Separation and identification of difluorobenzoic acids by Hplc/Ms

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SEPARATION AND IDENTIFICATION
OF DIFLUOROBENZOIC ACIDS
BY HPLC/MS

by

Zhen Wu

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of the requirements for the degree of

Master of Science

in

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ABSTRACT

A method was developed for the separation and identification of difluorobenzoic acids by high performance liquid chromatography / mass spectrometry (HPLC/MS) with a particle beam interface (PB). The development of this method included the optimization of the mobile phase and MS parameters for the best isocratic separation and detection of the analytes. A performance evaluation procedure for PB HPLC/MS operation was established. Ionization control and ion pairing techniques were employed in an attempt to achieve the chromatographic separation. Although the complete separation of all six isomers was not achieved, the combination of mass spectral detector with liquid chromatography allowed qualitative identification of all analytes.

The fragmentation mechanisms of the benzoic acid involve hydroxyl H/ortho H scrambling. This mechanism proposes that the carboxyl hydrogen and the ortho hydrogens of benzoic acids undergo partial exchange, and is used to suggest the formation of the m/e 123 ion formed during the fragmentation of the difluorobenzoic acids.
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CHAPTER 1

INTRODUCTION

Fluorinated benzoic acids are being used as ground water tracers for the Yucca Mountain Site Characterization Project and were used for hydrologic characterization of the WIPP site in Carlsbad N.M.. Tracers provide information about the ground water movement in the proposed repository areas. These projects require to use multiple tracers during one test, and therefore require that the multiple tracers can be separated chromatographically or mass spectrometrically. Among the sixteen fluorinated benzoic acids, there are six isomers of difluoro substituted benzoic acids. This work uses high performance liquid chromatography / mass spectrometry (HPLC/MS) with a particle beam interface (PB) to separate and identify these difluorinated benzoic acids. HPLC is used to separate the isomers in a sample mixture and introduce them to the mass spectrometer. No HPLC conditions currently exist that separate all six of these isomers.

Mass spectrometry (MS) is used to measure the molecular weight, structure and amount of analyte. MS as a detector provides more structural information than a conventional UV detector. For instance, individual ions detected by the MS can indicate the presence of a target analyte. The pattern of detected fragment ions can yield more definitive information regarding the chemical structure of the analyte, and therefore
provide positive identification of analytes in a complex mixture.

**Reverse Phase Liquid Chromatography**

HPLC is an analytical separation technique. This separation technique is based on the selective distribution of analytes between a liquid mobile phase and a stationary phase. An estimated 60 to 70 percent of analytical work by HPLC is carried out using a bonded, nonpolar, stationary phase, and polar mobile phase. This is referred to as reverse phase liquid chromatography (RPLC). The surface of silica gel is chemically modified by reacting a chlorosilane with the hydroxyl groups of the silica gel. The modified surface is usually further treated with trimethylchlorosilane or hexamethyldisilazane to eliminate most remaining unreacted hydroxyl groups. As a result, the hydrophilic silica gel surface is converted to a hydrophobic (nonpolar) surface. The surface of the bonded phase is stable in aqueous mobile phases at a pH range of 2.5-7.5. The mobile phases used for RP are mixtures of polar solvents such as methanol and water or acetonitrile and water.

The retention mechanism of the reversed phase is complex and not fully understood. The mobile phase interacts with the stationary phase forming a stagnant layer at the surface of the bonded phase. The equilibrium between this stagnant mobile phase layer and the stationary phase is interrupted by the passing analytes. The analytes compete with the stagnant mobile phase for part of the surface of the bonded phase. The analytes can interact with this mobile phase layer forming a bi-layer. The analytes can also interact directly with the surface and displace the mobile phase (displacement). The retained analytes are then eluted by a mixture of dispersive and polar interactions with the mobile
phase. The less polar analytes travel through the system more slowly. Analytes elute in order of decreasing polarity.

The option of manipulating both the mobile phase and the bonded stationary phase enables RPLC to separate a wide range of compounds with varying polarities and molecular weight. Moreover, by manipulating secondary chemical equilibrium through ionization control (pH) and ion pairing in the aqueous mobile phase, RPLC can be used to separate ionic and ionizable compounds, such as the fluorinated benzoic acids.

**Ionization Control**

Ionic compounds generally remain in the aqueous mobile phase. The ionic compounds elute earlier than the neutral compounds, and are often poorly retained on the RP column. By use of ionization control techniques, reversed phase chromatography can also be made to separate ionic compounds. The primary function of ionization control techniques is to shift the ionic equilibrium to a nonionic compound (undissociated) by adjusting the pH of the mobile phase. The nonionic compound is then readily chromatographed on a reversed phase column. Ionic compounds exist according to the following equilibrium:

\[
\text{nonionic} \rightleftharpoons \text{ionic}
\]

for the difluorobenzoic acids:

\[
F_2C_6H_3COOH + H_2O = F_2C_6H_3COO^- + H_3O^+ \quad (1.1)
\]

The pKa value of 2,6-DFBA is 2.85. At pH=pKa=2.85, the 2,6-DFBA will be 50% ionized. When the pH values are less than the pKa value, the excess protons drive the
equilibrium to the left. The nonionic species dominate the system. At a pH two units lower, pH=0.85, the 2,6-DFBA will be almost completely protonated (nonionized). The nonionic form of analyte is then readily retained on a reversed phase column. When the pH values are higher than the pKa value, the equilibrium moves to the right, and the ionic species dominate. At a pH two units higher, pH=4.85, the 2,6-DFBA will be almost completely ionized, and its retention is at a minimum.

As discussed earlier, even when the surface of the silica gel is chemically bonded, some silanol groups on the surface of the silica gel remain. Some analyte molecules adsorb to the remaining silanol group resulting in unsymmetrical peaks. The ionic modifier of the mobile phase also interacts with these residual silanol groups, and therefore minimize the interactions of the analytes with them. This often improves the peak shape dramatically.

**Ion Pairing**

Since the operating pH range for the bonded phase packing is 2.5-7.5, suppression of ionization of strong acids and bases is not possible. Moreover, protonated species can adsorb on silanol groups leading to peak tailing. Another method that eliminates the above problems is ion pair reversed phase chromatography. With ion pairing, the retention of ionic compounds is controlled by the addition of a counter ion to the mobile phase to form hydrophobic ion pairs as illustrated by equation 1.2:

$$A^{-} + B^{+} \rightarrow [A^{-} B^{+}]_{org}$$  \hspace{1cm} (1.2)

When a counter ion ($B^{+}$) is added to the mobile phase, it forms an ion pair ($A^{-} B^{+}$) with
an analyte component of opposite charge (\( A_{aq}^- \)). The ion pairs can be extracted into an organic layer and be separated as nonionic polar molecules\(^{10}\). There are two proposed mechanisms for ion pair chromatography. One assumes that the ion pairs are formed in the mobile phase and travel through the system as the complexed species. The separation of these ion pairs occurs by partitioning between the mobile phase and the stationary phase. Another suggested mechanism is that the counterion partitions into the stationary phase with its ionic group oriented toward the surface. The stationary phase then behaves like an ion exchange column\(^{10,11}\).

The counterions most commonly used for acids are tetra alkylammonium salts. Various parameters such as the length of the alkyl groups, the concentration of the counterion, the pH, and the type and the composition of organic modifiers are used to control retention and selectivity\(^{11}\).

**Mass Spectrometry**

The mass spectrometer is composed of an ion source, mass analyzer, and an ion detection system. All components are enclosed in a vacuum manifold (See Figure 1.1). After the sample is introduced into the vacuum manifold, it is ionized in the ion source. The ions are then focused into the mass analyzer where they are separated by their mass to charge ratio (m/e). The ions are then detected by an electron multiplier. The electron multiplier output signal is amplified and passed into the data system for processing and for display\(^{13}\).
Figure 1.1 Quadrupole Mass Spectrometer

**Ion Source**

A description of the ion source can be found in reference 13. Briefly stated: The ion source consists of the following components: an ion source block, a filament, an ion volume, and lenses. The ion source generates a beam of electrons, provides a site for these electrons to interact with sample molecules to form ions, and then focuses the ions into the analyzer assembly.

The ion source block is heated to vaporize the sample and to minimize the rate of deposition of the analytes. In the electron impact (EI) ionization mode, the ion source is typically maintained at 250°C. In the chemical ionization (CI) mode, the temperature is typically maintained at 200°C or less.

The filament is a rhenium wire that is heated to produce electrons. The filament is maintained at a negative potential relative to the ion volume, so that the electrons emitted
from the filament are accelerated into the ion volume. The difference in potential between
the filament and the ion volume is called the electron energy. The energetic electrons
produced by the filament are used for the ionization process. The ion volume, located in
the center of the ion source, is the site where sample molecules interact with energetic
electrons and ions are formed. The ion source block and the ion volume are maintained at
ground potential. When the appropriate potentials are applied to the lenses, ions are
extracted from the ion volume. Three ion source lenses together focus the ions into a
beam and inject them into the analyzer assembly".

**Ionization Techniques**

There are several ionization techniques. The two general used techniques are:
electron impact ionization (EI) and chemical ionization (CI). In the EI mode of operation,
the analyte vapor is bombarded with a beam of energetic electrons generated by the
filament. By exchange of energy during the collision, an electron is removed from the
molecule, and a radical cation called the molecular ion, \( M^+ \), is generated\( ^{12} \):

\[
M + e^- \rightarrow M^+ + 2e^- \quad (1.3)
\]

The molecular ions may dissociate by the elimination of a radical (1.4), or by the loss of a
neutral molecule (1.5)\( ^{12} \):

\[
M^+ \rightarrow A^+ + B \quad (1.4)
\]

\[
M^+ \rightarrow C^+ + D \quad (1.5)
\]

If the fragment ion (\( A^+ \), \( C^+ \)) has sufficient internal energy, then further decomposition
may occur and new fragment ions are formed.

The pathway of such a series of decomposition is called the fragmentation pathway. The molecular ion and any of the fragment ions may decompose by more than one pathway. The various fragmentation pathways together compose a fragmentation pattern characteristic of the compound under investigation. The extent to which fragmentation takes place is determined by the molecular structure as well as the amount of internal energy originally imparted to the molecular ion.

The internal energy imparted to the molecular ion is controlled by the filament potential that can be varied from 5-100 eV. The ionization potential of most organic molecules is in the range of 5-13 eV. When 70 eV is applied, the excess internal energy causes the molecular ion to further dissociate into fragment ions. At 70 eV, the characteristic fragmentation pattern is nearly independent of the electric field.

When a constant electric field is maintained, the probability of a specific bond cleavage depends on the bond strength, and the stability of the charged and uncharged fragments formed by the dissociation process. Certain fragments are the result of the presence of particular functional groups in the molecule and their interconnection. For example, an alkyl substituted benzene ring will always produce a prominent peak at m/z 91 (C₆H₅CH₂⁺). The same fragments are produced in the same relative abundance for any given compound when the electron voltage is constant.

Most ions formed under EI conditions have a charge of +1, and the mass to charge ratio of any fragment is therefore equal to the mass of that fragment. The mass to charge ratio of the molecular ion corresponds to the molecular weight of the analyte. Based on
the molecular weight and fragmentation pattern, EI mass spectra can be used to deduce the structure of the analytes and provide a positive identification for unknown compounds\textsuperscript{15}.

In the CI ionization mode of operation, ionization of molecules is a two-step process. Reagent gas (such as CH\textsubscript{4} or NH\textsubscript{3}) is introduced into the ion source at a pressure of approximately 0.5 torr. In the first step of the ionization process, the reagent gas is ionized by interaction with energetic (10eV to 150 eV) electrons emitted by the heated filament. Then the reagent gas ions formed react with reagent gas molecules to form a variety of secondary ions (such as CH\textsubscript{3}\textsuperscript{+} and C\textsubscript{2}H\textsubscript{5}\textsuperscript{+}). In the second step, positive analyte ions are formed by transfer of a proton from a secondary reagent gas ion to an analyte molecule, or by an ion association reaction in which an adduct ion is formed between a reagent gas ion and a sample molecule. The ionization process, when methane is used as the reagent gas, is as follows:

\begin{align*}
\text{CH}_{4} & + e^{-} \rightarrow \text{CH}_{4}^{+} + 2 e^{-} \\
\text{CH}_{4}^{+} + \text{CH}_{4} & \rightarrow \text{CH}_{3}^{+} + \text{CH}_{3} + \text{CH}_{2}^{+} \\
\text{CH}_{3}^{+} + \text{CH}_{4} & \rightarrow \text{C}_{2}\text{H}_{5}^{+} + \text{H}_{2} \\
\text{CH}_{3}^{+} + \text{M} & \rightarrow \text{MH}^{+} + \text{CH}_{4}
\end{align*}

CI produces little fragmentation and usually provides only molecular weight information. However, by changing the reagent gas, the degree of fragmentation can be controlled\textsuperscript{12, 16}.
Parent ions, Daughter ions, Metastable Ions

If a molecular ion of mass $m_1$ does not dissociate before arriving at the collector, it is recorded as a molecular ion $m_1^+$. This molecular ion is also called the parent ion\textsuperscript{17}.

If a reaction ($m_1^+ \rightarrow m_2^+$) occurs in the source, then $m_2^+$ travels to the analyzer as a mass, $m_2$, and is recorded as daughter ion $m_2^+$.\textsuperscript{17}

If a reaction ($m_1^+ \rightarrow m_2^+$) occurs after the source slit, but, before arrival at the collector, a metastable ion is formed. Metastable ions are very useful for determining fragmentation pathways. If an ion of mass $m_1$ decomposes to give an ion of mass $m_2$, then a metastable ion may be found at mass $m^* = m_2^2/m_1$. Therefore, observation of a metastable ion at this position ($m^*$) confirms this fragmentation pathway. In the mass spectrum of toluene, there are ions at $m/z$ 91 ($C_7H_7^+$), $m/z$ 65 ($C_5H_5^+$), and $m/z$ 46.4. The metastable ion $m/z$ 46.4 ($=65^2/91$) indicates that at least some of the ions at $m/z$ 65 are from the ion at $m/z$ 91 by ejection of $C_2H_2$.\textsuperscript{17}

Mass Analyzer

A quadrupole mass analyzer was used for this work. It consists of four parallel metal rods arranged as shown in Figure 1.2.\textsuperscript{13} The opposite rods are connected together, one pair being attached to the positive side of a DC source and the other pair to the negative terminal. In addition, a radio frequency (RF) voltage is applied to both pairs. Neither field acts to accelerate the positive particles ejected from the ion source. The combined fields, however, cause the particles to oscillate about their central axis of travel. At fixed voltages, only those ions with a certain mass-to-charge ratio can pass through the
array without being removed by collision with one of the rods. Mass scanning is achieved by varying the potentials of the two sources while keeping their ratio constant. At a given instant, when a particular set of RF and DC voltages is being applied, only ions of mass to charge ratios, e.g., (m/z) 18, are allowed to pass through the quadrupole; during the same time, all other ions are removed. At a later time, both RF and DC voltages change, and ions of the next mass to charge ratio (m/e) 19 are allowed to pass, while all other ions, including m/z 18, are removed. This procedure continues, with ions of one mass to charge ratio after another being transmitted, as the RF and DC voltages change. At the end of the scan, the RF and DC voltage are discharged to zero, and the process is repeated. By this technique, an entire mass spectrum is produced.\textsuperscript{18,19}

**Ion Detection System**

The ions, separated by the analyzer, are detected when they reach the collector. Among the several collectors available, the electron multiplier is the most sensitive and the
fastest responding detector. The positive ions leaving the analyzer are increased in energy by an accelerating voltage of -5000 volts. After each ion collides with the surface of the conversion dynode, one or more electrons are ejected from its surface. These electrons are then repelled from the conversion dynode and accelerated toward the continuous-dynode electron multiplier. As electrons pass through the electron multiplier, they strike the walls, causing the emission of more and more electrons. Thus, a cascade of electrons is created that finally results in a measurable current at the end of the electron multiplier. Typically, an electron multiplier has a gain of about $10^5$ (i.e., for each ion or electron, $10^5$ electrons are produced). If the current of ions entering the electron multiplier from the quadrupole filter is $10^{-12}$ A and the gain of the multiplier is $10^5$, then a current of $10^{-7}$ A leaves the electron multiplier through the anode.

HPLC/MS Techniques

Background

Many current methods for the analysis of nonpolar, volatile, thermally stable organic compounds are based on GC/MS. However, 80 to 90 percent of known organic compounds cannot be analyzed by GC/MS. For the analysis of polar, nonvolatile, organic compounds such as the fluorinated benzoic acids, GC methods are limited. Strongly acidic and polar carboxyl groups cause strong adsorption on the GC column and often produce tailing peaks. The thermal instability of the compounds causes the loss of the compounds in the chromatographic system. Therefore, derivatization is used to
improve the gas chromatographic characteristics of the polar compounds by increasing their volatility or their thermal stability. For example, some organic acids need to be esterified into volatile derivatives prior to GC analysis. The derivatization procedures usually require that the analytes are dissolved in an organic solvent. This requires a solvent exchange for the analysis of water samples. Also, the derivatization step required for GC/MS methods prolongs the analysis. The need for quick, sensitive methods for identification and determination of nonvolatile organic compounds has led to the widespread use of HPLC with UV/visible, refractive index, fluorescence, conductivity and electrochemical detectors. However, identification of compounds using these detectors is based on the retention time; these methods do not provide the positive compound identification capability of a mass spectrometer. The on-line coupling of the HPLC to MS combines the benefits of two well established and complementary techniques. However, these two techniques are fundamentally incompatible. A mass spectrometer requires a high vacuum and the ionization of molecular species in the gas phase, whereas, HPLC is used to analyze compounds that are not volatile enough to be analyzed by GC.

There are three fundamental compatibility problems that must be solved when interfacing liquid chromatography and mass spectrometry. The liquid stream cannot be introduced directly into the mass spectrometer at normal LC flow without overloading the vacuum system. The liquid has to be evaporated and the solvent has to be pumped away before ionization is possible. The nonvolatile and thermally liable molecules often used with HPLC, are difficult to vaporize without degrading them.

The on-line combination of LC and MS has been under investigation for about 20
years. During this time, a number of interfaces have been designed to solve the above dilemma. These interfaces can be classified into three categories:

Direct liquid introduction (DLI): LC effluent is split, only 1 to 10 ul/min of effluent enters the ion source. Analytes are ionized by chemical ionization in the ion source. Chemical ionization provides only molecular weight information. Therefore, this method provides little use for the analysis of unknown mixtures.

Liquid ion evaporation: These methods which include thermospray (TSP), electrospray (ESP), and ion spray (ISP) interfaces are a combination of sample introduction and ionization techniques. Analytes are ionized in solution with the help of heat (TSP), electrical field (ESP), electrical field and pneumatic force (ISP) while they are transported into MS. They are soft ionization methods, which give molecular weight information but substantially less structure information than that provided by EI spectra. This limits the usefulness of these methods for the analysis of unknown samples.

Solvent removal: These methods include moving belt (MB) and particle beam (PB) interfaces. Solvent is removed before the analytes are introduced into the ion source. With these methods the analytes can be ionized by both EI and CI. The MB interface is limited to a relatively narrow range of volatile compounds. Compounds with low volatility are difficult to remove from the belt, while compounds with high volatility may be lost from the belt before entering the ion source of MS. Also, the MB is difficult to operate.

Because the goal of this research is to separate the disubstituted benzoic acids, the particle beam interface is the interface of choice. The fragmenting capability of electron
impact ionization is required.

**Particle Beam (PB) Interface**

The PB interface was first introduced by Willoughby and Browner in 1984.\(^{25,26}\) This technique has shown promise for analyzing compounds with low volatility and thermal stability. The PB interface can be operated under both EI and CI ionization. The EI spectra obtained contain the fragmentation information necessary for matching with library spectra of reference compounds obtained from GC/MS. Therefore, PB/LC/MS can provide a positive identification of unknown compounds. Since the first commercial version of the PB interface introduced in 1988, this technique has been applied to a variety of environmental organic compounds such as diazo dyes, aromatic sulfonic acids, benzidine, and carbamate pesticides. The performance of this technique has been evaluated by several groups.\(^{27-35}\)

**Principle of PB Interface**

Figure 1.3 is a diagram of the PB interface.\(^1\) All the liquid effluent from the \(^{1}\) HPLC is forced into a fused silica tubing (O.D. = 350 \(\mu\)m) inside the nebulizer. A thermal pneumatic nebulizer is used to convert the LC effluent, typically 0.5 - 1 ml/min, into a very fine aerosol. As the aerosol passes through the slightly heated (64°C to 80°C) desolvation chamber, the volatile solvent is vaporized, leaving the less volatile analyte as an aerosol of submicrometer particles. The mixtures of helium gas, solvent vapor, and particles then enter a two-stage momentum separator. The momentum separator consists of one nozzle,
Figure 1.3 Particle Beam Interface (Reprinted With Permission from Extrel Co.)
two skimmers and two stage mechanical pumps (E2M18, Edwards High Vacuum).

Solvent vapor and particle mixtures pass through the nozzle at supersonic velocity into a subsonic area. The first skimmer allows a central particle beam with the higher momentum to be transmitted while the solvent vapor with low momentum is pumped away. The second skimmer removes more of the vapor while allowing the particles to pass. This process results in analyte enrichment and pressure reduction. The pressure is reduced from approximately 200 Torr in the desolvation chamber to 5-10 Torr in the first momentum separator stage, to 0.5 Torr in the second momentum separator stage, to $10^{-5}$ Torr in the mass spectrometer ion source. After the dry particles enter the ion source of the mass spectrometer, they collide with the hot (250°C) surface of the ion source and are flash vaporized. The vapor-phase analyte molecules are then ionized by either EI or CI processes. Ions generated are then focused into the mass analyzer by lenses for identification.

Transfer Efficiency

The transfer efficiency is defined as the ratio of the amount of analyte collected in the ion source to the total amount of analyte introduced. The transfer efficiency depends at least on the design of the interface, the nature of the mobile phase, the vapor pressure of the analyte, the concentration of the analyte, and the presence of coeluting carrier substances. The transfer efficiency of the PB interface is limited. Significant analyte losses often take place in the desolvation chamber and the momentum separator. The analytes are often either too volatile and are pumped away with the solvent vapor, or too
heavy and sediment in the desolvation chamber. The small particles resulting at low analyte concentration are more subject to turbulent losses\textsuperscript{31}. The factors that affect the transfer efficiency are complex.

The transfer efficiency of a Hewlett-Packard 59980A PB interface has been investigated. It was reported that analyte transport efficiencies are generally less than 20\% for most analytes in the PB interfaces\textsuperscript{32}. Transfer efficiencies up to 50\% are reported by the Extrel Manufature for the Extrel PB\textsuperscript{3}. Due to the poor transport efficiency of the analytes through the Particle beam interface, the response of PB LC/MS is often non linear at low concentration and the sensitivity of PB LC/MS is relatively poor.

\textbf{Non-Linearity}

The PB LC/MS interface is subject to variable transport efficiencies, resulting in a nonlinear response at low concentrations. The nonlinear behavior was first described by Bellar et al.\textsuperscript{32} and has since been observed in various laboratories\textsuperscript{33-40}. A model to predict the response factors and linear performance has been proposed by Apfel\textsuperscript{33}. The model assumes that the PB interface has a particle size cutoff level, below which small particles are pumped away in the momentum separator or lost in the system, and above which the larger particles are transferred quantitatively into the MS source. An initial aerosol entering the PB desolvation chamber has a given droplet size. The size and mass of the resulting desolvated particles depend only on the initial droplet size. The droplet size in turn depends on the analytes concentration, the sample bulk density, and the diameter of the nebulizer tip. As the sample concentration is reduced, the resulting particle's size is
also reduced. At some point, the mean size of the particle begins to pass the cut off level. This leads to a reduction in the response factor, and results in a calibration curve that is linear at the higher concentration but nonlinear at the lower levels.

**Carrier Effects**

During the investigation of the nonlinear behavior of the PB interface, Bellar et al noted enhanced ion abundance for some polar compounds when ammonium acetate was added to the mobile phase. Bellar describes this phenomenon of ion abundance enhancement as a “carrier effect,” and offered a possible explanation. It was proposed that analytes and the “carrier” form molecular aggregates, such as molecule-ammonium ion complexes, in the mobile phase and in the initial aerosol. These aggregates are formed through weak dipole-dipole interactions or hydrogen bonding interactions. The higher momentum aggregates persist in the desolvated particles and act as carriers, carrying the analyte particles through the PB momentum separator. The carrier increases the effective mass of the particles and reduces the vapor pressure of the analyte. Thus, vaporization of analyte from the droplets is reduced, and subsequently reducing loss of the analyte in the momentum separator. These weak complexes, after entry into the high vacuum of the ion source, are rapidly dissipated during flash vaporization of the particles on the hot (250°C) walls of the ion source. The carrier effect results in an increased transfer efficiency. This improves the sensitivity and reduces the nonlinear behavior of PB LC/MS. The “carrier” with a structure similar to the target analyte has been studied by Mattina. It was shown that the addition of 0.4M hydroxy succinic acid leads to
significantly improved sensitivity and linearity in the analysis of succinic acid (HOOCCH₂CH₂COOH). By using phenoxy acetic acid as the carrier in the analysis of a mixture of chlorophenoxy acids, a linear calibration curve and better detection limits are obtained.

Mass Spectra

Mass Spectra of Benzoic Acids

The mass spectra of benzoic acid and a number of methyl substituted benzoic acids have been studied by McLafferty⁴¹ and Aczel⁴² in late 1950s. It was concluded that the molecular ion (M⁺) is one of the most abundant peaks in the spectra of these acids. This is due to the high resonance stability of the benzoyl system⁴². The relative abundance of the molecular ion depends on the proximity of the methyl groups. Two other characteristic peaks are the ions due to the loss of the hydroxyl radical (M⁺-OH)⁺, and the ions due to the loss of the carboxyl radical (M⁺-COOH)⁺. Both ions decrease with the proximity of substituents to the carboxyl functional group⁴².

When there are hydrogens in the ortho position, the loss of mass 18 (M⁺-H₂O)⁺ becomes prominent. A similar ortho effect occurs with the loss of mass 46 (M⁺-COOH-H). A peak corresponding to an M⁺-44 (M⁺-CO₂) fragment is also observed in the spectra of all compounds. Aczel⁴² thinks the loss of CO₂ is primarily due to electron bombardment and to a minor extent of thermal decomposition in the system. It was proposed by McLafferty⁴¹ that the cleavage of the phenyl-carbonyl bond causes...
rearrangement of a hydrogen to the phenyl group, to give an M'–44 ion and a stable carbon dioxide molecule. He also observed that formation of the M'–44 ion is favored when the M'–18 ion is insignificant.

Aczel\textsuperscript{14} concludes that the position of the methyl substituents on the benzene ring are important in the formation of the fragment ions. The relative abundance of the peaks, which are mostly influenced by the ortho effect (M–H\textsubscript{2}O, M–COOH–H), can be used to determine the position of the substituted groups. 2,6 and 3,5 methyl benzoic acid can be easily determined in sample mixtures. At least, it can be determined whether the substituted group is in the ortho position. Table 1.1 lists peak and their relative abundance of six methyl substituted benzoic acids\textsuperscript{22}, only the ions present in significant amounts are included.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|c|c|}
\hline
\hline
3,4-Dimethylbenzoic acid & 88.1 & 6.11 & 37.4 & 17.6 & 10.6 & 100 & 17.2 \\
3,5-Dimethylbenzoic acid & 100 & 8.1 & 39.8 & 4.7 & 10.1 & 99.3 & 12.7 \\
2,3-Dimethylbenzoic acid & 100 & 6.6 & 29.4 & 96.7 & 9.8 & 94.6 & 71.5 \\
2,4-Dimethylbenzoic acid & 88.9 & 4.05 & 52.1 & 100 & 7.59 & 70.57 & 54.3 \\
2,5-Dimethylbenzoic acid & 98.3 & 5.1 & 32.6 & 90.1 & 10.7 & 100 & 90.4 \\
2,6-Dimethylbenzoic acid & 65.9 & 2.75 & 26.1 & 100 & 4.89 & 47.7 & 57.8 \\
\hline
\end{tabular}
\caption{Relative Abundance of Methyl Substituted Benzoic Acids}\textsuperscript{22}
\end{table}

In 1965, the fragmentation mechanism of benzoic acid was elucidated by Meyerson and Corbin\textsuperscript{13} using deuterium labeling of the carboxyl group, the 2,3,4 ring positions, and the analysis of the metastable peaks. The associated metastable peaks demonstrated that the molecular ions \([C\textsubscript{6}H\textsubscript{5}COOH]\textsuperscript{+} undergo sequential loss of OH and CO (See Figure 1.4). The molecular ion lost a hydroxyl group first, giving a metastable peak at 90.4 and
a daughter ion of m/e at 105; carbon monoxide is then lost to give a metastable peak at 56.5 and the daughter ion C₆H₅⁺.

![Diagram of fragmentation mechanism](image)

**Figure 1.4 Fragmentation Mechanism of Benzoic Acid**

It was also shown by deuterium labeling of the carboxyl group and the 2,3,4 ring positions that there is partial exchange of hydrogen between the carboxyl hydrogen and ortho hydrogens before OH is lost from the molecular ion⁴⁴. It was concluded that the loss of the hydroxyl radical from the benzoic acid molecular ion has at least two mechanisms⁴⁵⁻⁴⁹. In the first mechanism the loss is due to the simple cleavage of the C-OH bond (See Figure 1.4). The other mechanism proposes that this loss is from the rearranged species. This involves the transfer of a hydrogen atom from the ortho-ring positions to the carboxyl group (Figure 1.5). The C₇H₅O⁺ ions yield therefore includes two isomeric species B and E. Only B will lose carbon monoxide to give C₆H₅⁺. E is unusually stable and does not decompose further to give C₆H₅⁺.

The scrambling process in which the carboxyl hydrogen and the ortho hydrogens of benzoic acid undergo partial exchange has been studied by several groups⁴⁵⁻⁴⁹. Benoit⁴₆
studied the effect of substituents of (NH₂, OH, CH₃O, CH₃, F, Br, COOH, CN, NO₂) on the scrambling process of the molecular ions of para and meta substituted benzoic acids. The scrambling process occurs in all the substituted benzoic acids examined, except the para and meta nitro benzoic acids. He concluded that different molecular ions have different energy distributions. The different energy distributions cause different abundances of molecular ions and fragmentation ions. The energy of activation for the loss of hydroxyl from the molecular ion is dependent on the nature, but not the position of substituent. However, the amount of the H/D scrambling in the molecular ion of carboxyl-d₄ derivatives is dependent on both the nature and the position of the substituent. No
consistent correlation between the amount of scrambling and the electron withdrawing or
donating power of the substituent was detected. However, when the substituent is in the
para position, it more effectively inhibits the scrambling mechanism than when it is meta to
the carboxyl group. Therefore, there is an increase in the rate of hydroxyl loss by the
direct cleavage mechanism in the para substituted acid. In addition, substituents reduce
the abundance of molecular ions formed.

Mass Spectra of Methyl Benzoates

The mass spectra of methyl benzoate and substituted methyl benzoates appear to
be generally analogous to benzoic acid and substituted benzoic acid. The molecular ion of
the methyl benzoate is not as intense as in benzoic acid, but still very significant. The
predominant cleavage is at the bonds to the methoxy group. The (M-OCH₃)⁺ ion is the
base peak. The ion due to loss of COOCH₃ is also prominent (Figure 1.6). The ortho-
effect again is very striking for the loss of an extra hydrogen with the methoxy (M-OCH₃-
H)⁺, and for the loss of COOCH₃-H.

![Figure 1.6 Fragmentation Mechanism of Methyl Benzoates](image)

Shapiro and Beynon studied the mass spectrum of ethyl benzoates. They found
that after eliminating neutral \( \text{C}_2\text{H}_4 \), transferring a hydrogen atom from the ethyl group to
the carbonyl-oxygen, the carboxylic acid hydrogen can exchange with ortho position
hydrogen. (See Figure 1.7)

\[
\text{CH}_3\text{CH}_2\text{COOH} \quad \xrightarrow{-\text{CH}_2\text{CH}_2} \quad \text{CH}_3\text{CH}(...)\text{OH}
\]

Figure 1.7 McLaffery Rearrangement in Methyl Benzoates

**Mass Spectra of Fluorobenzene**

The mass spectra of the ring substituted mono-fluorinated benzenes have been
studied by McLafferty\(^2\). The carbon-fluorine bonds in the ring substituted fluorinated
aromatic compounds are very stable. This high stability makes the fluoride substituted
spectra very similar to the corresponding aromatic hydrocarbons. None of the fragment
ions formed with loss of the fluorine atom appear to be significant. Isomers that differ
only by the position of the substitution on the ring give qualitatively similar mass spectra.

A. M. Wirtz-Cordier\(^3\) studied the difluorobenzenes. He found that the molecular
ions abundance is related to the position of fluorine. (See Table 1.2).
Table 1.2 Molecular Ion Abundance of Fluoro Substituted Benzene

<table>
<thead>
<tr>
<th>Fluorine Position</th>
<th>Molecular Ion Abundance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Para difluoro benzene</td>
<td>27%</td>
</tr>
<tr>
<td>Ortho difluoro benzene</td>
<td>18.2%</td>
</tr>
<tr>
<td>Meta difluoro benzene</td>
<td>12.8%</td>
</tr>
</tbody>
</table>

Research Plan

A method of separation and identification of the difluorobenzoic acids using HPLC/MS with a particle beam interface was developed. Difluorobenzoic acids are 2,3-DFBA; 2,4-DFBA; 2,5-DFBA; 2,6-DFBA; 3,4-DFBA and 3,5-DFBA. Analytes were first separated by HPLC/UV to select the best isocratic mobile phase conditions. Ionization control and ion pairing techniques were used. The separated acids will then be introduced to the MS by particle beam interface for mass separation. A performance evaluation procedure of HPLC/MS with PB interface was carried out. The effects of mobile phase additives on fragmentation pattern, sensitivity and reproducibility for the difluorobenzoic acids was examined.
CHAPTER 2

EXPERIMENTAL

Particle Beam LC/MS Hardware

The Particle Beam LC/MS system used in this study consists of three parts: the HPLC, the particle beam interface, and the mass spectrometer. A Spectra-Physics (SP 8800, San Jose, California USA) HPLC equipped with a ternary gradient solvent delivery system was used. The injection system consisted of a Rheodyne 7125 injection valve fitted with a 20-μl sample loop. The column used was a Supelco C-18, 15.0 cm × 4.0 mm, packed with 5-μm spherical particles. The chromatographic outlet is connected to the particle beam interface by poly ether ketone tubing.

The interface is an Extrel thermabeam interface that provides a heated nebulizer with temperature control. The nebulizer was equipped with a fused silica capillary column. A 150-μm ID fused silica tubing was used in this study. The mass spectrometer is a Benchmark benchtop quadrupole system (Extrel Corporation, Pittsburgh, PA, USA). This instrument can be switched from LC/MS to GC/MS, and from a particle beam interface to a thermospray interface. The MS and interface system are controlled through a Sun Station Sparc I data system with Open Windows (ver2) and Extrel Ion Station software packages. The vacuum in the chamber is maintained by two turbo molecular...
pumps (Balzers TPH520, Balzers TPH190) that are backed by a mechanical roughing pump (2 stage rotary vanes, E2M8, Edwards High Vacuum). The vacuum in the momentum separator is maintained by two mechanical roughing pumps (2 stage rotary vanes, E2M18, Edwards High Vacuum).

Instrument performance was optimized as follow.

**PB MS Performance Evaluation Procedure**

**Vacuum Check**

The pressures in the ion source (10^-6) and desolvation chamber (10^-2) are checked to insure that there are no major leaks before turning on the filament and electron multiplier voltage. The intensities of ions due to air (m/e 28 N_2^+, 32 O_2^+, 40 Ar^+) and water (m/e 18 H_2O^+) are then checked. The abundance of m/e 28 should be less than that of m/e 18, and each should be less than 5% of m/e 69, otherwise, there may be an air leak in the system. Exceptions are when it is within 1 hour of venting.

**Tuning the MS**

Tuning is a process for optimizing the performance of the mass spectrometer. The goal of tuning is to maximize sensitivity while maintaining acceptable resolution, ensuring accurate mass assignment, and providing the desired relative abundance across the mass spectrum. Tuning is performed at the beginning of each day and after maintenance of the MS. The tune report is kept in a log book. The tuning log book provides information to
detect the start of a tuning problem and to monitor the gradual rise of the electron multiplier and lens voltages over time. These reports help in planning ion source cleaning or multiplier replacement. Perfluorotributylamine (PTA or PFTBA) is used as the tuning compound.

The mass assignment should be within +/− 0.2 amu of the correct value. The peak width at half the peak height should be 0.5 +/− 0.1 amu. In the positive EI mode, with the ion source temperature at 250°C, mass 69 is the base peak. Relative to 69, the peak at 219 amu is between 35% to 99%, and the peak at 502 should be higher than 1%. The relative abundances of the isotope should be close to the values of 1.08 for m/e 69, 4.32 for m/e 219, and 10.09 for m/e 502.

Tuning the PB Interface

The PB interface is connected and tuned in the following order: High purity helium is first introduced to the nebulizer to serve as the nebulizing gas. The HPLC mobile phase is then pumped through the nebulizer at 0.4 - 1 ml/min, and the nebulizer heater is turned on. The aerosol produced at the tip of the fused silica column is observed, while adjusting helium and mobile phase flow rates and the nebulizer temperature. A fine aerosol that is not too dry or wet is desired. If it is too dry or too wet, significant analyte losses will take place in the desolvation chamber. The desolvation chamber heater is turned on, and the isolation valves that isolate the roughing pumps from the vacuum chamber are opened. The PB interface is then connected to the desolvation chamber. The helium pressure, the temperature of the nebulizer and the desolvation chamber are adjusted to avoid liquid
drops condensing in the desolvation chamber. The typical operation conditions are given
in Table 2.1.

<table>
<thead>
<tr>
<th>Table 2.1 PB Interface Operation Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mobile Phase Flow Rate</td>
</tr>
<tr>
<td>Helium Flow Pressure</td>
</tr>
<tr>
<td>Nebulizer Temperature</td>
</tr>
<tr>
<td>Desolvation Chamber Temperature</td>
</tr>
</tbody>
</table>

After the preliminary tuning, caffeine is injected, and the interface is further
optimized by watching the abundance of the caffeine at m/e 194 while making fine
adjustments of the above parameters.

**PB MS Conditions**

The pressure of the ion chamber should be maintained at 1.5-2.5 × 10⁻³ torr while
the PB interface is connected. A clean ion volume is installed at the beginning of the
analysis. The ion source temperature was at 250 °C, and the electron energy was 70 eV.
The mass spectrometer is scanned from 100 to 350 amu at 1.5 sec/scans. All spectra were
acquired in the positive ion mode. The MS should be off until the solvent peak has eluted
from the column and passed through the PB interface. A solvent delay of 3 minutes is
usually used.

**Evaluation of Spectral Quality**

The separation work is based on the masses of the fragmentation ions and their
relative intensities. Variations in the LC/MS mass spectra have been found in this study. The variability in the mass spectra made it difficult to separate and identify the isomers. Efforts were made to find the cause and thereby control this variability. Three factors that are easily controlled were investigated. These include the temperature and cleanliness of the ion source as well as the concentration of the analyte.

The Temperature of the Ion Source

In PB LC/MS, the analyte particles must be vaporized upon impact with the ion source wall before they can be ionized by the electron beam. If there is insufficient thermal energy available to the particles as they impact the source wall, vaporization is not complete and ionization does not completely occur. This often results in peak tailing. However, at higher ion source temperatures (to 300 °C), thermal decomposition may occur.

2,3-DFBA was selected to evaluate the ion source temperature. Table 2.2 shows the relative abundance of three ions at three ion source temperatures. The total ion chromatogram peaks exhibited tailing at 200 °C. The peak shape of this acid became sharper upon heating towards 300°C but spectral quality suffered as seen in table 2.2. Thus, 250 °C was used in all experiments in this study. This temperature produced the better spectral quality while maintaining symmetrical chromatographic peaks.
Table 2.2 Effects of the Ion Source Temperature on the Spectra Quality

<table>
<thead>
<tr>
<th>2,3-DFBA</th>
<th>200 °C</th>
<th>250 °C</th>
<th>300 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>m/e 113</td>
<td>58.1</td>
<td>76.5</td>
<td>82.3</td>
</tr>
<tr>
<td>m/e 123</td>
<td>43.2</td>
<td>57.8</td>
<td>100</td>
</tr>
<tr>
<td>m/e 141</td>
<td>100</td>
<td>100</td>
<td>80.5</td>
</tr>
</tbody>
</table>

Cleanliness of the Ion Source

Among the six isomers, 2,6-DFBA was the only one that has a reference GC/MS mass spectrum (NIST). 2,6-DFBA was therefore selected to study the effects of the cleanliness of the ion source on the mass spectra. 2,6-DFBA is injected into a clean ion source with a clean ion volume. At the beginning of the experiment, the base peak of 2,6-DFBA was at m/e 141 (M-O H)^+. After six injections, the base peak of 2,6-DFBA shifted to m/e 114 (M-CO^-). The reason for base peak shifts is not clear. The change in the relative abundance over time is shown in Table 2.3. In all experiments that follow a clean ion volume was installed each day prior to analysis.

Table 2.3 Effects of the Cleanliness of the Ion Source on the Mass Spectra

<table>
<thead>
<tr>
<th>2,6-DFBA (25ppm)</th>
<th>m/e 114 (M-CO^-)</th>
<th>%RSD</th>
<th>m/e 141 (M-OH)^+</th>
<th>%RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Injection 1-3</td>
<td>62.7</td>
<td>4.40</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Injection 4-6</td>
<td>87.8</td>
<td>1.20</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Injection 7-9</td>
<td>100</td>
<td>0</td>
<td>91.7</td>
<td>5.74</td>
</tr>
<tr>
<td>Injection 19-21</td>
<td>100</td>
<td>0</td>
<td>59.0</td>
<td>5.15</td>
</tr>
<tr>
<td>Injection 22-24</td>
<td>100</td>
<td>0</td>
<td>59.5</td>
<td>12.8</td>
</tr>
</tbody>
</table>
Concentration

2,6-DFBA was also used to evaluate the effect of analyte concentration on the spectral quality. Three injections were made of 2,6-DFBA at four concentration levels. There is no significant influence of the concentration on the relative intensity of the 2,6-DFBA spectrum if the ion source is clean and other conditions are not changed. Experimental data are summarized Table 2.4.

<table>
<thead>
<tr>
<th>2,6-DFBA (25ppm)</th>
<th>m/e 114</th>
<th>%RSD</th>
<th>m/e 141</th>
<th>%RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 ppm</td>
<td>51.8</td>
<td>4.58</td>
<td>33.2</td>
<td>11.7</td>
</tr>
<tr>
<td>40 ppm</td>
<td>52.2</td>
<td>2.57</td>
<td>33.1</td>
<td>5.0</td>
</tr>
<tr>
<td>20 ppm</td>
<td>36.6</td>
<td>8.1</td>
<td>23.1</td>
<td>20.6</td>
</tr>
<tr>
<td>12.5 ppm</td>
<td>49.3</td>
<td>21.2</td>
<td>30.2</td>
<td>4.6</td>
</tr>
</tbody>
</table>

Liquid Chromatographic Condition (Experiment I)

Ionization Control

Separation of the difluorobenzoic acids was one of the primary goals in this study. Difluorobenzoic acids were first separated and detected by HPLC/UV to select the mobile phase condition. This work was performed on a HPLC system with a UV visible detector. Since the pKa value for the difluorobenzoic acids ranged from 2.85 to 3.85 at 298 K\(^{38}\) (See Table 2.5), ionization control techniques were first employed to separate these ionic compounds. The mobile phases were acidified with formic acid to suppress dissociation
Table 2.5 $pK_a$ (298K) value for difluorobenzoic acids

<table>
<thead>
<tr>
<th>Compound</th>
<th>$pK_a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,3-DFBA</td>
<td>3.29</td>
</tr>
<tr>
<td>2,4-DFBA</td>
<td>3.58</td>
</tr>
<tr>
<td>2,5-DFBA</td>
<td>3.30</td>
</tr>
<tr>
<td>2,6-DFBA</td>
<td>2.85</td>
</tr>
<tr>
<td>3,4-DFBA</td>
<td>3.83</td>
</tr>
<tr>
<td>3,5-DFBA</td>
<td>3.59</td>
</tr>
</tbody>
</table>

of the acids. Initially 70% MeOH and 30% H$_2$O with formic acid/0.01M ammonium acetate were tested. Increasing the amount of H$_2$O will increase the polarity of the mobile phase and will therefore increase the retention time. Mobile phases with increasing percentages of H$_2$O were then tested. The chromatographic conditions in experiment I are listed below. Retention times of difluorobenzoic acids in experiment I are given Table 2.6.

I. Column: Supelco C-18, 15.0cm × 4.0mm, packed with 5-um spherical particles.
Flowrate: 0.4ml/min
UV: 245nm
Mobile phase:
- 65% DI H$_2$O with 0.01M ammonium acetate pH=3.0
- 35% MeOH
- Analyte Concentration 20 ppm

II. Column: Supelco C-18, 15.0cm × 4.0mm, packed with 5-um spherical particles.
Flowrate: 1ml/min
UV: 250nm
Mobile phase:
- 65% DI H$_2$O with 0.01M ammonium acetate pH=3.0
- 35% MeOH
- Analyte Concentration 20 ppm

III. Column: Supelco C-18, 15.0cm × 4.0mm, packed with 5-um spherical particles.
Flowrate: 0.4ml/min
UV: 250nm
Mobile phase:
- 75% DI H₂O with 0.01M ammonium acetate pH=2.7
- 25% MeOH
Analyte Concentration 20 ppm

IV. Column: Supelco C-18, 15.0cm × 4.0mm, packed with 5-um spherical particles.
Flowrate: 0.4ml/min
UV: 250nm
Mobile phase:
- 75% DI H₂O with 0.01M ammonium acetate pH=2.7
- 25% Acetonitrile
Analyte Concentration 20 ppm

Table 2.6 Retention Times (minutes) of Difluorbenzoic Acids Under Ionization Control Conditions:

<table>
<thead>
<tr>
<th>Compound</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,6-DFBA</td>
<td>6.12</td>
<td>2.51</td>
<td>6.86</td>
<td>7.43</td>
</tr>
<tr>
<td>2,5-DFBA</td>
<td>13.08</td>
<td>3.87</td>
<td>10.99</td>
<td>11.47</td>
</tr>
<tr>
<td>2,3-DFBA</td>
<td>13.93</td>
<td>4.01</td>
<td>11.60</td>
<td>12.41</td>
</tr>
<tr>
<td>2,4-DFBA</td>
<td>15.48</td>
<td>4.28</td>
<td>12.35</td>
<td>12.86</td>
</tr>
<tr>
<td>3,4-DFBA</td>
<td>28.53</td>
<td>6.78</td>
<td>20.35</td>
<td>19.89</td>
</tr>
<tr>
<td>3,5-DFBA</td>
<td>28.53</td>
<td>7.02</td>
<td>20.98</td>
<td>19.89</td>
</tr>
</tbody>
</table>

1: Partially Resolved, 2: Co-elute

For the ionization control technique, a mixture of 25% MeOH and 75% H₂O with acetic acid/0.01M ammonium acetate at pH=2.7 (condition III) gives the best separation. There was no baseline separation of 3,4-DFBA and 3,5-DFBA using any of the tested conditions.
Ion Pairing

The operating pH range for the bonded phase is 2.5-7.5. In order to utilize ionization control optimally, a pH (below 2.5) two units lower than the pKa of DFBA is preferred for these compounds. Ion pairing technique was therefore employed to separate these compounds. Tetrabutyl ammonium acetate was selected as the ion pairing reagent.

The chromatographic conditions are listed as follow. Retention times of difluorobenoic acids in experiment II are given Table 2.7.

V. Column: Supelco C-18, 15.0cm x 4.0mm, packed with 5-um spherical particles.
   Flowrate: 0.4ml/min
   UV: 254nm
   Mobile phase:
   50% DI H2O with 0.05M tetra butylammonium acetate
   50% MeOH
   pH=7.4
   Analyte Concentration 20 ppm

VI. Column: Supelco C-18, 15.0cm x 4.0mm, packed with 5-um spherical particles.
   Flowrate: 0.4ml/min
   UV: 254nm
   Mobile phase:
   50% DI H2O with 0.075M tetra butylammonium acetate
   50% MeOH
   pH=7.4
   Analyte Concentration 20 ppm

VII. Column: Supelco C-18, 15.0cm x 4.0mm, packed with 5-um spherical particles.
    Flowrate: 0.4ml/min
    UV: 254nm
    Mobile phase:
    70% DI H2O with 0.075M tetra butylammonium acetate
    30% MeOH
    pH=7.4
    Analyte Concentration 20 ppm
Table 2.7 Retention Times (minutes) of Difluorobenzoic Acids under Ion Pairing Condition:

<table>
<thead>
<tr>
<th>Compound</th>
<th>V</th>
<th>VI</th>
<th>VII</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,6-DFBA</td>
<td>8.63</td>
<td>9.08</td>
<td>13.42</td>
</tr>
<tr>
<td>2,5-DFBA</td>
<td>9.12(^2)</td>
<td>9.90</td>
<td>16.90</td>
</tr>
<tr>
<td>2,3-DFBA</td>
<td>9.12(^2)</td>
<td>10.70(^2)</td>
<td>17.83</td>
</tr>
<tr>
<td>2,4-DFBA</td>
<td>9.12(^2)</td>
<td>10.70(^2)</td>
<td>19.02</td>
</tr>
<tr>
<td>3,4-DFBA</td>
<td>10.08(^2)</td>
<td>12.67(^2)</td>
<td>33.63(^1)</td>
</tr>
<tr>
<td>3,5-DFBA</td>
<td>10.08(^2)</td>
<td>12.67(^2)</td>
<td>34.47(^1)</td>
</tr>
</tbody>
</table>

For the ion pairing technique, a mixture of 30% MEOH and 70% H\(_2\)O with 0.075M tetrabutylammonium acetate at pH=7.4 (condition VII) gives the best separation. Again, no baseline separation of 3,4-DFBA and 3,5-DFBA was achieved.

PB LC/MS Experimental (Experiment II)

The optimum mobile phase conditions obtained in Experiment I were tested first, however, these were not good conditions for the PB interface. The PB interface requires a high percentage of organic solvent and a lower percentage of water. Gradient elution cannot be used for the same reason. Separation of the difluorobenzoic acids by a chromatographic system is not the primary concern of the experimental work. The mass spectra are obtained for each analyte, with the hope that coeluting analytes can be determined by mass spectra. In this experiment, difluorobenzoic acids are injected into the column individually.

To improve the transfer of the analytes into the mass spectrometer, a compound
that would function as a carrier was introduced. Ammonium acetate has been reported as a carrier that increases MS sensitivity. Ammonium acetate (0.01M) was added to the mobile phase in experimental condition VIII.

The function of ion pairing reagents in LC/MS is actually two fold. Ion pairing agents are used for the separation of ionic compounds. Ion pairing agents can also be good carriers, the analyte and ion pairing agent can form more stable ion pairs by chemical bonding, and therefore improve sensitivity and reproducibility of the spectra.

Tetrabutylammonium acetate was first investigated, but it caused clogging of the skimmer and nozzle area. Tetramethylammonium acetate was then investigated in experiment condition IX.

Condition VIII:

Column: Supelco C-18, 15.0cm x 4.0mm, packed with 5-um spherical particles.
Flowrate: 0.4 ml/min
Mobile phase:
- 70% MeOH with 0.01M ammonium acetate
- 30%DI H2O, pH=2.7 adjusted by formic acid
Helium pressure: 75psi
Nebulizer temperature: 90°C
desolvation Chamber temperature: 60°C
Ion Source temperature: 250°C
Ionization Mode: +EI
Scan range: 70 - 250 amu
Scan rate: 1.5 sec/scan
Electron Multiplier voltage: 1800ev
Analyte concentration: 20ppm

Two experiments, A and B, were done under this conditions at different days.

Condition IX:

Column: Supelco C-18, 15.0cm x 4.0mm, packed with 5-um spherical particles.
Flowrate: 0.4 ml/min

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Mobile phase:

95% MeOH with 100 ppm tetramethylammonium acetate
5% DI H₂O
Total pH = 7.2 adjusted by acetic acid

Helium pressure: 90 psi
Nebulizer temperature: 100°C
Desolvation Chamber temperature: 75°C
Ion Source temperature: 250°C
Ionization Mode: +EI
Scan range: 70 - 350 amu
Scan rate: 1.5 sec/scan
Electron Multiplier voltage: 1800 ev
Analyte concentration: 20 ppm

Two experiments, C and D, were done under ion pairing conditions (IX) at different days.

Detection Limits

Detection limits for difluorobenzoic acids were determined using ionization control condition VIII and ion pairing condition IX. An injection loop of 20 ul was used. With the mass spectrometer operated in selected ion monitoring mode, detection limits for each compound at two conditions are listed in table 2.8.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Detection Limits (ng)</th>
<th>Detection Limits (ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ionization Control Condition VIII</td>
<td>Ion Pairing Condition IX</td>
</tr>
<tr>
<td>2,6-DFBA</td>
<td>20</td>
<td>2</td>
</tr>
<tr>
<td>2,3-DFBA</td>
<td>20</td>
<td>2</td>
</tr>
<tr>
<td>2,4-DFBA</td>
<td>20</td>
<td>2</td>
</tr>
<tr>
<td>2,5-DFBA</td>
<td>20</td>
<td>2</td>
</tr>
<tr>
<td>3,4-DFBA</td>
<td>100</td>
<td>10</td>
</tr>
<tr>
<td>3,5-DFBA</td>
<td>100</td>
<td>10</td>
</tr>
</tbody>
</table>
CHAPTER 3

RESULTS AND DISCUSSION

HPLC/MS Mass Spectra

The mass spectra of difluorobenzoic acids and corresponding methyldifluoro benzoates have been studied. Difluorobenzoic acids are listed in Figure 3.1. Mass Spectra are presented in Appendix A. Due to the variation of the experimental conditions and the instability of the instrument, the relative intensities of the major peaks in each mass spectrum on the computer drawings may differ from what were observed here. Table 3.2-3.9 lists the precise relative intensity of the major peaks in each mass spectrum.

Mass Spectra of Difluorobenzoic Acid

The mass spectra of the difluorobenzoic acid isomers have not been previously studied. However, the mass spectra of benzoic acid and methyl substituted benzoic acid have been determined by McLafferty and Aczel. In this study all difluorobenzoic acids, like other substituted benzoic acids, have the major peaks at (M-OH)⁺ and (M-COOH)⁺.
Figure 3.1 Difluorobenzoic Acids
The molecular ion (M)$^+$, however, is much smaller for the difluorobenzoic acids. Besides these two ions, there is another ion at mass 123, possibly due to the loss of neutral oxygen fluorine, (M-OF)$^+$. It was mentioned earlier that the ortho hydrogen undergoes partial exchange with the hydroxyl hydrogen. So, it can be reasoned that the ortho fluorine acts as hydrogen, partially transferring to the carbonyl-oxygen, then OF is ejected as a neutral, forming the very stable FC$_6$H$_2$CO$^+$ ion. Figure 3.2 lists two possible mechanisms.

2,3-DFBA, 2,4-DFBA and 2,5-DFBA have one ortho position fluorine, the loss of OF becomes one of the main fragmentation pathways for these three acids. Mass 123 (M-OF)$^+$ is often the base peak.

Although 2,6-DFBA has two ortho position fluorines, mass 123 (M-OF)$^+$ is not the major peak. It has a unique fragmentation pattern with the base peak at mass 114. This corresponds to the loss of CO$_2$. It is proposed that the positive charge for the 2,6-DFBA molecular ion is predominantly located on the aromatic ring. The hydrogen of the carboxyl group migrates back to the C$_1$ position and CO$_2$ is removed as a neutral (See Figure 3.3). This fragmentation pathway is due to the stability of the aromatic species formed and the thermochemical stability of CO$_2$.

3,4-DFBA and 3,5-DFBA do not have ortho position fluorines. The loss of neutral OF is small.
Figure 3.2 Formation of (M-OF)$^-$ Ion
Figure 3.3 Formation of \((M-CO_2)^+\) Ion

Mass Spectra of Methyl difluorobenzoates

Under ion pairing reagent conditions, the difluorobenzoic acids interact with tetramethylammonium acetate in the mobile phase and form an \(R-COO^- \cdot N(CH_3)_4\) complexes. This complex loses \(N(CH_3)_4\) and forms methyl difluorobenzoate when heated in the ion source.

\[
F_2C_6H_4COOH + (CH_3)_4N(O_2CCH_3) \rightarrow F_2C_6H_4COO^- \cdot N(CH_3)_4 + HO_2CCH_3
\]

\[
F_2C_6H_4COOCH_3 + N(CH_3)_4
\]

The mechanism of the fragmentation of a methyl difluorobenzoate appears to be analogous to difluorobenzoic acids.

The relative intensity of the molecular ion \([F_2C_6H_4COOCH_3]^+\) is small. Two major peaks are \((M-31)^+\) and \((M-59)^+\). Mass 123, due to loss of \(FO\), is also observed in methyl difluorobenzoates. The fragmentation pathway is suggested in Figure 3.4.
Figure 3.4 Fragmentation Pathway of Methyl Difluorobenzoates II

Separation by HPLC/MS Mass Spectra

The difluorobenzoic acids could be separated into three groups (see Table 3.1).

Group 1 includes only the 2,6-DFBA. 2,6-DFBA contains two ortho position fluorines.

Group 2 consists of 2,3-DFBA, 2,4-DFBA and 2,5-DFBA. This group contains one ortho position fluorine. Group 3 consists of 3,4-DFBA and 3,5-DFBA. This group contains no ortho position fluorines.

2,6-DFBA is readily separated from the other five difluorobenzoic acids isomers by its unique fragmentation pattern. It has just one high intensity ion at m/e 114, which corresponds to the loss of CO₂ from the molecular ion. The relative intensities of the other ions are very small.

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Table 3.1 Difluorobenzoic Acids Separated Into Three Groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Compounds</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>2,6-DIBFA</td>
</tr>
<tr>
<td>Group 2</td>
<td>2,3-DIBFA, 2,4-DIBFA, 2,5-DIBFA</td>
</tr>
<tr>
<td>Group 3</td>
<td>3,4-DIBFA, 3,5-DIBFA</td>
</tr>
</tbody>
</table>

The other five difluorobenzoic acids have the same base peak. Sometimes the base peaks of group 2 and group 3 shift, and the reason for base peak shift is not clear. Regardless, Group 2 and Group 3 could be identified by m/e 123 (M⁺-FO); the relative intensity of mass 123 of group 3 is generally smaller than that of group 2. Separation of three isomers in group 2 is achieved by plotting m/e 113 vs. m/e 123. Three isomers can be identified under ionization condition. Three isomers are not separate clearly under ion pairing condition. Within group 3, 3,4-DFBA and 3,5-DFBA can be identified by at least one of two ions at m/e 113 (M⁺-CO₂H) and m/e 141 (M⁺-OH). 3,4-DFBA and 3,5-DFBA can be identified under both ionization control and ion pairing conditions.

Experiment II has two parts: acidic condition (VIII) and ion paring conditions (IX). Two experiments, A and B, were done under acidic conditions (VIII) at different days. Two experiments, C and D, were done under ion pairing conditions (IX) at different days. The separations of difluorobenzoic acids in each experiment are discussed as follow.

I. Acid Condition V(III)
Experiment A:
Table 3.2 lists the relative intensities of the six difluorobenzoic acids in experiment
A. Only 2,6-DFBA has high intensity ion at m/e 114, and therefore it can be separated from other five isomers easily. The other five difluorobenzoic acids all have the same base peak at m/e 141. Group 2 and Group 3 could be separated by m/e 123. The relative intensity of m/e 123 of group 2 is higher than 40%. The relative intensity of m/e 123 of group 3 is smaller than 10% (See Table 3.3 A).

Within group 2, 2,4-DFBA is separated from other two acids by its relative intensity of m/e 123 and m/e 113. The relative intensity of m/e 123 of 2,4-DFBA is about 75-85%, higher than that of 2,3-DFBA and 2,5-DFBA. The relative intensity of m/e 113 of 2,4-DFBA is about 55% smaller than that of 2,3-DFBA and higher than that of 2,5-DFBA (See Table 3.3 B).

Within group 3, 3,4-DFBA and 3,5-DFBA are separated by m/e 113. The relative intensity of m/e 113 of the 3,5-DFBA is higher than that of 3,4-DFBA. The relative intensity of m/e 113 of 3,5-DFBA is between 85% to 90%. The relative intensity of m/e 113 of 3,4-DFBA is between 70% to 75% (See Table 3.3 C).

Figure 3.5-3.7 plot the relative intensities of m/e 113 vs. m/e 123, m/e 114 vs. 123 and m/e 113 vs. M/e 114. The best mass separation is observed in the plot of m/e 113 vs. m/e 123. Although some variability is observed, these compounds are clearly separated. Separation of the Group 2 compounds is not observed in the other plots.
Table 3.2 The Relative Intensities of Difluorobenzoic Acids in Experiment A

<table>
<thead>
<tr>
<th>m/e</th>
<th>113</th>
<th>114</th>
<th>123</th>
<th>140</th>
<th>141</th>
<th>157</th>
<th>158</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>2,6-DFBA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Injection 1</td>
<td>100</td>
<td>76.9</td>
<td>23.1</td>
<td>26.9</td>
<td>34.6</td>
<td>11.5</td>
<td>15.4</td>
</tr>
<tr>
<td>Injection 2</td>
<td>100</td>
<td>85.95</td>
<td>21.02</td>
<td>26.33</td>
<td>38.6</td>
<td>14.05</td>
<td>17.58</td>
</tr>
<tr>
<td>Injection 3</td>
<td>100</td>
<td>81.8</td>
<td>18.2</td>
<td>20.5</td>
<td>34.1</td>
<td>9.1</td>
<td>15.9</td>
</tr>
<tr>
<td><strong>2,3-DFBA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Injection 1</td>
<td>78.9</td>
<td>42.1</td>
<td>47.4</td>
<td>68.4</td>
<td>100</td>
<td>31.6</td>
<td>47.4</td>
</tr>
<tr>
<td>Injection 2</td>
<td>78.2</td>
<td>34.4</td>
<td>46.9</td>
<td>71.9</td>
<td>100</td>
<td>37.5</td>
<td>40.6</td>
</tr>
<tr>
<td>Injection 3</td>
<td>80</td>
<td>40</td>
<td>46.7</td>
<td>66.7</td>
<td>100</td>
<td>33.3</td>
<td>40</td>
</tr>
<tr>
<td><strong>2,4-DFBA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Injection 1</td>
<td>52.9</td>
<td>32.4</td>
<td>83.3</td>
<td>81.3</td>
<td>100</td>
<td>21.6</td>
<td>32.4</td>
</tr>
<tr>
<td>Injection 2</td>
<td>56.8</td>
<td>32.4</td>
<td>79.7</td>
<td>77</td>
<td>100</td>
<td>21.6</td>
<td>32.4</td>
</tr>
<tr>
<td>Injection 3</td>
<td>54.9</td>
<td>31.37</td>
<td>78.43</td>
<td>76.47</td>
<td>100</td>
<td>19.61</td>
<td>31.37</td>
</tr>
<tr>
<td><strong>2,5-DFBA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Injection 1</td>
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<td>39.3</td>
<td>51.4</td>
<td>60</td>
<td>100</td>
<td>32.1</td>
<td>46.4</td>
</tr>
<tr>
<td>Injection 2</td>
<td>73.6</td>
<td>51</td>
<td>58.2</td>
<td>68.4</td>
<td>100</td>
<td>32.7</td>
<td>45.9</td>
</tr>
<tr>
<td>Injection 3</td>
<td>70.12</td>
<td>39.4</td>
<td>51.2</td>
<td>69.4</td>
<td>100</td>
<td>35.6</td>
<td>57.27</td>
</tr>
<tr>
<td><strong>3,4-DFBA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Injection 1</td>
<td>90.6</td>
<td>40</td>
<td>10.1</td>
<td>50</td>
<td>100</td>
<td>30</td>
<td>50</td>
</tr>
<tr>
<td>Injection 2</td>
<td>87.9</td>
<td>36.4</td>
<td>7.7</td>
<td>45.5</td>
<td>100</td>
<td>45.5</td>
<td>54.5</td>
</tr>
<tr>
<td>Injection 3</td>
<td>91</td>
<td>46.2</td>
<td>7.7</td>
<td>53.8</td>
<td>100</td>
<td>38.5</td>
<td>53.8</td>
</tr>
<tr>
<td><strong>3,5-DFBA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Injection 1</td>
<td>73.9</td>
<td>26.1</td>
<td>6.5</td>
<td>50</td>
<td>100</td>
<td>47.8</td>
<td>54.3</td>
</tr>
<tr>
<td>Injection 2</td>
<td>74.5</td>
<td>23.6</td>
<td>9.1</td>
<td>56.4</td>
<td>100</td>
<td>50.9</td>
<td>56.4</td>
</tr>
<tr>
<td>Injection 3</td>
<td>70.2</td>
<td>19.3</td>
<td>8.3</td>
<td>47.4</td>
<td>100</td>
<td>40.4</td>
<td>56.1</td>
</tr>
</tbody>
</table>
Table 3.3 Separation of Difluorobenzoic Acids in Experiment A

<table>
<thead>
<tr>
<th>Analytes</th>
<th>m/e 123</th>
<th>m/e 141</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>18-25%</td>
<td>&lt;40%</td>
</tr>
<tr>
<td>Group 2</td>
<td>&gt;40%</td>
<td>100%</td>
</tr>
<tr>
<td>Group 3</td>
<td>&lt;10%</td>
<td>100%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>m/e 113</th>
<th>m/e 123</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,3-DFBA</td>
<td>75-82%</td>
<td>45-48%</td>
</tr>
<tr>
<td>2,4-DFBA</td>
<td>50-58%</td>
<td>75-85%</td>
</tr>
<tr>
<td>2,5-DFBA</td>
<td>62-75%</td>
<td>50-60%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>m/e 113</th>
</tr>
</thead>
<tbody>
<tr>
<td>3,4-DFBA</td>
<td>70-75%</td>
</tr>
<tr>
<td>3,5-DFBA</td>
<td>86-92%</td>
</tr>
</tbody>
</table>

Figure 3.5 Relative Intensity of m/e 113 vs. m/e 123 in Experiment A
Figure 3.6 Relative Intensity of m/e 114 vs. m/e 123 in Experiment A

Figure 3.7 Relative Intensity of m/e 113 vs. m/e 114 in Experiment A

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Calculation of the composition of a mixture is done by solving a series of simultaneous equations:

\[ P_{m_1} R_1 X_1 + P_{m_2} R_2 X_2 + \ldots + P_{m_n} X_n = I_m \]

- \( P_m \): The relative intensity of a peak at a m/e value \( m \) in the spectrum of the pure component \( n \).
- \( R_n \): The response factors for the base peak in the spectrum of the pure component \( n \).
- \( X_n \): The concentration of the component \( n \).
- \( I_m \): The relative intensity of a peak at a m/e value \( m \) in the spectrum of the mixture.

In experiment A, 3,4-DFBA and 3,5-DFBA coelute. The relative intensities of pure component 3,4-DFBA and 3,5-DFBA are listed in Table 3.2. The response factors for 3,4-DFBA and 3,5-DFBA are 35.7 count/ng and 44.8 count/ng. The percentages of each DFBA in the mixture are then calculated by solving:

\[ 89.8 \times 35.7 \times X_{34} + 72.9 \times 44.8 \times X_{35} = I_{113} \]
\[ 100 \times 35.7 \times X_{34} + 100 \times 44.8 \times X_{35} = I_{141} \]

The values of \( X_n \) are given by:

- 3,4-DFBA: \( X_{34} = 0.002 I_{113} - 0.001 I_{141} \)
- 3,5-DFBA: \( X_{35} = -0.001 I_{113} + 0.001 I_{141} \)

The mole percentage of each component in the mixture:

- 3,4-DFBA: \( X_{34} / (X_{34} + X_{35}) \)
- 3,5-DFBA: \( X_{35} / (X_{34} + X_{35}) \)

In experiment A, 2,3-DFBA, 2,4-DFBA and 2,5-DFBA coelute, The relative intensities of pure component 2,3-DFBA, 2,4-DFBA and 3,5-DFBA are listed in Table 3.2.
the response factors for 2,3-DFBA, 2,4-DFBA and 2,5-DFBA are 145.6, 105.6 and 163.9 count/ng. The mole percentages of each DFBA in the mixture are then calculated by solving:

\[
79.0 \times 145.6 X_{23} + 54.9 \times 105.6 X_{24} + 69.3 \times 163.9 X_{25} = I_{113} \\
47.0 \times 145.6 X_{23} + 80.5 \times 105.6 X_{24} + 53.6 \times 163.9 X_{25} = I_{123} \\
100 \times 145.6 X_{23} + 100 \times 105.6 X_{24} + 100 \times 163.9 X_{25} = I_{141}
\]

The values of \(X_a\) are given by:

- 2,3-DFBA: \(X_{23} = 0.001 I_{113} + 5.962 \times 10^{-4} I_{123} - 0.001 I_{141}\)
- 2,4-DFBA: \(X_{24} = 3.767 \times 10^{-4} I_{113} + 5.537 \times 10^{-4} I_{123} - 5.579 \times 10^{-4} I_{141}\)
- 2,5-DFBA: \(X_{25} = -0.001 I_{113} - 8.864 \times 10^{-4} I_{123} + 0.001 I_{141}\)

The mole percentage of each component in the mixture:

- 2,3-DFBA: \(X_{23} / (X_{23} + X_{24} + X_{25})\)
- 2,4-DFBA: \(X_{24} / (X_{23} + X_{24} + X_{25})\)
- 2,5-DFBA: \(X_{25} / (X_{23} + X_{24} + X_{25})\)
Experiment B:

Table 3.4 lists the relative intensity of the difluorobenzoic acids in experiment B. All six isomers have the same base peak at 114. 2,6-DFBA could be separated from other two groups by the relative intensity of m/e 113, which is smaller than 40%. Group 2 could be separated from group 3 by mass 123. The relative intensity of m/e 123 of group 2 is higher than 40%. The relative intensity of m/e 123 for group 3 is less than 16% (See Table 3.5 A).

Within group 2, 2,3-DFBA can be separated from the other two acids by m/e 123. The relative intensity of m/e 123 of 2,3-DFBA is more than 65%, higher than that of 2,4-DFBA and 2,5-DFBA. The relative intensity of m/e 141 of 2,4-DFBA is above 90%, higher than that of 2,5-DFBA (See Table 3.5 B).

Within group 3, 3,4-DFBA and 3,5-DFBA can be separated by m/e 141. The relative intensity of m/e 141 of 3,4-DFBA is between 55% to 70%. The relative intensity of m/e 141 for 3,5-DFBA is between 20% to 25% (See Table 3.5 C).

Figure 3.8-3.10 plot the relative intensity of m/e 113 vs. m/e 123, m/e 141 vs. 123 and m/e 113 vs. m/e 141. Mass separation is observed only in the plot, m/e 141 vs. m/e 123. In spite of some variability, all six isomers are separable (Figure 3.9).
Table 3.4 The Relative Intensity of The Difluorobenzoic Acids in Experiment B.

<table>
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<th>m/e</th>
<th>11</th>
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<th>123</th>
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<td>12.4</td>
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Table 3.5 Separation of Difluorobenzoic Acids in Experiment B.

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<th>Analytes</th>
<th>m/e 113</th>
<th>m/e 123</th>
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<td>Group 1</td>
<td>&lt;40%</td>
<td>18-20%</td>
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<tr>
<td>Group 2</td>
<td>&gt;55%</td>
<td>&gt;40%</td>
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<td>Group 3</td>
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<td>&lt;16%</td>
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B

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<tr>
<th>Analytes</th>
<th>m/e 123</th>
<th>m/e 141</th>
</tr>
</thead>
<tbody>
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<td>2,3-DFBA</td>
<td>&gt;65%</td>
<td>78-93%</td>
</tr>
<tr>
<td>2,4-DFBA</td>
<td>&lt;55%</td>
<td>90-95%</td>
</tr>
<tr>
<td>2,5-DFBA</td>
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<td>&lt;75%</td>
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C

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<th>Analytes</th>
<th>m/e141</th>
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<td>3,4-DFBA</td>
<td>55-70%</td>
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<td>3,5-DFBA</td>
<td>20-25%</td>
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Figure 3.8 Relative Intensity of m/e 113 vs. m/e 123 in Experiment B
Figure 3.9 Relative Intensity of m/e 141 vs. m/e 123 in Experiment B

Figure 3.10 Relative Intensity of m/e 113 vs. m/e 141 in Experiment B
II. Ion Pairing Condition (XI)

Experiment C:

Table 3.6 lists the relative intensities of the six difluorobenzoic acids in experiment C. 2,6-DFBA has base peak at m/e 114. The other five difluorobenzoic acids all have the same base peak at m/e 141. Therefore, 2,6-DFBA can be separated from other five isomers easily. Group 2 and Group 3 could be separated by m/e 123. The relative intensities of m/e 123 of group 2 are higher than 20%. The relative intensity of m/e 123 for group 3 is smaller than 12% (See Table 3.7 A).

The three isomers in group 2 cannot be clearly identified by MS fragmentation pattern (See Figure 3.11-3.13)

Within group 3, 3,4-DFBA and 3,5-DFBA can be separated by m/e 113. The relative intensity of m/e 113 of 3,5-DFBA is higher than that of 3,4-DFBA. The relative intensity of m/e 113 of 3,5-DFBA is between 70% to 85%. The relative intensity of m/e 113 of 3,4-DFBA is between 55% to 69% (See Table 3.7 C).

Figure 3.11-3.13 plot the relative intensities of m/e 113 vs. m/e 123, m/e 114 vs. 123 and m/e 113 vs. m/e 141. No clear separation of the group 2 compounds is observed.
Table 3.6  The Relative Intensities of the Difluorobenzoic Acids in Experiment C

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<th>141</th>
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Table 3.7 Separation of Difluorobenzoic Acids in Experiment C.

A

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<tr>
<th>Analytes</th>
<th>m/e 114</th>
<th>m/e 123</th>
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B Group 2 cannot be separated

C

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<td>3,5-DFBA</td>
<td>70-85%</td>
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Figure 3.11 Relative Intensity of m/e 113 vs. m/e 123 in Experiment C
Figure 3.12 Relative intensity of m/e 114 vs. m/e 123 in Experiment C

Figure 3.13 Relative Intensity of m/e 113 vs. m/e 114 in Experiment C

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Experiment D:

Table 3.8 lists the relative intensities of the difluorobenzoic acids in experiment D. 2,6-DFBA has the base peak at m/e 114. Group 2 has base peak at m/e 123. Group 3 has base peak at m/e 141. 2,6-DFBA can be separated from other five isomers easily by comparing the base peaks. Group 2 and Group 3 could be separated by m/e 141. The relative intensities of m/e 141 of group 2 are 45%-76%. The relative intensities of m/e 141 of group 3 are 100% (See Table 3.9 A).

The three difluoro benzoates in group 2 could not be clearly separated.

Within group 3, 3,4-DFBA and 3,5-DFBA can be separated by m/e 113. The relative intensity of m/e 113 of the 3,5-DFBA is higher than that of 3,4-DFBA. The relative intensity of m/e 113 of 3,5-DFBA is between 80% to 90%. The relative intensity of m/e 113 of 3,4-DFBA is between 60% to 70% (See Table 3.9 C).

Figure 3.14-3.16 plots the relative intensities of m/e 113 vs. m/e 123, m/e 114 vs. 123 and m/e 113 vs. m/e 141. No clear separation of all compounds is observed in any of these plots.
Table 3.8 The Relative Intensities of The Difluorobenzoic Acids in Experiment D

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Table 3.9 Separation of Difluorobenzoic Acids in Experiment D.

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B Group 2 cannot be separated

C

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Figure 3.14 Relative Intensity of m/e 113 vs. m/e 123 in Experiment D

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Figure 3.15 Relative Intensity of m/e 114 vs. m/e 141 in Experiment D

Figure 3.16 Relative Intensity of m/e 113 vs. m/e 141 in Experiment D
CHAPTER 4

CONCLUSION

A method to separate and identify difluorobenzoic acids based on particle beam LC/MS with positive EI ionization was developed. This method provides positive identification of difluorobenzoic acids in a sample. The difluorobenzoic acids can be identified by the fragment pattern produced by EI ionization. Separation of the difluorobenzoic acids was achieved by plotting m/e 113 vs. m/e 123 under ionization condition. The instrument detection limits for the difluorobenzoic acids are in the 20ng to 100ng range at ionization control condition and 2ng to 10ng range at ion pairing condition.

Difluorobenzoic acids that differ only by the position of fluorine on the ring give similar mass spectra. Differences between the isomers are often insignificant when compared with the variability in the spectra observed between multiple injections of the same compound. In the future, a number of issues such as variations in spectral quality, reproducibility and sensitivity need to be addressed before a reliable method can be developed. It is also necessary to use an internal standard if quantitative measurements are to be made. Negative chemical ionization is often preferred for compounds containing...
electronegative atoms. Negative ion CI often improves the sensitivity further studies should be conducted. HPLC conditions that is suitable for PB interface needs to be studied further to achieve chromatographic separation.
APPENDIX A

Mass Spectra of Difluorbenzoic Acids

2,3-DFBA Mass Spectra

2,4-DFBA Mass Spectra
2,5-DFBA Mass Spectra

2,6-DFBA Mass Spectra

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3,4-DFBA Mass Spectra

3,5-DFBA Mass Spectra

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APPENDIX B

SEPARATION AND IDENTIFICATION
OF DIFLUOROBENZOIC ACIDS BY GC/MS

Introduction

The combination of gas chromatography and mass spectrometry is widely recognized as the sensitive, accurate, and versatile tool for the identification and quantitation of organic compounds in complex mixtures. The major limitation of GC/MS is that analytes must have sufficient volatility and thermostability to pass through the GC.

Compounds, containing function groups such as carboxyl, hydroxyl, carbonyl, thio and amino, tend to adsorb on the active surface of the stationary phase and produce asymmetrical peaks. The high polarities of these compounds and their tendency to form intermolecular hydrogen bonds make direct GC analysis either impossible or difficult. They may also undergo partial thermal decomposition causing loss of the sample in the chromatographic system. Derivatization is employed to overcome the above problems. Derivatization involves the replacement of hydrogen by silylation, alkylation and acylation reactions. By decreasing the polarity of functional groups, derivatization improves compound volatility, reduces adsorption losses, yields a more thermal stable product and
improves chromatographic performance\textsuperscript{22}.

Chemical derivatization is also important in the analytical work by the MS. It is important that the compound under investigation be vaporized in the ion source of the MS without thermal degradation or molecular rearrangement. Changes in the structure of the compound will result in false interpretation of mass spectrometric data.

Trimethyl silylation derivatives are the widely used. They increase volatility and protect analytes against thermal decomposition. They have very low polarity, providing excellent GC peaks. They are not easily adsorbed on surfaces and their high volatility permits quick removal from the ion source of the MS. Their mass spectra often exhibit abundant molecular ions, or evidence about the molecular weight of a compound, such as loss of a TMS methyl group producing an intense peak which corresponds to [M-15]\textsuperscript{+} \textsuperscript{22}.

**GC/MS Experimental**

Procedure for Extraction and Derivatization

The analyte is acidified with 1M phosphoric acid (pH=3.3) and extracted into hexane/ethyl acetate (5:1). After discarding aqueous layer, the organic layer is transferred into vials. The organic solvent is then removed by a stream of nitrogen. When the solvent has been removed and the vials are dry, 50ul of N, O-bis-trimethylsilytrifluoro acetamide (BSTFA) is added, the vial is capped tightly, and heated for 15 minutes at 90\textdegree C in the heat block. The vials are cooled and 100ul of ethyl acetate is added to dissolve the trimethylsilyl derivative. The entire content of each vial is transferred into GC auto-sampler vials.
General Parameters for GC/MS

The analysis is performed on an HP 5890/5970 GC/MS system equipped with a 7673 autosampler. The general parameters are as follow:

Column: Capillary Column, 30 meters, 0.25 mm i.d. coated with 5% methyl phenyl silicone, J&W Scientific (Type DB-5)
Temperatures: Injector-220°C
   Transfer Line- 290°C
   Oven
   Initial Temperature = 75°C for 1 min
      10°C/min to 150°C
      30°C/min to 250°C

Helium Carrier Gas: 25-30 ml/min
Mass spectrometer:
   Positive Electron impact ionization
   Scan range: 70 - 250 amu; scan rate: 1.5 sec/min;

GC/MS Mass Spectra

Difluorobenzoic acids are derivatized to their trimethylsily derivative by BSTFA.

The silylation reaction is as follow:

\[
\text{COOH} + \text{CF}_3\text{C}=\text{N-Si(CH}_3\text{)}_3 + \text{heat at } 90^\circ\text{C} \rightarrow \text{COOSi(CH}_3\text{)}_3 + \text{CF}_3\text{C}=\text{N-Si(CH}_3\text{)}_3
\]

The mass spectra of difluorobenzoic acid trimethylsily derivatives have the major peaks at 
[M-CH\text{3}]^+, [M-OSi(CH\text{3})_3]^+ and [M-COOSi(CH\text{3})_3]^+. The molecular ion (M)^+ is small.

The base peak is at [M-CH\text{3}]^+. In addition to these ions, there are two other ions at m/e
171, m/e 173. The possible fragmentation pathway of 171 is shown in Figure B-1. The fragmentation pathway of ion m/e 173 is not clear. The mass spectra of difluorobenzoic acid trimethylsily derivatives appear in Figure B-2.

Figure B-1 Fragmentation Pathway of Difluorobenzoic Acids Trimethylsily Derivatives
Separation by Mass Spectra and Retention Times

The GC/MS chromatogram of difluorobenzoic acids is shown in Figure B-3.

The relative intensities of the major ions of each difluorobenzoic acid trimethylsilyl derivative are listed in Table B-1. All six difluorobenzoic acids have the same base peak at m/e 215. 3,4-DFBA and 3,5-DFBA have major peaks at m/e 171 (more than 40%). While other difluorobenzoic acids have small peaks at m/e 171 (less than 5%). Therefore 3,4-DFBA and 3,5-DFBA can be differentiated from other four difluorobenzoic acids isomers by the mass fragment at m/e 171. 3,4-DFBA (Rt=8.30min) and 3,5-DFBA (Rt=7.79min) can then be separated by their retention times. 2,5-DFBA and 2,6-DFBA have close retention times. The relative intensity of ion at m/e 141 in 2,5-DFBA is smaller than 40%. The relative intensity of ion at m/e 141 in 2,6-DFBA is higher than 60%. 2,5-DFBA is then separated from 2,6-DFBA and other four difluorobenzoic acids isomers by the mass fragment at m/e 141 and its retention time. Although 2,4-DFBA and 3,4-DFBA coelute, they can be separated by m/e 171. The remaining isomers can be separated by
Figure B-2 Mass spectra of the Difluorobenzoic Acid Trimethylsilyl Derivatives

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their retention time. Thus, the difluorobenzoic acids can be separated using three criteria: relative abundances of ion at m/e 171, m/e 141 and retention times. With the information in table B-2, the difluorobenzoic acids can be separated.

**Table B-2** Separation of Difluorobenzoic Acids by Relative Abundances of Ion at m/e 171, m/e 141 and Retention Times Using GC/MS

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<tr>
<td>3,4-DFBA</td>
<td>&gt; 40%</td>
<td>&gt; 50%</td>
<td>8.30&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>2,5-DFBA</td>
<td>&lt; 5%</td>
<td>&lt; 40%</td>
<td>8.48</td>
</tr>
<tr>
<td>2,6-DFBA</td>
<td>&lt; 5%</td>
<td>&gt; 60%</td>
<td>8.53</td>
</tr>
<tr>
<td>2,3-DFBA</td>
<td>&lt; 5%</td>
<td>&gt; 50%</td>
<td>8.75</td>
</tr>
</tbody>
</table>

<sup>1</sup>: Partially Resolved, <sup>2</sup>: Co-elute
Mass Spectra of The Six Difluorobenzoic Acid Trimethylsilyl Derivatives

2,3-DFBA-TMS Mass Spectra

2,4-DFBA-TMS Mass Spectra

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2,5-DFBA-TMS Mass Spectra

2,6-DFBA-TMS Mass Spectra

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3,4-DFBA-TMS Mass Spectra

3,5-DFBA-TMS Mass Spectra
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