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Novel materials and methods for solid-phase extraction and liquid chromatography

by

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DISCLAIMER

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 widespread. 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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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.

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