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Dianna Lynn Ambrose
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Novel materials and methods for solid-phase extraction and high-performance liquid chromatography

by

Dianna Lynn Ambrose

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements of the degree of
DOCTOR OF PHILOSOPHY

Major: Analytical Chemistry

Major Professor: Dr. James S. Fritz

Iowa State University
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1997

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Major Professor

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For the Major Program

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For the Graduate College

This work is dedicated to my very understanding husband, John.

You encouraged me when I was frustrated,

made me laugh when I was down.

You were always there for me,

even when we were miles apart.

I WILL LOVE YOU ALWAYS!

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GENERAL INTRODUCTION

Dissertation Organization

This dissertation begins with a general introduction containing a literature review. References cited in the literature review are compiled in the reference section within the general introduction. This is followed by four research papers that have been accepted, submitted, or will soon be submitted for publication. A general conclusion section follows the four papers. Each paper is similar to the published version. Figures and tables are contained in the text of the paper at the appropriate location. References cited within each paper are listed after the conclusions of each paper.

Solid-Phase Extraction

Sample preparation is often the weakest link in an analytical determination. This step frequently introduces the major source of error and takes a significant amount of time. The major goal of sample preparation is to isolate and concentrate an analyte from matrix components. The extent of isolation, purification, and concentration of the analyte is determined by the complexity and composition of the matrix itself, the concentration of the analyte in the matrix, the selectivity and sensitivity required for the subsequent analysis; and the analytical objectives (screening, quantitative or qualitative analysis).

Sample dissolution followed by liquid-liquid extraction (LLE) was a popular sample preparation technique for many years. However, traditional LLE is tedious, time-consuming, and costly. LLE requires several sample handling steps and can cause many difficulties, including phase emulsions, handling large solvent volumes, and impure and wet extractions.

Other classical sample preparation techniques include centrifugation, filtration, distillation, and precipitation. In the early 1970's a simpler sample preparation technique was introduced, solid-phase extraction (SPE) [1]. A solid-phase extraction consists of bringing a liquid or gas sample in contact with a sorbent. The analyte is selectively adsorbed on the surface of the sorbent which is then easily separated from the sample. SPE has many advantages over the traditional LLE, including reduced analysis time through automation [2-6], decreased solvent usage and disposal, cleaner extracts, and no emulsions. The topic of SPE has been reviewed extensively [7-13]. SPE has become a widely used isolation technique with applications in different fields such as the quality control of pharmaceutical products, therapeutic drug monitoring and toxicology [14-22], pharmacokinetic and pharmacological studies, screening for forensic analysis [23-27], environmental analysis [28-36], food analysis [37-42], and drinking water analysis [43,44].

Historically, bonded-phase silica sorbents provide the broadest applicability for SPE [12]. Bonded phases with a large range of functionalities can be prepared and are commercially available. However, silica bonded sorbents have their limitations. The hydrophobic sorbents require a conditioning step with a wetting solvent to promote good surface contact with an aqueous sample solution. If the sorbent bed dries before loading the sample, low recoveries can result. Also, the pH sensitivity of silica-based sorbents restricts their usable range to approximately pH 2-8. Acidic solutions will hydrolyze the bonded phase and basic solutions will dissolve the base silica [45]. Another limitation of silica-based sorbents is the poor extraction of polar analytes. In addition, residual surface silanols can affect the recovery of basic compounds due to a strong ion exchange mechanism.

Poly(styrene-divinylbenzene) (PS-DVB) copolymers overcome many of the limitations of silica-bonded sorbents. PS-DVB resins offer a broader range of pH stability and increases the method development flexibility. Without silanols, only one predominant retention mechanism exists, resulting in simpler extraction protocols. In addition, PS-DVB resins provide greater retention of polar analytes. However, the hydrophobic PS-DVB polymers require a conditioning step and must remain wetted before sample loading [46,47].

Modified porous PS-DVB resins containing surface polar groups increase surface hydrophilicity and improve extraction efficiencies. Surface acetyl, hydroxymethyl, cyanomethyl, and sulfonate groups have been permanently affixed onto crosslinked polystyrene and the modified resins have been used to extract many types of organic analytes from aqueous solutions [46,48,49]. The capacity of the surface groups influences the SPE of analytes. For example, a 0.6 mmol sulfonate/g resin capacity was found to give the highest analyte capacity factors and promote water wettability without pretreatment [49]. However, the surface modified resins are unable to extract small, polar compounds successfully.

These same small, polar compounds can be extracted with a more classical sorbent, a molecular sieve. The first pure silica molecular sieve, Silicalite, was synthesized in the 1970's [50]. Silicalite is a polymorph of SiO_2 which exhibits a high degree of organophilic-hydrophobic character. It is capable of separating organic molecules out of water-bearing streams. The pores of Silicalite are microporous, 6 Å in diameter, which give the molecular sieve size exclusion properties. Molecules small enough to enter the channels are retained through hydrophobic interactions. The use of PS-DVB resins and Silicalite for SPE are

compared and contrasted in Chapter 1 of this dissertation. Their performances are evaluated for SPE of a wide variety of organic analytes from aqueous solutions.

Most recently, several novel polymeric resins have been synthesized which are ideal for analytical SPE. One particular polymer incorporates N-vinyl pyrrolidone to form a hydrophilic-lipophilic balanced copolymer with increased water wettability [51]. Another polymeric resin has been prepared through the polymerization of a Diels-Alder adduct of maleic anhydride with cyclopentadiene [52]. Hydrolysis of the polymer then converts the anhydride to carboxyl groups, which increase the water wettability of the polymer. No pretreatment of the polymer is needed prior to SPE.

Chapter 2 of this dissertation describes the performance of this novel polymeric resin for SPE. The performance was compared with a sulfonated PS-DVB resin and Silicalite.

The original method of performing a solid phase extraction was batchwise by mixing the sorbent and sample in a tube and separating both phases by centifugation or filtration. The method used today involves passing the sample through the sorbent which is packed in a column. Several manufacturers produce disposable SPE columns containing different types of sorbents packed between two frits made of polyethylene, stainless steel, or polytetrafluoroethylene (PTFE). The amount of sorbent in the cartridges ranges from 50 mg to 10 g and the volume of the corresponding sample reservoir ranges from 1 ml to 1L. The sample can be drawn through the cartridge through the use of a vacuum device, centrifugation, or positive air pressure.

A solid-phase extraction can consist of as many as 5 steps [7-13]. The first step involves wetting the sorbent. The research presented in the first 3 chapters of this dissertation will

show that this step is not necessary with certain adsorbents. This first step is critical when hydrophobic adsorbents are used. A solvent capable of wetting the surface of the sorbent is passed through the column to ensure good contact between the analyte in the sample and the SPE sorbent. Two solvents commonly used are methanol and acetone. These solvents have a polar end (-OH or -C=O) and a hydrophobic end ($-CH_3$). The hydrophobic portion coats the sorbent surface while the polar end promotes good sample contact.

The SPE sorbent is then conditioned with a solvent or buffer similar to the sample. Failure to carry out this stage causes the first portion of the sample to condition the sorbent, resulting in inefficient recoveries.

The third step in SPE is analyte extraction. An analyte can encounter many different attractive forces from the SPE sorbent and be extracted from the sample. These forces may be dispersive, dipole-dipole, hydrogen bonding, ionic, or covalent. The SPE applications in this dissertation employ hydrophobic interactions which are weak, non-specific, and dispersive forces between non-polar groups.

The fourth step is a wash step, necessary when the sample matrix is complex. By passing a suitable solvent through the SPE column, interfering matrix components can be removed while the analyte of interest remains adsorbed.

The final step in a solid-phase extraction is the elution of the analyte from the solid phase with an appropriate solvent. Common solvents used include acetone, acetonitrile, ethyl acetate, and buffers at a specific pH. Typically, 1 to 10 ml of these solvents are used for elution.

Although SPE cartridges are widely and successfully used, difficulties can arise in their routine application. First, a rapid sample flow rate can cause kinetic effects in the bed of 40-μm particles and reduce the recovery of certain analytes [53]. Secondly, channeling can occur when an adsorbent is not packed tightly into the cartridge, resulting in an incomplete isolation of the analyte of interest.

The development of SPE disks solves some of the problems encountered with cartridges. There are many disk configurations employed in SPE [53,54], the most common being packing-impregnated PTFE. These devices consist of a PTFE fibril network that hold bonded silica particles or resin particles in place. The 8 μm particles comprise approximately 90% of the weight of a disk. The SPE disks provide advantages not found in cartridges. The decreased back pressure encountered with the disks makes much higher flow rates possible, versus 1 ml/min. Also, the smaller particles improve mass transfer and the impregnation decreases channeling. Because of the reduced bed mass, less eluting solvent can be used with the disks, resulting in a more concentrated analyte and the elimination of an evaporation step.

Solid-Phase Semi-Micro Extraction

Heightened awareness of the pollution and hazards caused by hydrocarbons has resulted in international initiatives to eliminate the production and use of the organic solvents on which many current sample preparation methods depend [55]. This phasing out of solvent use induced a major change in analytical methodology [56]. Reducing or eliminating the use or organic solvents has become an irreversible trend. Membrane extractions use up to 90% less solvent than do traditional cartridges [57]. However, membranes are often eluted with

relatively large volumes of organic solvent, 1 to 10 ml, preventing the full advantages of membrane technology from being fully realized.

Recently, solid-phase micro-extraction (SPME) was introduced. SPME is a solvent-free sample preparation technique which employs a modified syringe housing a fused-silica fiber coated with a gas chromatographic stationary phase [55]. The extraction technique consists of two processes: partitioning of the analytes between the coating on the fiber and the sample and desorption of the concentrated analytes into an analytical instrument. In the first process, the coated fiber is exposed to the sample and target analytes are extracted from the sample matrix into the coating. The fiber can be used for direct and headspace sampling. The second process involves the transfer of the fiber with the concentrated analytes to a gas or liquid chromatograph [58] for desorption, followed by separation and quantitation.

SPME preserves all of the advantages of SPE such as simplicity, low cost, easy automation, and on-site sampling. At the same time, SPME eliminates the disadvantage of the use of solvents. However, SPME is an equilibrium extraction technique. Complete extraction of analytes is seldom achieved. Major modifications to the SPME device and/or sampling technique are necessary to achieve quantitative extraction [55]. In addition, careful calibration is needed for quantitation.

The third chapter of this dissertation discusses the use of semi-micro solid-phase extraction (SM-SPE). SM-SPE is an exhaustive extraction technique which dramatically reduces solvent use. The entire SPE process is miniaturized while retaining the speed and high analyte recoveries obtained with conventional SPE. SM-SPE was applied for the analysis of ideal aqueous samples and biological samples.

High-Performance Liquid Chromatography

A large variety of medications exist that provide relief from disease and suffering but may produce toxic effects. Therapeutic drug monitoring involves the measurement of the serum concentration of a wide spectrum of drugs to achieve optimum concentrations and results. Depending on the serum drug concentration, the dosage may have to be adjusted for a particular patient to realize the full benefits of a drug without toxic side effects. At the same time, therapeutic drug monitoring helps to provide an estimate of patient compliance in taking the medication as directed. Any therapeutic drug can become a drug of abuse. Therefore, urine screening is used in determining employee and athlete drug abuse.

The analysis of serum and urine using high-performance liquid chromatography (HPLC) presents many challenges. Approximately 10% of serum is composed of proteins [59]. These proteins can interfere with the analytical process by physical obstruction whereby the analyte peak of interest is masked or by binding with the drug. More importantly, proteins can irreversibly adsorb and/or precipitate onto the column packing materials. Back pressure increases, decreased column efficiency, changes in retention time and decreased column capacity may result. Protein precipitation occurs more readily in mobile phases containing organic modifiers. Thresholds for commonly used organic modifiers include 25% for acetonitrile, 20% isopropyl alcohol, and 10% for tetrahydrofuran [60]. These thresholds are pH dependent. Precipitation has been shown to occur more rapidly in organically modified buffers at $pH<5$ and is more pronounced at $pH<4$ [61]. Unlike serum, urine contains little protein. The main constituents of urine include water, urea, uric acid, and creatinine [59]. These and other endogenous compounds can make adequate resolution hard to attain and

cause difficulties in peak identification. Furthermore, urine components vary day to day and person to person which makes a clinical analysis even more difficult.

Another problem is the concentration of the analyte of interest. Drugs undergo metabolism in the human body, reducing the concentration of the parent drug. In some cases, both the parent drug and its metabolite(s) must be determined. Therapeutic serum drug concentrations range from 1 to 100 ppm. However, if non-compliance is an issue, the concentrations can be much lower.

Therefore, a major consideration in an HPLC analysis of biological fluids is sample preparation. Sample preparation is needed for analytical reasons (to increase sensitivity and specificity by concentrating the analytes of interest and removing interferences and operational reasons (to minimize detriment from the sample matrix). Some of the most commonly used sample preparation techniques include protein precipitation, liquid-liquid extraction (LLE), and solid-phase extraction (SPE).

Protein precipitation is widely used to remove proteins from biological fluids prior to injection of an aliquot onto an analytical column [62-64]. Precipitation agents, organic modifiers and concentrated acids, are added to the sample and the precipitate is removed by centrifugation. The disadvantages of precipitation methods are increased total analysis time and reduced recovery due to adsorption of the drugs onto the precipitated protein. Also, the addition of the precipitation agent causes sample dilution.

The two most common sample preparation techniques used for biological fluid analysis are LLE and SPE. LLE serves the dual function of sample clean up and deproteinization, interfering compounds and proteins are removed [65-68]. Like protein precipitation methods,

LLE is labor intensive and time consuming. It also requires a large volume of sample, at least 1 ml, and the addition of an internal standard due to analyte loss from the multiple sample manipulations.

SPE offers greater selectivity and specificity [69-71]. Through the proper choice of SPE adsorbent particles and mobile phase, the analytes of interest are extracted onto the sorbent materials while the interfering substances are selectively removed from the sample. Both clean-up and concentration of the sample are accomplished. SPE also produces cleaner extracts. There is a wide choice of instrumentation for automating SPE [72-74]. Automated SPE is advantageous in terms of its time saving capabilities. However, it dramatically increases the complexity and expense of instrumentation. Also, SPE requires the use of an internal standard.

One way to avoid protein adsorption and eliminate the need for sample preparation is to employ a direct injection technique. Reviews of direct injection techniques have been published [75-78]. Direct injection of biological samples onto HPLC columns substantially reduces analysis time and labor. Several direct injection methods have been devised which deal with the problem of proteins and other endogenous compounds. The methods include precolumn techniques, surfactant-containing mobile phases and restricted access media.

The precolumn technique is the most popular. Reviews have been published describing the practical technique and technical aspects of this methodology [79-84]. The technique has a tandem column design consisting of a precolumn, a switching valve, and an analytical column. It also includes two pumps, one to introduce the sample onto the precolumn and the other to elute the analytes concentrated on the precolumn to the analytical column for

separation. The precolumn technique has two steps. The first step involves the injection of the sample into a buffered 100% aqueous mobile phase that flows through the precolumn. Hydrophobic components of the sample are retained and hydrophilic components, like proteins, flow to waste. The second step involves the elution of the retained components onto the analytical column. As a result, the analyte is removed from the sample matrix and concentrated and the analytical column is not exposed to proteins. The pre-column commonly employs reversed-phase packing materials with a reversed-phase analytical column. Several studies compare the performance of various precolumn packing materials [85-88].

Reported column lifetimes range from 16 to 60 ml serum, or several hundred 20 μl injections [76]. The high precolumn lifetimes resulted from dilution of the sample and a cleanup of the precolumn with 70% acetonitrile prior to the next injection. In most cases precolumns, on-line filters, or guard columns were replaced to attain maximum analytical column lifetimes. Lifetimes of the precolumn system were found to decrease with increasing organic modifier concentrations in the analytical mobile phase [89].

There are many advantages of the precolumn injection technique in comparison to traditional sample preparation techniques. A precolumn technique saves time and does not require the addition of an internal standard [90]. It is also less costly with respect to supplies than SPE. Hundreds of injections can be made on the precolumn in comparison to the SPE column that is usually disposed after one sample.

An advantage of the precolumn technique over other direct injection techniques is the superior detection limit capabilities. The major disadvantage is the need for an additional pump, a column switching device, and timed computer control of events [76].

Use of surfactant mobile phases for the direct injection of serum and plasma samples on reversed-phase columns was first reported in 1985 [91-93]. The most common surfactant used is sodium dodecyl sulfate (SDS). The surfactant prevents the adsorption of proteins on packing material [94] and releases protein-bound drugs [95,96]. Both the stationary phase and proteins are bound by the surfactant, preventing adsorption. Although the use of submicellar concentrations of SDS has been suggested [94], this direct injection technique is mostly used with mobile phases containing micellar concentrations of SDS. There is a required minimum concentration of surfactant needed in the mobile phase for protein solubilization. Over 35mM SDS must be present to prevent protein precipitation [97].

The use of surfactant-containing mobile phases for direct injection has not been wide-spread. This may be due to a decrease in column efficiency which results from slow mass transfer from the poorly wetted stationary phase [98]. Column efficiency can be improved with the addition of organic modifiers to the mobile phase, column temperature elevation [98], and use of a lower concentration of surfactant [99]. Other problems encountered using surfactants are interferences from impurities in the surfactant reagents and the required sample pretreatment to dissociate strongly protein-bound drugs [100]. Most of these shortcomings can be resolved with the use of a precolumn.

Column lifetimes for direct injection techniques employing both a surfactant containing mobile phase and a precolumn have not been widely reported. One study reported 50-100 ml of serum can be injected before an increase in back pressure [101] and another noted protein was being sent to the analytical column via the precolumn even after an increase in SDS concentration in the application mobile phase [102].

A third type of direct injection technique involves the use of restricted access media (RAM). The topic of restricted access media has been reviewed [103-105]. A restricted access medium restricts the access of large molecules and retains small molecules. The packings are designed in two ways. One design incorporates a hydrophilic surface barrier at the external surface of the particles and another design includes the barrier inside the particles at the interface of the stationary phase.

The first design uses a microporous packing to remove large solutes by steric exclusion. Also, the outer surface bears hydrophilic ligands which weakly interact with the proteinaceous components. The stationary phase at the inside of the particles has a different surface chemistry due to the hydrophobic ligands used, which interact selectively with the analytes. Some examples and application of these types of packings include protein-coated n-octadecyl packings [106-108], internal-surface reversed phase (ISRP) packings [109-114], and dual zone packings [115].

In a second design, the surface barrier is located at the interface between the stagnant mobile phase and the stationary phase. The packings allow access of analytes to the stationary phase and exclude high molecular weight proteins. The internal surface exerts a dual chemical functionality with hydrophilic ligands externally and hydrophobic ligands internally. Careful control of the topography and distribution of the two different kinds of ligands is necessary to obtain the desired characteristics. Several examples and clinical applications of this type of packing include semipermeable surfaces [116,117], shielded hydrophobic [118,119] and mixed functional packings [120-123]. Most recently, a diol-bonded silica gel [124] was introduced, containing two different functions. The "binary-

layered phase" packing is so named because the bonded phase contains a hydrophilic function at the tip of the single bond and a hydrophobic function on the lower part of the bond.

Retention of analytes using restricted access media is controlled by the organic modifier content and pH of the mobile phase. The maximum organic modifier concentration used in the mobile phase is limited by protein precipitation, < 25% for acetonitrile, < 20% for 2-propanol, and < 10% for tetrahydrofuran [60]. However, once the proteins have passed through the column, the concentration of the organic modifier can be increased. Also, chemically bonded columns cannot by used for long periods of time at a pH of 3 or lower because of the hydrolysis of the bonded phase.

The major advantage of restricted access media over a precolumn technique is less sophisticated equipment is needed. One disadvantage is the higher detection limit of the restricted access media. Detection can be limited, depending on the analyte, at or above the ppm level. Also, column lifetimes can be shorter. However, the last two problems can be corrected through the use of a precolumn.

A novel restricted access medium, Silicalite is discussed in Chapter 4 of this dissertation. It requires no surface modifications prior to use, an advantage over other restricted access media. Many drugs and metabolites were separated and quantitated with percentage recoveries above 90%.

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SILICALITE AS A SORBENT FOR SOLID-PHASE EXTRACTION

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Abstract

A molecular sieve known as Silicalite was used as a sorbent for solid-phase extraction (SPE) of organic analytes from aqueous samples. Silicalite contains an intricate system of channels approximately 6Å in diameter, but unlike most other molecular sieves the channels of Silicalite are able to retain organic compounds by hydrophobic attraction. Small hydrophilic compounds, such as the lower alcohols, aldehydes, esters and ketones, are well extracted by Silicalite, thus adding a valuable new capability to conventional SPE. Extensive data are presented to define the scope and limitations of Silicalite for SPE. Breakthrough curves were used for several compounds to determine their loading capacity on Silicalite.

Introduction

Solid-phase extraction (SPE) is one of the most popular sample preparation techniques for the isolation and preconcentration of organic compounds from aqueous samples. It has many advantages over traditional liquid-liquid extraction. SPE is a multistage rather than a single stage process. Organics undergo multiple equilibrations, resulting in a more complete extraction. SPE also uses less organic solvent and is easily automated, saving time and money. It has been shown that modified porous polystyrene-divinylbenzene (PS-DVB) resins containing polar groups are superior for SPE of organic solutes from aqueous samples [1,2]. Most recently, Dumont and Fritz [3] introduced surface sulfonic acid groups to PS-DVB resins. These modified resins displayed excellent surface hydrophilicity and improved extraction efficiencies. However, these same resins were unable to extract small, polar organics successfully. Therefore, Silicalite was employed for SPE to complement polystyrene and bonded-phase silica adsorbents.

Silicalite is a molecular sieve, first synthesized in the early 1970's [4]. Unlike other molecular sieves, Silicalite is hydrophobic and extracts organic molecules from liquid and gaseous samples. Silicalite is a polymorph of silica with an unusual crystal structure. It has a tetrahedral framework of mostly 5-membered rings of silicon-oxygen tetrahedra. These tetrahedra form a three dimensional system of intersecting channels, 6Å in diameter, defined by rings of 10 oxygen atoms. The total pore volume is approximately 33 % [4]. For sorption, the diameter of a particular analyte must not exceed 6Å, roughly the kinetic diameter of benzene. Water molecules are not adsorbed because they associate into clusters of 10-12 molecules, producing a total diameter larger than 6Å [4]. Because of its unique

structure, Silicalite adsorbs organic analytes through hydrophobic interactions and possesses size exclusion properties.

To date, research involving Silicalite has concentrated on the mechanisms and kinetics of its adsorption and desorption of inert gases [5,6], hydrocarbons [7-17], aromatics [17-26], and alcohols [27,28]. Silicalite has also been used to remove n-butanol from fermentation liquors [29], ethanol from beer [30], and sulfur dioxide from stack gas [31]. In other applications, Silicalite has been used as a catalyst [32-35].

Silicalite has been embedded in polydimethylsiloxane, ethylene-propylene, polychloroprene, and nitrile butadiene rubbers to form composite membranes. These membranes were used in pervaporation studies of organic solvent/water systems [36-40], separation studies of carbon dioxide/methane and oxygen/nitrogen gas systems [41], and sorption of chlorinated hydrocarbons [42]. Silicalite increased the selectivity of alcohol adsorption and improved the separation properties of the polymers towards gaseous mixtures. Other composite membranes consist of a pure, thin layer of Silicalite on porous ceramic substrates [43,44]. The layer is formed through an in-situ synthesis and composed of highly intergrown Silicalite polycrystals. These membranes were used to study the permeation of single component and binary mixtures of various gases and showed high selectivities for specific conditions.

Silicalite has also been crystallized to form a non-composite membrane on a variety of surfaces, including Teflon [45], silver [45], stainless steel [45-49], Vycor [45], and alumina disks [46,49]. The permeability and selectivity of the membranes were measured for bicomponent gas mixtures [45]. The membranes were also used for pervaportation studies

of alcohol/water [46,48,49] and methanol/methyl-tert-butyl ether mixtures [47]. Silicalite crystals have also been embedded in silver and nickel matrices [50] and a large, single crystal has been placed in an epoxy resin and mounted in a specially designed permeability cell [51].

An extensive study on the scope and limitations of using Silicalite as a sorbent for SPE is lacking in the literature. Schultz-Sibbel et al [52] reported distribution coefficients and capacities for the adsorption of a variety of compounds by Silicalite from gas or water samples. Fritz and Ogawa [53] used Silicalite to concentrate low concentrations of aldehydes and ketones from aqueous samples prior to derivatization and separation by liquid chromatography.

In the present work, Silicalite was used as a sorbent for SPE. Aqueous solutions containing a wide variety of organic compounds were used to determine the extraction ability of Silicalite. Percentage recoveries of various organics extracted by Silicalite were compared with those using a lightly sulfonated porous PS-DVB resin-loaded membrane. Extraction capacities for small, polar organic compounds were also determined using Silicalite.

Experimental

Reagents and chemicals

Analytes studied were >99% pure and used as obtained from Fisher (Fisher Scientific, Pittsburgh, PA, USA) and Aldrich (Aldrich Chemical Company, Inc., Milwaukee, WI, USA). Laboratory distilled water was further purified using a Barnstead Nanopure II system (Sybron Barnstead, Boston, MA, USA). Silicalite from the UOP, Inc., were sieved to obtain 5 -10 μm particles. Experimental membranes containing these particles [54] were obtained from the

3M company (3M Co., St. Paul, MN, USA). Experimental Empore®-type membranes embedded with 8 μm PS-DVB sulfonated resin, 0.6 mequiv/g sulfonation capacity, were used as obtained from the 3M Company (3M Co., St. Paul, MN, USA) and can be purchased commercially as SDB-RPS.

Procedure for SPE

The apparatus for SPE consisted of a 30-ml glass syringe barrel fitted with a luer tip. A 1.5 ml polypropylene SPE column (P.J. Corbert Assoc., St. Louis, MO, USA) was connected to the glass reservoir via a universal adapter. The Silicalite particles and sulfonated resin-loaded membranes were used as the SPE adsorbents. Towards the end of this study, experimental Silicalite-loaded membranes were available and used to determine the effect of embedding Silicalite particles in a membrane on SPE efficiency. Each adsorbent was placed between two 20-μm polyethylene frits (P.J. Corbert Assoc.) in the SPE column. Additionally, the Silicalite particles were supported from the bottom with a piece of 2-μm filter paper. The bed heights measured approximately 0.5-cm. Positive pressure was used to force liquids through the adsorbents. Prior to use, 1-ml acetone and 1-ml deionized (DI) water were used to condition each column.

Samples were prepared by adding a 100-μl aliquot of a methanol solution containing 1000-ppm each of 2-3 analytes to 10 ml of DI water. The final concentration of each compound in the sample was about 10-ppm, except for all phenols which were 5-ppm. Air pressure was adjusted to provide a flow of 1-2 ml per minute (30-50 p.s.i.; 1p.s.i. = 6894.76 Pa). After loading, the glass reservoir was rinsed with 1-3 ml DI water and air was blown through the column to remove any remaining water. A 1-ml aliquot of acetone was used to

elute the compounds into a GC vial. Methanol was the eluting solvent when acetone was an analyte. An internal standard (100-μl of an acetone solution containing 150-ppm toluene) was added to the contents of the vial, which were then analyzed by gas chromatography. A Shimadzu (Kyoto, Japan) GC 14A equipped with an AOC-14 autoinjector, flame ionization detector and a C-R4A Chromatopac data analysis system was used to separate and quantitate the analytes. The GC column was a Supelco SPB-5 column, 15 m x 0.32 mm i.d. with a phase thickness of 1 μm. Recoveries were calculated as an average of 3 trials by comparing the relative peak areas with standards that were not subjected to SPE.

Breakthrough curve procedure

The HPLC column used was a 10 cm x 4.6 mm i.d. stainless steel column packed with Silicalite particles. The following packing procedure was used. Silicalite was added to degassed 2-propanol and sonicated under vacuum for 30 minutes. This step removed any trapped air from within the Silicalite and produced a slurry. A Shandon HPLC packing pump (Shandon Southern, Sewichley, PA, USA) was used at a pressure of 4000 p.s.i. to pack the slurry into the column. The HPLC system consisted of several components. A Gilson (Middleton, WI, USA) Model 302B HPLC pump equipped with a Model 802B Gilson manometric module and Scientific Systems (State College, PA, USA) Model LP-21 pulse dampener was used for feed solution delivery. Aqueous feed solutions containing 1500 ppm of the organic compounds of interest were pumped through at rates between 0.50 and 1.00 ml per minute. The effluent was monitored on-line with a Kratos 783 UV-Vis detector (Applied Biosystems, Ramsey, NJ, USA) or a Erma 7510 Refractive Index detector (Chrom Tech Inc., Apple Valley, MN, USA). Breakthrough curves were recorded by a Hitachi D-200

Chromato-Integrator (EM Science, Cherry Hill, NJ, USA).

Results and Discussion

Particles for solid-phase extraction

Porous crosslinked polystyrene resins have proved to be very effective for solid-phase extraction (SPE) of a wide variety of organic compounds from predominately aqueous samples. Their effectiveness is enhanced by chemical introduction of polar groups, such as acetyl [1] or sulfonated [2,3] on the resin surface. Incorporation of these surface-modified resins into membranes of the Empore® type is generally more efficient for SPE than the use of loose resin particles packed into small columns. However, polystyrene resins do not extract, or extract very poorly, small, polar organic compounds such as the lower alcohols, aldehydes, ketones and carboxylic acids. The major goal of this research was to fill this gap by using Silicalite for SPE of relatively small, polar organic analytes from aqueous samples.

Silicalite was expected to have a sieving effect based on the molecular size and configuration of the analytes. Molecules small enough to enter the 6Å diameter channels would be retained by interaction of the hydrophobic parts of the analyte with the hydrophobic interior of the Silicalite. Molecules that would not fit the channels would be extracted poorly or not at all. However, our early experiments showed that some relatively large molecules were strongly extracted into Silicalite from dilute aqueous solution. Studies by Choudhary and Akolekar [17] have shown that molecular configuration and flexibility (compressibility) need to be considered in addition to the critical molecular diameter. For planar molecules. their penetration into the elliptical channels will be easier if the orientation of the analyte

molecule aligns itself with the larger axis of the elliptical opening. Some molecules are sufficiently flexible that their bonds can be bent in a direction opposite to that of penetration into the Silicalite channels.

The ability of Silicalite (particles and membrane) and lightly sulfonated polystyrene resins (in a membrane) to extract various organic test compounds from aqueous samples was compared using identical small columns packed with the adsorbents. After the extraction step, the test compounds were eluted with 1.0 ml of acetone and the individual compounds determined quantitatively by gas chromatography. The percentage recoveries were calculated for the test compounds and used as a measure of SPE efficiency.

Extraction of alcohols

Percentage recoveries of alcohols with Silicalite particles and a lightly sulfonated PS-DVB resin-loaded membrane are compared in Table 1. The percentage recoveries of the normal (straight-chain) alcohols are plotted as a function of carbon number in Figure 1. The most striking difference between the two sorbents is in the C_1 - C_4 alcohols which are much more strongly retained by Silicalite. From a maximum around C_6 the Silicalite recoveries decrease gradually with increasing chain length. Recoveries using the sulfonated membrane remain almost constant between C_5 and C_{14}.

For alcohols with a n-alkyl group, increasing the chain length does not increase their effective diameter. The analytes can easily fit into Silicalite with their hydrocarbon chain parallel to the channels. The increased recoveries are thought to result from an increased interaction between paraffinic hydrogen atoms and the channel walls. As the length of the hydrocarbon chain becomes longer, extraction recoveries of the alcohols decrease. The longer

Table 1. Comparison of percentage recoveries of alcohols using Silicalite particles and a sulfonated PS-DVB resin-loaded membrane.

Compound	Silicalite	Membrane
Methanol	7	0
Ethanol	54	0
1-Propanol	84	0
1-Butanol	95	10
2-Butanol	94	6
tert-Butanol	95	87
1-Pentanol	92	99
2-Pentanol	94	85
1-Hexanol	99	91
Cyclohexanol	10	88
1-Octanol	89	94
1-Decanol	82	89
1-Dodecanol	75	89
1-Tetradecanol	58	87
3-Phenyl-1-Propanol	73	99
2,2-Dimethyl-3-Pentanol	89	86
3-Ethyl-2,2-Dimethyl-3-Pentanol	94	84
2-Ethyl-1-Hexanol	92	90

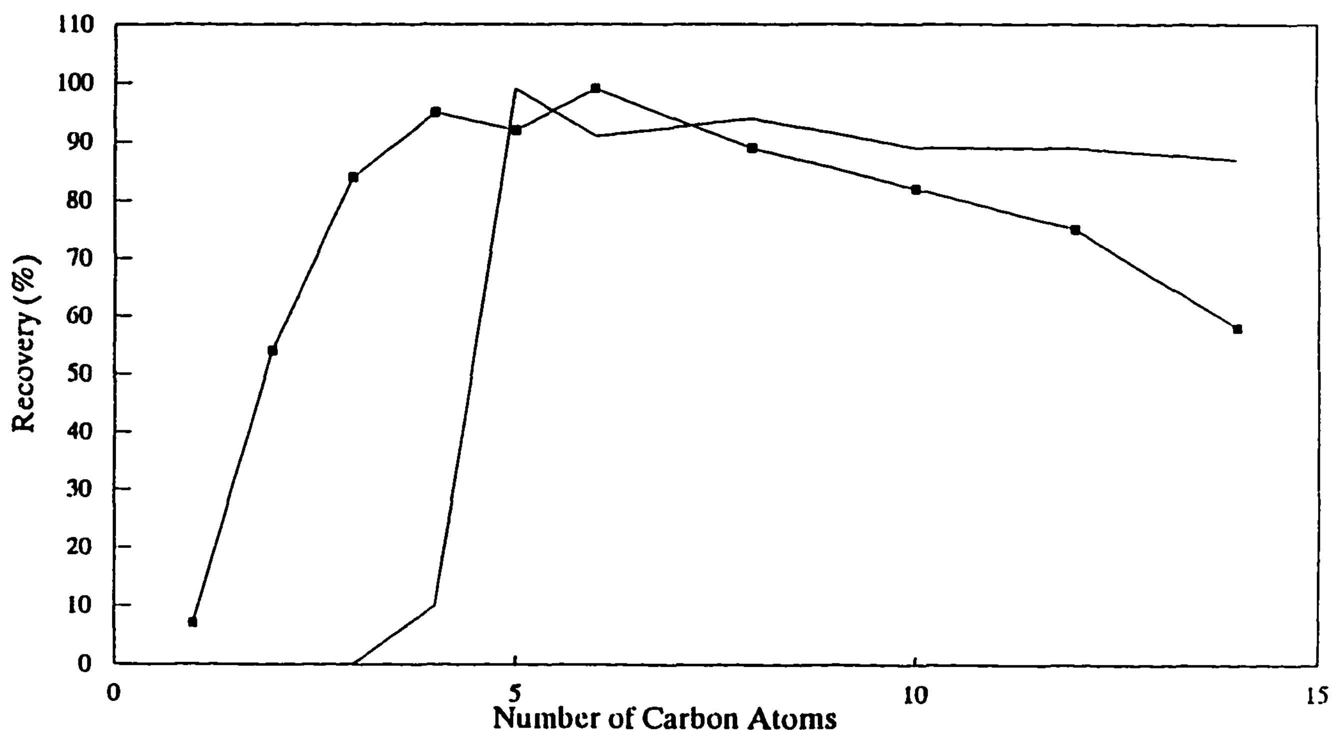

Figure 1. SPE percentage recoveries of n-alcohols using Silicalite particles (■) and a sulfonated PS-DVB resin-loaded membrane (solid line).

hydrocarbon chain lessens the chance of an analyte directly entering the channels of Silicalite. An analyte will be sorbed if the energy needed to straighten the chain is less than the energy of sorption by Silicalite [52]. Longer molecules need more energy to be straightened and, therefore, give lower extraction recoveries.

The shape of an analyte will also influence its ability to be retained by Silicalite. Alcohols with methyl or ethyl groups as side chains still gave high recoveries. However, cyclohexanol gave only a very low recovery. Not only does cyclohexanol have a kinetic diameter larger than 6Å, but it also has a rigid structure [18]. This rigidity precludes compression of the molecule and prevents entrance into the Silicalite channels.

Extraction of other alkyl compounds

Data for SPE of aldehydes and ketones, esters, carboxylic acids, amines, and chlorinated alkanes are given in Table 2. In several instances the lower members of a homologous series are retained much more strongly by Silicalite than by the sulfonated PS-DVB resin-loaded membrane. Examples include acetone, 2-butanone, methyl formate, methyl acetate, ethyl formate, ethyl acetate, propionic acid, butyric acid, propyl amine, and n-butyl amine.

As might be expected, recoveries of more bulky analyte molecules tended to be lower with Silicalite than with the sulfonated membrane. A good example is tert-butyl amine, which gave only 64% recovery using Silicalite compared to 84% using the sulfonated membrane. Tributyl amine, with its bulky three-dimensional structure, gave only 13% recovery using Silicalite compared to 66% recovery using the sulfonated membrane.

Extraction of phenols

Recoveries of phenols following SPE with Silicalite are listed in Table 3. Even phenols with moderately bulky substituents were extracted by Silicalite provided the molecular structure is favorable. Substituents in the 4-position offer the least resistance to penetration into the Silicalite channels. For 4-n-alkyl substituents the recoveries for C_1 to C_4

Table 2. Comparison of percentage recoveries of various test compounds from aqueous solutions using Silicalite particles and a sulfonated PS-DVB resin-loaded membrane

Class	Compounds	Silicalite	Membrane
Aldehydes	trans-Crotonaldehyde	91	24
	n-Valeraldehyde	100	91
	Hexanal	107	85
	Nonylaldehyde	84	74
	Benzaldehyde	89	94
	Salicylaldehyde	54	96
Ketones	Acetone	94	0
	2-Butanone	92	0
	2-Pentanone	90	88
	3-Pentanone	41	68
	4-Methyl-2-Pentanone	104	88
	2,4-Pentadione	31	27
	2-Hexanone	93	89
	3-Hexanone	81	89
Esters	Methyl formate	74	0
	Methyl acetate	85	4
	Ethyl formate	83	0
	Ethyl acetate	91	55
	Ethyl propionate	88	61
	Ethyl butyrate	90	75
	Hexyl acetate	79	88
	Methyl benzoate	68	94
	Pentyl benzoate	82	70

Table 2. (Continued)

Class	Compounds	Silicalite	Membrane
Chlorinated Alkanes	Chloroform	82	81
	1,2-Dichloroethane	83	77
	1,1-Dichloroethane	80	77
	1,2-Dichloropropane	85	85
Carboxylic Acids	Acetic	2	0
	Propionic	62	0
	Butyric	78	13
	Valeric	95	104
Amines	Propyl Amine	63	0
	n-Butyl Amine	72	52
	Tert-Butyl Amine	64	84
	Pentyl Amine	78	93
	Pyridine	77	35
	Tributyl Amine	13	66

Table 3. Comparison of percentage recoveries of phenols using Silicalite particles and a sulfonated PS-DVB resin-loaded membrane

Compound	Silicalite	Membrane
Phenol	66	84
o-Cresol	53	93
m-Cresol	97	93
p-Cresol	100	87
2-Chlorophenol	33	89
3-Chlorophenol	39	96
4-Chlorophenol	90	95
3-Nitrophenol	56	105
2,5-Dimethylphenol	54	92
2,3,5-Trimethylphenol	27	99
4-Ethylphenol	98	96
4-Propylphenol	98	98
4-Isopropylphenol	89	91
4-Butylphenol	83	95
4-Tert-Butylphenol	27	87

were 100%, 98%, 98%, and 83%, respectively. However, recovery was only 27% for the broader 4-tert-butyl phenol.

Slight differences in molecular configuration yielded sharp differences in recoveries. For example, recoveries of the cresols in Table 3 depended on the location of the methyl group. In the molecule p-cresol, the hydroxyl and methyl groups are attached to the benzene ring on the same axis. The kinetic diameter is very close to 6Å and p-cresol can enter Silicalite with its axis parallel to the channels. In m-cresol and o-cresol molecules, the two

groups are located on two different axes, producing a kinetic diameter larger than the size of the channels [18]. However, these molecules were still extracted by Silicalite. The elastic methyl group in the ortho or meta position can bend to align with the axis of the channels. The lower recovery of o-cresol may be the result of repulsion from the neighboring hydroxyl and methyl groups.

The presence of a large substituent in the 2- or 3-position lead to much lower SPE recoveries. Thus, 2-chlorophenol gave only 33% recovery and 3-chlorophenol gave 39% recovery. However, 4-chlorophenol gave 90% recovery. It is interesting to compare these results with HPLC of chlorinated phenols on a Silicalite column with 35% acetonitrile-65% water (v/v) as the mobile phase [55]. The elution order for monochlorophenols was 2, 3, 4.

Silicalite in a membrane

Silicalite particles were incorporated into an experimental membrane of the Empore® type. Several circles were cut from the membrane sheets and packed into a small column to the same height as that previously packed with loose Silicalite particles. Results of SPE with the loose particles and membrane column were then compared. The results in Table 4 showed much lower recoveries of ethanol and methyl acetate with the membrane compared to the loose particles. On the other hand, the recovery of cyclohexanol was much higher with the membrane column. In general, results were slightly better with the Silicalite membrane. For the alcohols, excluding methanol, ethanol and cyclohexanol, the average recovery was 90% with the membrane compared to 87% with the loose particles.

Table 4. Comparison of percentage recoveries of various analytes using Silicalite particles and a Silicalite-loaded membrane

Class	Compound	Particles	Membrane
Alcohols	Methanol	7	1
	Ethanol	54	5
	1-Propanol	84	86
	1-Butanol	95	93
	2-Butanol	94	98
	tert-Butanol	95	100
	1-Pentanol	92	100
	2-Pentanol	94	99
	1-Hexanol	99	99
	Cyclohexanol	10	53
	1-Octanol	89	89
	1-Decanol	82	78
	1-Dodecanol	75	73
	1-Tetradecanol	58	57
	3-Phenyl-1-Propanol	73	89
	2,2-Dimethyl-3-Pentanol	89	98
	3-Ethyl-2,2-Dimethyl-3-Pentanol	94	98
	2-Ethyl-1-Hexanol	92	98

Table 4. (Continued)

Class	Compound	Particles	Membrane
Ester	Methyl acetate	85	27
	Ethyl formate	83	99
	Ethyl acetate	91	99
Carboxylic Acid	Acetic Acid	2	0
	Propionic Acid	62	5
	Butyric Acid	78	84
	Valeric Acid	95	100
Phenol	p-Cresol	100	94
	o-Cresol	97	92
	4-Isopropylphenol	89	89

Breakthrough curves

Load capacity is another good indication of the extraction ability of Silicalite. Resin load capacity is the total number of moles or weight of analyte extracted by a given amount of resin. In this study, resin load capacities were determined through the use of breakthrough curves. Breakthrough curves were generated by passing a 1500 ppm aqueous solution of the analyte of interest through a column packed with Silicalite particles until breakthrough occurred. The curves were plotted as a ratio of effluent concentration, C, to influent concentration, C_o, versus the volume of effluent. The total number of moles of analyte adsorbed was calculated by multiplying the retention volume, V_R, by the concentration of the influent [56]. For this study, V_R was defined as the extrapolated volume from the curve at the point $C/C_o = 0.5$. Capacities for several small, polar organics were calculated from breakthrough curves and are given in Table 5.

Table 5. Silicalite loading capacities for various test compounds

Class	Compound	Capacity (mg cpd./g)	Pore filling (%)
Alcohol	Methanol	7	5
	Ethanol	18	12
	1-Propanol	69	45
	1-Butanol	85	55
Ester	Ethyl formate	14	8
	Ethyl acetate	119	69
	Ethyl propionate	118	70
	Ethyl butyrate	116	70

The resin load capacities tended to correlate with the recoveries obtained in the SPE studies. Also, capacities increased as the hydrocarbon chain length increased. The capacities (by weight) increased from methanol, 0.7%, to 1-butanol, 8.5%. Maddox [29] showed that Silicalite can adsorb up to 8.5% of its own weight of butanol from fermentation liquors. Silicalite also adsorbed approximately 12.0%, by weight, of the larger esters in Table 5. However, the analytes used in this study occupied only a fraction of the theoretical pore volume in Silicalite of 0.19 cm^3 per gram [4]. This could be caused by hydrogen bonding of the alcohols with water molecules in the aqueous solutions, preventing the alcohols from entering the pores of Silicalite. Also, small analytes may interact with each other to form associates in the sorbed phase. Schultz-Sibbel et al [52] concluded that Silicalite sorbed various gases in a condensed state. This could prevent the low energy sites of Silicalite from being occupied. In addition, the esters have a relatively rigid structure that may not allow favorable packing inside the channels of Silicalite.

Conclusions

Silicalite fills an important gap in solid-phase extraction with its ability to extract relatively small, polar molecules that are not well extracted by sorbent particles used in conventional SPE. Extracted compounds are retained by interaction of the hydrophobic part of the molecule with the hydrophobic interior channels of the Silicalite. Molecules with bulky substituents are partly or completely excluded from these channels due to size restrictions. However, the chemical structure of some large molecules is sufficiently flexible that the molecules may still fit into the channels and be extracted. Positional isomers were extracted in varying degrees, depending on how well they fit into the Silicalite channels. Large variations in loading capacity were observed. This is further evidence that Silicalite adsorbs organic compounds selectively.

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A NEW, HIGH-CAPACITY CARBOXYLIC ACID FUNCTIONALIZED RESIN FOR SOLID-PHASE EXTRACTION

A paper submitted to the *Journal of Chromatography A*, 1997

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Abstract

New high capacity carboxylic acid-functionalized resins prepared by Ring-Opening-Metathesis Polymerization (ROMP) were used for solid-phase extraction (SPE) of organic compounds from water. Two resins, exhibiting a capacity range of 3.0 and 3.75 mequiv COOH/g, have been employed in their beaded form as well as in the form of particle-loaded membranes. A large variety of organic compounds such as phenols, alcohols, aldehydes, ketones, carboxylic acids, esters, chlorinated hydrocarbons, amines, nitrosoamines as well as polyaromatic hydrocarbons was successfully extracted by these materials. For most compounds, a quantitative recovery was observed. The extraction efficiency of the new resins was compared to those of other high-performance materials such as Silicalite and Empore®-disks. The general advantages of the new materials, the mechanism of extraction, and the difference in extraction efficiency of the new particles when incorporated into membranes and columns are discussed.

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Introduction

Identification and quantification of organic compounds in water and other matrices are necessary for solving various environmental, biological or clinical problems. For this purpose, solid-phase extraction (SPE) [1-4] is a widely used method for sample concentration and clean-up. Although a number of solid sorbents are available, there is a need for new sorbents with a broader applicability for SPE. Despite the fact that standard silica-based materials are widely accepted and available, they suffer from the disadvantage of not being wettable by water alone, and therefore need a conditioning step prior to SPE. Additionally, these materials exhibit a significant pH stability. Surface modified polystyrene-divinylbenzene (PS-DVB) materials overcome both disadvantages [5,6]. Because of the ease of derivatization, sulfonic acid groups are introduced onto the surface of PS-DVB. These materials display enhanced surface hydrophilicity and improved extraction efficiencies [7]. However, these materials are unable to extract small, polar organic compounds successfully. Recently, Fritz et. al. described a new zeolite, Silicalite, which exhibits good extraction properties, especially for small molecules. The selective adsorption is believed to be based on a molecular sieve-like interaction into the small, apolar cavities of the material. This type of zeolite represents a useful tool to close the gap for the extraction of water soluble, low molecular weight analytes. Unfortunately, Silicalite tends to degrade in very basic solutions and does not possess any ion-exchange capabilities.

In this study, the use of a new organic polymeric material for SPE is described. The new material is a crosslinked poly(norbornene-5,6-dicarboxylic acid). A detailed description for the preparation of the resin using Ring-Opening-Metathesis Polymerization (ROMP) as

well as its mechanical and chemical properties are given elsewhere [8]. The most striking features of the polymerization technique employed are the reproducibility of the synthesis, the use of functional monomers, and access to high capacity resins (3-4 mequiv COOH/g). In addition to a high cation-exchange capability, the resin is characterized by a strong affinity for apolar compounds, a rather large particle diameter (40 ± 10 μm), resulting in a low back pressure (<15 psi) when used for SPE, and excellent chemical resistance against both acids and bases. The latter allows the re-use of the material after careful clean-up using 2N NaOH and HCl at least 30 times without any loss in performance. Additionally, the wettability of the resin is high, which makes pretreatment with an organic solvent prior to SPE unnecessary. An in-depth investigation of its extraction properties in comparison to other high-performance materials, such as Silicalite and Empore® extraction disks impregnated with sulfonated PS-DVB, demonstrates the advantages of the new resin. Many different types of analytes were studied to characterize the new material. For all types of compounds, both the low and high molecular weight homologues were investigated. Most of these were extracted effectively by the COOH-resin, even when the SPE bed dried out. Finally, for certain compounds, a comparison between two different forms of the new resin was made. For that purpose, the new resin was embedded into a matrix of Teflon fibrils to form an Empore®-type membrane.

Experimental

Reagents and chemicals

Analytes studied were >99% pure and used as obtained from Fisher (Fisher Scientific, Pittsburgh, PA, USA), Fluka (Fluka AG, Buchs, Switzerland) and Aldrich (Aldrich Chemical

Company, Inc., Milwaukee, WI, USA). All solvents and reagents used (acetone, methanol, ethyl acetate, triethyl amine, hydrochloric acid, trifluoroacetic acid) were of analytical grade. Laboratory distilled water was further purified using a Barnstead Nanopure II system (Sybron Barnstead, Boston, MA, USA). Silicalite from the UOP, Inc., was sieved to obtain 5-10 μm particles. Experimental membranes containing these particles were obtained from the 3M Company (St. Paul, MN, USA). Experimental Empore®-type membranes embedded with 8 μm PS-DVB sulfonated resin, 0.6 mequiv/g sulfonation capacity, were used as obtained from the 3M Company and can be purchased commercially as SDB-RPS. Porous, COOH functionalized resins (3-4 mequiv COOH/g, specific surface area 15-20 m^2/g) were prepared as described elsewhere [8]. Sheets of experimental COOH resin-loaded membranes containing these particles were obtained from the 3M Company.

Procedure for SPE

The apparatus for SPE consisted of a 30-ml glass syringe barrel fitted with a luer tip. A 1.5 ml polypropylene SPE column (P.J. Corbert Assoc., St. Louis, MO, USA) was connected to the glass reservoir via a universal adapter. The COOH resin particles (50 mg) were slurry packed in methanol into the SPE column and held between two 20-μm polyethylene frits (P.J. Corbert Assoc.) in the SPE column. Round disks of the COOH resin-loaded membrane were punched out of the membrane sheets and packed between two 20-μm polyethylene frits. The bed heights measured approximately 0.5-cm. Positive pressure was used to force liquids through the columns. Prior to use, 0.5 ml acetone and 1-ml deionized (DI) water were used to condition each column, then 3 ml of 0.1 M sulfuric acid was added to ensure the polymer was in the acidic form.

Samples were prepared by adding a 100-μl aliquot of a methanol solution containing 100 ppm each of 2-3 analytes to 10 ml of DI water. The final concentration of each compound in the sample was 1 ppm unless otherwise stated. Aqueous solutions containing the analytes were passed over the columns filled with the corresponding material. Except for the analysis of polyaromatic hydrocarbons, positive air pressure was adjusted to provide a flow of liquid through the adsorbents to 1-2 ml per minute (5-10 p.s.i.; 1p.s.i. = 6894.76 Pa). After loading, the glass reservoir was rinsed with 1-3 ml DI water and air was blown through the column to remove any remaining water. Elution of all non-basic compounds from the columns was performed with 0.5 ml acetone or ethyl acetate. Methyl amine (2N in acetone) was used to elute bases off the sorbents.

A Shimadzu (Kyoto, Japan) GC 14A equipped with an AOC-14 autoinjector, flame ionization detector and a C-R4A Chromatopac data analysis system was used to separate and quantify all analytes, except nitrosoamines and PAH's. The GC column was a Supelco SPB-5 column, 15 m x 0.32 mm with a phase thickness of 1 μm. To quantitate the nitrosoamines and PAH's, a GC 8030 (Fisons Instruments) with MD 800 mass spectrometer (Fisons Instruments) was used. The GC column consisted of a SE 54 capillary column (poly(5%-diphenyl-95%-dimethylsiloxane), 0.25 mm i.d. with a phase thickness of 0.1-0.15 μm. Recoveries, using the COOH resin, were calculated as an average of at least 3 trials by comparing the relative peak areas with standards that were not subjected to SPE.

Breakthrough curve procedure

The HPLC column used was a 10 cm x 4.6 mm i.d. stainless steel column packed with 500 mg of COOH resin particles. The particles were added to 10 ml of methanol and

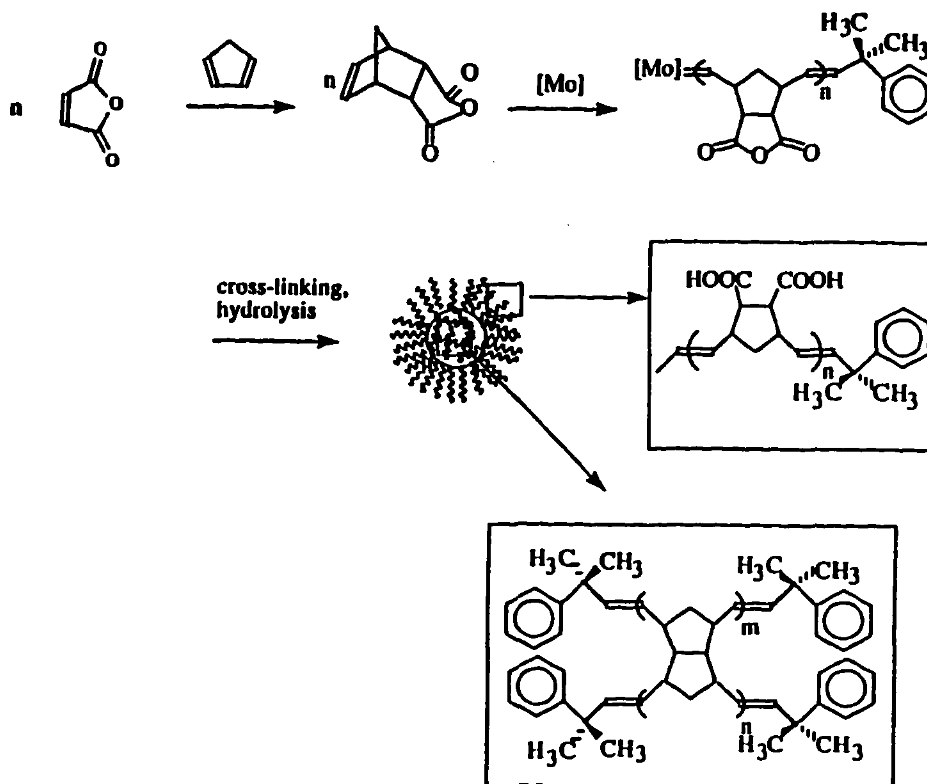
sonicated to produce a slurry. A Shandon HPLC packing pump (Shandon Southern, Sewichley, PA, USA) was used at a pressure of 2000 p.s.i. to pack the slurry into the column. The HPLC system consisted of several components. A Gilson (Middleton, WI, USA) Model 302B HPLC pump equipped with a Model 802B Gilson manometric module and Scientific Systems (State College, PA, USA) Model LP-21 pulse dampener was used for feed solution delivery. Aqueous feed solutions containing 150 ppm of the organic compounds of interest were pumped through at a rate of 1.00 ml per minute. The effluent was monitored on-line with a Kratos 783 UV-Vis detector (Applied Biosystems, Ramsey, NJ, USA) or a Erma 7510 Refractive Index detector (Chrom Tech Inc., Apple Valley, MN, USA). Breakthrough curves were recorded by a Hitachi D-200 Chromato-Integrator (EM Science, Cherry Hill, NJ, USA).

Results and discussion

Preparation of the COOH resin

Ring-Opening Metathesis Polymerization (ROMP) was used for the preparation of the new COOH functionalized resins [8]. The synthesis was performed by reacting molybdenum based Schrock-carbenes with the functional monomer norborn-2-ene-5,6-dicarboxylic acid anhydride. The resulting prepolymer was cross-linked using 1,4,4a,5,8,8a-hexahydro-1,4,5,8-exo-endo-dimethylnaphthalene to form particles (Scheme 1). The new polymers were found to consist of an inert, cross-linked interior and an exterior bearing the linear chains formed by the functional monomers [8]. As a consequence, all functionalities are readily available for SPE. Scheme 1 shows the synthetic pathway as well as the backbone and interior structure of the polymer. While the carboxylate groups provide sufficient hydrophilicity, the

poly-unsaturated character of the carrier chains as well as the entire backbone result in a significant reversed-phase character.



Scheme 1: Preparation of the COOH resin. For experimental details refer to [8]

ELMI-investigations reveal the material formed consist of irregularly shaped, agglomerated particles with a mean particle diameter of ca. 40 μm, which accounts for the low back pressure (5-10 psi) when used for SPE. The specific surface of the 3.75 mequiv COOH/g resin was 15 m^2/g, indicating a non-porous or microporous structure. Nevertheless, a significant non-permanent porosity must be assumed, as considerable swelling takes place upon treatment with any polar solvent such as methanol or water, leading to a particle with ca. 400 μl pore volume/g resin.

Particles for solid-phase extraction

Porous PS-DVB resins have proved to be very effective for SPE of a wide variety of organic compounds from predominately aqueous samples. Their effectiveness is enhanced by chemical introduction of polar groups, such as acetyl or sulfonic acid moieties on the resin surface. However, these modified PS-DVB resins do not extract, or extract very poorly, small polar organic compounds such as the lower alcohols, aldehydes, ketones and carboxylic acids. Silicalite is able to successfully extract these same compounds. Molecules small enough to enter the 6Å diameter channels are retained by interaction of the hydrophobic regions of the analyte with the hydrophobic interior of the Silicalite. Molecules that do not fit the channels are extracted poorly or not at all [9].

The new COOH functionalized resin was expected to possess characteristics of both the polystyrene resins and Silicalite. Hydrophobic analytes would be extracted through interactions with the hydrophobic region of the polymer, which is represented both by the cross-linked interior as well as by the unsaturated backbone bearing the carboxyl groups. More hydrophilic analytes would be retained by the carboxyl groups located at the outside of the particles, which increases the hydrophilicity of the entire resin. A comparison of the extraction behavior for different types of analytes between Silicalite, PS-DVB resin-loaded membranes, and the COOH resin (particles and membranes) is discussed in the following.

Extraction of phenols

Despite the fact that phenols are acidic, they may be concentrated on a weak cation exchanger by adjusting the pH below 2. In this case, the mode of retention is exclusively based on a reversed-phase (RP) mechanism. The use of polar sorbents or weak cation

exchangers using this concept has been reported [10,11]. Nevertheless, the efficiency of extraction strongly depends on the surface hydrophilicity combined with the RP-character. Percentage recoveries of 9 phenols using the three different sorbents are listed in Table 1. The average recovery of the phenols using the COOH resin was 99% with a relative standard deviation of 2%, which is higher than the recoveries using the other two sorbents (88%, Silicalite and 92%, PS-DVB membrane). Silicalite gave lower recoveries of many of the phenols because of their bulky structures.

Extraction of various alkyl compounds

Organic compounds, neither acidic nor basic, are usually extracted by alkyl-derivatized silica, PS-DVB or activated carbon [12,13]. Percentage recoveries of 13 alcohols using the three different sorbents are compared in Table 1. The most striking difference is in the C_1 - C_4 alcohols which are more strongly retained by Silicalite. From a maximum around C_6 the Silicalite recoveries decrease gradually with increasing chain length. The decrease results from the fact that the high molecular weight homologues are sterically hindered from entering the 6 Å cavities of Silicalite. Recoveries using the PS-DVB resin-loaded membrane remain almost constant between C_5 and C_{14}. The COOH resin gave recoveries similar to the sulfonated PS-DVB resin-loaded membrane, showing higher recoveries for propanol, butanol and pentanol than the Empore®-disk. In addition, the average recovery of the other alcohols was 92% for the two sorbents, which is higher than the 74% recovery using Silicalite. Carboxylic acids, which are soluble in water are extracted by Silicalite with significantly greater success. Those with more than 4 carbons show better recoveries with either the PS-DVB resin-loaded membrane or the COOH resin.

Table 1. Comparison of percentage recoveries of phenols and alcohols using Silicalite particles and sulfonated PS-DVB resin-loaded membrane (capacity: 0.6 mequiv/g) and the COOH polymeric resin (capacity: 3.0 mequiv/g).

Class	Compound	Silicalite	Membrane	COOH Polymer
Phenol	Phenol	66	84	101
	m-Cresol	97	93	100
	2-Chlorophenol	33	89	101
	4-Chlorophenol	90	95	102
	3-Nitrophenol	56	105	92
	2,5-Dimethylphenol	54	92	97
	4-Ethylphenol	98	96	102
	4-Propylphenol	98	98	103
	4-Tert-Butylphenol	27	87	97
Alcohol	1-Propanol	84	0	2
	1-Butanol	95	10	13
	2-Butanol	94	6	20
	1-Pentanol	92	99	82
	1-Hexanol	99	91	99
	Cyclohexanol	10	88	94
	1-Octanol	89	94	95
	1-Decanol	82	89	100
	1-Dodecanol	75	89	83
	1-Tetradecanol	58	87	84
	3-Phenyl-1-Propanol	73	99	95
	2-Ethyl-1-Hexanol	92	90	94

Data for the extraction of aldehydes, ketones, esters, carboxylic acids, and chlorinated alkanes are given in Table 2. In several instances the lower molecular weight members of a homologous series are retained much more strongly by Silicalite and the COOH resin than by the sulfonated PS-DVB resin-loaded membrane. Examples include 2-butanone, the C_2 to C_5 esters, as well as the chlorinated alkanes. Additionally, recoveries of the chlorinated alkanes and some carbonyl compounds shown in Table 2 are higher for the COOH based resin than for Silicalite. The ability of the COOH resin to extract smaller molecules as efficiently as Silicalite suggests a microporous structure. During its synthesis, the resin is highly swollen in a chlorinated solvent. In the course of the work-up, this solvent is removed. Template-like cavities may result, which accommodate small molecules like $CHCl_3$, $ClCH_2CH_2Cl$, Cl_2CHCH_3, as well as certain short chain esters. As a consequence, the COOH resin possesses the characteristics of both Silicalite and the sulfonated PS-DVB resin-loaded membrane.

Extraction of amines and N-nitrosoamines

Amines are usually extracted by weak cation exchangers [14-17]. Strongly basic amines may not be eluted quantitatively from strong cation exchangers. As expected, the COOH resin shows a high extraction capability as well as high recoveries for amines [8]. These findings agree with the results for the first few amines in Table 3, which are higher than those using the sulfonated membrane. The COOH resin possesses a significantly higher capacity (3.0 mequiv/g) than the sulfonated PS-DVB material (0.6 mequiv/g). Additionally, elution is achieved more easily because the ion pairing is weaker (pK_1 = 4.5, pK_2 = 8) than in the case of sulfonic acids (pK = -1). Both water soluble as well as water insoluble amines are

Table 2. Comparison of percentage recoveries of various test compounds using Silicalite particles, a sulfonated PS-DVB resin-loaded membrane and the COOH resin

Class	Compounds	Silicalite	Membrane	COOH Resin
Aldehydes	trans-Crotonaldehyde	91	24	8
	n-Valeraldehyde	100	91	100
	Hexanal	107	85	98
	Nonylaldehyde	84	74	89
	Benzaldehyde	89	94	84
	Salicylaldehyde	54	96	92
Ketones	2-Butanone	92	0	72
	2-Pentanone	90	88	94
	4-Methyl-2-Pentanone	104	88	92
	2-Hexanone	93	89	96
	3-Hexanone	81	89	93
Esters	Ethyl acetate	91	55	90
	Ethyl propionate	88	61	98
	Ethyl butyrate	90	75	98
	Hexyl acetate	79	88	100
	Methyl benzoate	68	94	100
	Pentyl benzoate	82	70	92
Chlorinated Alkanes	Chloroform	82	81	89
	1,2-Dichloroethane	83	77	85
	1,1-Dichloroethane	80	77	92
	1,2-Dichloropropane	85	85	92
Carboxylic Acids	Acetic	2	0	1
	Propionic	62	0	2
	Butyric	78	13	20
	Valeric	95	104	100

Table 3. Comparison of percentage recoveries of amines and N-nitrosoamines using Silicalite particles, a sulfonated PS-DVB resin-loaded membrane (capacity: 0.6 mequiv/g) and the COOH polymeric resin (capacity: 3.0 mequiv/g for amines and 3.75 mequiv/g for N-nitrosoamines). Elution was performed with 0.5 ml methyl amine (2M in acetone) for amines and 1 ml ethyl acetate:triethylamine (1 : 1) for N-nitrosoamines.

Class	Compounds	Silicalite	Membrane	COOH Polymer
Amines	Propyl Amine	63	0	96
	n-Butyl Amine	72	52	90
	Pentyl Amine	78	93	90
	Pyridine	77	35	90
N-Nitrosoamines	N-Nitrosoethylmethylamine	--	--	0
	N-Nitrosodiethylamine	--	--	47
	N-Nitrosopyrrolidine	--	--	7
	N-Nitrosomorpholine	--	--	6
	N-Nitrosopiperidine	--	--	40
	N-Nitrosobutylamine	--	--	101

extracted very well. By contrast, the neutral N-nitrosoamines in Table 3 are only extracted by hydrophobic interactions; thus only the water-insoluble, higher molecular weight homologues such as N-nitrosobutylamine are extracted quantitatively by the COOH resin.

Incorporation of the COOH resin into a membrane

Incorporation of the COOH resin into a membrane is generally a more efficient way to perform SPE [18-22]. In contrast to loose particles packed into a small column, a membrane represents a more dense packing. For that purpose, the COOH resin particles were enmeshed in a network of Teflon fibrils to form a strong, porous sheet. Several circles were cut from the membrane sheet and packed into a small column to the same height as that previously

packed with loose COOH resin particles. Results of SPE with the loose particles and membrane column were then compared. The results in Table 4 shows that the Teflon fibrils, which themselves possess a high extraction capacity for apolar compounds, did not affect recoveries of the various compounds.

Table 4. Comparison of percentage recoveries of various analytes using COOH resin particles and a COOH resin-loaded membrane (capacities: 3.0 mequiv/g).

Class	Compound	Particles	Membrane
Alcohols	1-Propanol	3	3
	1-Butanol	13	10
	2-Butanol	20	16
	1-Octanol	95	100
	1-Decanol	100	93
	1-Dodecanol	83	75
Esters	Ethyl acetate	90	95
	Ethyl propionate	98	96
	Ethyl butyrate	100	98
Ketone	2-Butanone	72	71
	2-Pentanone	94	96
	4-Methyl-2-Pentanone	85	92
Carboxylic Acids	Acetic Acid	1	trace
	Propionic Acid	2	2
	Butyric Acid	20	14
	Valeric Acid	106	93
Phenols	Phenol	101	96
	m-Cresol	100	95
	2-Chlorophenol	101	100

Extraction of polyaromatic hydrocarbons (PAH's)

Alkyl-derivatized silica or PS-DVB sorbents are usually employed to extract PAH compounds [23,24]. These apolar compounds may be used to characterize the reversed-phase properties of a resin. In this study, 16 EPA priority PAH's have been studied. 2-propanol was added to the aqueous sample to increase the solubility of the PAH compounds. The positive influence of 2-propanol on the stabilization of PAH solutions in water at the low ppb level has been reported earlier [25]. Table 5 shows the recoveries obtained using different amounts of 2-propanol. While addition of 2-propanol favors high recoveries for the high molecular weight homologues, high recoveries for the low molecular weight homologues are obtained in pure water. These findings are in accordance with the results reported by Borrull et. al. [25]. Generally, PAH compounds are extracted very well by the new resin at the level of 1 ppb. The average range of recovery for all three solvent systems, including the membrane, was found to be 83%-86%.

Breakthrough curves

Load capacity is another good indication of the extraction ability of a resin. It is defined as the total number of moles or weight of analyte extracted by a given amount of resin. In the case where an ion-exchange mechanism is present, it also gives a general idea of the accessibility of the functional groups. In the case where the extraction is based only on hydrophobic sorption, it is often related to the specific surface area of the resin. Resin load capacities were determined through the use of breakthrough curves. Breakthrough curves were generated by passing an aqueous solution of the analyte of interest through a column packed with the COOH resin particles until breakthrough occurred. The curves were plotted

Table 5. Percentage recoveries of various PAH compounds. **Conditions:** COOH resin and COOH resin-loaded membrane: 3.0 mequiv COOH/g; [1] sample size: 1000 ml water: 2-propanol = 90 : 10 (containing 1 ppb each of 16 PAH compounds), sampling rate: 7 mL/min., elution: 2 mL ethyl acetate; [2] sample size: 1000 ml water: 2-propanol = 85 : 15 (containing 1 ppb each of 16 PAH compounds), sampling rate: 7 mL/min., elution: 2 mL ethyl acetate; [3] sample size: 500 mL water (containing 1 ppm each of 16 PAH compounds), sampling rate: 3.5 mL/min., elution: 1 mL ethyl acetate.

Class	Compound	COOH resin particles[1]	COOH resin particles[2]	COOH membrane[3]
PAH	Naphthalene	31	18	99
	Acenaphthalene	82	56	92
	Acenaphthene	97	78	92
	Fluorene	103	94	91
	Phenanthrene	103	99	94
	Anthracene	97	88	76
	Fluoranthene	97	94	85
	Pyrene	100	94	84
	Benzo[a]anthracene	99	95	74
	Chrysene	87	91	76
	Benzo[b]fluoranthene	94	93	67
	Benzo[k]fluoranthene	94	91	70
	Benzo[a]pyrene	89	89	61
	Dibenzo[a,h]anthracene	82	97	77
	Benzo[g,h,i]perylene	36	83	98
	Indeno[1,2,3-cd]pyrene	79	100	86

Table 6. Loading Capacities for COOH resin (3.0 mequiv COOH/g) for Various Test Compounds

Compound	Capacity (mg cpd./g)
Phenol	2
4-Chlorophenol	40
2-Chlorophenol	5
m-Cresol	8
p-Cresol	8
Ethyl Acetate	20
Propyl Amine	42
Pyridine	10
Pentamethyldiethylentriamine	45
Dizabicyclooctane (DABCO)	46
Di-N-morpholinodiethylether	24
4-Methylmorpholine	17
4-Ethylmorpholine	17
N,N-Dimethylethanolamine	43
1,4-Dimethylpiperazine	3
N,N-Dimethylaniline	25
2,6-di-2-propylaniline	40
1-Naphthylamine	17

as a ratio of effluent concentration, C, to influent concentration, C_0, versus the volume of effluent. The total number of moles of analyte adsorbed was calculated by multiplying the retention volume, V_R, by the concentration of the influent [26]. For this study, V_R was defined as the extrapolated volume from the curve at the point $C/C_0 = 0.5$. Capacities for

several analytes were calculated from breakthrough curves and are given in Table 6. The highest capacities by weight were 4.0% for 4-chlorophenol, 4.2% for propylamine, 4.5% for pentamethyldiethylentriamine, and similar capacities for several other amines. Effective extraction behavior for basic compounds was also observed in the sampling of volatile amines from air and will be reported elsewhere [27].

Conclusions

A new polymeric resin has been developed that contains a high concentration of carboxyl groups. These groups provide good water wetting of the surface and thereby circumvent the need for pretreatment with an organic solvent prior to SPE. The carboxyl groups also enable analytes to be taken up by an ion-exchange mechanism as well as by sorption due to hydrophobic attraction. It has been found previously that resins with approximately 0.6 to 1.0 mequiv/g of sulfonic acid groups retain analytes well both by hydrophobic sorption and by ion exchange but that hydrophobic retention becomes very poor at sulfonate concentrations of ~2.0 mequiv/g or more [7]. By contrast, the new COOH resins with ion exchange capacities of 3.0 to 3.75 mequiv/g retain analytes by both hydrophobic sorption and ion exchange.

The new resin retains an unusually wide variety of organic analytes efficiently. The high COOH capacity favors high loadings of basic compounds, but apolar or acidic compounds such as phenols are also extracted efficiently. As a consequence, the new resin is widely applicable for SPE and may be used for the full screening of contaminated aqueous solutions. Restrictions in applications are presented only by a few non-basic water-soluble analytes,

which are extracted poorly or not at all. The physical nature of the resin allows its incorporation into a membrane without changing its extraction properties. As a consequence to its physical nature and relatively large particle size, the back pressure in packed SPE columns is unusually low, which is an advantage over many sorbents used in SPE.

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SEMI-MICRO SOLID-PHASE EXTRACTION OF ORGANIC COMPOUNDS FROM AQUEOUS AND BIOLOGICAL SAMPLES

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Abstract

A technique is described for performing solid-phase extractions on a semi-micro scale. Thin membrane disks 4 mm in diameter containing lightly sulfonated polystyrene or Silicalite particles are placed in the hub of a syringe needle. Aqueous samples (1-6 ml) are passed through the membrane disks and the extracted compounds are subsequently eluted with 20-50 μl of an organic solvent. Unlike solid-phase micro extraction (SPME) which uses a coated fiber, the present method is essentially a total extraction technique. Recoveries >90% were generally obtained for a wide variety of test compounds. The same test compounds in human urine, albumin, and human serum samples can be extracted without any pretreatment other than addition of a suitable surfactant. A "double-pass" technique was developed for convenient field sampling.

Introduction

Liquid-liquid extraction (LLE) has for many years played an important role in sample preparation. However, heightened awareness of the hazards and pollution caused by the use

of organic solvents has led to a search for alternative methodologies. Solid-phase extraction (SPE) is an attractive replacement for LLE [1]. For example, SPE is now specified in the drinking water regulations of the U.S. Environmental Protection Agency method 525.1 [2]. SPE is faster, extraction is more complete, and the amount of organic solvent used is but a small fraction of that is LLE.

In a typical SPE method, analytes are simultaneously extracted and concentrated by passing a sample through a cartridge containing appropriate sorbent particles. The analytes are quantitatively eluted off the sorbent with a small volume of organic solvent, typically 1 to 10 ml. The eluate is then analyzed, usually by gas chromatography. SPE is applicable to almost any analyte through the proper choice of sorbent and eluting solvent. Overall, SPE is complete, simple, inexpensive, portable, easily automated, and uses relatively little solvent. SPE works well both in an online and off-line set up [3].

The use of resin-loaded membranes has been a boon to SPE. In Empore® membranes, solid sorbent particles are enmeshed in a network of Teflon fibrils to form a strong, porous sheet [4]. Membranes require less eluting solvent and are generally more efficient than cartridges containing loose sorbent particles. However the full advantages of membranes for SPE has yet to be fully realized. Like their cartridge counterparts, membranes are often eluted with relatively large volumes of organic solvent, up to 10 ml. To obtain better sensitivity and detection limits, eluates are often evaporated down to a fraction of the original volume. This additional step reduces the speed of SPE and risks the chance of sample loss. Richard and Junk quantitatively eluted pesticides [5,6], polycyclic aromatic materials [6], and tributyltin chloride [7] from C18 cartridges using approximately 100 µl ethyl acetate. Since

then, elution volumes less than 0.5 ml have been used infrequently.

A technique known as solid-phase micro extraction (SPME) uses a modified syringe housing a fused-silica fiber coated with a gas chromatographic stationary phase [8-11]. SPME involves the partitioning of analytes between the coating and sample matrix, followed by thermal desorption of the analytes into an analytical instrument, typically a gas chromatograph. Although it is truly a micro technique, SPME is an equilibrium extraction technique [10]. Complete extraction of liquid samples is seldom achieved [8] and careful calibration is therefore needed for quantitation.

The goal of the present research was to miniaturize the entire SPE process while retaining the speed and high analyte recoveries obtained with conventional SPE. The semi-micro solid-phase extraction (SM-SPE) system developed reduces sample size to 1-6 ml and the volume of eluting solvent to only 20-50 μl. In addition, SM-SPE provides a more compact and portable system that allows easier on-site usage.

Experimental

Reagents and chemicals

Analytes studied were >99% pure and used as obtained from Fisher Scientific (Pittsburgh, PA, USA) and Aldrich Chemical Company, Inc. (Milwaukee, WI, USA). Laboratory distilled water was further purified using a Barnstead Nanopure II system (Sybron Barnstead, Boston, MA, USA). Sheets of experimental sulfonated polystyrene-divinyl benzene (PS-DVB) resin-loaded membranes, now commercially available as SDB-RPS, and experimental Silicalite-loaded membranes [12] were obtained from 3M Co. (St. Paul, MN, USA). Urine was

collected personally and both bovine serum albumin and human serum were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Procedure for SM-SPE

The apparatus used for SM-SPE is shown in Figure 1, which is drawn larger than actual scale to show the details. The apparatus consisted of a Hamilton 1000 series GASTIGHT 5-ml glass syringe with a Teflon luer lock (Cat. No. 81520) acting as the sample reservoir. The syringe was fitted with fluorocarbon hubbed, 22 gauge stainless steel needles (Cat. No. 90134), which served as the extraction columns. The bottom of each needle was gently tapered to a point by the university machine shop. A piece of stainless steel wire mesh, 4-mm in diameter, 228 μm thick, and 53.3% open pore volume, was machined into place just above the tapered bottom to support the membranes.

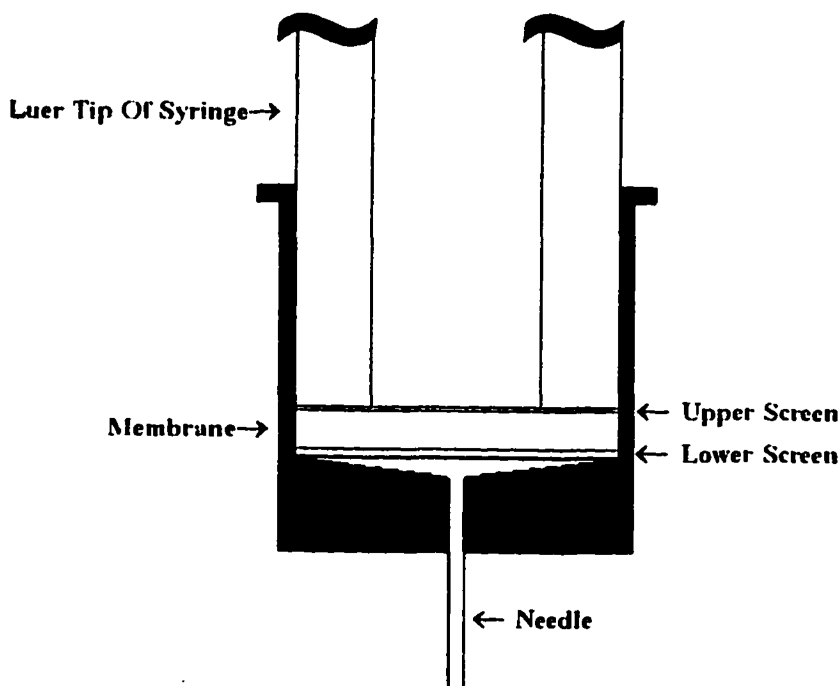

Figure 1. Device used for SM-SPE. The figure is drawn to scale and enlarged for clarity.

Three needles were used, each containing a different membrane. One needle was packed with the lightly sulfonated PS-DVB resin-loaded membrane, a second with the Silicalite-loaded membrane, and the third contained a combination of the two in a mixed membrane. The first two needles were packed by placing a 1 mm x 4 mm membrane disk, weighing 9-11 mg, of either membrane into the needle hub. The thickness of these membranes was reduced when the double pass sampling method was used. The mixed membrane needle was packed by placing a 0.5 mm x 4 mm disk of the Silicalite-loaded membrane on top of a disk of sulfonated PS-DVB resin-loaded membrane of the same size. Liquids were forced through the packed needles by pushing down on the syringe plunger. Prior to use, 250 µl acetone and 1 ml deionized (DI) water were passed through to condition each packed needle.

Single pass sampling

The procedure for loading the membranes was the same for all three needles. The plunger was removed from the syringe barrel and a packed needle locked into place. Samples were prepared by adding a 10 µl aliquot of methanol solution containing 100-ppm each of 2-3 analytes to 1 ml to 6 ml of DI water. The final concentration of each analyte in the sample was 0.17 to 1.0 ppm. For the mixed membrane experiments, samples were prepared by adding a 10 µl aliquot of a methanol solution containing 50 ppm of each analyte to 6 ml of DI water and adjusting the final concentration of each analyte to 0.083 ppm. The samples were manually pushed through the membranes.

Double pass sampling

Double pass sampling was used with the two needles containing each membrane separately. To reduce sampling time, the thickness of the Silicalite membrane was decreased

to 0.33 mm and the sulfonated PS-DVB resin-loaded membrane was reduced to 0.50 mm. An additional piece of stainless steel wire mesh was placed on top of each membrane to prevent movement. Samples were prepared by adding a 5 μl aliquot of a methanol solution containing 100 ppm each of 2-3 analytes to 1 ml DI water in a capped GC vial. The final concentration of each analyte was 0.5 ppm. The plunger remained in the syringe barrel and a packed needle locked into place. The packed needle pierced the cap septa and a 1 ml sample was drawn up through the membrane. The cap septa was punctured to release air pressure and decrease sampling time. The syringe barrel was immediately emptied by pushing down on the plunger, achieving a double pass through the membrane.

Elution and quantification

After loading was complete, the syringe barrel was rinsed with approximately 200 μl DI water and air was pushed through the membranes to remove any remaining water. A 20 μl -50 μl aliquot of acetone, ethyl acetate, or methylene chloride was used to elute the compounds into a capped GC vial. An internal standard, 2 μl of acetone solution containing 250 ppm toluene, was added to the contents of the vial, which were analyzed by gas chromatography using manual injection. A Shimadzu (Kyoto, Japan) GC 14A equipped with a flame ionization detector and a C-R4A Chromatopac data analysis system was used to separate and quantitate the analytes. The GC column used was a Supelco SPB-5 column, 15 mm x 0.32 mm with a phase thickness of 1 μm. Recoveries were calculated as an average of two trials by comparing the relative peak areas with standards that were not subjected to SM-SPE.

Biological samples

Fresh urine samples were diluted 1:1 with DI water. Sample volumes of 1.0 ml were made up by adding 10% v/v of methanol or an aqueous 30 mM sodium dodecyl sulfate (SDS) solution. Stock solutions containing either 1,000 ppm or 10,000 ppm bovine serum albumin were prepared in DI water; in some cases 10% v/v of an aqueous 30 mM SDS solution was added. Serum samples were prepared by diluting 1:2 with an aqueous 30 mM solution of SDS. A 10 μl aliquot of a methanol solution containing 50 ppm each of 2-3 analytes was added to each 1.0-ml sample. The final concentration of each analyte in the sample was 0.5 ppm. The samples were extracted with a 1 x 4 mm Silicalite disk as explained above in the single-pass procedure.

Results and Discussion

Extraction assembly

The device used for SM-SPE (Figure 1) must be carefully designed to provide efficient extraction and subsequent desorption on a small scale. The 1 x 4 mm membrane disks weigh only ~ 9 mg (PS-DVB) and 11 mg (Silicalite), of which 90% is estimated to be solid particles for the extraction. The disks are rather soft and need to be supported on a thin stainless steel screen that is machined into place in the needle hub (see Experimental). It was necessary to drill out the inside of the needle hub slightly so that a conical void was created just below the membrane disk and its mesh support. This was needed to assure a smooth flow of liquid through the membrane and out the needle. Without the void, the disk lay flat against the needle hub, causing the back pressure to rise and doubling the volume of elution solvent needed.

In the work described the needle containing a small membrane disk was connected to a small syringe. The aqueous sample was placed in the syringe barrel and forced manually through the needle assembly. Alternatively, air or gas pressure could be used to push the samples through.

SM-SPE of test compounds

Experimental Empore®-type membranes containing lightly sulfonated PS-DVB resins have been shown previously to effectively extract a wide variety of organic test compounds from aqueous samples [13]. Excellent recoveries were obtained with these membranes on a semi-micro scale, as indicated by the data in Table 1. The aqueous samples contained 0.17 to 1.0 ppm each of several test compounds. The average recovery for all test compounds was 97% for 1-ml samples and 95% for 6-ml samples. The relative standard deviation was 1.7%.

The volume of acetone required for elution varied from 20 to 50 μl. In some cases methylene chloride or ethyl acetate was used for the desorption step. With a 2-μl injection into a gas chromatograph, the fraction of the organic solvent eluate actually injected into the GC varies from about 0.1 to 0.04. In conventional SPE the fraction of eluate injected is often the order of 0.001.

In conventional SPE an experimental Empore®-type membrane loaded with Silicalite particles has been shown to extract smaller, more polar molecules than sulfonated PS-DVB resin-loaded membranes [13]. Silicalite also extracts molecules that are somewhat larger and more hydrophobic, but bulkier compounds are excluded from the 6-Å channels in the Silicalite and are poorly extracted. Data for SM-SPE of polar test compounds with Silicalite -loaded membrane disks are presented in Table 2. The average recovery for the 29

Table 1. SM-SPE recovery of organic compounds using a sulfonated PS-DVB resin-loaded membrane. Aqueous samples contained 0.17 to 1.0 ppm of each test compound. Acetone was used for desorption of extracted compounds except where noted (*eluted with methylene chloride, ** eluted with ethyl acetate).

Class	Compound	Vol. desorption solvent, µl	Avg. 1-ml sample	recovery, (%) 6-ml sample
Phenol	Phenol	40	92	91
	o-Cresol	40	102	102
	2,5-Dimethylphenol	40	101	98
	2-Chlorophenol	40	100	93
	4-Chlorophenol	40	92	98
	3-Nitrophenol	40	94	93
Aldehyde	n-Valeraldehyde	40	88	90
	Octylaldehyde	50	89	86
	Benzaldehyde	50	99	98
	Salicylaldehyde*	40	100	95
Alcohol	1-Pentanol**	20	102	95
	3-Phenyl-1-propanol*	20	92	91
	2-Ethyl-1-hexanol	20	94	94
	1-Octanol**	20	97	96
Ester	Ethyl acetoacetate	20	99	97
	Hexyl acetate	40	99	98
	Methyl benzoate	40	92	91
	Isopentyl benzoate	30	98	93
Ether	Anisole	50	98	98
Ketone	2-Pentanone	30	98	93
	4-Methyl-2-pentanone	30	100	94
	2-Hexanone**	30	100	100
	Acetophenone	40	99	99

Table 2. SM-SPE recovery of organic compounds using a Silicalite-loaded membrane. Aqueous samples contained 0.17 to 1.0 ppm of each test compound. Acetone (40 μl) was used for desorption.

Class	Compound	Average recovery, (%) 1-ml samples	6-ml samples
Alcohol	1-Propanol	85	85
	1-Butanol	97	94
	2-Butanol	97	98
	1-Pentanol	102	99
	2-Pentanol	102	99
	1-Hexanol	101	99
	1-Octanol	99	100
	2-Octanol	101	98
	2-Ethyl-1-hexanol	93	93
	3-Ethyl-2,2-dimethyl-3-Pentanol	94	94
Ester	Methyl acetate	96	95
	Ethyl acetate	97	94
	Ethyl propionate	98	96
	Ethyl butyrate	95	93
	Ethyl acetoacetate	99	96
Ketone	2-Butanone	98	96
	2-Pentanone	98	91
	4-Methyl-2-pentanone	100	97
	2-Hexanone	106	106
	3-Hexanone	98	92
	2-Heptanone	97	95

Table 2. (Continued)

Class	Compound	Average 1-ml samples	recovery, (%) 6-ml samples
Phenol	p-Cresol	99	94
	4-Isopropyl phenol	97	95
Aldehyde	trans-Crotylaldehyde	91	89
	cis-Crotylaldehyde	96	95
	Butyraldehyde	96	92
	n-Valeraldehyde	91	88
	Hexaldehyde	97	94
	Benzaldehyde	99	97

compounds tested was 97% on 1-ml samples and 95% on 6-ml samples with a relative standard deviation of 1.9%. All of the test compounds were eluted with 40 μl of acetone.

It is known that sulfonated PS-DVB resin-loaded membranes extract larger organic compounds and the Silicalite-loaded membrane extracts small, more polar compounds. Therefore, the membranes were mixed to determine whether they can successfully extract organics in tandem from aqueous solutions. The membrane packing order did not influence the recoveries, shown in Table 3. The first analyte in each pair was selectively extracted by the Silicalite-loaded membrane and extracted little, if at all, by the sulfonated PS-DVB resin-loaded membrane. The second analyte in each pair was more favorably extracted by the sulfonated PS-DVB resin-loaded membrane. All recoveries were about 90% or greater with a relative standard deviation of 2.3%. These results show that the membranes perform just as well in tandem as they do alone. This technique promises to expand the scope of organic compounds that are amenable to solid-phase extraction.

Table 3. SM-SPE using mixed membranes. Conditions: 0.5 mm thick sulfonated PS-DVB resin-loaded and 0.5 mm thick Silicalite-loaded membranes, single pass, 6 ml aqueous samples, 1 ml/min. Elution: 40 μl acetone, 10 s. Analyte concentration: 0.083 ppm.

Compound Pair	Average recovery, (%)
Methyl acetate	91
Methyl benzoate	95
2-Butanol	99
2-Cresol	102
1-Butanol	94
3-Nitrophenol	99
Ethyl butyrate	90
2,5-Dimethylphenol	93
Ethyl acetate	90
Salicylaldehyde	90
2-Pentanone	94
2-Chlorophenol	100
Ethyl propionate	96
Phenol	93

Analysis of biological samples

Extraction and quantification of organic compounds from biological samples such as urine and serum present several specific problems. Biological samples contain components such as proteins, lipids, saccharides, and salts in varying concentrations. The organic compounds to be extracted range in polarity and may associate with the matrix components. Human urine has been found to contain several hundred volatile organic compounds [14]. These organic volatiles consist of certain essential nutrients, intermediates, waste products, environmental contaminants, and other substances of low molecular weight involved in metabolism. Most of these volatiles are also present in serum [15].

Fresh urine, bovine serum albumin, and human serum samples were analyzed to determine the extraction ability of SM-SPE for samples of this type. Compounds previously identified in human urine [15,16] and serum [15,17,18] were added to the samples in low concentrations. A Silicalite-loaded membrane was used for these experiments for two reasons. One was that the analytes selected were more successfully extracted by Silicalite than by sulfonated PS-DVB resins. The other reason was that preliminary experiments showed that large biological compounds do not clog up the pores of Silicalite.

Results of these SM-SPE experiments are summarized in Table 4. Diluted urine samples required the addition of 10% v/v of methanol or, better, addition of 10% v/v of an aqueous 30 mM sodium dodecylsulfate (SDS) solution to reduce the back pressure. SDS binds to proteinaceous material and releases protein-bound drugs, etc. [19,20]. As a result, proteins are not adsorbed onto the membrane and thus do not interfere with the SPE process. The chromatograms of eluates from samples treated with SDS were much cleaner than those

Table 4. Recoveries of test compounds from various biological samples using a Silicalite-loaded membrane

		Average	Recovery, (%)	
Compound	Urine, SDS	1000 ppm Albumin	1000 ppm Albumin, SDS	Serum, SDS
1-Butanol	95	98	97	90
2-Butanol	--	102	96	87
1-Pentanol	89	99	95	101
2-Pentanol	91	--	--	--
1-Hexanol	--	101	100	100
2-Ethyl-1-Hexanol	--	96	92	93
1-Octanol	93	91	93	100
2-Octanol	--	91	93	100
2-Butanone	100	101	101	100
2-Pentanone	100	101	100	100
4-Methyl-2-Pentanone	97	100	95	88
2-Hexanone	100	93	100	99
3-Hexanone	88	--	--	--
2-Heptanone	94	98	90	100
2-Methylbutyraldehyde	59	--	--	--
n-Valeraldehyde	--	88	87	90
trans-2-Pentenal	93	--	--	--
Hexaldehyde	--	95	92	97
Benzaldehyde	99	97	94	97
Thiophene	81	--	--	--
R-Carvone	92	--	--	--

Table 4. (Continued)

		Average	Recovery, (%)	
Compound	Urine, SDS	1000 ppm, Albumin	1000 ppm, Albumin	Serum, SDS
Benzene	78	82	75	55
Toluene	65	79	67	76
Phenol	88	--	--	--
p-Cresol	100	--	--	--
Chloroform	58	75	58	68

containing methanol. The average recovery of 20 compounds from SDS-treated urine samples was 88% with a relative standard deviation (RSD) of 3%.

SM-SPE experiments were performed with 1,000 and 10,000 ppm samples of bovine serum albumin. No noticeable changes in back pressure were observed on sampling 1,000 ppm albumin samples. The average recovery of 17 test compounds was 94% (Table 4) with a relative standard deviation of 2%. However, sampling 10,000 ppm albumin required the addition of 10% v/v of an aqueous 30 mM SDS solution to prevent a serious increase in back pressure. Recovery of test compounds with added SDS averaged 88% with a RSD of 2% .

Serum samples required dilution (1:2) using an aqueous 30 mM solution of SDS. The serum samples were more viscous than the aqueous-albumin samples and contained more potential interferences. After many extractions with the same membrane, the back pressure did not increase, implying no fouling of the membrane by protein adsorption. The average recovery of 18 test compounds was 91% with a RSD of 2%.

Double pass sampling

A convenient manual technique for SM-SPE is to draw the sample via the needle tip up through the membrane. The sample is then pushed back through the membrane a second time and expelled out the needle. This double-pass technique should ensure a high degree of extraction. It is particularly convenient for field sampling; elution of the analytes immobilized on the membrane can be completed in the laboratory if desired.

Minor adjustments in the membrane-needle assembly were required for the double-pass technique to be practical. A second mesh screen was inserted above the membrane to hold it in place during the sample-draw step. Sampling was slow during the draw step due to the resistance of flow of 1-mm thick membranes. This problem was avoided by reducing the membrane thickness to 0.5 mm or in some cases to 0.33 mm.

Recovery data are given in Table 5 for samples extracted by a 0.5 mm x 4 mm sulfonated PS-DVB resin-loaded membrane using double-pass sampling. The sampling time for a 1-ml aqueous sample was only 45-60 s (draw time 30-45 s; push time 15 s). The average recovery for sample compounds was 89% with a RSD of 2%. Recovery data for double-pass extractions using a 0.33 x 4 mm Silicalite-loaded membrane are given in Table 6. In this case the sampling time was 50-85 s (draw, 30-45 s; push 20-30s). The average recovery for sample compounds was 99% with a RSD of 2%.

The amount of sorbent particles in these thinner membranes is quite small: 4.5 mg for 0.5 mm x 4 mm sulfonated PS-DVB and ~3.7 mg for 0.33 x 4 mm Silicalite. However, the analyte in a 1.0 ml sample containing 1 ppm is smaller yet: ~1 µg. With such small amounts of solid-extractant particles in the membranes it is useful to know the effect of analyte

Table 5. Recovery of test compounds using a sulfonated PS-DVB resin-loaded membrane (0.5 mm thick) and double-pass sampling. Conditions: 1.0 ml aqueous samples containing 0.5 ppm of each test compound. Draw time 30-45 s, push time 15 s. Elution with 20-50 μl acetone in 5-10 s.

Class	Compound	Recovery, (%)
Phenol	o-Cresol	60
	2,5-Dimethylphenol	76
	4-Chlorophenol	68
Aldehyde	Octylaldehyde	100
	Benzaldehyde	95
	Salicylaldehyde	100
Alcohol	3-Phenyl-1-propanol	85
	2-Ethyl-1-hexanol	93
	1-Octanol	99
Ester	Hexyl acetate	100
	Methyl benzoate	94
	Isopentyl benzoate	100
Ether	Anisole	81
Ketone	2-Hexanone	82
	2-Heptanone	100

Table 6. Recovery of test compounds using a Silicalite-loaded membrane (0.33 mm thick) and double-pass sampling. Conditions: 1 ml aqueous samples, 0.5 ppm of each test compound. Sampling times: draw 30-45 s, push 20-30 s. Elution with 40 μl acetone, 10 s.

Class	Compound	Recovery, (%)
Alcohol	1-Butanol	86
	1-Pentanol	100
	2-Pentanol	93
	1-Hexanol	103
	2-Ethyl-1-hexanol	95
	1-Octanol	101
	2-Octanol	103
Aldehyde	n-Valeraldehyde	102
	Hexaldehyde	99
	Benzaldehyde	100
Ketone	2-Butanone	92
	2-Pentanone	103
	4-Methyl-2-pentanone	102
	2-Hexanone	103
	3-Hexanone	99
	2-Heptanone	100
Ester	Ethyl propionate	102
	Ethyl butyrate	101

concentration in the sample on its percentage recovery in SM-SPE. Recoveries of 2-hexanone by double-pass SM-SPE with a 0.33 x 4 mm Silicalite-loaded membrane were as follows: 0.005 ppm, 91%; 0.01 ppm, 96%; 0.10 ppm, 103%; 1.0 ppm, 106%; 10 ppm, 84%; 100 ppm, 81%. Thus, recoveries >90% were obtained for 2-hexanone concentrations ranging from

0.005 ppm to 1.0 ppm. Some overloading and, therefore, lower recoveries occurred at 10 ppm and 100 ppm. Recoveries of 2-hexanone on the 0.5 x 4 mm PS-DVB resin-loaded membrane were lower: 0.005 ppm, 67%; 0.01 ppm, 70%; 0.10 ppm, 72%; 1.0 ppm, 79%; 10 ppm, 67%; 100 ppm, 54%.

Conclusions

Two basic approaches can be used for solid-phase extraction of solutes from liquid samples. In "conventional" SPE the liquid sample is passed through a mini-column or membrane containing the solid extractive particles. Extraction of the solutes tends to be rapid and essentially complete. A second approach is illustrated by SPME in which a fiber coated with the solid-phase extractant is placed in the sample liquid. An equilibrium is attained in which a fixed portion of each solute is taken up by the fiber. Often only a small fraction of each solute is actually extracted. However, all of the solutes on the fiber are thermally desorbed into a gas chromatograph for further analysis.

Semi-micro SPE retains many of the best aspects of both of these basic approaches. SM-SPE is a total extraction technique but the sample size has been reduced to 1-6 ml and the volume of eluting solvent to only 20-50 µl. This in turn reduced the amount of waste produced and lowered the time needed per run. SM-SPE also provides flexibility in sampling and extraction design. Two types of sampling techniques were used, single and double pass. Also, membrane disks of different compositions were mixed to achieve a multimodal extraction, which is difficult to accomplish in conventional SPE. Finally, the SM-SPE device could be brought on-site to perform extractions immediately, saving transport time and storage

space. The extracted compounds could be eluted immediately or the needle hubs could be stored in air-tight containers for later elution.

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THE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF DRUGS AND METABOLITES IN HUMAN SERUM AND URINE USING DIRECT INJECTION AND A UNIQUE MOLECULAR SIEVE

A paper to be submitted to the *Journal of Chromatography*

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Abstract

Silicalite is a molecular sieve that contains an intricate system of channels approximately 6 Å in diameter. These channels are hydrophobic and retain relatively small hydrophobic and hydrophilic molecules from aqueous and biological samples. Macromolecules, proteins, are not retained because they are sterically hindered from entering the channels. Therefore, biological fluids can be directly injected into the HPLC system, eliminating the need for sample preparation. The sample macromolecules elute with high recovery mostly at the extraparticulate void. Simultaneously, Silicalite allows various drugs and metabolites to enter the channels and be retained. Recoveries >90% were generally obtained for a wide variety of drugs and their metabolites from human serum and urine. The use of precolumns, column switching, and surfactant-containing mobile phases was not needed.

Introduction

The isolation and quantification of small molecules from biological samples using high-performance liquid chromatography (HPLC) presents many challenges. The major challenge is the removal of macromolecules, proteins, to avoid damage to chromatographic columns. Proteins in the biological fluids can precipitate or denature and adsorb onto the packing material, leading to back-pressure buildup, changes in retention time, and decreased column efficiency and capacity. Chromatographic systems utilizing mobile phases containing organic modifiers are especially prone to protein precipitation and its effects.

Often, sample preparation is the most vital step in a HPLC clinical analysis. Sample preparation separates an analyte of interest from proteinaceous material, allowing the total amount of the analyte (protein bound and free) to be determined and preventing or reducing the adsorption of protein and other interferences onto the analytical column. At the same time, the analyte is concentrated to improve sensitivity and detection capabilities. Some of the most commonly used sample preparation techniques include liquid-liquid extraction, protein precipitation, and solid-phase extraction. However, these methods are labor intensive, increase the total analysis time and reduce the total recovery of the analyte of interest.

One way to avoid protein adsorption and eliminate the need for sample preparation is to employ a direct injection technique. Reviews of direct injection techniques have been published [1-4]. Several direct injection methods have been developed which solve the problems of proteins and other endogenous compounds. These methods include

precolumn techniques, surfactant-containing mobile phases and restricted access media.

The precolumn technique has a tandem column design consisting of a precolumn, a switching valve, and an analytical column [5-10]. Drugs are removed from the sample matrix, which elutes to waste, and are concentrated onto the precolumn. As a result, the analytical column is not exposed to proteins. One disadvantage of this technique is the need for an additional pump, column switching device, and timed computer control of events.

Use of surfactant-containing mobile phases for the direct injection of serum and plasma samples on reversed-phase columns was first reported in 1985 [11-13]. The addition of a surfactant prevents the adsorption of proteins on the HPLC packing material [14] and releases protein-bound drugs [15]. Both the stationary phase and proteins are bound by the surfactant, preventing adsorption. The use of surfactant-containing mobile phases for direct injection has not been wide spread. This may be due to a decrease in column efficiency which results from slow mass transfer from the poorly wetted stationary phase [16]. Another problem encountered using surfactants is the interferences from impurities in the surfactant reagents.

The topic of restricted access media has been reviewed [17-19]. In general, a restricted access medium is a packing material having a hydrophobic interior covered by a hydrophilic barrier. There are several types of restricted access media including protein (bovine serum albumin)-coated ODS, internal-surface reversed phase, shielded hydrophobic phase, semipermeable surface, mixed functional phase, and a diol-bonded silica gel or "binary-layered phase". These packing materials are synthesized by attaching

various hydrophilic and/or hydrophobic groups to external and/or internal surfaces(s) of various high performance sorbent particles. The hydrophilic groups provide a barrier which macromolecules, such as proteins, cannot penetrate. Thus, the macromolecules pass through the column unretained. Small analytes are retained through interactions with the hydrophobic surfaces. A disadvantage of these packing materials is the modification process, often long and consisting of a number of tedious steps. Also, chemically bonded columns cannot be used for long periods at a pH of 3.0 or lower because of the hydrolysis of the bonded phase [20].

Silicalite is a molecular sieve, first synthesized in 1977 [21]. It is a polymorph of silica with an unusual crystal structure. Silicalite has a tetrahedral framework of mostly 5-membered rings of silicon-oxygen tetrahedra. These tetrahedra form a 3-D system of intersecting channels, 6 Å in diameter, defined by rings of 10 oxygen atoms. Because of its unique structure, Silicalite adsorbs organic analytes due to hydrophobic interactions and possesses size exclusion properties. Silicalite is highly water wettable and extracts organic molecules from water, either liquid or vapor phase, unlike other molecular sieves. For sorption, the diameter of a particular analyte must not exceed 6 Å, roughly the kinetic diameter of benzene. Mayer and Fritz have shown that Silicalite, when used as a sorbent for solid-phase extraction, is successful at extracting a wide variety of organic compounds from aqueous [22] and biological samples [23].

Because the external surface of Silicalite is relatively hydrophilic and the pore diameter is 6 Å, macromolecules cannot enter. In addition, the pores of Silicalite are hydrophobic and successfully adsorb small organic analytes from biological samples.

Therefore, Silicalite was used for the HPLC determination of drugs in human serum and urine using direct injection.

Experimental

Reagents and chemicals

The drugs and metabolites of interest were obtained from Sigma (Sigma Chemical Company, St. Louis, MO, USA). Potassium dihydrogen phosphate and acetonitrile were obtained from Fisher (Fisher Scientific, Pittsburgh, PA, USA). Laboratory distilled water was further purified using a Barnstead Nanopure II system (Sybron Barnstead, Boston, MA, USA). Human serum and urine were personally donated. Silicalite from the UOP, Inc., were sieved to obtain 5-8 μm particles.

Instrumentation

Approximately 1.5 grams of Silicalite was slurry packed into a 100 x 4.6 mm i.d. stainless steel column. The following packing procedure was followed. Silicalite was added to degassed 2-propanol and sonicated under vacuum for 30 minutes. This step removed any trapped air from within the Silicalite and produced a slurry. A Shandon HPLC packing pump (Shandon Southern, Sewichley, PA, USA) was used at a pressure of 3000 psi to pack the slurry into the column. The chromatographic system for the quantitation studies and isocratic elution consisted of an Isco syringe pump equipped with a Kratos 783 UV-Vis detector (Applied Biosystems, Ramsey, NJ, USA). Gradient elution was achieved using a chromatographic system consisting of a Waters Model 600E pump controller, Model 610 pump, and valve station. A Waters Model 996 photodiode array

was used for identifying the eluting compounds. Samples were manually injected using a Rheodyne system fitted with a 5 μl injection loop.

Recovery of serum and urine components from Silicalite

The recovery of serum and urine, including proteins and endogenous compounds, was calculated by comparing the peak areas obtained using a column packed with Silicalite with those obtained with an empty 100 x 4.6 mm i.d. column. The effects of mobile phase conditions, acetonitrile concentration and pH, on the recovery of the biological fluids were studied. The effluent from the columns was monitored at 236 nm and all recoveries were calculated as an average of three injections.

Recovery of drugs and metabolites from serum and urine

Aqueous standards were prepared by adding acetonitrile solutions of the drugs or metabolites to 1.0 ml of deionized water. Biological samples were prepared by adding the same acetonitrile solutions of the drugs or metabolites to 1.0 ml of serum or urine. The final concentration of each drug was in its therapeutic range [24,25]. The recovery of each drug and metabolite was calculated from the peak area ratio of the analytes dissolved in the biological fluids and in deionized water.

Determination of caffeine metabolites in human urine

Caffeine for oral administration was in the form of a diet cola, 2 liters. A 2 liter dose (180 mg caffeine) was administered over a 24 hour period, after which urine was collected and pooled. Concentrations of methylxanthines and methyluric acids in urine were determined using direct injection and isocratic elution. Working curves for the determinations of caffeine metabolites in urine were derived as follows. To a 500 μl

aliquot of urine was added 10 μl of a known concentration of a caffeine metabolite.

Results and Discussion

Recovery of serum components from Silicalite

The smallest, major serum protein is serum albumin [26], which constitutes nearly 60% of the total serum protein. Serum albumin has a molecular weight of approximately 66,000 daltons and its effective sphere radius has been estimated at 40 Å [27]. Therefore, serum albumin should be excluded from the pores of Silicalite and exit the column virtually unretained. The peak resulting from a 5 μl injection of human serum onto a 10 cm x 4.6 mm i.d. column is in Figure 1. The 5 μl aliquot of serum elutes with the injection peak. The percentage recovery of the serum using a pure aqueous buffer was 89% with a relative standard deviation (RSD) of 2%.

The recovery of serum using direct injection is highly dependent on the mobile phase, namely the organic modifier content and pH. Therefore, the effect of acetonitrile concentration in the mobile phase on serum recovery was studied. The aqueous mobile phase contained 20 mM potassium phosphate buffer (pH=6.9) with varying percentages of acetonitrile, 0 to 20% (v/v). The percentage recoveries of serum from Silicalite are located in Table 1. Complete recovery of serum was obtained with 20% acetonitrile in the mobile phase. The recovery of serum using 25% acetonitrile was lower, suggesting denaturization and precipitation. This trend can be seen by comparing Figures 2 a, b, and c. As the acetonitrile concentration in the mobile phase increased from 3 to 10% (v/v), the peak area of the serum peak increased. However, when the mobile phase contained

25% (v/v) acetonitrile the serum peak decreased. Therefore, the results in Table 1 and Figure 2 indicate that the acetonitrile content in the mobile phase should not exceed 25% when using a pH of 6.9. These findings agree with Pinkerton et al [28] who determined a protein precipitation cutoff of less than 25% acetonitrile.

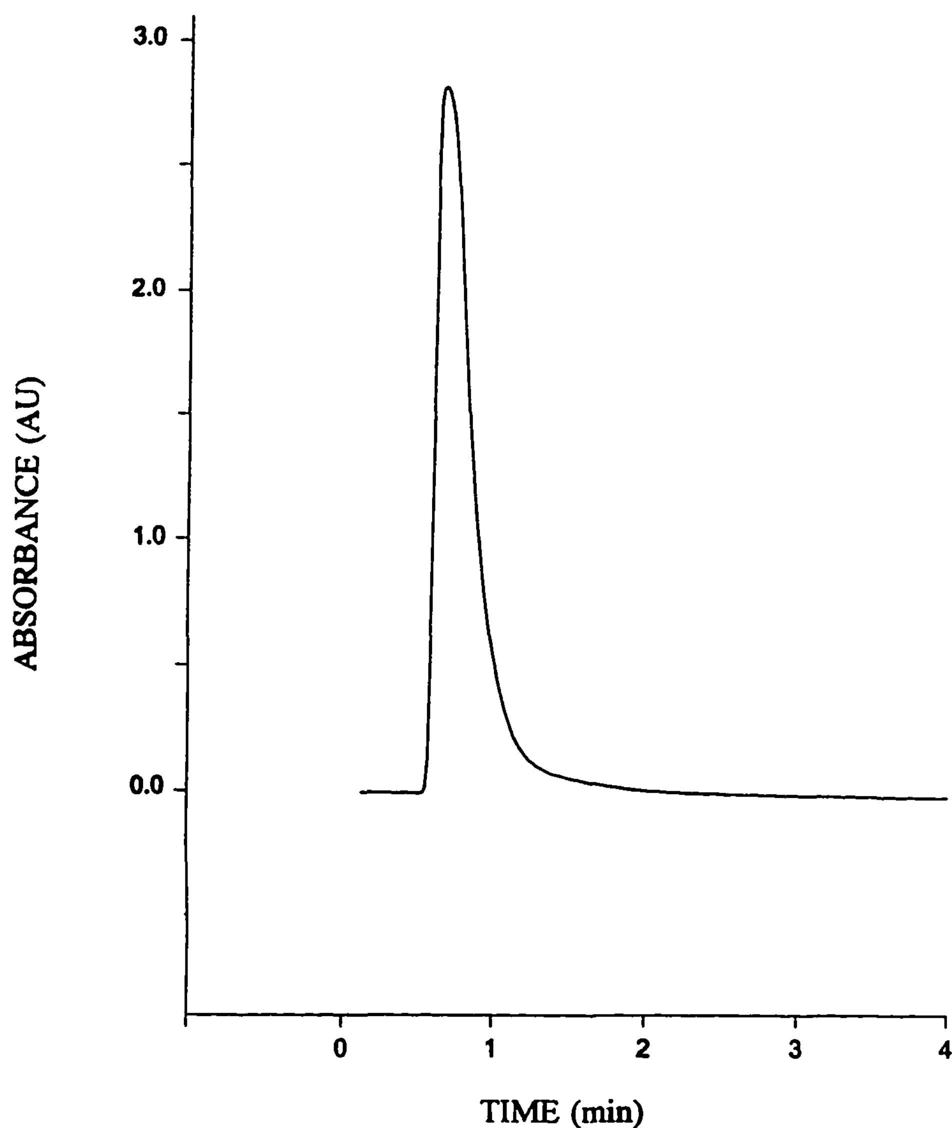

Figure 1. Elution of human serum. Conditions: column, Silicalite (5-10μm) 10 cm x 4.6 mm i.d.; mobile phase, aqueous 20mM phosphate buffer, pH=6.9; flow rate, 1.0 ml/min.; detection, 236 nm; injection volume, 5 μl.

Table 1. Effect of Acetonitrile Concentration on the Recovery of Serum Components from Silicalite. Mobile phase: 20 mM phosphate buffer (pH=6.9), Flow rate: 1.0 ml/min., Column: 10 cm x 4.6 mm i.d., Detection at 236 nm, injection: 5 μl.

Percentage Acetonitrile in Eluent	Recovery (%)
0	89
1	90
4	90
5	91
10	100
15	100
20	100
25	81

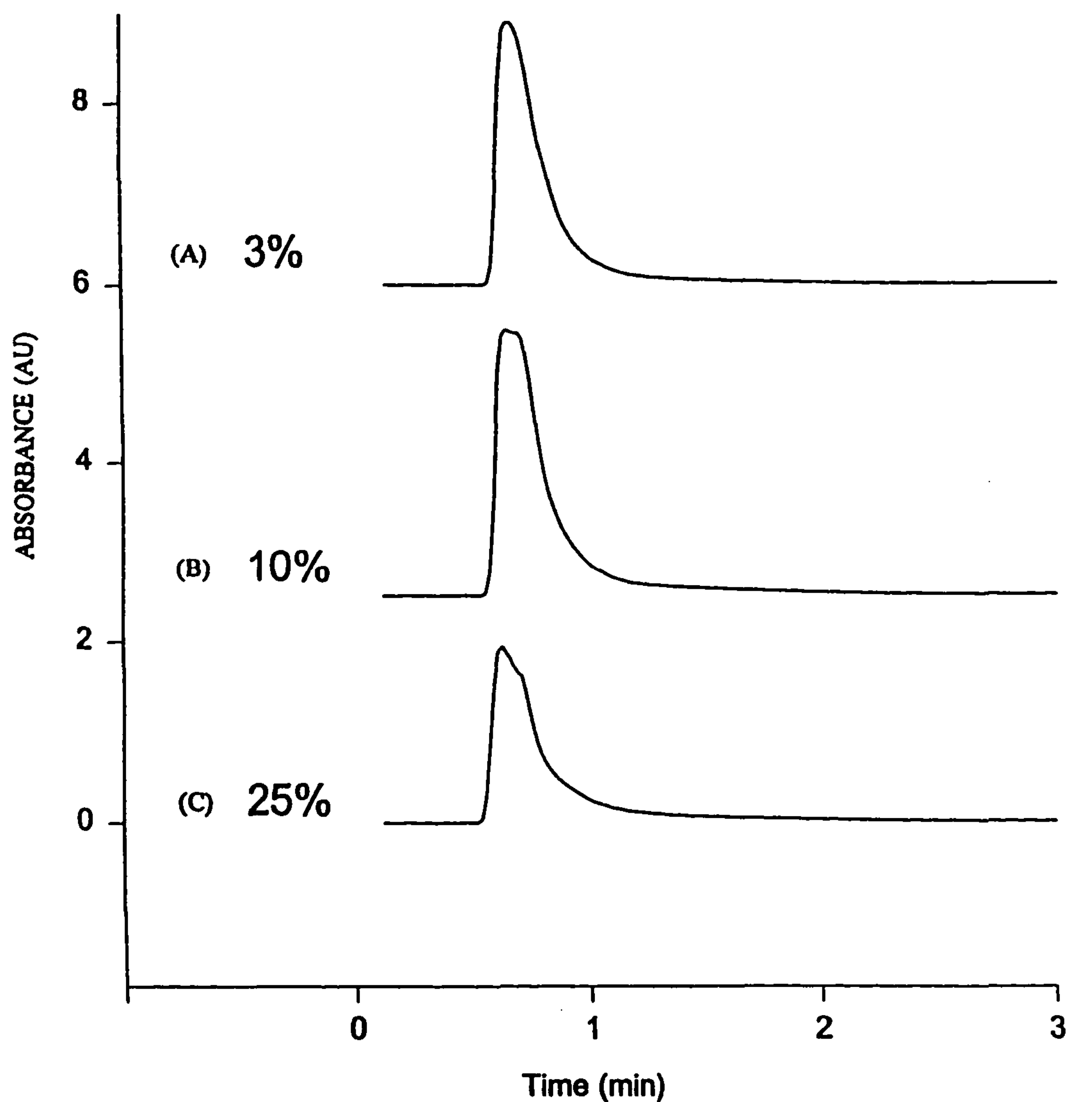

Figure 2. Effect of acetonitrile concentration on the elution of human serum. Conditions: column, Silicalite (5-10 μm) 10 cm x 4.6 mm i.d.; mobile phase, aqueous 20mM phosphate buffer, pH=6.9; (A) 3% (v/v) acetonitrile, (B) 10% (v/v) acetonitrile, (C) 25% (v/v) acetonitrile; flow rate, 1.0 ml/min.; detection 236 nm; injection volume, 5 μl.

Another concern when using direct serum injection is the mobile phase pH. Proteins are more prone to precipitate at their isoelectric pH, or the pH where the overall charge on the protein is neutral. The pI for serum albumin is 4.7 [2]. The effect of pH on the recovery of serum was investigated using a mobile phase pH range of 2.5 to 6.9, containing 5% and 20% acetonitrile. The percentage recoveries using serum under these conditions are in Table 2. As the pH of the mobile phase decreased, the recovery of serum decreased. This decrease may be due to defect hydroxyl groups or silanols in the matrix of Silicalite. At a pH lower than 4.7, serum albumin will have an overall positive charge, where the silanols will be ionized. As a result, there is an electrostatic attraction and proteins can adsorb onto Silicalite. Therefore, the mobile phase should contain less than 25% acetonitrile at a pH higher than 4.7 to avoid protein precipitation.

Table 2. Effect of pH on the Recovery of Serum Components from Silicalite
Mobile phase: 20 mM phosphate buffer/acetonitrile (95/5),
Flow rate: 1.0 ml/min., Column: 10 cm x 4.6 i.d.,
Detection 236 nm, Injection: 5 μl.

pH	Recovery (%)
6.9	91
5.5	93
5.0	91
4.7	92
4.0	89
3.5	87
3.0	87
2.5	88

Recovery of urine components from Silicalite

Unlike serum, urine contains little protein. The main constituents of urine are water, urea, uric acid creatinine, sodium, potassium, chloride, calcium, magnesium, phosphates, sulfates, and ammonia [29]. The influence of acetonitrile concentration and pH of the mobile phase on urine recovery from Silicalite was investigated using a pH of 2.5 and 4.5 and various percentages of acetonitrile. The percentage recoveries of urine from Silicalite are located in Table 3. Silicalite gave recoveries of over 90% for all conditions used in Table 3. These results show that the mobile phase may contain up to 70% acetonitrile, using a pH range of 2.5 to 4.5 without any denaturization or adsorption of endogenous urine components. Furthermore, this range can be extended to a pH of 7.8. The recovery of urine using a mobile phase pH higher than 8 was not assayed.

The influence of acetonitrile concentration in the mobile phase on the urine peak shape can be compared and contrasted in Figure 3. Using 0 to 5% (v/v), acetonitrile and a mobile phase pH of 2.5, the components of urine were resolved into 4 peaks, which were quite broad. These peaks represent uric acid, creatinine, and urea among other endogenous compounds. The peaks sharpen and elute as one when the acetonitrile concentration is increased to 15%. Increasing the acetonitrile concentration further to 25% (v/v) sharpens the urine peak further.

Recovery of drugs from serum

Most drugs in blood are bound to serum proteins to a different extent. For example, phenytoin, theophylline, and acetaminophen are 90%, 55%, and 30% bound, respectively [30]. There is an equilibrium between the bound and unbound drug. This equilibrium is

Table 3. Percentage Recovery of Urine from Silicalite
Mobile phase: 20 mM phosphate buffer, Flow rate:
1.0 ml/min., Column: 10 cm x 4.6 mm I.D. Silicalite column,
Detection: 236 nm, Injection: 5 μl.

Percentage Acetonitrile in Eluent	Recovery (%)	
	pH=2.5	pH=4.5
1	97	97
5	99	97
7	98	98
10	100	98
15	100	100
20	100	100
25	100	100
30	100	-
40	100	-
55	98	-
60	90	-
70	90	-

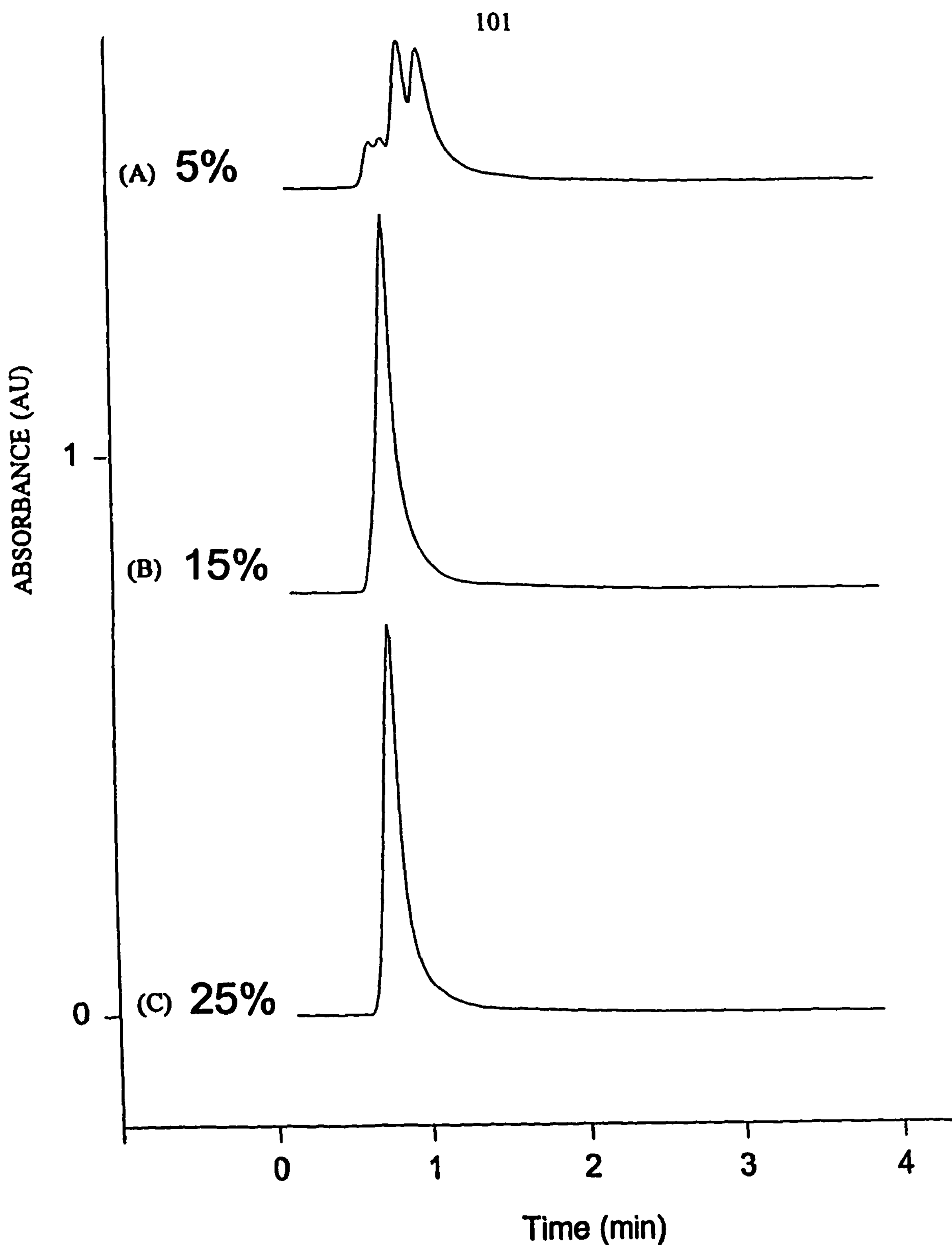

Figure 3. Effect of acetonitrile concentration on the elution of human urine. Conditions:column, Silicalite (5-10 μm) 10 cm x 4.6 mm i.d.; mobile phase, aqueous 20mM phosphate buffer, pH=4.5; (A) 5% (v/v) acetonitrile, (B) 15% (v/v) acetonitrile,(C) 25% (v/v) acetonitrile; flow rate, 1.0 ml/min.; detection 236 nm; injection volume, 5 μl.

disrupted with the addition of an organic solvent in the mobile phase. The organic solvent cleaves the protein binding and releases the drug [31]. The protein is partially denatured and the drug can be released without precipitation.

Each of the 15 drugs in Table 4 were quantitated individually in serum. The concentration of each drug was within its therapeutic range. A range of acetonitrile concentrations, 3 to 20% (v/v), were used in the mobile phase for the quantitation studies. In all determinations, the mobile phase consisted of 20 mM potassium phosphate buffer (pH=6.9) and was adjusted at a flow rate of 1.0 ml/min. The exact conditions used in each trial and percentage recoveries of each drug are located in Table 4. The average percentage recovery of the 15 drugs was 95% with a RSD of 3%. Almost complete recoveries with a low RSD indicate the total amounts of the 15 drugs can be determined under the mobile phase conditions employed regardless of the differences in their bindings to serum proteins.

Silicalite was expected to have a sieving effect based on the size and configuration of a molecule. Molecules small enough to enter the 6 Å diameter channels would be retained by interaction of the hydrophobic regions of the molecule with the hydrophobic interior of the Silicalite. Molecules that would not fit the channels would be extracted poorly or not at all. However, relatively large drugs, carbamazepine and phenytoin, were strongly extracted into Silicalite from serum. Studies by Choudhary and Akolekar [32] have shown that molecular configuration and flexibility (compressibility) need to be considered in addition to the critical molecular diameter. For planar molecules, penetration into the elliptical channels will be easier if the orientation of the analyte

Table 4. Percentage Recoveries of Various Drugs from Human Serum
Mobile phase:20 mM phosphate buffer (pH=6.9), Flow rate:1.0 ml/min., Column:10 cm x 4.6 mm i.d., Detection:254 nm, Inj.:5 μl.

Drug	ACN % (v/v) in mobile phase	Adjusted Ret. Time (Minutes)	Conc. of Drug (ppm)	Recovery (%)
CARDIAC				
Nifedipine	7	1.35	12	94
ANTIBACTERIAL				
Sulfapyridine	20	4.12	3	100
Sulfamethoxazole	10	7.42	2	95
Septra	10	5.63	8	92
ANOREXIC				
Deoxyephedrine	10	3.79	25	94
ANTI-INFLAMMATORY				
Acetaminophen	5	4.99	10	99
Ibuprofen	3	5.29	15	96
ANTICONVULSANT				
Ethosuximide	10	2.45	60	99
Theophylline	5	2.33	10	96
Primidone	10	2.16	10	95
Phenobarbital	13	3.58	5	91
Carbamazepine	20	15.22	7	90
Phenytoin	19	2.88	16	90
SEDATIVES				
Barbital	8	7.60	5	99
STIMULANTS				
Caffeine	11	2.99	20	92

molecule aligns itself with the axis of the elliptical opening. Some molecules are sufficiently flexible that their bonds can be bent in a direction opposite to that of penetration into the Silicalite channels.

Separation of seven drugs from human serum is shown in Figure 4b. Gradient elution was employed in which the concentration of acetonitrile in the mobile phase was varied from 5% to 50%. This was accomplished in the following steps: 5-20% in 2 minutes, 20-25% in 2 minutes, 25-30% in 4 minutes, 30-50% in 2 minutes, and 50% for 10 minutes. Incorporating 50% acetonitrile in the mobile phase did not promote protein denaturization nor adsorption because most of the serum proteins eluted off the column in the first minute. Instead, the last 2 steps in the gradient acted as a rinse, preventing build up of proteins and other macromolecules on the stainless steel frits and Silicalite. There is a slight increase in the background after 5 minutes. This may be due to the increase in acetonitrile concentration in the mobile phase removing excess endogenous compounds and protein from the stainless steel frits and/or Silicalite. The drugs are well resolved and elute in less than 16 minutes. Because the pores of Silicalite are very selective, this separation was not possible with isocratic elution, Figure 5. The later eluting peaks broadened badly and blended in with the background.

Recovery of drugs and metabolites from urine

Most circulating drugs undergo metabolism or biotransformation. As a result, a portion of an administered dose is excreted as one or more metabolite(s) of the drug. Drug metabolites are formed through a number of different pathways: oxidation, demethylation, and/or hydroxylation. Their concentrations vary human to human.

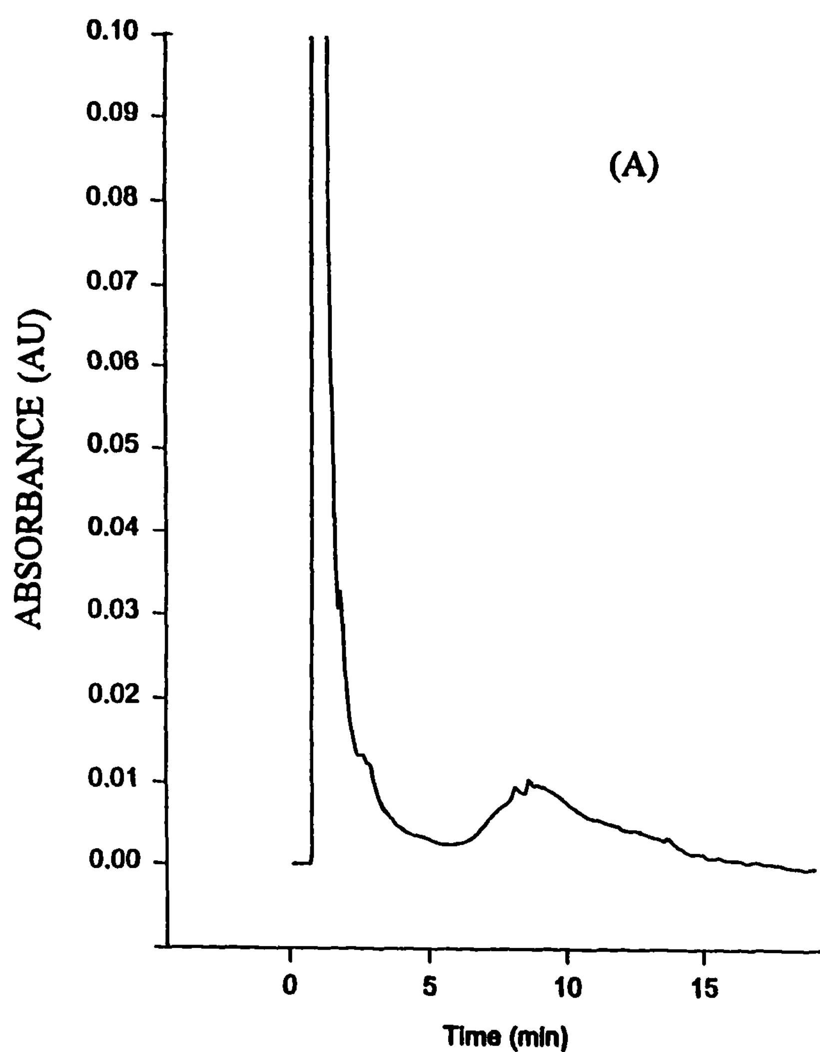

Figure 4a. Chromatogram of human serum blank. Conditions: column, Silicalite (5-10 μm) 10 cm x 4.6 mm i.d.; mobile phase, aqueous 20mM phosphate buffer (pH=6.9), gradient elution steps: 5-20% (v/v) acetonitrile in 2 min., 20-25% (v/v) acetonitrile in 2 min., 25-30% (v/v) acetonitrile in 4 minutes, 30-50% (v/v) in 2 minutes, and 50% (v/v) acetonitrile for 10 min.; flow rate, 1.0 ml/min.; detection 254 nm; injection volume, 20 μl.

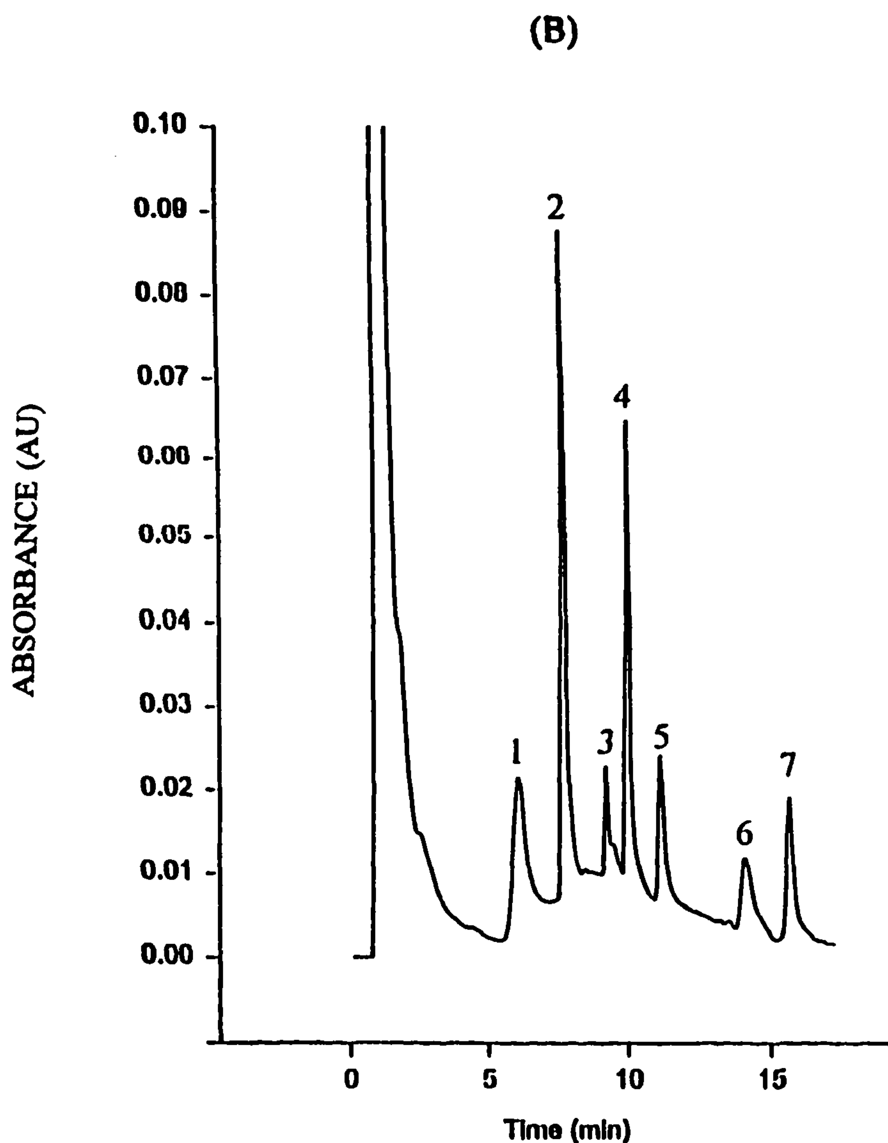

Figure 4b. Chromatogram of human serum spiked with 1. acetaminophen (2 ppm), 2. barbital (25 ppm), 3. primidone (15 ppm), 4. phenobarbital (20 ppm), 5. phenytoin (20 ppm), 6. sulfapyridine (2 ppm), 7. carbamazepine (2 ppm) (B). Conditions: column, Silicalite (5-10 μm) 10 cm x 4.6 mm i.d.; mobile phase, aqueous 20mM phosphate buffer (pH=6.9), gradient elution steps: 5-20% (v/v) acetonitrile in 2 min., 20-25% (v/v) acetonitrile in 2 min., 25-30% (v/v) acetonitrile in 4 minutes, 30-50% (v/v) in 2 minutes, and 50% (v/v) acetonitrile for 10 min.; flow rate, 1.0 ml/min.; detection 254 nm; injection volume, 20 μl.

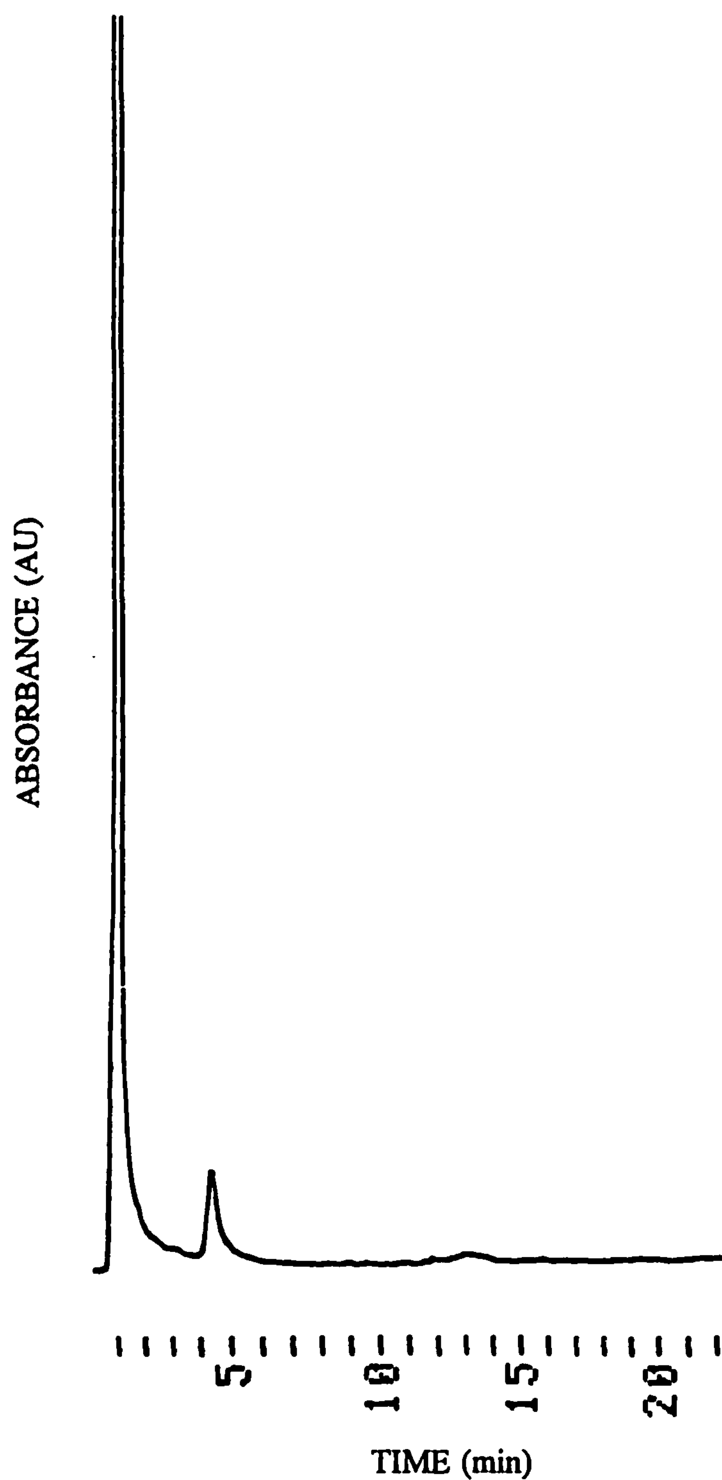

Figure 5. Chromatogram of human serum spiked with same mixture using isocratic elution, 20 mM phosphate buffer/acetonitrile (93/7). All other conditions same as in Figures 4a and 4b.

Each of 7 analytes (6 drugs and 1 nutrient) and some of their metabolites were quantitated individually from urine. The acetonitrile content of the mobile phase varied from 1 to 30% (v/v). In all determinations, the mobile phase consisted of 20 mM potassium phosphate buffer (pH=2.5) and was adjusted at a flow rate of 1.0 ml/min. The percentage recoveries of the analytes are located in Table 5. The average percentage recoveries of the parent drugs and metabolites were 97% and 95%, respectively with an average RSD of 3%.

The optimized separation of primidone, phenytoin, and carbamazepine and their metabolites in urine is located in Figure 6b. Often a mixture of these antiepileptic drugs is prescribed, including phenobarbital which is an active metabolite of primidone. Therefore, a selective and sensitive assay is required for the detection and quantification of both the parent drugs and their metabolites. The 6 analytes are well resolved and elute in less than 16 minutes. The limit of detection for the 3 drugs and their metabolites are located in Table 6.

Analysis of urine for caffeine metabolites

The adsorption of caffeine from the gastrointestinal tract is rapid but irregular [33,34] and its disposition in the human body is variable. Caffeine is extensively metabolized with only 2% of a dose excreted unchanged in the urine. The primary degradation of caffeine is N-demethylation and/or oxidation to theophylline (1,3-dimethylxanthine), paraxanthine (1,7-dimethylxanthine), theobromine (3,7-dimethylxanthine), and 1,3,7-trimethyluric acid. These compounds can degrade further to dimethylated uric acids, monomethylxanthines and monomethyluric acid.

Table 5. Percentage Recoveries of Various Metabolites from Human Urine
Mobile phase: 20 mM phosphate buffer (pH=2.5), Flow rate: 1.0 ml/min. Column: 10 cm x 4.6 mm i.d., Detection: 262 nm, Inj.: 5 μl.

Drug and Metabolite(s)	Conc. of Drug or Metabolite (ppm)	Recovery from Human Urine (%)
Caffeine	10	95
1-Methyluric Acid	8	81
1-Methylxanthine	8	97
1,7-Dimethylxanthine	7	100
7-Methylxanthine	6	95
1,3-Dimethylxanthine	5	97
3-Methylxanthine	6	100
3,7-Dimethylxanthine	2	100
1,3-Dimethyluric Acid	5	96
Phenytoin	3	91
5-(p-Hydroxyphenyl)-5-Phenylhydantoin	1	99
Primidone	4	95
Phenobarbital	4	98
Acetylsalicylic Acid	4	95
Salicylic Acid	8	85
O-Hydroxyhippuric Acid	9	90
Acetaminophen	3	94
p-Acetamidophenyl ß-D-Glucuronide	5	95
Phenylalanine	5	100
Phenylpyruvic Acid	8	87
Carbamazepine	0.5	100
Carbamazepine-10,11-epoxide	10	96

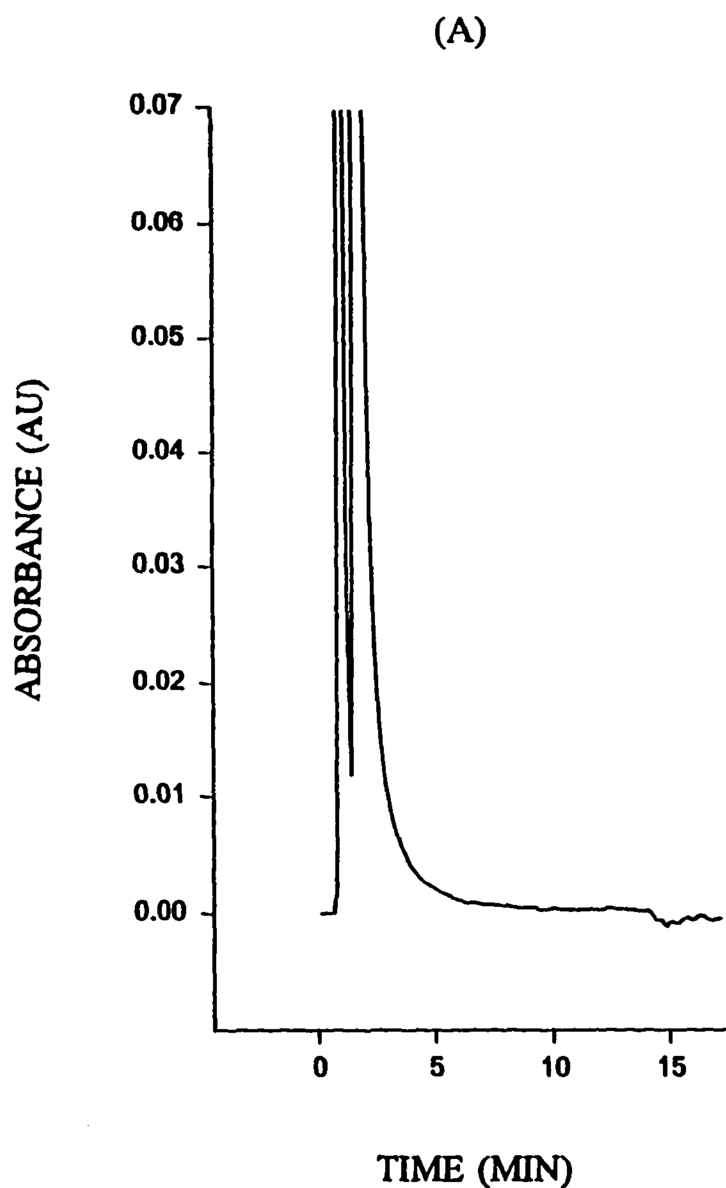

Figure 6a. Chromatogram of human urine blank. Conditions: column, Silicalite (5-10 μm) 10 cm x 4.6 mm i.d.; mobile phase, aqueous 20mM phosphate buffer (pH=6.9), gradient elution: 14% (v/v) acetonitrile for 5 min., 14-25% (v/v) acetonitrile in 1 min., 25-30% (v/v) acetonitrile in 2 min., 30-50% (v/v) acetonitrile in 3 min., and 50% (v/v) acetonitrile for 6 min.; flow rate, 1.0 ml/min.; detection 230 nm; injection volume, 5 μl.

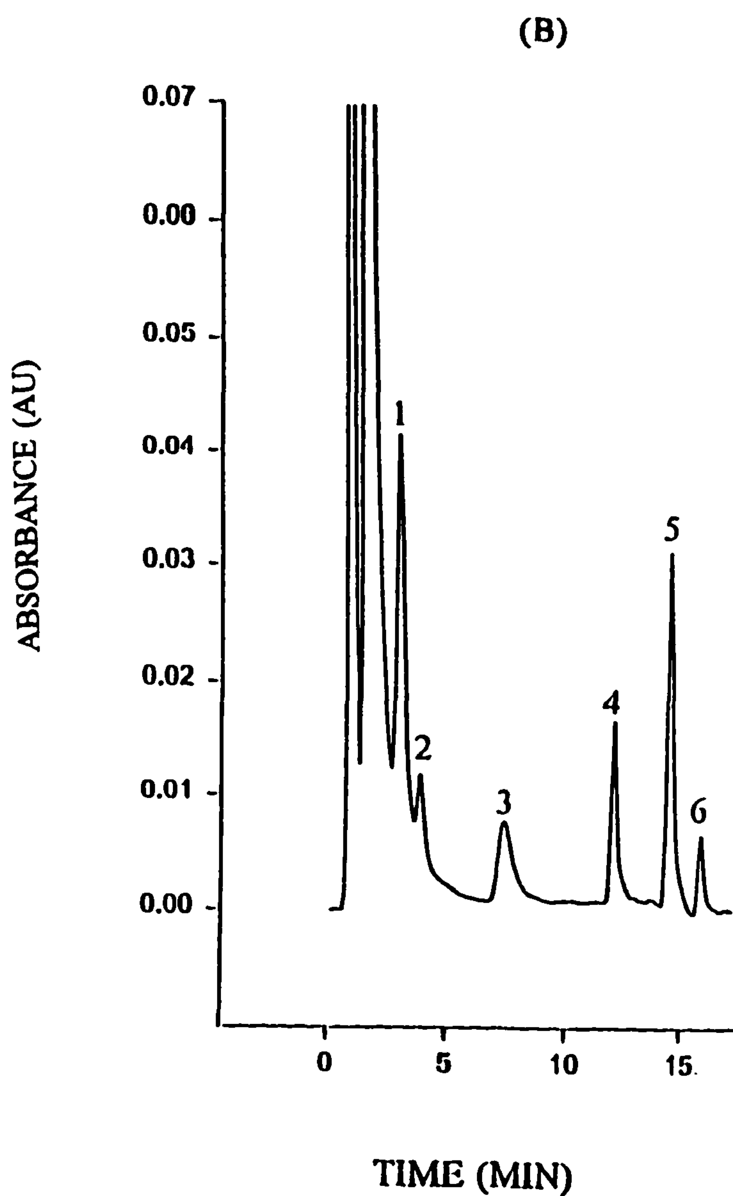

Figure 6b. Chromatogram of human urine spiked with 1. 5-(p-hydroxyphenyl)-5-phenylhydantoin (4 ppm), 2. primidone (8 ppm), 3. phenobarbital (12 ppm), 4. phenytoin (12 ppm), 5. carbamazepine-10,11-epoxide (10 ppm), and 6. carbamazepine (2 ppm) (B). Conditions: same as in Figure 6a.

Table 6. Absolute limits of detection in urine
Conditions same as in Figures 6a and 6b.

Drug and Metabolite	Absolute limit of detection (ng) in urine
Phenytoin	2
5-(p-Hydroxyphenyl)-5-Phenylhydantoin	1
Primidone	2
Phenobarbital	3
Carbamazepine	1
Carbamazepine-10,11-epoxide	5

A sensitive and specific assay is necessary to separate and quantitate these structurally related metabolites. Dumont and Fritz [35] have shown that Silicalite can separate positional isomers due to the selective channels imparting a size exclusion effect. A mixture of trimethylphenol isomers were well separated with the largest isomer, 2,4,6,-trimethylphenol, eluting first. Therefore, Silicalite was employed to separate and quantitate the major metabolites found in urine after consumption of a caffeinated cola.

After caffeine consumption (180 mg), urine samples were collected and pooled. The presence of methylxanthines and methyluric acids was determined using direct injection and isocratic elution. The resulting chromatogram, Figure 7c, shows the presence of 6 caffeine metabolites in urine. Peak identification was achieved by matching retention times in urine with the retention times of various metabolites in an aqueous standard. Caffeine, retention time of 29.7 min., was present in the urine at a concentration of 2 ppm. Using working curves, the amount of each metabolite was determined. The most abundant caffeine metabolite in urine was 1-methyluric acid, 50 ppm, Table 7. The peak

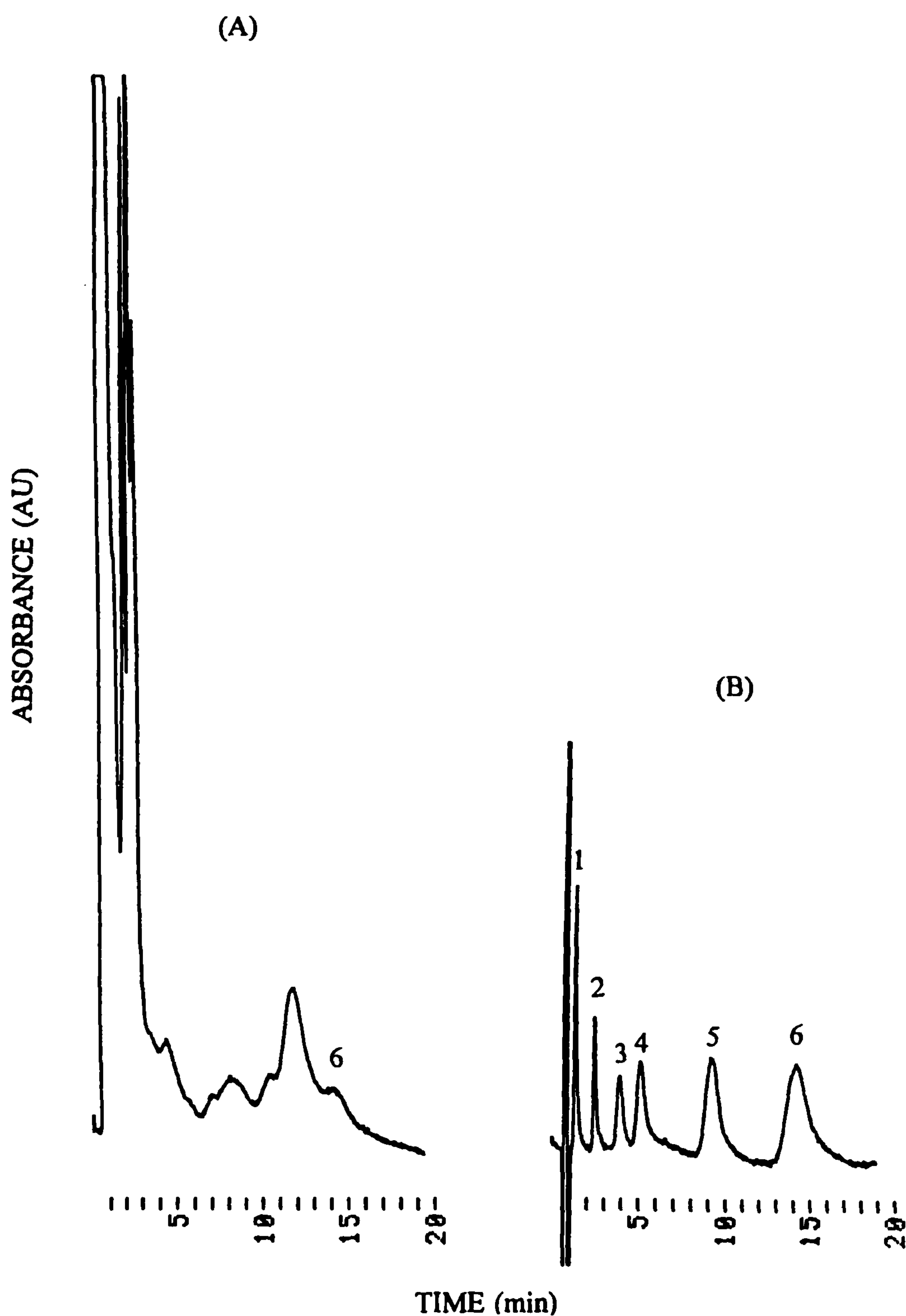

Figure 7a and 7b. Chromatogram of human urine blank (A), chromatogram of a standard mixture of 6 caffeine metabolites: 1. 1-methyluric acid, 2. 1,3-dimethyluric acid, 3. 3-methylxanthine, 4. 1-methylxanthine, 5. 3,7-dimethylxanthine, 6. 1,7-dimethylxanthine (B), Conditions: column, Silicalite (5-10 μm) 10 cm x 4.6 mm i.d.; mobile phase, 20 mM phosphate buffer/acetonitrile (95/5) pH=2.5; flow rate, 1.0 ml/min.; detection 270 nm; injection volume, 5 μl.

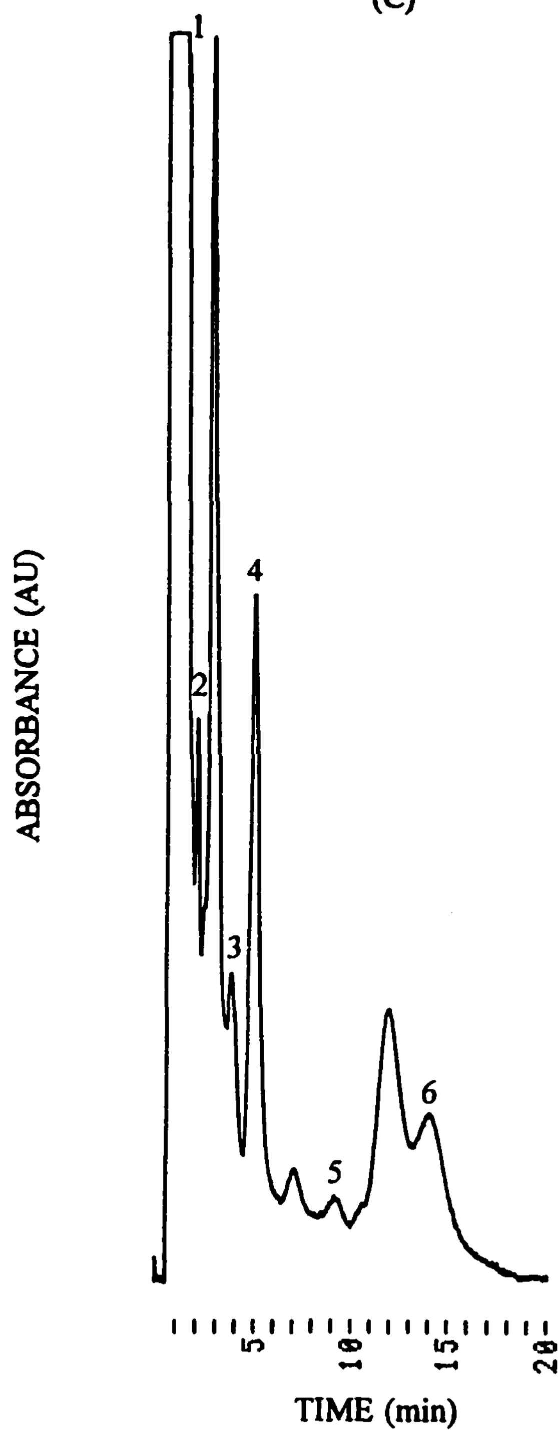

Figure 7c. Chromatogram of human urine after consumption of caffeinated cola. Conditions same as in Figures 7a and 7b.

Table 7. Quantitation of Major Caffeine Metabolites in Urine. Conditions same as in Figures 7a, 7b, and 7c.

Caffeine Metabolite	Amount of Metabolite Found in Urine (ppm)
1-Methyluric Acid	50
1,3-Dimethyluric Acid	9
3-Methylxanthine	1
1-Methylxanthine	6
3,7-Dimethylxanthine	1
1,7-Dimethylxanthine	4

between 3,7-dimethylxanthine and 1,7-dimethylxanthine is hippuric acid. Complete baseline resolution of 1,7-dimethylxanthine and hippuric acid was difficult. At a higher pH, 4.5, hippuric acid (pKa=3.8) eluted with the other endogenous components and baseline resolution of 1,7-dimethylxanthine was possible. However, the retention times of the acidic metabolites became too short and eluted very close to the urine peak.

Durability and stability of Silicalite

The durability of the Silicalite column was not exhaustively tested. The same column was used in analyzing both urine and serum. The back pressure remained constant and urine recovery from Silicalite did not decrease after at least 200 (5 μl) injections of urine using a pH range of 2.5 to 7.8 and an acetonitrile concentration range of 3 to 70% (v/v). However, the back pressure increased slightly when serum was injected using particular experimental conditions. The stainless steel frit was more prone to clogging when using a mobile phase pH of 5 or lower. After approximately 100 injections the front frit was

replaced, decreasing the pressure. Prolonged use of a mobile phase (pH=4 and lower) containing 20% (v/v) acetonitrile resulted in protein precipitation and accumulation at the head of the column. When this yellowish film was removed and the frit replaced, the back pressure decreased and the column resumed normal function and behavior.

Conclusions

Silicalite is an excellent stationary phase for direct injection of biological fluids. Macromolecules were excluded from the column and eluted with the void volume. When analyzing serum, the mobile phase should contain less than 25% acetonitrile at a pH of 4.7 or higher to prevent protein adsorption. The mobile phase may contain up to 55% acetonitrile at a pH range of 2.5 to 7.8 when analyzing urine without any degradation of the column. The direct injection technique is highly selective and sensitive at extracting various drugs and metabolites from human serum and urine. The stability and durability of the column depended on the mobile phase conditions employed. If an increase in back pressure occurred, replacing the stainless steel frit returned the pressure to its original level.

Acknowledgments

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GENERAL CONCLUSION

Silicalite fills an important gap in solid-phase extraction. Traditional SPE sorbents, including polystyrene and bonded-phase silica adsorbents, are successful at extracting many types of organic compounds. However, these resins are unable to extract low molecular weight, polar organic compounds efficiently. Because of its unique structure, Silicalite adsorbs organic analytes through hydrophobic interactions and possesses size exclusion properties. Small hydrophilic compounds, such as the lower alcohols, aldehydes, esters and ketones are well extracted by Silicalite, adding a valuable new capability to conventional SPE. Recoveries of various hydrophobic and hydrophilic compounds averaged 90% or higher, using Silicalite. Positional isomers were extracted in varying degrees due to steric hindrance when entering the selective pores of Silicalite. Load capacities were determined through the use of breakthrough curves. Silicalite adsorbed nearly 12 %, by weight, of ethyl acetate, ethyl propionate, ethyl butyrate.

A new COOH functionalized resin, prepared by ring-opening-metathesis polymerization (ROMP), possesses characteristics of both the sulfonated polystyrene resins and Silicalite. Hydrophobic analytes were extracted through interactions with the cross-linked interior as well as the unsaturated backbone bearing the carboxyl groups. More hydrophilic analytes were retained by the carboxyl groups located on the exterior of the particles. The carboxyl groups provide good water wetting of the surface of the resin and eliminate the need for pretreatment with an organic solvent prior to SPE. In addition, the carboxyl groups also enable analytes to be taken up by an ion-exchange mechanism. A comparison of the extraction behavior for different types of analytes between Silicalite, a

lightly sulfonated PS-DVB resins, and the new COOH resin was discussed.

All three types of resins were incorporated into an Empore®-type membrane. The membranes were strong, porous and simple to use. They also provided a more efficient extraction due to the elimination of channeling and require less eluting solvent.

The advantages of membrane technology were incorporated to create semi-micro solid-phase extraction (SM-SPE). SM-SPE involving placing thin membrane disks 4 mm in diameter, containing lightly sulfonated polystyrene or Silicalite particles, into the hub of a syringe needle. Aqueous samples can be passed through the membrane disks using a single or double pass technique. Extracted compounds were eluted with 20-50 μl of an organic solvent. SM-SPE was applied to analyze various biological samples without any degradation to the sorbents or reduction in extraction recoveries of various organic compounds. The semi-micro technique is a total extraction technique, reducing the sample size and volume of eluting solvent and, therefore, the amount of waste produced.

Silicalite has been shown to be a viable HPLC stationary phase for the direct injection of biological fluids. Because the external surface of Silicalite is relatively hydrophobic and the pore diameter is 6 Å, macromolecules contained in biological fluids cannot enter. At the same time, the pores of Silicalite are hydrophobic and successfully adsorb drugs and metabolites from the biological fluids. Protein precipitation was avoided by employing a mobile phase containing less than 25% acetonitrile at a pH of higher than 4.7 when analyzing serum. When analyzing urine, the acetonitrile content in the mobile phase can be increased to 55% and a pH range of 2.5 to 8 can be used. When these conditions are employed, hundreds of 5 μl aliquots of serum and urine can be injected

onto a Silicalite column without a noticeable change in back pressure, retention times, and column efficiency.