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DEVELOPMENT OF A CAPILLARY ELECTROPHORETIC METHOD FOR THE SEPARATION AND DETECTION OF RESIN ACIDS

by

Tracey Rigby

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

Department of Food Science and Agricultural Chemistry

McGill University

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SHORT TITLE

CAPILLARY ELECTROPHORESIS OF RESIN ACIDS

ABSTRACT

M.Sc. Tracey Rigby

A method for the separation and detection of standard resin acids (RAs), commonly found in pulp mill effluent and known to bioaccumulate in fish bile, was optimized using cyclodextrin modified electrokinetic capillary electrophoresis (CD-EKC) with ultra violet (UV) and laser induced fluorescence (LIF) detection. Optimal separation conditions were found with RA standards using UV detection at 214 nm, with a 72 mM sodium borate buffer pH 9.25, containing 35 mM β-cyclodextrin sulfobutyl ether (SβCD), 15 mM of methyl-β-cyclodextrin (MECD) and a 37 cm capillary with an internal diameter of 50μm. This resulted in a 12-min separation and the identification of 9 peaks, with a LOD of 10 ppm. To enable increased sensitivity, RAs were derivatized using the fluorescent label 4-BrMMc. Fluorometric analysis verified that the esters fluoresced optimally at 325 nm excitation wavelength and 400-nm emission. The mass spectroscopic (MS) data showed the reaction was complete and free from side reactions. RA esters were analyzed using CD-EKC with LIF detection at 325 nm excitation wavelength, and 400 nm emission. Due to conformational changes during derivatization, separation conditions were re-optimized using a 36 mM sodium borate buffer pH 9.25, with a SβCD concentration of 60 mM and 15 mM MECD, with a separation voltage of 10 kV, and a capillary length of 37 cm with an inner diameter of 50 μm. Under these conditions, 7 peaks were resolved, and the separation was completed in 15 min. Peaks were identified, and the LOD was 50 ppb. The results were cross-referenced using reverse phase HPLC equipped with UV or scanning fluorescence detector. A method for extracting resin acids from spiked fish bile and pulp mill effluent was developed, the extracted samples were derivatized, separated and identified using CD-EKC with LIF detection. The method of extraction and derivatization using CD-EKC was applied to biological samples of contaminated effluent and fish bile. Dehydroabietic acid, the most prevalent resin acid, as well as other chlorinated and unchlorinated RAs were detected and identified in all of the pulp mill effluent samples. No resin acids were detected in fish bile samples. Results were cross referenced using reverse phase HPLC with scanning fluorescence detection and/or gas chromatography (GC) with flame ionization (FID) analysis.

RESUME

M.Sc. Tracey Rigby

Une méthode de séparation et de détection de standards d'acides de résine (RAs), communément rencontrés dans l'éffluent de pulpe d'usine et connus pour s'accumuler dans la bile de poisson, a été optimisée en utilisant l'électrophorèse capillaire en mode électrocinétique avec cyclodextrine (CD-EKC) et détections en ultra violet (UV) et par fluorescence induite par un laser (LIF). Les conditions de séparation optimales ont été obtenues avec les standards de RA en utilisant la détection UV à 214 nm, avec un tampon de borate de sodium 72 mM pH 9.25, contenant 35 mM d'éther de sulfobutyl-β-cyclodextrine (SβCD), 15 mM de méthyl-β-cyclodextrine (MECD) et un capillaire de 37 cm avec un diamètre interne de 50μm. Ceci a résulté en une séparation de 12-min et l'identification de 9 pics, avec une LOD de 10 ppm. De façon a augmenter la sensibilité, les RAs ont été dérivatisés avec le marqueur fluorescent 4-BrMMc. L'analyse fluorométrique a permis de vérifier que les esters fluorescent de façon optimale à des longueurs d'onde d'excitation de 325 nm et d'émission de 400 nm. Les données de masse spectroscopie (MS) ont montré que la réaction a été totale, sans réactions secondaires. Les esters de RA ont été analysés par CD-EKC avec détection LIF à des longueurs d'onde d'excitation de 325 nm et d'émission de 400 nm. A cause des changements de conformation au cours de la dérivatisation, les conditions de séparation ont été ré-optimisées en utilisant un tampon de borate de sodium de 36 mM pH 9.25, avec une concentration de SβCD de 60 mM et de MECD de 15 mM, un voltage de séparation de 10 kV et une longueur du capillaire de 37 cm avec un diamètre interne de 50 μm. Sous ces conditions, 7 pic ont été résolus, et la séparation a été complétée en 15 min. Les pics ont été identifiés, et la LOD a été de 50 ppb. Tous le résultats ont été confirmés en utilisant l'HPLC en phase inverse équipée avec un détecteur UV ou un détecteur de fluorescence. Une méthode d'extraction des acides de résine de bile de poisson et d'éffluent de pulpe d'usine a été développée, les échantillons extraits ont été dérivatisés, séparés et identifiés par CD-EKC ave détection LIF. La méthode d'extraction et de dérivatisation par CD-EKC a été utilisée pour des échantillons biologiques d'éffluent contaminé et de bile de poisson. L'acide déhydroabiétique, l'acide de résine le plus important, ainsi que d'autres RAs chorinés et non-chlorinés ont été détectés et identifiés dans tous les échantillons d'éffluent. Aucun acide de résine n'a été détecté dans les échantillons de bile de poisson. Les résultats ont été confirmés par HPLC avec détection de fluorescence et/ou chromatographie en phase gazeuse (GC) avec analyse par ionisation de flamme (FID).

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TABLE OF CONTENT

LIST OF FIGURES

LIST OF TABLES

List of Abbreviations

CE	Capillary electrophoresis
RAs	Resin acids
CD	Cyclodextrins
SβCD	β-cyclodextrin sulfobutyl ether
MECD	Methyl-β-cyclodextrin
CD-EKC	Cyclodextrin modified electrokinetic capillary electrophoresis
UV	Ultra-violet
LIF	Laser-induced fluorescence detection
HPLC	High-performance liquid chromatography
GC	Gas-liquid chromatography
4-BrMMc	4-bromomethoxy coumarin
PME	Pulp mill effluent

1. INTRODUCTION

The persistence of toxic substances in our water resources continues to be of significant concern as we enter the new millenium. Many toxic substances tend to biomagnify in food webs, and persist at high levels in some areas of the ecosystem. Examples of familiar compounds which have been known to bioaccumulate, are PCB's, DDT, dioxins, methylmercury and many chlorinated substances (Escartin and Porte., 1999).

Resin acids (RAs), tricyclic diterpenoids with a carboxylic moiety are found in the oleoresin of coniferous trees (Dethlefs and Stan., 1996). RAs are released during chemical and mechanical pulping processes, with the resulting effluents constituting 70% of all of the compounds toxic to aquatic organisms. Effluent from industrial pulp mills is treated and released into rivers, lakes and coastal waters and is potentially harmful to aquatic life. RAs are very resistant to degradation, are hydrophobic in nature and therefore have relatively low solubility, RAs are known to bioaccumulate in aquatic species (Richardson *et al.*, 1992). RAs are classified into two main groups, abietanes and pimaranes. Abietanes consist of abietic acid, dehydroabietic acid, neoabietic acid and palustric acid, and are classified based on their isopropyl substituent on the C-13 carbon. Pimarines consist of pimaric acid, isopimaric acid, sandarocopimaric acid and levopimaric acid, and possess two substituents, vinyl and methylmoieties on the C-13 carbon. Chlorobleaching of wood using Cl_2 or ClO_2 results in the formation of 2 chlorinated resin acid structures, 12-chlorodehydroabietic acid, and 12-14 dichlorodehyroabietic acid. Effluent concentrations as high as 10, 000 ppm, to an almost undetectable concentration of 50 ppb have been measured in effluent waters. More specifically, a 96 hour LC 50 of 0.5 – 1.1 ppm has been determined for RA's in waters inhabited by fish (Richardson *et al.*, 1992). The most prevalent, toxic and most studied resin acid is dehydroabieitc acid (Johnsen *et al.*, 1998). Resin acids are considered to be the major contributors to the toxicity of pulp mill effluent towards fish and other aquatic organisms. Many studies have been performed to assess the effects of both long term and short term resin acid contamination and bioaccumulation in aquatic species (Johnsen *et*

al., 1995). Fish species inhabiting regions near pulp mills have been shown to produce a number of lethal and sub-lethal effects, including growth deficiencies, decreased reproductive fitness, neurotoxic effects, metabolic impairments, and decreased immune responses (Soimasuo *et al.*, 1998). Resin acids have structures which are very similar to hormones, and for this reason it is thought that they may be endocrine disrupters. Identification of chlorinated resin acids may be of particular concern as chlorinated compounds are more prone to bioaccumulation, are more toxic and are not detoxified in the body. High concentrations of these chemicals in fish tissue have also been shown to produce an off-taste in the fish.

Strict controls and government regulations have significantly decreased the concentration of these toxicants in released effluent. Pulp mill effluent is routinely treated prior to its release to surrounding waters in order to eliminate, or at least to reduce the concentrations of resin acids and other toxic compounds to levels which are no longer considered harmful (Shimp and Owens., 1993). As a result there have been many technological advancements in pulping, and bleaching which have effected in an improved quality of wastewaters. Modifications include alterations in cooking, substitution of chlorine dioxide for elemental chlorine, and biological treatment (Shimp and Owens., 1993). Such changes have had significant beneficial impact on effluent quality, as well as on the receiving environment.

Various analytical methods have already been developed to measure resin acids in environmental and biological samples, including treated and untreated pulp mill effluents, receiving waters, fish bile, and tissue samples. Methods, which have been developed to date include gas liquid chromatography (GLC), high performance liquid chromatography (HPLC), and spectrophotometric methods (Chow and Shepard., 1996) In addition to time-consuming analysis, current analytical methods are unable to separate completely all of the resin acids in a mixture. For this reason, it is necessary to develop monitoring programs that are sensitive, rugged, and simple and can be used to measure the toxic effects of RA's on fish species (Andersson and Forlin., 1988).

A correlation exists between bile resin acid levels in fish and exposure to resin acids in contaminated waters (Oikari., 1986). Concentrations of a toxicant and its metabolite can be 1,000-100,000 times higher in the bile than in water where the fish live (Leppanen *et al.*, 1999). Resin acid concentrations in tissues of exposed fish show similar correlation, however accumulated concentrations are lower. Fish bile analysis may be used as an accurate and sensitive marker for pulp and paper mill effluents (Andresson and Goran, 1994; Brumley *et al.*, 1998).

Capillary electrophoresis is a newly developed analytical separation method that can be applied to the analysis of a variety of samples. Cyclodextrin modified CE is one mode of CE which employs a mixture of negatively charged sulfobutylether-β-CD and neutral methyl-β-CD to effect a differential partitioning of the resin acid between the buffer and CD phase (Groom and Luong, 1997). Many CE separations have been developed for the separation and quantitation of pollutants, including polycyclic aromatic hydrocarbons, (PAH's), pentachlorphenols (PCP), phenols and chlorphenols (Brown *et al.*, 1996).

The aim of this research was to develop a novel separation utilizing cyclodextrin modified CE (CD-EKC), the specific objectives of this research were:

1. To optimize the separation of resin acid standards using CD-EKC with UV detection, to establish limits of detection.

2. To develop a fluorescent tagging method for the resin acid standards for separation and detection using the CD-EKC coupled with LIF detection.

3. To develop an extraction method for resin acids in both pulp mill effluent, and fish bile using spiked control samples, and contaminated samples.

4. To fluorescently tag the extracted resin acids and measure them using CD-EKC with LIF detection

5. To compare the methods of separations with conventional HPLC and GC results.

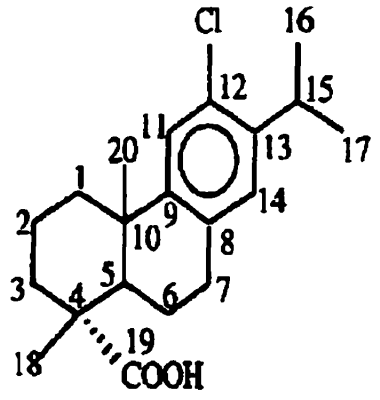
2. LITERATURE REVIEW

2.1. Resin Acids

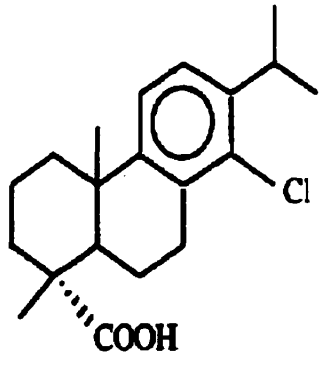
Resin acids (RAs), tricyclic diterpenoids with a carboxylic moiety are found in the oleoresin of coniferous trees. Figure 1 outlines the structures of the RA's, and gives their abbreviations as used in this thesis. RAs are released during chemical and mechanical pulping processes, with the resulting effluents constituting 70% of all of the compound toxic to aquatic organisms (Oikari, and Anas., 1985). Effluent from industrial pulp mills is treated and released into rivers, lakes and coastal waters and is potentially harmful to aquatic life. RAs are very resistent to degradation, are hydrophobic in nature and therefore have relatively low solubility, RAs are known to bioaccumulate in aquatic species. RAs are classified into 2 main groups, abietanes and pimaranes. Abietanes consist of abietic acid, dehydroabietic acid, neoabietic acid and palustric acid, and are classified based on their isopropyl substituent on the C-13 carbon. Pimarines consist of pimaric acid, isopimaric acid, sandarocopimaric acid and levopimaric acid, and possess two substituents, vinyl and methylmoieties on the C-13 carbon. Chlorobleaching of wood using Cl_2 or ClO_2 results in the formation of 2 chlorinated resin acid structures, 12-chlorodehydroabietic acid, and 12-14 dichlorodehyroabietic acid.

2.1.1. Resin acid Toxicity

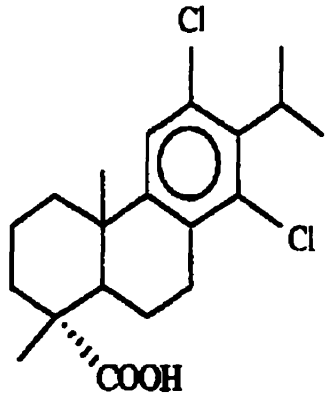
Resin acids constitute a major part of the compounds toxic to aquatic organisms in pulp mill effluent, therefore numerous and extensive studies have been performed both in the laboratory, and in the field to assess their effects on fish (Kennedy *et al.*, 1995). The most frequently studied, and the most lethal resin acid is dehydroabietic acid, this compound has been found to produce sub-lethal effects at concentrations of 5 to 20 µg/L (Johnsen *et al.*, 1998). A 96-h LC_{50} value ranging from 0.5-1.1 mg l^{-1} for aquatic organisms has been established. Fish species inhabiting regions near pulp mills have been shown to produce a number of lethal and sub-lethal effects, including liver damage, growth deficiencies, decreased reproductive fitness, neurotoxic effects, metabolic impairments, and decreased immune responses (Soimasuo *et al.*, (1995). Resin acids have structures, which are very similar to hormones, and for this reason it is thought that they



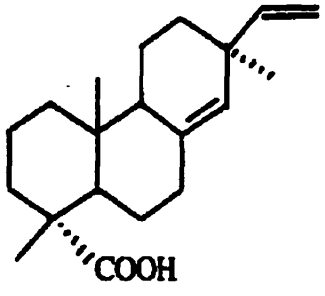
12-Chlorodehydroabietic acid
12-CDA



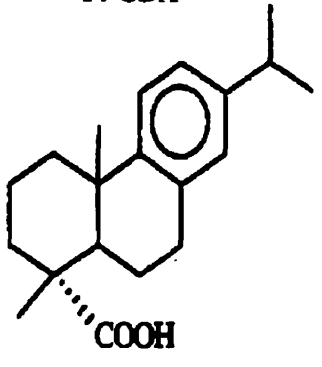
14-Chlorodehydroabietic acid,
14-CDA



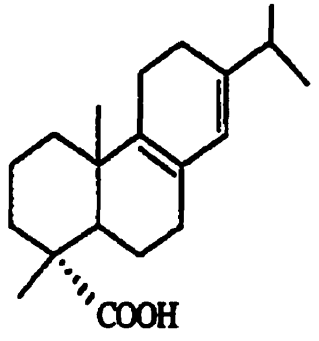
12,14 Chlorodehydroabieitc acid DCDA



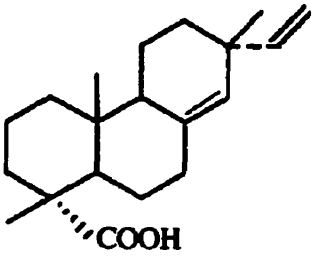
Pimaric acid
PIM,



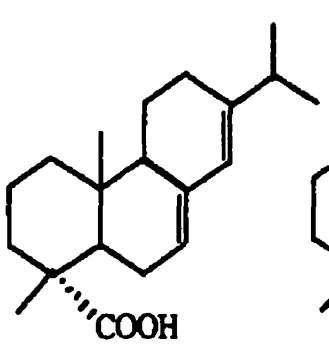
Dehydroabietic acid
DHA



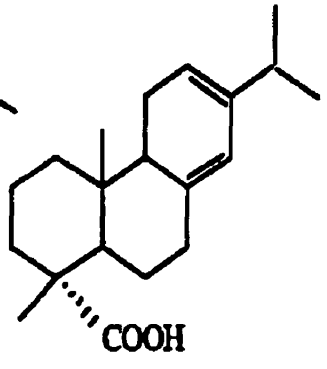
Palustric acid,
PAL,



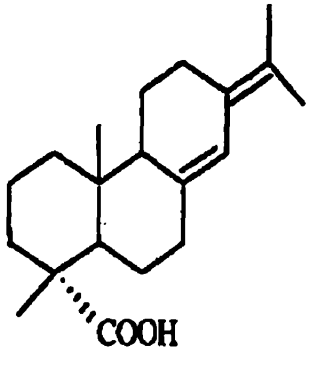
Sandarocopimaric,
SAND



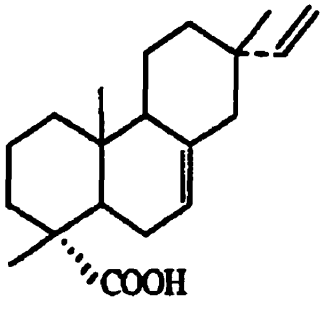
Abietic acid,
ABA



Levopimaric acid,
LEV



Neoabietic acid,
NEO,



Isopimaric acid
ISO

Figure 1. Chemical structures and peak assignments of selected resin acids

may be endocrine distruptors. Recent studies have shown that fish exposed to pulp mill effluent have consistently demonstrated reduced steroid levels, increased age to sexual maturation, and reduced expression of secondary sexual characteristics (Soimassuo *et al.*, 1995).

2.1.2. Pulp Mill Effluent Treatment Processes

Concern about discharged substances by the pulp and paper industry, in particular about chlorinated organic substances has resulted in worldwide governmental legislation and industrial regulations (Soimasuo *et al.*, 1998). Pulp mill effluents are composed of complex mixtures of organic and inorganic substances, which have remained chemically unknown or unidentified with potential harmful effects on the environment. The forestry industry has therefore introduced several alterations in the manufacturing of products, and treatment of wastewaters. These alterations include modified cooking, substitution of chlorine dioxide for elemental chlorine, and biological treatment. All of these processes have proven to have a beneficial effect on effluent quality as well as on the receiving environment (Soimasuo *et al.*, 1998).

2.1.3. Methods of Analysis of Resin Acids

There are many different methods which exist that are commonly used to measure resin acids in pulp mill effluent (Sharma *et al.*, 1996). These include colorimetric methods involving spectrometric analysis at 525 nm wavelength, GC methods which involve the extraction of resin and fatty acids with methyl *tert*-butyl ether followed by derivatization with diazomethane for analysis using flame ionization detection (FID). Richardson *et al.* (1992) have used HPLC for resin acid analysis, using dichloromethane or 4-bromomethoxycoumarin (4-BrMMc) to extract resin acids before HPLC analysis using both UV detection, and scanning fluorescence detection; solid phase extraction has also been used to isolate and concentrate resin acids from rosin, and paper mill deposits, and the electron capture detector has been used to measure pentafluorbenzyl esters of resin acids.

A satisfactory method must be fast and accurate to allow enough time to arrest the resin acid problem while it is still in the treatment stage (before being released to receiving waters). Another issue is sensitivity, because the LC50 of several resin acids is so low (< 0.1 ppm), it is necessary to use detection methods that will accurately measure very low concentrations of the analyte (Chow and Shepard., 1996). Fluorescence is one of the possibilities if RAs could be labeled with a fluorescent "tag" since they do not fluoresce naturally.

2.1.4. The Use of Fish Bile Metabolite Analysis as Exposure Biomarkers to PME

Fish bile analysis may allow for a more sensitive analysis of the effects of the levels of resin acids present in the receiving waters of pulp and paper mills (Krahn *et al.*, 1987). Exposure has been monitored through the measurement of biliary metabolites. The concentration of a toxicant may be 1,000-100,000 times higher in the bile than in the water the fish inhabit (Leppanen *et al.*, 1998). This technique has allowed researchers to show the extent of effluent toxicity on surrounding waters which are close to or below the detection limits of routine analytical methods (Oikari and Ojala, 1987).

2.2. Electrophoresis

Electrophoresis is an analytical method that is frequently applied for the separation and characterization of many proteins or nucleic acids. Its principle is that the charged particles of a sample migrate in an applied electrical field (Weston and Brown, 1997). If conducted in solution, samples are separated according to their surface net charge density. The most frequent applications, use gels (polyacrylamide, agarose) as a support medium. The presence of such a matrix adds a sieving effect so that particles can be characterized by both charge and size. Protein electrophoresis is often performed in the presence of a charged detergent like sodium dodecyl sulfate (SDS) which usually equalizes the surface charge and, therefore, allows for the determination of protein sizes on a single gel.

2.2.1. Capillary Electrophoresis

Capillary electrophoresis (CE), is a relatively newly developed separation technique which employs the use of narrow bore capillaries (20-100 μm) to perform a

highly efficient separation on a variety of analytes. Capillary electrophoresis offers a compliment to traditional techniques such as HPLC, and GC, or slab gel electrophoresis.

Table 1 tabulates the comparable terms commonly used in HPLC, which can be applied to CE. Many methods of analysis currently using these techniques will be converted to CE in the future because of the many advantages offered by it in terms of versatility, separation efficiency, sensitivity, analysis time and cost. CE is an analytical technique with a broad application range, enabling the user to analyze many sample types, from large complex macromolecules such as proteins and nucleic acids, to small solutes such as organic drugs, and inorganic ions and cations (Baker., 1995). Capillary electrophoresis is a method of analysis that is now considered to be accurate, precise, and dependable, it is being used routinely in the industry, at universities, and in government laboratories.

2.2.2. Components of a CE System

The principle components of a CE are a high voltage power supply, a fused silica capillary with an optical viewing window that passes through the optical center of a detector, a controllable high voltage power supply and two buffer reservoirs in which two electrodes, a cathode and anode are immersed. The system is connected to a data acquisition devise, and usually the system is controlled with a computer and system software.

The sample being analyzed is introduced to a properly conditioned capillary inlet with a timed pressure injection (0.5 psi), or a voltage controlled electrokinetic injection. A high electric field of up to 30 kV is applied to both ends of the capillary with the two ends of the capillary immersed in the anodic and cathodic buffer reservoirs. With the optical viewing window aligned with the detector, sample components pass by the detector window and are measured. The data is collected and a plot of detector response (absorbency) versus time, called an electropherogram is produced.

Electropherogram	Chromatogram
Applied Potential	Flow rate
Carrier electrolyte or buffer	Effluent or mobile phase
Injection mode (hydrostatic electrokinetic)	Injector
Migration time	Retention time
Electrophoretic velocity	Column capacity factor
Velocity	-N/A
Electroosmotic flow	-N/A
High-voltage power supply	Pump
Capillary	Column

Table 1. Comparison of Electrophoretic and Chromatographic Terms (Weston, 1997).

N/A not applicable

2.3. Principles of Separation

2.3.1. Electrophoretic Mobility Electrophoresis is the process by which ions migrate towards a cathode or anode when voltage is applied to them in an electrolytic solution. The rate of this movement is termed electrophoretic mobility μ_{ep} (cm^2/Vs), and relates to the speed at which ions migrate in a specific buffer, under an applied electric field.

$$\mu ep = \upsilon_{EP}/E = \upsilon_{EP} (L_d/t_m)/ (V/L_t) \ldots\ldots\ldots\ldots\ldots (1)$$

where,

υ_{EP} = Electrophoretic velocity of ion (cm/s)

E = Applied voltage (V/cm)

L_d = Length of the capillary to detector

L_t = Total capillary length

t_m = Migration time

Rates at which molecules move under high voltage separate depend on their charge to size ratio. Smaller molecules will migrate faster than larger sized ones, and ions with a higher charge will migrate faster than ones with a lower charge, neutral molecules will not be affected. The fractional drag of the buffer also effect mobility. Buffer viscosity and the size of the ion determine drag, therefore the higher the viscosity, or the larger the ion, the resistance is higher, and the net movement is slower. Ionic mobility CE is related to the charge to mass ratio as follows:

$$\mu_{ep} = (q/6\pi)\, \eta r \ldots\ldots\ldots\ldots\ldots (2)$$

where,

μ_{ep} = electrophoretic mobility

q = number of charges

η = solution viscosity

r = radius of the ion

2.3.2. Electrosomosis

Electrosmosis is one of the fundamentals processed that drives a CE separation. This phenomenon is the result of ionization of the acidic silanol groups on the inner lining of the capillary when it is in contact with a buffer solution. Electroosmotic flow (EOF) is effected by many factors, the primary one being the net charge on the capillary. At high pH, the silanol groups are dissociated, resulting in a negatively charged capillary surface. The negatively charged wall attracts positively charged ions from the buffer, creating an electrical double layer. When voltage is applied across the capillary, cations migrate away from the capillary wall, towards the cathode. This effect is often termed 'an electric pump' and the force created is so strong that if effectively pushes neutral ions towards the cathode, and also overwhelms anions, reversing their direction from the anode towards the cathode (Camilleri, 1998) . The order of migration is cations, neutrals and anions. EOF is defined by:

$$\upsilon_{eo} = \varepsilon\zeta/4\pi\eta \; E \quad \ldots\ldots\ldots\ldots\ldots\ldots (3)$$

where,

ε = dielectric constant

ζ = zeta potential (charge on capillary surface)

η = buffer viscosity

E = applied voltage (V/cm)

2.3.3. Effect of pH

EOF decreases with lower pH, is insignificant at pH below 4, and is strongest at pH above 9. Dongmei *et al.* (1999). At high pH, the silanol groups on the capillary are fully ionized, the linear velocity of EOF is around 2mm/s at pH 9 in 20 mm borate buffer solution, with a 50 μm i.d. capillary. The result is a volume flow of about 4 nL/s. In contrast, at pH 3 the EOF is much lower, measuring only about 0.5 nL/s (Kitagishi, 1997). EOF can therefore be controlled, or suppressed to effect a variety of different separations. pH is a very important factor involved in the separation, because it controls

the solutes charge state as well as the level of EOF. The overall migration time of a solute is therefore related to both the mobility of the solute, and the EOF.

2.3.4. Effect of Ionic Strength

Mobility is also dependant on ionic strength, utilizing buffers of lower ionic strength results in band broadening, and decreased resolution from adsorption of cations due to an increased EOF, and overloading effects on the capillary. Both EOF and mobility decrease with increased ionic strength, peaks become narrower, and resolution increases because these effects are reduced. Utilizing buffers of higher ionic strength also results in a higher current being generated, the result will be peak broadening due to joule heating effects. Lowering the running voltage can decrease these effects, however this will result in longer analysis times. Alternatively capillary diameter can be decreased, but this results in a shorter path length at the optical window, resulting in decreased sensitivity. Typical buffer concentrations range from 20 - 100 mM.

2.3.5. Potential Gradient/Field Strength

When voltage is applied across a capillary filled with buffer, current flows, the passage of this current causes the buffer temperature to increase by a process called 'Joule heating'. Heat is dissipated at the outside of the capillary wall, resulting in a temperature differential across the capillary wall, and temperature changes with time, due to ineffective heat dissipation. The rate of heat generation in a capillary can be approximated with the following equations:

$$dH/dt = iV/LA \quad \ldots\ldots\ldots\ldots\ldots\ldots (4)$$

$$dH/dt = kV^2/L^2 \quad \ldots\ldots\ldots\ldots\ldots\ldots (5)$$

where,

L = Capillary length

A = Cross sectional area of capillary

K = conductivity

The result of this temperature gradient is peak broadening. The effects of joule heating can be reduced by decreasing the applied potential, by utilizing capillaries with smaller diameters, by increasing the length of the capillary, or by using lower concentration buffers. Using a liquid cooling system that will effectively dissipate heat and allow higher running voltages to be used further reduces these affects. To determine the optimum balance between analysis speed, resolution, and run voltage and Ohm's Law plot can be performed by running at an increasing voltage, and monitoring the current generated at each applied voltage. These values can then be plotted, and a straight line should result, a positive deviation from linearity shows that the temperature removal capacity of the system has been exceeded. An optimal run voltage can be chosen based on this graph.

2.4. Modes of Capillary Electrophoresis

Capillary electrophoresis is comprised of four main separation modes, each having different operative and supportive characteristics. These separation modes are; capillary zone electrophoresis (CZE), capillary gel electrophoresis (CGE), capillary isoelectric focusing (CIEF) electrokinetic capillary electrophoresis (EKCD).

2.4.1. Capillary Zone Electrophoresis (CZE)

Capillary zone electrophoresis is the most basic form of capillary electrophoresis. This mode of separation is based on differences in charge to mass ratio of components being separated. A buffer solution is used to perform the separation under constant applied field strength throughout the length of the capillary, and components separate into discrete zones (Kitagishi, 1997). This separation is dependent on the nature of the sample, temperature, voltage applied, ionic strength, buffer pH and capillary diameter and length. EOF also plays a critical role in CZE. Separations of both large and small molecules, or of neutral ions, cations or anions can be accomplished using CZE.

2.4.2. Capillary Gel Electrophoresis (CGE)

Capillary gel electrophoresis involves using a capillary coated with a gel that acts as a molecular sieve to provide separation based on solute size. In this process, EOF is

eliminated and adsorption to the capillary wall is also greatly reduced. In CGE, the gel matrix acts to effectively impede solute migration based on molecular size. This is called the sieving effect. CGE can be used to separate solutes such as proteins and macromolecules, which have the same charge to mass ratio, but are of different size. Using CGE, separation occurs because larger molecules will be impeded during passage through a gel filled capillary, and smaller molecules will pass through easily (Baker, 1995). Under well-controlled conditions, mobility of the solute is inversely proportional to solute size.

2.4.3. Capillary Isoelectric Focusing

Capillary isoelectric focusing involves the separation of zwitterionic compounds such as peptides and proteins based on their isoelectric points. Separation occurs because of a pH gradient, which is established in the capillary. In this process, a coated capillary is used to eliminate EOF and decrease solute adsorption to the capillary. A pH gradient is established using carrier ampholytes that are zwitterionic, and are chosen so that the range of their isoelectric points comprises (Baker., 1995).

2.4.4. Capillary Electrokinetic Chromatography

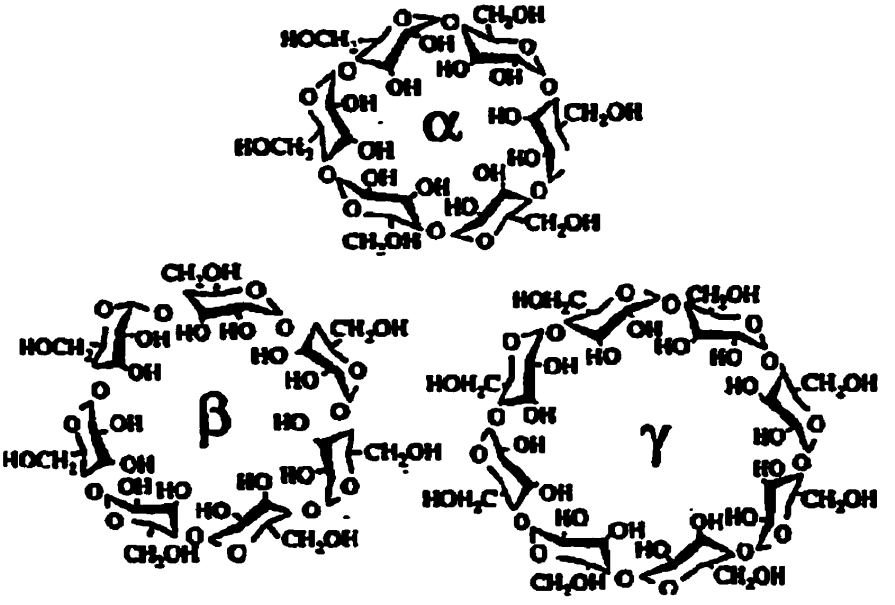
Capillary electrokinetic chromatography (EKC) is based on chromatographic principles using homogenous solutions containing an ionic "carrier", the unique characteristics of EKC is that it allows for the separation of both neutral and charged analytes (Otsuka and Terabe., 1996). There are various modes of EKC, one being micellar electrokinetic chromatography, which uses micellar solutions, or cyclodextrin modified capillary electrophoresis which uses cyclodextrins in solution.

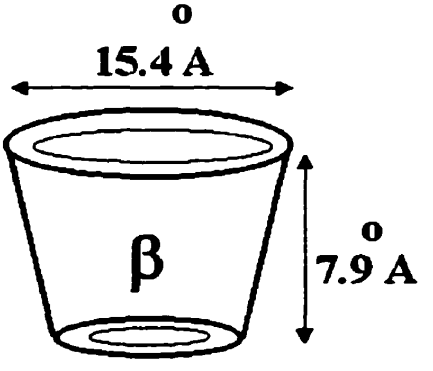
2.5. Cyclodextrins

Cyclodextrins (CD's) are cyclic oligosaccarides of D-glucose. Structurally CDs consists of 6, 7 or 8 glucose residues (α, β and γ, respectively). Figure 2 shows representative drawings of these structures, and Table 2 also outlines the physical

characteristic of the three CDs. The cyclic arrangement of the sugar residues creates a hydrophobic cavity such that molecules of the proper size shape and polarity enter the cavity forming inclusion complexes. Together, van der waals forces, hydrogen bonding, and solvent (hydrophobic) interactions account for the stable complexes that may be formed with chemical substances while in the apolar environment of the CD cavity. The complexes formed exist in equilibrium dependent upon the concentrations of the CD, the guest chemical and water (Baker., 1995). The rate at which the associated complex is formed is determined in the large part by the accessibility of the guest molecule to the CD cavity and the magnitude of the net thermodynamic driving force. Accessibility is dependent on the molecular geometry of the guest molecule, and the particle size. Complexes are usually formed rapidly, because even the most hydrophobic compounds are solvated by water to some degree, and therefore these hydrated portions are able to get past the hydrophilic hydroxyl groups at the entrance of the CD cavity (Baker., 1995). The actively interact with the hydroxyl groups on the rims of the CD torrid or they may shield the hydrophobic molecule from being repelled by the hydroxyl groups. When past the rim of the hydroxyls, the hydrating molecules of water are driven from the hydrophobic cavity, allowing the molecule to find it's most stable resting-place. After the molecule has entered the cavity, the "goodness of fit", as determined by the weak interactions taking place in the cavity, will make the final contribution to the degree of association component of the equilibrium process (Righetti. ,1996).

Cyclodextrins can be used in CE to provide a wide variety of separation selectivity's. The procedure of cyclodextrin modified capillary electrophoresis involves using a mixture of negatively charged sulfobutylether-β-cylcodextrin (SβCD) and neutral methyl-β-CD (MECD). The charged and neutral CD's act to effect a differential distribution (partitioning) of analyte between the buffer and CD phase in the capillary (Groom and Luong, 1997). Using this technique, one phase travels through the capillary at the same rate as the electrosmotic flow (neutral CD), and the other cyclodextrin phase (negatively charged) travels at a rate slower than the EOF. In the case of resin acids, each





MW 1135

Figure 2. (A) Structures of α,β, and γ cyclodextrins (B) and molecular weight and size of β cylclodextrin

Physical Characteristics	α	β	γ
Molecular Weight	972	1135	1297
Glucose Monomers	6	7	8
Internal Cavity Diameter (A°)	5	6	8
Water Solubility (g/100mL: at 25°C)	14.2	18.5	23.2
Surface Tension (mN/m)	71	71	71
Melting Range (° C)	255-260	255-265	240-245
Water of Crystallization	10.2	13-15	8-18
Water Molecules in Cavity	6	11	17

Table 2. Physical characteristics of α, β and γ cyclodextrins

RA will interact with the two phases to result in a baseline separation of very closely related resin acids Luong *et al.* (1999a).

2.6. Detection Modes in CE

The main types of detection used in CE, which are commercially available, include; ultraviolet (UV/VIS), photo-diode array (PDA), fluorescence, and laser induced fluorescence (LIF).

2.6.1. U.V. Detection

UV/VIS detectors are the most common and offer universal detection, meaning that they will be suitable to measure many types of analytes (Baker., 1995). These detectors will provide reasonable detection limits for most applications, however sensitivity may be a limiting factor in CE when trace analysis is required. One of the main factors, which reduce sensitivity in CE is the small inner diameter of the capillary, and therefore the small sample window that exists. This problem has been helped by the use of extended light capillaries, which are now commercially available. These capillaries are often called "bubble cells", and have windows, which are 3 of 4 times larger at the window, allowing for increased sensitivities (Weston and Brown., 1997).

2.6.2. Photodiode Array

Diode array detection allows for simultaneous multiple wavelength analysis of samples, and may be convenient to determine the optimal wavelength of a sample, as well it can provide information on substance purity.

2.6.3. Fluorescence Detection

Fluorescence occurs when an electron drops from an excited singlet into the ground state. In fluorescence, the light is given off for approximately 10^{-8} secs after the molecule has been excited (Baker., 1995). The energy that is given off in the form of fluorescence is less than the energy that is taken in, therefore the wavelength of light that is used for excitation is shorter than the wavelength of light that is given off, which is the emission wavelength. Therefore in fluorescence detection there are two wavelengths

specified, an excitation and an emission, and there are also 2 fluorescence spectras, which are associated with a specific compound which fluoresces. The excitation spectrum produced is a plot of intensity of emitted light versus wavelength of excited light, and the emission spectrum is a plot of intensity of emitted light versus wavelength of emitted light. The emission spectrum is approximately a mirror image the excitation spectrum, and maximum excitation wavelengths for excitation and emission can be determined from these spectras (Baker., 1995). Fluorescence detection is a sensitive technique, detection limits using fluorescence detection are one to three orders of a magnitude lower than those obtained using absorbance detection. Fluorescence is also a selective type of detection because only solutes which fluoresce, or which are fluorescently labeled can be detected.

2.6.4. Laser Induced Fluorescence Detection

The sensitivity of fluorescence detection is proportional to the intensity of emitted light. The greater the intensity of emitted light for a solute, the taller its peak will be, and accordingly there will be a higher signal-to-noise-ratio and increased sensitivity (Baker., 1995). The intensity of the light emitted is a function of the quantum efficiency of the fluorescing solute, as well as the number of excited molecules. Lasers produce a high-intensity radiation for excitation, and allow for a very high level of focusing. A laser can be focused to the inner diameter of the capillary, so that all of the light impinges right on the solutes. Laser induced fluorescence detection allows for very high sensitivities, detection limits of 10^{-18} - 10^{-21} have been reported (Baker., 1995).

Light from a laser is focused by a lens onto the window of the inner diameter of the capillary, a bandpass filter (or cutoff filter) is placed between the beam between the laser and the capillary. Emitted light is collected at right angles, to the incident beam, it is filtered through another band-pass-filter, and the signal is detected and amplified by a photomultiplier tube. The filter is used to minimize the chance of any scattered light from reaching the photomultiplier tube (Baker., 1995).

Many different commercial lasers are available, including He-Cd, argon, and helium – neon. In selecting a laser, there are three very important points to which need to be considered to optimize laser performance, and results, these are; excitation / emission maxima of solutes to be measured, laser output power, and the focusing ability of the laser (Baker., 1995). The laser wavelength should be at or near the excitation maxima of the solutes being analyzed, naturally fluorescing solutes in a multi-component solution will usually fluoresce at different emission maximas, so a compromise must be made when choosing laser wavelength. If a fluorescing agent is being used to tag the solutes, all of the solutes in solution will have the same fluorescence maxima, the fluorescent tag used can be chosen according to the laser used, and optimized for it. Laser output power effects sensitivity, higher laser power gives higher peak intensities, but if laser power is too high, it will cause photobleaching of the solutes being measured. Laser output power can be measured and optimized or adjusted using a power meter. A third and also very important factor involved in LIF detection is the ability to align and focus the laser light to a very small spot on the capillary.

2.7. Derivatization of resin acids

Resin acids are not naturally fluorescing compounds, therefore to obtain the sensitivity necessary for trace analysis, the resin acids would have to be derivatized and fluorescently tagged so the they could be detected using LIF detection. Richardson *et al.* (1992) has developed a method for derivatizing resin acids using both 4-bromomethyl-7-methoxycoumarin 4-Bromomethyl-7-acetooxycoumarin. The fluorescent label reacts with the carboxyl group of the resin acid in the presence of potassium carbonate to from an ester. Figure 3 shows the derivatization reaction for the conversion of abietic acid to it's derivatizing ester, using 4-BrMMc.

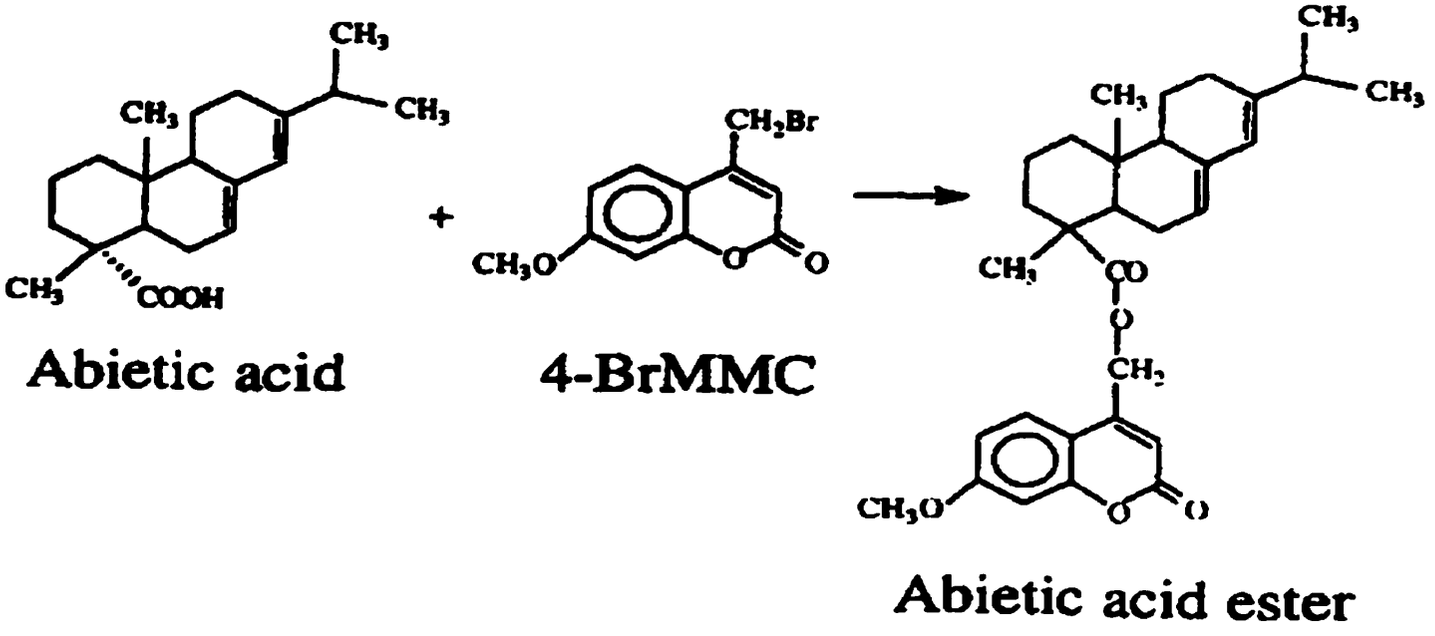


Figure 3. Derivatization reaction for the conversion of abietic acid to it's fluorescent derivative .

3. MATERIALS AND METHODS

3.1. Materials

β-Cyclodextrin sulfobutyl ether (SβCD), with an average degree of substitution (ds) of 4 , and a pK_a of 2 was purchased from CyDex Inc. (Overland Park, KS). Methyl–β–Cyclodextrin (MECD) with an average ds of 0.8 methyl groups per cyclodextrin, was purchased from Aldrich (Milwaukee, WI). The fluorogenic reagent 4-bromomehtyl-7-methoxycoumarin (4-BrMC) with a purity of 97% was purchased from Sigma Chemical Co. (St. Louis, MO). The resin acids (purity 95-99%) except for pimaric acid, (85-90%), chlorodehyroabietic acid, (85-95%), and abietic acid, (90-95%) were purchased from Helix Biotechnologies (Richmond, B.C.) and used without further purification. CDA contains equal mixtures of 12- and 14 -chloroisomers. Except for dehydroabietic acid (MW 300), chlorodehyroabietic acid (MW 336) and dichlorodehydrabietic acid (MW 370), the resin acids posses the same molecular weight of 302. Water was purified using a Zenopure Quadra 90 filtration system (Zenon Environmental, Burlington, ON.) with a specific resistance of 15 M μcm.

3.2 Instrumentation

All of the CE experiments were performed on a P/ACE 5500 capillary electrophoresis system (Beckman, Fullerton, CA). A 50 μm i.d capillary with a 37, 47 or 57 cm total length was used for all of the results presented. Capillary temperature was regulated at 25°C using a liquid coolant in a sealed cartridge. Experiments to monitor derivatized resin acids separation were performed using a Beckman LIF laser-induced fluorescence detector, module interfaced with an Omnichrome series 74 He-Cd laser (Melles Griot, Carlsbad, CA), 325 nm, 35 mW output. A 100 μm i.d. 140 μm. fused silica step-index optical fibre using a laser coupler (OZ Optics, Ottawa, ON) was used for transmission to the fluorescence detector. Fluorescence emission was measured using a 370 nm long-pass filter coupled with a 400 nm bandpass filter before the photomultiplier tube for the LIF detector. Using P/ACE Station software (Version 1.0 Beckman) facilitated data acquisition and analysis. The resulting signal was processed using a Dell Optiplex QXI computer for storage, data handling, and real time display of the

electropherograms. Sample injections were performed for 5 sec at 0.5 psi (3.44 kPa) injection at the capillary inlet. The separation voltage was applied using a 1-min ramping time, to prevent possible current breakdown. On-column UV detection was also performed using a Beckman modular UV detector operated at 214 nm.

The HPLC system consisted of a Waters (Waters, Milford, MA) model 590 pump, a Waters HPLC UV detector at 214 nm, (model 481), and a Waters scanning fluorescence detector (Model 747). The LC column used was not thermostated during operation, and all injections were made in duplicate using a Waters WISP model 710B autosampler, with an injection volume of 15 μL. The stock mobile phase was degassed by helium before use. The resin acid were separated using a C-18 Beckman octadeclysilane column, (4.6 mm x 15 cm, 5 μm), or a Supelco Supelcosil LC-8 column, (4.6 mm x 15cm, 3μm. The fluorescence characterization of the derivatized resin acids was measured with a Gilford Fluoro-IV spectrofluorometer (Gilford, Oberlin, OH). The detector PMT voltage was set at +550V.

3.3. Procedures

Solutions for CE experiments were prepared by diluting the appropriate concentrations of cyclodextrins with the stock buffer. All the solutions were sonicated, and filtered through 0.45 μm Millex-HV filter (Millipore, Bedford, MA) before use. Solutions of RAs for UV analysis were prepared by dissolving 10 mg of each RA in 10 mL of methanol (final concentration 1000 ppm), solutions were protected from the light by covering the vials with aluminum foil. New capillaries were conditioned using a high pressure rinse (20 psi) sequence of 5 min in 1 N HCl, 60 min in 1 N NaOH, 10 min in distilled H_20 and 30 min in running buffer. Before daily operation of the CE, the capillary was rinsed for 5 min in 1 N NaOH, 2 min in distilled H_20 and 5 mins in running buffer. This procedure was also used to recondition the capillary during daily use whenever necessary. The capillary was further conditioned by applying a 10 kV running voltage to the capillary for about 10 min prior to the first sample injection of the day.

3.4. Derivatization of the Resin Acids

Resin acids were derivatized according to the method established by Richardson *et al.* (1992). Stock solutions of RAs for derivatizations were prepared by dissolving 10 mg of each RA in 10 mL vial (final concentration 1,000 ppm). Standard solutions for derivatizations were prepared by mixing 100 μL of each of the ten resin acids (final concentration 33.3 ppm each RA) in 2 mL of acetone. For the derivatization, a 300 μl portion of this solution was mixed with 150 μL of 4-BrMMc (7.4 mM) and 5 mg of calcium carbonate (final concentration of each resin acid 3.3 ppm), and diluted with 2.550 mL of acetone in a light protected vial. Samples were sonicated in a ultrasonic bath for 20 mins at 25° C, and tightly sealed to ensure no loss of acetone between derivatization and analysis. After sonication, samples were filtered to remove calcium carbonate for analysis using a 0.45 μm filter. The derivatized resin acids were placed and stored in light-protected vials, (5 mL) during the course of the experiment.

3.5. Resin Acid Extraction Procedure from Pulp Mill Effluent

Extractions were performed according to a method reported by Oikari and Anas (1985) for the extraction of congugated and uncongugated resin acids from fish bile. Briefly, a 1mL sample of effluent was spiked with 10 μL of each resin acid (100 ppm each resin acid) the sample pH was adjusted to 3 using acetic acid. The sample was extracted 3-times using 1 mL aliquots of a 3:1 (v/v) hexane/acetone mixture (final concentration 33.3 ppm, each resin acid). A 300 μL portion of this sample was then mixed with 150 μL of 4-BrMMc (7.4 mM), and 5 mg of calcium carbonate, and diluted with 2.55 mL of acetone. The sample was then sonicated for 20 min at 25°C and filtered. A control sample was also run, which used 1 mL of distilled water spiked with 100 μL of acetone in which the extraction and derivatization was then performed as above. Contaminated effluent samples were also analyzed using this procedure.

3.6. Resin Acid Extraction Procedure from Fish Bile

3.6.1. Analysis of (Uncontaminated) Control Samples

Fish bile was extracted from rainbow trout supplied by Environment Canada (Ottawa, ON). Fish were sacrificed with a quick blow to the head, and the bile was extracted immediately from the gall bladder using a 20 gauge needle and syringe. After extraction the bile was frozen under nitrogen, and stored in light protected glass vials until analyzed. In this experiment, spiked bile samples were analyzed for free resin acids according to the same method developed by Oikari and Anas (1985). Because the total volume of bile available was small (approximately 500 μL), only 100 μL was used for each analysis. In this procedure 100 μL of bile was pH adjusted to 3 using acetic acid, and was spiked with 250 μL of resin acid mix (100 ppm concentration) and 750 μL of a 3:1 (v/v) hexane:acetone mixture, a sec extract using 250 μL of 3:1 (v/v) hexane/acetone was then taken. The extracts were combined, and 600 μL of the extract was mixed with 150 μL of 4-BrMMc (7.4 mM), and diluted with 2.25 mL of acetone.

3.6.2. Fish Experimental Approach and Sampling

Fish bile was collected from whitefish obtained from a field study performed at a pulp mill near Edmunston N.B. with the assistance of Noranda Technologies (Montreal, Qc), and Environment Canada. Whitesucker fish were used in the study, and these fish were obtained downstream from the pulp mill test site, meaning that they were theoretically free from any toxicants that would be released in the surrounding waters of the mill. Fish were netted and trapped and were sexed and placed in three separate stainless steel tanks, two of which contained circulating water that was pumped from the river and mixed with a 50% concentration of pulp mill effluent obtained from the site (one for male fish, one for female fish) a third tank was a control tank which contained only circulating water pumped in from a clean water source. The water in the tanks was continuously being mixed and circulated, the water temperature was maintained from 16-19 °C. The fish were not fed during the course of the study. After three days of exposure, the fish were sacrificed with a quick blow to the head, and the gallbladder was carefully removed, and the bile was evacuated into a vial. The samples were immediately

frozen in liquid nitrogen and stored until analyzed a total of 100 samples were collected, and each vial contained between 0.5 - 2.5 mL of bile, with an average quantity of 1 mL collected from each fish. Samples of the effluent used in the study (50:50, v/v, water/PME) were also taken for analysis.

3.6.3. Analysis of Bile Samples from Field Study

In this experiment, bile samples were analyzed for congugated and free resin acids according to the same method developed by Oikari and Anas (1985). Because the total volume of bile available from the study was high, samples were pooled to a volume of 5 mL. The bile was pH adjusted to 3 using acetic acid, and to release the congugated RAs, alkaline hydrolysis was performed using 0.5 M KOH in ethanol at 70 C for 2 hours. The Ras were then extracted using 3-times 1 mL aliquots of a 3:1 (v/v) hexane:acetone mixture. The extracts were combined, the extract was mixed with 150 μL of 4-BrMMc (7.4 mM), 5 mg of finely ground potassium carbonate and diluted with 1 mL of acetone. The samples were sonicated at 25°C for 25 min. The samples were filtered using a 45 μm filter, and evaporated to 500 μL under a stream of N_2.

4. RESULTS AND DISCUSSION

4.1. Development of a CD-EKC Capillary Electrophoretic UV Separation

Table 3 summarizes all of the key parameters tested and measured and in optimization process for the separation of the resin acids using cyclodextrin modified capillary electrophoresis with UV detection. Table 3 also details the optimized results for the separation. Luong *et al.* (1999a) have previously reported the separation of resin acids using CD-EKC. The aim of the present work was to further confirm, and possibly optimize these findings. It is important to also note that CDA contains equal mixtures of 12- and 14-chloroisomers, the isomer standards are not commercially available, therefore it was not possible to identify them separately. For the purpose of this study, the CDA isomers are identified as CDA 12, or 14.

4.1.1. Spectrophotometric Analysis

Initially, samples of each resin acid were dissolved in methanol and measured using a Beckman UV spectrophotometer. Figure 4 shows a sample of the absorbance spectrum for a selected resin acid, abietic acid. Samples of each of the resin acids were analyzed with a wavelength scan ranging from 200 - 400 nm. The experimental results (not shown) indicated that the UV absorbance of all of the resin acids was in the range of 220 - 260 nm. Some of the resin acids also showed an increased absorbance at a wavelength of 254 nm; however, other RAs showed no absorbance at this wavelength. Because simultaneous analysis of all of the eleven resin acids was desired, a running wavelength of 214 nm was chosen and used for all further CE experiments.

4.1.2. Effect of SBCD and MECD Concentrations on CD-EKC UV Separation

The concentration of SβCD and MECD in the separation was shown to have significant importance on the electrophoretic separation efficiency. Figure 5 shows the effect of varying the concentration ratios of SβCD and MECD on the CE separation efficiency. In this separation, a negatively charged cyclodextrin (SβCD) is used as a 'pseudostationary' phase with an uncharged (neutral) cyclodextrin (MECD). The neutral CD moves with the bulk flow of the EOF, while the negatively charged CD will move at

Parameter	Measured	Optimum Condition
Detection Wavelength (nm)	200,214.254	214
Limit of Detection (ppm)	1-100	10
CD Concentration (mM) SβCD:MBCD	Various	35:15
Buffer pH	Sodium borate 9.25 Sodium acetate 4.5 Sodium phosphate 7.0	Sodium borate pH 9.25
Buffer Ionic Strength (mM)	10,25,50,72	72
Separation Voltage (kV)	5,10,15,20	15
Capillary Length (cm)	27,37,47,57	37
Capillary Inner Diameter (μm)	20,50,75,360	50,360
Buffer Modifiers	Acetonitrile, Methanol	None

Table 3. Summary of conditions tested and parameters optimized for the UV separation of resin acids.

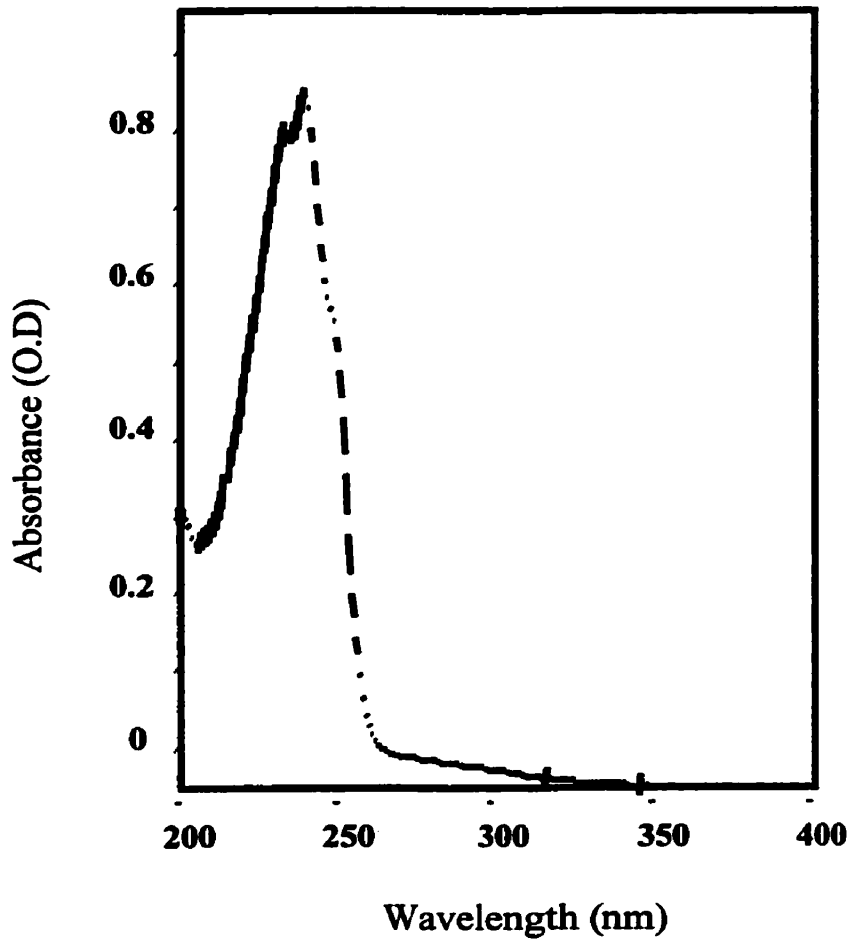


Figure 4. Spectrophotometric analysis of abietic resin acid (1ppm) dissolved in methanol.

a slower rate than the EOF. The benefit of having the charged CD in the separation buffer is that it provides two phases for the negatively charged resin acids to associate with the CDs. Each resin acid will effectively interact differently with the CD phase and as a result a multi-component separation of all resin acids is achieved by optimizing the concentrations and ratios of each of the CDs used (Luong *et al.*, 1999a).

To determine the concentration and ratio of each CD required to effectively separate each of the resin acids, a series of experiments were run in which a quantity of each of the CDs was added to each buffer (sodium acetate pH 4.5 or sodium borate pH 9.25) ranging in concentration from 0 to 40 mM of MECD or SβCD. Figure 5 shows the effect of various concentration ratios on the separation of resin acids using sodium borate buffer pH 9.25, with a 37 cm. capillary with an i.d. of 50 μm and a applied voltage of 15 kV. The electropherogram A clearly shows that with no CDs in the running buffer, the resin acids migrate as a bulk phase close to the EOF. It was also found that with concentrations of SβCD below 30mM the resin acids still co-eluted. In electropherogram B and C of Figure 5, we see a more clear separation of peaks by increasing the concentration of SβCD from 30 mM to 35 mM. From these experiments it was found that the ideal concentration of SβCD was 35 mM, increasing the concentration beyond this still resulted in a complete separation; however, the migration time of the peaks was also increased because the concentration of the negatively charged SβCD was increased. The concentration of MECD was not as critical for an efficient separation of all peaks. Various concentrations ranging from 0 - 40 mM were run, results showed that separation time was decreased with higher concentrations of MECD in the running buffer because it is neutral and results in a faster EOF because resin acids have less opportunity to interact with the charged CD; these results were similar those obtained by Luong *et al.* (1999a) for the separation of resin acids using CD-EKC with UV detection. Figure 5, electropherogram (D) shows the ideal concentrations of SβCD and MECD are 35 mM and 15 mM respectively. These concentrations and ratios result in the most efficient separation in terms of the separation of the resin acids, and the time of analysis.

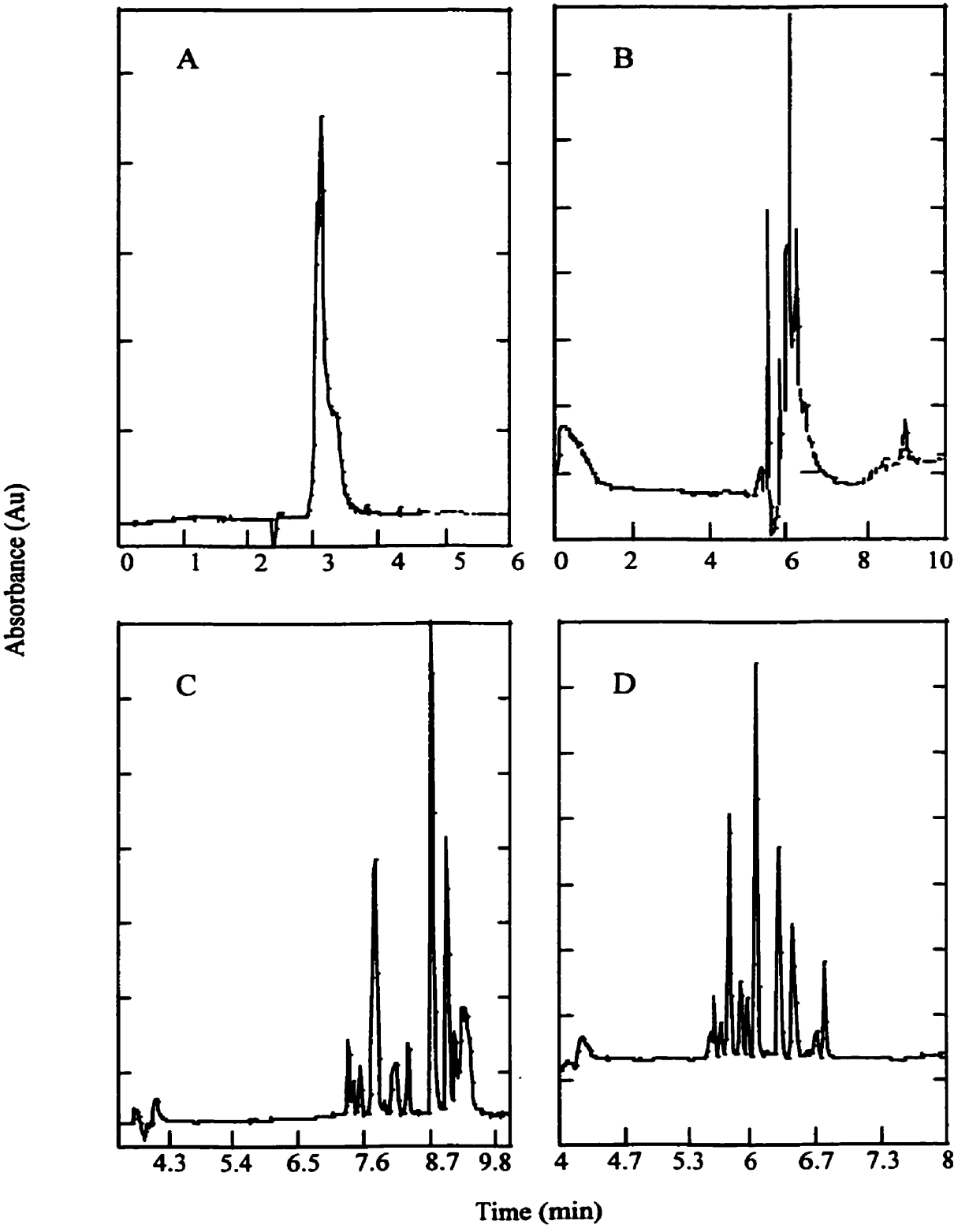


Figure 5. The effect of concentration ratios of SβCD and MECD on the separation of selected resin acids, using a 37 cm capillary 50 μm i.d. 5 sec pressure injection, with an applied voltage of 15 Kv. The resin acid concentration is 100 ppm. (A) 50 mM Na Borate pH 9.25 (B) 30:20 mM SβCD:MECD (C) 35:30 SβCD:MECD (D) 35:15 SβCD:MECD

4.1.3. Effect of Buffer Ionic Strength

The effect of buffer ionic strength on CE separation efficiency was examined by preparing a sodium borate buffer pH 9.25 containing 35 mM SβCD and 15 mM MECD sodium borate pH 9.25 at ionic strengths ranging from 10-72 mM. The separation was run using a 37 cm capillary, with a 50 μm i.d. and the separation voltage applied was 15 kV. Figure 6 shows that increasing buffer ionic strength did increase the resolution of peaks. Overloading, and adsorption effects on the capillary wall are reduced by increasing buffer ionic strength, increasing buffer ionic strength should effectively decrease separation time due to the increase in cationic charges on the interior of the capillary, and therefore increased EOF (Weston and Brown, 1997). This is also shown in Figure 6, although the EOF is similar at ionic strengths of 25 and 50 mM, we see almost identical EOF's. However, as the buffer ionic strength is increased to 72 mM, the EOF also decreases by approximately 1 min. Generally running buffers should range from 25 - 100 mM depending on the application; however, using buffers of high ionic strength also results in higher generated running currents, and eventually results in peak broadening, so a compromise must be made. The ideal concentration was found to be 72 mM, we were unable to try buffers of higher concentrations because we were limited by the effects of using SβCD and MECD (dissolved in water) in our running buffer. However, this was not of particular concern, as the effects of buffer ionic strength seemed to be less critical than other parameters.

4.1.4. Effect of Applied Voltage

Figure 7 shows how increasing running voltage directly acts to reduce analysis time, however this results in an increased current being generated across the capillary. If the capillary is not able to 'cool' itself at a fast enough rate, a temperature differential results across the capillary, yielding broadened peaks, and therefore a decrease in resolution of peaks. Because our buffer contains a charged cyclodextrin (SβCD), the current generated by it will also be greater, meaning that we are limited as to how much potential can be applied to the capillary for a separation. Reducing the inner diameter of the capillary will also reduce the generated current, however there is also a loss of

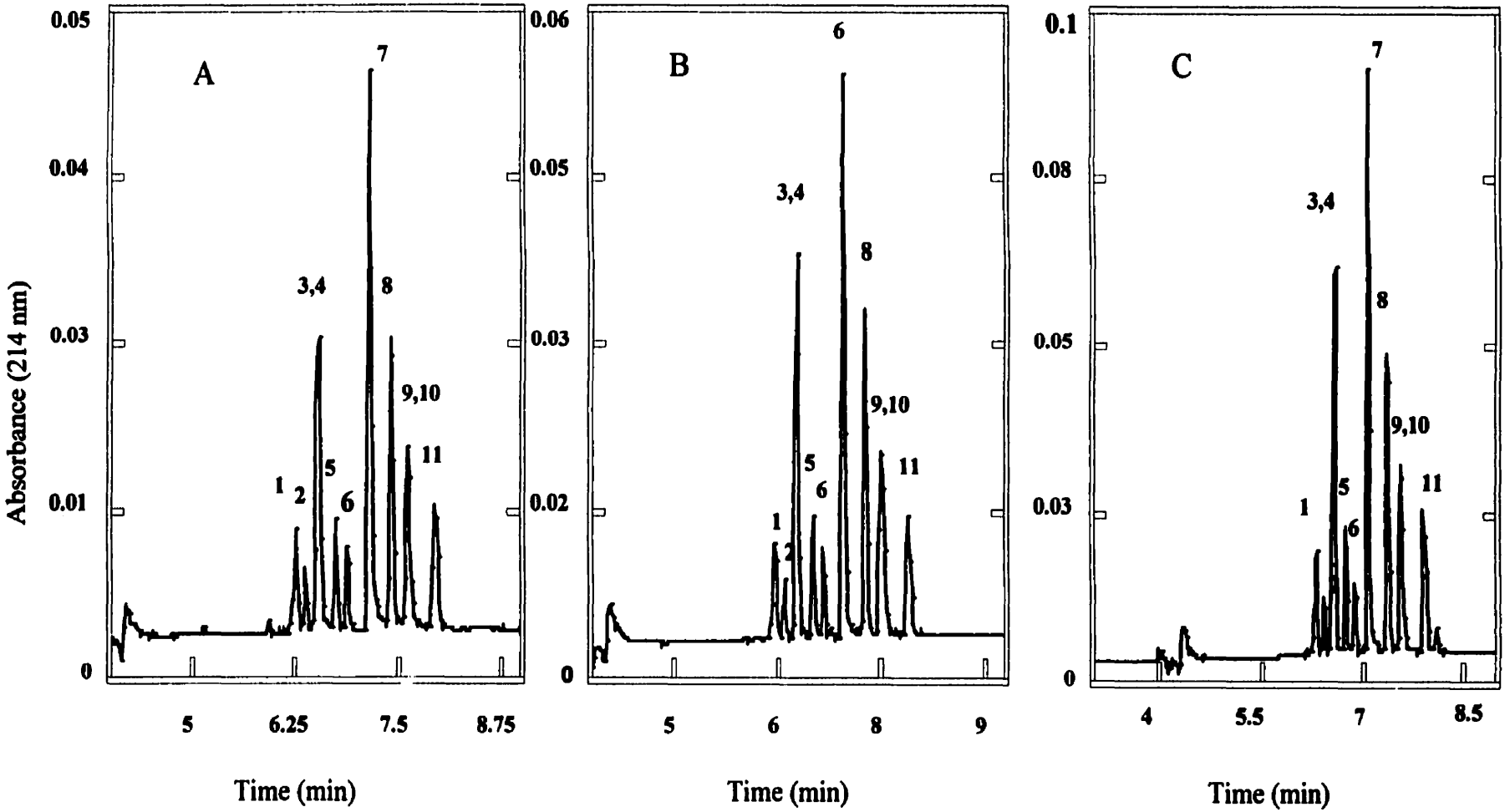


Figure 6. Effect of buffer ionic strength on CD-EKC UV separation of selected resin acids with a sodium borate buffer pH 9.25 containing of 35 mM SβCD 15 mM MECD, using a 37 cm capillary 50 μm i.d. 5 sec pressure injection, and an applied voltage 15 kv. (A) 25mM (B) 50 mM (C) 72 mM Na Borate buffer. The concentration of each resin acid is 100 ppm. Peak identification: Peak 1 NEO 2. LEV 3,4 PIM/SAND 5 ISO 6. PAL 7. DCDA 8. DHA 9,10 CDA (12,14), ABA, 11. CDA (12,14).

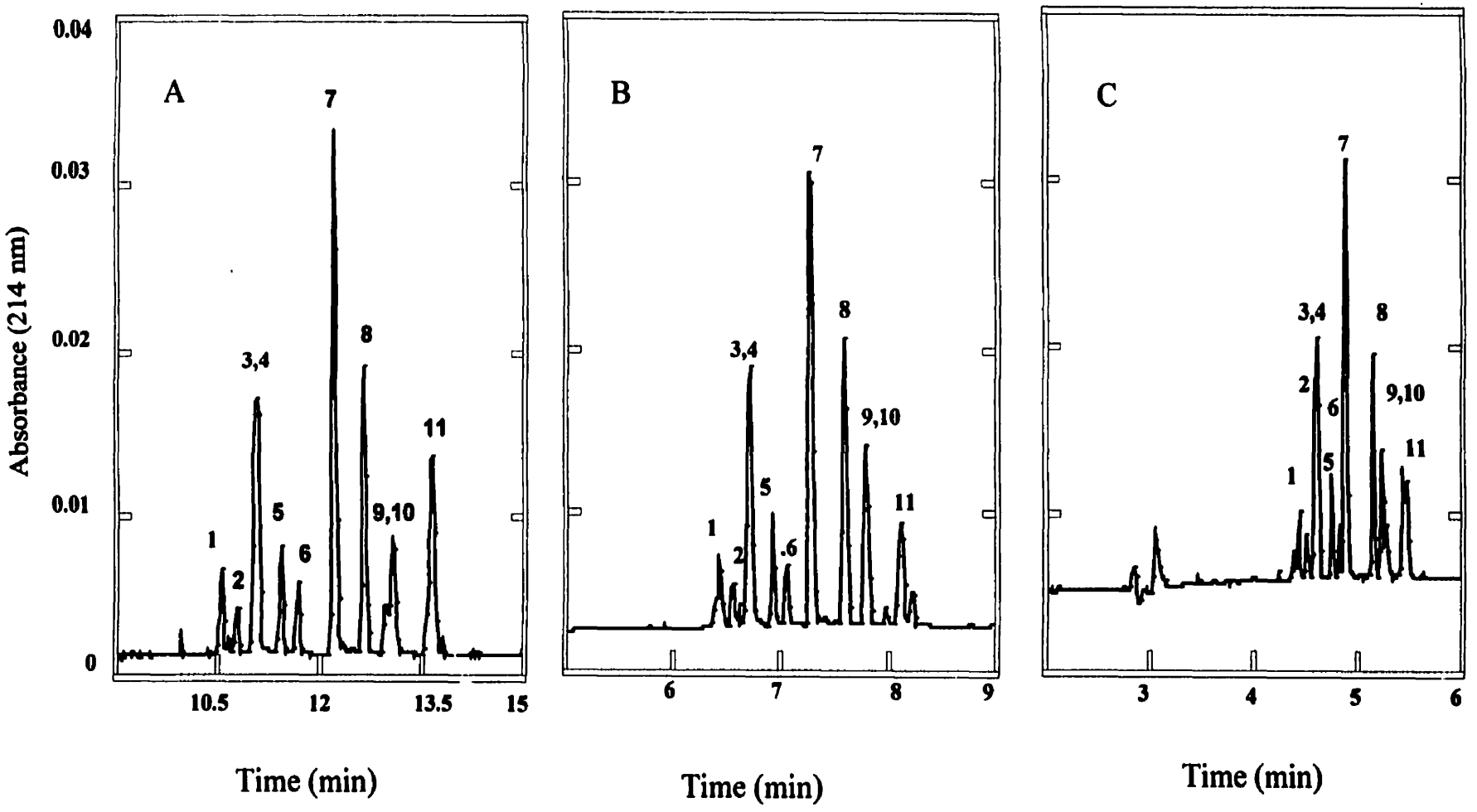


Figure 7. Effect of running voltage on CD-EKC UV separation of selected resin acids with a sodium borate buffer of pH 9.25 containing 35 mM SβCD 15 mM MECD using a 37 cm capillary 50 µm i.d. and a 5 sec pressure injection. Applied voltage:(A)10 (B) 15 kV (C) 20 kV,. The concentration of resin acids is 100 ppm. Peak identification: Peak 1 NEO 2. LEV 3,4 PIM/SAND 5 ISO 6. PAL 7. DCDA 8. DHA 9,10 CDA (12,14) ABA, 11. CDA (12,14).

sensitivity as the window becomes narrower. Also because there are so many components (RAs) to separate in this separation, some of the peaks will co-migrate because the voltage is too high. In Figure 7 the effect of increasing voltage on separation time is clearly shown using a sodium borate buffer pH 9.25 containing 35 mM SβCD and 15 mM MECD sodium borate, and applying a running voltages ranging from 10 to 20 kV. Electropherogram (Fig 7A) shows that at a separation voltage of 15 kV the separation takes 14 min, in comparison (Fig. 7B) shows the separation at a running voltage of 10 kV takes 8.5 min. Accordingly with an applied voltage of 20 kV (Fig. 7C), the separation is completed in less than 6 min which can be seen in Figure 7C. Based on these results, the optimum balance between analysis speed and peak resolution was found to be 15 kV.

4.1.5. Effect of Capillary Length

A series of experiments were run during the optimization process to determine that effect of capillary length on the separation quality. It was found that the separation time could be decreased from 12 to 8 min by shortening the capillary from 57 to 37 cm total length, and maintaining the voltage at 15 kV. All these experiments were performed using sodium borate buffer pH 9.25, 35 mM SβCD 15 mM MECD, with a 50 μm i.d. capillary. A comparison of Figure 8 to Figure 10 demonstrates that the separation quality is not affected, while separation run time is shortened. Decreasing the capillary a further 10-cm (27 cm) resulted in too high of a running current being generated, accordingly the optimal capillary length was chosen to be 37 cm.

4.1.6. Effect of capillary inner diameter

The narrow separation window (path length) of a capillary is a definate limitation in CE, especially in UV detection. Capillaries must remain narrow (20-100 μm) in order to control and minimize the amount of heat generated by high running voltages. To compensate for this, specialized extended light path capillaries exist which have a standard internal diameter but which have an extended light path at the capillary window (360 μm), allowing for increased sensitivities due to the longer pathlength. These

capillaries were tested during the optimization process, and they were found to result in twice the sensitivity achieved with a regular capillary of 50 μm i.d. (results not shown). Unfortunately these capillaries are also costly and furthermore, in LIF detection the more important aspect in achieving high sensitivity is focusing of the light and the capillary, meaning that pathlength is not necessarily a critical parameter. For this reason extended light path capillaries were not used past the optimization phase of this work.

4.1.7. The Use of Buffer Modifiers

A series of experiments were run involving the use of organic buffer modifiers using both sodium acetate and sodium borate (results not shown). Both acetonitrile and methanol were added to the running buffers in concentrations ranging from 2.5 - 10%. Each of the modifiers exhibited marked effects on the overall separation. The overall separation was longer, as expected due to the increase in viscosity of the buffer by the addition of solvent. Experiments showed that with the addition of 10% methanol or acetonitrile to a separation buffer of 50 mM sodium borate with a pH of 9.25, containing 35 mM SβCD, 15 mM MECD and using a 37 cm capillary (50 μm i.d.), and 15 Kv applied potential, a separation which was normally complete 8 min was completed in 25. Ideally the peaks which were co-eluting would become more baseline separated due to the change in buffer composition, and in theory the voltage applied could be increased to decrease overall separation time and optimize the separation. However, in this case, the peaks seemed to co-elute more with the use of modifiers, and changing applied voltage or concentration of modifiers was not beneficial. Furthermore, there was less reproducibility, and more erratic behavior from the capillary was exhibited. For this reason the use of buffer modifiers was discontinued, as it was shown that they could not improve the separation quality.

4.1.8. Effect of pH on Resin Acid Separation

The effect of pH on the separation of resin acids was assessed using a range of buffers, including sodium acetate at pH 4.50, sodium phosphate at pH 7.00, and sodium borate at pH 9.25. While different buffers were being tested, it was found that the

phosphate buffer consistently caused current crashes, and results were often not reproducible (results not shown) eventually the phosphate buffer was disregarded, and further studies concentrated on sodium acetate and sodium borate buffers only.

Figure 8 shows the separation and identification of selected resin acids using a sodium borate buffer of pH 9.25 with 35 mM SβCD and 15 mM MECD, the capillary used was 57 cm in length and the applied voltage was 15 kV, with a 5 sec pressure injection. The concentration of each resin acid was 100 ppm. The separation was 12 min in length, and using these conditions resulted in 9 resolved peaks. Peak identification was determined by running consecutive analysis and 'spiking' each sample with a concentration of one particular resin acid each time. The peak was identified when the signal increased at one specific peak, and the spiking was also verified in two different experimental sets to re-verify peak identifications (results not shown). Peak identifications are also shown in Figure 8. These results show that most of the resin acids were baseline separated except for pimaric and sandarocopimaric acid (peaks 3 and 4), and abietic acid and chlorodehydroabieitc acid (12 or 14) (peaks 9 and 10). Because there is no chemical standard available for the CDA 12 or 14 isomers, it was impossible to identify these two isomers. These results are comparable to results reported by Luong *et al.* (1999a), although the optimal SβCD concentration is higher, the sequence of peak migration is the same.

Figure 9 shows the separation performed under the exact same parameters as above, but using a sodium acetate buffer of pH 4.5. The electropherogram demonstrates the decreased effect of EOF at low pH, because of a resulting net decrease in movement of analytes in the capillary due to the lower number wall charges. Under these conditions, the separation was completed in only 25 min, and the separation window was also wider (10 min) as opposed to the less than 4 min separation window achieved using borate buffer. Accordingly, the peak identification, which resulted, was also different. The results show that most of the resin acids were baseline separated, except for

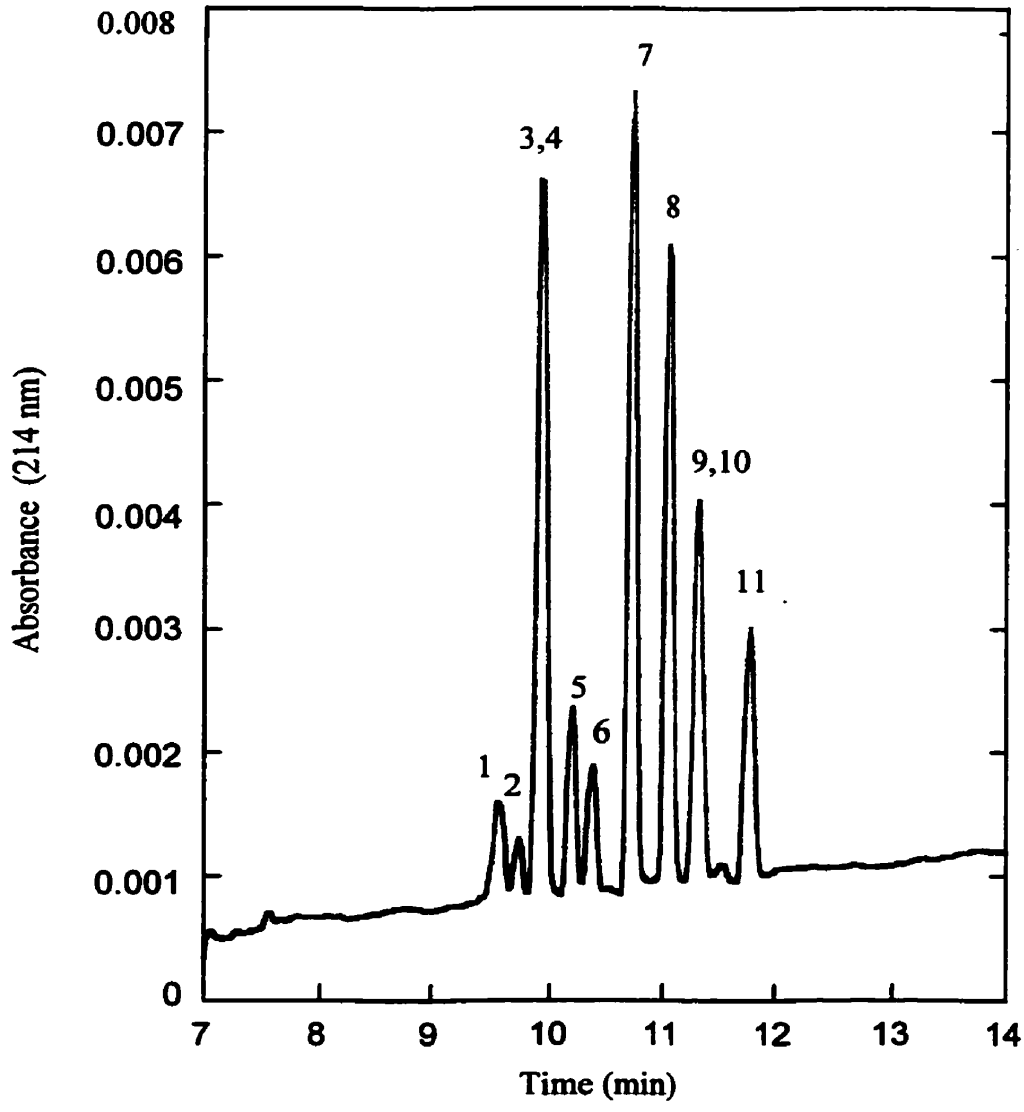


Figure 8. Separation of selected resin acids using a 50 mM sodium borate buffer, pH 9.25 containing 35 mM SβCD and 15 mM MECD. Using a 57 cm capillary with a 50 μm i.d. a 5 sec pressure injection. and a separation voltage of 15 kV. The concentration of each resin acid is 100 ppm. Peak identification: Peak 1 NEO 2. LEV 3,4 PIM/SAND 5 ISO 6. PAL 7. DCDA 8. DHA 9,10. CDA (12,14), ABA, 11. CDA (12,14)

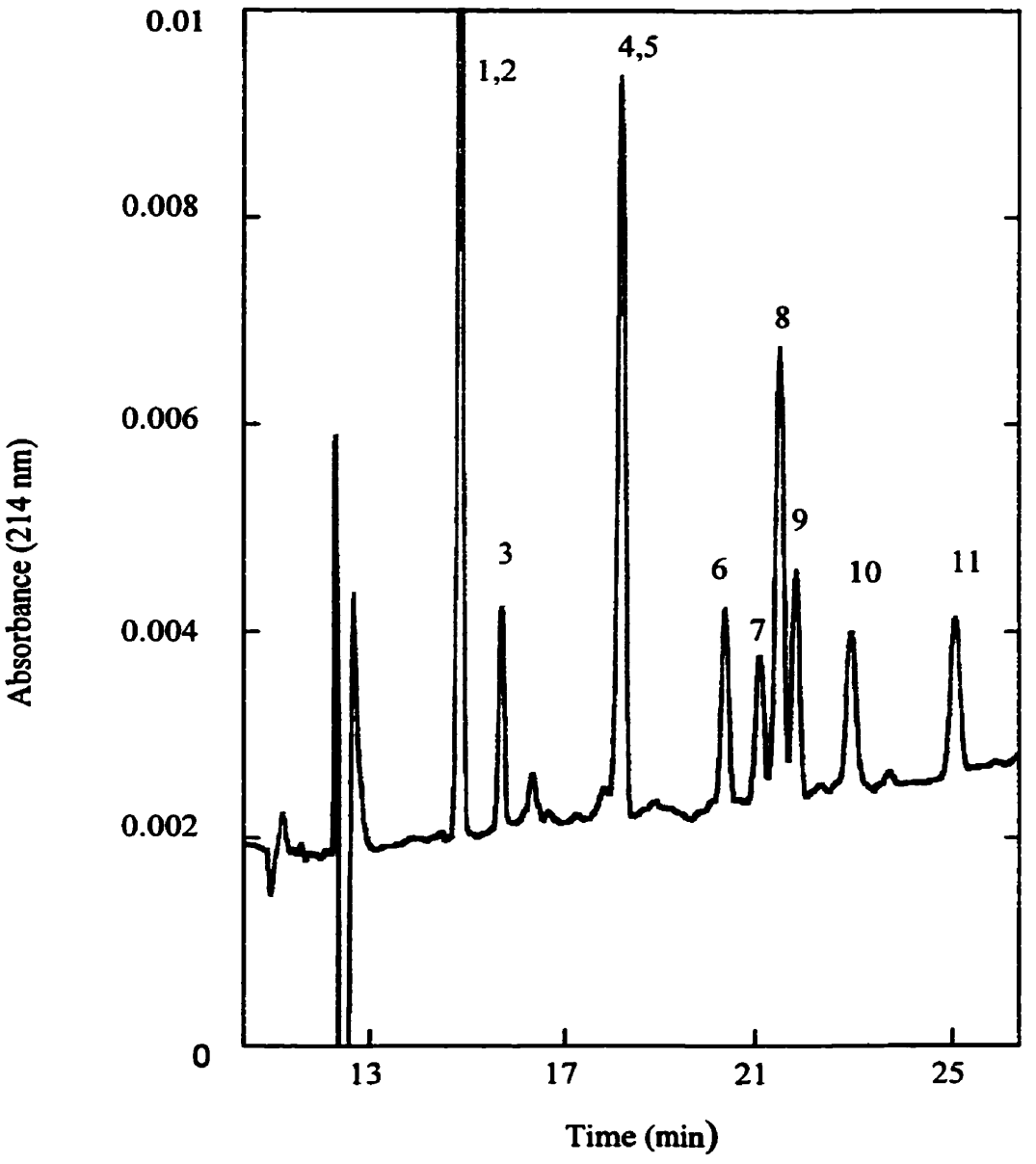


Figure 9. Separation of selected resin acids using a separation buffer of 50 mM sodium acetate buffer at pH 4.50, containing 35 mM SβCD and 15 mM MECD. Using a 57 cm capillary with a 50 μm i.d. a 5 sec pressure injection and a separation voltage of 15 kV. The concentration of resin acids is 100 ppm. Peak Identification: Peak 1,2. DCDA , CDA (12,14) 3. PIM 4,5 DHA PAL 6. ABA 7. LEV 8. SAND 9. CDA (12,14) 10. ISO 11. NEO.

chlorodehydroabietic acid (12 or 14) and dehydroabietic acid (peaks 1 and 2) which co-eluted, as did dehydroabietic acid and palustric acid (peaks 4 and 5). At this pH, the resin acids are expected to be neutral because their pka's range from 5.7-6.4 (Luong *et al.*, 1999a), SβCD however is still negatively charged with a pka of 2. It is this that was attributed to the different ionizations of each of the resin acids using a running buffer at pH 4.5, or at pH 9.25. Peak identification was performed by spiking each RA into the sample and recording the increase in peak height to the assigned peak.

4.1.9. Optimized Separation

As indicated previously, a summary of all of the parameters tested during the optimization process, as well as the optimal conditions found for the separation of the resin acids can be see in Table 3. The optimal separation condition using CD-EKC were found using a detection wavelength of 214 nm, a buffer of sodium borate 72 mM, pH 9.25, 35 mM SβCD 15 mM MECD and a separation voltage of 15 kV using a capillary of 37 cm total length with a 50 μm i.d. capillary. Figure 10 represents the optimized separation of the selected resin acids. These results are significant, in that a majority of the RAs were easily separated in a short period of time, significantly less timer than in methods involving GC or HPLC analysis, and more importantly that chlorinated compounds, which may be considered more toxic and more dangerous were also easily detected.

4.1.10. Comparison Using Reversed Phase HPLC with UV detection

A method for the quantitative analysis of resin acids in effluent and water samples using high performance liquid chromatography has already been developed (Chow and Shepard, 1996). This method was optimized by using a C18 column (4.6 mm x 5 cm, 3 μm), a mobile phase of 76% methanol and 24% distilled water which contained 1% acetic acid. The flow rate was 1ml/min, and the detection wavelength selected was 214 nm. Figure 11 shows the result of the separation of the resin acids which took over 80 min. The resin acids were not completely resolved and less resolved peaks were observed than with the developed CE method. Seven peaks in total were defined, the elution of

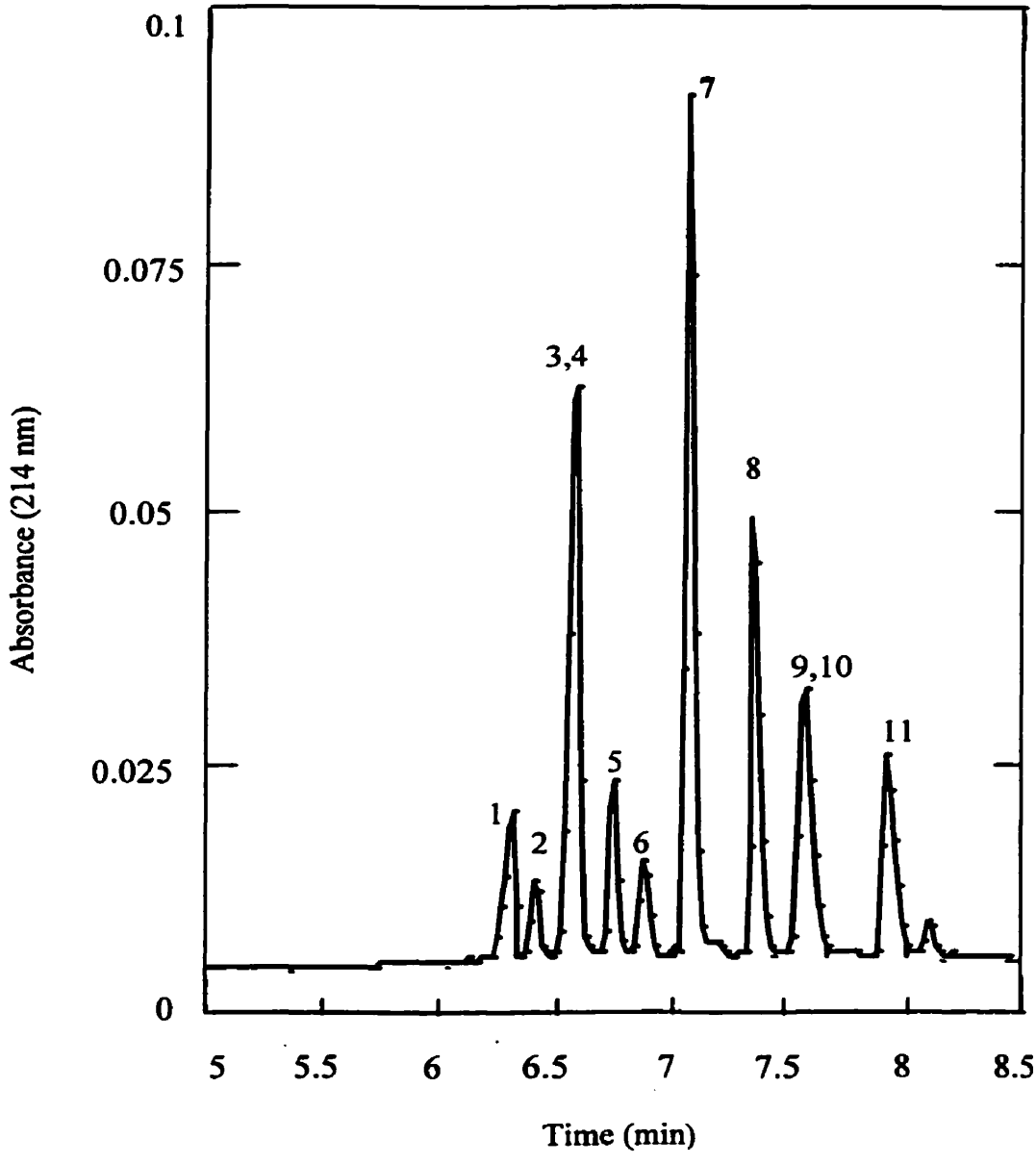


Figure 10. Optimized conditions for the separation of selected resin acids using a separation buffer of 72 mM sodium borate of pH 9.25, containing 35 mM SβCD 15 mM MECD. With a 37 cm capillary 50 μm i.d. separation, a 5 sec pressure injection, and a separtation voltage of 15 kV. The concentration of resin acids is 100 ppm. Peak identification: Peak 1 NEO 2. LEV 3,4 PIM/SAND 5 ISO 6. PAL 7. DCDA 8. DHA 9,10 CDA (12,14), ABA, 11. CDA (12, 14).

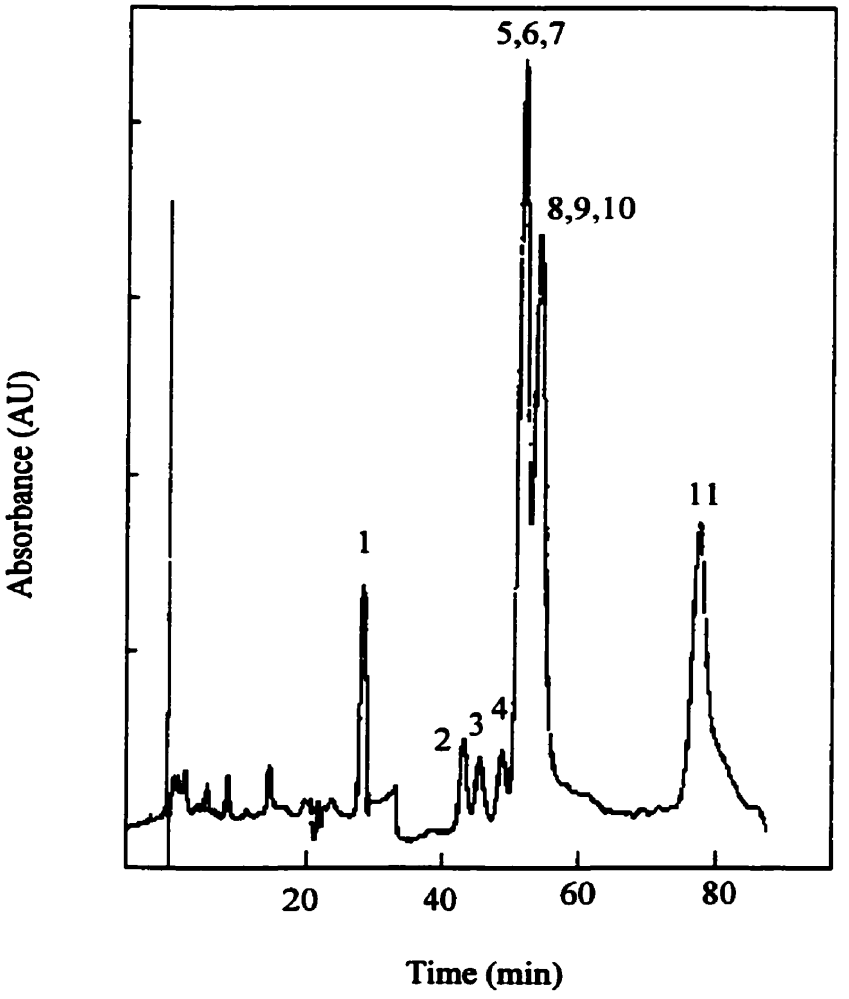


Figure 11. HPLC UV detection of selected resin acids. Detection 214 nm C18 column 50 mm i.d. mobile phase: MeOH:H_20 containing 1% Acetic acid (76:24) 1 mL/min flow rate the resin acid concentration was 100 ppm. Peak Identification 1 DHA 2 CDA (12,14) 3 CDA (12,14) 4 LEV 5,6,7 PIM, NEO, SAND 8,9,10 ABA,PAL, ISO 11 DCDA

dehydroabietic acid (peak 1), followed by CDA (12 or 14) (peak 2) CDA (12 or 14) (peak 3), and with the elution of levopimaric acid (peak 4) pimaric, neoabietic, sandarocopimric (peaks 5,6 and 7) abietic, palustric and isopimaric acid co-eluted (peaks 8,9 and 10) and finally peak 11 dichlorodehydroabietic acid. An effort was made to alter run conditions, including mobile phase composition, the utilization of buffer modifiers or column type, but none of these changes resulted in an improved separation. Poor resolution of peaks can generally be attributed to the very similar chemical structures of resin acids, as mentioned before, resin acids have very similar chemical structures, differing only by the location of the double bond molecular weights, and pKa's (Luong *et al.*, 1999a).

4.2. Characterization of Derivatized Resin Acids

4.2.1. Derivatization of Resin Acids

Although a successful separation was achieved using UV detection, it was necessary to develop a more sensitive separation method that could be applied to the detection of trace quantities, (in the ppb range) of Ras in effluent of fish bile. As discussed earlier, LIF detection is ideal for achieving low detection limits because it allows for a highly sensitive and highly selective sample analysis. Because resin acids do not fluoresce naturally, it was necessary to develop a method of fluorescently tagging the resin acids so that they could be analyzed for LIF detection with CE. Resin acides have been previously derivatized using bromomethyl coumarin compounds containing substituents on the aromatic ring have been previously used to form esters with its carboxylic acid (Richardson *et al.*, 1992). The reaction must be performed in an aprotic dipolar solvent such as acetone, and uses potassium carbonate as a catalyst to enhance the reaction rate. The derivatization reaction was performed using 4-BrMMC in the presence of acid potassium carbonate to form a fluorescent compound as shown previously in Figure 3. Acetone was used as a solvent as it was found to be most compatible with the CE system, a 1:1 (v/v) ratio of coumarin to resin acid was used because it was found that excess amounts of derivatizing agents resulted in overloading of the capillary and sometimes caused current crashes. The reaction was completed in 25 min in a sonicating water bath at 25°C. Reactions were performed in light protected amber glass vials

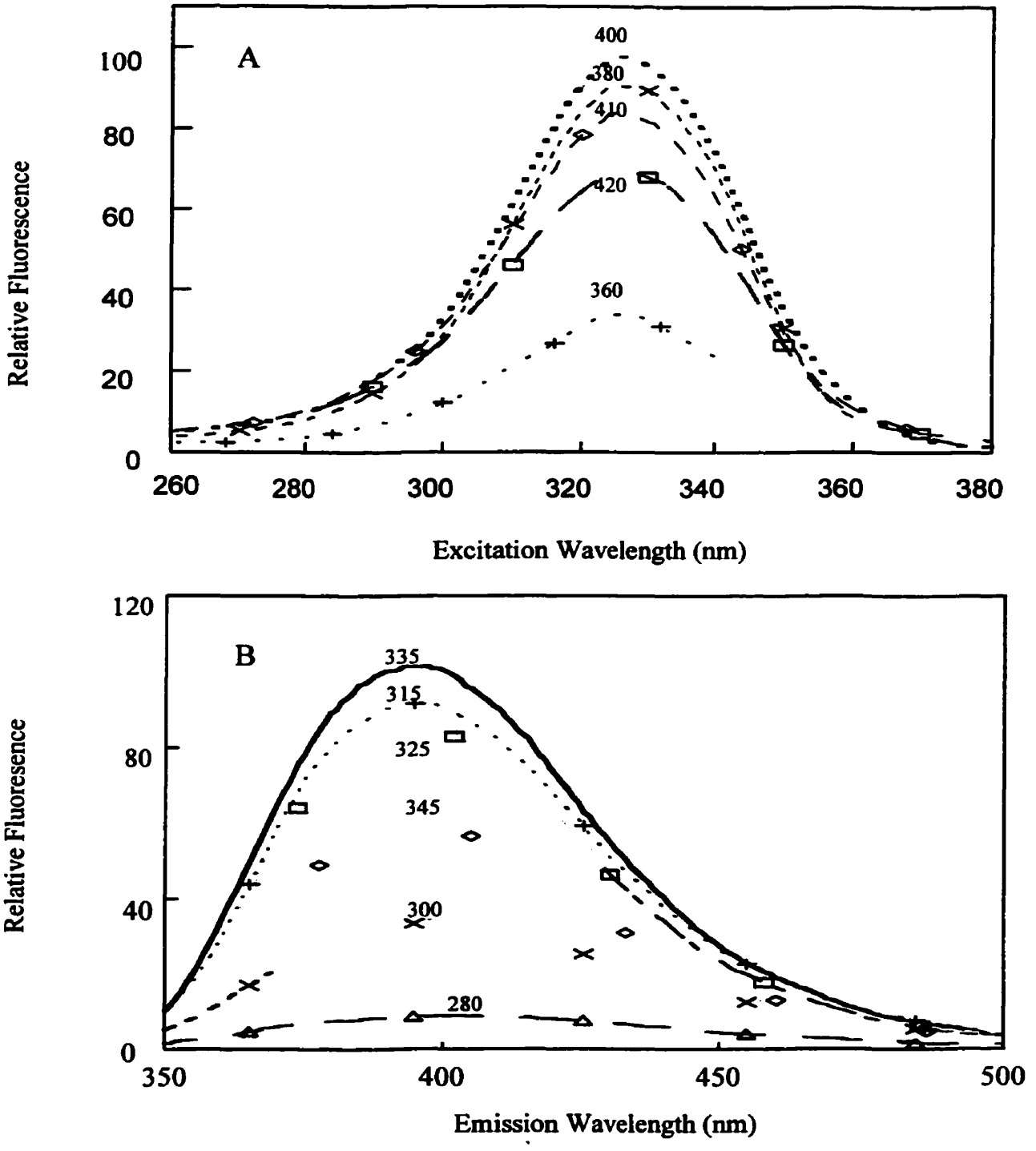


Figure 12. Maximum excitation (A) and emmission (B) fluorescent spectra for derivatized abietic resin acid ester

covered with aluminum foil. Samples were filtered after derivatization using a 0.45 μm filter to remove the potassium carbonate after analysis.

4.2.2. Verification of Resin Acid Ester Excitation and Emission Maximas

To determine the exact excitation and emission maxima, each derivatized resin acid ester was scanned using a Gilford spectrofluorometer. Results showed that each derivatized ester was found to fluoresce optimally at an excitation wavelength of 325 nm, and the resulting emission maxima was found at 400 nm. Figure 12 shows the excitation and emission maximas obtained with a 1 ppm solution of abietic acid. These conditions were ideal for the CE instrument used in our lab, which has an external helium cadmium laser with an excitation emission of 335 nm and which can be fitted with a 400 nm bandpass filter. The signal of 4-BrMMC in the resulting product was shown to have little effect on fluorescence, as the signal for 1μg/mL of 4-BrMMC was less than 5 RFU (relative fluorescent unit).

4.2.3. Verification of Derivatization Using Electrospray Ionization Mass Spectrometry

The derivatized esters were also analyzed using mass spectrometry to confirm ester formation. Results are shown in Figure 13, a major peak is seen at 491 m/z (mass to charge ratio), and the absence of the abietic acid peak at 302 m/z was observed. An underivatized abietic acid sample was run initially (results not shown) to confirm a peak at 302 m/z. The absence of this signal in the derivatized samples confirms that the reaction proceeded to completion, and the lack of other signals show also that the reaction produces no side reactions. Unreacted coumarin used in two-fold excess showed a doublet at 269, and 271 m/z respectively, one peak corresponding to the ^{79}Br, and the other signal corresponded to the ^{82}Br signal. These results are similar to those reported by Luong *et al.* (1999b) and Richardson *et al.* (1992), and further confirmed that although the resin acids do not fluoresce naturally, could be easily and efficiently derivatized using 4-BrMMc in acetone, under the presence of finely ground potassium carbonate.

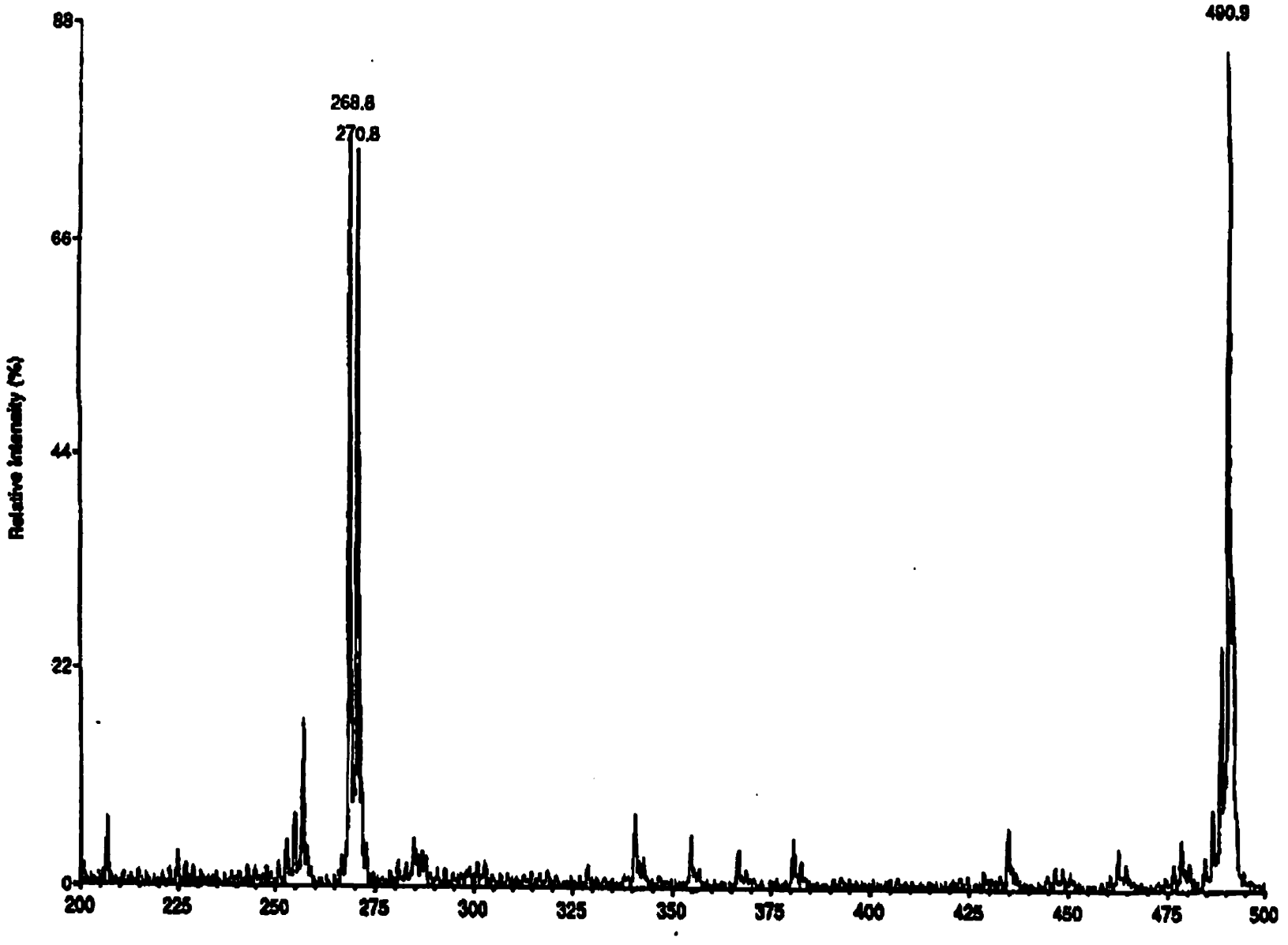


Figure 13. Electrospray ionization mass spectrometry spectrum of derivitized abietic resin acid ester.

4.2.4. Optimization of Laser Light Output from CE

As was expected, a large amount of effort and time was put into optimizing the laser light path between the laser source and the capillary window. The resin acids were analyzed using CD-EKC equipped with an LIF detector, with excitation energy of 325 nm, and an emission setting of 400 nm. Because the intensity of light emitted from the laser is a function of laser power, a power meter was first used to measure and maximize the power output from the laser. Power is 'lost' at each break in the light path, and directly correlates to detection sensitivity. Therefore, measurements were taken and adjustments were made to the sets screws of the laser head to maximize power output. A fiber optic patchcord, specially fitted to attach to the laser transmits the light to another optical fiber in the LIF detector and this light is transmitted through the fiber optic in the LIF detector to the capillary. The intensity of power output from the laser could also be verified on a daily basis by placing a white paper in front of the light path to confirm the presence of an intense purple light. Eventually it was understood that the input and output ends of the patchcord itself had to be protected from environmental particles such as dust or dirt that would contaminate it and burn the ends when the laser light was turned on. Contamination of the fiber optic resulted in a huge loss in laser light intensity and the patchcords had to be sent back to the manufacturers for repolishing of the ends. A system was developed where if the system was not in use, the patchcord stayed attached to the laser and the LIF detector, or if the patchord had to be removed, it was carefully capped with a protective cover and removed to a safe place. It was then discovered that the most critical point of contact for detection sensitivity occurred at the point of contact between the laser light output of the LIF detector fiber optic and the capillary. In order to achieve any amount of sensitivity, the capillary and light output had to be focused correctly, therefore the capillary position was carefully adjusted underneath a microscope to observe that it was properly aligned with the mirror of the capillary.

Another important factor in achieving sensitivity in detection was by the PMT setting of the CE unit itself. Adjustments were necessary to optimize the PMT settings for each new capillary in order to achieve the best signal to noise ratio. To do this,

acetone was first run through the capillary under high pressure (20 psi) and at time 2 min zeroed, then a high concentration (1 ppm) of derivatized abietic acid was run through the capillary using a high pressure (20 psi) rinse, and the number recorded at time 2 min. The PMT setting was then adjusted to allow for the best signal to noise ratio for that particular capillary.

4.2.5. Separation of Chlorodehydroabietic Acid

After confirmation was made that the resin acids were completely derivatized, and that the laser light output as well as the capillary alignment was optimized, each of the resin acids was analyzed separately. The separation was performed using a sodium borate buffer of pH 9.25 containing 35 mM SβCD, and 15 mM MECD with a 37 cm capillary that had a 50 μm i.d. Figure 14 shows the electropherogram for chlorodehydroabietic acid, which has 2 observable peaks (CDA 12 and 14). Coumarin was observed to migrate from the capillary after the resin acid peaks occurred (approximate time 25 min). A blank sample was prepared using the same derivatization reaction containing acetone with no dissolved resin acids and was also analyzed (results not shown) , the electropherograms clearly show the EOF of the acetone at time 11 min, as well as the coumarin at time 25 min, but no other peaks were observed, indicating that we were actually measuring the extracted CDA peaks.

4.2.6. Separation of Selected Resin Acids Using CD-EKC with LIF Detection

The next step was to perform the separation of a mixture of all of the resin acids. After derivatization, the concentration of resin acids was 10 ppm, a 1 mL volume of each resin acid (10 in total) was combined resulting in a final resin acid concentration of 1 ppm for each resin acid in the mixture. The sample was initially analyzed using the optimized protocol which was developed with the UV detector, using a 37 cm capillary with a 50 μm i.d. containing 35 mM SβCD 15 mM MECD in a sodium borate buffer of 72 mM. Unfortunately successive runs showed the resin acids eluted as a group, and were very poorly separated (results not shown).

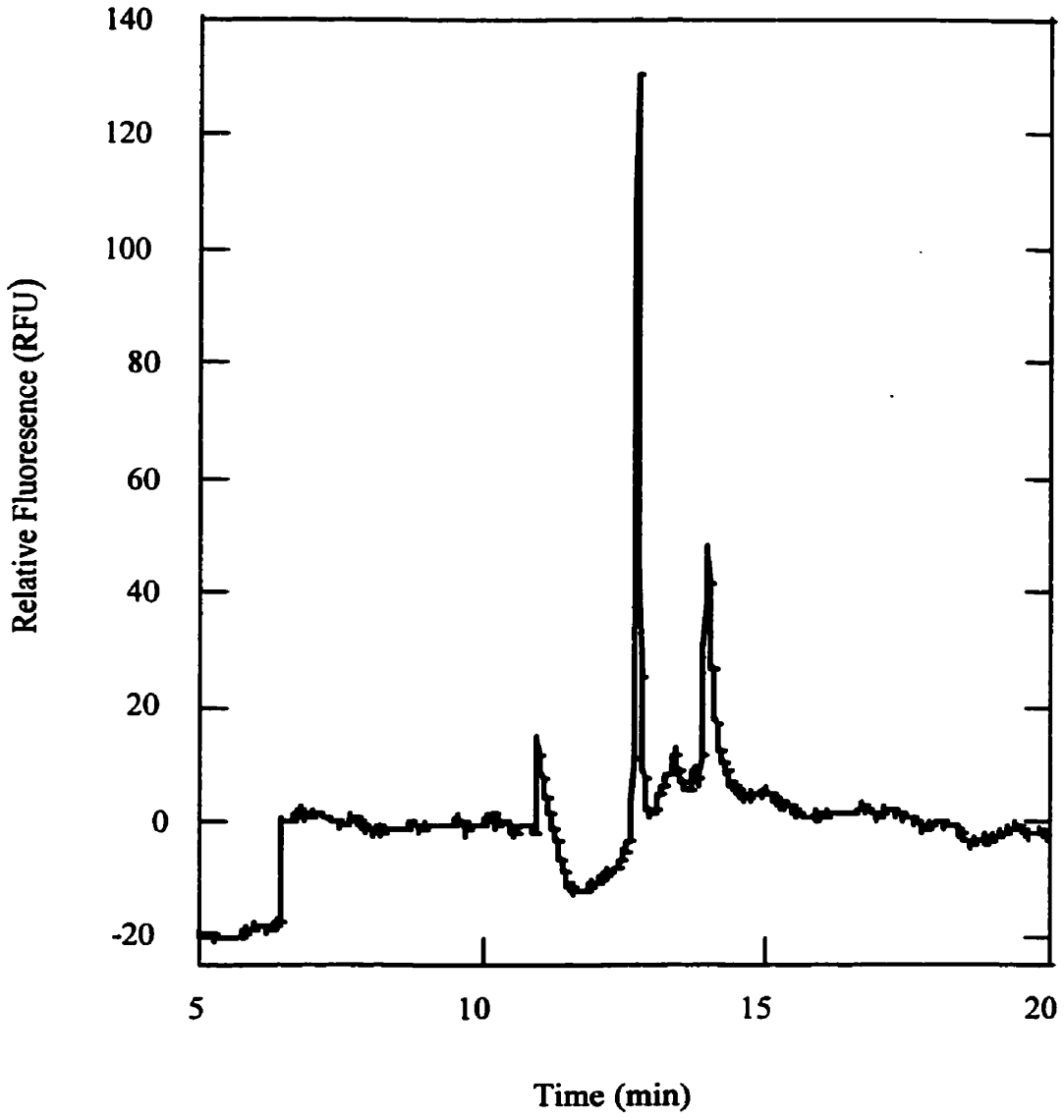


Figure 14. Electropherogram for the CD-EKC electropherogram of 1 ppm of CDA rsin acids ester. λ_{exc} 325 nm - λ_{emm} 400 nm 50 mM Na-Borate, pH 9.25 35 mM SBCD 15 mM MECD 37 cm capillary 50 μm i.d. 5 sec injection 10 kV applied potential 1000 ppb RA

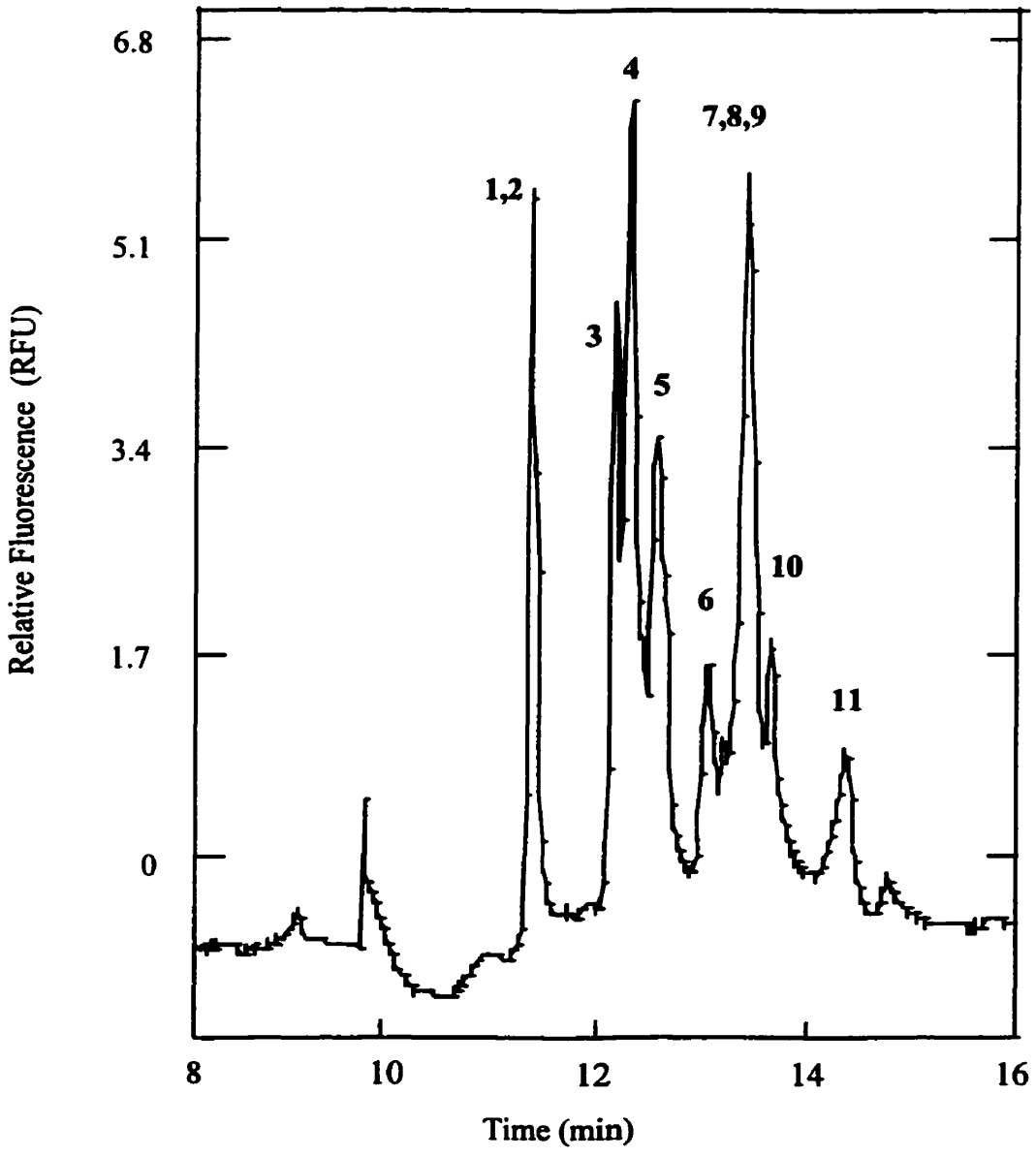


Figure 15. Optimized conditions for the LIF separation of selected resin acids using a 36 mM Na-Borate, pH 9.25 containing 60 mM SβCD 15 mM MECD. 37 cm Capillary 50 μm i.d. 5 sec injection and an separation voltage of 10 kV. LIF detection, λ_{exc} 325 nm - λ_{emm} 400 nm The concentration of each resin acid is 1000 ppb. Peak identification: Peaks 1,2 DCDA, CDA (12,14), Peak 3. PIM , Peak 4.PAL, Peak 5. DHA Peak 6. ISO Peak 7. ABA , Peak 8.,9 SAN CDA (12,14) Peak 10. LEV Peak 11. NEO.

These results suggested that during the derivatization reaction, the resin acids had undergone conformational changes that caused them to interact and behave differently in the capillary For this reason, the SβCD and MECD concentrations were adjusted to improve and optimize the separation.. Figure 15 shows an optimized separation for all of the resin acids in the mixture, using a 36 mM sodium borate solution pH 9.25 containing 60 mM SβCD and 15 mM MECD. The separation voltage had to be reduced to 10 kV as a result of the increased SβCD concentration which resulted in a higher current (> 100 μAu) being generated. The ionic strength of the running buffer was also reduced to 36 mM in order to accommodate the increase in SβCD (as SβCD is dissolved in water). Table 4 also summarizes the optimal conditions used for the separation. The final separation was not as baseline separated as in the UV separation, but sensitivities were much higher, enabling detection of resin acids at much lower concentrations (up to 1000 times more sensitivity achieved) than capable with UV detection. Seven peaks were resolved, and the separation was completed in 15 min; the first two resin acids to migrate were dichlorodehydroabietic acid and one of the chlorodehydroabietic acid isomers (peaks 12 and 14), and the last peak to migrate was neoabietic acid. Peaks were also identified according to the previous method of 'spiking' used for the UV separation. Again, the speed of analysis, the small sample volume required as well as the ability to separate chlorinated compounds, including the isomers of CDA make this method very appealing as an alternative to traditional separation methods.

The separation was also attempted with a sodium acetate buffer of pH 4.50, and a sodium phosphate buffer of 7.00, and resulted in an identical elution profile, which confirmed that the resin acids were no longer charged, and were migrating based on their hydrophobic interactions with the 2 phases of the cyclodextrins. Again these buffers sometimes gave erratic results, and the acetate buffer also resulted in a longer separation time (20 min). The addition of organic modifiers, as well as variations of the parameters tested in the UV optimization failed to improve the overall quality of the separation.

Parameter	Optimum
Excitation wavelength	λ_{exc} 325 nm
Emmission wavelength	λ_{emm} 400 nm
Limit of Detection (LOD)	50 ppb
CD Concetration (mM) SβCD:MECD	60 MM SβCD 15 mM MECD
Buffer Ionic Strength/pH	Na Borate 36 mM pH 9.25
Separation Voltage	10 kV
Capillary Length/ Inner Diameter (μm)	37 cm / 50 μm i.d.

Table 4. Summary of conditions tested and optimized for the LIF separation of resin acids.

4.2.7. Measurement of Detection Limits for Resin Acid Esters

To establish detection limits for each resin acid ester, a standard sample for each resin acid was analyzed using CD-EKC. The separation was performed using a sodium borate buffer of pH 9.25 containing 35 mM SβCD, and 15 mM MECD with a 37-cm capillary that had a 50 μm i.d. and a 5 sec injection time was used. A calibration plot of peak area versus concentration was made for each derivatized resin acid by running each sample diluted in acetone at different concentration ratios. The detection limit was then established as the concentration at which the peak area was equal to three times the standard deviation of the peak area versus the concentration plot. The detection limit for each of the derivatized resin acid esters was different, the detection limit of the conjugated esters such as abietic, neoabietic, palustric and levopimaric acids was between 20 - 50 ppb (μg/L). The ester of the non-conjugated resin acid esters such as dehydroabietic, isopimaric and pimaric was lower, at 10 to 20 ppb. Utilizing CD-EKC with LIF detection therefore resulted in upto a 1000 foldincrease in detection sensitivity, as the limit of detection was determined to be 10 ppm using CD-EKC with UV detection.

4.2.8. Comparison Using HPLC Fluorescence Detection

Derivatized resin acids have been previously separated using reverse phase HPLC with a scanning fluorescence detector by Richardson *et al.* (1992). Based on this method, the separation was optimized and compared to CD-EKC results. Figure 16 shows the optimized separation of the selected resin acids using a LC-8 column (15 cm x 4.6 mm, 3 μm), with a mobile phase containing 55% acetonitrile (w/v) and 45% (w/v) water, and a flow rate of 1 mL/min. The separation resulted in the elution of seven peaks, with some of the peaks co-eluting. Coumarin and acetone eluted first followed by dehydroabietic acid, the two CDA isomers eluted as a doublet peak with levopimaric and neoabietic closely following it. Most of the other peaks co-eluted, and dichlorodehydroabietic acid eluted last at approximately 120 min. Limits of detection were also performed, with a detection limit of 10 ppb being calculated. Efforts were taken to improve the separation resolution, but again due to the similar chemical structures of the resin acids, the separation could not be improved upon.

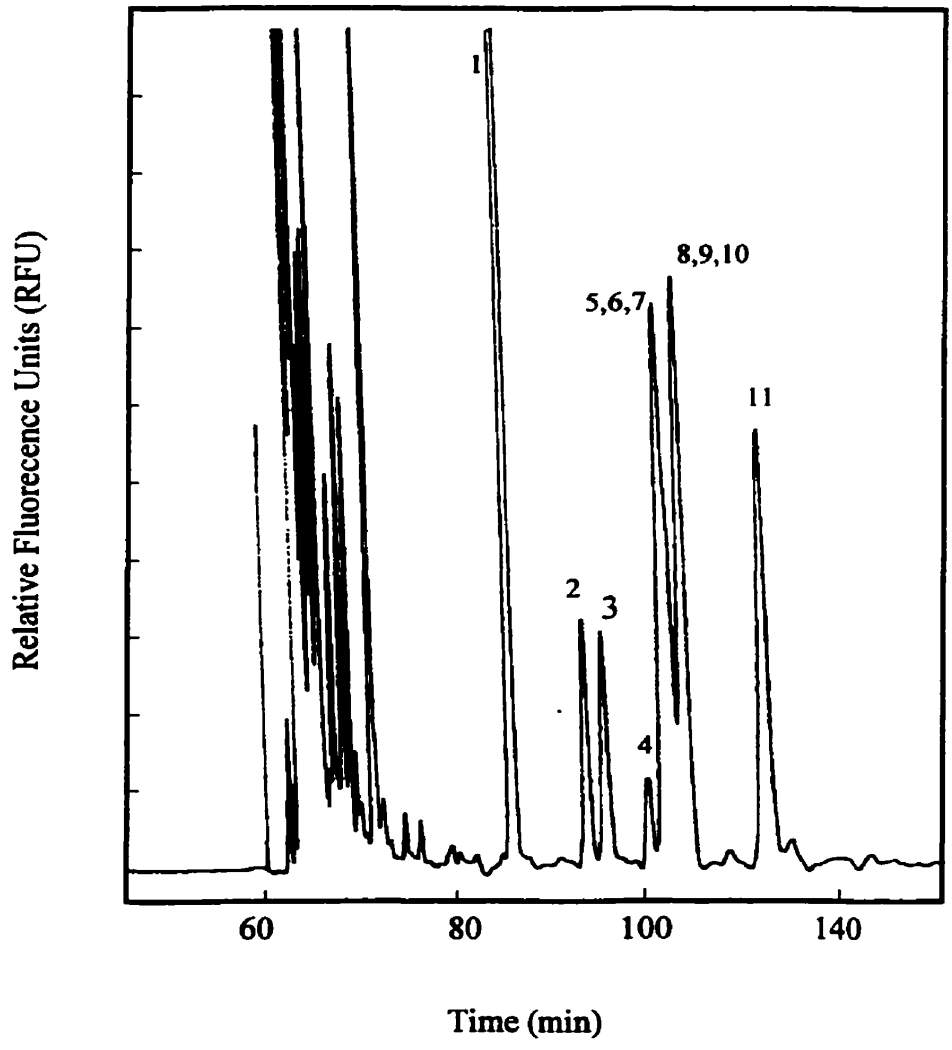


Figure 16. HPLC fluorescence detection of 11 resin acids Run Conditions l_{exc} 325 nm - l_{emm} 400 nm LC-8 Column (15cm x 4.6 mm, 3 mm) Mobile Phase: Acetonitrile : H_20 (55 : 45) 1 mL/min flow rate 250 ppb resin aicds. Peak Identification: 1 DHA 2 CDA (12,14) 3 CDA (12,14) 4 NEO5,6 PAL, ISO 7,8,9 ,10 ABA,SAND,LEV,PIM 11 DCDA

4.3. Application of CD-EKC Separation to Samples Extracted from PME and Fish Bile

One of the goals of this research was to apply the developed methods to 'real' samples, which would contain concentrations of resin acids. Both pulp mill effluent samples and fish bile samples could contain varying concentrations of resin acids. Effluent samples would contain concentrations ranging from more than 100 ppm in the case of untreated effluent samples from pulp mills, or in the low ppm for treated effluent samples. Trace concentrations in the ppb range could be found in fish bile, which have been exposed, to resin acid contaminated water. For this reason, the research focused on the development of a method, which would ideally be applicable to both types of samples, and ideally these methods would also allow for a high range of detection capability. Because the derivatization reaction and CD-EKC LIF analysis is more complicated, as well as costly, it may be quicker, easier and more economical to run the samples initially using CD-EKC with UV analysis. If there are no peaks detected, then the samples could be derivatized and analyzed using CD-EKC with LIF detection.

4.3.1. Developments of an Extraction Method to Release Resin Acids from Bile and Pulp Mill Effluent Samples

Oikari and Anas (1985) have successfully developed a method for the extraction of free and congugated resin acids from pulp mill effluent and fish bile, and various modified versions of this extraction have also been developed by Soimasuo *et al.* (1998). In this research, various methods of extraction were tested, both chemical and enzymatic. Solvent extractions included the use of methyl-tert-butyl ether (MTBE), or a hexane acetone mixture combined with potassium hydroxide to release conjugated resin acids (in the case of bile samples) and enzymatic methods which used β-gluoconidase and sulfatase to release congugated resin acids, followed by a solvent extraction with ethyl acetate. After the extractions were performed, samples were derivatized and analysis attempted using CD-EKC LIF detection. and HPLC equipped with fluorescence detection. The extraction, which was found to be most reliable and compatible with the developed derivatization method used hexane:acetone in a 3:1 (v/v) ratio. Samples were

not hydrolyzed at this point, because naturally no congugated resin acids would be found in effluent or spiked bile.

4.3.2. Spiking of Effluent and Bile Samples

Samples of effluent were obtained from a hardwood pulp mill, which contained no traces of resin acids, and bile samples taken from rainbow trout which had been laboratory reared and free from any environmental contamination were used as model samples for spiking. Each of the samples were spiked with a known concentration of a mixture of resin acids, and extracted using hexane:acetone. After solvent extraction, a portion of the extract was used to perform the derivatization. Samples were sonicated and filtered, and evaporated under nitrogen to concentrate the sample before analysis. Samples were analyzed using both HPLC and CE. Figure 17 shows the electropherograms for extracted and derivatized bile and effluent samples. Results were very positive, as the electropherograms showed a very similar migration profile as seen when analyzing standard resin acid samples with CE-EKC LIF detection. A comparison was also made using HPLC with fluorescent detection and again, results were very similar to those obtained previously with standards. Numerous samples of both bile and effluent were run to optimize the extraction procedure, and lower the limit of detection. Controls were run by spiking effluent and bile with acetone, and performing the extraction and derivitization with the developed procedure, the samples were measured with both HPLC and CE both analyses showed no peaks (results not shown).

4.3.3. CD-EKC UV analysis of contaminated PME samples

To determine whether the developed extraction and derivatization procedure could be applied to samples known to contain concentrations of resin acids, samples of softwood pulp mill effluent were obtained from Noranda Technologies. Initially a portion of the effluent sample was extracted using the method developed. Because it was suspected that this sample contained a very high concentration of resin acids, the extracted sample was first analyzed using CD-EKC with UV detection. Figure 18A shows a large peak with an absorbance signal at 8.75 min. Because dehydroabietic acid is the most prevalent resin acid found in effluent, it was suspected that this peak may be

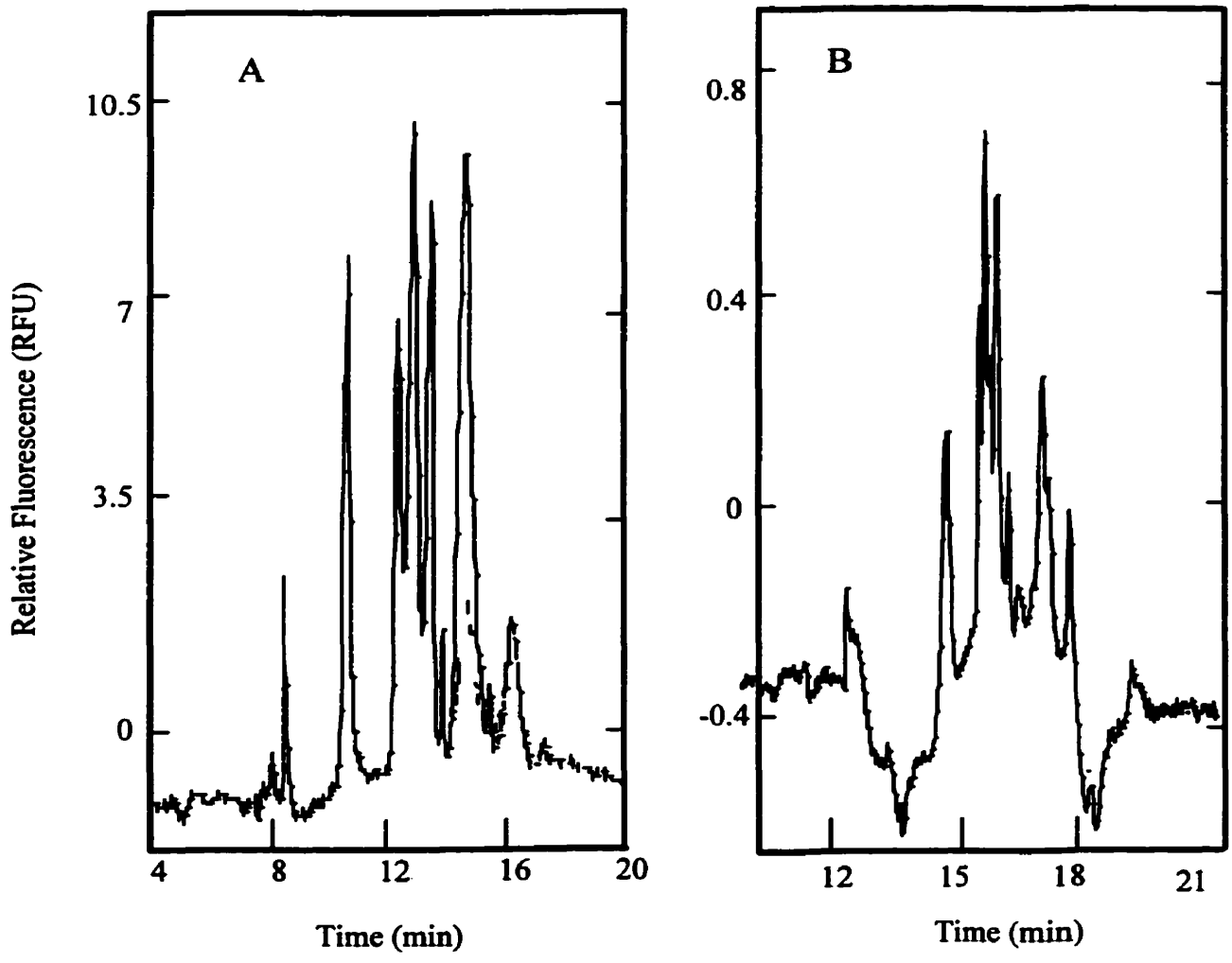


Figure 17. CD-EKC LIF Analysis of spiked model (control) samples: (A) pulp mill effluent sample from a hard-wood pulp mill (A) supplied by *PAPRICAN* and (B) fish bile from unexposed fish supplied by Environment Canada.

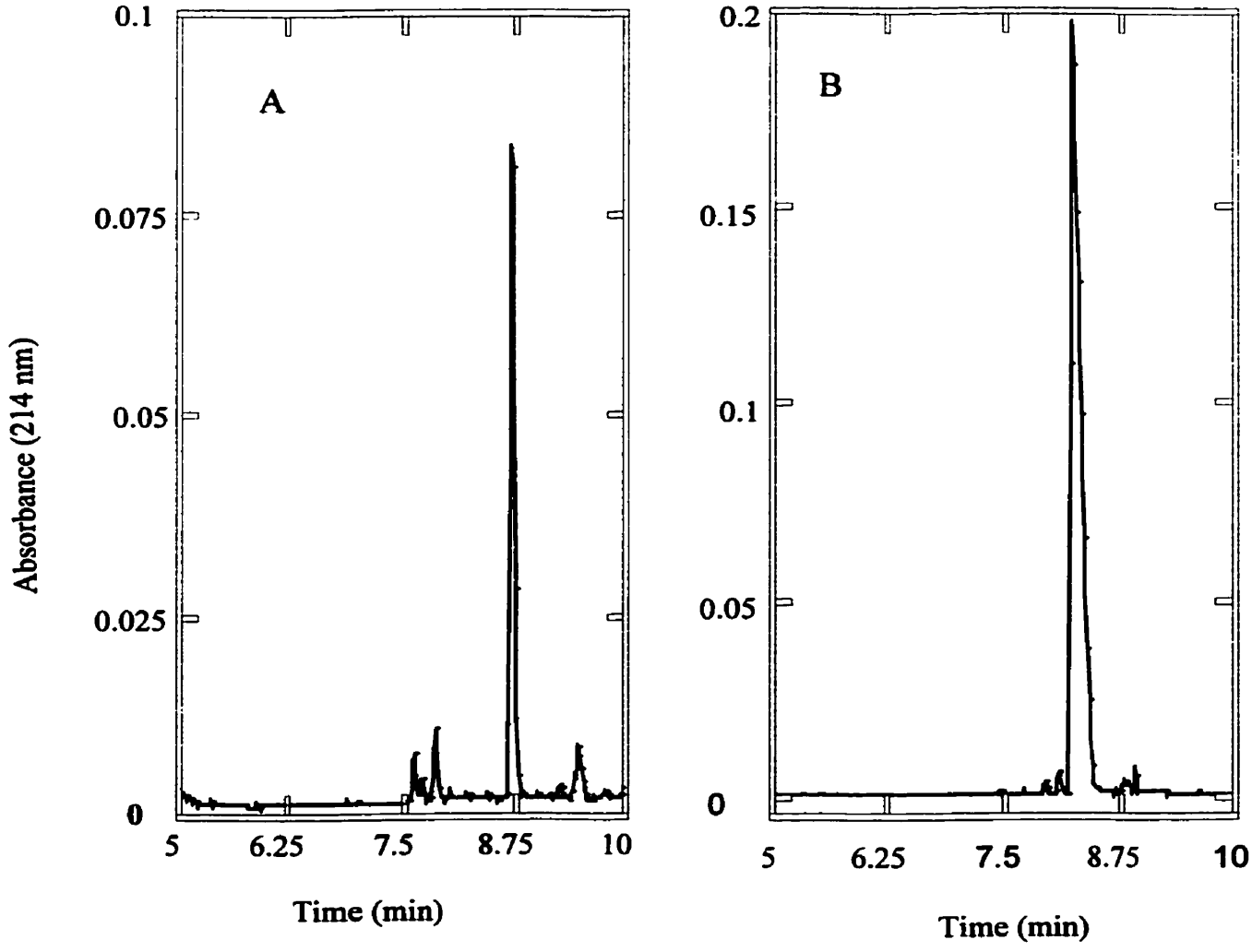


Figure 18. CD -EKC /UV Analysis of Contaminated PME samples obtained from pulp mill effluent samples (Noranda). (A) Extracted resin acid peak identified as DHA (B) Sample A spiked with known concentration of DHA.

dehydroabietic acid. To confirm this, a second extraction was performed, this time the sample was spiked with a known concentration of dehydroabietic acid. The sample was extracted and analyzed on CE. The resulting electropherogram Figure 18B show that at approximately 8.50 min, there was a large absorbance signal that was double what had been observed in the unspiked sample. Control samples were also run, resulting in no peaks. Based on this information it was thought that the peak could be an extracted resin, in particular because of the time of elution and in the fact that dehydroabietic constitutes approximately 80% of all resin acids found in effluent it was thought that the peak may be dehydroabietic acid.

4.3.4. CD-EKC LIF Analysis of Contaminated PME Samples

To confirm the presence of dehydroabietic acid as well as detect other resin acids that may be present in the effluent at lower concentrations, a portion of the extracted sample was derivatized according to the developed method and run with CD-EKC LIF detection. Figure 19A shows the results, a large peak was seen at approximately 13 min, and a smaller peak was also observed at time 11 min. To confirm this result, a portion of the sample was spiked with derivatized dehydroabietic acid, and the corresponding peak increased in relative fluorescence Figure 19B The smaller peak remained at the same fluorescence level.

4.3.5. HPLC Fluorescence Detection of Contaminated PME Samples

To further confirm these results, the sample was cross-referenced with HPLC equipped with fluorescence detection. Figure 20 shows the results. The HPLC chromatogram shows a large peak at 60 min, and as well showed 2 other peaks which looked like CDA 12 and 14 isomers, as well there is a peak that looks like dichlordehydroabietic acid, and eluted at the same time as the standard resin acids did (Fig. 16).

4.3.6. CD EKC LIF Analysis of Effluent for Comparison with GC Analysis

To further confirm the identification of extracted resin acids from pulp mill effluent samples, a sample of contaminated effluent was obtained through *Paprican* that

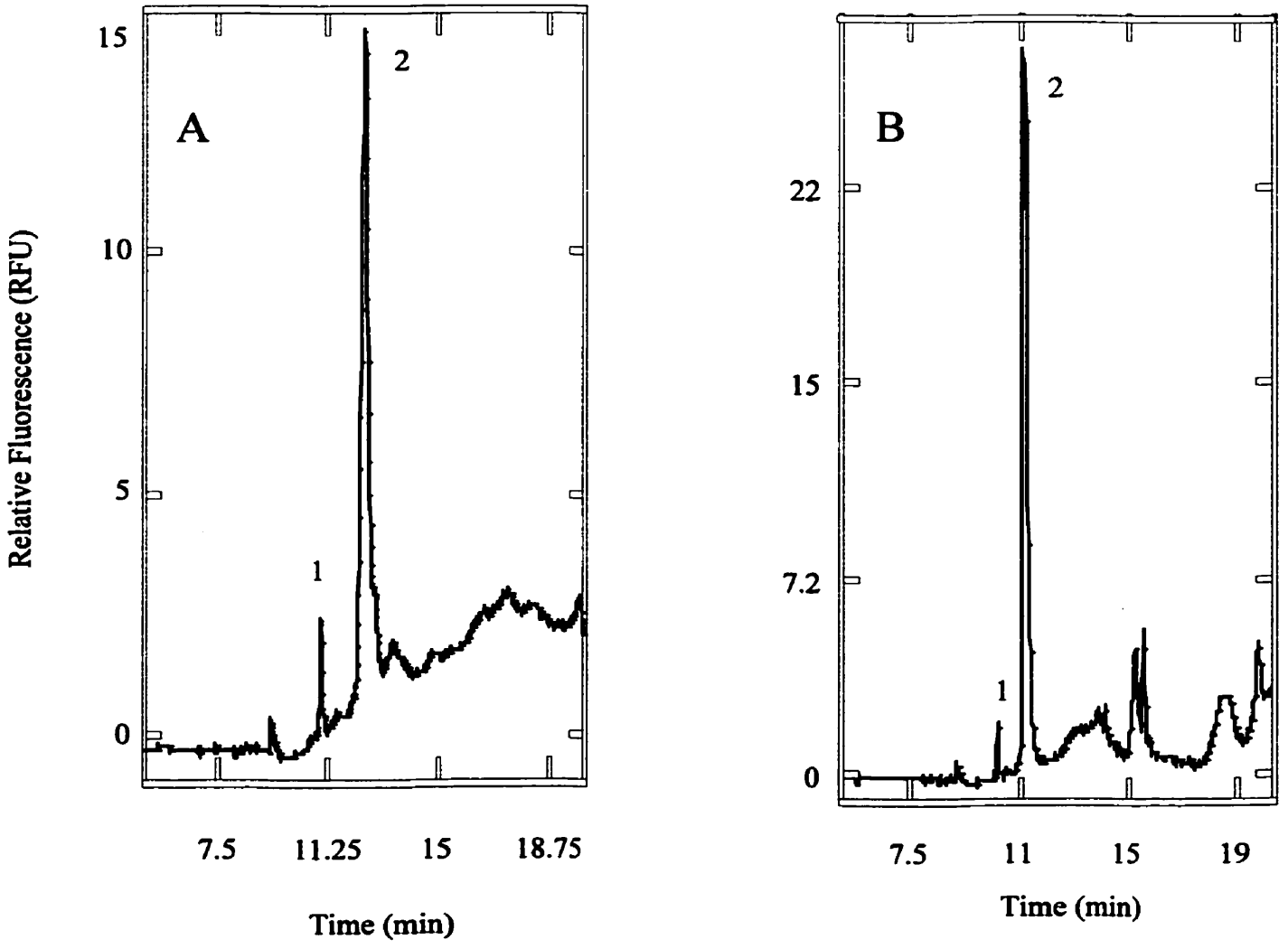


Figure 19. CD -EKC /LIF Analysis of Contaminated PME samples obtained from pulp mill effluent samples (Noranda). (A) Extracted resin acids identified as (1) CDA and (2) DHA (B) Sample A spiked with known concetration of DHA

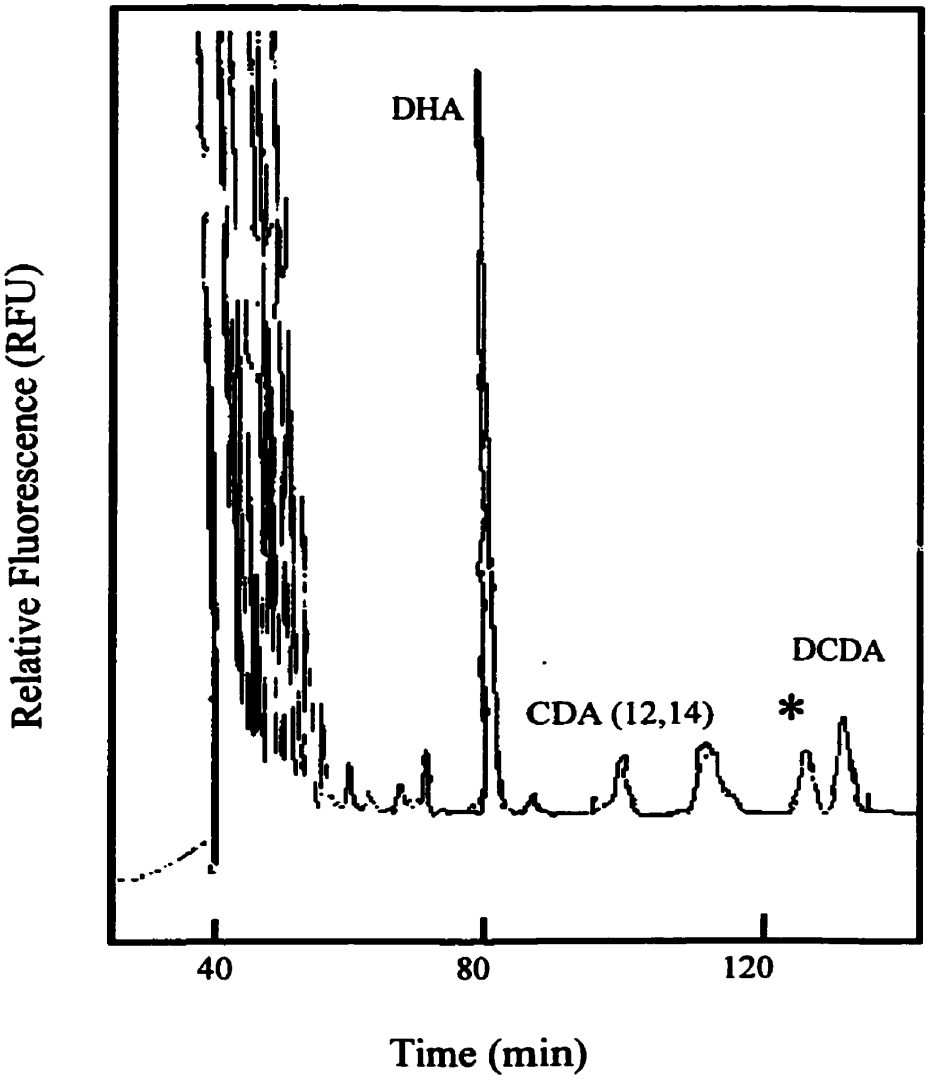


Figure 20. HPLC fluorescence detection of contaminated pulp mill effluent sample (NORANDA). Run conditions: λ_{exc} 325 nm - λ_{emm} 400 nm LC-8 Column (15cm x 4.6 mm, 3 µm) Mobile Phase: Acetonitrile : H_20 (55 : 45) 1 mL/min flow rate. * unidentified contaminant extracted from PME.

Compound	Concentration (ug/L)
Pimaric acid	282
Sandarocopimaric acid	452
Isopimaric Acid	969
Palustric Levopimaric acid	309
Dehydroabietic acid	3145
Abietic acid	605

Table 5. Comparative GC (FID) analyis results for pulp mill effluent sample

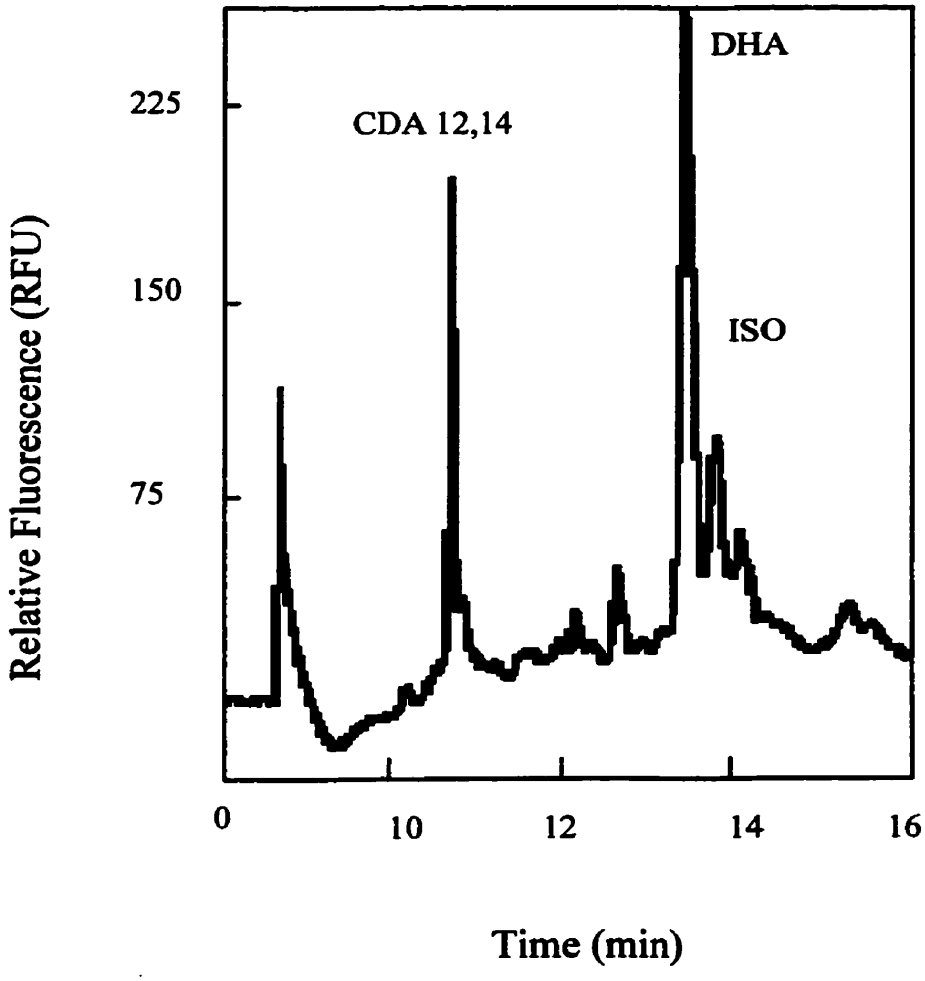


Figure 21. CD EKC fluorescence detection of contaminated pulp mill effluent sample (PAPRICAN). Run conditions: λ_{exc} 325 nm - λ_{emm} 400 nm.

could be used as another cross-reference. Results for the GC/FID analysis of the contaminated pulp mill effluent sample obtained by *Paprican* are shown in Table 5. CD-EKC LIF detection was used because the results showed maximum concentration of 3145 ppb (dehydroabietic acid), and a minimum of 282 ppb (pimaric acid), which required a low limit of detection. Figure 21 show the result of CD-EKC LIF analysis of the extracted and derivatized sample. The electropherogram shows a strong peak fluorescence at approximately 14 min, as well a peak at time 11 min which matched the profile for one of the CDA isomers. Another peak was observed to migrate from the capillary immediately after dehydroabietic acid at time 14.5 min, and was identified as isopimaric. Interestingly, the concentration of dehydroabietic and isopimaric were also the highest concentrations found by GC analysis. Each of the suspected peaks (CDA, DHA and ISO) were then spiked into the solution (one at a time), and the samples analyzed. Again, an increase of relative fluorescence was observed in each peak (results not shown).

4.3.7. HPLC Fluorescence detection of contaminated PME samples

The sample was also run using HPLC fluorescence detection to compare the elution profile and peak concentrations. Figure 22 shows a strong fluorescence at 50 min, confirming that this peak was dehydroabietic acid. The two isomers of chlorodehydroabietic acid were also observed.

4.3.8. Results of Field Exposure Studies Using Whitesucker fish (Catostomus commersoni)

Samples of fish bile obtained from field exposure studies performed in Edmonston NewBrunswick were also extracted and analyzed for resin acids. The fish had been exposed for 3 consecutive days to a 50:50 (v/v) mixture of river water, and effluent which had been untreated and therefore should contain very high concentrations of toxic compounds, including resin acids. Samples of the effluent which the fish were exposed to were also taken for analysis in the laboratory.

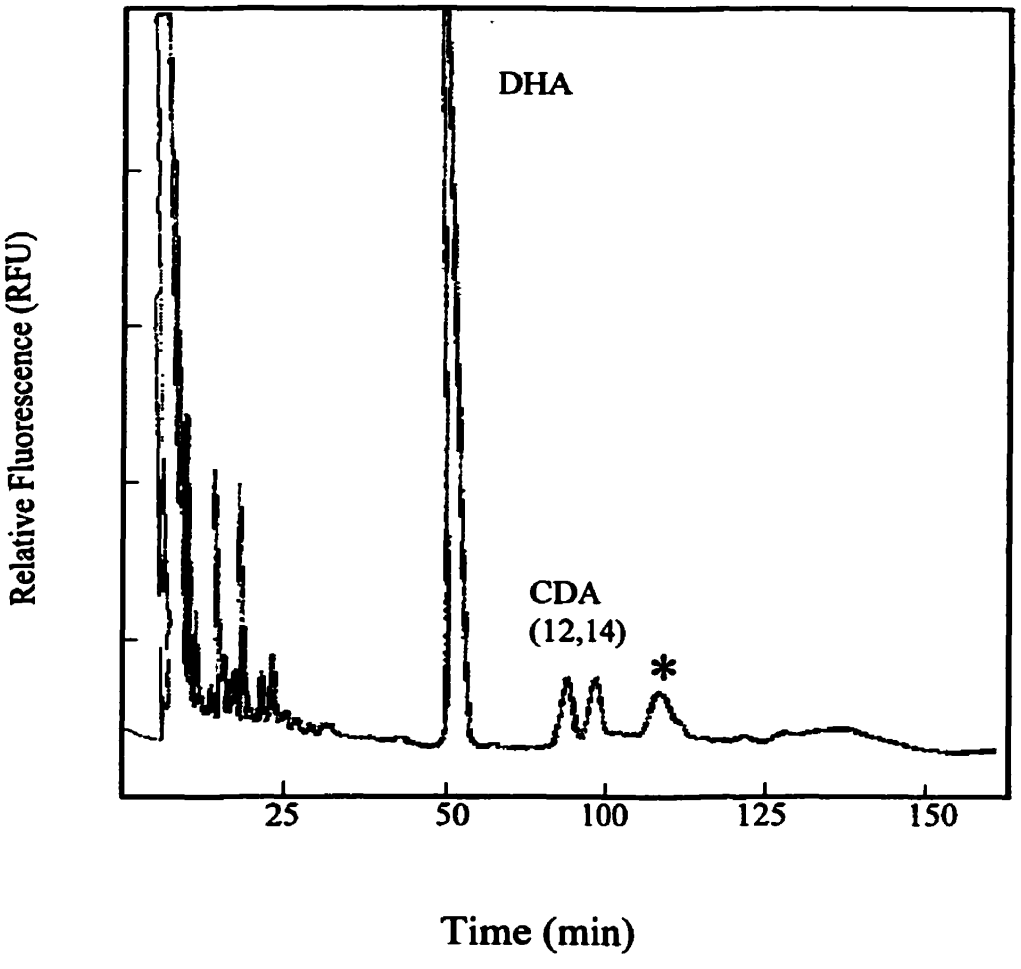


Figure 22. HPLC fluorescence detection of contaminated pulp mill effluent sample (PAPRICAN). Run conditions: λ_{exc} 325 nm - λ_{emm} 400 nm LC-8 Column (15cm x 4.6 mm, 3 μm) Mobile Phase: Acetonitrile : H_20 (55 : 45) 1mL/min flow rate. * Unidentified contaminant found in PME.

4.3.9. CD-EKC LIF Analysis of Contaminated Fish Bile Samples

Samples of fish bile were pooled to a volume of 5 mL, extracted and derivatized according to the developed methods. Samples were initially run with LIF detection because it was anticipated that the final concentrations of resin acids which may have bioaccumulated in the bile, would be in the ppb range. Congugated resin acids were released into free resin acids prior to extraction using KOH. Approximately 80 % of all resin acids present in the bile sample will be in the uncongugated form therefore this step is essential. Various samples were extracted and derivitized and analysis attempted using CD-EKC with LIF detection, unfortunately there were never any discernable peaks, including the peak of dehydroabietic acid, which had been seen in virtually all of the effluent samples that were know to contain concentrations of resin acids. Control samples, of fish which had only been exposed to river water were also extracted and derivatized however the electropherograms looked identical to the samples of bile obtained from exposed fish (results not shown).

4.3.10. HPLC Fluorescence Detection of Contaminated PME Samples

To confirm the presence or absence of resin acids in the samples of bile, the samples were cross referenced using HPLC equipped with a scanning fluorescent detector. Again, no peaks were observed in both the control and exposed fish bile samples (results not shown).

4.3.11. Analysis of Effluent Used in Exposure Study

Samples of the effluent which was used in the exposure study were also analyzed using both HPLC with scanning fluorescence, and CD-EKC LIF analysis. No resin acids were detected in the effluent using both methods of analysis. The fact that no resin acids were measurable in the effluent using both methods led us to believe that the concentrations of resin acids which the fish were exposed to during the trial were very low, considering the LOD using HPLC was determined to be 25 ppb. Although in theory the resin acids would bioconcentrate in the fish bile, and therefore even if the levels were low in the effluent, the concentrations measured in the bile may be much

higher. It was hypothesized that the levels in the effluent may have been below the levels dangerous to the fish (5 ppb for dehydroabietic acid) To further confirm the usefulness and validity of using this method to analyze fish bile and assess the level of resin acids which may be in the surrounding environment, more studies which were beyond the scope of this research would be necessary.

5. CONCLUSION

This study has illustrated the suitability of using capillary electrophoresis with cyclodextrin modified electrokinetic chromatography as a novel method for the analysis of and detection of several resin acids commonly found in pulp mill effluent, and known to bioaccumulate in the bile of fish which inhabit regions near pulp mills.

A CD-EKC UV method with SβCD and MECD was optimized using resin acid standards, resulting in the baseline separation 9 resin acids. To increase method sensitivity, the developed method was then applied to the analysis of resin acids standards which could be reacted with 4-BrMMc to form fluorescent resin acid esters which fluoresced optimally at 325 nm excitation, and 400 nm emission respectively. The limit of detection was increased by a factor of 200 using LIF detection, and a separation of 7 resin acids resulted. The research performed also showed that resin acids could be detected in both bile and effluent, and readily extracted using a 3:1 (v/v) solution of hexane/acetone. Samples which were suspected to contain high concentrations of the resin acids in the ppm range could be easily detected using CE equipped with a UV detector at 214 nm. Samples which contained lower concentrations of resin acids could be derivatized using 4-BrMMc , and analyzed using CD-EKC LIF detection. An effluent sample from a soft-wood pulp mill processing plant was cross referenced with results from standard GC analysis show comparable results for three of the resin acids in the sample, and results obtained from HPLC analysis also showed comparable results. Samples of white sucker fish bile obtained from an effluent exposure study performed near a pulp mill in New Brunswick were also analyzed using the developed methods. Although it was assumed that the fish were exposed to effluent contaminated with a high or even lethal quantity of resin acids, laboratory analysis showed that there were no resin acids detectable in either the fish bile of exposed fish, or in the effluent used in the exposure study. Control samples of fish bile from the exposure study showed the same results.

Future work could involve further exposure studies either in the laboratory under more rigorously controlled conditions, or in field studies. Additional work would also concentrate on method validation for the proposed methodologies presented in this thesis.

REFERENCES

Andersson, T. and Forlin, L. (1988). Physiological disturbances in fish living in coastal water polluted with bleached kraft pulp mill effluents. *Can. J. Aquatic Sci.* **45**, 1525-1536.

Andreasson, M. and Goran, D. (1994). Toxicity of bile from fish exposed to PCP-spiked sediment. *Aquatic Toxicol.* **30**, 171-182.

Baker, D.R. (1995).In *Capillary Electrophoresis.* John Wiley & Sons, Inc., New York, N.Y.

Brown, S.R., Luong, J.H.T., Szolar, O., Halasz, A. and Hawari, J. (1996). Cyclodextrin-modified capillary electrophoresis: determination of polycyclic aromatic hydrocarbons in contaminated soils. *Anal. Chem.* **68**, 287-292.

Brumley, C.M., Haritos, V.S., Ahokas J.T. and Holdway, D.A. (1998). The effects of exposure duration and feeding status on fish bile metabolites: Implications and biomonitoring. *Ecotoxicol. Environ. Safety* **39**, 147-153.

Camilleri, P. (1998). In *Capillary Electrophoresis Theory and Practice, Second Edition.* CRC Press, Washington, D.C.

Chow, S.Z. and Shepard, D. (1996). High performance liquid chromatographic determination of resin acids in pulp mill effluent. *Tappi J.* **79**, 173-179.

Dethlefs, F. and Stan, H.J. (1996). Determination of resin acids in pulp mill EOP bleaching process effluent. *J. Anal. Chem.* **356**, 403-410.

Dongmei, L., Shilin F. and Lucy, C.A. (1999). Prediction of electrophoretic mobilities. 3. Effect of ionic strength in capillary zone electrophoresis. *Anal. Chem.* **71**, 687-699.

Escartin, E. and Porte, C. (1999). Biomonitoring of PAH in high altitude mountain lakes through the analysis of fish bile. *Environ. Sc. Technol.* **33**, 406-409.

Groom, C. and Luong, J.H.T. (1997). Sulfobutyl-β-cyclodextrin-mediated capillary electrophoresis for separation of chlorinated and substituted phenols. *Electrophoresis* **18**, 1166-1172.

Johnsen, K. Mattsson, K., Tana, J., Stuthridge, T.R., Hemming, J. and Lehtinen K.J. (1995). Uptake and elimination of resin acids and physiological responses in rainbow trout exposed to total mill effluent from an integrated newsprint mill. *Environ. Toxicol. Chem.* **14**, 1561-1568.

Johnsen, K. Mattsson, K., Tana, J., Stuthridge, T.R., Hemming, J. and Lehtinen K.J. (1998). Experimental field exposure of brown trout to river water receiving effluent from an integrated newsprint mill. *Ecotoxicol. Environ. Safety* **40**, 184-193.

Kennedy, C.J. Sweeting, R.M., Johansen, J.A., Farrell, A.P. and Mckeown, B.A. (1995). *Environ. Toxicol. Chem.* **6**, 977-982.

Kitaghishi, K. (1997). In *Handbook of Capillary Electrophoresis Applications.* H. Shintani, and J. Polansky (Eds.), Blackie Academic and Professional, New York, N.Y., pp. 1-40.

Krahn, M, Burrows, D.G., MacLeod, W.D. and Malins, D.C. (1987). Determination of individual metabolites of aromatic compounds in hydrolyzed bile of English sole *(Parophrys vetulus)* from polluted sites in Puget Sound, Washington. *Arch. Environ. Contamin. Toxicol.* **16**, 511-522.

Leppanen, H., Marttinen, S. and Oikari, A. (1999). The use of fish bile metabolite analyses as exposure biomarkers to pulp and paper mill effluents. *Chemosphere* **36**, 2621-2634.

Luong, J.H.T., Rigby, T., Male, K.B. and Bouvrette, P. (1999a). Separation of resin acids using cyclodextrin modified capillary electrophoresis. *Electrophoresis* **20,** 1546-1554.

Luong, J.H.T., Rigby, T., Male, K.B. and Bouvrette, P. (1999b). Derivatization of resin acids with a fluorescent label for cyclodextrin - modified electrophoretic separation. *J. Chromatogr. A* **849**, 255-266.

Oikari A. (1986). Metabolite of xenobiotics in the fish bile in waterways polluted by pulpmill effluents. *Bull. Environ. Contam. Toxicol.* **36**, 426-429.

Oikari, A. and Anas, E. (1985). Chlorinated phenolics and their congugates in the bile of trout. *Bull. Enviro. Contamin. Toxicol.* **35**, 802-809.

Oikari, A. and Ojala K.T. (1987). Tracing of xenobiotic contamination in water with the aid of fish bile metabolites: A field study with caged rainbow trout (*Salmo Gairdneri*). *Aquatic Toxicol.* **9**, 327-341.

Otsuka, K. and Terabe, S. (1996) Micellar electrokinetic chromatography. In *Capillary Electrophoresis Guidebook, Methods in Molecular Biology Series*, Vol. 52, K.D. Altria (Ed.), Humana Press, Totowa, N.J., pp. 125-128.

Richardson, D.E., Bremner, J.B. and O'Grady, B.V. (1992). Quantitative analyses of total resin acids by high performance liquid chromatography of their coumarin ester derivatives. *J. Chromatogr.* **595,** 155-162.

Righetti, P.G. (1996). In *Capillary Electrophoresis in Analytical Biotechnology*. CRC Press, Washington, D.C.

Sharma, C., Mohanty, S.K. and Rao, N.J. (1996). Gas chromatographic analysis of chlorophenolic, resin and fatty acids in effluents from bleaching processes of agricultural residues. *International J. Environ. Anal. Chem.* **64**, 289-299.

Shimp, R.J. and Owens, J.W. (1993). Pulp and paper technologies and improvements in environmental emissions to aquatic environments. *Toxicol. Environ. Chem.* **40**, 213-233.

Soimasuo, M.R, Lappivaara, J. and Oikari, A. (1995). Biomarker responses along a poluution gradient: Effects of pulp and paper mill effluents on caged whitefish. *Aquatic Toxicol* **31**, 329-345.

Soimasuo, M.R, Lappivaara, J. and Oikari, A. (1998). Confirmation of in situ exposure of fish to secondary treated bleached kraft mill effluent using a laboratory simulation. *Environ. Toxicol. Chem.* **17**, 1371-1370.

Weston, A. and Brown, P.R. (1997). In *HPLC and CE: Principles and Applications.* Academic Press, San Diego, CA.