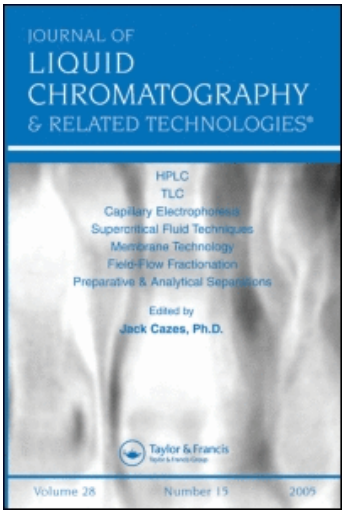

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Louis J. Glunz[a]; John A. Perry[a]; Benedict Invergo[a]; Hans Wagner[ab]; Ted J. Szczerba[a]; John D. Rateike[a]; Peter W. Glunz[a]

[a] Regis Chemical Company, Austin Avenue, Illinois [b] Hercules Research Center, Wilmington, DE

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THE SEMIPERMEABLE SURFACE, A NEW RESTRICTED ACCESS MEDIUM

LOUIS J. GLUNZ, JOHN A. PERRY*, BENEDICT INVERGO, HANS WAGNER, TED J. SZCZERBA, JOHN D. RATEIKE, AND PETER W. GLUNZ**

Regis Chemical Company
8210 Austin Avenue
Morton Grove, Illinois 60053

ABSTRACT

The Internal Surface Reversed Phase (ISRP)--an elegant invention of Dr. Thomas C. Pinkerton--was the first example of a Restricted Access Medium (RAM). These materials allow the determination of small molecules in the presence of large biomolecules, for instance the direct injection of serum into an HPLC column without requiring prior removal of protein. A new RAM is the SemiPermeable Surface (SPS), based on the doctoral research of Carla Desilets under Dr. Fred Regnier at Purdue University, developed to its present form at the Regis Chemical Company. The SPS has two phases: an outer and an inner, each independently synthesized, each covalently bound to the silica surface of the packing particle. The outer is a hydrophilic polyoxyethylene polymer; the inner is any of several common hydrophobic reversed phases (at present, C8, C18, nitrile, phenyl). The outer phase forms a semipermeable surface that prevents large biopolymers from reaching the inner phase. Small molecules, however, can and do interact with either the outer or the inner phase. They are retained by a unique mechanism--a combination of hydrogen bonding at the outer polyoxyethylene surface and hydrophobic interaction at the inner reversed phase. Aside from its experimentally variable selectivity--a completely new property--SPS also shows excellent chromatographic efficiency and roughly twice the retentivity of comparable RAM materials.

Present address, * *Postboks 154, 1900 Fetsund, Norway;*
** *Hercules Research Center, Wilmington, DE 19894.*

INTRODUCTION

Serum is commonly analyzed for both drugs--including substances that may become drugs and drugs that have been submitted for certification--and the metabolites of these drugs. Analysis of serum for drugs is required when the concentration levels of drugs must be either detected or monitored in either humans or animals. In a larger sense, fluids must be analyzed for smaller molecules--toxins, for instance (1)--in the presence of larger.

Unfortunately, reversed-phase HPLC can't be used in its ordinary form for direct serum injections; proteins precipitate and ruin the column. Early attempts to circumvent this difficulty were cumbersome, slow, and expensive (for a review, see, for instance, ref. 2).

The Internal Surface Reversed Phase (ISRP), invented by Thomas C. Pinkerton (3), allows the direct automatable analysis of serum and body fluids without requiring the prior removal of protein (see the original paper, 4; and reviews, for instance refs. 5-6). For the first ISRP, Pinkerton bonded glycine - phenylalanine - phenylalanine (GFF) to 80A-pore spherical silica. In use, the hydrophilic, non-denaturing glycine covers the outside surfaces of the packing, the only surfaces that serum proteins can reach; the selective and hydrophobic GFF covers the inner.

The GFF packing was typically afforded a minimum of 800 injections, usually provided adequate selectivity (comparing favorably with the popular octadecylsilyl (6-8)), and additionally could be used to separate peptides (9). Recently, a new version of it has increased its chromatographic efficiency to over 60,000 plates per meter; its

retentivity to more than double that of the original CFF, as measured by the retention of carbamazepine (10); and its reproducibility of production (6).

The ISRP set a benchmark for performance of Restricted Access Media (RAM)(a term originated by Fred Regnier (11)), of which it is an example. RAM packings permit chromatography of small molecules in the presence of large biomolecules such as protein. By one means or another, RAM packings prevent access of large biomolecules to some inner surface.

Recently, RAMs have appeared that function similarly to the ISRPs. These include the Shielded Hydrophobic Phase (SHP)(12) and the SemiPermeable Surface (SPS).

The SHP consists of a hydrophilic, polymeric, water-solvated phase, covalently bonded to silica. Small analytes such as drugs can reach hydrophobic regions embedded in the SHP (12), but proteins cannot. An acceptable column life of over 1,000 injections has been reported (12). The concept of the SemiPermeable Surface stems from work of Carla Desilets. In the laboratory of Dr. Fred Regnier at Purdue University, Desilets showed that oxyethylene-based nonionic surfactants are readily adsorbed in monolayers on conventional reversed phase packings (11,13). Such a packing displays RAM function. Given the injection of serum, the layer of hydrophilic polymer prevents serum proteins from making contact with hydrophobic sites.

In Figure 1 is shown a chromatogram that resulted from a direct injection of human serum onto a C8 column that Desilets had coated with Brij-700 surfactant (Code: P-100-AE-18). In the chromatogram, diuretics are seen separated from the proteins and each other.

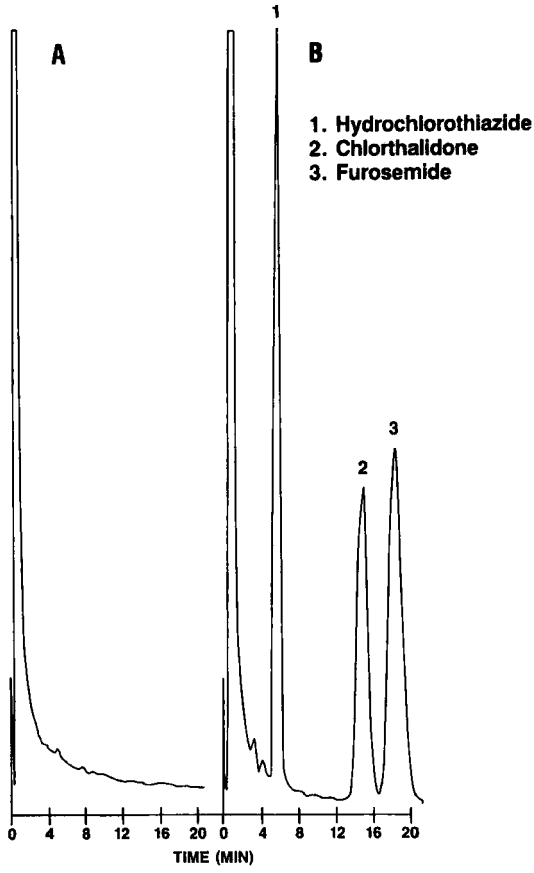

Figure 1. Carla Desilets showed that an oxyethylene-surfactant-coated conventional C8 stationary phase functions as a Restricted Access Medium. Chromatogram A shows immediate excellent protein recovery from an injection of human serum; B, separation of diuretics from serum proteins and each other. Diuretics: 1. hydrochlorothiazide (50 ug/mL); 2. chlorthalidone (100 ug/mL); 3. furosemide (100 ug/mL). Column dimensions: 5 cm x 4.6. mm i.d. Mobile phase: 97/3 0.05M phosphate buffer, pH 6.5/isopropanol. Flow rate: 1.0 mL/min. Sample volume: 25 uL. Detection: 254 nm, 0.04 a.u.f.s.

Protein recoveries from that material were excellent: 97 $\pm$ 3% as determined by the Bradford method. Similar results were obtained using several other detergents on C8 and C18 packings. Although there was some loss of chromatographic efficiency with these coated columns, the major disadvantage was the leaching of the detergent layer from the column.

Proceeding from this work, methods were developed at Regis Chemical for bonding hydrophilic polymers to the silica surface. With this bonding, the polymer forms an outer semipermeable surface, at the same time leaving an inner layer for the chromatography of smaller molecules.

EXPERIMENTAL

Materials

Mobile phases (pH 6.8) were prepared with HPLC-grade water, KH_2PO_4 salt used at 0.1 M concentration, and acetonitrile (EM Science, Cherry Hill, NJ, U.S.A.). Carbamazepine and phenobarbital were purchased from Sigma (St. Louis, MO, U.S.A.).

Chromatographic experiments were carried out with standard commercial equipment. The Kratos (Ramsey, NJ, U.S.A.) system used included a Model 783G detector (flow-cell, 12 ul internal volume) set at 254 nm and a Kratos Spectroflow 400 pump set to produce 0.6 mL/min. The internal diameter of the capillary tubing throughout the system was 0.01 in. Samples, all 10 ul in volume, were injected with a Model 7010 injection valve (Rheodyne, Calabasa, CA, U.S.A.).

SPS packings and columns were manufactured at Regis (the synthesis of SPS packings is being reported elsewhere (14)). The SHP column was purchased from Supelco (Bellefonte, PA, U. S.A.).

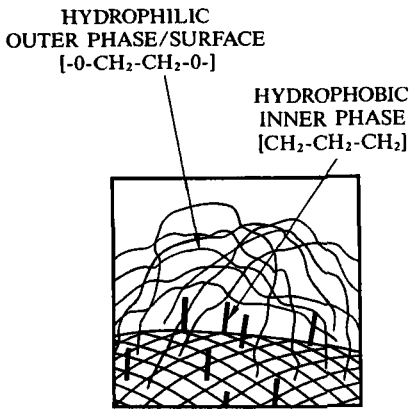

Figure 2. SemiPermeable Surface (SPS) media consist of particles that bear two phases: an outer semipermeable surface that is also a hydrophilic phase, and an inner hydrophobic phase. The outer semipermeable surface prevents large molecules such as proteins from reaching the inner phase. Small molecules interact with the outer phase/surface, particularly if they are hydrophilic; small molecules also can and do penetrate the outer surface, reach the inner phase, and interact with it, particularly if they are hydrophobic. The chemical nature of both the outer SPS phase/surface and the SPS inner phase can be varied independently.

RESULTS AND DISCUSSION

The Semipermeable Surface

As suggested in Figure 2, SemiPermeable Surface particles have two phases, an outer and an inner. The outer phase is a polyoxyethylene polymer that is covalently bonded to the surface of the silica particle and that forms a hydrophilic outer phase/surface. The inner phase consists of one of the common reversed phases that are used in chromatography.

The outer semipermeable surface prevents large molecules such as proteins, or biopolymers, or other normally globular polymeric material, from reaching the inner

phase. Small molecules, however, can and do penetrate the semipermeable surface and interact with the inner phase.

A major difference of SPS from other restricted access packings is its inherent versatility. With the SPS, the outer and inner phases are synthesized independently, and thus can be controlled independently (14). The density of the outer phase/surface can be varied to change the penetration threshold; the inner phase can be varied from that of the particle surface itself to any of the reversed phases that are commonly used on chromatography. Thus, for a given separation with SPS media, either inner or outer phase can be optimized independently.

Another distinguishing characteristic of SPS is its unique retention mechanism. As Desilets and Regnier first suggested (11, 13), small molecules appear to be retained by a combination of hydrogen bonding at the polyoxyethylene phase/surface and hydrophobic interaction at the inner reversed phase. Depending on its hydrophobicity and hydrogen-bonding capability, a small molecule can penetrate into and partition with different regions of the hydrophilic layer and the reversed phase below.

In Table 1 are presented the retention times found with SPS C1, C4, C8, and C18 inner surfaces for some hydrophobic solutes--a group of aromatic hydrocarbons. As might be expected, retentions increase from C1 through C18. However, hydrophilic solutes tend neither to penetrate deeply into the reversed phase nor therefore to distinguish between the successively longer paraffinic chains of the C1, C4, C8, and C18 reversed phases. The retentions shown in Table 2 differ among the hydrophilic solutes; but for a given hydrophilic solute, the retentions do not differ from one SPS alkyl reversed phase to the next. As mentioned, both patterns agree with the earlier comments of Desilets and Regnier concerning the overall mechanism (11,13). In Table

TABLE 1
Aromatic Hydrocarbons on 15 cm SPS Columns

	Retention Times (min.)*			
	C1	C4	C8	C18
Benzene	2.51	2.60	2.93	3.24
Naphthalene	3.56	3.94	4.76	6.32
Phenanthrene	5.56	6.71	8.85	14.38
Chrysene	9.45	12.24	17.98	37.76
	Capacity Factors*			
Benzene	0.6	0.7	1.0	1.3
Naphthalene	1.2	1.6	2.2	3.5
Phenanthrene	2.5	3.5	4.9	9.3
Chrysene	4.9	7.2	11.0	26.0

* Mobile phase, at 1.0 mL/min: 75/25 methanol/water. Void time taken as time from injection to injection-caused deflection from base line.

TABLE 2
Selected Compounds on 15 cm SPS Columns

	Retention Times (min.)			
	C1	C4	C8	C18
Tartaric acid	2.40	2.50	2.29	1.93
Citric acid	3.19	3.61	3.83	2.73
Barbital	2.11	2.08	2.03	1.89
Secobarbital	3.66	4.33	4.56	4.65
Methyl Paraben	2.57	2.65	2.67	2.83
Propyl Paraben	3.18	3.56	3.91	4.51

TABLE 3

Comparing Retention Times (min) of Selected Drugs

Conventional	-------SPS 5PM-------					
	C8	C18	C8	C18	CN	PHENYL
Toluic acid	2.6	3.0	2.2	1.8	2.3	2.2
Caffeine	2.6	2.4	1.8	1.6	1.8	1.8
Trimethoprin	4.9	4.6	3.9	3.2	4.2	3.9
Phenobarbital	11.7	10.9	10.0	9.9	10.1	9.8
Sulfinpyrazone	26.0	27.7	15.1	13.4	15.4	15.2
Carbamazepine	36.7	41.8	15.1	13.6	15.2	14.9
Methyl salicylate	62.6	92.9	29.4	32.6	20.5	20.9

3, the retention times over conventional C8 and C18 columns and over the 4 SPS phases--C8, C18, CN, and phenyl--are compared for 7 hydrophilic drugs. In general, the conventional alkane phases retain the drugs longer than the SPS phases. Notice too that the SPS C18 does not retain either sulfinpyrazone or carbamazepine longer than the SPS C8. Table 4 shows SPS column reproducibility and typical efficiency.

A characteristic of SPS columns that follows from this retention mechanism is the excellent peak shapes obtained for basic drugs. In Figure 3, for instance, see the excellent peak shape obtained for the basic drug sulfapyridine on the SPS-5PM-C8 SPS column.

Already synthesized and tested are C1, C4, C8, C18, CN, and phenyl inner phases. In addition, SPS silica packings have been made with the current hydrophilic polymer for the outer phase/surface but for the inner surface, only the bare support. This opens to SPS applications the possible uses of silica for normal-phase adsorption chromatography for smaller molecules in the presence of globular biopolymers such as proteins.

TABLE 4

Lot-to-lot Reproducibility, SPS C8

	Retentions, minutes			
	--------Lots---------			
	A	B	C	D
Toluic acid	2.2	2.1	2.2	2.2
Caffeine	1.8	1.9	1.8	1.7
Trimethoprin	3.9	5.0	3.9	3.8
Phenobarbital	10.0	10.4	10.8	10.6
Sulfinpyrazone	15.1	14.2	16.3	15.7
Carbamazepine	15.1	18.3	16.6	15.9
Methyl salicylate	29.4	33.2	35.7	36.5
	Plates, 10^3/meter			
	61.1	67.8	66.6	61.3

Column dimensions: 15 cm x 4.6 mm i.d.; mobile phase flow rate: 1.0 mL/min; mobile phase composition: 80/20 0.1 M KH_2PO_4, pH 6.8/CH_3CN; detection, 254 nm; sample volume: 10 microliters.
Column efficiency measured separately on dibutyl phthalate peak, using a 10 microliter volume of a solution containing 75 mg dibutyl phthalate per 100 mL of mobile phase; the mobile phase was methanol/water 70/30, used at 0.5 mL/min.; the dibutyl phthalate capacity factor was 3.7.

Direct-Injection Serum Analysis

A sample of human serum that contained phenobarbital and carbamazepine was injected into a 15-cm SPS C8 column. The packing pore diameter was 100 A; the particle diameter, 5 microns. The chromatogram is shown in Figure 4. In that chromatogram, the efficiency measured on the carbamazepine peak was over 60,000 plates per meter--about equal to that of the improved GFF (6). If not outstanding, such

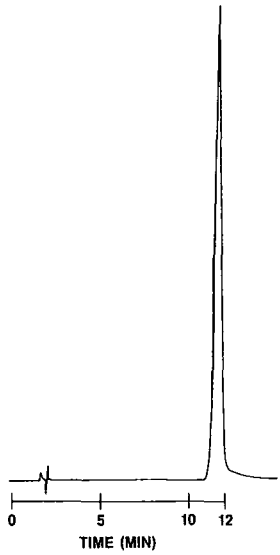

Figure 3. The hydrophilic SPS outer phase is well suited for the chromatography of basic drugs, as demonstrated here by a chromatogram of sulfapyridine. Column: 15 cm x 4.6 mm I.D. SPS-5PM-S5-100-C8. Mobile phase composition: 95/5 0.1 M KH_2PO_4 buffer, pH 6.8/acetonitrile; and flow rate: 1.0 mL/min.

efficiency is nevertheless acceptable (a Regis Rexchrom 15-cm C18 column provides 85,000 plates per meter). Note also the carbamazepine retention: approximately 27 minutes (in comparison with 12.5 min with newly revised version of GFF, and 15 min with the SHP). Over 400 serum injections were made on this column without any buildup in backpressure or change in performance.

Figures 5 and 6 show other chromatograms of drugs in human serum, both sets at therapeutic levels. In Figure 5, theophylline is shown well separated from serum proteins, as well as from caffeine and acetomenophen. In Figure 6, 5 barbiturates are

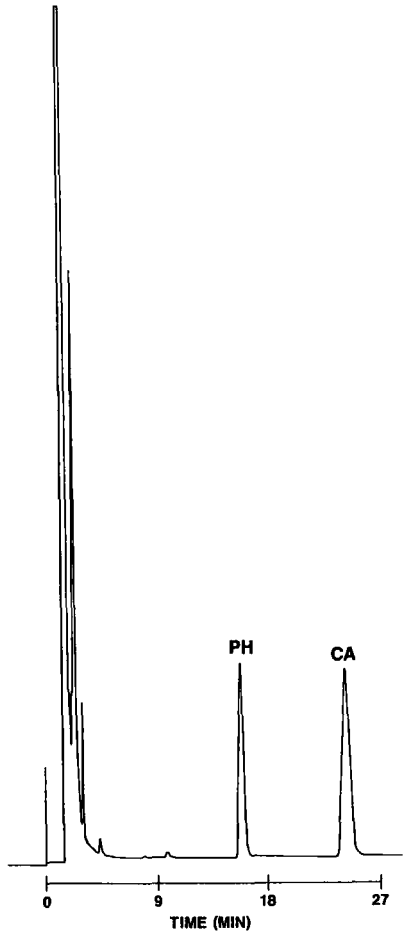

Figure 4. In this chromatogram, phenobarbital and carbamazepine are shown to have been separated from each other and from serum proteins by injection onto an SPS C8 column. Chromatographic efficiency of the carbamazepine peak: over 60,000 plates per meter. The SPS retention of the carbamazepine is also roughly 50% greater than any other RAM materials available at this time, at least twice that of the ISRP packings. Column dimensions: 15 cm x 4.6 mm I.D. Packing: 100 A pore diameter, 5 micron particle diameter. Mobile phase: 80/20 phosphate buffer, pH 6.8/acetonitrile; at 0.6 mL/min. Sample volume: 10 uL. Concentrations in ug/uL: phenobarbital, 160; carbamazepine, 60.

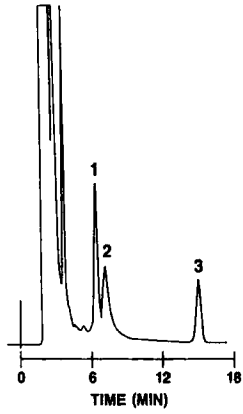

Figure 5. The 15 cm SPS C8 separates theophylline (1) from serum very cleanly, and could be used to separate it from caffeine (2) and acetomenophine (3) as well. Column: 15 cm x 4.6 mm I.D. SPS-5PM-S5-100-C8. Mobile phase: 0.01 M KH_2PO_4 buffer, pH 7.0, at 0.5 mL/min. Sample volume: 10 uL. Concentrations in ug/mL: theophylline, 15; caffeine, 15; acetomenophine, 10. Detection: 254 nm.

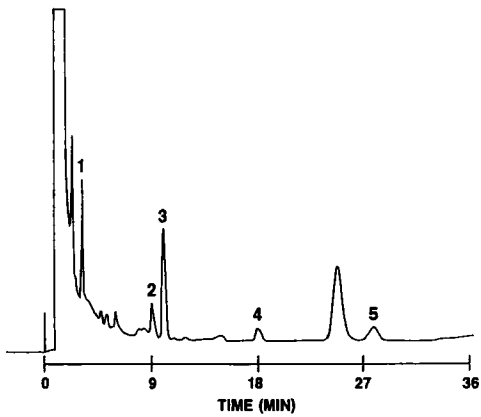

Figure 6. On injection into an SPS C8 column, barbiturates at therapeuitic levels in serum are resolved from each other and from serum proteins. Column: 15 cm x 4.6 mm I.D. SPS-5PM-S5-100-C8. Mobile phase: 0.1 M KH_2PO_4 buffer, pH 6.8, at 1.0 mL/min. Sample volume: 10 uL. Concentrations: 15 ug/mL. Detection: 254 nm. Barbital, 1; Butabarbital, 2; Penobarbital, 3; Amobarbital, 4; Secobarbital, 5. The substance causing peak "X" comes from the particular lot of serum used, is not present in all lots of serum.

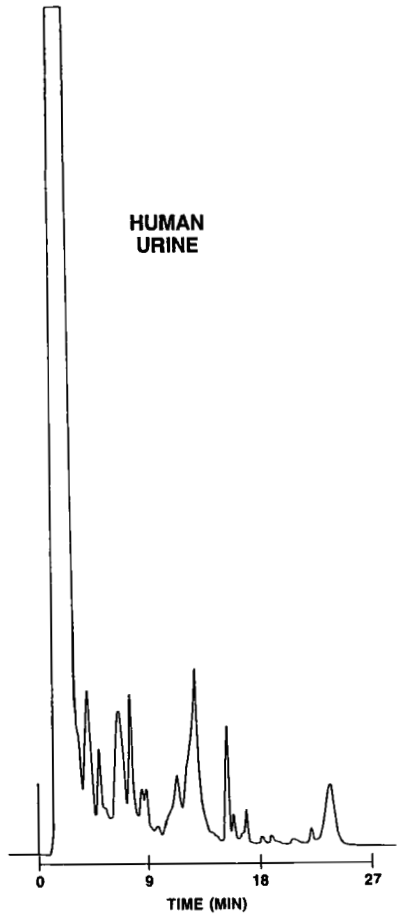

Figure 7. Human urine. Column: 15 cm x 4.6 mm I.D. SPS-5PM-S5-100-C8. Mobile phase: A: H_2O, pH 2.5; B: acetonitrile. Gradient: 99% A to 50% A in 30 min. Flow rate: 1.0 mL/min. Sample volume: 50 uL. Detection: 214 nm.

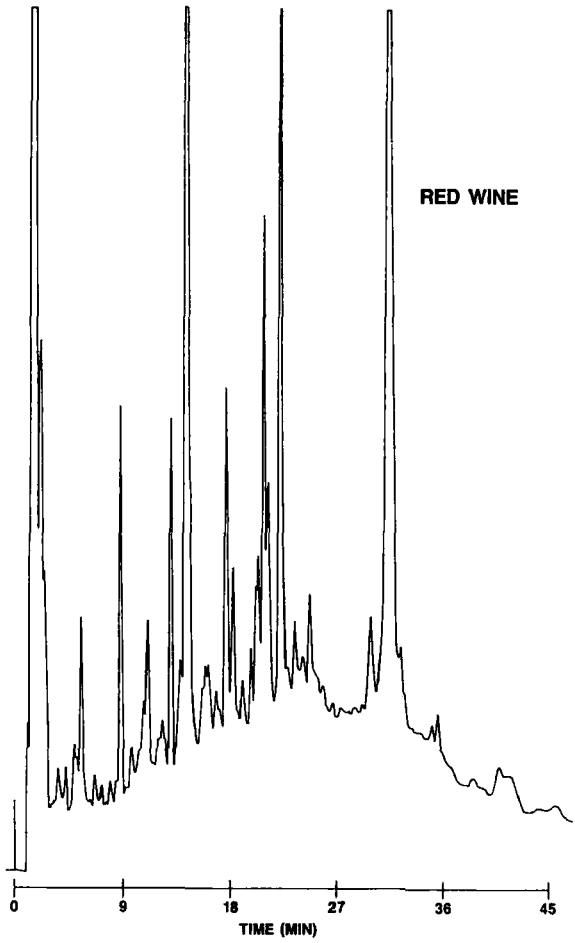

Figure 8. Red wine. Column: 15 cm x 4.6 mm I.D. SPS-5PM-S5-100-C8. Mobile phase: A: H_2O, pH 2.5; B: acetonitrile. Gradient: 99% A to 50% A in 30 min. Flow rate: 1.0 mL/min. Sample volume: 50 uL. Detection: 214 nm.

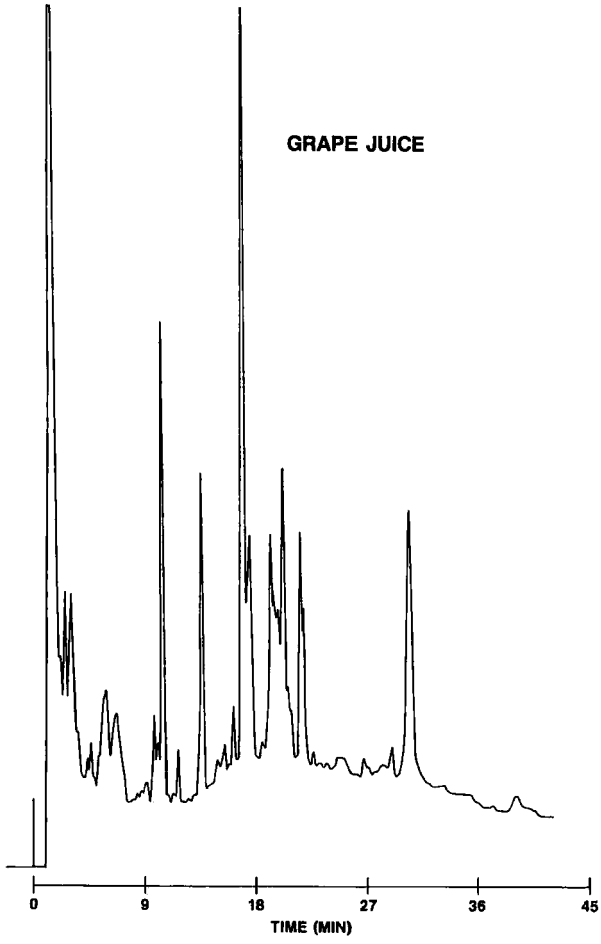

Figure 9. Grape juice. Column: 15 cm x 4.6 mm I.D. SPS-5PM-S5-100-C8. Mobile phase: A: H_2O, pH 2.5; B: acetonitrile. Gradient: 99% A to 50% A in 30 min. Flow rate: 1.0 mL/min. Sample volume: 50 uL. Detection: 214 nm.

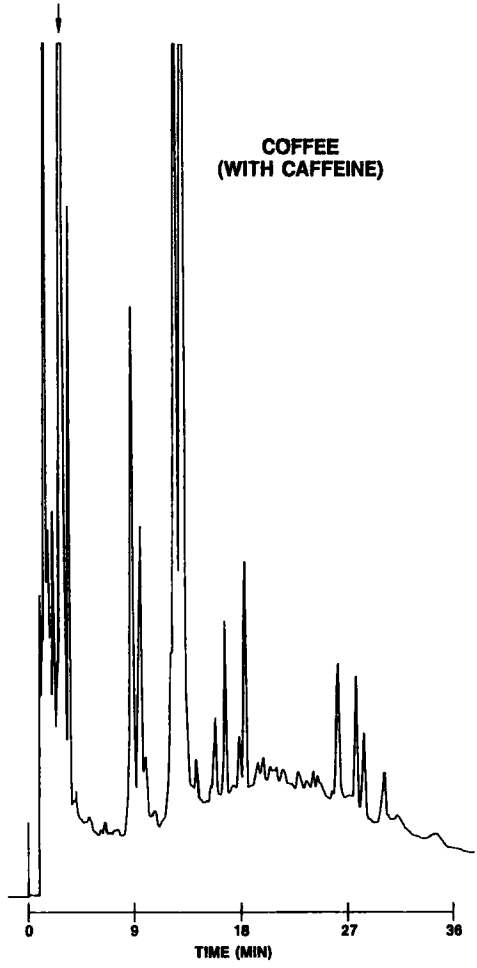

Figure 10. Coffee with caffeine. Column: 15 cm x 4.6 mm I.D. SPS-5PM-S5-100-C8. Mobile phase: A: H_2O, pH 2.5; B: acetonitrile. Gradient: 99% A to 50% A in 30 min. Flow rate: 1.0 mL/min. Sample volume: 50 uL. Detection: 214 nm.

shown nicely separated from each other; the early-eluting barbital is usably separated from the serum proteins.

A New Capability:

Direct Injection of Natural Materials

Materials of natural origin present at least two difficulties to chromatography: Each such material possesses far too many components to be resolved by one method applied to the sample in one injection. If, however, pretreatment is used to render the sample less complex, then that very pretreatment alters the original composition and renders it unknowable. Thus, the successful chromatographgic examination of natural materials requires both the highest possible resolution and direct injection. SPS columns offer potential in this area.

SPS columns can receive a wide variety of natural materials by direct injection, and show a surprising resolution of the very many components in each. The examples shown here--human urine, red wine, grape juice, and caffeine-containing coffee (caffeine identified by arrow)(Figures 7-10)--merely illustrate the many types of materials that may successfully be injected and resolved.

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