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CHARACTERIZATION OF THE WARNING ODOR OF THE NORTH AMERICAN PORCUPINE; CAPILLARY ELECTROPHORESIS OF THE PRIORITY POLLUTANT PHENOLS

by

GUANG LI

A dissertation submitted to the Graduate Faculty in Chemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

1995

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ABSTRACT

CHARACTERIZATION OF THE WARNING ODOR OF THE NORTH AMERICAN PORCUPINE; CAPILLARY ELECTROPHORESIS OF THE PRIORITY POLLUTANT PHENOLS

by

GUANG LI

Adviser: Professor David C. Locke

This dissertation consists of three parts: (I) Characterization of the Warning Odor of the North American Porcupine; (II) Separation of the Priority Pollutant Phenols by Capillary Zone Electrophoresis; and (III) Nonionic Surfactants as Buffer Additive for Improving Separation Selectivities in Capillary Electrophoresis.

Part One reports for the first time the chemical composition of the porcupine's warning odor. Thirty-five compounds are identified in the headspace odor extract by GC/MS. The major components include acetates (C14 and C16), terpenoids, and lactones. The principal component responsible to the unique odor is an enantiomer of δ-decalactone (tentatively the R- enantiomer).

Part Two studies the application of capillary zone electrophoresis (CZE), a newly developed separation technique, to the analysis of EPA priority pollutant phenols. Using a simple mathematical model, the buffer pH optimization can be

greatly simplified. Under the optimized conditions, the eleven phenols can be resolved in less than 15 min using a 75 μm i.d. capillary and 22.5 kV applied voltage. Using a 30 μm i.d. capillary, complete separation can be achieved in less than 3 min.

Part Three discusses the potential of nonionic surfactants, Tween 40 and Brij 35, for improving separation selectivity in capillary electrophoresis. The addition of as little as 0.5% of the surfactants to running buffer effectively improves the separation selectivities of the priority pollutant phenols, which shows unique advantages compared to other buffer modifiers.

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TABLE OF CONTENTS

		Page
PART ONE	CHARACTERIZATION OF THE WARNING ODOR OF THE NORTH AMERICAN PORCUPINE	1
	I. Introduction	2
	II. Experimental	23
	1. Gas Chromatography-Mass Spectrometry (GC-MS) Analysis	23
	2. Collection of Porcupine Quills	23
	3. Collection of Headspace Volatile Components from Porcupine Quills	24
	4. Solvent Desorption of Volatiles from Tenax	26
	5. Methylation of the Acidic Components in the Volatile Extract	26
	6. Isolation of the Acidic Components from the Odor Extract	26
	7. Fractionation of Total Organics from Porcupine Quills on Silica Gel	27
	8. Gas Chromatography-Olfactory Detection	27
	9. Determination of Enantiomeric Composition of Decalactones	30
	III. Results	31

1. Identification of Volatile Components in the Methanol Extract 31

2. Identification of Acidic Components in the Odor Extract 36

3. Identification of Components in Each Fraction from Silica Gel Cleanup 40

4. GC-Organoleptic Evaluation 45

5. Transesterification by Methylation Reagents 47

6. Identification of Other Components in the Headspace of Porcupine Quills 52

7. Enantiomeric Compositions of Decalactones in Porcupine's Warning Odor 55

8. Summary of the Experimental Pathways and Results 58

IV. Discussion 60

PART TWO SEPARATION OF THE PRIORITY POLLUTANT PHENOLS BY CAPILLARY ZONE ELECTROPHORESIS 64

I. Introduction 65

II. Basic Theory of Capillary Electrophoresis 70

1. Electrophoretic Mobility 70

2. Electroosmotic Flow 70

3. Micellar Electrokinetic Capillary Chromatography (MECC) 77

III. Experimental 82

1. Instrument 82

2. Chemicals 82

3. Capillary Conditioning 83

IV. Results and Discussion 84

1. Structures of the Priority Pollutant Phenols 84

2. Optimization of Buffer pH 86

3. Effect of Buffer Concentration 94

4. Effect of Applied Voltage 97

5. Effect of Sample Injection Time 99

6. Optimized Separation Conditions 101

7. Quantitation 103

8. Rapid CZE Analysis Using Capillary with Smaller Internal Diameter 105

PART THREE NONIONIC SURFACTANTS AS BUFFER ADDITIVE FOR IMPROVING SEPARATION SELECTIVITIES IN CAPILLARY ELECTROPHORESIS 109

I. Introduction 110

II. Experimental 112

1. Instrument 112

2. Chemicals 112

3. Capillary Conditioning 113

III. Results and Discussion 114

REFERENCES 129

LIST OF TABLES

PART ONE

Table 1.1.	Some examples of insect semiochemicals and their functions in insect behavior.	5
Table 1.2.	Some example of mammalian semiochemicals and their functions	8
Table 1.3.	Types of messages which could be conveyed by mammals by means of olfaction	10
Table 1.4.	Identification of volatile components in the headspace of porcupine quills	33
Table 1.5.	Acidic components in the headspace of porcupine quills identified as their methyl derivatives	39
Table 1.6.	Odor characteristics of the enantiomers of decalactones	62

PART TWO

Table 2.1.	pK_a Values of the priority pollutant phenols and the measured electrophoretic mobilities of A^-.	89
Table 2.2.	Regression coefficients (r) for calibration curves and limit of detection (LOD)	104

LIST OF FIGURES

Part One

Figure 1.1. Structure and shape of cyclodextrin molecules. 21

Figure 1.2. The experimental device for collecting quill headspace volatiles. 25

Figure 1.3. Experimental setup for GC-Organoleptic evaluation of components in quill headspace extract. 29

Figure 1.4. Chromatogram of volatile components in the headspace of porcupine quills. 32

Figure 1.5. Linear relationship of retention time *vs.* carbon number among homologous series. 35

Figure 1.6. Extracted Ion Chromatograms (EIC) for free fatty acids and their methyl esters. 38

Figure 1.7. Total Ion Chromatogram (TIC) of the methanol fraction from silica gel cleanup. 42

Figure 1.8. Mass spectra of δ-decalactone. 44

Figure 1.9. Chromatogram from GC-Organoleptic evaluation of quill headspace volatiles in methanol. 46

Figure 1.10. TIC of the methanol extract treated with methylating reagents. 48

Figure 1.11. Mass spectra of 1-dodecanol acetate and 1-dodecanol. 49

Figure 1.12. Mass spectra of 1-tetradecanol acetate and 1-tetradecanol. 50

Figure 1.13. Mass spectra of 1-hexadecanol acetate and 1-hexadecanol. 51

Figure 1.14. Mass spectra of several representative acyclic terpenoids found in the headspace volatiles of porcupine quill. 53

Figure 1.15. TIC of quill headspace volatiles in methanol. 54

Figure 1.16. Chiral separation of δ-decalactone enantiomers on γ-DEX 120 column. 56

Figure 1.17. Chiral separation of γ-decalactone enantiomers on β-DEX 120 column. 57

Figure 1.18. Summary of experimental pathways and results. 59

Part Two

Figure 2.1. Schematic diagram of a capillary electrophoresis device. 67

Figure 2.2. Illustration of electroosmotic flow. 72

Figure 2.3. Comparison of the electroosmotic flow profile and laminar flow profile. 73

Figure 2.4. Illustration of migration directions of cations, neutral species, and anions under the influence of electroosmotic flow. 75

Figure 2.5. Illustration of the separation mechanism of micellar electrokinetic capillary chromatography. 79

Figure 2.6. Structures of the eleven priority pollutant phenols. 85

Figure 2.7. Electropherograms of the eleven priority phenols by CZE at six different pH buffers. 91

Figure 2.8. Dependence of overall mobility on pH. 92

Figure 2.9. The effect of buffer concentration on resolution and current. 96

Figure 2.10. The effect of applied voltage on column efficiency. 98

Figure 2.11. The dependence of peak width on sample injection time. 100

Figure 2.12. The CZE separation of the eleven priority pollutant phenols under optimized conditions. 102

Figure 2.13. Comparison of current-voltage profiles of the 30 μm and 75 μm capillaries. 106

Figure 2.14. Electropherogram of the eleven priority pollutant phenols using a 50 cm x 30 μm i.d. capillary. 108

Part Three

Figure 3.1. Structures of Tween 40 and Brij 35. 116

Figure 3.2. The effect of Tween 40 on electroosmotic mobility, electrophoretic mobility, and migration time. 119

Figure 3.3. The effect of Brij 35 on electroosmotic mobility, electrophoretic mobility, and migration time. 120

Figure 3.4. Effect of Tween 40 on the resolutions of three closely migrated solute pairs. 123

Figure 3.5. Effects of nonionic surfactants as buffer additives on the separation of the priority pollutant phenols by CE. 124

Figure 3.6. Change of peak shape and migration time of pentachlorophenol as the concentration of Tween 40 increases. 127

PART ONE

Characterization of the Warning Odor of the North American Porcupine

I. Introduction

For most species in the animal kingdom, communication is an essential part in their life. An animal must have a knowledge of the surrounding world in order to survive. This is most obvious for animals such as honeybees, ants, and humans, that live in complex societies [1]. Communication is the mechanism through which the social animals interact with each other and by which they are organized according to their relative status and functions. Although the need is less obvious, communication is also essential in the lives of animals that appear to live alone. For example, communication brings individuals together prior to mating; additional communication is usually necessary to stimulate and guide the mating process.

The purpose of communication is the exchange of information between individuals and their surroundings. Communication can be defined as "the phenomenon of one organism producing a signal that, when responded to by another organism, confers some advantage (or the statistical probability of it) to the signaler or his group" [2].

Communication may be conducted in different modalities, including sight, sound, smell, electromagnetic waves, etc. Human beings gather information about the outside world largely through sight and sound. For many other living creatures, the world is a much different place: for them chemical signals are the primary source of information. Chemical compounds from other organisms or events in the environment provide their basic knowledge of the world [3]. Chemical signals may be a very primitive form of communication [1,3,4]; they probably played an important role in

the evolution of single-celled organisms. For most animals, sensitivity to chemicals in the environment is critical for survival. The early detection of predator odor is an important way for animals to avoid being attacked. On the other hand, predators locate their food by tracking the odor from their targets. Social animals usually keep away from predators when member(s) detect imminent danger and then release(s) alarm signals to the entire group. Chemical communication in insects is the earliest studied as well as the most understood in animal communications. Studies of communication of ants, honeybees, and moths, etc., have shown that chemical communication is the primary mode of information transfer in the insect world [3,4].

Semiochemistry and Semiochemicals

Since communication by chemical signals is an important form of animal communication, it is of great interest to investigate the chemical nature of these special chemical compound(s). This research field is called semiochemistry. According to Albone, "the science of semiochemistry, derived from the Greek word for sign or signal -semeion- deals with the chemistry of those substances, known as semiochemicals, by means of which organism interacts with organism in the shared natural environment" [4]. Semiochemicals may be classified into two major categories: pheromones, those occurring between same species, and allelochemics, those occurring between different species. A pheromone is the chemical message emitted by one organism to stimulate a physiological or behavioral response in some specific way in another member of the same species. Allelochemics are the chemical

compounds by which organisms of one species affect the growth, health, behavior, or population of organisms of another species.

Scientists have known for a long time that odor played an important role in animal communication. It was, however, not until a few decades ago could the first semiochemical be isolated and identified. It was achieved in the study of a sex attractant of the female silkworm moth *Bombyx mori*. This pioneer research, which cost half a million female silkworm moths and took about twenty years to isolate the components and identify the chemical structure, led to the discovery of the target compound: bombykol [5]. With the rapid developments in analytical technology and instruments in the last few decades, more and more insect semiochemicals have been identified. The sensitivity of analysis has been significantly improved to the point that only a few to a few hundred insects are required to identify insect semiochemicals [3]. Some of the insect semiochemicals and their functions are summarized in Table 1.1.

Table 1.1. Some examples of insect semiochemicals and their functions in insect behavior.

Insect	Chemical (Common name)	Function	Reference
Female silkworm moth *Bombyx mori*	(10E,12Z)-Hexadien-1-ol (Bombykol)	Sex attractant	[5]
Japanese beetle *Popillia japonica*	(R,Z)-5-(1-Decenyl)dihydro-2(3H)-furanone (Japonilure)	Sex attractant	[6]
Myrmicine ant *Atta sexens*	D-β-Hydroxydecanoic acid (Myrmicacin)	Herbicide	[7]
Cockroach *Nauphoeta cinerea*	2-Methylthiazolidine 4-Ethylguaiacol	Sex attractant	[8]
Fruit fly *Bactrocera visenda*	3-Methyl-2-butenyl acetate	.....	[9]
Carrion beetle *Nerodes surinamensis*	α and β -Necrodols	Defense	[10]
Spider *Linyphia triangularis*	(R)-3-Hydroxybutyric acid and its dimer	Sex attractant	[11]
Rove beetle *Bledius spectabilis, Platystethus arenarius, Oxytelus piceus*	Acetates, lactones, alkenes, etc.	Defense	[12]
Rice bug *Leptocorisa oratorius*	(E)-2-Octenal n-Octyl acetate	Repellent Alarm	[13]

Chemical Communication in Mammals

Study of chemical communication in insects has been a great achievement, and applications of these studies, for example insect control with semiochemicals [14], have been fruitful. However, advances in the study of chemical communication in mammals have been relatively slow. This is mainly because the mammalian systems are characterized by a much greater complexity.

The main difference between chemical communication in insects and in mammals lies in the different chemoreceptor systems [15,16]. For insects there are specific receptor systems which are tuned to react to just one or a few closely related chemicals. Mammalian chemoreceptors, on the other hand, are tuned to detect a wide range of substances rather than the few specific compounds. To the animals themselves, each system is equipped to meet the requirement of survival in the natural environment.

The complexity of mammalian behavior causes even more difficulty for the study of mammalian semiochemistry. With their constant body temperature and enlarged brain, mammals are less dependent on external environments [3]. Many mammals incorporated highly evolved sensory structures because of the enlarged brain. They may gather information through various sensory organs and obtain an integrated conclusion about the surrounding environment and events. They are somewhat able to control their response to a chemical signal. They make use of semiochemicals in ways similar to other creatures, but they also send messages that

possess more functions. All these factors make it difficult to determine the precise biological effect of a semiochemical on a mammal.

Some of the mammalian communication systems that have been studied and chemicals which have been found to have behavioral effects are summarized in Table 1.2 [17].

Table 1.2. Some example of mammalian semiochemicals and their functions [17].

Animal	Chemical (Source)	Behavior
Boar	androstenol and androstenone (subaxillary gland)	induces lordosis
Deer	cis-4-hydroxydodec-6-enoic acid lactone (urine)	sniffing licking
Marmoset monkey	butyrate esters of long-chain alcohols (circumgenital gland)	subspecies identity
Hamster	dimethyl disulfide (vagina)	elicits attraction
Rhesus monkey	short-chain aliphatic acids (vagina)	sexual activity
Dog	methyl-p-hydroxybenzoate (vagina)	sexual activity
Rabbit	cis-Undec-4-enal (vagina)	"rabbit odor" heart rate
Reindeer	short chain acids; ketones (interdigital)	sniffing
Pronghorn	isovaleric acid (subauricular)	territorial marking, courtship (m/f)
Reindeer	aldehyde, alcohols (tarsal)	.....

The behavioral responses of mammals to semiochemicals are more complicated. Mammalian semiochemicals mediate more complex behaviors than observed in insects. Depending on each animal species, chemical signals can transmit various messages. A list of the known functions is summarized in Table 1.3 [18].

Table 1.3. Types of messages which could be conveyed by mammals by means of olfaction [18].

Intraspecific communication	Interspecific communication
Individual appraisal	Individual
Group membership appraisal	Species membership
Age appraisal	Prey
Social status appraisal	Predator
Sex appraisal	Warning
Reproductive stage indication	Defense
Trail marking	
Territory marking	
Identification of home range	
Warning	
Defense	
Alarm	
Submission	
Gregariousness	

Warning Odor from the North American Porcupine

The North American Porcupine (*Erethizon dorsatum*), like other animals, has its own defensive mechanism to protect itself from being attacked by predators. To most of us, this animal is famous because of its legendary defense, the bristling coat of quills. Driving quills into an enemy's body is, however, the last step of the defense mechanism of porcupine. Before using this ultimate weapon, the animal warns its encounters by means of sight, sound, and smell [19]. The white fluorescent coloration of porcupine stands out in the night, which serves as the first warning. The porcupine's second warning comes from an ominous shivery tooth clacking, which is easy to hear at close distance.

When the warnings by sight and sound have failed, a porcupine sends the third, most immediate signal: a warning odor. According to Roze [19], "The warning smell, released only during times of stress, is so invasive that it is impossible to stay in a closed space such as a car or small room with an aroused adult porcupine. One's eyes begin to water and the nose runs. The degree of smell production is proportional to the size of the animal and its degree of arousal".

Each of the preliminary warning signals reduces the likelihood of the ultimate confrontation between porcupine and predator, detrimental to both. If the ultimate confrontation does happen, the naive attacker will probably end up with a faceful of quills and will have learned a good lesson, and those warning signals will help it to remember the painful experience in its next encounter.

It is not uncommon for animals to send warning signals before using their ultimate defensive weapons. One such example is the "Case of the Poisonous Birds" [20]. Hooded pitohuis, a bird found in New Guinea jungle, has a strong sour smell around its body. The skin and feathers of the bird contain homobatrachotoxin, one of the most powerful poisonous substances known. Injection of the skin or feather extracts into mice caused the mice to develop hindleg prostration and paralysis, leading to convulsions and death in as little as fifteen minutes. The poison is probably the defense weapon of the bird. "Presumably such would-be predators as snakes and possums would be driven off after one bitter, mouth-puckering lick of the pitohui's feathers, and the bird's sour smell and bold, orange-and-black coloration would help them remember the experience" [20]. The poison homobatrachotoxin itself has no odor, so the bird has evolved some unidentified sour smelling chemical to warn off predators before they can take a bite. Warning signals are also wide-spread in insects that are well defended. One example is the bees that defend themselves by stings and carry conspicuous warning coloration. Huheey summarized such a multiple-line defense mechanism in insects [21]: (i) warning coloration, acting at long range to avoid contact; (ii) aversive taste or odors or both, acting upon contact; (iii) toxinosis (usually) acting only upon ingestion.

Porcupine's warning odor is released from the lipids around the quills at the lower back of porcupine. Quills in this area are long and hard, thus they attack enemies most effectively. Porcupine quills have antibiotic properties because wounds by quills rarely become infected. Chemical analysis of quill lipids revealed that there

is an unusually high content of fatty acids in the lipids [19,22,23]. Palmitic, palmitoleic, and oleic acids were found among the major fatty acid components. Fatty acids are known to have antibiotic properties, which accounts for the antibiotic feature of porcupine quills. This chemical analysis, however, focused on the nonvolatile acidic components of the quills. The volatile components of porcupine quills that are responsible for the warning smell of porcupine have only now been characterized. The subject of this part of the thesis was to establish the chemical nature of the warning smell.

Odor Characterization-Headspace Analysis Technique

Chemical communication in animals involves mainly the use of the olfactory organ, hence characterization of chemical components of the odor emitted by the animal is an essential step in the study of chemical communication of animals.

Odor is composed of volatile chemical compounds emitted from an odor source. To reach a receptor, odor molecules must travel over space from the source to the receptor, which can happen only when the molecules are in gas phase. So the vapor above an odorant is the ultimate concern for odor characterization. Consequently, the condensed phase of scent-emitting substances contains important information about the formation and release of volatile compounds into the gas phase. For an accurate understanding of a scent, both the volatile and nonvolatile fractions should be studied.

Odor characterization is most commonly carried out by means of headspace analysis, in which the gas phase over an odorant is collected and analyzed. A distinctive feature of headspace analysis is that the chemical information in the gas phase is used to determine the nature and composition of the condensed phase [24]. At this point headspace analysis shares the same principle with an animal's olfactory communication mechanism in that animals obtain information from surroundings by means of smell.

A major concern of headspace analysis is the sensitivity. Because the extremely high sensitivity and selectivity of animal olfactory organs, the odor threshold for many substances is so low that even the most sensitive instrument cannot match the animal's detection limit. For this reason direct analysis of vapor phase is rarely done, and preliminary concentration of the volatile components is usually required. The dynamic headspace sampling technique is commonly employed to increase the overall sensitivity.

When using the dynamic headspace method to collect volatile compounds, a continuous flow of gas sweeps over the surface of the odor-emitting substance, carrying the headspace volatiles to a trap where they are accumulated. Many factors may affect the collecting efficiency, including length of sampling time, flow rate, temperature, volatility of the compounds, sample matrix, design of device, etc. The selection of the trap is critical, the choice is made depending on analytical requirements and the nature of the sample. A polymer sorbent cartridge is the most frequently used for trapping volatiles. Typical sorbent materials include Tenax,

Porapak Q, charcoal, etc., each designed to fit special analytical requirements. Tenax efficiently adsorbs a wide range of organic compounds, has low retention of water, and is thermally stable, permitting the use of high desorption temperatures. Tenax is the choice for most applications. As an alternative to a sorbent cartridge, cryogenic trapping is also a popular technique for preconcentrating volatiles, especially for on-line collection and analysis by GC. Low temperature is achieved using dry ice or liquid nitrogen, depending on the volatility of the sample.

There have been several systems designed specifically for the collection of volatile semiochemicals from plants and animals [25-34]. Heath and Manukian described a system for the collection of volatiles produced by plants and insects that minimizes stresses on the plant or insect in an environment that is free from chemical impurities [30]. A volatile trapping technique based on dynamic solvent effect was introduced by Apps et al. [31,32]. Volatiles were accumulated on the solvent film formed on a sintered glass bed which was immersed in a solvent. The loaded concentrator was then transferred to a modified GC injector for desorption. The technique gave sensitive and precise determination of volatiles, and was considered a suitable technique for the analysis of mammalian chemical signals [33,34].

Once collected on a sorbent cartridge, volatiles are then desorbed using thermal desorption or solvent elution. The thermal desorption technique transfers volatiles from the sorbent directly into the GC column, so it generally requires modification of the GC injection port. Since thermal desorption is kinetically slow, volatile solutes spread out which results in a wide solute band. It is usually necessary to force

volatiles to reconcentrate at the GC column inlet to provide a narrow solute plug. This is achieved by cryogenic focusing at column inlet end. Thermal desorption provides maximum sensitivity for detection since all trapped volatiles on the sorbent are transferred to the GC column. It is a preferred technique when solvent interference may cause problems, because no solvent is involved in the desorption process. It is also suitable for analysis of thermally labile compounds because a lower desorption temperature or temperature programming can be used for such compounds.

A simpler method for volatile desorption from a sorbent is solvent desorption. Volatiles on the sorbent particles are eluted with a suitable organic solvent, by either continuously passing the solvent through the sorbent cartridge or soaking the sorbent in a vial containing solvent for a period of time. This method has minimal instrumental requirements, with no modification of the GC. Because only a portion of the solution is injected into GC, multiple injections can be made with the same sample. This is important for comparing analytical results from different columns and/or detectors. However, the volatiles are diluted in the solvent which may produce problems of detectability.

GC-Organoleptic Evaluation

Semiochemicals are often released from an organism in extremely small amounts and in very complex mixtures. It is usually necessary to separate and identify all the components in order to locate the active components. The most sensitive and selective analytical tool is gas chromatography coupled with mass spectrometry (GC-

MS). GC-MS has been involved in virtually all chemical analyses of semiochemicals. Preliminary identifications of unknown solutes can be obtained from the mass spectra. However it is often difficult to reveal the biologically active components by GC-MS results alone, because semiochemicals exist in such tiny quantities and so many components make up the scent mixtures. For the purpose of characterization of chemical signals, it is the ultimate goal to find the specific component(s) that cause(s) a specific response in animals. Thus it is necessary to perform a biological assay at each stage of isolation, fractionation and analysis to guide the search for active compounds.

Bioassay with insects is fairly straightforward. Usually the behavioral response of insects exposed to semiochemicals can be visually observed. For example, silkworm moths fan their wings when Bombykol is present; ants exactly follow the trail of extracts from other ant's abdomens. The bioassay in mammals, however, is not as simple. Quite often visual observations are not adequate to reveal the response by mammals, and a specially designed bioassay is necessary. Some examples of the trials include food intake [35], heartbeat [36], metabolites and excretions [37], length of oestrus [38], number of animals being trapped [39,40], etc.

For odor analysis the most direct bioassay is the use of the olfactory organ-the nose. Because of the complex nature of odor, it is necessary to use high resolution capillary column gas chromatography. The sample capacity on capillary columns is inherently small, and the conventional fraction collection followed by the olfactory evaluation technique is not quite compatible with capillary column GC. A solution to

this problem is to sniff each GC effluent peak directly at the outlet end of a wide-bore capillary column. The success of this bioassay method, which is sometimes called GC-Organoleptic Evaluation or Smell GC [41], depends on the experience of human nose as well as instrumental design. Because of individual differences, the description of the odor characteristics for the same solute can be quite different, especially when the amount of solutes is at low levels. For a more objective evaluation, it is necessary to perform the experiment with several persons. Nonetheless, the GC-Organoleptic Evaluation technique is an effective technique for odor characterization, especially for samples of a very limited amount.

Enantiomeric Analysis of Flavor Compounds

It is well established that enantiomers can be very different in sensory properties. A well-known example is carvone; the R enantiomer smells like spearmint, and the S enantiomer smells like caraway [42]. Because the biosynthetic process usually provides an asymmetric environment, the occurrence of optically active compounds in natural materials can be expected. In fact, "optical activity is not only possible, it is probable" [43]. Because most synthetically produced chiral compounds are racemic mixtures, it is useful for authenticity control of natural products to determine the optical purity of chiral compounds in the mixture [43,44].

Enantiomers have identical physical and chemical properties in a symmetric environment. Some asymmetry has to be involved for the chromatographic separation and identification of enantiomers. Enantiomers can be separated indirectly or directly.

Indirect chiral separation requires the use of a chiral derivatizing reagent to convert the optical isomers to diastereomeric isomers, which are no longer enantiomers of each other and can be separated on a nonchiral stationary phase. Direct chiral separation requires a column with a chiral stationary phase to introduce the enantioselective interaction. Direct chiral separation is more attractive because no prior derivatization is required.

In order for enantiomers to be chromatographically separated on a chiral stationary phase (CSP), two conditions must be fulfilled. Diastereomeric adsorbates must be formed between the CSP and at least one of the analyte enantiomers, and they must differ in their free energy of formation [45]. The separation factor, α_{RS}, is thermodynamically related to the difference of free energies of formation, $\Delta(\Delta G)°$.

$$-\Delta(\Delta G)° = RT\ln\alpha_{RS} \tag{1.1}$$

$$\alpha_{RS} = \exp[-\Delta(\Delta G)°/RT] \tag{1.2}$$

Equation 1.2 indicates the temperature dependence of the chiral separation factor, α_{RS}. Lower temperature is necessary to achieve reasonable separation of enantiomers, especially for enantiomers with small values of $\Delta(\Delta G)°$. At this point, HPLC, capillary electrophoresis (CE), and supercritical fluid chromatography (SFC) are advantageous compared to GC because these techniques are normally operated at ambient or near-ambient temperatures. On the other hand, if $\Delta(\Delta G)°$ is large enough, a higher separation temperature can be used to shorten analysis time. This concern is particularly useful for direct GC chiral separation, because a temperature lower than the optimum elution temperature will result in a prolonged analysis time and wide

peak. Thus the development of new CSPs has been a very active area for chiral separations.

Enantioselective analysis is of special interest to flavor and fragrance research because these deal largely with naturally occurring materials. Because numerous enantiomeric pairs have been found to have quite different sensory characteristics, it is necessary to obtain a more accurate knowledge of the natural material by studying the enantiomeric composition. Various approaches for chiral separation of flavor compounds by GC have been surveyed, including direct chiral separation with cyclodextrin-containing chiral stationary phases [46], enantioselective multidimensional GC [44,47], and indirect chiral separation by GC [48], etc. The sensory properties of various classes of optical isomers have been summarized [43,49].

Naturally occurring cyclodextrins (CDs) have been increasingly used as chiral stationary phases, especially in GC and capillary electrophoresis. Cyclodextrins are macrocyclic polymers of glucose that contain from six to twelve D-(+)-glucopyranose units which are bonded via α-(1,4)-linkages [50]. The structure of cyclodextrins is shown in Figure 1.1 [51]. These molecules are shaped like a truncated cone, with a chiral inner surface. The radius of the cavity is determined by the number of glucose units. Enantioselectivity is achieved when enantiomers get in and out of the chiral cavity, based on a host-guest complexation mechanism. A major factor that controls enantioselectivity is the size of the cavity. It is generally necessary to try the separation using a few different chiral stationary phases of different cyclodextrins to provide maximum interaction difference for the two enantiomers.

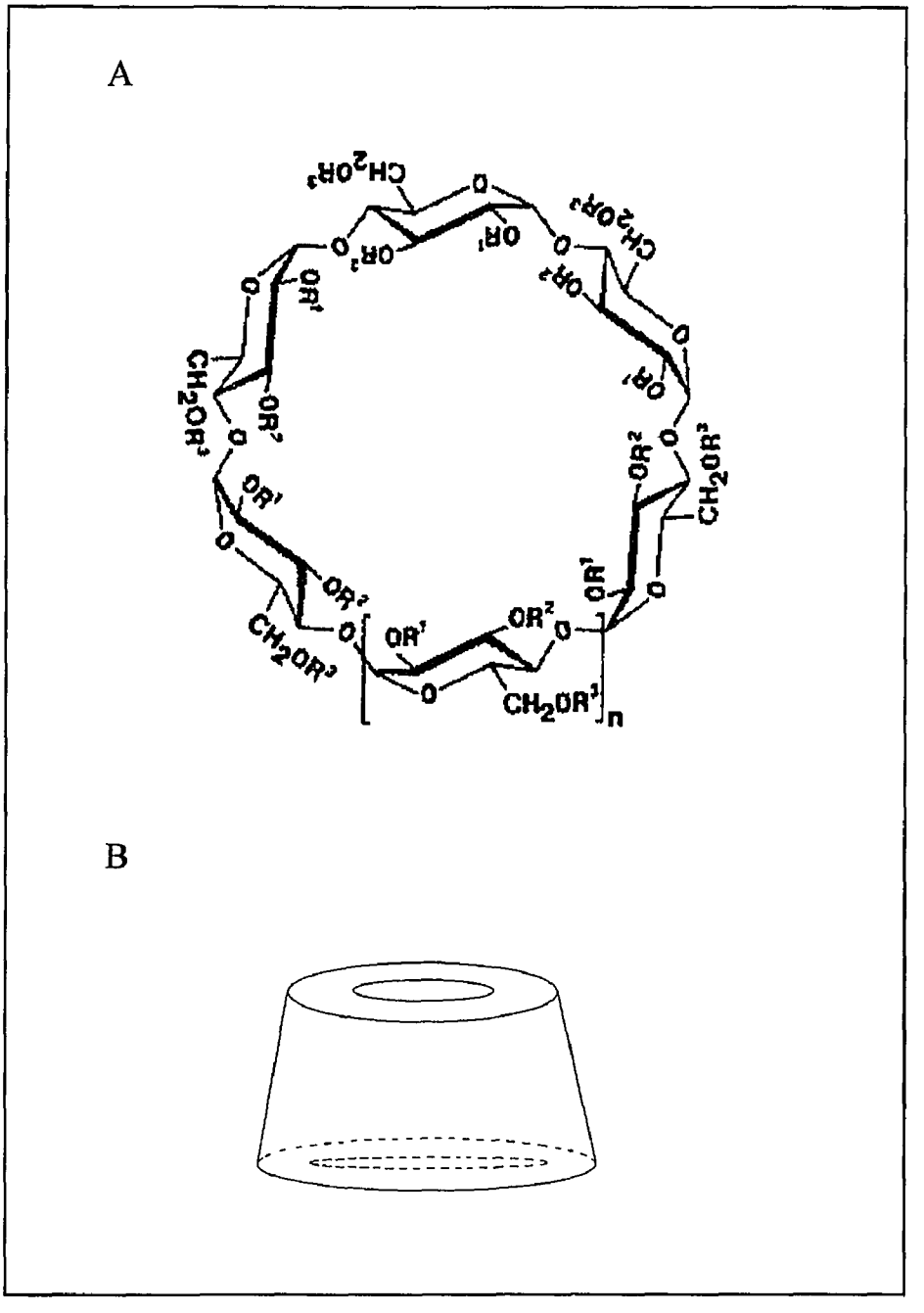


Figure 1.1. A: Structure of cyclodextrins [51]. For non-derivatized CDs, R^1-R^3 = hydrogen atoms. n =1, 2, 3 corresponds to α-, β-, and γ- CD, respectively. B: Truncated cone shape of CD molecule.

Once the right stationary phase is selected, experimental parameters must be further optimized to enhance selectivity and shorten analysis time. For GC this generally means optimizing the temperature program and carrier gas velocity. There is a greater choice of parameters for HPLC, e.g., buffer pH, solvent gradient and additives might all be useful variables. Although there have been many theoretical studies of chiral separation mechanisms and interaction models, most chiral separations are still not predictable. One exception is the direct chiral separation on Pirkle-type stationary phases [45,52]. These are chiral stationary phases designed on the basis of molecular interaction models, so the chiral recognition mechanism is known. Chiral selection on a Pirkle phase results from donor-acceptor (π-π) or charge-transfer interaction between solutes and stationary phase molecules. According to the "three-point interaction rule", the absolute configuration can be assigned to each enantiomer on the chromatogram. However for most naturally occurring chiral stationary phases including cyclodextrins, the three point interaction rule does not apply. The elution order of two enantiomers has to be determined by experiment, which normally requires the use of pure enantiomer standards.

II. Experimental

1. Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

Separation and identification were carried out mainly with a Hewlett-Packard (HP) 5890/5988A GC/MS equipped with a HP 1000 data system. A NBS-Wiley mass spectral library containing 85,000 mass spectra of commonly occurring organic compounds was installed in the data system for computer search of unknown spectra from samples. The columns were either a 50 m x 0.32 mm i.d. HP cross linked Carbowax 20M column or a 30 m x 0.25 mm i.d. DB-5 bonded methyl-phenyl silicone column (J&W Scientific).

A HP5890/5971A GC/MSD was also used for sample analysis. Data acquisition and processing with this system were controlled by the HP Chemstation software package, which included a NBS spectral library of 75,000 entries. A 30m x 0.25 mm bonded DB-5 column was used as the analytical column on this instrument.

2. Collection of Porcupine Quills

Porcupine quills were collected by Dr. Uldis Roze (Biology Department, Queens College). His previous observations have demonstrated that the lipid around the quills is the source of the odor [19]. After capture, porcupines were anesthetized by injection of ketamine·HCl. Immediately after the animals lost their defensive abilities, quills around different skin areas were smelled and collected. These fresh

quills were sealed in separate 40 ml precleaned sample vials and stored in a freezer until analyzed.

3. Collection of Headspace Volatile Components from Porcupine Quills

The device used for collecting headspace volatiles is shown in Figure 1.2. Fresh quills were placed in a glass container such as a vacuum filtration flask or an air sampling vial (Supelco #6-4712). A thermal mantle was placed under the sample container to keep the sample warm (approximately 40 to 50°C), which aids the release of volatiles from the lipids. A charcoal cartridge trap was placed in front of the purging air inlet to remove volatile organic impurities from air. The purified air was drawn with a portable vacuum pump (SKC, Model 224-PCXR7) at a speed of 1 L/min over the surface of quills, and passed into a Tenax cartridge for a period of time, typically 5 to 10 hours. When the collection was finished, the Tenax cartridge was removed and stored in a freezer until analysis.

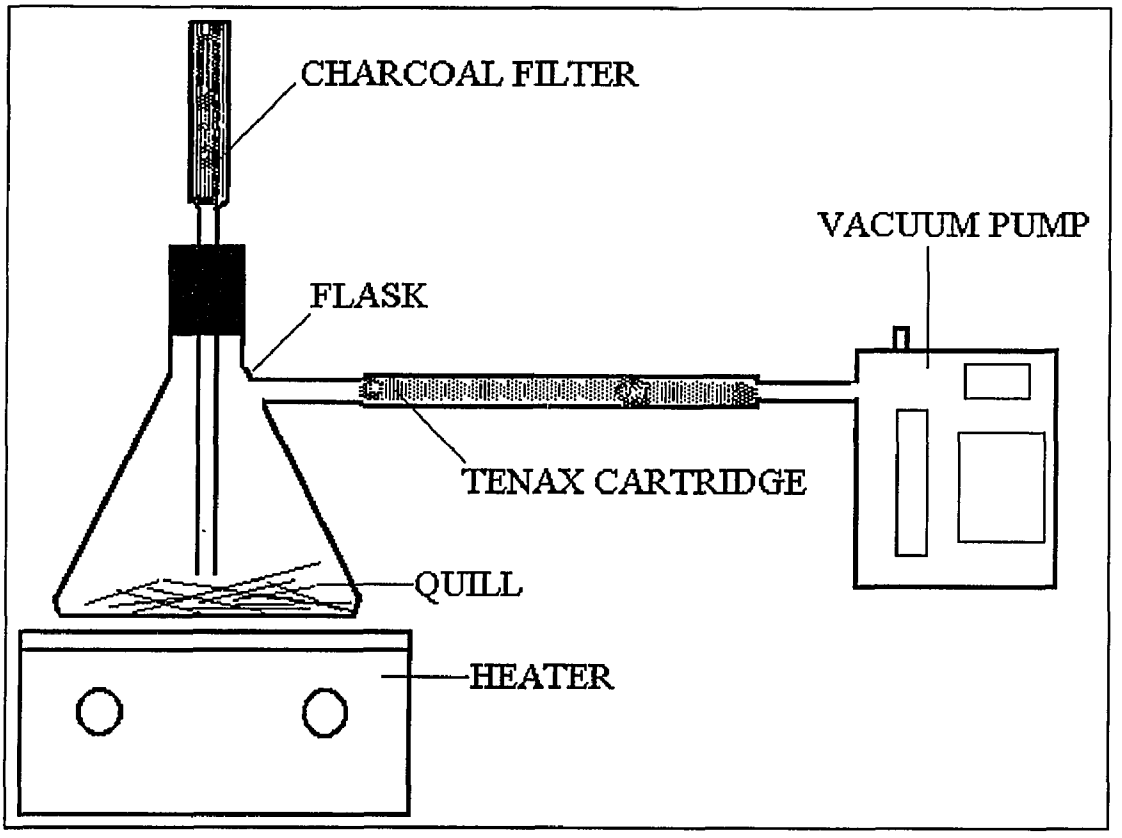


Figure 1.2. The experimental device for collecting quill headspace volatiles.

4. Solvent Desorption of Volatiles from Tenax

The Tenax sorbent was transferred to a small glass pipette tip. The volatile compounds on Tenax were eluted with 0.5 ml of methanol into a chilled 1 ml micro vessel (Supelco, #3-3293), which was then sealed and stored in a freezer.

5. Methylation of the Acidic Components in the Volatile Extract

The methanol solution of quill headspace volatiles collected on Tenax was treated with a transesterification reagent, methanolic (m-trifluoromethylphenyl) trimethylammonium hydroxide (Meth-Prep II, Alltech, #18007). The mixture was allowed to stand for 30 min, and then analyzed by GC/MS. A blank solution was prepared by adding the same concentration of Meth-Prep II solution to methanol.

Diazomethane was also used to methylate the fatty acids in the headspace extracts. Diazomethane was generated by mixing 1-methyl-3-nitro-1-nitrosoguanidine (Aldrich, #12,994-1) with cold NaOH solution. For safety reasons the reaction was carried out in a special diazomethane generator (Aldrich, #10,100-1). The gaseous diazomethane was collected in cold ether, and a portion of the solution was added to the methanol extract of quill headspace volatiles.

6. Isolation of the Acidic Components from the Odor Extract

To a chilled microvessel that contained 100 μL of methanol extract of porcupine odor, 100 μL of 1 N NaOH was added. After mixing, the odor of the mixture was checked organoleptically. After a 24 hour reaction, the basic and neutral

components were extracted into hexane (2 x 500 μL). The remaining aqueous solution was cooled and acidified to pH<2 with 1 N HCl. The mixture was extracted again with hexane (2 x 500 μL). Both of the hexane extracts were concentrated using a stream of nitrogen to the final volume of 50 μL. The odor properties of each solution were examined; the components in the solution were identified by GC/MS.

7. Fractionation of Total Organics from Porcupine Quills on Silica Gel

To a sample vial containing approximately 2 g quills, 15 mL of hexane solvent (Spectro Grade, Aldrich, #24887-8) was added. The vial was vigorously shaken for 2 min and allowed to stand for 30 min with occasional shaking. The vial was centrifuged for 5 min and the upper clear solution was separated. The quill was reextracted with another 15 ml of hexane. The hexane extracts were combined and added to a dry-packed silica gel (activated by adding 5% of H_2O) column, followed by addition of 10 mL each of hexane/ether (50/50) and methanol. Three portions of column effluents were collected, which were concentrated to 1 mL final volume with a stream of nitrogen. The odor of each concentrated fraction was recorded, and the solution was analyzed by GC/MS.

8. Gas Chromatography-Olfactory Detection

Preliminary experimental results showed that the porcupine warning odor is a very complex mixture of organic compounds. In order to determine which of the components contribute to the odor, olfactory detection after GC separation was

performed. A 30 m x 0.53 mm i.d. (megabore) Supelco SPB-5 column was used for the analysis in order to be able to inject more sample (6 μL). Instead of passing into the detector, the outlet column end was conducted outside of GC oven. To avoid condensation of the column effluent, the portion of the column outside the GC oven was heated by a specially designed heating device shown in Figure 1.3, using a short length of Teflon tubing. One end of the Teflon tubing was inserted inside the GC oven; the other end was connected to a copper "T" unit. The column was inserted through the Teflon tubing and through a silicone septum at the other end of the copper "T". The side arm of the "T" was connected to a vacuum pump. During the GC temperature program the hot air in the oven was pumped by the vacuum into the copper "T" so the unit could be heated. After sample injection, the GC effluents were sniffed and a description of the odors and corresponding runtime were recorded.

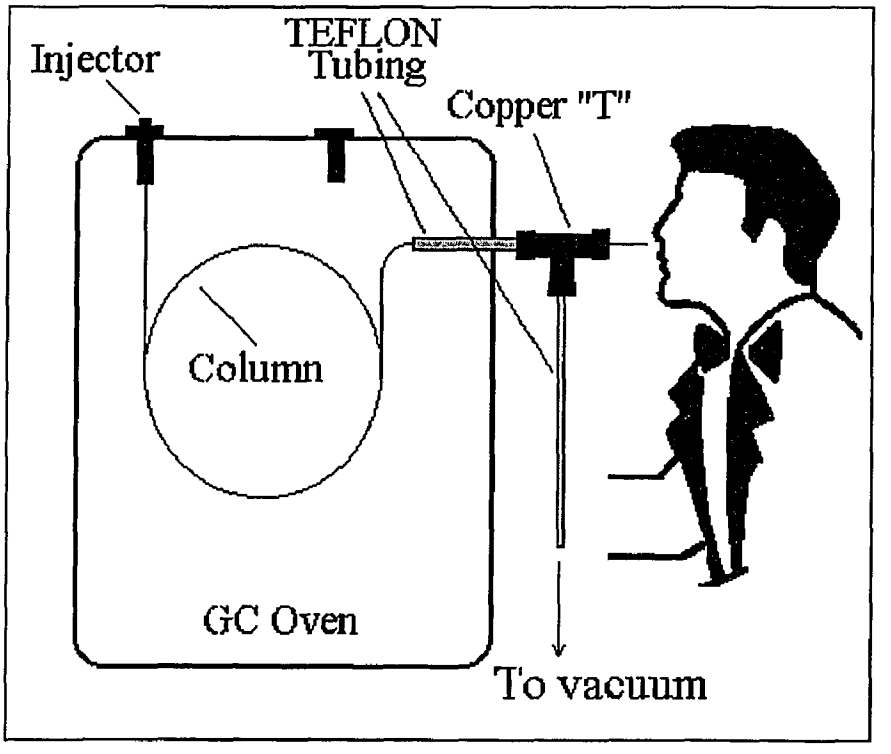


Figure 1.3. Experimental setup for GC-Organoleptic evaluation of components in quill headspace extract.

9. Determination of Enantiomeric Composition of Decalactones

Chiral separation of γ- and δ- decalactones was carried out directly on cyclodextrin-embedded capillary GC columns. A set of three chiral columns, α-, β-, and γ- DEX 120, were obtained as a gift from Supelco (Bellefonte, PA). These columns were coated with 20% permethylated cyclodextrin in SPB-35 (poly 35% diphenyl/65% dimethylsiloxane). Separation and identification was performed using both a HP5890/5988A GC/MS and a HP5880 GC/FID. Chiral separation conditions were selected by testing the separation of the racemic mixtures of each lactone to achieve the optimized resolution. The methanol solutions of the headspace volatiles collected on Tenax were subsequently analyzed to identify the enantiomeric compositions of decalactones.

III. Results

1. Identification of Volatile Components in the Methanol Extract

Immediately after desorbing the volatiles from Tenax with methanol, it was known that the odoriferous compounds were successfully collected in the methanol, because the methanol extracts possessed the characteristic porcupine odor. The methanol solution was then analyzed by GC/FID and GC/MS. A representative chromatogram is shown in Figure 1.4. Identification of the corresponding components is given in Table 1.4. In addition to searching the mass spectral library by computer, the identifications were also confirmed by comparing the mass spectra and retention times of authentic compounds whenever such compounds were available.

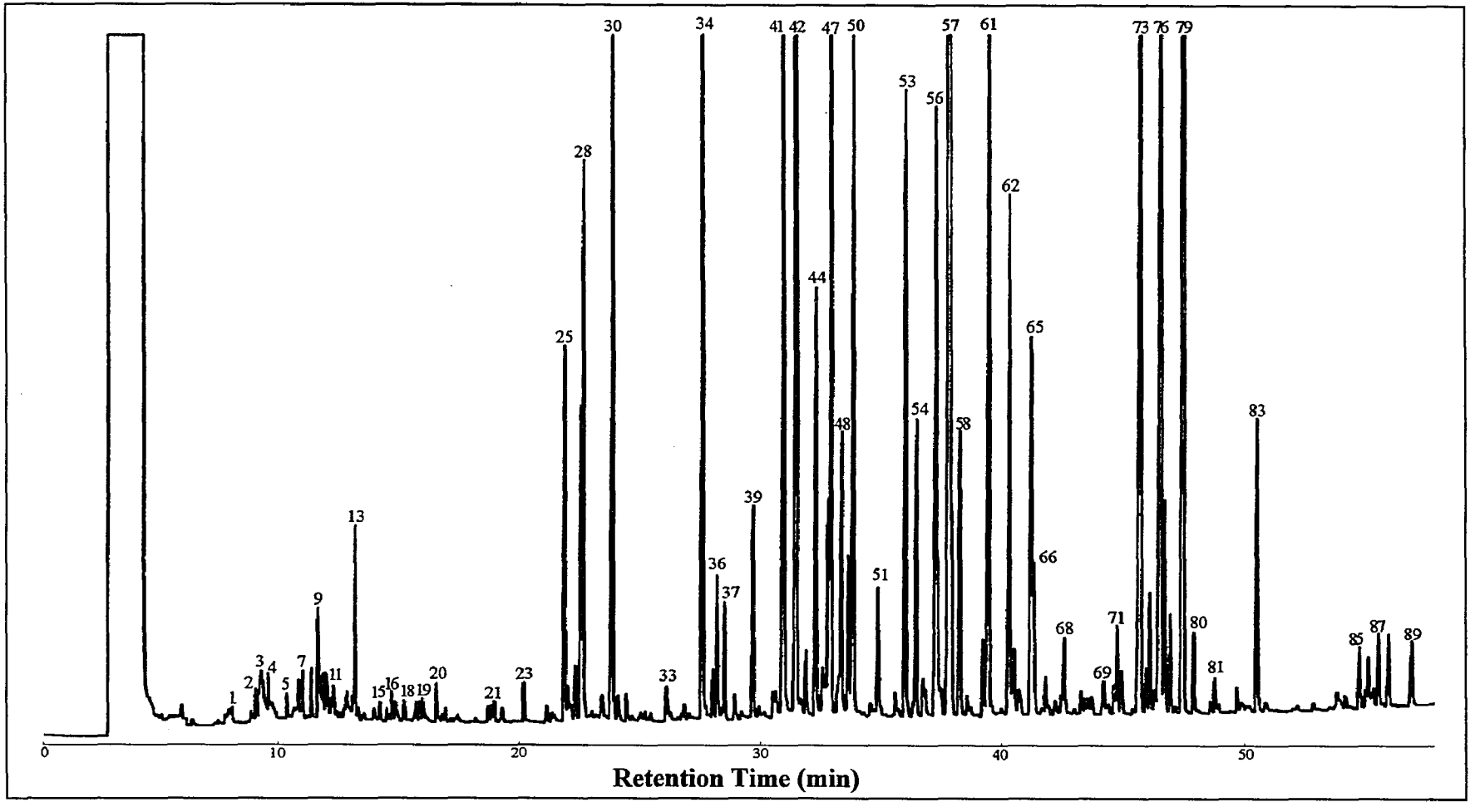


Figure 1.4. Chromatogram of volatile components in the headspace of porcupine quills.
Sample: methanol desorption product from Tenax; column: 60 m x 0.25 mm DB-5; detector: FID; GC temperature: 65°C for 2 min, 10°C/min to 150°C, held for 2 min, then 2°C/min to 270°C.

Table 1.4. Identification of volatile components in the headspace of porcupine quills

Peak #	Peak Identification	A% of Total
1	Heptanal	<0.1
2	2-Heptenal	<0.1
3	Hexanoic acid*	0.1
5	6-Methyl-5-hepten-2-one	<0.1
7	Heptanoic acid*	0.1
8	2-Nonenal	0.1
9	Unidentified fatty acid	0.2
13	Octanoic acid*	0.4
18	γ-Octalactone	0.1
19	Nonanoic acid*	0.1
20	δ-Octalactone	0.2
21	Decanoic acid*	0.1
23	Dodecanal	0.1
25	Geranylacetone*	1.1
27	1-Dodecanol*	0.9
28	γ-Decalactone*	1.8
30	δ-Decalactone*	2.8
34	Dihydroapofarnesol	5.3
36	1-Dodecanol acetate*	0.5
37	Tetradecanal	0.4
39	1-Tetradecene	0.8
41	cis-9-Tetradecen-1-ol	3.8
42	1-Tetradecanol*	5.3
43	γ-Dodecalactone	0.2
44	Unidentified terpenoid	1.6
47	Unidentified terpenoid	3.3
48	δ-Dodecalactone*	1.2
50	Farnesol*	3.2
51	Farnesal	0.5
53	Unidentified	2.4
54	Unidentified	1.1
56	1,13-Tetradecadiene	3.0
57	1-Tetradecanol acetate*	14.2
58	Hexadecanal	1.2
61	Farnesyl acetate	4.0
62	1-Octadecene	2.1
65	1-Hexadecanol*	1.7
73	Alkyl (C16) acetate	6.8
76	Alkyl (C16) acetate	8.9
79	1-Hexadecanol acetate*	8.7

* Confirmed by mass spectrum and retention time of authentic compounds.

From Table 1.4 it can be seen that porcupine odor consists mostly of aliphatic compounds, including acids, alcohols, aldehydes, acetates, and lactones, isoprenoids, etc. Tetradecyl acetate, hexadecyl acetates, and decalactones are among the major peaks. Most of these compounds belong to various homologous families as shown by the empirical linear relationship rule in GC. This rule states that for a homologous series differing only in $-CH_2-$ units, a linear relationship exists between the logarithm of isothermal retention time ($\log t_R$) and carbon number; or between retention time (t_R) and carbon number for linear programmed temperature analysis. Figure 1.5 shows the linear relationships for various class of compounds, which serves as a useful verification to the identification of these compounds.

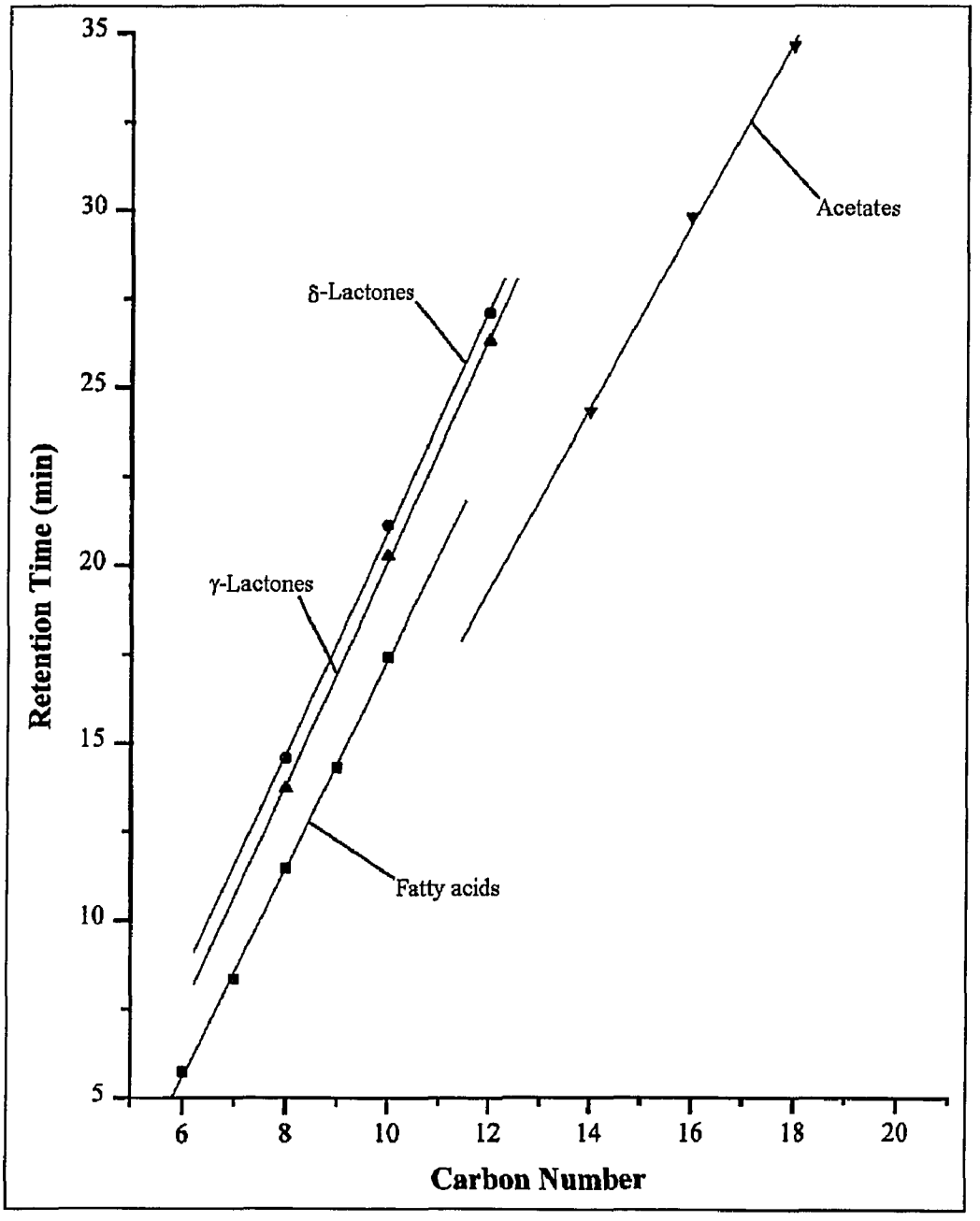


Figure 1.5. Linear relationship of retention time *vs.* carbon number among homologous series. Column : 30 m x 0.25 mm DB-5; detector: MS; GC temperature: 60°C for 2 min, 10°C/min to 280°C.

2. Identification of Acidic Components in the Odor Extract

The first qualitative impression of porcupine odor was that it was fatty acid-like [19], so initial attention was given to acidic components in the odor extract. Because acidic compounds usually do not chromatograph well, especially on less polar columns, the fatty acids were converted to their corresponding methyl esters with Meth-Prep II or diazomethane and subsequently analyzed by GC/MS. The extracted ion chromatogram (EIC) technique was used to locate the methyl esters in the total ion chromatogram (TIC). With this method, the intensity of a specific ion was extracted from the spectrum at each scan and plotted versus retention time. Only the spectra that contain that specific ion will have a significant intensity in the EIC. When the selected ion is characteristic of a class of compounds, the search for these compounds can be greatly simplified. The mass spectra of fatty acids with more than two carbon atoms are distinguished by the peaks at m/z of 60 and 73. Correspondingly, the methyl esters of these fatty acids have peaks at m/z of 74 and 87 in their spectra. Thus ions of m/z 74 and 87 were extracted to get an EIC for identification of methyl esters, and as a comparison, ions of m/z 60 and 73 were used to identify free fatty acids from the original volatile extract. The chromatograms are given in Figure 1.6. It can be seen that the odor extract contains a series of straight chain free fatty acids with carbon number ranges from 6 to 18. Although several fatty acids were found directly from the original odor extract, it is clear from Figure 1.6 that conversion of free fatty acids to methyl esters significantly increases the analytical sensitivity for these compounds. Again the retention time of each nonbranched methyl esters increases nearly linear

with carbon number. Those peaks eluting between the two adjacent nonbranched methyl esters should be either branched or unsaturated fatty acid methyl esters. The identification of the acidic components from their methyl esters is given in Table 1.5.

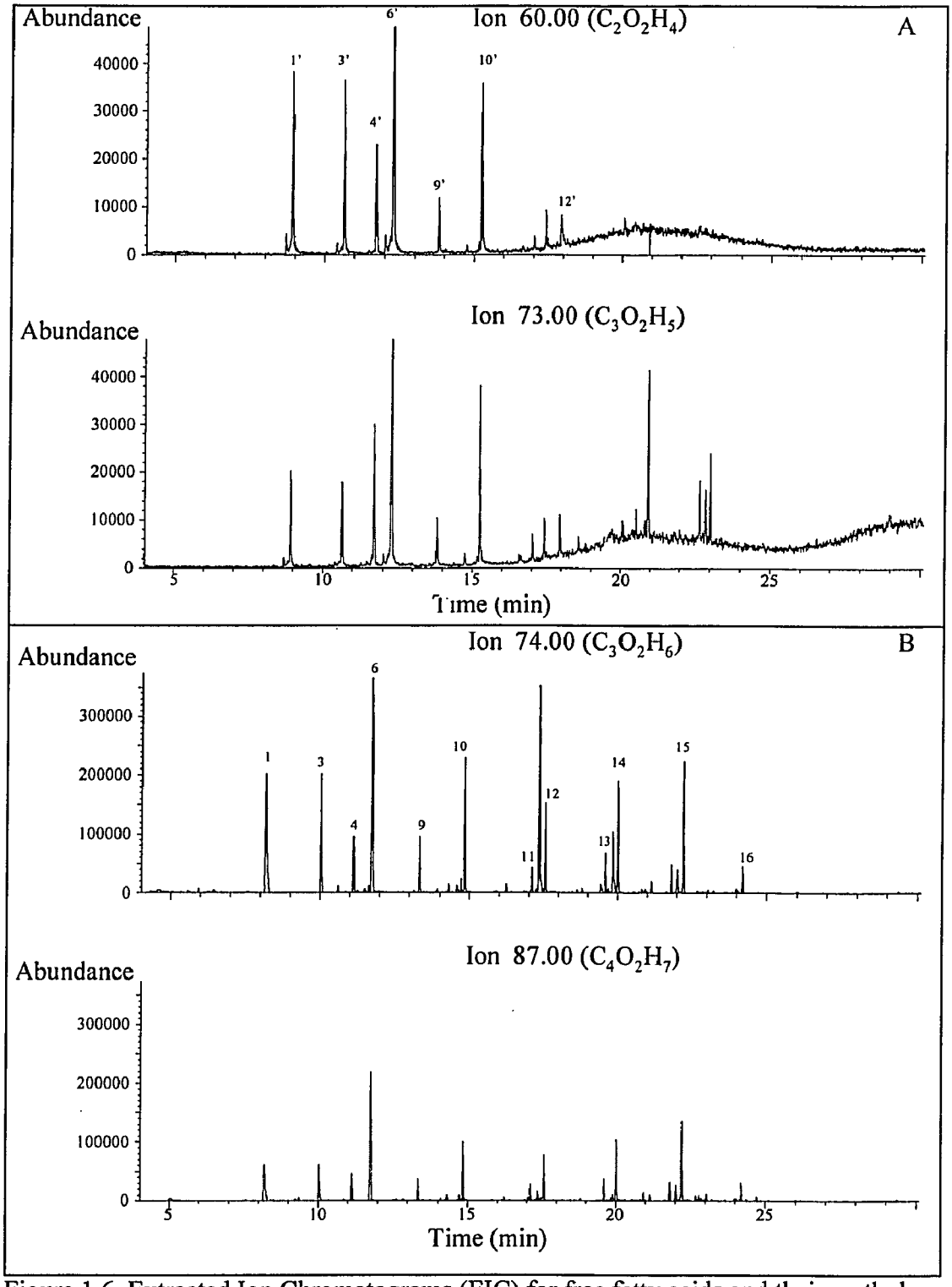


Figure 1.6. Extracted Ion Chromatograms (EIC) for free fatty acids and their methyl esters. A: m/z 60 and 73 were extracted to identify free fatty acids in the original headspace sample; B: m/z 74 and 87 were extracted to identify methyl esters in the headspace sample treated with methylating reagent (diazomethane).

Table 1.5. Acid components in the headspace of porcupine quills identified as their methyl derivatives

Peak ID	Ret. Time (min)	Identification of Methyl Esters
1	8.19	Hexanoic
2	9.35	4-Oxopentanoic
3	10.02	Heptanoic
4	11.13	6-Methylheptanoic
5	11.51	Benzoic
6	11.75	Octanoic
7	12.21	Methyl nicotinate
8	12.76	Benzeneacetic
9	13.35	Nonanoic
10	14.85	Decanoic
11	17.10	Unidentified
12	17.58	Dodecanoic
13	19.59	Unidentified
14	20.01	Tetradecanoic
15	22.20	Hexadecanoic
16	24.19	Octadecanoic

After the methylation of fatty acids in the extract, the odor of the sample was examined by several staff members again to see if there was any change in the odor. The result was: the basic note of porcupine odor remained unchanged but there was a new more fruity-sweetish smell, which is apparently due to the formation of methyl esters. This eliminated the possibility of fatty acids being the principal odor component.

The exclusion of fatty acids as the principal components contributing to the specific porcupine odor was also confirmed by treatment of odor extract with base: addition of 1 N NaOH to the extract did not eliminate the porcupine odor from the extract right away. However the porcupine odor indeed disappeared from the NaOH treated solution after standing for several hours. After separating the basic and neutral from acidic components, it was found that the porcupine odor appeared in the acidic fraction. This experiment suggested that the odor might originate from an ester-type compound.

3. Identification of Components in Each Fraction from Silica Gel Cleanup

The concentrated hexane fraction did not have any special odor, and no significant solute peaks were found by GC. This indicates that there was not a large amount of nonpolar components in the porcupine quills. The hexane/ether fraction was smelly, however it was not porcupine-like. Many compounds were tentatively identified by GC/MS from this fraction, including alcohols, aldehydes, alkenes, ketones, acetates, etc.

Special attention was given to the methanol fraction because it possessed the characteristic porcupine odor. This fraction was further concentrated to 100 μL and analyzed by GC/MS. The chromatogram and identification of the solutes are shown in Figure 1.7.

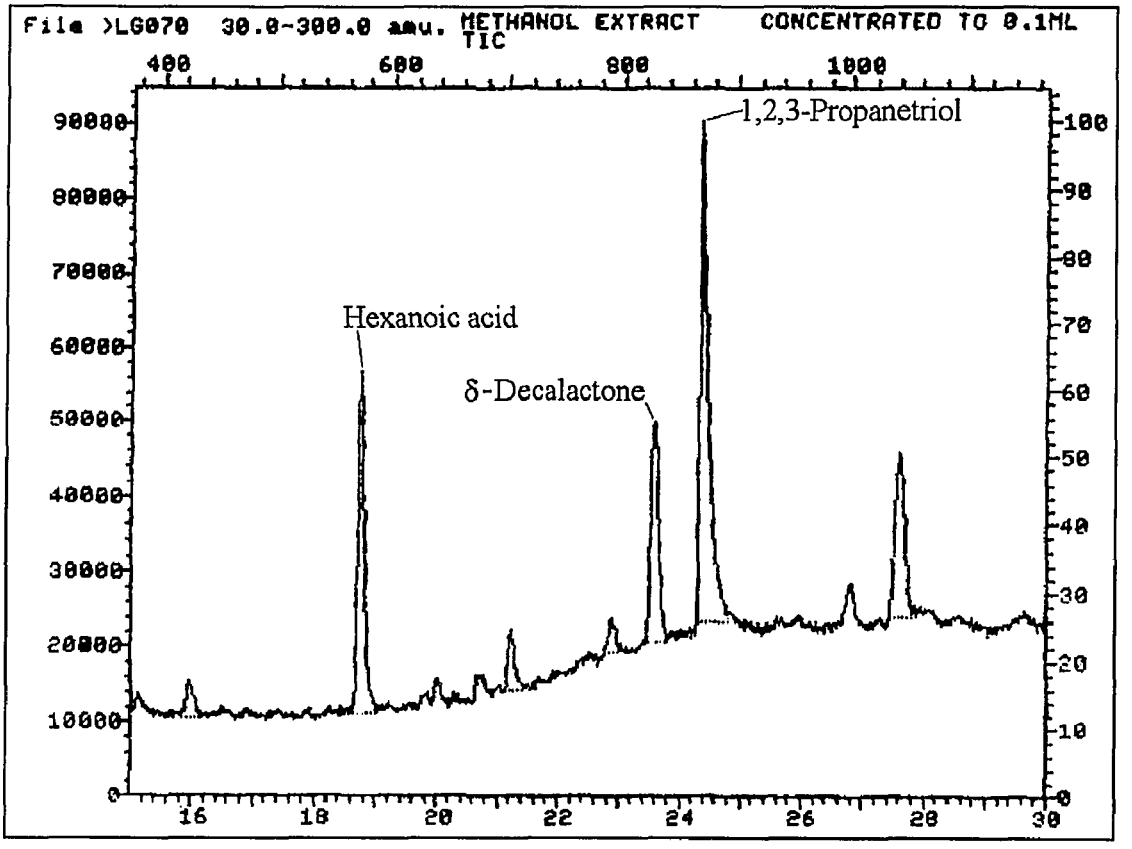
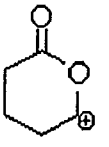


Figure 1.7. Total Ion Chromatogram (TIC) of the methanol fraction from silica gel cleanup.

The chemical composition of the methanol fraction is relatively simple. Among the identified solutes, the peak at retention time of 23.6 min attracted most attention. This compound was identified as δ-decalactone. The mass spectrum of this unknown peak and the standard spectrum of δ-decalactone are given in Figure 1.8(A) and 1.8 (B), respectively. The base peak of 99 is the characteristic ion for δ-lactones, which corresponds to elemental composition of $C_5H_7O_2$. This fragment is formed by the cleavage of hydrocarbon side chain from the lactone ring to give a stable six-member ring structure:



$[C_5H_7O_2]^+$, m/z = 99

Similarly, γ-lactones have a base peak of m/z 85 ($C_4H_5O_2$) in their mass spectra. The identification of δ-decalactone was confirmed by comparing the mass spectrum and GC retention time of the authentic compound (Aldrich Chemicals) with the unknown peak. We then examined the odor properties of the δ-decalactone standard. Indeed, the standard was very similar to the porcupine's odor, except that it also gave a strong coconuty smell. This was the first clue that led to the discovery of δ-decalactone as the major odor contributor.

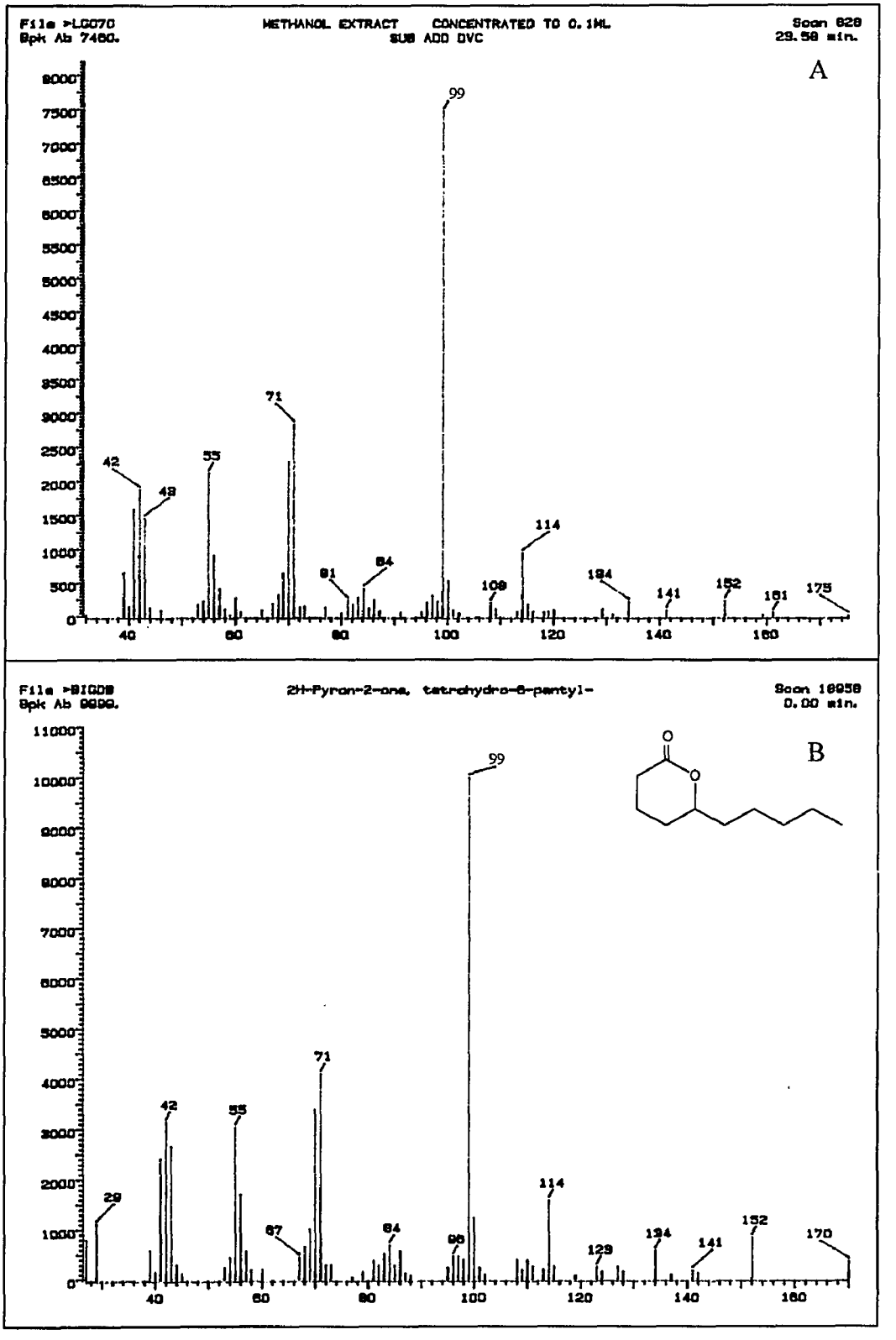


Figure 1.8. Mass spectra of δ-decalactone. (A) identified from quill volatile; (B) standard spectrum from NBS-Wiley spectral library.

4. GC-Organoleptic Evaluation

The complexity of porcupine odor prompted us to perform gas chromatography with "smell detection" to find out the odor characteristics of each GC peak. Initial attempts were not successful because very little odor could be smelled along the retention range of the solute peaks. We attributed the lack of odor to the column end being kept at room temperature. When vaporized solute molecules reach the end of the column at room temperature, these molecules recondense on the wall of the column before reaching the nose. For this reason we designed a simple device (described in the Experimental section) to heat the piece of column outside the GC oven. With this experimental setup, more compounds can be detected by nose. The results of this experiment are illustrated in Figure 1.9. This shows that odoriferous components eluted between 5-16 min, though peaks within this retention range are relatively small. Some small peaks, e.g. fatty acids, gave strong pungent acidic odors. The characteristic porcupine odor was detected in several places on the chromatogram, but the most significant one occurred near 15 min, which corresponds to the retention time of δ-decalactone. So the GC-Organoleptic Detection results confirm the suggestion of δ-decalactone being responsible for the porcupine odor.

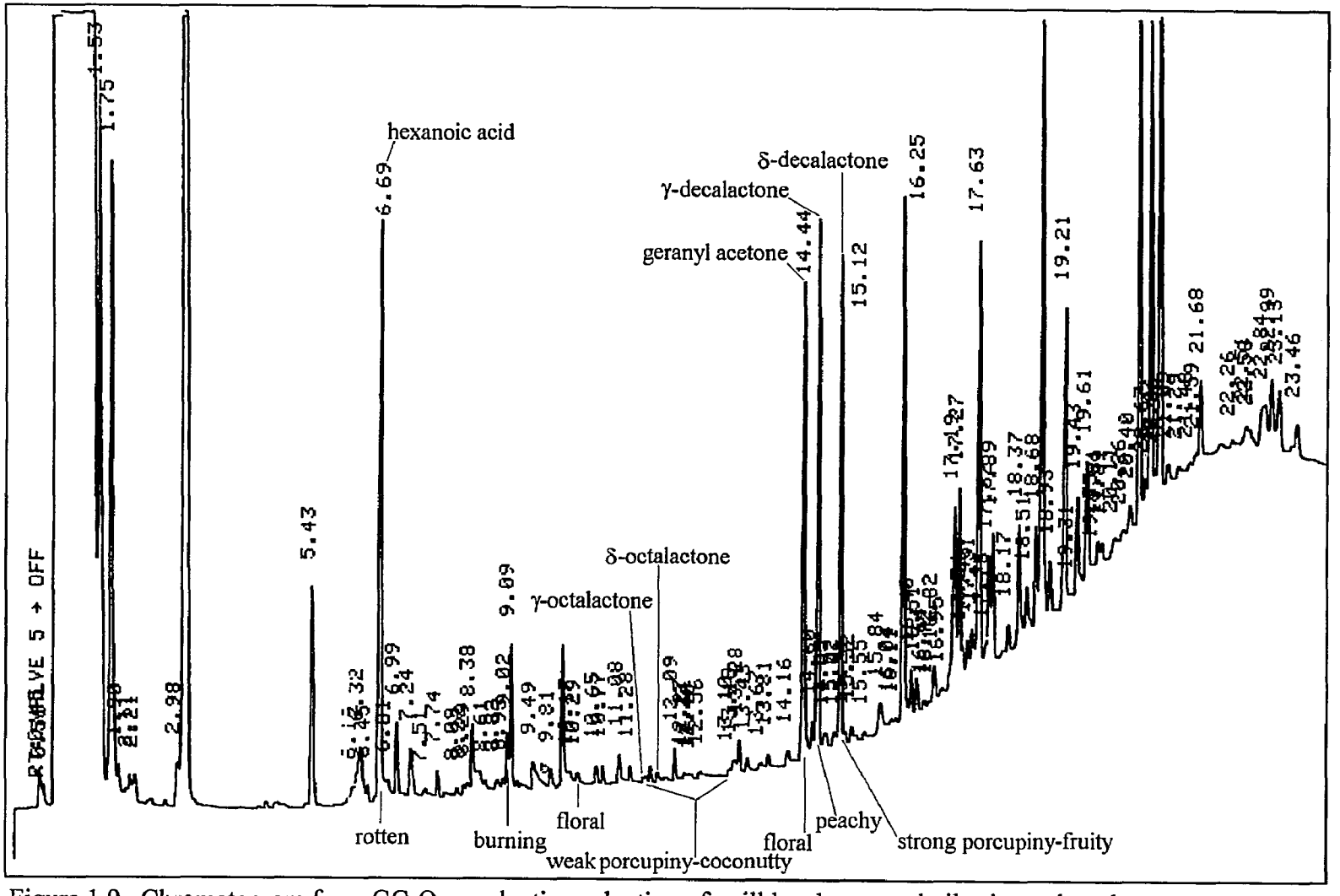


Figure 1.9. Chromatogram from GC-Organoleptic evaluation of quill headspace volatiles in methanol.

5. Transesterification by Methylation Reagents

It is interesting to notice the change in the major peaks of acetates after the methanol extract was treated with the trans-methylating reagent, Meth-Prep II. In Figure 1.10 chromatograms of (a) the original methanol extract of quill headspace volatiles on Tenax, (b) the methanol extract treated with Meth-Prep II, and (c) the methanol extract treated with diazomethane are shown together in order more clearly to observe the changes. It can be see that all the acetate peaks disappeared when the extract was treated with Meth-Prep II. A large peak at t_R = 19.51 min, identified as 1-tetradecanol, was detected instead. Apparently Meth-Prep II caused transesterification reactions of the acetates. For acetates, the reaction products will be methyl acetates (which is a very volatile compound that cannot be seen in the chromatogram due to solvent delay time) and the corresponding alcohols. However such reaction could not be seen in the diazomethane-treated sample in Figure 1.10(c). Diazomethane is not a transesterifying reagent, so acetates remained to be the major peaks in this sample. The mass spectra of the acetates and the corresponding alcohol products are compared and shown in Figure 1.11-1.13. It can be seen that the spectra of the acetates and corresponding alcohols are quite similar. The major differences are m/z 43, which corresponds to the fragments of CH_3CO- and C_3H_7-, and m/z 61, possibly of $[CH_3C(OH)_2]^+$ (protonated acetic acid), in the spectra of acetates.

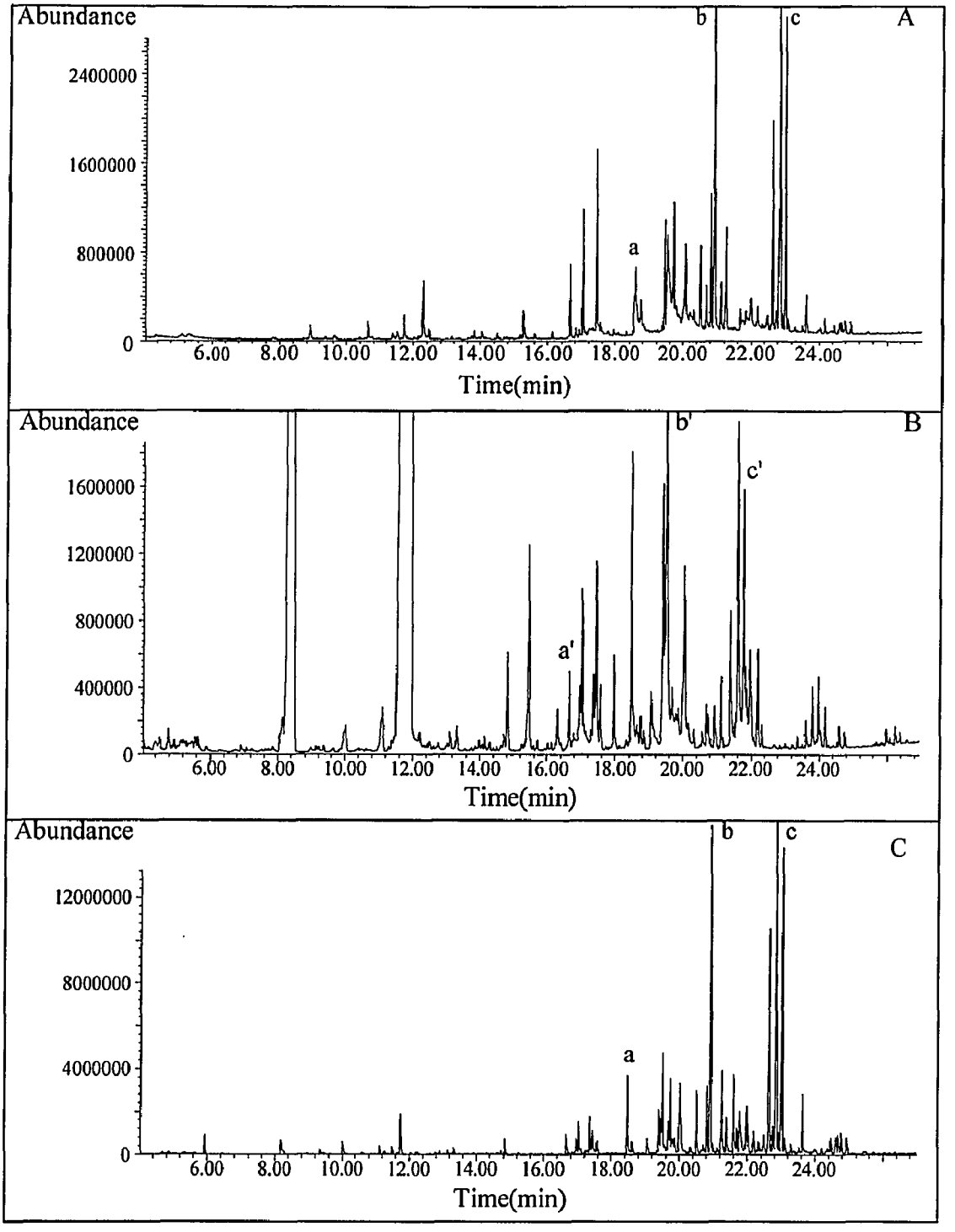


Figure 1.10. TIC of the methanol extract treated with methylating reagents. (A) the original headspace extract; (B) the original extract treated with Meth-Prep II; (C) the original extract treated with diazomethane. Peak a, b, and c are dodecyl, tetradecyl, and hexadecyl acetates; peak a', b', and c' are the corresponding alcohols.

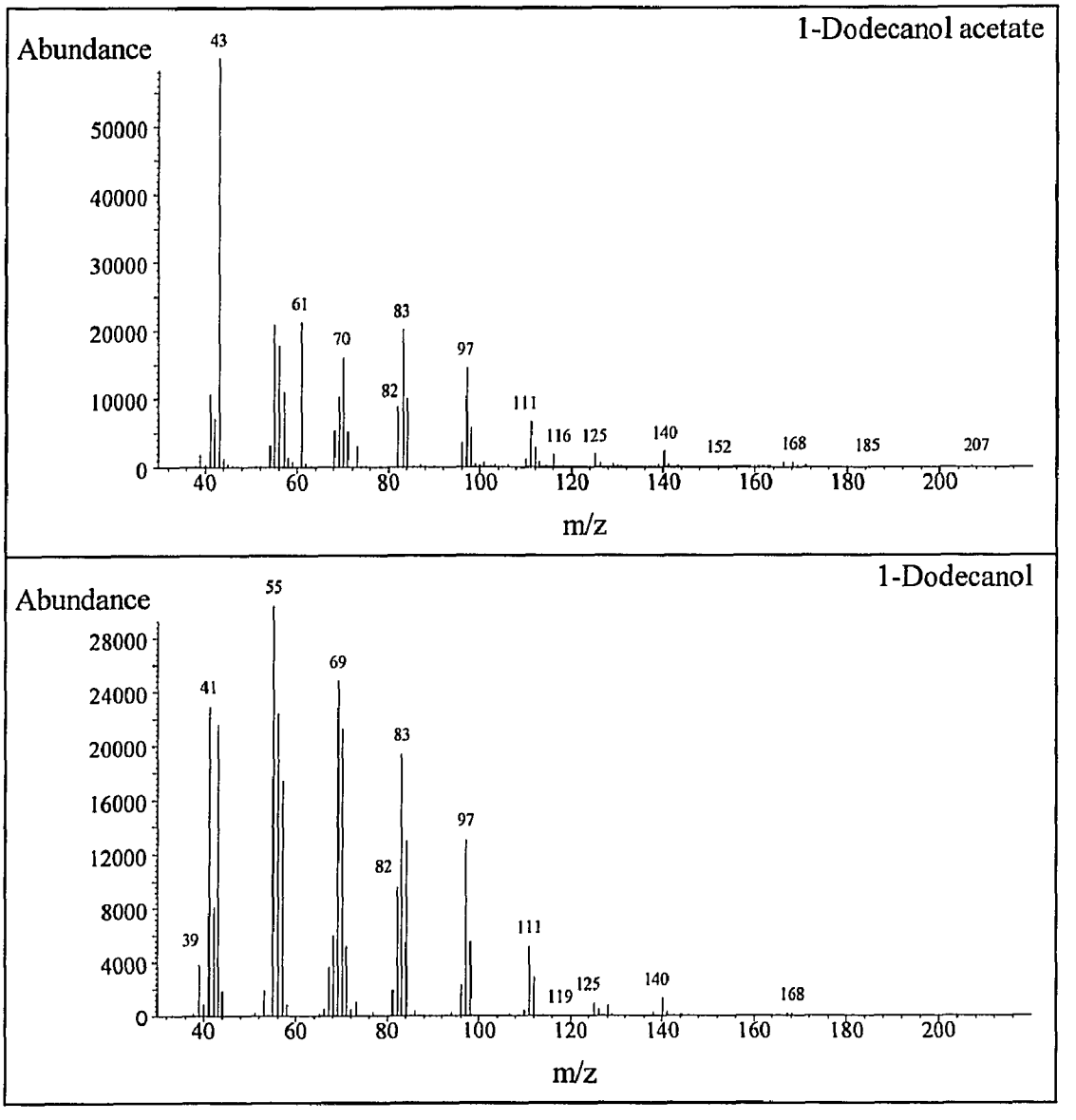


Figure 1.11. Mass spectra of 1-dodecanol acetate and 1-dodecanol.

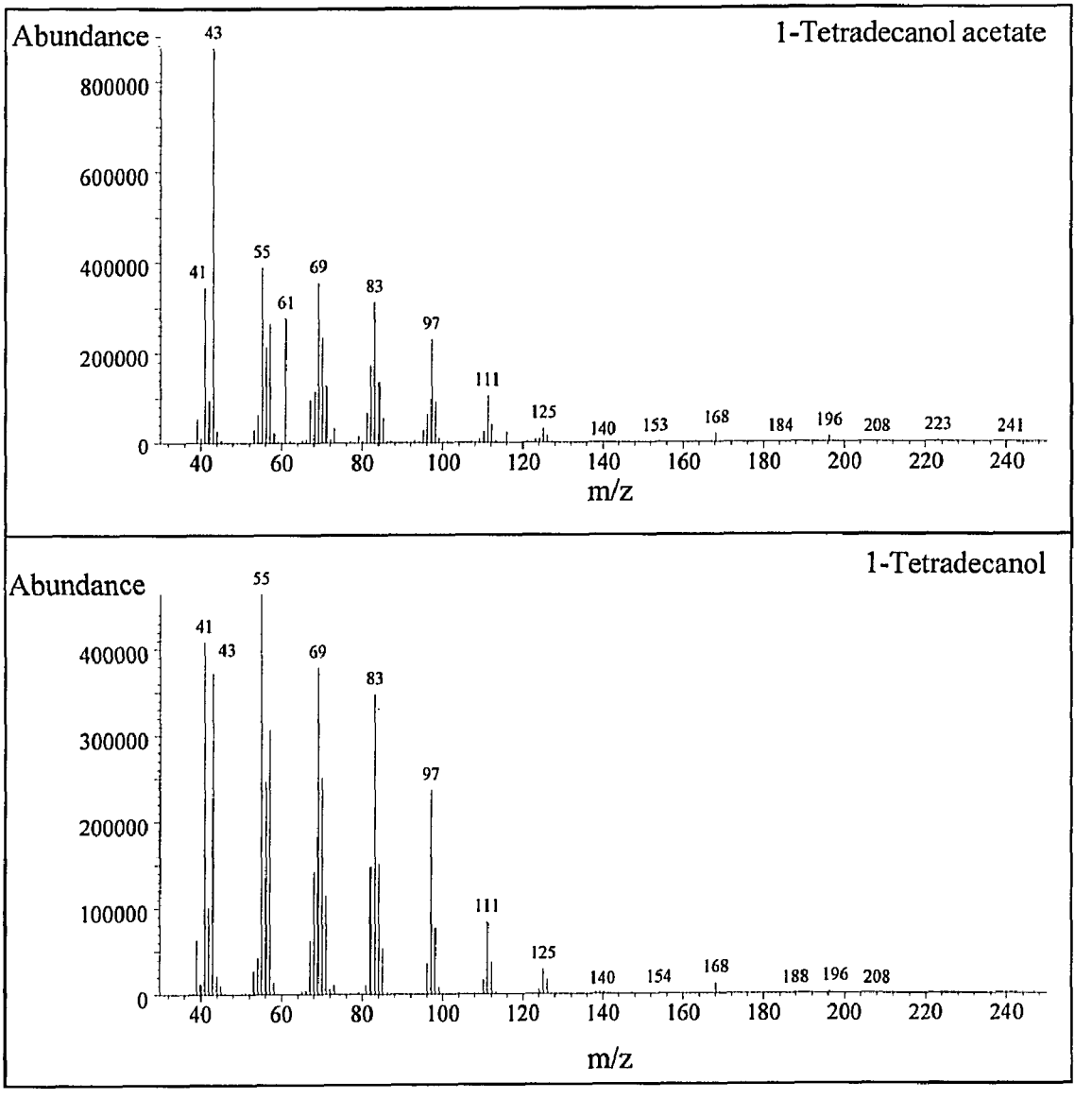


Figure 1.12. Mass spectra of 1-tetradecanol acetate and 1-tetradecanol.

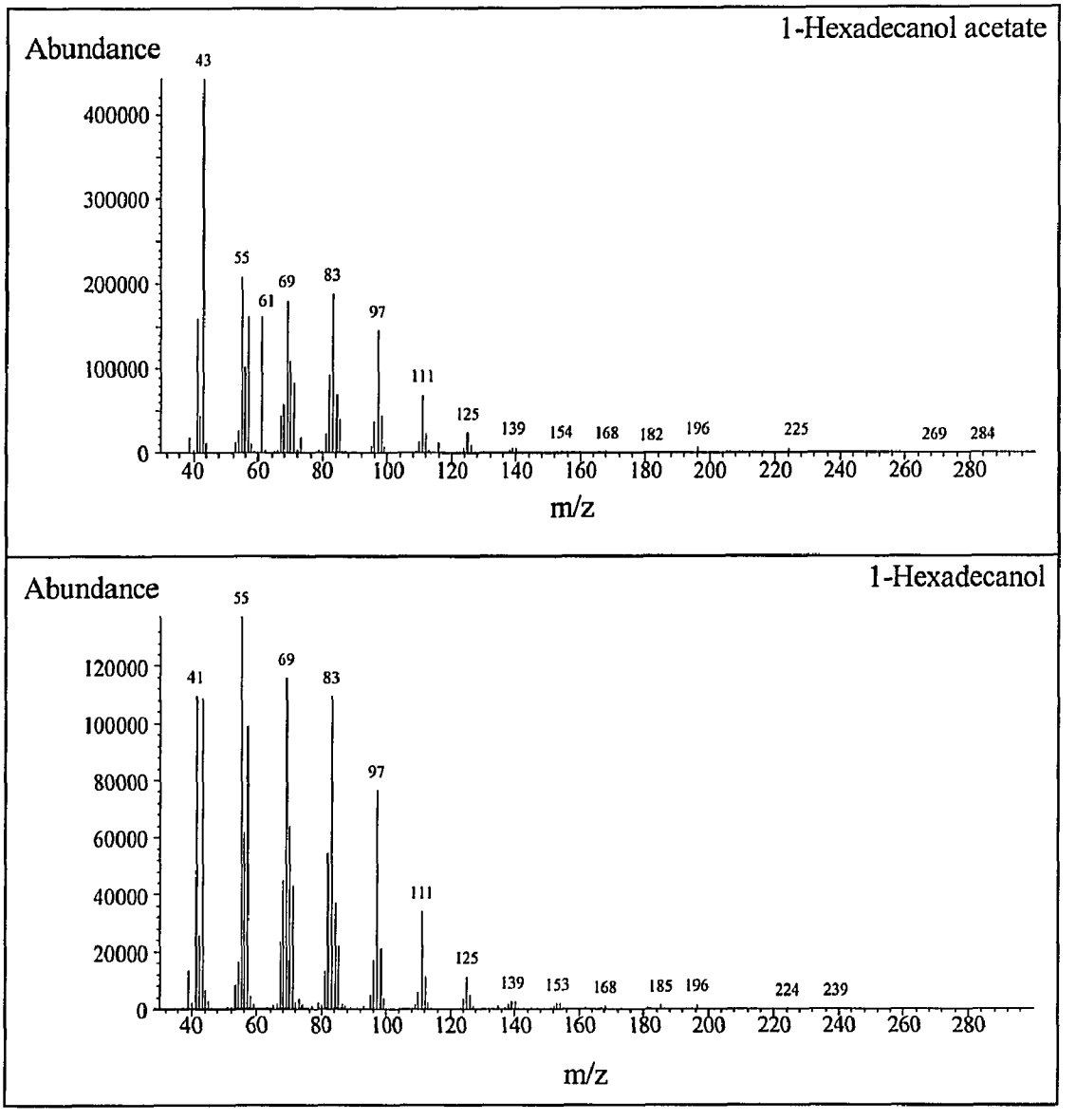


Figure 1.13. Mass spectra of 1-hexadecanol acetate and 1-hexadecanol.

6. Identification of Other Components in the Headspace of Porcupine Quills

Some acyclic terpenoids were identified from the odor extract, including geranylacetone, farnesol and its acetate, dihydroapofarnesol, etc. The chemical structures of this type of compound all contain a terminal isopentenyl unit, $(CH_3)_2C=CHCH_2-$. In their mass spectra, a strong peak at m/z 69 is characteristic of acyclic terpenoids and arises from the loss of the terminal isopentenyl group by allylic cleavage. Similarly m/z 138 which corresponds to two isopentenyl units, or a monoterpene, is also present. The mass spectra of some of the compounds are shown in Figure 1.14.

In addition to the terpenoids shown in Figure 1.14, more components with similar spectra were found in the porcupine's headspace. In the chromatogram given in Figure 1.15, all the peaks marked with a "*" belong to this class. Because their spectra are very similar and information about these compounds in commercial mass spectral library is very limited, it is difficult to identify the exact structure of these compounds by mass spectrum alone. The combination of GC/MS/IR as well as other spectroscopic technique should be helpful to determine their identities.

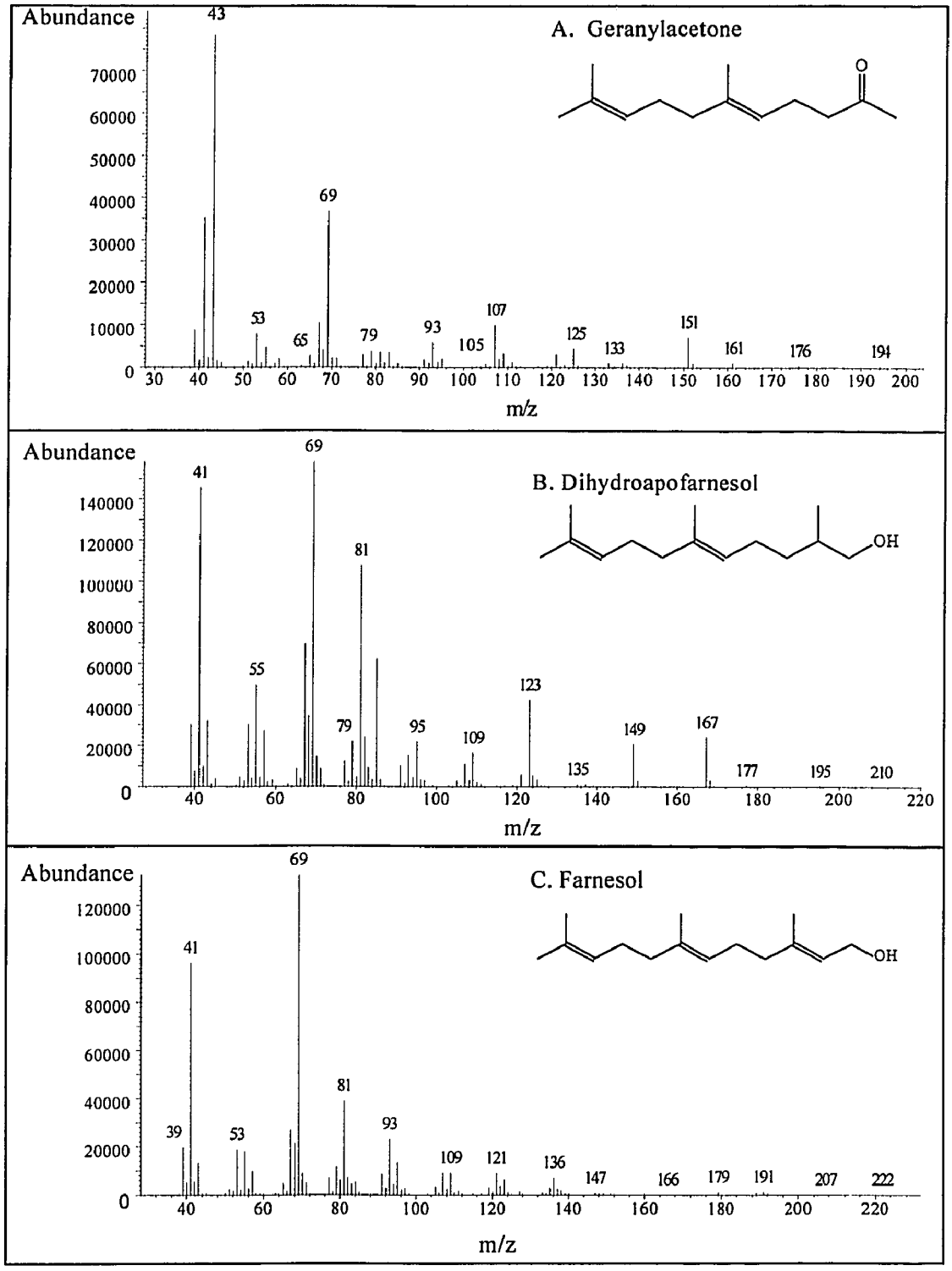


Figure 1.14. Mass spectra of several representative acyclic terpenoids found in the headspace volatiles of porcupine quill.

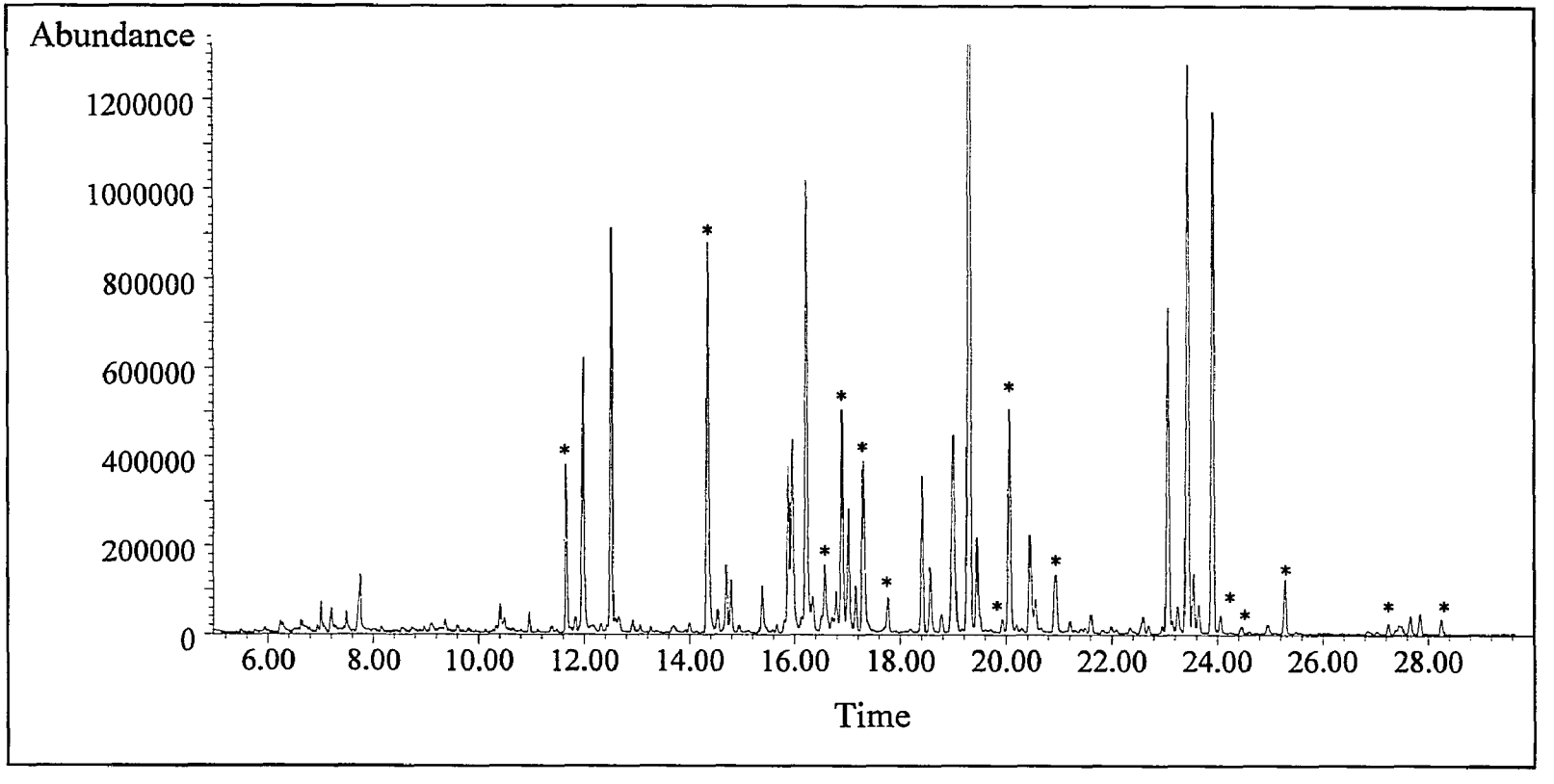


Figure 1.15. TIC of quill headspace volatiles in methanol. Peaks labeled with "*" are identified as acyclic terpenoids.

7. Enantiomeric Compositions of Decalactones in Porcupine's Warning Odor

The accumulating attention paid to δ-decalactone sparked our interest in establishing the enantiomeric composition of this compound, as well as of the other lactone, γ-decalactone. Direct separation of decalactone enantiomers was attempted on three cyclodextrin columns. The γ-decalactone enantiomers can be well resolved on the β-DEX 120 column at 130°C. Chiral separation of δ-decalactones is more difficult; the best resolution was obtained on the γ-DEX 120 column at 115°C. None of the columns can separate the enantiomers of both δ-decalactone and γ-decalactone. Following the optimized experimental conditions the porcupine odor extract was also analyzed to determine the enantiomeric compositions of the decalactones. The chromatograms of the enantiomers of δ-decalactone and γ-decalactone are shown in Figure 1.16. and Figure 1.17, respectively. It can been seen that neither of the lactones is racemic in the odor extract. From the published results of direct separation of decalactones on same type of chiral columns it is tentatively assumed the excess enantiomers are *R*-δ-decalactone, and *R*-γ-decalactone. Because of the lack of pure enantiomer standard, currently we cannot describe the sensory property of each enantiomer. Nonetheless, it should be reasonable to assume that one of the δ-decalactone enantiomer smells porcupiny, and the other smells coconuty.

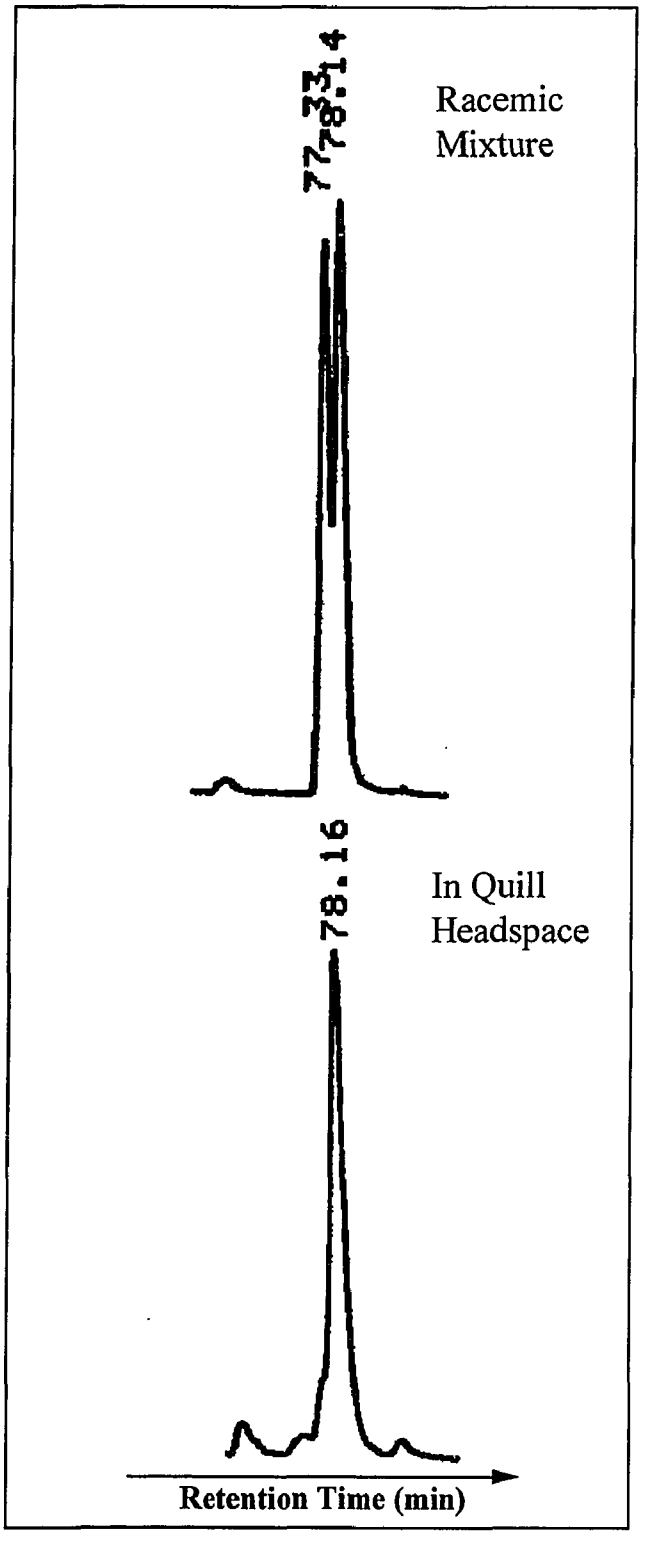


Figure 1.16. Chiral separation of δ-decalactone enantiomers on γ-DEX 120 column. GC temperature program: 70°C to 115°C at 10°C/min, hold until the solutes eluted from the column. Linear velocity of helium: 40 cm/s at 70°C.

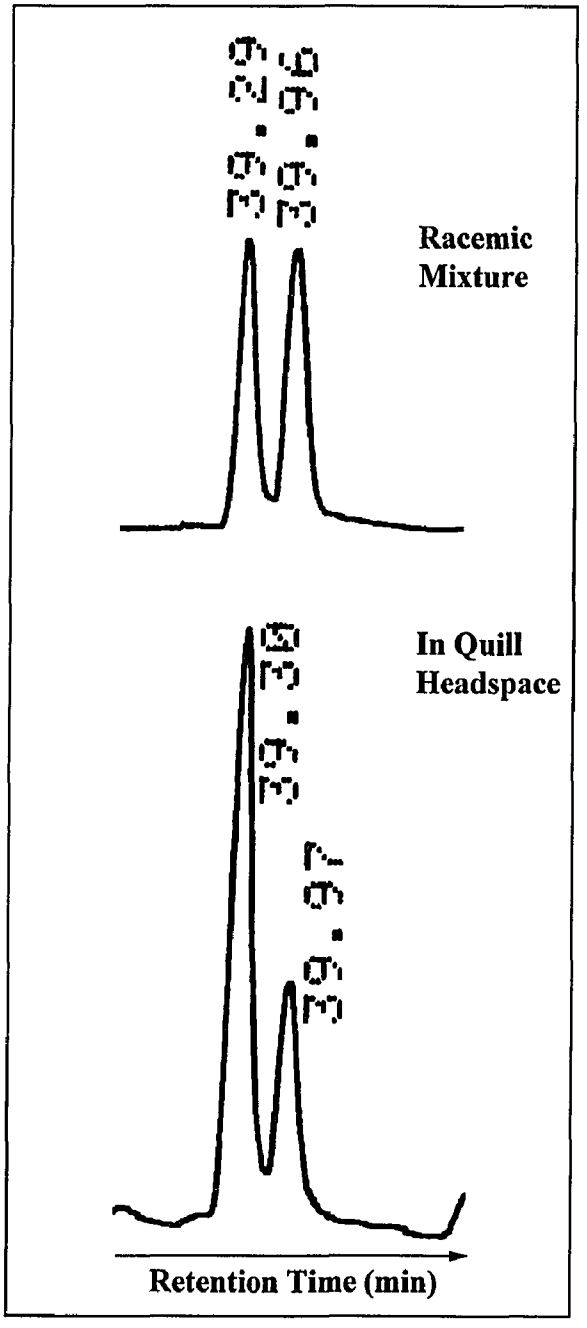


Figure 1.17. Chiral separation of γ-decalactone enantiomers on β-DEX 120 column. GC temperature program: 70°C to 130°C at 10°C/min, hold until the solutes eluted from the column. Linear velocity of helium: 43.5 cm/s at 70°C.

8. Summary of the Experimental Pathways and Results

As a summary, the experiments involved in this project and corresponding results that lead to the final conclusion are shown in Figure 1.18.

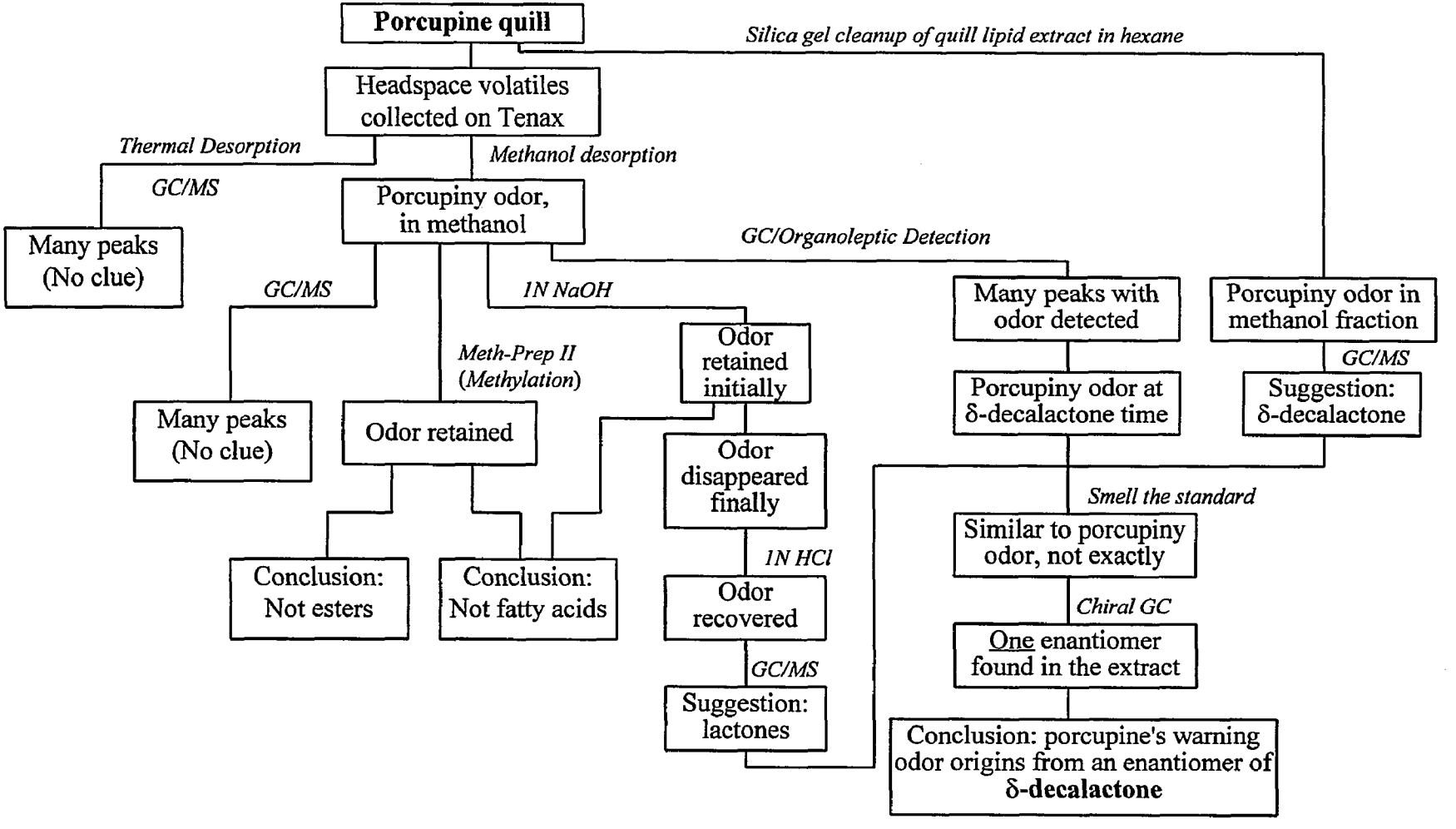


Figure 1.18. Summary of experimental pathways leading to the conclusion that δ-decalactone enantiomer is the principal odor component.

IV. Discussion

The results obtained from this study suggest that one of the δ-decalactone enantiomers, tentatively the R- form, is the active component of the porcupine's warning odor. Lactones are common in plant as well as in animal products [53]. They have been found in many fruits, and most lactones possess pleasant fruity odors. Some lactones are also responsible for animal odors. A known example is cis-4-hydroxydodec-6-enoic acid lactone, a lactone identified from the blacktailed deer tarsal gland, which has a unique garlic-like odor[54]. However, it is still somewhat a surprise to us that the porcupine's warning odor is from δ-decalactone.

Like many other lactones, δ-decalactone is widespread in fruits and vegetables such as apricot [55], coconut [56], peach [57], raspberry [58]; dairy products such as oil [59], milk [60,61], butter [62,63], and cheese [64,65]. The odor characteristics are described as coconuty, fruity-sweet, peachy, etc. When our experimental results led our attention to δ-decalactone, we obtained δ-decalactone standard from Aldrich Chemicals. The odor of δ-decalactone indeed reminded us of the porcupines, but it also resembled strongly the odor of coconut.

The multiple clues on δ-decalactone and odor difference between the porcupine sample and the commercial standard prompted us to determine the enantiomeric composition of δ-decalactone in the quill sample. As expected the δ-decalactone in porcupine's warning odor consists mainly of one enantiomer, while the commercial standard is a racemic mixture. It is reasonable to explain the odor difference via the

enantiomeric composition. Unfortunately, because the pure enantiomers are not available, currently we are not able to identify the absolute structure of the lactone in the sample. Several reports on chiral separation of lactones have been published using both indirect [48,66-70] and direct [44,46,47,71] separation techniques. The sensory properties of many optical isomers including lactones have been studied by Mosandl et al (69,70). Their descriptions of the odor characteristics of the enantiomers of both γ- and δ- decalactones are given in Table 1.6. However we still cannot conclude which of the δ-decalactone enantiomers should account for the porcupine's warning smell based on those authors' sensory evaluation of decalactone enantiomers.

Considering the biosynthesis of natural compounds, it can be expected to find an enantiomeric excess of a chiral compounds. The microbiological action on lipids may lead to the formation of fatty acids, including the lactone precursors--hydroxy fatty acids [53,59,72,73]. Further enzymatic action on the hydroxy acids would result in the formation of lactones [53,74,75]. Because of the high enantioselectivity of enzymes, the biosynthesis products are usually of high enantiomeric purity.

Table 1.6. Reported odor characteristics of the enantiomers of decalactones [69,70].

Compound	Config.	$[\alpha]_D^{20}$ (in MeOH)	Odor Characteristics (1% in propylene glycol, smelling strips)
γ-Decalactone	4R	+48.5	strong, fatty-sweet fruity note, some reminscene to coconut, caramel
	4S	-48.1	soft, sweet coconut note with fruity-fatty aspects
δ-Decalactone	5R	+61.0	fruity-sweet, milky note
	5S	-60.0	fruity-sweet, creamy peach note with a fatty buttery tonality, more intensive than (R)(+) antipode

Several alkyl acetates appeared to be the major peaks in the porcupine headspace, with mainly C_{14} and C_{16} alkyl groups. Although acetates have been found in some insects [9,12,13,76,77], in general they are rarely observed in mammalian sources [4]. The only known example is the house mouse. It was found that acetates can cause significant behavioral response in mice, so they are sometimes important animal semiochemicals [38,78,79]. Our experimental results show that acetates account for up to 39% of the total content in the headspace of porcupine quills. The odors of acetates are relatively weak, mostly fruity-sweet. Although they are rich in the headspace, acetates are not the major contributor to the porcupine's unique warning odor.

The warning odor of porcupine was initially described as fatty acid-like [19]. As expected, a series of fatty acids was identified from the headspace of quills. Although all of the acids occurred as minor components in the headspace volatiles, their existence may still affect the overall odor characteristics because of the low odor threshold of fatty acids. In fact many of the odors detected at the early stage of the chromatogram by GC-Organoleptic Detection experiment correspond to the fatty acid peaks. These odors were described as pungent, burning, fatty, etc., during the sniffing experiment. It was concluded from the experimental results that although fatty acids are not the principal components to the warning odor of porcupine, they might contribute to the pungent animal-like odor of the porcupine.

PART TWO

Separation of the Priority Pollutant Phenols by Capillary Zone Electrophoresis

I. Introduction

Electrophoresis is a technique that separates charged molecules based on differential migration in an applied potential field. When a sample is placed in buffer and an electrical field is applied, sample solutes will migrate in a direction and at a speed determined by their charge and electrophoretic mobility. Since most biomolecules such as amino acids and proteins dissociate in solution to form charged species, electrophoresis has become an effective analytical tool in biochemical research.

Because the voltages needed for electrophoresis are generally high, a considerable amount of Joule heat is generated in the system and separation efficiency in free solution is limited by thermal diffusion and convection. For this reason, electrophoresis has been traditionally performed in anti-convective media, such as polyacrylamide or agarose gels.

An alternative to overcome the problems of thermal diffusion and convection is to perform free zone electrophoresis in capillary tubes. Capillaries with smaller internal diameters have a larger surface to volume ratio, which allows more efficient heat dissipation. Initial attempts involved the use of 200 - 500 μm i.d. glass and Teflon tubes [1-3], which showed some unique advantages of using small inner diameter tubes. The major advance of capillary electrophoresis was made by Jorgenson and Lukacs [3-5], who performed electrophoresis in 75 μm fused silica capillaries. Separation efficiency of approximately 250,000 theoretical plates for

dansylamino acid isoleucine was achieved with a 1 m capillary and applied potential of 30,000 V. Jorgenson also described the basic separation theory and factors related to separation efficiency.

A schematic diagram of a CE instrument is given in Figure 2.1. The capillary filled with buffer is placed between two buffer reservoirs, and high voltage is applied across the capillary. An on-line detector, most often UV or fluorescence, is placed near the outlet end of the capillary. Sample is injected at the inlet end of the capillary by special injection techniques, including hydrodynamic, electromigration, sample splitter, etc. The whole instrument is usually kept in a safety chamber, e.g., a plexiglass box, to avoid electrical shock from the high voltage.

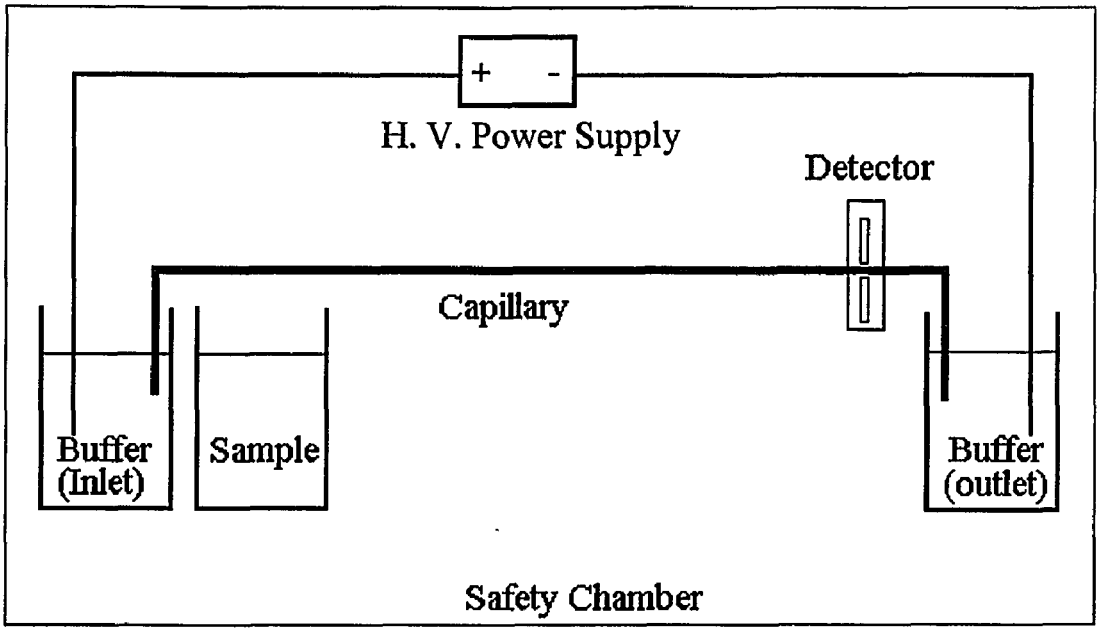


Figure 2.1. Schematic diagram of a capillary electrophoresis apparatus.

While capillary zone electrophoresis is a great success in the separation of charged species, neutral molecules cannot be separated since the electrophoretic mobility for neutral species is zero. An important advance for the separation of neutral molecules by capillary electrophoresis was made by Terabe [6,7], who added ionic micelles to the electrophoresis buffer. The micelles are considered to form a pseudo stationary phase in the buffer solution; solute partitioning between micelles and buffer solution results in differential migration velocities for neutral molecules. This difference in velocity leads to the separation of neutral species in a manner very similar to liquid chromatography, but offers much higher separation efficiency. This separation technique is termed "Micellar Electrokinetic Capillary Chromatography" (MECC) [7] to emphasize the partitioning mechanism.

Phenols are important environmental pollutants due to their common use and toxicity. Among the substituted phenols, some chlorinated and nitrated phenols are of special concern. Eleven phenols appear on the US EPA priority pollutant list [8]. The analysis of these compounds has been widely studied using GC and HPLC [9-14].

Capillary electrophoresis has also been employed for the separation of certain phenols. Isomers of chlorinated phenols were separated by both MECC [15] and CZE [16,17]. The electrophoretic behavior of chlorophenol congeners was investigated and the separation was optimized as function of buffer pH, concentration and applied voltage by Gonnord and Collet [17]. Ong *et al.* [18] separated the eleven priority pollutant phenols by MECC using a 180 μm i.d. capillary at 10 kV. Recently Chao

and Whang [19] showed the separation of the eleven priority pollutant phenols as an application of CZE coupled with laser-induced indirect fluorimetry.

Buffer pH is a critical parameter for the separation of weakly acidic or basic solutes by CE. The extent of dissociation, which determines the overall electrical charge of the solute, is governed by the pH of the buffer. So the selection of buffer pH can greatly affect the separation, especially for mixtures of solutes with a wide range of pK_a, as is the case for the eleven priority pollutant phenols. Compared with techniques such as HPLC and MECC, a unique feature of CZE is the ability of the operator to predict the dependence of electrophoretic mobility on pH if a few simple parameters are known [17,20]. Thus it is possible to estimate the migration/pH profiles, which greatly simplifies the pH optimization process. Based on a few initial experiments, Smith and Khaledi [20] calculated the electrophoretic mobilities of several substituted phenols and optimized the separation pH using a window diagram.

II. Basic Theory of Capillary Electrophoresis

1. Electrophoretic Mobility

In capillary electrophoresis the migration velocity v of a solute is given by

$$v = \mu_{ep}E = \mu_{ep}V/L \tag{2.1}$$

where μ_{ep} is the electrophoretic mobility, E is the potential field strength, V is the voltage applied across the capillary, and L is the capillary length. The migration time t for a solute is given by

$$t = L/v = L^2/\mu_{ep}V \tag{2.2}$$

Under ideal experimental conditions in capillary electrophoresis the contribution to band broadening can be considered solely due to longitudinal diffusion. The separation efficiency, in terms of the total number of theoretical plates, N, is given by

$$N = \mu_{ep}V/2D \tag{2.3}$$

where D is the diffusion coefficient of the solute. Equation 2.3 reveals that separation efficiency is proportional to applied voltage but not capillary length. Thus it is desired theoretically to apply a voltage as high as possible to achieve the highest efficiency in the shortest time.

2. Electroosmotic Flow

Another fundamental phenomenon of CE is electroosmotic flow (EOF), the bulk flow of liquid in the capillary under the influence of the potential field. EOF results from the capillary surface charge, as illustrated in Figure 2.2. Under normal

operating conditions the surface of the capillary wall has an excess of negative charge resulting from ionization of the surface silanol groups. Positive counterions to these anions build up in the solution adjacent to the capillary wall. When an electric field is applied, this layer of positive charge is drawn toward the negative electrode. Since these positive ions are solvated they drag solvent to move with them, resulting in the bulk flow of liquid toward the negative electrode.

In contrast to the conventional laminar forced flow profile, a unique feature of electroosmotic flow in capillaries is the "plug" shape flat flow profile, as illustrated in Figure 2.3[21]. It is clearly seen from Figure 2.3 that this flat flow profile itself leads to minimal zone broadening comparing to laminar flow. This is one of the reasons for the inherent high separation efficiency of CE.

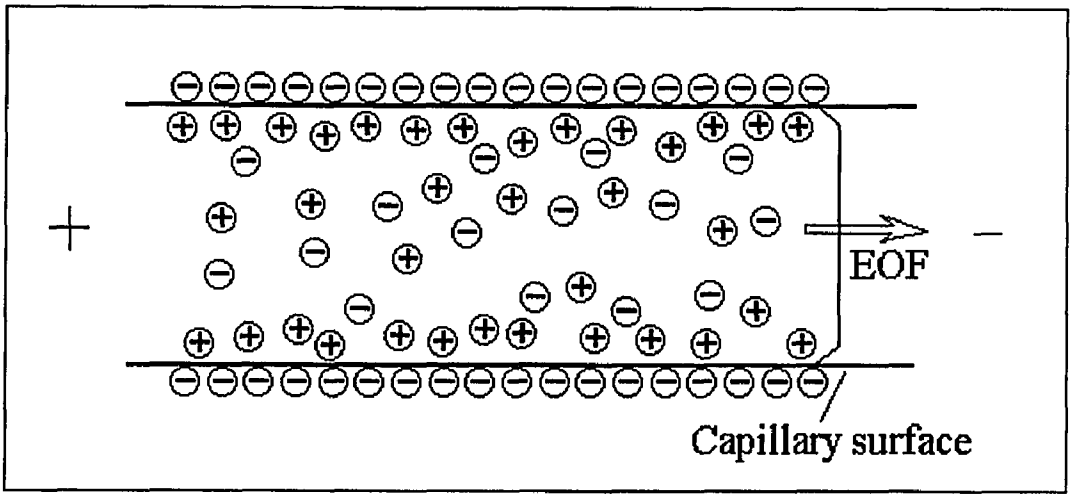


Figure 2.2. Illustration of electroosmotic flow.

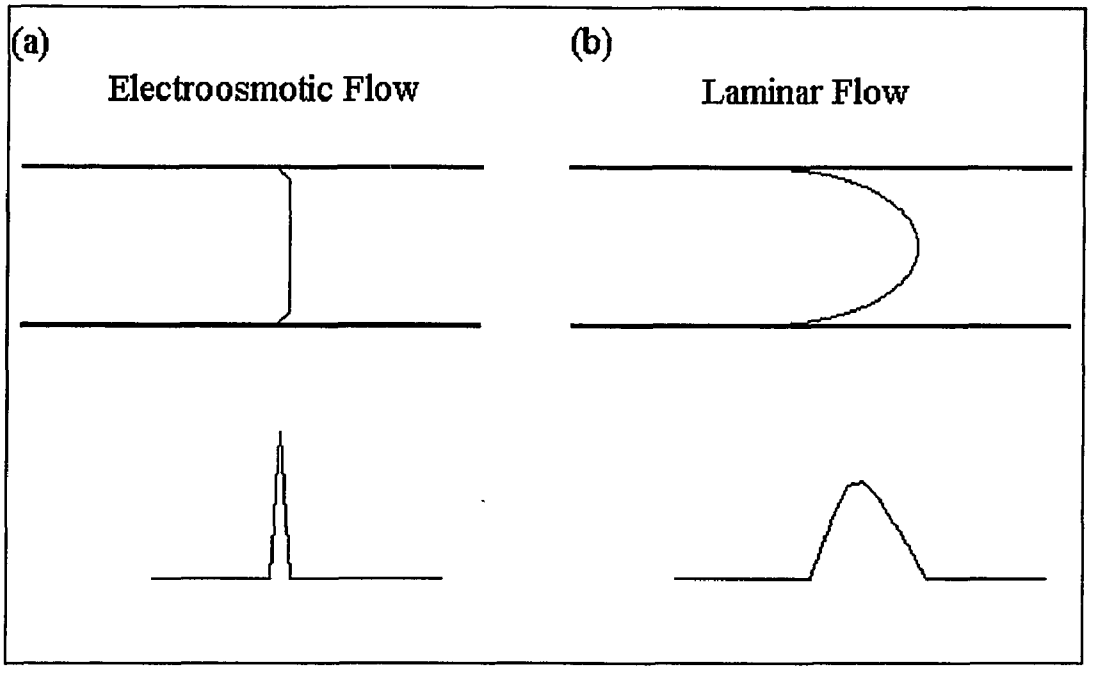


Figure 2.3. Comparison of the electroosmotic flow profile (a) and laminar flow profile (b). The flat flow profile in (a) yields a much narrower solute band, which is one of the factors in the high separation efficiency of capillary electrophoresis.

Another useful feature of EOF is that under normal conditions it causes all species to move in the same direction, regardless of charge. When a high voltage is applied across the capillary, anions and cations migrate electrophoretically in opposite directions while neutral species do not migrate electrophoretically, as is shown in Figure 2.4 [22]. The magnitude of EOF is so large that it even carries the anions to the cathode, although the direction of electrophoretic mobility of anion is towards the anode. Thus anions, cations and neutral species can be analyzed in one run.

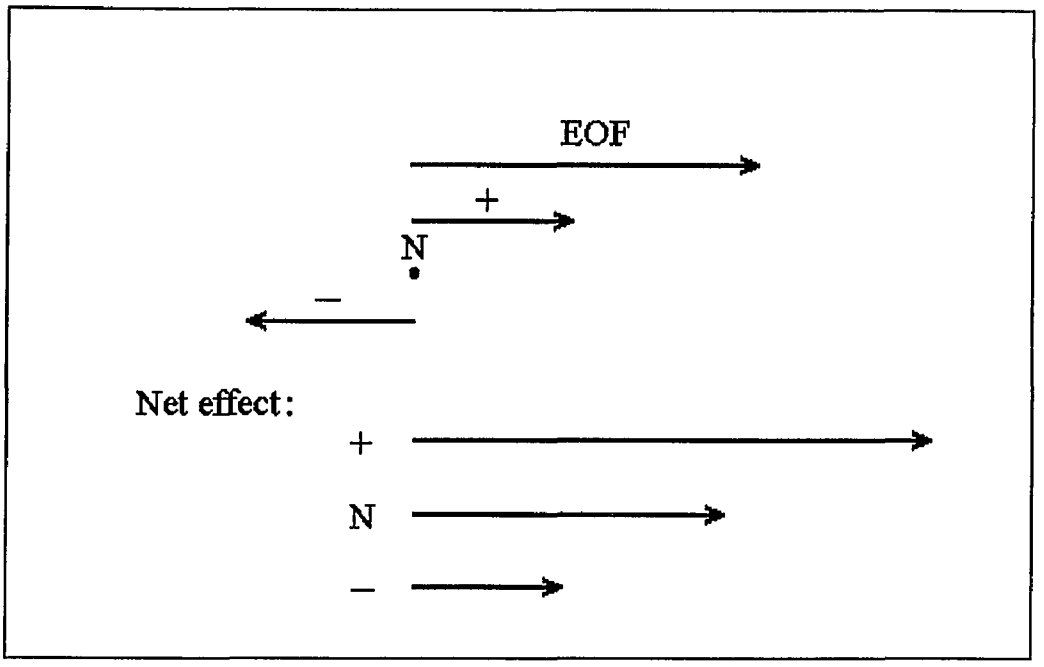


Figure 2.4. Illustration of migration directions of cations (+), neutral species(N), and anions (-), under the influence of electroosmotic flow [22].

When electroosmotic flow is present, the basic relationships need to be modified. With the electroosmotic flow (μ_{eo}), equation 2.1 and 2.2 can be rewritten as

$$v = (\mu_{ep} + \mu_{eo})V/L \tag{2.4}$$

and

$$t = L^2/(\mu_{ep} + \mu_{eo})V \tag{2.5}$$

Similarly equation 3 for theoretical plates can be rewritten as

$$N = (\mu_{ep} + \mu_{eo})V/2D \tag{2.6}$$

In separation science the ultimate goal is the resolution of sample components. Resolution can be defined as

$$R_s = \frac{2(t_2 - t_1)}{W_1 + W_2} = \frac{t_2 - t_1}{4\sigma} \tag{2.7}$$

where W is the baseline peak width, σ is the standard deviation of the peak. In CE the relationship between resolution and mobility can be expressed as [5]

$$R_s = 0.177\Delta\mu_{ep}\left[\frac{V}{D(\bar{\mu}_{ep} + \mu_{eo})}\right]^{1/2} \tag{2.8}$$

where $\Delta\mu_{ep}$ is the mobility difference of two solutes, and $\bar{\mu}_{ep}$ is the average of the mobility values of the two solutes. It is clear that a large component of electroosmotic flow in the same direction as the electrophoretic migration will decrease the actual resolution of two zones. In fact, it may be seen that the best resolution will be obtained when the electroosmotic flow just balances the electrophoretic migration or

$$\mu_{eo} = -\bar{\mu}_{ep} \tag{2.9}$$

at which point substances with extremely small differences in mobility may be resolved. However the expense for such resolution is the infinite analysis time, as can be seen from equation 2.5. Nonetheless the ability to control EOF is necessary so that adequate separation can be achieved within shortest analysis time.

3. Micellar Electrokinetic Capillary Chromatography (MECC)

CZE shows tremendous power for the separation of charged molecules; however, this technique cannot be applied to separate mixtures of neutral species directly. A breakthrough on the problem was the use of micelles to form a pseudo stationary phase in electrophoresis buffer, which was first introduced by Terabe [6,7].

An illustration of separation using MECC is given in Figure 2.5 [22]. At concentrations in aqueous solution above critical micelle concentration (CMC), surfactant molecules aggregate to form micelles, with the hydrophobic tail groups towards inside and the charged hydrophilic head at the surface. Sodium dodecyl sulfate (SDS) is the most widely used surfactant in MECC. When a potential field is applied across the capillary, negatively charged micelles have a electrophoretic mobility towards the anode. However the strong electroosmotic flow brings the micelles to the cathode. These micelles function as a moving "pseudo stationary phase", resulting in a fast-moving aqueous phase and a slow moving pseudo stationary phase in the capillary. Neutral solutes can partition between the two phases in a manner similar to that in liquid chromatography. Differential partitioning of solutes results in the difference of retention and consequently the separation. Charged

species, on the other hand, migrate under the influence of electrical field. So MECC is the only operational mode of CE which can separate both ionic and nonionic solutes simultaneously.

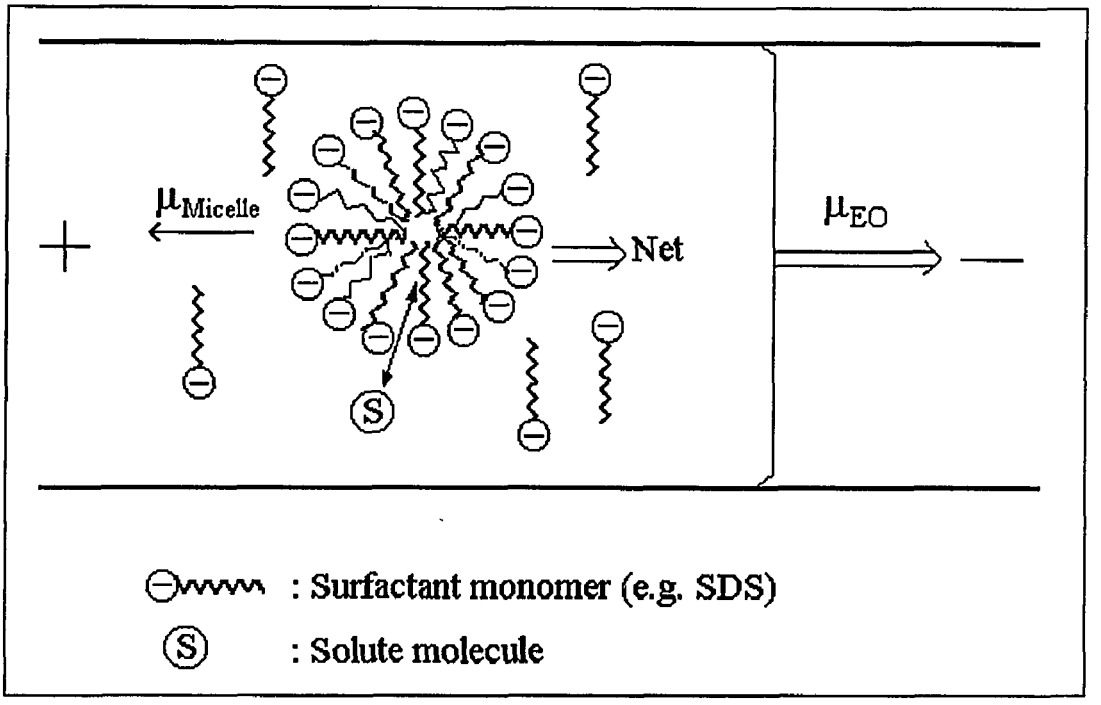


Figure 2.5. Illustration of the separation mechanism of micellar electrokinetic capillary chromatography.

Because the separation mechanism of neutral solutes with MECC is chromatographic-like, basic relationships of chromatography can be applied with some modification. The capacity factor, k', the ratio of amount of solute in the micelle to those in mobile phase, is given by

$$k' = \frac{(t_r - t_0)}{t_0\left(1 - \frac{t_r}{t_m}\right)} = K\left(\frac{V_s}{V_m}\right) \tag{2.10}$$

where t_r is the retention time of solute, t_0 is the retention time of unretained solute, t_m is the retention time of micelle, V_m is the volume of the micellar phase, V_s is the volume of the mobile phase, K is the partition coefficient. Resolution in MECC is given by [7]

$$R_s = \frac{N^{1/2}}{4}\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{k_2'}{k_2' + 1}\right)\left(\frac{1 - (t_0 / t_m)}{1 - (t_0 / t_m)k_1}\right) \tag{2.11}$$

where $\alpha = K_2' / K_1'$. Equation 2.11 is essentially a modification of the conventional chromatographic description of resolution in consideration of the movement of stationary phase. If the stationary phase does not move, i.e. $t_m \rightarrow \infty$, the last term in equation 2.11 equals to 1 and the equation changes back to its original form.

It can be seen that the separation mechanism in MECC is very much similar to reverse-phase HPLC in that neutral solutes are separated based on the differential partitioning between running buffer and stationary phase. A very useful feature of micelles in MECC is that they are dynamic structures, which allows for fast solute movement in and out the micelle. This is important for efficient mass transfer of solutes between stationary phase and running buffer. So MECC often gives much

higher separation efficiency than HPLC. One of the drawbacks of MECC as well as other capillary electrophoresis techniques compared to HPLC is that the concentration detection limit is not as good as in HPLC because of the low allowable injection volume (nanoliters). So the two separation technologies complement each other.

III. Experimental

1. Instrument

CZE was carried out using an Isco Model 3850 Electropherograph with an adjustable wavelength UV detector set at 210 nm. An uncoated fused silica capillary 100 cm long (65 cm from injector to detector) and 75 μm i.d. was used. Sample injection was carried out by applying a vacuum at the outlet buffer beaker for a controlled period of time. The electropherograms were recorded on a Spectra-Physics SP-4600 or a Shimadzu CR-6A integrator.

2. Chemicals

All the eleven phenols tested in this work were obtained from Aldrich chemical Co. (Milwaukee, WI). HPLC grade water (Fisher Scientific, Fair Lawn, NJ) or distilled, deionized water was used to prepare buffers and samples. The buffers of different pH were prepared as follows: NaH_2PO_4, 47.1 mM and Na_2HPO_4, 2.9 mM, pH = 5.6; NaH_2PO_4, 7 mM and Na_2HPO_4, 43 mM, pH = 7.6; Na_3PO_4, 12.5 mM and $Na_2B_4O_7$, 37.5 mM, pH = 9.3; Na_3PO_4, 25 mM and $Na_2B_4O_7$, 25 mM, pH = 9.8; Na_3PO_4, 30 mM and $Na_2B_4O_7$, 20 mM, pH = 10.5; Na_3PO_4, 37.5 mM and $Na_2B_4O_7$, 12.5 mM, pH = 11.4; Na_3PO_4, 50 mM, pH = 12.4. All buffers were filtered through 0.45 μm syringe filters and degassed with high purity helium prior to analysis.

Phenol stock solutions were prepared separately by weighing and dissolving in HPLC grade methanol. A mixture of 11 phenols was prepared by diluting each of the

stock solutions in methanol to give a concentration of 100 mg/l. This mixture was stored at 4°C and used to make test solutions by further dilution with the running buffer to the appropriate concentration.

3. Capillary Conditioning

Each day the capillary was first conditioned by filling with 1 M NaOH and soaking for 10 min. The capillary was then washed with deionized water followed by running buffer. At the end of the day the column was washed with water followed by 0.1 M NaOH. The column was left filled with 0.1 M NaOH overnight for the next day's use.

IV. Results and Discussion

1. Structures of the Priority Pollutant Phenols

The structures of the eleven priority pollutant phenols are shown in Figure 2.6.

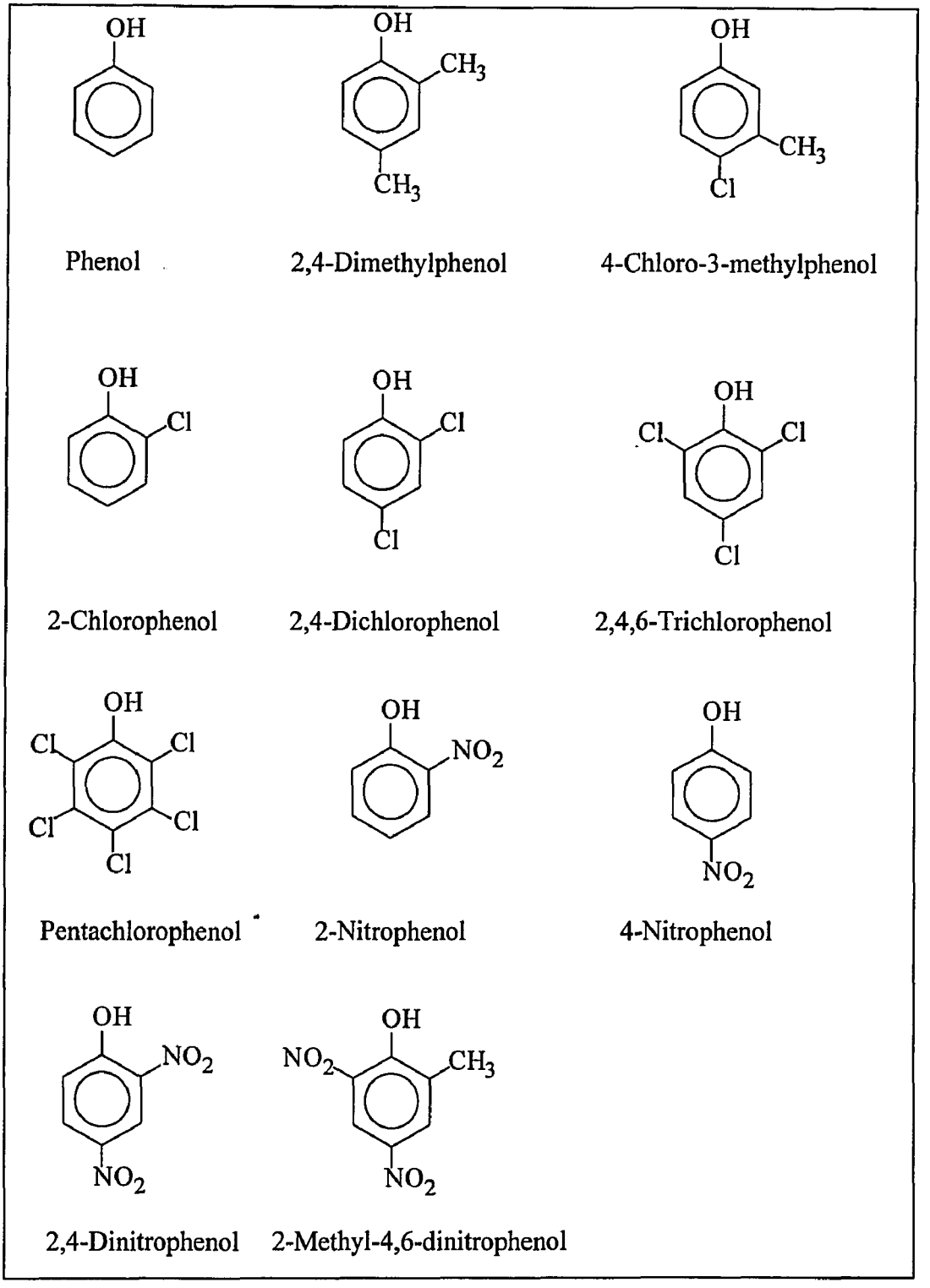


Figure 2.6. Structures of the eleven priority pollutant phenols.

2. Optimization of Buffer pH

All the phenols in this study are weak acids. In aqueous solution the phenols are partially dissociated:

$$HA = H^+ + A^-$$

$$K_a = [H^+][A^-]/[HA] \tag{2.12}$$

If such a solution is subject to electrophoresis in the absence of electroosmotic flow, the ionic species A^- will migrate under the electrical field at the electrophoretic mobility μ_A. The electrophoretic mobility of the neutral species μ_{HA} is zero. If the dissociation equilibrium is fast, the overall mobility of this solute can be considered as an weighted average of the mobilities of the two species:

$$\mu_{ep} = \frac{[HA]}{[HA]+[A^-]}\mu_{HA} + \frac{[A^-]}{[HA]+[A^-]}\mu_A \tag{2.13}$$

$$= \frac{[A^-]}{[HA]+[A^-]}\mu_A \tag{2.14}$$

$$= \frac{K_a}{K_a+[H]}\mu_A \tag{2.15}$$

The usefulness of equation 2.15 is that it provides the possibility of calculating the overall mobility of a particular solute, and subsequently predicting separation profiles of the mixture. If the values of pK_a and μ_A of a solute remain constant over the operating pH range, the overall mobility of the solute is a sole function of pH. If the pK_a and μ_A of each solute in a mixture are known, the overall mobilities of each solute at any pH can be calculated and separation of the mixture can be predicted.

In aqueous buffers the value of pK_a can be considered constant if the capillary is well thermostatted to keep the buffer temperature constant. The electrophoretic mobility, μ_A, depends on the charge and size of the solute as well as the concentration and viscosity of the buffer [17,23], and can be considered essentially constant if buffers with similar ion compositions and concentrations are used. The pK_a values are generally available in the literature. The electrophoretic mobilities, μ_A, on the other hand, usually have to be determined experimentally. Thus, as suggested by Smith and Khaledi [20], if the value of pK_a is available, only one buffer solution is needed to determine values of μ_A which can be used subsequently to predict the migration behavior of each solute over the entire pH range.

In this study, the relationship between the electrophoretic mobility and pH for each phenol was calculated as discussed above. The values of pK_a were taken from the literature [18,24]. The μ_A of each solute was determined by measuring the apparent migration time (t_{app}) in the buffer of pH = 11.4 and subtracting the electroosmotic migration time, t_{MEOH}. The following equation was used:

$$\mu_A = - (1 + [H^+]/pK_a) [(1/t_{app}) - (1/t_{MEOH})]lL/V \qquad (2.16)$$

where l = 65 cm, the effective column length; L = 100 cm, the total column length; V = 20 kV, the applied voltage. The negative sign accounts for the fact that the direction of electrophoretic flow of anions is opposite that of electroosmotic flow. The migration time of methanol, t_{MEOH}, was used to calculate the electroosmotic flow. The values of μ_A and pK_a are listed in Table 2.1. This measured μ_A of each solute was then

used with equation 2.15 to calculate the overall electrophoretic mobilities at other pH values.

Table 2.1. pK_a Values of the priority pollutant phenols and the measured electrophoretic mobilities of A^-.

Peak I.D.	Compound	pK_a[a]	μ_A(x 10^{-4} cm^2/Vs)[b]
a	2,4-Dimethylphenol	10.59	3.98
b	Phenol	9.89	3.64
c	4-Chloro-3-methylphenol	9.54	3.17
d	Pentachlorophenol	4.50	3.35
e	2,4,6-Trichlorophenol	7.42	3.64
f	2,4-Dichlorophenol	7.85	4.04
g	2-Methyl-4,6-dinitrophenol	4.70	3.55
h	2-Chlorophenol	8.48	3.47
i	2,4-Dinitrophenol	4.00	3.87
j	4-Nitrophenol	7.15	4.10
k	2-Nitrophenol	7.23	3.95

a. From references [18, 24].
b. Measured at pH = 11.4.

To verify the calculated results, the electrophoretic mobility/pH relationship was determined experimentally using running buffers of various pH values. The phenol mixture was separated in each buffer solution under same experimental conditions. Electropherograms at each pH are shown in Figure 2.7. The experimental electrophoretic mobilities were calculated from migration times of each solute using the following equation.

$$\mu_{ep} = - [(1/t_{app}) - (1/t_{MEOH})]lL/V \qquad (2.17)$$

The calculated and the measured curves of μ_{ep} *versus* pH for the eleven phenols are shown in Figure 2.8. It can be seen that in the pH range of about pH 9 - 11 all the phenols are separated; the best separation of all phenols was obtained at pH 9.8, and this buffer was used for all the following experiments.

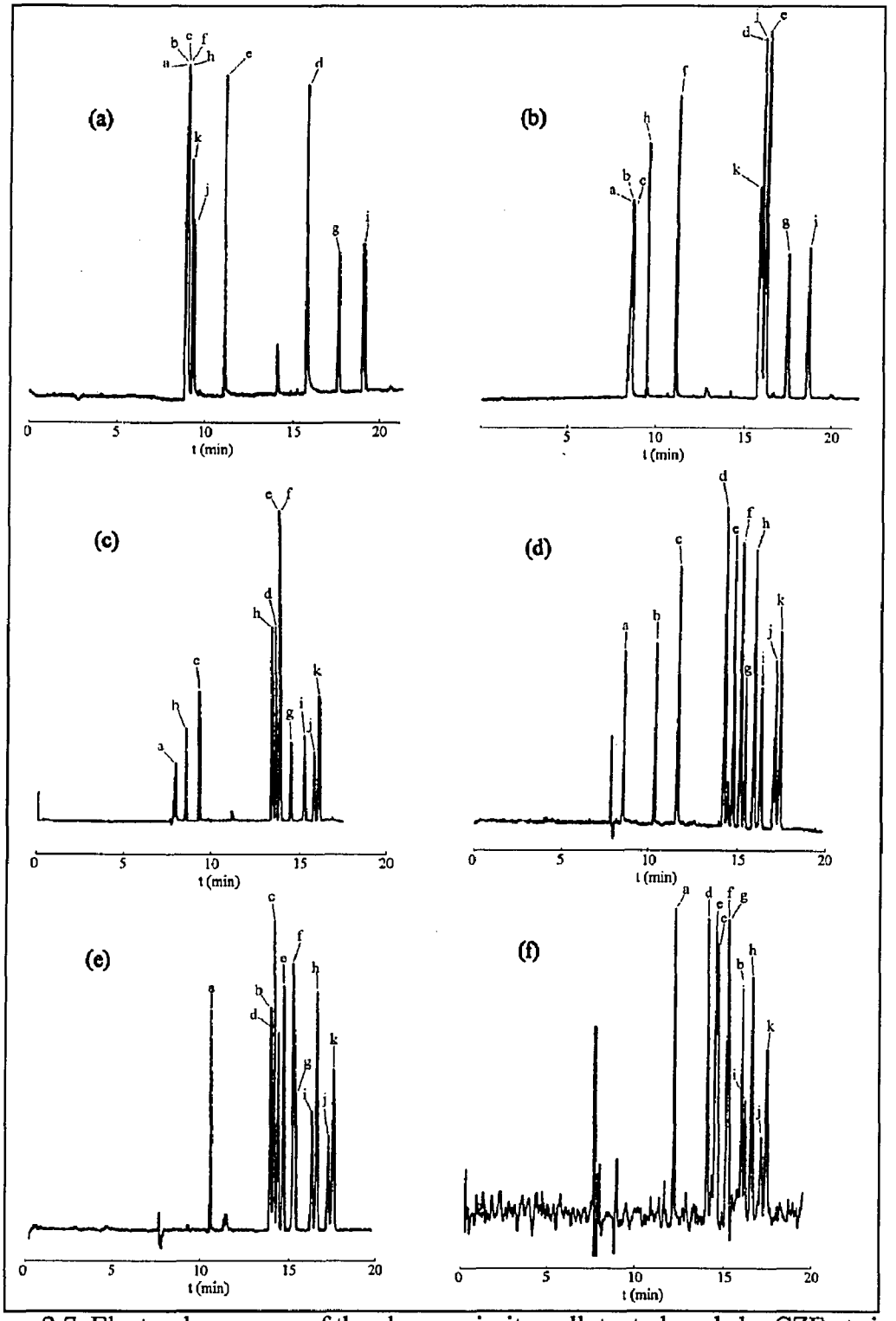


Figure 2.7. Electropherograms of the eleven priority pollutant phenols by CZE at six different pH buffers: pH = (a) 5.6; (b) 7.6; (c) 9.1; (d) 9.8; (e) 10.5; (f) 11.4. All buffer concentrations are half of the stock buffer concentrations given in Experimental section. Peak identifications as in Table 2.1.

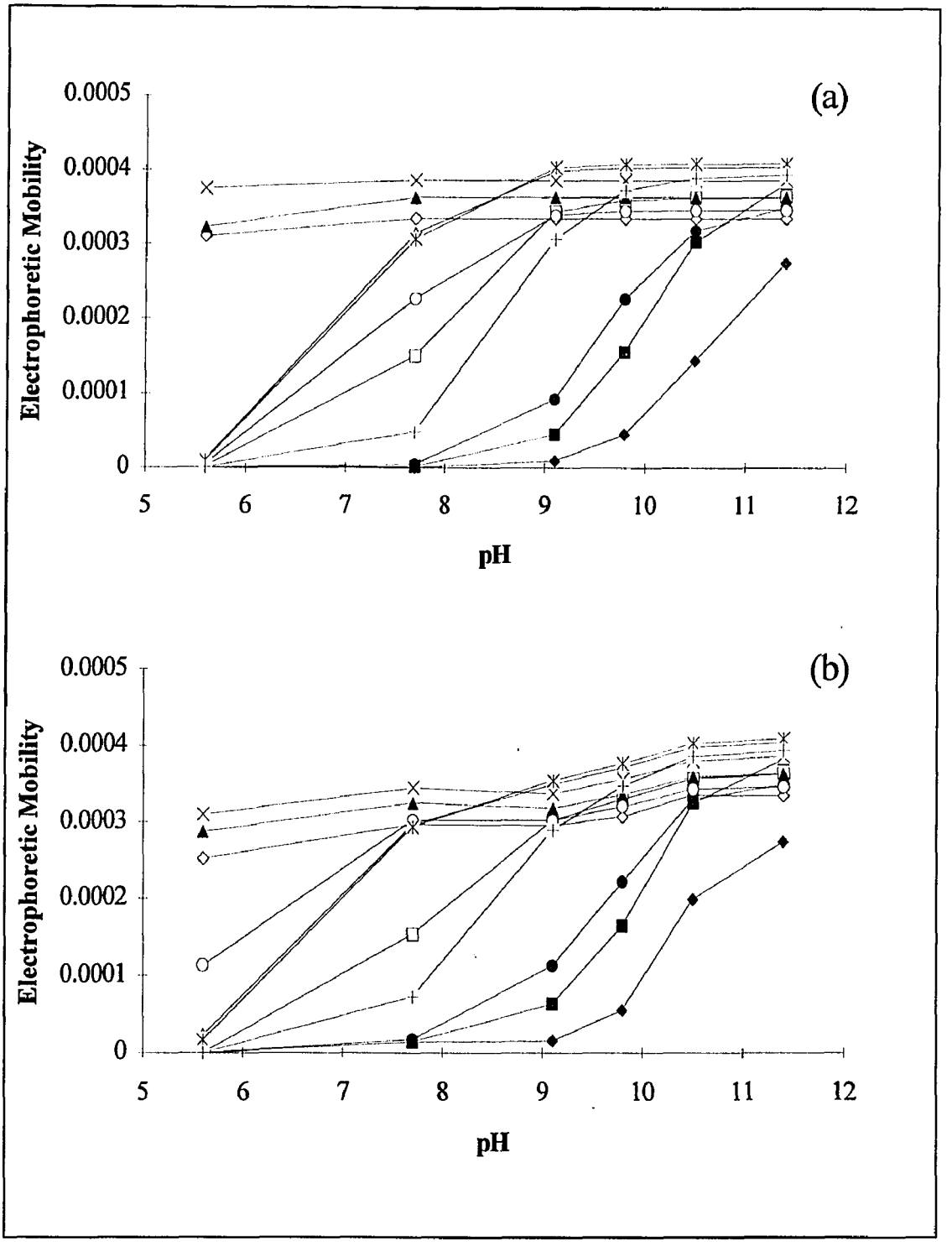


Figure 2.8. Dependence of overall mobility on pH: (a) Calculated; (b) Experimental. Units of mobility: cm^2/Vs. ■ = Phenol; □ = 2,4-Dichloro-; ◆ = 2,4-Dimethyl-; ◇ = Pentachloro-; ▲ = 2-Methyl-4,6-dinitro-; △ = 4-Nitro-; ● = 4-Chloro-3-methyl-; ○ = 2,4,6-Trichloro-; × = 2,4-Dinitro-; ∗ = 2-Nitro-; + = 2-Chloro-.

Comparison of the calculated and experimental μ_{ep} *versus* pH plots shows that the electrophoretic behavior of most of the phenols follows equation 2.15. The calculated and experimental order of decreasing mobilities, i.e. the orders of peaks in the electropherogram, are the same at most pH values, although some differences in the absolute mobilities exist. The discrepancies are more obvious in the lower pH region, probably because either the actual μ_A values differ more at low pH values from the values used for calculation, or the migration time reproducibilities are relatively not as good at lower pH values. For the purpose of pH optimization, however, these simple calculations can serve as useful guidelines. The prediction of the electrophoretic mobility/pH relationship can be considered a general method for optimizing pH for the separation of weakly acidic and basic solutes, as long as the ionization equilibrium occurs over a practical pH range. For solutes of unknown pK_a, measurement of μ_{ep} in two buffers of different pH values should allow determination of both pK_a and μ_A.

For the separation of a complex mixture, both pK_a and μ_A need be considered, as shown in equation 2.15. At pH near pK_a, selectivity is determined mainly by the difference in pK_a between solutes. At pH >> pK_a, separation is mainly based on differences in μ_A of each solute. The relative contribution of pK_a and μ_A to mobility at different pH values is easily seen in Figure 2 for the four chlorinated phenols. Because of the electron-withdrawing effect of chlorine, the order of pK_a for these phenols is pentachloro- < trichloro- < dichloro- < chloro-phenol. Thus in the lower pH range (6 to 7.5), the order of electrophoretic mobilities are opposite that of pK_a (i.e.

same order as K_a). At pH > 9.5, the order of electrophoretic mobilities changes completely to the same order as that of μ_A. Presumably, the more chlorine atoms on the benzene ring, the bulkier the molecule and thus the smaller μ_A. This change in migration order with pH is also observed for the nitrophenols. To achieve separation of a pair of solutes, there must be sufficient difference in the pK_a or the μ_A. If both quantities are similar for the two solutes, adjustment of pH will not significantly improve of the separation of the two solutes. For example, a baseline separation of 2-nitrophenol and 4-nitrophenol was rarely achieved over the entire pH range studied. In this case, other means must be employed to differentiate the values of either pK_a or μ_A, e.g., by the addition of organic buffer modifiers [25] or surfactants.

3. Effect of Buffer Concentration

The concentration of buffer can affect the CZE separation in several ways. The electroosmotic flow increases with decreasing buffer concentration, producing shorter analysis times. On the other hand, a certain minimum buffer concentration is necessary for uniform distribution of the electrical field and for adequate buffering capacity. Higher buffer concentrations are also required to minimize solute adsorption at the capillary wall. However, increasing the buffer concentration is limited by Joule heating. The optimum buffer concentration has to be determined experimentally.

In this study, pH 9.8 buffers of various concentrations in the range of 10 to 50 mM were prepared by diluting the stock pH 9.8 buffer solution. At pH 9.8, the slowest migrating three solute pairs, 2,4-dichlorophenol/2-methyl-4,6-dinitrophenol,

2-chlorophenol/2,4-dinitro-phenol, and 4-nitrophenol/2-nitrophenol, have relatively small resolution values, R_s. The resolution of these three pairs was studied as a function of buffer concentration. Figure 2.9(a) is the plot of R_s *versus* buffer concentration. R_s decreases with increasing buffer concentration, more rapidly at concentrations higher than 20 mM. However, all three pairs show baseline separation ($R_s > 1.4$) at buffer concentrations up to 30 mM.

The relationship of current and buffer concentration is shown in Figure 2.9(b). Theoretically the current should increase linearly with buffer concentration. However, positive deviation of current from linearity was observed at buffer concentrations higher than 30 mM, probably because of Joule heating.

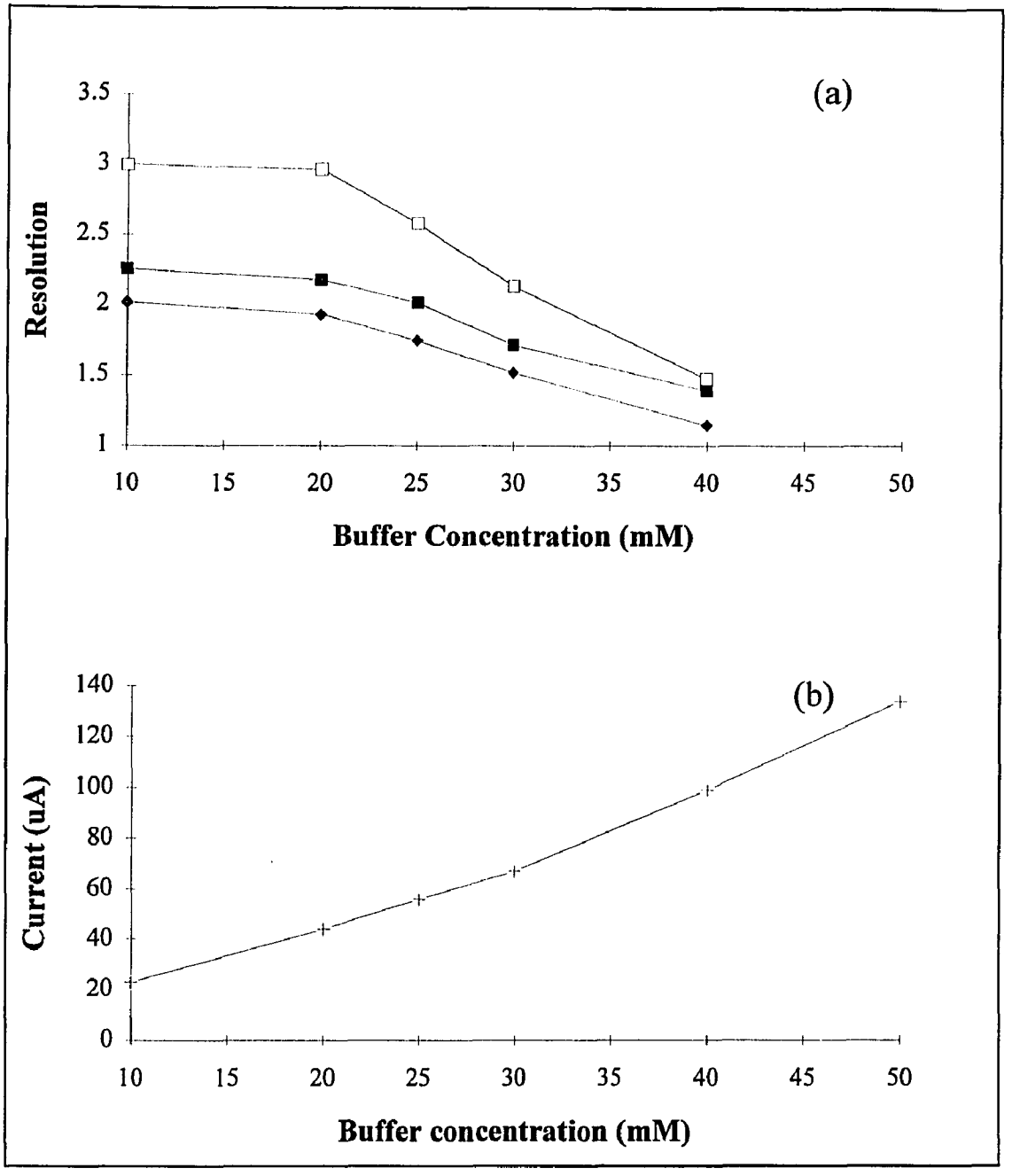


Figure 2.9. The effect of buffer concentration on resolution and current: (a) Resolution *versus* buffer concentration; (b) Current *versus* buffer concentration. ■ = 2,4-Dichloro-/2-Methyl-4,6-dinitro-; □ = 2-Chloro-/2,4-Dinitro-; ◆ = 4-Nitro-/2-Nitro-.

4. Effect of Applied Voltage

In principle the plate number in CE is proportional to applied voltage [17]. However, applied voltage increases are limited by Joule heating which results in zone broadening and a decrease in efficiency. An optimum voltage range exists within which higher efficiency and shorter analysis times can be achieved without generation of detrimental amounts of heat.

For the phenol separation, the voltage range studied was 10 to 30 kV. The theoretical plate number for each solute was measured for each phenol at each applied voltage. Representative results are shown in Figure 2.10(a). The theoretical plate numbers roughly increase with increasing applied voltage, pass through a maximum near 22.5 kV, and then decrease with the further increase in voltage. Again, the current-applied voltage curve in Figure 2.10(b) shows a nonlinear increase in current for voltages higher than about 20 kV because of Joule heating.

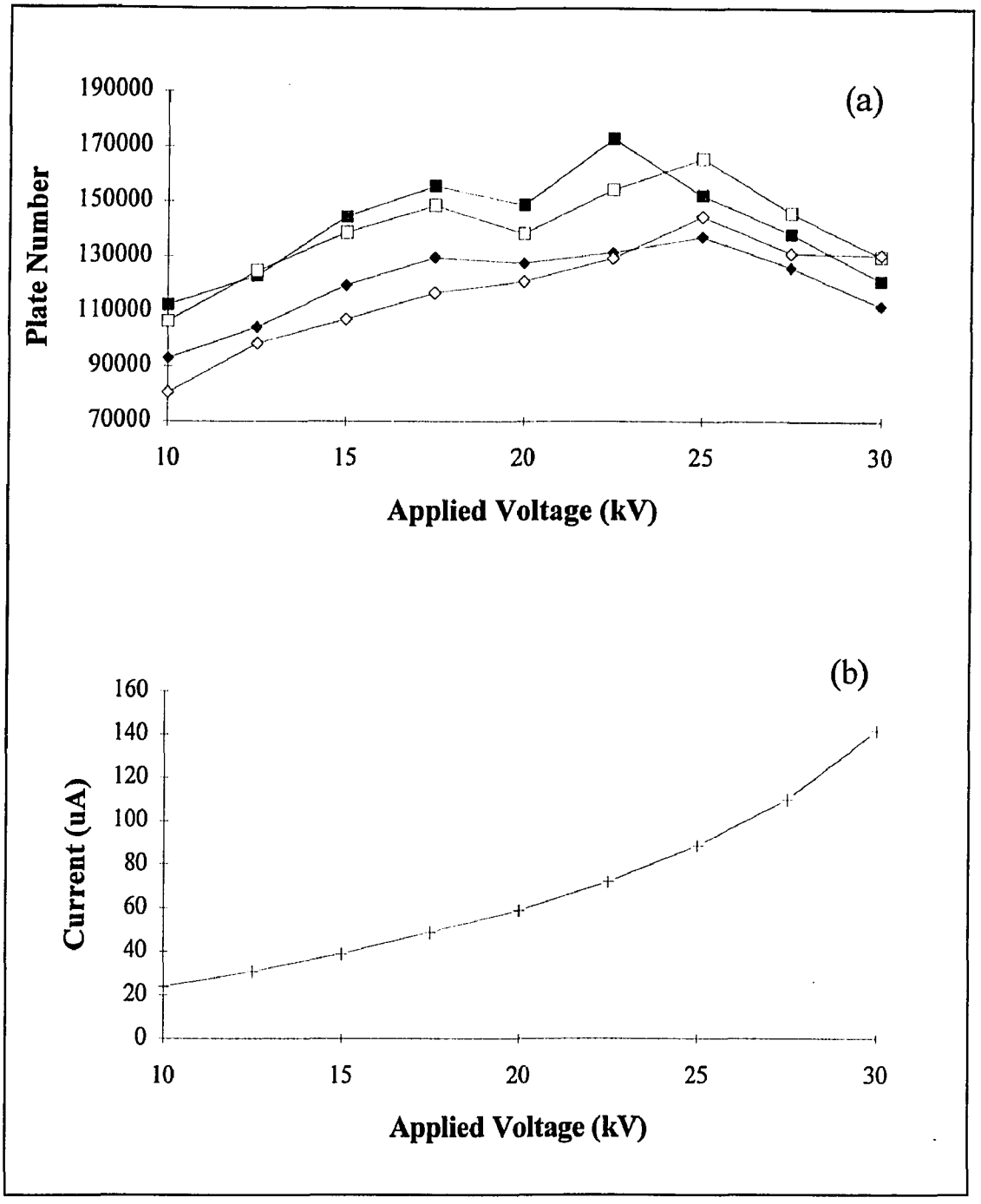


Figure 2.10. The effect of applied voltage on column efficiency: (a) Plate number *versus* applied voltage; (b) Current *versus* applied voltage. □ = Pentachloro-; ■ = 4-Chloro-3-methyl-; ◆ = 4-Nitro-; ◇ = 2-Nitro-.

5. Effect of Sample Injection Time

Samples were injected by applying vacuum at the column outlet buffer beaker. The amount injected depends on the strength of the applied vacuum, the capillary length and i.d., and the sample viscosity. Within limits, peak height increases with the absolute amount of sample introduced; however, the maximum allowable sample volume introduced using vacuum or pressure injection is limited by the initial band width. When this initial band width becomes a significant factor in overall band broadening, any further increase in injection time will be detrimental to the separation.

Vacuum injection times were varied from 2 s to 60 s. The resulting peak widths of several phenols are plotted in Figure 2.11. The peak widths of all solutes increase with injection time. However, the rate of increase of peak widths with injection time was relatively small for injection times less than 20 s. With some sacrifice of peak width, the injection time could be made as long as 40 s. For injection times larger than 40 s, dramatic increases of peak widths were found. Thus injection times were restricted to 20 s or less.

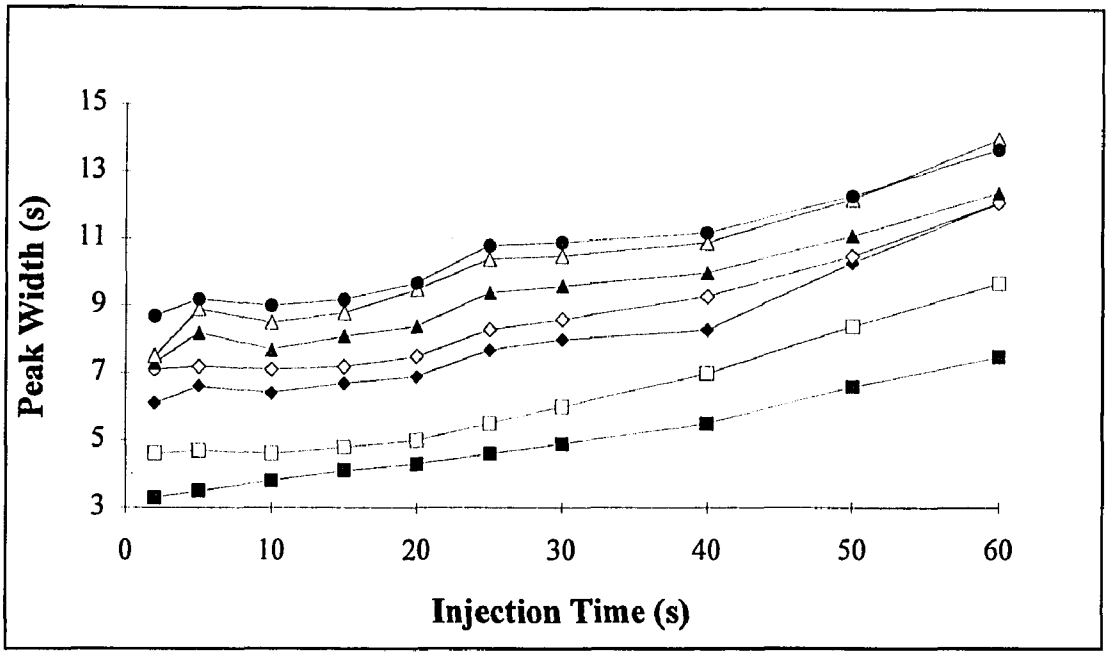


Figure 2.11. The dependence of peak width on sample injection time. ■ = 2,4-Dimethyl-; □ = 4-Chloro-3-methyl-; ◆ = Pentachloro-; ◇ = 2,4-Dichloro-; ▲ = 2,4-Dinitro-; △ = 4-Nitro; ● = 2-Nitro-.

6. Optimized Separation Conditions

Taking into consideration the experimental results described above, the optimum conditions for the separation of the eleven phenols were determined to be pH 9.8 buffer, with $[Na_3PO_4] = [Na_2B_4O_7] = 10$ mM , applied voltage 22.5 kV, current 53 μA, solute concentration 25 mg/l, and vacuum injection time 10 s. The electropherogram obtained using these conditions is shown in Figure 2.12. The eleven phenols are separated in less than 15 min.

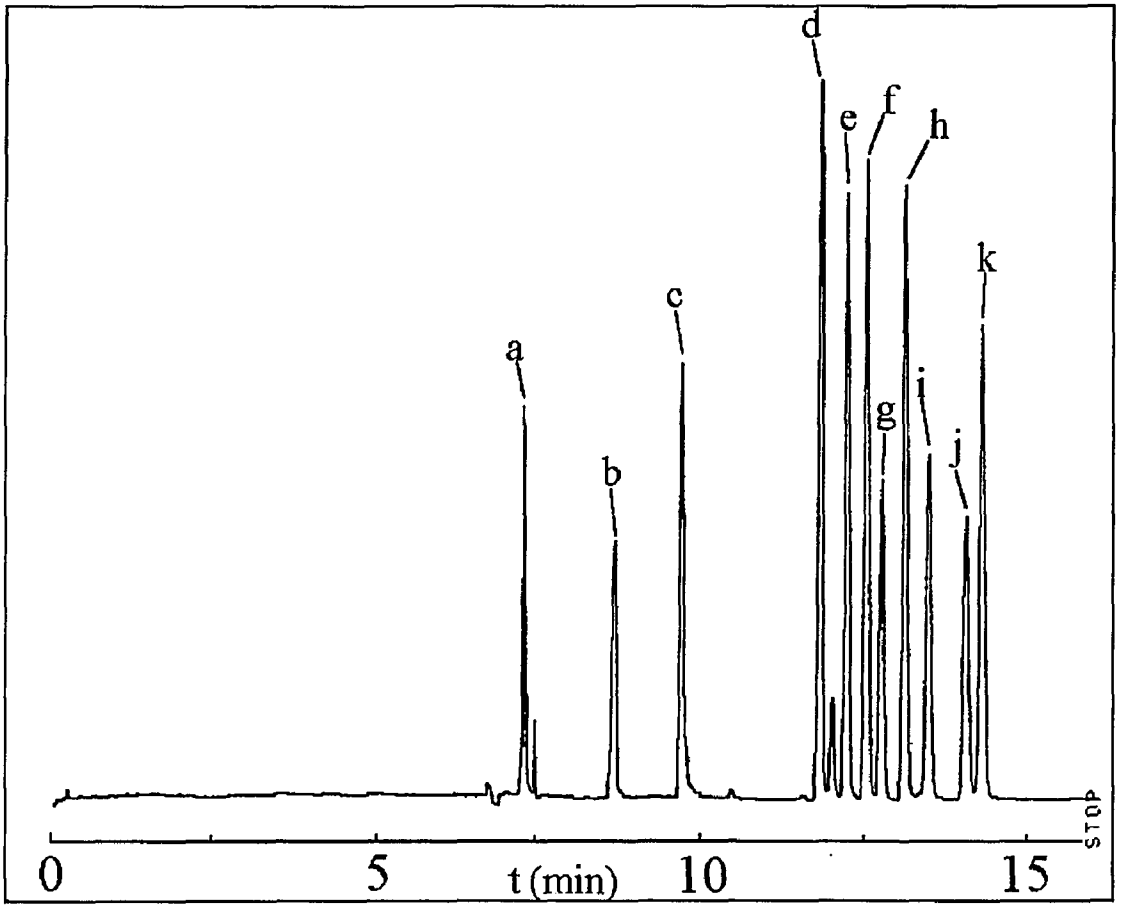


Figure 2.12. The CZE separation of eleven priority phenols under optimized conditions. Buffer: $[Na_3PO_4] = [Na_2B_4O_7] = 10$ mM (pH = 9.8); applied voltage 22.5 kV; current 53 μA; solute concentration 25 mg/l; vacuum injection time 10 s.

7. Quantitation

Phenol mixtures at 5 different concentration levels from 5 mg/l to 50 mg/l were injected in triplicate. The calibration curves for each solute were obtained using the averaged peak areas. The correlation coefficients (r) are listed in Table 2. The results show that good linearities can be obtained for concentrations up to at least 50 mg/l. The limits of detection at the wavelength used (210 nm) were found to be between 0.3 mg/l and 0.6 mg/l (signal/noise = 3), and are also listed in Table 2.2.

Table 2.2. Regression coefficients (r) for calibration curves and limit of detection (LOD)

Compound	r [a]	LOD (mg/l) [b]
2,4-Dimethylphenol	0.9980	0.33
Phenol	0.9995	0.38
4-Chloro-3-methylphenol	0.9995	0.34
Pentachlorophenol	0.9996	0.27
2,4,6-Trichlorophenol	0.9994	0.29
2,4-Dichlorophenol	0.9993	0.27
2-Methyl-4,6-dinitrophenol	0.9996	0.59
2-Chlorophenol	0.9999	0.27
2,4-Dinitrophenol	0.9970	0.54
4-Nitrophenol	0.9998	0.51
2-Nitrophenol	0.9996	0.32

a. At five concentration levels range from 5 to 50 mg/l, the average of three replicate injections each level.

b. S/N = 3, the average of three replicate injections.

8. Rapid CZE Analysis Using Capillary with Smaller Internal Diameter

While separation under the optimized experimental parameters was achieved, we considered the possibility of reducing analysis time. In CE it is desired to apply a voltage as high as possible to achieve high efficiency and short analysis time. However the practical upper limit of applied voltage is often determined by Joule heating. Joule heating is caused by the resistance to electrical current through the buffer electrolyte. Since the current is proportional to the square of the radius of the capillary, a smaller internal diameter of the capillary will greatly reduce the Joule heating. Capillaries with reduced size have larger surface-to-volume ratio, so they are more efficient for heat dissipation. A comparison of the current-voltage relationship for two capillaries with different sizes is given in Figure 2.13. The positive deviation for the 75 μm i.d. capillary indicates that the accumulation the Joule heat inside the capillary increases the conductivity of the electrolytes and generates increasing amounts of current. For the 30 μm i.d. capillary, however, current increase with voltage remains linear over the entire voltage range studied. The narrower bore capillary dissipates Joule heat more efficiently because of the larger ratio of surface area-to-volume.

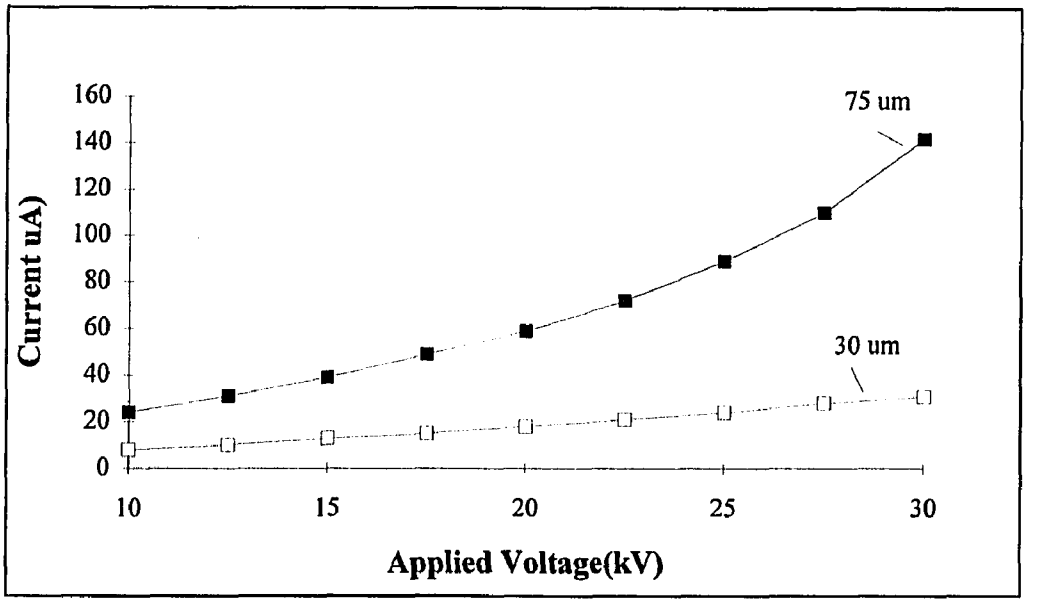


Figure 2.13. Comparison of current-voltage profiles of the 30 μm and 75 μm capillaries.

The linear current-voltage profile of the 30 μm capillary allows the application higher electrical fields. A electropherogram of the separation of the phenol mixture is shown in Figure 2.14. Good separation was achieved with a total analysis time of less than 3 min when 30 kV was applied.

Though narrower bore capillaries are the choice for rapid analysis, the tradeoff is the loss of sensitivity, as can be seen in Figure 2.14. The signal-to-noise ratio is less than that obtained with a 75 μm i.d. capillary. In CE the maximum amount of sample injected is limited by the length of sample plug at the capillary inlet end. The same length of sample plug results in a smaller sample volume for the smaller i.d. capillary because volume is proportional to the square of radius. Thus the selection of capillary dimension will depend on application requirements. In practical experiments, one must consider the balance of separation efficiency, analysis time, and sensitivity.

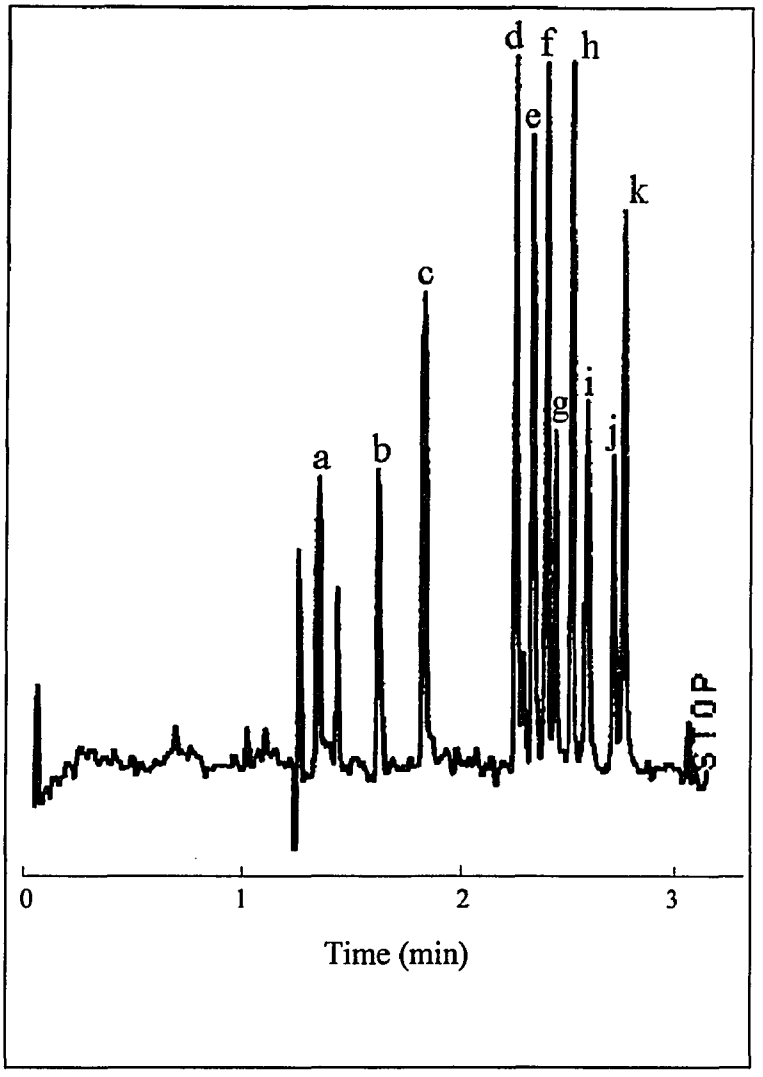


Figure 2.14. Electropherogram of the eleven priority pollutant phenols using a 50 cm x 30 μm i.d. capillary. Buffer: $[Na_3PO_4] = [Na_2B_4O_7] =$ 25 mM (pH = 9.8); applied voltage 30 kV; current 31 μA; concentration of solute 25 mg/l; vacuum injection time 10 s.

PART THREE

Nonionic Surfactants as Buffer Additives for Improving Separation Selectivities in Capillary Electrophoresis

I. Introduction

Separation by CZE is based on differences in the electrophoretic mobilities of solutes. Although CZE is inherently a highly efficient separation technique, it is necessary to carefully study experimental conditions to achieve satisfactory separation selectivity. Among the experimental variables, buffer selection is probably the most important. For weakly acidic and basic solutes the effective electrophoretic mobilities can be modified by optimizing buffer pH to maximize the mobility differences and achieve highest resolution between closely migrated solute pairs. Capillary length and diameter, applied voltage, buffer concentration, temperature control, etc. are important factors in separation efficiency and analysis time, but in general they are not the primary concerns in improving separation selectivity.

A simple and effective method of modifying solute mobilities is the use of buffer additives, including surfactants [1-9], organic buffer modifiers [10-13], polymers [14], etc. Surfactants are probably the most widely used buffer modifiers for separation improvement in CE. In addition to their function as a pseudophase in micellar electrokinetic capillary chromatography (MECC), ionic surfactants have been widely used as buffer additives for controlling the magnitude and/or reversal of EOF, reducing wall adsorption of protein molecules, modifying solute-micelle interactions, etc. While the majority of studies that involve surfactants have focused on the use of ionic surfactants, several nonionic surfactants have been used as buffer modifiers [1-7]. Octyl glucoside and 3-[3-(chloroamidopropyl)dimethylammonio]-1-propane-

sulfonate (CHAPS) were added to the buffer for the enhancement of separation of closely related species by Swedberg [1]. These surfactants are effective only at or above the CMC, so they are considered to function by a MECC mechanism. Matsubara and Terabe separated peptides [2] and dansylamino acids [3] using a nonionic surfactant additive Tween 20 in which the formation of a micellar pseudophase contributed to the separation. The concentration of the surfactant additive required for satisfactory separations was high (>0.1M).

While many studies have been done on the improvement of separation of mixtures of small molecules, rarely have nonionic surfactants been used for such purpose. In this work the potential of using a nonionic surfactant as a buffer additive for the enhancement of resolution was explored. Two nonionic surfactants, Tween 40 and Brij 35, were studied for the separation of the eleven priority pollutant phenols.

II. Experimental

1. Instrument

CZE was carried out on an Isco Model 3850 Electropherograph with an adjustable wavelength UV detector set at 210 nm. An uncoated fused silica capillary 100 cm long (65 cm from injector to detector) and 75 μm i.d. was used. Sample injection was carried out by applying a vacuum at the outlet buffer beaker for 10 s. The electropherograms were recorded on Spectra-Physics SP-4600 or Shimadzu CR-6A integrators.

2. Chemicals

All eleven phenols tested in this work were obtained from Aldrich Chemical Co. (Milwaukee, WI). HPLC grade water (Fisher Scientific, Fair Lawn, NJ) or distilled, deionized water (Milli-Q, Millipore Corp., Bedford, MA) was used to prepare buffers and samples. The nonionic surfactants, polyoxyethylene(20)sorbitan monopalmitate (Tween 40) and polyoxyethylene(23)lauryl ether (Brij 35), were obtained from Sigma Chemical (St. Louis, MO). The stock buffer consisted of 25 mM Na_3PO_4 and 25 mM $Na_2B_4O_7$, pH = 9.8. The buffer solution was filtered through a 0.45 μm syringe filter. The running buffers that contained nonionic surfactants were prepared by pipeting the appropriate amount of surfactant liquid and diluting the buffer to a volume such that the concentrations of both electrolytes were 12.5 mM.

Phenol stock solutions were prepared separately by weighing and dissolving in HPLC grade methanol. A mixture of 11 phenols was prepared by diluting each of the stock solutions in methanol to give a concentration of 100 mg/l. This mixture was stored at 4°C and was used to make test solutions by further dilution to 25 mg/l with the running buffer.

3. Capillary Conditioning

Each day the capillary was first conditioned by filling with 1 M NaOH and soaking for 10 min. The capillary was then washed with deionized water followed by running buffer. At the end of the day the column was washed with water followed by 0.1 M NaOH. The column was left filled with 0.1 M NaOH overnight for the next day's use.

III. Results and Discussion

In capillary zone electrophoresis, solutes are separated based on differential electrophoretic mobilities. Resolution of a closely migrating solute pair can be enhanced by enlarging the differences between mobilities of two solutes. In our previous work [15], the separation of the eleven EPA priority pollutant phenols by CZE was studied with emphasis on buffer pH selection. The pK_a values of the eleven priority pollutant phenols range from 4 to 10.6, so in aqueous solution they are all at least partially dissociated. The overall mobility (μ) of each phenol can be calculated by the following equation:

$$\mu = \mu_A K_a/(K_a + [H^+]) \qquad (3.1)$$

where μ_A is the electrophoretic mobility of the ionized species and K_a the dissociation constant of the acidic solute. To a first approximation, electrophoretic mobility is a function only of pH. Thus the selectivities can be improved by adjusting buffer pH. It was found that all eleven phenols could be separated at pH = 9.8 in about 15 min. However, the resolution of solutes with longer migration times was relatively small at higher pH values. In fact the 4-nitrophenol/2-nitrophenol pair had very close migration times at all the pH values. Because both the μ_A and K_a values of these two components are very close, their overall electrophoretic mobilities are also similar, as expected from equation 3.1. Thus a change in pH will not significantly improve resolution.

Two nonionic surfactants, Tween 40 and Brij 35 were selected as buffer additives for the separation of the phenols. The structures of these two surfactants are shown in Figure 3.1. The phenol mixture was separated in a series of phosphate/borate (pH = 9.8) buffers that contained various concentrations of each selected surfactant. The electroosmotic mobility (μ_{eo}) and electrophoretic mobility (μ_{ep}) of each solute was calculated from the migration time (t) of each solute using the following equation:

$$\mu_{eo} = (1/t_{MEOH})lL/V \tag{3.2}$$

$$\mu_{ep} = -[(1/t) - (1/t_{MEOH})]lL/V \tag{3.3}$$

where l = 65 cm, the effective column length; L = 100 cm, the total column length; V = 20 kV, the applied voltage. The negative sign accounts for the fact that the direction of electrophoretic flow of anions is opposite that of electroosmotic flow. The migration time of methanol, t_{MeOH}, was used to calculate the electroosmotic flow.

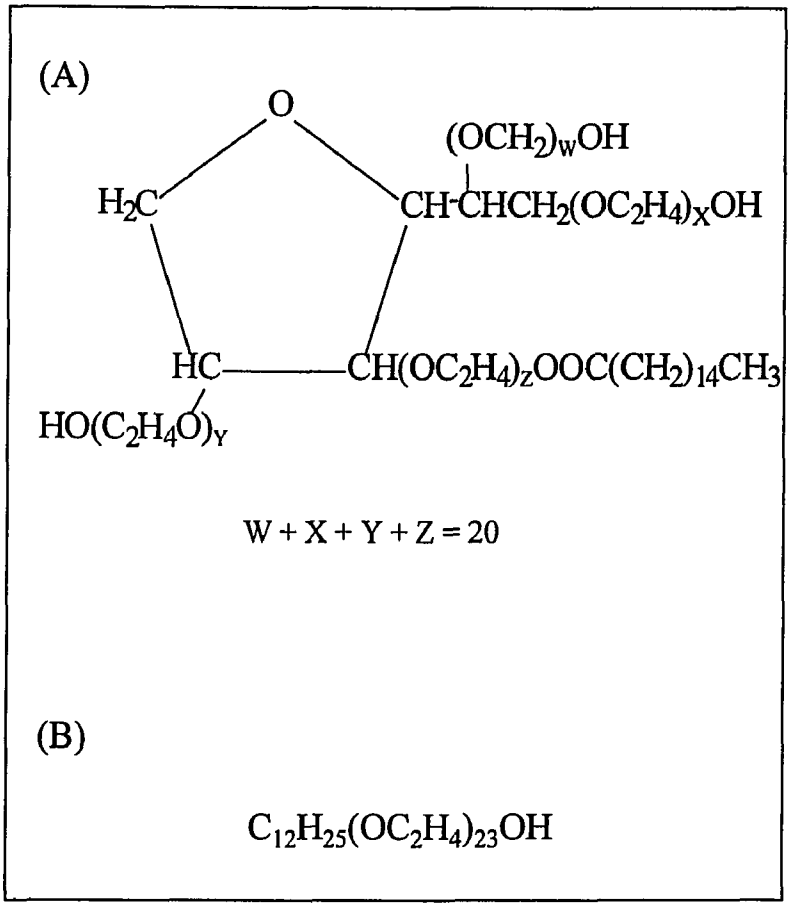


Figure 3.1. Structures of (A) Tween 40, and (B) Brij 35.

Organic solvents have been widely used as buffer modifiers to enhance resolution [10-13]. It is generally recognized that the organic solvent changes the zeta potential, and thus modifies (usually decreases) the electroosmotic flow and improves resolution. The analysis time consequently increases. To understand the role of nonionic surfactants on separation, the dependence of electroosmotic flow on the concentration of Tween 40 was studied using the methanol peak as an EOF marker. Figure 3.2(a) shows the changes of electroosmotic flow with increase in surfactant concentration. It was observed that the presence of Tween 40 slightly reduced (less than 10%) the electroosmotic flow within the concentration range studied. This probably results from the same effect as that of organic solvents as buffer modifiers.

In contrast to EOF, the changes of electrophoretic flow were more significant. A plot of μ_{ep} *versus* C_{Tween} is given in Figure 3.2(b), using the calculated values of electrophoretic mobility. It can be seen that for all solutes the electrophoretic mobility decreases as the percentage of Tween 40 in the buffer increases. For most solutes the mobility values decrease more rapidly at Tween 40 concentrations less than 1%.

The influence of nonionic surfactant on analysis time results from a combination of changes of electroosmotic mobility and electrophoretic mobility. For CZE of negatively charged species, a decrease in electroosmotic flow results in an increase in migration time. On the other hand, a decrease in electrophoretic mobility reduces the migration time. The opposite trends in migration time should offset each other. The influence of Tween 40 on solute migration time is given in Figure 3.2(c). It can be seen that the change in migration time is solute dependent. However the total

run time did not change much within the concentration range of Tween 40 studied, which is advantageous over the use of other organic buffer modifiers.

The effect of another nonionic surfactant, Brij 35, was also studied. Similar to the study of Tween 40, the dependence of EOF, electrophoretic mobility, and analysis time on the concentration of Brij 35 are shown in Figure 3.3. It can be seen that although the two surfactants are quite different in structure, their influences on the separation of phenols are very similar.

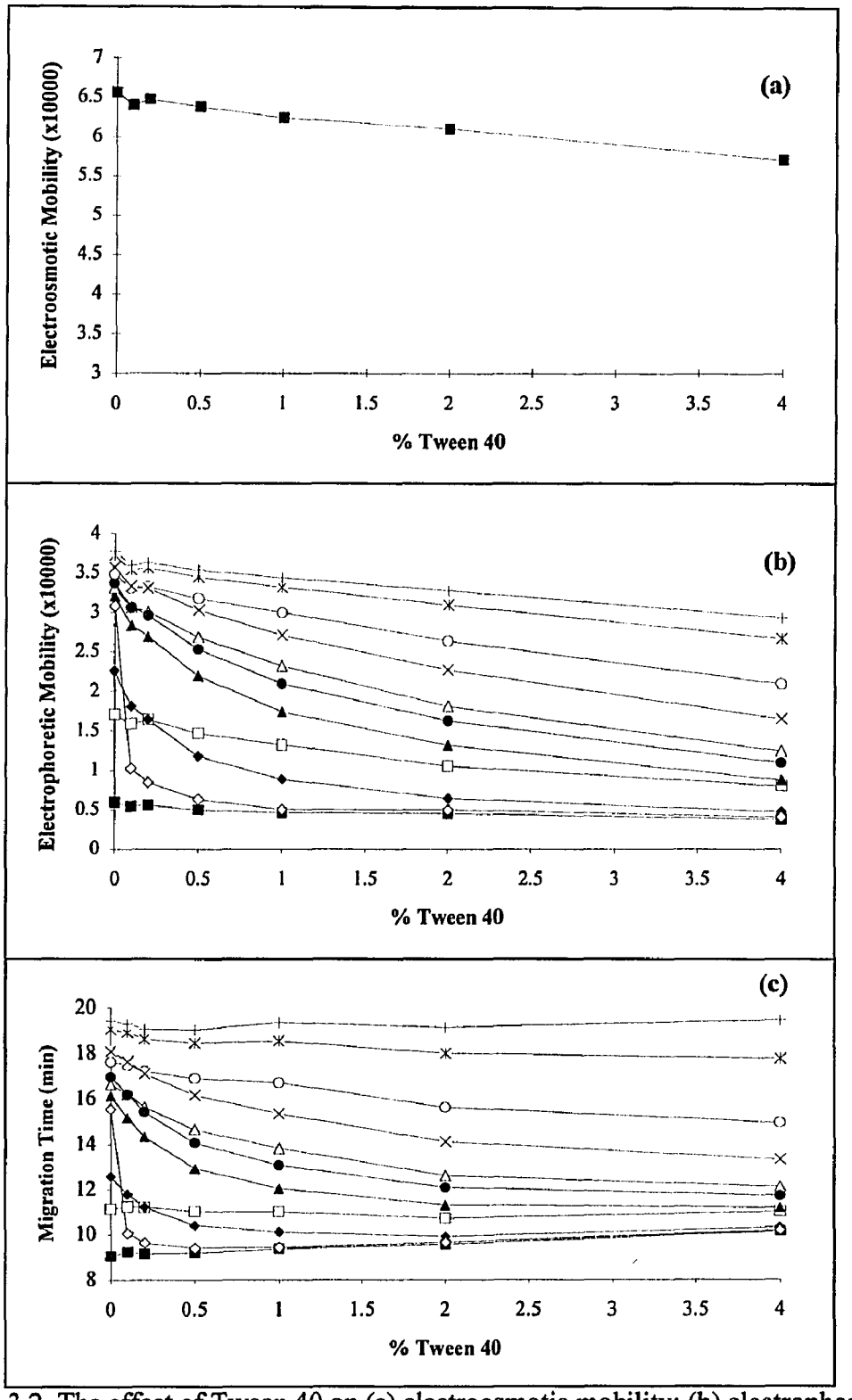


Figure 3.2. The effect of Tween 40 on (a) electroosmotic mobility; (b) electrophoretic mobility; and (c) migration time. ■ = Phenol; □ = 2,4-Dichloro-; ◆ = 2,4-Dimethyl-; ◇ = Pentachloro-; ♦ = 2-Methyl-4,6-dinitro-; △ = 4-Nitro-; ● = 4-Chloro-3-methyl-; ○ = 2,4,6-Trichloro-; × = 2,4-Dinitro-; ∗ = 2-Nitro-; + = 2-Chloro-.

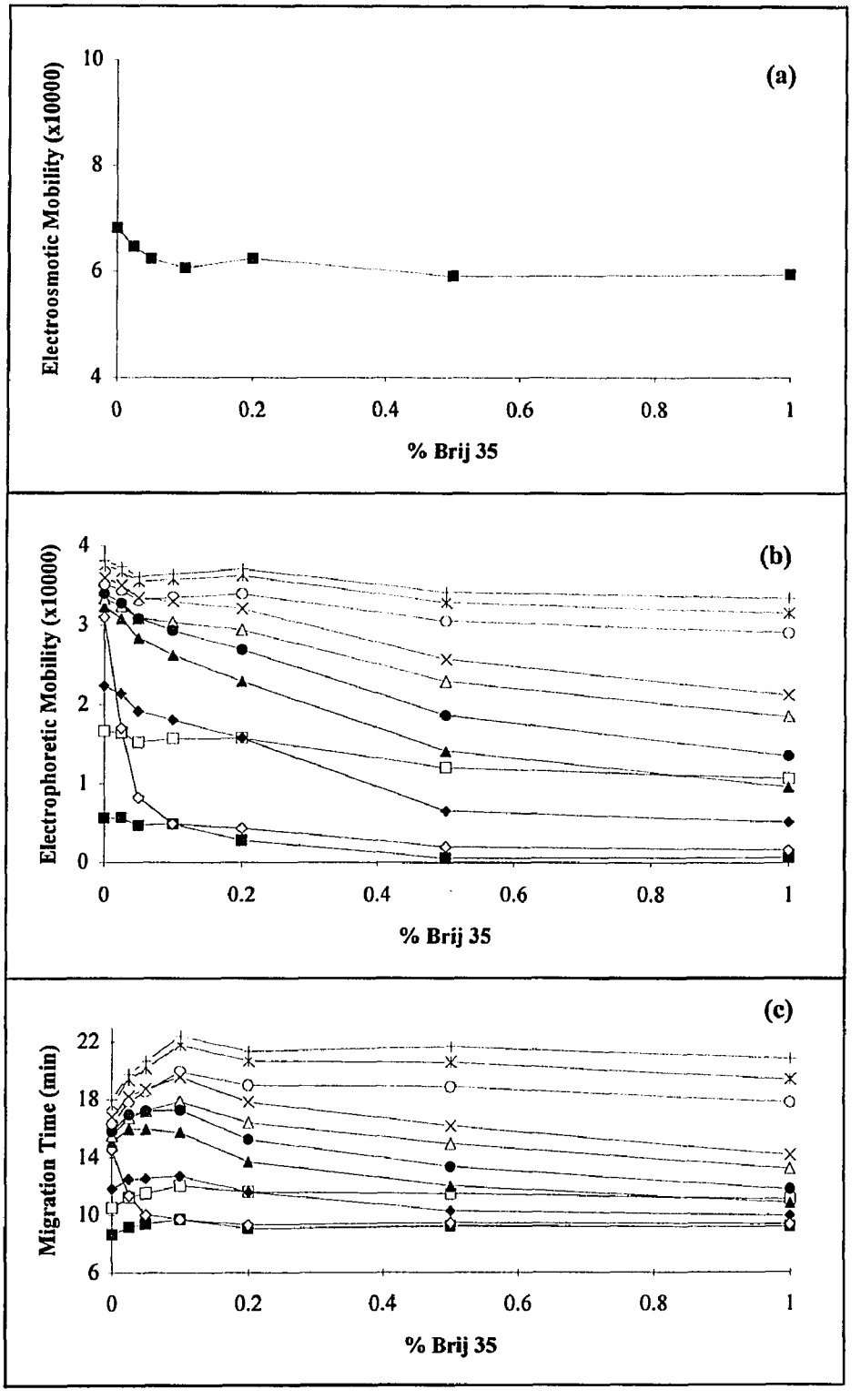


Figure 3.3. The effect of Brij 35 on (a) electroosmotic mobility; (b) electrophoretic mobility; and (c) migration time. The figure legends are the same as those in Figure 3.2.

An important feature revealed in Figure 3.2(b) and Figure 3.3(b) is that the rate of decrease in mobility differs for each solute, which makes it possible to adjust selectivity and improve resolution by changing the concentrations of these surfactants in the buffer solution. When no buffer additive is used for the separation at pH = 9.8, the resolution of the three slowest migrating solute pairs, 2,4-dichlorophenol/2-methyl-4,6-dinitrophenol, 2-chlorophenol/2,4-dinitrophenol, and 4-nitrophenol/2-nitrophenol, is relatively small compared to other solutes. It was our primary goal to improve the resolution of these solute pairs. The effect of the addition of Tween 40 on resolution is shown in Figure 3.4. Although 4-nitrophenol and 2-nitrophenol are a solute pair difficult to separate over the entire buffer pH range because of their very close pK_a and μ_A values, their resolution improved significantly with increasing percentage of Tween 40. However, the resolution of 2,4-dichlorophenol/2-methyl-4,6-dinitrophenol goes through a minimum at about 0.1% of Tween 40 and then increases with higher concentrations of Tween 40, which results from the change in migration order between the two solutes. A similar migration order change was also observed for the 2-chlorophenol/2,4-dinitrophenol pair. It was concluded from Figure 3.2(b) that good separation of the mixture of eleven phenols could be achieved with the concentration of Tween 40 in the range of 0.5% to 2%. Electropherograms for the separation of the phenol mixture (a) without surfactant additive, (b) with 0.5% of Tween 40, and (c) with 0.5% of Brij 35 in borate/phosphate buffer are given in Figure 3.5. Comparing Figure 3.5(b) and 3.5(c) it can be seen that though the two nonionic

surfactants differ in structure, their impacts on the separation are much the same in terms of change in migration order.

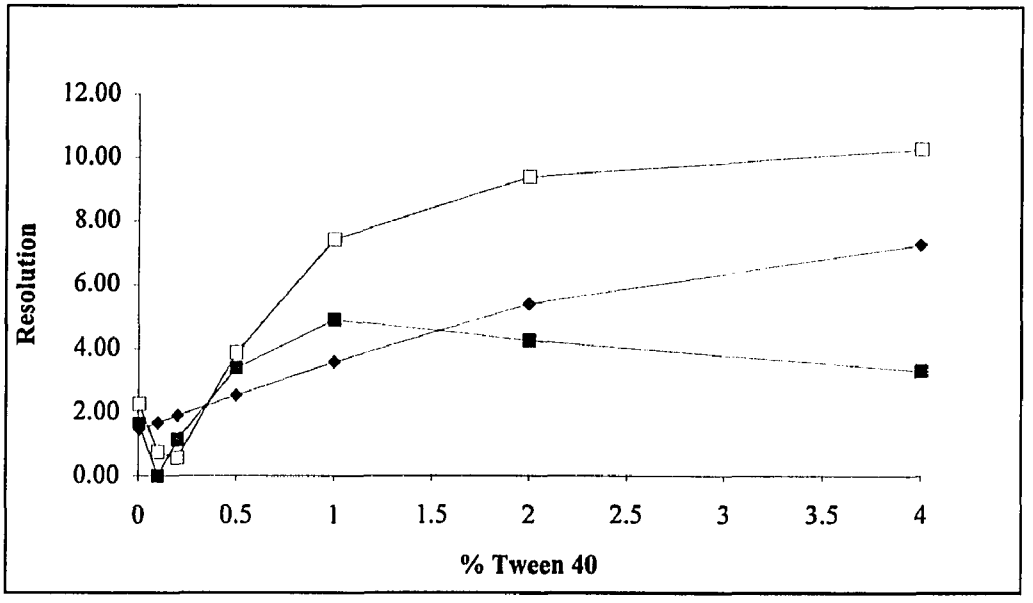


Figure 3.4. Effect of Tween 40 on the resolutions of three closely migrated solute pairs. □ = 2-Chloro-/2,4-Dinitro-; ◆ = 4-Nitro-/2-Nitro-; ■ = 2,4-Dichloro-/2-Methyl-4,6-dinitro-.

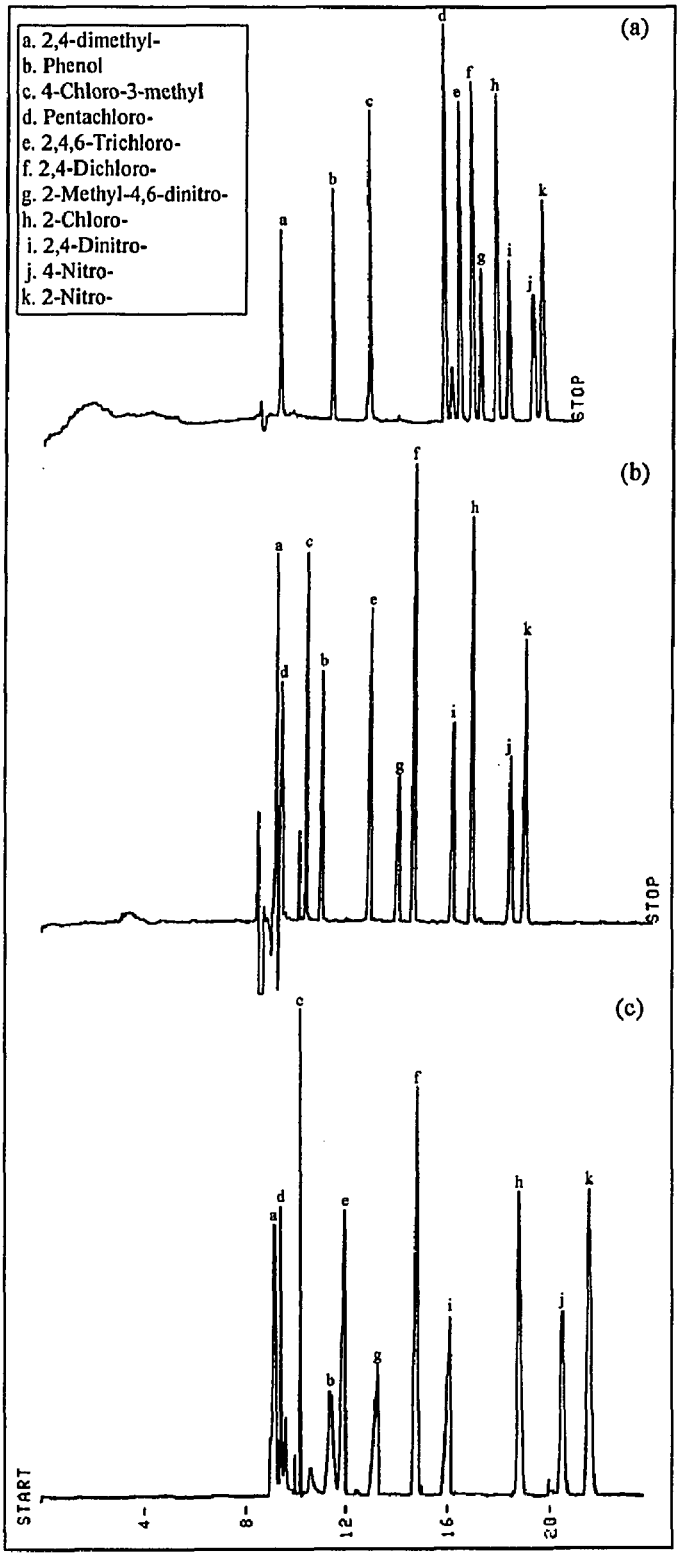


Figure 3.5. Effects of nonionic surfactants as buffer additives on the separation of the EPA priority pollutant phenols by CE. (a) no additive; (b) 0.5% Tween 40; (c) 0.5% Brij 35.

The most surprising result from the experiment is the migration behavior of pentachlorophenol. The electrophoretic mobility of pentachlorophenol decreased rapidly with the addition of Tween 40 in buffer and became invariant at about 0.5% of Tween 40. In Swedberg's study[1], the enhancement of resolution by the addition of non-ionic and zwitterionic surfactants was explained by an apparent MECC mechanism. Since the CMC value of Tween 40 in water is reported to be 0.0029% (w/v) [16], micelle formation might contribute to the migration behavior of the phenols. Micelles formed by nonionic surfactants carry no electrical charge, and migrate at electroosmotic flow rate. The sharp decrease of electrophoretic mobility suggests pentachlorophenol has a stronger interaction with the micelle than other phenols. However based on the pK_a values of the eleven phenols, pentachlorophenol (pK_a = 4.5) is the second strongest acid in the series. In the pH = 9.8 buffer, pentachlorophenol can exist only in the ionic form. So partitioning in and out of the non-ionic micelle seems not to be a reasonable explanation. Another solute, 4-chloro-3-methylphenol, shows similar migration behavior, but not as striking as pentachlorophenol.

In addition to the dramatic shift of migration time, pentachlorophenol also shows anomalous peak shape at certain concentration range of the surfactants. In Figure 3.6, electropherograms of pentachlorophenol peak with the concentration of Tween 40 between 0 to 0.2% are shown. It is likely that pentachlorophenol has a strong interaction with the micelles of Tween 40. Similar peak shape distortion of

pentachlorophenol was also observed when Brij 35 was used as buffer additive. Currently we do not have a reasonable explanation of these distorted peaks.

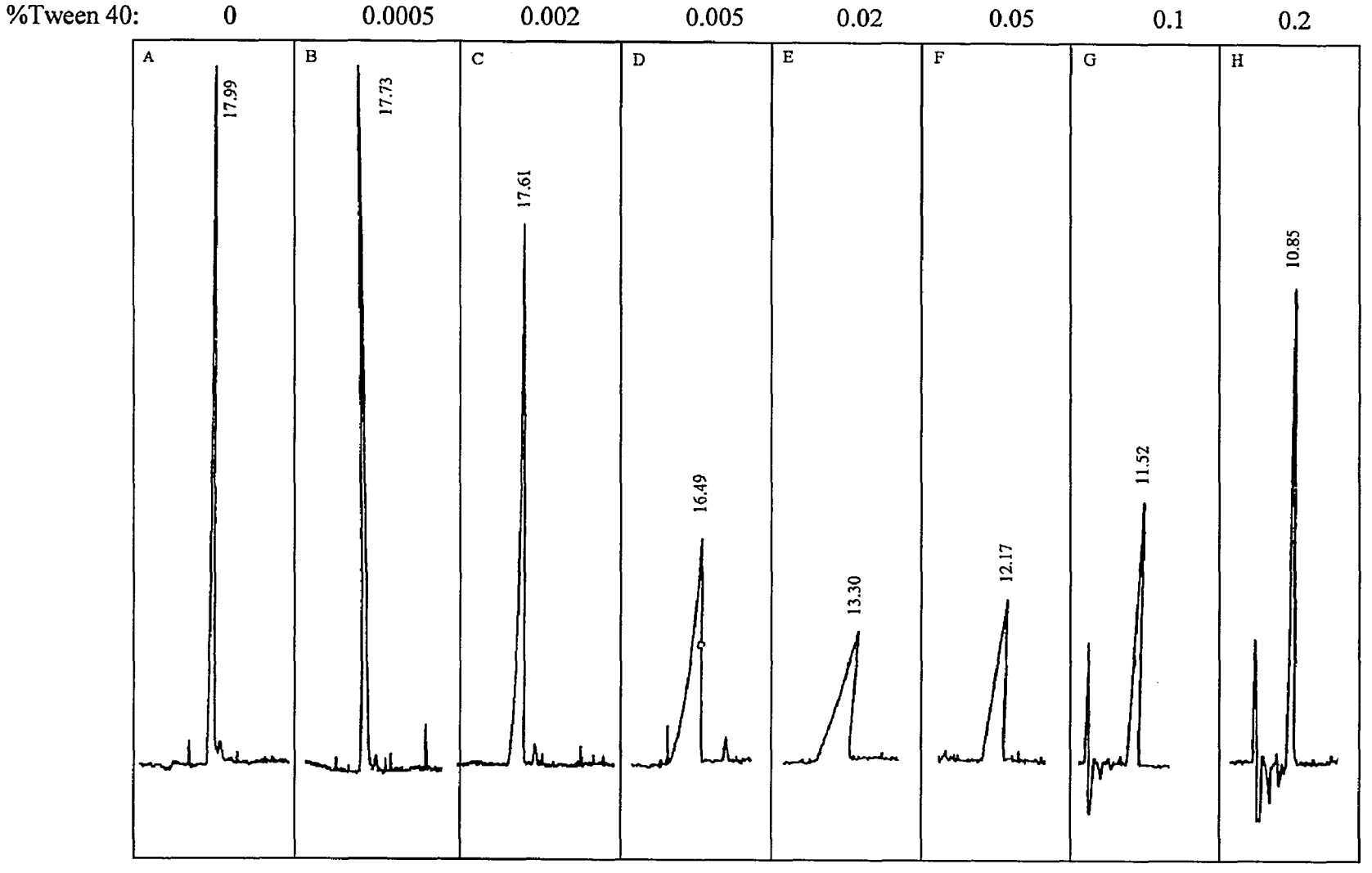


Figure 3.6. Change of peak shape and migration time of pentachlorophenol as the concentration of Tween 40 increases.

This work indicates that the use of nonionic surfactants in CZE is a very useful approach for the enhancement of selectivity and resolution of complex mixtures. Several potential advantages of this approach can be summarized:

(1) Nonionic surfactants do not increase conductivity and do not contribute to Joule heating.

(2) Only small amounts of surfactant are necessary to produce significant changes in selectivity, so buffer compositions are not changed significantly.

(3) Low concentrations of nonionic surfactant have little effect on magnitude of EOF, so run time is not changed much, which overcomes the drawback of the use of additives that work by modifying EOF.

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