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ADVANCES IN FLOW EXTRACTION TECHNIQUES: APPLICATIONS IN FORENSIC TOXICOLOGY

by

Kristina L. Peterson

A dissertation submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

University of Washington

1997

Approved by _____
Chairperson of Supervisory Committee

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Doctoral Dissertation

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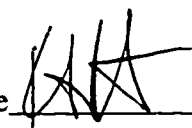
Signature 

Date 5-28-97

University of Washington

Abstract

ADVANCES IN FLOW EXTRACTION TECHNIQUES: APPLICATIONS IN FORENSIC TOXICOLOGY

by Kristina L. Peterson

Chairperson of the Supervisory Committee: Professor Gary D. Christian
Department of Chemistry

The goal of this work was to advance existing flow extraction techniques by incorporating repeatable fluid handling systems. The work contained in this dissertation describes the development of novel microscale extraction methods to isolate organic compounds from biological matrices encountered in forensic toxicology. Two new extraction techniques were developed; a sequential injection liquid extraction manifold and a fiber optic fluorescence detector for monitoring a heterogeneous immunoassay. The merits of each were compared to conventional methods in forensic toxicology.

Conventional liquid solvent extraction was enhanced with sequential injection extraction. The sequential injection manifold took advantage of existing liquid extraction protocols and applied them to a new method of manipulating immiscible solvents in thin tubing, that combined the merits of flow injection extraction and sequential injection analysis. This method was used to extract both weakly basic and acidic drugs of therapeutic interest from urine.

The merits of conventional solid phase extractions were shown with commercially available columns filled with a chemically modified solid phase to isolate the

biotransformation products of cocaine. A novel flow cell designed to trap beads and monitor them with fiber optics, representing a combined extraction and detection system, was developed. An immunoassay using commercially available reagents was applied to this system to determine the feasibility of using flow injection renewable surface immunoassay in a quantitative immunoassay.

Both of the techniques developed here require computer control, and enclosed and small volume samples. The advantages to such methods are that they can be readily automated and keep potentially hazardous solvents and samples away from the operator, and the produce less waste.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGMENTS

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Most importantly, she would like to thank Jamie Ann Ginter whose confidence in her abilities never waivers and whose friendship and love are irreplaceable.

DEDICATION

The author wishes to dedicate this dissertation to her mother, Judy Peterson.

CHAPTER 1: INTRODUCTION TO SAMPLE PREPARATION AND ANALYTICAL TECHNIQUES

1.1 SAMPLE PREPARATION

The complexity of most samples makes them unsuitable for direct injection into sophisticated analytical instrumentation such that sample preparation is usually required. Sample preparation includes a variety of techniques to remove matrix interferents and concentrate the solutes (1-2). These steps are essential to ensuring both that the sample is in a matrix compatible with the detector, and that the solute concentration is in the linear range of the detector. Conventional liquid solvent extraction with volumetric glassware is widely used for the sample preparation of aqueous biological samples. Solvent extraction has the potential to impart selectivity by separating various compounds which may interfere with the analysis, away from the compounds of interest. Also, solvent extraction may increase detectability by concentrating the solute in a smaller volume. However, solvent extraction is usually the bottle neck in a laboratory because it is time consuming and difficult to automate (3). While sample preparation is necessary in any analysis that begins with a complex sample, the techniques developed in this work were applied to the field of forensic toxicology.

Forensic toxicology involves the use of toxicological studies or analyses in legal proceedings. Toxicology is the study of substances and their concentrations that are harmful to biological systems and as such toxicology measures the effects of a substance via a biological response. Analytical toxicology encompasses the detection

and identification of the previously mentioned substances with laboratory instruments. The facet of forensic toxicology that detects and identifies those substances associated with death is postmortem forensic toxicology. The postmortem forensic toxicology analysis of biological fluids is used in a death investigation to support other findings regarding possible causes of death.

Postmortem forensic toxicology samples include many biological fluids in various amounts, including but not limited to blood, urine, hair, sweat and sebum. This work has focused on urine because it is readily attainable in most circumstances. Sample preparation is required with urine because it contains a variety of endogenous molecules that can clog analytical instruments. It must be buffered to a suitable pH for extraction because it has a pH range from 5.5 to 7 that affects the degree of ionization of compounds and their subsequent extraction (3).

The selectivity and recovery of the extraction technique must be considered along with the overall analysis. Some investigations require only the simple confirmation of one drug to support evidence found at a death scene, while at the other end a complete screen for unknown poisons may be required in a case of death by unknown causes. Many different classes of organic compounds may be present in a sample and the concentration of each may be significant. Extraction methods that isolate a variety of compounds derived from a parent drug, usually with a range of functional groups and consequently variable polarity, are needed. Some drugs are excreted relatively unchanged, while others are rapidly metabolized and found in the urine at trace levels making the recovery of the analyte more important in some cases (1).

Some compounds are present more often than others because they are routinely abused and therefore seen in death investigation cases. Other compounds appear in death investigation cases because they are commonly prescribed for therapeutic reasons. This work has been limited to the more commonly abused drugs; cocaine and opiates, and those therapeutic; barbiturates and selective serotonin reuptake inhibitors as model compounds. However, the extraction methods presented here could be extended to other compounds of similar structure.

Advances in analytical instrumentation such as gas chromatography and mass spectrometry have increased the resolution and identification of complex mixtures and have been used extensively in forensic toxicology (1). However, while rapid developments have been made in data processing and analytical instrumentation, sample preparation methods have remained relatively unchanged. The methods described in this dissertation, sequential injection extraction (SIE), solid phase extraction (SPE) and flow injection renewable surface technology (FIRST), are sample preparation systems that rely on flow based extraction. SIE uses liquid solvents and SPE and FIRST use sorbents that can be considered solvents that have been immobilized on solid phases. These are not chromatographic methods because analytes are partitioning into a very small number of theoretical plates and are recovered from the solvent so that they can undergo further analysis. Background information on the topics discussed in this work is presented below.

1.2 EXTRACTION PRINCIPLES

In any type of extraction, where a compound, I, transfers between phases at the phase boundary, the degree of extraction depends on the volume-phase ratio, the

number of extraction steps, and the distribution coefficient, K_D (4). The distribution law is independent of concentration and predicts that, at equilibrium, the distribution coefficient K_D, is equal to a ratio of the solute activity [I], in the two immiscible phases. Assuming an extraction of component I from aqueous to organic phases, I_a -> I_o:

$$K_D = [I]_o / [I]_a \qquad (1.1)$$

$$\{I\} = \gamma_I [I] \qquad (1.2)$$

At low solute concentration, the activity coefficient, γ_I, goes to one and the K_D is related to the ratio of the solute solubility in the two phases, where the distribution coefficient reduces to a ratio of the concentrations of the analyte in both phases. The distribution ratio, D is represented as:

$$D = \{I\}_o / \{I\}_a \qquad (1.3)$$

When there are no side reactions, $K_D = D$.

Experimentally, the extraction efficiency, %E is measured as an indicator of the distribution ratio. The %E represents the amount of analyte, I, in the organic phase as compared to the total amount of I in both phases:

$$\%E = 100\, V_o\{I\}_o / (V_o\{I\}_o + V_a\{I\}_a \qquad (1.4)$$

$$\%E = 100\, D / (D+(V_a/V_O)) \qquad (1.5)$$

Deviations occur at very high concentrations due to limited solubility in a given volume. The dissolution rate, d_r, describes the rate at which %E approaches K_D. The dissolution rate is a first order process that depends on the diffusion coefficient, d; the solute surface area, A; the concentration of the solute at the surface, $\{I\}_{surface}$; the concentration of the solute in bulk, $\{I\}_{bulk}$ and the volume of dissolution, V.

$$d_r \propto dA(\{I\}_{surface}-\{I\}_{bulk}) / V \qquad (1.6)$$

The diffusion coefficient, d, describes the average distance, x_{rms}, at which the solute molecules travel in time, t, as estimated through the Einstein/Smouochowski equation (5).

$$2dt = \langle x_{rms} \rangle^2 \qquad (1.7)$$

The diffusion coefficient depends on molecular size and shape. Vigorous mixing via shaking breaks the phases into smaller units, increasing the surface area, and reduces $\{I\}_{surface}$ -$\{I\}_{bulk}$ to 1. It also gives the molecules additional kinetic energy.

In spite of the theoretical potential of solvent extraction, there are practical concerns that limit the technique (1). The physical parameters discussed above indicate that optimal extraction conditions are vigorous mixing, a large distribution ratio, a large diffusion coefficient and a large organic-to-aqueous phase ratio. Conventional solvent extraction involves shaking the two phases, but this often leads to emulsion formation. Samples are often centrifuged to separate the phases based on density, but this adds time to the analysis and does not always result in complete separation. Increasing phase ratio by increasing the volume of high purity organic reagents used in solvent

extraction is not desirable because the solvents are often expensive, potentially hazardous to the operator's health, and flammable, making waste disposal expensive. Conventional solvent extraction often uses disposable glassware, adding to the disposal cost. Currently available batch techniques cannot be readily automated or interfaced to analytical instruments, making the procedure labor intensive and open to human error. Many of these limitations can be addressed with flow based extraction techniques that use immobilized solvents, as shown in the following sections.

1.3 FLOW INJECTION ANALYSIS

Flow injection analysis (FIA), a commonly used tool for modulating impulse responses, has improved both liquid and solid extraction by its ability to handle small amounts of fluids, its repeatability and its ease of computer control and automation (6). FIA was first introduced by Ruzicka and Hansen in 1975 (7). A basic flow injection manifold consists of a unidirectional pump, injection valve, reaction coil and detector (Figure 1.1). A sample is introduced into a flowing carrier or reagent stream and detected downstream after being physically modulated by transforming from a plug into a bullet due to flow transport (Figure 1.2) and chemically modulated by its diffusional mixing with the adjacent carrier or reagent at the front and rear boundaries. FIA's attributes include timed, and reproducible physical and chemical modulation.

In a homogeneous system, where the sample and reagent have the same physical interaction with the wall material, the physical modulation can be measured by the maximum dispersion coefficient, D_{max} (7).

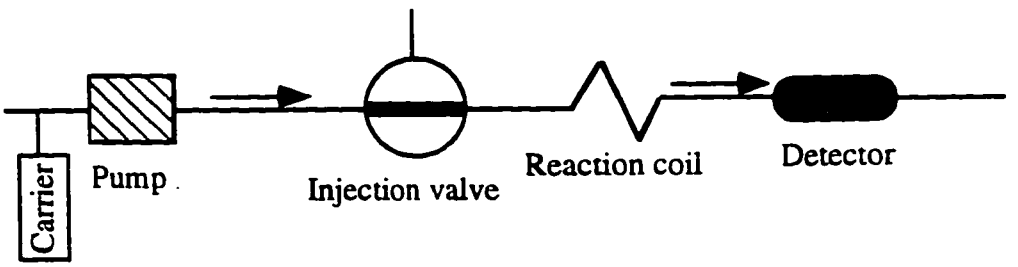


Figure 1.1 FIA Manifold

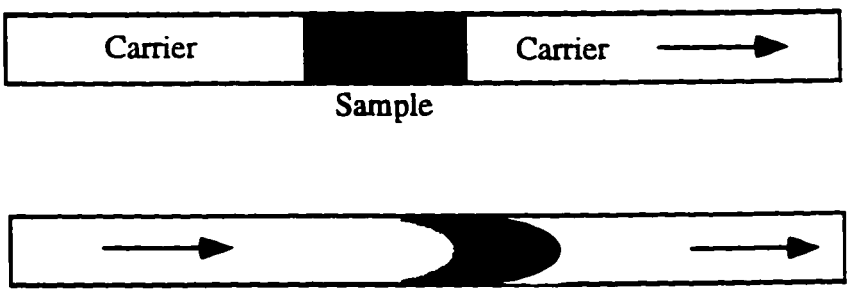


Figure 1.2 FIA Flow Profile

$$D_{max} = C_{max} / C_t \qquad (1.8)$$

If there is no reaction between the sample and the reagent stream, then D_{max} measures the degree of sample dilution in the reagent, or the ratio of the original concentration, C_{max}, to the concentration, C_t at a given time, t. The resulting output is a concentration gradient (Figure 1.3).

FIA provides a repeatable way to monitor aqueous chemistries because each step is automated and can speed up analysis time because non-equilibrium measurements can be made. FIA tends to use less reagents and disposable glassware than the batch methods it replaces and as an enclosed system, is not subject to outside contamination and protects the user from any harmful reagents or samples.

1.4 SEQUENTIAL INJECTION ANALYSIS

Sequential injection analysis (SIA) is a variant of FIA that uses a bi-directional pump and multiposition selection valve (Figure 1.4). The bi-directional pump in SIA adds versatility to FIA system without compromising reproducibility. Samples and reagents can be introduced in any order, depending upon the requirements of the analysis (Figure 1.5). SIA does not require continuous pumping of the carrier stream and the pump can alternate back and forth to promote mixing via shaking (8-9). The repeatable control of fluids in both FIA and SIA make them suitable techniques to couple with solvent extraction.

1.5 FLOW INJECTION EXTRACTION

In order for FIA to be useful in extraction, it requires two phases. In 1978, Karlberg and Thelander developed flow injection extraction (FIE, 10). A second pump was added to a FIA manifold and organic and aqueous carriers and formed a stream of alternating segments (Figure 1.6). Aqueous samples were injected into the segmented carrier stream and the solute partitioned into the two phases at the phase boundary of each segment (10). The phases were separated by physical means downstream, and the analyte of interest was detected in one phase. The segmentation of organic and aqueous phases in FIE leads to discontinuous profiles, where the dispersion process is limited at the phase boundaries. The same parameters used to optimize solvent extraction are of concern in FIE.

The enrichment factor, EF, is a measure of the preconcentration ability of a FIE system (6).

$$EF = V_o\{I\}_o / V_a\{I\}_a \qquad (1.9)$$

It compares the amount of solute in the organic phase after extraction to the amount of solute in the original aqueous phase and approaches the volumetric ratio as %E approaches 100.

Observations of the two phases revealed that each phase interacted differently with the tubing material. In non-polar Teflon tubing, the organic segments wetted the walls, forming a film that surrounded the aqueous segments (Figure 1.7). The thickness of the organic film influences extraction by affecting the mass transfer of analytes into and out of the film (11). Early FIE work measured the film thickness per unit length, d_{film},

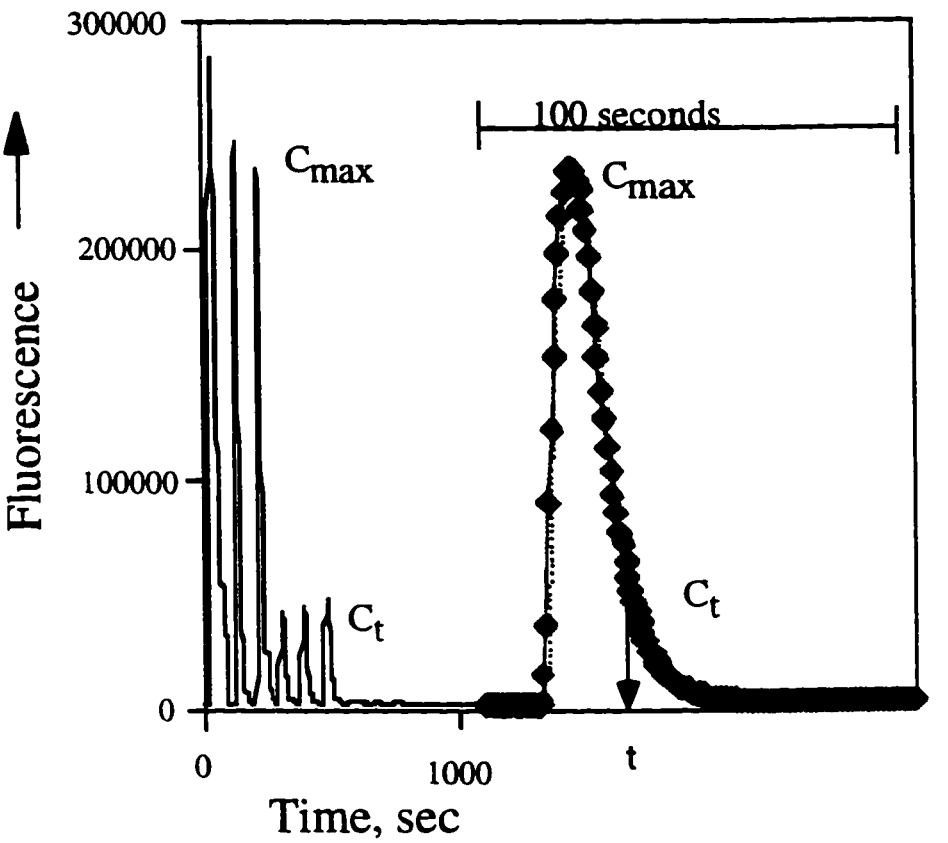


Figure 1.3 FIA Concentration Profile

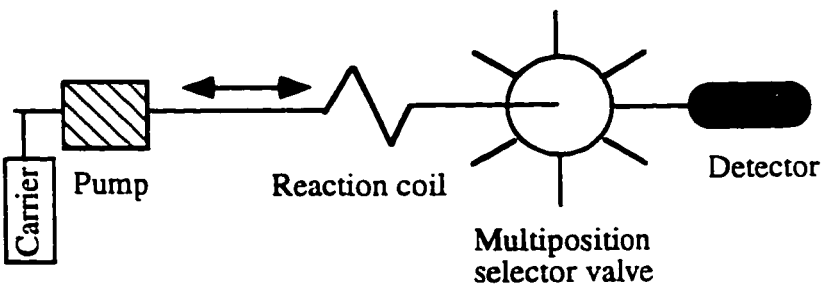


Figure 1.4 SIA Manifold

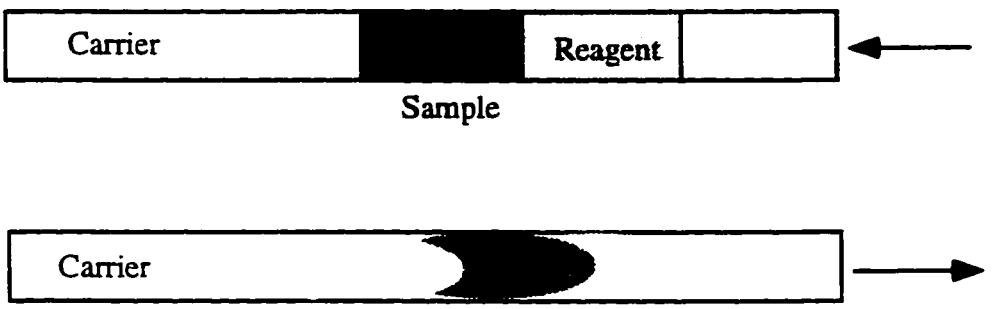


Figure 1.5 SIA Flow Profile

and its dependence on the experimental variables flow velocity, u, and tubing diameter, d_{tube}, and the organic solvent characteristics viscosity, η, and surface tension, γ (11). This information led to a general relationship of

$$d_{film} = k\, d_{tube}\, (u\, \eta/\gamma)^a \qquad (1.10)$$

where k and a are constants between about 0.5 and 0.66 (11-12). Literature values for the solvent parameters are useful for predicting relative film thickness over a series of analytes, but the equation is not recommended for predicting absolute film thickness. In a typical FIE system, the film thickness is on the order of 30 to 100 μm in 0.5 mm inner diameter tubing. As seen in equation 1.7, if solute molecules have a diffusion coefficient of 500 μm^2/sec it would take less than 1 second for a solute to diffuse through the film. The fast mass transport of the analytes and thin film will lead to a small difference between the concentration on the surface and in the bulk. Several researchers have measured the progression of an extraction by FIE (13-15).

Recently Lucy and coworkers illustrated and exploited the temporal as well as spatial resolution of analytes due to the organic film present in FIE (16-18). As a portion of the organic phase wets the walls it slows down the overall flow rate of the organic solvent and the analytes in it, relative to those in the aqueous phase (18). Using the resulting differential flow velocities between organic and aqueous phases, they showed that unextracted compounds present in a faster moving aqueous phase are separated in time from those extracted into a film-forming organic phase. Detection of the time-resolved compounds was done either in a homogenate produced by mixing the aqueous and organic segments with methanol (16) or in the aqueous phase after back

extraction (18). This is an important improvement as it makes unnecessary the physical separation of the two phases and therefore the use of a phase separator, which has been a weak step in the overall procedure due to fouling, by interfering compounds and carryover from highly concentrated samples (6).

Reports by Facchin and Pasquini using glass tubing (19) and by Luo et al., with Teflon tubing (20), show the usefulness of thinly coated solvent wall extraction and the benefits of combining air segmentation with extraction. These reports, essentially used FIA to coat the inner wall of the tubing with and organic solvent and then passed an aqueous sample through the tubing, where analytes were extracted at the phase boundary. Other recent advances in FIE have increased the precision and spatial resolution of the organic and aqueous phases (6, 12). In FIE, flow replaces shaking in conventional solvent extraction. However, current on-line detection of compounds is not suitable for complex samples. In these cases, the two phases must be recovered and separated for further analysis.

The advantages of FIE were developed into a new technique in the present study by using a bi-directional piston pump and multiposition selection valve to design a sequential injection extraction (SIE) system. SIE uses the physical characteristics of FIE with an SIA manifold. Instead of injecting a sample into a segmented stream, samples are injected immediately after an organic segment and both are pushed through the tubing together. In microtubing, the differences in the affinity of the two phases for the wall material causes one phase to flow inside the other, resulting in a large surface area of contact for mass transfer. The difference in the affinities for the tubing material in SIE replaces density separation via centrifugation in conventional solvent extraction.

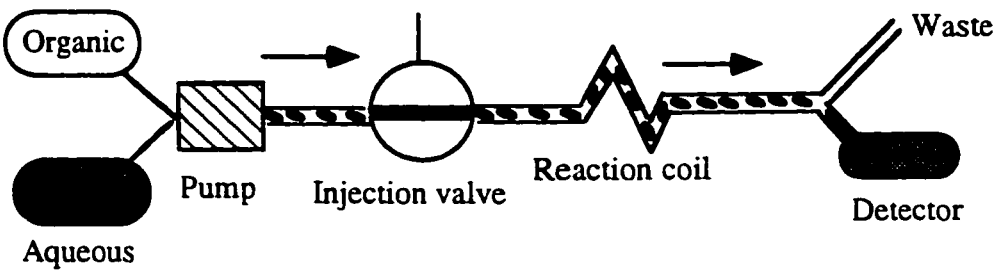


Figure 1.6 FIE Manifold

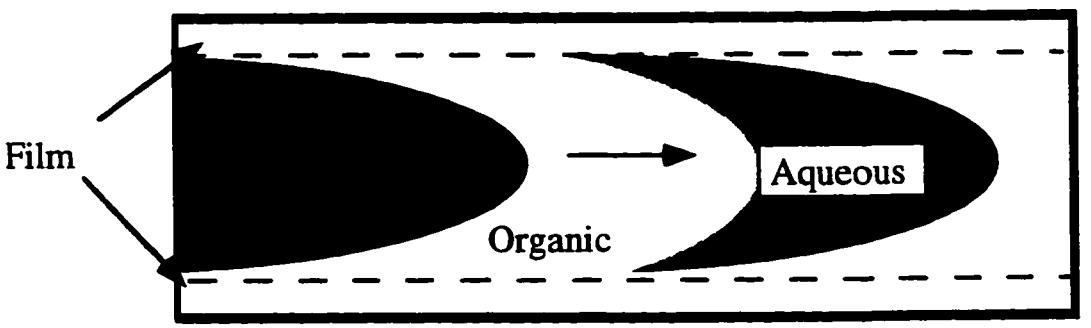


Figure 1.7 Organic Solvent Wetting the Walls of Teflon Tubing

1.6 SOLID PHASE EXTRACTION

Solid phase extraction (SPE) is another way to addresses many of the practical problems encountered in conventional liquid extraction and has been widely used in forensic toxicology (21-23). A growing number of chemically modified particles allow for the selective isolation of classes of compounds. The availability of phases with combinations of partition mechanisms has expanded the range of compounds isolated in each analysis (22). SPE has been used in the extraction of biotransformation products along with the parent drug in order to determine routes of administration (24) or to link pharamocokinetic studies to information on abused drugs and to establish markers for drug use in cases where the primary metabolite may have degraded (25).

In SPE, the solvent is immobilized on solid microparticles (Figure 1.9). As a liquid sample flows over the microparticle surface, the solute partitions between liquid $\{I\}_l$ and solid phases $\{I\}_s$ as determined by the distribution ratio,

$$D = \{I\}_s / \{I\}_l \qquad (1.11)$$

The action of flow over the immobilized solvent provides the contact area between phases that would be due to shaking in conventional solvent extraction, without forming emulsions. Since one phase is immobilized, flow also separates the two phases, so centrifugation is not required. In conventional SPE, by manipulating the pH and polarity of the elution solvent compounds are desorbed from the microparticles with relatively non-hazardous solvents. The eluent is collected and the concentration of analytes in the eluent is measured. Less than 100% recovery occurs for those compounds which either do not bind to the material in the adsorption step or do not elute from the column in the desorption step.

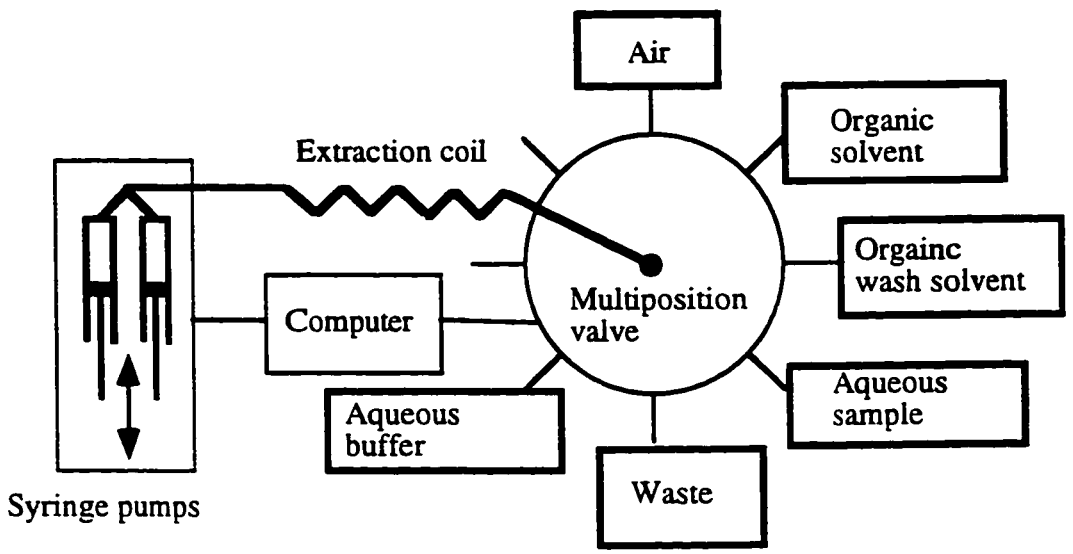
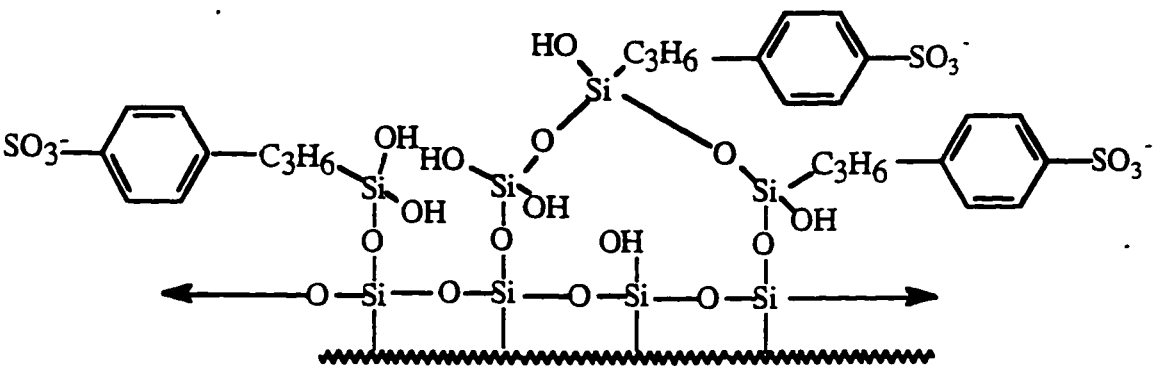


Figure 1.8 SIE Manifold

A range of chemically bonded phases that provide selectivity based on their partition mechanism are available. Ion exchange phases separate molecules based on their charge, partition phases use differences in polarity between molecules, affinity phases use a variety of natural biospecific interactions, and phases with controlled pore size separate molecules due to their size (26). Binding is based on a combination of non-covalent interactions including van der Waals, hydrophobic, pi-pi and Coulombic. These interactions are disrupted by changing the salt concentration, pH, or polarity of the solvent.

The substrate of the microparticles are characterized by their shape, hydrophobicity, stability of the bonded phases at various pH values and pore size as summarized in Table 1.1. Extractions on solid particles are limited to the availability of suitable chemistries which will however continue to grow. As the popularity of SPE has increased, automated systems and those with robotic control have been introduced to reduce labor (23, 27).

Silica is the most widely used substrate in commercially available prepacked solid phase extraction columns (ca. 100 mg). Columns of microparticles are packed in 1 to 5 mL plastic tubes with frits at the bottom and top of the packing material. While commercially available columns provide a way for multiple labs to follow the same protocol, the degree of quality control in manufacturing the columns must be taken into account and internal standards should be extracted along with samples. The bed volume in these columns imposes sample volume limitations and the geometry of the columns makes them unsuitable to direct detection, so compounds must be desorbed, collected and detected. Due to potential carry over and decreased performance, the columns are not reusable (28).



Silica Microparticle

Figure 1.9 Silica Microparticle Modified with Propyl Benzoylsulfonate

The extraction kinetics for a sample flowing over solid phase microparticles are based on the distance the analyte must travel into the bead and residence time on the column. Typically this is not a problem as flow rates are on the order of 1 mL/minute and bed volume is on the order of 1 to 5 mL so that analytes have more than 1 minute to diffuse over the particles with diameters that range from 35 to 150 μm. However, this requires that the flow rate be consistent, especially at low bed volumes. This is often achieved via vacuum manifolds or centrifugation.

BENZOYLSULPHONATE MODIFIED SILICA BEADS

In this work, adsorption SPE was performed on commercially available columns filled with benzoylsulphonate modified silica beads (Figure 1.9). Both ion exchange and partition mechanisms were chosen to isolate a variety of cocaine related compounds. The details of this assay are discussed in Chapter 3.

1.7 FLOW INJECTION RENEWABLE SURFACE TECHNOLOGY

The utility of solid phases have been recognized and incorporated into FIA systems (29). The solid phase provides good surface area for contact and can preconcentrate analytes, but fouling has been a drawback to these systems. Flow injection renewable surface technology (FIRST) uses a novel flow cell, the jet ring cell to combine SPE and FIA (30-32) and overcome the fouling problems in previous work by regenerating the reaction surface for each analysis.

1.7.1 THE JET RING CELL

The jet ring cell is illustrated in Figure 1.10. As shown in A, microparticles flow through conventional FIA microtubing and are held in the jet ring cell at a point where

Table 1.1 Commercially Available Particles

Backbone	Ligand types	Ligand capacity	Particle, shape and size	Pore diameter, A	Exclusion of MW
Silica	Ion exchange, partition,	mg/g	30-60 μm, hard and irregular	25-250	10^3 to 10
Glass	Size		10-150 μm, hard and spherical	700	
Agarose	Affinity, size, ion exchange, partition,	mg/mL	45-165 μm, soft and spherical		10^4 to 10^7
Polystyrene	Affinity, size, ion exchange, partition,		10-1200 μm, soft and spherical	40-800	10^3 to 10^8

the end of the tubing is pressed against a coverslip or other flat surface. Sample or reagent solutions flow over the microparticles and escape through the small gap between the end of the tube and the coverslip. Analytes that bind to the bead surface are detected directly via chromophores or fluorophores or indirectly via a competing reaction with a labeled species (30). After detection, the tube is lifted away from the coverslip, forming a gap that is large enough for the microparticles to flow out of the cell and to waste (Figure 1.10B). In FIRST a new solid phase is used for each analysis. Binding does not have to be reversible, as in column based SPE, because analytes are detected on the microparticles.

Any of the particles listed in Table 1.1 that meet the mechanical requirements of the jet ring cell can be used in FIRST. The particles must be larger than the gap (ca. 30 μm) so they will be trapped but cannot exceed the diameter of the smallest conduit used in the FIRST system, or it will become clogged. The microtubing and valve inner diameters are on the order of 0.5 mm so that beads made out of rigid material (i.e., glass) over 150 μm clog the system. Soft spherical particles are preferred over irregularly shaped rigid particles because the latter scratch the inside of the valve, making silica inappropriate (33).

FIRST has a number of advantages for performing extraction. In the jet ring cell the solute is concentrated on the beads, enhancing low limits of detection. The design of the jet ring cell allows for continuous monitoring as the extraction proceeds, providing a way to monitor the kinetics of the extraction. Kinetic measurements can save time by eliminating time consuming equilibration steps and may provide additional information, such as binding kinetics (34). The small amounts of beads (ca. 0.5 mg, 10,000 beads) trapped in each sample can be released and flushed to waste so that the

technique is renewable. FIRST has been used to determine kinetics of antigen to antibody binding, as an electrochemical sensor and in non-aqueous titration (34-36).

1.7.2 Flow Injection Renewable Surface Immunoassay

Bioligand interactions are specifically well suited to FIRST because they are specific and sensitive. For the same reasons, they play a large role in forensic toxicology for the qualitative analysis of drugs of abuse. Flow injection renewable surface immunoassays (FIRSI) in particular, show advantages over previous flow injection immunoassays because the reagents are expensive and the kinetic measurements are important (32-34, 37). Immunoassays depend on the affinity of an antibody to specific sites or epitopes, on an antigen and have been extensively studied (38).

In FIRSI, antigens bind to antibodies that are immobilized on microparticles. One advantage to using particles is that they offer greater surface area for binding compared to wells and have been used elsewhere in immunoassays to decrease the analysis time (39). Antibodies make excellent reagents in FIRST because they have two distinct regions and can therefore be immobilized on microparticles while still maintaining their binding capabilities (40). Figure 1.11 illustrates the antibody structure and antigen binding. The F_c region is relatively constant composition for each species and is easily anchored to Protein G coated agarose microparticles (41). This leaves the F_{ab} region, that has a variable composition specific for binding the antigen, free. The complementarity between antibody and antigen is a combination of van der Waals, electrostatic and hydrophobic forces and is disrupted with increasing pH, salt concentration, temperature or solvent polarity (38).

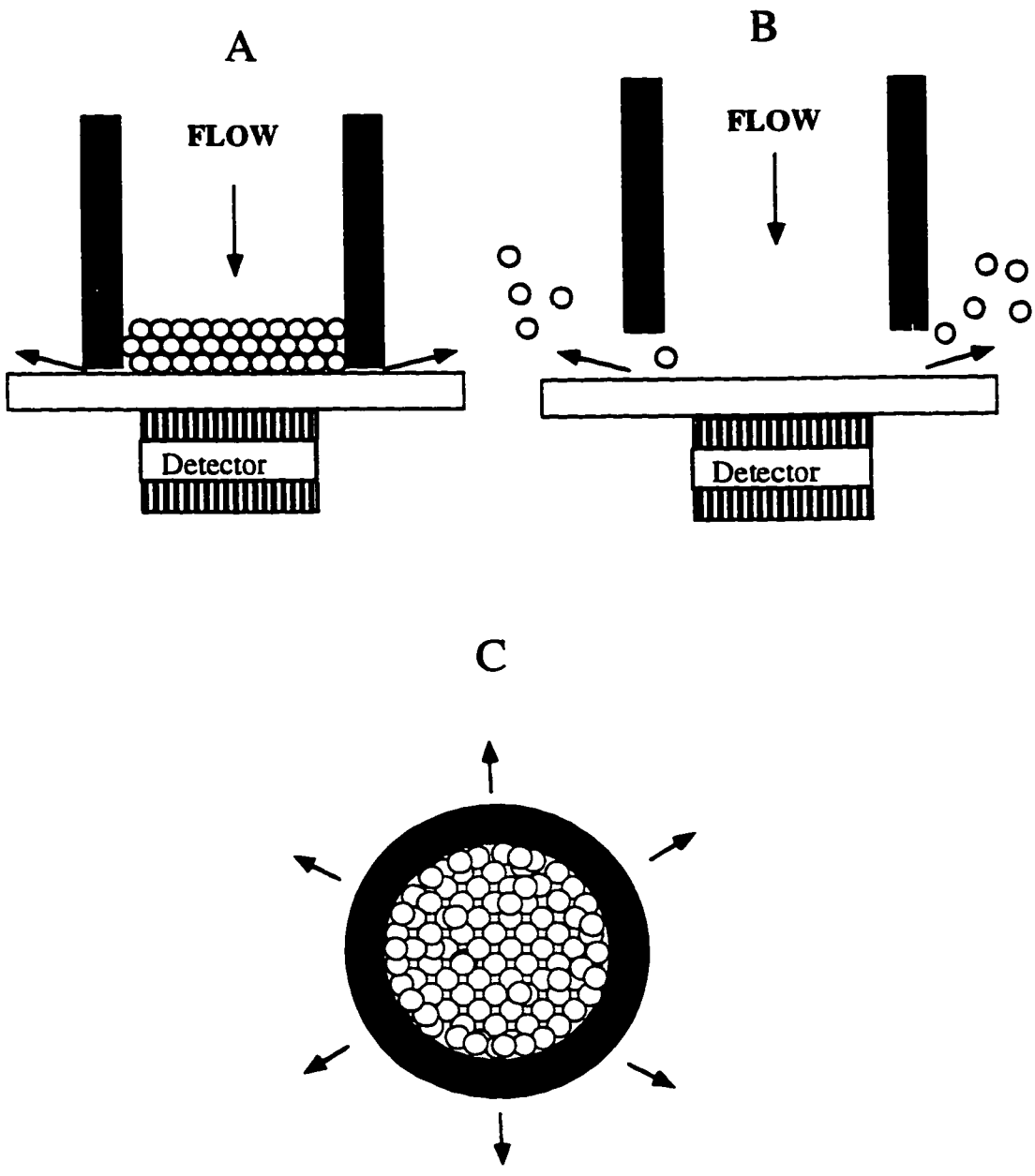


Figure 1.10 Jet Ring Cell

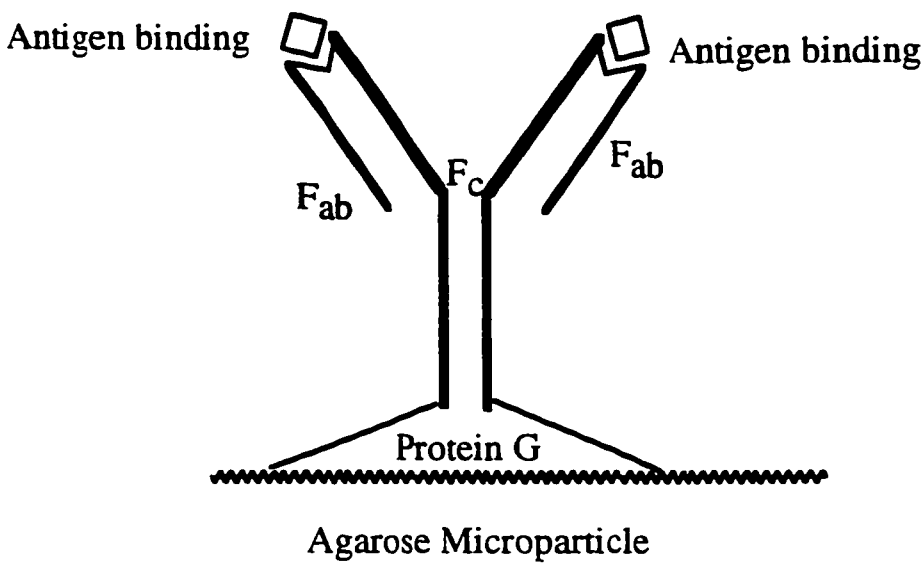


Figure 1.11 Antibody Structure and Antigen Binding

The agarose particles used in FIRSI are 35-165 μm in diameter and are easily trapped in the jet ring cell. Aqueous samples flow through the agarose backbone comprised of a polysaccharide copolymer of D-galactose and 3-6 anhydro-L-galactose (41). The hydroxy groups on the sugar backbone serve as an easy point for derivatization. Proteins G, a component of bacterial cell walls with high affinities for antibodies, has been covalently liked to agarose as a method for purifying immunoglobulins (41).

A drawback to bioligands is that they require storage at 4 ℃ in an aqueous media. If the solutions are left at room temperature, bacteria grow that can interfere with analysis. Some biological agents come with NaN_3 to prohibit microbial growth, but NaN_3 is a known toxin and should be handled with care. Also the bioligands need to be at a pH, salt concentration and low temperature that will preserve its structure and therefore function. For these reasons, microparticles with bioligands are sold as slurries and the columns are packed in house.

While it would be possible to detect the antigens directly, most antigens do not posses selective chromophores or native fluorescence. Therefore, indirect methods that involve the competition between the antigen and a labeled antigen for antibody sites are typically used. In a competitive immunoassay, a known amount of labeled antigen A* competes with the unknown quantity of antigen, A, for a limited number of antibody sites (38). The binding is reversible and can be described from an overall rate equation that takes into account the multi step nature of antibody to antigen binding.

$$A + Ab <-> A\text{-}Ab \quad (1.12a)$$

$$A^* + Ab <-> A^*\text{-}Ab \quad (1.13a)$$

The association constants, for both labeled and unlabeled antigens can be expressed in a similar manner.

$$K^* = \{AbA^*\}/\{A^*\}\{Ab\} \quad (1.12b)$$

$$K = \{AbA\}/\{A\}\{Ab\} \quad (1.13b)$$

When no unlabeled antigen is in solution the analytical signal obtained can be represented as S_0. That will depend on absolute values of K* and Ab and A* (38). When some unlabeled antigen is in the sample the signal decreases in proportion to the amount of unlabeled antigen that inhibits the label antigen binding and a signal is observed. The affinities and kinetics of labeled and unlabeled antigens for the antibody determine pattern of the of the response.

Due to the indirect detection and matrix effects discussed above, immunoassays are not suitable for quantitation. A second more specific chromatographic method is required to confirm and quantify substances. In this way, immunoassays serve as a first step in the analysis of a complex sample. The binding mechanism allows the efficient extraction of the antigen from solution.

1.8 SUMMARY TO CHAPTER 1

Each of the background techniques discussed above were applied to extraction in this work. Extraction is necessary to prepare complex samples for introduction into sophisticated analytical instruments. Extraction is based on some difference in partition

ability, and the compound of interest will determine the type of solvent to use in the extraction. Since, extraction is limited by physical concerns this thesis explores the immobilization of solvents and repeatable flow control as a way to reduce the analysis time while using small volumes and moving closer to interfacing extraction with analysis. The feasibility of this methodology was demonstrated by adapting existing extractions used in forensic toxicology to sequential injection extraction and flow injection renewable surface technology.

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CHAPTER 2: SEQUENTIAL INJECTION EXTRACTION FOR SAMPLE PREPARATION

Sequential injection extraction (SIE) was developed with the goal of reducing waste by using smaller volumes of aqueous sample and organic solvent than in conventional solvent extraction. The goals were to utilize established extraction chemistries in a renewable flow format for automated sample preparation, to eliminate the need for disposable volumetric glassware and to interface the extraction with instrumental methods. This extraction system was designed to use sample volumes applicable to those used in drug screens from biological fluids, 100 μL - 5 mL. SIE was demonstrated with both an extraction for acidic and basic compounds in urine samples. After extraction, each eluent was collected and analyzed with liquid chromatography, to resolve structurally similar compounds. For this second purpose, the aqueous and organic phases must be separated after extraction to ensure compatibility of the extracting phase with the analytical instrument.

SIE utilizes the wetting properties of organic solvents on Teflon tubing and the resulting differential migration of aqueous and organic phases as described in Chapter 1 to form a mobile extracting zone. SIE uses a simplified, robust, manifold design that brings sequentially loaded organic solvent and aqueous sample into contact with each other and later resolves them, without the conventional FIE components of phase segmentor and separator. The experimental parameters important in designing an SIE system, including viscosity, flow rate, solvent composition, and film thickness, and the difference in air or liquid propulsion, are discussed below, along with an application of the method.

2.1 EXPERIMENTAL DESIGN

The SIE manifold is illustrated in Figure 2.1. It is comprised of 1 mL and 5 mL digital syringe pumps (Cavro Scientific Instruments, Inc., Sunnyvale, CA), a 0.8 mm inner diameter six meter long extraction coil made of Teflon FEP (fluorinated ethylene propylene; Upchurch Scientific, Oak Harbor, WA) and an eight port multiposition selector valve, MPV (Valco Instruments, Houston, TX). FEP differs slightly from the more common Teflon (PTFE), in that it is a copolymer of ethylene and propylene moieties. Physically it is more transparent and less flexible than PTFE, and has similar wetability properties. The SIE instruments were controlled with a 386 PC using Quick BASIC software (Microsoft, Redmond, WA). The HPLC instrumentation consisted of an HP1040 Series II diode array detection system (Hewlett Packard, San Fernando, CA) and Gilson model 305/7 piston pumps (Gilson, Middleton, WI) and a 5 μm Lichrospher RP-8 column (Merck, Darmstadt, Germany).

Experiments were conducted in Teflon FEP tubing to determine the relative physical characteristics of the organic solvents. Additionally, viscosity measurements were performed on all solvents using an Ostwald viscometer (1) in a 25° C water bath. 1-Octanol, alone and with varying fractions of either butyl chloride or methylene chloride, were investigated as potential SIE solvents for their film forming abilities and extraction selectivity, respectively. Mixtures were 20, 33, 40, 50, 60, and 80% (v/v) butyl chloride in octanol and 33, 50 and 66% (v/v) methylene chloride in octanol.

The wetting properties of these solvents and their dependence on flow velocity were observed over flow velocities commonly used in FIE, from 1 to 8 cm/second. A 45 μL volume of organic phase (ORG) was sandwiched between two 45 μL zones of aqueous methylene blue, pH 12. At this pH, methylene blue does not extract into the

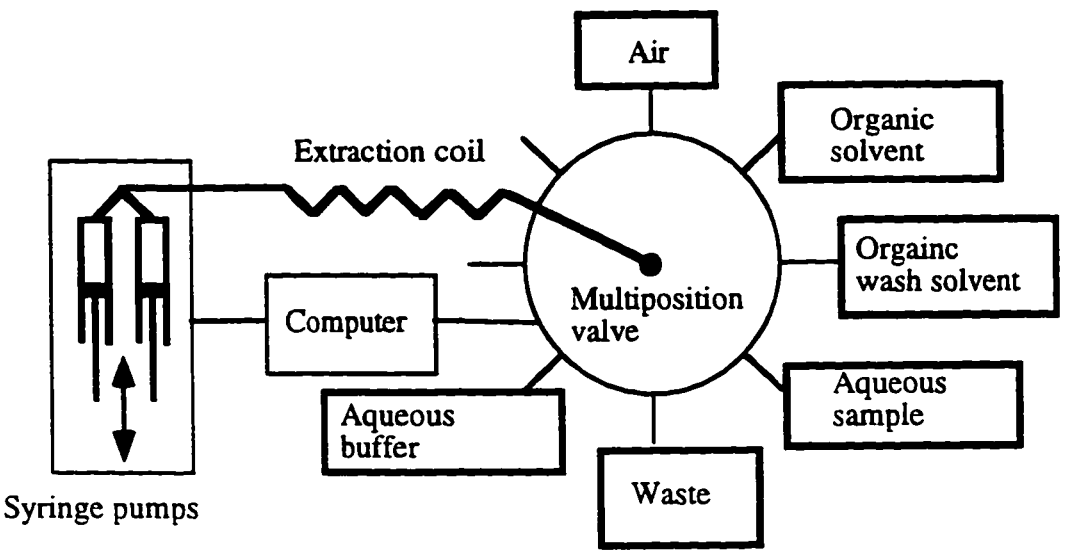

Figure 2.1 SIE Manifold

organic phase and the phase boundary is easily identified. Figure 2.2 shows the initial position of the ORG and the aqueous phases (AQ1, AQ2) in the extraction coil. In Figure 2.2, the zones have traveled a distance, L, from the load position. At this point of zone inversion, the front of AQ2 has traveled through the organic solvent and reached the tail of AQ1. The time to inversion and the flow velocity of AQ1 were used to calculate the length of tubing in which 45 µL of organic solvent has deposited on the tubing as a film. L serves as an average bulk measure of the dynamic process. A second set of experiments was performed where air replaced AQ2, so that L represented the length of tubing for ORG (50 µL) to be deposited when using air as the propellant instead of water.

2.1.1 Solvent Composition

The solvent composition is a critical parameter for the successful application of SIE, determining the difference in flow velocity between the organic and aqueous phases and the chemical selectivity and efficiency of the extraction. Due to hydrophobic interactions, a fraction of the organic solvent, V_{org}, forms a stationary film, V_{film}, on Teflon tubing whose volume determines the overall flow velocity of the organic solvent, u_{org} (2).

$$u_{org} = \frac{(V_{org} - V_{film}) \bullet (u_{aq})}{V_{org}} \qquad (2.1)$$

Therefore, in a continuously pumping segmented stream of aqueous and organic phases, the flow velocity of analytes in the organic phase is slower than that of analytes traveling in the aqueous phase (2). SIE, however, uses discrete zones of immiscible phases, not a continuous carrier stream. By loading the faster moving aqueous sample

behind a slower moving organic solvent plug and using air propulsion as diagrammed in Figure 2.2, the differential migration serves as a mechanism for both bringing into contact and separating immiscible phases. A separated phase may be collected for subsequent operations and measurement.

As discussed in Chapter 1, the equation used to determine the parameters important in extraction is

$$d_{film} = k\, d_{tube}\, (u\, \eta/\gamma)^a \qquad (2.2)$$

where d_{film} is the film thickness, d_{tube} is the tubing diameter, u is the flow velocity, η is the organic phase viscosity, and γ is surface tension, and a is a constant between 0.5 and 0.66.

Figure 2.3 compares solvent mixtures of 0, 40, 60 and 100% (v/v) butyl chloride in octanol and L. Equation 2.2 predicts an increase in the film thickness, experimentally measured as a decrease in L, with increasing organic solvent viscosity. At a flow velocity of 3 cm/second, L is 407 cm for the most viscous solvent, 100% butyl chloride and decreases to 138 and 94 cm with smaller fractions of butyl chloride in octanol to 28 cm for 1-octanol.

Table 2.1 lists the viscosity of each solvent mixture as calculated from literature values (3-4) using the mole fractions, X_a and X_b (5)

$$\text{viscosity of mixture} = (\eta_a)^{X_a}(\eta_b)^{X_b} \qquad (2.3)$$

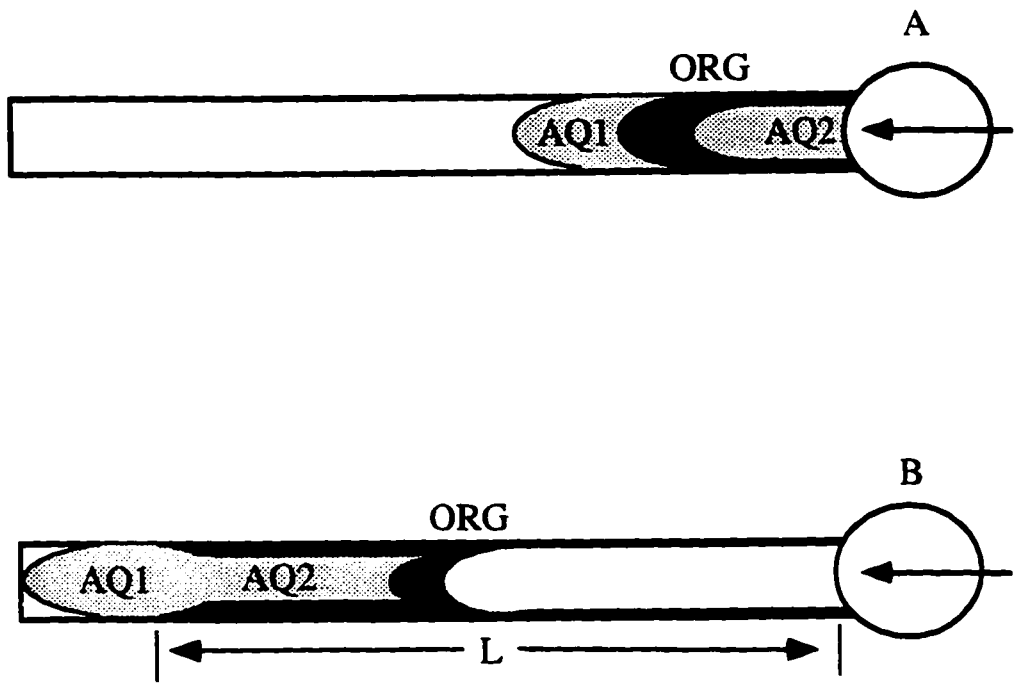

Figure 2.2 Zone Inversion

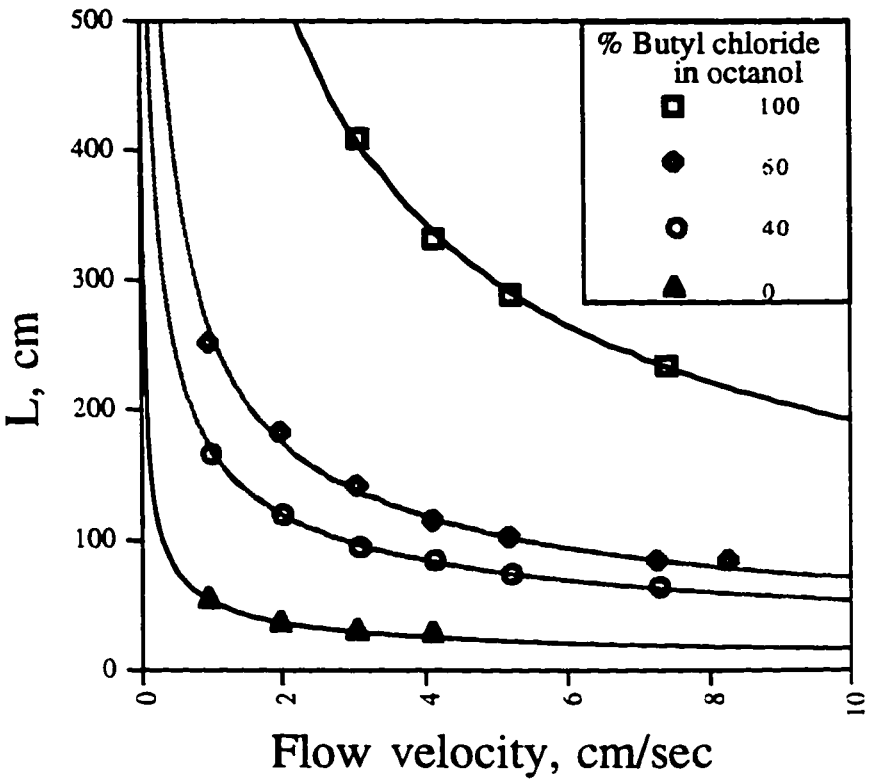

Figure 2.3 Zone Inversion as a function of Flow Velocity for Mixtures of Butyl Chloride in Octanol

and determined experimentally with an Ostwald viscometer (1). Solvents with low viscosities 0.5 cPoise did not have a sufficient difference in flow velocity from that of water, making SIE less efficient, as it requires more time and longer extraction coils.

Also, without rigorous temperature control, butyl chloride and methylene chloride produced solvent degassed in the tube which interfered with the flow processes due to their low boiling points. On the other hand, highly viscous solvents like octanol were difficult to wash out of the tubing and produced the least repeatable results. Solvent mixtures, with intermediate viscosities, near 1 cPoise, displayed intermediate properties: sufficient difference in flow velocity from water without adhering too strongly to the tubing and good extraction that proved to be optimal.

2.1.2 Flow Velocity

Figure 2.3 illustrates the dependence of the zone inversion value, L, on flow velocity for mixtures of butyl chloride and octanol. As the flow velocity increases, AQ2 penetrates AQ1 in a shorter distance. At a constant injected volume of organic phase (45 μL 60% (v/v) butyl chloride in octanol), L decreased from 248 to 80 cm, over a range of velocities. Since the injected volume of ORG was constant, this trend indicates a greater thickness of the organic stationary film at higher flow velocities. Assuming the organic solvent forms a uniform film on the tubing, the corresponding film thicknesses as calculated from L, increased from 7 to 23 μm and is comparable to thicknesses obtained by other researchers (6-7). In experiments with flow velocities above 8 cm/second, air bubbles form in the solvent producing a discontinuous film and irreproducible results.

2.1.3 Propulsion Method

This dependence of the film thickness on air or water propulsion becomes an important parameter for solvent recovery in mixed air and aqueous propelled flow systems. More than just a means of separating segments, with suitable solvents, air or liquid propulsion may be used to control film thickness. Illustrated in Figure 2.2, the ORG is first propelled into the extraction coil by AQ2, and it forms a film whose thickness is inversely proportional to the interfacial tension of these two liquids (Equation 2.2). Later, ORG is propelled by air, leaving a film inversely proportional to the surface tension of the organic solvent. The difference in the film thickness between air and liquid systems is proportional to the length of the segment of ORG that trails AQ2, which is used for the back extraction. The fraction of ORG left in the tubing contains unrecovered analytes, and it is washed out by the wash solvent.

Film thickness is dependent on the interfacial or surface tension of the organic solvent in liquid or air propelled systems, respectively. It is appropriate to consider viscosity along with either interfacial or surface tension to viscosity as a film thickness parameter, η/γ. Table 2.2 lists the literature values for η and γ, and calculated η/γ for unmixed solvents. These values predict an octanol film will be three times thicker when formed via liquid propulsion than with air and suggest that there would be no effect from the propulsion method on the film thickness of the chlorinated solvents.

The results of experiments comparing air and liquid propulsion for a 1:1 (v:v) octanol:butyl chloride mixture are presented in Figure 2.4. Approximately 1.6 times greater tubing length was required to completely deplete the organic solvent with air propulsion than with water over the range of flow velocities examined. This corresponds to a thickness parameter intermediate between butyl chloride and octanol.

Table 2.1 Viscosities of Solvent Mixtures

% (v/v) Butyl chloride in octanol	Predicted viscosity, cPoise	Experimental viscosity (+/-5%), cPoise
0	7.288	7.1
20	4.122	3.2
33	2.79	2.1
40	2.332	1.6
50	1.754	1.2
60	1.319	0.94
80	0.746	0.58
100	0.422	0.422*
% (v/v) Methylene chloride in octanol	**Predicted viscosity, cPoise**	**Experimental viscosity (+/-5%), cPoise**
0	7.288	7.1
33	2.777	2.7
50	1.741	1.00
66	1.241	0.69
100	0.416	0.422*

*given (Riddick et al.)

If the viscosity of the organic solvent limits the L to a narrow range, the contact time can be increased by increasing the number of cycles per analysis. The extraction procedure could be repeated with a smaller volume of the aqueous sample in fractions. The fractions would be collected and analyzed together. Alternatively, the program could be changed to incorporate more than one forward and reverse stroke for each extraction step, that would increase the time for the extraction to occur.

2.2 APPLICATION OF SEQUENTIAL INJECTION ANALYSIS

The applicability and relevance of SIE to forensic toxicology was determined by taking a well established method for liquid extraction using conventional glassware and applying similar solvent to SIE. Typically in extraction procedures for both acidic and basic compounds are necessary from an unknown sample. Therefore, extractions for both acidic and basic compounds were designed using SIE. In SIE, however, both of the extraction methods can be performed one after the other on the same sample.

From the experiments on solvent composition, flow rate and air or liquid propulsion, the following general experimental protocol was designed. Figure 2.5 illustrates each phase of the extraction method. First, (a) organic wash solvent (WS) is loaded into the extraction coil and separated from the organic phase that has been loaded into the extraction coil by an air segment (5 cm). Organic phase coats the tubing wall as it is followed by the aqueous sample (AQ). AQ pushes through ORG as it is injected into the extraction coil (b). Next, air is pumped into the coil, propelling AQ further through ORG (c), until all of the organic solvent has deposited on the tubing, ensuring complete contact of the two phases during step (d). In the next step, the pump changes direction pushing AQ back through ORG and out of the valve to waste

Table 2.2 Physical Properties of Pure Solvents

	Octanol	Butyl chloride	Methylene chloride
Surface tension (mN/m), γ (liquid)	27.530	22.500	26.520
Interfacial tension (mN/m), γ (air)	8.520	25.000	28.310
Viscosity (cP), η	7.288	0.422	0.416
Film thickness parameter, η/γ			
(s/m*m) liquid propulsion	0.265	0.019	0.016
(s/m*m) air propulsion	0.855	0.017	0.015

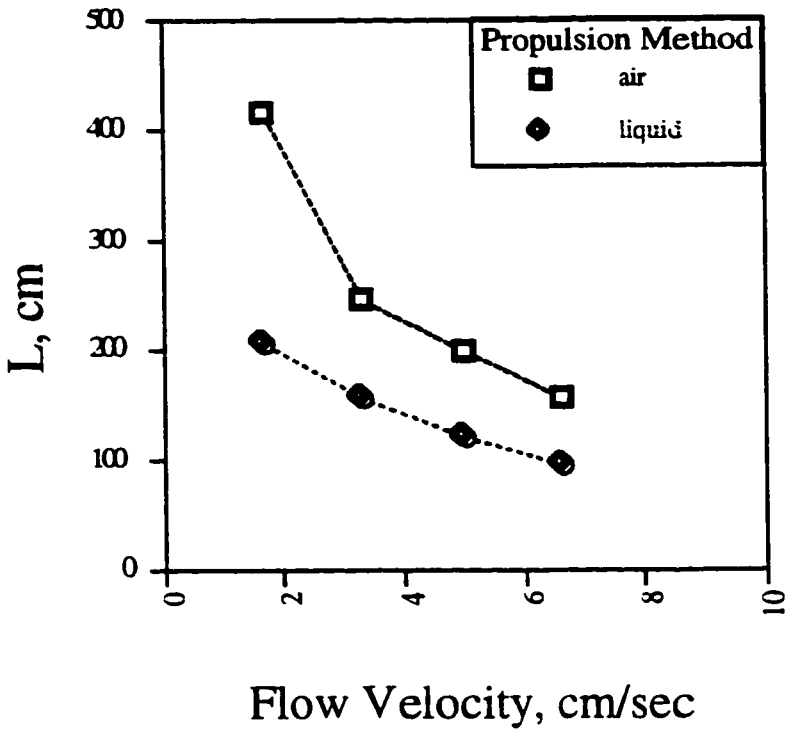

Figure 2.4 Zone Inversion as a function of Flow Velocity and Propulsion Method

(e), with analytes extracted into ORG. Due to the difference in air and liquid movement, a fraction of the ORG remains coated on the tubing, while the rest forms a segment that is aligned next to the valve head (f). A second aqueous segment, buffered to an optimal pH for back extraction, is aspirated into the holding coil and passes through ORG as shown in step a, and the same sequence is repeated. During this second cycle, analytes are extracted out of ORG and into the aqueous segment, which is collected through the collection port and analyzed by high pressure liquid chromatography (HPLC). Finally, the wash solvent initially introduced washes ORG off the tubing and to waste, leaving the tubing ready for the next extraction.

This SIE system was used to extract both weak acid/neutral and basic drugs of therapeutic interest from urine. Blank urine was spiked (0 - 50 μg/mL) with a mixture of anticonvulsants (phenobarbital, secobarbital, pentobarbital, amobarbital), serving as the model acid/neutral compounds, and with serotonin reuptake inhibitors, SSRIs (venlafaxine, paroxetine, sertraline, norsertraline), serving as model basic compounds. SSRIs are available for use as antidepressants (8) and are excreted at less than 5% for venlafaxine and less than 1% paroxetine, sertraline.

Using the general SIE method described above, compounds were extracted from 500 μL urine samples into 50 μL 4:1 (v:v) butyl chloride:octanol and back extracted into 100 μL 0.45 M NaOH, for isolation of acid/ neutral compounds or 0.18 M H_3PO_4 for the isolation of basic compounds. Cyclopal (10 μg/mL) was added as the internal standard for quantification. For optimal isolation of the basic compounds, 100 μL of pH 9 borate buffer (9) was added to each 500 μL urine sample.

Sample and extracting solvent were loaded into the extraction coil at a flow velocity of 3.3 cm/second. Air propelled the zones further into the coil at the same flow velocity for a total of 220 cm, to ensure complete zone inversion. A 400 μL portion of acetonitrile, loaded initially, washed the organic film to waste, leaving the extraction coil ready for the next extraction cycle. All aqueous extracts were collected and analyzed by HPLC.

For comparison, all separations were performed using conventional glassware and solvent extraction. Aliquots of 500 μL of urine and aqueous standards were extracted into 50 μL of 4:1 (v:v) butyl chloride:octanol and back extracted with 100 μL of 0.45 M NaOH to isolate acidic compounds. To isolate basic compounds, 100 μL of pH 9 borate buffer (9) was added to 500 μL aliquots of urine and aqueous standards and they were extracted into 50 μL of 4:1 (v:v) butyl chloride:octanol and back extracted with 0.18 M H_3PO_4. Each sample was vortex mixed and then centrifuged (2500 rpm, 5 minutes) to ensure separation of the phases and analyzed by HPLC.

Extracts were analyzed by HPLC on a 5 μm Lichrospher RP-8 column (Merck, Darmstadt, Germany) The mobile phase was 35% (v/v) acetonitrile in 0.05 M KH_2PO_4 (pH 2.5). At a mobile phase flow rate of 1.5 mL/minute, the barbiturates and SSRIs eluted from the column in 4 to 15 minutes and were detected with a photodiode array (190-400 nm).

2.3 RESULTS: A COMPARISON TO CONVENTIONAL SOLVENT EXTRACTION

Table 2.3 lists the absolute recovery of compounds from both water and urine standards and compares SIE to liquid extraction with conventional glassware under the

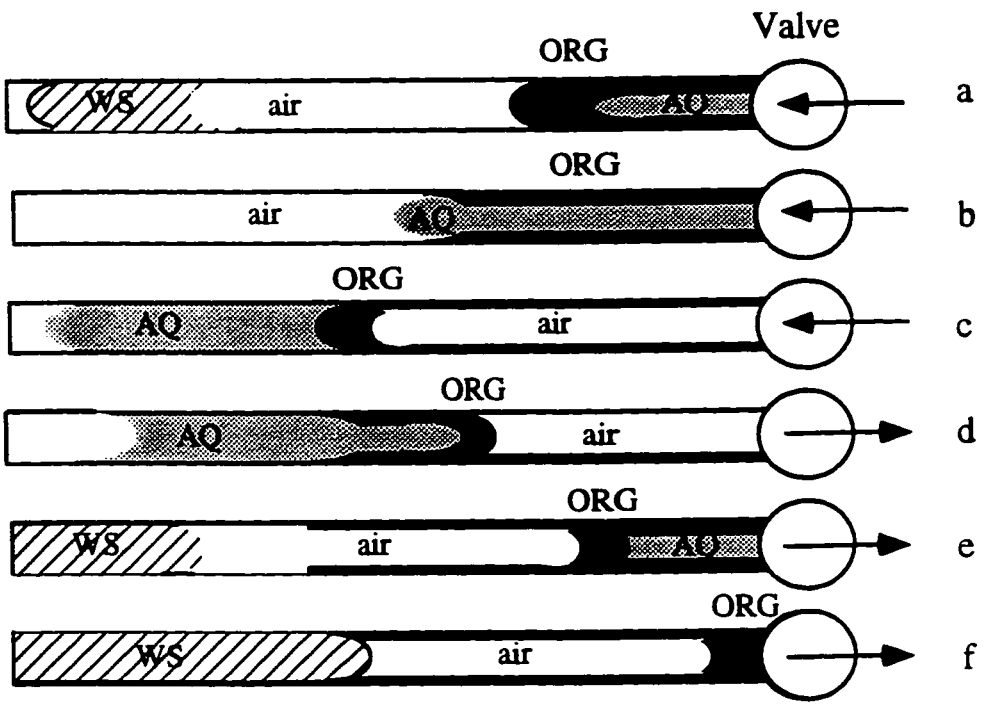

Figure 2.5 Method Steps

same conditions. When extracting the standards under conditions appropriate for basic drug extraction, the selective serotonin reuptake inhibitors were recovered more efficiently from water than urine using either SIE or conventional glassware extraction. This matrix effect was not seen with the barbiturates where the recovery was comparable between urine and aqueous standards using SIE and urine with conventional glassware extraction but slightly lower with aqueous standard and conventional glassware. Absolute recoveries for all compounds were less than 50% but these values were comparable to the amount extracted with conventional glassware extraction where samples were centrifuged and extracted under equilibrium conditions, representing maximum extraction, SIE took considerably less time, 3 minutes per analysis, as compared to 20 minutes for centrifugation and manual pipetting.

2.3.1 Efficiency Factor

Preconcentration is dependent on the phase ratios between the original sample and the extraction and back extraction solvents. In this method, the initial phase ratio was 10:1 aqueous-to-organic, followed by a 2:1 aqueous-to-organic back wash, leading to a maximum preconcentration factor of 5. However, the recovery was limited by the lower extractability by the octanol, and the maximum enrichment factor achieved was 2.5. Better extractability could be achieved with unmixed chlorinated solvents, but the difference in flow velocity versus that of the aqueous phase for pure butyl chloride was not great enough to mix and separate the two phases in the tubing dimensions tested, as discussed in the section on solvent mixtures. This system was not optimized for extraction efficiency but rather for speed and minimum waste production; therefore, chemical selectivity to ensure detection in the therapeutic range was acceptable. Modifiers such as butyl chloride and methylene chloride to octanol can enhance the

Table 2.3 Percent Recovery of Acidic and Basic Compounds from Urine and Water

	Urine - SIE	Aqueous - SIE	Urine - CEx	Aqueous - CEx
Venlafaxine	29	36	21	37
Paroxetine	30	51	20	38
Norsertraline	25	50	16	29
Sertraline	20	47	16	28
Phenobarbital	11	9	13	10
Amobarbital	24	27	22	16
Pentobarbital	23	25	23	16
Secobarbital	27	32	25	18

extraction efficiency but these modifiers must have low water solubility, being 0.11 and 1.30% by weight (3), respectively, for butyl chloride and methylene chloride, otherwise they will create a homogenate with the organic film and sample and not maintain a two phase system.

Phase separation was determined by the coextraction of basic compounds into the extract for acid/neutral compound and the coextraction of acid/neutral compounds into extract for basic compounds. Under conditions for basic extraction, less than 2% of the barbiturates coextracted from water or urine. Less than 1% of the SSRIs coextracted under conditions for acidic/neutral drugs. Figure 2.6 is a chromatogram of the eight component mixture before extraction and shows the resolution of the analytes.. Table 2.4 identifies the peaks at each retention time.

2.3.2 High Performance Liquid Chromatography

SIE based chromatographic analysis was linear over the range of concentrations tested (0-25 μg/mL phenobarbital, amobarbital, pentobarbital and secobarbital), with $r^2 \geq 0.99$. The relative coefficients of variation are presented in Table 2.5 and were less than 10% for all compounds except phenobarbital with 50 μg/mL standards (n=9), using amobarbital and paroxetine as an internal standards for the barbiturates and SSRIs, respectively. Importantly, no carryover of analytes was seen during these experiments, with a maximum concentration of 50 μg/mL. The compounds examined did not adhere to the Teflon tubing and the acetonitrile thoroughly removed any remaining organic film., No analytes were present in blank samples that were run just after the highest concentration sample, 50 μg/mL. Additionally, analytes were

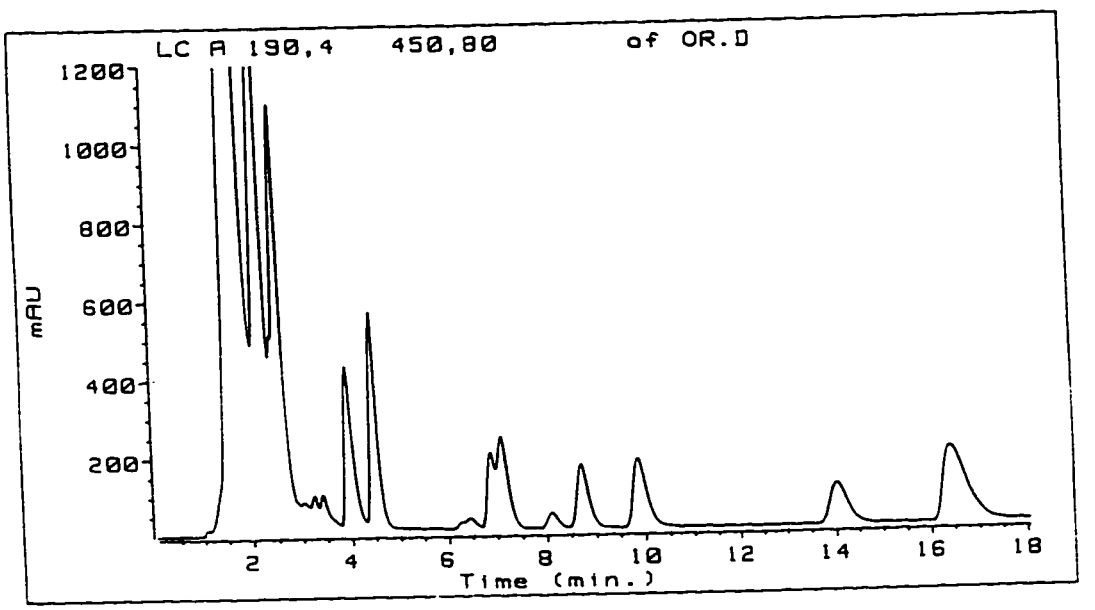

Figure 2.6 Chromatogram

Table 2.4 Retention Times for Figure 2.6

Compound	Retention time, min.
Venlafaxine	3.9
Phenobarbital	4.4
Amobarbital	6.8
Pentobarbital	7.1
Secobarbital	8.7
Paroxetine	9.9
Norsertraline	14.0
Sertraline	16.4

Table 2.5 Repeatability of Extraction

	Relative Standard Deviation for Recoveries in Table2.3			
	Urine - SIE	**Aqueous - SIE**	**Urine - CEx**	**Aqueous - CEx**
Venlafaxine	5	12	1	7
Paroxetine	*	*	*	*
Norsertraline	4	10	4	1
Sertraline	5	9	7	5
Phenobarbital	9	22	3	4
Amobarbital	*	*	*	*
Pentobarbital	4	3	1	0
Secobarbital	4	3	1	1

***Relative Internal Standard**

measured before and after adding a sample directly in the Teflon and showed identical signals.

The advantages and disadvantages of SIA relative to FIA have been discussed elsewhere (10) and such arguments can be extended to SIE in comparison with FIE. In SIE, incorporating bi-directional digital syringe pumps and a multiposition selector valve increases the versatility of the system. Several reagents can be stacked into a holding coil and reagent volumes are reduced because the continuous pumping of reagents is unnecessary, unlike FIE. With SIE, changing the manifold configuration has allowed the extension of flow based extractions to preparative systems.

2.4 SUMMARY TO CHAPTER 2

The SIE system described here combines the advantages of sequential injection and the coated surface technique of solvent extraction (11). The coated surface technique uses a FIA pump to load organic solvent into thin tubing but does not use the difference in flow velocity between the aqueous and organic phases. SIE allows the automated extraction of single liquid samples while keeping the organic solvent volume for each sample to a minimum, and does not compromise repeatability or recovery as compared to conventional glassware extraction with the same volume and shows no carryover. It operates without phase separators or segmentors by stacking the reagents in such a way that the faster moving phase overcomes the slower one as the two travel through the tubing. In comparison to conventional liquid/liquid extraction, SIE does not require volumetric glassware, manual pipetting or centrifugation, yet uses established chemistries. The robustness of design and ease of changing reagents

makeSIE a useful technique for automating batch analysis of liquid/liquid extractions and therefore applicable to many fields.

This renewable surface technique shows no evidence of carryover and therefore eliminates concerns of the irreversible adsorption and loss of analytes. In addition as a completely enclosed system, SIE isolates potentially hazardous organic solvents from the operator and reduces contact with biohazardous samples, which is an advance over conventional extraction as shown with the selected application. This extraction method can be used broadly for preparative extractions as liquid/liquid extraction protocols are generally available and with appropriate solvent mixtures can be readily adapted to SIE. Because SIE results in bulk separation of two immiscible solvents, discrete zones of both aqueous and organic solvents are resolved from the system and analytes are evenly distributed within the zones; therefore, detection of any volume element of the zone will yield the same results. While these samples were collected in test tubes, the effluent could be easily interfaced to chromatographic methods. Further advancements in sensing multicomponent systems directly in Teflon tubing will add to the utility of SIE.

This work would also benefit from the study of the two phase in the injection valve and how it contributes to the mechanisms of extraction. The role of mixing at the injection valve was not measured here. This work only evaluated extraction in one tubing material and it is possible that other solvents may exhibit similar properties as those examined here with different tubing materials. An evaluation of a variety of tubing materials and their effect on extraction efficiency would be beneficial. This extraction method could include ion pairing reagents in the organic phase to facilitate

UV detection of those analytes without chromophores. This would save time, since a second derivatization step would be unnecessary.

2.5 CHAPTER 2 END NOTES

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CHAPTER 3: SOLID PHASE EXTRACTION FOR THE ANALYSIS OF POLAR BIOTRANSFORMATION PRODUCTS OF COCAINE

The merits of solid phase extraction (SPE) were introduced in Chapter 1. In this work, SPE was used to isolate cocaine and its metabolites from urine. Cocaine is one of the most abused drugs and therefore is of forensic interest. Cocaine is an alkaloid present in the plant *Erythroxylum coca*. The leaves are dried to a paste and then cocaine is extracted as the hydrochloride salt, which is from 15 to 20% pure. Cocaine is used medicinally in the salt form as a topical local anesthetic. In the salt form, cocaine is soluble in aqueous solution and can be titrated easily to control its effects. Illicitly, cocaine is available in its free base form, commonly known as "crack." The free base form is insoluble in water and can be vaporized easily so that smoking is a common route of administration.

Once injected, cocaine undergoes extensive biotransformation. The biotransformation and degradation reactions of cocaine have been well studied (1-8). Initial methods to extract cocaine from aqueous matrices used liquid solvent extraction (9-10). Because these methods use the hydrophobic interactions of cocaine with the non-polar solvent, cocaine and related compounds with similar functional groups are efficiently extracted but those compounds with more polar functional groups are poorly recovered. In cases where the sample is collected or tested long after the drug was administered, degradation of the parent drug or primary metabolite may have proceeded far beyond the limit of detection, increasing the importance of other byproducts with more polar functional groups. The following is a review of the biotransformation of cocaine.

3.1 ROUTES OF COCAINE BREAKDOWN

3.1.1 ESTER HYDROLYSIS

Cocaine detoxification proceeds primarily via hydrolysis of the phenyl and methyl esters, to produce the monoesters ecgonine methyl ester and benzoylecgonine, respectively (Figure 3.1). Less than 5% of a cocaine dose is excreted in urine as the parent compound with approximately 40-80 % as benzoylecgonine and ecgonine methyl ester (11-12). The majority of benzoylecgonine is formed from cocaine by chemical hydrolysis at physiological pH (2, 7). Hepatic and serum esterases are responsible for the conversion of cocaine to ecgonine methyl ester and recent evidence suggests they may also be albeit to a much lesser extent, responsible for benzoylecgonine production (13).

Further breakdown of the remaining ester function of benzoylecgonine or ecgonine methyl ester produces ecgonine. It seems reasonable that the majority of ecgonine is formed from ecgonine methyl ester, whose methyl ester will hydrolyze chemically at physiological pH, at a rate similar to that of the methyl ester of cocaine, given their common structural features (14).

3.1.2 N-DEMETHYLATION AND FURTHER CONVERSION

Norcocaine, the N-demethylation product of cocaine, has been identified as an *in vivo* metabolite in the brains of animals given cocaine (15, 16). Human experiments with subjects ingesting cocaine radiolabelled with ^{14}C on the N-methyl group measured radioactive CO_2 in breath as an N-demethylated byproduct (4). While not a major excretion product, representing only 2.4 to 6.2% of the administered cocaine dose, N-demethylation of cocaine is important due to the pharmacological activity of

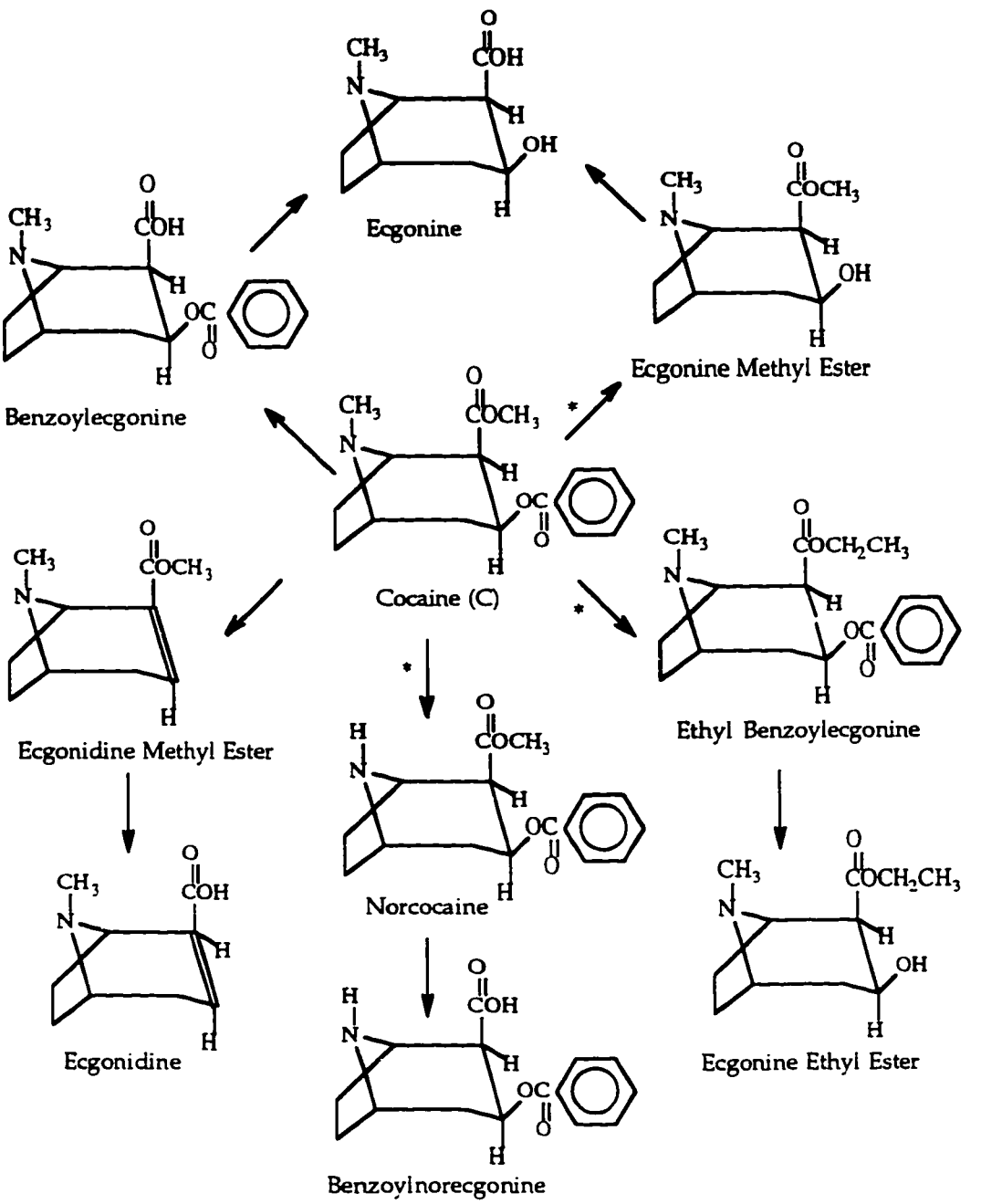

Figure 3.1 Cocaine Breakdown (* represents an enzyme mediated process)

norcocaine being similar to that of cocaine (15).

Hepatic and serum esterases used to study the formation of ecgonine methyl ester from cocaine were found to have similar affinity for norcocaine (2, 4), suggesting by analogy to cocaine, enzymatic hydrolysis to norecgonine methyl ester as the likely route of metabolism. A later report has identified norecgonine methyl ester in the urine of a cocaine user (17). Given its structural similarities to cocaine, norcocaine presumably hydrolyzes chemically at physiological pH to benzoylnorecgonine via the same mechanisms as benzoylecgonine is formed from cocaine (2, 18). Benzoylnorecgonine has been detected in the urine in animal studies (16, 19) and is pharmacologically inactive (15). Norecgonine, the resulting product of benzoylnorecgonine and norecgonine methyl ester hydrolysis, was not detected in the same animal studies mentioned above (16, 20), possibly as a result of limitations of the methodology.

3.1.3 Transesterification

Ethyl benzoylecgonine, commonly referred to as ethyl cocaine or cocaethylene, results from the enzymatic transesterification of cocaine in the presence of ethanol and is formed in the liver (13, 21-23). Reports on one isozyme found in human liver showed that it not only produces ethyl benzoylecgonine from cocaine but also catalyzes the hydrolysis of cocaine to benzoylecgonine (23-24). A follow up study identified methyl esterases in the rat brain, lung, kidney and heart (13). A site dependent postmortem study in humans found no predictable time dependent changes in the concentrations of cocaine, benzoylecgonine and cocaethylene (25).

Ethyl benzoylecgonine is of toxicological significance due to its neurotoxicity (21, 26). It has been detected with some frequency along with cocaine in the urine of

trauma victims (27-28). Analogous to the metabolism of cocaine, at physiological pH, chemical hydrolysis of the ethyl ester function of ethyl benzoylecgonine would produce benzoylecgonine, and enzymatic hydrolysis would form ecgonine ethyl ester. As early as 1979, ethyl benzoylecgonine and ecgonine ethyl ester were qualitatively identified in human urine (29), but their significance was not recognized at that time.

3.1.4 Arylhydroxylation

The phenyl groups on cocaine, benzoylecgonine, and ethyl benzoylecgonine serve as substrates for various enzymatic aromatic hydroxylation and methoxylation reactions. Although identified in the urine of animals (10, 19) and later in cocaine users (17, 30-32), very little is known regarding the significance of these analytes due to their poor recoveries, and apparent low concentrations. Hydroxycocaine was reported as early as 1979 in human bile (33).

3.1.5 Pyrolysis

The administration of cocaine by smoking can produce specific chemical markers. Ecgonidine methyl ester is a major pyrolysis product of cocaine formed during smoking (34-35) and has been identified in the urine of human research subjects (35) as well as in the urine of street cocaine users (17, 33). An experiment comparing various routes of administration determined that subjects receiving cocaine by inhalation excreted ecgonidine methyl ester in the urine at about half of the amount of cocaine excreted in the urine (35). In the same study, those subjects who were administered cocaine via intravenous or intranasal routes exhibited much lower amounts of ecgonidine methyl ester in relation to cocaine (35). However, Cone et al. found ecgonidine methyl ester at much lower concentrations than cocaine and

recommended it usefulness as a marker for cocaine smoking is limited to just after smoking (36). The main objection to this work was that the cocaine smoking device did not reach the same temperature as with street "crack" pipes.

Ecgonidine, the corresponding ester hydrolysis product of ecgonidine methyl ester, is also found in human urine (17). However, it was not measured in the aforementioned study (35). Its origin, therefore, as another pyrolysis product, as opposed to an *in vivo* hydrolysis product of ecgonidine methyl ester, has not been established. N-demethylation of these compounds results in norecgonidine methyl ester and norecgonidine; the former has been detected in the urine (17). No evidence exists to support any of these compounds attributed to cocaine smoking as pharmacologically active.

3.1.6 Other Alkaloids

Other alkaloids are extracted along with cocaine from the leaves of the erythroxylum coca plant (35, 37-39). Of these congeners, the most significant is cinnamoylcocaine which was present in more than half of the illicit cocaine samples examined in one study at levels at least 1% the amount of cocaine (37). Cinnamoylcocaine is frequently detected in the urine of cocaine users. Hydrolysis of the methyl or benzoyl esters of cinnamoylcocaine produces, respectively, cinnamoylecgonine and ecgonine methyl ester, together with cinnamic acid. Hydroxylation of the phenyl group of cinnamoylcocaine, analogous to the formation of hydroxycocaine, results in several other hydroxylated cinnamoylcocaine and cinnamoylecgonine derivatives. Cinnamoylcocaine might also be expected to be subject to transesterification in the presence of ethanol to produce its ethyl analog, ethyl

cinnamoylecgonine. There is no evidence to suggest that these congeners are pharmacologically active or contribute to the toxic effects of cocaine use.

3.2 ANALYTICAL METHODS TO DETECT COCAINE BREAKDOWN PRODUCTS

Previous methods to increase the range of analyte polarity in solvent extractions have either increased the ionic strength (40) of the sample to salt out organic compounds or increased the polarity of the organic solvent by using mixed solvent systems such as a 9:1 chloroform:isopropanol (41). Alternatively, the polarity of the analytes can be reduced by derivatizating them via extractive alkylation (42-43). This also facilitates liquid chromatography and UV detection of the analytes.

Solid phase extraction methods for cocaine metabolites have developed as solid microparticles with both ionic and hydrophobic groups have become available (36, 44-51). Each of the above methods has its merits. Hydrolyzing all of the cocaine related compounds to ecgonine and then extracting it via a very polar method simplifies the choice of extraction solvent but this removes any biotransformation information (52). Using an anion exchange column preferentially isolates benzoylecgonine and ecgonine (44). However, the majority of articles including the work described use a solid phase extraction performed with a combination of cation exchange and non-polar interactions to isolate transformation products of cocaine that could not be extracted with liquid solvents due to their range of polarity (53).

3.3 EXPERIMENTAL PROTOCOL

The extractions were performed on commercially available strong cation exchange (SCX) propylbenzenesulfonyl bonded solid phase extraction columns (Varian, Walnut Creek, CA) installed on a vacuum manifold (Varian, Walnut Creek, CA). The benzoyl moiety interacts with the phenyl group via a combination of hydrophobic and pi-pi interactions and the sulfate interacts with the amino terminus via ion exchange, to extract cocaine and related compounds, such as ecgonine, ecgonine methyl ester and benzoylecgonine from complex matrices (Figure 3.2). Analytes without phenyl groups can be isolated, binding to the column via weaker hydrophobic interactions with the tropane ring and ionic interactions.

The columns must be preconditioned so that the active groups are available for binding. The 100 mg SCX columns were activated by washing with methanol (2 mL) and deionized water (2 mL). This was followed by two washes of 0.2 M H_3PO_4 (500 µL) in succession. The column was not allowed to dry between the washes and the addition of the sample. When dry, the reactive sites are prone to fold up and therefore give less surface area for extraction.

Urine (1 mL) was diluted to 5 mL with deionized water so that the samples could freely flow through the columns. This is necessary because urine varies in composition, and particulate matter is less likely to clog the SPE cartridge in diluted samples. Calibration samples consisted of urine from a drug free volunteer spiked with standards (1, 5, 50 and 100 µg/mL ecgonine, ecgonine methyl ester, benzoylecgonine, and cocaine, Radian, Austin, TX). N-methyl trideutero analogs of benzoylecgonine and ecgonine were added as internal standards (Radian, Austin, TX). Because urine pH varies with diet, 0.2 M H_3PO_4 (500 µL) was added to acidify the sample to pH 2

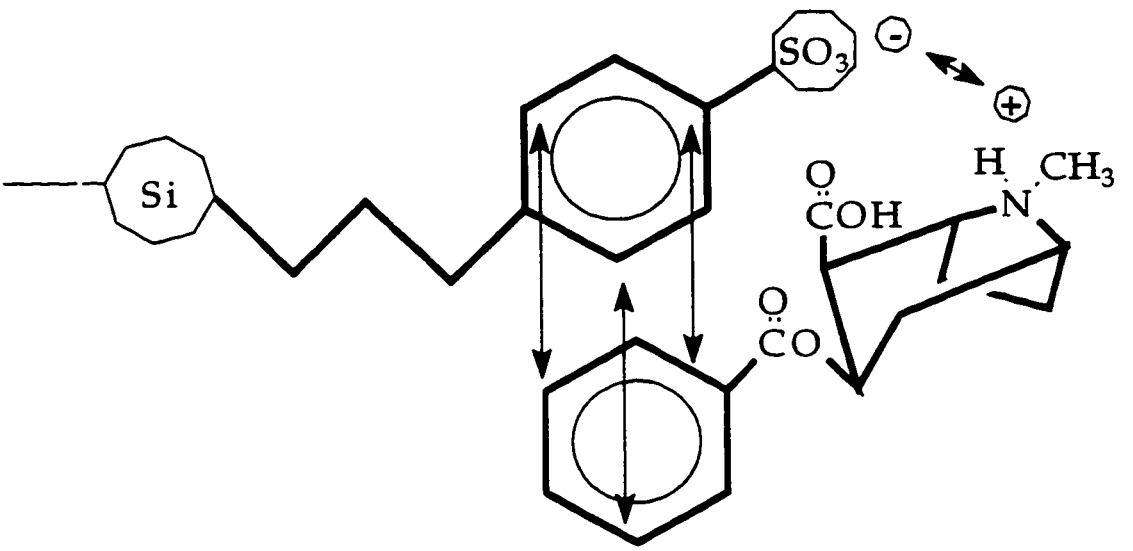

Figure 3.2 Binding Interactions

to 2.5. pH was checked and additional aliquots of 0.2 M H_3PO_4 were added until the pH of the sample fell in this range. The samples were mixed on a vortex mixer for 30 seconds just before adding them to the extraction column to ensure complete mixing.

The samples were added to the columns, flowing through at a rate of about 1 mL/minute, and the columns were allowed to air dry for 30 seconds after the sample had passed through. After washing with 0.2 M H_3PO_4 (1 mL) and methanol (1 mL), the columns were air dried for another 30 seconds. These wash samples were collected and found to be blank when analyzed for the compounds of interest.

The analytes were eluted in off the column by washing with a basic solution of three washes of 3% ammoniacal methanol (3 x 2 mL). Ammoniacal methanol was prepared fresh daily by diluting ammonium hydroxide (3 mL) to 100 mL with methanol. Each batch of elution solvent was allowed to flow at atmospheric pressure before the next was added. The majority of the analytes washed off the column in 6 mL. Samples of the eluent after 6 mL did not show an increase recovery.

The extracts were dried under flowing air, at 50° C in a water bath, reconstituted in ethanol (100 µl), and chilled in an ice bath.

After the extraction any acids were converted to propyl esters, via reaction with diazopropane, making them amenable to gas chromatography (Figure 3.3). This was a modification of an earlier method using diazoethane (10). Formation of the propyl esters is preferred over the ethyl esters due to the potential presence of ethyl benzoylecgonine as formed from the *in vivo* reaction of ethanol and cocaine (21). The method is a one step process and alkyl derivatives have minimal interference with subsequent chromatography.

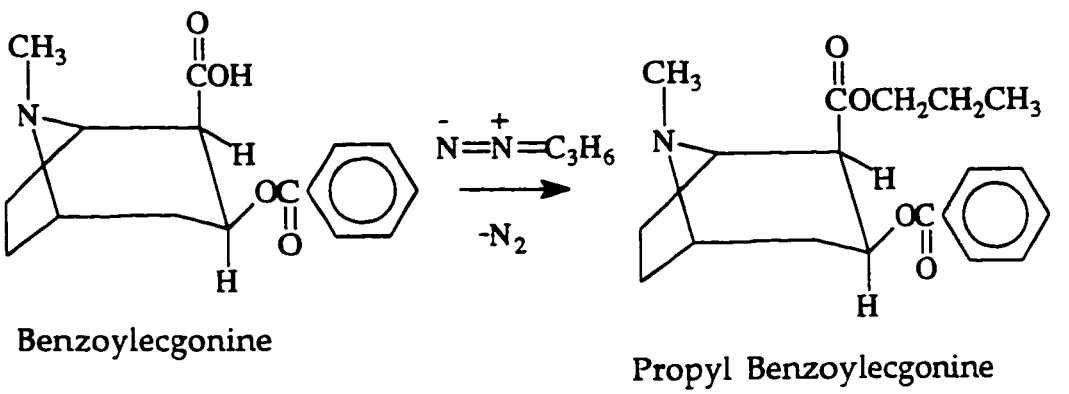

Figure 3.3 Derivatization to Propyl Ester

Diazopropane was prepared *in situ* based on the method described by Jindal and Vestergaard. Chilled diethyl ether (5 mL) and 10 M KOH (2 mL) were added to a disposable test tube containing 20 mg N-propyl-N'-nitro-N-nitrosoguanidine (Sigma Chemical Co., St. Louis, MO). This material is a known carcinogen and appropriate precautions should be taken. The mixture was kept in an ice bath and the reaction was allowed to proceed for 20 minutes. Diazopropane gas was evolved from the aqueous layer and dissolved in the ether layer, causing the ether layer to turn pale yellow.

The sample extract reconstituted in ethanol was treated with 500 µL of ethereal diazopropane. The solution was vortex mixed, kept on ice for 15 minutes, then evaporated to dryness under flowing air in a fume hood, at 50° C.

A back extraction was required to reduce interference in subsequent chromatograms. The derivatized extracts were redissolved in chloroform:isopropanol, 4:1, (1 mL) and vortex mixed together with pH 9 sodium borate buffer (200 µL). After centrifuging for five minutes at 2000 RPM, the organic layer was removed and dried under air at 50° C.

The final extract was reconstituted in 75 µL ethanol and a 2 µL aliquot was injected into a Hewlett-Packard 5890 gas chromatograph equipped with a 5970 mass selective detector. The column was 30 m/0.32 µm i.d. 5% phenylmethylsilicone (Econocap BP-5, Alltech, Deerfield, IL). Separation was achieved via a temperature program from 100-295° C over 15 minutes, holding the final temperature for 7 minutes. GC/MS was performed in an electron impact mode. Selected ion (m/z 199, 227, 230, 303, 331, 334) and total ion chromatograms were obtained for the standards. The molecular ions of propyl ecgonine (m/z 227), ecgonine methyl ester

(m/z 199), propyl benzoylecgonine (m/z 331) and cocaine (m/z 303), along with those of the internal standards, N-methyl trideutero propyl ecgonine (m/z 230) and N-methyl trideutero propyl benzoylecgonine (m/z 334), were used to create calibration curves.

Calibration curves were plotted with the data from spiked urine samples (0, 1, 5, 50, and 100 μg/mL of ecgonine, ecgonine methyl ester, benzoylecgonine, and cocaine. N-methyl trideutero ecgonine and benzoylecgonine served as the internal standard for ecgonine and ecgonine methyl ester, and benzoylecgonine and cocaine, respectively. The method was linear over this range ($r^2 > 0.96$). The method was not linear at concentrations greater than 100 μg/mL, possibly due to saturation of the available binding sites on the extraction column. Smaller fractions of the samples that were above this concentration were reanalyzed.

To test inter sample precision, reproducibility experiments (n=5) were carried out using of 5 μg/mL samples. The coefficient of variation for ecgonine methyl ester was 6% and for ecgonine was 7% when using N-methyl trideutero internal standards.

Absolute recovery of the analytes from the extraction columns was determined by comparing spiked urine samples (10 μg/mL) extracted as above and unextracted standards. Recovery of ecgonine was found to be 65%, that of ecgonine methyl ester was less than 50%, and benzoylecgonine and cocaine were 85% and 95%, respectively, which is similar to other reports using SPE cartridges (36). The coefficients of variation reported above for ecgonine and ecgonine methyl ester imply that the method is reproducible under the specified conditions, employing deuterated internal standards. The limit of quantitation for all analytes was 0.5 μg/mL. The

patient samples discussed below validate the use of the method in this range, for postmortem human urine.

Samples were selected from a case database at the Washington State Toxicology Laboratory in which the urine had previously been determined to be positive at a level above 300 ng/mL for benzoylecgonine by immunoassay (EMIT, Syva, San Jose, CA). The concentrations of ecgonine, ecgonine methyl ester, benzoylecgonine and cocaine were measured in urine samples from 29 cases of cocaine related deaths by treating the samples in the same manner as the standards. Ecgonine, ecgonine methyl ester, benzoylecgonine, and cocaine were quantified by comparison with the calibration curve. Analytes for which standards were not available were identified by comparison with literature data and consideration of their mass spectra. The abundance of the base peak ions for these compounds (m/z 82 or 124) was compared to the base peak ion abundance of the trideutero ecgonine (m/z 85) or trideutero benzoylecgonine standards (m/z 85). Figure 3.4 illustrates a total ion chromatogram from a urine sample prepared using this method. Figure 3.5 shows a reconstructed chromatogram, indicating relative retention behavior of the compounds identified. Peaks were selected by the molecular ion of each analyte and identified by their mass spectrum. The mass spectrum of propyl ecgonine is illustrated in Appendix A. The chromatographic peaks of Figure 3.5 are identified in Table 3.1 together with the molecular ions used to select them from the total ion chromatogram (Figure 3.4).

3.4 QUANTITATIVE ANALYSIS OF COCAINE RELATED COMPOUNDS

Table 3.2 shows the concentrations of ecgonine, ecgonine methyl ester, benzoylecgonine and cocaine in the urine samples. Cocaine concentrations in these

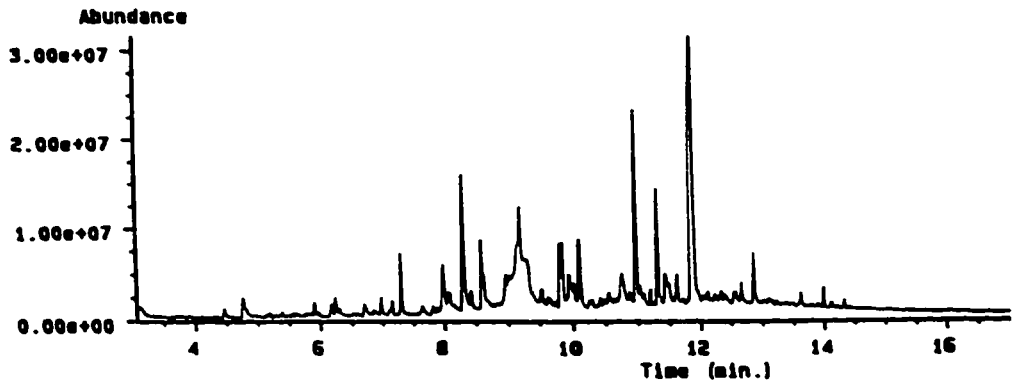

Figure 3.4 Total Ion Chromatogram

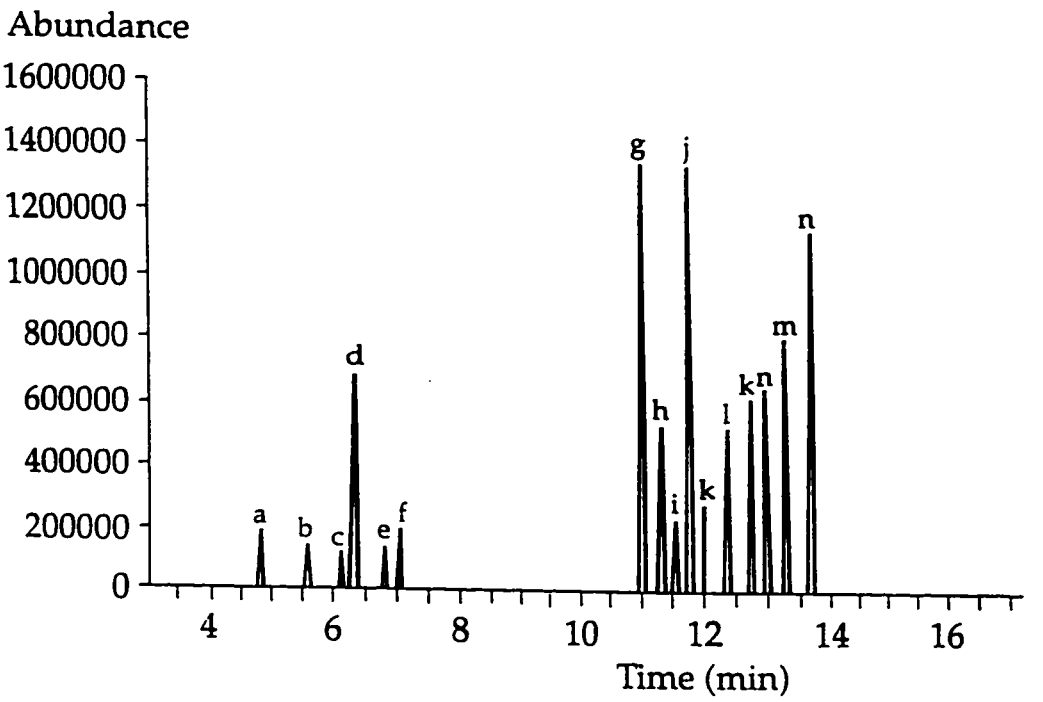

Figure 3.5 Reconstructed Chromatogram

Table 3.1 Peak Identification for Figure 3.5

Peak	Analyte (molecular ion used to select analyte (m/z))
a	Ecgonidine methyl ester (181)
b	Ecgonine methyl ester (199)
c	Ecgonine ethyl ester (213)
d	Propyl ecgonidine (209)
e	Propyl N-methyl trideutero ecgonine* (230)
f	Propyl ecgonine (227)
g	Cocaine (303)
h	Ethyl benzoylecgonine (317)
i	Propyl N-methyl trideutero benzoylecgonine* (334)
j	Propyl benzoylecgonine (331)
k	Cinnamoylcocaine (329)
l	Benzoylnorecgonine (317)
m	Norcocaine (289)
n	Propyl cinnamoylecgonine (357)
	*internal standard

Table 3.2 Concentration in Urine Samples

Concentrations (µg/mL) of E, EME, BE, and C in Patient Urine Samples (n=29)

CASE	ANALYTE Ecgonine	Ecgonine Methyl Ester	Benzoylecgonine	Cocaine
A	-	-	•	•
B	-	-	1.8	•
C	-	-	2.8	•
D	•	-	6.3	•
E	-	-	12.4	•
F	•	1.4	18.3	18.5
G	•	2.2	21.2	•
H	0.5	-	17.3	•
I	1.5	4.5	30.6	9.8
J	1.7	5.3	51.2	29.6
K	2.7	•	•	•
L	3.4	•	2.2	•
M	5.5	4.9	17.7	8.8
N	6.5	4.7	6.6	61.1
O	6.5	28.5	84.1	82.9
P	6.6	8.2	122.5	130.6
Q	13.4	16.9	128.6	129.4
R	13.5	12.8	52.1	26.0
S	14.1	21.0	192.6	214.3
T	15.5	25.4	223.5	272.5
U	19.5	60.7	162.0	122.8
V	21.2	63.8	288.2	282.5
W	21.9	14.2	194.8	254.3
X	22.4	34.0	167.3	453.7
Y	25.1	23.1	206.8	303.0
Z	30.8	28.6	249.7	304.5
AA	38.3	65.2	266.5	640
BB	64.7	109.4	83.8	92.1
CC	104.4	177.5	1074	1221

• indicates presence identified, but at less than 0.5 µg/mL
- not detected

samples were typically very high, and many came from subjects where the deaths were attributed to acute cocaine toxicity. Benzoylecgonine was identified in every case, with two at less than 500 ng/mL. In those two cases where the benzoylecgonine concentration was less than 500 ng/mL, several other cocaine metabolites were also identified (Table 3.3). Ecgonine methyl ester concentrations ranged from about 0.5 to 180 μg/mL, and ecgonine concentrations ranged from about 0.5 to 100 μg/mL.

The prevalence of ecgonine is an important finding since it supports the hypothesis that this compound is the final hydrolysis product following cocaine ingestion. The concentrations of ecgonine were also significant, and similar in magnitude to those of ecgonine methyl ester. From this study it is impossible to identify the specific routes of formation of ecgonine. However, the readily hydrolyzed methyl ester function of ecgonine methyl ester is labile at pH of above 6, making ecgonine methyl ester the most likely precursor of ecgonine in typical cases. Under moderate conditions of pH, benzoylecgonine will also be a stable product and a reliable marker for cocaine use. At higher pH, as encountered if a urine sample is adulterated with a base such as bleach, hydrolysis of the benzoyl ester of benzoylecgonine is accelerated, thus ecgonine may be the only residual marker of cocaine use.

For those compounds for which a standard was unavailable, quantitation was not possible. Table 3.3, however, presents data to illustrate the relative abundance of prominent ions from these analytes, allowing some assessment of their relative concentration in these extracts. The peak height of a common fragment ion (m/z = 82) was compared to the peak height of a fragment ion (m/z = 85) present in the N-methyl trideutero standards. N-methyl trideutero propyl ecgonine was used at the internal

standard for compounds without a benzoyl group and N-methyl trideutero propyl benzoylecgonine was used for the compound with a benzoyl group.

N-demethylated products norcocaine and benzoylnorecgonine were present in many samples. Norecgonine methyl ester and norecgonine were not detected in the urine samples examined here. This may be an indication of the lower affinity for the N-demethylated product by the cholinesterases. It also supports the hypothesis of previous studies on norcocaine (2, 4) that the only N-demethylated compounds seen *in vivo* result from the breakdown of norcocaine and not the N-demethylation of other cocaine metabolites, like ecgonine methyl ester.

In addition to the analytes identified in many arylhydroxy and arylmethoxy substituted cocaine, benzoylecgonine, cinnamoylcocaine and cinnamoylecgonine derivatives were tentatively identified (Appendix A). The significance of some of these has been described elsewhere (17, 30-32). They appear to be present in much smaller amounts than the other analytes.

These data suggest that ecgonine ethyl ester is a metabolite of ethyl benzoylecgonine, as all of the samples positive for ecgonine ethyl ester were also positive for its parent compound, ethyl benzoylecgonine. The data cannot confirm benzoylecgonine as a metabolite of ethyl benzoylecgonine because it is not a unique breakdown product.

Some consideration of the significance of ecgonidine methyl ester and ecgonidine is also possible based on the above results. Approximately 50% of the urine cases sampled contained moderate levels of ecgonidine and ecgonidine methyl ester; none of the cases contained ecgonidine ethyl ester. Ecgonidine methyl ester is believed to be

Table 3.3 Relative Abundance Ratios of Analyte Fragment Ion (m/z 82) to Fragment Ion of Internal Standard (m/z 85) as found in Patient Urine Samples

CASE	Ecgonidine Methyl Ester	Ecgonidine	Ecgonine Methyl Ester	Ecgonine Ethyl Ester	Ecgonine	Cocaine	Ethyl Benzoyl-ecgonine	Benzoyl-ecgonine	Benzoyl-norecgonine	Norcocaine**	Cinnamoyl-cocaine	Cinnamoyl-ecgonine
A	-	-	-	-	-	0.5	-	0.1	-	-	-	-
B	-	-	-	-	-	*	-	0.4	-	-	-	-
C	-	-	0.2	-	0.1	*	-	0.4	-	*	*	*
D	-	-	*	-	0.1	0.1	0.1	1.0	-	-	-	*
E	-	0.3	0.1	-	0.2	*	-	1.0	-	-	-	-
F	0.2	0.2	0.2	0.1	0.4	1.9	0.9	2.4	-	-	*	*
G	-	0.7	0.2	-	0.4	0.1	0.3	3.1	*	0.1	-	0.4
H	-	0.6	0.2	0.2	0.6	*	0.1	1.5	-	-	-	0.2
I	0.1	0.6	0.7	-	0.7	0.8	0.5	4.3	*	0.8	0.1	0.8
J	*	0.4	0.5	-	0.8	3.0	1.9	10.6	0.5	0.5	1.7	1.1
K	-	-	*	-	0.1	0.1	-	0.4	-	-	-	-
L	-	0.1	-	-	0.1	0.1	-	0.7	-	-	-	0.1
M	-	-	1.4	0.9	0.3	1.0	1.3	2.0	*	0.6	0.2	-
N	0.3	0.3	1.4	1.1	0.5	3.1	3.1	3.2	-	1.2	2.2	0.4
O	1.2	1.3	3.5	0.5	1.5	11.1	6.5	12.6	0.8	0.5	12.4	4.6
P	0.3	1.6	0.7	‡	1.5	16.4	6.6	21.1	1.0	5.8	10.8	14.6
Q	0.7	3.5	1.7	-	2.2	13.1	6.0	18.8	0.3	2.7	4.6	12.3
R	0.8	5.8	2.0	0.8	3.0	2.5	1.8	9.7	-	1.2	0.7	4.1
S	0.6	1.3	4.6	1.1	2.0	11.0	3.0	15.1	0.4	14.4	36.	1.9
T	1.4	3.1	2.4	‡	2.8	30.9	24.4	42.1	2.5	6.1	38.1	‡
U	4.3	4.7	6.5	3.2	3.7	1.6	18.8	23.1	0.3	4.7	16.7	16.5
V	1.7	1.8	7.6	0.4	4.0	36.6	10.3	37.2	-	33.6	31.6	10.8
W	0.4	3.0	1.2	‡	4.4	27.6	24.1	49.7	3.1	5.5	1.9	16.4
X	1.0	1.1	13.4	2.9	2.4	19.1	4.8	18.0	1.0	14.2	20.8	2.7
Y	0.6	0.7	4.3	2.3	1.2	21.0	20.7	20.4	0.8	11.4	23.4	7.0
Z	1.4	1.7	5.5	‡	3.5	31.9	20.3	32.7	*	33.2	11.8	20.3
AA	1.1	1.5	9.6	4.2	4.0	28.5	9.1	28.4	*	2.5	26.7	20.0
BB		0.6	7.4	0.6	8.5	9.2	2.5	11.0	-	5.6	1.4	0.5
CC	7.5	7.8	31.3	2.2	12.6	108.4	42.4	102.4	1.1	109.5	155.4	20.3

* indicates presence identified, ratio less than 0.1

\- not detected

‡ not determined

**Norcocaine ratio calculated with fragment ion (m/z 124)

exclusively a pyrolysis product of cocaine formed in the smoking of crack cocaine smoking (34-35). Evidence supporting the identification of ecgonidine methyl ester as a pyrolysis product and not a biotransformation product lies the absence of the corresponding ethyl analog, ecgonidine ethyl ester, in cases where ethyl benzoylecgonine was present. There are no published reports regarding the toxicity of these pyrolysis products.

Cinnamoylcocaine and cinnamoylecgonine are routinely encountered in the urine of cocaine users, and cinnamoylcocaine is known to be present in illicit cocaine samples. We noted the presence of cinnamoylecgonine in most urine samples containing cinnamoylcocaine. The main significance of these congeners is to indicate that the subject had been using low grade illicit as opposed to pharmaceutical grade cocaine.

3.5 SUMMARY TO CHAPTER 3

The above method demonstrated good linearity from 0 - 100 µg/mL and was useful in studying a wide variety of compounds associated with cocaine use. Unfortunately, the more polar compounds ecgonine and ecgonine methyl ester, were the most difficult to wash off the columns and collect for further detection. While the method isolates several compounds of interest, elution of 100% of these compounds with larger solvent volumes is not practical due to a labor intensive drying step. The reversible binding needed in SPE will make 100% isolation of a range of compounds on one phase difficult. Incorporating direct detection will greatly increase the reliability of analysis.

The derivatization procedure used in this analysis could be improved as well. Even if 100% of analytes are recovered, 100% derivatization is unlikely. Incorporating a derivatization of both the groups either before chromatography (43) or after (54) may improve the analysis.

While this method was applied to urine analysis, it would be useful to have a method for the other biological fluids. Steps would have to be incorporated to precipitate proteins if the method was used on whole blood samples.

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CHAPTER 4: FIRST FIBER OPTIC FLUORESCENCE DETECTION FOR FLOW INJECTION RENEWABLE SURFACE TECHNIQUE: CONFIGURATIONS AND LIMITATIONS

The advantages of solid phase extraction over conventional solvent extraction and the practical limitations of commercially available packed solid phase extraction columns were discussed earlier in this work. While the mechanism in solid phase extraction of immobilizing solvents on a sorbent to eliminate the need for liquid solvents is elegant, as currently designed the elution steps are time consuming and on-column detection is not feasible. A novel technique, the flow injection renewable surface technique (FIRST), overcomes these limitations by using a disposable reaction surface in a controlled sequential injection system.

FIRST was developed to use a disposable solid phase as a way to study the interactions of biomolecules without having to regenerate a reaction surface, a step that had proven to be a major contributor to analysis time (1). Spherical microparticles or beads were reproducibly controlled in a sequential injection manifold. Successful FIRST hinges on both the repeatable manipulation of fluids and beads and the detection of analytes as they bind to the beads. As such, a major advancement was made in FIRST with the introduction of the jet ring cell, because it serves as both a flow cell to hold the beads and an optical cell to monitor them (2). The jet ring cell was well suited to the epiluminescent microscope used for detection. However, the applications to FIRST can be extended with the use of other detectors and light sources. This chapter describes and characterizes a novel and versatile flow cell coupled to fiber optics to facilitate detection.

A suitable FIRST flow cell must have a flow geometry that reproducibly traps and releases beads while at the same time leaving a space for fluid to perfuse over the bead surface. Figure 4.1 shows a previous version of a FIRST cell, the jet ring cell (2). In A) the beads are held in place in a hollow tube by a narrow gap, while reagent perfuses over the beads and out to waste through the space between the end of the tube and the detector window. The beads are released by pulling the hollow tube away from the detector window, increasing the gap to a size greater than the bead diameter, as seen in B. The size of the gap can be adjusted so that it is just smaller than the bead diameter by grinding the end of the tube to desired roughness. It was determined that the gap could be reduced so that beads with a minimum diameter of 30 μm do not escape out the gap, while maintaining regular fluidic profiles (3). The radial geometry of the flow at the end of the jet ring cell allows reagent to perfuse the beads evenly. The usefulness of FIRST will be extended as more versatile and commercially available flow cells are developed.

While electrochemical measurement has already been published (4) optical measurements still need do be worked out, especially fluorescence. In addition to the flow requirements, a FIRST cell must be designed in such a way that the analyte is measured as it binds to the bead surface. Figure 4.1C illustrates the view of the detector in a jet ring cell. This design succeeds because the beads are held against the detector window and the flow must perfuse over the beads to reach the outlet. Thus, any beads in the detector window will come in contact with reagent and analyte.

The initial FIRST experiments have been carried out with an epiluminescent microscope (2) as the detector. However, FIRST detection has also included a bifurcated fiber optic cable linked to a spectrophotometer for reflectance or fluorescence

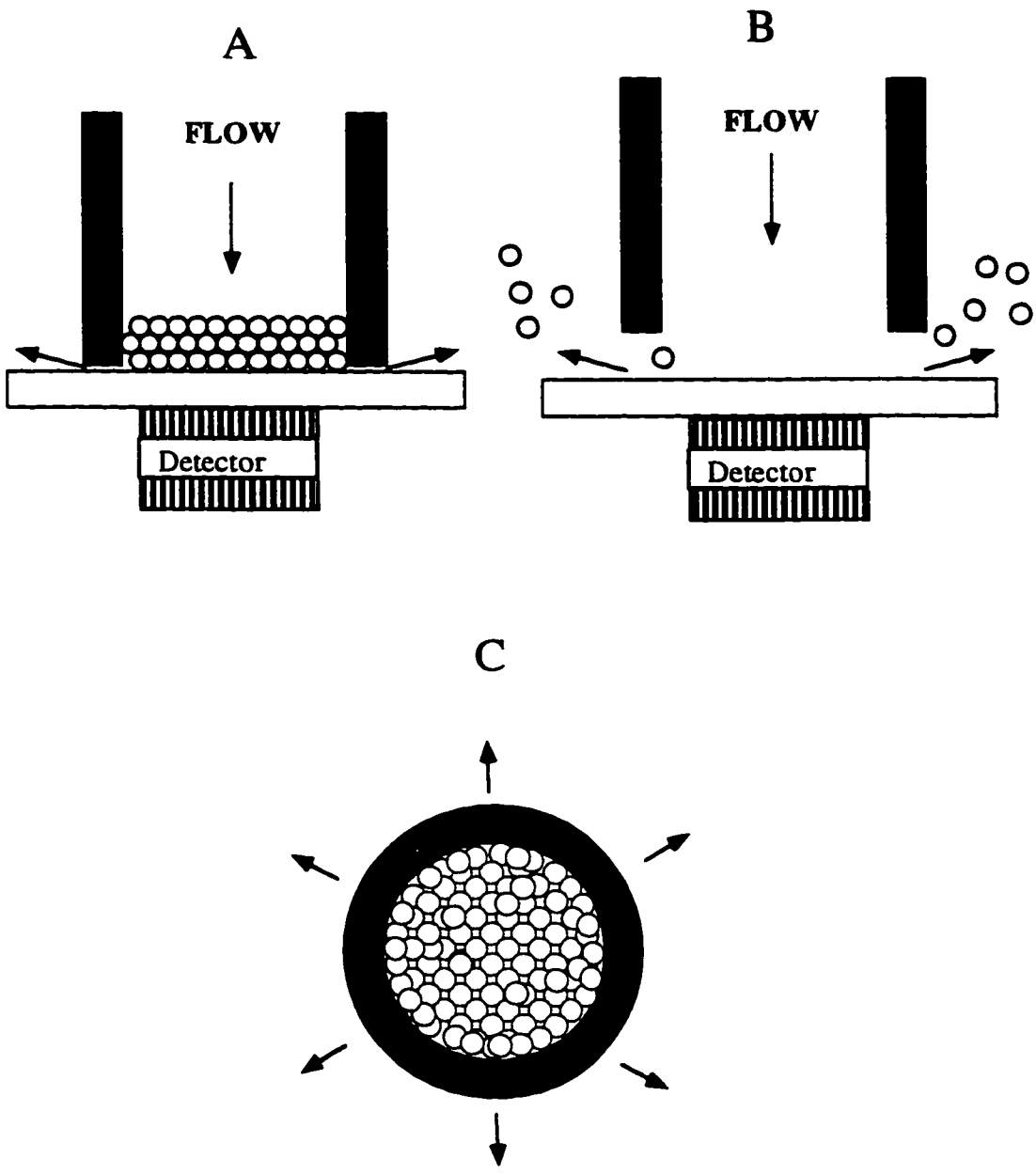


Figure 4.1 Jet Ring Cell

measurements (5-6). The advantage to using fiber optics are that they can be interfaced to a variety of light sources and detectors, depending upon the application or resources (7).

In this work, a jet ring flow cell with leaky piston geometry, originally designed for transmittance measurements by Ivaska (8) was characterized for fluorescence measurements by considering several flow and fiber optic configurations. The flow cell geometry was evaluated with respect to the repeatability of bead packing, bead volume, and perfusion of reagents. Additionally, the flow cell was designed to use standard fittings, making it low cost, versatile and rugged. Since the cell was designed to be used for fluorescence measurements, the optical geometry was required to excite and collect the emission from fluorophores in view of the detection window. The following components and reagents were used in the flow cell characterization studies.

4.1 FIRST MANIFOLD

Figure 4.2 shows the FIRST manifold comprised of an 8 port multiposition selector valve (Valco Instrument Co. Inc., Houston, TX) fitted with standard fittings (Upchurch Scientific, Harbor, WA) and 0.8 mm inner diameter Teflon tubing (Valco Instrument Co. Inc., Houston, TX). The valve and syringe pumps (1 and 5 mL, Cavro, Sunnyvale, CA) were controlled with a 386 personal computer (ADPS, Computer World). Programs to control the manifold and collect data were written in house (Quick Basic, Microsoft, Redmond, WA) and used a Real Time Devices ADA 1100-2 interface card (Real Time Devices, State College, PA).

In addition to standard fittings, a novel dual lumen injection device was fitted to the valve. It consisted of two hypodermic needles (21 gauge) glued in tandem into a

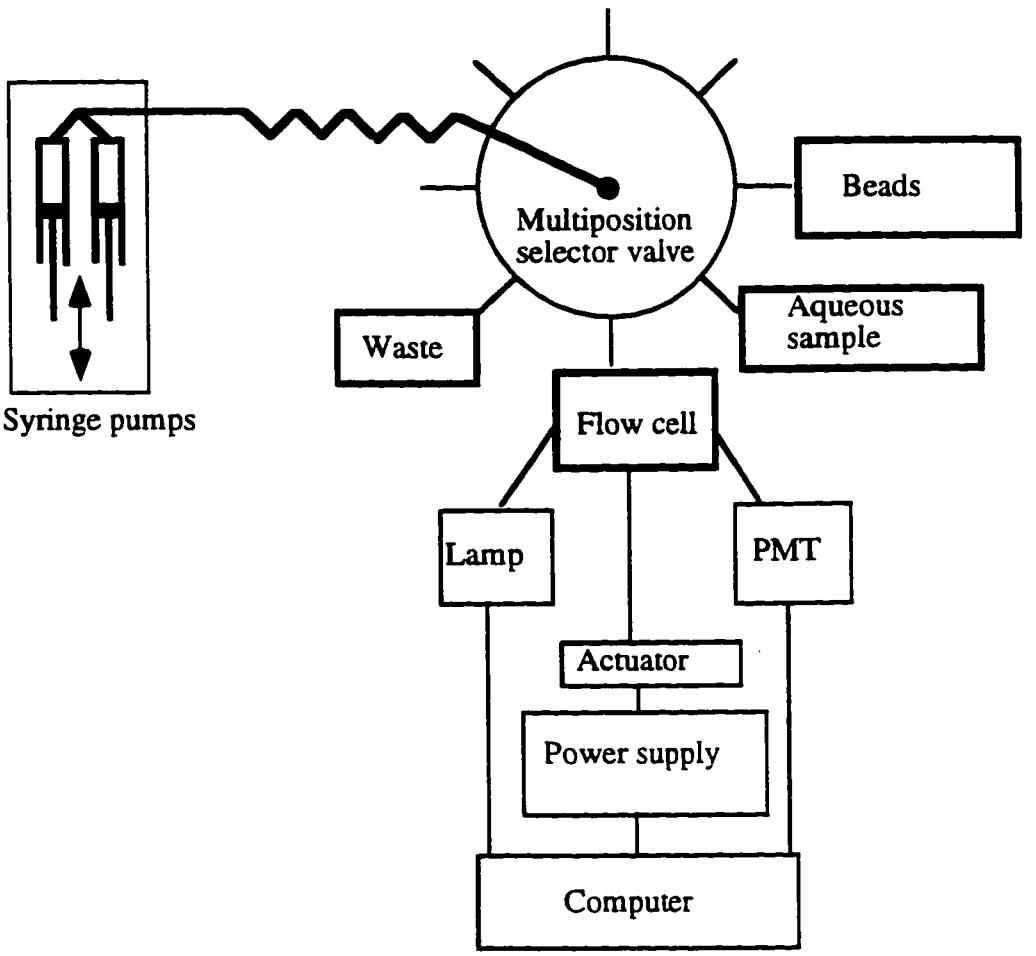


Figure 4.2 FIRST Manifold

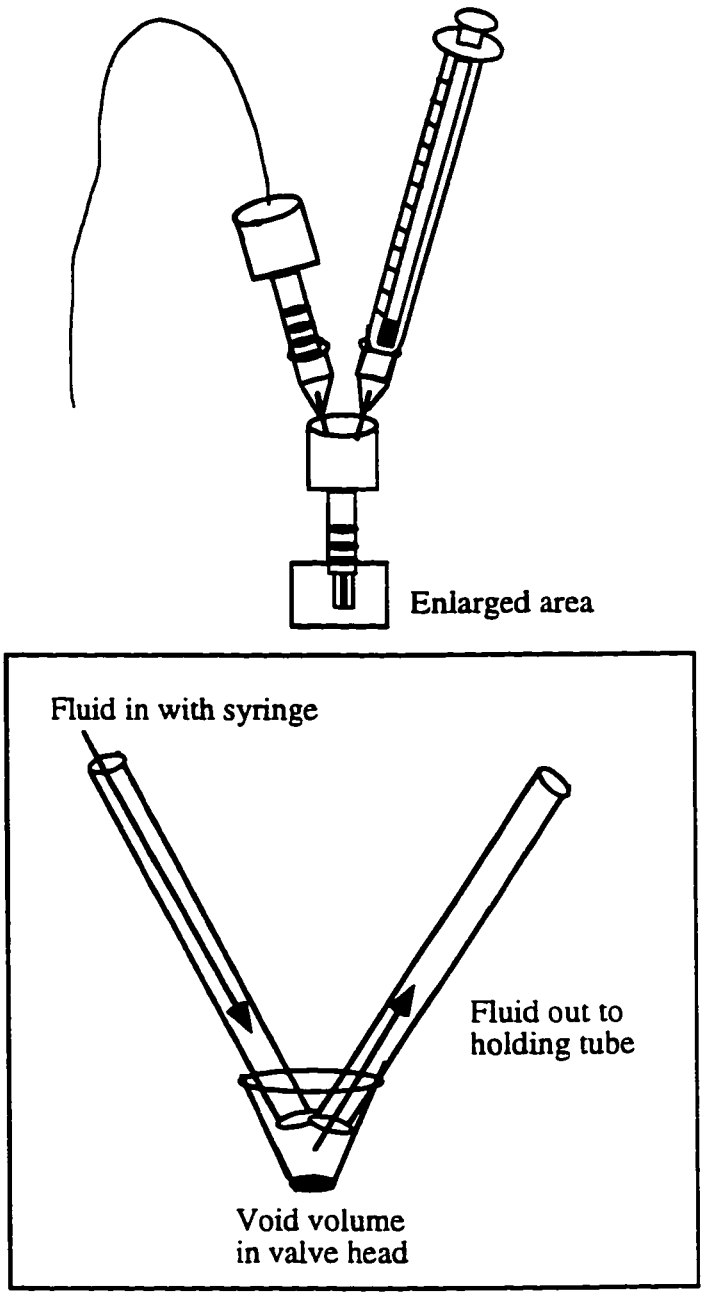


Figure 4.3 Dual Lumen Injection Device

standard fitting and then sawed off to a blunt tip and polished (Figure 4.3). Reagent that is injected into one needle flows through the dead volume at the end of the valve head (ca 1 μL) that serves as a conduit between the two needles, and is stored in tubing connected to the other needle. When the valve is at the dual injection device position, it draws in sample from the tubing. This design is useful for loading in very small volumes of reagent, as the reagent is directly injected into the valve and does not have to travel through connector tubing.

4.1.1 Flow Design

Figure 4.4 shows the optimized version of the jet ring cell for fluorescent measurement using a "leaky piston" geometry. All lines were drilled with a #51 drill bit equivalent to 0.067 inch (1.58 mm) and the ports were threaded so that they can be fitted with standard finger tight fittings. Fluids were pumped into the cell at positions B or C, depending on the flow configuration, and went out to waste at E.

The piston, shown at position A, was a 1.5 mm diameter stainless steel rod actuated by 24 volt solenoids (Guardian Electric, Woodstock, IL). The middle diagram shows the piston in the "down position," with the beads trapped in approximately 4 μL volume. The right hand side shows the piston in the "up" position, with beads flowing out to waste.

4.1.2 Optical Design

An 8 V (20 W) halogen lamp was used as the excitation source (Osram, Germany). A 485 nm (22 nm) bandpass filter was placed between the lamp and a 1 mm diameter plastic fiber optic (Crofon, Dupont, DE). The jacket was stripped off the other end of the fiber and it was inserted into 1 mm inner diameter PEEK tubing

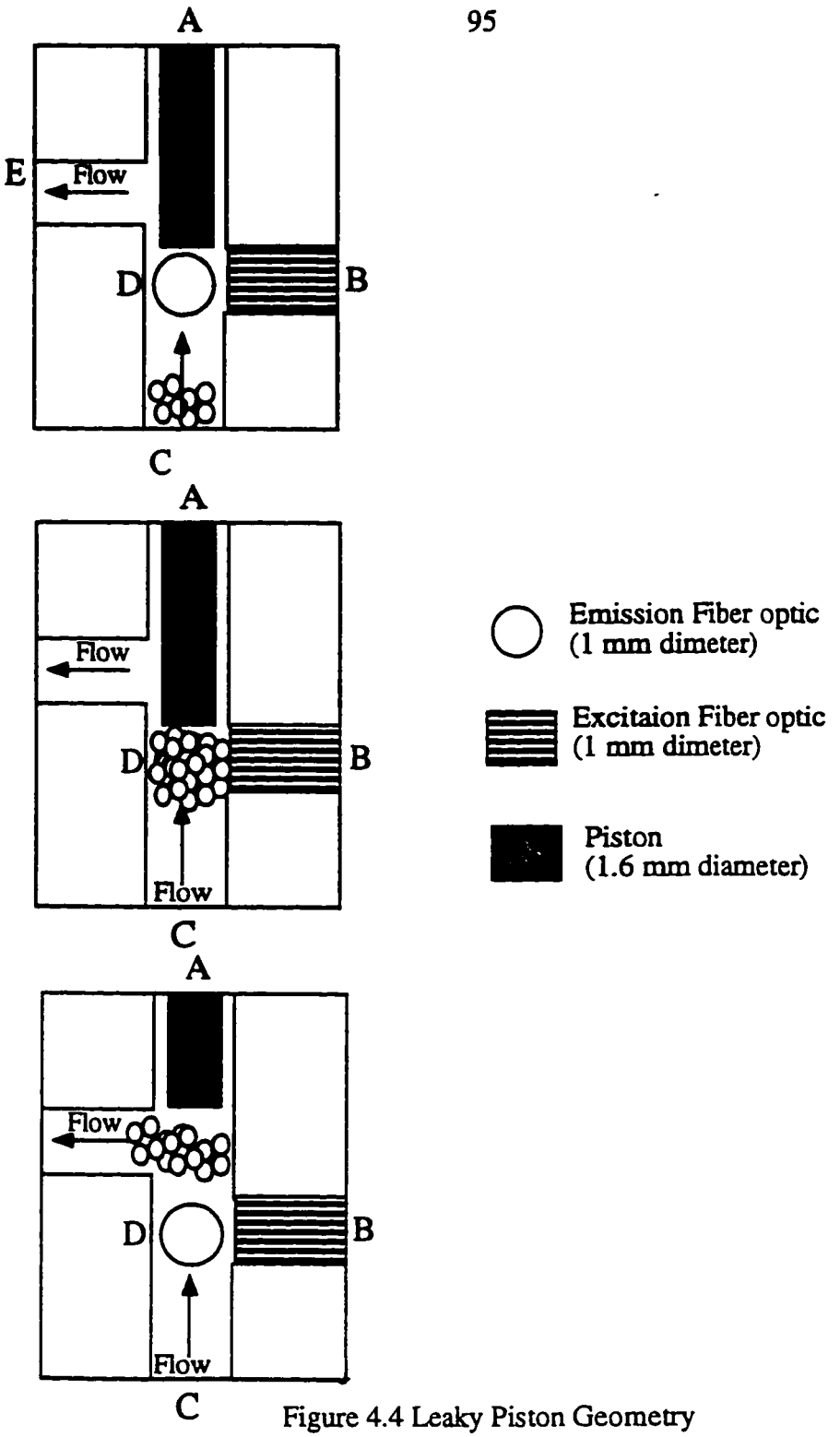


Figure 4.4 Leaky Piston Geometry

(Upchurch Scientific, Oak Harbor, WA) and fit into the flow cell at position B. A second fiber optic was prepared in the same manner and inserted at position D in the flow cell, at 90° to the first, the other end of which was fitted against a 520 nm long pass filter (Optometrics, Ayer, MA) and then against the window (diameter 5 mm) of a photomultiplier tube (PMT) H5783 (Hamamatsu Corp., Bridgewater, NJ).

Video images were taken with a color CCD camera (OSCAR World Precision Instruments Inc., Sarasota, FL) fitted to a microscope (Carl Zeiss, Oberkochen, Germany) with a 1.6 objective and recorded on a Panasonic Omnivision VHS.

4.2 REAGENTS

A working solution of diethylaminoethyl (DEAE) Sepharose (Sigma Chemical Co. St. Louis, MO) anion exchange beads was prepared by diluting the bead stock (66% solution of gel) in twenty times the volume of pH 7.4 0.1 M phosphate buffered saline (PBS, 9). This dilution factor has been used in other FIRST applications as the beads are present in a concentration that can reproducibly be pulled in with the syringe pumps. The bead concentration of this suspension was approximately 30 μg/mL. It is difficult to reproducibly inject small volumes of concentrated bead suspensions. In most of this work, the beads are kept in suspension between runs by stirring in a rotating flask, tilted at a 45° angle.

A 1 mg/mL stock solution of fluorescein isothiocyanate (FITC, Sigma Chemical Co. St. Louis, MO) was prepared in pH 9.4 carbonate buffer (0.1 M $NaHCO_3$/0.1 M Na_2CO_3). Dilutions were made with pH 7.4 PBS and carbonate buffer. Fluorescently labeled beads were made by adding FITC solutions (100 μL) to 100 μL of stock DEAE beads and mixing for 1 hour before being diluted to 2 mL.

A working solution of Protein G labeled Sepharose beads (Zymed Laboratories Inc., South San Francisco, CA) were prepared in the same manner and used for experiments to illustrate no binding of FITC.

4.3 FLOW CELL DESIGN

4.3.1 Leaky Piston Flow Geometry

Figure 4.4 illustrates the sequence of events used in these experiments. The cell was loaded with different volumes of bead suspension from C, at a flow rate of 2 ml/min. The beads were trapped in the detection area with the piston in the "down" position, as the beads cannot pass around the outside of the piston. The sample or reagent was then pushed over the beads at 0.5 mL/min and monitored by the fiber optic at position D at 5 Hz, linked to the PMT, unless otherwise noted. The piston was pulled "up" and the beads washed out to waste, through position E, at 5 mL/min with 1 mL of carrier buffer.

A second flow geometry with similar optical properties to that in Figure 4.4 was found to have irreproducible flow patterns as revealed by comparing the variation (CV $\geq$ 10%) with that of the other detector configurations (CV $\leq$ 5%). Figure 4.5 shows the cell with the flow entering at B, and then making a turn above the detector window. The beads would not always reach into the far corner as shown in the center diagram and were not tightly packed across the entire cell.

4.3.2 Leaky Piston Optical Geometry

Two Fibers

In addition to the flow geometries discussed above, a variety of fiber optic arrangements are possible with the leaky piston cell. In the final version (Figure 4.4), the excitation fiber was placed in position B, 90° to the piston. This configuration was evaluated alongside that shown in Figure 4.5, with the excitation fiber 180° from the piston, because preliminary experiments in solution showed an increased sensitivity due to reflection of the excitation light off the end of the piston. A third configuration, similar to that in Figure 4.5, had the excitation fiber at D and the emission collected at C was used to determine if any emitted light could reflect off the piston and improve sensitivity.

The emission intensities of fluorescein in solution situated around the beads using the geometry of fibers 90° and 180° to the piston were roughly equal. The emission signal was collected with the CCD camera and plotted as the light intensity across the cell diameter using NIH image software (Figure 4.6). The profile covers 1.8 mm across the flow cell and the area and the maximum signal (ca 150 units) of the profiles are about equal in each case. The reflective profile is slightly asymmetric in the geometry shown in Figure 4.5, as the light spreads out to position B on one side and against the wall of the flow cell on the other.

The configuration shown in Figure 4.4 was chosen as it had suitable optical properties and an unimpeded flow geometry, resulting in a more laminar flow profile. The results from the configuration in Figure 4.5 were irreproducible. This is due to the problems with bead packing as discussed above. No improvements were observed when the emission was collected 180° to the piston because it suffered from the same

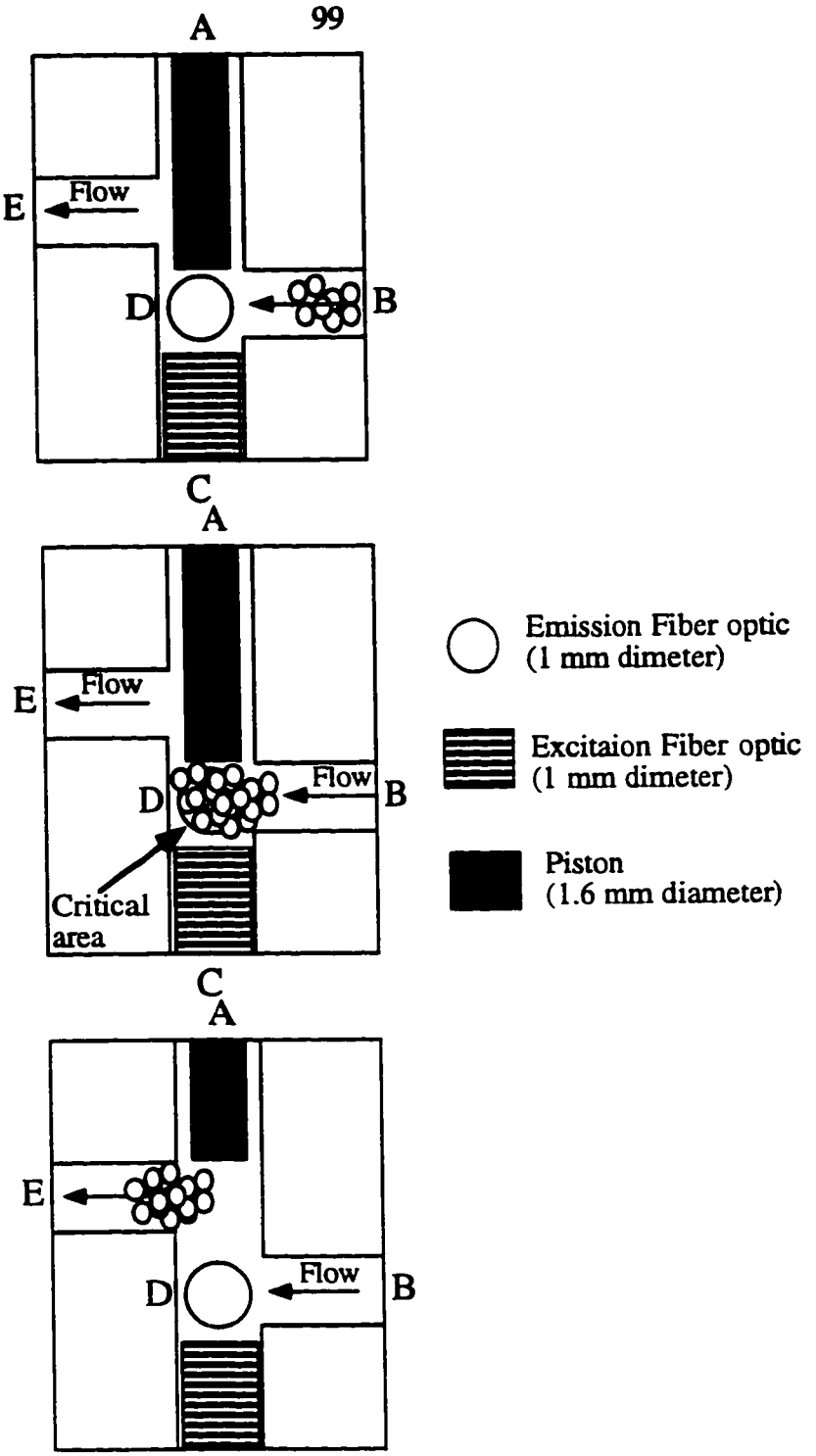
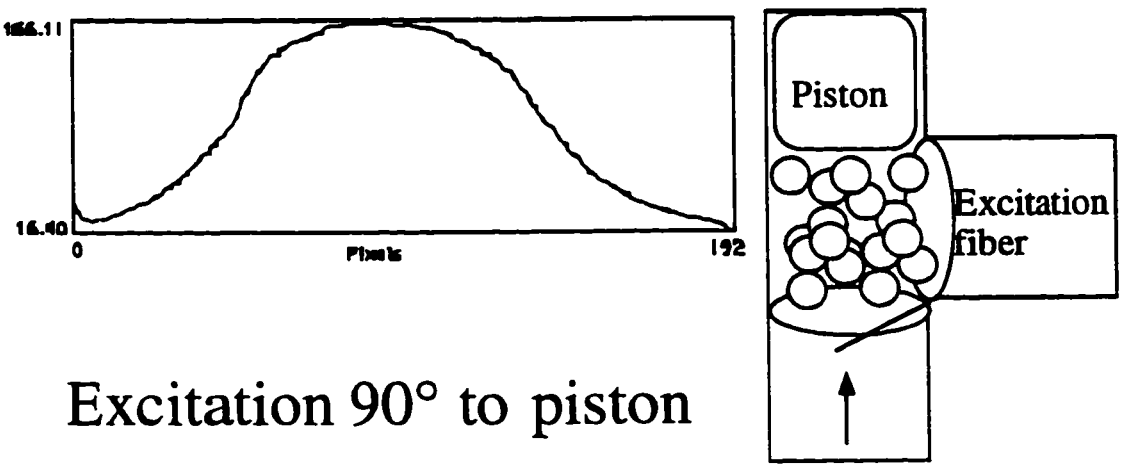
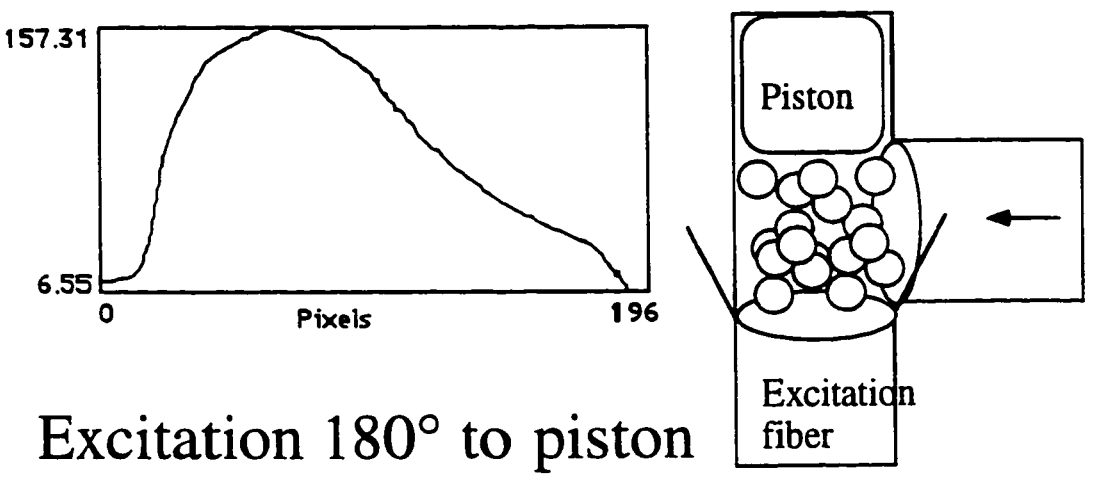


Figure 4.5 Leaky Piston Geometry with Corner



Excitation 90° to piston



Excitation 180° to piston

Figure 4.6 Comparative Light Profiles with Reflective and Non-reflective Excitation

problems with irreproducibility and there was no increase in reflection because the cell was filled with beads.

LEAKY PISTON GEOMETRY WITH DETECTION AT 360°

Since the area closest to the excitation fiber will receive the highest intensity of light, experiments were conducted with excitation and emission at the same position. The common end of a bifurcated fiber optics with diameters of 200 and 500 μm (Ocean Optics, Dunedin, FL) was inserted at position C in Figure 4.4. The fluorescence signal resulting from this arrangement was an order of magnitude lower than either of the 90° arrangements discussed above. Presumably, this is due to the smaller size of the fibers, as compared to the larger fibers that can be used when only one is fit into 0.16 mm diameter. The collection optics of the epiluminescent microscope reduced this problem in the initial FIRST applications (10).

MOVING TUBE GEOMETRY

The leaky piston was also used to compare the signal to a moving tube geometry that was close to the original moving tube used in FIRST applications by other researchers (5). Experiments were conducted with the flow going through a 0.8 mm inner diameter tube at position A, held against the bifurcated fiber optics at C. This configuration gave a signal two to three times less than that of the leaky piston as described above. Because part of the cell volume is taken up by the walls of the hollow tube, the cell volume is two times smaller with a tube geometry than a leaky piston, resulting in less fluorophore in the range of the detector.

4.4 CHARACTERIZATION OF THE LEAKY PISTON FLOW CELL

Once the optimal flow and optical geometries of the leaky piston flow cell were determined (Figure 4.4), it was necessary to characterize the cell more precisely. From other work on solid phase fluorescence, it was known that the amount of scattered light can significantly contribute to measurements in a non-homogeneous system (11-12). Past experience in FIA lead to investigations of the effects of flow rate, bead packing and binding kinetics.

4.4.1 Influence of Background Scattered Light on the Void Volume

Figure 4.7 shows the amount of scattered light changing with the introduction of solid particles. In the first 40 seconds, DEAE beads filled the flow cell and increased the amount of scattered light by diffusing the excitation light. The signal was not repeatable as the beads filled the cell but came to a stable baseline once the cell was filled. The amount of scattered light reaching the detector can be reduced by adjusting the optical filters. Using a more narrow band pass filter on the excitation light and a sharper cut off filter before the PMT will reduce the wavelength region of the filters that overlap.

FIRST requires discrimination between the fluorescence coming from the fluid around the beads, and that coming from analytes bound to the beads. Figure 4.7 shows a flow profile of 100 µL fluorescein isothiocyante (FITC) over the packed beads without an anion exchange and the same sample in an empty flow cell. The signal in a packed cell is slightly higher than in an empty cell because of the background scattering. The profiles are close in shape and maximum, showing that while some of the cell volume is occupied by the bead material, most of the cell volume is filled with fluid, which is expected for agarose beads. Agarose beads are transparent and more

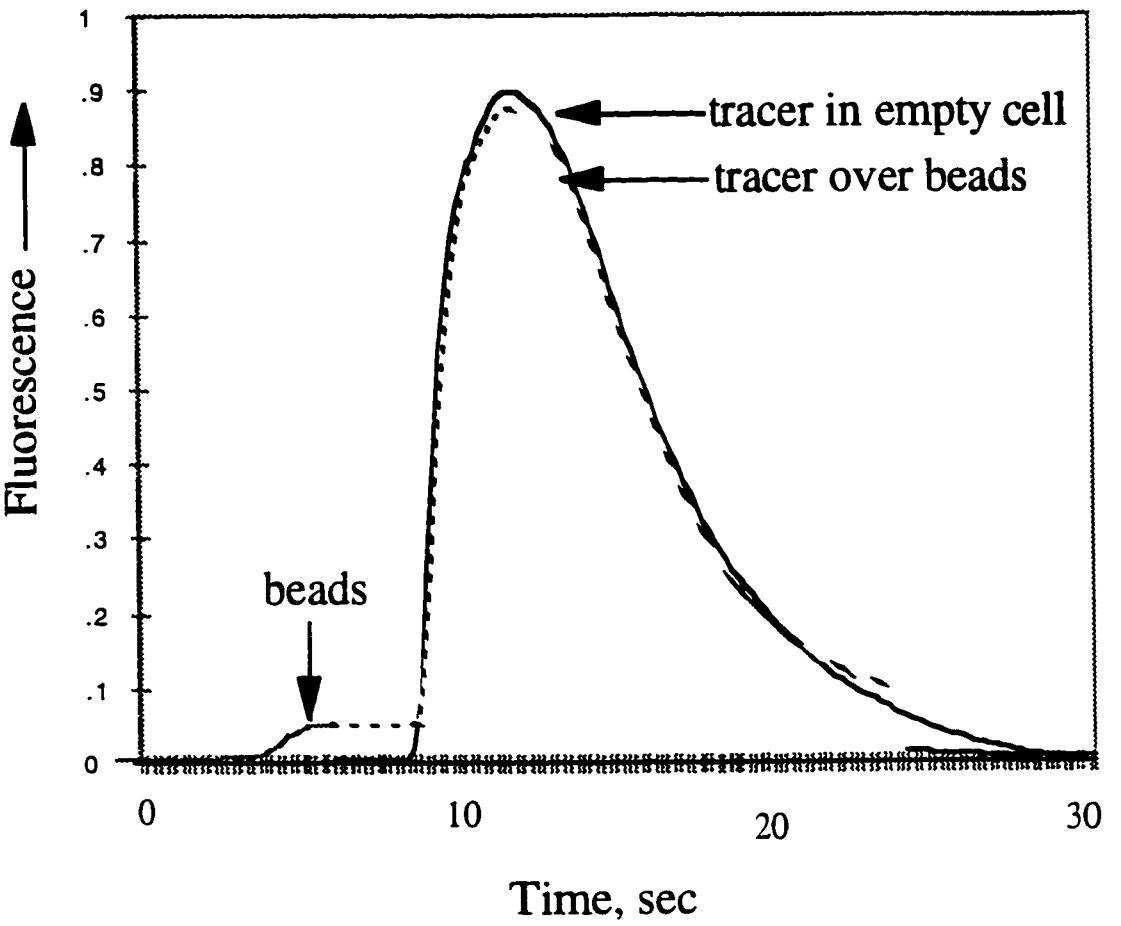


Figure 4.7 Flow Profile with Leaky Piston Geometry

than 80% water by volume. The contribution of reagent in solution to the overall measurements is reduced by using the data just after the tracer peak passes through the flow cell. At this point, very little of the tracer solution remains in the flow cell so that the signal is due to the bound analytes.

4.4.2 Repeatability of Solid Phase Manipulation with FITC Labeled Beads

FIRST depends on the repeatable manipulation of the beads as they provide the reaction surface. Previous applications of FIRST used approximately 10,000 beads per analysis so that small deviations in the bead amount do not significantly contribute to the number of binding sites. The leaky piston flow cell uses about three times this many beads. In order to evaluate the repeatability of bead manipulation in the leaky piston cell, 200 μL of a DEAE bead suspension that had soaked in 100 μL FITC (66 μg/mL) to just saturate the beads was pumped into the flow cell. Figure 4.8 shows 4 replicates with a coefficient of variation at the maximum of 1%. The repeatability of other concentrations of FITC labeled beads was examined and showed coefficients of variation less than 10%. When these results are compared to the signal from injections in an empty flow cell, which are consistently less than 5% CV, it suggests that somewhat more than half of the variation is due to the beads, 60-65%.

4.4.3 Repeatability of FITC Binding to Beads

Since FIRST measures the analytes as they bind to the bead surface, experiments were conducted to determine the signal change and repeatability of binding. FITC solutions (100 μL) of different concentrations (0.3, 0.6, 3, 6 μg/mL) were perfused over 200 μL of DEAE bead suspension and held in the cell. The average values of the flow profiles are shown in Figure 4.9, coefficient of variation (CV) was less than 10%

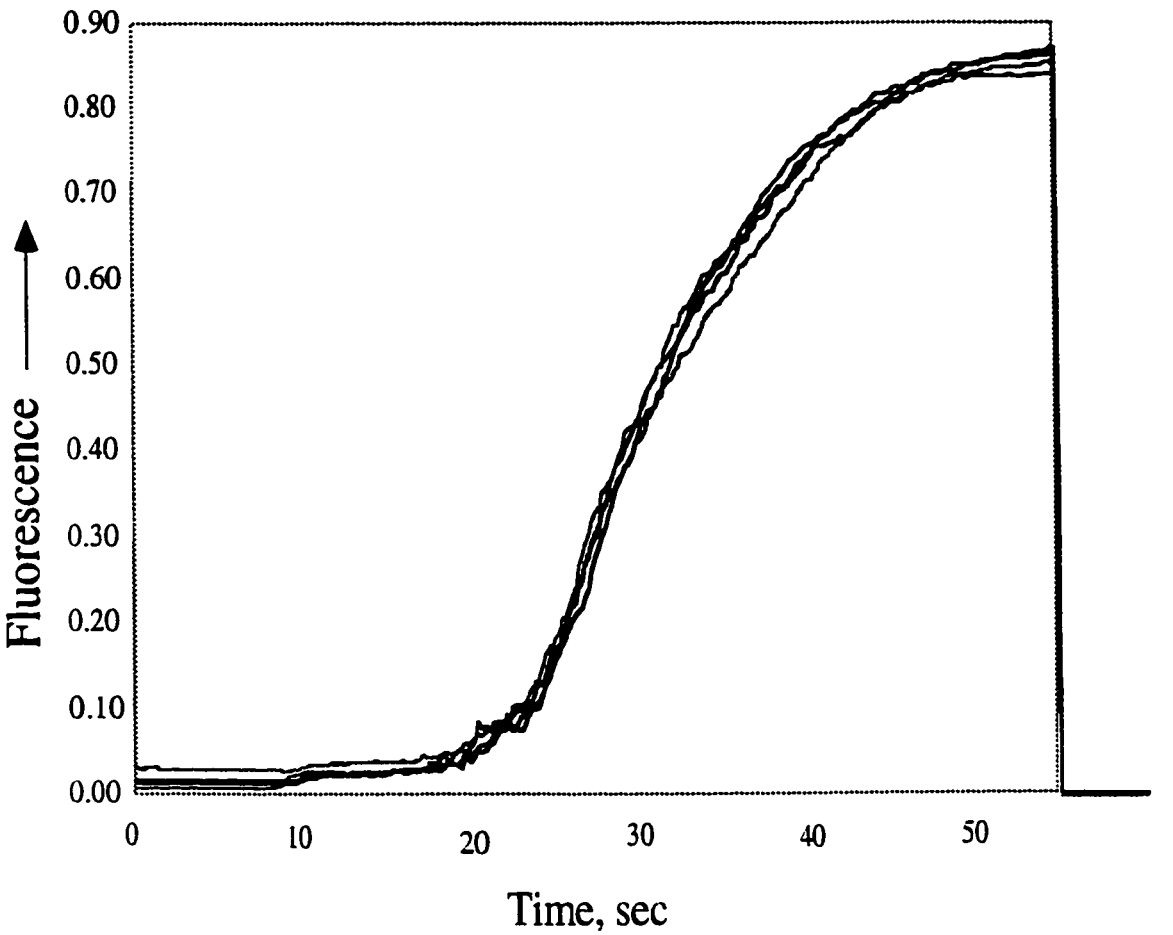
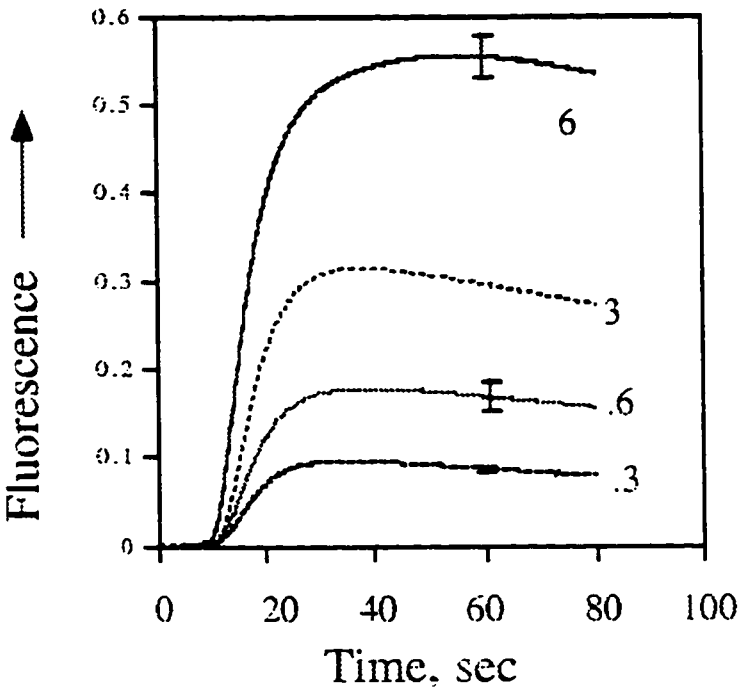


Figure 4.8 Repeatability of Fluorescence Signal from FITC Labeled Beads



Concentration, µg/mL	CV%	(n)
0.3	4	4
0.6	9	4
3		1
6	4	3

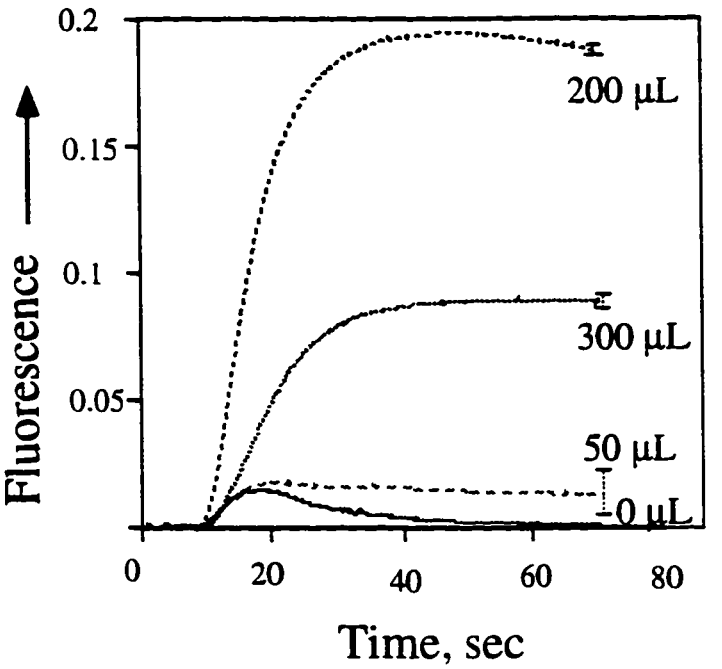
Figure 4.9 Flow Profiles of Fluorescence Signal with Changing FITC Concentration

for each concentration. As expected, the maximum signal increased as the FITC concentration increased. The repeatability was not significantly effected by the binding as compared to the signal from prelabeled beads with FITC (6 μg/mL) that had 10% CV on the same day (n=3).

The repeatability experiments used 200 μL of the bead suspension (1:20) because when packed, this volume just filled the flow cell. Using more than 200 μL of the suspension results in some of the beads sitting outside of the detector window. Figure 4.10 shows a tracer of 100 μL FITC (6 μg/mL) flowing over different bead volumes. As the volume of the bead solution was increased from 50 to 200 μL the signal increased as the number of binding sites increased. With a 300 μL bead suspension the signal drops because a significant amount of the FITC bound to the beads outside of the detector window. The signal increases with increasing bead amount until the bead volume exceeds the cell volume. This same effect was present with other flow cell geometries so that it could not be overcome with this cell design.

This affect should not be underestimated as it can effect results in several ways. If the concentration of analyte in the sample is low, larger volumes may be required to make sure the analytes will bind to the beads in view of the detector window. If larger sample volumes are not available, the number of binding sites on the beads may be reduced or a mixture of modified beads and blank beads may be used to fill the flow cell.

Additionally, experiments that used less than 200 μL of the bead suspension were not repeatable, as shown in Figure 4.10. The beads must entirely fill the flow cell or they may shift around and reorder during the analysis and severely hamper the



Volume, µL	CV%	(n)
0		(1)
50	64	(3)
200	3	(4)
300	2	(3)

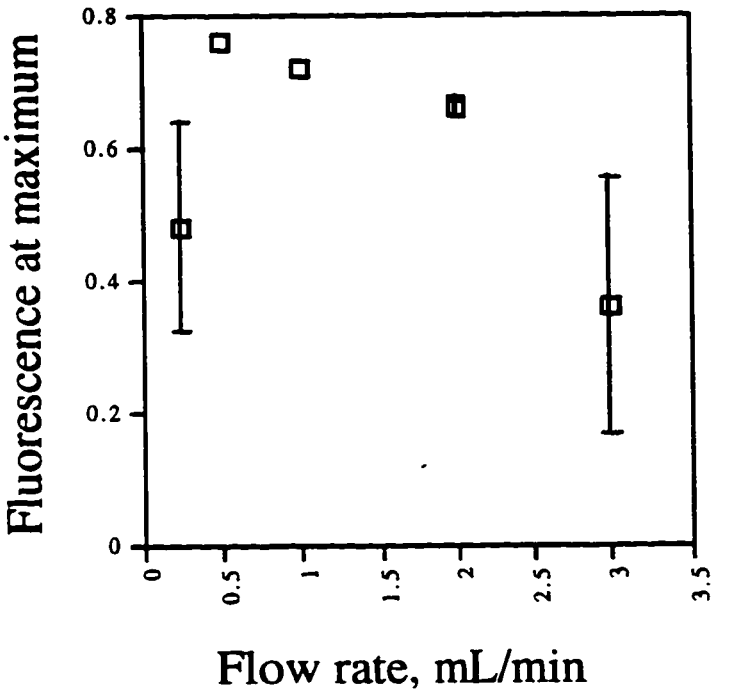
Figure 4.10 Flow Profiles as a function of Bead Amount

reproducibility. The coefficient of variation went from 64% for 50 μL of the bead suspension to 3% with 200 μL (n=3). The repeatability was best with a filled flow cell (ca 200 μL suspension or about 6 mg gel) and is limited by the packing of the beads.

The above results were very different than with the jet ring cell, where changing the amount of beads changed the amount of signal but did not significantly alter the repeatability (3). Experiments with an epiluminescent microscope to characterize this type of cell using fluorescent beads showed that increasing the number of beads had a direct effect on the signal (3). With the moving tube geometry, the first beads loaded into the system will be the first to pack against the detection window, with successive layers settling further away from the detector. This differs from the leaky piston geometry, where beads fill on the side closest to the outlet and then towards the inlet across the detection window. The bead volume must be above a threshold volume to fill the cell. A partially filled flow cell prohibits repeatable analysis because a variety of light paths are possible. However, the bead volume cannot exceed the cell volume or it will compromise detection as some analytes may be bound outside the detection window.

4.4.4 Effects of Flow Rate

The effect of flow rate on the binding of FITC to DEAE Sepharose was tested as a way to improve the repeatability. If the binding is diffusion limited, it should increase with increased contact time. The flow rate did not affect the binding. This was examined by stopping the flow at the tracer maximum but did not result in an increases in signal. Figure 4.11 illustrates that while the amount of FITC bound was not affected by the flow rate, there was a large impact on the repeatability. At high flow rates, over 3 mL/min, imprecision was due to beads escaping around the piston. Agarose beads



Flow rate	CV%	(n)
0.25	33	(3)
0.5	1	(2)
1	1	(3)
2	2	(3)
3	54	(4)

Figure 4.11 Effect of Flow Rate on Repeatability

are not hard spheres but can be deformed along the side of the piston at high pressures. At slow flow rates one source of imprecision may be that the beads are not packed tightly against each other and they diffuse back around the flow cell.

4.5 RESULTS OF CELL CONFIGURATION AND CHARACTERIZATION

The leaky piston flow cell yields the most reproducible results when the geometry of flow is symmetrical along the axis of the jet ring cell - resulting in symmetrical deposition of retained beads. In this configuration, the excitation and emission fibers are 90° to the piston. Since this configuration was optimized for fluorescence measurements, it would be logical to extend the capabilities of the cell to include a full circle of fibers for collection 90° to the excitation fiber and thus increase the signal.

A cell with three emission fibers was constructed and experiments revealed a three-fold increase in signal. However, the physical distance between each port was very small so that it could not be made out of standard fittings, and the cell was prone to leaking. Also, at least one side of the flow cell should be without fittings and transparent for imaging.

Changing the bead amount has the largest influence on the repeatability. Changing the FITC amount does not change the repeatability. Future advances in bead loading and suspension control will help the most to improve the repeatability.

The flow rate has upper and lower limits. At high flow rates, the beads escape around the piston and at low flow rates the beads do not pack tightly. The cell volume has both upper and lower limits with respect to the acceptable bead volume as well. In

order for analytes to be detected on the beads, the beads must be in the window of the detector.

The 360° excitation and emission moving tube geometry that is suitable for an epiluminescent microscope is not appropriate when using fiber optics because only the analytes on the beads closest to the fiber optic can be detected, and the fibers cannot focus onto different volume elements. Also, the fiber optics cannot pick up as much light due to smaller numerical apertures than an objective lens of comparable size, so fiber diameter must be on the order of the cell area.

In this detection format of monitoring a heterogeneous mechanism comprised of beads and interstitial liquid, the fluorescence signal is complicated by the scattered excitation light. This effect can be reduced by using more precise optical filters.

4.6 CHAPTER 4 END NOTES

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CHAPTER 5: DEVELOPMENT OF AN IMMUNOCHEMICAL METHOD TO "FIRST"

In the previous chapter, FIRST was demonstrated to be a reliable and quick technique for the preconcentration and detection of a model analyte on a solid phase. Other researchers have applied FIRST to immunoassays, resulting in flow injection renewable surface immunoassays (FIRSI; 1-2). As discussed in Chapter 1, the selectivity of immunoassays is due to the specific binding of an antibody to an antigen. For this reason, antibodies are particularly well suited as sorbents in FIRST where detection depends on the selectivity of the immobilized solvent. The work presented here discusses those immunoassays used in forensic toxicology and evaluates their potential application to FIRSI with the leaky piston cell.

Immunochemical methods are widely used in forensic toxicology and several commercial automated immunoassay analyzers are available. Automated immunoassays are rapid; analyzers can process on the order of 100 samples per hour (3). The cost per analysis depends on the purchase price of the instrument along with the costs associated with maintenance, reagents and quality control procedures. The specificity and selectivity of immunoassays make them an excellent screening tool for the presence of a variety of drugs in biological fluids. The specificity of an immunoassay however, is limited by poorly understood matrix effects (4). Because immunoassays are used to measure very low concentrations, they are sensitive to slight disturbances in sample and buffer pH.

5.1 CURRENTLY AVAILABLE METHODS

In order to evaluate the usefulness of FIRSI in forensic toxicology, the literature was examined for antigens of interest in forensic toxicology which have suitable chromophores or fluorophores for detection. Some reports were found for morphine (5-9), lysergic acid (LSD, 10-11) and derivatizing agents for cocaine metabolites (12). However, none of these analytes had very high quantum efficiency and most had excitation wavelengths that would require costly UV optics. For these reasons, direct detection of compounds was disregarded and indirect detection was pursued.

Commercially available competitive immunoassay reagents and antigens were evaluated for their ability to be transferred to FIRSI. These methods are based on competition between an antigen with a label and the antigen in solution for a limited amount of antibody (Figure 5.1). The analytical signal is generated from the label, not directly from the antigen in the sample and is proportional to the amount of unlabeled antigen bound to the antibody. Indirect methods are simpler than direct measurements because the detection system always monitors the label and the same label may be used in immunoassays for different compounds.

Table 5.1 lists the competitive methods that were reviewed along with the detection principle and separation requirements. Heterogeneous assays require that the portion of antigen that is bound to the antibody be separated from the portion that is not bound to antibody before the signal is detected. In homogenous immunoassays, the signal is generated while the bound and unbound fractions are both in solution.

The heterogeneous immunoassays listed below, where one reagent was immobilized on a solid phase, could not be used in FIRSI because solutions of the

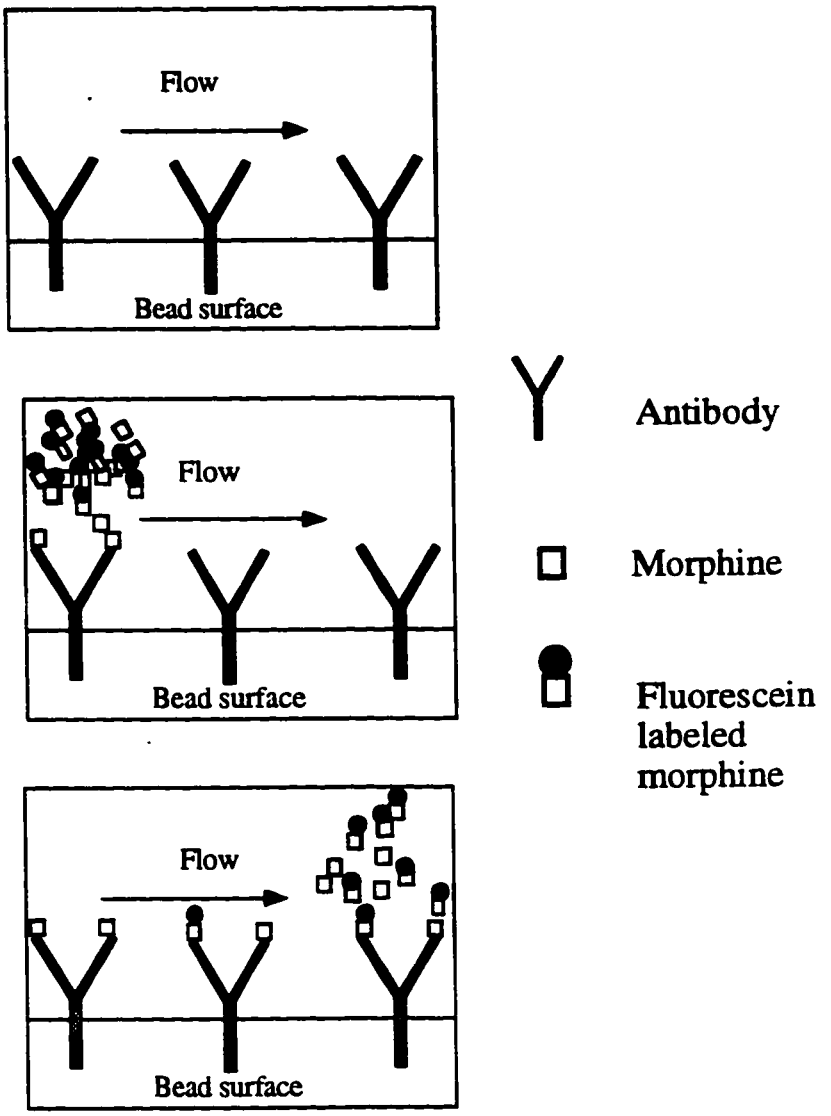

Figure 5.1 Competitive Immunoassay

Table 5.1 Competitive Immunoassays Available for Drugs of Abuse Testing

Company	Immunoassay	Format
Diagnostic Products Corporation (Los Angeles, CA)	Coat-A-Count	heterogeneous radioimmunoassay
Roche Diagnostic Systems (Somerville, NJ)	Abuscreen	heterogeneous radioimmunoassay
STC Technology (Bethlehem, PA)	Micro-Plate EIA	heterogeneous enzyme immunoassay
Behring Diagnostics (San Jose, CA)	EMIT II	homogeneous enzyme immunoassay
Boehringer Manheim (Concord, CA)	CEDIA DAU	homogeneous enzyme immunoassay
Abbott Laboratories (Abbott Park, IL)	TDx/TDxFLx/AxSYM	homogeneous fluorescence polarization immunoassay

reagents were not commercially available. Reagents of this type included those sold by STC and Diagnostic Products Corporation, where the analyte is covalently bound to the test tube or the walls of a well, respectively. In these methods, a limited amount of labeled antibody competes for the immobilized antigen and antigen in solution. The amount of labeled antibody bound to the immobilized antigen is measured via the label, after the antibody bound to the antigen in solution is decanted.

Batch heterogeneous methods, like the ones mentioned above, can require manual centrifugation that can be time consuming and may not be readily automated. Homogeneous immunoassays do not require a separation step, instead the luminescent properties of the label are somehow changed upon binding to the antibody. Of the homogeneous immunoassay systems, reagents from Behring Diagnostics, Boehringer Mannheim, and Abbott Laboratory were evaluated as to the ability of detecting a signal from the competitive reaction and the availability of reagents in solution, as described below.

EMIT II™, available through Behring Diagnostic, is a competitive enzyme immunoassay. If no unlabeled antigen is in a sample, the antibody binds to the antigen labeled with the enzyme (glucose-6-phosphate dehydrogenase), rendering it inactive (3). When unlabeled antigen, A, is present in the sample, it binds to some of the antibody, leaving a fraction of the enzyme unbound. The free enzyme is active and reduces the substrate nicotinamide adenine dinucleotide (NAD^+) to NADH. The colorimetric signal is directly proportional to the concentration of unlabeled antigen in the sample.

In order to use the EMIT II™ reagents in FIRSI, the antibody would be immobilized on the beads. The labeled antigen, A*, and unlabeled antigen, A, could compete for sites on the bead. However, the presence of labeled or unlabeled antigen bound to the beads would not give a signal. When the labeled antigen binds to the antibody on the beads the enzyme loses its activity and does not form a colored product. A signal could, however, be detected downstream in solution with a flow through cell, because as the unlabeled antigen binds to the beads, the labeled antigen flows through the cell and the enzyme activity could be monitored colorimetrically.

In another competitive enzyme immunoassay, CEDIA™ by Boehringer Manheim, the antibody does not block the active site of the enzyme, as in EMIT, but instead prohibits the enzyme from associating into an active form. CEDIA™ uses recombinant DNA technology to produce the enzyme β-galactosidase as inactive fragments, one of which is bound to analyte (13-14). If no unlabeled antigen is in solution, the antibody binds to the labeled antigen and the enzyme fragment cannot associate with the other fragments in solution to form an active enzyme. When unlabeled antigen is present in the sample, a fraction of the antibody binds to it, leaving some of the labeled antigen free to associate with the other enzyme fragments. The active enzyme generates a colorimetric signal by cleaving the substrate, chlorophenol red-β-D-galactopyranoside, that is directly proportional to the concentration of unlabeled antigen.

Attempts were made to use the CEDIA™ reagents in FIRSI. The inactive fragment of the enzyme that was not bound to the antigen, E_A, was immobilized on the bead surface. The enzyme labeled antigen, E_D, and substrate were mixed and added to the bead suspension, but no active enzyme was formed. Immobilization of E_A somehow

prohibited the association of the enzyme fragments; the complete enzyme requires complementary binding of two of each of the subunits.

ADx™/TDx™/FLx™ available through Abbott Laboratories, is a fluorescent polarization immunoassay (FPIA, 15). Fluorescence polarization is a technique where the fraction of polarized light that is emitted by molecules in a solution is measured when the solution is excited with polarized light. In this method, fluorescein labeled antigen competes with unlabeled antigen for the antibody. The degree to which the fluorescein labeled antigen emits polarized light depends on the speed at which the fluorescein labeled antigen is rotating in solution. If no unlabeled antigen is in the solution, the labeled antigen will be bound to the antibody and will rotate slowly. If instead, the concentration of unlabeled antigen is large, most of the labeled antigen will remain unbound and will rotate rapidly, emitting light in many directions, so that the polarization value is inversely proportional to the concentration of unlabeled antigen.

The Abbott reagents have been used by other researchers in a stopped-flow fluorimmunoassay (16). These reagents are shown below to also be suited to FIRSI because the antibody can be immobilized on the bead surface and competition between the labeled antigen and the unlabeled antigen for the antibody occurs as the solution passes over the bead surface. The amount of antigen bound to the antibody on the bead surface can be measured as an increase in fluorescence, as seen below. In addition, the principles in the Abbott system are somewhat universal; reagents are available for a variety of compounds of interest in forensic toxicology including cocaine metabolite (benzoylecgonine), barbiturates, benzodiazepines, amphetamines, cannabinoids, phencyclidine, and propoxyphene.

A FIRSI was developed using Abbott reagents, to determine the feasibility of applying FIRSI in forensic toxicology.

5.2 OPIATES

Opiates were chosen as model analytes because immunoassays for opiates are widely used. Opiates include the natural and semisynthetic alkaloidal derivatives of opium poppy Papever somniferum; morphine, codeine and heroin. Some opiates are prescribed therapeutically as analgesics and others are commonly abused (17). Biotransformation of these compounds occurs primarily in the liver via hydrolysis, oxidation, n-dealkylation and conjugation with glucuronic acid, as illustrated in Figure 5.2 (17). More than 50% of the morphine is excreted in urine as morphine-3-glucuronide (18) which posses no pharmacological activity. Morphine-6-glucuronide, however, is more pharmacologically potent than morphine (19-20). Heroin, an abused drug, is rapidly converted to monoacetyl morphine, which serves as a specific marker of heroin use.

5.3 EXPERIMENTAL PARAMETERS

5.3.1 Reagents

Immobilization of Antibody on Protein G Labeled Agarose

Protein G labeled agarose beads were purchased as a 50% gel suspension in 10 mM PBS, pH 7.4 (Zymed Laboratories Inc., South San Francisco, CA). Recombinant-protein G is covalently bound to Sepharose 4B (35-165 μm, Pharmacia, Sweden). A working bead suspension was prepared fresh daily, as a 1:20 dilution of bead stock in pH 7.4 PBS.

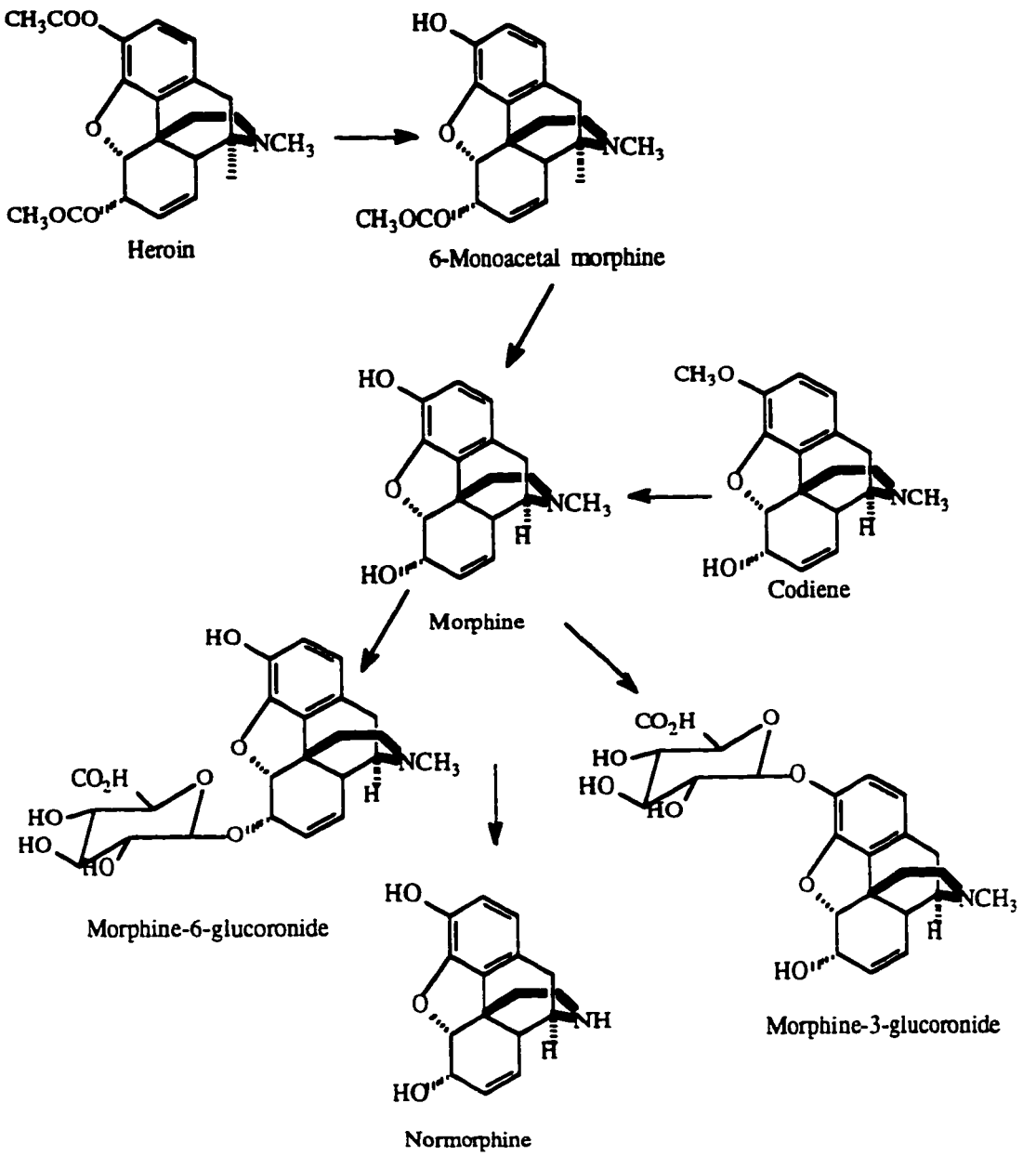

Figure 5.2 Morphine Metabolism

Antibodies were purchased as part of a TDx™ kit (Abbott Laboratories, Abbott Park, IL). The antiserum is less than 1% opiates sheep antisera (%v/v, 15) and may contain antibodies to other compounds, as it is not purified. Opiates antisera stock was added to the bead suspension prepared above, to a final 1:40 dilution of the antisera stock. The suspension was mixed by rotating on a stirrer for over one hour at room temperature, so that the antibody could bind to the Protein G. The opiates fluorescein tracer solution was less than 0.01%(%v/v, 15) and was used as purchased.

The antibodies bound to the Protein G via noncovalent interactions as described in Chapter 1 (21). Because the sample flows over the immobilized antibodies on the beads, loosely bound antibodies can wash off the beads with time. It was for this reason that some FIRSI experiments by other researchers have included a procedure to covalently link the antibodies to Protein G (22). When using the Abbott reagents however, the results were unfavorable as the antibodies subject to the covalent immobilization protocol were no longer able to bind labeled morphine. The cause of inactivity was not determined but presumably could be attributed to modification of the antibodies' F_{ab} region by the immobilization reagents to hinder binding or the antibody could have been lost in the repeated wash steps. The immobilization protocol is discussed below.

Briefly, the Protein G labeled beads were mixed with the antiserum, washed twice with 0.1 M borate buffer pH 8.2 to remove any interfering compounds, then spun and decanted. A 10 mM dimethyl pimilimidate (DMP, Pierce, Rockford, IL) solution in borate buffer was added and allowed to mix with the proteins for one hour to bridge the amines in the two proteins. The beads were washed with 10 mM ethanolamine (Sigma Chemical Co., St. Louis, MO) for five minutes to react with any excess DMP and stop

the reaction. Finally, the beads were washed with borate and then phosphate buffers to remove excess ethanolamine. Antibody coated beads prepared in this manner did not bind labeled antigen and therefore, the non-covalent link was chosen to suffice.

A stock solution of morphine was prepared by diluting a 10 mg/mL morphine sulfate solution (Sigma Chemical Co., St. Louis, MO) to 1 μg/mL in pH 7.4 PBS. Dilutions were made to final concentration from 1.25 to 350 ng/mL. Samples were prepared by adding 25 μL of the fluorescein labeled morphine to 100 μL of the appropriate concentration of the morphine dilution.

5.3.2 FIRSI Protocol

The leaky piston flow cell, associated optics and manifold were the same as those described in the previous chapter. The antibody coated beads were kept from settling out of suspension by stirring on a rotating flask. A 200 μL portion of the bead suspension was pulled into the holding coil and then pumped into the flow cell at 2 mL/min with a volume of 400 μL carrier buffer (pH 7.4 PBS). The piston was in the "down" position so that the beads were trapped within view of the detector. Then, a 125 μL portion of sample as prepared above was pulled into the holding coil and then pumped over the beads at 0.5 mL/min and the signal was collected at 5 Hz. After the sample passed through the flow cell, the piston was raised to the "up" position and the beads were pumped to waste.

5.4 RESULTS AND DISCUSSION

The signal from the fluorescein labeled morphine preconcentrating on the beads was collected as the sample flowed through the leaky piston cell. Figure 5.3 shows the

flow profiles of several samples that had been spiked with varying concentration of unlabeled morphine (0, 2.5, 12.5 and 25 ng/mL). First a baseline is collected as the antibody coated beads entered the cell and then a fluorescence signal is measured as the beads were perfused with sample.

Figure 5.4 illustrates the dependence of the percent of the labeled morphine that is bound to the antibodies on the amount of unlabeled antigen in the sample. Graphical representation of the competition curve in conventional coordinates does not illustrate the concentration response well. Instead, the data are usually presented in a semi-logarithmic plot as seen in Figure 5.5.

5.4.1 Cutoff Value

Because immunoassay methods are subject to matrix effects, they are usually calibrated to be linear in a region that is defined as a cutoff value. Samples are determined to be positive for an analyte above the cutoff of negative below the cutoff value. A second method is then used to confirm and quantify the analyte concentration. Practically, the cutoff value should not be lower than the limit of detection in the confirmation method, or false positives will be common and should be at a level that could not likely result from the presence of any other compounds. For morphine in urine, a cutoff above 1000 ng/mL is set, as it meets the above requirements .

Confirmation methods include sample preparation steps, such as solid phase or liquid extraction followed by chromatographic and mass spectrometric identification. HPLC methods with either UV (23-24), native fluorescence (25) or coulometric (9) detection methods have been described, with detection limits on the order of 1 ng/mL for morphine and between 1 and 100 ng/mL for the other metabolites. Morphine can

also be detected at a limit of detection of 6 ng/mL with a kinetic spectrofluorometric method that does not require chromatographic step but cannot simultaneously measure the metabolites (26).

In an immunoassay, the detection limit is defined as the lowest detectable signal, B_{DL}, that is different by a significant amount from the signal resulting from the blank, B_o. The

$$B_{DL} = B_o - ts\,(1/n + 1/n_o)^{1/2} \qquad (5.1)$$

value of B_{DL} is determined from the value from the Student t test at 95% confidence, t, the standard deviation, *s*; and from n_o or n measurements used to determine an average value for the blank and sample, respectively (4). A detection limit of 0.068 arbitrary fluorescent units was calculated, when the average blank signal from 5 runs was 0.122 fluorescent units, the standard deviation was 0.0305 and a value of 2.78 was used for the Student t test (27). From Figure 5.5, this corresponds to 55% of the labeled morphine bound and a concentration between the 12.5 and 25 ng/mL unlabeled morphine standards.

5.4.2 Sensitivity

The sensitivity of the immunoassay determines the range of concentrations in which the immunoassay results are useful and is defined as the concentration of unlabeled antigen that gives a signal half of the value of that with no unlabeled antigen. The sensitivity depends on the concentration of labeled antigen and the amount of antibody.

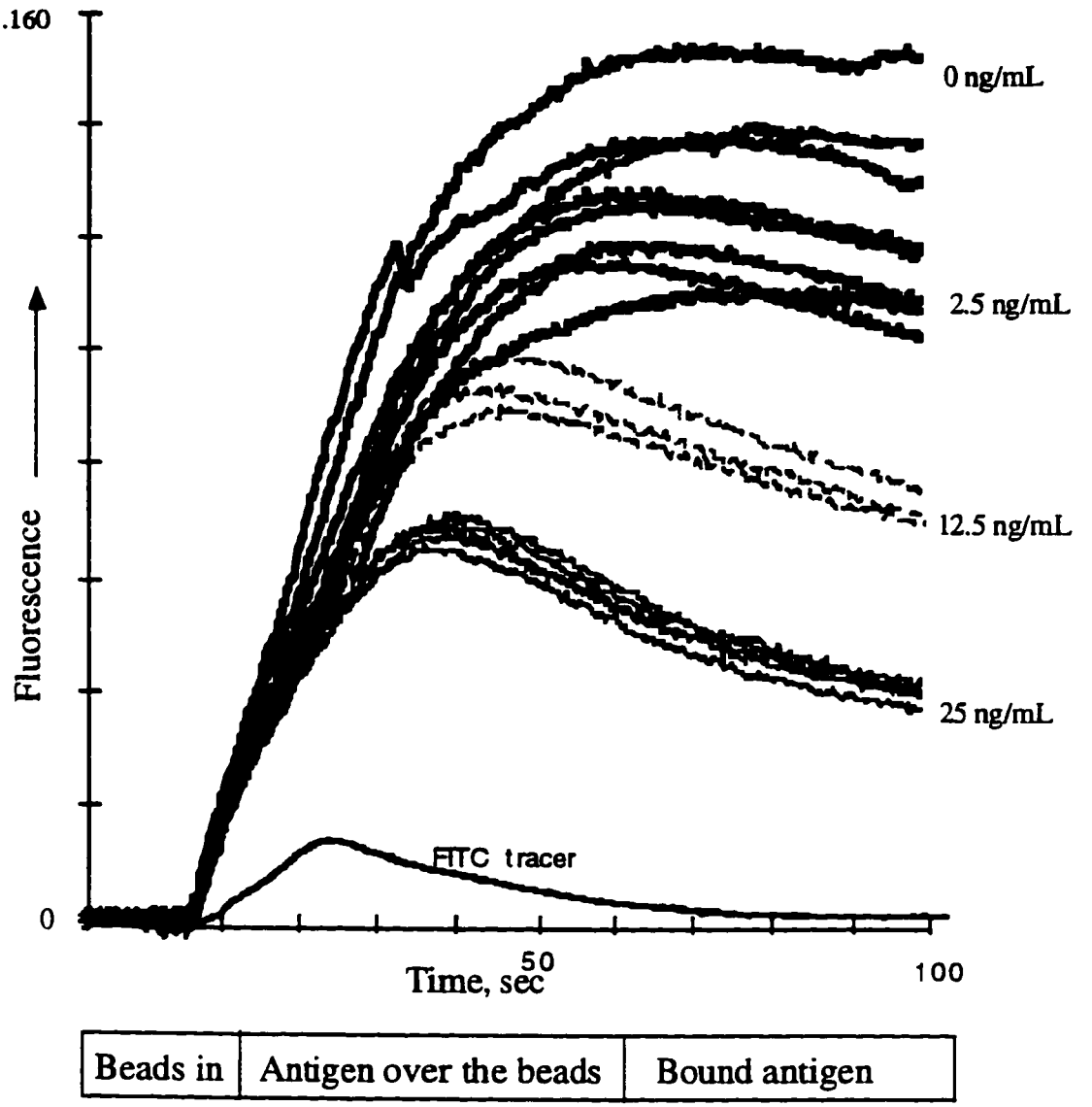

Figure 5.3 Immunoassay Flow Profiles

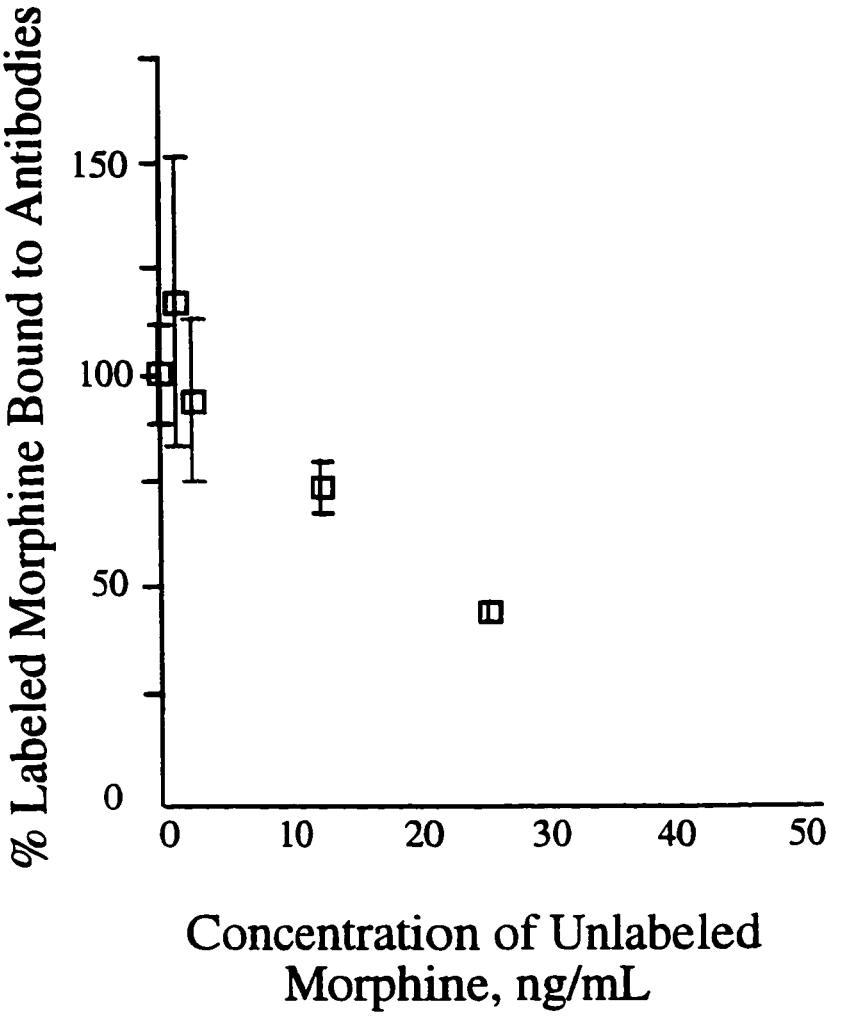

Figure 5.4 Percent Labeled Morphine Bound vs. Concentration of Unlabeled Morphine

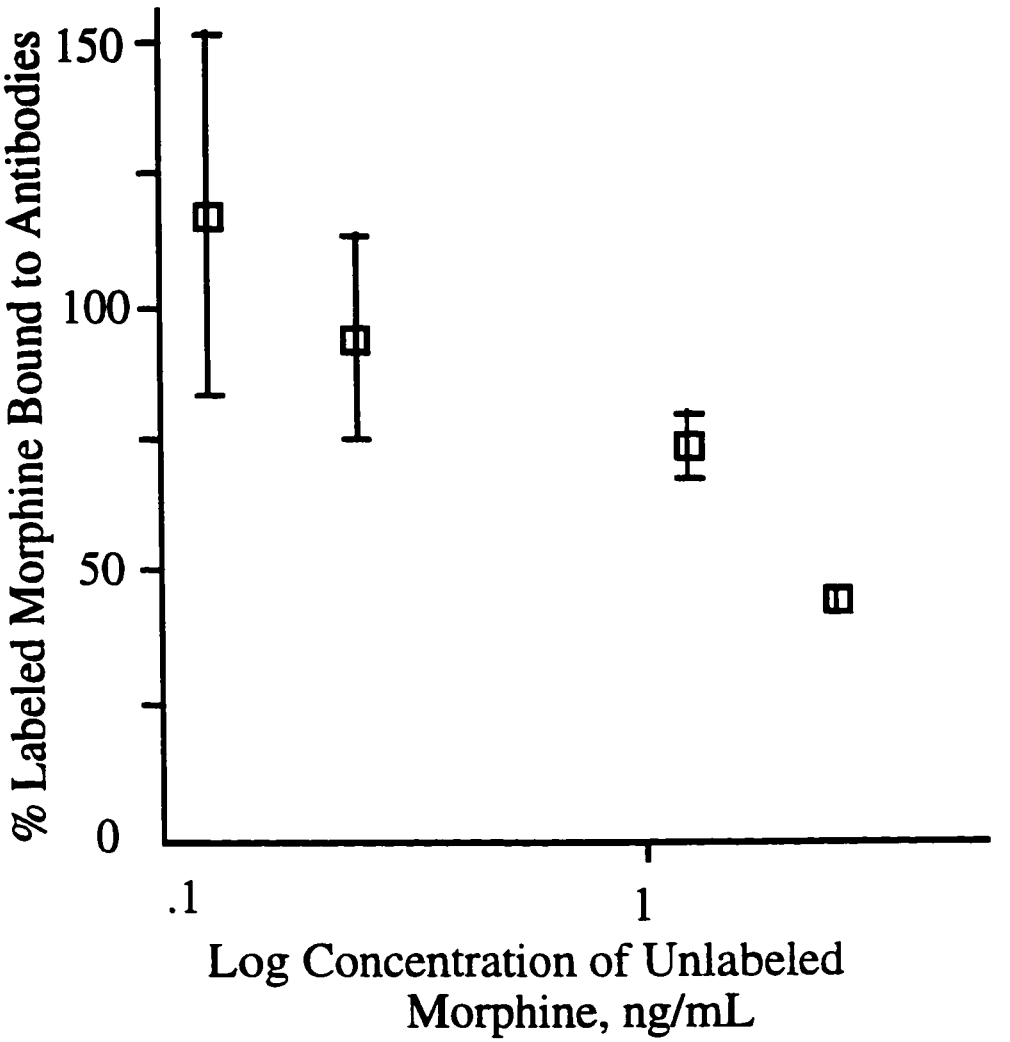

Figure 5.5 Percent Labeled Morphine Bound vs. Log Concentration of Unlabeled Morphine

Immunoassays are useful within two orders of magnitude of the sensitivity (4). The method developed here has a working range from about 2 to 200 ng/mL which is much lower than needed in practice. In order to use this FIRSI, original samples could either be diluted so that the concentration of the sample used in the analysis was in the working range of the immunoassay or more concentrated reagents could be used in the immunoassay to reduce the sensitivity. The concentrations of the fluorescein labeled morphine used here was limited to the availability of the commercial reagent in the Abbott kit. However, other researchers have synthesized their own fluorescein labeled morphine (28-29) along with other fluorescent conjugates (30).

5.4.3 Cross Reactivity

Another aspect to consider when designing an immunoassay it the degree of cross reactivity the antibody has for compounds other than the antigen of interest. Cross reactivity depends on the specificity of the antibody to antigen binding and therefore on the position and method of haptenization (20). The antibodies used in developing this immunoassay have been tested for their cross reactivity to other opiates as well as a variety of compounds that are used concurrently with opiates (15). Other work in FIRSI has shown it to be feasible for carrying out cross reactivity studies (2) in order to determine which compounds may interfere with the analysis.

5.5 SUMMARY TO CHAPTER 5

These feasibility studies using the Abbott reagents presented here have shown that FIRSI has a place in the forensic toxicology laboratory. Reagents originally designed for a homogeneous immunoassay were applied to a heterogeneous FIRSI to preconcentrate the analytes in the detection window. Compared to other heterogeneous

immunoassays, FIRSI does not suffer from the time consuming manual centrifugation steps, as the jet ring cell is able to separate solid particles from the liquid sample in an automated fashion. Fluorometric measurements like the one described here will continue to grow as multianalyte systems are possible with different fluorescent labeled analytes.

This work has outlined one aspect of FIRST that will continue to benefit from advances in technology. Temperature control will have a great impact on the repeatability of this immunoassay and other bioligands used in FIRST because temperature affects both binding and fluorescence (31). Additionally, narrowing the size distribution of the beads may result in more repeatable bead packing.

5.6 CHAPTER 5 END NOTES

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CHAPTER 6: CONCLUSIONS

Each of the extraction based methods discussed in this work has helped to advance the overall chemical analysis of complex samples by enhancing sample preparation. Novel microscale extraction methods to isolate organic compounds from biological matrices encountered in forensic toxicology were developed that incorporated repeatable fluid handling systems. Sequential injection analysis was used, as it is a way to automate handling of liquids and solid suspensions in order to reduce operator error in manual handling and the exposure to hazardous samples and reagents.

The work presented here began with the design of an extraction system that combined conventional liquid/solvent extraction protocols and reagents with a new method of manipulating immiscible phases. The difference in the affinity of organic and aqueous phases, along with flow was used to contact and separate immiscible phases via sequential injection extraction. SIE is a universal method that can be used in a variety of fields to extract a class of compounds away from a complex matrix as demonstrated by the isolation of weakly basic and acidic drugs of therapeutic interest from urine.

The benefits of conventional solid phase extraction were demonstrated by a method to isolate the biotransformation products of cocaine. Later, a manifold for the flow injection renewable surface technique was designed and characterized, representing a combined extraction and detection system. An immunoassay using commercially available reagents was applied to this system to determine the feasibility of using flow injection renewable surface immunoassay in a quantitative immunoassay.

Further advances in extraction methods will improve the overall chemical analysis of samples.

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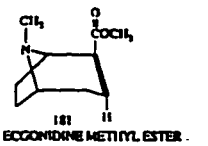
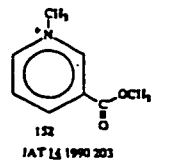
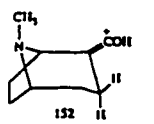
APPENDIX A: MASS SPECTRA OF COCAINE RELATED COMPOUNDS

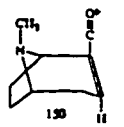
The following mass spectra were acquired as part of the work discussed in Chapter 3. Each spectra is accompanied by the chemical structure and mass of the parent compound and related fragments. For those fragment structures that were previously published in the Journal of Analytical Toxicology (JAT), the reference is listed below the fragment ion.

ECGONIDINE METHYL ESTER









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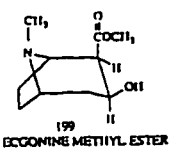

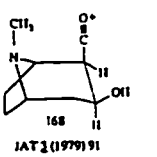

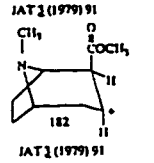

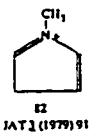

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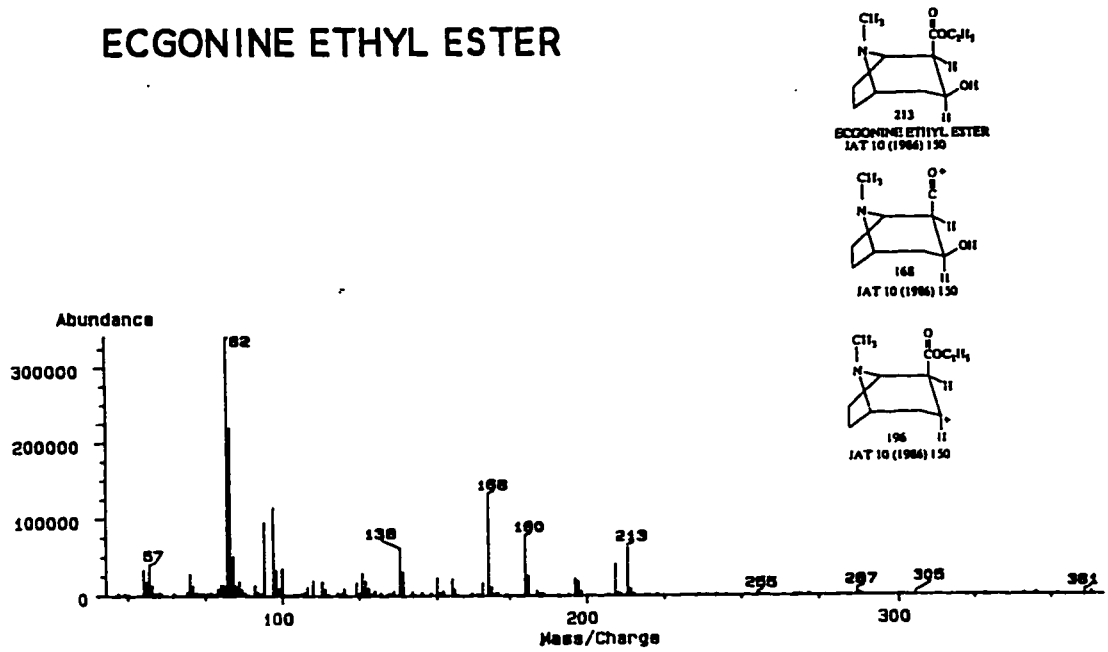

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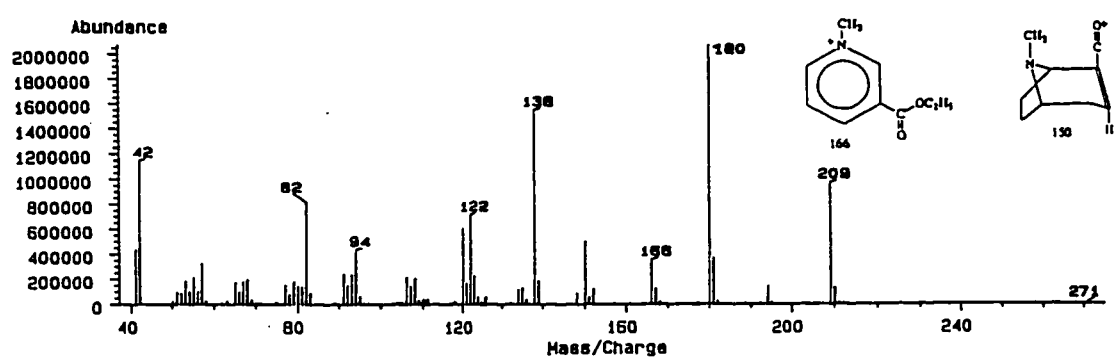

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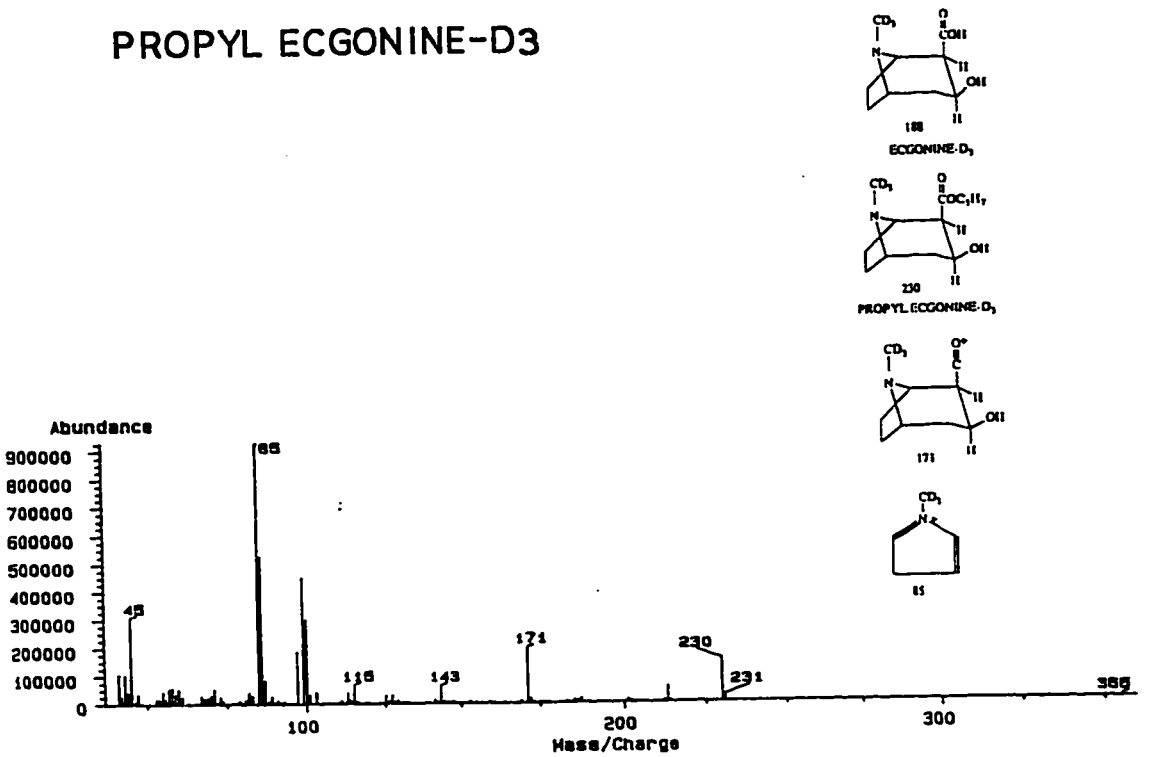

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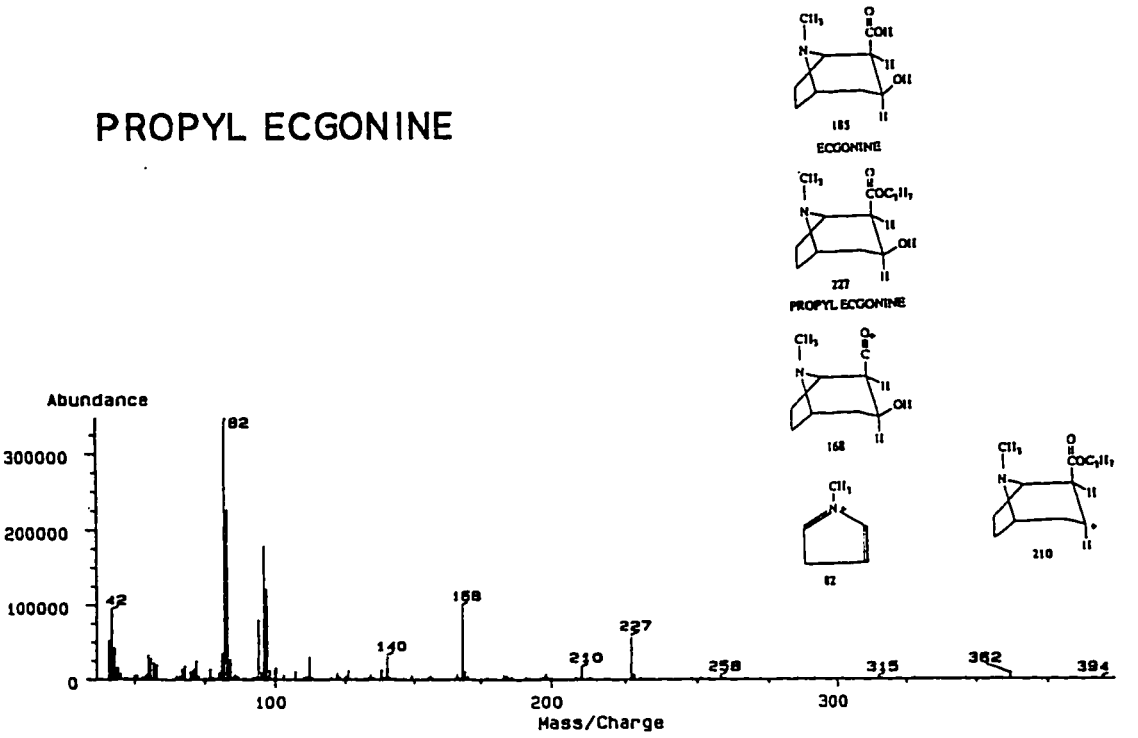
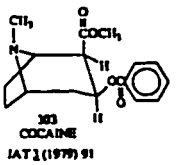
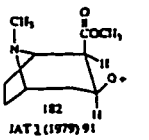
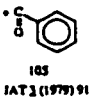
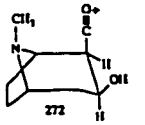


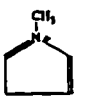
COCAINE

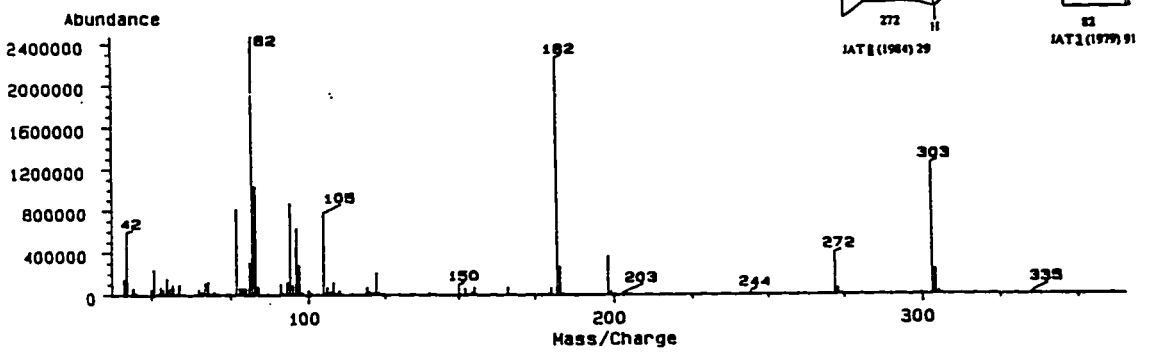












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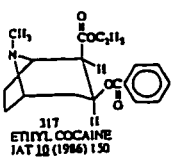

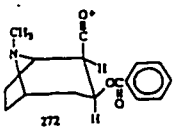

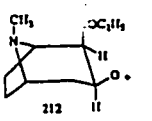

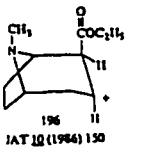

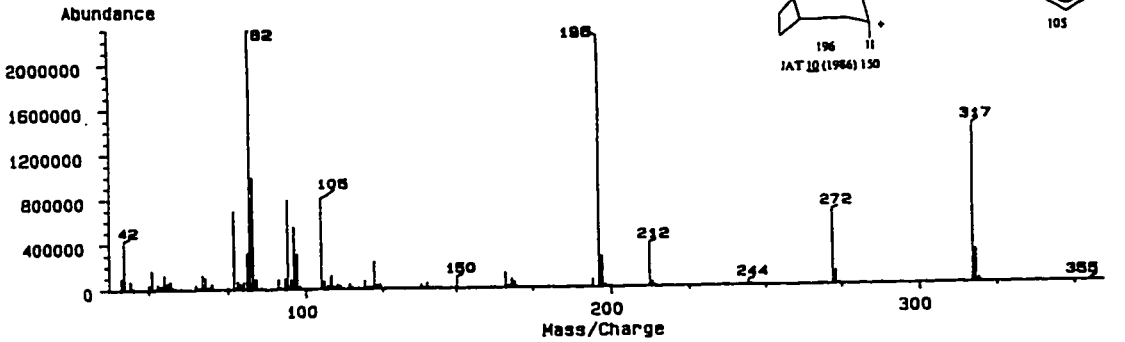

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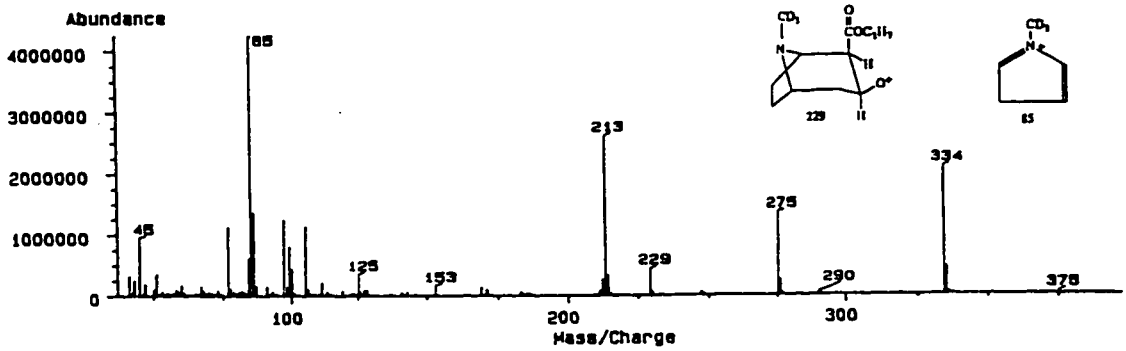

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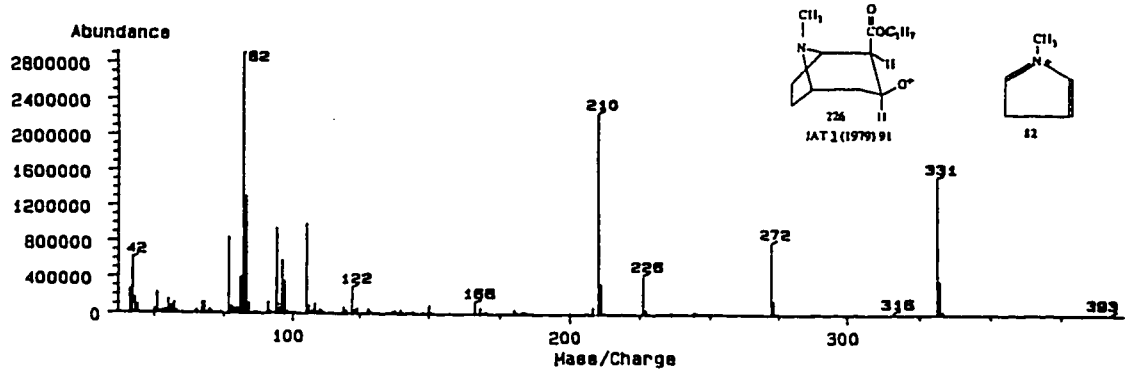
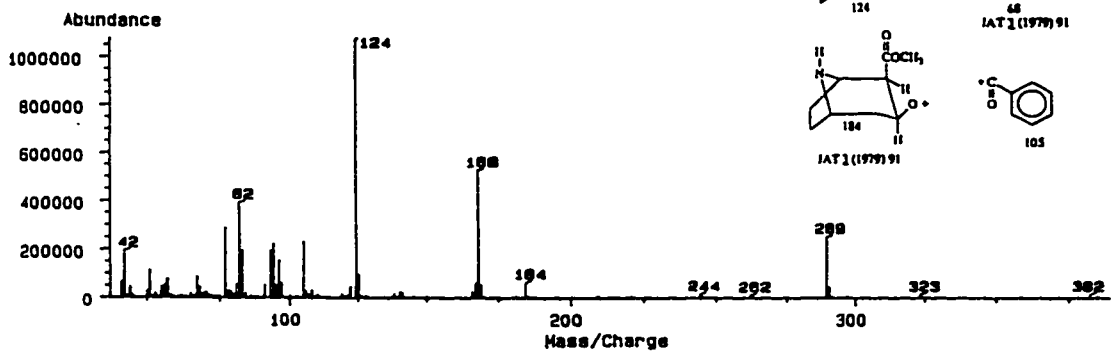
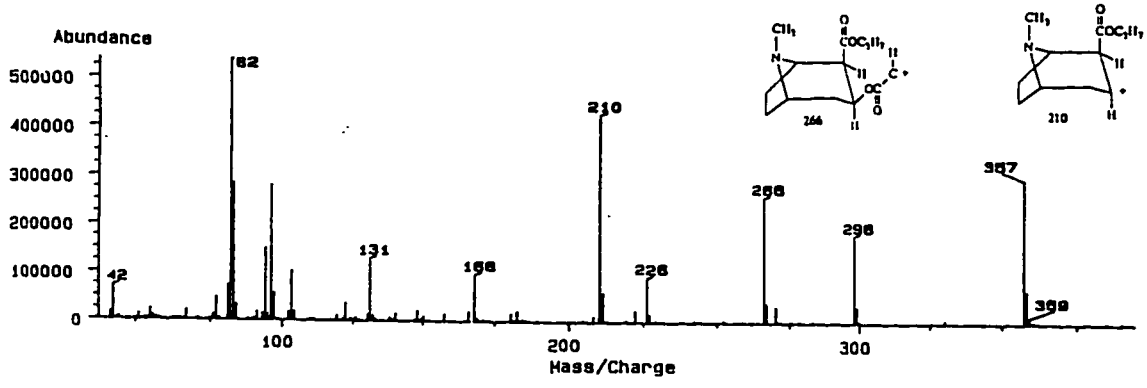
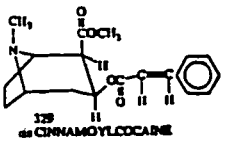


NORCOCAINE

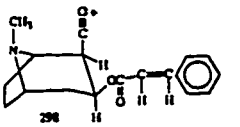


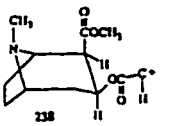
PROPYL CIS/TRANS CINNAMOYLBENZOYLECGONINE

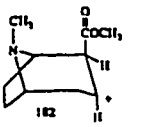


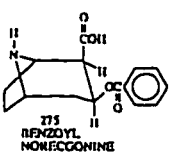


CIS/TRANS CINNAMOYLCOCAINE

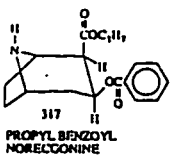


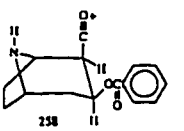


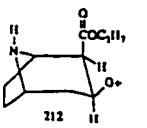


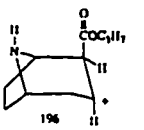


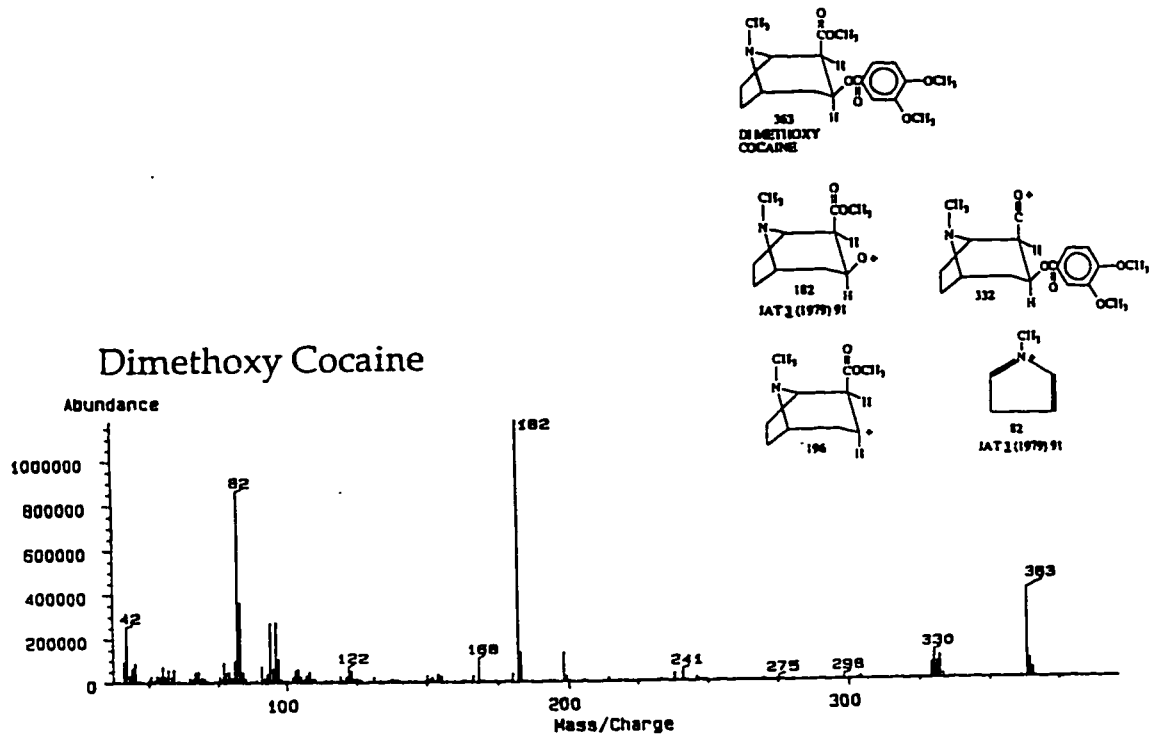
PROPYL BENZOYLNORECGONINE

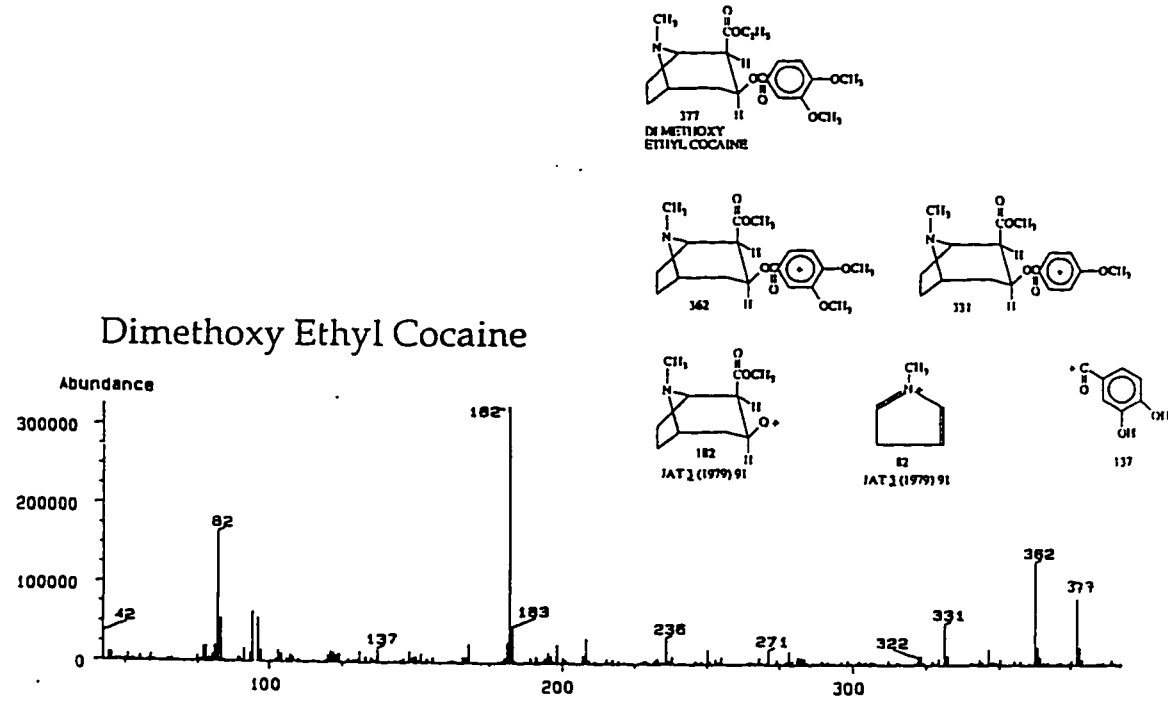









Dimethoxy Cocaine
Abundance
1000000
800000
600000
400000
200000
0
42
82
122
168
182
241
275
298
330
383
100
200
300
Mass/Charge


Dimethoxy Ethyl Cocaine
Abundance
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200000
100000
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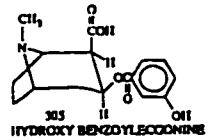
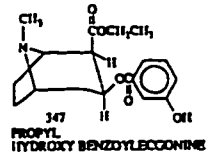
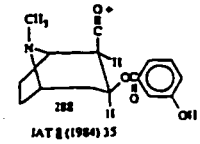
137
183
236
271
322
331
362
377
100
200
300

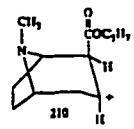


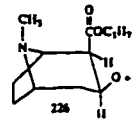
HYDROXY BENZOYLECGONINE

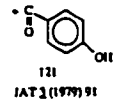


PROPYL HYDROXY BENZOYLECGONINE

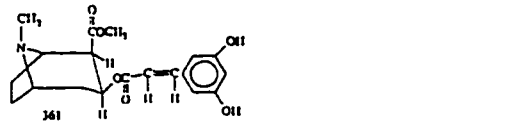




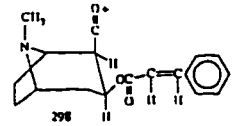


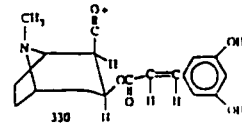


Hydroxy Benzoylecgonine

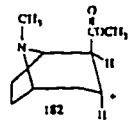


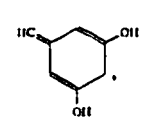
DIHYDROXY cis CINNAMOYLCOCAINE

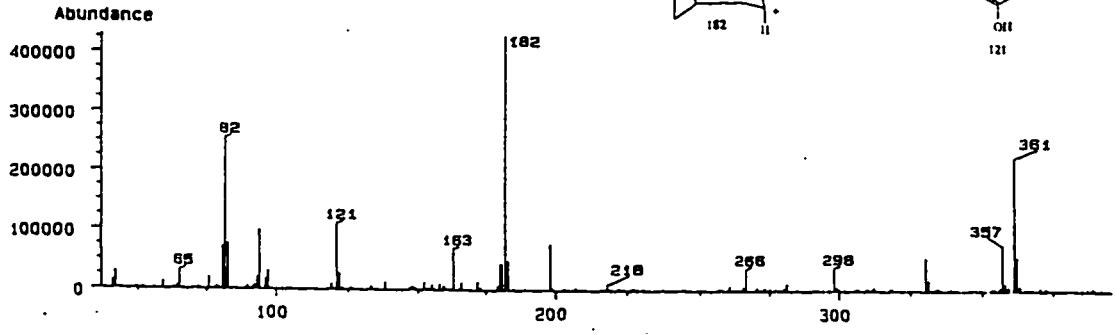


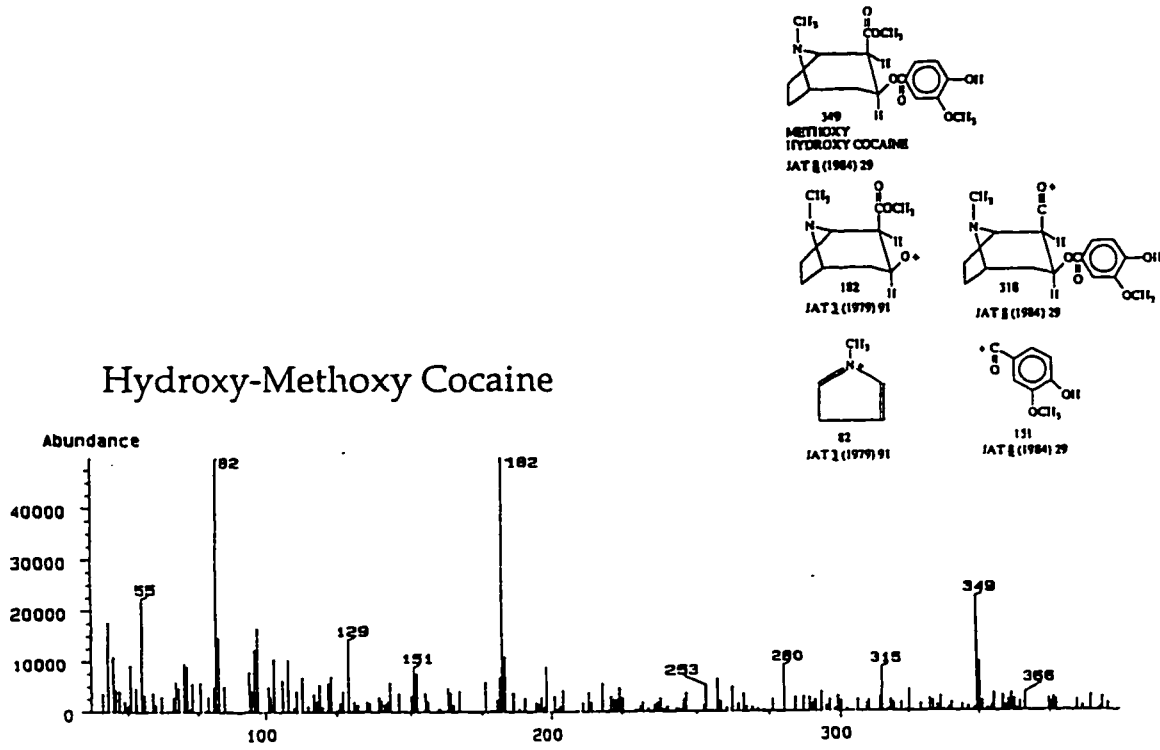


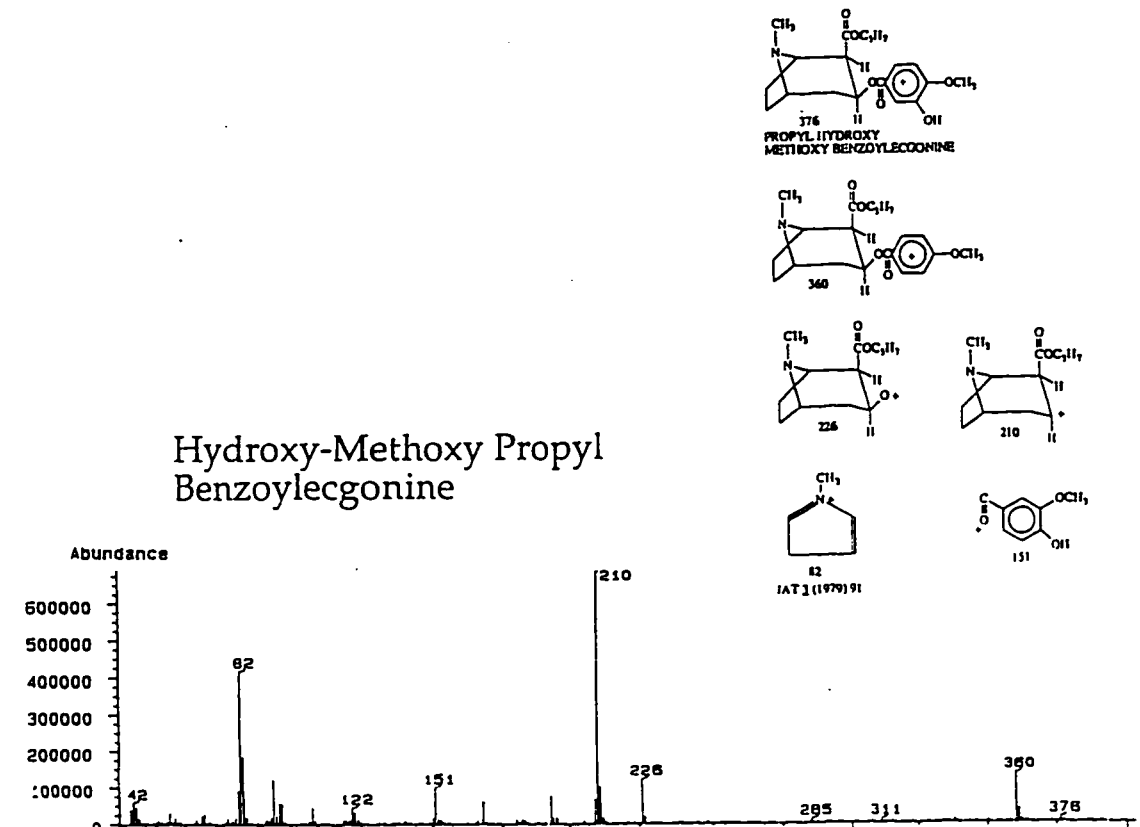
Dihydroxy Cinnamoylcocaine








Hydroxy-Methoxy Cocaine
Abundance
82
182
55
129
151
253
280
315
349
366
40000
30000
20000
10000
0
100
200
300
METHOXY HYDROXY COCAINE
JAT 8 (1984) 29
182
JAT 3 (1979) 91
318
JAT 8 (1984) 29
82
JAT 3 (1979) 91
151
JAT 8 (1984) 29


Hydroxy-Methoxy Propyl Benzoylecgonine
Abundance
210
82
42
122
151
226
285
311
360
376
600000
500000
400000
300000
200000
100000
0
100
200
PROPYL HYDROXY METHOXY BENZOYLECGONINE
360
226
210
82
JAT 3 (1979) 91
151

VITA

Kristina L. Peterson

University of Washington

1997

Kristina L. Peterson was born on December 8, 1969 to Judy and Larry Peterson in San Diego, California USA. She grew up under the influence of two sisters Debra and Dana, and many excellent educators at public schools in San Diego and graduated from Patrick Henry High School in 1988. Kristina received her Bachelor of Science Degree in Chemistry from the University of California at Los Angeles in June 1992. In May 1997, she completed the requirements for the degree of Doctor of Philosophy in Analytical Chemistry at the University of Washington in Seattle.