THE EFFECTS OF METHANOL AND ISOPROPANOL ON THE CHIRAL SEPARATION OF DRONABINOL AND THREE RELATED IMPURITIES: A 3 X 3 X 3 FACTORIAL DESIGN

THESIS

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TABLE OF CONTENTS

Page

LIST OF TABLES

Table **Page**

LIST OF FIGURES

Figure **Page**

ABSTRACT

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May 2010

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The enantioseparations of Δ^9-THC and Δ^8-THC on amylose tris dimethylphenylcarbamate CSP was investigated using mobile phases containing 1-3% methanol with 1-7% IPA in n-heptane. A factorial design was used to study the main and interaction effects of these alcohols as measured by the retention time, selectivity, and resolution of each pair of enantiomers, as well as the critical pair: (+)-Δ^9-THC and (-)-Δ^8-THC. Methanol did not significantly affect the enantioseparation of the critical pair, while the interaction effect was significant in all cases. An elution order reversal for Δ^8-THC was noted at 1% IPA with 2-3% methanol, and excluding these conditions, Δ^9-THC resolution was greater than 5.0 in all other cases.

CHAPTER I

INTRODUCTION

The Research Problem

Dronabinol active pharmaceutical ingredient (API) was manufactured by Austin Pharma Labs (Round Rock, Texas) and characterized by Cerilliant Corporation (Round Rock, Texas). High-performance liquid chromatography (HPLC) methods were developed and validated to provide evidence of the purity, potency, related substances, and enantiomeric composition of this API. Proof of these characteristics is essential to demonstrating the overall quality of this product.[1,2]

The method for characterization of enantiomers, or chiral analysis, was performed using a chiral stationary phase (CSP) and a mobile phase containing a mixture of two alcohols. Though this method was successfully validated, the effects of the alcohols on the separation, or resolution, of the enantiomers, were not fully investigated. Resolution of sample components is arguably the most important requirement of an HPLC method to ensure accurate identification and quantification.

In this study, the effects of the independent variables, isopropanol (IPA) and methanol content in the mobile phase, will be characterized by measuring the dependent variable, resolution, for Dronabinol and three related chiral impurities. Unlike previous in-house studies of this chiral method,[3] a controlled, factorial design will be implemented

to study deliberate small incremental changes in the percentages of the two alcohols across a predetermined range of mixtures. Also unique to this experiment is the analysis of interaction effect of the alcohols, if present, the use of methanol in this type of system, and the design of the method protocol with regard to instrument parameters.

Enantiomers

Stereospecificity

Isomers are molecules that share the same chemical formula but have different structures. Stereoisomers are isomers that have the same functional groups only connected in different geometries, whereas constitutional isomers have the same atoms only connected in different orders. Enantiomers are pairs of stereoisomers that are nonsuperimposable mirror images of each other, whereas diastereoisomers are not restricted to pairs and are nonsuperimposable non-mirror images. Enantiomers contain one or more chiral centers and are often described as being related to each other like the right and left hand – mirror images identical in both shape and appearance – but not superimposable.[4] Enantiomers are also referred to as optical isomers due to their unique ability to rotate plane-polarized light, and are generally denoted using the following terms: D- and L-, (*R*) and (*S*), or (+) and (-).

Analysis of Enantiomers

Optical isomers are chemically and physically impossible to differentiate by most analytical techniques, whereas diastereomers have distinct physical properties.[5,6] Only by methods that employ plane-polarized light or a chiral substrate, probe, or reactant in the analysis conditions can enantiomers be investigated or characterized. Optical rotation is a useful technique that measures the direction and degree that an enantiomer rotates

plane-polarized light. Its application is limited because the relationship between response and structure is not well-defined and therefore the results are useful primarily when literature values or historical data are available for comparison.[5] Precise identification and quantification of enantiomers requires analytical methods that exploit other chiral attributes.

Myriad analytical methods developed to identify and quantitate enantiomers utilize liquid chromatography (LC).[7] These methods generally operate in one of two ways: derivitization with a chiral reagent or use of a CSP. There are advantages and disadvantages to both approaches, the most important of which involve ease of sample preparation, and preservation of sample integrity. The use of chiral mobile phase additives is an infrequently encountered practice that will not be discussed here.

In the first technique, which is referred to as an indirect method, enantiomers are chirally derivitized to form diastereomers.[7] Unlike enantiomers, they have distinct physical properties and are therefore readily separated using conventional chromatographic equipment. However, some derivitizations require lengthy or complicated sample preparation that should be performed only by experienced analysts. Because derivitization alters the original sample, important information about the sample may be lost in the process. Also, impurities in the reagent can lead to complicated or confounding results due to the generation of secondary and tertiary structures through side reactions.[7]

In the second approach, a very specific chromatographic stationary phase is used to separate the enantiomers, which is considered to be a direct method.[7] This costly specialized piece of equipment comprises a chiral substrate embedded in its pores that

interacts preferentially with one enantiomer, thereby facilitating separation of the pair. One major benefit of using this technique to separate enantiomers is that sample preparation is as simple as dissolving the sample in an appropriate solvent, which results in optimum preservation of sample information.

Relevance and Safety of Enantiomers in Drugs

Stereospecificity of Biomolecules

Nearly all biologically relevant molecules, such as amino acids, enzymes, and steroids have one or more chiral centers and thus the potential to exist as enantiomers.[6] However, as in the case of the amino acids found in proteins, aside from the achiral glycine, only the L-enantiomer of these molecules is naturally occurring.[5] Because enzymes are proteins made up solely of L-amino acids, they have chiral centers in and around active sites. The presence of these centers does not necessarily indicate a high degree of specificity, however. For example, chymotrypsin is a digestive enzyme that catalyzes both peptide and ester bond hydrolysis.[5] The stereospecificity of an enzyme is enhanced significantly when chiral centers contribute to both substrate recognition and catalysis.[5]

An enzyme's geometric specificity, even more so than its stereospecificity, can render one optical isomer of a substrate useless while the other one is readily catalyzed.[5] Similarly, some enzymes form single enantiomer products, regardless of the substrate's chiral configuration. Benzoylformate decarboxylase is an example of an enzyme that produces a specific enantiomer; it makes (*R*)-benzoins but no significant amount of (*S*)-benzoins.[8] The human body, with innumerable processes governed by enzymes, also exhibits some level of specificity towards enantiomers.

Chiral Active Pharmaceutical Ingredients

The stereospecificity of numerous molecular mechanisms within the human body means that though one enantiomer of an API in a drug elicits a physiological effect, the other enantiomer may have a different or a diminished effect, or none at all. An API could produce a beneficial response, but have an enantiomer that produces a harmful response, as in the extreme example of thalidomide. With thalidomide, one enantiomer acts as a pain reliever, while the other one is a teratogen.[5,9] This enantiomeric drug was briefly prescribed in Europe as a sedative and subsequently used to treat morning sickness in thousands of pregnant women until being linked to severe birth defects.[10]

Regulation of Drugs

Approval of thalidomide for the U.S. market was avoided only because the FDA inspector refused to approve the drug application without sufficient safety data, which were not provided.[11] The drug was still distributed in the US under the guise of investigational research. Clearly the harmful enantiomer of this API and the implications of the chemistry it conferred were not understood by the manufacturer. To avoid similar mistakes in the future, the Kefauver Harris Amendment to the US Federal Food, Drug, and Cosmetic Act was passed. This amendment granted power to the FDA to cultivate and enforce a safer regulatory system for the approval, manufacturing, and advertising of drug products.[11]

In a trend of increasing governmental control over drugs in the light of safeguarding Americans, the Controlled Substances Act became law in 1970. This Act endowed the FDA and the DEA with the authority to classify drugs into one of five different schedules, and basically determine the legality of drugs and therefore

availability to the public.[12] This scheduling has hampered research into any legitimate medical benefits of Schedule I controlled substances, which are deemed to have the highest abuse potential and no currently accepted medical use. Marijuana is currently classified as a Schedule I drug. However, trans-Δ^9-tetrahydrocannabinol (Δ^9-THC), the most active component in Marijuana,[13,14] in isolated, purified form, is an approved API known as Dronabinol, and only classified as a Schedule III drug.

Tetrahydrocannabinol

Initial Characterization of Δ^9-THC

Due to potent psychoactive effects and a negative stigma, use of the cannabis plant within the US and numerous other countries has been completely prohibited or severely restricted.[11] The legal status of cannabis did not deter some researchers, including Raphael Mechoulam and S. Loewe, from investigating the plant's active ingredients.[15] Mechoulam has spent over 40 years studying natural and synthetic cannabinoids. The term cannabinoid refers to any compound that is extracted, derived, or related to those from the cannabis plant. Δ^9-THC was isolated from plant extracts and identified in 1964 by Mechoulam and co-workers.[16] The potent pharmacological activity of Δ^9-THC was noted at the outset of cannabinoid studies, and originally attributed to both enantiomers: (-)-trans-Δ^9-tetrahydrocannabinol ((-)-Δ^9-THC) and (+)-trans-Δ^9-tetrahydrocannabinol ((+)-Δ^9-THC) (see Figure 1.1).[13,14,17]

Differential Potency of Enantiomers

In early cannabinoid experiments, Δ^9-THC was primarily synthesized as a racemate, or a mixture containing an equal amount of the enantiomers.[13] After pharmacological studies emerged indicating that (+)-Δ^9-THC was far less potent or

possibly inactive compared to (-)-Δ^9-THC,[18] synthesis and characterization efforts shifted focus largely to the (-)-enantiomer of Δ^9-THC.[19,20] Patents on the synthesis and therapeutic use of Δ^9-THC were first issued in the late 60's and early 70's, and included its regioisomer, trans-Δ^8-tetrahydrocannabinol (Δ^8-THC), which only differs from Δ^9-THC by the location of a double-bond.[21,22]

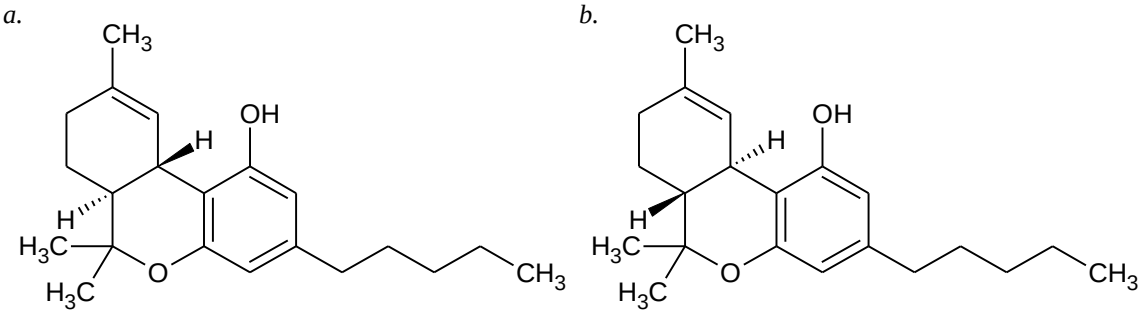


Figure 1.1. Δ^9-THC Structures. a. (+)-enantiomer and b. (-)-enantiomer.

Diastereomers of Δ^9-THC

The similarity in structure means that Δ^8-THC is also enantiomeric. The shifted location of the double-bond in the structures of the Δ^8-THC enantiomers, (-)-trans-Δ^8-tetrahydrocannabinol ((-)-Δ^8-THC) and (+)-trans-Δ^8-tetrahydrocannabinol ((+)-Δ^8-THC), makes them diastereomers of Δ^9-THC (see Figure 1.2). While Δ^8-THC and Δ^9-THC enantiomers are indistinguishable in an achiral environment, characterization of the regioisomers has been well established.[23,24] Separation of these diastereomers is accordingly documented in a United States Pharmacopeia (USP) monograph using achiral analysis conditions on conventional equipment.[25]

USP monographs are recognized by the FDA through the Food, Drug, and Cosmetic Act as the official regulatory analytical procedures for analysis of specific APIs and drug products.[26] Verifying a USP monograph, which in most cases requires some

minor adjustments to the given methodology and a few brief studies, is far less intensive and less expensive than developing and validating every aspect of an alternative analytical procedure from scratch. After documented justification of its intended use, the USP monograph for Dronabinol could enable compliance with US FDA laws regarding the manufacture of Δ^9-THC.

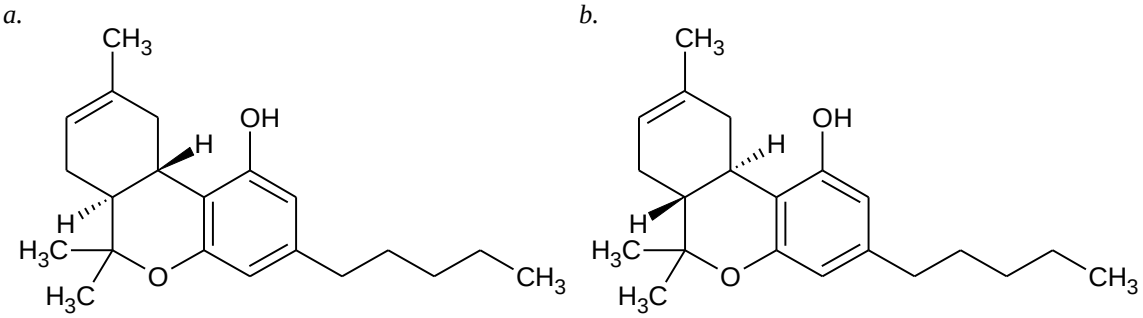


Figure 1.2. Δ^8-THC Structures. a. (+)-enantiomer and b. (-)-enantiomer.

Chiral Analysis

Chiral analytical methods emerged from the need to verify the products of asymmetric syntheses that could then be used to produce larger quantities of single enantiomers for pharmacological studies.[18,20] As the science of chiral chromatography matured, and the importance of the characterization of drug enantiomers became accepted[27] and then regulated,[2] interest in chiral separations of myriad chiral drugs, including cannabinoids, increased. The evaluation of enantiomers is now considered an essential component of the drug development process,[1,2] and because of its flexibility and wide range of applications, LC is the most commonly utilized analytical technique for drug analysis.[7]

Because the enantiomer of Dronabinol is documented to have inferior pharmacological activity,[15] FDA guidelines prescribe that the enantiomeric impurity must

be quantified in the API, especially for clinical-trial material.[2] Though the enantiomeric nature of Dronabinol has been recognized for decades, there is no USP monograph for chiral analysis of this API, and no validated method for this material was reported in the literature. Relevant literature describe a number of chiral analyses of Δ^9-THC and related cannabinoids, but not a one outlines a single method for accurate separation of the four Δ^9- and Δ^8-THC diastereomers simultaneously.

The most commonly used and well-characterized LC stationary phases (ie- C18, C8, phenyl, etc) are achiral and therefore do not effectively separate enantiomers such as (+)-Δ^9-THC and (-)-Δ^9-THC without derivitization or use of a chiral additive in the mobile phase. Thus a CSP must be utilized to directly quantify these and related cannabinoid enantiomers. Studies investigating the enantioseparations of various compounds on polysaccharide-based CSPs date back to the mid-1980's, when researchers in Japan, like Yoshio Okamoto, described the preparation and use of phenylcarbamate-derived cellulose and amylose coated silica packings.[28] Various enantioseparations of cannabinoids have been achieved using amylose tris 3,5-dimethylphenylcarbamate (ADMPC) CSP (see Figure 1.3).[28-31]

The ADMPC column is widely associated with normal phase chromatography due to the prevalent use of non-polar alkane-based mobile phases, as reported in texts and the literature. However, chiral discrimination on ADMPC is attributed to both normal phase and reversed phase characteristics.[29,30] In normal phase chromatography, retention is described as a process of adsorption, wherein the analyte displaces a polar moeity/alcohol from a polar silanol (or bonded-phase ligand) adsorbent site on the surface of the column packing.[7] Polar analytes and solvent molecules attach, or localize, onto the adsorbent

sites, forming strong associations with the column packing. Due to the localization of polar solvent molecules in the mobile phase, changes in selectivity are achieved by varying the alcohol component. In contrast, reversed phase chromatography is characterized as a process of differential partitioning of the analyte between a polar mobile phase and a non-polar column packing based on relative hydrophobicities.[7] Without changing stationary phases, solvent localization has the greatest effect on selectivity in normal phase chromatography, whereas pH, salt concentration, and dipolarity of the mobile phase have the greatest effect on selectivity in reversed phase chromatography.

The most frequently utilized mobile phase combination for polysaccharide-based CSPs is an alkane/alcohol mixture, usually consisting of n-hexane and either IPA or ethanol. N-hexane/IPA and n-hexane/ethanol mobile phases are so common that numerous studies have compared them in the enantioseparations of related compounds, including cannabinoids, using ADMPC.[28-37] Column stability in and the selectivity afforded by alkane/alcohol mixtures was noted from the outset of these investigations.[28] Though the list of solvents not recommended for the coated version of the CSP includes those that could dissolve the polysaccharide coating like methylene chloride and tetrahydrofuran,[7] polar organic phase eluents including acetonitrile and some alcohols are permitted by the manufacturer[38] and described in the literature.[34,35,37] The latest generation of these columns features immobilized CSPs and thus the column stability in a wider range of solvents is purportedly improved.[39,40]

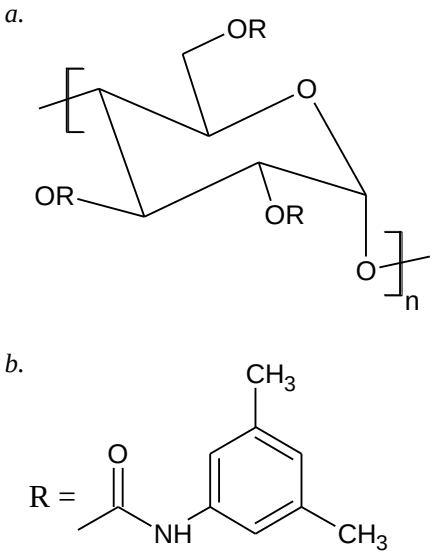


Figure 1.3. Amylose Tris 3,5-Dimethylphenylcarbamate Structures. a. each amylose backbone unit is bonded to three disubstituted carbamate groups (R), b. the carbamate moeity.

Clearly elucidating the chiral retention mechanism of ADMPC would enable precise prediction of enantioseparations of new compounds. A well-demonstrated, working description of the chiral discrimination afforded by ADMPC has not yet emerged in the literature, though many empirical trends have been reported. Most of these studies compare the enantioselectivities achieved for families of chiral compounds in different percentages of IPA or ethanol in n-hexane mobile phases.[28-37] In the current study, the effects of IPA and methanol in n-heptane mobile phases are investigated in the simultaneous enantioseparation of the Δ^9- and Δ^8-THC diastereomers on ADMPC using a factorial design.

CHAPTER II

METHOD

Experimental Design

A 3 x 3 x 3 factorial experiment was designed to study the main and interaction effects of mobile phase modifiers on the separation of two sets of related enantiomers, Δ^8-THC and Δ^9-THC. The independent variables included % IPA in the mobile phase, % methanol in the mobile phase, and experimental set (see Table 2.2). The effects of IPA and methanol were analyzed across three levels. Experimental set was used as an independent variable to allow partitioning of the effect of time (ie- deterioration) on the detector, column, and system, as well as any other uncontrollable variation between the three experimental sets.

Specimen

Mobile Phase Solutions

For each experimental set, four 1 L solutions were prepared in n-heptane with the following percentages (v/v) of IPA and methanol, respectively: 1) 1%, 1%; 2) 1%, 5%; 3) 7%, 1%; and 4) 7%, 5%. These solutions were used individually and mixed (by the instrument) to obtain a total of nine mobile phases containing varying percentages of IPA and methanol at the levels desired for this experiment (see Table 2.2).

Table 2.1. Chiral Separation of Enantiomers.
Independent variables were % IPA, % methanol, and experimental set. Dependent variables were determined from the chromatograms. Four resolution sample chromatograms were collected for each mobile phase composition in each setup.

Retention Time, Peak Width, Capacity Factor, Theoretical Plates, Selectivity, Resolution		IPA in Mobile Phase								
		1%			4%			7%		
Experimental Set		#1	#2	#3	#1	#2	#3	#1	#2	#3
Methanol in Mobile Phase	1%	n=4	n=4	n=4						
		average (n=12)								
	2%									
	3%									

The solvents used in this experiment were HPLC grade or better. N-heptane and IPA were purchased from Sigma-Aldrich (St. Louis, MO), and methanol was purchased from Honeywell (Morristown, NJ). One liter of each mobile phase was prepared at a time. Solvents were measured out using Class A graduated cylinders directly into 1 liter mobile phase bottles. The solutions were prepared by following the same order of addition of ingredients each time to minimize mixing issues due to the limited miscibility of methanol and n-heptane. 100 mL of n-heptane was added to a bottle, followed by the appropriate volume of IPA. This solution was mixed thoroughly, followed by addition of the appropriate volume of methanol. After mixing again, the remainder of n-heptane was added and the solution mixed completely to obtain the final solution.

Table 2.2. Mobile Phase Compositions.
Four solutions (A-D) were mixed by the instrument to achieve nine mobile phases containing IPA and methanol at three levels each (v/v) in n-heptane. Each of the nine cells describes the solutions and proportions mixed to obtain the desired alcohol levels in the mobile phases.

Key to solutions: *Solution* *%IPA* *%Methanol* *A* *1* *1* *B* *1* *3* *C* *7* *1* *D* *7* *3*		IPA in Mobile Phase		
		1%	4%	7%
Methanol in Mobile Phase	1%	*A x 100%*	*A x 50%* *C x 50%*	*C x 100%*
	2%	*A x 50%* *B x 50%*	*A x 25%* *B x 25%* *C x 25%* *D x 25%*	*C x 50%* *D x 50%*
	3%	*B x 100%*	*B x 50%* *D x 50%*	*D x 100%*

Column Flush Solution

Ethanol was of the highest grade available and purchased from Pharmco Products/Aaper Alcohol (Brookfield, CT). Partially hydrated ethanol was obtained by exposing a lint-free towel-covered container of anhydrous ethanol to moist air for 24 hours, which was then mixed with an equal amount of anhydrous ethanol to produce a so-called “half-saturated” ethanol solution. This solution served as both a cleaning solution to remove strongly retained impurities, as well as an initial equilibration solution, to prepare the column for the experiments.

Analyte Solutions

The high purity Δ^8-THC and Δ^9-THC materials used in these experiments were provided by Cerilliant Corporation (Round Rock, Texas). Three sample solutions were

prepared from these materials. One solution was used for peak identification of Δ^8-THC enantiomers, one was used for peak identification of Δ^9-THC enantiomers, and one was used to measure the resolution obtained from the different mobile phases. Analysis of the n-heptane blank provided a baseline signal for each system.

Sample solutions were prepared in n-heptane using Class A volumetric flasks and pipettes. The resolution sample solution contained all four diastereomers and was prepared such that the final concentration of each analyte was 10 μg/ml. Identification solutions were prepared such that the final concentration of the negative enantiomer was twice that of the positive enantiomer (ie- 20 and 10 μg/ml, respectively). All solutions were stored in volumetric flasks sealed with Teflon tape, in the freezer, with approximately ten milliliters removed for each set of experiments.

Instrumentation

HPLC

Components of the Liquid Chromatograph System

HPLC was the analytical chemistry technique utilized to measure the dependent variables, including the resolution of Δ^8-THC and Δ^9-THC, as influenced by the independent variables, percentage of IPA and percentage of methanol in the n-heptane mobile phase. The apparatus, or LC instrument, was an Agilent 1100 HPLC system (Santa Clara, CA) equipped with a quaternary pump, an inline degasser, a 100 vial autosampler with a 100 μL flow-through sample loop, a thermostatted column compartment, and a diode array detector (DAD) (see Figure 2.1). The column used was a Chiralpak AD-H 4.6 x 250 mm analytical column with 5μ particle size that contained amylose tris-3,5-dimethylphenylcarbamate coated silica gel packing and was purchased

from Chiral Technologies (Exton, PA). To collect and analyze the data, Agilent Chemstation for LC Control and Analysis software version A.08.03 was used. For the experiments described herein the column and instrument was the same; only the mobile phase was intentionally varied between experiments.

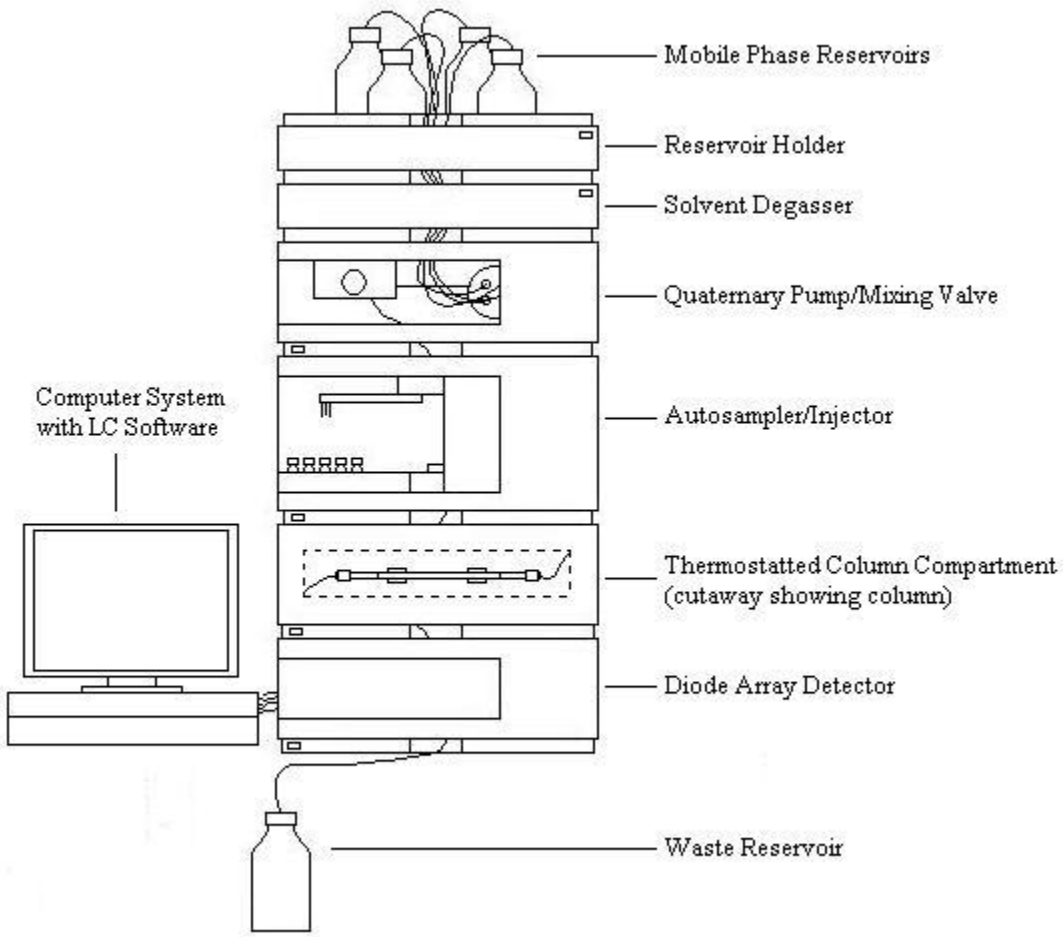


Figure 2.1. Components of a Liquid Chromatograph Instrument. Diagram illustrating the modular Agilent 1100 LC system used in these experiments.

In an LC instrument, the pump and mixing valve generate a constant flow of the mobile phase through the system, past the sample injector, through the column, to the detector, and finally to a waste container. For a sample to be analyzed, it is injected into the path of the mobile phase, and then carried through the column, where it may or may not interact with the column packing, and finally to the detector (see Figure 2.2). To aid

in the application of this technique to a wide variety of compounds, the solvents or mobile phase, and column are easily changed out, and myriad combinations are available for users. A particular combination of mobile phase and a column on an LC instrument is often referred to as a "system" when intact.

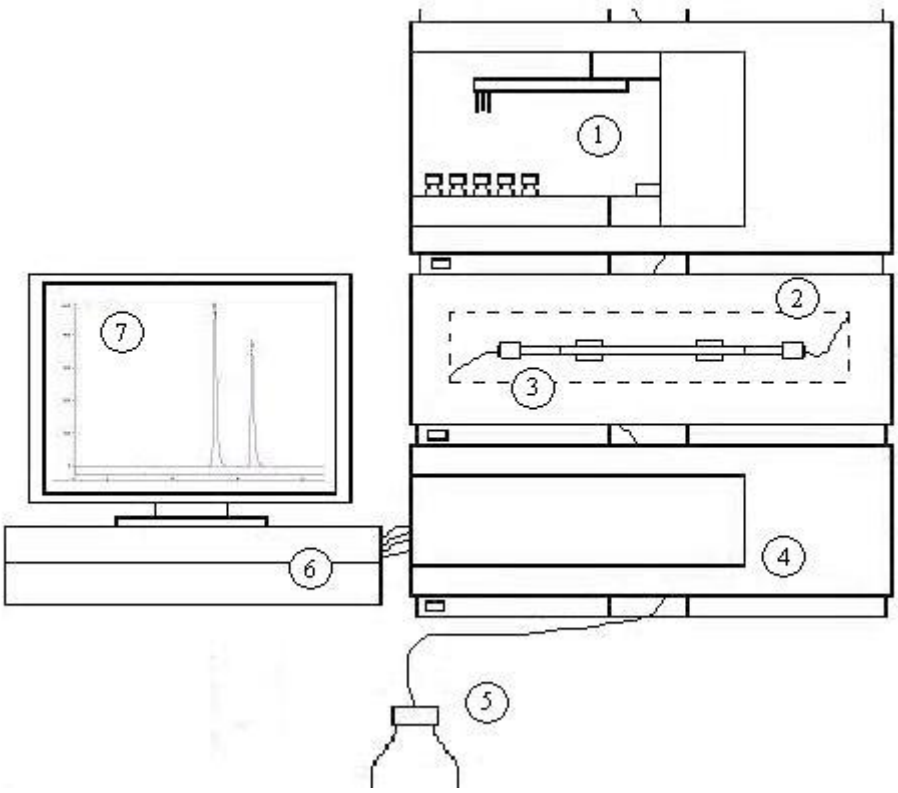


Figure 2.2. Path of Sample Through LC Instrument. The sample is injected by the autosampler (1) and carried by mobile phase to the column (2) where it interacts with CSP and is separated (3) into distinct bands before it exits the column. Mobile phase carries the analytes to the detector (4) and finally to the waste (5). The software (6) processes the detector signal and creates a chromatogram (7) from that information.

Generating a Chromatogram

For each analysis, an aliquot of sample was injected by the autosampler into the system immediately upstream from the column. Then the mobile phase carried the sample through the column and to the detector where the sample absorbed UV light as it passed through the detector cell. Ideally, the sample components (Δ^8-THC and/or Δ^9-

THC enantiomers) were partitioned between being dissolved in the mobile phase and being associated with the chiral moieties that coat the particles of the column packing, or the stationary phase. Thus the analytes were separated into distinct bands while traveling through the length of the column. Once these bands exited the column and passed through the detector, they were recorded electronically by the software as a peak, in direct proportion to the amount of light absorbed.

For each sample injected, a chromatogram was generated by the software corresponding to light absorbance over time (see Figure 2.3). A peak in the chromatogram at a specific time (ie- the retention time) represents the elution of analyte(s) after that amount of time within the system. The degree of separation of the analytes in these systems was influenced by the alcohol content of the mobile phase, and measured through the resolution of the analytes, as determined from the chromatograms.

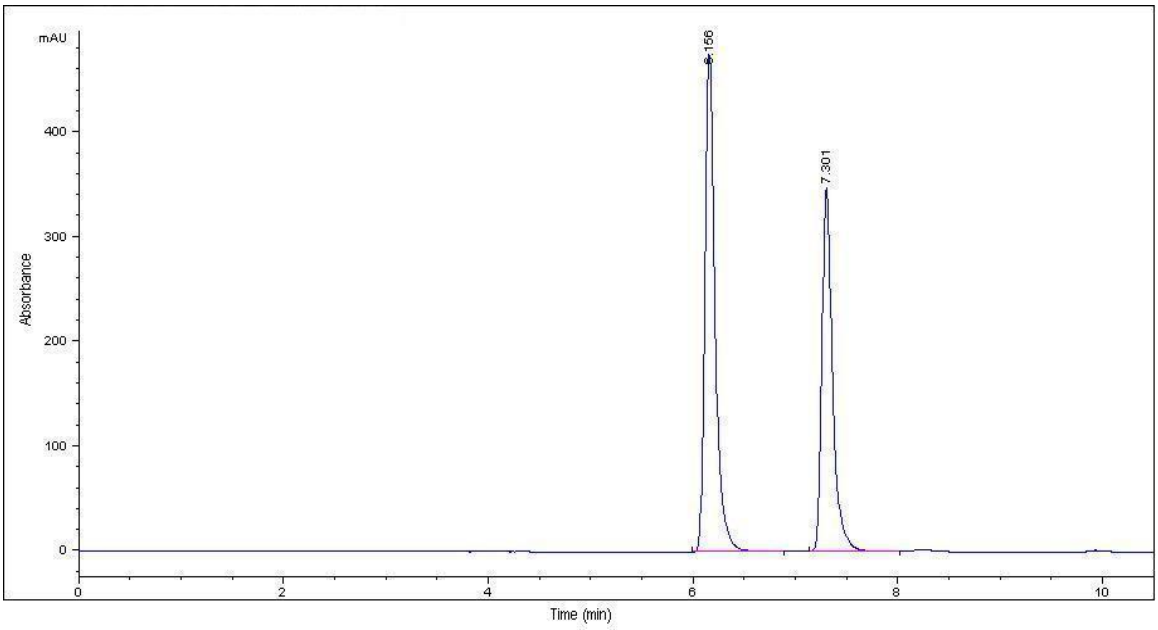


Figure 2.3. Example Chromatogram. A pair of enantiomers with approximate retention times of 6.2 and 7.3 minutes exhibit baseline resolution.

Analytical Parameters

Peak Identification

Retention times obtained for the peaks in the Δ^8-THC and Δ^9-THC identification solution chromatograms were used to determine the identity and elution order of the analytes in the resolution sample chromatograms. Identity of the negative enantiomer was assigned to the larger of the two peaks based on the sample preparation. Where baseline resolution was not obtained between enantiomers, information from all identification injections (ie- entire experimental set) was used to make the best possible determination of elution order to aid in data analysis.

Capacity Factor

The capacity factor, k', also known as the retention factor, was calculated from the chromatograms using the following equation:

$$k' = \frac{t_r - t_v}{t_v} \qquad \text{(equation 2.1)}$$

where t_r is the retention time of the analyte, and t_v the void time. Void time is defined as the retention time of a compound that is not retained on ADMPC, and was determined from solvent impurities in the blank chromatograms. The capacity factor provides an estimate of the retention of the analyte in each system. Because the column and instrument were the same for all analyses, the capacity factors were directly compared to ascertain the influence of the mobile phase composition on the retention of each analyte.

Selectivity Factor

The level of discrimination of two analytes provided by the CSP was calculated from the capacity factors using the following equation:

$$\alpha = \frac{k'_2}{k'_1} \qquad \text{(equation 2.2)}$$

where k'_1 is the capacity factor of the earliest-eluting enantiomer, and k'_2 the other enantiomer, as calculated by equation 2.1. α is always greater than 1, and provides an estimate of relative migration rates of a pair of analytes in each system.

Theoretical Plates

Column efficiency was determined by calculating the theoretical plate count for a given analyte. Theoretical plates, N, is defined empirically by the following equation:

$$N = 16\left(\frac{t_r}{W}\right)^2 \qquad \text{(equation 2.3)}$$

where W is the peak width at baseline and t_r the retention time of the analyte. The effect of peak tailing on theoretical plates was mitigated by using a form of the equation for N based on peak width at half-height, $W_{h/2}$:

$$N = 5.54\left(\frac{t_r}{W_{h/2}}\right)^2 \qquad \text{(equation 2.4)}$$

Peak Resolution

The ability of each mobile phase to separate pairs of analytes was determined based on resolution of the peaks. Without resolution greater than 0.6, there was no measurable distinction between pairs of analytes. Resolution, R, of two peaks is defined empirically by the following equation:

$$R = \frac{2(t_2 - t_1)}{(W_1 + W_2)} \qquad \text{(equation 2.5)}$$

where t_1 and t_2 are the retention times of the two analytes, and W_1 and W_2 the peak widths at baseline. For these experiments, resolution was calculated using the USP recommended formula for resolution:[41]

$$R = \frac{2(t_2 - t_1)}{1.70(W_{1,h/2} + W_{2,h/2})} \qquad \text{(equation 2.6)}$$

which included the peak width, $W_{1,h/2}$ and $W_{2,h/2}$, measured at half the peak height by the Chemstation software.

Procedure

Column Preparation

To remove any strongly retained impurities a column equilibration/cleaning procedure was used prior to each set of experiments. This procedure included a 3 hour flush of the column with a half-saturated ethanol solution at 0.2 ml/min, then a 3 hour flush with the four solutions at a 1:1:1:1 ratio (equivalent to 2% methanol, 4% IPA in n-heptane; see Table 2.2) at 1 ml/min at room temperature to prime the column packing for the mobile phases. To saturate the column packing with mobile phase and prepare it for analysis of the samples, prior to each sequence of injections, the column was flushed for 2.5 hours with the new mobile phase composition at 1 ml/min and 40ºC.

Analysis Conditions

For all analyses, the column temperature was 40ºC, the mobile phase flow rate was 0.7 ml/min, the sample injection volume was 5 µl, the analysis wavelength was 228 nm, and the injection run time was 25 minutes. Only the mobile phase composition

varied from experiment to experiment. The order of experiments was randomized with respect to the mobile phase composition. The same samples (ie- from the same stock solution) were analyzed using each mobile phase, and followed the same automated sequence of seven injections on a single column (ie- unique, serially number). Fresh crimp-top vials of samples were prepared for each mobile phase injection sequence.

Mobile Phase Injection Sequence

Multiple injections of the resolution sample were performed before and after the identification solution injections for two reasons: 1) to evaluate the equilibration of each mobile phase system over an extended period of time, and 2) to allow peak identification in the case of shifting retention times in a poorly equilibrated system. The automated injection sequence used for determining resolution provided by each mobile phase (ie- 4% IPA, 1% methanol mobile phase) was as follows:

- Injection 1: Blank (n-heptane)
- Injection 2: Resolution Sample
- Injection 3: Resolution Sample
- Injection 4: Δ^8-THC Peak Identification Solution
- Injection 5: Δ^9-THC Peak Identification Solution
- Injection 6: Resolution Sample
- Injection 7: Resolution Sample

Because there were nine different mobile phase compositions (see Table 2.1), and seven injections per mobile phase, a total of 63 injections were performed for each experimental set. Three experimental sets were performed over the course of approximately two weeks, for a grand total of 189 injections including 108 injections of resolution sample.

Experimental Set Sequence

The flushes and sample sequences were programmed into the software and, with the exception of the initial cleaning/equilibration method, automated in the experimental set sequence to minimize variability. The transition between the ethanol flush and the mobile phase analyses required the replacement of the solutions on the instrument, which had to be performed manually. The series of flushes and mobile phase injection sequences used to collect data for the entire experimental set was as follows:

- Ethanol Flush

Manual: replace ethanol with mobile phase solutions

- Initial System (1:1:1:1) Mobile Phase Flush
- Mobile Phase 1 Flush
- Mobile Phase 1 Injection Sequence (7 injections)
- Mobile Phase 2 Flush
- Mobile Phase 2 Injection Sequence (7 injections)
- Mobile Phase 3 Flush
- Mobile Phase 3 Injection Sequence (7 injections)
- Mobile Phase 4 Flush
- Mobile Phase 4 Injection Sequence (7 injections)
- Mobile Phase 5 Flush
- Mobile Phase 5 Injection Sequence (7 injections)
- Mobile Phase 6 Flush
- Mobile Phase 6 Injection Sequence (7 injections)
- Mobile Phase 7 Flush
- Mobile Phase 7 Injection Sequence (7 injections)
- Mobile Phase 8 Flush
- Mobile Phase 8 Injection Sequence (7 injections)
- Mobile Phase 9 Flush
- Mobile Phase 9 Injection Sequence (7 injections)

The order of the mobile phase injection sequences in the experimental set sequence outlined above was arbitrarily labeled 1-9 for illustrative purposes. The actual order of the mobile phases was chosen randomly, using a random sequence generator[42] and is summarized in Table 2.3. Each mobile phase injection sequence required approximately five and a half hours to run including the flush, and each experimental set required approximately 55 hours total instrument time.

Table 2.3. Random Order of Mobile Phases.
Mobile phases are designated (n,m) where n is the IPA level and m is the methanol level in the mobile phase. IPA levels (n) for the mobile phases (see Table 2.2) are 1=1%, 2=4%, 3=7%, whereas methanol levels (m) are 1=1%, 2=2%, 3=3%.

		Order of Mobile Phase Injection Sequences								
		1	2	3	4	5	6	7	8	9
Experimental Set	1	(2,2)	(3,2)	(2,1)	(1,1)	(2,3)	(3,3)	(1,2)	(3,1)	(1,3)
	2	(3,2)	(3,3)	(2,3)	(3,1)	(1,3)	(1,1)	(2,2)	(2,1)	(1,2)
	3	(2,3)	(1,1)	(2,2)	(3,2)	(1,3)	(3,3)	(3,1)	(1,2)	(2,1)

Hypotheses

The experimental design was chosen primarily to evaluate the main and interaction effects of IPA and methanol in resolving the four analytes of interest on ADMPC. Also of interest is the appropriateness of using the LC instrument to in-line mix mobile phases containing small percentages of modifiers given that normal phase systems notoriously require lengthy equilibration times. Some effects of the alcohols on retention time, resolution, selectivity, and elution order of the four analytes can be predicted from literature findings. However no literature described the use of more than one alcohol in an enantioseparation on a CSP.

Mobile Phase Alcohol Effects

The elution strength of the alcohols used in alkane/alcohol chiral systems is attributed primarily to polarity, as observed in classic normal phase systems, though some studies indicate steric bulk is a more important determining factor.[7,29,36] Ethanol is generally accepted to be a stronger eluent than IPA, which follows either argument, due to its higher polarity and smaller size. Likewise, methanol is more polar and smaller than ethanol, so logically it is likely to be an even stronger eluent in these systems than IPA.

Retention Time and Elution Order

Overall, retention time will decrease with increasing alcohol, as is commonly seen in normal phase systems. More specifically, similar to trends noted in the previous enantioseparations of related cannabinoids, the stronger solvent (in this case, methanol) will likely have a larger effect on retention time, or in other words, will reduce retention time more markedly, than IPA.[29-32] The unusual elution order reversal of Δ^8-THC enantiomers noted by Levin and coworkers in mobile phases containing small percentages of ethanol (0.5-2%) in n-hexane,[29,31] leads to the prediction that the effect of methanol on Δ^8-THC retention, especially the negative enantiomer, is likely to be larger than that observed for Δ^9-THC. The effect of methanol on Δ^8-THC retention is predicted to be larger than that observed for IPA as well. Also, Δ^9-THC will be retained on the column longer than Δ^8-THC, with the positive enantiomer eluting first in all instances except for Δ^8-THC in the 1% methanol containing mobile phase due to the predicted elution order reversal.

Conformational changes induced by alcohols in the CSP are indicated as the primary cause of elution order reversal of enantiomers in several experiments.[29,31,36,43,44]

A solid-state Nuclear Magnetic Resonance (NMR) study reported significant changes in conformation of ADMPC at very low percentages of IPA.[36] If interaction effects between IPA and methanol in the mobile phase are present, based on these observations, they do seem likely to occur at the low alcohol percentages (2-10% of total volume) examined in this experiment.

Resolution and Selectivity

A decrease in retention time of enantiomers due to increasing eluent strength generally results in the decreased resolution of enantiomers,[7] probably due to decreased residence time in the CSP and increased competition of the alcohol for achiral hydrogen-bonding sites. This trend does not necessarily translate to an analogous decrease in selectivity, as chiral discrimination by the CSP depends on both chiral and achiral interactions, with some chiral interactions being more dominant.[29,44,45] When Δ^9-THC and Δ^8-THC were analyzed on ADMPC using n-hexane/IPA mobile phase in one study, resolution decreased whereas selectivity increased after the alcohol modifier was changed from 2% to 5%.[29] Of the six cannabinoids examined in that investigation, Δ^8-THC was the only one that could not be effectively resolved using both n-hexane/IPA and n-hexane/ethanol mobile phases.[29]

The data discussed indicate that with increasing IPA in the mobile phase, overall THC selectivity will improve, but that this will have a stronger effect on Δ^9-THC than Δ^8-THC.[3,29] Additionally, resolution is likely to decrease with increasing alcohol content, and this will be more pronounced for methanol as well as for Δ^8-THC in general. Interaction effects in the mobile phase are likely to take place at low concentrations of

methanol, and to effect resolution but not necessarily the selectivity of Δ^8-THC, depending on the extent to which any elution order reversal occurs.

Instrument Setup/Experimental Design Effects

Use of an alcohol known to be miscible with alkanes only at low concentrations in an alkane-based mobile phase is not common practice. Observations made during initial method development led to the unusual selection of methanol for the validated method. During investigations of a system that employed an n-heptane/IPA mobile phase, a THC sample in methanol was analyzed because no other sample of that particular material was readily available at the time. The sample solvent was different from the mobile phase, which had a marked effect on the system. Repeated injections of this sample resulted in an increase of enantioseparation that was not duplicated when the same material in n-heptane was analyzed repeatedly in the same system, nor in any other alkane/alcohol system attempted.[3]

Column equilibration was of the greatest concern when conceiving the instrumental setup and sequence parameters for the design of this study. Insufficient in-line mixing of the solutions and insufficient equilibration time pose the greatest threat to column equilibration. Either of these problems would result in larger standard deviations in the retention time for effected cells (summarized according to the Table 2.2 layout). Each cell in Table 2.2 corresponds to a unique combination of IPA and methanol in n-heptane, for a total of which nine will be investigated. If in-line mixing of the mobile phases is insufficient to produce repeatable results, the cells specified in Table 2.2 to be composed of multiple solutions will have the highest retention time standard deviations.

Incomplete equilibration will be most likely and most evident in the solutions containing the smallest percentages of alcohol modifiers.

The effect of experimental set is predicted to be insignificant. However, the extended analysis times (>50 hours per experimental set) and the 40ºC column temperature could potentially degrade the column and negatively impact the enantioseparations. Aging of the lamp should be mentioned, but is not of major concern here as long as the lamp is relatively new.

Statistical Approach

Multiple analysis of variance (MANOVA) was used to test the hypotheses arising out of the experimental design, and to evaluate the data derived therefrom. Follow-up analysis of variance (ANOVA) tests were performed to test à priori polynomial trends of the interaction effect, and post-hoc comparisons of the main effects.

CHAPTER III

RESULTS

Data Collection and Processing

Chromatograms were recorded and processed using the Agilent Chemstation software. A batch processing method was applied to all chromatograms obtained from an injection sequence to ensure consistent integration of peaks. Batch-processing minimized the error (or noise) introduced into measurement of the retention times and peak widths.

The chromatograms obtained from injecting n-heptane were used to show that each system was free of interfering peaks and served as the baseline for subsequent analyses. Where minor extraneous or artifact peaks were present, only the four analytes of interest were integrated and analyzed to determine the performance of each system. Analytes that co-eluted were assigned identical retention times and peak widths.

Once the peaks in the resolution sample injections were identified based on retention times, chromatographic parameters were calculated as outlined above in the section Analytical Parameters. Of special interest were the resolution and selectivity, which were determined for several pairs of analytes. Separation of the (-)-Δ^8-THC and (+)-Δ^9-THC diastereomers was considered to be most important given that the entire study emerged from a lack of sufficient chromatographic conditions to separate these analytes. In contrast, separation of the (+)-Δ^8-THC and (-)-Δ^9-THC diastereomers was

considered to be least important due to the fact that the level of (+)-Δ^8-THC present in a Cerilliant Dronabinol sample would not likely be quantifiable (< 0.01%) and was therefore of the least concern for method development.

Descriptive Statistics

Measured Parameters

Retention time (t_r) and peak width at half-height ($W_{h/2}$) data, as obtained from the Chemstation software, were summarized for each experimental set, as seen in Appendix A. Identification solutions were used only qualitatively to identify peaks and thus were not included. Void times (t_v) were determined by averaging the values from the blanks for each level across all three experiments. Representative chromatograms for the experimental sets are presented in the Figures 3.1-3.3. Refer to Table 2.3 for the order of analysis of the mobile phases.

Though differences between experimental set data were apparent, overall retention characteristics, such as elution order and number of peaks resolved, remained fairly constant. One notable difference between experimental sets occurred for the mobile phase containing 1% of each alcohol. Whereas only two peaks were resolved in the first two experiments (Figures 3.1a and 3.2a), three peaks were at least partially resolved in the third experiment (Figure 3.3a).

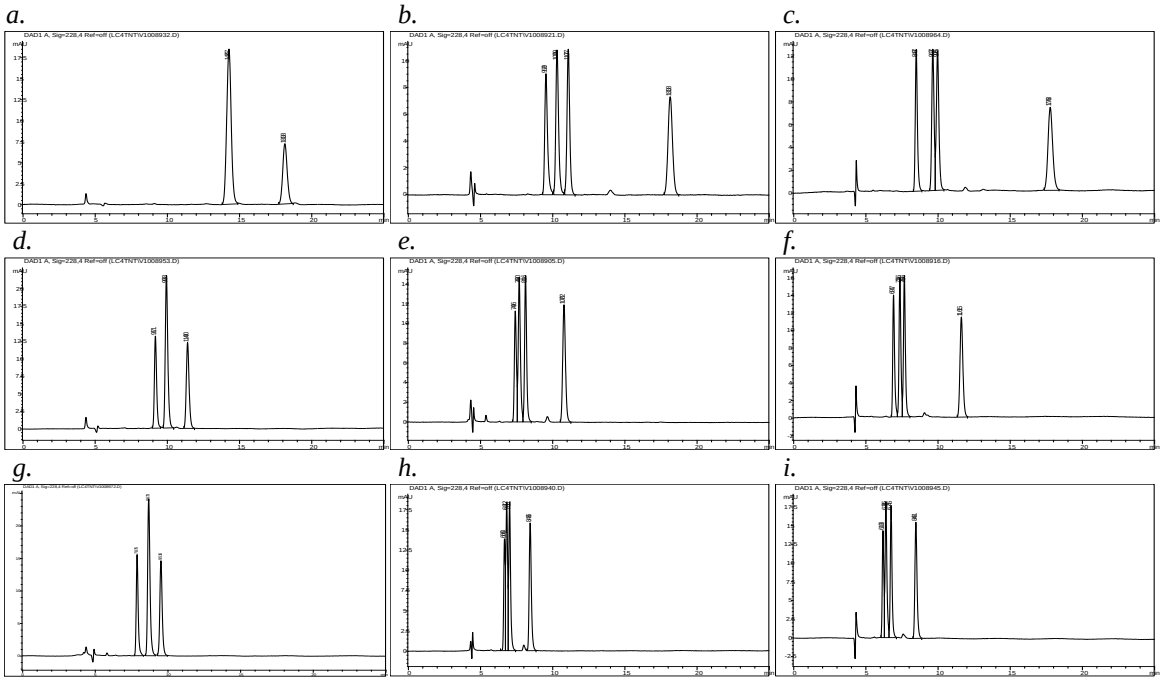


Figure 3.1. Chromatograms for Experimental Set 1. Figures a-i are representative chromatograms from the analysis of mobile phases across all experimental levels (n,m): a. (1,1), b. (2,1), c. (3,1), d. (1,2), e. (2,2), f. (3,2), g. (1,3), h. (2,3), i. (3,3).

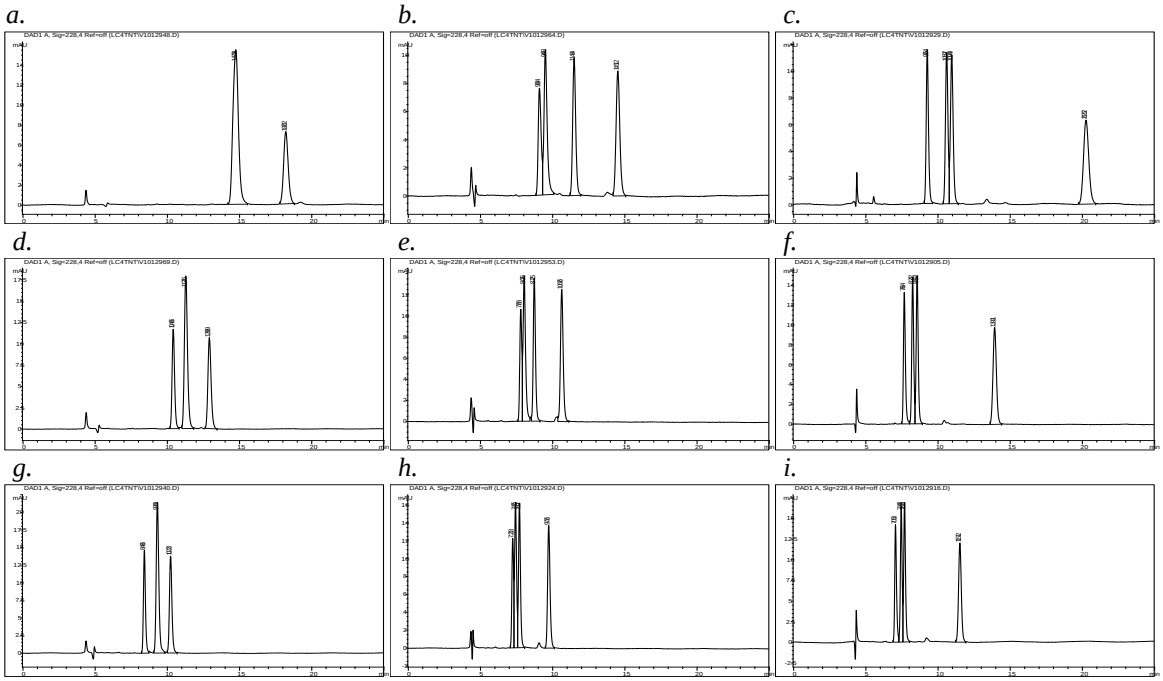


Figure 3.2. Chromatograms for Experimental Set 2. Figures a-i are representative chromatograms from the analysis of mobile phases across all experimental levels (n,m): a. (1,1), b. (2,1), c. (3,1), d. (1,2), e. (2,2), f. (3,2), g. (1,3), h. (2,3), i. (3,3).

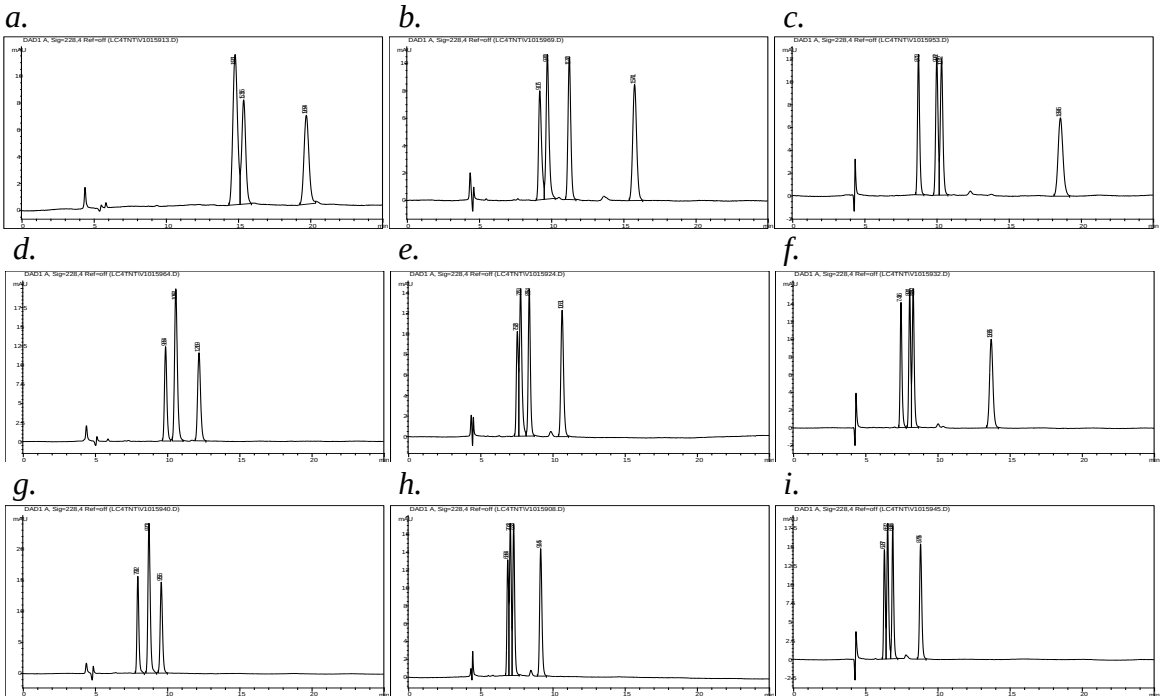


Figure 3.3. Chromatograms for Experimental Set 3. Figures a-i are representative chromatograms from the analysis of mobile phases across all experimental levels (n,m): a. (1,1), b. (2,1), c. (3,1), d. (1,2), e. (2,2), f. (3,2), g. (1,3), h. (2,3), i. (3,3).

Retention Time

Retention time cell means were summarized for each analyte as presented in Tables 3.1-3.4 as well as Figure 3.4. In general, retention time decreased with increasing alcohol content, which is characteristic for normal phase chromatography. Notable exceptions included that of Δ^9-THC with respect to IPA content. (-)-Δ^9-THC exhibited longer retention time's at the highest % IPA in the mobile phase than at the lower two levels of IPA for 1 and 2% methanol. Similarly, (+)-Δ^9-THC had longer retention time's at 7% IPA compared to 4% IPA for 1 and 2% methanol.

Overall, the (-)-enantiomers were retained longer than their (+)-enantiomer counterparts, except in the case of Δ^8-THC, which underwent an elution order reversal at 1% IPA and either 2% or 3% methanol. (-)-Δ^8-THC co-eluted with the (+)-enantiomers at 1% of both alcohols in the first two experiments, but was partially resolved in the third

experiment. Also at 1% IPA, the (+)-enantiomers co-eluted regardless of methanol content.

Table 3.1. Retention Time Cell Means for (+)-Δ^8-THC.
Retention times in minutes summarized by cells (n=4) and over all three runs (n=12).

		IPA in Mobile Phase								
		1%			4%			7%		
Experimental Set		#1	#2	#3	#1	#2	#3	#1	#2	#3
Methanol in Mobile Phase	1%	14.253	14.662	14.887	9.526	9.082	9.180	8.485	9.265	8.720
		14.601 (n=12)			9.263 (n=12)			8.823 (n=12)		
	2%	9.965	11.277	10.565	7.398	7.791	7.524	6.905	7.669	7.430
		10.602 (n=12)			7.571 (n=12)			7.335 (n=12)		
	3%	8.674	9.311	8.711	6.640	7.234	6.863	6.193	7.057	6.288
		8.899 (n=12)			6.912 (n=12)			6.512 (n=12)		

Table 3.2. Retention Time Cell Means for (-)-Δ^8-THC.
Retention times in minutes summarized by cells (n=4) and over all three runs (n=12).

		IPA in Mobile Phase								
		1%			4%			7%		
Experimental Set		#1	#2	#3	#1	#2	#3	#1	#2	#3
Methanol in Mobile Phase	1%	14.253	14.662	15.485	11.076	11.482	11.222	9.960	10.967	10.321
		14.800 (n=12)			11.260 (n=12)			10.416 (n=12)		
	2%	9.195	10.408	9.857	8.109	8.733	8.347	7.652	8.560	8.273
		9.820 (n=12)			8.396 (n=12)			8.161 (n=12)		
	3%	7.875	8.409	7.941	6.891	7.697	7.273	6.758	7.683	6.868
		8.075 (n=12)			7.317 (n=12)			7.103 (n=12)		

Table 3.3. Retention Time Cell Means for (+)-Δ^9-THC.
Retention times in minutes summarized by cells (n=4) and over all three runs (n=12).

		IPA in Mobile Phase								
		1%			4%			7%		
Experimental Set		#1	#2	#3	#1	#2	#3	#1	#2	#3
Methanol in Mobile Phase	1%	14.253	14.662	14.887	10.295	9.490	9.709	9.635	10.614	9.993
		14.601 (n=12)			9.831 (n=12)			10.080 (n=12)		
	2%	9.965	11.277	10.565	7.670	8.029	7.749	7.340	8.249	8.033
		10.602 (n=12)			7.816 (n=12)			7.874 (n=12)		
	3%	8.674	9.311	8.711	6.790	7.426	7.043	6.397	7.452	6.513
		8.899 (n=12)			7.086 (n=12)			6.788 (n=12)		

Table 3.4. Retention Time Cell Means for (-)-Δ^9-THC.
Retention times in minutes summarized by cells (n=4) and over all three runs (n=12).

		IPA in Mobile Phase								
		1%			4%			7%		
Experimental Set		#1	#2	#3	#1	#2	#3	#1	#2	#3
Methanol in Mobile Phase	1%	18.144	18.093	19.808	18.130	14.497	15.776	17.758	20.283	18.577
		18.682 (n=12)			16.134 (n=12)			18.866 (n=12)		
	2%	11.433	12.909	12.175	10.751	10.640	10.622	11.462	13.940	13.660
		12.173 (n=12)			10.671 (n=12)			13.021 (n=12)		
	3%	9.522	10.230	9.572	8.397	9.731	9.150	8.496	11.523	8.802
		9.775 (n=12)			9.092 (n=12)			9.607 (n=12)		

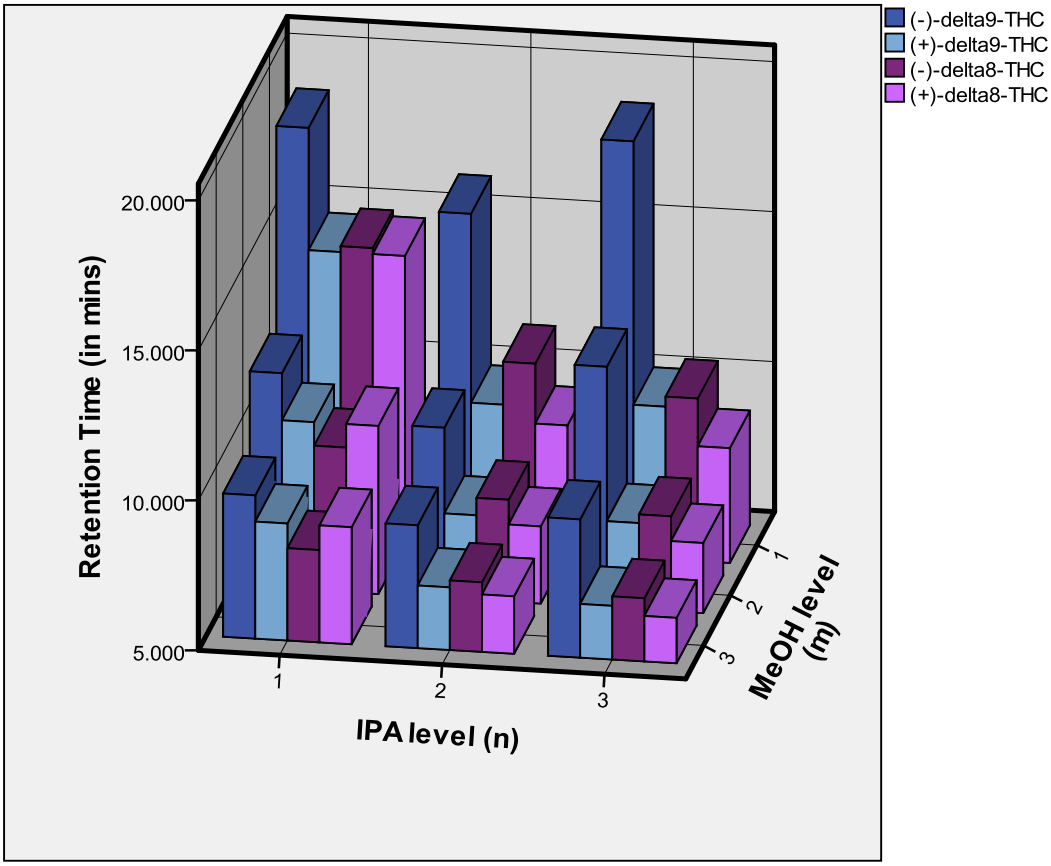


Figure 3.4. Retention Time Cell Means. Results are summarized for each mobile phase over all three experiments.

Derived Parameters

RT's and peak widths were used to calculate capacity factor, theoretical plates, selectivity, and resolution as described previously in Chapter II (see equations 2.1, 2.2, 2.4, and 2.6), and presented in Appendix B. For each analyte, the capacity factor and theoretical plates cell means are summarized in Tables 3.5-3.8 and 3.9-3.12, respectively. For designated pairs of analytes, selectivity and resolution cell means are summarized in Tables 3.13-3.17 and 3.18-3.22, respectively.

Capacity Factor, k'

The capacity factor equation (see equation 2.1) in essence normalized the retention time of an analyte by the system void time. Thus, trends for the capacity factors

(see Figure 3.5) were similar to those seen for analyte retention time's. As alcohol content increased, k' decreased, with the same notable exceptions for Δ^9-THC. The negative enantiomers had larger capacity factors than their positive enantiomer counterparts, except for Δ^8-THC due to the elution order reversal discussed previously. k' values between 2 and 5 are generally considered ideal/acceptable for most chromatographic separations.[46]

Table 3.5. Capacity Factor Cell Means for (+)-Δ^8-THC.
Capacity factor, k', summarized by cells (n=4) and over all three runs (n=12).

		IPA in Mobile Phase								
		1%			4%			7%		
Experimental Set		#1	#2	#3	#1	#2	#3	#1	#2	#3
Methanol in Mobile Phase	1%	2.437	2.536	2.590	1.294	1.187	1.210	1.061	1.250	1.118
		2.521 (n=12)			1.230 (n=12)			1.143 (n=12)		
	2%	1.413	1.731	1.558	0.794	0.889	0.825	0.673	0.858	0.801
		1.567 (n=12)			0.836 (n=12)			0.777 (n=12)		
	3%	1.099	1.253	1.108	0.617	0.761	0.671	0.542	0.757	0.565
		1.153 (n=12)			0.683 (n=12)			0.621 (n=12)		

Table 3.6. Capacity Factor Cell Means for (-)-Δ^8-THC.
Capacity factor, k', summarized by cells (n=4) and over all three runs (n=12).

		IPA in Mobile Phase								
		1%			4%			7%		
Experimental Set		#1	#2	#3	#1	#2	#3	#1	#2	#3
Methanol in Mobile Phase	1%	2.437	2.536	2.734	1.667	1.765	1.702	1.419	1.664	1.507
		2.569 (n=12)			1.711 (n=12)			1.530 (n=12)		
	2%	1.226	1.520	1.387	0.967	1.118	1.024	0.854	1.074	1.005
		1.378 (n=12)			1.036 (n=12)			0.978 (n=12)		
	3%	0.905	1.034	0.921	0.700	0.874	0.771	0.682	0.913	0.710
		0.954 (n=12)			0.782 (n=12)			0.768 (n=12)		

Table 3.7. Capacity Factor Cell Means for (+)-Δ^9-THC.
Capacity factor, k', summarized by cells (n=4) and over all three runs (n=12).

		IPA in Mobile Phase								
		1%			4%			7%		
Experimental Set		#1	#2	#3	#1	#2	#3	#1	#2	#3
Methanol in Mobile Phase	1%	2.437	2.536	2.590	1.479	1.285	1.338	1.340	1.578	1.427
		2.521 (n=12)			1.367 (n=12)			1.449 (n=12)		
	2%	1.413	1.731	1.558	0.860	0.947	0.879	0.779	0.999	0.947
		1.567 (n=12)			0.896 (n=12)			0.908 (n=12)		
	3%	1.099	1.253	1.108	0.653	0.808	0.715	0.593	0.855	0.622
		1.153 (n=12)			0.726 (n=12)			0.690 (n=12)		

Table 3.8. Capacity Factor Cell Means for (-)-Δ^9-THC.
Capacity factor, k', summarized by cells (n=4) and over all three runs (n=12).

		IPA in Mobile Phase								
		1%			4%			7%		
Experimental Set		#1	#2	#3	#1	#2	#3	#1	#2	#3
Methanol in Mobile Phase	1%	3.376	3.363	3.777	3.365	2.490	2.798	3.314	3.927	3.508
		3.505 (n=12)			2.885 (n=12)			3.583 (n=12)		
	2%	1.768	2.126	1.948	1.607	1.580	1.576	1.778	2.378	2.310
		1.947 (n=12)			1.588 (n=12)			2.155 (n=12)		
	3%	1.304	1.475	1.316	1.045	1.370	1.228	1.115	1.869	1.191
		1.365 (n=12)			1.214 (n=12)			1.392 (n=12)		

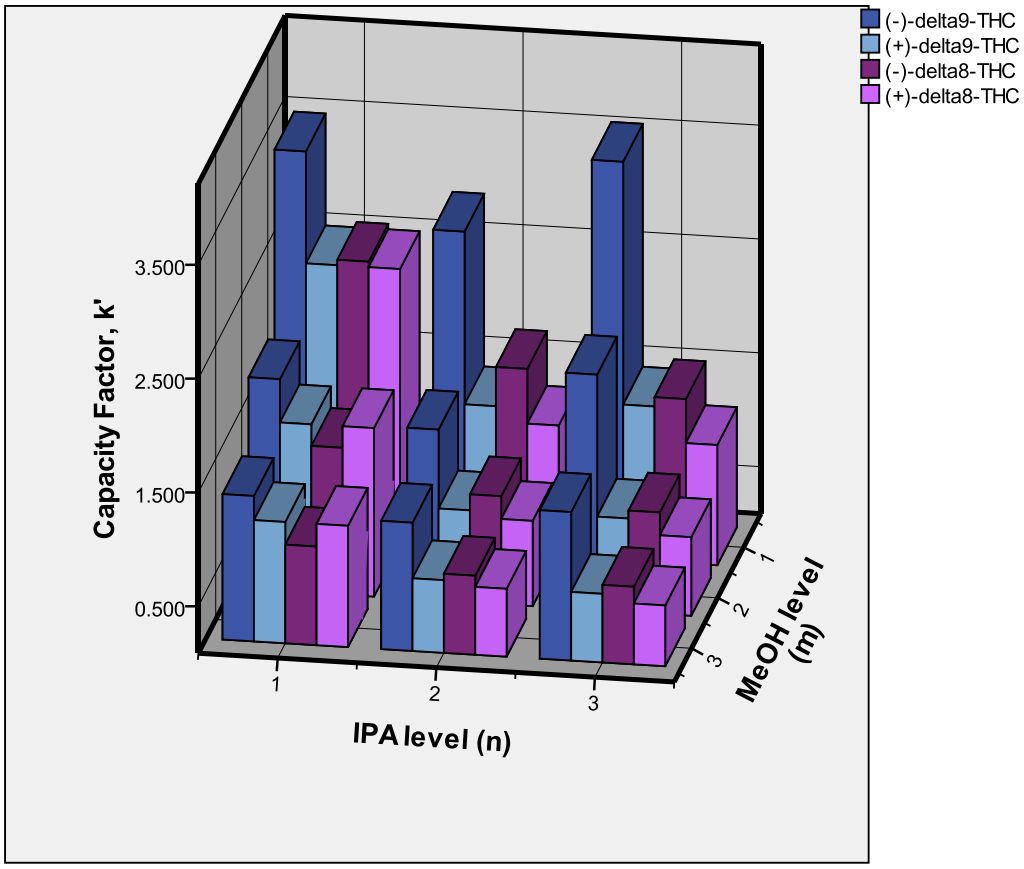


Figure 3.5. Capacity Factor Cell Means. Results are summarized for each mobile phase over all three experiments.

Theoretical Plates, N

Fewer trends were obvious from the cell means for theoretical plates than for retention time or k' cell means as illustrated in Figures 3.6-3.7. The theoretical plates for (+)-Δ^8-THC were fairly consistent (~14500) for 3% methanol, regardless of IPA content. Also for 1% IPA, Δ^8-THC enantiomers exhibited higher efficiencies at the methanol levels that resulted in elution order reversal: 2% or 3% methanol. The (+)-Δ^9-THC cell mean for 4% IPA and 3% methanol contains only eleven results due to one instance in which the Chemstation software could not calculate a peak width for this peak.

(-)-Δ^9-THC results for 7% IPA and 2% methanol in experiment 1 had an unusually large standard deviation due to two of the four results (standard deviation = 4695; relative standard deviation=37.7%). The first and last injection for that mobile phase exhibited shorter, wider peaks than any other injections for that mobile phase in any of the experiments. Also, the retention time for (-)-Δ^9-THC in one of these two injections was shorter than the other injections for that experiment. Unlike the (-)-Δ^9-THC peak, the other analyte peaks in these four injections presented uniform chromatography.

Table 3.9. Theoretical Plates Cell Means for (+)-Δ^8-THC.
Theoretical plates summarized by cells (n=4) and over all three runs (n=12).

		IPA in Mobile Phase								
		1%			4%			7%		
Experimental Set		#1	#2	#3	#1	#2	#3	#1	#2	#3
Methanol in Mobile Phase	1%	10229	7006	9607	12759	8714	9847	17038	17258	17535
		8947 (n=12)			10440 (n=12)			17277 (n=12)		
	2%	15910	13613	15007	11736	9540	9788	15752	16625	17690
		14843 (n=12)			10355 (n=12)			16689 (n=12)		
	3%	14946	13519	14947	18304	12144	12868	13019	16772	14246
		14471 (n=12)			14439 (n=12)			14679 (n=12)		

Table 3.10. Theoretical Plates Cell Means for (-)-Δ^8-THC.
Theoretical plates summarized by cells (n=4) and over all three runs (n=12).

		IPA in Mobile Phase								
		1%			4%			7%		
Experimental Set		#1	#2	#3	#1	#2	#3	#1	#2	#3
Methanol in Mobile Phase	1%	10229	7006	14281	17425	14688	16204	15673	16012	15966
		10505 (n=12)			16106 (n=12)			15884 (n=12)		
	2%	17967	18020	17779	17632	16303	16706	18141	18408	17194
		17922 (n=12)			16880 (n=12)			17914 (n=12)		
	3%	18303	18632	18750	12801	16509	15785	17002	17747	17558
		18562 (n=12)			15032 (n=12)			17436 (n=12)		

Table 3.11. Theoretical Plates Cell Means for (+)-Δ^9-THC.
Theoretical plates summarized by cells (n=4) and over all three runs (n=12).

		IPA in Mobile Phase								
		1%			4%			7%		
Experimental Set		#1	#2	#3	#1	#2	#3	#1	#2	#3
Methanol in Mobile Phase	1%	10229	7006	9607	14977	10170	12278	16508	16583	16701
		8947 (n=12)			12475 (n=12)			16597 (n=12)		
	2%	15910	13613	15007	13237	10241	9746	16871	17686	17730
		14843 (n=12)			11075 (n=12)			17429 (n=12)		
	3%	14946	13519	14947	9018	11122	11151	13011	17393	14515
		14471 (n=12)			10559 (n=11)			14973 (n=12)		

Table 3.12. Theoretical Plates Cell Means for (-)-Δ^9-THC.
Theoretical plates summarized by cells (n=4) and over all three runs (n=12).

		IPA in Mobile Phase								
		1%			4%			7%		
Experimental Set		#1	#2	#3	#1	#2	#3	#1	#2	#3
Methanol in Mobile Phase	1%	16447	16382	16006	16462	15132	16202	14764	14813	14659
		16278 (n=12)			15932 (n=12)			14745 (n=12)		
	2%	17447	16864	17361	15694	16163	15865	12446	17087	16980
		17224 (n=12)			15907 (n=12)			15504 (n=12)		
	3%	17381	17697	17773	17020	16841	16629	16222	17851	17084
		17617 (n=12)			16830 (n=12)			17053 (n=12)		

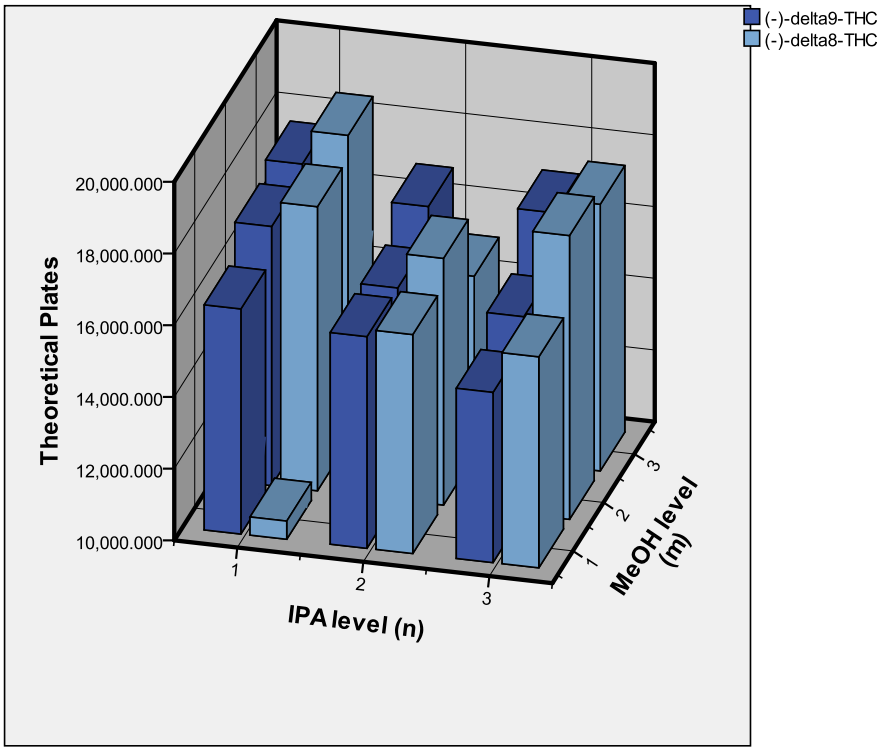


Figure 3.6. (-)-Enantiomer Theoretical Plates Cell Means. Results are summarized for each mobile phase over all three experiments.

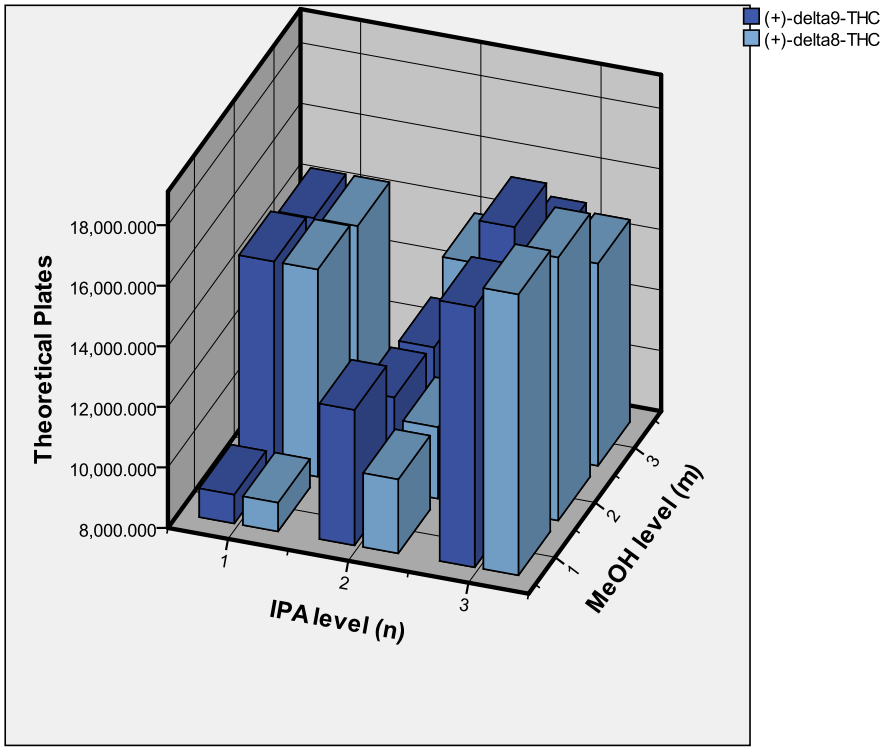


Figure 3.7. (+)-Enantiomer Theoretical Plates Cell Means. Results are summarized for each mobile phase over all three experiments.

Selectivity, α

Column selectivity was greatest for Δ^9-THC at all levels except for 1% IPA and 3% methanol. As indicated in Figure 3.8, Δ^8-THC underwent an elution order reversal under these conditions. At 2% and 3% methanol with 1% IPA, selectivity was greatest for the negative enantiomers. Outside of the Δ^8-THC elution order reversal, most pairs followed a trend of increasing selectivity with increasing IPA and decreasing methanol in the mobile phase. The critical pair, (+)-Δ^9-THC and (-)-Δ^8-THC, appeared to follow both increasing and decreasing trends within each level of IPA with the highest selectivity achieved at 4% IPA and 1% methanol (1.258) followed closely by 1% IPA and 3% methanol (1.209).

Table 3.13. Selectivity Cell Means for (+)-Δ^9-THC and (-)-Δ^8-THC.
Selectivity for the critical pair, (+)-Δ^9-THC and (-)-Δ^8-THC peaks, summarized by cells (n=4) and over all three runs (n=12).

		IPA in Mobile Phase								
		1%			4%			7%		
Experimental Set		#1	#2	#3	#1	#2	#3	#1	#2	#3
Methanol in Mobile Phase	1%	1.000	1.000	1.056	1.127	1.373	1.272	1.059	1.054	1.056
		1.019 (n=12)			1.258 (n=12)			1.056 (n=12)		
	2%	1.152	1.138	1.124	1.124	1.180	1.165	1.097	1.075	1.061
		1.138 (n=12)			1.156 (n=12)			1.078 (n=12)		
	3%	1.214	1.211	1.202	1.071	1.082	1.078	1.152	1.067	1.142
		1.209 (n=12)			1.077 (n=12)			1.120 (n=12)		

Table 3.14. Selectivity Cell Means for (+)-Δ^8-THC and (+)-Δ^9-THC.
Selectivity for (+)-enantiomer peaks summarized by cells (n=4) and over all three runs (n=12).

		IPA in Mobile Phase								
		1%			4%			7%		
Experimental Set		#1	#2	#3	#1	#2	#3	#1	#2	#3
Methanol in Mobile Phase	1%	1.000	1.000	1.000	1.143	1.083	1.105	1.263	1.262	1.276
		1.000 (n=12)			1.110 (n=12)			1.267 (n=12)		
	2%	1.000	1.000	1.000	1.083	1.065	1.066	1.156	1.164	1.183
		1.000 (n=12)			1.071 (n=12)			1.168 (n=12)		
	3%	1.000	1.000	1.000	1.059	1.062	1.065	1.094	1.130	1.099
		1.000 (n=12)			1.062 (n=12)			1.108 (n=12)		

Table 3.15. Selectivity Cell Means for (-)-Δ^8-THC and (-)-Δ^9-THC.
Selectivity for (-)-enantiomer peaks summarized by cells (n=4) and over all three runs (n=12).

		IPA in Mobile Phase								
		1%			4%			7%		
Experimental Set		#1	#2	#3	#1	#2	#3	#1	#2	#3
Methanol in Mobile Phase	1%	1.385	1.326	1.381	2.019	1.411	1.644	2.335	2.360	2.328
		1.364 (n=12)			1.691 (n=12)			2.341 (n=12)		
	2%	1.442	1.398	1.405	1.663	1.414	1.539	2.081	2.213	2.299
		1.415 (n=12)			1.538 (n=12)			2.198 (n=12)		
	3%	1.440	1.426	1.429	1.493	1.567	1.593	1.634	2.047	1.678
		1.432 (n=12)			1.551 (n=12)			1.786 (n=12)		

Table 3.16. Selectivity Cell Means for (+)-Δ^8-THC and (-)-Δ^8-THC.
Selectivity for Δ^8-THC peaks summarized by cells (n=4) and over all three runs (n=12).

		IPA in Mobile Phase								
		1%			4%			7%		
Experimental Set		#1	#2	#3	#1	#2	#3	#1	#2	#3
Methanol in Mobile Phase	1%	1.000	1.000	1.056	1.289	1.487	1.406	1.338	1.331	1.348
		1.019 (n=12)			1.394 (n=12)			1.339 (n=12)		
	2%	1.152	1.138	1.124	1.217	1.257	1.242	1.269	1.251	1.255
		1.138 (n=12)			1.239 (n=12)			1.258 (n=12)		
	3%	1.214	1.211	1.202	1.134	1.148	1.149	1.260	1.206	1.255
		1.209 (n=12)			1.144 (n=12)			1.240 (n=12)		

Table 3.17. Selectivity Cell Means for (+)-Δ^9-THC and (-)-Δ^9-THC.
Selectivity for Δ^9-THC peaks summarized by cells (n=4) and over all three runs (n=12).

		IPA in Mobile Phase								
		1%			4%			7%		
Experimental Set		#1	#2	#3	#1	#2	#3	#1	#2	#3
Methanol in Mobile Phase	1%	1.385	1.326	1.458	2.276	1.938	2.092	2.472	2.488	2.458
		1.390 (n=12)			2.102 (n=12)			2.473 (n=12)		
	2%	1.252	1.228	1.250	1.869	1.669	1.792	2.283	2.380	2.440
		1.243 (n=12)			1.777 (n=12)			2.368 (n=12)		
	3%	1.187	1.177	1.188	1.599	1.694	1.718	1.882	2.185	1.917
		1.184 (n=12)			1.670 (n=12)			1.994 (n=12)		

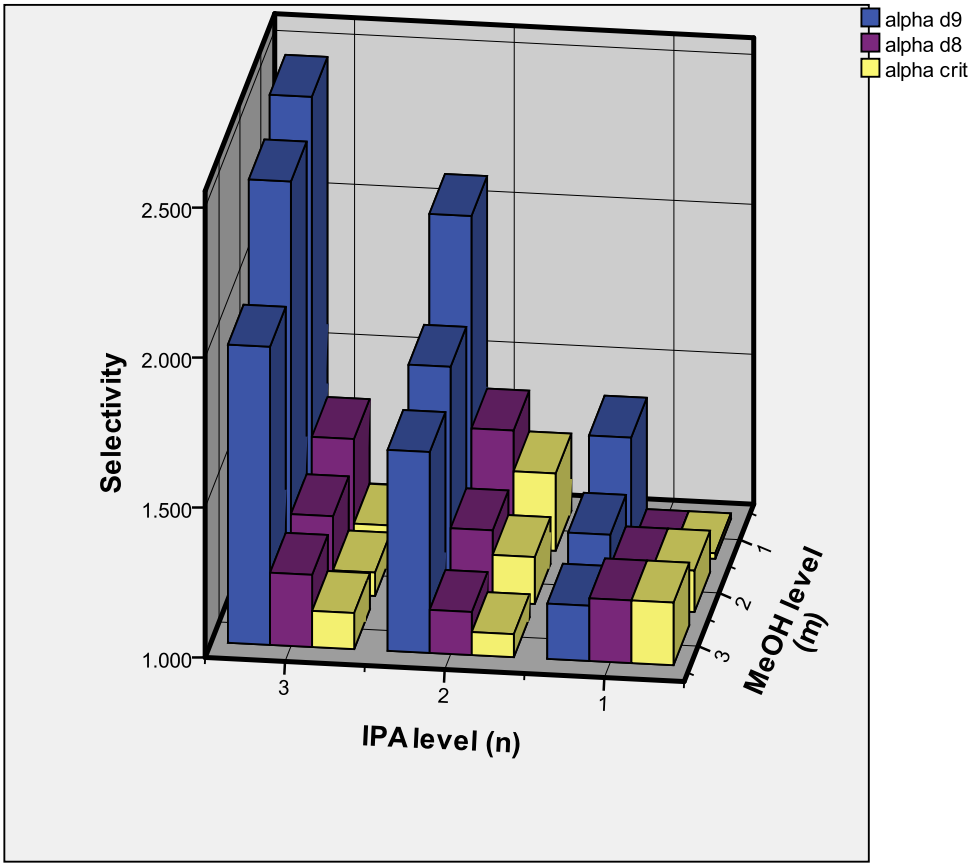


Figure 3.8. Selectivity Cell Means. Selected results are summarized for each mobile phase over all three experiments.

Resolution, R

Peak resolution of the five pairs followed similar patterns to column selectivity. Figure 3.7 contains a bar chart summary of resolutions for the critical pair, Δ^9-THC, and Δ^8-THC. Analogous to column selectivity, the maximum resolution achieved for the critical pair was with 4% IPA and 1% methanol (3.97) followed closely by the elution order reversal at 1% IPA and 3% methanol (3.09). As noted for theoretical plates, the (+)-Δ^9-THC cell mean for 4% IPA and 3% methanol contains only eleven results due to one instance in which the Chemstation software could not calculate a peak width for this peak.

Table 3.18. Resolution Cell Means for (+)-Δ^9-THC and (-)-Δ^8-THC.
Resolution for (+)-Δ^9-THC and (-)-Δ^8-THC peaks summarized by cells (n=4) and over all three runs (n=12).

		IPA in Mobile Phase								
		1%			4%			7%		
Experimental Set		#1	#2	#3	#1	#2	#3	#1	#2	#3
Methanol in Mobile Phase	1%	0.00	0.00	1.06	2.32	5.27	4.30	1.05	1.04	1.03
		0.35 (n=12)			3.97 (n=12)			1.04 (n=12)		
	2%	2.61	2.49	2.21	1.72	2.38	2.09	1.38	1.24	0.97
		2.44 (n=12)			2.06 (n=12)			1.20 (n=12)		
	3%	3.09	3.18	2.98	0.74	1.04	0.92	1.67	1.01	1.67
		3.09 (n=12)			0.91 (n=11)			1.45 (n=12)		

Table 3.19. Resolution Cell Means for (+)-Δ^8-THC and (+)-Δ^9-THC.
Resolution for (+)-enantiomer peaks summarized by cells (n=4) and over all three runs (n=12).

		IPA in Mobile Phase								
		1%			4%			7%		
Experimental Set		#1	#2	#3	#1	#2	#3	#1	#2	#3
Methanol in Mobile Phase	1%	0.00	0.00	0.00	2.28	1.07	1.47	4.10	4.41	4.44
		0.00 (n=12)			1.60 (n=12)			4.32 (n=12)		
	2%	0.00	0.00	0.00	1.01	0.75	0.73	1.95	2.38	2.59
		0.00 (n=12)			0.83 (n=12)			2.31 (n=12)		
	3%	0.00	0.00	0.00	0.62	0.71	0.71	0.93	1.78	1.05
		0.00 (n=12)			0.68 (n=11)			1.25 (n=12)		

Table 3.20. Resolution Cell Means for (-)-Δ^8-THC and (-)-Δ^9-THC.
Resolution for (-)-enantiomer peaks summarized by cells (n=4) and over all three runs (n=12).

		IPA in Mobile Phase								
		1%			4%			7%		
Experimental Set		#1	#2	#3	#1	#2	#3	#1	#2	#3
Methanol in Mobile Phase	1%	6.89	5.42	7.55	15.58	7.09	10.75	17.27	18.38	17.52
		6.62 (n=12)			11.14 (n=12)			17.72 (n=12)		
	2%	7.21	7.06	6.97	8.99	6.27	7.64	11.77	15.84	16.04
		7.08 (n=12)			7.63 (n=12)			14.55 (n=12)		
	3%	6.31	6.57	6.28	5.61	7.54	7.28	7.33	13.34	8.11
		6.39 (n=12)			6.81 (n=12)			9.59 (n=12)		

Table 3.21. Resolution Cell Means for (+)-Δ^8-THC and (-)-Δ^8-THC.
Resolution for Δ^8-THC peaks summarized by cells (n=4) and over all three runs (n=12).

		IPA in Mobile Phase								
		1%			4%			7%		
Experimental Set		#1	#2	#3	#1	#2	#3	#1	#2	#3
Methanol in Mobile Phase	1%	0.00	0.00	1.06	4.61	6.25	5.65	5.10	5.41	5.42
		0.35 (n=12)			5.50 (n=12)			5.31 (n=12)		
	2%	2.61	2.49	2.21	2.75	3.18	2.93	3.34	3.63	3.54
		2.44 (n=12)			2.95 (n=12)			3.50 (n=12)		
	3%	3.09	3.18	2.98	1.53	1.85	1.73	2.66	2.79	2.77
		3.09 (n=12)			1.70 (n=12)			2.74 (n=12)		

Table 3.22. Resolution Cell Means for (+)-Δ^9-THC and (-)-Δ^9-THC.
Resolution for Δ^9-THC peaks summarized by cells (n=4) and over all three runs (n=12).

		IPA in Mobile Phase								
		1%			4%			7%		
Experimental Set		#1	#2	#3	#1	#2	#3	#1	#2	#3
Methanol in Mobile Phase	1%	6.89	5.42	7.97	17.29	11.82	14.36	18.36	19.40	18.56
		6.76 (n=12)			14.49 (n=12)			18.78 (n=12)		
	2%	4.43	4.16	4.51	10.10	8.01	8.82	14.41	16.86	17.03
		4.37 (n=12)			8.98 (n=12)			15.55 (n=12)		
	3%	2.96	2.93	3.01	5.92	7.92	7.65	8.54	14.25	9.42
		2.96 (n=12)			7.28 (n=11)			10.74 (n=12)		

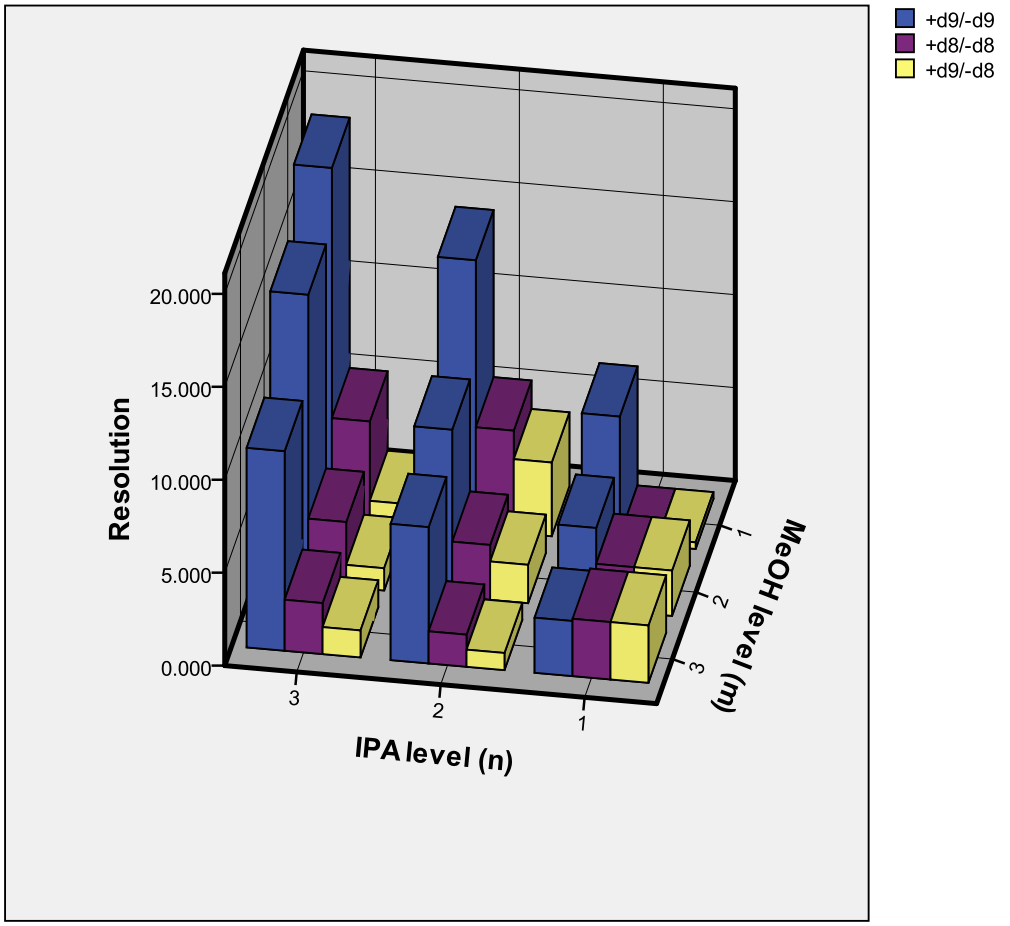


Figure 3.9. Resolution Cell Means. Selected results are summarized for each mobile phase over all three experiments.

Inferential Statistics

As noted, a qualitative investigation of the results indicated some variations between experimental sets, such as the chromatograms presented in Figures 3.1a, 3.2a, and 3.3a. However obvious these differences appeared on the surface, statistical analysis provided a deeper examination of the data to determine the probability of true divergence. Predictive Analytics Software (PASW, formerly SPSS) version 18.0 (Chicago, Illinois) was used to perform in-depth statistical analyses of the collected data. The results of these analyses were summarized for selected dependent variables.

Reduced Model

Three iterations of the 3 x 3 factorial study of the two alcohols were utilized to provide some idea of the repeatability and therefore suitability of this design for characterizing the chiral separation. In an ideal situation, all possible mobile phase orders would be analyzed to account for uncontrolled variations. The amount of resources required would be too large to make such a design feasible and lamp or column decay would likely add significantly to variation over such a lengthy period of use. Slight differences between experimental set data due to uncontrollable variations were anticipated, but the magnitude of such effects could not be inferred since no data were available from which to derive these hypotheses. The degradation of system components was expected to be negligible, or if detected, constant.

When selecting an appropriate model for statistical analysis of the data, a full factorial model was considered. The full factorial model for the three factors experimental set, methanol, and IPA is the following:

$$\text{exp} + \text{MeOH} + \text{IPA} + \text{MeOH*IPA} + \text{MeOH*exp} + \text{IPA*exp} + \text{exp*MeOH*IPA} \quad \text{(Design 3.1)}$$

where exp represents the experiment setup effect in the model, MeOH is the methanol effect, MeOH*IPA represents the two-way interaction effect of methanol and IPA, MeOH*exp is the two-way interaction of methanol and experimental set, IPA*exp is the two-way interaction of IPA and experiment setup, and exp*MeOH*IPA is the three-way interaction effect of experiment, methanol, and IPA.

Inclusion of 2-way and 3-way interaction effects in the model serves only to unnecessarily complicate the interpretation of the effects of the alcohols on the separation. The differences between experimental sets does aid in interpreting the usefulness of the experimental approach and to a lesser degree, the repeatability of the analyses. The risk in ignoring these higher level interactions is that the chosen model may not adequately describe the main or interaction effects of methanol and IPA on the dependent variables. Though interaction effects between alcohols and experimental sets could appear to be significant, the inclusion of the interactions involving experiment set offers little useful information with regard to characterizing the separation of the analytes on the CSP in this study. Because the experiment was performed multiple times with consistent results, the risk of incorrectly ignoring experimental set interaction effects is considered low, and therefore acceptable.

To test the assumption that the effect of experimental set could be adequately described using a reduced statistical model in the analysis of the data, ANOVA of retention time for each experiment individually was compared to ANOVA of retention

time over all experiments combined. Individual analyses by experiment were performed using the following reduced model which does not include an experiment term:

$$\text{MeOH} + \text{IPA} + \text{MeOH*IPA} \qquad \text{(Design 3.2)}$$

All components of the model were significant at an alpha level (designated as α_s) of 0.01 ($p<0.001$) for the retention time of all four analytes for each experiment, as analyzed individually. The adjusted R^2 value for the model was 1.000 for all four analytes in all experiments. See Appendix C for source tables.

The same model (Design 3.2) was applied to the data from all experiments combined, and again all components of the model were significant at $\alpha=0.01$ ($p\leq0.001$) for the retention time of all four analytes (see Appendix C for source table). The adjusted R^2 value for the model was lowest for (-)-Δ^9-THC at 0.995 and highest for (+)-Δ^8-THC at 0.999. While these values indicate that the model describes most of the variation in the samples, another model was tested that included an experimental set term. ANOVA for retention time over all experiments was performed using the following model:

$$\text{exp} + \text{MeOH} + \text{IPA} + \text{MeOH*IPA} \qquad \text{(Design 3.3)}$$

Again, all components of the model were significant at $\alpha_s=0.01$ ($p\leq0.001$) for the retention time of all four analytes (refer to Table 3.26). For this model, the adjusted R^2 value was again lowest for (-)-Δ^9-THC at 0.995 and highest for the remaining analytes at 0.999.

A lack of fit test was performed for the ANOVA of each dependent variable to further verify the inclusion of necessary terms in the reduced model.

Retention Time

MANOVA for the retention times of the four analytes was performed using α_s=0.01. Levene's test for homogeneity of variance indicated that the null hypothesis of equal group variances was rejected for all analytes ($p<0.001$) as seen in Table 3.23. Because cell sizes were equal in all cases, this was not considered to represent a threat to theoretical conclusions. The Pillai's trace multivariate test of overall differences among groups was statistically significant for each factor ($p<0.001$) as summarized in Table 3.24. Although significant, partial eta-square values for experiment (0.493) and IPA-methanol interaction (0.514) indicate that the effect sizes of these relationships with retention time were weaker than those for methanol (η^2=0.674) and IPA (η^2=0.903). A lack of fit test was performed for the fitted model and results (see Table 3.25) were significant for all four analytes ($p<0.001$).

Table 3.23. Levene's Test for Retention Time.
Retention time error variances were significantly different across groups at α_s=0.01.

	F	df1	df2	Sig.
(-)-Δ^9-THC	328.285	26	81	.000
(-)-Δ^8-THC	39.857	26	81	.000
(+)-Δ^9-THC	67.182	26	81	.000
(+)-Δ^8-THC	44.412	26	81	.000

Table 3.24. Multivariate Test of Overall Differences for Retention Time.
Overall retention time differences were significant for each factor at α_s=0.01.

Effect		Value	F	Hypothesis df	Error df	Sig.	Partial Eta Sq.	Observed Power
exp	Pillai's Trace	.986	23.082	8.000	190.000	.000	.493	1.000
	Wilks' Lambda	.134	40.668	8.000	188.000	.000	.634	1.000
	Hotelling's Trace	5.562	64.660	8.000	186.000	.000	.736	1.000
	Roy's Largest Root	5.397	128.169	4.000	95.000	.000	.844	1.000
IPA	Pillai's Trace	1.806	221.089	8.000	190.000	.000	.903	1.000
	Wilks' Lambda	.001	920.575	8.000	188.000	.000	.975	1.000
	Hotelling's Trace	311.106	3616.603	8.000	186.000	.000	.994	1.000
	Roy's Largest Root	306.863	7288.004	4.000	95.000	.000	.997	1.000
Methanol	Pillai's Trace	1.349	49.210	8.000	190.000	.000	.674	1.000
	Wilks' Lambda	.003	381.452	8.000	188.000	.000	.942	1.000
	Hotelling's Trace	191.322	2224.120	8.000	186.000	.000	.990	1.000
	Roy's Largest Root	190.774	4530.876	4.000	95.000	.000	.995	1.000
IPA x Methanol	Pillai's Trace	2.056	25.644	16.000	388.000	.000	.514	1.000
	Wilks' Lambda	.002	122.269	16.000	287.812	.000	.792	1.000
	Hotelling's Trace	61.769	357.103	16.000	370.000	.000	.939	1.000
	Roy's Largest Root	54.898	1331.288	4.000	97.000	.000	.982	1.000

Table 3.25. Lack of Fit Test for Retention Time.
The F-values were significant for the fitted model for each analyte at α_s=0.01.

Analyte	Source	Sum of Squares	df	Mean Square	F	Sig.	Observed Power
(-)-Δ^9-THC	Lack of Fit	80.902	16	5.056	1118.816	.000	1.000
	Pure Error	.366	81	.005			
(-)-Δ^8-THC	Lack of Fit	4.660	16	.291	136.328	.000	1.000
	Pure Error	.173	81	.002			
(+)-Δ^9-THC	Lack of Fit	7.444	16	.465	220.715	.000	1.000
	Pure Error	.171	81	.002			
(+)-Δ^8-THC	Lack of Fit	4.731	16	.296	144.363	.000	1.000
	Pure Error	.166	81	.002			

Tests of between-subjects effects showed that retention time was significantly related to all of the factors ($p \leq 0.001$), where the weakest associations occurred for (-)-Δ^9-THC, (-)-Δ^8-THC, (+)-Δ^9-THC, and (+)-Δ^8-THC with experimental setup (η^2=0.141, 0.677, 0.472, 0.564, respectively), and the strongest associations with methanol (η^2=0.943, 0.989, 0.974, 0.979, respectively). Refer to the source table below (Table

3.26). By this model, the retention time of (-)-Δ^9-THC, (-)-Δ^8-THC, (+)-Δ^9-THC, and (+)-Δ^8-THC was more closely related to IPA (η^2=0.472, 0.958, 0.968, 0.985, respectively) than the interaction of IPA and methanol (η^2=0.200, 0.901, 0.804, 0.908, respectively). Adjusted R^2 values were 0.995 for (-)-Δ^9-THC and 0.999 for (-)-Δ^8-THC, (+)-Δ^9-THC, and (+)-Δ^8-THC.

Table 3.26. Tests of Between-Subjects Effects for Retention Time.
Between-subjects retention time differences were significant for each factor at α_s=0.01.

Source	Analyte	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Sq.	Observed Power
Model	(-)-Δ^9-THC	20021.060	11	1820.096	2172.433	.000	.996	1.000
	(-)-Δ^8-THC	10294.576	11	935.871	18781.957	.000	1.000	1.000
	(+)-Δ^9-THC	9875.658	11	897.787	11436.742	.000	.999	1.000
	(+)-Δ^8-THC	9243.058	11	840.278	16645.627	.000	.999	1.000
exp	(-)-Δ^9-THC	13.362	2	6.681	7.974	.001	.141	.844
	(-)-Δ^8-THC	10.145	2	5.073	101.803	.000	.677	1.000
	(+)-Δ^9-THC	6.796	2	3.398	43.285	.000	.472	1.000
	(+)-Δ^8-THC	6.343	2	3.171	62.824	.000	.564	1.000
IPA	(-)-Δ^9-THC	72.577	2	36.288	43.313	.000	.472	1.000
	(-)-Δ^8-THC	111.476	2	55.738	1118.605	.000	.958	1.000
	(+)-Δ^9-THC	233.828	2	116.914	1489.343	.000	.968	1.000
	(+)-Δ^8-THC	318.754	2	159.377	3157.205	.000	.985	1.000
Methanol	(-)-Δ^9-THC	1343.342	2	671.671	801.694	.000	.943	1.000
	(-)-Δ^8-THC	416.705	2	208.352	4181.418	.000	.989	1.000
	(+)-Δ^9-THC	290.365	2	145.182	1849.452	.000	.974	1.000
	(+)-Δ^8-THC	225.434	2	112.717	2232.888	.000	.979	1.000
IPA x Methanol	(-)-Δ^9-THC	20.362	4	5.090	6.076	.000	.200	.931
	(-)-Δ^8-THC	43.970	4	10.992	220.607	.000	.901	1.000
	(+)-Δ^9-THC	31.313	4	7.828	99.724	.000	.804	1.000
	(+)-Δ^8-THC	48.384	4	12.096	239.615	.000	.908	1.000
Error	(-)-Δ^9-THC	81.268	97	.838				
	(-)-Δ^8-THC	4.833	97	.050				
	(+)-Δ^9-THC	7.615	97	.079				
	(+)-Δ^8-THC	4.897	97	.050				
Total	(-)-Δ^9-THC	20102.328	108					
	(-)-Δ^8-THC	10299.409	108					
	(+)-Δ^9-THC	9883.273	108					
	(+)-Δ^8-THC	9247.955	108					

Subsequent post-hoc comparisons between groups were performed using F statistics and both Tukey's honestly significant difference (HSD) and Scheffé simultaneous confidence intervals based on Student's t distribution (see Appendix D). Tukey's HSD was used as a conservative means for pairwise comparisons, and Scheffé's was used as a conservative range test; both tests are subject to the equality of variances assumption. Results indicated that differences between levels of IPA, methanol, and experiment were significant for analyte retention times. Homogeneous subsets per Tukey's HSD and Scheffé's tests are presented in Tables 3.27-3.29 for the different levels of each factor.

For (-)-Δ^9-THC, 4% IPA was significantly different from both 1 and 7% IPA ($p<0.001$). For (-)-Δ^9-THC, experiments 1 and 2 were significantly different ($p\leq0.001$), but experimental set 3 indicated no significant difference from the others (Tukey's test: $p=0.100, 0.139$, respectively). 1% IPA for (+)-Δ^9-THC was significantly different from both 4 and 7% IPA ($p<0.001$), which were not indicated to be significantly different. In contrast, all IPA levels were significantly different for both Δ^8-THC enantiomers ($p<0.001$). All three levels of methanol were significantly different for all four analytes ($p<0.001$), and all three experiments were significantly different for (-)-Δ^8-THC and (+)-Δ^8-THC ($p<0.001$), as well as (+)-Δ^9-THC (Scheffé test: $p\leq0.002$).

Table 3.27. Homogeneous Subsets of Retention Time by IPA Level.
Means for groups in homogeneous subsets were based on observed values. All levels for Δ^8-THC were significantly different at α_s=0.01.

Analyte/Test		IPA (%)	N	Subset		
				1	2	3
(-)-Δ^9-THC	Tukey HSD	4	36	11.96594		
		1	36		13.54283	
		7	36		13.83100	
		Sig.		1.000	.379	
	Scheffe	4	36	11.96594		
		1	36		13.54283	
		7	36		13.83100	
		Sig.		1.000	.413	
(-)-Δ^8-THC	Tukey HSD	7	36	8.56000		
		4	36		8.99106	
		1	36			10.89814
		Sig.		1.000	1.000	1.000
	Scheffe	7	36	8.56000		
		4	36		8.99106	
		1	36			10.89814
		Sig.		1.000	1.000	1.000
(+)-Δ^9-THC	Tukey HSD	4	36	8.24442		
		7	36	8.24722		
		1	36		11.36717	
		Sig.		.999	1.000	
	Scheffe	4	36	8.24442		
		7	36	8.24722		
		1	36		11.36717	
		Sig.		.999	1.000	
(+)-Δ^8-THC	Tukey HSD	7	36	7.55683		
		4	36		7.91525	
		1	36			11.36717
		Sig.		1.000	1.000	1.000
	Scheffe	7	36	7.55683		
		4	36		7.91525	
		1	36			11.36717
		Sig.		1.000	1.000	1.000

Table 3.28. Homogeneous Subsets of Retention Time by Methanol Level.
Means for groups in homogeneous subsets were based on observed values. All levels were significantly different for each analyte at α_s=0.01.

Analyte/Test		Methanol (%)	N	Subset		
				1	2	3
(-)-Δ^9-THC	Tukey HSD	3	36	9.49119		
		2	36		11.95478	
		1	36			17.89381
		Sig.		1.000	1.000	1.000
	Scheffe	3	36	9.49119		
		2	36		11.95478	
		1	36			17.89381
		Sig.		1.000	1.000	1.000
(-)-Δ^8-THC	Tukey HSD	3	36	7.49811		
		2	36		8.79253	
		1	36			12.15856
		Sig.		1.000	1.000	1.000
	Scheffe	3	36	7.49811		
		2	36		8.79253	
		1	36			12.15856
		Sig.		1.000	1.000	1.000
(+)-Δ^9-THC	Tukey HSD	3	36	7.59081		
		2	36		8.76400	
		1	36			11.50400
		Sig.		1.000	1.000	1.000
	Scheffe	3	36	7.59081		
		2	36		8.76400	
		1	36			11.50400
		Sig.		1.000	1.000	1.000
(+)-Δ^8-THC	Tukey HSD	3	36	7.44106		
		2	36		8.50267	
		1	36			10.89553
		Sig.		1.000	1.000	1.000
	Scheffe	3	36	7.44106		
		2	36		8.50267	
		1	36			10.89553
		Sig.		1.000	1.000	1.000

Table 3.29. Homogeneous Subsets of Retention Time by Experiment.
Means for groups in homogeneous subsets were based on observed values. All levels were significantly different for Δ^8-THC and (+)-Δ^9-THC at α_s=0.01.

Analyte/Test		Experiment	N	Subset		
				1	2	3
(-)-Δ^9-THC	Tukey HSD	1	36	12.67692		
		3	36	13.12458	13.12458	
		2	36		13.53828	
		Sig.		.100	.139	
	Scheffe	1	36	12.67692		
		3	36	13.12458	13.12458	
		2	36		13.53828	
		Sig.		.122	.165	
(-)-RT Δ^8-THC	Tukey HSD	1	36	9.09519		
		3	36		9.50944	
		2	36			9.84456
		Sig.		1.000	1.000	1.000
	Scheffe	1	36	9.09519		
		3	36		9.50944	
		2	36			9.84456
		Sig.		1.000	1.000	1.000
(+)-Δ^9-THC	Tukey HSD	1	36	9.00192		
		3	36		9.24475	
		2	36			9.61214
		Sig.		1.000	1.000	1.000
	Scheffe	1	36	9.00192		
		3	36		9.24475	
		2	36			9.61214
		Sig.		1.000	1.000	1.000
(+)-Δ^8-THC	Tukey HSD	1	36	8.67089		
		3	36		8.90767	
		2	36			9.26069
		Sig.		1.000	1.000	1.000
	Scheffe	1	36	8.67089		
		3	36		8.90767	
		2	36			9.26069
		Sig.		1.000	1.000	1.000

Tests of between-subjects effects by ANOVA were evaluated at α_s=0.01 using polynomial contrasts. Homogeneity of variance was evaluated by the Bartlett-Box F test which indicated that the null hypothesis of equal variances was rejected for all of the analytes (p<.001). Because cell sizes were equal, this was not considered to be a threat to the validity of the data. Source table results (see Table 3.30) indicated that quadratic IPA by linear methanol terms comprised the highest order interaction effect that described (-)-Δ^9-THC retention time with significantly better fit than lower order terms (p<0.001). For (-)-Δ^8-THC, (+)-Δ^9-THC, and (+)-Δ^8-THC both a quadratic IPA by linear methanol term and a linear IPA by quadratic methanol term described a significantly improved fit (p<0.001). However, a quadratic IPA by quadratic methanol term did not (p=0.028, p=0.085, and p=0.024, respectively), as seen in Table 3.30 below.

Table 3.30. Trend Analysis for Retention Time.
Polynomial contrasts were used to evaluate trends in retention time at α_s=0.01, where "L" designates the linear term and "Q" designates the quadratic term.

Source	Analyte	Sum of Squares	df	Mean Square	F	Sig.	Eta Sq.	Observed Power
Error (Within + Residual)	(-)-Δ^9-THC	81.27	97	.84				
	(-)-Δ^8-THC	4.83	97	.05				
	(+)-Δ^9-THC	7.61	97	.08				
	(+)-Δ^8-THC	4.90	97	.05				
exp(L)	(-)-Δ^9-THC	3.61	1	3.61	4.31	.041	.043	.299
	(-)-Δ^8-THC	3.09	1	3.09	61.99	.000	.390	1.000
	(+)-Δ^9-THC	1.06	1	1.06	13.52	.000	.122	.850
	(+)-Δ^8-THC	1.01	1	1.01	19.99	.000	.171	.965
exp(Q)	(-)-Δ^9-THC	9.75	1	9.75	11.64	.001	.107	.781
	(-)-Δ^8-THC	7.06	1	7.06	141.63	.000	.594	1.000
	(+)-Δ^9-THC	5.73	1	5.73	73.05	.000	.430	1.000
	(+)-Δ^8-THC	5.33	1	5.33	105.66	.000	.521	1.000
IPA(L)	(-)-Δ^9-THC	1.49	1	1.49	1.78	.185	.018	.104
	(-)-Δ^8-THC	98.40	1	98.40	1974.85	.000	.953	1.000
	(+)-Δ^9-THC	175.21	1	175.21	2232.01	.000	.958	1.000
	(+)-Δ^8-THC	261.34	1	261.34	5176.97	.000	.982	1.000

Table 3.30. Continued.

Source	Analyte	Sum of Squares	df	Mean Square	F	Sig.	Eta Sq.	Observed Power
IPA(Q)	(-)-Δ^9-THC	71.08	1	71.08	84.84	.000	.467	1.000
	(-)-Δ^8-THC	13.07	1	13.07	262.32	.000	.730	1.000
	(+)-Δ^9-THC	58.61	1	58.61	746.68	.000	.885	1.000
	(+)-Δ^8-THC	57.42	1	57.42	1137.44	.000	.921	1.000
Methanol(L)	(-)-Δ^9-THC	1270.87	1	1270.87	1516.89	.000	.940	1.000
	(-)-Δ^8-THC	390.95	1	390.95	7845.92	.000	.988	1.000
	(+)-Δ^9-THC	275.64	1	275.64	3511.27	.000	.973	1.000
	(+)-Δ^8-THC	214.80	1	214.80	4255.13	.000	.978	1.000
Methanol(Q)	(-)-Δ^9-THC	72.47	1	72.47	86.50	.000	.471	1.000
	(-)-Δ^8-THC	25.75	1	25.75	516.77	.000	.842	1.000
	(+)-Δ^9-THC	14.73	1	14.73	187.63	.000	.659	1.000
	(+)-Δ^8-THC	10.63	1	10.63	210.64	.000	.685	1.000
IPA(L) x Methanol(L)	(-)-Δ^9-THC	0.37	1	0.37	0.44	.507	.005	.000
	(-)-Δ^8-THC	34.94	1	34.94	701.11	.000	.878	1.000
	(+)-Δ^9-THC	17.41	1	17.41	221.81	.000	.696	1.000
	(+)-Δ^8-THC	34.50	1	34.50	683.40	.000	.876	1.000
IPA(Q) x Methanol(L)	(-)-Δ^9-THC	16.67	1	16.67	19.89	.000	.170	.964
	(-)-Δ^8-THC	4.63	1	4.63	92.90	.000	.489	1.000
	(+)-Δ^9-THC	12.28	1	12.28	156.48	.000	.617	1.000
	(+)-Δ^8-THC	10.97	1	10.97	217.22	.000	.691	1.000
IPA(L) x Methanol(Q)	(-)-Δ^9-THC	2.82	1	2.82	3.37	.070	.034	.224
	(-)-Δ^8-THC	4.16	1	4.16	83.45	.000	.462	1.000
	(+)-Δ^9-THC	1.38	1	1.38	17.58	.000	.153	.938
	(+)-Δ^8-THC	2.65	1	2.65	52.55	.000	.351	1.000
IPA(Q) x Methanol(Q)	(-)-Δ^9-THC	0.50	1	0.50	0.60	.441	.006	.000
	(-)-Δ^8-THC	0.25	1	0.25	4.97	.028	.049	.353
	(+)-Δ^9-THC	0.24	1	0.24	3.02	.085	.030	.196
	(+)-Δ^8-THC	0.27	1	0.27	5.29	.024	.052	.378
Model	(-)-Δ^9-THC	1449.64	10	144.96	173.03	.000		
	(-)-Δ^8-THC	582.29	10	58.23	1168.59	.000		
	(+)-Δ^9-THC	562.30	10	56.23	716.31	.000		
	(+)-Δ^8-THC	598.91	10	59.89	1186.43	.000		
Total	(-)-Δ^9-THC	1530.91	107	14.31				
	(-)-Δ^8-THC	587.13	107	5.49				
	(+)-Δ^9-THC	569.92	107	5.33				
	(+)-Δ^8-THC	603.81	107	5.64				

Selectivity

Column selectivity for Δ^9-THC, Δ^8-THC, and the critical pair were analyzed by MANOVA at α=0.01. Levene's test for homogeneity of variance (see Table 3.31) indicated that the null hypothesis was rejected for these pairs ($p<0.001$), but this was not considered to be a threat to validity of the data because the cell sizes were equal. The Pillai's trace multivariate test of overall differences among groups was statistically significant for each factor ($p\leq0.001$), as illustrated by the source table, Table 3.32. Partial eta-square for experiment (0.107) indicated a weak though significant relationship with selectivity. IPA appeared to have the strongest relationship to selectivity (η^2=0.859), followed in strength by the IPA-methanol interaction (η^2=0.713) and then methanol (η^2=0.643). See Table 3.33 for a summary of results from the lack of fit test performed for the three pairs of interest where all results were significant ($p<0.001$).

Table 3.31. Levene's Test for Selectivity.
Column selectivity error variances were significantly different across groups at α_s=0.01.

	F	df1	df2	Sig.
Δ^9-THC	136.593	26	81	.000
Δ^8-THC	1964.502	26	81	.000
critical pair	1594.758	26	81	.000

The ANOVA results summarized in Table 3.34 suggested that column selectivity for all three pairs was significantly related to IPA and the IPA-methanol interaction ($p<0.001$). With regard to methanol effects, only selectivity for Δ^9-THC and Δ^8-THC were significantly different ($p<0.001$), whereas there was a failure to reject the null hypothesis for the critical pair ($p=0.036$) with a 61.5% risk of Type II error. Results

indicated a failure to reject the null hypothesis for experimental setup for Δ^9-THC ($p=0.427$), Δ^8-THC ($p=0.021$), and the critical pair ($p=0.058$) as seen in Table 3.34.

Table 3.32. Multivariate Test of Overall Differences for Selectivity.
Overall selectivity differences were significant for each factor at $\alpha_s=0.01$.

Effect		Value	F	Hypothesis df	Error df	Sig.	Partial Eta Sq.	Observed Power
Intercept	Pillai's Trace	1.000	265071.969	3.000	95.000	.000	1.000	1.000
	Wilks' Lambda	.000	265071.969	3.000	95.000	.000	1.000	1.000
	Hotelling's Trace	8370.694	265071.969	3.000	95.000	.000	1.000	1.000
	Roy's Largest Root	8370.694	265071.969	3.000	95.000	.000	1.000	1.000
exp	Pillai's Trace	.215	3.849	6.000	192.000	.000	.107	.880
	Wilks' Lambda	.791	3.937	6.000	190.000	.000	.111	.890
	Hotelling's Trace	.257	4.022	6.000	188.000	.000	.114	.898
	Roy's Largest Root	.224	7.167	3.000	96.000	.000	.183	.918
IPA	Pillai's Trace	1.717	194.162	6.000	192.000	.000	.859	1.000
	Wilks' Lambda	.001	1068.642	6.000	190.000	.000	.971	1.000
	Hotelling's Trace	339.652	5321.221	6.000	188.000	.000	.994	1.000
	Roy's Largest Root	337.081	10786.602	3.000	96.000	.000	.997	1.000
Methanol	Pillai's Trace	1.286	57.636	6.000	192.000	.000	.643	1.000
	Wilks' Lambda	.013	249.648	6.000	190.000	.000	.887	1.000
	Hotelling's Trace	54.348	851.450	6.000	188.000	.000	.965	1.000
	Roy's Largest Root	53.911	1725.140	3.000	96.000	.000	.982	1.000
IPA x Methanol	Pillai's Trace	2.138	60.186	12.000	291.000	.000	.713	1.000
	Wilks' Lambda	.001	250.070	12.000	251.638	.000	.895	1.000
	Hotelling's Trace	85.014	663.585	12.000	281.000	.000	.966	1.000
	Roy's Largest Root	77.595	1881.671	4.000	97.000	.000	.987	1.000

Table 3.33. Lack of Fit Test for Selectivity.
The F-values were significant for the fitted model for the three pairs at $\alpha_s=0.01$.

Analyte	Source	Sum of Squares	df	Mean Square	F	Sig.	Observed Power
Δ^9-THC	Lack of Fit	.638	16	.040	437.758	.000	1.000
	Pure Error	.007	81	.000			
Δ^8-THC	Lack of Fit	.094	16	.006	3703.870	.000	1.000
	Pure Error	.000	81	.000			
Critical Pair	Lack of Fit	.150	16	.009	4524.029	.000	1.000
	Pure Error	.000	81	.000			

The relationship of Δ^9-THC selectivity to IPA effects was strongest (η^2=0.966), followed by methanol (η^2=0.795), and then IPA-methanol (η^2=0.442). Δ^8-THC selectivity followed a different pattern, having the strongest relationship to the IPA-methanol interaction (η^2=0.868), then IPA (η^2=0.849), and then the weakest relationship with methanol (η^2=0.364). Selectivity of the critical pair had a weaker relationship to IPA (η^2=0.425) than to the IPA-methanol interaction effect (η^2=0.743). Adjusted R^2 values for the model were 0.998, 0.999, and 0.999, respectively for Δ^9-THC, Δ^8-THC, and the critical pair.

Table 3.34. Tests of Between-Subjects Effects for Selectivity.
Between-subjects selectivity differences were significant for IPA and methanol at α_s=0.01.

Source	Analyte Pair	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Sq.	Observed Power
Model	Δ^9-THC	371.334	11	33.758	5077.527	.000	.998	1.000
	Δ^8-THC	161.919	11	14.720	15232.212	.000	.999	1.000
	Crit. Pair	136.872	11	12.443	8022.004	.000	.999	1.000
exp	Δ^9-THC	.011	2	.006	.858	.427	.017	.067
	Δ^8-THC	.008	2	.004	4.018	.021	.076	.464
	Crit. Pair	.009	2	.005	2.936	.058	.057	.316
IPA	Δ^9-THC	18.346	2	9.173	1379.732	.000	.966	1.000
	Δ^8-THC	.527	2	.264	272.851	.000	.849	1.000
	Crit. Pair	.111	2	.056	35.913	.000	.425	1.000
Methanol	Δ^9-THC	2.495	2	1.247	187.604	.000	.795	1.000
	Δ^8-THC	.054	2	.027	27.769	.000	.364	1.000
	Crit. Pair	.011	2	.005	3.436	.036	.066	.385
IPA x Methanol	Δ^9-THC	.510	4	.128	19.189	.000	.442	1.000
	Δ^8-THC	.617	4	.154	159.624	.000	.868	1.000
	Crit. Pair	.435	4	.109	70.040	.000	.743	1.000
Error	Δ^9-THC	.645	97	.007				
	Δ^8-THC	.094	97	.001				
	Crit. Pair	.150	97	.002				
Total	Δ^9-THC	371.979	108					
	Δ^8-THC	162.013	108					
	Crit. Pair	137.022	108					

Post-hoc comparisons for between-groups differences indicated that for Δ^9-THC selectivity, all levels of IPA and methanol were significantly different ($p<0.001$). Refer to Appendix D for post-hoc analysis tables and Tables 3.35-3.36 for Scheffé and Tukey's homogeneous subsets based on observed means. For Δ^8-THC, selectivity at 1% IPA was significantly different from the other levels ($p<0.001$). According to Tukey's HSD, the difference between 4% and 7% IPA was not significant ($p=0.015$). Δ^8-THC selectivity in 1% methanol was also significantly different from the other levels ($p<0.001$), while the difference between 2% and 3% methanol, where entantiomeric elution order reversed, was not significant (Tukey's test: $p=0.141$). The critical pair exhibited significantly different selectivity at each level of IPA ($p\leq0.001$).

ANOVA polynomial contrasts ($\alpha_s=0.01$) revealed that the interaction effect on Δ^9-THC selectivity was expressed by quadratic IPA and methanol terms significantly better than lower order terms ($p<0.001$). Table 3.37 presents the sources for these trend analyses. Similar to trend analysis results for retention time of both Δ^8-THC enantiomers, polynomial contrasts for the selectivity of Δ^8-THC indicated that both quadratic IPA by linear methanol and linear IPA by quadratic methanol terms described a significantly improved fit for the interaction effect ($p\leq0.001$), but not the quadratic IPA by quadratic methanol term ($p=0.048$). The highest order term that significantly improved the model's fit for selectivity of the critical pair was quadratic IPA by linear methanol ($p<0.001$). Homogeneity of variance was evaluated with the Bartlett-Box F test with a significant result for Δ^9-THC selectivity ($p<0.001$), and no results for Δ^8-THC or the critical pair due to at least one cell with zero variance.

Table 3.35. Homogeneous Subsets of Selectivity by IPA Level.
Means for groups in homogeneous subsets were based on observed values. All levels were significantly different for Δ^9-THC and the critical pair at α_s=0.01.

Analyte Pair/Test		IPA (%)	N	Subset		
				1	2	3
Δ^9-THC	Tukey HSD	1	36	1.2724		
		4	36		1.8496	
		7	36			2.2783
		Sig.		1.000	1.000	1.000
	Scheffe	1	36	1.2724		
		4	36		1.8496	
		7	36			2.2783
		Sig.		1.000	1.000	1.000
Δ^8-THC	Tukey HSD	1	36	1.1218		
		4	36		1.2585	
		7	36		1.2793	
		Sig.		1.000	.015	
	Scheffe	1	36	1.1218		
		4	36		1.2585	
		7	36		1.2793	
		Sig.		1.000	0.021	
Critical Pair	Tukey HSD	7	36	1.0849		
		1	36		1.1218	
		4	36			1.1636
		Sig.		1.000	1.000	1.000
	Scheffe	7	36	1.0849		
		1	36		1.1218	
		4	36			1.1636
		Sig.		1.000	1.000	1.000

Table 3.36. Homogeneous Subsets of Selectivity by Methanol Level.
Means for groups in homogeneous subsets were based on observed values. All levels were significantly different for Δ^9-THC at α_s=0.01.

Analyte Pair/Test		Methanol (%)	N	Subset		
				1	2	1
Δ^9-THC	Tukey HSD	3	36	1.6161		
		2	36		1.7960	
		1	36			1.9883
		Sig.		1.000	1.000	1.000
	Scheffe	3	36	1.6161		
		2	36		1.7960	
		1	36			1.9883
		Sig.		1.000	1.000	1.000
Δ^8-THC	Tukey HSD	3	36	1.1976		
		2	36	1.2116		
		1	36		1.2503	
		Sig.		.141	1.000	
	Scheffe	3	36	1.1976		
		2	36	1.2116		
		1	36		1.2503	
		Sig.		.167	1.000	

Table 3.37. Trend Analysis for Selectivity.
Polynomial contrasts were used to evaluate trends in column selectivity at α_s=0.01, where "L" designates the linear term and "Q" designates the quadratic term.

Source	Analyte Pair	Sum of Squares	df	Mean Square	F	Sig.	Eta Sq.	Observed Power
Error (Within + Residual)	Δ^9-THC	0.64	97	0.01				
	Δ^8-THC	0.09	97	0.00				
	Critical Pair	0.15	97	0.00				
exp(L)	Δ^9-THC	0.00	1	0.00	0.40	.530	.004	.000
	Δ^8-THC	0.01	1	0.01	6.29	.014	.061	.457
	Critical Pair	0.01	1	0.01	3.75	.056	.037	.255
exp(Q)	Δ^9-THC	0.01	1	0.01	1.32	.254	.013	.075
	Δ^8-THC	0.00	1	0.00	1.75	.189	.018	.102
	Critical Pair	0.00	1	0.00	2.12	.149	.021	.128
IPA(L)	Δ^9-THC	18.21	1	18.21	2739.57	.000	.966	1.000
	Δ^8-THC	0.45	1	0.45	462.15	.000	.827	1.000
	Critical Pair	0.02	1	0.02	15.75	.000	.140	.907

Table 3.37. Continued.

Source	Analyte Pair	Sum of Squares	df	Mean Square	F	Sig.	Eta Sq.	Observed Power
IPA(Q)	Δ^9-THC	0.13	1	0.13	19.89	.000	.170	.964
	Δ^8-THC	0.08	1	0.08	83.55	.000	.463	1.000
	Critical Pair	0.09	1	0.09	56.08	.000	.366	1.000
Methanol (L)	Δ^9-THC	2.49	1	2.49	375.07	.000	.795	1.000
	Δ^8-THC	0.05	1	0.05	51.75	.000	.348	1.000
	Critical Pair	0.01	1	0.01	6.86	.010	.066	.500
Methanol (Q)	Δ^9-THC	0.00	1	0.00	0.14	.708	.001	.000
	Δ^8-THC	0.00	1	0.00	3.79	.054	.038	.258
	Critical Pair	0.00	1	0.00	0.02	.899	.000	.002
IPA(L) x Methanol (L)	Δ^9-THC	0.22	1	0.22	33.59	.000	.257	.999
	Δ^8-THC	0.25	1	0.25	258.05	.000	.727	1.000
	Critical Pair	0.05	1	0.05	30.90	.000	.242	.998
IPA(Q) x Methanol (L)	Δ^9-THC	0.03	1	0.03	4.90	.029	.048	.347
	Δ^8-THC	0.35	1	0.35	363.72	.000	.789	1.000
	Critical Pair	0.38	1	0.38	245.05	.000	.716	1.000
IPA(L) x Methanol (Q)	Δ^9-THC	0.13	1	0.13	19.05	.000	.164	.956
	Δ^8-THC	0.01	1	0.01	12.73	.001	.116	.823
	Critical Pair	0.00	1	0.00	3.13	.080	.031	.205
IPA(Q) x Methanol(Q)	Δ^9-THC	0.13	1	0.13	19.21	.000	.165	.958
	Δ^8-THC	0.00	1	0.00	4.00	.048	.040	.275
	Critical Pair	0.00	1	0.00	1.08	.300	.011	.063
Model	Δ^9-THC	21.36	10	2.14	321.31	.000		
	Δ^8-THC	1.21	10	0.12	124.78	.000		
	Critical Pair	0.57	10	0.06	36.47	.000		
Total	Δ^9-THC	22.01	107	.21				
	Δ^8-THC	1.30	107	.01				
	Critical Pair	0.72	107	.01				

Resolution

Peak resolution of Δ^9-THC, Δ^8-THC, and the critical pair were analyzed at α_s=0.01. As summarized in Table 3.38, Levene's test for homogeneity of variance indicated that the null hypothesis of equal group variances was rejected for these pairs ($p<0.001$). Because cell sizes were equal, this was not considered to represent a threat to the validity of the data. The Pillai's trace multivariate test of overall differences among groups was statistically significant for each factor ($p<0.001$). Refer to Table 3.39 for a summary of the data. Partial eta-square for experiment, though significant, implied a weak relationship with peak resolution (0.161). Resolution appeared to have the strongest relationship with IPA (η^2=0.776), followed by the IPA-methanol interaction (η^2=0.659) and then methanol (η^2=0.571). A lack of fit test was performed for the designated pairs with all significant results ($p<0.001$) as summarized in Table 3.40.

Table 3.38. Levene's Test for Resolution.
Peak resolution error variances were significantly different across groups at α_s=0.01.

	F	df1	df2	Sig.
Δ^9-THC	25.661	26	80	.000
Δ^8-THC	465.723	26	80	.000
critical pair	1497.851	26	80	.000

Table 3.39. Multivariate Test of Overall Differences for Resolution.
Overall peak resolution differences were significant for each factor at α_s=0.01.

Effect		Value	F	Hypothesis df	Error df	Sig.	Partial Eta Sq.	Observed Power
exp	Pillai's Trace	.321	6.065	6.000	190.000	.000	.161	.990
	Wilks' Lambda	.682	6.601[b]	6.000	188.000	.000	.174	.995
	Hotelling's Trace	.460	7.134	6.000	186.000	.000	.187	.997
	Roy's Largest Root	.448	14.188[c]	3.000	95.000	.000	.309	.999
IPA	Pillai's Trace	1.553	110.003	6.000	190.000	.000	.776	1.000
	Wilks' Lambda	.005	408.441[b]	6.000	188.000	.000	.929	1.000
	Hotelling's Trace	86.065	1334.000	6.000	186.000	.000	.977	1.000
	Roy's Largest Root	84.768	2684.311[c]	3.000	95.000	.000	.988	1.000
Methanol	Pillai's Trace	1.143	42.200	6.000	190.000	.000	.571	1.000
	Wilks' Lambda	.037	132.041[b]	6.000	188.000	.000	.808	1.000
	Hotelling's Trace	21.310	330.299	6.000	186.000	.000	.914	1.000
	Roy's Largest Root	21.078	667.478[c]	3.000	95.000	.000	.955	1.000
IPA x Methanol	Pillai's Trace	1.976	46.313	12.000	288.000	.000	.659	1.000
	Wilks' Lambda	.002	207.069	12.000	248.992	.000	.879	1.000
	Hotelling's Trace	74.741	577.169	12.000	278.000	.000	.961	1.000
	Roy's Largest Root	68.515	1644.364[c]	4.000	96.000	.000	.986	1.000

Table 3.40. Lack of Fit Test for Resolution.
The F-values were significant for the fitted model for each pair at α_s=0.01.

Analyte Pair	Source	Sum of Squares	df	Mean Square	F	Sig.	Observed Power
Δ^9-THC	Lack of Fit	211.152	16	13.197	89.693	.000	1.000
	Pure Error	11.771	80	.147			
Δ^8-THC	Lack of Fit	7.555	16	.472	1094.858	.000	1.000
	Pure Error	.035	80	.000			
Critical Pair	Lack of Fit	21.572	16	1.348	4343.959	.000	1.000
	Pure Error	.025	80	.000			

Similar to column selectivity, ANOVA tests for between-subjects effects showed that peak resolution was significantly effected by IPA and IPA-methanol interaction for all three pairs ($p<0.001$). Again, with regard to methanol effects, only results for Δ^9-THC and Δ^8-THC were significantly different ($p<0.001$), whereas there was a failure to reject

the null hypothesis for the critical pair (p=0.579). There was also a failure to reject the null hypothesis for Δ^9-THC resolution in relation to experimental setup (p=0.458), though both Δ^8-THC and the critical pair had significant differences due to experiment (p<0.001 and p=0.006, respectively). The data are summarized in Table 3.41.

Table 3.41. Tests of Between-Subjects Effects for Resolution.
Between-subjects resolution differences were significant for each factor at α_s=0.01.

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Sq.	Observed Power
Model	Δ^9-THC	13473.005	11	1224.819	527.459	.000	.984	1.000
	Δ^8-THC	1265.662	11	115.060	1455.357	.000	.994	1.000
	Critical Pair	494.036	11	44.912	199.639	.000	.958	1.000
exp	Δ^9-THC	3.655	2	1.828	.787	.458	.016	.061
	Δ^8-THC	2.391	2	1.196	15.123	.000	.240	.993
	Critical Pair	2.464	2	1.232	5.476	.006	.102	.640
IPA	Δ^9-THC	1922.921	2	961.461	414.046	.000	.896	1.000
	Δ^8-THC	69.963	2	34.982	442.472	.000	.902	1.000
	Critical Pair	21.540	2	10.770	47.873	.000	.499	1.000
Methanol	Δ^9-THC	724.200	2	362.100	155.936	.000	.765	1.000
	Δ^8-THC	26.588	2	13.294	168.148	.000	.778	1.000
	Critical Pair	.248	2	.124	.550	.579	.011	.042
IPA x Methanol	Δ^9-THC	90.029	4	22.507	9.693	.000	.288	.996
	Δ^8-THC	152.113	4	38.028	481.006	.000	.952	1.000
	Critical Pair	105.278	4	26.320	116.992	.000	.830	1.000
Error	Δ^9-THC	222.923	96	2.322				
	Δ^8-THC	7.590	96	.079				
	Critical Pair	21.597	96	.225				
Total	Δ^9-THC	13695.928	107					
	Δ^8-THC	1273.252	107					
	Critical Pair	515.633	107					

Relationship patterns were nearly identical to those that described selectivity. The relationship of Δ^9-THC to IPA was strongest, followed by methanol, and then the IPA-methanol interaction (η^2=0.896, 0.765, and 0.288, respectively). Δ^8-THC had the strongest relationship to the IPA-methanol interaction, followed by IPA, then methanol (η^2=0.952, 0.902, 0.778, respectively). Resolution of the critical pair had a weaker

relationship with IPA than the IPA-methanol interaction (η^2=0.499, 0.830, respectively). Adjusted R^2 values for the model for Δ^9-THC, Δ^8-THC, and the critical pair were 0.982, 0.993, and 0.953, respectively.

Follow-up post-hoc comparisons between groups showed that all levels of IPA and methanol were significantly different for resolution of the Δ^9-THC and Δ^8-THC pairs ($p<0.001$). Tables 3.42-3.44 present homogeneous subsets while Appendix D contains complete post-hoc analysis source tables. All levels of IPA were significantly different for the critical pair (Scheffé's test: $p\leq0.003$), while no levels of experimental set were (Tukey's test: $p\geq0.016$). Experiment setup 1 was significantly different from setups 2 and 3 for Δ^8-THC (Scheffé's test: $p\leq0.001$), which did not significantly differ from each other (Tukey's test: $p=0.687$).

Table 3.42. Homogeneous Subsets of Resolution by IPA Level.
Means for groups in homogeneous subsets were based on observed values. All levels were significantly different for each pair at α=0.01.

Analyte Pair/Test		IPA (%)	N	Subset		
				1	2	3
Δ^9-THC	Tukey HSD	1	36	4.69655		
		4	35		10.33287	
		7	36			15.02322
		Sig.		1.000	1.000	1.000
	Scheffe	1	36	4.69655		
		4	35		10.33287	
		7	36			15.02322
		Sig.		1.000	1.000	1.000
Δ^8-THC	Tukey HSD	1	36	1.95871		
		4	35		3.44004	
		7	36			3.85214
		Sig.		1.000	1.000	1.000
	Scheffe	1	36	1.95871		
		4	35		3.44004	
		7	36			3.85214
		Sig.		1.000	1.000	1.000

Table 3.42. Continued.

Analyte Pair/Test		IPA (%)	N	Subset		
				1	2	3
Critical Pair	Tukey HSD	7	36	1.23004		
		1	36		1.95871	
		4	35			2.35472
		Sig.		1.000	1.000	1.000
	Scheffe	7	36	1.23004		
		1	36		1.95871	
		4	35			2.35472
		Sig.		1.000	1.000	1.000

Table 3.43. Homogeneous Subsets of Resolution by Methanol Level.
Means for groups in homogeneous subsets were based on observed values. All levels were significantly different at α_s=0.01.

Analyte Pair/Test		Methanol (%)	N	Subset		
				1	2	3
Δ^9-THC	Tukey HSD	3	35	6.98558		
		2	36		9.63202	
		1	36			13.34206
		Sig.		1.000	1.000	1.000
	Scheffe	3	35	6.98558		
		2	36		9.63202	
		1	36			13.34206
		Sig.		1.000	1.000	1.000
Δ^8-THC	Tukey HSD	3	35	2.54072		
		2	36		2.96337	
		1	36			3.72182
		Sig.		1.000	1.000	1.000
	Scheffe	3	35	2.54072		
		2	36		2.96337	
		1	36			3.72182
		Sig.		1.000	1.000	1.000

Polynomial contrasts were evaluated with ANOVA for trends at α_s=0.01. The data summarized in Table 3.45 show that the quadratic IPA by quadratic methanol term provided significantly better fit than any lower order combination of terms for Δ^9-THC ($p=0.003$), Δ^8-THC ($p<0.001$), as well as the critical pair

(p=0.001). The Bartlett-Box F test was used to determine homogeneity of variance, and again, the result for Δ^9-THC was significant ($p<0.001$) but could not be calculated for Δ^8-THC or the critical pair due to the presence of one or more cells with zero variance.

Table 3.44. Homogeneous Subsets of Resolution by Experiment.
Means for groups in homogeneous subsets are based on observed values. Only two levels were significantly different for Δ^8-THC at α_s=0.01.

Analyte Pair/Test		Experiment	N	Subset		
				1	2	3
Δ^8-THC	Tukey HSD	1	35	2.89427		
		3	36		3.14331	
		2	36		3.19815	
		Sig.		1.000	.689	
	Scheffe	1	35	2.89427		
		3	36		3.14331	
		2	36		3.19815	
		Sig.		1.000	.713	
Critical Pair	Tukey HSD	1	35	1.64508		
		3	36	1.91570		
		2	36	1.96299		
		Sig.		.015		
	Scheffe	1	35	1.64508		
		3	36	1.91570		
		2	36	1.96299		
		Sig.		.021		

Table 3.45. Trend Analysis for Resolution.
Polynomial contrasts were used to evaluate trends in peak resolution at α_s=0.01, where "L" designates the linear term and "Q" designates the quadratic term.

Source	Dependent Variable	Sum of Squares	df	Mean Square	F	Sig.	Eta Sq.	Observed Power
Error (Within + Residual)	Δ^9-THC	222.92	96	2.32				
	Δ^8-THC	7.59	97	.08				
	Critical Pair	21.60	96	.22				
exp(L)	Δ^9-THC	3.15	1	3.15	1.36	.247	.014	.078
	Δ^8-THC	1.51	1	1.51	19.26	.000	.166	.958
	Critical Pair	1.57	1	1.57	6.97	.010	.068	.508

Table 3.45. Continued.

Source	Dependent Variable	Sum of Squares	df	Mean Square	F	Sig.	Eta Sq.	Observed Power
exp(Q)	Δ^9-THC	.52	1	.52	.23	.636	.002	.000
	Δ^8-THC	.96	1	.96	12.21	.001	.112	.804
	Critical Pair	.92	1	.92	4.08	.046	.041	.281
IPA(L)	Δ^9-THC	1919.52	1	1919.52	826.63	.000	.896	1.000
	Δ^8-THC	64.53	1	64.53	824.42	.000	.895	1.000
	Critical Pair	9.56	1	9.56	42.48	.000	.307	1.000
IPA(Q)	Δ^9-THC	3.40	1	3.40	1.46	.229	.015	.084
	Δ^8-THC	5.51	1	5.51	70.37	.000	.420	1.000
	Critical Pair	11.98	1	11.98	53.26	.000	.357	1.000
Methanol (L)	Δ^9-THC	716.16	1	716.16	308.41	.000	.763	1.000
	Δ^8-THC	26.43	1	26.43	337.61	.000	.777	1.000
	Critical Pair	.01	1	.01	.04	.838	.000	.002
Methanol (Q)	Δ^9-THC	6.76	1	6.76	2.91	.091	.029	.188
	Δ^8-THC	.56	1	.56	7.14	.009	.069	.521
	Critical Pair	.24	1	.24	1.05	.307	.011	.061
IPA(L) x Methanol (L)	Δ^9-THC	54.00	1	54.00	23.25	.000	.195	.985
	Δ^8-THC	84.27	1	84.27	1076.57	.000	.917	1.000
	Critical Pair	16.17	1	16.17	71.87	.000	.428	1.000
IPA(Q) x Methanol (L)	Δ^9-THC	6.77	1	6.77	2.91	.091	.029	.188
	Δ^8-THC	60.18	1	60.18	768.79	.000	.888	1.000
	Critical Pair	83.62	1	83.62	371.67	.000	.795	1.000
IPA(L) x Methanol (Q)	Δ^9-THC	6.68	1	6.68	2.88	.093	.029	.185
	Δ^8-THC	6.16	1	6.16	78.73	.000	.448	1.000
	Critical Pair	2.36	1	2.36	10.49	.002	.099	.727
IPA(Q) x Methanol (Q)	Δ^9-THC	22.14	1	22.14	9.54	.003	.090	.676
	Δ^8-THC	2.99	1	2.99	38.14	.000	.282	1.000
	Critical Pair	2.59	1	2.59	11.52	.001	.107	.776
Model	Δ^9-THC	2741.73	10	274.17	118.07	.000	n.d.	n.d.
	Δ^8-THC	253.08	10	25.31	323.32	.000	n.d.	n.d.
	Critical Pair	130.56	10	13.06	58.03	.000	n.d.	n.d.
Total	Δ^9-THC	2964.66	106	27.97				
	Δ^8-THC	260.67	107	2.44				
	Critical Pair	151.16	106	1.44				

CHAPTER IV

DISCUSSION

Statistical Assumptions

The underlying assumptions for ANOVA include but are not limited to: independent observations, homogeneity of variances, and randomly distributed residuals.[47] Where a reduced model is used, deviations from the full factorial model must not exclude any terms necessary to accurately describe the phenomena. While departures from the underlying assumptions could jeopardize the integrity of the inferential data, some violations of the assumptions are not considered fatal, particularly where a balanced design is utilized with equally sized groups.[48] An alpha level of 0.01 was selected due to the controlled nature of this study, in that factor levels were fixed and not random. Investigation of the underlying assumptions included qualitative and quantitative techniques.

Residuals were analyzed using several different methods including the observed by predicted by residuals plots (see Figures 4.1-4.3). In these plots, the observed by predicted patterns follow an overall linear/diagonal form, suggesting a significant relationship between the predictors and the dependent variables. Some spacing is noted in the upper portions of the retention time plots. The standardized residuals graphs are mostly amorphous clouds, with no dominant increasing or decreasing trends in error

according to observation or prediction, though some spread due to the noted spacing is apparent.

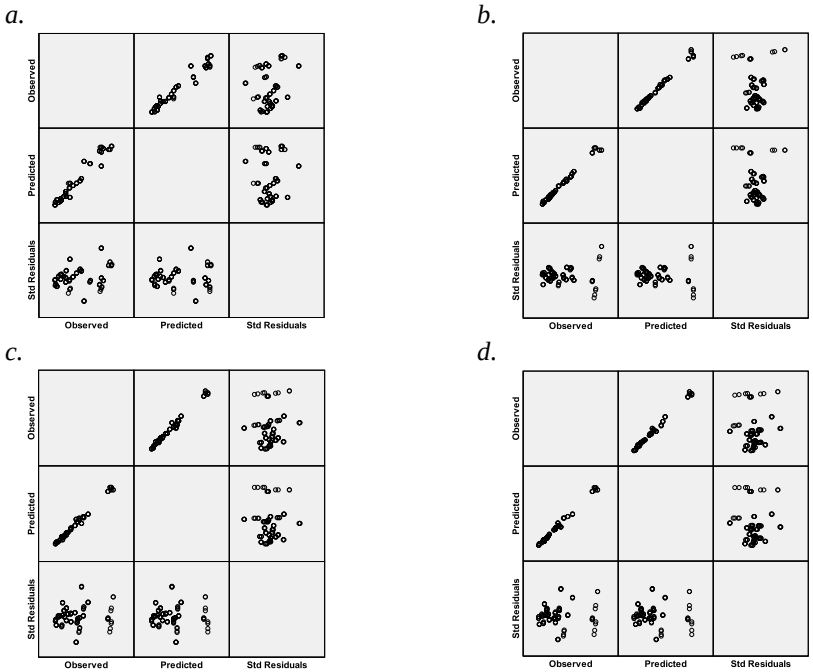


Figure 4.1. Observed by Predicted by Residual Plots for Retention Time. Observed by predicted plots for retention time appeared linear while standardized residuals plots were amorphous for: a. (-)-Δ^9-THC, b. (-)-Δ^8-THC, c. (+)-Δ^9-THC, d. (+)-Δ^8-THC.

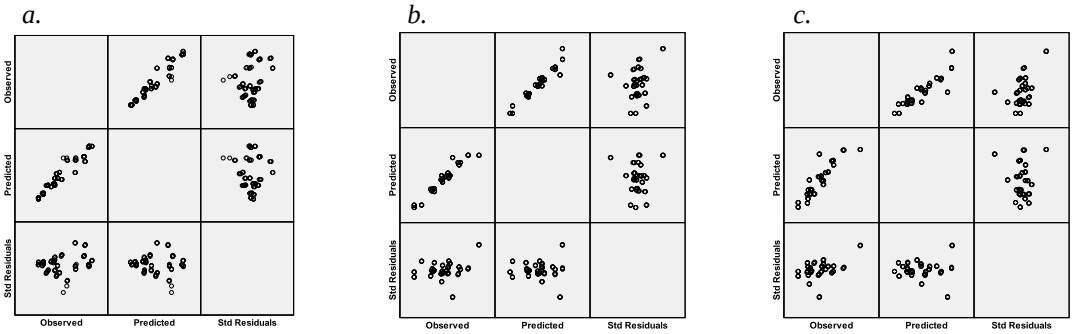


Figure 4.2. Observed by Predicted by Residual Plots for Selectivity. Observed by predicted plots for selectivity appeared linear while standardized residuals plots were amorphous for: a. Δ^9-THC, b. Δ^8-THC, c. critical pair.

Normal and detrended normal Q-Q plots were also evaluated for the dependent variables. These plots are presented in Figures 4.4-4.6. The normal Q-Q plots suggest that the results did not conform to normal Gaussian distributions. With the exception of

selectivity and resolution for Δ^8-THC, several results were skewed right, or in the positive direction. All of the cases fell within 1.5 standard deviations of the hypothetical mean, indicating no obvious outliers. Of the three dependent variables investigated, retention time results consistently had the largest standard deviations.

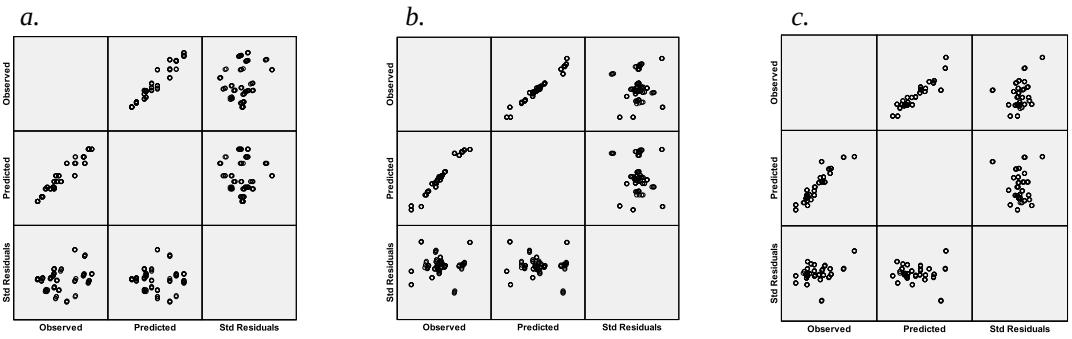


Figure 4.3. Observed by Predicted by Residual Plots for Resolution. Observed by predicted plots for resolution appeared linear while standardized residuals plots were amorphous for: a. Δ^9-THC, b. Δ^8-THC, c. critical pair.

The assumption of homogeneity of variance for the omnibus ANOVA was evaluated by Levene's test (see Tables 3.23, 3.31, and 3.38) because the data did not meet the assumption of normality, and this test is less sensitive to non-normal distributions.[48] Significant results for each dependent variable indicated that this assumption was not met. Because equal group sample sizes were used, this is less of a threat to the validity of the inferential statistics than would be the case if an unbalanced design was used.

Lack of fit tests were used to evaluate the reduced model (see Design 3.3) for missing terms. Significant results in all cases ($p<0.001$) implied that the necessary terms were present in the reduced model. Further, these results suggested that no error was due to misfitting of the model and that all error was pure error.

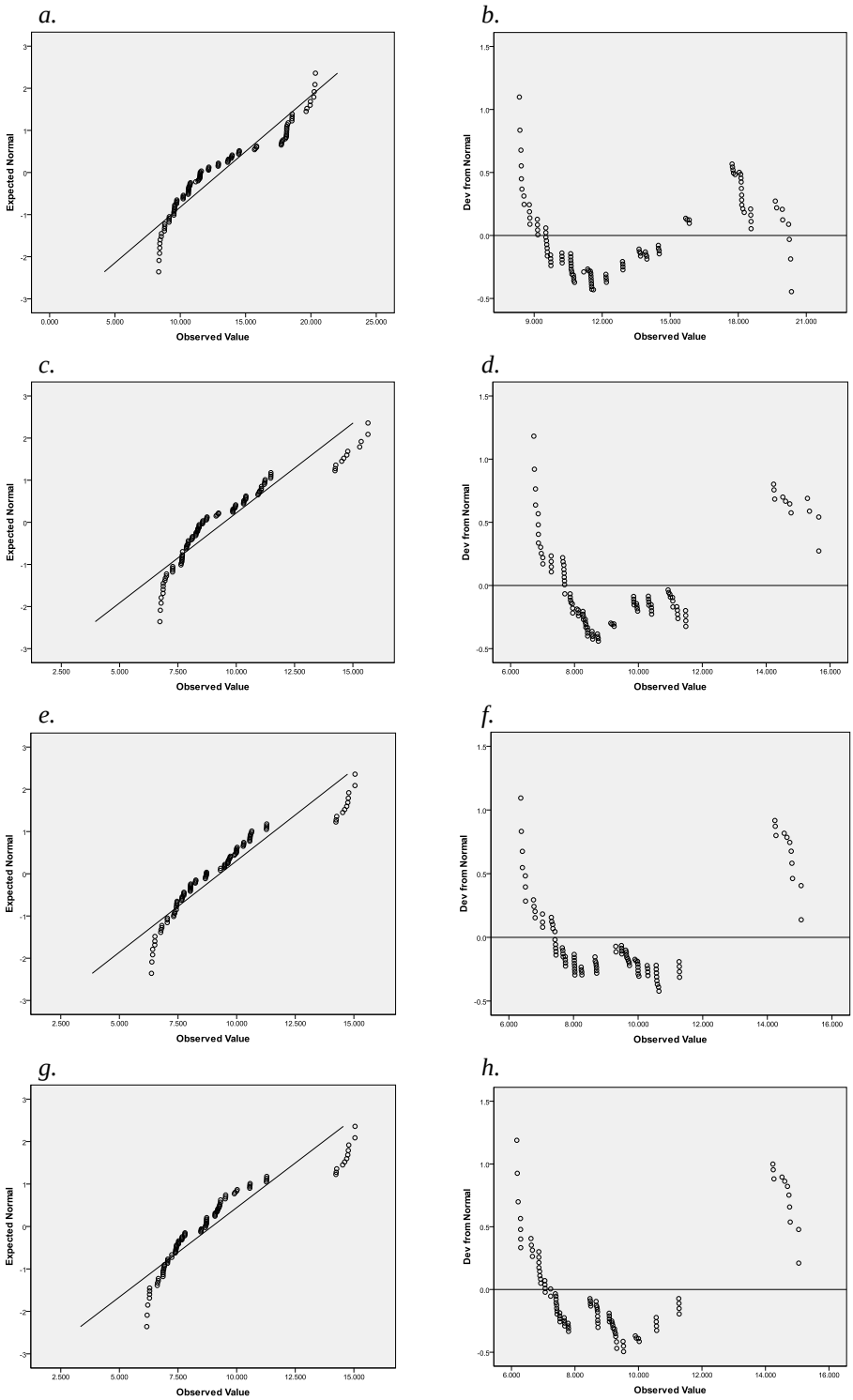


Figure 4.4. Q-Q Plots for Retention Time. Normal and detrended normal plots, respectively, for: a.-b. (-)-Δ^9-THC, c.-d. (-)-Δ^8-THC, e.-f. (+)-Δ^9-THC, g.-h. (+)-Δ^8-THC.

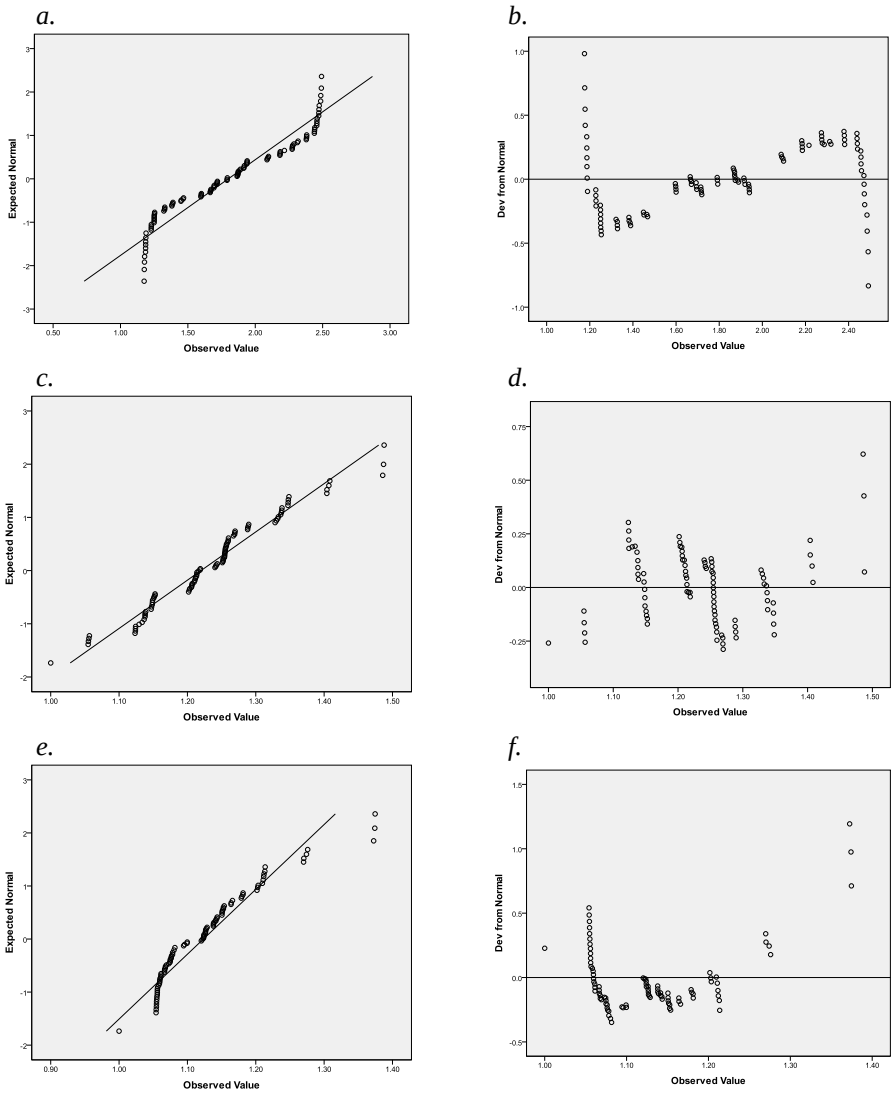


Figure 4.5. Q-Q Plots for Selectivity. Normal and detrended normal plots, respectively, for: a.-b. Δ^9-THC, c.-d. Δ^8-THC, e.-f. critical pair.

Experiment Effect: Mobile Phase Preparation

The experimental design included using the LC to mix various proportions of four manually prepared solutions to study a total of nine mobile phases. Column equilibration is a significant concern with regard to chiral analyses. %RSD of analyte retention time is generally accepted as a good measure of equilibration. The largest RSD obtained for the four resolution sample injections performed in any single run was 1.6%, indicating that column equilibration was not a problem.

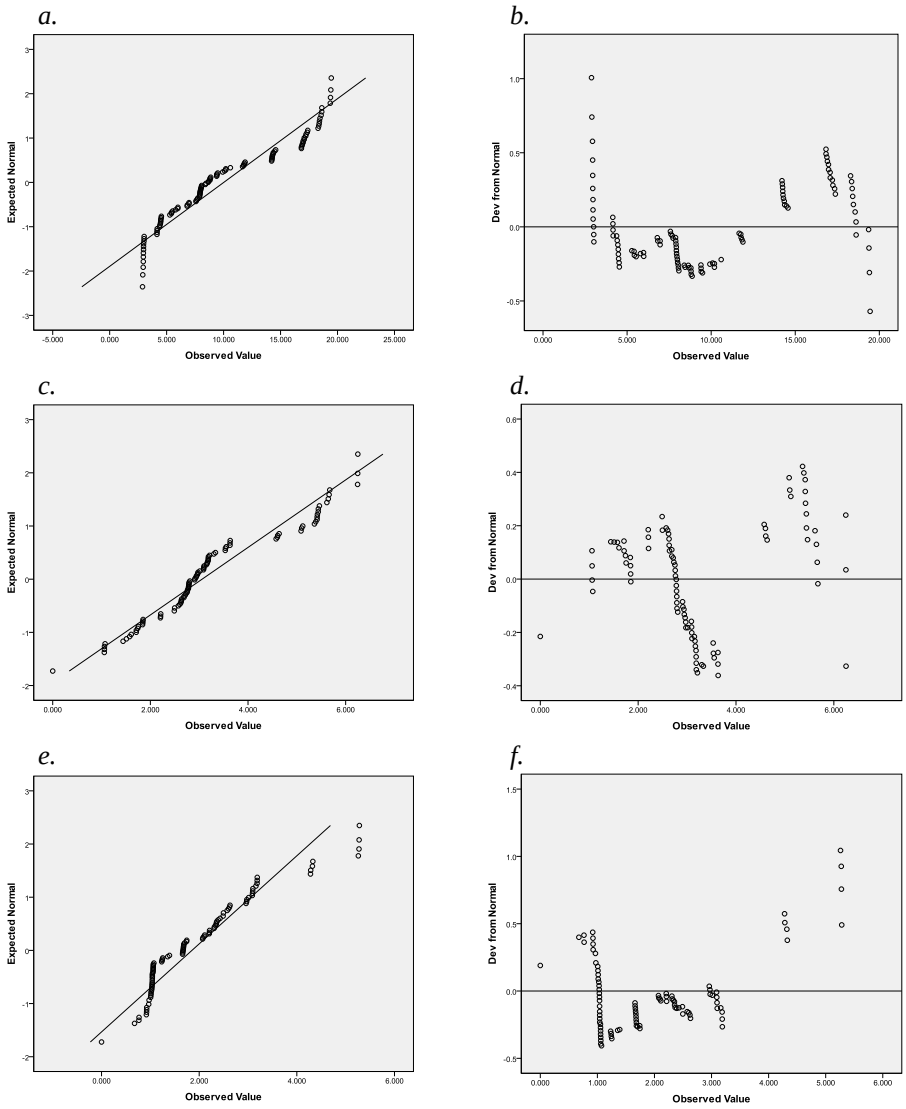


Figure 4.6. Q-Q Plots for Resolution. Normal and detrended normal plots, respectively, for: a.-b. Δ^9-THC, c.-d. Δ^8-THC, e.-f. critical pair.

Ideally, each experiment could have been performed using the exact same set of mobile phase solutions (ie- only one preparation of the four solutions for all three experiments). However, this was not feasible as concentration changes due to evaporation were considered imminent during storage time between experiments, and so a new set of solutions was prepared for each experiment. Because solution preparation is inherently subject to numerous uncontrollable variations such as measurement imprecision and daily temperature and humidity fluctuations, the

automated mixing of the mobile phases likely imparted greater reproducibility to the experiment. Slight variations in the mobile phase solutions prepared on different days were averaged across the results for each separate experimental set due to the mixing.

Overall, as a result of in-line mobile phase mixing, variation due to mobile phase preparation within each experiment was minimized, reflecting the LC's ability to mix the solutions accurately, while variation between experiments reflected the normal tendency for slight variations in manual solution preparation. In other words, the statistical significance of the experiment effect in the results likely indicated that the analyst did not prepare the four individual solutions as reproducibly as the LC mixed them.

Mobile Phase Alcohol Effects

This study was designed with the primary purpose of investigating the effects of IPA and methanol on the enantioseparation of Dronabinol and three related chiral impurities. These effects were characterized using a handful of chromatographic parameters and inferential statistics. Statistical results and trends were compared to predictions made prior to data collection based on information found in the literature. Specific instances where qualitative or descriptive data and inferential statistics strongly agreed were noted. Numerous graphs summarizing the results were also evaluated with some directly generated from the inferential analyses and others generated as a means to investigate the data from alternative perspectives.

Both the descriptive and the inferential statistics for the data presented intriguing empirical trends for the enantioseparation of the four analytes. Data for several variables

for Δ^9-THC, Δ^8-THC, and the critical pair are summarized in Table 4.1. Results from the post hoc and trend analyses are summarized in Table 4.2.

Table 4.1. Chromatographic Parameters of Δ^9-THC and Δ^8-THC Using IPA and Methanol Mixtures in n-Heptane.
Capacity factors, retention times, selectivity, and resolution were summarized by mobile phase composition over all experiments, where the (+) or (-) subscript denotes the enantiomer.

	Independent Variables		Dependent Variables					
	% IPA	% Methanol	$k'_{(+)}$	$k'_{(-)}$	$t_{r(+)}$	$t_{r(-)}$	α	R
Δ^9-THC	1	1	2.52	3.51	14.601	18.682	1.39	6.76
	4	1	1.37	2.89	9.831	16.134	2.10	14.49
	7	1	1.45	3.58	10.080	18.866	2.47	18.78
	1	2	1.57	1.95	10.602	12.173	1.24	4.37
	4	2	0.90	1.59	7.816	10.671	1.78	8.98
	7	2	0.91	2.16	7.874	13.021	2.37	15.55
	1	3	1.15	1.37	8.899	9.775	1.18	2.96
	4	3	0.73	1.21	7.086	9.092	1.67	7.28
	7	3	0.69	1.39	6.788	9.607	1.99	10.74
Δ^8-THC	1	1	2.52	2.57	14.601	14.800	1.02	0.35*
	4	1	1.23	1.71	9.263	11.260	1.39	5.50
	7	1	1.14	1.53	8.823	10.416	1.34	5.31
	1	2	1.57	1.38	10.602[#]	9.820[#]	1.14	2.44
	4	2	0.84	1.04	7.571	8.396	1.24	2.95
	7	2	0.78	0.98	7.335	8.161	1.26	3.50
	1	3	1.15	0.95	8.899[#]	8.075[#]	1.21	3.09
	4	3	0.68	0.78	6.912	7.317	1.14	1.70
	7	3	0.62	0.77	6.512	7.103	1.24	2.74
Critical Pair	1	1	-	-	-	-	1.02	0.35*
	4	1	-	-	-	-	1.26	3.97
	7	1	-	-	-	-	1.06	1.04
	1	2	-	-	-	-	1.14	2.44
	4	2	-	-	-	-	1.16	2.06
	7	2	-	-	-	-	1.08	1.20
	1	3	-	-	-	-	1.21	3.09
	4	3	-	-	-	-	1.08	0.91
	7	3	-	-	-	-	1.12	1.45

*partial resolution (1.06) obtained in experiment 3; [#]denotes elution order reversal.

Table 4.2. Summary of Results for Inferential Statistics.

Results for the post hoc and trend analyses of retention time, selectivity, and resolution are summarized for the main and interaction effects of the alcohols.

Retention Time				Selectivity				Resolution			
Analyte	Main Effects[a,b] IPA	Main Effects[a,b] Methanol	Interaction Effect[c,d]	Analyte Pair	Main Effects IPA	Main Effects Methanol	Interaction Effect	Analyte Pair	Main Effects IPA	Main Effects Methanol	Interaction Effect
(-)-Δ^9-THC	1 7 4	1 2 3	i^Q x m^L	Δ^9-THC	7 4 1	1 2 3	i^Q x m^Q	Δ^9-THC	7 4 1	1 2 3	i^Q x m^Q
(+)-Δ^9-THC	1 4 7	1 2 3	i^Q x m^L i^L x m^Q	Critical Pair	4 1 7	not analyzed	i^Q x m^L	Critical Pair	4 1 7	not analyzed	i^Q x m^Q
(-)-Δ^8-THC	1 4 7	1 2 3	i^Q x m^L i^L x m^Q	Δ^8-THC	4 7 1	1 2 3	i^Q x m^L i^L x m^Q	Δ^8-THC	7 4 1	1 2 3	i^Q x m^Q
(+)-Δ^8-THC	1 4 7	1 2 3	i^Q x m^L i^L x m^Q								

[a]Main effects are presented as homogeneous subsets of % alcohol with the highest result (as determined by post hoc analysis) on the top row and the lowest on the bottom row; [b]Where the main effect result was not significantly different due to the treatment, post hoc analysis was not performed; [c]Interaction effect is presented as the highest order term that provided significantly better fit by trend analysis, where i = IPA, m = methanol, Q = quadratic, and L = linear; [d]Two results are presented where multiple forms of the interaction term were significant and of equivalent higher order.

Main Effects on Retention Time

The main effects of IPA and methanol on retention time for each analyte were determined from the data. Results were compared to predictions where applicable. The profile plots in Figure 4.7 illustrate the main effects on retention time of each alcohol across all three levels. Overall, (-)-Δ^9-THC was retained substantially longer than the other analytes, and in all cases except for the elution order reversal, (-)-enantiomers were retained longer than (+)-enantiomers.

Decreasing Retention Time with Increasing Alcohol

Figure 4.7 profile plots and the chromatograms presented in Figures 3.1-3.3 indicate that overall retention time trended downward when alcohol content trended upward, which is a typical normal phase effect. The one notable exception was Δ^9-THC retention time with respect to 7% IPA in the mobile phase. At 7% IPA, (-)-Δ^9-THC retention time noticeably increased, as did (+)-Δ^9-THC retention time (at lower methanol levels). Post-hoc analyses (see Tables 3.27 and 4.2) presented just two significantly different IPA subsets for (-)-Δ^9-THC retention time, with 1% and 7% ranked together, above 4%. (+)-Δ^9-THC retention time was also grouped into two subsets, with the lower subset containing 4 and 7% IPA.

For both Δ^9-THC enantiomers, retention time was inversely related to methanol content as indicated by the results for each enantiomer as presented in Tables 3.27 and 4.2, wherein the highest percentage of alcohol resulted in the lowest retention time. Δ^8-THC retention time was also inversely related to methanol content, as well as IPA content. The homogeneous subsets for both Δ^8-THC enantiomers (see Tables 3.27, 3.28, and 4.2) indicated that all levels of either alcohol were significantly different at α_s=0.01.

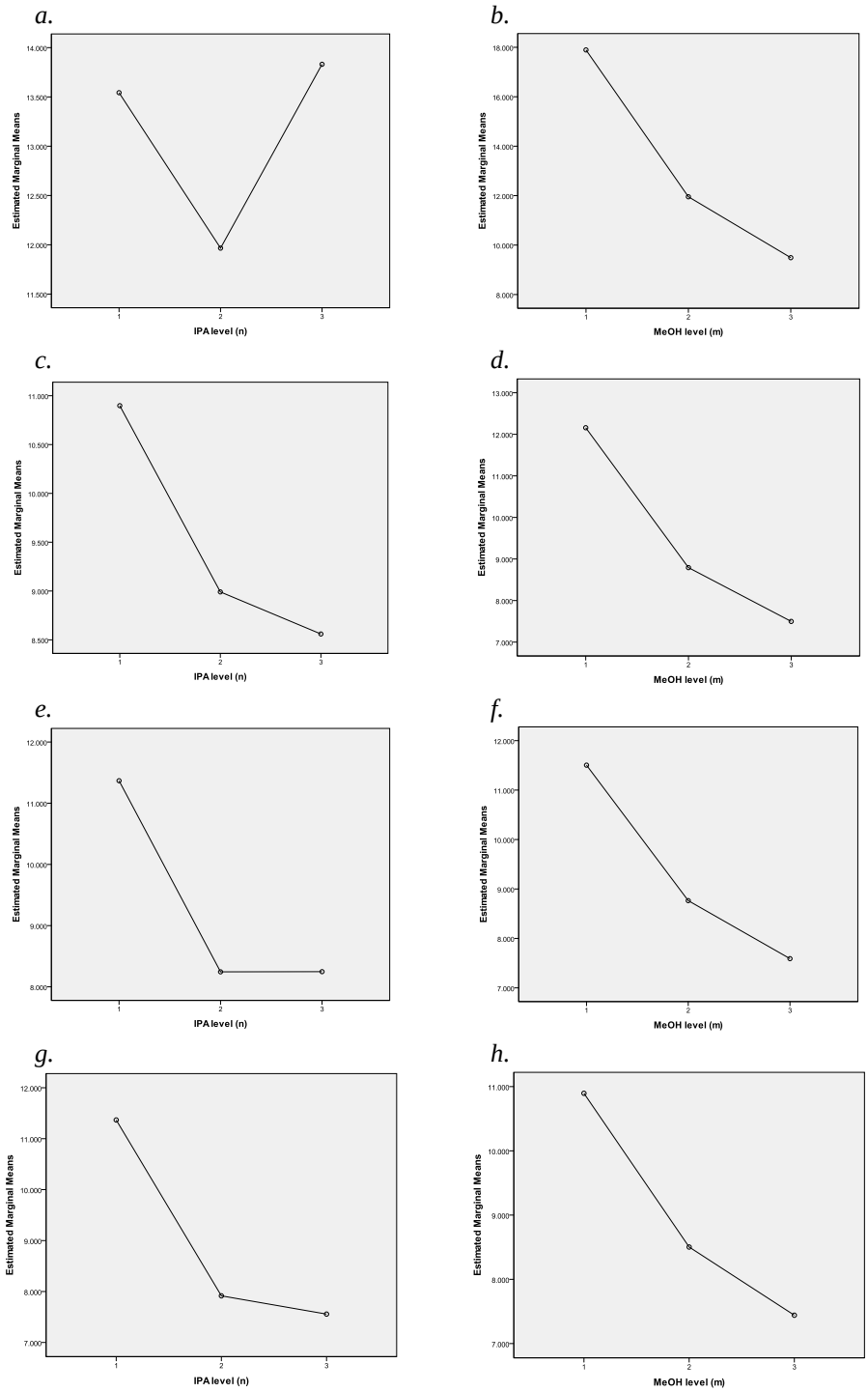


Figure 4.7. Profile Plots for Main Effects on Retention Time. Main effects versus level of IPA and methanol, respectively for: a.-b. (-)-Δ^9-THC, c.-d. (-)-Δ^8-THC, e.-f. (+)-Δ^9-THC, g.-h. (+)-Δ^8-THC.

Mobile Phase Strength: Methanol versus IPA

Some predictions for the effects of methanol on retention were supported by the data. The effects of each alcohol, as determined from differences in the observed means given in Tables 3.27 and 3.28, were compared. Changes in retention time across the levels of IPA were larger for each of the (+)-enantiomers than the corresponding (-)-enantiomers, with the largest retention time difference (3.8 minutes) observed for (+)-Δ^8-THC. In contrast, changes in retention time averages across the levels of methanol were smaller for each of the (+)-enantiomers than the corresponding (-)-enantiomers, wherein (-)-Δ^9-THC showed the largest retention time difference (8.4 minutes).

To aid in data interpretation, the molarity of IPA and methanol in each mobile phase was calculated based on density at room temperature (0.785 and 0.791 g/mL, respectively) and molecular weight (60.10 and 31.03 g/mol, respectively). Levels 1-3 of each alcohol corresponded to 0.131, 0.523, and 0.914 M IPA, or 0.255, 0.510, and 0.765 M methanol, respectively. Considering that the IPA content in the mobile phase varied from 0.131 to 0.914 M (Δ0.784 M) or 1 to 7% while the methanol content only varied from 0.255 to 0.765 M (Δ0.510 M) or 1 to 3%, evidence suggests that methanol had a stronger effect on retention time than IPA.

The methanol effect on retention time was larger for each of the (-)-enantiomers, but more so for Δ^9-THC, than for Δ^8-THC, as was predicted. The effect of methanol on the retention time of Δ^8-THC enantiomers was also expected to be stronger than the IPA effect on Δ^8-THC retention time. While the difference in (-)-Δ^8-THC retention time was larger for methanol (4.7 minutes) than IPA (2.3 minutes), the difference for (+)-Δ^8-THC was slightly smaller for methanol (3.5 minutes) compared to IPA (3.8 minutes). Given

the unequal ranges for the alcohol levels, the methanol effect on Δ^8-THC retention appeared to be stronger than the IPA effect on the basis of retention time change per change in molarity.

Main Effects on Selectivity and Resolution

The main effects of the alcohols on selectivity and resolution of Δ^9-THC, Δ^8-THC, and the critical pair were determined from the data. Profile plots for main effects of the alcohols on these dependent variables are presented in Figures 4.8 and 4.9. Overall for the three pairs, enantioseparation was greatest for Δ^9-THC and least for the critical pair. A failure to reject the null hypothesis in the ANOVA for the main effect of methanol on both the selectivity and resolution of the critical pair (Tables 3.34 and 3.41, respectively) indicated that differences observed by varying methanol content alone were no larger than that expected by chance (α_s=0.01). The remaining between-subjects effects were significant for the alcohols and thus information from post-hoc analyses was used to compare their main effects on selectivity and resolution and to à priori predictions.

Selectivity was expected to improve as IPA content increased, with a more pronounced effect on Δ^9-THC than Δ^8-THC. The graphs in Figure 4.8 illustrate these anticipated trends. Post-hoc analyses for IPA resulted in three homogeneous subsets for Δ^9-THC and the critical pair, and two subsets for Δ^8-THC (see Tables 3.35, 3.36, and 4.2). For Δ^9-THC, selectivity improved with increasing IPA, with a maximum difference of 1.01 units between subsets. Δ^8-THC selectivity was significantly lower at 1% IPA than the other levels. The critical pair had the lowest selectivity at 7% IPA and the highest at 4% IPA with the smallest measured IPA main effect on selectivity (0.08 units).

As methanol content increased, the selectivity of Δ^8-THC and Δ^9-THC decreased, which had the largest effect on the latter pair (0.37 units). Although the methanol effect on selectivity of the critical pair was statistically non-significant, the observed power for this test was only 0.385, indicating a low confidence in this result (desired power $\geq$ 0.80) and a 61.5% risk of Type II error.

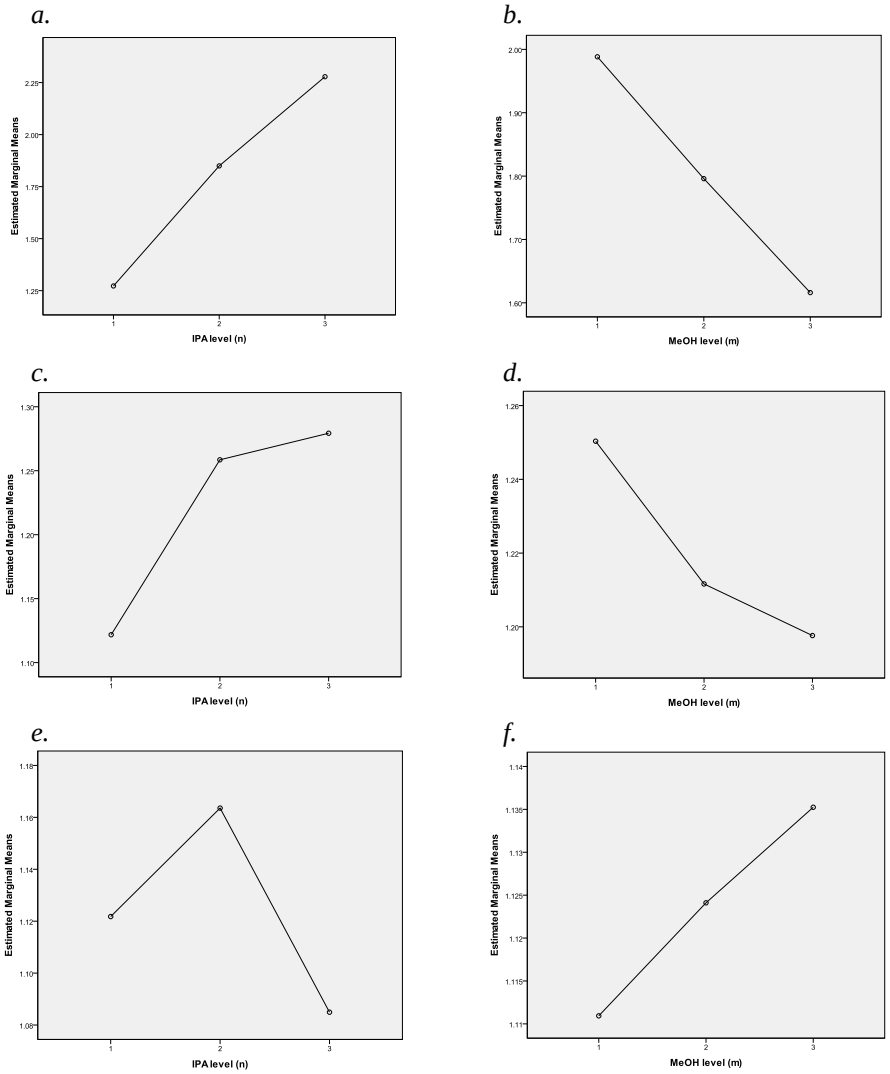


Figure 4.8. Profile Plots for Main Effects on Selectivity. Main effects versus level of IPA and methanol, respectively for: a.-b. Δ^9-THC, c.-d.Δ^8-THC, e.-f. critical pair.

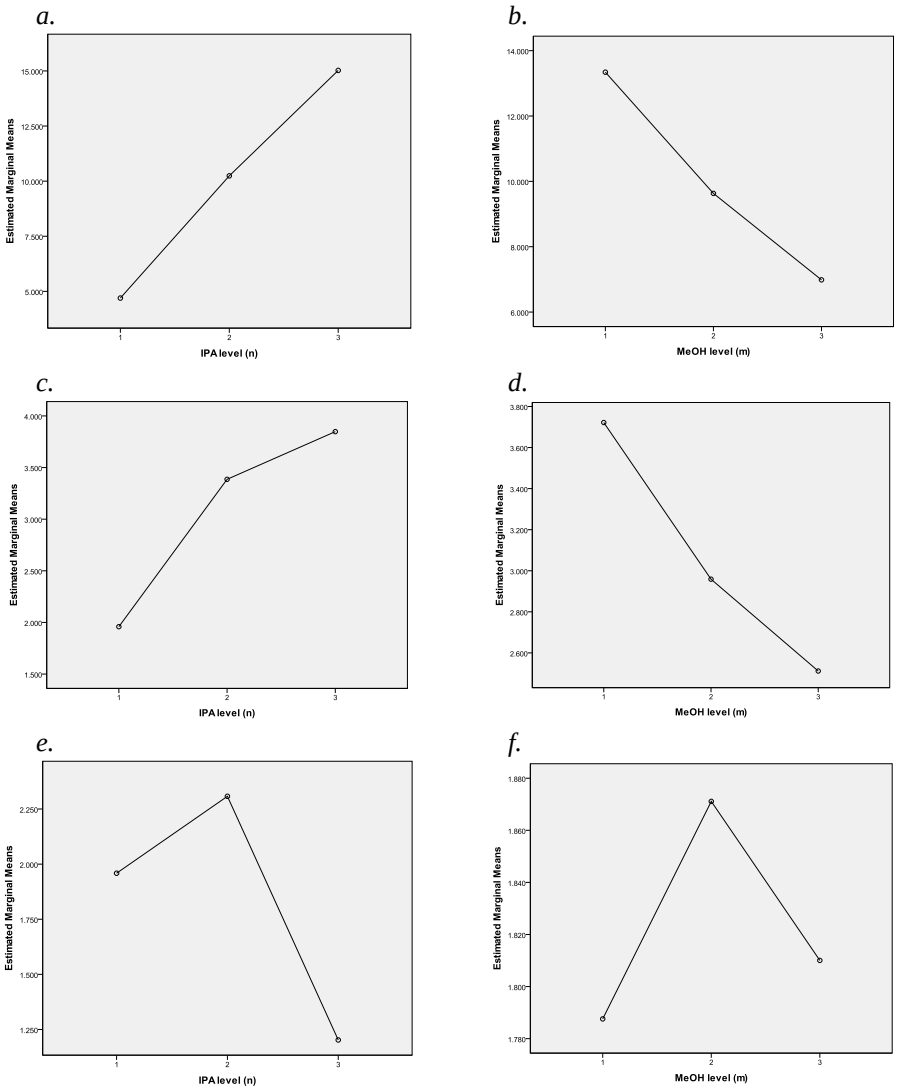


Figure 4.9. Profile Plots for Main Effects on Resolution. Main effects versus level of IPA and methanol, respectively for: a.-b. Δ^9-THC, c.-d.Δ^8-THC, e.-f. critical pair.

Based on literature data, resolution was predicted to be inversely related to alcohol content, particularly for % methanol. The profile plots for Δ^9-THC and Δ^8-THC in Figure 4.9 indicate that while resolution did diminish as methanol content increased, it improved with increasing IPA content. Data from the resolution homogeneous subsets for IPA (see Tables 3.42 and 4.2) revealed that all IPA levels were significantly different for the three pairs of interest, with the largest difference in main effect evident for Δ^9-THC (10.6 units). Like selectivity, resolution of the critical pair was best at 4% IPA and

lowest at 7% IPA and had the smallest measured difference between levels (1.1 units). According to post-hoc analyses (see Tables 3.43 and 4.2) all three levels of methanol were significantly different for the Δ^9-THC and Δ^8-THC pairs. The largest resolution main effect for methanol was on Δ^9-THC (6.4 units).

Additionally, the decrease in resolution due to increasing alcohol content was predicted to be more evident for Δ^8-THC than Δ^9-THC. For both selectivity and resolution, the largest difference due to IPA or methanol content was noted for Δ^9-THC, but this relationship followed a positive trend. Δ^8-THC selectivity and resolution did exhibit the largest significant decrease due to the main effects of the alcohols. However, a direct comparison of the alcohol effects across the levels studied here was not reliable, considering the actual molar concentrations of each alcohol. The IPA levels chosen for the factorial study did not mirror the methanol levels because the experimenter anticipated methanol's superior strength in this type of system.

Interaction Effects

Interaction effects were predicted to significantly impact resolution but not necessarily selectivity. The anticipated elution order reversal at 1% methanol was expected to effect resolution more noticeably than selectivity. Results from ANOVA indicated that the two-way methanol by IPA interaction term was significant (α_s=0.01) for retention time, selectivity, and resolution as summarized in Tables 3.26, 3.34, and 3.41, respectively. The absence of strictly parallel lines in the profile plots confirms this visually (see Figures 4.10-4.12).

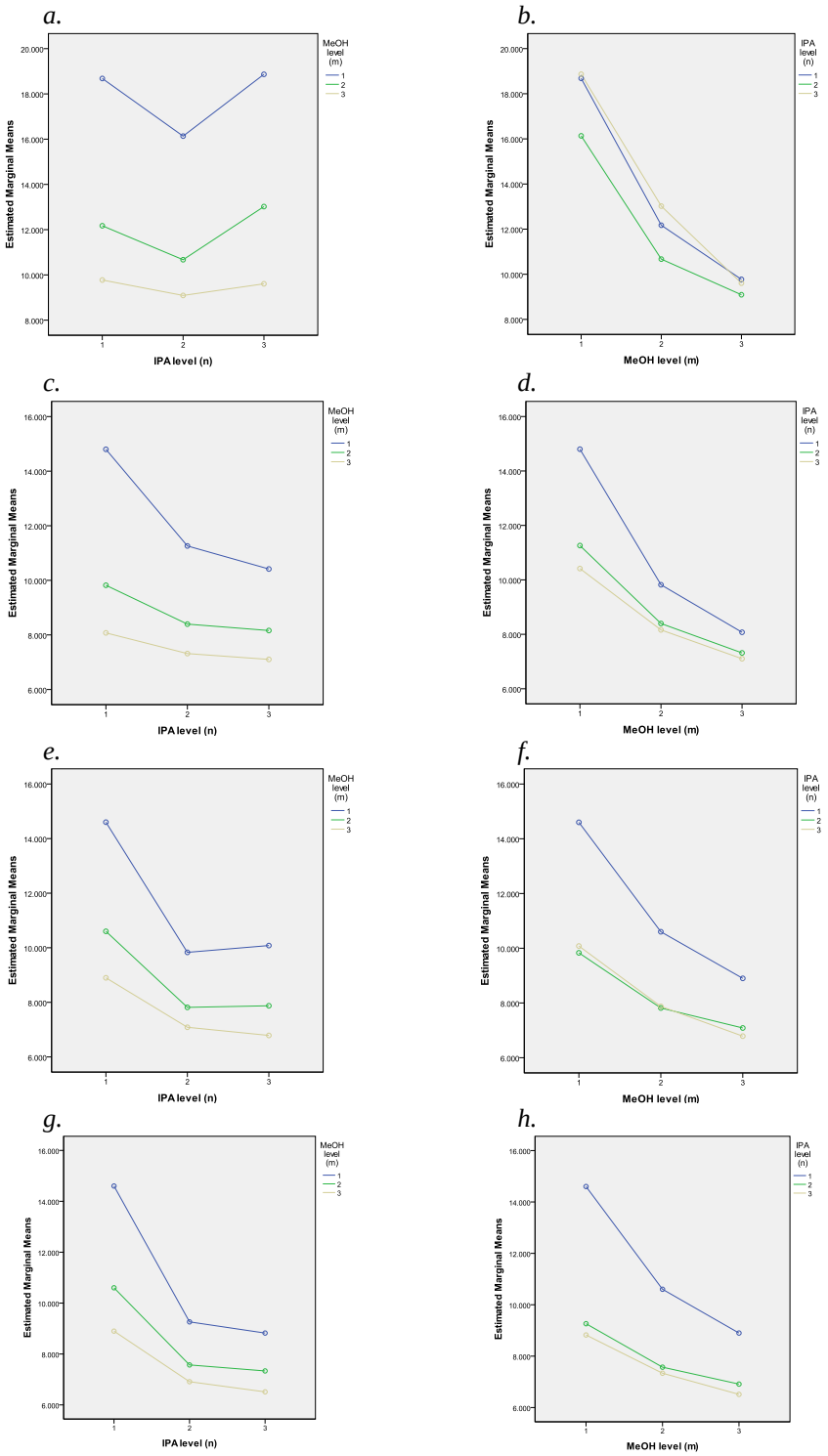


Figure 4.10. Profile Plots for Retention Time Results. Retention time results plotted for IPA by methanol, or methanol by IPA, respectively for: a.-b. (-)-Δ^9-THC, c.-d. (-)-Δ^8-THC, e.-f. (+)-Δ^9-THC, g.-h. (+)-Δ^8-THC.

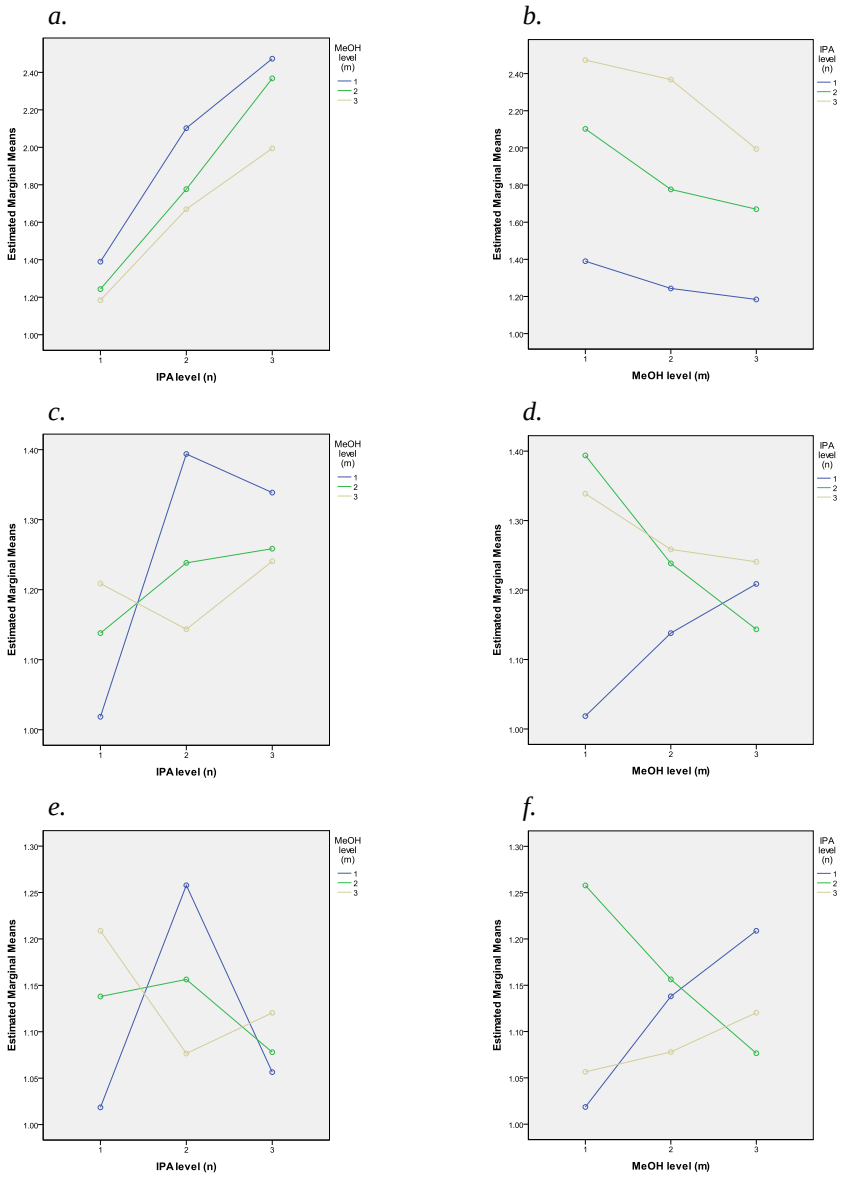


Figure 4.11. Profile Plots for Selectivity Results. Selectivity plotted for IPA by methanol, or methanol by IPA, respectively for: a.-b. Δ^9-THC, c.-d.Δ^8-THC, e.-f. critical pair.

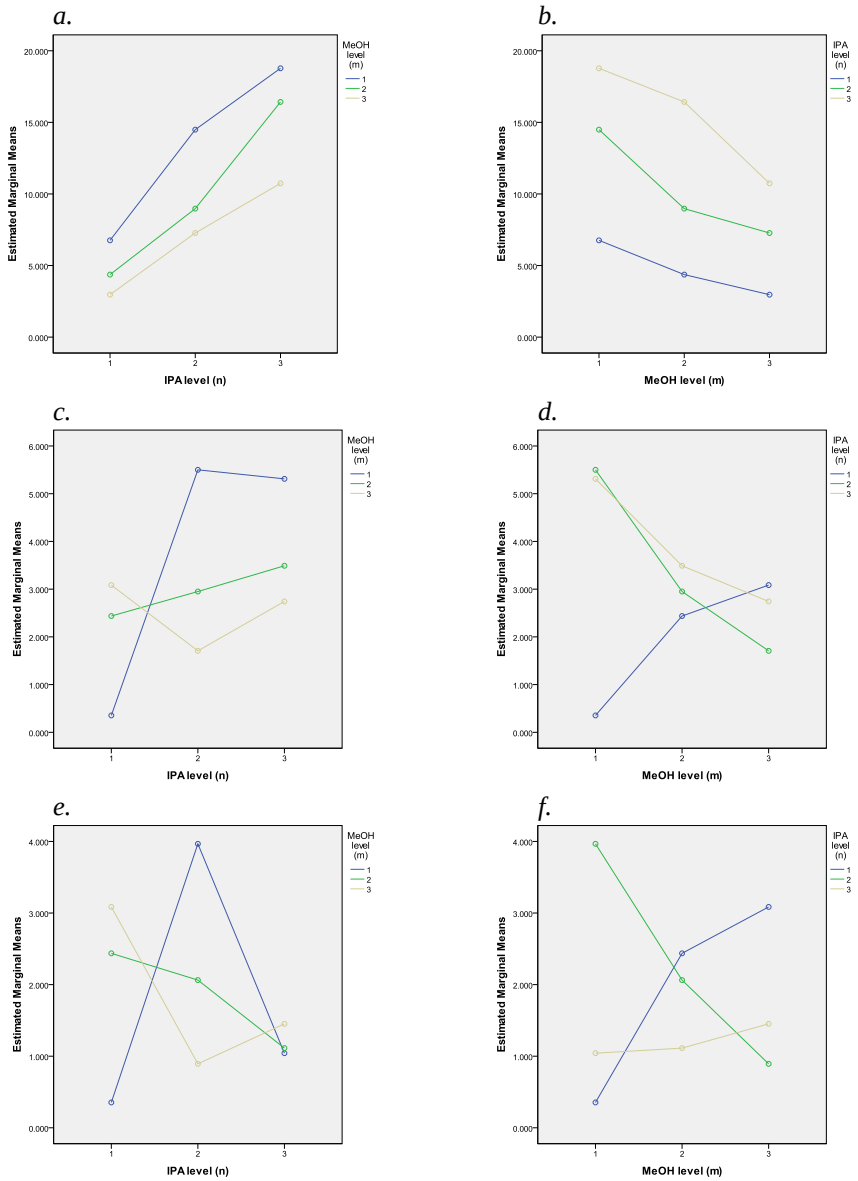


Figure 4.12. Profile Plots for Resolution Results. Resolution results plotted for IPA by methanol, or methanol by IPA, respectively for: a.-b. Δ^9-THC, c.-d.Δ^8-THC, e.-f. critical pair.

Strength of the Interaction Effect

Partial eta-squared values for the main and interaction effects are summarized for retention time, selectivity, and resolution in Table 4.3. Adjusted R^2 values for the model are included to aid in comparing the dependent variables. In each case where partial eta-squared was 10% or less, there was a failure to reject the null hypothesis, indicating that

few substantially weak relationships exhibited significant effects on the tested variables. However, in the case of (-)-Δ^9-THC retention time, both experiment and alcohol interaction terms were significant, but partial eta-squared indicated that only 14.1 and 20.0%, respectively, of the variation in these parameters was explained by either effect alone.

Table 4.3. Partial Eta-Squared Summarized for Dependent Variables.
η^2 summarized for dependent variables by effect for ANOVA at α_s=0.01. The adjusted R^2 is also provided for each analysis.

Dependent Variable		R^2	Main and Interaction Effects			
			Experiment	IPA	Methanol	IPA x Methanol
Retention Time	(-)-Δ^9-THC	.995	.141	.472	.943	.200
	(-)-Δ^8-THC	.999	.677	.958	.989	.901
	(+)-Δ^9-THC	.999	.472	.968	.974	.804
	(+)-Δ^8-THC	.999	.564	.985	.979	.908
α	Δ^9-THC	.998	.017*	.966	.795	.442
	Δ^8-THC	.999	.076*	.849	.364	.868
	Critical Pair	.999	.057*	.425	.066*	.743
Resolution	Δ^9-THC	.982	.016*	.896	.765	.288
	Δ^8-THC	.993	.240	.902	.778	.952
	Critical Pair	.953	.102**	.499	.006*	.830

**failed to reject null hypothesis; **Tukey's HSD post-hoc analysis indicated no significant difference between levels (α_s=0.01)*

The relationship between retention time and the alcohol interaction was weaker for (-)-Δ^9-THC (η^2=0.200) and (+)-Δ^9-THC (η^2=0.804) than for (+)-Δ^8-THC (η^2=0.908) and (-)-Δ^8-THC (η^2=0.901). The interaction effect described just 44.2% and 28.8% of variation for selectivity and resolution, respectively, of the Δ^9-THC pair, compared to 86.8% and 95.2% of the variation for the Δ^8-THC pair. Like Δ^8-THC, the selectivity and resolution of the critical pair was strongly related to the alcohol interaction (η^2=0.743 and 0.830, respectively).

Trend Analysis for the Interaction Effect

While Tukey's HSD and Scheffé's tests evaluated differences between levels for the experiment, IPA, and methanol factors, these tests did not provide information regarding the alcohol interaction effect. Interaction effects were anticipated, and therefore à priori ANOVA trend analyses were planned to elucidate polynomial trends. Refer to Tables 3.29, 3.37, and 3.45 for source tables and Table 4.2 for a summary. Conclusions for these analyses were mixed.

Results from retention time trend analyses indicated that for (-)-Δ^9-THC, quadratic IPA by linear methanol terms best fit the model ($p<0.001$). The interaction effect for retention time of the remaining analytes was best described with both a quadratic IPA by linear methanol term ($p<0.001$) as well as a linear IPA by quadratic methanol term ($p<0.001$). Rejection of the quadratic IPA by quadratic methanol term as a significantly better fit for (-)-Δ^8-THC ($p=0.028$), (+)-Δ^9-THC ($p=0.085$), and (+)-Δ^9-THC ($p=0.024$) was associated with a substantial amount of Type II risk (64.7, 80.4, and 62.2%, respectively). These conflicting results point to the quadratic by quadratic interaction term as the best fit for retention time of these analytes, but for error in the model or bias unaccounted for in the experimental design, only inconclusively.

Interaction effects on the selectivity of the Δ^8-THC pair followed the pattern of the retention time data, where both a quadratic IPA by linear methanol term ($p<0.001$) as well as a linear IPA by quadratic methanol term ($p=0.001$) provided a significantly improved fit, but not the quadratic by quadratic term ($p=0.048$ with a 72.5% risk of Type II error). Unlike retention time results, the quadratic by quadratic term was clearly indicated as a significantly better fit than lower order terms for selectivity of the Δ^9-THC

pair ($p<0.001$). Similar to retention time of (-)-Δ^9-THC, the interaction term for selectivity of the critical pair took the form of quadratic IPA by linear methanol ($p<0.001$). Surprisingly, a quadratic IPA by quadratic methanol interaction term best described the reduced model for resolution of the Δ^9-THC pair ($p=0.003$), the Δ^8-THC pair ($p<0.001$), and the critical pair ($p=0.001$). Surface areas graphs illustrate the different relationships between alcohol content and resolution of each pair of analytes (see Figures 4.13-4.15). These surface areas indicate that the resolution of the critical pair more closely resembles that of the Δ^8-THC pair.

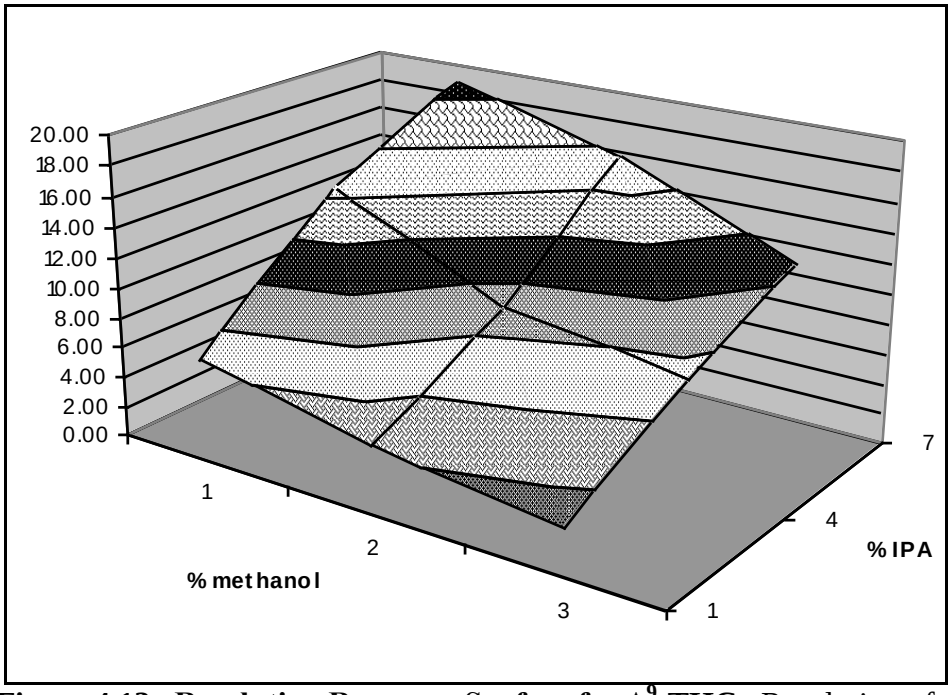


Figure 4.13. Resolution Response Surface for Δ^9-THC. Resolution of Δ^9-THC is illustrated by mobile phase levels.

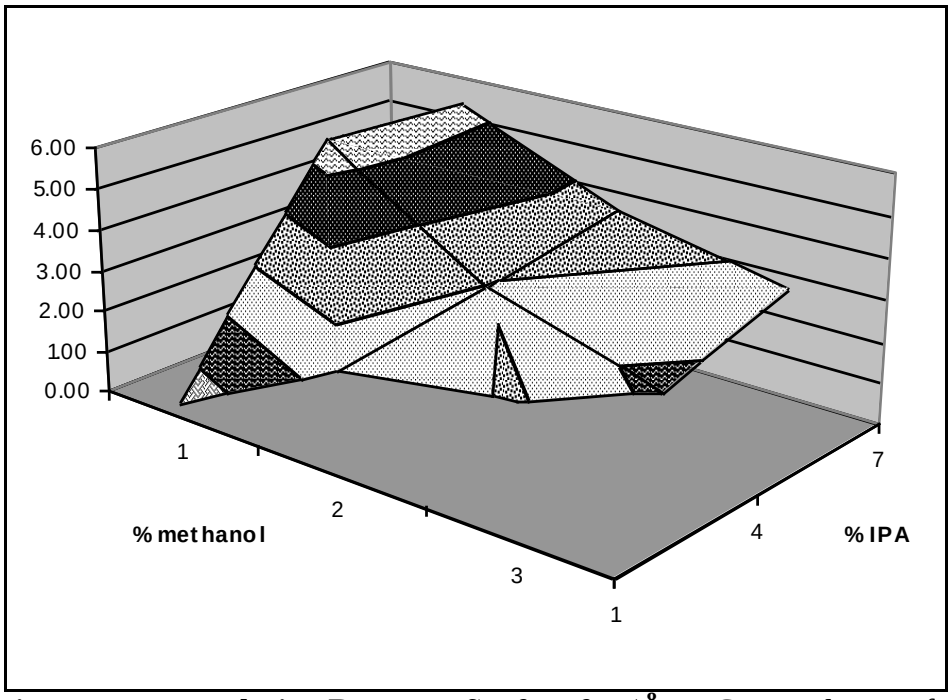


Figure 4.14. Resolution Response Surface for Δ^8-THC. Resolution of Δ^8-THC is illustrated by mobile phase levels.

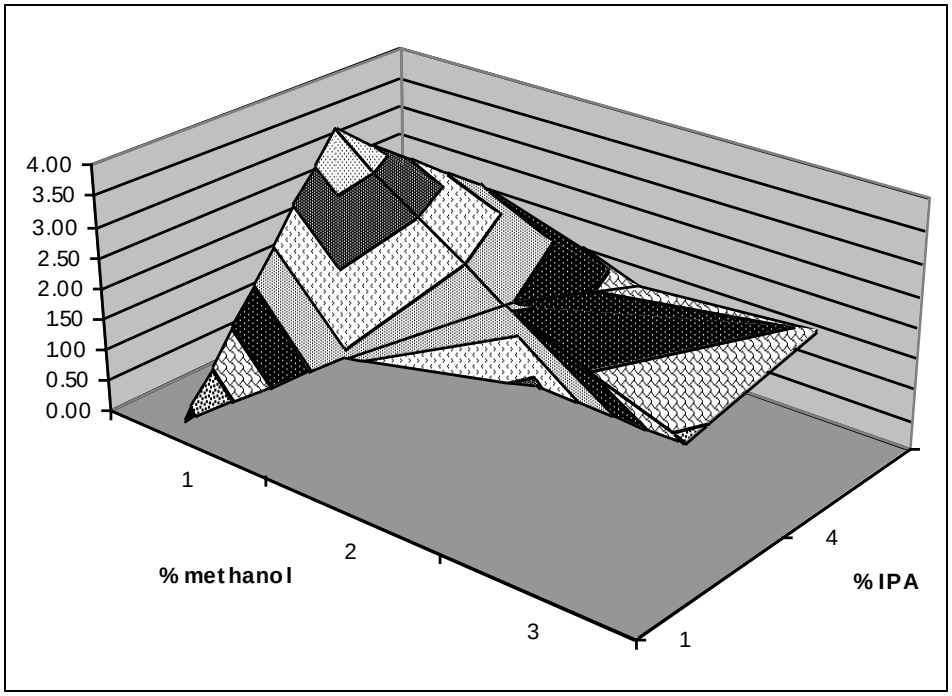


Figure 4.15. Resolution Response Surface for Critical Pair. Resolution of the critical pair is illustrated by mobile phase levels.

Elution Order Reversal for Δ^8-THC

Based on the literature, a reversal in elution order was predicted for Δ^8-THC enantiomers in mobile phases that contained 1% methanol. The elution order reversal did occur and under two conditions: 1% IPA with 2% methanol and 1% IPA with 3% methanol, signifying a potential change in the CSP's recognition of these two enantiomers. At 1% of each alcohol the Δ^8-THC enantiomers were partially resolved (elution order: (+)/(-)) in one of the three experiments (Table 3.21; resolution = 1.06), and otherwise co-eluted with (+)-Δ^9-THC (see Figures 3.1-3.3). Though the elution reversal resulted in critical pair resolutions greater than 2.2, the elution of (-)-Δ^8-THC prior to (+)-Δ^9-THC makes the application of this mobile phase questionable with regard to Dronabinol samples wherein the (+)-Δ^9-THC impurity peak could be lost or obfuscated by the tail of the (-)-Δ^8-THC impurity peak. The elution order reversal in the 1% IPA with 3% methanol mobile phase resulted in critical pair selectivity (1.21) greater than that of the Δ^9-THC pair (1.18).

Alcohol Molar Ratio Graphs

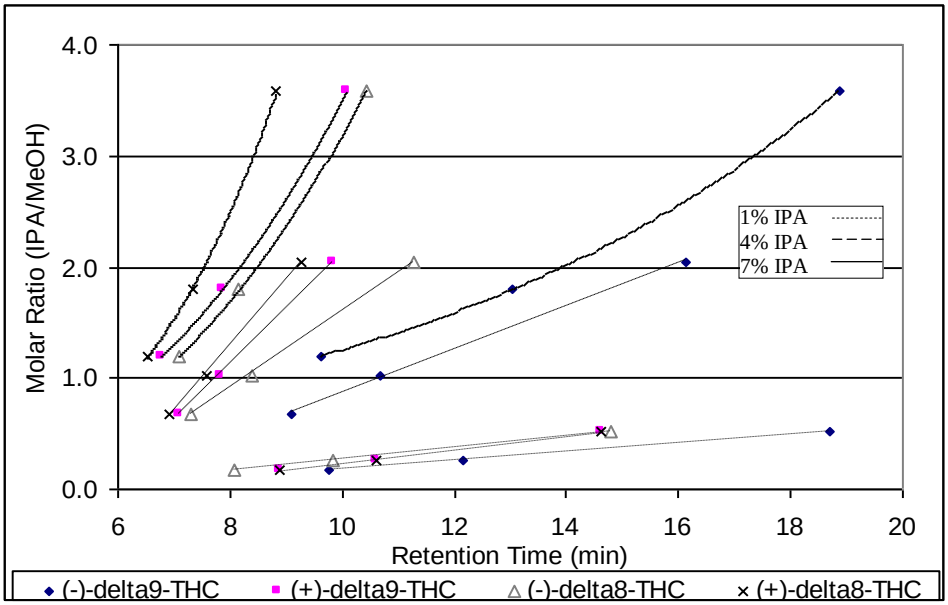
To investigate the effects of the alcohols in terms of molar concentrations, retention time, selectivity and resolution data were plotted versus the molar ratio of mobile phase alcohol content. The methanol-to-IPA molar ratio ranged from 0.28 to 5.85 in the mobile phase, whereas the IPA-to-methanol molar ratio ranged from 0.17 to 3.59. Trends were noted by levels of each alcohol. These qualitative analyses were used to further scrutinize and describe the chiral discrimination afforded by ADMPC.

Retention Time versus Alcohol Ratio

Retention times for each analyte versus the molar ratio of mobile phase alcohol content were plotted by IPA level, as shown in Figure 4.16. In the graphs, which depict inverses of the same retention time information, the relationship between retention time and either molar ratio appeared to approach a linear function by level of IPA. In general, the graph supports the inverse relationship between retention time and alcohol content, with the main exception of (-)-Δ^9-THC at 7% IPA.

As seen in Figure 4.16a, where IPA was favored (molar ratio of IPA:methanol > 1), the retention time for (+)-Δ^9-THC was relatively unchanged between the 4 and 7% IPA levels at either 1 or 2% methanol. This observation agrees with the homogeneous subsets results from the post hoc analysis of (+)-Δ^9-THC retention time for IPA (see Table 4.2). At 7% IPA, the critical pair exhibited especially similar, but not identical, retention times across the levels of methanol. In this case, some level of discrimination of these diastereomers occurred that was independent of the change in methanol content, as supported by the ANOVA results for a lack of significant methanol effect on selectivity or resolution of the critical pair (see Tables 3.34, 3.41, and 4.2). In contrast, at 4% IPA the discrimination of the critical pair changed noticeably with methanol content. That is, while (+)-Δ^9-THC retention time was essentially the same in 4 and 7% IPA where IPA was favored, the discrimination of this analyte from Δ^8-THC enantiomers changed noticeably.

a.



b.

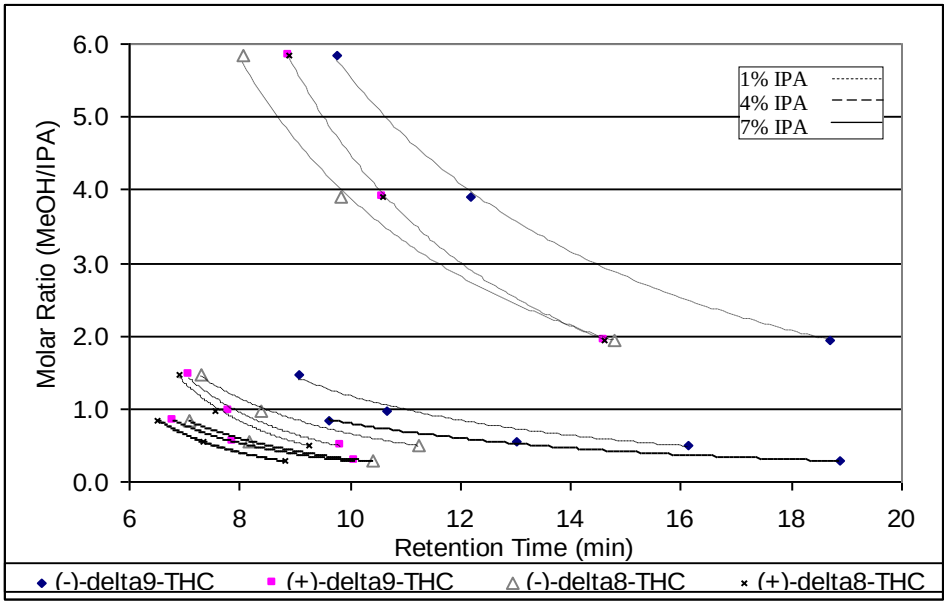


Figure 4.16. Retention Time Versus Alcohol Ratio by IPA Level. Retention time of each analyte versus alcohol ratio for: a. methanol/IPA, b. IPA/methanol, where MeOH = methanol.

As illustrated in Figure 4.16b, in solutions where methanol was favored (molar ratio of methanol:IPA > 1), the (+)-enantiomers had similar retention times and co-eluted at 1% IPA. This trend is more interesting when considered in light of (-)-Δ^8-THC retention. Though the (+)-enantiomers co-eluted, the extent of chiral discrimination

between (-)-Δ^8-THC and the (+)-Δ^8-THC improved with increasing methanol content in 1% IPA, favoring the retention of the (+)-enantiomers (as well as the elution order reversal). This decrease in the retention of (-)-Δ^8-THC compared to the (+)-enantiomers in 1% IPA suggests that the increasing methanol preferentially blocked or distorted the chiral cavity such that (-)-Δ^8-THC was excluded.

A closer look at Figure 4.16b indicates that the retention of Δ^8-THC also appeared to follow a somewhat linear trend with respect to the methanol:IPA molar ratio by methanol level (see Figure 4.17). Δ^8-THC retention time decreased with the ratio, regardless of which alcohol was in excess, indicating a strong relationship between methanol content and the retention time of both Δ^8-THC enantiomers, which is supported by ANOVA results (see Tables 3.26 and 4.3). According to the crossed trend lines, which clearly delineate the elution order reversal, somewhere between 1 and 4% IPA at a molar ratio of 2.5-3 in favor of methanol, the Δ^8-THC enantiomers would likely co-elute.

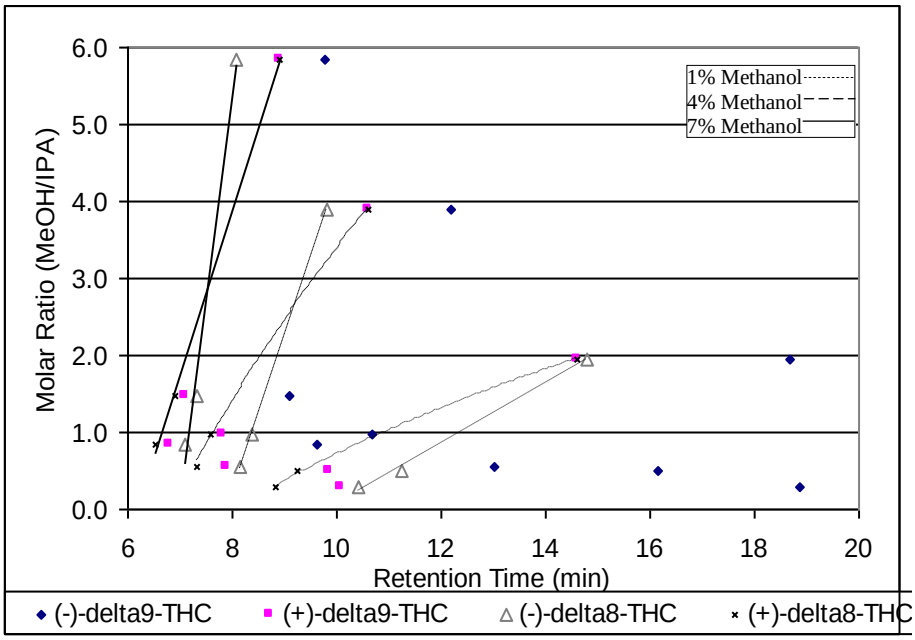


Figure 4.17. Retention Time Versus Alcohol Ratio by Methanol Level. Retention time of each analyte versus alcohol ratio for methanol/IPA.

Retention time results suggest that as a general trend, the smaller, higher polarity methanol more efficiently disrupted associations between the (-)-enantiomers and the chiral stationary phase. This methanol effect on retention is clearly illustrated in Figure 7.16b at 1% IPA, with further evidence of this effect noted in Figure 7.16a. When IPA was favored, the (+)-Δ^9-THC retention was unchanged by IPA, but decreased by methanol, with the corresponding effect on (-)-Δ^8-THC larger than that for (+)-Δ^8-THC.

Selectivity and Resolution versus Alcohol Ratio

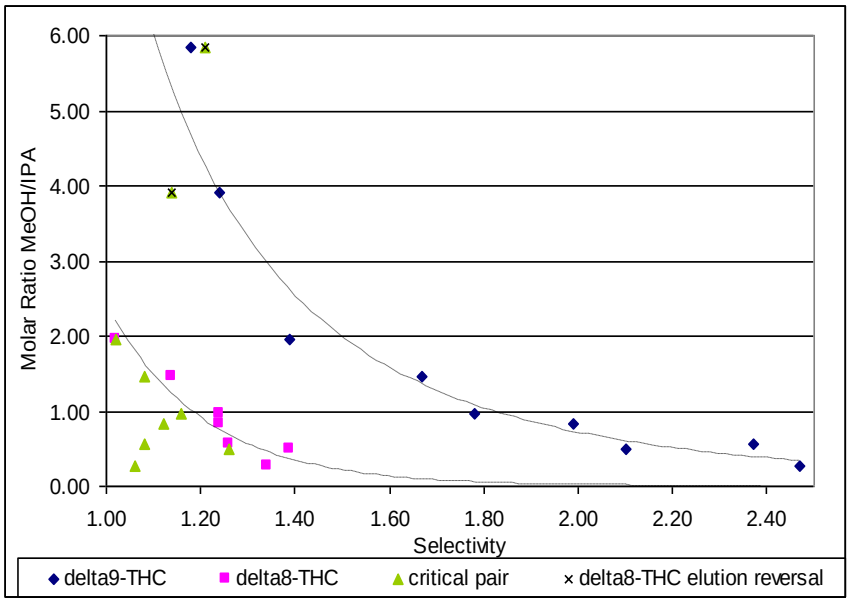
Selectivity and resolution data were plotted versus molar ratios of the alcohols in the mobile phases (see Figure 4.18 and 4.19). The trends in these graphs indicate that the selectivity and resolution of Δ^9-THC and Δ^8-THC were closely related to the ratios of these low alcohol concentrations, excluding the elution order reversal. On a molar ratio basis, higher relative concentrations of IPA were associated with higher selectivity and resolution, especially for Δ^9-THC. The opposite was true for methanol where higher relative concentrations were associated with lower selectivity and resolution for Δ^9-THC.

Enantioseparation of Δ^9-THC and Δ^8-THC

Reproducible chiral discrimination of varying degrees was achieved on Chiralpak ADMPC with 1-7% IPA and 1-3% methanol under prescribed chromatographic conditions. In general, the retention of the (-)-enantiomers was favored over the (+)-enantiomers under all conditions except for the elution order reversal conditions. Analyte retention time was inversely related to alcohol content except for Δ^9-THC enantiomers in 7% IPA. Methanol was stronger than IPA in this experiment, and appeared to more efficiently displace (-)-enantiomers as well as Δ^8-THC from ADMPC versus (+)-

enantiomers and Δ^9-THC, respectively. The methanol effect was statistically significant in all cases except for the selectivity and resolution of the critical pair.

a.



b.

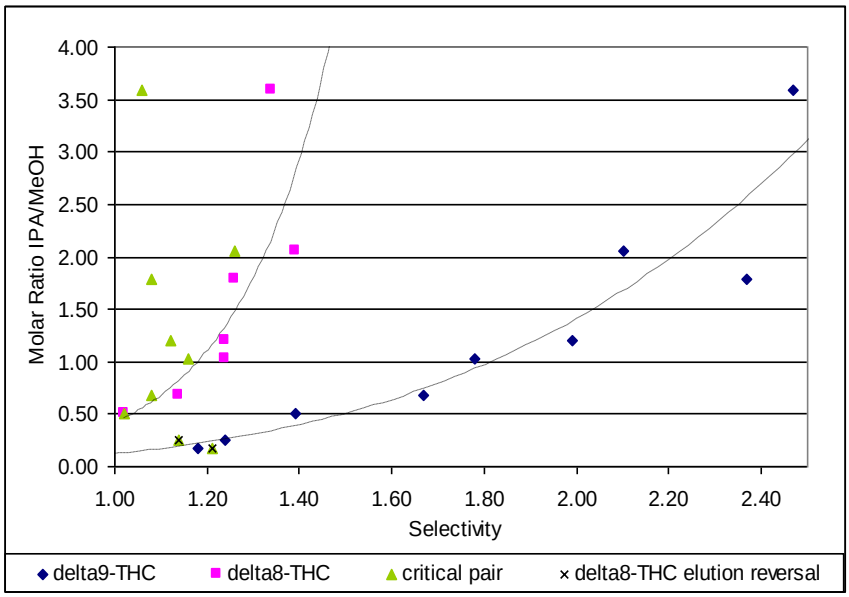
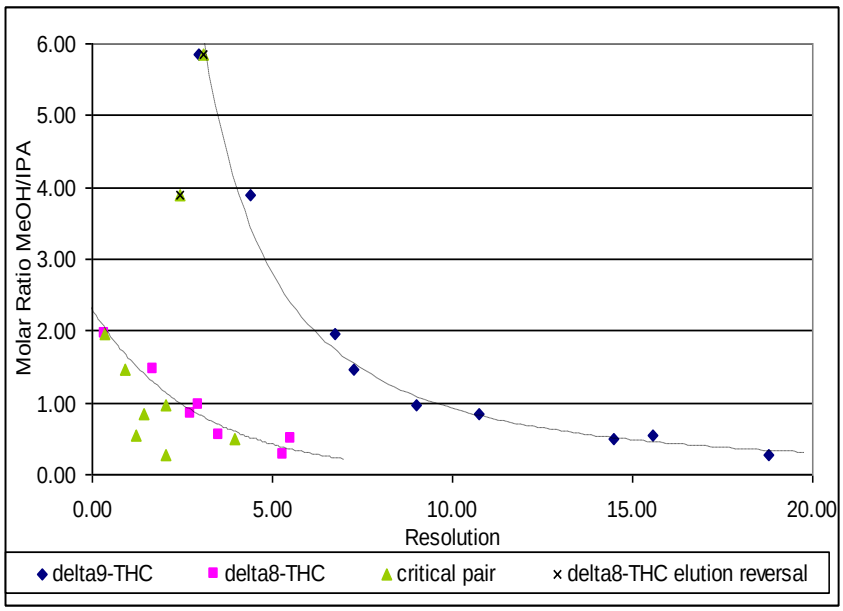


Figure 4.18. Selectivity Versus Alcohol Ratio by IPA Level. Selectivity of each pair versus alcohol ratio for: a. methanol/IPA, b. IPA/methanol.

a.



b.

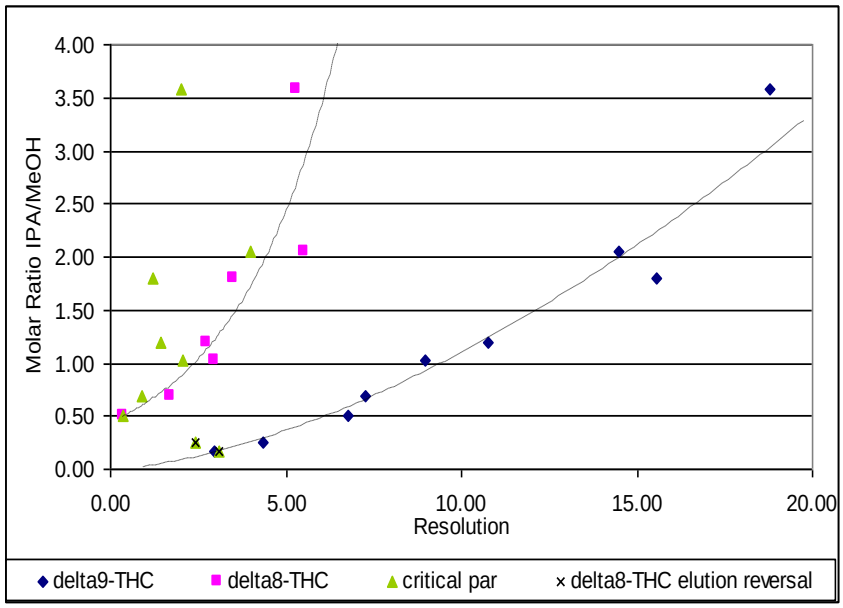


Figure 4.19. Resolution Versus Alcohol Ratio by IPA Level. Resolution of each pair versus alcohol ratio for: a. methanol/IPA, b. IPA/methanol.

The alcohol interaction effect was statistically significant in all cases, and for resolution was best described with quadratic IPA and methanol terms. Partial eta-squared values indicated that interaction effect was more strongly related to the resolution of the critical pair and Δ^8-THC, than Δ^9-THC. This statistical trend is reflected by the response

surfaces where Δ^9-THC resolution looks relatively flat compared to response surfaces for the other pairs. These response surfaces also indicate that the resolution of the critical pair more closely resembled that of Δ^8-THC. Of the three pairs evaluated, the enantioseparation of Δ^9-THC was best and the critical pair the worst, excluding the elution order reversal. Enantioseparations of Δ^9-THC and Δ^8-THC improved as the molar ratio of IPA-to-methanol increased, except where Δ^8-THC's elution order reversed.

Specifically, peak resolution was greater than 2.8 for Δ^9-THC in all cases (selectivity $\geq$ 1.18) and, excluding 1% IPA with 1% methanol, greater than 1.4 for Δ^8-THC (selectivity $\geq$ 1.12). Resolution greater than 1.6 was attained consistently for the critical pair under the following conditions: 4% IPA with 1-3% methanol and 1% IPA with 2% methanol (selectivity $\geq$ 1.12). An elution order reversal of the Δ^8-THC enantiomers occurred at 1% IPA with 2% methanol and 1% IPA with 3% methanol. Excluding the elution reversal conditions, Δ^9-THC had a resolution greater than 5.0 in all other mobile phases.

With unique exceptions in many cases, generalized retention characteristics were summarized according to the descriptive evaluation of the data as supported by the statistical results. While retention behaviors observed here are directly applicable in the chiral analysis of Dronabinol and its three related impurities, generalizations to a larger body of molecules is yet unknown. Further investigations involving column temperature on the chiral discrimination could result in greatly improved resolution of the critical pair. To allow a wider base for generalizations regarding the chiral mechanisms afforded under these conditions, related chiral cannabinoids or synthetic intermediates could be investigated.

CHAPTER V

CONCLUSIONS

Δ^9-THC and Δ^8-THC enantiomers were successfully separated on ADMPC using various mobile phases containing mixtures of 1-3% methanol and 1-7% IPA by volume in n-heptane under typical chromatographic conditions. These low level alcohol mixtures resulted in significant differences in the selectivity and resolution of these regioisomers, supporting further optimization of the mobile phase concentration for resolution of the critical pair. Statistical analyses revealed that the interaction between these alcohols contributes significantly to the discrimination between the analytes on ADMPC. An elution order reversal was noted for Δ^8-THC in 1% IPA with 2-3% methanol, including one case where the selectivity of this pair surpassed that of Δ^9-THC.

The Agilent LC's automated solvent delivery and mixing capability facilitated the investigation of nine mobile phases by mixing different proportions of the four manually prepared solutions. This use of the instrument allowed a much more efficient approach to investigating the alcohol mobile phase modifiers, resulting in far fewer analyst hours spent preparing and changing out mobile phases on the instrument as well as overall reduced experiment times.

APPENDIX A

SUMMARY TABLES BY EXPERIMENTAL SETS

Table A.1. Retention Time Summary for Experimental Set 1.
Retention time (t_r) in minutes and width at half height ($W_{h/2}$) data were provided by the software. Void time (t_v) in minutes was determined from the blank chromatograms.

Mobile Phase (n,m)		(+)-Δ^8-THC		(-)-Δ^8-THC		(+)-Δ^9-THC		(-)-Δ^9-THC	
	t_v	t_r	$W_{h/2}$	t_r	$W_{h/2}$	t_r	$W_{h/2}$	t_r	$W_{h/2}$
(1,1)	4.147	14.233	0.330	14.233	0.330	14.233	0.330	18.157	0.333
	4.147	14.245	0.327	14.245	0.327	14.245	0.327	18.151	0.333
	4.147	14.267	0.337	14.267	0.337	14.267	0.337	18.138	0.333
	4.147	14.267	0.333	14.267	0.333	14.267	0.333	18.130	0.333
(2,1)	4.153	9.531	0.198	11.080	0.198	10.304	0.198	18.152	0.333
	4.153	9.529	0.200	11.077	0.198	10.299	0.198	18.138	0.337
	4.153	9.515	0.198	11.068	0.196	10.281	0.198	18.108	0.333
	4.153	9.529	0.198	11.080	0.198	10.295	0.198	18.120	0.337
(3,1)	4.117	8.472	0.153	9.942	0.187	9.616	0.176	17.728	0.343
	4.117	8.484	0.153	9.954	0.187	9.634	0.177	17.745	0.343
	4.117	8.487	0.153	9.965	0.188	9.637	0.176	17.768	0.347
	4.117	8.497	0.153	9.977	0.187	9.651	0.177	17.791	0.343
(1,2)	4.130	9.901	0.184	9.136	0.158	9.901	0.184	11.361	0.202
	4.130	9.933	0.182	9.171	0.160	9.933	0.182	11.400	0.202
	4.130	10.001	0.189	9.231	0.163	10.001	0.189	11.475	0.204
	4.130	10.024	0.189	9.241	0.165	10.024	0.189	11.497	0.207
(2,2)	4.123	7.381	0.158	8.076	0.143	7.654	0.153	10.730	0.202
	4.123	7.406	0.161	8.116	0.145	7.680	0.156	10.782	0.202
	4.123	7.400	0.162	8.118	0.145	7.670	0.157	10.748	0.202
	4.123	7.404	0.162	8.125	0.142	7.675	0.162	10.745	0.202
(3,2)	4.127	6.887	0.129	7.630	0.131	7.314	0.133	11.187	0.297
	4.127	6.894	0.129	7.642	0.135	7.325	0.133	11.520	0.211
	4.127	6.917	0.131	7.666	0.136	7.356	0.133	11.615	0.213

Table A.1. Continued.

Mobile Phase (n,m)		(+)-Δ^8-THC		(-)-Δ^8-THC		(+)-Δ^9-THC		(-)-Δ^9-THC	
	t_v	t_r	$W_{h/2}$	t_r	$W_{h/2}$	t_r	$W_{h/2}$	t_r	$W_{h/2}$
(3,2)	4.127	6.922	0.129	7.668	0.133	7.363	0.133	11.527	0.287
(1,3)	4.133	8.665	0.167	7.864	0.137	8.665	0.167	9.515	0.171
	4.133	8.665	0.167	7.866	0.137	8.665	0.167	9.514	0.169
	4.133	8.673	0.167	7.875	0.137	8.673	0.167	9.516	0.169
	4.133	8.693	0.167	7.893	0.137	8.693	0.167	9.542	0.171
(2,3)	4.107	6.614	0.111	6.941	0.156	6.762	n.d.	8.348	0.150
	4.107	6.626	0.113	6.961	0.148	6.776	0.174	8.372	0.150
	4.107	6.660	0.123	7.011	0.139	6.812	0.139	8.436	0.153
	4.107	6.659	0.116	7.009	0.140	6.810	0.166	8.431	0.153
(3,3)	4.017	6.168	0.128	6.727	0.121	6.368	0.132	8.417	0.155
	4.017	6.183	0.128	6.745	0.123	6.385	0.132	8.461	0.157
	4.017	6.210	0.128	6.779	0.121	6.417	0.132	8.547	0.158
	4.017	6.210	0.127	6.781	0.123	6.418	0.132	8.557	0.158

Note: n.d. = not determined, whereas the parameter could not be calculated by the software

Table A.2. Retention Time Summary for Experimental Set 2.
Retention time (t_r) in minutes and width at half height ($W_{h/2}$) data were provided by the software. Void time (t_v) in minutes was determined from the blank chromatograms.

Mobile Phase (n,m)		(+)-Δ^8-THC		(-)-Δ^8-THC		(+)-Δ^9-THC		(-)-Δ^9-THC	
	t_v	t_r	$W_{h/2}$	t_r	$W_{h/2}$	t_r	$W_{h/2}$	t_r	$W_{h/2}$
(1,1)	4.147	14.607	0.417	14.607	0.417	14.607	0.417	18.046	0.330
	4.147	14.521	0.417	14.521	0.417	14.521	0.417	17.861	0.327
	4.147	14.739	0.413	14.739	0.413	14.739	0.413	18.202	0.337
	4.147	14.780	0.403	14.780	0.403	14.780	0.403	18.263	0.337
(2,1)	4.153	9.082	0.228	11.482	0.224	9.488	0.222	14.477	0.277
	4.153	9.076	0.230	11.477	0.222	9.484	0.222	14.481	0.277
	4.153	9.084	0.230	11.484	0.222	9.493	0.222	14.512	0.277
	4.153	9.086	0.228	11.485	0.224	9.495	0.220	14.517	0.277
(3,1)	4.117	9.245	0.165	10.930	0.203	10.581	0.193	20.221	0.390
	4.117	9.254	0.167	10.949	0.205	10.597	0.193	20.252	0.393
	4.117	9.275	0.165	10.986	0.205	10.632	0.195	20.310	0.393
	4.117	9.284	0.167	11.002	0.203	10.645	0.195	20.347	0.393

Table A.2. Continued.

Mobile Phase (n,m)	t_v	(+)-Δ^8-THC		(-)-Δ^8-THC		(+)-Δ^9-THC		(-)-Δ^9-THC	
		t_r	$W_{h/2}$	t_r	$W_{h/2}$	t_r	$W_{h/2}$	t_r	$W_{h/2}$
(1,2)	4.130	11.280	0.227	10.412	0.182	11.280	0.227	12.916	0.236
	4.130	11.276	0.227	10.406	0.184	11.276	0.227	12.909	0.236
	4.130	11.269	0.229	10.399	0.182	11.269	0.229	12.897	0.231
	4.130	11.283	0.227	10.415	0.182	11.283	0.227	12.914	0.233
(2,2)	4.123	7.783	0.187	8.718	0.160	8.021	0.187	10.614	0.196
	4.123	7.788	0.188	8.725	0.162	8.025	0.188	10.626	0.198
	4.123	7.796	0.188	8.743	0.162	8.034	0.187	10.656	0.196
	4.123	7.797	0.188	8.747	0.160	8.035	0.185	10.665	0.198
(3,2)	4.127	7.661	0.140	8.550	0.148	8.238	0.145	13.903	0.251
	4.127	7.664	0.140	8.553	0.148	8.242	0.146	13.921	0.251
	4.127	7.675	0.140	8.567	0.149	8.256	0.146	13.963	0.251
	4.127	7.677	0.140	8.570	0.149	8.260	0.147	13.971	0.251
(1,3)	4.133	9.309	0.187	8.410	0.145	9.309	0.187	10.236	0.180
	4.133	9.309	0.187	8.409	0.145	9.309	0.187	10.232	0.182
	4.133	9.309	0.191	8.406	0.145	9.309	0.191	10.223	0.182
	4.133	9.316	0.189	8.411	0.145	9.316	0.189	10.227	0.180
(2,3)	4.107	7.233	0.155	7.697	0.141	7.427	0.165	9.739	0.178
	4.107	7.234	0.155	7.697	0.141	7.427	0.164	9.736	0.176
	4.107	7.233	0.154	7.697	0.141	7.425	0.167	9.726	0.176
	4.107	7.234	0.154	7.697	0.141	7.425	0.167	9.723	0.176
(3,3)	4.017	7.062	0.128	7.690	0.135	7.459	0.133	11.541	0.204
	4.017	7.060	0.128	7.687	0.136	7.457	0.133	11.534	0.204
	4.017	7.053	0.128	7.679	0.136	7.448	0.133	11.512	0.202
	4.017	7.051	0.129	7.677	0.136	7.445	0.133	11.505	0.202

Table A.3. Retention Time Summary for Experimental Set 3.
Retention time (t_r) in minutes and width at half height ($W_{h/2}$) data were provided by the software. Void time (t_v) in minutes was determined from the blank chromatograms.

Mobile Phase (n,m)		(+)-Δ^8-THC		(-)-Δ^8-THC		(+)-Δ^9-THC		(-)-Δ^9-THC	
	t_v	t_r	$W_{h/2}$	t_r	$W_{h/2}$	t_r	$W_{h/2}$	t_r	$W_{h/2}$
(1,1)	4.147	14.694	0.356	15.292	0.300	14.694	0.356	19.634	0.367
	4.147	14.761	0.358	15.355	0.302	14.761	0.358	19.694	0.367
	4.147	15.045	0.358	15.646	0.309	15.045	0.358	19.960	0.370
	4.147	15.048	0.358	15.647	0.309	15.048	0.358	19.942	0.370
(2,1)	4.153	9.157	0.217	11.202	0.207	9.679	0.207	15.683	0.290
	4.153	9.175	0.218	11.220	0.207	9.700	0.207	15.741	0.290
	4.153	9.199	0.218	11.238	0.207	9.732	0.207	15.839	0.293
	4.153	9.190	0.218	11.228	0.209	9.724	0.204	15.842	0.290
(3,1)	4.117	8.720	0.155	10.317	0.192	9.992	0.181	18.552	0.360
	4.117	8.713	0.155	10.312	0.192	9.982	0.181	18.545	0.360
	4.117	8.724	0.155	10.326	0.193	9.997	0.183	18.564	0.363
	4.117	8.724	0.155	10.328	0.192	9.999	0.183	18.566	0.360
(1,2)	4.130	10.573	0.202	9.864	0.175	10.573	0.202	12.188	0.218
	4.130	10.567	0.202	9.858	0.175	10.567	0.202	12.180	0.216
	4.130	10.561	0.204	9.854	0.173	10.561	0.204	12.169	0.218
	4.130	10.559	0.204	9.851	0.173	10.559	0.204	12.164	0.218
(2,2)	4.123	7.520	0.179	8.338	0.152	7.745	0.185	10.611	0.198
	4.123	7.521	0.179	8.341	0.152	7.746	0.185	10.615	0.198
	4.123	7.528	0.179	8.354	0.152	7.753	0.185	10.631	0.200
	4.123	7.527	0.179	8.355	0.152	7.752	0.184	10.632	0.198
(3,2)	4.127	7.423	0.130	8.263	0.148	8.023	0.142	13.625	0.244
	4.127	7.425	0.132	8.267	0.148	8.027	0.142	13.640	0.247
	4.127	7.436	0.132	8.280	0.149	8.041	0.142	13.685	0.249
	4.127	7.437	0.132	8.281	0.149	8.042	0.142	13.690	0.247
(1,3)	4.133	8.712	0.169	7.943	0.135	8.712	0.169	9.581	0.169
	4.133	8.713	0.169	7.943	0.137	8.713	0.169	9.580	0.169
	4.133	8.703	0.164	7.932	0.137	8.703	0.164	9.555	0.169
	4.133	8.717	0.169	7.944	0.137	8.717	0.169	9.573	0.169

Table A.3. Continued.

Mobile Phase (n,m)	t_v	(+)-Δ^8-THC		(-)-Δ^8-THC		(+)-Δ^9-THC		(-)-Δ^9-THC	
		t_r	$W_{h/2}$	t_r	$W_{h/2}$	t_r	$W_{h/2}$	t_r	$W_{h/2}$
(2,3)	4.107	6.865	0.146	7.275	0.136	7.046	0.155	9.162	0.167
	4.107	6.861	0.145	7.270	0.136	7.041	0.156	9.151	0.167
	4.107	6.864	0.139	7.274	0.136	7.043	0.158	9.145	0.167
	4.107	6.862	0.140	7.272	0.137	7.041	0.159	9.140	0.167
(3,3)	4.017	6.284	0.124	6.863	0.121	6.509	0.127	8.786	0.158
	4.017	6.287	0.124	6.866	0.123	6.512	0.128	8.795	0.158
	4.017	6.290	0.124	6.870	0.123	6.516	0.127	8.811	0.160
	4.017	6.291	0.124	6.871	0.121	6.516	0.127	8.814	0.158

APPENDIX B

SUMMARY TABLES FOR DERIVED DATA

Table B.1. Capacity Factor Summary.
The capacity factor, k', was calculated from the void time (t_v) and retention time (t_r) for each analyte.

Mobile Phase (n,m)	(+)-Δ^8-THC			(-)-Δ^8-THC			(+)-Δ^9-THC			(-)-Δ^9-THC		
	Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 3
(1,1)	2.432	2.523	2.544	2.432	2.523	2.688	2.432	2.523	2.544	3.379	3.352	3.735
	2.435	2.502	2.560	2.435	2.502	2.703	2.435	2.502	2.560	3.377	3.307	3.749
	2.441	2.554	2.628	2.441	2.554	2.773	2.441	2.554	2.628	3.374	3.390	3.814
	2.441	2.564	2.629	2.441	2.564	2.773	2.441	2.564	2.629	3.372	3.404	3.809
(2,1)	1.295	1.187	1.205	1.668	1.765	1.697	1.481	1.284	1.330	3.370	2.486	2.776
	1.294	1.185	1.209	1.667	1.763	1.701	1.480	1.283	1.335	3.367	2.487	2.790
	1.291	1.187	1.215	1.665	1.765	1.706	1.475	1.286	1.343	3.360	2.494	2.814
	1.294	1.188	1.213	1.668	1.765	1.703	1.479	1.286	1.341	3.363	2.495	2.814
(3,1)	1.058	1.246	1.118	1.415	1.655	1.506	1.336	1.570	1.427	3.306	3.912	3.507
	1.061	1.248	1.117	1.418	1.660	1.505	1.340	1.574	1.425	3.311	3.920	3.505
	1.062	1.253	1.119	1.421	1.669	1.508	1.341	1.583	1.428	3.316	3.934	3.509
	1.064	1.255	1.119	1.424	1.673	1.509	1.344	1.586	1.429	3.322	3.943	3.510
(1,2)	1.397	1.731	1.560	1.212	1.521	1.388	1.397	1.731	1.560	1.751	2.127	1.951
	1.405	1.730	1.559	1.221	1.520	1.387	1.405	1.730	1.559	1.760	2.126	1.949
	1.422	1.729	1.557	1.235	1.518	1.386	1.422	1.729	1.557	1.778	2.123	1.946
	1.427	1.732	1.557	1.238	1.522	1.385	1.427	1.732	1.557	1.784	2.127	1.945
(2,2)	0.790	0.888	0.824	0.959	1.114	1.022	0.856	0.945	0.878	1.602	1.574	1.573
	0.796	0.889	0.824	0.968	1.116	1.023	0.863	0.946	0.879	1.615	1.577	1.574
	0.795	0.891	0.826	0.969	1.120	1.026	0.860	0.948	0.880	1.607	1.584	1.578
	0.796	0.891	0.825	0.970	1.121	1.026	0.861	0.949	0.880	1.606	1.586	1.578

Table B.1. Continued.

Mobile Phase (n,m)	(+)-Δ^8-THC			(-)-Δ^8-THC			(+)-Δ^9-THC			(-)-Δ^9-THC		
	Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 3
(3,2)	0.669	0.856	0.799	0.849	1.072	1.002	0.772	0.996	0.944	1.711	2.369	2.302
	0.671	0.857	0.799	0.852	1.073	1.003	0.775	0.997	0.945	1.792	2.373	2.305
	0.676	0.860	0.802	0.858	1.076	1.006	0.783	1.001	0.949	1.815	2.384	2.316
	0.677	0.860	0.802	0.858	1.077	1.007	0.784	1.002	0.949	1.793	2.386	2.317
(1,3)	1.096	1.252	1.108	0.903	1.035	0.922	1.096	1.252	1.108	1.302	1.476	1.318
	1.096	1.252	1.108	0.903	1.034	0.922	1.096	1.252	1.108	1.302	1.475	1.318
	1.098	1.252	1.106	0.905	1.034	0.919	1.098	1.252	1.106	1.302	1.473	1.312
	1.103	1.254	1.109	0.910	1.035	0.922	1.103	1.254	1.109	1.309	1.474	1.316
(2,3)	0.611	0.761	0.672	0.690	0.874	0.772	0.647	0.809	0.716	1.033	1.372	1.231
	0.613	0.762	0.671	0.695	0.874	0.770	0.650	0.809	0.715	1.039	1.371	1.228
	0.622	0.761	0.671	0.707	0.874	0.771	0.659	0.808	0.715	1.054	1.368	1.227
	0.622	0.762	0.671	0.707	0.874	0.771	0.658	0.808	0.715	1.053	1.368	1.226
(3,3)	0.536	0.758	0.564	0.675	0.915	0.709	0.585	0.857	0.620	1.096	1.873	1.187
	0.539	0.758	0.565	0.679	0.914	0.709	0.590	0.857	0.621	1.106	1.872	1.190
	0.546	0.756	0.566	0.688	0.912	0.710	0.598	0.854	0.622	1.128	1.866	1.194
	0.546	0.755	0.566	0.688	0.911	0.711	0.598	0.854	0.622	1.130	1.864	1.194

Table B.2. Theoretical Plates Summary.
The theoretical plate count, N, was calculated for each analyte.

Mobile Phase (n,m)	(+)-Δ^8-THC			(-)-Δ^8-THC			(+)-Δ^9-THC			(-)-Δ^9-THC		
	Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 3
(1,1)	10306	6798	9438	10306	6798	14394	10306	6798	9438	16471	16567	15856
	10513	6718	9418	10513	6718	14322	10513	6718	9418	16460	16528	15953
	9929	7056	9784	9929	7056	14204	9929	7056	9784	16436	16162	16122
	10169	7452	9788	10169	7452	14205	10169	7452	9788	16422	16270	16093
(2,1)	12837	8790	9865	17348	14556	16224	15003	10119	12112	16462	15132	16202
	12576	8627	9813	17339	14807	16276	14989	10111	12165	16048	15141	16322
	12794	8642	9865	17666	14825	16329	14937	10130	12245	16382	15206	16189
	12831	8798	9845	17348	14564	15989	14977	10319	12587	16016	15216	16532
(3,1)	16986	17392	17534	15659	16060	15996	16538	16651	16883	14799	14893	14712
	17034	17011	17506	15697	15803	15981	16413	16702	16850	14828	14712	14701
	17047	17505	17550	15565	15910	15858	16610	16469	16533	14525	14796	14489
	17087	17122	17550	15770	16273	16030	16471	16509	16539	14905	14850	14735
(1,2)	16041	13680	15178	18523	18132	17601	16041	13680	15178	17524	16594	17317
	16502	13670	15160	18201	17719	17580	16502	13670	15160	17645	16576	17616
	15512	13416	14848	17768	18086	17974	15512	13416	14848	17529	17269	17263
	15584	13687	14842	17377	18142	17963	15584	13687	14842	17090	17018	17248
(2,2)	12090	9597	9778	17670	16448	16670	13864	10193	9710	15632	16246	15911
	11723	9507	9780	17356	16070	16682	13427	10094	9712	15784	15956	15923
	11560	9527	9799	17365	16136	16734	13222	10226	9730	15684	16375	15653

Table B.2. Continued.

Mobile Phase (n,m)	(+)-Δ^8-THC			(-)-Δ^8-THC			(+)-Δ^9-THC			(-)-Δ^9-THC		
	Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 3
(2,2)	11572	9529	9796	18138	16557	16738	12435	10451	9833	15675	16073	15974
(3,2)	15790	16589	18063	18794	18489	17269	16754	17882	17685	7860	16997	17274
	15822	16602	17529	17752	18502	17286	16804	17655	17703	16514	17041	16894
	15446	16650	17581	17602	18314	17108	16947	17715	17765	16474	17144	16734
	15951	16659	17586	18415	18327	17112	16979	17492	17769	8937	17164	17019
(1,3)	14915	13729	14722	18254	18637	19178	14915	13729	14722	17153	17915	17806
	14915	13729	14726	18263	18632	18622	14915	13729	14726	17557	17510	17802
	14942	13160	15601	18305	18619	18571	14942	13160	15601	17565	17479	17709
	15011	13460	14739	18389	18641	18627	15011	13460	14739	17250	17884	17776
(2,3)	19669	12064	12249	10967	16509	15853	n.d.	11225	11448	17159	16584	16675
	19048	12067	12404	12255	16509	15831	8402	11362	11286	17258	16953	16635
	16242	12221	13509	14094	16509	15848	9329	10951	11008	16842	16918	16613
	18256	12224	13309	13886	16509	15609	9324	10951	10864	16822	16908	16595
(3,3)	12864	16863	14228	17123	17976	17822	12893	17425	14552	16337	17731	17131
	12927	16854	14241	16660	17699	17263	12962	17415	14339	16090	17710	17166
	13040	16820	14255	17389	17662	17283	13093	17373	14584	16211	17993	16800
	13246	16551	14260	16838	17653	17864	13097	17359	14584	16249	17971	17240

Note: n.d. = not determined

Table B.3. Selectivity Factor Summary.
The selectivity factor, α, was calculated from the k' for five pairs of analytes.

Mobile Phase (n,m)	(+)-Δ^9-THC, (+)-Δ^8-THC			(-)-Δ^9-THC, (-)-Δ^8-THC			(-)-Δ^8-THC, (+)-Δ^8-THC			(-)-Δ^9-THC, (+)-Δ^9-THC			(-)-Δ^8-THC, (+)-Δ^9-THC		
	Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 3
(1,1)	1.00	1.00	1.00	1.39	1.32	1.39	1.00	1.00	1.06	1.39	1.32	1.47	1.00	1.00	1.06
	1.00	1.00	1.00	1.39	1.32	1.39	1.00	1.00	1.06	1.39	1.32	1.47	1.00	1.00	1.06
	1.00	1.00	1.00	1.39	1.32	1.38	1.00	1.00	1.06	1.39	1.32	1.45	1.00	1.00	1.06
	1.00	1.00	1.00	1.38	1.32	1.38	1.00	1.00	1.06	1.38	1.32	1.45	1.00	1.00	1.06
(2,1)	1.14	1.08	1.11	2.03	1.40	1.64	1.29	1.48	1.41	2.28	1.92	2.10	1.13	1.37	1.28
	1.14	1.08	1.11	2.03	1.40	1.65	1.29	1.48	1.41	2.28	1.92	2.10	1.13	1.37	1.28
	1.14	1.08	1.11	2.02	1.41	1.66	1.29	1.48	1.41	2.29	1.92	2.11	1.13	1.37	1.27
	1.14	1.08	1.11	2.02	1.41	1.66	1.29	1.48	1.41	2.28	1.92	2.11	1.13	1.37	1.27
(3,1)	1.26	1.26	1.28	2.32	2.37	2.34	1.33	1.33	1.35	2.45	2.50	2.47	1.06	1.05	1.06
	1.26	1.26	1.28	2.32	2.37	2.34	1.33	1.33	1.35	2.45	2.50	2.47	1.06	1.05	1.06
	1.26	1.26	1.28	2.31	2.36	2.34	1.33	1.33	1.35	2.45	2.49	2.47	1.06	1.05	1.06
	1.26	1.27	1.28	2.31	2.36	2.34	1.33	1.33	1.35	2.45	2.49	2.47	1.06	1.05	1.06
(1,2)	1.00	1.00	1.00	1.45	1.39	1.41	1.15*	1.14*	1.12*	1.26	1.22	1.25	1.15*	1.14*	1.12*
	1.00	1.00	1.00	1.45	1.39	1.41	1.15*	1.14*	1.12*	1.26	1.22	1.25	1.15*	1.14*	1.12*
	1.00	1.00	1.00	1.45	1.39	1.41	1.15*	1.14*	1.12*	1.26	1.22	1.25	1.15*	1.14*	1.12*
	1.00	1.00	1.00	1.45	1.39	1.41	1.15*	1.14*	1.12*	1.25	1.22	1.25	1.15*	1.14*	1.12*
(2,2)	1.09	1.07	1.06	1.68	1.42	1.52	1.22	1.26	1.23	1.89	1.68	1.76	1.12	1.18	1.16
	1.08	1.07	1.06	1.68	1.42	1.52	1.22	1.26	1.23	1.89	1.68	1.76	1.12	1.18	1.16
	1.08	1.07	1.06	1.67	1.42	1.52	1.22	1.26	1.23	1.88	1.69	1.76	1.13	1.19	1.16

Table B.3. Continued.

Mobile Phase (n,m)	(+)-Δ^9-THC, (+)-Δ^8-THC			(-)-Δ^9-THC, (-)-Δ^8-THC			(-)-Δ^8-THC, (+)-Δ^8-THC			(-)-Δ^9-THC, (+)-Δ^9-THC			(-)-Δ^8-THC, (+)-Δ^9-THC		
	Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 3
(2,2)	1.08	1.07	1.06	1.66	1.42	1.52	1.22	1.26	1.23	1.88	1.69	1.76	1.13	1.19	1.16
(3,2)	1.15	1.17	1.18	1.99	2.23	2.30	1.26	1.26	1.26	2.18	2.40	2.45	1.10	1.08	1.06
	1.15	1.17	1.18	2.08	2.23	2.31	1.26	1.26	1.26	2.28	2.40	2.45	1.10	1.08	1.06
	1.15	1.17	1.18	2.09	2.23	2.31	1.26	1.26	1.26	2.28	2.40	2.45	1.09	1.08	1.06
	1.15	1.17	1.18	2.06	2.23	2.31	1.26	1.26	1.26	2.25	2.40	2.45	1.09	1.08	1.06
(1,3)	1.00	1.00	1.00	1.43	1.42	1.44	1.21*	1.21*	1.20*	1.18	1.18	1.19	1.21*	1.21*	1.20*
	1.00	1.00	1.00	1.43	1.42	1.44	1.21*	1.21*	1.20*	1.18	1.18	1.19	1.21*	1.21*	1.20*
	1.00	1.00	1.00	1.43	1.42	1.44	1.21*	1.21*	1.20*	1.18	1.18	1.19	1.21*	1.21*	1.20*
	1.00	1.00	1.00	1.43	1.42	1.44	1.21*	1.21*	1.20*	1.18	1.17	1.19	1.21*	1.21*	1.20*
(2,3)	1.06	1.06	1.07	1.49	1.56	1.62	1.13	1.15	1.15	1.59	1.68	1.75	1.07	1.08	1.08
	1.06	1.06	1.07	1.49	1.56	1.61	1.13	1.14	1.15	1.59	1.68	1.75	1.07	1.08	1.08
	1.06	1.06	1.07	1.48	1.55	1.61	1.14	1.15	1.15	1.59	1.68	1.74	1.07	1.08	1.08
	1.06	1.06	1.07	1.48	1.55	1.61	1.14	1.14	1.15	1.59	1.68	1.74	1.07	1.08	1.08
(3,3)	1.10	1.13	1.10	1.64	2.01	1.69	1.27	1.20	1.26	1.90	2.14	1.93	1.16	1.06	1.15
	1.10	1.13	1.10	1.65	2.01	1.69	1.27	1.20	1.26	1.90	2.14	1.93	1.16	1.06	1.14
	1.10	1.12	1.10	1.66	2.01	1.69	1.27	1.20	1.26	1.92	2.14	1.94	1.16	1.06	1.14
	1.10	1.12	1.10	1.66	2.01	1.69	1.27	1.20	1.26	1.92	2.14	1.94	1.16	1.07	1.15

** denotes an elution order reversal*

Table B.4. Peak Resolution Summary.

The resolution, R, was calculated for five pairs of analytes. The (-) sign is merely indicative of an elution order reversal.

Mobile Phase (n,m)	(+)-Δ^8-THC, (+)-Δ^9-THC			(-)-Δ^8-THC, (-)-Δ^9-THC			(+)-Δ^8-THC, (-)-Δ^8-THC			(+)-Δ^9-THC, (-)-Δ^9-THC			(+)-Δ^9-THC, (-)-Δ^8-THC		
	Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 3
(1,1)	0.00	0.00	0.00	6.96	5.42	7.66	0.00	0.00	1.07	6.96	5.42	8.04	0.00	0.00	1.07
	0.00	0.00	0.00	6.96	5.28	7.63	0.00	0.00	1.06	6.96	5.28	8.00	0.00	0.00	1.06
	0.00	0.00	0.00	6.80	5.43	7.47	0.00	0.00	1.06	6.80	5.43	7.94	0.00	0.00	1.06
	0.00	0.00	0.00	6.82	5.54	7.44	0.00	0.00	1.06	6.82	5.54	7.91	0.00	0.00	1.06
(2,1)	2.30	1.06	1.45	15.67	7.03	10.61	4.60	6.25	5.67	17.39	11.76	14.21	2.31	5.26	4.33
	2.28	1.06	1.45	15.53	7.08	10.70	4.58	6.25	5.66	17.24	11.78	14.30	2.31	5.28	4.32
	2.28	1.06	1.48	15.66	7.14	10.83	4.64	6.25	5.64	17.34	11.83	14.37	2.35	5.28	4.28
	2.28	1.07	1.49	15.48	7.12	10.88	4.61	6.24	5.62	17.21	11.89	14.57	2.33	5.27	4.28
(3,1)	4.09	4.39	4.45	17.28	18.43	17.55	5.09	5.39	5.41	18.39	19.45	18.61	1.06	1.04	1.03
	4.10	4.39	4.44	17.29	18.30	17.55	5.09	5.36	5.42	18.35	19.38	18.62	1.03	1.04	1.04
	4.11	4.43	4.43	17.16	18.34	17.43	5.10	5.44	5.42	18.29	19.36	18.46	1.06	1.04	1.03
	4.11	4.42	4.44	17.35	18.45	17.56	5.12	5.46	5.44	18.42	19.41	18.56	1.05	1.06	1.03
(1,2)	0.00	0.00	0.00	7.27	7.05	6.96	-2.63	-2.50	-2.21	4.45	4.16	4.52	-2.63	-2.50	-2.21
	0.00	0.00	0.00	7.24	7.01	6.99	-2.62	-2.49	-2.21	4.49	4.15	4.54	-2.62	-2.49	-2.21
	0.00	0.00	0.00	7.19	7.12	6.97	-2.57	-2.49	-2.21	4.41	4.16	4.48	-2.57	-2.49	-2.21
	0.00	0.00	0.00	7.13	7.08	6.96	-2.60	-2.50	-2.21	4.38	4.17	4.47	-2.60	-2.50	-2.21
(2,2)	1.03	0.75	0.73	9.05	6.27	7.64	2.72	3.17	2.91	10.19	7.96	8.80	1.68	2.36	2.07
	1.02	0.74	0.73	9.04	6.21	7.64	2.73	3.15	2.91	10.19	7.93	8.81	1.70	2.35	2.08

Table B.4. Continued.

Mobile Phase (n,m)	(+)-Δ^8-THC, (+)-Δ^9-THC			(-)-Δ^8-THC, (-)-Δ^9-THC			(+)-Δ^8-THC, (-)-Δ^8-THC			(+)-Δ^9-THC, (-)-Δ^9-THC			(+)-Δ^9-THC, (-)-Δ^8-THC		
	Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 3
(2,2)	1.00	0.75	0.73	8.92	6.29	7.61	2.75	3.18	2.94	10.09	8.05	8.79	1.75	2.39	2.10
	0.98	0.75	0.73	8.96	6.30	7.65	2.79	3.21	2.94	9.92	8.08	8.87	1.74	2.43	2.11
(3,2)	1.92	2.38	2.60	9.78	15.78	16.09	3.36	3.63	3.55	10.60	16.83	17.07	1.41	1.25	0.97
	1.94	2.38	2.58	13.19	15.83	16.00	3.33	3.63	3.54	14.35	16.83	16.98	1.39	1.24	0.97
	1.96	2.39	2.60	13.31	15.87	15.98	3.30	3.63	3.53	14.48	16.91	16.98	1.36	1.24	0.97
	1.98	2.39	2.60	10.81	15.89	16.07	3.35	3.64	3.53	11.66	16.88	17.08	1.35	1.23	0.97
(1,3)	0.00	0.00	0.00	6.31	6.61	6.34	-3.10	-3.19	-2.98	2.96	2.97	3.02	-3.10	-3.19	-2.98
	0.00	0.00	0.00	6.34	6.56	6.29	-3.09	-3.19	-2.96	2.97	2.94	3.02	-3.09	-3.19	-2.96
	0.00	0.00	0.00	6.31	6.54	6.24	-3.09	-3.16	-3.01	2.95	2.88	3.01	-3.09	-3.16	-3.01
	0.00	0.00	0.00	6.30	6.57	6.26	-3.10	-3.19	-2.97	2.96	2.90	2.98	-3.10	-3.19	-2.97
(2,3)	n.d.	0.71	0.71	5.41	7.53	7.33	1.44	1.84	1.71	n.d.	7.93	7.73	n.d.	1.04	0.93
	0.61	0.71	0.70	5.57	7.57	7.30	1.51	1.84	1.71	5.80	7.99	7.69	0.68	1.04	0.92
	0.62	0.70	0.71	5.74	7.53	7.26	1.58	1.85	1.75	5.99	7.89	7.61	0.77	1.04	0.92
	0.63	0.70	0.70	5.71	7.52	7.23	1.61	1.85	1.74	5.98	7.88	7.57	0.77	1.04	0.92
(3,3)	0.90	1.79	1.05	7.20	13.36	8.11	2.64	2.81	2.78	8.40	14.25	9.40	1.67	1.01	1.68
	0.91	1.79	1.05	7.21	13.31	8.08	2.63	2.79	2.76	8.45	14.23	9.39	1.66	1.01	1.66
	0.94	1.78	1.06	7.46	13.34	8.07	2.69	2.79	2.76	8.64	14.27	9.41	1.68	1.01	1.67
	0.94	1.77	1.05	7.44	13.32	8.19	2.69	2.78	2.79	8.68	14.26	9.49	1.67	1.01	1.68

Note: nd = not determined; (-) denotes an elution order reversal

APPENDIX C

RETENTION TIME BY EXPERIMENT SOURCE TABLES

Table C.1. Tests of Between-Subjects Effects for Retention Time in Experiment 1. Between-subjects retention time differences were significant for each factor at α_s=0.01.

Source	Analyte	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Sq.	Observed Power
Model	(-)-Δ^9-THC	6337.058	9	704.118	131948.964	.000	1.000	1.000
	(-)-Δ^8-THC	3161.077	9	351.231	529427.200	.000	1.000	1.000
	(+)-Δ^9-THC	3104.828	9	344.981	519021.208	.000	1.000	1.000
	(+)-Δ^8-THC	2901.058	9	322.340	545140.939	.000	1.000	1.000
IPA	(-)-Δ^9-THC	2.410	2	1.205	225.846	.000	.944	1.000
	(-)-Δ^8-THC	34.741	2	17.370	26183.367	.000	.999	1.000
	(+)-Δ^9-THC	70.566	2	35.283	53083.042	.000	1.000	1.000
	(+)-Δ^8-THC	97.259	2	48.630	82242.317	.000	1.000	1.000
Methanol	(-)-Δ^9-THC	546.923	2	273.462	51245.661	.000	1.000	1.000
	(-)-Δ^8-THC	135.547	2	67.774	102158.520	.000	1.000	1.000
	(+)-Δ^9-THC	109.465	2	54.732	82344.503	.000	1.000	1.000
	(+)-Δ^8-THC	83.240	2	41.620	70388.076	.000	1.000	1.000
IPA x Methanol	(-)-Δ^9-THC	2.373	4	.593	111.157	.000	.943	1.000
	(-)-Δ^8-THC	12.777	4	3.194	4814.822	.000	.999	1.000
	(+)-Δ^9-THC	7.555	4	1.889	2841.659	.000	.998	1.000
	(+)-Δ^8-THC	13.923	4	3.481	5886.847	.000	.999	1.000
Error	(-)-Δ^9-THC	.144	27	.005				
	(-)-Δ^8-THC	.018	27	.001				
	(+)-Δ^9-THC	.018	27	.001				
	(+)-Δ^8-THC	.016	27	.001				
Total	(-)-Δ^9-THC	6337.202	36					
	(-)-Δ^8-THC	3161.095	36					
	(+)-Δ^9-THC	3104.846	36					
	(+)-Δ^8-THC	2901.074	36					

Table C.2. Tests of Between-Subjects Effects for Retention Time in Experiment 2. Between-subjects retention time differences were significant for each factor at α_s=0.01.

Source	Analyte	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Sq.	Observed Power
Model	(-)-Δ^9-THC	7020.695	9	780.077	184627.986	.000	1.000	1.000
	(-)-Δ^8-THC	3655.720	9	406.191	232163.251	.000	1.000	1.000
	(+)-Δ^9-THC	3498.911	9	388.768	226879.148	.000	1.000	1.000
	(+)-Δ^8-THC	3275.105	9	363.901	221534.099	.000	1.000	1.000
IPA	(-)-Δ^9-THC	79.633	2	39.817	9423.771	.000	.999	1.000
	(-)-Δ^8-THC	31.456	2	15.728	8989.560	.000	.999	1.000
	(+)-Δ^9-THC	83.507	2	41.753	24366.718	.000	.999	1.000
	(+)-Δ^8-THC	111.534	2	55.767	33949.529	.000	1.000	1.000
Methanol	(-)-Δ^9-THC	324.531	2	162.265	38404.803	.000	1.000	1.000
	(-)-Δ^8-THC	125.019	2	62.510	35728.118	.000	1.000	1.000
	(+)-Δ^9-THC	77.860	2	38.930	22719.036	.000	.999	1.000
	(+)-Δ^8-THC	61.184	2	30.592	18623.709	.000	.999	1.000
IPA x Methanol	(-)-Δ^9-THC	18.272	4	4.568	1081.147	.000	.994	1.000
	(-)-Δ^8-THC	10.295	4	2.574	1471.003	.000	.995	1.000
	(+)-Δ^9-THC	11.388	4	2.847	1661.522	.000	.996	1.000
	(+)-Δ^8-THC	15.010	4	3.753	2284.498	.000	.997	1.000
Error	(-)-Δ^9-THC	.114	27	.004				
	(-)-Δ^8-THC	.047	27	.002				
	(+)-Δ^9-THC	.046	27	.002				
	(+)-Δ^8-THC	.044	27	.002				
Total	(-)-Δ^9-THC	7020.809	36					
	(-)-Δ^8-THC	3655.767	36					
	(+)-Δ^9-THC	3498.958	36					
	(+)-Δ^8-THC	3275.149	36					

Table C.3. Tests of Between-Subjects Effects for Retention Time in Experiment 3. Between-subjects retention time differences were significant for each factor at α_s=0.01.

Source	Analyte	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Sq.	Observed Power
Model	(-)-Δ^9-THC	6744.209	9	749.357	187490.615	.000	1.000	1.000
	(-)-Δ^8-THC	3482.439	9	386.938	96817.782	.000	1.000	1.000
	(+)-Δ^9-THC	3279.362	9	364.374	92354.944	.000	1.000	1.000
	(+)-Δ^8-THC	3071.626	9	341.292	87279.462	.000	1.000	1.000
IPA	(-)-Δ^9-THC	29.465	2	14.732	3686.077	.000	.996	1.000
	(-)-Δ^8-THC	46.470	2	23.235	5813.810	.000	.998	1.000
	(+)-Δ^9-THC	82.665	2	41.333	10476.236	.000	.999	1.000
	(+)-Δ^8-THC	111.564	2	55.782	14265.283	.000	.999	1.000
Methanol	(-)-Δ^9-THC	489.328	2	244.664	61215.416	.000	1.000	1.000
	(-)-Δ^8-THC	157.362	2	78.681	19687.192	.000	.999	1.000
	(+)-Δ^9-THC	105.052	2	52.526	13313.386	.000	.999	1.000
	(+)-Δ^8-THC	82.472	2	41.236	10545.404	.000	.999	1.000
IPA x Methanol	(-)-Δ^9-THC	24.248	4	6.062	1516.728	.000	.996	1.000
	(-)-Δ^8-THC	23.143	4	5.786	1447.700	.000	.995	1.000
	(+)-Δ^9-THC	14.891	4	3.723	943.555	.000	.993	1.000
	(+)-Δ^8-THC	21.115	4	5.279	1349.953	.000	.995	1.000
Error	(-)-Δ^9-THC	.108	27	.004				
	(-)-Δ^8-THC	.108	27	.004				
	(+)-Δ^9-THC	.107	27	.004				
	(+)-Δ^8-THC	.106	27	.004				
Total	(-)-Δ^9-THC	6744.317	36					
	(-)-Δ^8-THC	3482.547	36					
	(+)-Δ^9-THC	3279.469	36					
	(+)-Δ^8-THC	3071.732	36					

Table C.4. Tests of Between-Subjects Effects for Retention Time in All Experiments. Between-subjects retention time differences were significant for each factor at α_s=0.01 using the statistical model design 3.2.

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Sq.	Observed Power
Model	(-)-Δ^9-THC	20007.698	9	2223.078	2325.741	.000	.995	1.000
	(-)-Δ^8-THC	10284.431	9	1142.715	7552.657	.000	.999	1.000
	(+)-Δ^9-THC	9868.862	9	1096.540	7533.331	.000	.999	1.000
	(+)-Δ^8-THC	9236.716	9	1026.302	9040.009	.000	.999	1.000
IPA	(-)-Δ^9-THC	72.577	2	36.288	37.964	.000	.434	1.000
	(-)-Δ^8-THC	111.476	2	55.738	368.395	.000	.882	1.000
	(+)-Δ^9-THC	233.828	2	116.914	803.208	.000	.942	1.000
	(+)-Δ^8-THC	318.754	2	159.377	1403.846	.000	.966	1.000
Methanol	(-)-Δ^9-THC	1343.342	2	671.671	702.689	.000	.934	1.000
	(-)-Δ^8-THC	416.705	2	208.352	1377.084	.000	.965	1.000
	(+)-Δ^9-THC	290.365	2	145.182	997.417	.000	.953	1.000
	(+)-Δ^8-THC	225.434	2	112.717	992.850	.000	.953	1.000
IPA x Methanol	(-)-Δ^9-THC	20.362	4	5.090	5.326	.001	.177	.884
	(-)-Δ^8-THC	43.970	4	10.992	72.653	.000	.746	1.000
	(+)-Δ^9-THC	31.313	4	7.828	53.781	.000	.685	1.000
	(+)-Δ^8-THC	48.384	4	12.096	106.545	.000	.811	1.000
Error	(-)-Δ^9-THC	94.630	99	.956				
	(-)-Δ^8-THC	14.979	99	.151				
	(+)-Δ^9-THC	14.410	99	.146				
	(+)-Δ^8-THC	11.239	99	.114				
Total	(-)-Δ^9-THC	20102.328	108					
	(-)-Δ^8-THC	10299.409	108					
	(+)-Δ^9-THC	9883.273	108					
	(+)-Δ^8-THC	9247.955	108					

APPENDIX D

POST-HOC ANALYSIS SOURCE TABLES

Table D.1. Post-Hoc Analysis of IPA for Retention Time.
Retention time differences for some IPA levels are significant.

Analyte/Test		(I) IPA (%)	(J) IPA (%)	Mean Difference (I-J)	Std. Error	Sig.	99% Confidence Interval	
							Lower Bound	Upper Bound
(-)-Δ^9-THC	Tukey HSD	1	4	1.57689	.215744	.000	.93323	2.22054
			7	-.28817	.215744	.379	-.93182	.35549
		4	1	-1.57689	.215744	.000	-2.22054	-.93323
			7	-1.86506	.215744	.000	-2.50871	-1.22140
		7	1	.28817	.215744	.379	-.35549	.93182
			4	1.86506	.215744	.000	1.22140	2.50871
	Scheffe	1	4	1.57689	.215744	.000	.90628	2.24749
			7	-.28817	.215744	.413	-.95877	.38244
		4	1	-1.57689	.215744	.000	-2.24749	-.90628
			7	-1.86506	.215744	.000	-2.53566	-1.19445
		7	1	.28817	.215744	.413	-.38244	.95877
			4	1.86506	.215744	.000	1.19445	2.53566
(+)-Δ^9-THC	Tukey HSD	1	4	3.12275	.066039	.000	2.92573	3.31977
			7	3.11994	.066039	.000	2.92292	3.31697
		4	1	-3.12275	.066039	.000	-3.31977	-2.92573
			7	-.00281	.066039	.999	-.19983	.19422
		7	1	-3.11994	.066039	.000	-3.31697	-2.92292
			4	.00281	.066039	.999	-.19422	.19983
	Scheffe	1	4	3.12275	.066039	.000	2.91748	3.32802
			7	3.11994	.066039	.000	2.91467	3.32522
		4	1	-3.12275	.066039	.000	-3.32802	-2.91748
			7	-.00281	.066039	.999	-.20808	.20247
		7	1	-3.11994	.066039	.000	-3.32522	-2.91467
			4	.00281	.066039	.999	-.20247	.20808
(-)-Δ^8-THC	Tukey HSD	1	4	1.90708	.052614	.000	1.75011	2.06405
			7	2.33814	.052614	.000	2.18117	2.49511
		4	1	-1.90708	.052614	.000	-2.06405	-1.75011
			7	.43106	.052614	.000	.27409	.58803
		7	1	-2.33814	.052614	.000	-2.49511	-2.18117
			4	-.43106	.052614	.000	-.58803	-.27409

Table D.1. Continued.

Analyte/Test		(I) IPA (%)	(J) IPA (%)	Mean Difference (I-J)	Std. Error	Sig.	99% Confidence Interval	
							Lower Bound	Upper Bound
(-)-Δ^8-THC	Scheffe	1	4	1.90708	.052614	.000	1.74354	2.07063
			7	2.33814	.052614	.000	2.17460	2.50168
		4	1	-1.90708	.052614	.000	-2.07063	-1.74354
			7	.43106	.052614	.000	.26751	.59460
		7	1	-2.33814	.052614	.000	-2.50168	-2.17460
			4	-.43106	.052614	.000	-.59460	-.26751
(+)-Δ^8-THC	Tukey HSD	1	4	3.45192	.052957	.000	3.29392	3.60991
			7	3.81033	.052957	.000	3.65234	3.96833
		4	1	-3.45192	.052957	.000	-3.60991	-3.29392
			7	.35842	.052957	.000	.20042	.51641
		7	1	-3.81033	.052957	.000	-3.96833	-3.65234
			4	-.35842	.052957	.000	-.51641	-.20042
	Scheffe	1	4	3.45192	.052957	.000	3.28731	3.61653
			7	3.81033	.052957	.000	3.64572	3.97494
		4	1	-3.45192	.052957	.000	-3.61653	-3.28731
			7	.35842	.052957	.000	.19381	.52303
		7	1	-3.81033	.052957	.000	-3.97494	-3.64572
			4	-.35842	.052957	.000	-.52303	-.19381

Table D.2. Post-Hoc Analysis of Methanol for Retention Time.
Retention time differences for all methanol levels are significant.

Analyte/Test		(I) Methanol (%)	(J) Methanol (%)	Mean Difference (I-J)	Std. Error	Sig.	99% Confidence Interval	
							Lower Bound	Upper Bound
(-)-Δ^9-THC	Tukey HSD	1	2	5.93903	.215744	.000	5.29537	6.58268
			3	8.40261	.215744	.000	7.75896	9.04627
		2	1	-5.93903	.215744	.000	-6.58268	-5.29537
			3	2.46358	.215744	.000	1.81993	3.10724
		3	1	-8.40261	.215744	.000	-9.04627	-7.75896
			2	-2.46358	.215744	.000	-3.10724	-1.81993
	Scheffe	1	2	5.93903	.215744	.000	5.26842	6.60963
			3	8.40261	.215744	.000	7.73201	9.07322
		2	1	-5.93903	.215744	.000	-6.60963	-5.26842
			3	2.46358	.215744	.000	1.79298	3.13419
		3	1	-8.40261	.215744	.000	-9.07322	-7.73201
			2	-2.46358	.215744	.000	-3.13419	-1.79298

Table D.2. Continued.

Analyte/Test		(I) Methanol (%)	(J) Methanol (%)	Mean Difference (I-J)	Std. Error	Sig.	99% Confidence Interval	
							Lower Bound	Upper Bound
(+)-Δ^9-THC	Tukey HSD	1	2	2.74000	.066039	.000	2.54298	2.93702
			3	3.91319	.066039	.000	3.71617	4.11022
		2	1	-2.74000	.066039	.000	-2.93702	-2.54298
			3	1.17319	.066039	.000	.97617	1.37022
		3	1	-3.91319	.066039	.000	-4.11022	-3.71617
			2	-1.17319	.066039	.000	-1.37022	-.97617
	Scheffe	1	2	2.74000	.066039	.000	2.53473	2.94527
			3	3.91319	.066039	.000	3.70792	4.11847
		2	1	-2.74000	.066039	.000	-2.94527	-2.53473
			3	1.17319	.066039	.000	.96792	1.37847
		3	1	-3.91319	.066039	.000	-4.11847	-3.70792
			2	-1.17319	.066039	.000	-1.37847	-.96792
(-)-Δ^8-THC	Tukey HSD	1	2	3.36603	.052614	.000	3.20906	3.52300
			3	4.66044	.052614	.000	4.50347	4.81741
		2	1	-3.36603	.052614	.000	-3.52300	-3.20906
			3	1.29442	.052614	.000	1.13745	1.45139
		3	1	-4.66044	.052614	.000	-4.81741	-4.50347
			2	-1.29442	.052614	.000	-1.45139	-1.13745
	Scheffe	1	2	3.36603	.052614	.000	3.20249	3.52957
			3	4.66044	.052614	.000	4.49690	4.82399
		2	1	-3.36603	.052614	.000	-3.52957	-3.20249
			3	1.29442	.052614	.000	1.13087	1.45796
		3	1	-4.66044	.052614	.000	-4.82399	-4.49690
			2	-1.29442	.052614	.000	-1.45796	-1.13087
(+)-Δ^8-THC	Tukey HSD	1	2	2.39286	.052957	.000	2.23487	2.55086
			3	3.45447	.052957	.000	3.29648	3.61247
		2	1	-2.39286	.052957	.000	-2.55086	-2.23487
			3	1.06161	.052957	.000	.90362	1.21961
		3	1	-3.45447	.052957	.000	-3.61247	-3.29648
			2	-1.06161	.052957	.000	-1.21961	-.90362
	Scheffe	1	2	2.39286	.052957	.000	2.22825	2.55747
			3	3.45447	.052957	.000	3.28986	3.61908
		2	1	-2.39286	.052957	.000	-2.55747	-2.22825
			3	1.06161	.052957	.000	.89700	1.22622
		3	1	-3.45447	.052957	.000	-3.61908	-3.28986
			2	-1.06161	.052957	.000	-1.22622	-.89700

Table D.3. Post-Hoc Analysis of Experiment Setup for Retention Time.
Retention time differences for some experiment setups are significant.

Analyte/ Test		(I) Experiment setup	(J) Experiment setup	Mean Difference (I-J)	Std. Error	Sig.	99% Confidence Interval	
							Lower Bound	Upper Bound
(-)-Δ^9-THC	Tukey HSD	1	2	-.86136	.215744	.000	-1.50502	-.21771
			3	-.44767	.215744	.100	-1.09132	.19599
		2	1	.86136	.215744	.000	.21771	1.50502
			3	.41369	.215744	.139	-.22996	1.05735
		3	1	.44767	.215744	.100	-.19599	1.09132
			2	-.41369	.215744	.139	-1.05735	.22996
	Scheffe	1	2	-.86136	.215744	.001	-1.53197	-.19076
			3	-.44767	.215744	.122	-1.11827	.22294
		2	1	.86136	.215744	.001	.19076	1.53197
			3	.41369	.215744	.165	-.25691	1.08430
		3	1	.44767	.215744	.122	-.22294	1.11827
			2	-.41369	.215744	.165	-1.08430	.25691
(+)-Δ^9-THC	Tukey HSD	1	2	-.61022	.066039	.000	-.80724	-.41320
			3	-.24283	.066039	.001	-.43986	-.04581
		2	1	.61022	.066039	.000	.41320	.80724
			3	.36739	.066039	.000	.17037	.56441
		3	1	.24283	.066039	.001	.04581	.43986
			2	-.36739	.066039	.000	-.56441	-.17037
	Scheffe	1	2	-.61022	.066039	.000	-.81549	-.40495
			3	-.24283	.066039	.002	-.44810	-.03756
		2	1	.61022	.066039	.000	.40495	.81549
			3	.36739	.066039	.000	.16212	.57266
		3	1	.24283	.066039	.002	.03756	.44810
			2	-.36739	.066039	.000	-.57266	-.16212
(-)-Δ^8-THC	Tukey HSD	1	2	-.74936	.052614	.000	-.90633	-.59239
			3	-.41425	.052614	.000	-.57122	-.25728
		2	1	.74936	.052614	.000	.59239	.90633
			3	.33511	.052614	.000	.17814	.49208
		3	1	.41425	.052614	.000	.25728	.57122
			2	-.33511	.052614	.000	-.49208	-.17814
	Scheffe	1	2	-.74936	.052614	.000	-.91290	-.58582
			3	-.41425	.052614	.000	-.57779	-.25071
		2	1	.74936	.052614	.000	.58582	.91290
			3	.33511	.052614	.000	.17157	.49865
		3	1	.41425	.052614	.000	.25071	.57779
			2	-.33511	.052614	.000	-.49865	-.17157

Table D.3. Post-Hoc Analysis

Analyte/ Test		(I) Experiment setup	(J) Experiment setup	Mean Difference (I-J)	Std. Error	Sig.	99% Confidence Interval	
							Lower Bound	Upper Bound
(+)-Δ^8-THC	Tukey HSD	1	2	-.58981	.052957	.000	-.74780	-.43181
			3	-.23678	.052957	.000	-.39477	-.07878
		2	1	.58981	.052957	.000	.43181	.74780
			3	.35303	.052957	.000	.19503	.51102
		3	1	.23678	.052957	.000	.07878	.39477
			2	-.35303	.052957	.000	-.51102	-.19503
	Scheffe	1	2	-.58981	.052957	.000	-.75441	-.42520
			3	-.23678	.052957	.000	-.40139	-.07217
		2	1	.58981	.052957	.000	.42520	.75441
			3	.35303	.052957	.000	.18842	.51764
		3	1	.23678	.052957	.000	.07217	.40139
			2	-.35303	.052957	.000	-.51764	-.18842

Table D.4. Post-Hoc Analysis of IPA for Selectivity.
Column selectivity differences for most IPA levels are significant.

Analyte Pair/Test		(I) IPA (%)	(J) IPA (%)	Mean Difference (I-J)	Std. Error	Sig.	99% Confidence Interval	
							Lower Bound	Upper Bound
Δ^9-THC	Tukey HSD	1	4	-.57889	.019156	.000	-.63604	-.52174
			7	-1.00611	.019156	.000	-1.06326	-.94896
		4	1	.57889	.019156	.000	.52174	.63604
			7	-.42722	.019156	.000	-.48437	-.37007
		7	1	1.00611	.019156	.000	.94896	1.06326
			4	.42722	.019156	.000	.37007	.48437
	Scheffe	1	4	-.57889	.019156	.000	-.63843	-.51935
			7	-1.00611	.019156	.000	-1.06565	-.94657
		4	1	.57889	.019156	.000	.51935	.63843
			7	-.42722	.019156	.000	-.48677	-.36768
		7	1	1.00611	.019156	.000	.94657	1.06565
			4	.42722	.019156	.000	.36768	.48677
Δ^8-THC	Tukey HSD	1	4	-.13500	.007326	.000	-.15686	-.11314
			7	-.15722	.007326	.000	-.17908	-.13536
		4	1	.13500	.007326	.000	.11314	.15686
			7	-.02222	.007326	.009	-.04408	-.00036
		7	1	.15722	.007326	.000	.13536	.17908
			4	.02222	.007326	.009	.00036	.04408

Table D.4. Continued.

Analyte Pair/Test		(I) IPA (%)	(J) IPA (%)	Mean Difference (I-J)	Std. Error	Sig.	99% Confidence Interval	
							Lower Bound	Upper Bound
Δ^8-THC	Scheffe	1	4	-.13500	.007326	.000	-.15777	-.11223
			7	-.15722	.007326	.000	-.17999	-.13445
		4	1	.13500	.007326	.000	.11223	.15777
			7	-.02222	.007326	.012	-.04499	.00055
		7	1	.15722	.007326	.000	.13445	.17999
			4	.02222	.007326	.012	-.00055	.04499
Critical Pair	Tukey HSD	1	4	-.04111	.009364	.000	-.06905	-.01318
			7	.03694	.009364	.000	.00901	.06488
		4	1	.04111	.009364	.000	.01318	.06905
			7	.07806	.009364	.000	.05012	.10599
		7	1	-.03694	.009364	.000	-.06488	-.00901
			4	-.07806	.009364	.000	-.10599	-.05012
	Scheffe	1	4	-.04111	.009364	.000	-.07022	-.01201
			7	.03694	.009364	.001	.00784	.06605
		4	1	.04111	.009364	.000	.01201	.07022
			7	.07806	.009364	.000	.04895	.10716
		7	1	-.03694	.009364	.001	-.06605	-.00784
			4	-.07806	.009364	.000	-.10716	-.04895

Table D.5. Post-Hoc Analysis of Methanol for Selectivity.
Column selectivity differences for some methanol levels are significant.

Analyte Pair/ Test		(I) Methanol (%)	(J) Methanol (%)	Mean Difference (I-J)	Std. Error	Sig.	99% Confidence Interval	
							Lower Bound	Upper Bound
Δ^9-THC	Tukey HSD	1	2	.19278	.019156	.000	.13563	.24993
			3	.37139	.019156	.000	.31424	.42854
		2	1	-.19278	.019156	.000	-.24993	-.13563
			3	.17861	.019156	.000	.12146	.23576
		3	1	-.37139	.019156	.000	-.42854	-.31424
			2	-.17861	.019156	.000	-.23576	-.12146
	Scheffe	1	2	.19278	.019156	.000	.13323	.25232
			3	.37139	.019156	.000	.31185	.43093
		2	1	-.19278	.019156	.000	-.25232	-.13323
			3	.17861	.019156	.000	.11907	.23815
		3	1	-.37139	.019156	.000	-.43093	-.31185
			2	-.17861	.019156	.000	-.23815	-.11907

Table D.5. Continued.

Analyte Pair/ Test		(I) Methanol (%)	(J) Methanol (%)	Mean Difference (I-J)	Std. Error	Sig.	99% Confidence Interval	
							Lower Bound	Upper Bound
Δ^8-THC	Tukey HSD	1	2	.03833	.007326	.000	.01648	.06019
			3	.05111	.007326	.000	.02925	.07297
		2	1	-.03833	.007326	.000	-.06019	-.01648
			3	.01278	.007326	.194	-.00908	.03464
		3	1	-.05111	.007326	.000	-.07297	-.02925
			2	-.01278	.007326	.194	-.03464	.00908
	Scheffe	1	2	.03833	.007326	.000	.01556	.06111
			3	.05111	.007326	.000	.02834	.07388
		2	1	-.03833	.007326	.000	-.06111	-.01556
			3	.01278	.007326	.224	-.00999	.03555
		3	1	-.05111	.007326	.000	-.07388	-.02834
			2	-.01278	.007326	.224	-.03555	.00999

Table D.6. Post-Hoc Analysis of IPA for Resolution.
Peak resolution differences for all IPA levels are significant.

Analyte Pair/ Test		(I) IPA (%)	(J) IPA (%)	Mean Difference (I-J)	Std. Error	Sig.	99% Confidence Interval	
							Lower Bound	Upper Bound
Δ^9-THC	Tukey HSD	1	4	-5.63632*	.361731	.000	-6.71578	-4.55685
			7	-10.32667*	.359174	.000	-11.39851	-9.25483
		4	1	5.63632*	.361731	.000	4.55685	6.71578
			7	-4.69035*	.361731	.000	-5.76982	-3.61089
		7	1	10.32667*	.359174	.000	9.25483	11.39851
			4	4.69035*	.361731	.000	3.61089	5.76982
	Scheffe	1	4	-5.63632*	.361731	.000	-6.76098	-4.51165
			7	-10.32667*	.359174	.000	-11.44339	-9.20995
		4	1	5.63632*	.361731	.000	4.51165	6.76098
			7	-4.69035*	.361731	.000	-5.81502	-3.56569
		7	1	10.32667*	.359174	.000	9.20995	11.44339
			4	4.69035*	.361731	.000	3.56569	5.81502
Δ^8-THC	Tukey HSD	1	4	-1.48133*	.066745	.000	-1.68051	-1.28215
			7	-1.89342*	.066274	.000	-2.09119	-1.69565
		4	1	1.48133*	.066745	.000	1.28215	1.68051
			7	-.41209*	.066745	.000	-.61127	-.21291
		7	1	1.89342*	.066274	.000	1.69565	2.09119
			4	.41209*	.066745	.000	.21291	.61127

Table D.6. Continued.

Analyte Pair/ Test		(I) IPA (%)	(J) IPA (%)	Mean Difference (I-J)	Std. Error	Sig.	99% Confidence Interval	
							Lower Bound	Upper Bound
Δ^8-THC	Scheffe	1	4	-1.48133*	.066745	.000	-1.68885	-1.27381
			7	-1.89342*	.066274	.000	-2.09947	-1.68737
		4	1	1.48133*	.066745	.000	1.27381	1.68885
			7	-.41209*	.066745	.000	-.61961	-.20457
		7	1	1.89342*	.066274	.000	1.68737	2.09947
			4	.41209*	.066745	.000	.20457	.61961
Critical Pair	Tukey HSD	1	4	-.39601*	.112591	.002	-.73200	-.06001
			7	.72867*	.111796	.000	.39506	1.06229
		4	1	.39601*	.112591	.002	.06001	.73200
			7	1.12468*	.112591	.000	.78869	1.46067
		7	1	-.72867*	.111796	.000	-1.06229	-.39506
			4	-1.12468*	.112591	.000	-1.46067	-.78869
	Scheffe	1	4	-.39601*	.112591	.003	-.74607	-.04595
			7	.72867*	.111796	.000	.38109	1.07626
		4	1	.39601*	.112591	.003	.04595	.74607
			7	1.12468*	.112591	.000	.77462	1.47474
		7	1	-.72867*	.111796	.000	-1.07626	-.38109
			4	-1.12468*	.112591	.000	-1.47474	-.77462

Table D.7. Post-Hoc Analysis of Methanol for Resolution.
Peak resolution differences for all methanol levels are significant.

Analyte Pair/ Test		(I) Methanol (%)	(J) Methanol (%)	Mean Difference (I-J)	Std. Error	Sig.	99% Confidence Interval	
							Lower Bound	Upper Bound
Δ^9-THC	Tukey HSD	1	2	3.71004*	.359174	.000	2.63820	4.78187
			3	6.35648*	.361731	.000	5.27701	7.43595
		2	1	-3.71004*	.359174	.000	-4.78187	-2.63820
			3	2.64644*	.361731	.000	1.56698	3.72591
		3	1	-6.35648*	.361731	.000	-7.43595	-5.27701
			2	-2.64644*	.361731	.000	-3.72591	-1.56698
	Scheffe	1	2	3.71004*	.359174	.000	2.59332	4.82675
			3	6.35648*	.361731	.000	5.23181	7.48114
		2	1	-3.71004*	.359174	.000	-4.82675	-2.59332
			3	2.64644*	.361731	.000	1.52178	3.77111
		3	1	-6.35648*	.361731	.000	-7.48114	-5.23181
			2	-2.64644*	.361731	.000	-3.77111	-1.52178

Table D.7. Continued.

Analyte Pair/ Test		(I) Methanol (%)	(J) Methanol (%)	Mean Difference (I-J)	Std. Error	Sig.	99% Confidence Interval	
							Lower Bound	Upper Bound
Δ^8-THC	Tukey HSD	1	2	.75845*	.066274	.000	.56068	.95623
			3	1.18110*	.066745	.000	.98192	1.38028
		2	1	-.75845*	.066274	.000	-.95623	-.56068
			3	.42265*	.066745	.000	.22347	.62183
		3	1	-1.18110*	.066745	.000	-1.38028	-.98192
			2	-.42265*	.066745	.000	-.62183	-.22347
	Scheffe	1	2	.75845*	.066274	.000	.55240	.96451
			3	1.18110*	.066745	.000	.97358	1.38862
		2	1	-.75845*	.066274	.000	-.96451	-.55240
			3	.42265*	.066745	.000	.21513	.63017
		3	1	-1.18110*	.066745	.000	-1.38862	-.97358
			2	-.42265*	.066745	.000	-.63017	-.21513

Table D.8. Post-Hoc Analysis of Experiment Setup for Resolution.
Peak resolution differences for some experiment setups are significant for Δ^8-THC.

Analyte Pair/ Test		(I) Experiment Setup	(J) Exp. Setup	Mean Difference (I-J)	Std. Error	Sig.	99% Confidence Interval	
							Lower Bound	Upper Bound
Δ^8-THC	Tukey HSD	1	2	-.30388*	.066745	.000	-.50306	-.10470
			3	-.24904*	.066745	.001	-.44822	-.04986
		2	1	.30388*	.066745	.000	.10470	.50306
			3	.05484	.066274	.687	-.14293	.25261
		3	1	.24904*	.066745	.001	.04986	.44822
			2	-.05484	.066274	.687	-.25261	.14293
	Scheffe	1	2	-.30388*	.066745	.000	-.51140	-.09636
			3	-.24904*	.066745	.002	-.45656	-.04152
		2	1	.30388*	.066745	.000	.09636	.51140
			3	.05484	.066274	.711	-.15122	.26089
		3	1	.24904*	.066745	.002	.04152	.45656
			2	-.05484	.066274	.711	-.26089	.15122
Critical Pair	Tukey HSD	1	2	-.31791	.112591	.016	-.65390	.01808
			3	-.27062	.112591	.047	-.60661	.06537
		2	1	.31791	.112591	.016	-.01808	.65390
			3	.04729	.111796	.906	-.28633	.38091
		3	1	.27062	.112591	.047	-.06537	.60661
			2	-.04729	.111796	.906	-.38091	.28633
	Scheffe	1	2	-.31791	.112591	.022	-.66797	.03215
			3	-.27062	.112591	.061	-.62068	.07944
		2	1	.31791	.112591	.022	-.03215	.66797
			3	.04729	.111796	.914	-.30029	.39488
		3	1	.27062	.112591	.061	-.07944	.62068
			2	-.04729	.111796	.914	-.39488	.30029

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VITA

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