-Arcand (PAPRICAN), and Dr. Ronald J. Neufeld (McGill University Department of Chemical Engineering).

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1.0 Introduction

1.1 Structure and Nature of Resin and Fatty Acids (RFAs)

Resin and fatty acids (RFAs) are two classes of wood extractives, compounds extractable from wood by organic solvents. These natural components of wood are released into pulp and paper mill effluents primarily during debarking and pulping. They are closely associated due to their similarities in chemical structure and toxicity. RFAs are many-carbon hydrocarbon structures with a single carboxylate group.

Resin acids (RAs) are a class of diterpenoid carboxylic acids naturally occurring primarily in the bark of many softwood species (e.g. spruce, pine). Hardwood species (e.g. maple, birch) have a lower RA content. The parent compound of many RAs is abietic acid. Several RAs (e.g. neoabietic, levopimaric, palustric) isomerize to abietic acid in low-pH or high-temperature conditions (NCASI, 1986). Other RAs typically released in pulp and paper processes include dehydroabietic, pimaric, and isopimaric acids. Chlorinated RAs are found in kraft bleach plant effluents. Figure 1 displays the chemical structures of selected resin acids.

Fatty acids (FAs) are naturally occurring long-chain carboxylic acids. Derived from acetic acid, tree FAs typically have even-numbered carbon chains. FAs are a food source for plants, and are precursor molecules for many commercially important substances, such as pharmaceuticals and foodstuffs (e.g. fats) (Trung, 1993). Typical FAs released in pulp and paper processes include oleic, linoleic, linolenic, and stearic acids. Figure 2 shows the chemical structures of selected fatty acids.

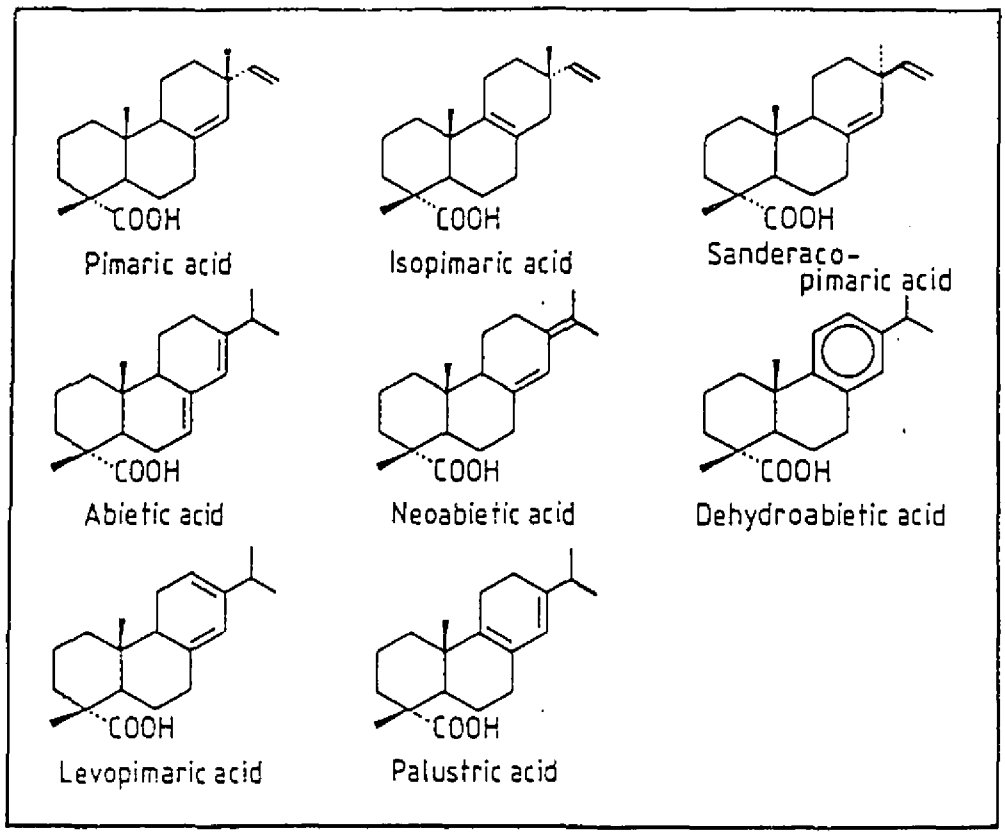


Figure 1. Structure of Selected Resin Acids (Holmbom, 1992).

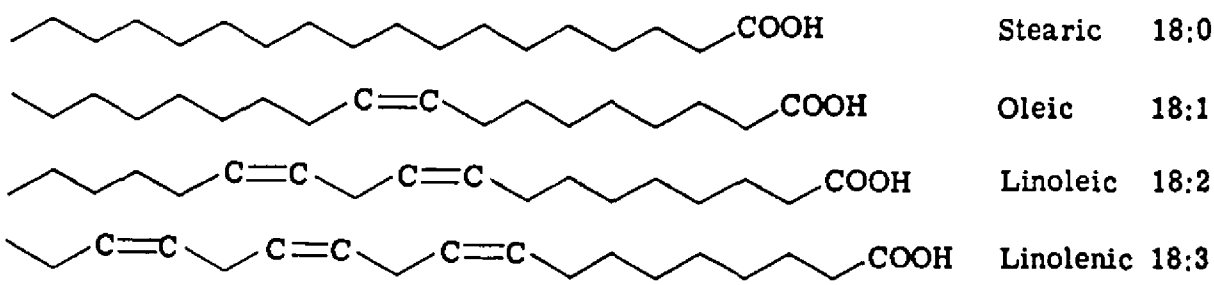


Figure 2. Structure of Selected Fatty Acids (Bonner & Varner, 1965).

1.2 RFAs and Toxicity

The toxicity of mill effluents is of prime importance to pulp and paper mills. Recent Canadian and provincial legislation has required all discharged effluents to be acutely non-toxic, i.e. less than 50% of the population of rainbow trout (*Salmo gairdneri*) or water fleas (*Daphnia magna*) killed after 96 hours of exposure to 100% effluent (Fisheries Act, 1992; Ontario Ministry of the Environment, 1993). This legislation has stirred Canadian interest in secondary treatment facilities and toxicity monitoring methods (Jamieson, 1992).

The acute and sublethal toxicity of whole mill effluents have been measured by bioassay. Leach and Thakore (1976) found that mechanical pulp mill effluents have LC_{50} values of 4-10% v/v. In their review of pulp and paper mill effluent toxicity, Walden and Howard (1976) reported that whole kraft mill effluent caused incipient sublethal changes in a variety of aquatic species at values of 0.1-0.6% v/v.

RFAs have been known to be toxic to fish since 1936 (Hagman, 1936). However, only in the last twenty to thirty years have RFAs been identified as major sources of toxicity in whole mill effluents.

Leach and Thakore (1976) found that RAs were responsible for 70% of the acute toxicity measured in a mechanical pulping effluent sample. Five individual RA concentrations were correlated with median lethal time (i.e. LT_{50}). RFA concentrations are generally higher in chemithermomechanical pulping (CTMP) effluents, due to the low total volumes of effluent generated by the CTMP process and the release of FAs not observed in thermomechanical pulping (TMP) processes (Liu *et al*, 1993). A more

recent correlation between acute toxicity and total RFA concentration in TMP and CTMP effluents was produced by Roy-Arcand, Archibald, and Méthot (1993). This correlation is given in Figure 3.

Effluents from bleached kraft pulps mills have multiple toxic factors. RFAs and chlorinated RFAs are the principal acutely toxic components in kraft pulping effluents, as reported by Priha and Talka (1986). Other compounds responsible for toxicity in kraft chlorination effluents are: tri- and tetrachlorocatechol, 2,6-dichlorohydroquinone, chloroguiacols, chlorosyringols, chlorovanillins, and various polychlorodihydroxybenzenes (Walden & Howard, 1981).

RFAs are the principal toxic factors in other types of pulp mill effluents. The toxicity in chlorination effluents from a sulphite mill was attributed to chlorinated forms of dehydroabietic acid (Walden & Howard, 1981). Leach and Thakore (1976) stated that RAs were the principal toxicants of unbleached whitewater, as well as woodroom, bleached kraft whole mill, and debarker effluents.

RAs are much more acutely toxic than FAs. In their 1981 survey, Walden and Howard listed the toxic contribution of RAs as intermediate to major, that of chlorinated RAs as intermediate, and that of FAs as minor to intermediate. The 96-hour LC_{50} values for rainbow trout exposed to individual RFAs are given in Table 1 (Lee *et al*, 1990). FAs are clearly less toxic than RAs, by about an order of magnitude in terms of LC_{50} values.

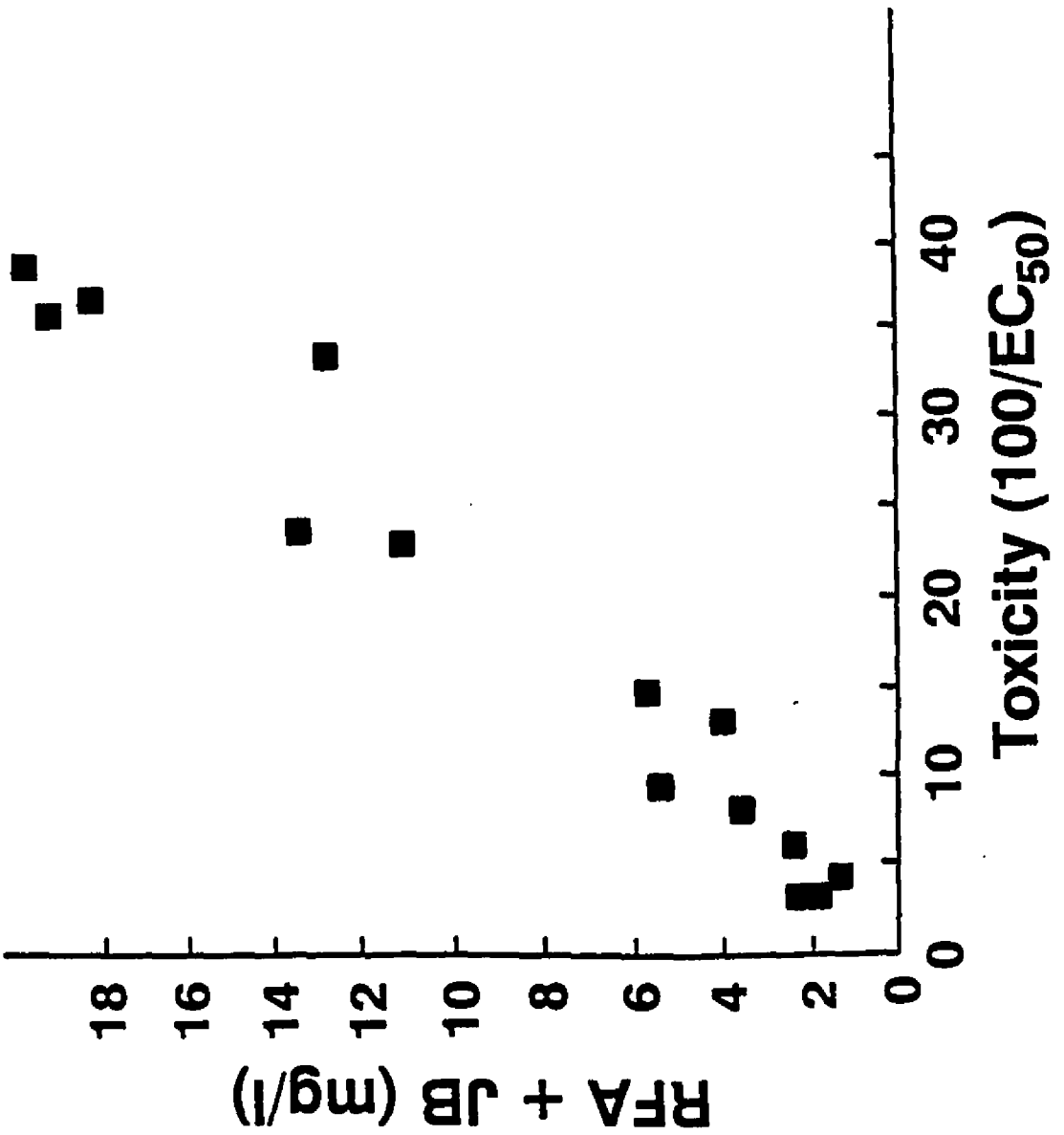


Figure 3. Correlation between Acute Effluent Toxicity and RFA/JB Concentration for TMP/CTMP effluents (Roy-Arcand, Archibald, & Méthot, 1993). JB = juvabione.

RFA	96-hr LC_{50} (mg/L)
Dehydroabietic	0.8-1.7
Levopimaric	1.1
Neoabietic	0.6-0.7
Chlorodehydroabietic	0.6-0.9
Dichlorodehydroabietic	0.6-1.2
Abietic	0.7-1.5
Sandaracopimaric	0.4
Palustric	0.5-0.6
Pimaric	0.7-1.2
Isopimaric	0.4-1.0
Oleic	3.2-8.0
Linoleic	2.0-4.5
Linolenic	3.0-6.0

Table 1. Acute Toxicity (96-hour LC_{50}) for Selected Resin and Fatty Acids.

Chemical analyses have been suggested as a possible alternative to bioassays for toxicity measurement (Chung *et al*, 1979). The measurement of recognized toxic compounds would be easier to implement on mill sites and would eliminate the broad variations often observed in bioassay results due to the sensitivity of individual fish stocks. However, toxicity tends to be underestimated by such analyses. Leach and Thakore (1976) recommended that toxicity be estimated by measurements of RA concentration in effluents in which RAs were the major toxic factors.

1.3 RFAs in Pulp and Paper Production

In addition to contributing toxicity to mill effluents, RFAs impact pulp and paper production in other important ways.

RFAs are a major component of pitch, a highly adhesive compound that can be deposited onto paper during the papermaking process. Pitch deposition leads to decreased runnability of the paper machines. The non-white colour of pitch damages the appearance of the final paper product.

RFAs also form a major component of tall oil, a valuable by-product of kraft pulping. Tall oil is a recovery product from RFA soap skimmings released during pulping. The skimmings are purified by distillation to form tall oil. Tall oil is used in printing, coating, paper sizing, adhesives, rubber manufacture, soap and detergent manufacture, mineral flotation, asphalt and cement manufacture, lubrication, and road oiling. Depending on the degree of refining, tall oil contains between 80-95% RFAs by weight (McSweeney *et al.* 1987).

Rosin is a naturally occurring resin that consists mainly of abietic acid. Rosin is used extensively in the varnish and plastic industries (Swann, 1951).

1.4 Non-Colourimetric Assays for RFAs

1.4.1 GC Assays

GC assays generally consist of 4 principal steps: 1) separation of RFAs from other components (e.g. using solvent extraction, or an extraction filter membrane); 2) concentration of the RFA-containing phase; 3) derivatization of RFAs, usually to their corresponding methyl esters; and 4) analysis by GC. Operation of the gas chromatograph involves many variables, most notably the column dimensions, stationary phase material, type and flowrate of carrier gas, and static and ramp temperatures. All GC assays give characteristic peaks for RFAs and are capable of detecting RFAs in concentrations of less than 1 ppb. Figure 4 is a sample chromatogram given by the procedure outlined by Voss and Rapsomatiotis (1985). (See Section 4.5.3.) Table 2 summarizes the operating conditions for published GC-based RFA assays.

1.4.2 GC-MS Assays

GC-MS assays are almost identical to the GC assays discussed above, except for the additional mass spectroscopy (MS) unit that allows for molecular weight determination. Table 3 provides operating conditions for a sampling of GC-MS assays.

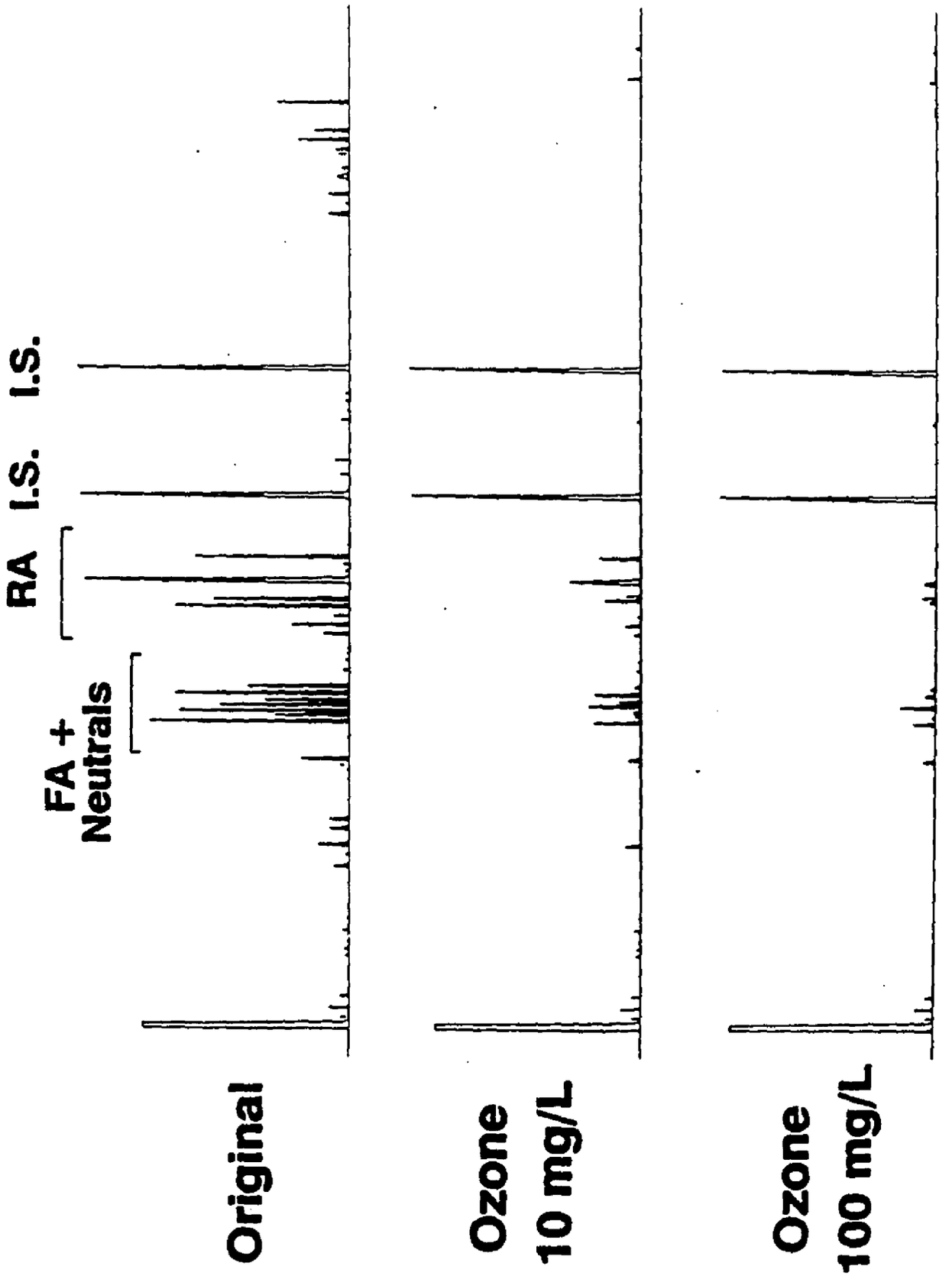


Figure 4. Sample GC Chromatogram of CTMP effluent, before and after ozonation (Roy-Arcand, Archibald, & Méthot, 1994). JB = juvabione. I.S. = internal standard.

Reference	Separation Technique	Concentration Technique	Derivatization Technique	GC Stationary Phase	GC Carrier Gas	Column Temp.
Zinkel *et al* (1968)	Purified RFA standards	None	Formation of trimethylsilyl esters w/ hexamethyldisilazane and trimethylsilyl chloride	Any nonpolar column is suitable	Helium	260-270°C
Bardyshev & Bulgakov (1975)	TLC to remove non-RFAs	None	Methylation w/ diazomethane	Brick / DEGS	Electrolytic Hydrogen	N/A
Zinkel & Engler (1977)	Purified RFA standards	None	Methylation w/ diazomethane, dialkyl acetals of dimethylformamide	Chromosorb W-AW	Helium	200-230°C
McMahon (1980)	Extraction w/ pet. ether / acetone / methanol	Evaporation	Methylation w/ diazomethane	10% SP-2310 on Chromosorb W-AW	Helium	160-230°C
Dorris *et al* (1982)	Tall oil solution fractionated by TLC	None	Methylation w/ diazomethane	10% OV-1 on Chromosorb W-AW	Helium	260°C
Richardson & Bloom (1982)	Adsorption on XAD-2 resin, elution w/ DCM or diethyl ether	Dehydration, evaporation	Methylation w/ diazomethane	Apolar 10CP, SP-2340	Hydrogen	160-250°C
Voss & Rapsomatiotis (1985)	Extraction w/ MTBE	Evaporation	Methylation w/ diazomethane	OV-17	Helium	150-260°C
NCASI (1985)	Extraction w/ diethyl ether	Evaporation	Ethylation w/ diisopropyl - ethylamine in DCM	Fused silica DB-5	Helium	280°C
Gref (1988)	Purified RFA standards	None	None	Non-polar fused-silica	Hydrogen	210 or 230°C

Table 2. Summary of Sample GC Assays for RFAs.

Reference	Separation Technique	Concentration Technique	Derivatization Technique	GC Stationary Phase	GC Carrier Gas	Column Temp.
Keith (1976)	Extraction w/ chloroform	Evaporation	Methylation w/ MethElute	Carbowax / terephthalic acid	Helium	100-200°C
Vine (1980)	None	None	Purified FA methyl ester standards	SE 30	Helium	190-230°C
Turoski *et al* (1981)	Extraction w/ DCM	Evaporation	Methylation w/ diazomethane	SP-2340	Helium	120-250°C
Mayr *et al* (1982)	Pure RFA and rosin standards	None	Methylation w/ diazomethane	SE-30	Hydrogen	250-270°C
Turner & Wallin (1985)	Extraction w/ DCM	Evaporation	Methylation w/ N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA)	Fused silica	Air	N/A
Lee *et al* (1990)	Extraction w/ MTBE	Evaporation	Conversion to pentafluorobenzyl esters	DB-17, DB-5	Helium	210-290°C
Nimz & Morgan (1993)	Extraction w/ ethyl acetate	None	On-line conversion to trimethylsilyl esters w/ bis(trimethylsilyl) trifluoroacetamide (BSTFA)	DB-1701	Helium	50-300°C

Table 3. Summary of Sample GC-MS Assays for RFAs.

1.4.3 HPLC Assays

Lam and Grushka (1978) developed a method for the fluorescence labelling of FAs with 4-bromomethyl-7-methoxy-coumarin (Br-Mmc) using 18-crown-6 ether as a catalyst. The FA derivatives were separated on a reversed-phase HPLC system. The fluorescence of the derivatives were obtained in either methanol/water or acetonitrile. Detection limits for this assay ranged from 9 to 90 pmole. This procedure was later improved by Lloyd (1979), who recommended the use of a parameter he dubbed intrinsic fluorescence sensitivity to compare fluorescence variations with solvents.

Tsuchiya *et al* (1982) modified this assay by replacing Br-Mmc with 4-bromomethyl-7-acetoxycoumarin (Br-Mac) as the fluorescence reagent. Because the fluorescent derivatives were indifferent to the HPLC solvent used, a gradient elution technique was added. The derivatives were separated on a reversed-phase HPLC system. Detection limits were in the low femto mole range.

Farinotti *et al* (1983) identified 4-bromomethyl-6,7-dimethoxycoumarin (Br-DMC) as a superior fluorescence labelling agent, giving both quantum yields and fluorescence sensitivity 50% greater than Br-Mmc. FAs were detectable in picomole amounts.

Another HPLC technique using 9-bromomethylacridine (Br-MAC) as the fluorophore was automated for on-line analysis of blood plasma by van der Horst *et al* (1990). The detection limit was about 30 nM, and the precision was ± 3%.

An HPLC technique for a quantitative analysis of total RAs in effluent was developed by Richardson *et al* (1992). The RAs were extracted from effluent by

passage through C_{18} solid-phase cartridges, and reacted with fluorophores Br-Mmc and Br-Mac in the presence of potassium carbonate. The Mmc esters were detected by UV absorption at 318 nm; the Mac esters were detected by fluorescence spectrophotometry. Detection limits were reported as low as 1 µg/L.

1.4.4 Miscellaneous Assays

Ruus (1965) developed a wet chemistry technique to determine RAs in waste water from forest product industries. The procedure involved the extraction of an acidified effluent sample with petroleum ether, vacuum evaporation of the solvent phase, solution of the RAs in acetone, and addition of cyclohexylamine to form an amine salt precipitate. The weight of precipitate formed was directly related to total RA concentration. Some FAs, excluding oleic and linoleic acids, were also detected by this method, which was accurate to ± 3-4%.

Dünges (1977) developed a thin-layer chromatographic (TLC) assay for FAs that used Br-Mmc as the fluorescence labelling agent, in the same way Br-Mmc was used in HPLC assays (See Section 1.4.3). He was able to measure picomole amounts of fluorescent FA esters.

Li *et al* (1994) developed an immunoassay for detecting one specific RA, dehydroabietic acid (DeAB), in pulp mill effluents. An amine derivative of DeAB was conjugated with carrier proteins to form both a means of immunization for rabbit specimens and a coating antigen. An enzyme linked immunosorbent assay using a biotin-streptavidin system was developed with a 50% inhibition concentration (IC_{50}) of

20.2 ppb and a detection limit of 1.9 ppb. Optimized conditions for this assay have not yet been reported.

Current work by D. Leclerc of PAPRICAN's Vancouver laboratory is focused on the determination of RFAs with Fourier transform infrared analysis (FT-IR) (Leclerc, 1993). The assay would involve extraction of RFAs using an organic solvent, concentration of the solvent phase, and FT-IR analysis. Preliminary results are promising, and publication of a complete procedure is forthcoming.

1.5 Colourimetric Assays

1.5.1 Colourimetric Assays for Fatty Acid Soaps

Ayers (1956) developed a colourimetric assay for the estimation of higher fatty acids (i.e. C_7-C_{18}). Aqueous solutions of potassium soaps were mixed with either copper nitrate or cobalt nitrate to form a coloured precipitate. Copper soap precipitates were blue in colour; cobalt soap precipitates were pink. The precipitates were extracted from the solutions with chloroform. The absorbance of the chloroform phase was measured at 675 nm for copper soaps, 525 nm for cobalt soaps.

Ayers reported an interference caused by the concentration of NaCl or KCl in the soap solution. She ruled out the possibility that the presence of chloride increased the solubility of metallic soaps in water, but was unable to identify the nature of the chloride ion interference.

Iwayama (1959) improved on Ayers' assay by replacing the copper or cobalt nitrate solution with copper-triethanolamine reagent. Iwayama was able to apply this

assay to determine FAs in fatty oils and non-ionic surface-active agents.

1.5.2 Colourimetric Assays for Surface-active Anions

1.5.2.1 Longwell and Maniece

Longwell and Maniece (1955) developed an assay for the determination of anionic detergents (e.g. alkylaryl sulphonates, lauryl sulphates) in sewage, sewage effluents, and river waters. The surface-active anions were extracted from the samples (pH 10.0) with chloroform. The chloroform phase was washed with a neutral 350 mg/L methylene blue (MB) solution, followed by an acidic MB solution, to remove interferences caused by salts. The absorbance of the resulting blue RFA-anion complexes in the chloroform phase was measured at 650 nm.

Longwell and Maniece made several improvements over an earlier, similar assay developed by Degens *et al* (1953). They performed their solvent extraction at an alkaline pH, as opposed to the acidic pH reported by Degens *et al*. They also included a washing step with an acidic MB solution.

1.5.2.2 Tomlinson and Sebba

Tomlinson and Sebba (1955) developed a method to measure surfactant ions by ion flotation. An aqueous mineral flotation liquor was added to a 300 mg/L aqueous solution of crystal violet dye in an ion flotation cell. Air was gently bubbled at the bottom of the cell. The bubbles buoyed surfactant-dye complexes to the surface, forming a froth. The absorbance of the crystal violet was measured at 590 nm before

and after flotation. The colour lost by the dye solution was directly proportional to the surfactant ion concentration in the sample. Potassium oleate was used as the standard.

Tomlinson and Sebba recognized that other cationic dyes would likely outperform crystal violet in this assay. Still, they identified two essential criteria for such an organic dye: 1) the dye must have no surface reactivity 2) the pH chosen must allow the dye to exist in cationic form and the surfactant to exist in anionic form. They further recommended lower dye concentrations to increase assay sensitivity.

1.5.2.3 Gregory

Gregory (1966) developed an assay for residual anionic surface-active reagents in mineral flotation liquors. Liquors were mixed with an alkaline 25 g/L solution of cupric triethylenetetraamine complex to form an adduct that was extracted into an isobutanol-cyclohexane mixture. The absorbance of the organic phase at 435 nm was directly related to total surfactant ion concentration. This assay was sensitive to 0.2 mg/L of long chain (i.e. C_{14}-C_{22}) carboxylates and anionic non-soapy surface-active reagents.

Gregory recommended the addition of EDTA to remove dissolved cations that could interfere with the assay by precipitating carboxylate soaps.

1.5.3 Cationic Dye Assays for RFAs

Cationic dye assays for RFAs operate on the identical principles used in the assays for surface-active anions discussed in Section 1.5.2.

1.5.3.1 Copper Acetate Assay (B. Sitholé)

The copper acetate assay (Sitholé, 1993) uses the cupric (Cu^{2+}) ion to complex with the carboxylic acid groups of RFAs. RFAs are extracted from effluent (pH 9.0) using an organic solvent, methyl-t-butyl ether (MTBE), which preferentially extracts RFAs. The solvent is concentrated from 100 mL to 1 mL in a rotary evaporator under a partial vacuum, a time-consuming step made necessary by the relatively low extinction coefficient of the RFA-copper complex. The concentrate is mixed with aqueous cupric acetate solution, and the blue-coloured complexes are extracted into hexane. The absorbance of the hexane phase at 680 nm is directly proportional to the total RFA concentration in the effluent.

This assay may be applied to other pulp and paper materials, such as pitch deposits, wood chip extracts, and whitewater.

1.5.3.2 Methylene Blue Assay (as developed by B. Dawe, Dow Forestry Products, Ste. Catharine's, Ontario)

The methylene blue (MB) assay uses this organic cationic dye to form a complex with the carboxylic acid groups of RFAs. RFAs in effluent are mixed with a poorly polar organic solvent, dichloromethane (DCM), and an aqueous MB solution. The highly photosensitive RFA-MB complexes formed (See Appendix B.6.) are then extracted into the DCM phase. The light absorbance of this DCM phase at 655 nm (i.e. the absorbance maximum for RFA-MB complexes) is linearly related to the total RFA concentration in the effluent.

It is important to note that this procedure did not adjust the pH of the effluent, nor concentrate the solvent phase. This procedure was originally developed by B. Dawe for in-house analysis of highly concentrated (i.e. 1000+ mg/L) RFA solutions. Since only microlitres of such concentrated effluents were required for these analyses, other effluent components did not noticeably affect the performance of the assay. The use of the assay developed by Dawe was never successfully broadened to include real mill effluents.

1.5.4 Carpenter's RA Assay

Carpenter's assay for resin acids (Carpenter, 1965) involves the spectrophotometric measurement of an unstable, coloured mixed anhydride intermediate. The intermediate, pink to violet in colour, is the product of a highly exothermic reaction between abietic acid and acetic anhydride in an acidic medium. Most resin acids isomerize to abietic acid at low pH (NCASI, 1986). The acid-soluble coloured compound is highly unstable. Its absorbance at 525 nm must be measured immediately after colour formation. Carpenter reported a linear relationship between absorbance and an abietic acid content of 0-6 mg abietic acid. This method was later automated using flow injection technology by Kester *et al* (1992).

2. Rationale for Project

Effluent emission regulations have become increasingly stringent on pulp and paper mills in recent years. Secondary biological treatment facilities are being constructed at nearly all pulp and paper mills across Canada to produce effluents with zero acute toxicity, among other characteristics, prior to discharge. Toxic breakthroughs resulting from process or biotreatment upsets pose even greater challenges to mills to maintain environmental compliance.

None of the current methods to monitor effluent toxicity is sufficiently simple, rapid, and inexpensive for routine monitoring at mill sites, including standard 96-hour tests using rainbow trout or water fleas (e.g. *Daphnia magna*). Researchers have identified a strong, direct relationship between the acute toxicity of TMP and CTMP combined and mill stream effluents and their total RFA content (See Section 1.2).

Current analytical techniques for measuring RFAs are expensive, complicated, and time-consuming, requiring highly trained technicians and sophisticated equipment. Although these assays provide results in less time than toxicity testing, the procedures are long and labour-intensive. A skilled technician takes one day to process 6 to 8 samples using an established GC technique.

An assay is desirable for the daily monitoring of RFA concentration as a measure of acute toxicity in mill effluents. The assay would be used to test effluents before and after secondary treatment, to ensure good system performance and environmental compliance and to identify and locate RFA-related toxic breakthroughs. This assay would be performed on site by mill personnel.

3. Objectives

The objectives of this research were:

1) To develop and optimize an assay for quantifying RFAs in mill effluent that satisfied the following criteria:
   a) 1 mg/L of total RFA (i.e. less than 96-hour EC_{50} of most RFAs in certain aquatic species) can be quantified (Leach and Thakore, 1976)
   b) would not require expensive, sophisticated equipment
   c) less time-consuming than current GC assays
   d) useable at mill sites
2) To compare the performance of this assay against an accepted GC assay by applying both to a wide variety of mill effluents
3) To correlate RFA concentration as measured by this newly developed assay with acute effluent toxicity as measured by the Microtox microbial acute toxicity assay
4) To develop a standard procedure useable at the mill

4. Materials and Methods

4.1 Chemicals and Reagents

Pure RAs (98%+) were purchased from Helix Biotech (Vancouver). Pure FAs and 98+% pure methylene blue crystals were from Aldrich Chemicals (Milwaukee). All RFAs were used without further purification. All organic solvents were HPLC grade and supplied by Fisher Scientific (Montreal): dichloromethane, isopropyl alcohol, methyl-t-butyl ether, hexane. Pure turpentine was supplied by Recochem (Montreal). All other chemicals were supplied by Anachemia (Montreal).

4.2 Mill Effluent Sampling and Handling

Mill effluent samples were sent by member companies to PAPRICAN Pointe-Claire in 20 L pails. Upon reception, samples were immediately transferred to disposable centrifuge tubes and frozen at -20°C, with no pre-treatment. Assay performance was shown to be unaffected by the freezing of samples. However, RFA degradation was observed in some effluents kept for over three days at 4°C. All unfrozen effluent samples were used within this critical time period.

4.3 Preparation of Methylene Blue (MB) Reagent

Forty mg of 98+% MB crystals and 10 g of sodium bicarbonate were dissolved in 1 L of distilled water. The pH of the solution was adjusted to 8.15 by the addition of 2 M NaOH or 2 N H_2SO_4; generally no pH adjustment was necessary. The solution was filtered through AAWP Millipore filter paper (0.45 μm pore size).

To a chilled 1 L separatory funnel were added 500 mL MB solution and 500 mL chilled DCM (boiling point of DCM = 40°C). The funnel was agitated vigorously for 1 minute, then settled for 4 minutes. The shaking/settling sequence was performed three more times. The final settling time was increased to 9 minutes.

As the DCM warmed to room temperature, the highly volatile solvent would cause a pressure build-up in the separatory funnel. Venting via the petcock of the inverted funnel was insufficient in such cases. The funnel would be refrigerated immediately prior to further handling at room temperature.

The 25-minute shaking/settling sequence was performed two additional times, each with 500 mL of fresh, chilled DCM. The DCM layer was withdrawn and discarded at the end of every sequence. The MB reagent was stored in an amber glass bottle, tightly sealed, and refrigerated in the dark.

A coloured background test was performed on this MB solution. In a 16 x 160 mm culture tube, 3 mL MB reagent and 3 mL DCM were vortex mixed for 45 s, then settled for 4 min. This shaking/settling sequence was repeated. The absorbance of the DCM phase was measured at 655 nm. If the total absorbance of the solution was less than 0.250 A.U. (i.e. the absorbance plateau) the solution was deemed acceptable. If not, an additional extraction of the MB reagent with DCM was performed.

The MB reagent had a shelf life of approximately four months if exposure to air and light were minimized by using amber glass jars and storage at 4°C. Use of expired MB reagent resulted in increased background colour. Coloured background controls were run with each experiment.

4.4 Preparation of Stock RFA Solutions

4.4.1 Preparation of RFA Solutions in DCM

Pure RFAs were weighed into a tared 4 mL screw-top glass vial with Teflon liner, and sufficient chilled DCM (approx. 3-3.5 mL) was added to produce known concentrations between 700-1500 mg/L for RAs, 3500-9000 mg/L for FAs. These stock solutions were stored in the dark at 4°C. Stock solutions were diluted to appropriate sample concentrations with chilled DCM.

4.4.2 Preparation of Aqueous RFA Solutions

Stock solutions of RFAs were made by dissolving pure RFA into isopropyl alcohol, producing concentrations of between 100 and 300 mg/L. For use as standards, these stock solutions were diluted in 20 mM tris base buffer solution to values between 0.5 and 100 mg/L. The volume of isopropyl alcohol added did not exceed 5% of total volume and was shown to have no effect on total background.

4.5 Use of Existing Assays

4.5.1 Copper Acetate Assay

Fifty ml of effluent (pH 9.0) were extracted twice with 50 mL methyl-t-butyl ether (MTBE). The MTBE phase was concentrated to 1 mL with a rotary evaporator. Two mL of a 5% cupric acetate solution and 5 mL hexane were added and shaken vigourously for 1 minute. The absorbance of the hexane layer was measured at 680 nm.

4.5.2 Carpenter's RA Assay (Carpenter, 1965)

A 1 L sample of effluent was acidified with 10 mL of 50% sulfuric acid. The effluent was extracted three times with 35 mL of petroleum ether. The organic phase was evaporated to dryness in a rotary evaporator, then cooled. The organic solutes were re-constituted with 3 separate 10 mL aliquots of chloroform and transferred to a separatory funnel.

Ten mL of 65% sulfuric acid was added and shaken. Two mL of acetic anhydride were added, while the funnel was cooled with running water. The acid phase, pink or violet in colour when RFAs were present in the effluent, was separated. The colour formation step was repeated until no further colour was produced. The pooled acid phase was filtered through a Millipore filter (pore size 0.45 μm) and topped to 50 mL with 65% sulfuric acid. Its absorbance was measured at 525 nm.

4.5.3 GC Assay (Voss and Rapsomatiotis, 1985)

Fifty mL of effluent (pH 9.0) were extracted twice with 50 mL methyl-t-butyl ether (MTBE). The MTBE phase was concentrated to 0.3 mL with a rotary evaporator. The concentrated extract was methylated using diazomethane under a steady stream of nitrogen. The extract was then analyzed by a split capillary 30m DB-1 fused silica GC column with a flame ionization detector. The injector and detector were at 250°C and 290°C, respectively. The oven was at 150°C for 6 minutes, then ramped 10°C/min to 200°C and 2°C/min to 280°C. Heneicosanoic acid and tricosanoic acid were used as internal standards.

4.5.4 Modified Microtox Assay (15-minute)

Acute effluent toxicity was measured using the standard 15-min Microtox assay with some modifications (Roy-Arcand, Archibald, & Méthot, 1994). The standard luminescence assay procedure given by Microbics Corporation (Carlsbad, Ca.) was followed, except that the Microtox reagent (i.e. suspensions of *Photobacterium phosphoreum)* was reconstituted and diluted using 10 mM tris buffer (pH 7.3) with 2% NaCl. A phenol solution was used as a positive toxicity control.

4.6 Washing Glassware

Glassware was soaked in a warm 2 g/L solution of commercially available sodium carbonate / metasilicate (i.e. Alcojet brand) detergent for a minimum of 30 min. Persistent MB stains were removed with a methanol or 1 N HCl rinse. Cuvettes were rinsed sequentially with warm tap water, deionized water, methanol, distilled/deionized water, and wash grade acetone.

5.0 Results and Discussion: Development of MB Assay

5.1 Survey of Existing Colourimetric RFA Assays

5.1.1 Copper Acetate Assay

The copper acetate assay (Sitholé, 1993), developed by Bruce Sitholé of the Pulp and Paper Research Institute of Canada (PAPRICAN), satisfies most of the criteria outlined in Section 3. A calibration curve was constructed using abietic acid standard solutions (Figure 5), as opposed to the oleic acid used by Sitholé (1993).

The copper acetate assay was reproducible, but offered minimal time savings over the conventional GC assay and was unsatisfactory in terms of the lower detectability limit for effluents. (N.B. The copper acetate assay was developed for a wide variety of pulp and paper matrices, including pitch and whitewater.) The bottleneck step is the concentration of the organic extract phase by rotary evaporation. This step requires a rotary evaporator and between 20 minutes to several hours per sample. However, no derivatization or GC analysis was required. The lower detectability limit of 3-4 mg/L is relatively high. While the sensitivity could theoretically be increased by the concentration of a greater volume of extracting solvent, this step would add greatly to the total sample processing time.

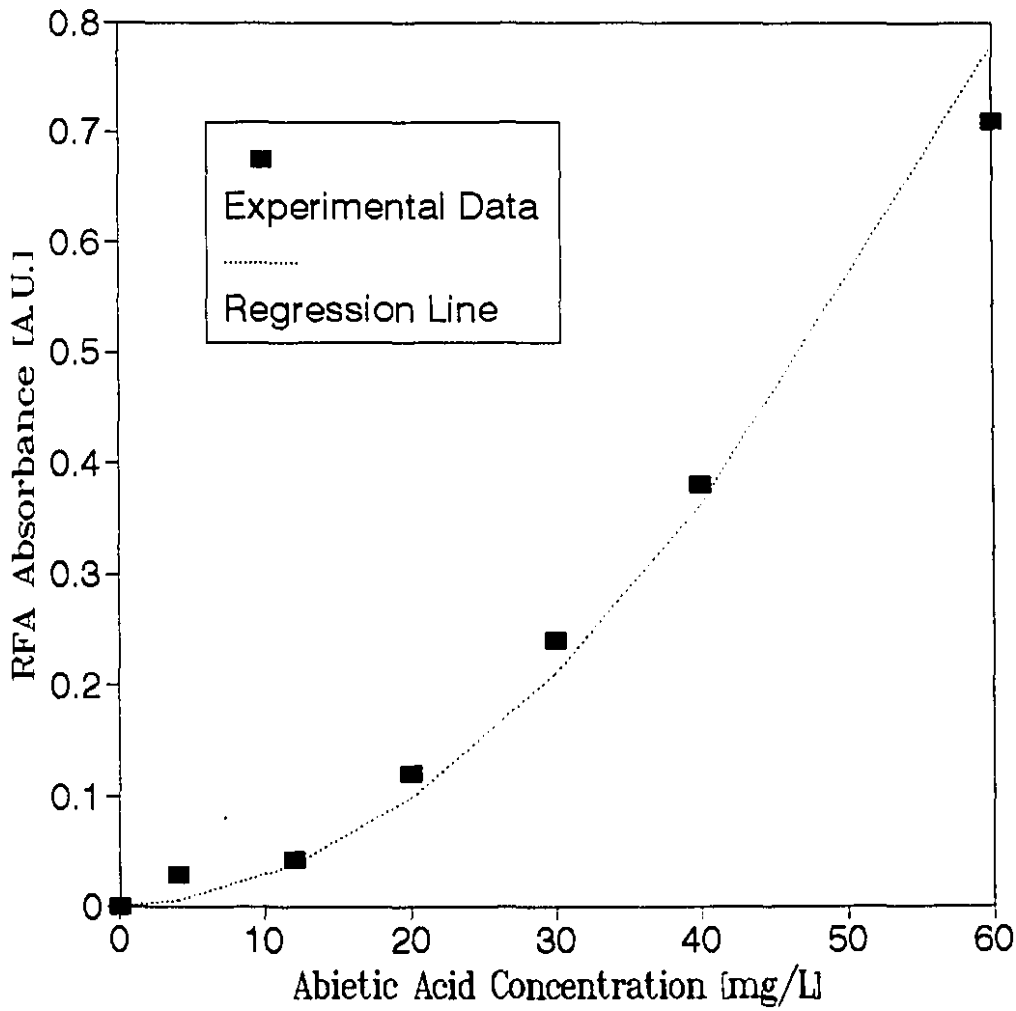


Figure 5. Calibration response curve for copper acetate assay using aqueous abietic acid standards. A regression curve has been fit for the range of 0-60 mg/L abietic acid (r^2 = 0.994).

5.1.2 Carpenter's RA Assay

Carpenter's RA assay (Carpenter, 1965) was found to be a reliable method for the quantification of RAs in effluent, but it offered little time savings over the conventional GC assay. The procedure was even more labour-intensive than the GC assay, and its highly exothermic complex-forming reaction poses some hazards. A calibration curve (Figure 6) constructed using abietic acid standards in water and chloroform agrees well with those presented by Carpenter (Carpenter, 1965). The complicated procedure and the inability to detect FAs therefore make it unsuitable for routine in-mill use.

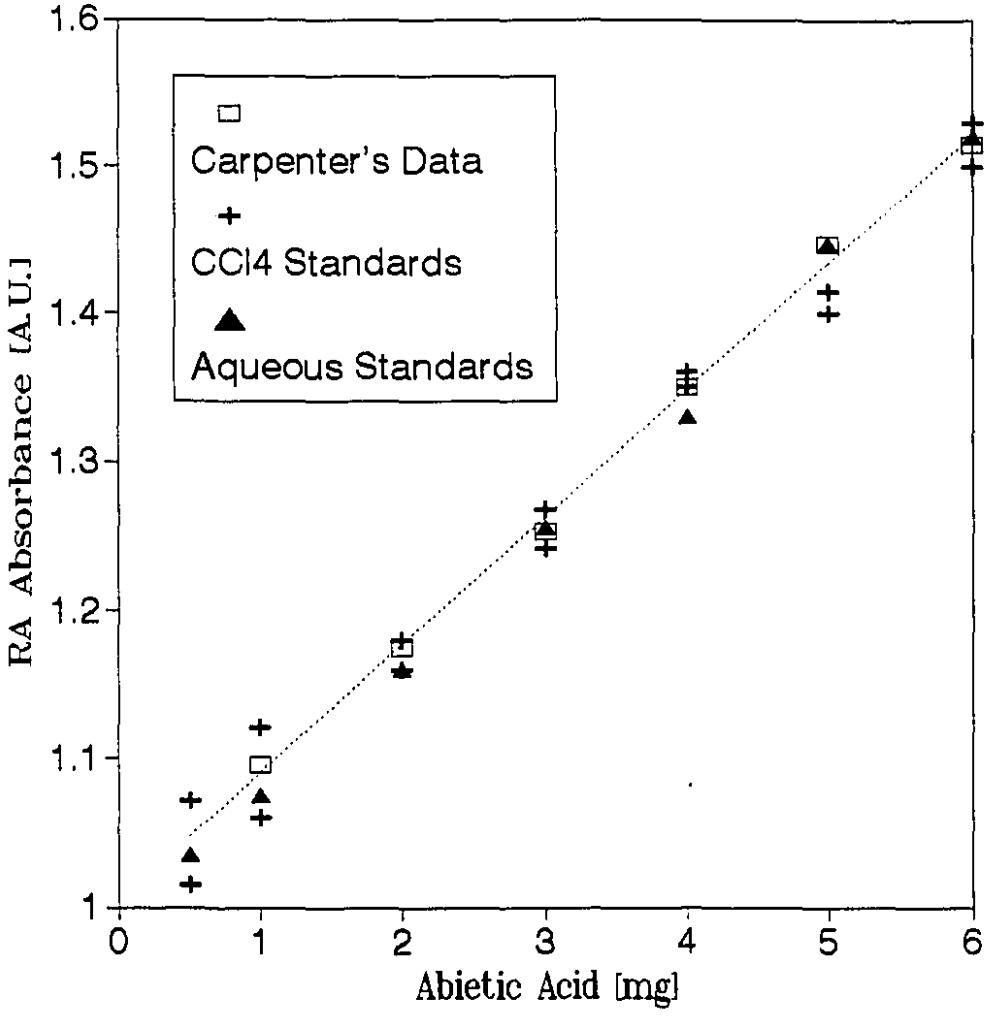


Figure 6. Calibration curves for Carpenter's RA assay, using published (Carpenter, 1965) and experimental data for aqueous abietic acid standards, and experimental data for chloroform abietic acid standards. ($r^2 = 0.998$)

5.1.3 Dawe's Methylene Blue Assay

The assay procedure developed by Dawe (1993) was used on forestry product samples with high RFA concentrations (i.e. in the order of g/L). Because of this high concentration, only microlitres of sample were required. Thus, no pH adjustment was necessary. Although Dawe recommended the extraction of the MB reagent with DCM, work conducted by Dow Forestry Products primarily used non-extracted MB reagent.

5.2 Methylene Blue Assay

The methylene blue assay, originally developed by Dawe, was modified for use on mill effluent, which had less concentrated RFAs than Dawe's samples. The following were used: 3 mL of MB reagent, 3 mL of effluent sample, 6 mL of DCM.

Several problems were noted. Coloured background tests performed on non-extracted MB reagent gave absorbance values exceeding 0.40 A.U. Secondly, Dawe's instructions for the preparation of an aqueous oleic acid solution were incomplete, as saponification of oleic acid occurred at the recommended pH of 11.0, making these solutions inappropriate for testing.

A final problem, the major influence of the effluent samples' NaCl content on the MB and RFA-MB complex formation and partition coefficient actually precludes the use of Dawe's assay on a variety of effluent samples. This problem was not discovered until after the work presented in Sections 5.2.5 was completed, whereupon a major modification to the procedure was introduced. However, this problem does not alter the useful conclusions reached in this section.

5.2.1 Modified Methylene Blue Assay

Dawe had applied the methylene blue assay to a limited set of RFA solutions. However, the objective of the present study was to develop an assay suitable for many diverse types of samples. Thus, a common basis of comparison including the pH was required for all samples. Effluent samples were buffered with tris base buffer, and the pH was set at 8.15. This pH was chosen for the following reasons. First, for the MB cations to complex effectively with RFAs, the carboxyl groups of the RFAs should be in anionic form (Tomlinson & Sebba, 1955) i.e. pH should be above 7.0. Acidic pH values have been shown to catalyze the degradation of DCM, leading to RFA isomerization (NCASI, 1986). Secondly, a pH of 8.15 identical to that of the bicarbonate-buffered MB reagent (See Appendix A.) would mean that changes in sample volume or buffering would not affect the assay pH (Dawe, 1993). Thirdly, Voss and Rapsomatiotis (1985) showed that RFA extractions from effluent using methyl-t-butyl ether, a polar organic solvent similar to dichloromethane, were nearly 100% efficient for pH values between 8.0 and 9.0. Finally, pH values above 10.0 caused undesirable saponification reactions, as noted previously.

MB assay performance is reproducible within a pH range of 7.0 to 9.0 Abietic acid standard solutions assayed within this range gave values of RFA concentration ± 5% of the mean value.

5.2.2 Miniaturized MB Assay

The formation of emulsions between mill effluent samples and DCM creates

problems when attempting to withdraw the organic extract phase (NCASI, 1984). A scaling-down of the assay was attempted to facilitate emulsion breakdown using the high centrifugal force available to an inexpensive microcentrifuge. Forces in excess of 10,000 x g were expected to reduce and compact the emulsion layer, thus facilitating the withdrawal of the DCM layer. Other incentives for miniaturizing the assay were reduced solvent and chemical consumption, the short centrifugation time necessary, and the convenience of disposable microcentrifuge tubes.

A sample of mill effluent was buffered to a final concentration of 20 mM tris base, and the sample adjusted to 8.15 with 2 M NaOH or 2 N H_2SO_4. Three 1.5 mL microcentrifuge tubes were each filled with 0.3 mL of treated sample and 0.3 mL MB reagent. The tubes were shaken in a microcentrifuge tube rack for 1 minute, then 0.6 mL DCM added. The tubes were shaken for 1 min., centrifuged (10,000 x g, 5 min.), and the DCM phases from the 3 tubes withdrawn using a 3 mL plastic syringe and pooled in a 3 mL glass cuvette. The absorbance of this DCM phase was measured at 655 nm.

Two problems were encountered with this assay. Shaking the samples in the microcentrifuge rack resulted in poor, non-uniform mixing. Secondly the plungers of the plastic syringes were attacked by DCM. Both problems led to irreproducible results.

5.2.3 Improved Miniaturized Assay

Larger glass culture tubes were used to improve mixing. To a 16 x 160 mm

tube was added 0.9 mL treated sample, 0.9 mL MB reagent, and 1.8 mL DCM, followed by 5 min. mixing on a vortex. The resulting emulsion was Pasteur-pipetted into three separate 1.5 mL microcentrifuge tubes, which were centrifuged (10,000 x g, 5 min.). The DCM phases were pipetted into a glass cuvette, and the absorbance measured at 655 nm.

The transfer of effluent/DCM emulsions from glass tubes to microcentrifuge tubes resulted in significant losses through adherence to the glass tube. More importantly, in a series of mill effluent samples tested with the modified microcentrifuge method, the emulsion layer was poorly compacted, hindering the withdrawal of the extract phase. Because of these problems, work resumed on a non-miniaturized assay.

5.2.4 Optimized MB Reagent Concentration

The use of a more dilute MB reagent was expected to reduce coloured background. MB cations, which would still be present in considerable excess over RFAs, would complex with RFA cations to the same degree, presumably leaving RFA absorbance unchanged. However, the coloured background, a function of total MB concentration, would be reduced. In theory, the useful operating range of the MB assay would be increased, since the background:RFA absorbance ratio would be lower at low sample RFA concentrations.

To determine the optimum MB concentration, a set of aqueous standards containing between 0 and 20 mg/L abietic acid (AB) were tested with MB reagents of

various concentrations (i.e. 40, 20, and 13.3 mg/L MB) to determine the effect of MB concentration on RFA absorbance. As Figure 7 shows, all MB reagent concentrations below 40 mg/L affected RFA absorbance. Both coloured background and RFA absorbance decreased with decreasing MB concentration. Using MB concentrations below 40 mg/L increased the linear operating range of this assay, but as Table 4 shows, the coloured background:RFA absorbance ratio actually increased with decreasing MB concentration. Therefore, the MB concentration was kept at 40 mg/L.

[AB Acid] (mg/L)	[MB] = 40 mg/L	[MB] = 20 mg/L	[MB] = 13.3 mg/L
2	0.83	1.51	2.19
5	0.48	0.66	0.58
20	0.19	0.22	0.23

Table 4. Effect of MB Reagent Dilution on Coloured Background: RFA Absorbance Ratio

A calibration curve (Figure 8) was constructed for this assay, using aqueous abietic acid solutions as standards. The curve is of the form $x = ky^2$, but is linear for abietic acid concentrations between 0 and 10 mg/L. The correlation coefficient (r^2) for this linear range is 0.989. These results compare very closely to those of Dow Forestry Products (Dawe, personal interview, 1993).

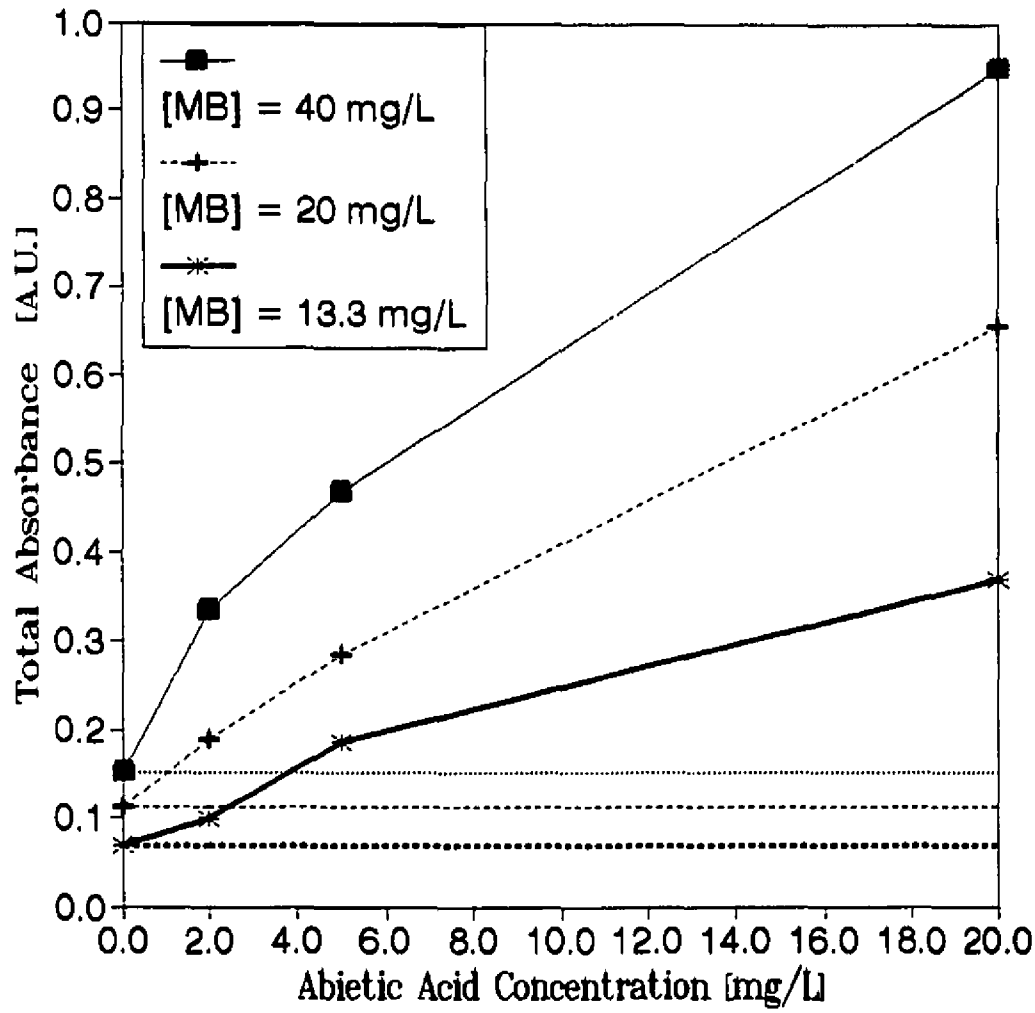


Figure 7. Effect of MB concentration on total absorbance of aqueous abietic acid standards. MB reagent with the original MB concentration of 40 mg/L was diluted. The dashed horizontal lines indicate the coloured background for each dilution of MB reagent.

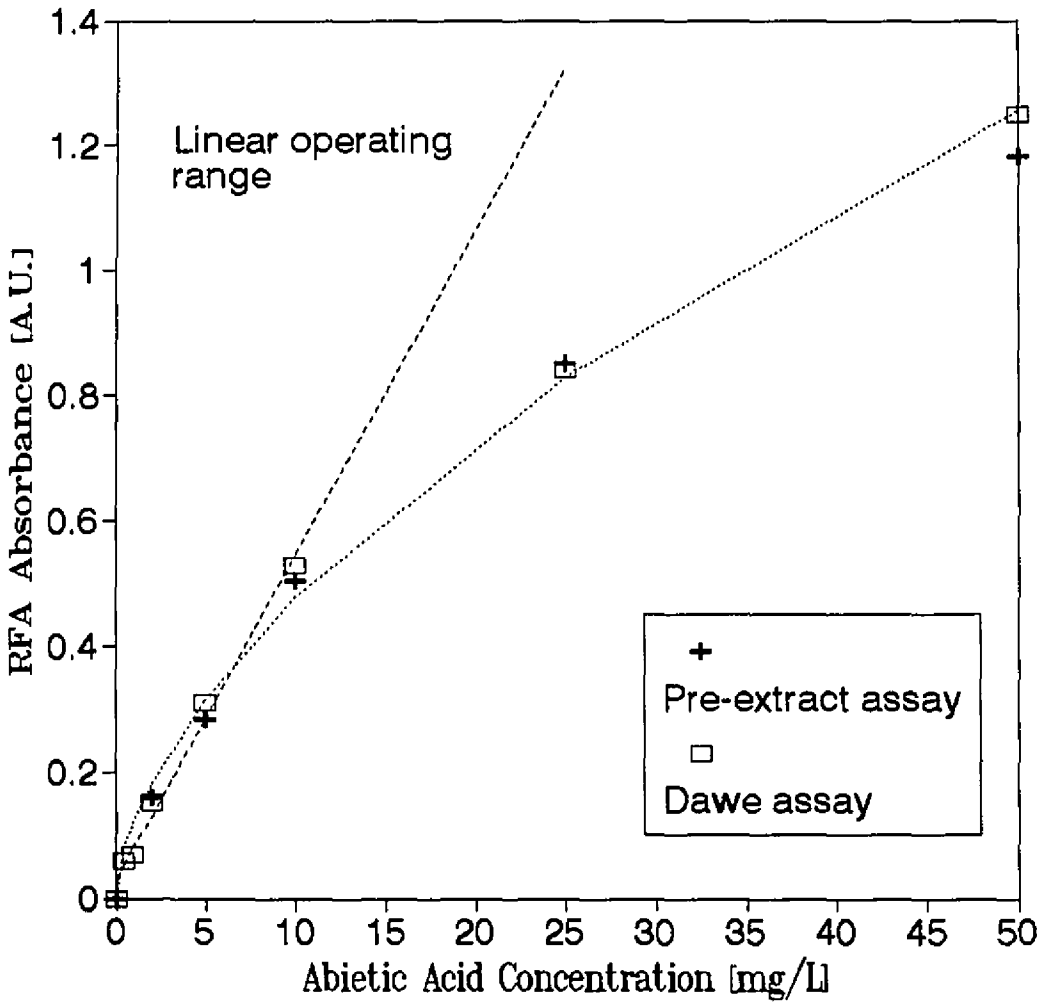


Figure 8. Comparison of Dawe assay and MB pre-extraction assay (Section 6) using abietic acid standards. (The pre-extraction assay involves an extraction of RFAs into DCM prior to mixing with MB reagent.) The data are fit to the equation: $y = 0.12 * x^{0.6}$ ($r^2 = 0.995$). The linear operating range is between 0-10 mg/L abietic acid ($r^2 = 0.989$).

5.2.5 Effect of Effluent NaCl Concentration on Total Absorbance

Calibration curves were constructed using BCMP samples spiked with abietic acid. Two representative curves are shown in Figure 9. The curve for Sample 93/10/14 shows that RFA absorbance was unaffected by spike concentration. The curve for Sample 93/10/01 bears a similar shape to the calibration curve of Figure 8. However, both samples produced values of RFA absorbance much higher than anticipated for unspiked BCMP samples, which contained between 10-20 mg/L total RFA as measured by the GC assay. That range of RFA concentration corresponds to RFA absorbances of 0.50-0.70 A.U. according to Figure 8. Clearly the source of these discrepancies had to be identified.

A method was sought to reduce or eliminate the emulsion layer, suspected as the major cause of the discrepancies. First, three solvents other than DCM were tried: MTBE, hexane, and turpentine. The RFA-MB complex was insoluble in all of these solvents. Chloroform and diethyl ether were rejected due to their highly toxic and explosive nature.

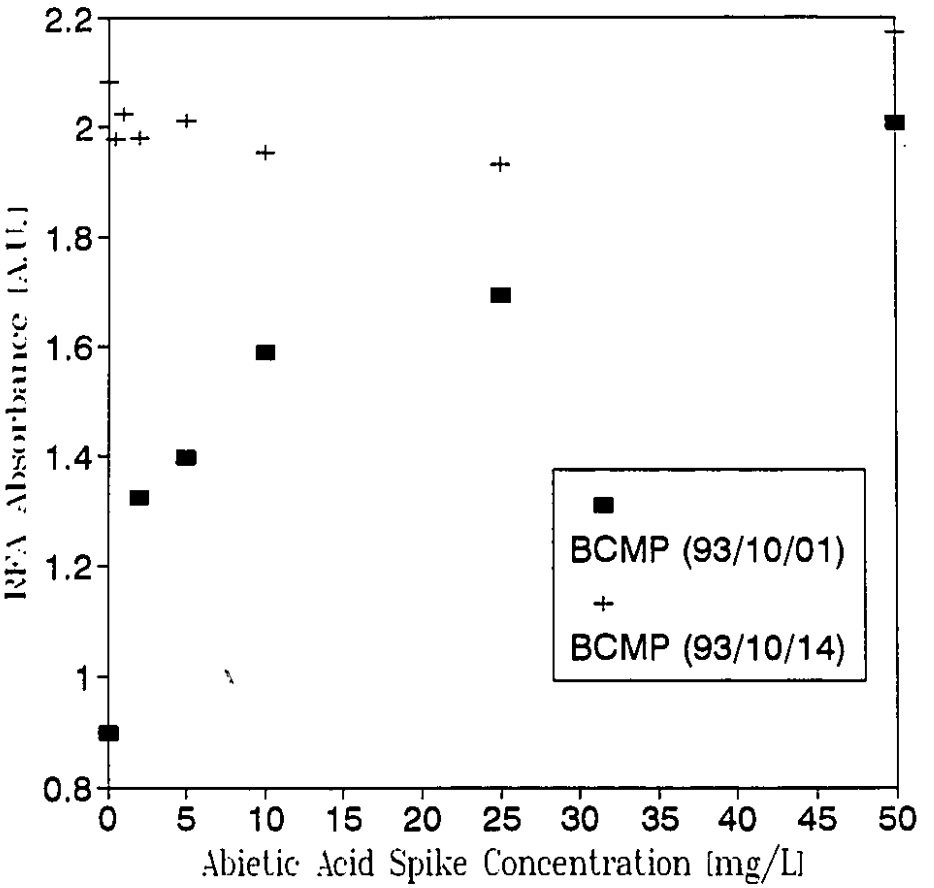


Figure 9. Representative data from MB assay trials on two BCMP effluent samples spiked with abietic acid. An unspiked BCMP sample was expected to contain 20-25 mg/L total RFA.

A common procedure to break emulsions is to add NaCl (M.G. Paice, personal interview, 1993). Before testing mill effluents spiked with NaCl, assays were run on NaCl-spiked synthetic abietic acid solutions and distilled water as emulsion-free controls.

Figure 10 illustrates the relationship between NaCl concentration and RFA absorbance. Results clearly indicate that total absorbance increases with increasing NaCl concentration, even at levels common in mill effluents. Increased colour transfer was likely due to a clustering of excess chloride ions around MB cations, cancelling their net charge and affecting their complexation to the carboxylate groups of the RFAs. The resulting electrically neutral, uncomplexed MB chromophores partitioned more easily into DCM. Because the mill effluents tested typically contained about 50 mg/L NaCl, this "salt effect" previously observed but left unexplained by Ayers (1956) was identified as the cause of the previous inaccurate results.

Methods of desalting the effluent (e.g. desalting columns, extraction filter disks, dialysis techniques) were considered, but all lead to increases in cost, complexity, and RFA losses. Therefore a reversal of steps in the current procedure was tried.

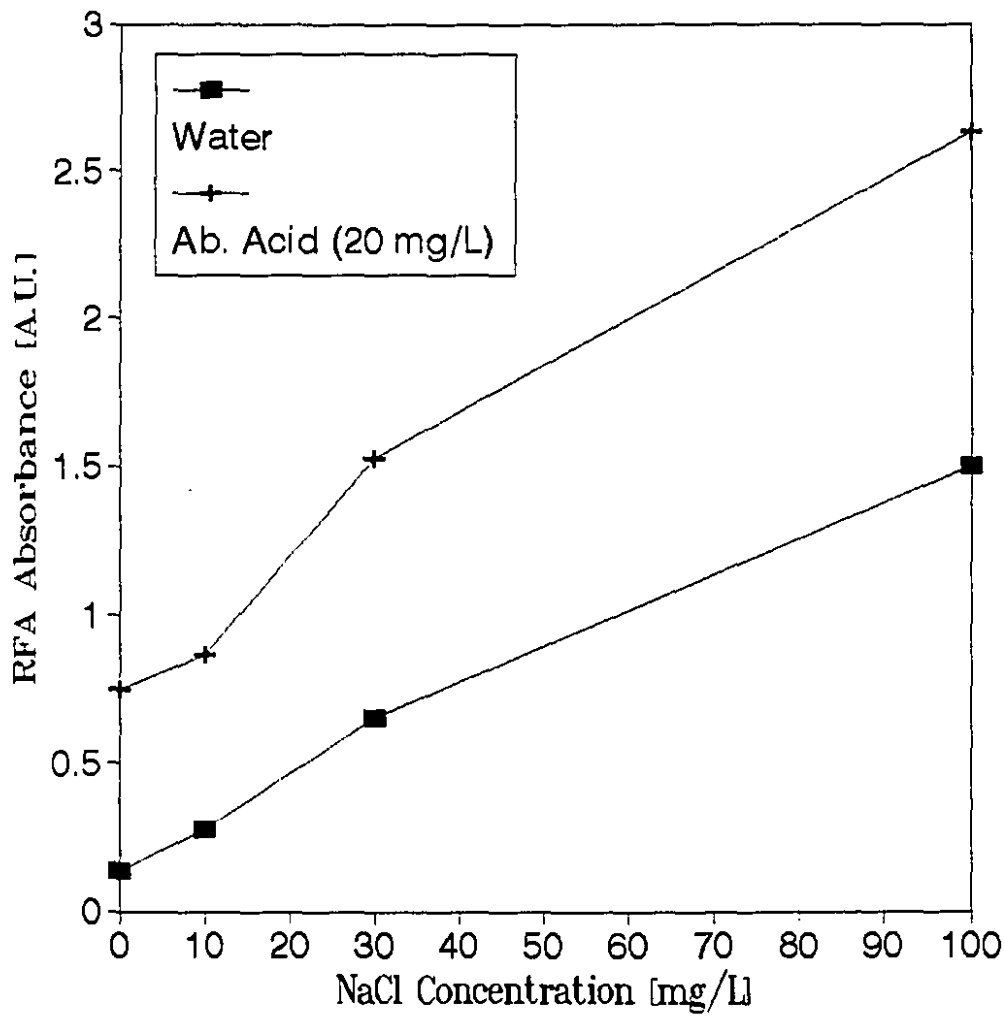


Figure 10. Effect of NaCl concentration on partition coefficient of MB chromophore. Water and 20 mg/L aqueous abietic acid solution were spiked with varying amounts of NaCl.

5.3 Final MB Assay

5.3.1 Eliminating the "Salt Effect"

To prevent the salt effects on complex formation and chromophore extraction, contact between the chloride ions in mill effluent and MB cations was avoided by a reversal of steps in the existing procedure. First 3 mL of mill effluent were extracted with 3 mL of DCM. Then this RFA-bearing DCM phase was mixed with 3 mL of MB reagent to form the RFA-MB complexes. This "pre-extraction" sequence effectively rendered the assay "salt-insensitive." Two samples of BCMP refiner effluent were tested: 1) no NaCl added (52 mg/L already present) 2) 52 mg/L NaCl added (104 mg/L total NaCl). The RFA absorbances of the two samples using the new "pre-extraction" method were both statistically equal to 0.803 A.U. i.e. 20% lower than the value given by the unmodified assay. The salt effect was clearly identified and effectively eliminated by the pre-extraction assay.

The pre-extraction assay was subsequently modified only slightly to arrive at the final procedure (See Section 7.).

5.3.2 Optimized Aqueous-to-Organic Ratios

This "pre-extraction" MB assay used only 3 mL of DCM to extract RFAs, as opposed to the 6 mL mentioned in Section 5.6. The effluent-to-DCM and MB-to-DCM ratios were both doubled, which would presumably result in greater RFA absorbance for a given sample. However, because MB and RFAs were no longer mixed in a common aqueous phase, RFA-MB complex formation was presumably

somewhat less efficient. It was therefore important to determine how these new ratios and the presence of DCM during complex formation affected assay performance.

The MB:DCM ratio (i.e. aqueous:organic ratio) was held constant at 1:1 while the effluent-to-DCM ratio was varied. The effluent-to-DCM ratio of 1:1 was used as a basis for comparison. The theoretical absorbance was defined as the absorbance resulting from a 1:1 extraction multiplied by the effluent-to-DCM ratio. For example, an effluent-to-DCM ratio of 4:1 should result in an absorbance 4 times greater than that from a 1:1 extraction. Figure 11 compares the theoretical absorbance to the actual absorbance.

A high effluent-to-DCM ratio was desirable in order to conserve solvent. Unfortunately, ratios greater than 1.0 produced values of RFA absorbance much greater than 0.8 A.U. (i.e. out of the linear operating range). The actual absorbances were significantly less than the theoretical absorbances, indicating less efficient extractions. Furthermore, emulsion layers were thicker and more troublesome at these higher ratios.

An effluent-to-DCM ratio of 0.33 produced similar values for theoretical and actual absorbance. Using low ratios would expand the linear operating range of the assay beyond 10 mg/L, but this benefit would be negated by the far greater quantities of solvent that would be consumed.

Based on these results, it was decided to keep both the effluent-to-DCM and MB-to-DCM ratios at 1.0.

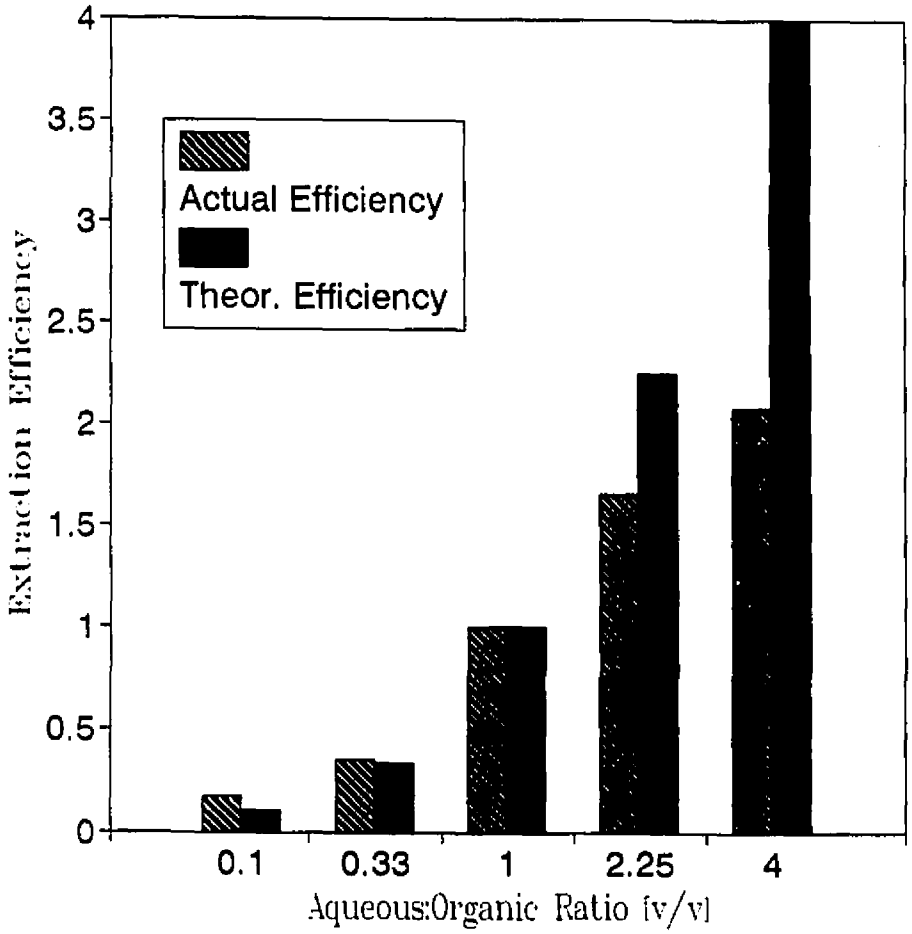


Figure 11. Comparison of actual and theoretical RFA extraction efficiencies for different aqueous-to-organic ratios. BCMP effluent samples were used. A 1:1 ratio was arbitrarily taken as standard (100% efficient).

5.3.3 Optimized Mixing Times

The "pre-extraction" assay involved two stages of mixing: 1) RFA extraction mixing (mill effluent and DCM) 2) RFA-MB complex formation mixing (RFA-rich DCM and MB). Optimum mixing times for each were selected as those times resulting in less than 5% standard deviation for a given set of RFA standards. For RFA extraction, two sequences of 90 s of vortex mixing and 3.5 min. of settling were required. For RFA-MB complex formation, two sequences of 45 s of vortex mixing and 4 min. of settling were needed.

5.3.4 Determination of Linear Operating Range

The linear operating range for this assay had previously been determined as 0 to 10 mg/L total RFA (or 0.0-0.8 A.U. RFA absorbance). This range required confirmation for the "pre-extraction" assay. Another calibration curve (Figure 8) was constructed using aqueous abietic standards. As Figure 8 shows, the "pre-extraction" calibration curve corresponds quite closely with its predecessor.

Although Figure 8 shows the operating range of the Dawe and "pre-extraction" MB assay, its applicability to mill effluents needed to be demonstrated. Effluents contain salts, suspended solids, and surface-active and apolar organic compounds that may affect RFA partition coefficients in DCM.

In Figure 12, a 6 mL sample of BCMP combined effluent was pre-extracted with 6 mL of DCM. The pre-extract was diluted sequentially with DCM and mixed with MB reagent.

Figure 12 shows the relationship between the RFA-MB complex concentration and its absorbance at 655 nm. Although the entire curve is close to linear (for the regressed linear curve, $r^2 = 0.978$), there is an inflection point at an RFA absorbance of about 0.6 A.U. The correlation coefficient (r^2) for the RFA absorbance range of 0.0-0.6 A.U. is 0.992. It should be noted that this curve only demonstrates the linearity of the MB complex concentration and complex absorbance. It does not assess the linearity of DCM extraction of RFAs from effluent. Turner and Wallin (1985) have reported DCM extraction to be linear in the 0-200 mg/L RFA range.

Thus the completely linear operating range for the pre-extraction MB assay, like the earlier Dawe assay, is up to RFA absorbance values of 0.600 A.U. or total absorbance values (i.e. RFA absorbance + background) of less than 0.85 A.U. Therefore, the RFA concentration for easy measurement in full strength effluent samples is 0.5-4.0 mg/L total RFA. More highly concentrated effluent samples should be diluted with distilled water before assay.

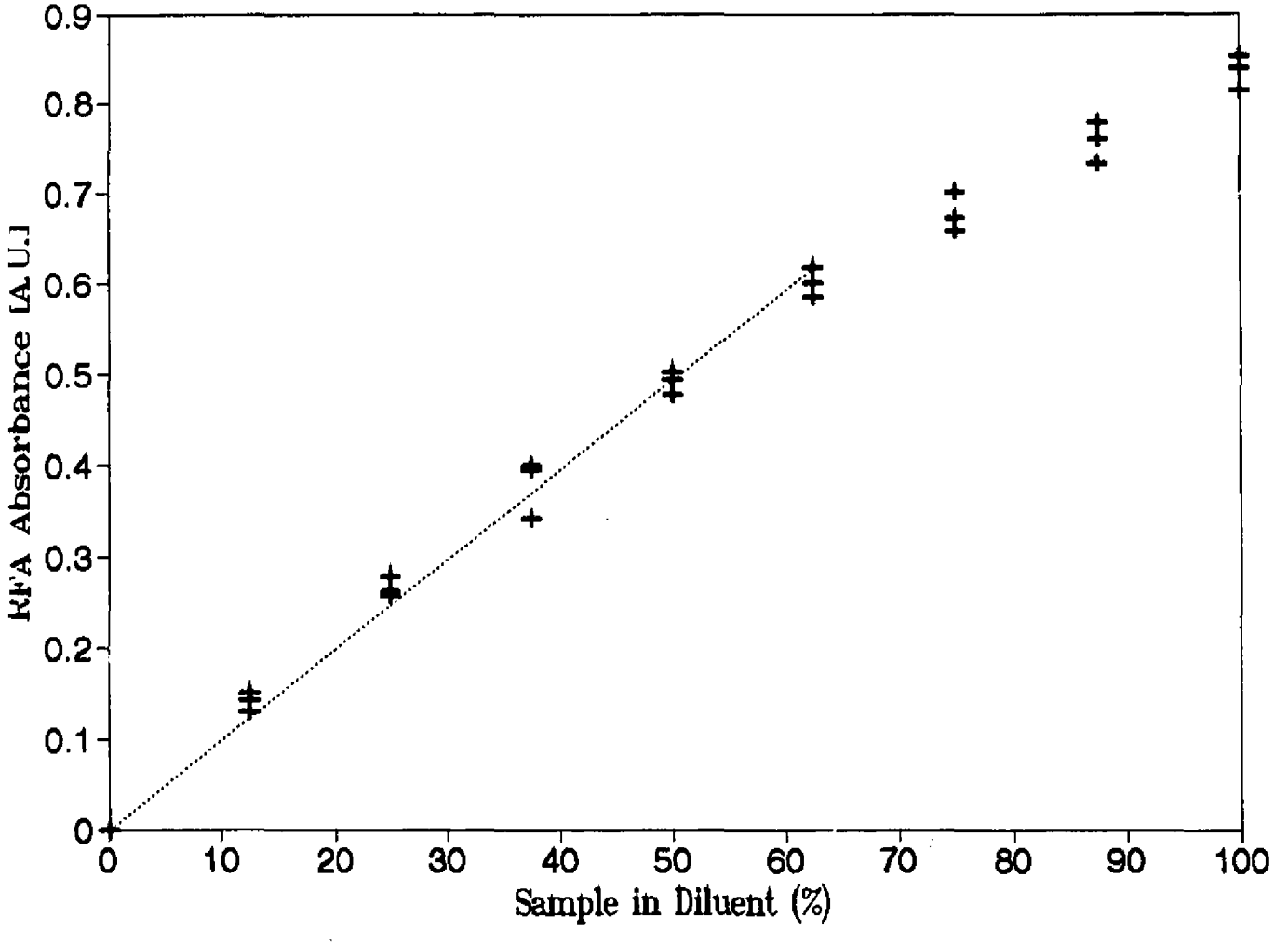


Figure 12 . Linearity of the RFA-MB complex absorbance-concentration relationship, as measured by a Lambda-3 spectrophotometer in the pre-extraction MB assay. Pre-extracts of a BCMP combined effluent sample were diluted in varying amounts of DCM. The total background equalled 0.224 A.U. for these experiments. The relationship is linear for dilution factors of less than 0.7 (r^2 = 0.985).

5.3.5 Determination of Extinction Coefficients

Because individual calibration curves are impractical for the great number and variety of mill effluents, it was decided to correlate RFA absorbance and RFA concentration by measuring the extinction coefficients of individual RFA-MB complexes. Extinction coefficients were expressed in units of A.U. / mM RFA.

Because great variations were expected in the percentages of individual RAs and FAs comprising the total RFAs in different effluents, two things were done:

1. The individual extinction coefficients of the common available RAs and FAs were determined to see how different they were i.e. how much effect different RFA compositions would have on the MB complex extinction coefficient for the total mixture.
2. A general extinction coefficient was calculated, assuming equal quantities of the 9 RFAs measured. This coefficient would permit conversion of the RFA absorbance to mg/L of total RFA.

A set of RFAs was chosen to represent the RFA composition of a typical mechanical pulping effluent. RAs outnumbered FAs six to three in this set. Chlorinated RAs were excluded, as these by-products of bleaching operations are minor and present in only kraft effluents. Juvabiones (JB), toxicants chemically similar to RFAs, were also excluded as they are present mainly in fir species. Individual pure RFAs were dissolved directly into DCM (See Section 4.4.1). Three mL each of RFA-rich DCM and MB reagent were mixed and centrifuged, and the absorbance of the DCM phase measured (655 nm).

Table 5 lists the extinction coefficients for the representative set of RFAs. The arithmetic mean of the extinction coefficients was 45.6 A.U./mM. Table 5 also shows the derivation of the conversion factor from RFA absorbance to total RFA concentration in mg/L, which was calculated to be 6.44. Thus, for undiluted effluent mixed 1:1 with DCM in the "pre-extraction" MB assay, multiplying the OD_{655} (less background) by 6.44 would give the total RFA content of the effluent in mg/L.

RFA	Extinction Coefficient (A.U./mM)	Mol. Wt.
Abietic	36.5 ± 5.9	302.44
Dehydroabietic	33.1 ± 5.9	288.43
Neoabietic	48.1 ± 11.3	302.44
Isopimaric	44.9 ± 9.5	302.44
Levopimaric	40.1 ± 6.1	302.44
Palustric	23.4 ± 4.5	302.44
Oleic	67.3 ± 7.0	282.45
Linoleic	64.5 ± 8.1	280.44
Linolenic	52.3 ± 8.5	278.44
AVG	45.6 ± 7.4	293.55

[RFA] = RFA ABS * $(MW/E.C.)_{AVG}$ = RFA ABS * 6.44 [mg/L]

Table 5. Extinction Coefficients of RFAs.

5.3.6 Determination of Effluent Background

Presumably because of the DCM:water partition coefficient of MB, there is an irreducible water (blank) background, referred to as coloured background. In addition there is an effluent background (i.e. background due to non-RFAs), whose magnitude

had to be determined by performing multiple extractions with DCM on a set of effluent samples. The number of extractions required to reach the background plateau would be incorporated into the final procedure to obtain a quantitative RFA value from effluents.

Three types of effluent were tested. Figure 13 shows absorbances measured over 5 extractions for BCMP refiner effluent, which yielded results representative of all three types of effluent tested. The absorbance measured after the first extraction is the total absorbance. The absorbance plateau measured after the third extraction is termed total background. Total background is the sum of coloured background and effluent background.

A total background plateau was reached for each replicate at the third extraction. Thus, effluent samples must be successively extracted twice with fresh DCM to give a quantitative yield of RFAs. Neither the total absorbance nor the total background plateau was the same for different batches of the same type of effluent. No correlation could be made between effluent type and total background. Thus total backgrounds had to be determined for each sample. Variations between values for total background are presumably due to the amount of emulsion layer solids in the individual replicates. It is difficult to control the amount of emulsion layer solids transferred into each individual effluent sample. This variability will be discussed further in Section 5.4.0

However, the difference between the total absorbance and total background, termed RFA absorbance, was reproducible for all replicates of a given type of effluent.

Thus, regardless of the values of the total absorbance and total background, the RFA absorbance and hence the RFA concentration was still determined in a reproducible fashion.

In total, three extractions are required to run the assay: the first and second for complete RFA recovery and the third for determining total background. It was shown that RFAs were preferentially extracted by DCM in the first two extractions, and that total background reaches a plateau after the third extraction.

In the final procedure, 1.5 mL each of the first and second pre-extracts were pooled. The absorbance of this pooled DCM phase minus the absorbance of the third pre-extract equalled the true RFA absorbance. Because the mass of RFAs extracted in the first two extractions was additive, RFA absorbance was multiplied by 2.08 to account for dilution by both pooling and addition of tris base buffer. Further multiplication by 6.44 (the conversion factor obtained in Table 5) and by the appropriate effluent dilution factor yielded total RFA concentration.

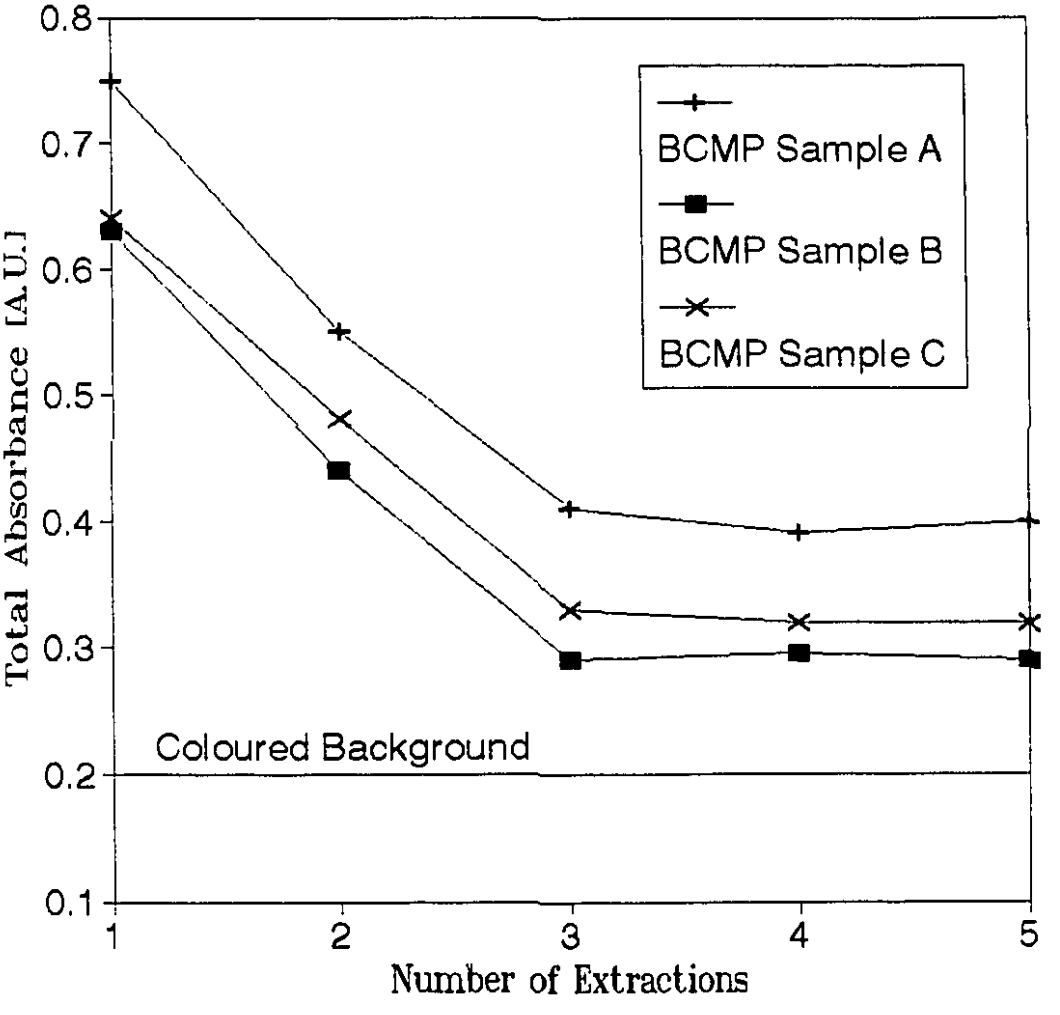


Figure 13. Repeated methylene blue extractions from BCMP refiner effluent using DCM, representative of BCMP, Kraft. and CTMP samples tested. Coloured background of 0.200 A.U. was typical for MB reagent.

5.3.7 Determination of Method Detection Limit

According to standard methods (Greenberg *et al*, 1992), the method detection limit (MDL) is the lowest constituent concentration that, when processed through the complete method, produces a signal with a 99% probability that it is different from a blank. The constituent (i.e. RFA) should be added to the appropriate matrix (i.e. virtually RFA-free effluent) to make a concentration near the estimated MDL. This solution would be assayed 7 times. The mean would be 3.14 s above the blank, where s is the standard deviation of the 7 samples. In this work, the solutions tested were not true replicates of one another (Table 6A); they did however provide a wide variety of effluents to which the MB assay could be applied. Furthermore, although all of these samples contained virtually no RFA as measured by the GC assay and could then be considered as blanks, they were distinguishable in other ways. Each solution was tested only in triplicate, to simulate eventual mill procedures. Because of their low concentrations, the RFA spikes added to the RFA-free matrices were incorporated into the calculations for MDL estimation.

The RFA-free samples included effluents subjected to secondary treatment (e.g. activated sludge, aerated lagoon). Other samples whose RFAs were extracted via extraction filter disks were used. These samples were provided by Harel (1994), whose work examined extraction filter disks and their efficiency in removing RFAs from effluent. Harel showed that Empore SDB (i.e. polystyrene divinyl benzene) filter disks were capable of over 99% RFA removal.

Table 6A gives the RFA concentrations as measured by the MB assay for these

selected samples. (N.B. A glossary of terms is included in Table 6B.) The method detection limit was calculated as function of the standard deviation of the RFA concentrations for each triplicate of data (Greenberg *et al*, 1992):

MDL = Average of Blanks + s where:
Average of Blanks = 0.502 (from Table 6A)
s = Average of S.D. + [Standard Deviation of S.D.*$t_{\alpha/2,\ n-1}/(2n)^{1/2}$]
$t_{\alpha/2,\ n-1}$ = probability factor based on t-distribution for α = 99%
n = number of samples

MDL = 0.502 + [0.0746 + 0.0254*2.65/$(2*14)^{0.5}$] = 0.589 mg/L total RFA

Effluent Sample	[RFA](mg/L)	Standard Deviation
Biotreated BCMP	0.53, 0.41, 0.53	0.0693
Biotreated BCMP	0.39, 0.50, 0.52	0.0700
Biotreated BCMP	0.44, 0.55, 0.51	0.0557
SDB Filtrate (BCMP)	0.40, 0.48, 0.54	0.0702
SDB Filtrate (BCMP)	0.60, 0.51, 0.51	0.0520
SDB Filtrate (BCMP)	0.50, 0.45, 0.58	0.0656
SDB Filtrate (BCMP)	0.63, 0.51, 0.53	0.0643
SDB Filtrate (BCMP)	0.52, 0.58, 0.49	0.0458
Biotreated Kraft E-stage	0.54, 0.43, 0.50	0.0557
Biotreated Kraft E-stage	0.41, 0.39, 0.59	0.1102
RFA-Spiked Biotreated BCMP	1.05, 0.85, 0.85	0.1155
RFA-Spiked Biotreated Kraft E-stage	0.79, 0.92, 0.90	0.0700
RFA-Spiked Biotreated Kraft E-stage	0.83, 0.97, 0.91	0.0702
RFA-Spiked Biotreated Kraft E-stage	0.96, 0.70, 0.82	0.1301
	$\bar{x}$ = 0.502	$\bar{x}$=0.0746, S.D.=0.0254

Table 6A. Determination of Method Detection Limit for MB Assay

Basement:	a process stream overflow that had leaked/seeped into the mill basement
BCMP:	bleached chemimechanical pulping
BCTMP:	bleached chemithermomechanical pulping
E-stage:	an alkaline extraction stage, in which pulp is treated with NaOH
Maple/Aspen:	BCTMP refiner effluent, generated from a maple/aspen feedstock
$[RFA]_{MB}$:	RFA concentrations determined by "pre-extraction" methylene blue assay
$[RFA]_{GC}$:	RFA concentrations determined by the GC assay of Voss and Rapsomatiotis (1985)
SDB Filtrate:	a mixture of BCMP refiner and WW/WR effluents, the RFAs of which had been removed by Empore SDB extraction filter disks
TMP:	thermomechanical pulping
TSS:	total suspended solids obtained from a 50 mL sample of a mixture of BCMP refiner and WW/WR effluents, re-suspended in 10 mL distilled water
Total Combined:	all process effluents pooled prior to primary and secondary treatment
Total Mill Tanker:	total pulping mill effluent
WW/WR:	whitewater/woodroom effluent

Table 6B. Glossary of Terms in Tables 6A and 8.

A method detection limit of 0.589 mg/L means that a solution containing that amount of total RFA would not be interpreted as a blank in 99% of all MB assay trials.

Note that this limit applies to the "pre-extraction" MB assay with no concentration step and is much better than the 3-4 mg/L limit of the copper acetate assay, but poorer than conventional GC assays that provide detection down to the μg/L range. Effluents containing about 0.5-1 mg/L total RFA are generally non-toxic (See Table 1), and the most important purpose of this assay is to serve as an indirect monitor of toxicity.

To ensure further that this assay could detect low levels of RFA, the effluent-

to-DCM ratio was increased to 5:1 for RFA-free effluent samples. Results obtained using this higher effluent-to-DCM ratio are given in Table 7. Using the procedures discussed, the method detection limit was calculated. These slight modifications reduced the lower detectability limit to $0.218+[0.0229 + 0.0130*4.514/(2*4)^{0.5} = 0.261$ mg/L total RFA. These low-RFA modifications to the MB assay procedure are incorporated in the final procedure (Section 7).

Effluent Sample	[RFA](mg/L)	Standard Deviation
Biotreated BCMP	0.25, 0.26, 0.26	0.0058
Biotreated Kraft E-stage	0.20, 0.25, 0.18	0.0361
Biotreated Kraft E-stage	0.18, 0.23, 0.18	0.0289
Biotreated Kraft E-stage	0.20, 0.19, 0.23	0.0208
	$\bar{x}$ = 0.218	$\bar{x}$=0.0229, S.D.=0.0130

Table 7. Determination of Method Detection Limit for Low-RFA MB Assay

5.3.8 RFA Spike Recovery

A mixed RFA spike (34.8% abietic acid, 34.0% levopimaric acid, 31.2% linoleic acid) in isopropyl alcohol was added to 4 virtually RFA-free effluent samples. (See Section 5.5.) The spike increased total RFA concentration in the sample by 1 mg/L. Using an average weighted on these 3 RFAs, the RFA absorbance was expected to increase by 0.157 A.U. Spike recovery was determined as the percentage of expected absorbance that was measured experimentally. Results included in Table 8 reveal that spike recovery exceeded 80% in all cases. Note that these results were also

used in the calculation of the lower detectability limit and the method detection limit of the MB assay (See Section 5.3.7).

5.4 Comparison between MB Assay and GC Assay

Organizations such as the Association of Standard Testing Methods (ASTM), the Canadian Pulp and Paper Association (CPPA), and the Technical Association of the Pulp and Paper Industry (TAPPI) do not currently recognize a standard RFA measurement method. The GC assay developed by Voss and Rapsomatiotis (1985) was selected as a basis of comparison for the MB assay (See Section 1.3.1.). This GC assay is widely used in the research community, including PAPRICAN. RFA concentrations measured using the MB and GC assays are presented in Table 8, and a glossary of terms is included in Table 6B.

The MB assay was much more economical than the GC assay, in terms of both time and money. About 15-30 samples per worker per day may be tested using the MB assay. Most mills already own the equipment needed to run this assay, including a spectrophotometer or colourimeter. Additional purchases of specialized glassware and chemicals would total less than $500.

SAMPLE	$[RFA]_{MB}$ mg/L	$[RFA]_{GC}$ mg/L	MICROTOX $100/EC_{50}$	SPIKE RECOVERY %
BCMP				
Refiner	14.3 ± 1.1	12.6		
Refiner	19.0 ± 0.9	16.0		
WW/WR	25.7 ± 1.0	21.9		
WW/WR	15.1 ± 0.9	13.2		
Combined	12.0 ± 0.5	10.4	14.9	
Combined	25.1 ± 1.6	22.9	33.0	
Biotreated	0.50	0.19	1.80	91.5
Biotreated	1.02 ± 0.31	0.71	1.64	
Biotreated	0.85 ± 0.22	0.71	1.64	
SDB Filtrate	0.50	0.45		
SDB Filtrate	0.54	0.04		
SDB Filtrate	0.51	0.40*		
SDB Filtrate	0.56	0.40*		
SDB Filtrate	0.53	0.40*		

$[RFA]_{MB}$: RFA concentrations determined by "pre-extraction" methylene blue assay
$[RFA]_{GC}$: RFA concentrations determined by the GC assay of Voss and Rapsomatiotis (1985)

* This RFA concentration was not measured explicitly, but was estimated using an extraction efficiency of 99% for the extraction filter disk.

Table 8. Comparison between MB and GC Assays.

SAMPLE	$[RFA]_{MB}$ mg/L	$[RFA]_{VOSS}$ mg/L	MICROTOX $100/EC_{50}$	SPIKE RECOVERY %
TSS	5.1 ± 0.84	3.5		
TSS	4.7 ± 0.40	3.6		
Kraft				
E stage effluent #1	0.75 ± 0.3	0.31	2.03	
Biotreated #1	0.50	0.05	< 1.0	87.1
E stage effluent #2	13.5 ± 1.4	11.2	13.81	
Biotreated #2	0.74 ± 0.2	0.31	1.06	90.3
E-stage effluent #3	1.74 ± 0.48	1.50	3.35	
Biotreated #3	0.46	0.05	0.83	82.6
TMP				
Refiner	20.0 ± 1.1	17.3		
Filter Press	14.1 ± 0.4	13.5		
Pre-Clarifier	3.3 ± 0.3	2.6		
Post-Clarifier	2.8 ± 0.2	2.2		

Table 8 (continued). Comparison between MB and GC Assays.

SAMPLE	$[RPA]_{MB}$ mg/L	$[RPA]_{VOSS}$ mg/L	MICROTOX $100/EC_{50}$
BCTMP			
Total Mill Tanker	28.7 ± 2.9	24.2	19.2
Total Mill Tanker	33.3 ± 3.5	29.3	19.2
PreBleach	42.0 ± 4.8	37.8	6.57
PreBleach	37.1 ± 2.1	34.8	6.57
Total Combined	90.5 ± 7.5	81.0	
Total Combined	95.1 ± 6.0	88.3	
Total Combined	102.5 ± 8.4	N/A**	
Interstage Wash	108.3 ± 9.9	97.9	34.4
Interstage Wash	112.0 ± 8.7	97.0	34.4
Maple/Aspen	33.0 ± 2.5	41.4	52.9
Maple/Aspen	32.4 ± 2.9	31.7	52.9
Chip Reject	25.6 ± 5.8	N/A**	
Basement	15.4 ± 6.3	10.6	

** These samples contained unidentified compounds that reacted with the internal standard of the GC assay; hence the GC results were unacceptable.

Table 8 (continued). Comparison between MB and GC Assays.

On average, the MB assay measured RFA concentrations that were about 15% higher than the GC results, but the two assays were clearly and consistently measuring the same or a very similar group of compounds. A plot of MB results versus GC results (Figure 14) has a correlation coefficient of 0.998. There are several possible explanations for the 15% greater values obtained with the MB assay. First, this additional 15% could represent RFAs that escape detection by the GC assay by: 1) appearing as unidentified peaks on the gas chromatogram (which are not counted); 2) escaping derivatization during the methylation step and thus not appearing on the chromatogram; 3) not being extracted as efficiently by methyl-t-butyl ether as by DCM. Second, this 15% could represent RFAs that adsorb onto emulsion layer solids in the GC assay extraction. When an emulsion layer is encountered in the course of a GC assay, the effluent is typically centrifuged, and only the supernatant is assayed, resulting in a loss of RFAs. Note that the GC assay, involving the extraction of 50 mL of sample into 100 mL of MTBE, is more greatly influenced by emulsion layer solids than the MB assay, given the greater quantities involved. However, there is no direct correlation between total effluent background, which may be linked to emulsion layer solids, and RFA concentration, as is evidenced by Figure 15. Thus, total background does not consist of RFAs not extracted after two extractions of effluent with DCM. Finally the 15% higher values in the MB assay could represent absorbance caused by non-RFA organic molecules that complex with MB and can be extracted into DCM, although there are no obvious candidates among known effluent components. Thus it is debatable which assay better measures the "true" total RFA

content of an effluent.

The use of the average extinction coefficient generated in this work (i.e. 45.6 A.U./mM) may be a source of significant error in the results for the MB assay. The only samples that gave lower RFA concentrations with the MB assay than the GC assay were the Maple/Aspen effluent samples from a BCTMP mill, and these samples were exceptional in that over 80% of their RFAs were FAs, as opposed to the more typical case in which 33% of total RFAs were FAs. Thus an extinction coefficient more heavily weighted towards RAs was not appropriate. If an extinction coefficient weighted 80% FAs/20% RAs (i.e. 56.6) were used, the two RFA concentrations would have been calculated as 41.0 mg/L, much closer to the GC-measured values of 41.4 mg/L. Thus for future work at mill sites, a preliminary GC analysis of the effluents of interest is recommended, in order to determine the appropriate weighting of the extinction coefficient. The values in Table 5 may then be used to calculate a more relevant average RFA extinction coefficient.

However, given the wide variety of effluents tested with the MB assay, one would expect that other FA-rich samples would have generated higher values of RFA concentration with the MB assay than the GC assay. Nonetheless, the average extinction coefficient applied in the calculations should be adjusted slightly for effluents having RFA compositions vastly different from mechanical pulping effluents. The data provided in Table 5 allows for customized overall extinction coefficients to be derived. For example, effluents resulting from a softwood furnish may require the use of an extinction coefficient more heavily weighted towards FAs.

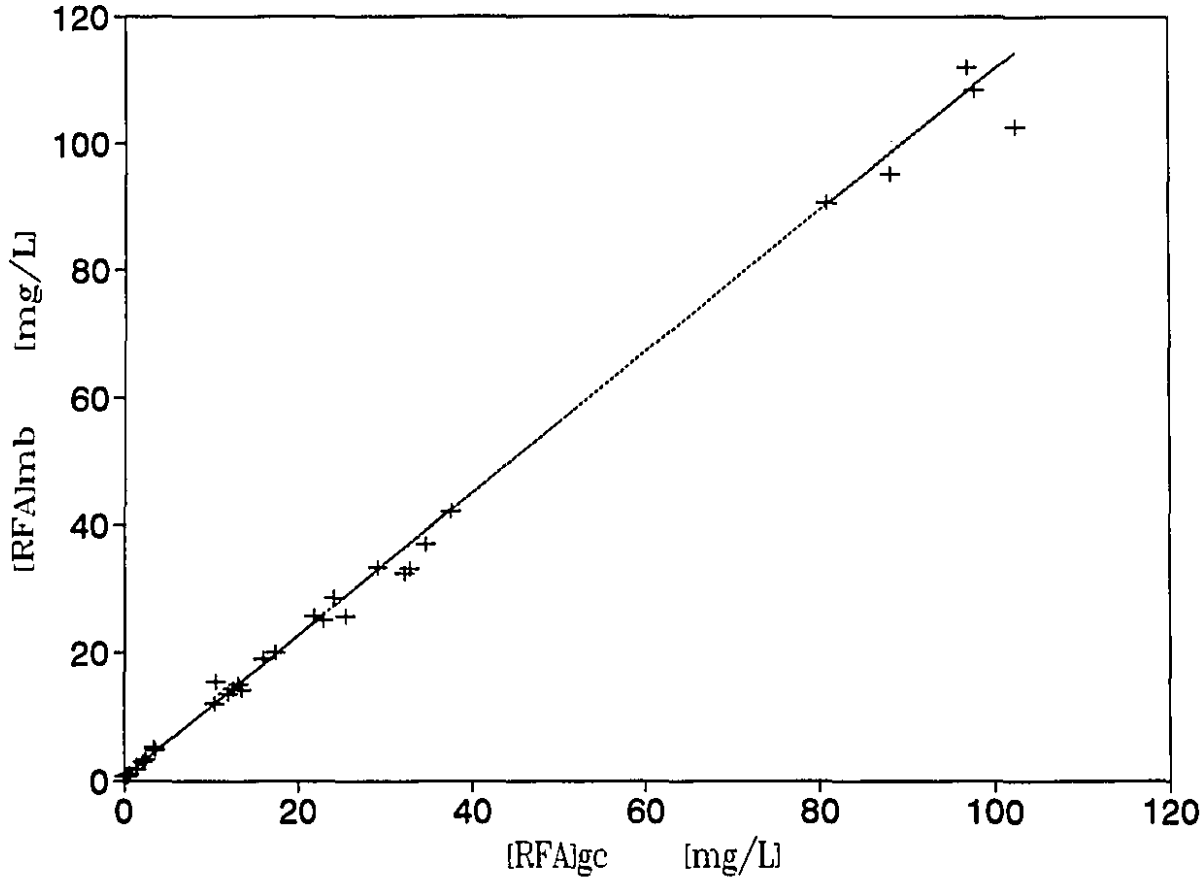


Figure 14. Comparison of MB pre-extraction assay and a standard GC assay for their measurement of total RFA concentration in various mill effluents (r^2 = 0.999).

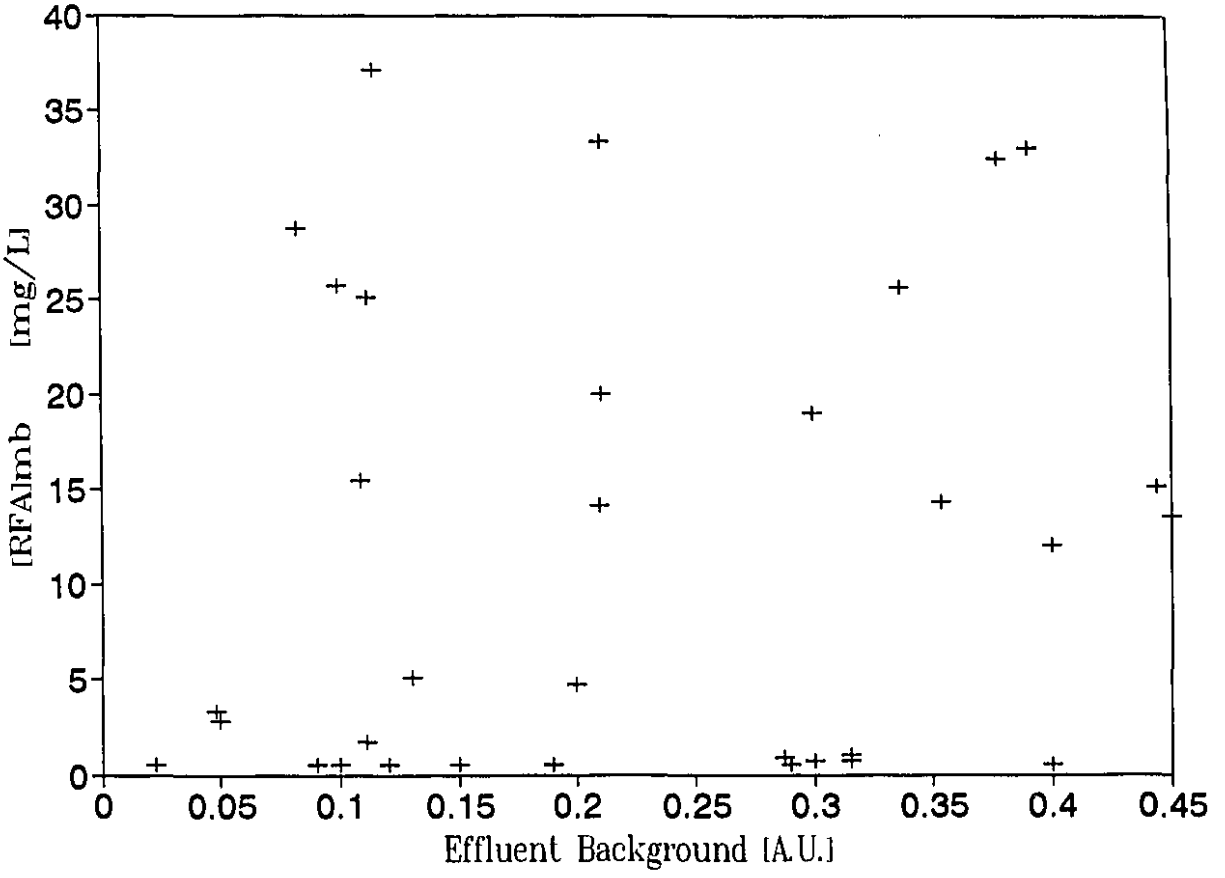


Figure 15. Relationship between the total RFA concentration in various mill effluents and effluent background absorbance in the MB assay.

5.5 RFAs and Acute Effluent Toxicity

Both RFA concentration and acute toxicity were measured in eighteen effluent samples. RFA concentration was measured using the MB assay. Toxicity was measured using the 15-minute Microtox assay and was expressed in toxicity units (i.e. $100/EC_{50}$). The results are presented in Figure 16.

Two sets of data points were clearly outliers. The Maple/Aspen samples from a BCTMP mill were unusually toxic, given that the RFA content in these samples was 80% FA, 20% RA. The presence of a non-RFA toxicant was likely. The Pre-Bleach sample taken from the same mill was much less toxic than anticipated. These unexpected results may be due to a recent re-routing of streams at the mill site.

The regression curve presented in Figure 16 has a correlation coefficient (r^2) of 0.986. This curve was not linear, as had been predicted by the literature (Priha & Talka, 1986). However, the data presented in Figure 16 were obtained from effluents from different mills with different pulping methods and at different sampling points. Previous work in this laboratory strongly correlated RFA concentration and effluent toxicity for numerous specific and combined effluent streams from TMP and CTMP mills (Roy-Arcand & Archibald, 1993; See Section 1.2).

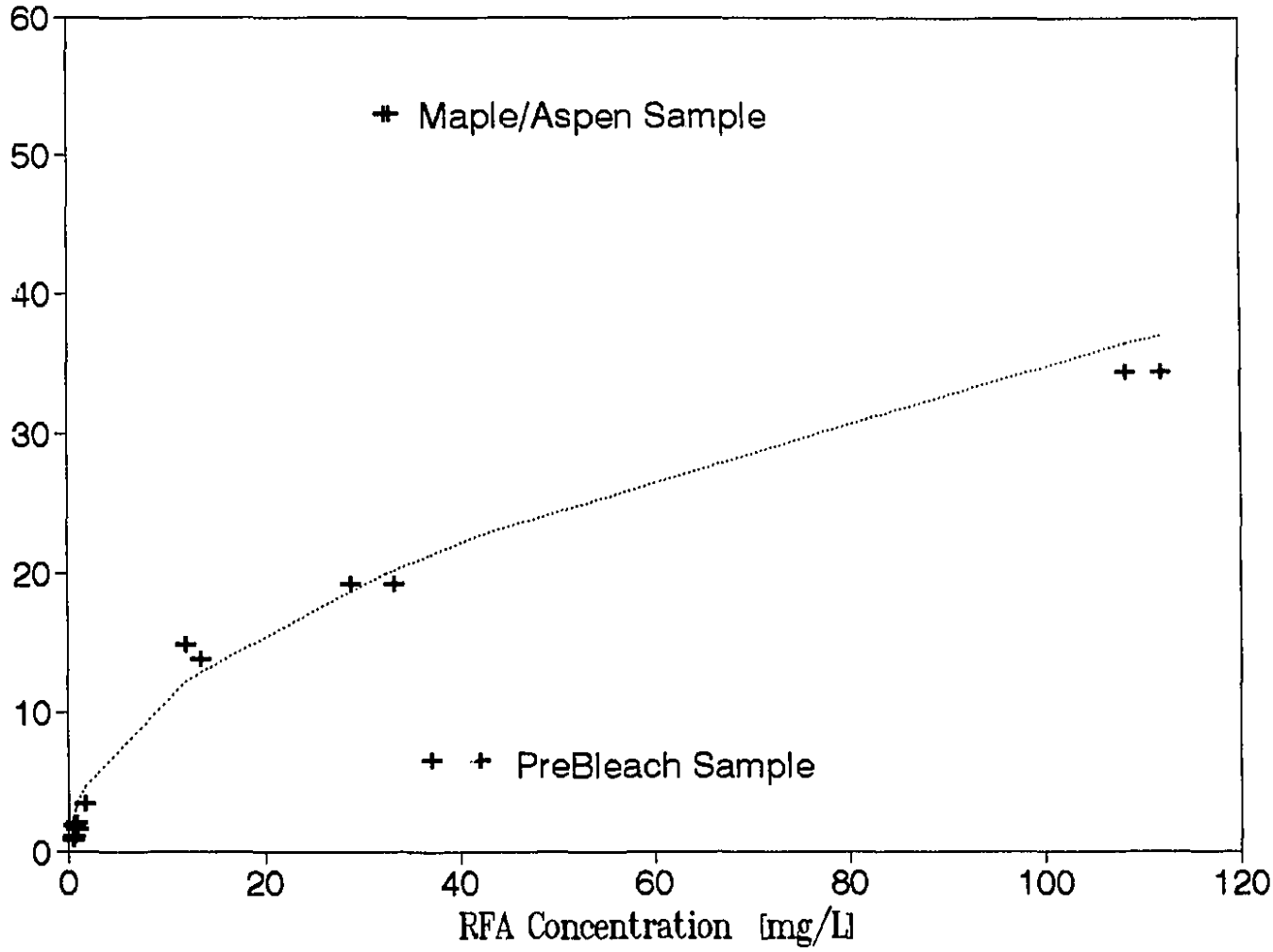


Figure 16. Relationship between total RFA concentration (measured by MB assay) and acute effluent toxicity (measured by Microtox) in all effluent types, fit to the curve $y = 3.5 * x^{0.5}$ ($r^2 = 0.986$), excluding the BCTMP samples of Maple/Aspen and PreBleach

6.0 Final Procedure

6.1 Outline of Procedure

A block diagram of this procedure is given in Figure 17. Effluent samples are diluted in distilled water to total estimated RFA concentration of 0.5-4 mg/L. Multiple dilutions may be required for samples whose approximate total RFA concentration is unknown. Experiments should be performed in triplicate.

Tris-base buffer (0.5 M, pH 8.15) is added to a tris concentration of 20 mM (1:25 v/v). The pH is adjusted to 8.15 by the addition of 2 M NaOH or 2 N H_2SO_4. Three mL of the effluent sample are extracted three times with 3 mL of fresh, chilled DCM. All extractions for a given effluent sample are performed in the same 16 x 160 mm culture tube (Tube #1) fitted with a rigid nylon cap. Tube #1 undergoes two sequences of vortex mixing for 90 s and settling for 3.5 min. Tube #1 is centrifuged (2500 x g, 5 min). Then 1.5 mL of the DCM phase are transferred to a new culture tube (Tube #2). A thick emulsion layer was typically observed at the interface. This layer can be a source of contamination and should not be disturbed. The DCM left in Tube #1 can then be withdrawn and discarded. The extraction is repeated, and 1.5 mL of the final DCM phase are pooled into Tube #2. Residual DCM in Tube #1 is withdrawn and discarded. After a third extraction, 2 mL of DCM are transferred to a separate tube (Tube #3). The content of Tube #1 (i.e. the extracted aqueous phase) is discarded. (See Appendix B for more detailed operational considerations.)

Three mL of MB reagent are added to Tube #2. Two mL of MB reagent are added to Tube #3. Each tube undergoes two sequences of vortex mixing for 45 s and settling on ice for 4 min. The tubes are centrifuged (2,500 x g, 5 min).

Each DCM phase is transferred to a 1.5 mL glass cuvette (path length = 10 mm), and its absorbance is measured at 655 nm on a calibrated spectrophotometer compared to DCM alone. Tube #2 gives a value for total absorbance. Tube #3 gives a value for total background.

Total RFA concentration is calculated from:

RFA absorbance = Total absorbance - Total background
Total RFA concentration = 2.08 * RFA absorbance * 6.44 * DF
where:
2.08 = Dilution factor due to pooling of pre-extracts and due to addition of tris buffer
6.44 = Conversion factor between RFA absorbance and total RFA concentration in mg/L (See Table 2 for derivation.)
DF = Dilution factor for effluent sample

6.2 Special Modifications for Dilute Samples

Increase volume of pH-adjusted effluent sample to 10 mL. Decrease volume of DCM in every extraction to 2 mL. Decrease volume of DCM sampled in determination of total background (Tube #3) to 1.5 mL. Calculate total RFA concentration as per usual, but divide final result by 2.5-3.0.

6.3 Preparation of Methylene Blue Reagent (1 L)

Forty mg of 98+% MB crystals (Aldrich) and 10 g of sodium bicarbonate are dissolved in distilled water to a final volume of 1 L. The pH is adjusted to 8.15 and the solution filtered through AAWP Millipore filter paper (0.45 μm pore size).

To a chilled 1 L separatory funnel are added 500 mL of this MB reagent and 500 mL chilled DCM. The funnel is shaken vigorously for 1 minute, then settled for 4 min. The shaking/settling sequence is performed three more times. The final settling time is 9 min. Periodic refrigeration is required to eliminate pressure build-up.

The 25-minute shaking/settling sequence is performed two additional times, each with 500 mL of fresh, chilled DCM. The DCM layer is withdrawn and discarded at the end of every sequence. The MB reagent is stored in an amber glass bottle, tightly sealed, and refrigerated in the dark.

6.4 Coloured Background Test

A coloured background test is performed on the MB reagent. In a 16 x 160 mm culture tube, 3 mL MB reagent and 3 mL DCM are vortex mixed for 45 s, then settled for 4 min. This shaking/settling sequence is repeated. The absorbance of the DCM phase is measured at 655 nm. If the total absorbance of the MB solution DCM extract is less than 0.250 A.U., the solution is acceptable. If not, an additional extraction of the MB reagent with DCM should be performed.

6.5 Important Notes

Due to the volatility and toxicity of DCM (See Appendix C for a listing of the physical/chemical properties of DCM.), all glassware and reagents should be kept on packed ice throughout the assay. Rigid nylon (Polypenco) culture tube screw caps were used due to their resistance to DCM.

The RFA-MB complex formed is extremely photosensitive (See Appendix B.6.). Direct exposure to ambient light levels will cause the blue complex to turn wine-red or purple within 10 min. The RFA-MB complex should be kept shielded from direct sources of light. Successful methods of shielding samples include wrapping the tubes in aluminum foil, or storing them in a box. The useful life of the RFA-MB complex is about 20 hours with appropriate light shielding and refrigeration.

The waste DCM generated by this assay should be disposed following the appropriate guidelines for hazardous materials, specifically organic solvents.

Rapid Assay:

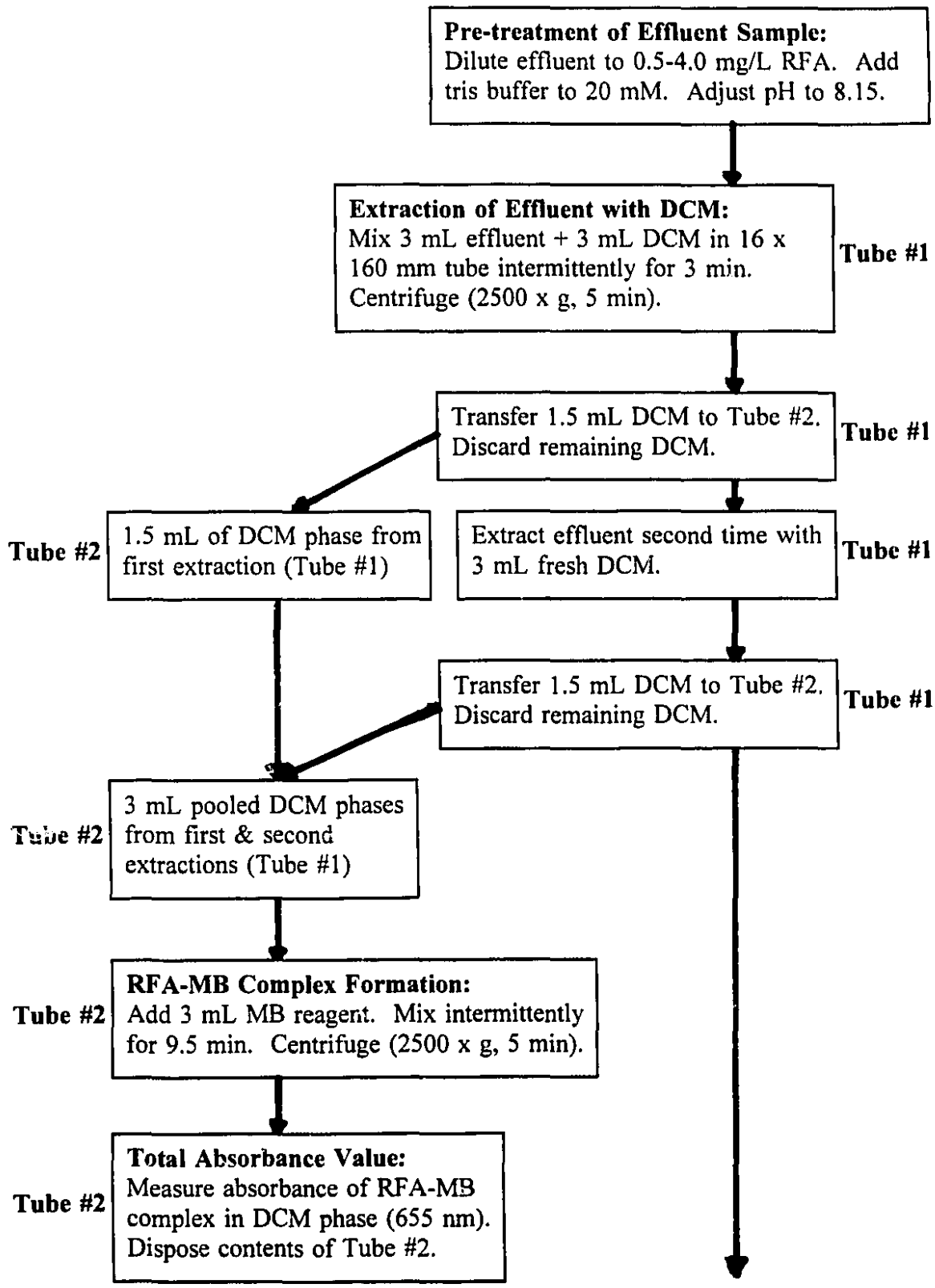
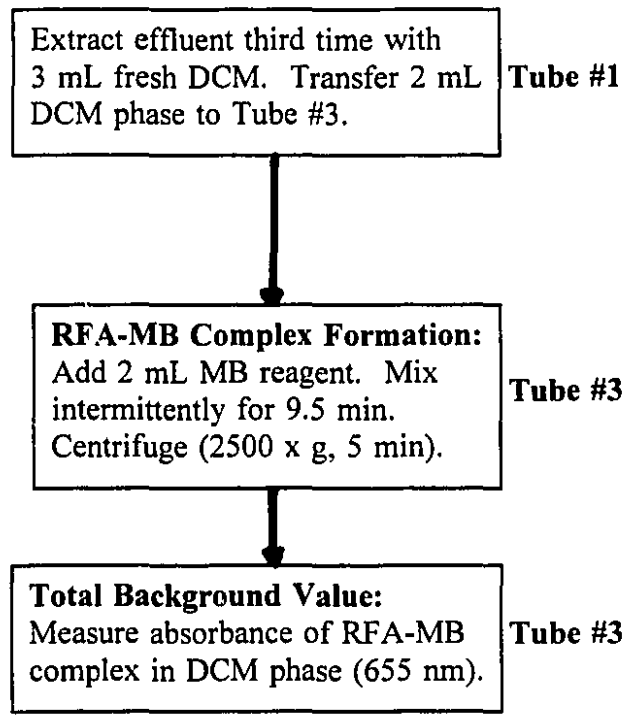


Figure 17. Block diagram of MB assay final procedure.



Calculations:

RFA ABSORBANCE = TOTAL ABSORBANCE - TOTAL BACKGROUND

RFA [mg/L] = 2.08 x 6.44 x RFA ABSORBANCE x DILUTION FACTOR

Figure 17 (continued). Block diagram of MB assay final procedure.

7. Conclusions

The objectives for this study have been met in the development of the methylene blue assay for RFAs in pulp and paper mill effluents. This assay:

* Is reproducible to ± 10-15% total RFA.
* Has a method detection of limit of 0.589 mg/L total RFA for low-RFA samples, and a method detection limit of 0.261 mg/L total RFA for more highly concentrated RFA samples.
* Is more economical than current GC-based RFA assays.
* Is less time-consuming than current GC-based RFA assays.
* Is easily implemented at mill sites.

The performance of the methylene blue RFA assay has been tested against an existing GC assay, and an excellent correlation exists between RFA concentrations obtained.

The methylene blue RFA assay may be used to monitor acute effluent toxicity indirectly in thermomechanical and chemithermomechanical mills. However, non-RFA components may account for too high a percentage of the acute toxicity in many kraft effluents for the MB assay to be used per se for acute toxicity monitoring. However, the MB assay may be used for RFA determination in these effluents. A correlation exists between RFA concentration as measured by the methylene blue assay and toxicity as measured by Microtox.

8. References

Ayers, C.W. "Estimation of the higher fatty acids C_7-C_{18}." *Analytica Chimica Acta*, Vol. 15, 1956. p. 77.

Bardyshev, I.I. & Bulgakov, A.N. "Chromatographic method for analysis of resin acids." *Khromat. Anal. Khim. Drev.*, 1975. p. 101.

Bennett, D.H., Reeser, D., Campbell, A.G. & Falter, C.M. "Pre-treatment of CTMP effluent by lime to reduce resin acids, sulphite, and toxicity." *Proceedings of the 1988 TAPPI Environmental Conference*, 1988. p. 199.

Bonner, J. & Varner, J.E. *Plant Biochemistry*. New York: Academic Press, 1965. p. 324.

Canadian Centre for Occupational Health and Safety (CCOHS). *Cheminfo: Methylene chloride*. Cheminfo database entry. November 1994.

Carpenter, W.L. "Colorimetric measurement of resin acid soaps in pulp and paper mill wastes." *Tappi*, Vol. 48, No. 11, November 1965. p. 669.

Chung, L.T.K., Meier, H.P. & Leach, J.M. "Can pulp mill effluent toxicity be estimated from chemical analyses?" *Tappi*, Vol. 62, No. 12, December 1979. p. 71.

Dawe, B. "Methylene blue test for organic acids in pulp mill effluent." *Dow Forest Products Research* communication, June 1993.

Dawe, B. Personal interview. Montreal-Ste. Catharine's. July 1993.

Degens, P.N., Evans, H.C., Kommer, J.D. & Winsor, P.A. *Journal of Applied Chemistry*, Volume 3, 1953. p. 54.

Dorris, G.M., Douek, M. & Allen, L.H. "Operating variables in the analysis of tall oil acids by capillary gas chromatography." *Journal of the American Oil Chemists' Society*, Vol. 59, No. 11, November 1982. p. 494.

Dünges, W. "4-bromomethyl-7-methoxycoumarin as a new fluorescence label for fatty acids." *Analytical Chemistry*, Vol. 49, No.3, March 1977. p. 442.

Farinotti, R., Siard, P., Bourson, J., Kirkiacharian, S., Valeur, B., & Mahuzier, G. "4-bromomethyl-6,7-dimethoxycoumarin as a fluorescent label for carboxylic acids in chromatographic detection." *Journal of Chromatography*, Vol. 269, 1983. p. 81.

Fisheries Act: Canada Gazette Part II, Vol. 126, No. 11, May 7 1992. Ottawa: Supply and Services Canada. p.1985.

Greenberg, A.E., Clesceri, L.S., & Eaton, A.D, Eds. Standard Methods for the Examination of Water and Wastewater, 18th ed. Maryland: American Public Health Association, 1992. p.1-1 to 1-23.

Gref, R. "Gas chromatography analysis of underivatized resin acids." *Journal of Chromatography*, Vol. 448, 1988. p. 428.

Gregory, G.R.E.C. "The determination of residual anionic surface-active reagents in mineral flotation liquors." *Analyst*, Vol. 91, April 1966. p. 251.

Hagman, N. "Resin acids in fish mortality." *Finnish Paper and Timber*, Vol. 18, 1936. p. 32.

Harel, G. "Extraction sur membranes des acides gras et résineux dans les effluents de pâte mécanique." *Projet de fin d'études (B. Ing.)*. École Polytechnique de Montréal, Département de génie chimique. 1994.

Holloran, M.F., Willes, R.F., Palmiere, M.J. & Wilson, D.G. "Estimating effluent toxicity from molecular-chlorine-gas-free bleaching in terms of toxicity equivalence." *Water Pollution Research Journal of Canada*, Vol. 27, No. 3, 1992. p. 539.

Holmbom, B. "Analysis of resin and fatty acids in pulp and paper mill effluents and in environmental samples." Symposium on methods of analysis of wood and environmental samples, New Zealand, May 7-8, 1992.

Iwayama, Y. "New colorimetric determination of higher fatty acids." *Journal of the Pharmacological Society of Japan*, Vol. 79, No. 4, 1959. p. 552.

Jamieson, S. "In environmental monitoring, what you don't know can hurt you." *Pulp and Paper Canada*, Vol. 93, No. 2, 1992. p. 9.

Keith, L.H. "Identification of organic compounds in unbleached treated kraft paper mill wastewaters." *Environmental Science and Technology*, Vol. 10, No. 6, June 1976. p. 555.

Kester, M.D., Horner, J.A., Nicolidakis, H., Wade, A.P. & Wearing, J.T. "Comparison of flow injection methods for determination of abietic acid." *Pulp and Paper Research Institute of Canada Miscellaneous Report No. 234*, August 1992.

Lam, S. & Grushka, E. "Labelling of fatty acids with 4-bromomethyl-7-methoxycoumarin via crown ether catalyst for fluorometric detection in high-performance liquid chromatography." *Journal of Chromatography*, Vol. 158, 1978. p. 207.

Leach, J.M. & Thakore, A.N. "Toxic constituents in mechanical pulping effluents." *Tappi*, Vol. 59, No. 2, February 1976. p. 129.

Leclerc, D. "Determination of resin and fatty acids with Fourier transform infrared analysis." *PAPRICAN internal communication*, 1993.

Lee, H.-B., Peart, T.E. & Carron, J.M. "Gas chromatographic and mass spectrometric determination of some resin and fatty acids in pulpmill effluents as their penta-fluorobenzyl ester derivatives." *Journal of Chromatography*, Vol. 498, 1990. p. 367.

Li, K., Chester, M., Kutney, J.P., Saddler, J.N. & Breuil, C. "Production of polyclonal antibodies for the detection of dehydroabietic acid in pulp mill effluents." *Analytical Letters*, Vol. 27, No. 9, 1994. p. 1671.

Liu, H.W., Lo, S.N. & Lavallée, H.C. "Secondary treatment of a CTMP effluent." *Journal of Pulp and Paper Science*, Vol. 19, No. 5, September 1993. p. J219.

Lloyd, J.B.F. "Fatty acid esters of 4-hydroxymethyl-7-methoxycoumarin: fluorescence characteristics and their variation in high-performance liquid chromatographic solvents." *Journal of Chromatography*, Vol. 178, 1979. p. 249.

Longwell, J. & Maniece, W.D. "Determination of anionic detergents in sewage, sewage effluents and river waters." *Analyst*, Vol. 80, March 1955. p. 167.

Mayr, M., Lorbeer, E. & Kratzl, K. "Gas chromatographic separation of diterpene acids on glass capillary columns of different polarity." *Journal of the American Oil Chemists' Society*, Vol. 59, No. 1, January 1982. p. 52.

McMahon, D.H. "Analysis of low levels of fatty and resin acids in kraft mill process streams." *Tappi*, Vol. 63, No. 9, September 1980. p. 101.

McSweeney, E.E., Arlt, H.G., & Russell, J. *Tall Oil and Its Uses.* New York City: Pulp Chemicals Association, 1987.

National Council of the Paper Industry for Air and Stream Improvement (NCASI). "Procedures for the analysis of resin and fatty acids in pulp mill effluents." *Technical Bulletin No. 501*, August 1986.

Nimz, E.L. & Morgan, S.L. "On-line derivatization for complex fatty acid mixtures by capillary gas chromatography/ mass spectrometry." *Journal of Chromatographic Science*, Vol. 31, April 1993. p. 145.

Ontario Ministry of the Environment. *Draft effluent limits regulation for the pulp and paper sector.* February 1993. p.5.

Paice, M.G. Personal interview. Pointe-Claire. December 1993.

Priha, M.H. & Talka, E.T. "Biological activity of bleached kraft mill effluent (BKME) fractions and process streams." *Pulp and Paper Canada*, Vol. 87, No. 12,

1986. p. T447.

Richardson, D.E. & Bloom H. "Analysis of resin acids in untreated and biologically treated thermomechanical pulp effluent." *Appita*, Vol. 35, No. 6, May 1982. p. 477.

Richardson, D.E., Brenner, J.B. & O'Grady, B.V. "Quantitative analysis of total resin acids by high-performance liquid chromatography of their coumarin ester derivatives." *Journal of Chromatography*, Vol. 595, 1992. p. 155.

Rogers, I.H. "Isolation and chemical identification of toxic components of kraft mill wastes." *Pulp and Paper Magazine of Canada*, Vol. 74, No.9, September 1973. p. 111.

Rogers, I.H., Mahood, H., Servizi, J. & Gordon, R. "Identifying extractives toxic to aquatic life." *Pulp and Paper Canada*, Vol. 80, No. 9, September 1979. p. T287.

Roy-Arcand, L., Archibald, F.S. & Méthot, M. *Unpublished toxicity data.* Pulp and Paper Research Institute of Canada, 1993.

Roy-Arcand, L., Archibald, F.S. & Méthot, M. "The combination of ozone and activated sludge for treatment of CTMP primary clarifier effluent." *Pulp and Paper Research Institute of Canada Pulp and Paper Report No. 1072*, April 1994.

Ruus, L. "Determination of resin acids in waste water from forest product industries." *Svensk Papperstidning*, Vol. 68, 1965. p. 34.

Sitholé, B.B. "A rapid spectrophotometric procedure for the determination of total resin and fatty acids in pulp and paper matrices." *Tappi Journal*, Vol. 76, No. 10, October 1993. p. 123.

Swann, M.H. "Colorimetric determination of rosin and rosin esters." *Analytical Chemistry*, Vol. 23, No. 6, June 1951. p. 885.

Thornton, J. "Dissolved and colloidal substances in the production of wood-containing paper." *Ph. D. Thesis submitted to Department of Forest Products Chemistry, Abo Akademi University*, 1993.

Tomlinson, H.S. & Sebba, F. "Determination of surfactant ions by ion flotation." *Analytica Chimica Acta*, Vol. 27, 1962. p. 596.

Trung, T. "Determination of fatty acids by flow injection analysis." *B.Sc. Thesis submitted to Department of Chemistry, University of British Columbia*, 1993.

Tsuchiya, H., Hayashi, T., Naruse, H., & Takagi, N. "High-performance liquid chromatography of carboxylic acids using 4-bromomethyl-7-acetoxycoumarin as

fluorescence reagent." *Journal of Chromatography*, Vol. 234, 1982. p. 121.

Turner, S.A. & Wallin, B.K. "The analysis of toxic and nonconventional pollutants by FSOT GC/MS." *Tappi Journal*, Vol. 68, No. 8, August 1985. p. 108.

Turoski, V.E., Kuehnl, M.E. & Vincent B.F. "Determination of resin and fatty acids in paper mill effluents by GC/MS." *Tappi*, Vol. 64, No. 5, May 1981. p. 117.

van der Horst, F.A.L., Post, M.H., Holthuis, J.J.M. & Brinkman, U.A.T. "Automated high-performance liquid chromatographic determination of plasma free fatty acids using on-line derivatization with 9-bromomethylacridine based on micellar phase-transfer catalysis." *Journal of Chromatography*, Vol. 500, 1990. p. 443.

Vine, J. "Analysis of fatty acid methyl esters by high-resolution gas chromatography-chemical ionisation mass spectrometry." *Journal of Chromatography*, Vol. 196, 1980. p. 415.

Voss, R.H. & Rapsomatiotis, A. "An improved solvent-extraction based procedure for the gas chromatographic analysis of resin and fatty acids in pulp mill effluents." *Journal of Chromatography*, Vol. 346, 1985. p. 205.

Walden, C.C. & Howard, T.E. "Toxicity of pulp and paper mill effluents - a review." *Pulp and Paper Magazine of Canada*, Vol. 82, No. 4. p. 115.

Zinkel, D.F. & Engler, C.C. "Gas-liquid chromatography of resin acid esters." *Journal of Chromatography*, Vol. 136, 1977. p. 245.

Zinkel, D.F., Lathrop M.B. & Zank, L.C. "Preparation of gas chromatography of the trimethylsilyl derivatives of resin acids and the corresponding alcohols." *Journal of Gas Chromatography*, Vol. 6, March 1968. p. 158.

Appendix A: Dawe's Assay Procedure

This is a modification of a method developed by Bob Dawe for analyzing surfactants used in oil field applications, which in turn was loosely based on that described by Greenberg *et al.*, in "Standard Methods for the Examination of Water and Wastewater" 15th Ed.: American Public Health Association, Washington, D.C., 1980, Section 512A.

EXPERIMENTAL: Consult the appropriate MSDS for the chemicals used in this method.

Methylene blue (20 mg) and sodium bicarbonate (5.00 g) were dissolved in water to give 500 mL of deep blue solution (pH 8.15) (SEE NOTE 1 BELOW). Dichloromethane was reagent grade. Oleic acid (500 mg) was dissolved in 20 mL 1 N sodium hydroxide then diluted to 500 mL to give a nominally 1000 ppm oleic acid solution.

Dichloromethane (5.00 mL) was transferred to a 20 mL disposable glass vial having a screw top. Methylene blue solution (4.00 mL) was added. The solution to be analyzed was added via microliter syringe. The vial was tightly capped, shaken vigorously about thirty times then centrifuged for 2-3 minutes to assist in separating the two layers. The bottom layer, sufficient to fill a UV-vis sample cell to the appropriate depth, was withdrawn with a disposable pipette (plastic pipettes work well due to their hydrophobic nature) and placed in a UV-vis sample cell. Care was taken to avoid inadvertent transfer of any of the top (aqueous) layer to the sample tube. The absorption of the sample at 650 nm was measured (SEE NOTE 1 BELOW.)

A calibration curve using oleic acid was prepared by using 0-50 microliters of 1000 ppm oleic acid in the above procedure. A non-linear calibration curve, useful to

about 50 micrograms of added oleic acid, was obtained.

Samples of mill water were examined. Dilute samples (ca. 100 ppm oleic acid eq.) required 200-250 microliters of solution, while with more concentrated samples 10-20 microliter aliquots were needed to give absorbance readings in the useful range of .2-.75 units. Concentration of organic acids in the samples were reported as oleic acid equivalents.

NOTE 1: It is possible to lower the zero point for the calibration curve by doing a methylene chloride extraction of the methylene blue/ sodium bicarbonate. This removes any methylene chloride-soluble impurities present in the methylene blue.

NOTE 2: The samples, after addition of the resin/organic acids, are somewhat photo-sensitive. The methylene blue/organic acid complex that transfers to the methylene chloride layer is initially blue, but on exposure to light begins to turn purple to wine-red. Consequently, it is advisable to keep the sample vials inside the centrifuge until ready for analysis, take out one at a time and make the absorbance measurement as quickly as possible.

Taken from (Dawe, 1993).

Appendix B: Operational Considerations

B.1 "Teflon-lined" Caps

"Teflon-lined" Bakelite screw caps are widely sold for use in assays involving highly aggressive solvents (e.g. concentrated sulfuric acid in the COD test). Such "Teflon-lined" caps were used for this assay at first. However, during experiments involving multiple extractions of effluent with DCM, the total liquid volume in the 15 mL glass culture tube was increased from 6 mL to 12 mL. Highly irreproducible results were immediately observed, with standard deviations exceeding 30% of the reading. Coloured background tests gave absorbance values typically above 0.400 A.U., more than twice the norm.

Solvent leakage from the tubes was ruled out as the prime cause for the higher values of coloured background. Although 45% of all tubes used lost over 10% of their initial mass of DCM in an overnight leakage test, no correlation was found between DCM leakage and coloured background absorbance values.

"Teflon-lined" caps were found to be the source of interference. The circular disk liner forming the actual cap-glass seal in each Bakelite plastic cap is actually Teflon-coated rubber. The rubber side of the liner is fixed to the Bakelite by a resinous adhesive, the Teflon side forming the cap-glass seal. Individual components of caps were immersed in DCM. Then 3 mL of each DCM solution were mixed with 3 mL of MB reagent, then centrifuged. The RFA absorbances of two of these DCM phases were significant: that from the rubber liner (1.76 A.U.), and that from the adhesive (0.29 A.U.). Clearly the rubber and adhesive were being leached by the DCM. Under a microscope, tiny pores and scratches were observed in the Teflon coatings of problem Teflon caps. It is believed that DCM had passed through these

micropores, leaching out rubber and adhesive components that complexed with MB.

B.2 Glass Centrifuge Tubes with Ground Glass Stoppers

Ground glass stoppers were regarded as a suitable alternative to Teflon screw caps. No culture tubes were available that were compatible with glass stoppers; so centrifuge tubes were used. Centrifuge tubes with ground glass stoppers are much more expensive than culture tubes, and their conical tips created a dead zone for mixing.

Ground glass stoppers also did not provide an airtight seal for the highly volatile DCM, which bubbled and leaked through the seal. Manual pressure was constantly applied to the stopper, and the tubes could not be inverted without significant leakage. Second, MB stained the ground glass in the stoppers and throats of the centrifuge tubes. Glassware was soaked in 1 N HCl for a 20 minutes to remove MB stains.

Results with glass centrifuge tubes with ground glass stoppers were nonetheless acceptable. Their much higher cost (i.e. about $150.00 / dozen) prompted a search for a more economical alternative.

B.3 Glass Culture Tubes with Solid Nylon Caps

The standard "Pyrex" or "Kimax" glass culture tubes used with the problem "Teflon-lined" Bakelite caps are inexpensive, readily available, and mechanically strong enough to withstand centrifugal forces below 3,000 x g.

PAPRICAN machinist Steven Horvath constructed two types of screw caps to fit these culture tubes. Caps made of pure Teflon proved too flexible and slippery to

provide a tight seal on the tubes. However, Polypenco brand nylon rod (0.75" diameter) was easy to machine completely resistant to DCM, and relatively inexpensive. The rigid nylon screw caps (12 threads per inch) produced from it provided an airtight seal, and they remained fixed in place during vortex mixing. The combination of these nylon caps and the original glass culture tubes proved ideal for this assay.

B.4 Centrifuge

A small bench-top centrifuge accepting 15 mL, 16 mm O.D. cylindrical tubes and producing 2500 x g was eventually selected for use in the MB assay, because of its economy and adequate performance. If available, an enclosed, refrigerated centrifuge is preferable.

B.5 Soap

The cleaning of glassware typically involved a soak in a solution of standard commercial sodium carbonate / metaphosphate mechanical washing (i.e. Alcojet brand) detergent. High detergent concentrations resulted in soapy films that were persistent despite repeated rinsings. Soap solutions were added to water and to abietic acid standards to determine the effect of soap on assay performance. This soapy residue interfered with the MB assay in two ways: 1) the soap complexed with MB ions 2) the soap affected the partition coefficient between uncomplexed MB chromophores and DCM. Detergent concentrations of 2 g/L or lower were suitably dilute for easy rinsing.

B.6 Photosensitivity of RFA-MB Complexes

The RFA-MB complexes will turn from blue to wine-red or purple in colour after only 10 minutes exposure to ambient light levels (i.e. 150 lux and higher), regardless of RFA-MB complex concentration. This colour change is rapid and drastic, discernable to the naked eye. It is also irreversible; thus a colour change represents a failed experiment.

It is recommended to keep all samples shielded from direct exposure to light (e.g. light source in the fume hood, sunlight from a nearby window). The experiments should be performed with the minimum amount of light required for a safe working environment. Background light levels are typically insufficient to cause the colour change. Colour change does not occur even after 20 hours if the sample is appropriately shielded.

Appendix C: Properties of Dichloromethane (CH_2Cl_2)

C.1 Physical/Chemical Properties

Molecular Weight =	84.94 g/mol
Density @ 20°C =	1.326 g/mL
Vapour Pressure @ 20°C =	43-46.5 kPa
log K_{OW} =	1.25
Henry's Law constant =	227.9 Pa/m^3
Solubility in Water @ 20°C =	20 g/L
Odour Threshold =	150 ppm (detection) - 230 ppm (recognition)

C.2 Toxicological Information

LC_{50} (guinea pig) =	11600 ppm (6-hour exposure)
LC_{50} (rat)=	57000 ppm (15-minute exposure)
LC_{50} (mouse)=	16186 ppm (8-hour exposure)
LD_{50} (oral, rat)=	2100-3000 mg/kg
Sub-lethal Effects:	Mild CNS depression, headache, nausea, dizziness, drowsiness, incoordination, confusion, unconsciousness
Carcinogenicity:	After 2 years' exposure to DCM via inhalation, increased incidence of mammary gland tumours was observed in rats (1000-4000 ppm), lung and liver tumours in mice (2000-4000 ppm). DCM is listed as "possibly causing cancer" under the Canadian Environmental Protection Act.

C.3 Equipment Considerations

Soluble in DCM:	Rubber, wax, polyethylene, resinous adhesives, Nalgene labware
Resistant to DCM:	Glass, Teflon, metal, Bakelite, polypropylene, Polypenco nylon
Safety Clothing:	Goggles, latex gloves, lab coat or coveralls, safety work boots
Safety Equipment:	Fume hood with adequate ventilation, spill containment provisions

Appendix C was taken from the Cheminfo database (CCOHS, 1994).

Appendix D: List of Abbreviations

A.U.:	absorbance unit
AAWP:	white plain
AB:	abietic acid
ABS:	absorbance
APPITA:	Australian Pulp and Paper Industry Technical Association
ASTM:	Association of Standard Testing Methods
BCMP:	bleached chemimechanical pulp
BCTMP:	bleached chemithermomechanical pulp
BKME:	bleached kraft mill effluent
bp:	(normal) boiling point
Br-DMC:	4-bromomethyl-6,7-dimethoxycoumarin
Br-MAC:	9-bromomethylacridine
Br-Mac:	4-bromomethyl-7-acetoxycoumarin
Br-Mmc:	4-bromomethyl-7-methoxycoumarin
BSTFA:	bis(trimethylsilyl)trifluoroacetamide
CCOHS:	Canadian Centre for Occupational Health and Safety
CNS:	central nervous system
COD:	chemical oxygen demand
CPPA:	Canadian Pulp and Paper Association
CTMP:	chemithermomechanical pulp
DCM:	dichloromethane, or methylene chloride
dd:	distilled and deionized
DeAB:	dehydroabietic acid
DF:	dilution factor
E.C.:	extinction coefficient
E-stage:	caustic extraction stage
EC_{50}:	effective concentration for 50% of population
EDTA:	ethylenedinitilotetraacetic acid
EGS:	ethylene glycol succinate
FA:	fatty acid
FSOT:	fused-silica open tubular
FT-IR:	Fourier transform infrared analysis
g:	acceleration due to gravity
GC:	gas chromatography
GLC:	gas-liquid chromatography
HPLC:	high-performance liquid chromatography
I.S.:	internal standard
IC_{50}:	concentration inhibitory to 50% of population
JB:	juvabione
K_{OW}:	octanol/water partition coefficient
LC_{50}:	lethal concentration to 50% of population
LD_{50}:	lethal dosage to 50% of population
LT_{50}:	lethal exposure time to 50% of population
Mac:	methyl-7-acetoxycoumarin
MB:	methylene blue

Mmc:	methyl-7-methoxycoumarin
MDL:	method detection limit
MS:	mass spectroscopy
MSDS:	material safety data sheet
MSTFA:	N-methyl-N-trimethylsilyl-trifluoroacetamide
MTBE:	methyl-t-butyl ether
MW:	molecular weight
NCASI:	National Council of the Paper Industry for Air and Stream Improvement
O.D.:	outer diameter
OD_{655}:	optical density at 655 nm
PAPRICAN:	Pulp and Paper Research Institute of Canada
ppb:	part per billion
ppm:	part per million
r^2:	correlation coefficient
RA:	resin acid
RFA:	resin and fatty acid
S.D.:	standard deviation
SDB:	polystyrene **d**ivinyl **b**enzene
TAPPI:	Technical Association of the Pulp and Paper Industry
Theor.:	theoretical
TLC:	thin layer chromatography
TMP:	thermomechanical pulp
TSS:	total suspended solids
UV:	ultraviolet
v/v:	volume-to-volume ratio
vis:	visible (light)
WR:	woodroom effluent
WW:	white water