2008

Enantiomeric separations and microorganism studies with analytical separation techniques

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Enantiomeric separations and microorganism studies with analytical separation techniques

by

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A dissertation submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Analytical Chemistry

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Ames, Iowa

2008

This dissertation is dedicated to

My Heavenly Father, who guides me through my whole life;

My parents, who raised me and loved me unconditionally;

My teachers, who educated me and shaped me into a Scientist.

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PART II. MICROORGANISM STUDIES WITH CAPILLARY ELECTROPHORESIS

ABSTRACT

This dissertation is divided into two parts: Part I involves enantiomeric separations by high performance liquid chromatography (HPLC) and capillary electrophoresis (CE); Part II describes a series of microorganism studies using CE.

In Part I, Chapter 1 gives an overview of enantiomeric separations using HPLC and CE. Chapter 2 presents enantiomeric separations on a new synthetic LC chiral stationary phase (CSP), i.e., a pentaproline-based CSP. The enantiomeric separation ability of this new CSP was evaluated by injecting 194 racemates on the LC column. The chiral recognition mechanism was discussed and sample loading was briefly tested. Chapter 3 shows the enantiomeric separations of three groups of synthetic chiral compounds using CE: furan derivatives, fused polycycles and isochromene derivatives. Cyclodextrin-modified micellar capillary electrophoresis (CD-MCE) was utilized in this study. The reason for using this complicated method rather than the simplest capillary zone electrophoresis (CZE) was discussed and different types of cyclodextrin selectors were compared.

In Part II, Chapter 4 serves as an introduction to this part of the dissertation: a review of work mainly from our group on microorganism studies using CE, focusing on the detection of microbial contamination and further evaluating the possibility of using CE to replace the traditional sterility test outlined in the *U.S.Pharmacopeia.* Chapter 5 presents a successful single-cell detection approach using a modification of a previously established procedure from our group, and thereby greatly widens the practicality and effectiveness of

the method. Chapter 6 is a CE study on the use of ionic liquids (ILs) in the detection of microbial contamination. By using dicationic ILs as auxiliary buffer additives, we are able to further reduce the possibility of lysing cells by surfactants (such as CTAB).

PART I. ENANTIOMEIC SEPARATIONS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND CAPILLARY ELECTROPHORESIS

CHAPTER 1

Introduction

The presence of stereogenic centers in many compounds often results in enantiomers that can differ substantially in their biological, pharmacological or toxicological profiles [1]. The Unite States Food and Drug Administration (FDA) issued guidelines for the development of stereoisomeric drugs in 1992 and the pharmacological effect of both enantiomers of chiral drugs must now be evaluated [2]. As a result, the determination of stereochemical composition is an important issue. High-performance liquid chromatography (HPLC), gas chromatography (GC), supercritical fluid chromatography (SFC), thin-layer chromatography (TLC) and capillary electrophoresis (CE) are five analytical methods used so far for the separation and analysis of chiral compounds. In Part I, enantiomeric separations using HPLC and CE are presented.

1.1 Enantiomeric Separations by High Performance Liquid Chromatography

HPLC is the most widely used method for enantiomeric separations in industry because of its robustness, reproducibility, and capability for both analytical and preparative scale separations. Till now, more than one hundred chiral stationary phases (CSPs) have been commercialized. Based on their structures, the most common CSPs can be classified into three categories: macrocyclic, polymeric, and π-π association CSPs [3]. Common

macrocyclic CSPs include macrocyclic glycopeptides, cyclodextrins, and chiral crown ethers. Polymeric CSPs include natural polymers (such as proteins and polysaccharides) and synthetic polymers. For π-π association CSPs, π-π interactions between the analytes and the CSP are required. If the CSP has a π-acid moiety (i.e., a π-electron deficient group), the analytes must have a complementary π-basic group (i.e., a π-electron rich group) in order to be separated.

Another way to classify CSPs is based on the source or origin of their chiral selectors, according to which, CSPs can be grouped into three main categories: naturally occurring, semi-synthetic, and synthetic CSPs [3]. Examples of naturally occurring chiral selectors are cyclodextrins, macrocyclic glycopeptides, amino acids, and proteins. Semisynthetic CSPs are usually derivatives of naturally occurring CSPs, such as derivatized linear carbohydrates, modified macrocyclic glycopeptides, and derivatized cyclodextrins. As the name indicates, synthetic chiral selectors are synthesized by researchers, and examples include methacrylate polymers, a few π-π complex compounds, and chiral crown ethers. Today, the semisynthetic chiral selectors dominate the field of enantiomeric separations, although many important contributions are still made by natural and synthetic selectors.

1.2 Enantiomeric Separations by Capillary Electrophoresis

Capillary electrophoresis is a rapidly expanding separation technique that provides high resolution, high efficiency and flexibility. CE is typically used to separate ionized

molecular species based on their charge and frictional forces [4]. The first enantiomeric separation using CE was reported by Gassmann in 1985 for the separation of dansyl amino acid enantiomers [5]. Since then, CE has been developed substantially for the separation of enantiomers in pharmaceutical, clinical, and environmental fields. In CE, the separation of enantiomers is achieved mainly by the direct separation method referred as capillary zone electrophoresis (CZE). In this method, the chiral selector is simply added to the achiral background electrolyte (BGE) where enantiomers can be resolved by these chiral recognition agents. Chiral agents utilised in CE include copper-amino acid complexes, chiral crown ethers, chiral micelles, antibiotics, proteins and cyclodextrins (CDs) or their derivatives. Among them, CDs and their derivatives are most widely investigated and applied for the enantioseparation of a large number of analytes.

Naturally occuring α-, β-, γ- CDs are cyclic oligomers of α-1,4-linked D-glucose units, which comprise six, seven or eight glucose units respectively [6-8]. They can be prepared by the treatment of starch with cyclodextrin glycosyltransferase. The native CD is a truncated cone with a relatively hydrophobic open cavity and a hydrophilic exterior because of hydroxy group (Figure 1) [8]. Therefore, nonpolar molecules or parts of the molecules will form inclusion complexes with hydrophobic cavity in the aqueous solutions [8-12]. Besides native CDs, various (hydrophobic or hydrophilic) derivatives are also used for chiral separations. These modified CDs can exhibit very different properties than the native ones, and the aim of derivatization is to: (i) increase the solubility of CDs in aqueous buffer; (ii)

change the hydrophobicity of the cavity; (iii) attach catalytic groups to the binding site; (iv) allow the analysis of uncharged compounds in CE [10]. Charged/ chargeable substituents of the cyclodextrin hydroxyl groups on the CD structure can expand the separation window in comparison with neutral CDs. Native or uncharged CDs can be used for ionic enantiomers with enhanced selectivity because of their high hydrogen bonding capability. In the charged mode, the CDs are suitable for neutral enantiomers because of the formation of inclusion-complexes with the analytes. Since two enantiomers posses similar physicochemical properties, for a successful enantiomeric separation in CE, selective modification of the effective mobilities of the two enantiomers is necessary. This can be achieved by forming transient diastereomeric complexes with CDs where hydrophobic, hydrogen bonding, π-π, dipole, van der Waals and electrostatic interactions in the case of charged analytes and CDs can be involved [13-23].

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a b

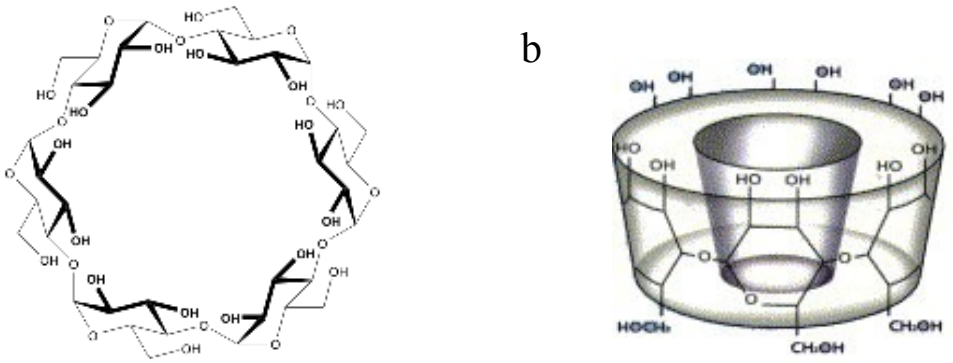

Figure 1. Structure of a) α-cyclodextrin and b) the toroidal shape of a cyclodextrin molecule (from Ref. [6])

CHAPTER 2

Evaluation of Pentaproline-based Chiral Stationary Phase by High-Performance Liquid Chromatography

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Chromatographia 67 (2008) S13- S32

Abstract

A pentaproline-based chiral stationary phase was prepared and the selectivity of the column was evaluated with 194 racemic compounds in three mobile phase modes: normal-phase mode, polar organic mode and reversed-phase mode. 94 racemates out of 194 were separated and the normal-phase mode proved to be the separation mode of broadest applicability. The column is stable in all common organic solvents and no degradation in column performance was observed in any mode even after more than 1000 injections. A brief sample loading test was performed on the 250 mm × 4.6 mm column and 13.2 mg of α-Methyl-9-anthracenemethanol was baseline separated. Retention behavior in the normal-phase mode and the effect of analyte structure on retention and enantioselectivity are discussed.

2.1 Introduction

As many enzymes and cell surface receptors possess “handedness”, the enantiomers of a racemic pair of compounds may be adsorbed, activated, and/or degraded in different manners. The presence of stereogenic centers in many compounds often makes enantiomers differ substantially in their biological, pharmacological or toxicological profiles [1, 2]. Therefore, the separation of enantiomers is an important step to enable the evaluation and study of individual enanatiomers. High-performance liquid chromatography (HPLC), gas chromatography (GC), thin-layer chromatography (TLC), capillary electrophoresis (CE) and supercritical fluid chromatography (SFC) are five analytical methods used so far for the separation and analysis of racemic compounds [3-7]. Among them, HPLC has a reputation for good reproducibility, selectivity, a large selection of chiral stationary phases (CSPs), automation, qualification and quantification of analytes, online structural determination by coupling with spectroscopic instruments, and preparative capabilities [8]. As a result, considerable efforts have been made in the development of chiral stationary phases in the past decades. In previous articles, we reported a new class of promising chiral stationary phases made from proline peptides, in which we described the preparation procedure of several stationary phases and also the effect of peptide length and linker on the enantioselectivity with a small library of 53 racemates [9-11]. Prior to this, most studies have focused on the proline monomer and enantiomeric separations in ligand exchange chromatography [12-21]. In these cases, Cu^{2+} is added to the mobile phase in order to

facilitate the adsorption of chiral analytes that can act as bidentate ligands (such as other amino acids and α-hydroxyacids). However, broad enantioselectivity and normal phase separations have not been demonstrated for this approach in HPLC. Proline is a unique amino acid (Figure 1) that contains a cyclic structure which stabilizes polyproline in a helical conformation that sterically hinders nonspecific hydrogen bonding with the analytes [22]. Thus it is not surprisingly that this amino acid has been studied as chiral selector. In a recent paper, Zhang et al. found that in the entire range of IPA/DCM solvent mixtures (0-100% IPA), VCD and ECD spectra indicated that the decaproline oligomer is in the polyproline II (PPII) conformation with its all peptide bonds in the trans configuration [23]. In this article, we report the chromatographic performances of a pentaproline chiral stationary phase by screening it with 194 racemates available in our lab. Pentaproline was chosen as a compromise from previous studies which showed that longer proline oligomers had good enantioselectivity but poor efficiency, while short oligomers (diproline and triproline) CSPs had good efficiencies but poorer enantioselectivities. In this work, the retention behavior, enantioselectivity, effect of analyte structures, and the effect of analyte loading are examined.

2.2 Experimental

2.2.1 Chemicals

Amino acid derivatives were purchased from NovaBiochem (San Diego, CA). All other chemicals and solvents were purchased from Aldrich (Milwaukee, WI), Fluka

(Ronkonkoma, NY), or Fisher Scientific (Pittsburgh, PA). HPLC grade Kromasil silica gel (particle size 5 μm, pore size 100 Å, and surface area 298 m^2/g) was purchased from Akzo Nobel (EKA Chemicals, Bohus, Sweden).

Preparation of 3-methylaminopropyl silica gel (MAPS)

MAPS was prepared from Kromasil silica gel (5 μm spherical silica, 100 A° , 298m^2/g) and 3-(methylamino)propyltrimethoxysilane according to a procedure described for the preparation of 3-aminopropyl silica gel (APS) [24]. The surface methylamino concentration is 0.561 mmol/g, based on elemental analysis data of nitrogen in % (w/w) (C, 3.10, 3.09; H, 0.73, 0.76; N, 0.80, 0.77).

Preparation of Pivalyl-Pro-Pro-Pro-Pro-Pro-MAPS

The stationary phase is prepared using the Fmoc solid phase peptide synthesis [25]. To 0.80 g of MAPS prepared above (the surface methylamino concentration was 0.561 mmol/g) were added mixtures of Fmoc-Pro-OH (3 equiv., 0.45 g), *O*-(7-azabenzotriazol-1-yl)-*N',N',N',N'*-tetramethyl uronium hexafluorophosphate (HATU) (3 equiv., 0.51 g), and N,N-diisopropylethylamine (DIPEA) (3 equiv., 0.17 g) in 8mL of DMF. After agitating for 6 h, the resulting silica was filtered and washed with DMF, methanol, and DCM, and then the unreacted free methylamine group on the silica gel was end-capped by reacting with acetic anhydride and pyridine in DCM. The surface Pro

concentration was determined to be 0.49 mmol/g based on the Fmoc cleavage method. The Fmoc protecting group was removed by treatment of the silica with 10mL of 20% (v/v) piperidine in DMF for 1 h. The deprotected silica, H-Pro-MAPS, was collected by filtration and washed with DMF, methanol, and DCM. Then the next module, Fmoc-Pro-OH, was coupled to the resulting silica following an identical reaction sequence and yielded the Fmoc-Pro-Pro-Fmoc-MAPS. From the second to the fifth module, the surface Fmoc concentration was determined to be the followings, 0.46, 0.44, 0.41, 0.39 mmol/g based on the Fmoc cleavage method. The Fmoc protecting group of Fmoc-$(Pro)_5$-MAPS was removed by treatment with 10mL of 20% (v/v) piperidine in DMF for 1 h, then end-capped by reacting with trimethylacetyl chloride (0.60 g, 5 mmol) and DIPEA (0.65 g, 5 mmol) in 10mL of dry DCM for 1 h. The desired enantioselective stationary phase was collected and washed with DMF, methanol, and DCM.

2.2.2 Column Evaluation

The chromatographic experiments were done on either a HP 1050 or Agilent 1100 HPLC system with a UV variable wavelength detector (VWD), an auto sampler, and computer-controlled HP ChemStation for LC data processing software. Retention factor (k') is equal to $(t_R - t_0)/t_0$, in which t_R is the retention time and t_0 is the dead time. The separation factor (R) equals k_2'/k_1', the ratio of the retention factors of the two enantiomers. Dead time t_0 was measured with 1,3,5-tri-*tert*-butylbenzene as the void volume marker [26]. Flow rate at

1 mL min^{-1}. UV detection was carried out at 214 or 254 nm for most probe compounds. Column dimension: 250 mm × 4.6 mm.

2.3 Results and Discussion

Structure of the pivalyl end-capped pentaproline chiral stationary phase is shown in Figure1. As mentioned previously, the direct linking of the peptide to 3-methylaminopropyl silica gel enhanced the enantioselectivity compared to linkage through a 6-methylaminohexanoic acid linker [11]. This is because the later contains within the structure of the chiral selector a functional group (CO-NH) that is able to form nonspecific hydrogen bonding interactions with many analytes. Though in general, the separation factors increase with increasing numbers of proline units, the pentaproline-based CSP evaluated in this study may be the best compromise due to its greater efficiency as well as for practical reasons such as price of reagents and stationary phase synthesis yields. This column was tested in three mobile phase modes: the normal-phase, polar organic and reversed-phase modes. In each mode, 194 racemic analytes were injected. These analytes cover a wide range of structural diversity, containing functional groups such as hydroxyl, carbonyl, oxime, amine, ester, amide and sulfoxide.

2.3.1 Mobile Phase Modes

Tables 1-3 list the chromatographic data for the enantiomeric separations. The mobile

phase solvent composition was chosen to adjust the retention factor to a value of less than 10. This column separated 74 compounds in the normal-phase mode, 44 compounds in the polar organic mode and 47 compounds in the reversed-phase modes. In total, 94 out of 194 compounds were separated and among them, 36 were baseline separated (Figure 2).

2.3.2 Interactions for Chiral Recognition

As described in previous work, proline is a unique amino acid that contains a cyclic structure which restricts rotation around the nitrogen-α-carbon and therefore, stabilizes polyproline in a helical conformation. This sterically hinders nonspecific hydrogen bonding with the analytes, as compared to other oligopeptides [9]. Enantiorecognition on this proline peptide CSP seems to be based mainly on hydrogen bonding and steric hindrance. However, this column's ability to separate enantiomers that contain no hydrogen bond donor groups (such as analytes 1, 2, 33, 39, 47, 57, 59, 61, and so on) indicates that other interactions (such as dipole/dipole, induced dipole/dipole, and dispersion forces) might contribute to the enantioselectivity as well.

2.3.3 Loading Test

Sample loading was examined by injecting α-Methyl-9-anthracenemethanol (analyte 7) into the column in the normal-phase mode and 13.2 mg of this racemate could be baseline separated which indicates that this type of stationary phase may be applied for preparative

enantioseparations. Figure 3 showed the chromatograms of the separations of 2.5 μg (Figure 3a) and 13.2 mg (Figure 3b) of analyte 7. As seen from Figure 3b, considerably more sample could be injected while maintaining baseline separated. However, due to the solubility problem the highest analyte concentration tested was 66 mg mL^{-1} with maximum injection volume 200 μL.

2.3.4 Retention Behavior

General speaking, the retention of analytes in the normal-phase mode will decrease when the percentage of polar solvent increases. However, for all analytes studied, the plot of mobile phase composition versus retention factor produced a U-shaped retention plot. Figure 4 shows representative plots for 10 analytes including analytes with/without hydrogen-bonding donor groups. The solvent system in the study uses dichloromethane (DCM) and isopropanol (IPA). DCM was chosen over heptane for the purpose of faster elution. The analyte was more strongly retained at both high concentrations of DCM and at high concentrations of IPA in the mobile phases. This U-shaped retention behavior is often observed in the reversed-phase mode, especially for highly water-soluble analytes such as organic salts [27-31]. The possible reasons for this unusual behavior in the normal-phase mode were indicated by Zhang et al. on a similar CSP column using the same solvent compositions [23]. Based on their spectroscopic results, Zhang claimed that the competition between the solvent and analyte molecules for the CSP changed with increasing IPA content.

In the range of 0-20% IPA, as IPA% increased, the CSP favored IPA molecules for hydrogen bonding more than the analyte molecules which led to decreased retention times of the analytes. When IPA% approached 60%, the interaction between IPA molecules and CSP reached saturation. Thus as IPA% increased furthermore, the analyte molecules interacted directly with the IPA bound CSP resulting in increased retention times.

2.3.5 Effect of Analyte Structure

Small differences in the structures of the analyte may greatly affect the enantioseparation. Figure 5 illustrates an example involving analytes 6, 9 and 29, which all have hydrogen bonding donor groups. Compared to analyte 6 that has one hydrogen-bonding donor, analyte 9 has an additional carbonyl near the stereogenic center which may introduce additional dipole-dipole interactions with this CSP. Not surprisingly, as seen from the chromatograms, analyte 9 was more retained and better separated compared to analyte 6 in the same mobile phase conditions. Likewise, compared to analyte 9, analyte 29 has one more hydrogen-bonding group and as a result was much longer retained and baseline separated.

2.4 Conclusions

The pentaproline based chiral stationary phase is effective for enantiomeric separation in three mobile phase modes, including the normal-phase mode, polar organic mode, and the reversed-phase mode. In total, 48% of the racemates (94 out of 194) were separated. Overall,

the normal-phase mode produced the most separations. The chiral recognition is mainly based on hydrogen bonding and steric interactions. However, the enantioseparations of analytes incapable of forming hydrogen bonds indicate that other interactions (dipople/dipole, induced dipole/dipole, dispersion forces) may play an important role in the enantioselectivity. The structure of the analytes studied has a great effect on the polyproline stationary phase's separation ability, particularly the position and polarity of the subsituent. Some analytes exhibited an unusual U-shaped retention behavior in the normal-phase mode. Sample loading tests with α-Methyl-9-anthracenemethanol (analyte 7) was performed and 13.2 mg of the analyte could be baseline separated in a single run which indicates the potential application of this CSP for preparative separations.

Acknowledgements

Support of this work by the National Institute of Health, General Medical Science NIH RO1 GM 53825-11 and NIH RO1 GM 63812-01 is gratefully acknowledged.

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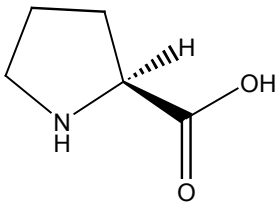

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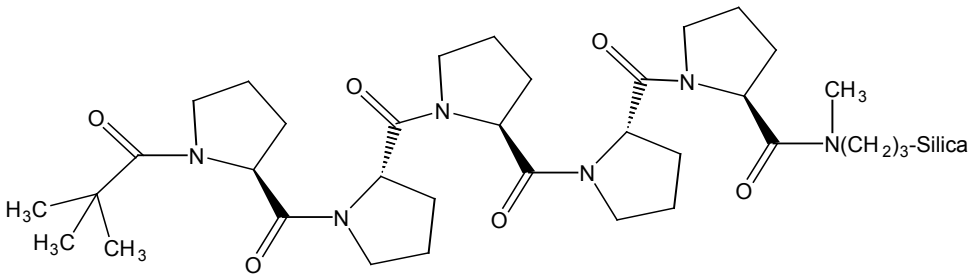


Figure 1. Structure of the stationary phase in this study: Pivalyl-Pro-Pro-Pro-Pro-Pro-MAPS

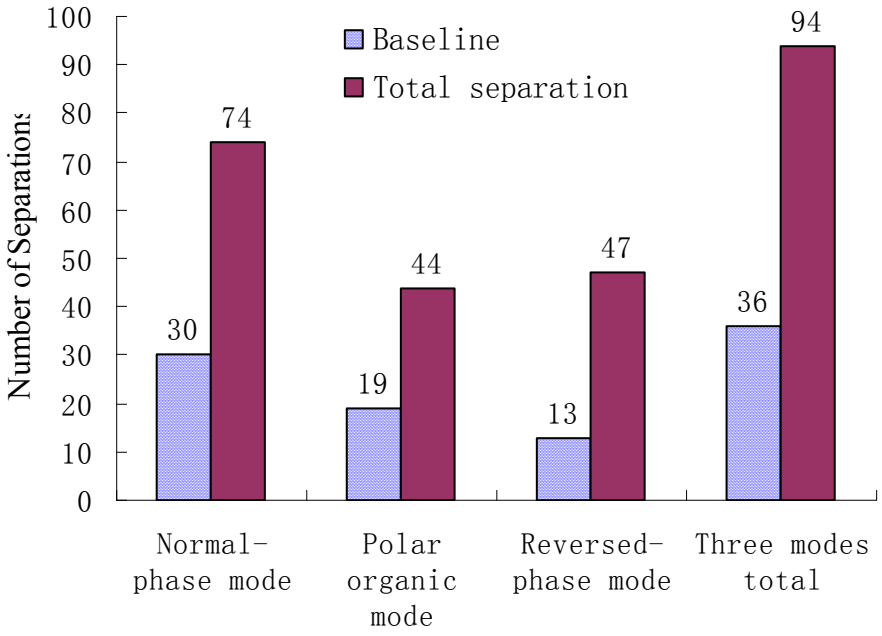


Figure 2. Enantiomeric separation summary in all three modes and the combined results on the pentaproline column. The CSP was packed as 250 mm × 4.6 mm HPLC column. All separations were performed at ambient temperature (~ 23 ºC). Flow rate: 1 mL min^{-1}

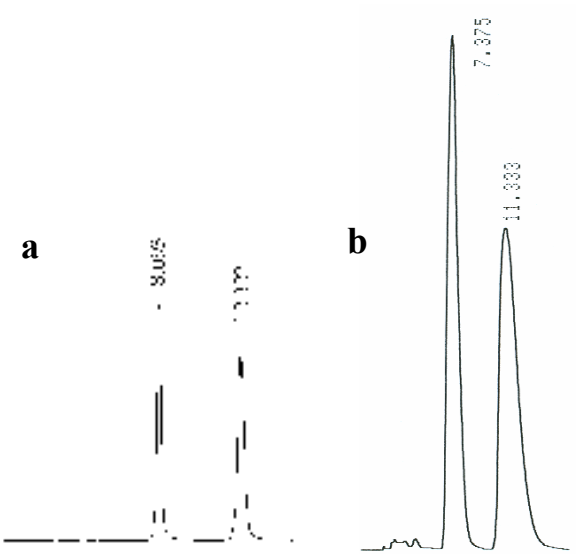


Figure 3. The effect of sample loading on the separation of α-Methyl-9-anthracenemethanol (analyte 7) with (a) 2.5 µg and (b) 13.2 mg of analyte injection. Mobile phases: IPA/Heptane = 30/70. Flow rate: 1 mL min^{-1}

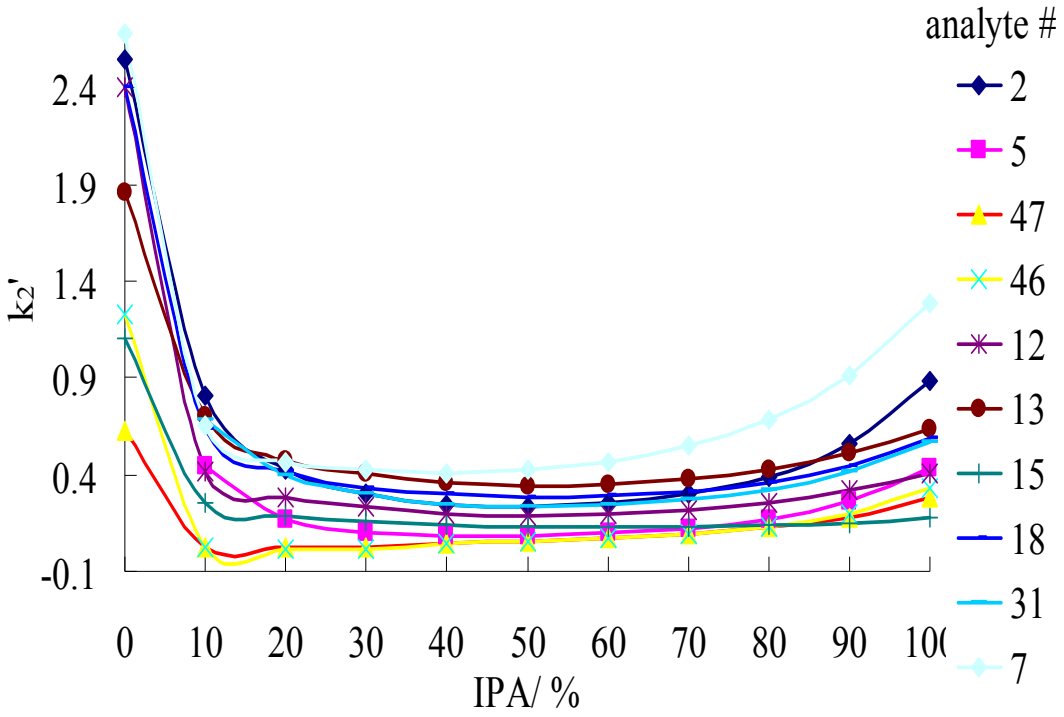


Figure 4. The normal-phase retention behavior of ten analytes. Flow rate: 0.5 mL min^{-1}. UV detection at 254 nm. T = 23 ºC

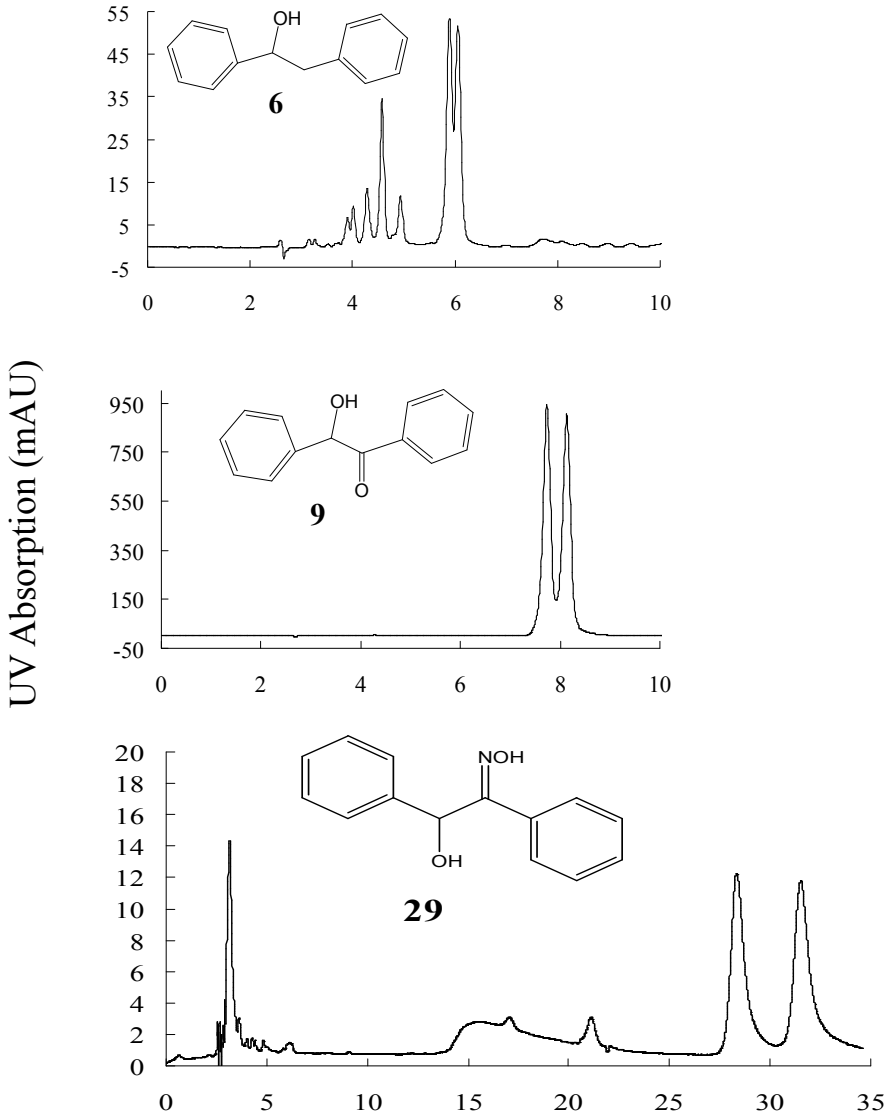


Figure 5. Comparison of the separations for analytes with small differences in structure: analytes 6, 9 and 29. Mobile phase: 20/80 Ethanol/Heptane. Flow rate: 1 mL min^{-1}. UV detection at 254 nm. T = 23 ºC

Table 1. Enantiomeric separations in the normal-phase mode

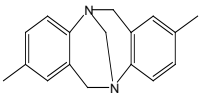
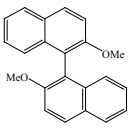
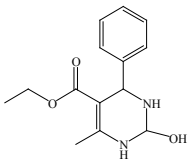
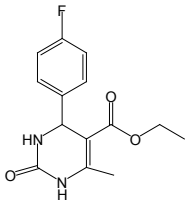
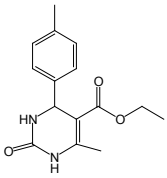
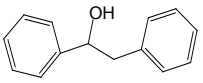
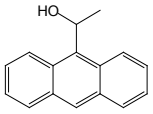
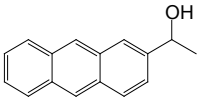
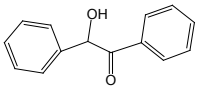
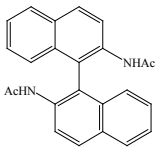
#	Analyte name	Analyte structure	k_1'[a]	α[b]	Rs	Mobile phase[c]
1	Troger's Base		0.48	1.23	1.2	1% IH
2	2,2'-Dimethoxyl-1,1'-binaphthyl		5.84	1.1	0.8	1% IH
3	Ethyl 2-Hydroxy-4-methyl-6-phenyl-1,6-dihydro-5-pyrimidinecarboxylate		0.67	1.6	1.5	60% EH
4	4-(4-Fluoro-phenyl)-6-methyl-2-oxo-1,2,3,4-tetrahydro pyrimidine-5-carboxylic acid ethyl ester		2.88	1.32	1.5	20% IH
5	Ethyl 6-methyl-4-(4-methylphenyl)-2-oxo-1,2,3,4-tetrahydro-5-pyrimidinecarbosylate		5.27	1.55	2.8	10% IH
6	Benzyl phenyl carbinol		8.23	1.11	1.5	3% IH
7	α-Methyl-9-anthracenemethanol		1.62	1.88	6.5	30% IH
8	1-Anthracen-2-yl-ethanol		9.19	1.09	1.2	10% IH
9	Benzoin		6.99	1.17	1.5	10% IH
11	N-2'-Acetylamino-[1,1']binaphthalenyl-2-yl)-acetamide		4.07	1.79	1.6	20% IH

Table 1. Enantiomeric separations in the normal-phase mode (*Continued*)

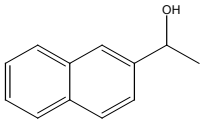
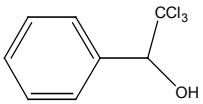
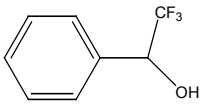
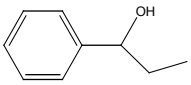
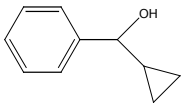
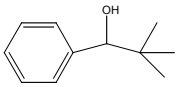
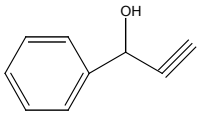
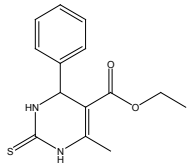
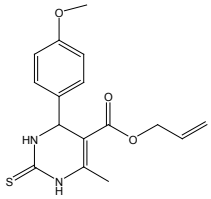
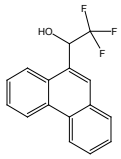
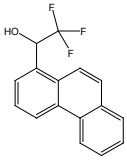
#	Analyte name	Analyte structure	k_1'[a]	α[b]	Rs	Mobile phase[c]
12	α-Methyl-2-naphthalenemethanol		4.56	1.11	1.1	10% IH
13	α-(Trichloromethyl)benzyl alcohol		2.98	1.14	1.4	30% IH
14	α-(Trifluoromethyl)benzyl alcohol		2.69	1.14	1.4	30% IH
15	1-Phenyl-1-propanol		3.74	1.16	1.0	3% IH
16	α-Cyclopropylbenzyl alcohol		6.05	1.07	1.0	3% IH
17	2,2-Dimethyl-1-phenyl-1-propanol		4.65	1.03	0.5	1% IH
18	1-Phenyl-2-propyn-1-ol		5.42	1.04	0.6	30% IH
19	6-Methyl-4-phenyl-2-thioxo-1,2,3,4-tetrahydro-pyrimidine-5-carboxylic acid ethyl ester		5.41	1.60	2.4	30% IH
20	4-(4-Methoxy-phenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydro-pyrimidine-5-carboxylic acid allyl ester		2.02	1.31	1.5	60% EH
21	2,2,2-Trifluoro-1-(9-phenanthryl)ethanol		2.19	1.19	1.5	60% EH
22	2,2,2-Trifluoro-1-(1-phenanthryl)ethanol		2.18	1.19	1.5	60% EH

Table 1. Enantiomeric separations in the normal-phase mode (*Continued*)

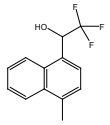
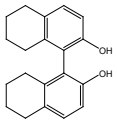
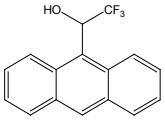
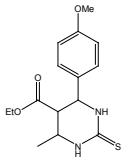
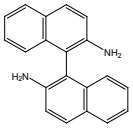
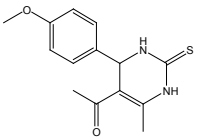
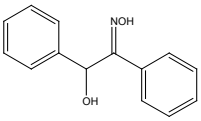
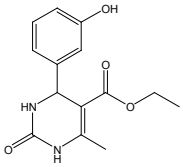
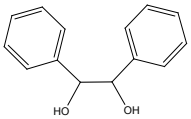
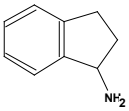
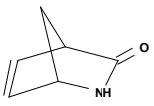
#	Analyte name	Analyte structure	k_1'[a]	α[b]	Rs	Mobile phase[c]
23	2,2,2-Trifluoro-1-(4-methylnaphthalen -1-yl) ethanol		4.08	1.3	3.1	30% IH
24	5,5',6,6',7,7',8,8'-Octahydro(1,1'binaphthalene) -2,2'-diol		4.07	1.46	3.5	60% EH
25	1-(9-Anthryl)-2,2,2-trifluoroethanol		2.58	1.95	5.1	60% EH
26	4-(4-Methoxy-phenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydro-pyrimidine-5-carboxylic acid ethyl ester		2.14	1.54	3.1	60% EH
27	2,2'-Diamino-1,1'-binaphthalene		6.23	1.21	2.3	60% EH
28	1-(2-Mercapto-6-(4-methoxyphenyl)-4-methyl-1,6-dihydropyrimidin-5-yl)-ethanone		2.13	1.54	3.2	60% EH
29	α-Benzoin oxime		4.16	1.23	2.4	60% EH
30	Ethyl 4-(3-hydroxyphenyl)-6-methyl-2-oxo-1,2,3,4-tetrahydro-5-pyrimidine carboxylate		3.10	1.54	2.6	60% EH
31	1,2-Diphenyl-1,2-ethanediol		3.08	1.25	2.3	30% IH
32	1-Aminoindan		0.52	1.15	1.0	1% IH
33	(1S)-(+)-2-Azabicyclo[2.2.1]-hept-5-en-3-one(1R)-(-)-2-Azabicyclo[2.2.1]-hept-5-en-3-one		2.69	1.17	0.7	10% IH

Table 1. Enantiomeric separations in the normal-phase mode (*Continued*)

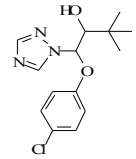
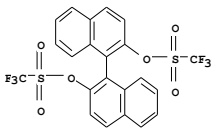
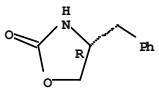
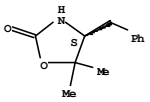
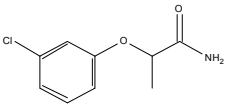
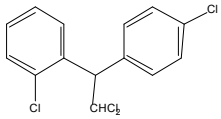
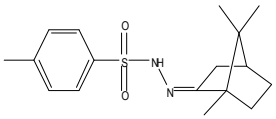
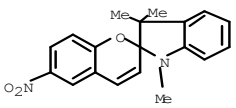
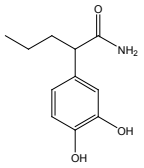
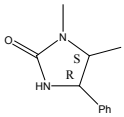
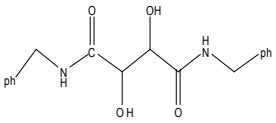
#	Analyte name	Analyte structure	k_1'[a]	α[b]	Rs	Mobile phase[c]
34	Baytan		1.74	1.13	1.2	10% IH
35	(R/S)-(-/+)-1.1'-Bi-2-naphthol bis(trifluoromethanesulfonate)		3.74	1.06	0.5	1% IH
36	(R/S)-(+/-)-4-Benzyl-2-oxazolidinone		8.19	1.17	1.1	10% IH
37	(R/S)-(-/+)-4-Benzyl-5, 5–dimethyl -2-oxazolidinone		8.41	1.10	0.9	3% IH
38	2-(3-chlorophenoxy)propionamide		2.07	1.17	1.5	20% IH
39	1-(2-chlorophenyl)-1-(4-chlorophenyl)-2,2-dichloroethane		3.14	1.11	1.3	1% IH
40	(±)Camphor p-tosyl hydrazon		5.11	1.15	1.4	10% IH
41	1',3'-Dihydro-1',3',3'-trimethyl-6-nitrospiro[2H-1-benzopyran-2,2'-(2H)-indole]		3.94	1.05	0.3	1% IH
42	DL-3,4-dihydroxyphenyl-alfa-propylacetamide		5.17	1.23	1.7	60% EH
43	1,5-Dimethyl-4-phenyl-2-imidazolidinone		5.77	1.07	0.5	3% IH
44	N,N'-Dibenzyl-tartramide		0.94	2.23	1.9	60% IH

Table 1. Enantiomeric separations in the normal-phase mode (*Continued*)

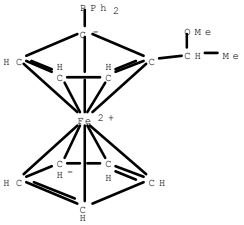
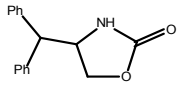
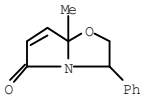
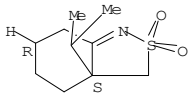
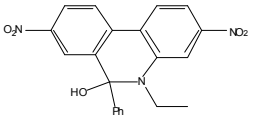
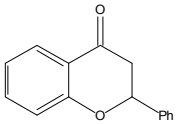
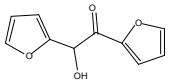
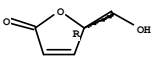
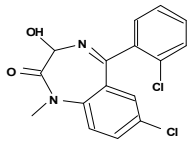
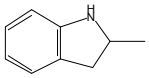
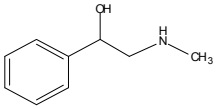
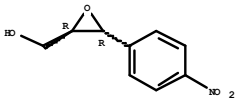
#	Analyte name	Analyte structure	k_1'[a]	α[b]	Rs	Mobile phase[c]
45	Ferrocene, 1-(diphenylphosphino)-2-[(1R)-1-methoxyethyl]-(R)-(9Cl)		2.94	1.11	0.7	1% IH
46	4-(Diphenylmethyl)-2-oxazolidinone		4.76	1.24	1.5	10% IH
47	2,3-Dihydro-7a-methyl-3-phenylpyrrolo[2,1-b]oxazol-5(7aH)-one		4.23	1.04	0.6	1% IH
48	10,10-Dimethyl-3-thia-4-azatricyclo[5.2.1.01,5]dec-4-ene-3,3-dioxide		1.83	4.94	3.4	30% IH
49	5-Ethyl-5,6-dihydro-3,8-dinitro-6-phenyl-6-phenanthridinol		5.97	1.05	0.7	1% IH
50	Flavanone		2.86	1.06	0.3	1% IH
51	Furoin		1.50	1.12	1.4	60% EH
52	(R/S)-(+/-)-5-hydroxymethyl-2(5H)-furanone		1.34	1.12	0.8	30% IH
53	Lormetazepam		1.05	1.25	0.8	60% IH
54	2-Methyllindoline		2.30	1.19	1.5	1% IH
55	α-(Methylaminomethyl)benzyl alcohol		1.27	1.18	1.5	1% IH
56	(2S/R,3S/R)-3-(4-Nitrophenyl) glycidol		2.18	0.46	1.1	60% EH

Table 1. Enantiomeric separations in the normal-phase mode (*Continued*)

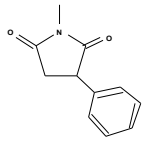
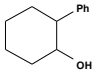
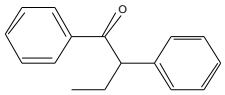
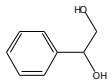
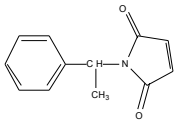
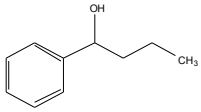
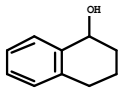
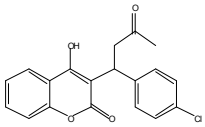
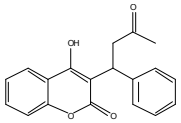
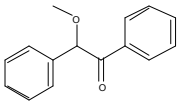
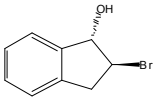
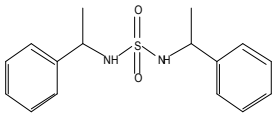
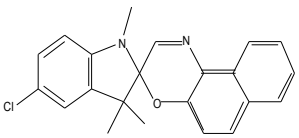
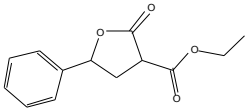
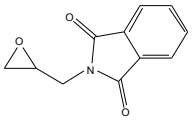
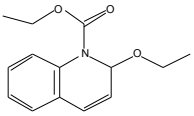
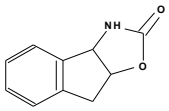
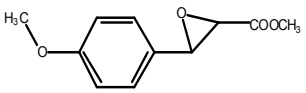
#	Analyte name	Analyte structure	k_1'[a]	α[b]	Rs	Mobile phase[c]
57	Phensuximide		3.96	1.21	1.4	10% IH
58	trans-2-Phenyl-1-cyclohexanol		2.65	1.08	1.3	1% IH
59	2-phenylbutyrophenone		0.56	1.24	0.9	1% IH
60	1-Phenyl-1,2-ethanediol		3.20	1.1	1.5	30% IH
61	N-(1-Phenylethyl) maleimide		2.66	1.5	1.7	10% IH
62	1-Phenyl-1-butanol		6.81	1.08	1.6	1% IH
63	1,2,3,4-Tetrahydro-1-naphthol		8.16	1.02	0.4	1% IH
64	3-(α-Acetonyl-4-chlorobenzyl)-4-hydroxycoumarin		4.54	1.04	0.4	1% IH
65	Warfarin 3-(α-Acetonylbenzyl)-4-hydroxycoumarin		4.45	1.06	0.7	1% IH
66	Benzoin methyl ether		1.90	1.08	0.5	1% IH
67	trans-2-bromo-1-indanol		5.69	1.08	1.4	10% IH
68	(R,R)or(S,S)-N,N'-Bis(2-methylbenzyl)sulfamide		2.79	1.14	1.1	30% IH

Table 1. Enantiomeric separations in the normal-phase mode (*Continued*)

#	Analyte name	Analyte structure	k_1'[a]	α[b]	Rs	Mobile phase[c]
69	5-Chloro-1,3-dihydro-1,3,3-trimethylspiro[2H-indole-2,3'-(3H)naphth[2,1-b](1,4)oxazine]		0.69	1.25	0.5	1% IH
70	2-Carbethoxy-gamma-phenyl-gamma-butyrolactone		7.03	1.06	0.9	3% IH
71	N-(2,3-Epoxypropyl)-phthalimide		5.68	1.04	0.5	1% IH
72	N-Ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline		0.61	1.06	0.2	1% IH
73	(3a(R,S)-cis)-(±)-3,3a,8,8a-Tetrahydro-2H-indeno[1,2-d]oxazol-2-one		5.26	1.22	1.3	30% IH
74	Methyl trans-3-(4-methoxyphenyl) glycidate		4.00	1.05	0.6	1% IH

[a] Retention factor of the least retained enantiomer. [b] Separation factor. [c] Mobile phases: I for IPA, H for heptane, E for ethanol, 1% IH for 1% I in H.

Table 2. Enantiomeric separations in the polar orgainc-phase mode

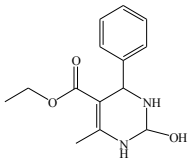
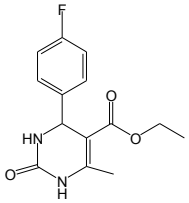
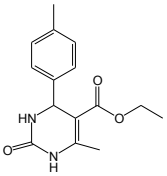
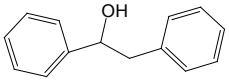
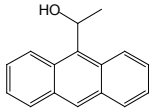
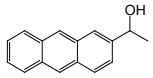
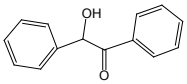
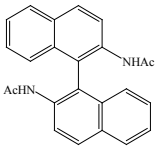
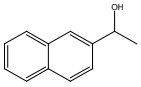
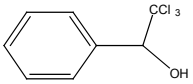
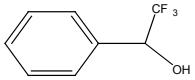
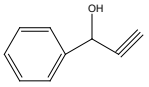
#	Analyte name	Analyte structure	k_1'[a]	α[b]	Rs	Mobile phase[c]
3	Ethyl 2-Hydroxy-4-methyl-6-phenyl-1,6-dihydro-5-pyrimidinecarboxylate		0.73	1.45	1.9	95% AM
4	4-(4-Fluoro-phenyl)-6-methyl-2-oxo-1,2,3,4-tetrahydro pyrimidine-5-carboxylic acid ethyl ester		0.68	1.39	1.5	95% AM
5	Ethyl 6-methyl-4-(4-methylphenyl)-2-oxo-1,2,3,4-tetrahydro-5-pyrimidinecarbosylate		0.65	1.44	1.5	95% AM
6	Benzyl phenyl carbinol		0.31	1.11	0.4	95% AM
7	α-Methyl-9-anthracenemethanol		0.93	1.97	3.8	95% AM
8	1-Anthracen-2-yl-ethanol		0.97	1.08	0.8	95% AM
9	Benzoin		0.26	1.11	0.3	95% AM
11	N-2'-Acetylamino-[1,1']binaphthalenyl-2-yl)-acetamide		0.13	2.75	1.5	95% AM
12	α-Methyl-2-naphthalenemethanol		0.56	1.08	0.7	95% AM
13	α-(Trichloromethyl)benzyl alcohol		0.78	1.16	1.4	95% AM
14	α-(Trifluoromethyl)benzyl alcohol		0.48	1.17	1.3	95% AM
18	1-Phenyl-2-propyn-1-ol		0.69	1.06	0.4	95% AM

Table 2. Enantiomeric separations in the polar orgainc-phase mode (*Continued*)

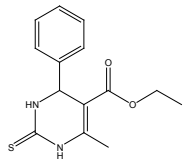
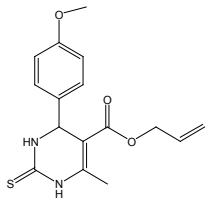
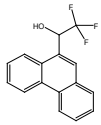
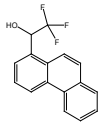
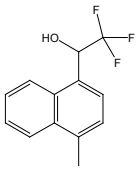
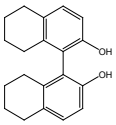
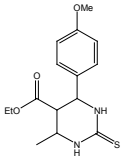
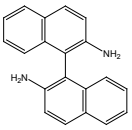
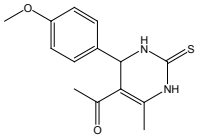
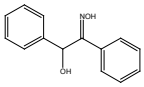
#	Analyte name	Analyte structure	k_1'[a]	α[b]	Rs	Mobile phase[c]
19	6-Methyl-4-phenyl-2-thioxo-1,2,3,4-tetrahydro-pyrimidine-5-carboxylic acid ethyl ester		0.84	1.55	2.0	95% AM
20	4-(4-Methoxy-phenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydro-pyrimidine-5-carboxylic acid allyl ester		0.83	1.43	1.5	95% AM
21	2,2,2-Trifluoro-1-(9-phenanthryl)ethanol		1.74	1.19	1.5	95% AM
22	2,2,2-Trifluoro-1-(1-phenanthryl)ethanol		1.74	1.19	1.5	95% AM
23	2,2,2-Trifluoro-1-(4-methylnaphthalen -1-yl) ethanol		0.84	1.3	1.5	95% AM
24	5,5',6,6',7,7',8,8'-Octahydro(1,1'binaphthalene)-2,2'-diol		4.84	1.26	3.7	95% AM
26	4-(4-Methoxy-phenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydro-pyrimidine-5-carboxylic acid ethyl ester		0.83	1.58	2.3	95% AM
27	2,2'-Diamino-1,1'-binaphthalene		1.28	1.13	1.4	95% AM
28	1-(2-Mercapto-6-(4-methoxyphenyl)-4-methyl-1,6-dihydropyrimidin-5-yl)-ethanone		0.83	1.57	2.7	95% AM
29	α-Benzoin oxime		2.98	1.25	2.9	95% AM

Table 2. Enantiomeric separations in the polar orgainc-phase mode (*Continued*)

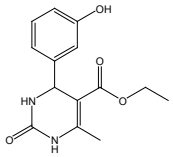
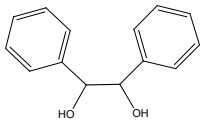
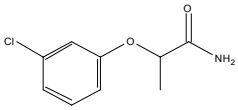
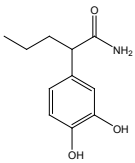
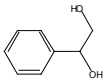
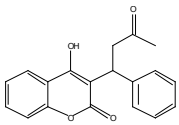
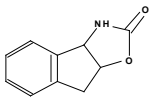
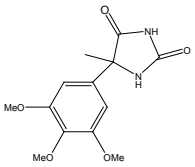
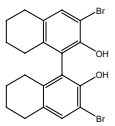
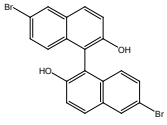
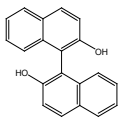
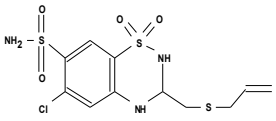
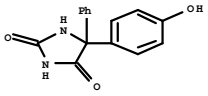
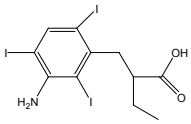
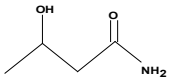
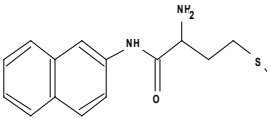
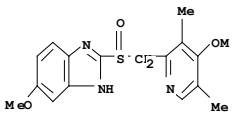
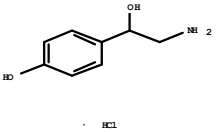
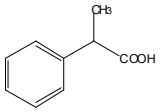
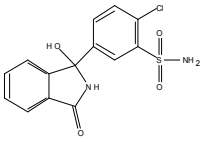
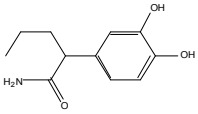
#	Analyte name	Analyte structure	k_1'[a]	α[b]	Rs	Mobile phase[c]
30	Ethyl 4-(3-hydroxyphenyl)-6-methyl-2-oxo-1,2,3,4-tetrahydro-5-pyrimidinecarboxylate		1.35	1.45	1.5	60% AM
31	1,2-Diphenyl-1,2-ethanediol		0.65	1.28	1.5	95% AM
38	2-(3-chlorophenoxy)propionamide		0.17	1.22	0.9	95% AM
42	DL-3,4-dihydroxyphenyl-alfa-propylacetamide		0.64	1.21	1.4	60% AM
60	1-Phenyl-1,2-ethanediol		0.71	1.13	1.3	95% AM
65	Warfarin 3-(α-Acetonylbenzyl)-4-hydroxycoumarin		3.62	1.20	0.4	95% AM
73	(3a(R,S)-cis)-(±)-3,3a,8,8a-Tetrahydro-2H-indeno[1,2-d]oxazol-2-one		0.33	1.26	1.3	95% AM
75	5-Methyl-5-(3,4,5-trimethoxyphenyl)hydantoin		1.221	1.12	1.0	95% AM
76	3,3'-Dibromo-5,5',6,6',7,7',8,8'-octahydro(1,1'-binaphthalene)-2,2'-diol		1.09	1.14	1.3	95% AM
77	6,6'-Dibromo-1,1'-bi-2-naphthol		7.47	1.25	3.1	60% AM
78	1,1'-Bi-2-naphthol		3.65	1.34	2.9	60% AM

Table 2. Enantiomeric separations in the polar orgainc-phase mode (*Continued*)

#	Analyte name	Analyte structure	k_1'[a]	α[b]	Rs	Mobile phase[c]
79	Althiazide		6.10	1.11	1.1	95% AM
80	DL-5-(4-hydroxyphenyl)-5-phenylhydantoin		3.02	1.04	0.5	60% AM
82	(3-[3-Amino-2,4,6-triiodophenyl] -2-ethyl-propanoic acid		4.77	1.37	0.8	60% AM
83	Lactamide		0.98	1.08	0.5	95% AM
84	,-Methionine a-Naphthylamide		0.23	1.70	0.6	95% AM
85	Omeprazole		0.58	1.49	1.4	95% AM
86	DL-Octopamine hydrochloride		0.59	1.48	1.5	95% AM
87	2-Phenylpropionic acid		2.84	1.46	O.7	60% AM
88	Chlorthalidone		0.87	1.04	0.3	60% AM
89	3,4-dihydroxyphenyl-2-propylace tamide		0.64	1.21	1.4	60% AM

[a] Retention factor of the least retained enantiomer. [b] Separation factor. [c] Mobile phases: A for acetonitrile, M for methanol. 1% AM for 1% A in M.

Table 3. Enantiomeric separations in the reversed-phase mode

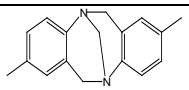
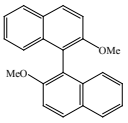
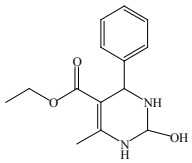
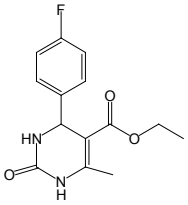
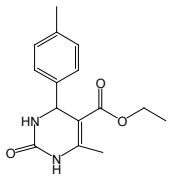
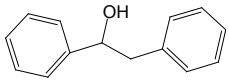
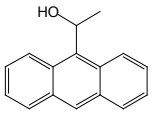
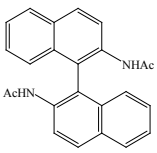
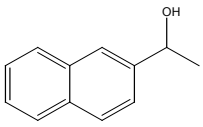
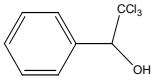
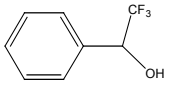
#	Analyte name	Analyte structure	k_1'[a]	α[b]	Rs	Mobile phase[c]
1	Troger's Base		3.09	1.11	1.1	30% MW
2	2,2'-Dimethoxyl-1,1'-binaphthyl		2.58	1.08	1.0	50% MW
3	Ethyl 2-Hydroxy-4-methyl-6-phenyl-1,6-dihydro-5-pyrimidine carboxylate		1.68	1.37	2.9	30% MW
4	4-(4-Fluoro-phenyl)-6-methyl-2-oxo-1,2,3,4-tetrahydro pyrimidine -5-carboxylic acid ethyl ester		1.76	1.26	1.5	30% MW
5	Ethyl 6-methyl-4-(4-methylphenyl)-2-oxo-1,2,3,4-tetrahydro-5-pyrimidinecarbosylate		2.16	1.38	2.4	30% MW
6	Benzyl phenyl carbinol		2.43	1.06	0.5	10% MW
7	α-Methyl-9-anthracenemethanol		2.08	1.37	1.5	60% MW
11	N-2'-Acetylamino-[1,1']binaphthalenyl-2-yl)-acetamide		3.54	1.17	1.4	30% MW
12	α-Methyl-2-naphthalenemethanol		5.94	1.04	0.6	1% MW
13	α-(Trichloromethyl)benzyl alcohol		3.91	1.05	0.8	30% MW
14	α-(Trifluoromethyl)benzyl alcohol		1.73	1.04	0.3	30% MW

Table 3. Enantiomeric separations in the reversed-phase mode (*Continued*)

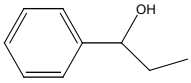
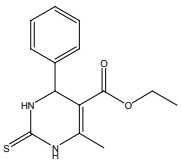
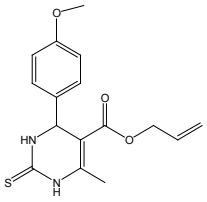
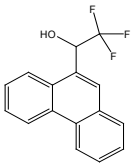
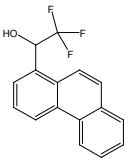
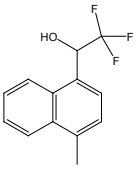
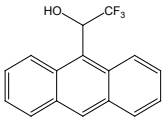
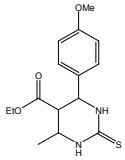
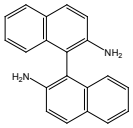
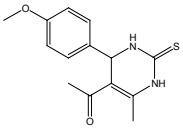
#	Analyte name	Analyte structure	k_1'[a]	α[b]	Rs	Mobile phase[c]
15	1-Phenyl-1-propanol		0.77	2.63	2.3	1% MW
19	6-Methyl-4-phenyl-2-thioxo-1,2,3,4-tetrahydro-pyrimidine-5-carboxylic acid ethyl ester		3.4	1.46	3.6	30% MW
20	4-(4-Methoxy-phenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydro-pyrimidine-5-carboxylic acid allyl ester		5.17	1.34	3.0	30% MW
21	2,2,2-Trifluoro-1-(9-phenanthryl)ethanol		3.88	1.09	1.3	60% MW
22	2,2,2-Trifluoro-1-(1-phenanthryl)ethanol		3.88	1.09	1.3	60% MW
23	2,2,2-Trifluoro-1-(4-methylnaphthalen -1-yl) ethanol		1.68	1.10	1.1	60% MW
25	1-(9-Anthryl)-2,2,2-trifluoroethanol		4.82	1.39	4.3	60% MW
26	4-(4-Methoxy-phenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydro-pyrimidine-5-carboxylic acid ethyl ester		2.54	1.35	3.8	30% MW
27	2,2'-Diamino-1,1'-binaphthalene		3.53	1.09	1.0	60% MW
28	1-(2-Mercapto-6-(4-methoxyphenyl)-4-methyl-1,6-dihydropyrimidin-5-yl)-ethanone		2.57	1.33	3.3	30% MW

Table 3. Enantiomeric separations in the reversed-phase mode (*Continued*)

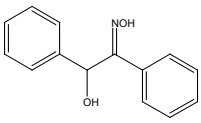
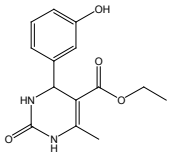
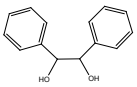
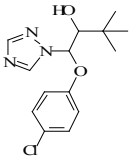
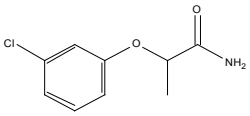
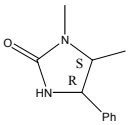
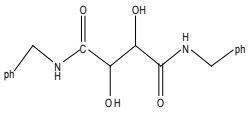
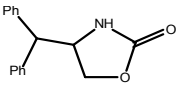
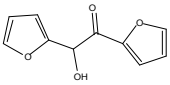
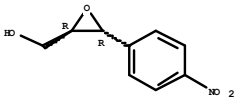
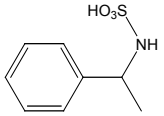
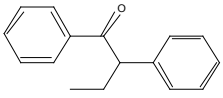
#	Analyte name	Analyte structure	k_1'[a]	α[b]	Rs	Mobile phase[c]
29	α-Benzoin oxime		3.26	1.06	0.6	10% MW
30	Ethyl 4-(3-hydroxyphenyl)-6-methyl -2-oxo-1,2,3,4-tetrahydro-5-py rimidinecarboxylate		3.54	1.52	4.2	30% MW
31	1,2-Diphenyl-1,2-ethanediol		1.08	1.10	1.0	10% MW
34	Baytan		2.23	1.09	0.5	1% MW
38	2-(3-chlorophenoxy)propiona mide		1.68	1.07	0.7	1% MW
43	1,5-Dimethyl-4-phenyl-2-imid azolidinone		1.10	1.05	0.5	1% MW
44	N,N'-Dibenzyl-tartramide		0.89	1.24	1.4	1% MW
46	4-(Diphenylmethyl)-2-oxazoli dinone		2.78	1.07	0.6	1% MW
51	Furoin		1.20	1.06	0.5	1% MW
56	(2S/R,3S/R)-3-(4- Nitrophenyl) glycidol		0.85	1.05	0.4	30% MW
57	(±)-Phenethylsulfamic acid		0.10	1.32	0.4	10% MW
59	2-phenylbutyrophenone		6.00	1.04	0.5	30% MW

Table 3. Enantiomeric separations in the reversed-phase mode (*Continued*)

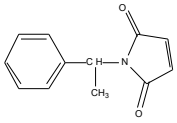
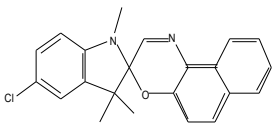
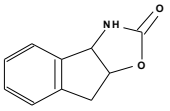
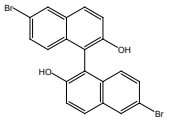
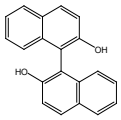
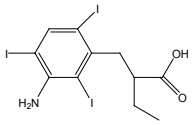
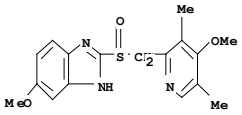
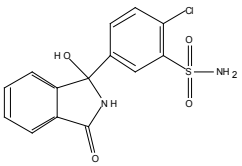
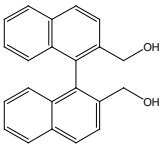
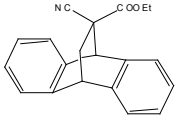
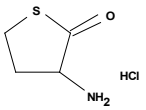
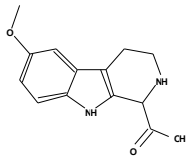
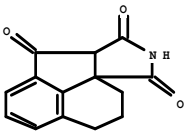
#	Analyte name	Analyte structure	k_1'[a]	α[b]	Rs	Mobile phase[c]
61	N-(1-Phenylethyl) maleimide		0.73	1.05	0.4	1% MW
69	5-Chloro-1,3-dihydro-1,3,3-trimethylspiro[2H-indole-2,3'-(3H)naphth[2,1-b](1,4)oxazine]		2.89	1.04	0.3	60% MW
73	(3a(R,S)-cis)-(±)-3,3a,8,8a-Tetrahydro-2H-indeno[1,2-d]oxazol-2-one		2.19	1.05	0.6	1% MW
77	6,6'-Dibromo-1,1'-bi-2-naphthol		7.84	1.16	2.6	90% MW
78	1,1'-Bi-2-naphthol		5.50	1.16	2.1	90% MW
82	(3-[3-Amino-2,4,6-triiodophenyl]-2-ethyl-propanoic acid		5.21	1.07	0.3	90% MW
85	Omeprazole		3.31	1.06	0.4	10% MW
88	Chlorthalidone		8.50	1.04	0.3	10% MW
90	1,1'-Binaphthyl-2,2'-dimethanol		2.59	1.08	1.0	50% MW
91	Ethyl 11-cyano-9,10-dihydro-endo-9,10-ethanoanthracene-11-carboxylate		9.31	1.10	0.9	10% MW
92	DL-Homocysteine thiolactone hydrochloride		0.08	1.34	0.3	1% MW

Table 3. Enantiomeric separations in the reversed-phase mode (*Continued*)

#	Analyte name	Analyte structure	k_1'[a]	α[b]	Rs	Mobile phase[c]
93	6-Methoxy-1,2,3,4-tetrahydro-9H-pyrido[3,4-b]indole-1-carboxylic acid		1.67	1.11	0.7	1%MW
94	3a,4,5,6-Tetrahydro-succininido[3,4-b]acenaphthen-10-one		2.42	1.04	0.4	1%MW

[a] Retention factor of the least retained enantiomer. [b] Separation factor. [c] Mobile phases: M for methanol, W for water, 1% MW for 1% M in W.

CHAPTER 3

Enantioseparations of Furan Devatives, Fused Polycles and Isochromenes by Cyclodextrin-Modified Micellar Capillary Electrophoresis

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Electrophoresis 26 (2005) 4164-4171

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Journal of Liquid Chromatography and Related Technologies 31 (2008) 2035-2052

Abstract

The enantiomeric separations of highly hydrophobic furan derivatives, polycycles and isochromenes were performed and optimized using cyclodextrin-modified micellar capillary electrophoresis. The most effective chiral selector for the enantiomeric separation of these analytes was hydroxypropyl-γ-cyclodextrin. The effects of cyclodextrin and sodium dodecyl sulfate concentration and organic modifier were examined in order to optimize the separation conditions. The ratio of cyclodextrin to surfactant concentration affected the enantiomeric separation significantly, with increases in the derivatized cyclodextrin concentration generally enhancing resolution. Addition of an organic solvent modifier to the run buffer served to increase the analytes' solubility and enhance the separation efficiency. A highly acidic pH was necessary to effectively suppress the electroosmotic flow when operating in the reverse polarity mode.

3.1 Introduction

Furans (Figure 1), one of the representative five-membered ring heterocycles, can be found in a variety of natural products and pharmaceuticals, including antibiotics, anti-leukemic agents, flavor and fragrance compounds, and insecticides [1-3]. Furan derivatives are important not only because they are known to be pharmaceutically active, but also because of their role as versatile intermediates in many synthetic processes [4-8]. Only a few enantiomers of furan derivatives have been separated using CE and cyclodextrin additives in the normal polarity mode [9]. In addition, some furan enantiomers have been separated by CE with a 1% (w/v) chiral polymeric surfactant as the chiral selector [10]. However, these chiral furan derivatives were relatively soluble in aqueous solution and could be separated without the addition of an organic modifier in the run buffer.

Fused polycycles (Figure 1) exist widely in the natural world. Dipuupehetriol, a fused polycycle, has been found in a Verongid sponge [11]. Aureol and its derivatives have been isolated from the Caribbean sponge *Smenospongia aurea* [12]. In addition, fused polycycles are known to possess beneficial therapeutic activities [13-15]. Fluoranthene-PAH exhibits environmental prevalence, and mutagenic and carcinogenic activities [16-19].

Isochromenes derivatives (Figure 2) make up an important class of heterocycles because of their biological activity including antibiotic and anti-tumor properties [20-25]. For example, isochromene carboxamides exhibited excellent activity against human ovarian cancer cell line SKOV3 [26]. Several pyrano-isochromenes displayed *in vitro* selective

cytotoxicity against human lung cancer cell line A549 and liver cancer cell line Bel7402 [27]. In addtion, isochromene derivatives are versatile intermediates in synthesis of more complex or important compounds [28-32].

Recently, Yao, Yue, Huang, Della Cà, Larock and co-workers have synthesized a series of new highly hydrophobic, chiral furans, fused polycycles and isochromenes [33-35]. Often different enantiomers of a compound have different biological properties. Thus, the enantiomeric separation of these compounds and subsequent evaluation of their properties are often desirable.

These highly hydrophobic compounds are difficult to separate in the traditional Capillary Zone Electrophoresis (CZE) mode due to the aqueous nature of the method. Cyclodextrin-modified micellar capillary electrophoresis (CD-MCE) was therefore utilized to separate these neutral and highly hydrophobic compounds [36, 37]. First reported in the early 1990s [38, 39], CD-MCE allows the separation of hydrophobic analytes *via* a two-pseudophase system: charged micelles form a pseudophase to enhance the solubility of the neutral hydrophobic analytes, while cyclodextrins form a pseudophase with a different mobility from the micelles to provide enantioselectivity. In order to achieve the separation, the enantiomers must differ in their association with the two pseudophases. To our knowledge, no other CE enantioseparations of these specific furan derivatives, polycyles and isochromenes have been reported.

3.2 Experimental

3.2.1 Chemicals

The chiral furan derivatives, polycycles and isochromenes used in this study were synthesized as reported previously, and their structures were confirmed by ^{1}H-NMR and ^{13}C-NMR spectroscopy [33-35]. Hydroxypropyl-β-cyclodextrin (HP-β-CD) and hydroxypropyl-γ-cyclodextrin (HP-γ-CD) were acquired from Aldrich Chemical Company (Milwaukee, WI, USA), with degrees of substititution of 0.8 and 0.6 respectively. Sodium dodecyl sulfate (SDS), sodium phosphate, sodium hydroxide, 85% phosphoric acid and acetonitrile were all purchased from Fisher Scientific (St. Louis, MO, USA).

3.2.2 Methods

Purchased from Polymicro Technologies (Phoenix, AZ, USA), the bare fused-silica capillaries used in these experiments were 37-40 long (30 cm to the detector), with inner diameters of 50 μm and outer diameters of 358 μm. The capillaries were conditioned before their first use by rinsing with 1 M sodium hydroxide for 5 mins, water for 5 mins, sodium hydroxide for 1 min and finally water for 1 min. Between each run, 1 min phosphoric acid, sodium hydroxide, water, and run buffer rinses were performed. Buffer solutions of 5 mM sodium phosphate buffer were made from deionized water, and adjusted to a pH 2.5 with 85% phosphoric acid, followed by the addition of ACN. Finally, SDS and the CD were dissolved in the buffer solution. This run buffer solution was freshly prepared in order to

prevent the slow hydrolysis of SDS. Samples were prepared by dissolving in ACN. All buffer and sample solutions were sonicated for 5 minutes prior to their first run. The capillary was maintained at a temperature of 25ºC. All separations were performed in the reverse polarity mode with an applied voltage of -20 kV. Data analysis was done with Beckman System Gold software. The reproducibility of the analytes' migration time was 3-8% RSD.

For furan derivatives, the separations were performed on a Beckman P/ACE 2100 (Fullerton, CA, USA) and detection was accomplished by UV absorbance at 214 nm. Samples were injected for 5 seconds via hydrodynamic pressure of 0.5 psi and the run buffer contained 20% ACN. The separations of the polycycles were performed on a Beckman P/ACE 5000 (Fullerton, CA, USA) and detection was achieved by UV absorbance at 254 nm. Samples were injected for 2 seconds via hydrodynamic pressure of 0.5 psi and the run buffer contained 10% ACN. The separations of isochromenes were carried on a Beckman P/ACE MDQ with a photodiode array detector (Fullerton, CA, USA). Samples were injected for 5 seconds via hydrodynamic pressure of 0.5 psi and the run buffer contained 20% ACN.

3.3 Results and Discussion

3.3.1 Effect of Different Types of Cyclodextrins

The negatively charged CDs sulfated-β-CD and carboxymethyl acid β-CD were initially investigated as chiral CE run buffer additives. Without the use of organic modifiers, no enantioseparations with these CDs were achieved in either the normal or reverse polarity

modes. This result is likely due to the fact that CDs solublize these highly hydrophobic analytes. Therefore the analytes reside almost completely within the CD cavity, and lack the required equilibrium between the CD and the run buffer for a separation to occur. However, addition of organic modifier (up to 20% ACN or MeOH) also did not produce any separations, indicating that these CDs do not provide sufficient selectivity to separate these enantiomers. Thus, CD-MCE was considered to be necessary to separate these highly hydrophobic neutral compounds.

HP-β-CD and HP-γ-CD were chosen as potential chiral selectors and successfully separated many of these analytes. These neutral cyclodextrin derivatives are promising for CD-MCE, because they provide these analytes an enantioselective environment with very different mobility than the negatively charged micelles alone. In addition, their high water solubility allows them to be used at high concentrations. Separation conditions were optimized by taking into account resolution, efficiency and peak shape as will be discussed in the following section. The respective electropherograms for the furan derivatives, polycycles and isochromenes are summarized in Tables 1, 2 and 3. In total, of the 34 synthetic compounds, 28 were separated using HP-γ-CD, and the rest 6 compounds were separated by HP-β-CD. Overall, HP-γ-CD produced good separations, because it has a cavity large enough to allow these relatively large compounds to bind to the CDs and form inclusion complexes.

3.3.2 Effect of Cyclodextrin and SDS Concentration

The absolute concentrations and the ratio of the cyclodextrin to SDS had a great effect on the enantiomeric separation of all 34 compounds studied. In general, at a constant SDS concentration, increased resolution but longer migration times were obtained when the concentration of the hydroxypropyl-substituted cyclodextrin was increased. It was also found that an increasing level of CD at a constant SDS concentration eventually resulted in a reversal of the analyte's migration direction in the reverse polarity mode. On the contrary, increasing the SDS concentration at a constant CD concentration decreased the resolution and reduced the migration time. Maintaining the ratio of CD and SDS concentrations constant, and increasing the concentration of both additives increased the resolution. However, an increase in the migration time was also observed. Figure 3 and 4 give brief illustrations of this point. It is known that chiral selectivity can be manipulated by changing the relative concentration of the chiral selector and surfactant, and thus changing the residence time that an analyte spends with each additive [39]. The optimized concentration and ratio of the surfactant and cyclodextrin derivatives are given in Tables 1, 2 and 3.

3.3.3 Effect of Organic Modifier and Run Buffer pH

A pH of 2.5 was chosen to suppress the electroosmotic flow (EOF) so that the EOF was nearly zero. This allowed the use of a high CD concentration without reversing the migration direction of the analyte. The data in Tables 1, 2 and 3 indicate that a relatively high

CD concentration was necessary for most enantioseparations.

The primary effect of an organic modifier on separation of the chiral furan derivatives, polycycles and isochromenes is to increase the analytes' solubility in the run buffer. These synthetic compounds are very hydrophobic and tend to precipitate out of solution during electrophoresis. Acetonitrile (ACN) was chosen over methanol (MeOH) because the analytes were much more soluble in ACN than in MeOH, thus providing much stronger signals.

Although micellar capillary electrophoresis is particularly useful for highly hydrophobic analytes, it has been observed that systems utilizing pseudophases sometimes have a lower efficiency than traditional capillary zone electrophoresis. This effect may be due to both a thermal effect [40] and the slow mass transfer of the analytes between the micelle and the analyte-cyclodextrin complex [41]. Asymmetrical peaks, which were observed in Table 1, are often seen for solutes that are poorly soluble or insoluble in the bulk solution. Hence, the solutes can only reside in the micelle and/or cyclodextrins, and the bulk solution inhibits or forms a barrier to the transfer of solutes between the two pseudophases. The addition of ACN in the run buffer produced increased separation efficiencies and reduced migration times. This may be due to the combined effects of ACN competing with the analyte for the CD cavity and increasing the analyte solubility in the bulk solvent, thus enhancing the mass transfer. However, it was also observed that a slightly higher CD concentration was needed to retain the same resolution when the run buffer contained ACN. Figure 5 gives several examples of how ACN affected the efficiency and migration time in

the separation of chiral polycycles.

3.3.4 Effect of Analyte Structure

Small differences in the structures of these compounds greatly affect their enantioseparations. In the case of the furan derivatives, these analytes have widely varying subsituents on the furan ring. For example, furan 4 has an iodide group and was separated with good resolution, while furan 11 has no iodide substituent and could not be separated. Of the twelve furans separated, half of them had an iodide group on the furan ring. It is well known that organic halides have strong affinities for the CD cavity. Steric repulsion may also play an important role in chiral recognition [42]. Furan 14 could not be separated with HP-β-CD, while furan 12, which differs in structure only by the addition of a phenyl group to its chiral center, was separated using HP-β-CD.

Hydrogen bonding interactions may greatly affect separations. For example, furan 2, which has a carboxylic acid group attached to the furan ring, was base-line separated, while furan 11 showed no enantioselectivity under the same separation conditions. Obviously, a carboxylic acid group at this position is important for the separation.

All of the polycycle analytes have a similar base molecular skeleton, as well as the same location of their stereogenic centers. There are varying degrees and types of substitution elsewhere in the molecule (Figure 1 and Table 2). These differences in the structure produced large effects on the enantiomeric separations, which are illustrated using

the following examples. Polycylce 1 which has a propanoyl substituent at the 6 position could be separated with HP-γ-CD, while polycycle 10 without such a group could not. However, using HP-β-CD in the run buffer produced a separation for polycycle 10. Similarly, the HP-γ-CD chiral additive could separate polycycle 7, but not 12. Again, HP-β-CD could separate polycycle 12. The methylenedioxy group at the 8 and 9 positions of the polycycle ring system greatly enhanced the enantioseparation because of increases in both the selectivity and efficiency. Polycycle 7 with a methylenedioxy group was near base-line separated, while polycycle 12 which has the same base structure produced the worst separation of all twelve polycycles.

Another interesting observation involves polycycles 10 and 12, which are structural isomers. Both could be separated with an HP-β-CD additive, but polycycle 10 provided much greater resolution using the same CD and SDS concentration (due to greater chiral selectivity and inspite of lower efficiency). Polycycles 4 and 5 are also structural isomers (Table 2). The different position of the methylenedioxy substituent had a great effect. Polycycle 4 was base-line separated in a much shorter time than polycycle 5, which had a resolution of only 0.8. This also was due to higher selectivity for polycycle 4.

3.4 Conclusions

Twelve chiral furan derivatives, twelve chiral polycycles and ten isochromenes were separated using either HP-γ-CD or HP-β-CD in conjunction with SDS micelles. In general,

HP-γ-CD separated more of these relatively large hydrophobic molecules presumably due to its larger cavity. An organic modifier played an important role in enhancing the separation by improving the separation efficiency. Without an organic modifier, very slow mass transfer and precipitation of these highly hydrophobic analytes may occur. The addition of ACN to the run buffer also increased the solubility of all analytes and, therefore, improved the UV detectability as well. Higher efficiencies were usually achieved with lower concentrations of SDS and CDs. However, optimized enantiomeric separations sometimes required higher concentrations to be used. The structure of the analytes studied also has a great effect on the CDs' ability to separate these enantiomers, particularly the position and size of the substituents.

Acknowledgements

We gratefully acknowledge the support of this work by the National Institutes of Health, NIH RO1 GM53825-08, NIH RO1 GM53825-11 and NIH RO1 GM070620.

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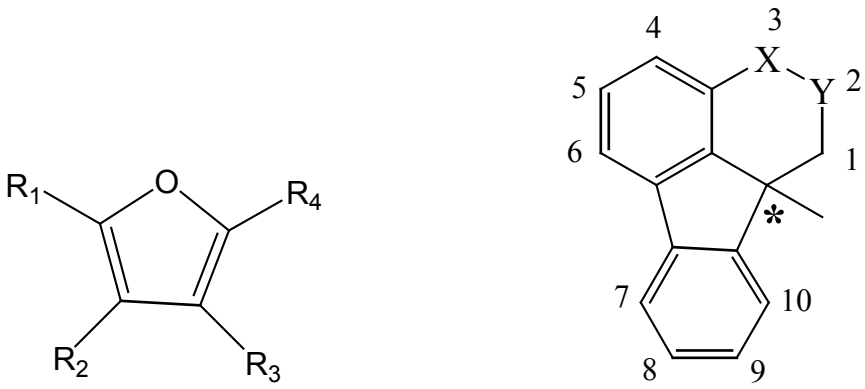


Figure 1. General structures of the chiral furan derivatives (left) and the chiral polycycles (right). **R**(**1-4**) = H or aromatic or aliphatic or bicyclic group; one of which contains a stereogenic center. X, Y = C, O, N. The carbon marked with an asterisk is the stereogenic center.

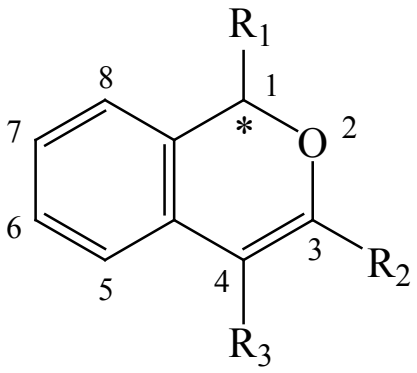
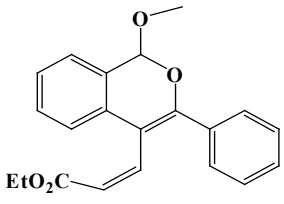


Figure 2. General structures of the chiral isochromene derivatives. **R**$_{(1\text{-}2)}$ = aromatic or aliphatic group; **R$_3$** = bromine, iodine or sulfur or vinylic group. The carbon marked with an asterisk is the stereogenic center.



Ishochromene 4

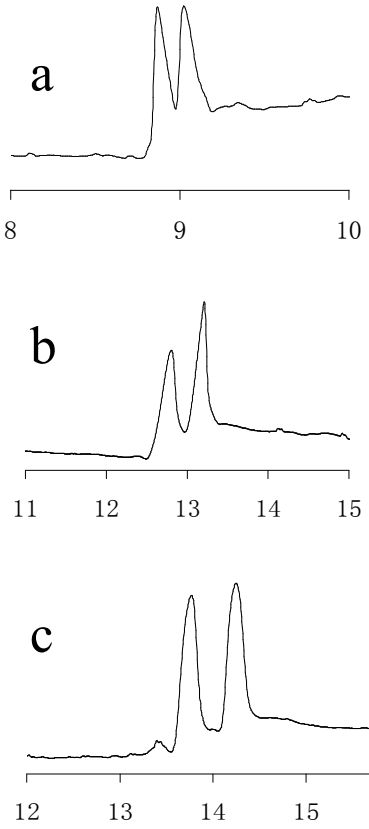
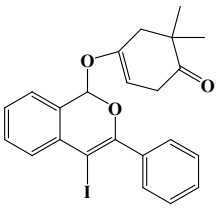


Figure 3. Comparison of the enantiomeric separations of isochromene 4 (Table 3) with constant 150 mM SDS concentration and different selector concentrations in the run buffer. a) 120 mM HP-γ-CD; b) 150 mM HP-γ-CD; c) 165 mM HP-γ-CD.



Isochromene 1

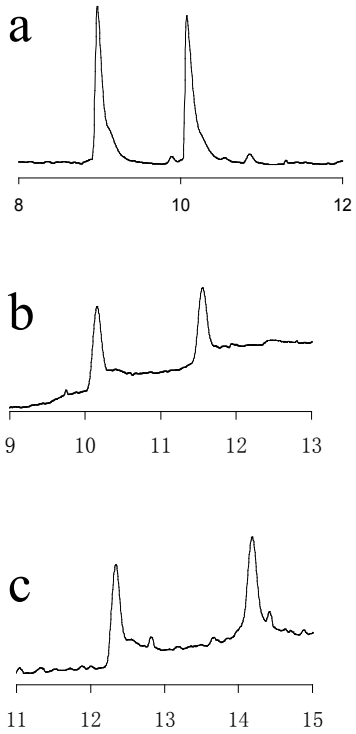


Figure 4. Comparison of the enantiomeric separations of compound 1 (Table 1) at a constant ratio of [SDS]/[HP-γ-CD] = 1.5, but at different absolute concentrations in the run buffer. a) 120 mM SDS, 80 mM HP-γ-CD; b) 150 mM SDS, 100 mM HP-γ-CD; c) 180 mM SDS, 120 mM HP-γ-CD.

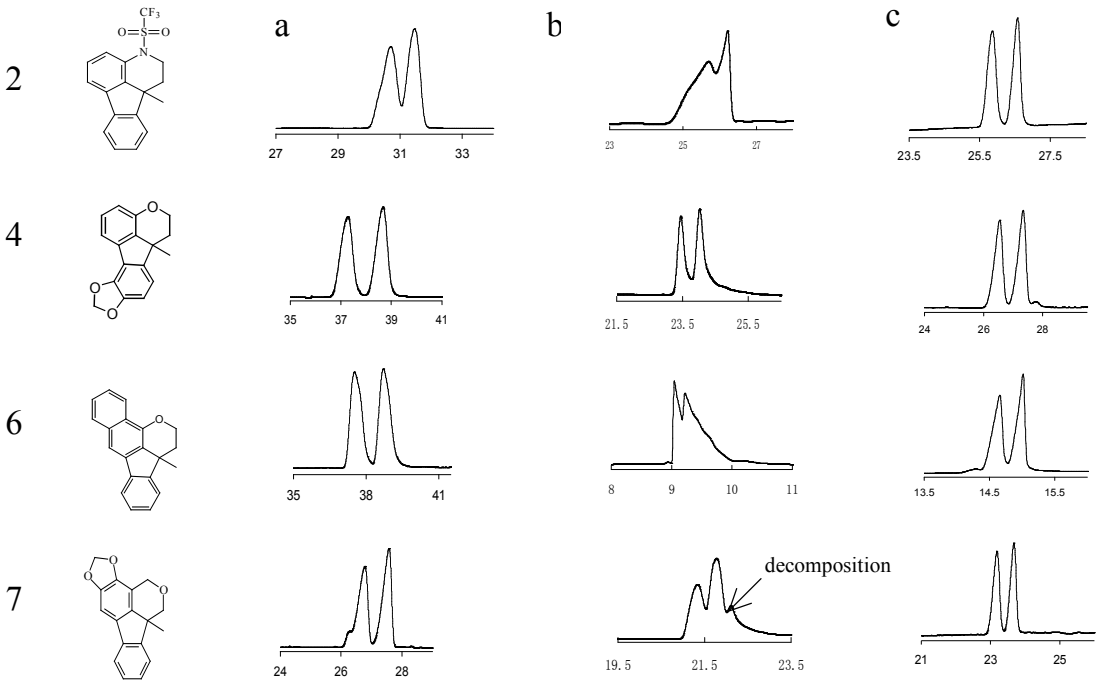


Figure 5. Comparison of separations a) 200 mM SDS, 180 mM HP-γ-CD; b) 200 mM SDS, 180 mM HP-γ-CD with 10% ACN; c) 200 mM SDS, 188~191 mM HP-γ-CD with 10% ACN in the run buffer.

Table 1. Optimized enantioseparations of furan derivatives using HP-γ-CD, HP-β-CD and SDS in 20% ACN run buffer.[a]

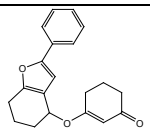
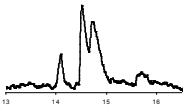
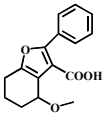
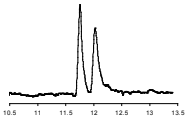
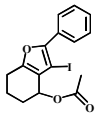
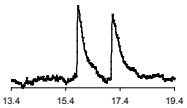
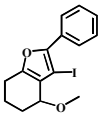
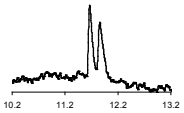
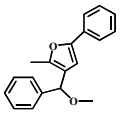
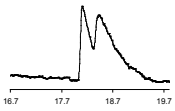
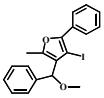
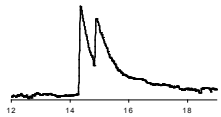
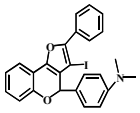
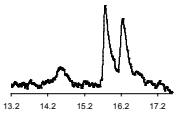
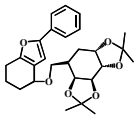
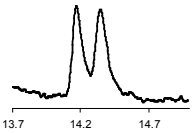
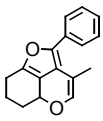
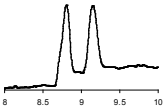
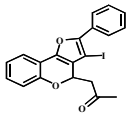
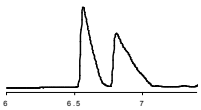
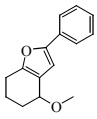
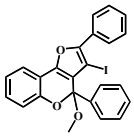
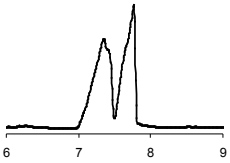
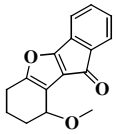
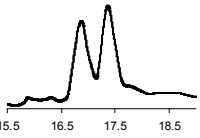
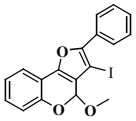
#	Structure	[SDS] (mM)	**[HP-γ-CD]** (mM)	[CD]/ [SDS]	R_s[b]	t_{m2}/t_{m1}[c]	N[d]	Electropherogram (in mins)
1		120	100	0.83	0.7	1.015	60,000	
2		120	100	0.83	1.5	1.023	112,000	
3		150	125	0.83	1.6	1.080	8,000	
4		120	100	0.83	1.1	1.016	85,000	
5		150	150	1.00	0.6	1.019	32,000	
6		200	220	1.10	0.7	1.038	10,000	
7		200	220	1.10	1.2	1.031	34,000	
8	 diastereomers	200	150	0.75	1.1	1.013	125,000	
9		200	175	0.875	1.7	1.040	34,000	
10		200	175	0.875	1.5	1.037	41,000	
11		-	-	-	-	-	-	No separation

Table 1. Optimized enantioseparations of furan derivatives using HP-γ-CD, HP-β-CD and SDS in 20% ACN run buffer.[a] (*Continued*)

#	Structure	[SDS] (mM)	**[HP- β -CD]** (mM)	[CD]/ [SDS]	R_s[b]	t_{m2}/t_{m1}[c]	N[d]	Electropherogram (in mins)
12		200	175	0.875	1.1	1.056	10,000	
13		200	175	0.875	1.0	1.029	19,000	
14		-	-	-	-	-	-	No separation by HP- β -CD

a) All separations performed with 5 mM phosphate buffer pH 2.5, -20 kV, 37 cm capillary (30 cm to detector) with 50 µm I.D.

b) R_s: separation resolution

c) t_m: migration time. EOF mobility could not be determined due to its suppression at pH 2.5, and therefore α values could not be calculated.

d) N: the number of theoretical plates obtained for the first detected peak on a 30 cm length (to the detector) capillary.

Table 2. Optimized enantioseparations of polycycles using HP-γ-CD, HP-β-CD and SDS in 10% ACN run buffer.[a]

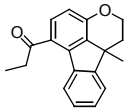
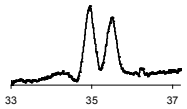
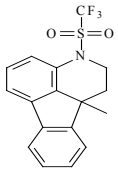
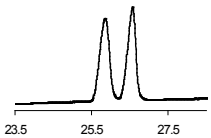
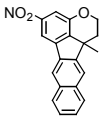
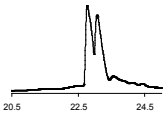
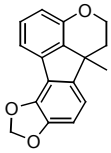
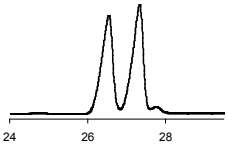
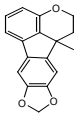
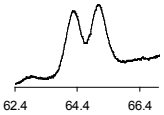
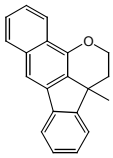
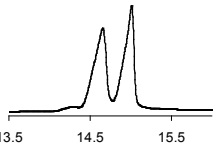
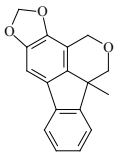
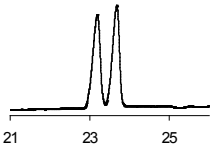
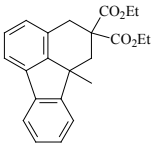
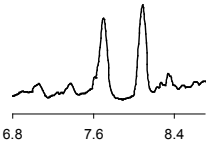
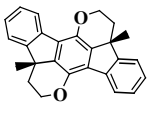
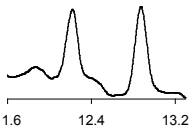
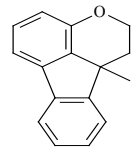
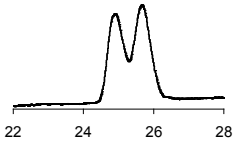
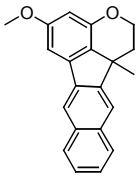
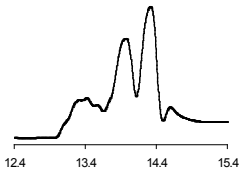
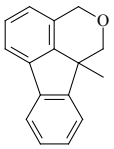
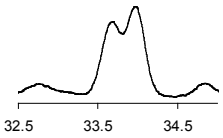
#	Structure	[SDS] (mM)	**[HP-γ-CD]** (mM)	[CD]/ [SDS]	R_s[b]	t_{m2}/t_{m1}[c]	N[d]	Electropherogram (in mins)
1		200	191	0.955	1.1	1.015	85,000	
2		200	188	0.94	1.5	1.027	41,000	
3		200	190	0.95	0.7	1.013	54,000	
4		200	191	0.955	1.5	1.030	37,000	
5		200	210	1.05	0.8	1.013	80,000	
6		200	192	0.96	1.3	1.024	38,000	
7		200	188	0.94	1.4	1.021	63,000	
8		200	160	0.80	2.6	1.049	48,000	
9		200	190	0.95	2.7	1.053	38,000	

Table 2. Optimized enantioseparations of polycycles using HP-γ-CD, HP-β-CD and SDS in 10% ACN run buffer [a] (*Continued*)

#	Structure	[SDS] (mM)	**[HP-β-CD]** (mM)	[CD]/ [SDS]	R_s [b]	t_{m2}/t_{m1} [c]	N [d]	Electropherogram (in mins)
10		200	188	0.94	0.8	1.031	11,000	
11		200	185	0.925	0.9	1.025	17,000	
12		200	188	0.94	0.5	1.009	38,000	

a) All separations performed with 5 mM phosphate buffer pH 2.5, -20 kV, 37 cm capillary (30 cm to detector) with 50 µm I.D.

b) R_s: separation resolution

c) t_m: migration time. EOF mobility could not be determined due to its suppression at pH 2.5, and therefore α values could not be calculated.

d) N: the number of theoretical plates obtained for the first detected peak on a 30 cm length (to the detector) capillary.

Table 3. Optimized enantioseparations of isochromene derivatives using HP-γ-CD, HP-β-CD and SDS in 20% ACN run buffer [a]

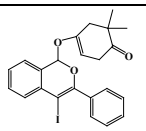
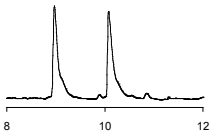
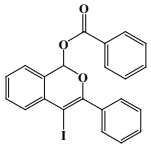
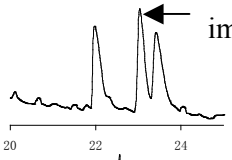
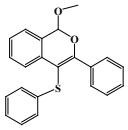
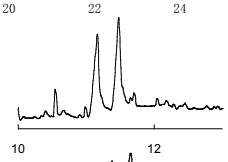
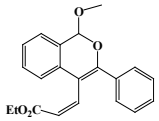
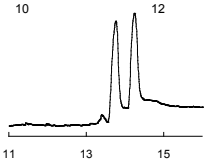
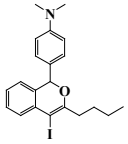
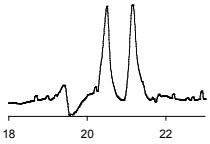
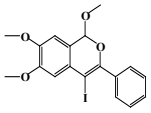
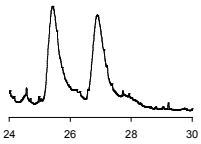
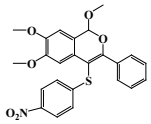
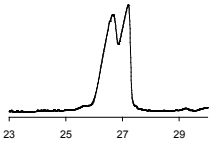
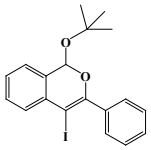
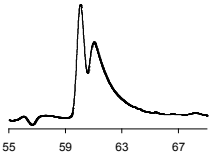
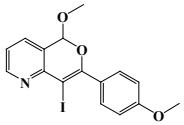
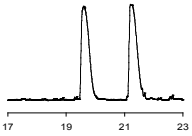
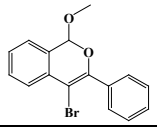
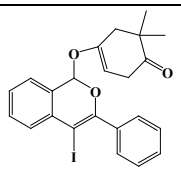
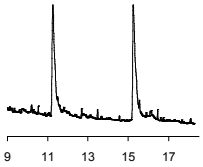
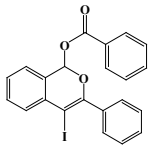
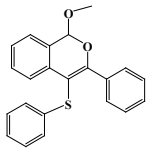
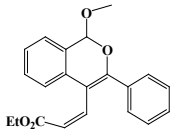
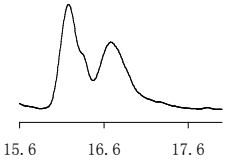
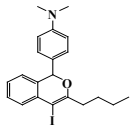
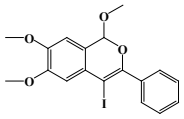
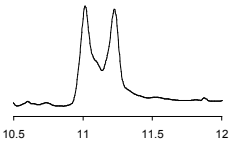
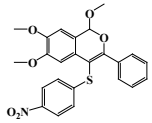
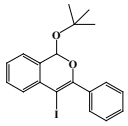
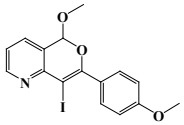
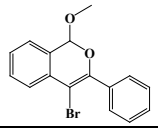
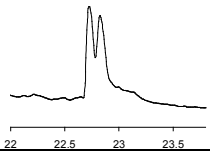
#	Structure	[SDS] (mM)	**[HP-γ-CD]** (mM)	[CD]/ [SDS]	R_s [b]	t_{m2}/t_{m1} [c]	N[d]	Electropherogram (in mins)
1		120	80	0.67	2.8	1.123	9,000	
2		200	215	1.08	3.5	1.065	49,000	 impurity
3		150	150	1.00	1.5	1.028	41,000	
4		150	165	1.10	1.3	1.035	21,000	
5		200	220	1.10	1.5	1.032	44,000	
6		200	220	1.10	1.7	1.058	14,000	
7		200	220	1.10	0.5	1.020	9,000	
8		150	180	1.20	0.5	1.016	47,000	
9		150	120	0.80	3.5	1.084	28,000	
10		-	-	-	0.0	-	-	No separation

Table 3. Optimized enantioseparations of isochromene derivatives using HP-γ-CD, HP-β-CD and SDS in 20% ACN run buffer [a] (*Continued*)

#	Structure	[SDS] (mM)	**[HP-β-CD]** (mM)	[CD]/ [SDS]	R_s [b]	t_{m2}/t_{m1} [c]	N[d]	Electropherogram (in mins)
1		150	100	0.67	12.0	1.355	19,000	
2		-	-	-	0.0	-	-	No separation
3		-	-	-	0.0	-	-	No separation
4		200	180	0.90	1.0	1.031	24,000	
5		-	-	-	0.0	-	-	No separation
6		150	100	0.67	1.2	1.019	82,000	
7		-	-	-	0.0	-	-	No separation
8		-	-	-	0.0	-	-	No separation
9		-	-	-	0.0	-	-	No separation
10		150	100	0.67	0.7	1.004	614,000	

a) All separations performed with 5 mM phosphate buffer pH 2.5, -20 kV, 40 cm capillary (30 cm to detector) with 50 µm I.D.

b) R_s: separation resolution

c) t_m: migration time. EOF mobility could not be determined due to its suppression at pH 2.5, and therefore α values could not be calculated.

d) N: the number of theoretical plates obtained for the first detected peak on a 30 cm length (to the detector) capillary.

PART II. MICROORGANISM STUDIES WITH CAPILLARY ELECTROPHORESIS

CHAPTER 4

Review

A Rapid Capillary Electrophoresis Method for the Detection of Microbial Contamination: An Alternative Approach for Sterility Testing

A paper submitted to *Pharmacopeia Forum*

Ye Bao, Andrew W. Lantz, Daniel W. Armstrong

Abstract

A review is presented on the capillary electrophoresis (CE) methodology for sterility testing as a possible alternative to the traditional direct inoculation method and more recent molecular techniques involving DNA testing, PCR and antibody-based methods. Topics discussed include basic CE theory, CE characterization of bacteria and fungi, CE sterility testing method development and the appropriate experimental procedure. Sample preparation and/or preconcentration procedures for CE analyses are considered. Finally the use of this CE approach to analyze actual consumer (eye care) products is demonstrated.

4.1 Introduction

Sophisticated instrumental techniques for the analysis and characterization of microorganisms are becoming more common. Although these newer, often experimental approaches (e.g. differential staining, phage typing, comparison of DNA sequences, PCR, mass spectrometry) may not replace traditional methods involving cultures, microscopy, and so forth in the immediate future, their development and use will continue to grow. In particular, after emerging in the early 80's, capillary electrophoresis (CE) has become a well established analytical technique. CE is well known to produce rapid, high efficiency analysis and separation of a wide variety of analytes from small ions, molecules, to proteins, DNA and RNA. Generally CE requires minimal sample preparation, and sample consumption is minuscule [1-6]. These advantages may also be extended to the analysis of microorganisms. As a result, the interest in CE as an analytical technique to analyze various types of intact biological cells has grown tremendously over the last decade [7-12]. In this review, we will limit our consideration to the application of CE as a rapid, effective approach to determine the sterility of samples.

Capillary electrophoresis is typically used to separate ionized molecular species based on their charge and frictional forces [13]. Charged species move in the direction of the oppositely charged electrode in a conductive liquid medium (i.e., electrophoresis) when an electric field is applied. The movement through the surrounding solution imposes frictional forces on the molecules/ ions. The net mobility of a molecule is estimated by Eq. (1):

$$\mu = q / f \qquad (1)$$

where μ is the eletrophoretic mobility, q the net charge and f the friction coefficient (which is equal to $6\pi\eta R$, where R is the particle radius and η is viscosity). Since the frictional forces are directly related to the size of an analyte, its mobility is directly controlled by its charge-to-size ratio. This simple equation was derived for spherical particles (often molecules) [13]. As for very large particles such as virus, bacteria and fungi, more complicated factors need to be included (see next section) [14].

Sterility testing (testing for microbial contamination) is a crucial and often necessary procedure in food, pharmaceutical, and medical industries for safety and quality control [15-16]. Infected food, medicine or biological samples (i.e. blood or plasma) may cause serious problems for patients and consumers in general. Among the varied approaches to detect the presence of microbial contaminations, the direct inoculation method is a universally accepted technique [17]. In this method, an aliquot of sample is placed in a sterilized growth medium and allowed to incubate for a prescribed period of time. The medium is then checked for the presence of microbes under a microscope; a positive result indicates that the original sample is contaminated. However, there are several shortcomings in this standard method [18-20]: first, it suffers from long analysis time (14 days); second, it may only detect the microbes that can grow on the particular medium used; furthermore, it is difficult to discern the number/ concentration of microbes in the original sample. To overcome these drawbacks, molecular techniques have attracted substantial attention as

alternatives for the detection of microbial contamination. There are mainly three types of molecular techniques: nucleic acid hybridization, amplification (PCR, polymerase chain reaction) and immunoassay [21-24]. Though these methods lessen the analysis time considerably, they are confined to the detection of specific microorganisms and require significant personnel training to perform these complex experiments. Hence, they are not particularly useful for general sterility tests where a simple, rapid and definitive yes or no answer is needed.

Recently, capillary electrophoresis (CE) has been explored as an analytical technique for the separation, identification, quantification and characterization of microorganisms [25-33]. In 1999, the separation of several bacteria with the use of polymer additives in the run buffer was reported [25]. Shintani et al. coupled CE with a laser-induced fluorescence (LIF) system for the identification of Salmonella enteritidis [30]. By staining *S. enteritidi*s cells with a cell-permeable nucleic acid stain or a salmonellae-specific polyclonal antibody, they successfully detected as few as three cells. More recently, Valcárcel and co-workers successfully separated eight different types of bacteria in 25 mins based on the differential mobility of bacteria in the capillary by using specific ions (calcium and myoinositol hexakisphosphate) to interact with the bacterial surface, and applied the method in the identification and quantification of bacteria contamination in several food samples [32]. Buszewski successfully separated five species of bacteria over a short distance (8.5 cm) using trimethylchlorosilane or divinylbenzene – modified capillaries with suppressed EOF [33].

Interested readers can find more information in several reviews that have been reported on the analysis of microorganisms by CE [8-10, 34-36].

When using CE to detect general microbial contamination, the separation, identification or characterization of the microorganisms are not needed. There is a need for an efficient/effective method capable of providing a simple, rapid and binary (yes/no) answer in regards to the presence/absence of any/all microorganisms. This method should combine the best elements of broad applicability of culture techniques and the fast analysis time of molecular techniques. Having such a rapid and reliable screen would eliminate the need for more complex or time-consuming testing of samples. Once the "clean" samples have passed, only those that appear to be contaminated can be more exhaustively analyzed or eliminated.

4.2 CE Characterization of Microorganism

In CE, molecules of a single compound are individually identical, thus have the same mass-to-charge ratios, in other words, same electrophoretic mobilities. Therefore, these individual molecules reach the detector at nearly the same time, producing a single signal. However, this is not the case for microorganisms. Unlike molecules which have dimensions on the scale of a few to several Angstroms, microorganisms typically have at least one dimension that ranges from tens of nanometers to a few micrometers. Consequently, the electrophoretic process can be more complicated [9, 10]. The microorganisms may obtain charge upon protonation or deprotonation of ionizable functional groups on the surface, or

through the adsorption of ions from the surrounding solution. Also microbes are amphoteric: they have a negative net charge at high pH, while a positive net charge at low pH. In addition, they can aggregate to form clusters, or attach to other different types of microbes, or secrete substances that cover their surfaces and therefore alter their mobilites (these problems can be ameliorated by sonication and preparation of fresh microbial samples) [37-38]. Frequently, a single species of microorganism will exhibit a distribution of sizes, shapes and charges (all of which are affected by their growth cycle and the nature of their environment/ growth medium), and hence different electrophoretic mobilites. This property is often referred as electrophoretic heterogeneity (EH) [28, 39]. Also, microbes are sensitive to extremes of pH, osmolarity differences, or electric fields, all of which may lyse cells [27]. To achieve successful CE runs, the above properties and behaviors of microorganisms need to be taken into consideration.

4.3 Detection of Microbial Contamination by CE

Recently, a rapid, widely applicable CE method for the detection of microbial contamination was reported [40]. After modification of the original method, single cell detection was achieved which provided a solid foundation for an *in situ* sterility test [41]. In the original method, the capillary is initially rinsed and filled with run buffer containing dilute cationic surfactants (i.e., CTAB). Three injections are then made (i.e., microbial sample, run buffer, and finally blocking agent composed of nutrient broth), followed by

electrophoresis. The possible mechanism is shown in Figure 1: the capillary wall is initially dynamically coated with the positively charged CTAB. Upon application of a voltage to the capillary, the cationic surfactants CTAB coat (or are dynamically adsorbed by) the microbes and sweep them out of the original sample zone until they encounter the blocking agent where cellular aggregation occurs. This method utilizes cationic surfactants and blocking agent to sweep and stack all cells into a single, sharp peak. The presence or absence of this peak signal is used to designate sample sterility. This method can be applied to a variety of bacteria and fungi, and also mixtures of different types of microorganisms and the analysis can be done within 15 minutes (for example see Figure 2). Another advantage of this method is that if the sample contains any microbes, they always come out at a fixed time right in front of the blocking agent (BA). Any slight variations in microbial migration times are due to inherent EOF fluctuations. Detection of the microbes can be achieved by UV-vis or laser-induced fluorescence (LIF). However, in this original method the natural fluorescent property of nutrient broth created an interference which limited the detection sensitivity to ~50 cells.

In order to achieve a single cell detection which is a prerequisite of a "sterility test", a modification of the method has been made to replace the fluorescent nutrient broth blocking agent with a zwitterionic surfactant, caprylyl sulfobetaine [41]. This surfactant appears to be universally effective as a blocking agent for bacteria and fungi, yet has no fluorescence

signal. By applying the modified method, a single cell detection was achieved *in vitro* which provided a solid foundation for the "real-world" sterility test.

The experimental procedure is as follows: The bacteria were initially grown in the liquid broth and then plated on agar plates and stored under refrigeration until needed. When needed for experiments, a single colony was taken from the agar plates and then grown in the liquid broth. When in the stationary phase of growth in their respective media, the bacteria were harvested with cell contration ~10^8 colony forming units (CFU)/mL and centrifuged down, then the excess broth removed, and washed with working solution once (1 mM TRIS, 0.33 mM citric acid), and finally suspended in the working solution of the same volume as the broth that was originally removed. For the single cell detection, firstly 1 mL diluted bacterial sample solution (~10^4 CFU/ mL) was fluorescently tagged by the addition of 1 µL BacLight fluorescent dye solution, allowing 30 mins for the stain of cells at room temperature (The orginal fluorescence dye is powder, simply add 2 µL of DMSO to the powder to make the dye solution). At the same time, CTAB (1 mg/mL), zwitterionic surfactant (caprylyl sulfobetaine) solutions (5 mg/mL) with glass vials were autoclaved to make sure no other bacterial contaminants in these reagents and the capillary was rinsed with the sterile working solution. Once the bacteria sample was ready, the sample vial was vortexed for 30 s to prevent the aggregation of individual cells. Then a tiny drop (~ 2 µL) of the above bacteria solution was applied onto a sterile microscope slide, and smeared into smaller drops using sterile pipet tip. Secondly, these drops were inspected visually under the

microscope (40x lens) until one drop containing a single cell was found and then this drop was quickly injected into the capillary by siphoning effect, followed by the injections of run buffer and blocking agent- caprylyl sulfobetaine. The electrophoresis was then carried out at – 2kV. Fluorescence emission from the stained cell was detected at 516 nm. The signal-to-noise ratios of all tested single cell were between 5 and 9 (Figure 3). Furthermore, detection of live/dead cells can be achieved by staining the microbial sample with different dyes. To further reduce the possibility of lysing cells by surfactants (such as CTAB), dicationic ionic liquids may be used as auxiliary buffer additives and thus lower the required CTAB concentration from 1 mg/mL to 0.25 mg/mL [42].

Most recently, CE and DNA-FISH (Fluorescence *In Situ* Hybridization) were used to detect *Salmonella Typhimurium* in a mixed culture [43]. In the experiment, the fluorescein-labeled peptide nucleic acid probe Sal23S15 (Am-ACC TAC GTG TCA GCG-Cbx) was used. This probe selectively binds to rRNA present in the 23S ribosomal subunit of *Salmonella* species. Detection of 3 injected *S. Typhimurium* cells against a background of ~ 300 injected *E. coli* cells indicated the possibility of single-cell detection of specific pathogens using this combined technique. Figure 4A shows the electropherogram for the Sal23S15 hybridized culture of *E. coli.* No significant signal was produced by *E. coli,* indicating both the selectivity of the probe and a lack of nonspecific binding of the probe to the surfaces of non-target cells. In contrast, strong fluorescent signals were obtained from hybridized *Salmonella Typhimurium* as seen in Figure 4B. This sample consisted of

undiluted *E. coli* and *Salmonella*, both of which were allowed to react with the peptide nucleic acid probe. These data demonstrate the successful combination of capillary electrophoresis and fluorescence *in situ* hybridization to detect specific types of sample contamination. The Sal23S15 probe was effective as a genus specific probe for the detection of *Salmonella* species in the sample injection.

Sample preparation is an essential step for any/all sterility tests. As described previously, the migration and aggregation of the microbes depend strongly on the ionic compositions of the solutions throughout the capillary. Therefore, the sample must be pretreated to remove other ionic interferences (such as proteins) in the original sample matrix. In the case of diluted sample solution, sample pre-concentration may be accomplished by centrifugation down to several micro-liters. For even larger sample volumes, membrane filtration may be applied, in which a sample is passed through a membrane with proper pore size. The cells are thus accumulated on the membrane and then can be washed off with small amount of solvent and centrifuged if necessary [17]. Furthermore, by using capillaries of larger inner diameters (~ 200 μm), the injected sample volume could reach several microliters [40]. Recently, an effective bacteria sample preconcentration by large-volume sample stacking method with LIF detection using CE was reported [44]. A 60-fold enhancement in detection sensitivity was obtained when a long sample plug of up to 39.6% of the capillary volume was injected.

4.4 Testing of Consumer Products

The applicability of consumer eye care products to this CE-based test for microbial contamination was examined in our laboratory. Bausch and Lomb ReNu MultiPlus® Multi-Purpose Solution and Visine® eye drops were chosen as sample matrices and inoculated with lab cultured *Candida albicans*. Injections of spiked samples (without any special preparation treatments) produced numerous peaks and broad bands of cells, many of which remained in the original sample zone (Figure 5A). Contact lens solutions and eye drops often contain a variety of constituents ranging from simple salts to surfactants and long-chain organic polymers for lubrication. For example, besides purified water, the major components of ReNu MultiPlus® solutions include 9% w/w polyhexamethylene, 1% NaCl, 1% poloxamine, and 0.9% sodium borate. Visine® drops also contain 1% w/w polyethylene glycol, 1% povidone, and 0.1% dextran. These matrix components may interfere with the ability of CTAB to effectively coat the surface of the microorganisms and extract the cells out of the sample plug. Furthermore, the increased ionic strength of the eye solutions increases the conductivity of the sample zone, thus lowering the electric field in this region and limiting the migration velocity of the cells. Therefore, it is helpful to remove these compounds from solution prior to CE analysis. In order to isolate the contaminating cells from the sample matrix, inoculated eye care solutions were centrifuged and the supernatant was decanted. The remaining pelleted cells were washed in working buffer, recentrifuged and resuspended in buffer once again for analysis. Successful removal of matrix interferences

resulted in a sharp peaks for fungal contaminants detected at the appropriate location (i.e. front of the blocking agent) *via* CE analysis (Figures 5B and 5C).

The sterility of Bausch and Lomb ReNu MultiPlus® Multi-Purpose Solution was tested using fluorescence detection. A fresh 5 mL aliquot of ReNu solution was transferred to an autoclaved vial using a sterile transfer pipette and centrifuged to pellet any cells present. The supernatant was decanted and any potential microbial contaminants were resuspended in ~250 µL of sterile working buffer and stained with BacLight Green dye. The sample was analyzed using the CE sterility test method. No measurable signal was detected for the injected samples (Figure 6A). This process was repeated 3 times. Based on the single cell detection capabilities demonstrated in the previous paragraph using BacLight Green dye, these results indicate that the injected samples of ReNu solution were indeed sterile. The ability of this technique to detect low levels of cellular contaminants in ReNu solution was examined by exposing 5 mL of the lens solution to a non-sterile inoculation loop. This solution was then concentrated down to approximately 250 µL via centrifugation, resuspended in sterile working buffer, and stained with BacLight Green dye. A relatively small, but easily detectable, peak was obtained for these trials (Figure 6B), indicating that microbial contamination was present from exposure to the ambient non-sterile environment.

4.5 Conclusions

Capillary electrophoresis is becoming a valuable technique in the separation,

characterization, identification and quantification of microorganisms given its properties of high efficiency and short analysis times. Recently, the *in vitro* detection of single cells of various bacteria and fungi was accomplished using a three-injection CE method with S/N between 5 and 9. These results indicate that a rapid and reliable alternative sterility testing method using CE is feasible. Dilute samples may be preconcentrated off-column by centrifugation or membrane filtration, or online-preconcentration by large-volume sample stacking using CE. Commercial consumer eye-care products were evaluated using this CE-based sterility test for the first time. The test results indicated that these eye care products were indeed sterile. Furthermore the rapid contamination of these products by brief exposure to ambient conditions was easily detected. Future work will focus on biological samples (such as animal blood and urine) to assess the accuracy and broad applicability of this CE method.

Acknowledgements

We gratefully acknowledge the support of this work by the National Institutes of Health, NIH RO1 GM53825-11.

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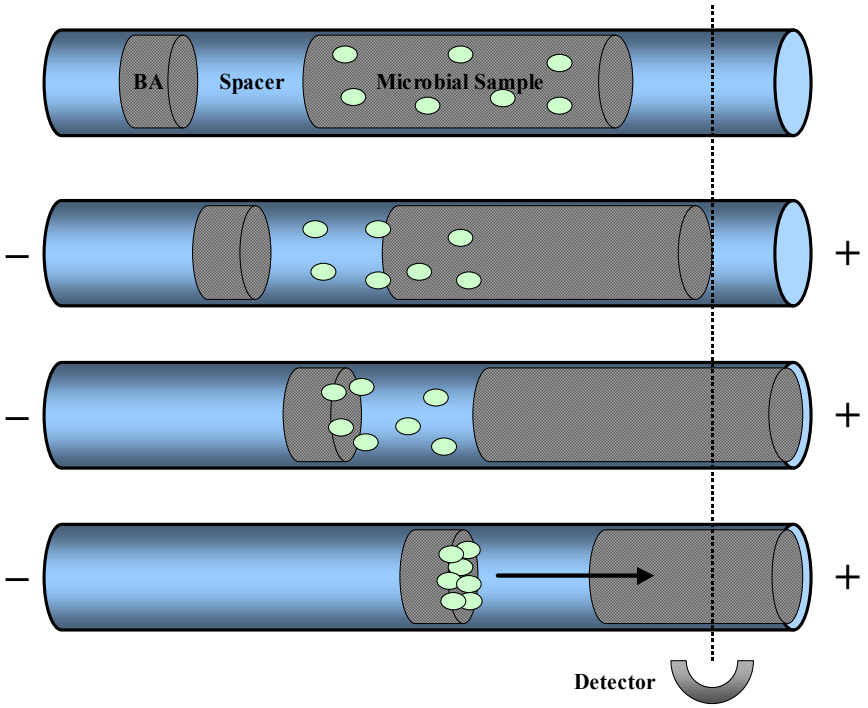

Figure 1. Schematic of CE/microfluidic-based test for microbial contamination. The entire capillary is initially filled with running buffer containing CTAB surfactant. Three injections are made prior to the run: (1) a large plug of sample containing microorganisms, (2) a spacer plug of running buffer and CTAB, and (3) a short plug of blocking agent (BA). Cells present in the sample are represented by ovals. The top capillary shows the relative locations of the solutions after the three injections have been made. The subsequent three capillary figures show the movement of the injected microbes after the voltage is applied. Reprinted from reference 41 with permission.

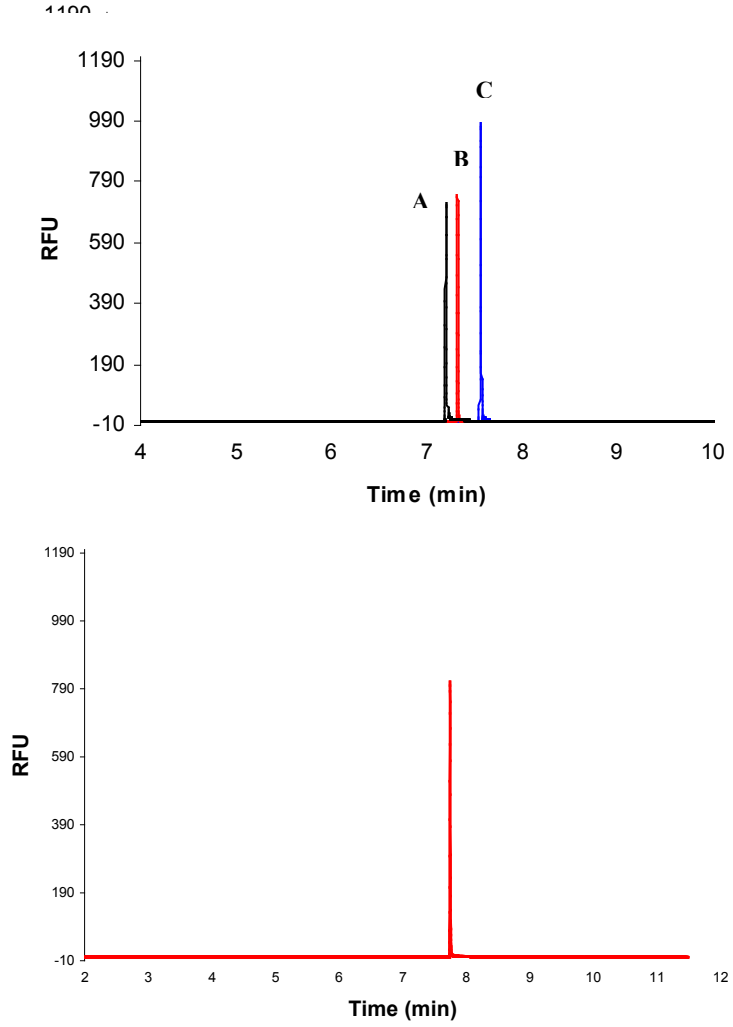

Figure 2. Three separate overlaid electropherograms showing S. aureus (A), S. subterreanea (B) and E. coli (C) individually (a) and in a mixed sample (b). Conditions: capillary, 30 cm long (20 cm to detector), 100 µm inner diameter. The running buffer is 1 mM TRIS/0.33 mM citric acid with 1 mg/mL CTAB, pH 7. The sample buffer is 1 mM TRIS/0.33 mM citric acid, pH 7. The nutrient broth concentration is 8 g/L. The bacteria sample concentration is ~ 10^8 CFU/mL. The voltage is -2 kV. The sample is injected for 6 s at 0.5 psi, the spacer injected for 5s at 0.4 psi, and the nutrient broth for 1.5 s at 0.2 psi. Laser-induced fluorescence detection was used, and the monitored wavelength was 517 nm. The bacteria were all stained with BacLight. Reprinted from reference 40 with permission.

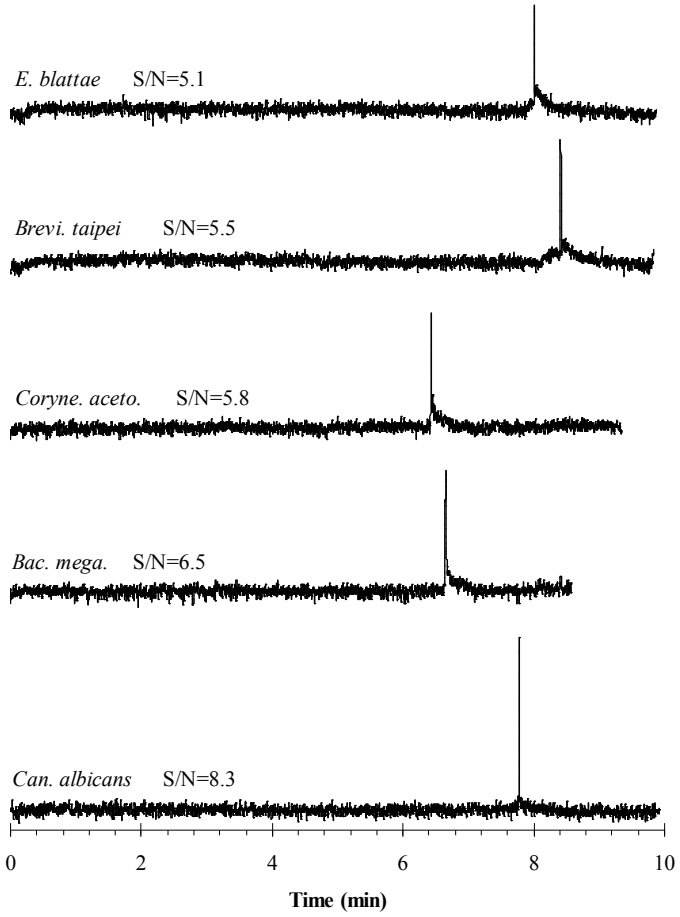

Figure 3. Electropherograms of single cells of various bacteria and fungi using the revised CE-based sterility test. Conditions: capillary, 30 cm long (20 cm to detector), 100 µm inner diameter. The running buffer is 1 mM TRIS/0.33 mM citric acid with 1 mg/mL CTAB, pH 7. The sample buffer is 1 mM TRIS/0.33 mM citric acid, pH 7. LIF detection at 516 nm. Concentration of blocking agent, 5 mg/mL. Reprinted from reference 41 with permission.

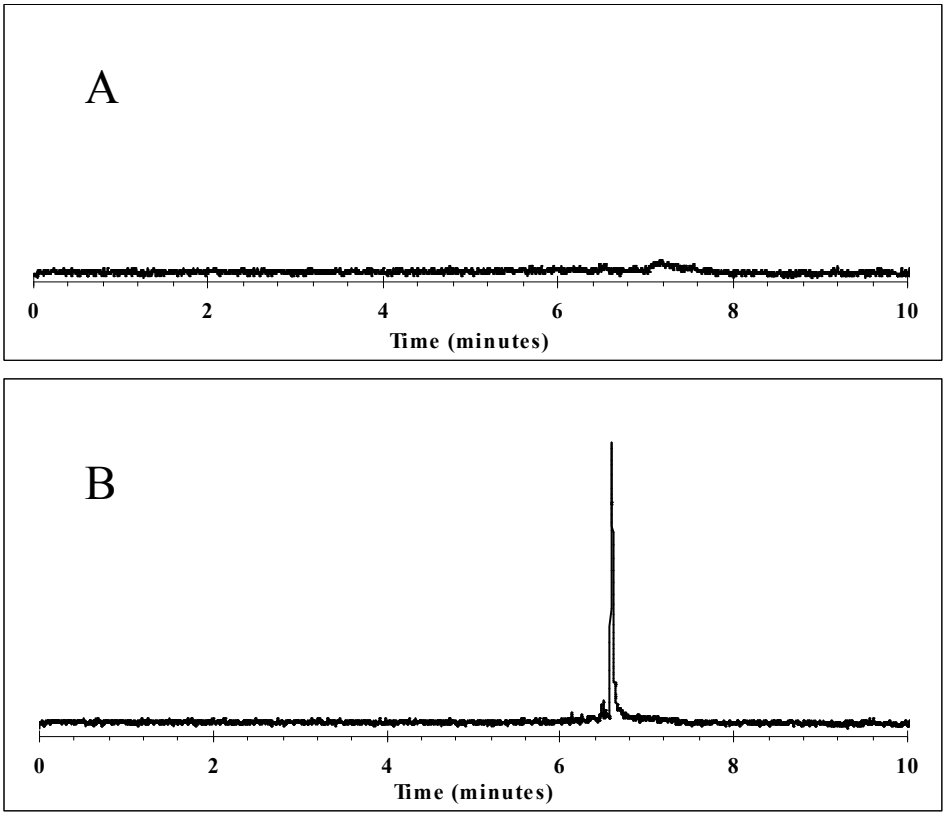

Figure 4. Electropherograms of A) ~10^8 CFU/mL *E. coli*, B) ~10^8 CFU/mL *Salmonella* Typh. and ~10^8 CFU/mL *E. coli*. All samples were hybridized with *Salmonella* specific Sal23S15 PNA probe.

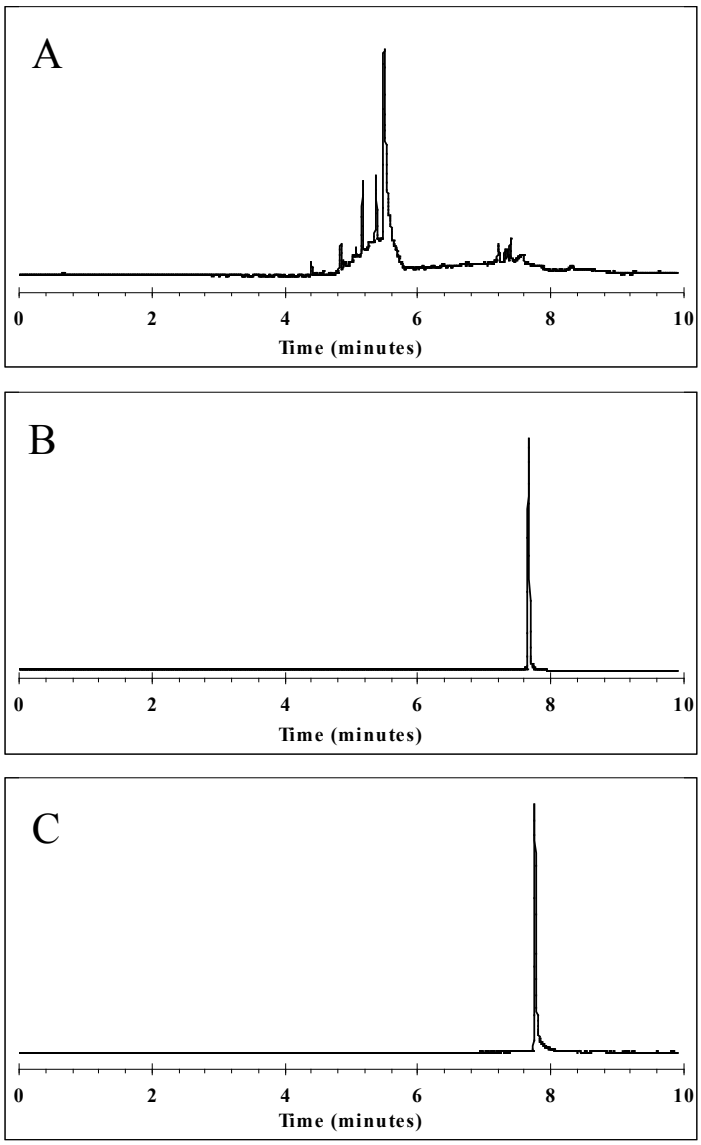

Figure 5. Electropherograms of *Candida albicans* in A) ReNu MultiPlus® solution, B) ReNu MultiPlus® solution after resuspension in working buffer, and C) Visine® solution after resuspension in working buffer.

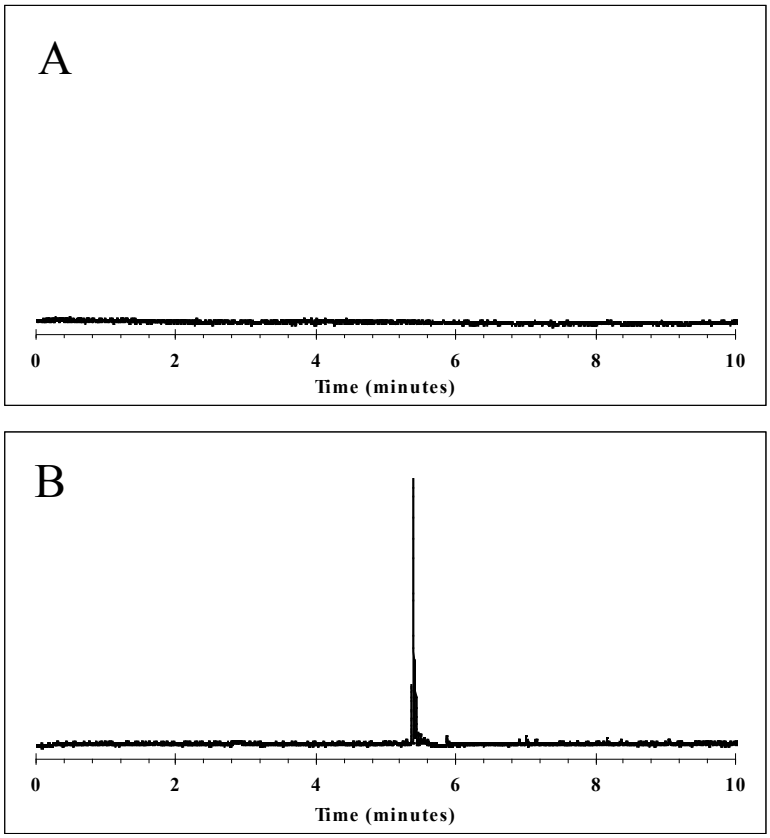

Figure 6. CE-based sterility test of ReNu MultiPlus® solution with A) an uncontaminated sample, and B) a sample inoculated with a non-sterilized instrument.

CHAPTER 5

Single Cell Detection: A Rapid Test of Microbial Contamination Using Capillary Electrophoresis

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Analytical Chemistry 79 (2007) 1720-1724

Abstract

Single cells of bacteria and fungi were detected using a capillary electrophoresis based test for microbial contamination in laboratory samples. This technique utilizes a dilute cationic surfactant buffer to sweep microorganisms out of the sample zone and a small plug of "blocking agent" to negate the cells' mobility and induce aggregation. Analysis times are generally under 10 minutes. Previously, a nutrient broth media was reported as an effective blocking agent, however the natural background fluorescence from the nutrient broth limited the detection sensitivity to ~50 cells. In order to enhance the sensitivity of the technique down to a single cell, an alternative synthetic blocking agent was sought. Various potential blocking agents were screened including salts, polypeptides, small organic zwitterions, and surfactants. Zwitterionic surfactants are shown to be attractive alternatives to a nutrient broth blocker, and mimic the nutrient broth's effects on cellular aggregation and mobility. Specifically, caprylyl sulfobetaine provided the sharpest cell peaks. By substituting caprylyl sulfobetaine in place of the nutrient broth the fluorescence of the blocker plug is reduced by

as much as 40x. This reduction in background noise enables the detection of a single microorganism in a sample, and allows this technique to be potentially used as a rapid sterility test. All single cells analyzed using this technique displayed signal-to-noise ratios between 5 and 9.

5.1 Introduction

The detection of microbial contamination in test samples is a crucial component of safety and quality control in the food [1], pharmaceutical [2], and medical industries [3], as well as in the public sector (e.g., homeland security [4] and water treatment [5]). Pathogenic bacteria and fungi can cause serious diseases in patients and consumers when introduced internally *via* food or medical products. Therefore, there is a great need for rapid methods of analysis that test for the presence or complete absence of microorganisms. Current standard methods involve inoculating a sterile growth medium with an aliquot of sample, and allowing it to incubate over several days or weeks. The medium is then examined for the presence of bacteria or fungi [6]. While this technique is capable of detecting the presence of a single cell in the original aliquot, it is very time-consuming to complete. Several variations of this method have been developed to compensate for its poor speed, however these tests still require days for definitive results [6]. Furthermore, all methods that utilize growth media have a fundamental shortcoming: they may only detect organisms capable of growth on the particular medium and under specific experimental

conditions. Therefore, current methods of sterility tests are often considered flawed [7].

As a means of overcoming the shortcomings of standard microbiological methods, molecular techniques have attracted considerable attention for the analysis of microorganisms. These methods include nucleic acid hybridization or amplification (PCR, polymerase chain reaction) as a means of microbial identification [8, 9], and immunoassay techniques [10]. However, these methods are highly specific (i.e., not particularly useful for general sterility test purposes), and are relatively complex procedures requiring significant training to perform. Recently, capillary electrophoresis (CE) has been explored as a technique for the separation and identification of microorganisms [11]. Traditionally used as an analytical technique for the separation of molecules by their mass-to-charge ratio, CE possesses unique attributes (aqueous running buffers, fast analysis time, and low sample requirements) that make it an attractive approach for "biocolloid" analysis. In the late 1980s, Hjerten et al. demonstrated that CE of viruses and bacteria was possible by examining the flow of the tobacco mosaic virus and *Lactobacillus casai* through a capillary with an applied electric field [12]. Since then, several studies have been published on the analysis of microorganisms using CE, including a mobility study on the human rhinovirus [13], and separations of several different bacteria strains utilizing dilute polymer additives [14] and coated capillaries [15, 16].

Few publications currently exist, however, concerning the development of a rapid sterility test with CE [17, 18]. Recently, our research group reported a versatile, rapid CE

method for the examination of bacterial contamination [18]. A wide variety of bacteria are compatible with this method, and analysis times are typically less than 10 minutes. Microbial entities may be detected using either ultraviolet-visible (UV-Vis) or laser-induced fluorescence (LIF) detection. However, the natural fluorescence of some of the reagents in these experiements limited the detection sensitivity of the method to ~50 cells. In order to be used successfully as a "sterility test" a detection limit of a single cell is an absolute requirement. This technical note reports the successful completion of this study. By attaining single cell detection, this method is now capable of indicating the presence or complete absence of microorganisms in a sample-the primary requirement of a sterility test.

5.2 Materials and Methods

5.2.1 Materials

Buffer additives, including tris(hydroxymethyl)aminomethane (TRIS), cetyltrimethylammonium bromide (CTAB), sodium dodecyl sulfate (SDS), sodium hydroxide, sodium chloride, and hydrochloric acid were obtained from Aldrich Chemical (Milwaukee, WI). Citric acid was purchased from Fisher Scientific (Fair Lawn, NJ). 3-(Decyldimethyl-ammonio)propanesulfonate or caprylyl sulfobetaine (SB3-10), as well as octyl sulfobetaine (SB3-8), lauryl sulfobetaine (SB3-12), myristyl sulfobetaine (SB3-14), and palmityl sulfobetaine (SB3-16) were all ordered from Sigma (St. Louis, MO). Taurine, betaine, sarcosine, triethylamine N-oxide, and all peptides and poly amino acids were

purchased from Sigma. Molecular Probes, Inc. (Eugene, OR) supplied the BacLight Green fluorescent dye. Nutrient and brain heart infusion broths were products of Difco Laboratories (Franklin Lakes, NJ), while luria broth was obtained from Sigma. *Brevibacterium taipei* (ATCC no. 13744), *Corynebacterium acetoacidophilum* (ATCC no. 13870), *Escherichia blattae* (ATCC no. 29907), *Bacillus cereus* (ATCC no. 10702), *Bacillus subtilis* (ATCC no.12695), *Candida albicans* (ATCC no. 10231), *Rhodotorula* (ATCC no. 20254), and *Bacillus megaterium* (ATCC no. 10778) were all purchased from American Type Culture Collection (Manassas, VA). Uncoated fused silica capillaries with inner and outer diameters of 100 μm and 365 μm respectively, were purchased from Polymicro Technologies (Phoenix, AZ). All microorganisms examined in this study are rated biosafety level one. Therefore, standard microbiological practices may be employed.

5.2.2 Methods

Analyses were performed on a Beckman Coulter P/ACE MDQ capillary electrophoresis system equipped with photodiode array and 488 nm laser-induced fluorescence detectors (Fullerton, CA). Fluorescence emission from BacLight Green stained cells was detected at 516 nm, while Mie scattering was detected at 449 nm. Capillaries used were 30 cm in total length (20 cm to the detector) with an inner diameter of 100 μm. New capillaries were initially conditioned with the following rinses: 1 N NaOH, 1 N deionized water, 1 N HCl, and running buffer each for 3 min. Between runs, the capillaries were

washed with 1 N NaOH, deionized water, and running buffer for 1 min each. Working buffers were prepared by adding appropriate amounts of TRIS and citric acid to deionized water to produce a 10 mM TRIS/3.3 mM citric acid solution, and diluting this solution 10x to a final concentration of 1 mM TRIS/ 0.33 mM citric acid. pH was adjusted to 7 using dilute sodium hydroxide or hydrochloric acid. The final running buffers were prepared by dissolving CTAB in the working buffer to a concentration of 1 mg/mL. Blocking solutions contained the blocking agent of interest at varying concentrations in working buffer adjusted to pH=7. These solutions were all made fresh daily. All bacteria and fungi were grown as specified by the supplier. All cell concentrations were approximated by serial dilutions and plate-count methods. Initially, the microorganisms were grown in the appropriate liquid broth, and then plated on agar growth media and stored under refrigeration. All broths and agar were autoclaved (Primus autoclave, Omaha, NE) for 1 hr prior to inoculation. For experiments, fresh liquid broth was inoculated with a single microbe colony that was extracted from the agar plate. These cells were grown at 30-37 ºC under gentle agitation for approximately 24 hrs, producing a cellular concentration of ~10^8 colony forming units (CFU)/mL. The microorganisms were centrifuged down, and the excess broth was removed. The cells were then washed with working TRIS/citric acid buffer, recentrifuged, and finally resuspended in fresh buffer for analysis. All samples were vortexed for 30 sec and sonicated briefly prior to analysis to disperse cellular aggregates. Serial dilutions of the microbial solutions were made using working buffer when necessary. BacLight Green fluorescent dye was used to stain the

cells for LIF detection. This dye was initially prepared in DMSO to produce a 1 mM solution, as directed by the manufacturer. The cells were stained by adding 1 μL of dye solution per 1 mL of microbial solution (to a final concentration of 1 μM) and incubating the cells at room temperature for at least 30 min. Experiments were performed with varying amounts of BacLight to ensure cells were saturated with the fluorescent dye to maximize the signal-to-noise ratio at this concentration.

After all wash cycles, the capillary was filled with running buffer containing CTAB. Three injections were made prior to the run: 1) sample plug consisting of microorganisms, 2) spacer plug of running buffer, and 3) plug containing blocking agent. Unless otherwise noted, sample injections were made for 5 s at 0.5 psi (158 nL), spacer injections for 4 s at 0.5 psi, and blocker injections for 2 s at 0.1 psi. For single cell analysis, microbial solutions were diluted down to ~10^4 CFU/mL and stained with BacLight Green dye as described above. A small drop (~2 μL) of this solution was applied to a sterile microscope slide, and using an autoclaved micro-utensil the drop was smeared across the slide to produce numerous drops of smaller volume. These drops were then inspected visually by microscopy until a drop that contained only a single microorganism was identified and isolated. This entire drop was then injected into the capillary *via* capillary action (in place of the sample plug mentioned above). All run buffers, solutions, and vials used in the CE analysis were autoclaved prior to the run. Run voltage was set to –2 kV in reverse polarity (current: -1.4 μA), due to reversal of the electroosmotic flow (EOF) by CTAB.

5.3 Results and Discussion

5.3.1 Blocking Agent

As reported previously, a CE/microfluidic-based test for the presence of microbes can be accomplished by filling the capillary with a dilute cationic surfactant (CTAB) buffer and injecting a series of plugs consisting of the microbial sample, running buffer spacer, and blocking agent (see Figure) [18]. Upon application of a voltage to the capillary, the cationic surfactant migrates through the sample zone. Since the electrophoretic mobility of a biocolloid is based on its surface charges [11], the coating of the microorganisms' surface by CTAB results in a cationic migration of the cells. These bacteria and/or fungi then traverse the sample and spacer zones until they come in contact with the blocking agent, at which point cellular aggregation occurs and their electrophoretic mobility is lost. Effectively, the microorganisms change direction twice during the separation. However, the natural fluorescence of the original nutrient broth blocking agent hindered the method's detection sensitivity, with a limit of detection of ~50 cells. It was clear that if this technique was to be an effective sterility test, the background problem had to be eliminated while preserving the unique aggregation and mobility effects on the microorganisms.

An alternate blocking agent was sought based on the following observations of the synergistic qualities of the CTAB/nutrient broth system: (a) in the presence of both CTAB *and* nutrient broth microorganisms often formed aggregates, however this seldom occurred in the presence of only one of these additives; (b) CTAB and nutrient broth do not precipitate

when combined, indicating that a unique mechanism involving both CTAB and the nutrient broth is likely responsible for cellular aggregation (not simply a co-precipitation); and (c) the cells lose their electrophoretic mobility in the presence of both CTAB and nutrient broth, signifying neutralization or masking of the surface charge on the microorganisms. Cellular aggregation may be induced in several ways including neutralization of surface charges on the cells or coating the microorganism with a polymeric or uncharged material [19-21]. Growth media (including nutrient broth) are often composed of beef or other protein extracts such as peptone, containing various water-soluble protein derivatives obtained by acid or enzyme hydrolysis of natural protein. It was initially hypothesized that the active agent in nutrient broth responsible for cellular aggregation was a hydrolyzed protein or peptides with multiple charges, either zwitterionic or simply ionic. Due to the previous success of surfactant coatings on the surface of bacteria [18], we postulated that this agent could potentially also be a surface-active species.

Numerous compounds were examined as potential blocking agents prior to method optimization and single cell analysis (Table 1). All additives were tested with concentrations ranging from 1 mM to 50 mM, with the exceptions of poly-L-glutamic acid and poly-L-arginine which were examined up to 10 mM due to their high molecular weights. To confirm that the focusing effect of the cells seen at the blocking agent front is not simply due to a stacking effect by the high ionic strength of the nutrient broth, small plugs of TRIS-citrate (up to 50 mM TRIS/ 16.7mM citrate) and sodium chloride (up to 50 mM) were

substituted in place the nutrient broth. While some peak focusing occurred, these agents were not effective at preventing the cells from passing through the blocker plug. Poly-L-glutamate and poly-L-arginine were then examined to test the effectiveness of acidic and basic long chain polypeptides as blocking agents. These amino acid polymers appeared to have no effect on the migration of the microorganisms, allowing the cells to pass completely through the blocker plug. Shorter peptides of 3-6 amino acid units with either nonionizable side chains (polyglycine and polyleucine) or both acidic and basic functional groups (Arg-Gly-Glu-Ser) were then assessed. Interestingly, while these peptides only blocked a small percentage of the cells in the sample, these agents proved to be more effective at negating the cells' electrophoretic mobility than the longer polypeptides. Therefore, it was theorized that small zwitterionic molecules might be responsible for the observed cell aggregation. Small zwitterionic molecules, such as sarcosine, taurine, betaine, and trimethylamine N-oxide, belong to a class of compounds known as osmolytes (or reverse denaturants). These molecules are well known to be involved in the stabilization and folding of proteins' tertiary and quaternary structures, and therefore may have a significant effect on the charge of proteins on the surface of microorganisms [22]. All four small zwitterionic molecules examined did indeed step a significant percentage (though not all) of certain microorganisms from passing through the blocker plug. However, these agents were not as effective as the nutrient broth, nor were they universal in their effect. For example, sarcosine blocked a majority of *Corynebacterium acetoacidophilum* cells while having little effect on

the mobility of *Escherichia blattae*.

The apparent universal ability of the surfactant CTAB to efficiently coat and sweep microorganisms out of the sample zone directed our study toward zwitterionic surfactants. Anionic surfactants, such as SDS, were also examined however these surfactants precipitate in the presence of the cationic CTAB buffer additive. A series of zwitterionic sulfobetaine surfactants of varying carbon chain length (from 8 to 16) was evaluated. All five of the zwitterionic surfactants evaluated successfully induced aggregation of the cells in the presence of CTAB and negated the electrophoretic mobility of microorganisms. In addition, these blocking agents appear to be universal for the bacteria and fungi tested in this study. Surprisingly, the length of the carbon chain on the surfactant tail appears to have a significant effect on the ability of the blocking agent to focus the cells. While all the sulfobetaine surfactants focus the cells into a single peak, the sharpest peaks were obtained using caprylyl sulfobetaine (SB3-10). Equimolar solutions of surfactants with more (SB3-12, -14, -16) or fewer (SB3-8) than 10 carbons in their tail produced significant peak tailing, resulting from stray cells passing through the blocker plug. Based on these studies SB3-10 was chosen as a synthetic alternative blocking agent for further studies of this CE-based sterility test.

5.3.2 Single Cell Detection

Substituting the zwitterionic surfactant SB3-10 in place of the nutrient broth greatly reduced the background fluorescence of the blocker plug. Electropherograms of ~25 cells of

Brevibacterium taipei illustrate how the background signal from the blocker plug is decreased by a factor of ~40 by switching from nutrient broth (Figure 2A) to SB3-10 (Figure 2B) blocking agent. This background reduction allows a single cell initially residing in the sample plug to be detected, which is an essential characteristic of a true sterility test. Samples containing a single cell of one of several bacteria or fungi species were tested in order to confirm the detection limit of the method. Figure 3 shows example electropherograms of single cell detection for four bacteria (both gram negative and positive) and one fungus. Small variations in the migration times of the cells (and blocker plug) are the result of slight changes in the EOF. All single cell runs in this study displayed signal-to-noise ratios (S/N) between 5 and 9 and were repeated at least 3 times. Slight background fluorescence exists from the SB3-10 blocker plug behind the cell peak. This fluorescence occurs even when BacLight dye is not introduced into the capillary, indicating that this signal is likely due to slight fluorescence from the SB3-10 or trace impurities present in the zwitterionic surfactant solid. Simple recrystalization of the SB3-10 did not significantly lower the background fluorescence. The background fluorescence signal however is directly proportional to the concentration of SB3-10 in the blocker plug. Therefore, the background noise may be minimized by limiting the concentration of blocking agent used. For most samples containing a relatively low concentration of microbial contamination ($<10^6$ CFU/mL), an SB3-10 concentration of 5 mg/mL is sufficient and exhibits negligible background fluorescence (see Figures 2 and 3). More turbid solutions of cells may require

higher concentrations of blocking agent to adequately (and rapidly) stack all microorganisms into a single peak and negate their mobility. However, at such high cell counts the detection limit of this technique no longer becomes an issue. The effect of increasing blocking agent concentration on a solution of *Corynebacterium acetoacidophilum* (~10^8 CFU/mL) is shown in Figure 4. The peak tailing in Figure 4A is the result of cells passing through the blocker plug due to an insufficient SB3-10 concentration. Simply doubling the concentration of the blocking agent (Figure 4B) eliminates the tailing.

5.3.3 Sample Preparation

Prior to CE analysis, samples must be prepared in such a manner so that the contaminating microorganisms are included in the injected volume. In most real-world scenarios, the original sample volumes are quite large and the number of bacteria or fungi cells present may be very low. Centrifugation is a rapid and effective technique for concentrating moderate volume microbial solutions down to several microliters. For significantly larger volumes membrane filtration may be used, where a sample is passed through a porous membrane capable of sequestering colloidal particles and microorganisms. The cells may then be removed from the membrane with a wash solution and concentrated further *via* centrifugation. These sampling techniques are also widely used and necessary for current sterility tests such as plating/streaking by direct inoculation, and are described in detail elsewhere [6]. In order to further ensure that the sample injected into the capillary

would contain any potential microbial contamination, the volume of the sample injection may be increased by using larger inner diameter capillaries. As shown in a previous publication, sample injections of several microliters may be accomplished by using a 200 μm I.D. capillary with this CE-based sterility test [18]. Because the migration of the microorganisms within the capillary depends heavily on the conductivity and composition of the sample and buffer solutions, proper sample preparation must also include removal of ionic species and other interferences in the original sample matrix. This may easily be accomplished by a series of wash cycles that involve centrifugation of the sample and resuspension of the microbial pellet into sterile working buffer.

5.4 Conclusions

The development of single cell detection is the last significant hurdle to the development of a rapid, microfluidic sterility test for use in public and private industries. By substituting an easily obtained, relatively inexpensive synthetic compound (SB3-10) in place of the nutrient broth blocking agent, the background noise was decreased by a factor of 40 allowing a single cell present in the sample zone to be detected. This small procedural change has a huge impact on the practicality and effectiveness of the method. Future work that is necessary prior to commercial applications includes the assessment of “real world” samples using this technique, and a statistical analysis of the method’s robustness and accuracy.

Acknowledgement

We gratefully acknowledge the support of the National Institutes of Health and Pfizer, Inc. for support of this work.

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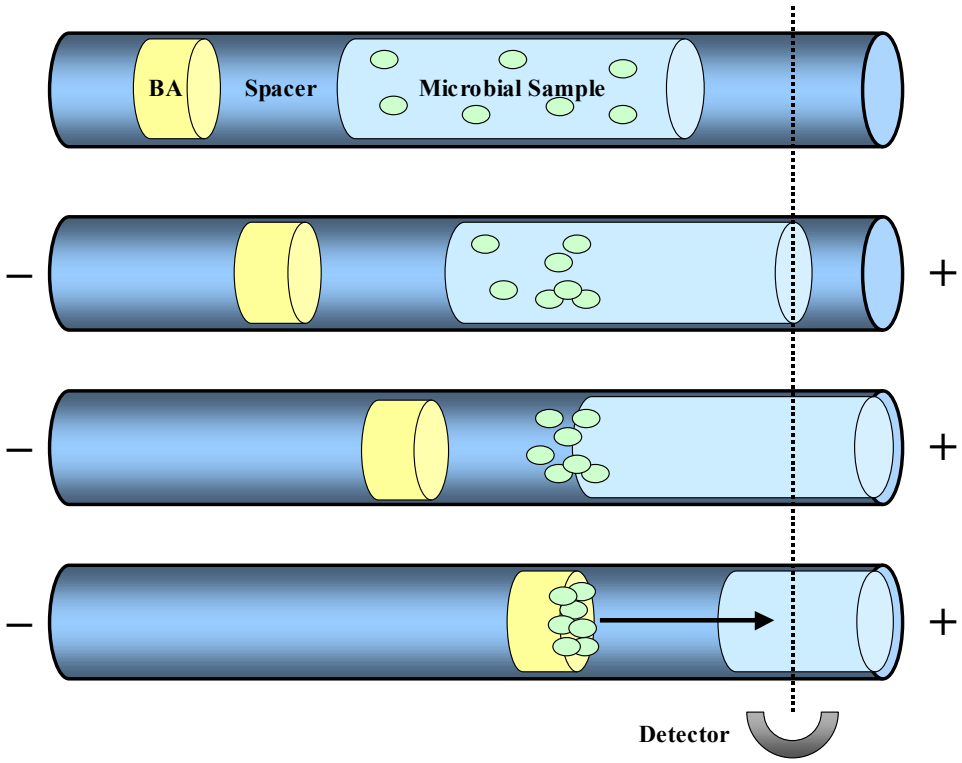

Figure 1. Schematic of CE/microfluidic-based test for microbial contamination. The entire capillary is initially filled with running buffer containing CTAB surfactant. Three injections are made prior to the run: 1) a large plug of sample containing microorganisms, 2) a spacer plug of running buffer and CTAB, and 3) a short plug of blocking agent (BA). Cells present in the sample are represented by ovals. See Experimental and Results sections for details.

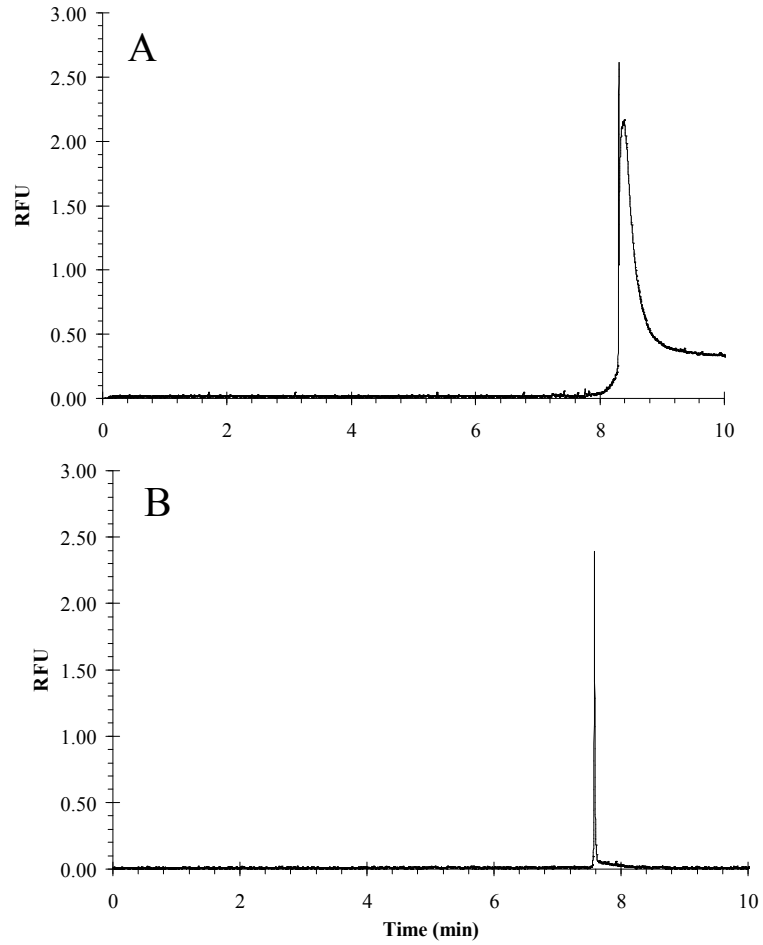

Figure 2. Electropherograms of ~25 cells of *Brevibacterium taipei* using A) 8 mg/mL of nutrient broth and B) 5 mg/mL of SB3-10 as a blocking agent. LIF detection at 516 nm. See Experimental section for method details.

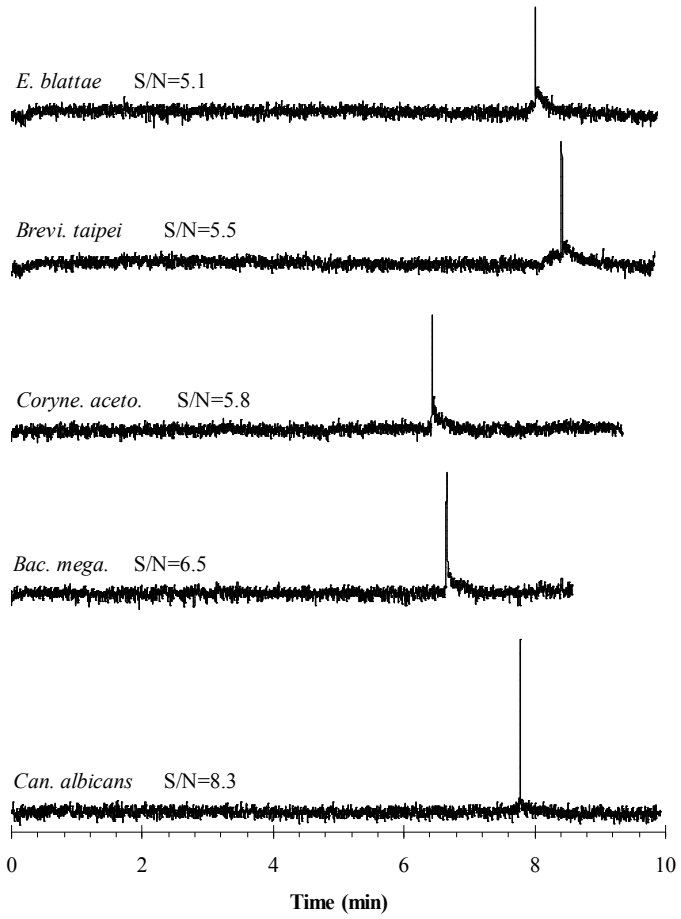

Figure 3. Electropherograms of single cells of various bacteria and fungi using the revised CE based sterility test. LIF detection at 516 nm. Concentration of SB3-10 blocking agent, 5 mg/mL. See Experimental section for method details.

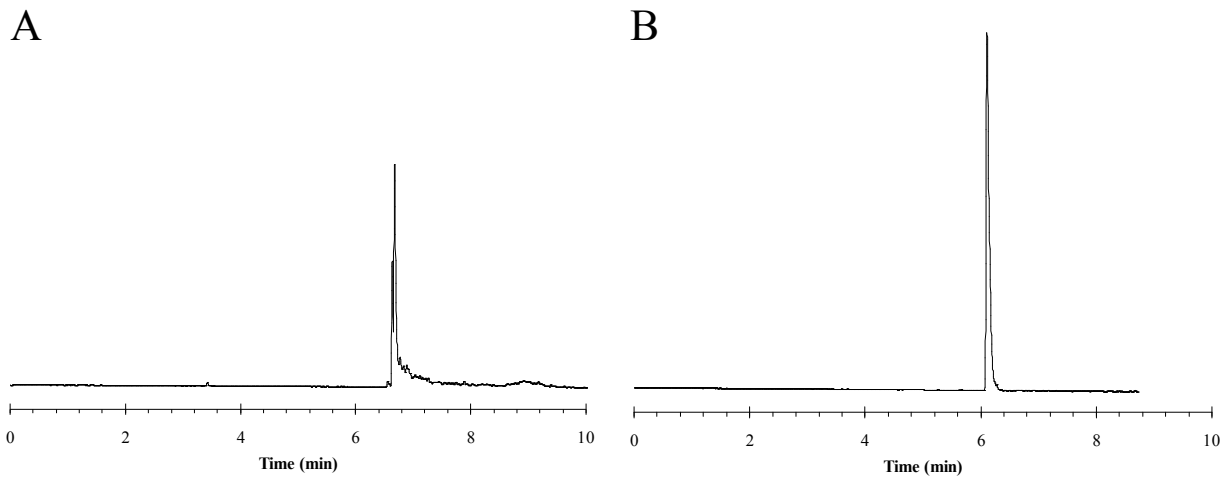

Figure 4. Effect of SB3-10 blocking agent concentration on high cell count samples. *Corynebacterium acetoacidophilum* at ~10^8 CFU/mL. A) 5 mg/mL SB3-10, B) 10 mg/mL SB3-10. Detection by Mie scattering at 449 nm. See Experimental section for method details.

Table 1. List of potential blocking agents tested.

Salts	TRIS-citrate
	NaCl
Peptides	Poly-L-glutamate (MW~10000)
	Poly-L-arginine (MW~40000)
	Gly-Gly-Gly-Gly-Gly-Gly
	Arg-Gly-Glu-Ser
	Gly-Gly-Gly
	Leu-Leu-Leu
Zwitterions	Triethylamine N-oxide
	Sarcosine
	Taurine
	Betaine
Zwitterionic Surfactants	Octyl sulfobetaine (SB3-8)
	Caprylyl sulfobetaine (SB3-10)
	Lauryl sulfobetaine (SB3-12)
	Myristyl sulfobetaine (SB3-14)
	Palmityl sulfobetaine (SB3-16)

CHAPTER 6

The Use of Ionic Liquids in the Detection of Microbial Contamination by Capillary Electrophoresis

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Electrophoresis 29 (2008) 2587-2592

Abstract

A rapid test of whether a laboratory sample contains any microorganisms is important and necessary for many areas of science and technology. Currently, most of the standard procedures for the detection of aerobic bacteria, anaerobic bacteria, and fungi, require the preparation of microbial cultures in respective growth media, which are dramatically slow. Different approaches providing fast analysis such as CE are becoming more desired. To compensate for the natural electrophoretic heterogeneity of microbes, various buffer additives were examined to stack all bacteria and fungi in a sample plug into a single peak. This peak was removed from the molecular contaminants in the sample to prevent false positives. Both cationic surfactants and ionic liquids (ILs) were investigated as run buffer additives and they are both widely applicable to different species of bacteria and fungi. Given that high concentrations of surfactants can potentially lyse cells, dicationic ILs are attractive auxiliary buffer additives for use in CE-based sterility tests. The analysis can be completed in 10 minutes, thus providing a great advantage over traditional direct inoculation methods that

require several weeks to complete.

6.1 Introduction

Developing a method capable of determining whether microbial contamination is present in a sample is important in many areas of science and technology, as well as the food/beverage and pharmaceutical industries [1]. Numerous approaches exist to test for the presence of potentially harmful microorganisms. Among them, the direct inoculation method and adaptations of this technique (such as membrane filtration) are universally accepted procedures for the determination of microbial contamination [2]. However, these methods involve growing the contaminant on a growth media, and thus suffer from long analysis times. Other approaches such as hybridization [3], amplification [4], and immunoassay [5] need considerably less analysis time, but these techniques are very complex to carry out and require significant personnel training. Also, the reagents and materials for these types of techniques are relatively expensive and they are selective for specific microorganisms. Hence they are not useful as a general sterility test in which the presence or absence of any/all microbes must be discerned.

In the last few years, sophisticated instrumental techniques for the analysis and characterization of microorganisms have become more common. In particular, methods based on capillary electrophoresis (CE) seem to be very promising [6-16]. CE is well known for rapid, high efficiency analyses and small sample consumption. However, most

microorganisms are generally characterized as colloidal particles based solely on their size, which typically have at least one dimension ranging in size from tens of nanometers to a few micrometers. The electrophoretic process is much more complicated for these particles, since their electrophoretic mobilities can no longer be estimated due to the varying surface and often non-fixed charges. Frequently a single species of bacteria or fungi will have a distribution of electrophoretic mobilities, due to their varying size and surface charges. This quality is referred to as electrophoretic heterogeneity (EH) [9, 10]. Also many microorganisms are susceptible to extremes of pH, high electric fields, or osmolarity differences, which may lyse cells.

Recently, our group developed a CE method to indicate the presence or complete absence of microbes in a sample by producing a single peak for all cells regardless of their EH or the composition of the sample [7]. It was observed that the addition of cationic surfactant, cetyltrimethylammonium bromide (CTAB), to the run buffer resulted in sweeping of bacteria and once the swept bacteria encountered the nutrient broth section (another injection plug that serves as a blocking agent) in the capillary, aggregation of the bacteria occurred (see Results and Discussion for details). This method may be used in biomedical and industrial applications as a test for microbiological contamination in pharmaceutical products, foods, beverages, as well as devices and containers. Most recently, we achieved single cell detection in laboratory samples using a modification of this method by replacing nutrient broth with a non-fluorescent blocking agent and using laser-induced fluorescence

detection [8]. In this method, cationic surfactant CTAB is still required to sweep the microbes. However, high concentrations of CTAB may lyse the cells and result in lower bacterial peak heights. To insure that no microorganism is lysed in the samples, lowering the CTAB concentration to a minimum yet sufficient concentration to sweep all cells is an important goal. This could be accomplished if other less surface-active additives could be added to replace some or all of the CTAB surfactant.

In recent years, interest in ionic liquids in scientific and industrial applications has increased due to their numerous desirable properties. Ionic liquids are thermally stable, nonvolatile, nonflammable and are good solvents for both organic and inorganic compounds [17-23]. The reported applications of ILs in CE mainly involve separations: ILs have been used as buffer modifiers or as the primary background electrolyte for the separation of achiral and chiral compounds [19, 24-28]. In these studies, monocationic ILs were investigated as potential buffer additives. It should be noted that when ILs are dissolved in aqueous solutions, they can no longer be considered ionic liquids, but rather just another dissolved salt. When added to the run buffer, the cations dissociated from monocationic ILs tend to interact with microorganisms in a manner similar to CTAB surfactant and sweep them into a single peak, regardless of the electrophoretic heterogeneity or the microbial composition of the sample. On the other hand, dicationic ILs [29-31] may be used as auxiliary buffer additives for the purpose of lowering CTAB concentration that are required. They are not as surface active and tend to be less disruptive of cell walls.

6.2 Experimental

6.2.1 Chemicals

Tris(hydroxymethyl)aminomethane (TRIS), citric acid, dimethyl sulfoxide (DMSO), sodium hydroxide, hydrochloric acid, and cetyltrimethylammonium bromide (CTAB) were all acquired from Aldrich (Milwaukee, WI). YM, tryptic soy, brain heart infusion and nutrient broths were purchased from Difco Laboratories (Franklin Lakes, NJ). Bare fused silica capillaries were purchased from Polymicro Technologies (Phoenix, AZ, USA). *Bacillus cereus* (ATCC #10702), *Brevibacterium Taipei* (ATCC #13744), *Corynebacterium acetoacidophilum* (ATCC #13870), *Escherichia blattae* (ATCC #29907), *Escherichia coli* (ATCC #10798), *Pseudomonas flourescens* (ATCC #11150), *Salmonella subterreanea* (ATCC #BAA-836), *Candida albicans* (ATCC #10231), *Cryptococcus albidus* (ATCC #10666), S*accharomyces cerevisiae* (ATCC #4003818), *Rhodotorula* (ATCC #20254) were all obtained from American Type Culture Collection (Manassas VA).

The monocationic IL, 1-hexadecyl-3-vinylimidazolium bromide (IL **0** in Table 1), was synthesized by reacting 1 molar equivalent of 1-vinylimidazole and 1-bromohexadecane. The mixture was heated to 65^{o}C and stirred for 4hrs. The bromide salt was then dissolved in water and heated to 50^{o}C for hot extraction. Eight extractions were preformed with ethyl acetate to remove impurities. The IL was then allowed to crystallize in the aqueous solution to further purify the product. The IL was then filtered and dried under vacuum.

The dication 1,6-Di(3-vinylimidazolium)hexane bromide (ILs **2**, Table 1) was

synthesized by reacting 2 molar equivalents of 1-vinylimidazole and 1 molar equivalent of 1,9 dibromohexane. The reaction mixture was stirred at room temperature until the ionic liquid solidified. The bromide salt was then dissolved in water and eight extractions were preformed with ethyl acetate to remove impurities. The IL was then placed under vacuum overnight to ensure complete dryness. IL's with the C12 and C3 linkers were made in an analogous manner.

(Pentane-1,5-diyl)-bis(trimethylammonium) dibromide (ILs **4**) was synthesized by adding 1,5-Dibromopentane (10 mmol, 2.30 g) to 30 mL of trimethylamine solution (31-35% in ethanol, ~4.2 M) cooled with ice-salt bath. The mixture was stirred with ice-salt bath for 1 h and then at room temperature overnight. The solvents were evaporated at reduced pressure, and the residue was purified by recrystallization in ethanol to obtain the product as white solid, yield: 92%, m.p.: 260°C (dec.), ^{1}H NMR (300 MHz, DMSO-d6): δ 1.28 (p, 2H, J = 7.6 Hz), 1.71 (m, 4H), 3.04 (s, 18H), 3.27 (m, 4H).

(Dodecane-1,12-diyl)-bis(trimethylammonium) dibromide (ILs **5**) was made in the same manner using 1,12-Dibromododecane (10 mmol, 3.28 g) as the linker. Yield: 89%, m.p.: 190-191°C, ^{1}H NMR (300 MHz, DMSO-d6): δ 1.24 (broad, 16H), 1.64 (m, 4H), 3.02 (s, 18H), 3.25 (m, 4H).

6.2.2 Methods

The CE experiments were performed on a Beckman Coulter P/ACE MDQ with a

photodiode array detector (Fullerton, CA, USA). The bare fused-silica capillaries used in these experiments was 30 cm long (20 cm to the detector), with inner diameters of 100 μm and outer diameters of 365 μm. The capillaries were conditioned before their first use by rinsing with 1M sodium hydroxide for 5 min, water for 5 min, sodium hydroxide for 1 min and finally water for 1 min. Between each run, 0.5 min 1 M phosphoric acid, 1 M sodium hydroxide, water, and run buffer rinses were performed. DMSO was used as a neutral marker with a concentration of ~ 10 μL/0.5 mL. The working solution of 1 mM TRIS, 0.33 mM citric acid was prepared from 10x dilution of 10 mM TRIS, 3.3 mM citric acid, and then adjusted to the desired pH with 0.1 M sodium hydroxide or 0.1 M hydrochloric acid when needed. Surfactants or ionic liquids were then added to this working solution to obtain actual run buffer solutions.

All bacteria and fungi were grown in medium according to the instructions supplied by the manufacturer. When needed, the microorganisms were withdrawn with the broth and centrifuged, after which the excess broth was removed and the microorganisms were suspended in the working concentration TRIS/citric acid buffer (pH 7). The resulting concentration of cells was about 10^8 CFU/mL. These were then used as samples for analysis. The lowest number of microbes that could be detected with UV was about 200 cells, comparable to the previous studies [7, 8]. Prior to the separation, the capillary was filled with run buffer. The following three-injections *via* hydrodynamic pressure of 0.5 psi was performed: the sample of microbes was injected for 5 s, followed by the injection of run

buffer for 5 s, and finally nutrient broth of 8 g/L was injected for 1 s. All separations were performed in the reverse polarity mode. Data analysis was done with Beckman System Gold software.

6.3 Results and Discussion

6.3.1 Three-injection method

The goal of our research was to develop a rapid method capable of determining whether any microbial contamination is present in a sample, regardless of the individual species, its electrophoretic heterogeneity, or having a combination of different types of microbes in the sample. As stated previously, we performed a series of experiments using CTAB as a run buffer additive to achieve a single peak for bacteria by injecting three plugs in sequence, namely bacteria sample, run buffer containing CTAB, and nutrient broth segment [9]. Figure 1 shows the schematic of this three-injection method. Prior to each run, the capillary is filled with run buffer containing cationic surfactants or cationic ILs in the running buffer. Thus when the voltage is applied, the EOF direction is reversed (i.e., towards the anode) as is the flow of the sample plug and nutrient broth segment. The run buffer additive (*i.e.* cationic surfactants) residing at the front of the microbes (on the anodic side) in the capillary migrates towards the cathode while the microbes move towards the anode, and when they encounter the cationic surfactants, they are coated by (or dynamically adsorb) the surfactants. Then the microbes reverse direction and move towards the cathode. As the swept

microbes traveled through the buffer spacer (2nd injection region), they are removed from any potential contaminants in the sample region. Upon reaching the front of nutrient broth segment (which serves as a blocking agent), the microbes begin to aggregate and form a large macroparticle. Once the macroparticle is formed, it quickly loses mobility and thus migrates at the same speed and direction as that of EOF. Figure 2 shows an example electropherogram of this three-injection method for the fungi S*accharomyces cerevisiae* where: A was obtained at UV 280 nm (this wavelength was used to detect the relative position of fungi peak (*S.cerevisiae*) to the nutrient broth segment), showing that the *S.cerevisiae* peak is at the front edge of the nutrient broth; B shows the detection at UV 214 nm, where the absorbance of nutrient broth greatly increased; C was also obtained at UV 214 nm, when EOF marker, DMSO, was injected in place of the microbial sample to represent interfering molecular contaminants in the sample; D is the overlaid electropherograms of B and C, showing that the yeast peak has moved away from the EOF marker (interfering neutral contaminants in the microbial sample) and was stopped by the blocking agent.

In the previous studies, CTAB seems to be efficient in sweeping the bacteria. However, there are some potential problems with this surfactant. CTAB may lyse cells when its concentration exceeds 2 mg/mL and this results in lower peak heights which makes this method less sensitive and potentially inaccurate [9]. Therefore in our current work, ionic liquids and other cationic surfactants were examined as potential alternative additives to the run buffer.

6.3.2 Buffer additives

6.3.2.1 Cationic surfactants (CTAB and Octadecyltrimethylammonium)

In this study, we examined four fungi (*Candida albicans*, *Cryptococcus albidus*, S*accharomyces cerevisiae*, and *Rhodotorula*)) with 6 mg/mL CTAB using this three-injection method. As fungi are less fragile and bigger than bacteria, higher surfactant concentrations may theoretically be used without lysing these cells. Post-run microscope inspection indicated that these fungi cells were not lysed.

Octadecyltrimethylammonium bromide (OTAB), an analog of CTAB, was examined as one possible buffer additive for both bacteria and fungi. A concentration as low as 0.6 mg/mL was efficient enough for sweeping fungi/bacteria. However, one drawback of this surfactant was its low solubility in water (saturation concentration ~ 0.7 mg/mL), which hindered the use of higher concentrations needed to sweep larger numbers of microbes to the nutrient broth front.

6.3.2.2 Monocationic ILs

1-Hexadecyl-3-vinylimidazolium bromide （IL **0** in Table 1）was chosen due to its structural similarity to CTAB which worked effectively for a wide variety of bacteria and fungi. This monocationic IL was also applicable to a variety of different bacteria and fungi (Figure 3a). These experiments indicated the possibility that this set of conditions may apply broadly to many microorganisms. When four bacteria and four fungi were combined in one

sample and subjected to this method, a single peak was still obtained, as shown in Figure 3b. In total, regardless of whether the sample contains many different types of microorganisms, or just a single species, the same sharp peak will occur at the same point in the capillary in each instance as indicated by the relative peak positions of sample to nutrient broth (slight variations in the EOF is inherent in CE). This behavior provides the foundation for a contamination or sterility test. Also note that it takes less than 10 minutes for any combined bacterial band to migrate to the detector. Hence, it could be used to obtain rapid information as to the presence or absence of microorganisms in a sample. Though this 1.5 mg/mL ILs concentration alone worked well for a large variety of microbes, the saturation concentration of this ILs is as low as 1.7 mg/mL, and therefore we could not test higher ILs concentrations.

6.3.2.3 Dicationic ILs

Several different dicationic ILs (Table 1) were tested as an alternative buffer additive to CTAB. Compared to CTAB or other cationic surfactants, dicationic ILs are not surface active and therefore should not interfere with the cell and rupture the cell membrane. Thus, higher concentrations of dicationic ILs could be used if needed without damaging the cells. If successful, dicationic ILs could be the ideal buffer additives for the purpose of this study. Unfortunately, all the dicationic ILs examined did not sweep microbes, which indicates the importance of the hydrophobic interaction between long alkyl tail of CTAB and the surface of the microbes that could not be achieved by dicationic ILs. However, by adding dicationic

ILs and CTAB together in the run buffer, the CTAB concentration could be significantly lowered to 0.25 mg/mL (1/4 of the CTAB concentration in the previous study) and therefore greatly reduce the potential for cell lysis. CTAB played two roles in the electrophoresis when it was the only buffer additive: first, these surfactants electro-statically coated the capillary wall and reversed the EOF; second, the surfactants swept and stacked microbes into a single peak in the front of the nutrient broth blocking segment. When mixed with CTAB, the dicationic ILs competed with CTAB to coat the capillary wall and therefore the amount of CTAB used as an additive was greatly lowered. Clearly, dicationic ILs can be used as auxiliary buffer additives in conjunction with cationic surfactants. This allows the possibility of greatly decreasing the concentration of the cationic surfactant while maintaining good results that usually require higher surfactant concentration.

6.3.2.3.1 Effect of chain length and head group of dicationic ILs

ILs **1-5** (Table 1) were dissolved separately in buffer to make a concentration of 2.74mM (1 equivalent to 1 mg/mL CTAB) and examined for their ability to reverse EOF. All five dicationic ILs were able to reverse EOF. The ability of dicationic ILs to reverse EOF (indicated by faster EOF in reverse polarity mode) increases with longer chain lengths (i.e. IL **3** reverses EOF to a greater extent than IL **1**; IL **5** than IL **4**). When mixed with CTAB, both IL **2** (C6-chain) and ILs **3** (C12-chain) decreased the required CTAB concentration to 0.25 mg/mL. Overall, long chain length was desired for the purpose of short analysis time.

In addition, the minimum concentration of IL **5** required to reverse EOF is lower than that of IL **3**. Compared to the head groups of IL **5**, IL **3** had positive charge dispersed in the aromatic head groups which made weaker the electro-static interaction with silanol group on the capillary wall and thus less easy to reverse the EOF. Among five dicationic ILs tested, IL 5 was the best to reverse EOF given its long chain length and head groups with high positive charge density.

6.3.2.3.2 Optimization of the analysis

In a previous report, it was shown that 2.74 mM CTAB (i.e. 1 mg/mL) was optimal to sweep bacteria [7]. In this study, dicationic ILs were used in conjunction with CTAB as auxiliary buffer additives. The purpose of using dicationic ILs is to minimize the CTAB concentration which may lyse cells. In this section, various concentration combinations of IL **5** and CTAB in the run buffer were tested to study their effects on the system. Figure 4 shows that the optimal combination was 2.74 mM dicationic ILs and 0.25 mg/mL CTAB . When the concentration of the IL was decreased to 1.37 mM, peak tailing appeared. Further decreasing in the ILs concentration led to broadening of the peak which indicated insufficient sweeping effect (A, B in Figure 4). However, when the IL concentration was increased to 5.48 mM, some cells were swept through the nutrient broth segment since this blocking segment was not able to stop them (G in Figure 4). With the IL concentration fixed at 5.48 mM and the CTAB concentration decreased to 0.20 mg/mL, insufficient sweeping occurred as indicated

by multiple peaks in front of the nutrient broth (H in Figure 4). With the IL concentration fixed at 2.74 mM and keeping increasing CTAB concentration to 0.30, 0.50 mg/mL, efficient sweeping occurred (E, F in Figure 4). However, when decreasing CTAB concentration to 0.15 mg/mL, some loss of cells occurred during sweeping as indicated by small bacterial peaks in front of the major bacterial peak (C in Figure 4). In conclusion, the minimum CTAB concentration that can be achieved is 0.25 mg/mL when used in conjunction with 2.74 mM IL **5**.

6.4 Conclusions

A rapid method is needed for the detection of the existence of microorganisms in samples. Standard culture methods are universally accepted procedures for sterility test, but they require long analysis time. In this study, different buffer additives including ILs were tested to provide a quick answer regarding the presence or absence of microorganisms. These buffer additives are applicable to a wide range of bacteria and fungi, even to samples containing a variety of different bacteria and fungi mixture. Though dicationic ILs could not replace CTAB or other surfactants that may lyse cells, they could be used as auxiliary buffer additives to lower CTAB concentrations to 1/4 of that needed in cases without the IL additives and thus, lower the risk of lysing cell. Future work will focus on applying this method to “real world” samples for diagnosis.

Acknowledgements

We gratefully acknowledge the support of this work by the National Institutes of Health, NIH RO1 GM53825-11.

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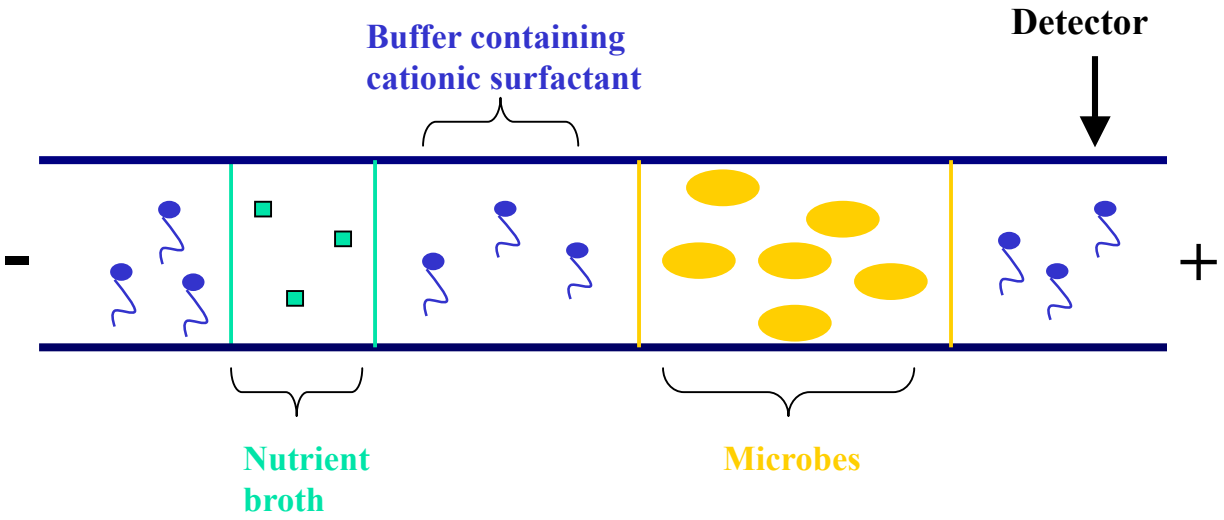


Figure 1. Schematic of the three-injection method. The capillary is firstly filled with run buffer containing CTAB or other surfactants prior to the run. Three injections are then made: 1) a plug of microbial sample, 2) run buffer as a spacer, 3) nutrient broth segment as blocking agent. Finally electrophoresis begins. Cells are represented by ovals.

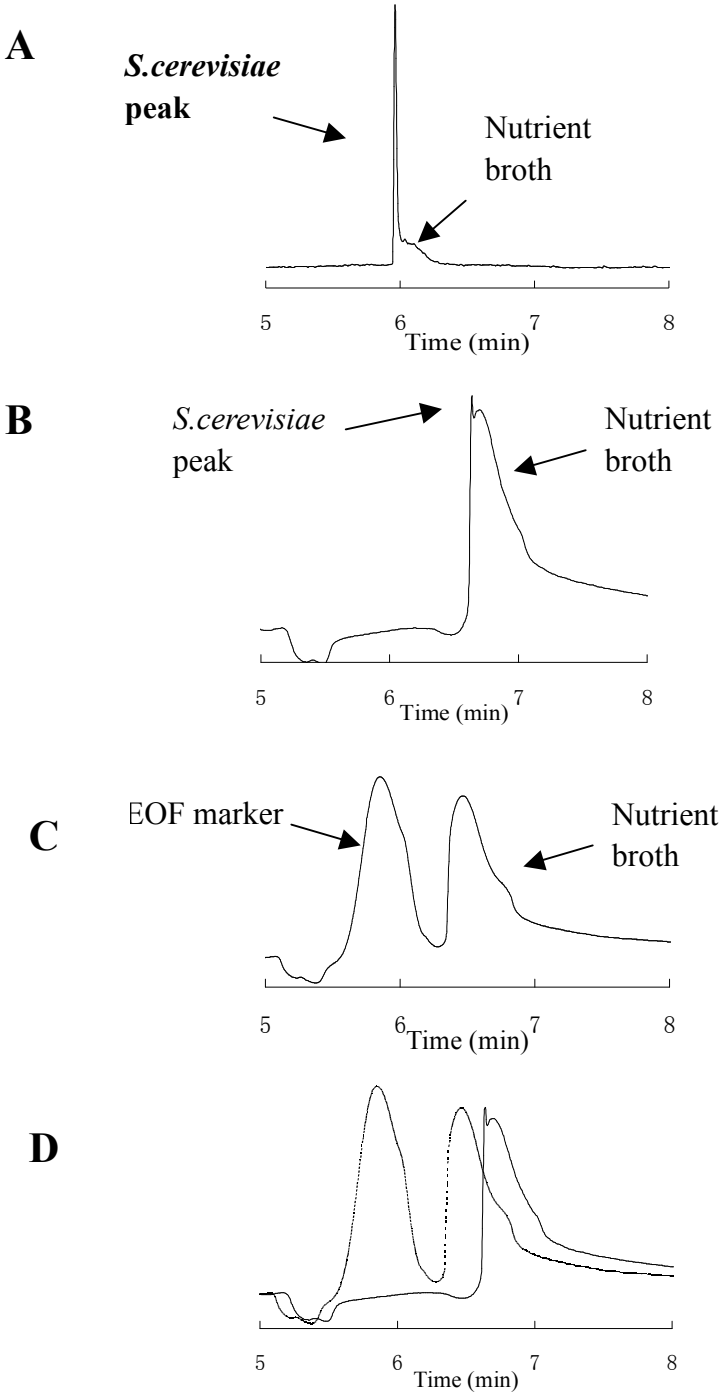
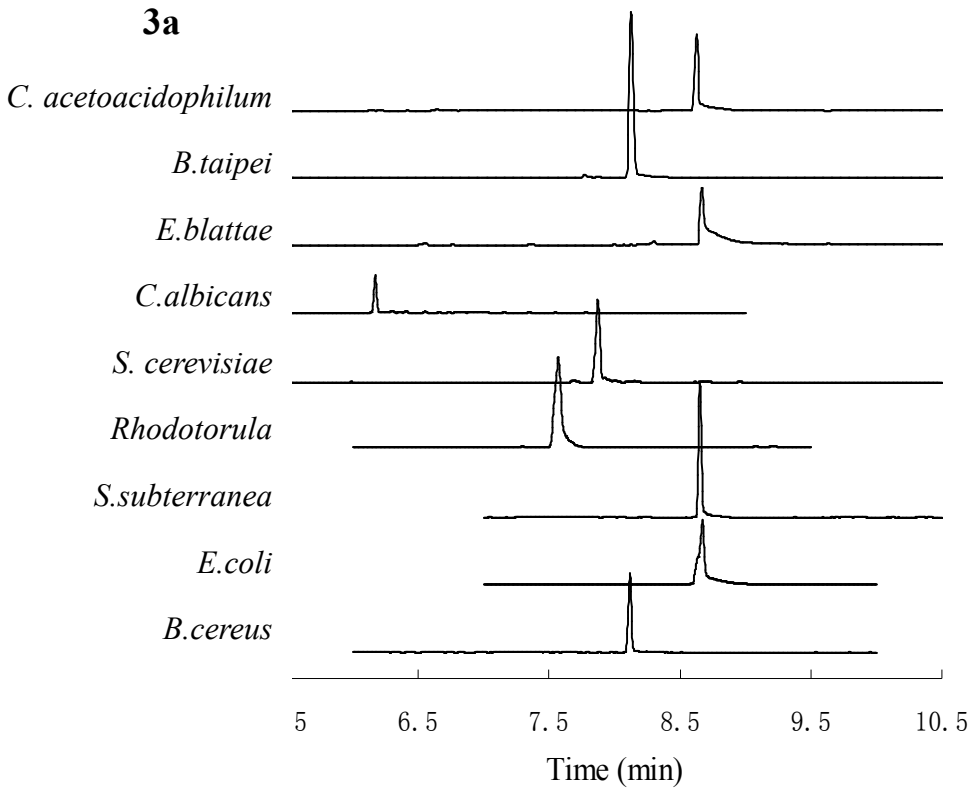


Figure 2. Example electropherograms obtained using the three-injection method. Conditions: Running buffer is 1 mM TRIS/0.33 mM citric acid with 6 mg/mL CTAB, pH 7. Sample buffer is 1 mM TRIS/0.33 mM citric acid, pH 7. Nutrient broth concentration is 8 g/L. Voltage is -2kV. See Results and discussion for details.



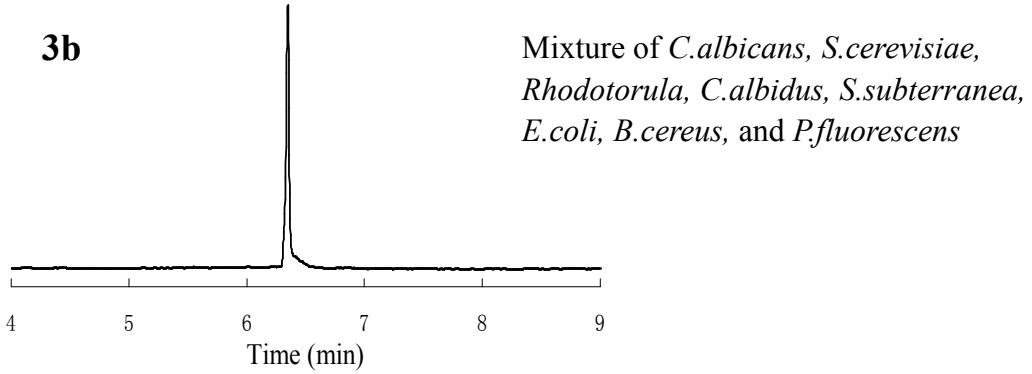


Figure 3a) Six different bacteria and three fungi successfully swept by monocationic ILs. Conditions: Running buffer is 1 mM TRIS/0.33 mM citric acid with 1.5 mg/mL 1-hexadecyl-3-vinylimidazolium bromide (IL **0**), pH 7. Dectection wavelength is 340 nm. Voltage is – 2kV. **3b)** The electropherogram for a mixture of four different fungi and four different bacteria. Conditions are the same as in Figure 3a.

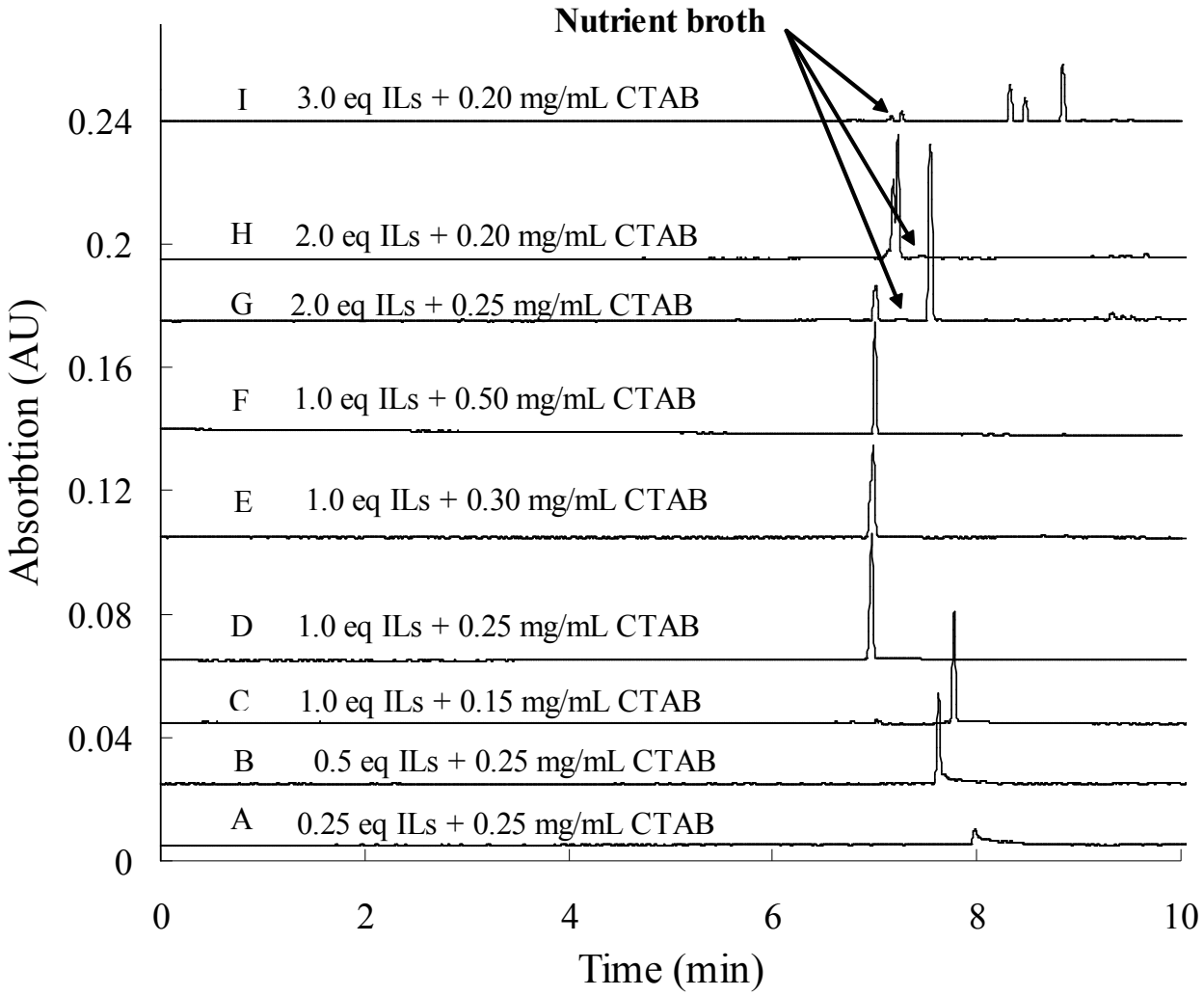
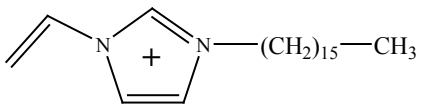
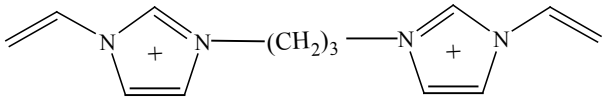
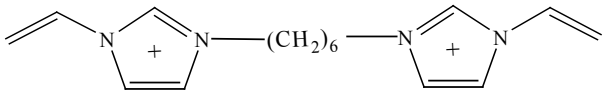
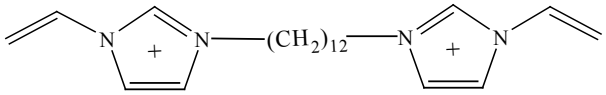
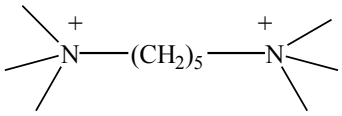
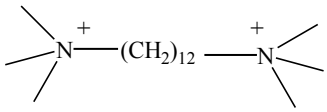


Figure 4. Optimization of dicationic ILs **5** and CTAB concentrations with *E. coli*. Detection for Electropherograms A-F was at 449 nm to eliminate the UV interference from nutrient broth segment. Detection for Electropherograms G-I was achieved at 340 nm to show the relative position of microbial peaks to the nutrient broth peak. Voltage: -4kV.

Table 1. Monocationic and dicationic ILs tested. Anion is bromide in all ILs

IL 0	
IL 1	
IL 2	
IL 3	
IL 4	
IL 5	

CHAPTER 7

General Conclusions

In the first part of the dissertation, Chapter 2 shows the HPLC evaluation of a new pentaproline-based column in three mobile phase modes (normal-phase, polar organic and reversed-phase) by injecting 194 racemates into the column. Overall, the best separations were obtained in the normal-phase mode. The enantiomeric separations of 94 racemates indicated good resolving ability of this CSP (48% success rate). The enantiomeric selectivity on this proline peptide CSP is mainly due to hydrogen bonding and steric interactions. However, this column's ability to separate enantiomers that are incapable of forming hydrogen bonds indicates that other interactions (such dipole/dipole, induced dipole/dipole, and dispersion forces) might contribute to the enantiorecognition as well.

Chapter 3 demonstrates the CE enantiomeric separations of three groups of newly synthesized racemates: racemic furans, racemic isochromenes, and racemic fused polycycles. Cyclodextrin-modified micellar capillary electrophoresis (CD-MCE) was chosen over the traditional simpler method capillary zone electrophoresis (CZE) because of the solubility problems of these highly hydrophobic analytes in CZE. In general, HP-γ-CD separated more of these relatively large molecules presumably due to its well fit cavity size. The addition of an organic modifier not only helped to solubilize the analytes, but also improved separation efficiency by facilitating mass transfer. The structure of the analytes also has a great effect on

the CDs' ability to separate these enantiomers, particularly the position and size of the substituents.

In the second part of the dissertation, Chapter 4 reviews microorganism studies using capillary electrophoresis, focusing on CE methodology for sterility testing as a possible alternative to the traditional direct inoculation method outlined in the *U. S. Pharmacopeia.* Topics include basic CE theory, CE characterization of bacteria and fungi, CE sterility testing method development and experimental procedure. Future efforts will be devoted to the use of this approach with "real-world" samples such as blood.

Chapter 5 presents a single-cell detection achieved by modification of a previously reported procedure. In the previously procedure, a nutrient broth media was used as an effective blocking agent, however, the natural background fluorescence from the nutrient broth limited the detection sensitivity to ~ 50 cells. By replacing the nutrient broth with non-fluorescent zwitterionic surfactants as blocking agents, especially caprylyl sulfobetaine, single-cell detections of bacteria and fungi were achieved with signal-to-noise ratios between 5 and 9. This success provides a solid foundation for sterility testing using CE.

Chapter 6 investigates different buffer additives (such as surfactants and ionic liquids (ILs)) in CE-based sterility testing. These buffer additives showed broad applicability to bacteria and fungi, even to samples containing a mixture of bacteria and fungi. This study showed that though dicationic ILs could not replace surfactants that may lyse cells, they could be used as auxiliary buffer additives to lower surfactant concentration to 25% of that

needed in the previous study, thus greatly lowering the chance of cell lysis.

ACKNOWLEDGEMENT

The studies carried out in this dissertation were under Professor Armstrong's supervision and support. Dr. Armstrong has been a great mentor and a role model, whose broad knowledge, creative way of thinking, scientific attitude, patience with students, wonderful presentation and interpersonal skills have inspired me greatly and will benefit me in the future as well. It has been my privilege working in his group and I want to thank him for that.

I also want to thank Dr. Robert S. Houk, Dr. Jacob W. Petrich, Dr. Klaus Schmidt-Rohr, and Dr. Yan Zhao for their time serving on my committee and their stimulating questions and constructive advice.

During these years, I received generous assistance from our research group members, visiting scholars, and collaborate researchers. The Departments of Chemistry at Iowa State University and University of Texas at Arlington also provided countless support. I really appreciate all that they have done for me.

Without all these guidance and support, it would have been impossible for me to finish my Ph.D. study. I want to take this opportunity to sincerely thank everyone.