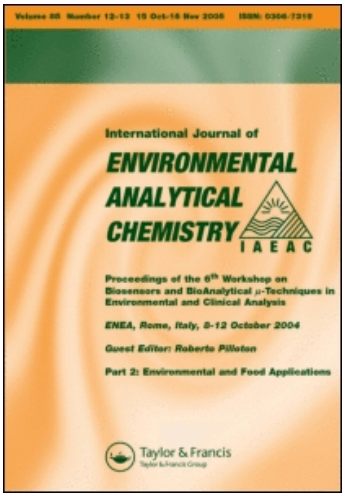

International Journal of Environmental Analytical Chemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713640455

The Use of the PREP™ System for Handling of Body Fluids

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To cite this Article Williams, Reed C.(1984) 'The Use of the PREP™ System for Handling of Body Fluids', International Journal of Environmental Analytical Chemistry, 18: 1, 37 — 49
To link to this Article: DOI: 10.1080/03067318408076990
URL: http://dx.doi.org/10.1080/03067318408076990

Intern. J. Environ. Anal. Chem., 1984, Vol. 18, pp. 37–49
0306-7319/84/1802-0037 $18.50/0

Printed in Great Britain

The Use of the PREP™ System for Handling of Body Fluids†

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(*Received November 25, 1983*)

The Du Pont PREP™ automated sample processor is a centrifugally based, microprocessor controlled instrument that was designed for extraction of samples from biological fluids. Extraction takes place in cartridges containing either organic resins or bonded silica packings as extraction sorbants. This paper will discuss the application of several lipophilic and ion exchange sorbants to the extraction of biological samples from body fluids. The advantages of these different types of sorbants will be compared and their performance with automated sample preparation will be shown. A variety of applications including the extraction of benzodiazepine, barbiturate, aminoglycoside and anticonvulsant drugs and their metabolites from serum, urine, and tissue homogenates will be discussed.

KEY WORDS: Body fluids, PREP™ system, automated sample preparation

INTRODUCTION

Sample preparation is an important part of any analytical method; the accuracy and precision of the assay are often dependent upon it. In past years solvent extraction has been the standard technique used in sample preparation, but recently the development of small particle resins and bonded silicas has revolutionized sample preparation techniques. Disposable catridges containing these

†Presented at the International Workshop on Handling of Environmental and Biological Samples in Chromatography, 24–25 November, 1983, Lausanne.

sorbants can now be used in the Du Pont PREP™ automated sample processor. The purpose of this paper is to give applications and to compare advantages of both small particle resins and bonded silicas and to give examples of the precision and extraction efficiency which can be obtained with automation.

EXPERIMENTAL

All samples discussed in this paper were extracted with the Du Pont PREP™ automated sample processor. The PREP™ (Diagnostic and Bio Research Systems Division, Du Pont) is a centrifugally based, microprocessor controlled instrument.[1,2] The extraction takes place within prepared cartridges which fit into a 12-place reversing rotor in the sample processor. An extraction cartridge consisting of a resin column, effluent cup, sample recovery cup, and a cap is shown in Figure 1. Sample aliquots are buffered and placed in the reservoir of the resin column; the remainder of the extraction sequence is done automatically with the PREP™. This sequence includes extraction onto the sorbant within the column, a rinse with a wash solvent, an elution into the sample recovery cup, and evaporation to dryness. This entire process is done in 10 to 30 minutes depending on the chosen extraction program. The dried extract can then be reconstituted and analyzed by the appropriate analytical technique.

Since this automated extraction procedure was used for all samples, the extraction variables discussed in these applications will be

- the extraction sorbant in the cartridges
- the sample buffer
- the wash solvent
- the elution solvent

The sample is buffered to a pH which will ensure the maximum extraction of the analyte onto the sorbant in the cartridge. The elution solvent is chosen to give the maximum elution from the sorbant. The purpose of the wash solvent is to remove any potential interfering extract components before the elution into the sample recovery cup.

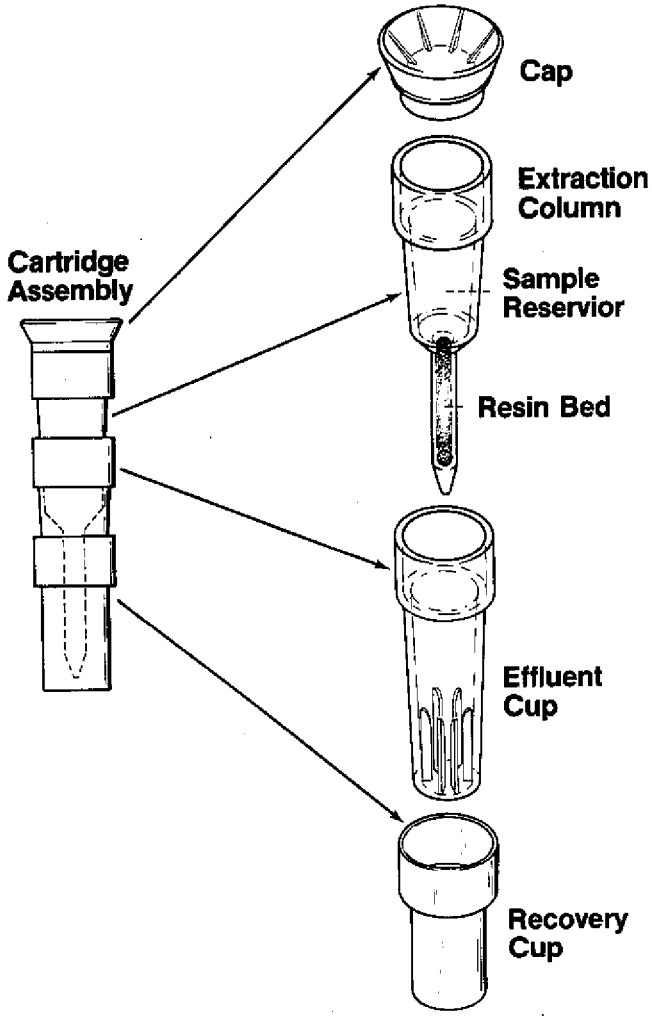

FIGURE 1 Extraction Cartridge for PREP™ Automated Sample Processor.

Cartridges containing four different types of extraction sorbants were used for these studies. Two of these sorbants, a C-18 bonded silica and a cross-linked polystyrene resin, were used for extraction of lipophilic compounds. An anion exchange resin and a cation exchange resin were used for extraction of acidic and basic components. Each cartridge and its use and applications will be discussed in more detail.

Lipophilic extractions

A. *Type W Cartridge:* Lipophilic extraction sorbants, sometimes called reverse phase sorbants, are the most convenient and widely used of the new extraction materials. Perhaps the first of these materials to be commercially available were cross-linked polystyrene resins. The PREP™ Type W cartridge contains a small particle (200–400 mesh) cross-linked polystyrene divinylbenzene resin. The cartridge comes preactivated and ready to use and method development is relatively simple. The sample and internal standard are added to the cartridge along with the appropriate buffer. The wash solvent usually is water and the elution solvent is usually a water miscible organic solvent such as acetone or methanol.

A typical application for these lipophilic cartridges is the extraction and analysis of the anticonvulsant drugs phenobarbital, phenytoin, carbamazepine, and primidone.[1] They are all reasonably water insoluble and found in patient serum in the range between one to 50 micrograms per milliliter. The concentration of the drugs in the serum is usually monitored to ensure the right dosage for proper therepeutic effect. For the extraction procedure, the serum is buffered to pH 4.6 with 0.3 M KH_2PO_4 where the drugs and internal standard are efficiently extracted onto the lipohilic polystyrene sorbant in the Type W cartridge. The cartridge is washed with water to remove excess protein and the drugs eluted from the cartridge into a recovery cup with acetone, an organic solvent in which the drugs are soluble. The extraction solution is automatically evaporated to dryness. The dried extract is reconstituted and analyzed by reverse phase HPLC with a C-8 column with the chromatographic conditions shown in Figure 2.

The extraction efficiency and reproducibility that can be typically expected for these drugs with the Type W cartridge and the PREP™

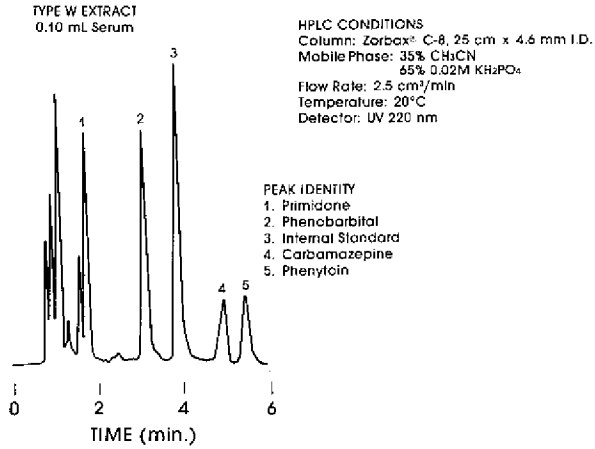

FIGURE 2 HPLC Chromatograms of Anticonvulstant Drug Extract.

automated sample processor is shown in Table I. The absolute recovery ranges from 96–102% with coefficients of variation from 3–6%. If an internal standard is added to the sample and the extraction recovery measured relative to it, then recoveries are between 97 and 101% and CV is 2–4%. This level of recovery and precision is typical for a wide variety of applications when using reproducibly manufactured cartridges. One of the major advantages of using these cartridges with automated extraction procedures is that material differences and technician error are removed from the extraction procedure and it is possible to obtain the same extraction efficiency and reproducibility from one lab to another.

TABLE I
Recovery of Anticonvulsant Drugs in PREP™ Extracts

Drug	Concentration (μg/mL)	Serum Sample Volume (mL)	Absolute Recovery	CV	Relative Recovery	CV
Phenobarbital	10	0.2	102%	3.6%	101%	2.5%
Phenytoin	5	0.2	96%	5.9%	97%	2.4%
Carbamazepine	10	0.2	102%	4.5%	101%	1.8%
Primidone	15	0.2	99%	4.5%	100%	4.2%

Another typical application for lipophilic extraction is the extraction and analysis of benzodiazepene drugs.[3] The benzodiazepene drugs are found in serum and are relatively water

insoluble at a basic pH. The serum samples are buffered to pH 9.1 with 0.1 M K_2HPO_4 and extracted onto Type W cartridges. The cartridges are washed with 1 ml of water and eluted with 2 ml of acetone. The extract solutions are evaporated to dryness, reconstituted, and analyzed by HPLC with conditions shown in Figure 3. The data in Table II show the extraction efficiency which can be expected of different concentrations in the serum. There appears to be no significant difference in recovery between concentrations from 50 nanograms to 25 micrograms per milliliter. It is typical that extraction efficiency is independent of sample concentration for both the lipophilic and ion exchange cartridges. However, when extracting trace quantities it should be remembered that small amounts of some compounds may irreversibly adsorb onto glass or metal when evaporated to dryness and appropriate precautions and materials should be used to avoid this.

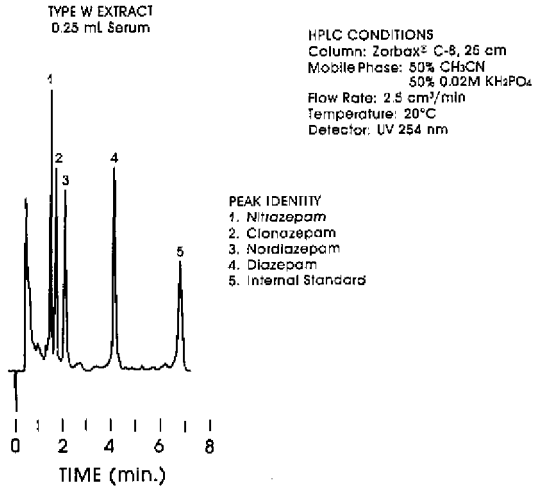

FIGURE 3 HPLC Chromatogram of Benzodiazepine Drug Extract.

TABLE II
Recovery of Benzodiazepine Drugs in PREP™ Extracts

	Concentration (μg/mL)	Serum Sample Volume (mL)	Nitrazepine	Clonazepam	Nordiazepam	Diazepam
High Level	25	0.25	90%	92%	93%	85%
Medium Level	4	0.5	95%		94%	90%
Low Level	0.05	2.0	90%	90%	98%	91%

An unusual application of the lipophilic cartridges is the extraction of the barbiturate drugs secobarbital and amobarbital from tissue homogenates.[4] Samples (0.25 grams) of homogenized liver or brain are placed in the Type W cartridge with 1 ml of pH 4 acetate buffer. A small plug of glass wool is used as a filter. The samples are extracted onto the cartridge in the PREP™; the cartridges are washed with 1 ml of water and eluted with 1 ml of methylene chloride. The dried extracts were reconstituted and analyzed by GC on a 6 ft. column of 3% OV-101 at 180°C with a N-P detector. Precision studies showed that the within day coefficient of variation for samples with a concentration of above 30 mg/Kg were less than 5%.

These lipophilic cartridges have also been applied to the environmental study of the effect of fuel oil spills on salt water fish.[5] A group of English sole were kept captive in flow-through seawater aquaria and exposed to salt water containing fuel oil. A second group of sole were exposed only to fresh salt water. Samples (100 milligrams) of the livers from the two groups were homogenized in 0.5 ml of 1.15% potassium chloride solution. These samples were then extracted in PREP™ automated sample processor with Type W cartridge. The samples were added to cartridges along with 1 ml of pH 2.5 buffer and one gram of 200 mesh glass beads which acted as filter bed. The samples were extracted and the cartridges were washed with water and eluted first with a 1:1 mixture of acetone and methanol and then with a (75:25:2) mixture of methylene chloride, isopropanol, and water. The dried extracts were reconstituted and analyzed by HPLC with a reverse phase C-18 column and gradient elution of the mobile phase from water to methanol. A fluorescence spectrometer was used as a sensitive detector. Comparison of the chromatograms from the samples clearly showed the presence of aromatic hydrocarbons and metabolites in the fish exposed to fuel oil; these aromatic hydrocarbons were not present in the control fish.

B. *Type OD Cartridge:* The second type of extraction sorbant which is widely used for extraction of lipophilic compounds from aqueous solutions is octadecyl (or C-18) bonded silica. The PREP™ Type OD cartridge contains an octadecyl silica which is of approximately 20% organic content by weight. The cartridges are dry packed and must be activated before use by washing with 1 milliliter of a water

miscible organic solvent such as acetone or methanol. The cartridges are convenient to use and method development is relatively simple. The aqueous sample must be buffered to promote the water insolubility of the analyte. After extraction of the lipophilic components, the cartridge is usually washed with water and then the analytes are usually eluted with an organic solvent in which they are readily soluble. The method development procedures used with the Type OD cartridge are similar to those used with the Type W cartridge. Since the applications for the two cartridges are also similar, further specific examples will not be discussed. However, there are differences between the two types of cartridges which should be noted.

- The use of C-18 bonded silica should be limited to solvents with pH between 2 and 11 or the chemically bounded stationary phase may be stripped from the packing. The polystyrene resin used in Type W cartridge has no such limitation and can be used with strong acids and bases.
- There are subtle differences in extraction characteristics of the two packings which are caused by the effect of the silica substrate in the C-18 packing and by chemical differences of the aromatic polystyrene and aliphatic octadecyl stationary phases. This may result in different extraction efficiencies for some compounds and in different interference backgrounds for the same samples.

These differences are important enough so that the Type W and Type OD cartridges can be considered complementary to each other for use in extraction of lipophilic compounds.

Ion exchange extractions

Ion exchange sorbants can be used in two areas of application where the lipophilic polystyrene and C-18 silica sorbants will often fail.

- The extraction of very water souble compounds from aqueous solution.
- The selective extractions of acidic or basic water insoluble compounds from very complex samples with many potential interferences.

Anion and cation exchange sorbants which are compatible with both aqueous and organic solvents are now commercially available. These materials can be applied to extraction of both water soluble and insoluble acidic and basic compounds.

C. *Type AS Cartridge:* The Type AS cartridge contains an anion exchange sorbant which is an organic resin with quarternary amine groups in the chloride form. Each cartridge contains approximately 0.4 milliequivalents of ion exchange capacity and is compatible with both organic and aqueous solvents. Since it is not a chemically bonded packing it can be used with solvents containing strong acids and bases without fear of stripping the stationary phase. The general method development procedure is to extract acidic compounds from aqueous solution at a basic pH. The cartridges can be washed with water or an organic solvent such as methanol to remove interferences. The sample can then be eluted from the resin with either of the following types of solvents.

- An aqueous solvent containing a high ionic content.
- An organic solvent containing a volatile organic acid such as formic or acetic acid.

The advantage of using the volatile organic solvents and volatile acid modifiers is that the extract can be evaporated to dryness. This allows concentration of sample as well as compatibility with analytical techniques such as gas chromatography.

The extraction of VMA from urine is a good example of an application of the Type AS cartridge to a water soluble acidic compound. VMA is 4-hydroxy-3-methoxymandelic acid and is a catecholamine metabolite found in urine. An elevated level of the metabolite is indicative of certain diseases and testing for the metabolite in urine is sometimes necessary. Urine samples are processed by placing half milliliter aliquots in the cartridge along with one milliliter of 0.01 NaOH and the internal standard. The samples are processed in the PREP™ sample processor where each cartridge is washed with 1 milliliter of methanol to remove lipophilic interferences and then eluted with 2 ml of a mixture of 70% methanol and 30% formic acid. The extract solutions can be evaporated to dryness, reconstituted and analyzed by the appropriate analytical

method such as HPLC with periodate reaction detection.[6] Recovery of the VMA averages about 92% from aqueous samples.

The Type AS cartridge can also be applied to the extraction of water insoluble acidic compounds from solution. An example of this is the urinary metabolite (11-norΔ^9-tetrahydrocannabinol-9-carboxylic acid) of tetrahydrocannabinol (THC). This metabolite is normally found in urine in the glucuronide conjugated form and must be changed to the free form by basic hydrolysis with 10 N KOH. The hydrolyzed urine is then changed to a pH of 9 and three milliliters placed in the cartridge along with the internal standard. The cartridges are then processed, washed with two milliliters of methanol and eluted with 2 milliliters of a mixture of 90:10:4 ethyl acetate/MeOH/glacial acetic acid. The ethyl acetate is used because it gives cleaner extract than just methanol for this sample. The extract solution is evaporated to dryness in a glass tube, reconstituted, derivatized and analyzed by GC with mass spectrometer or flame ionization detection.[7] The chromatogram in Figure 4 shows the clean background of a sample chromatographed by GC with FID detection. Normal liquid extractions or extraction with Type OD or W cartridge give dirty extract samples with many interfering peaks. However, the Type AS cartridge gives clean extracts which are equivalent to those of acid-base, liquid-liquid back extractions.

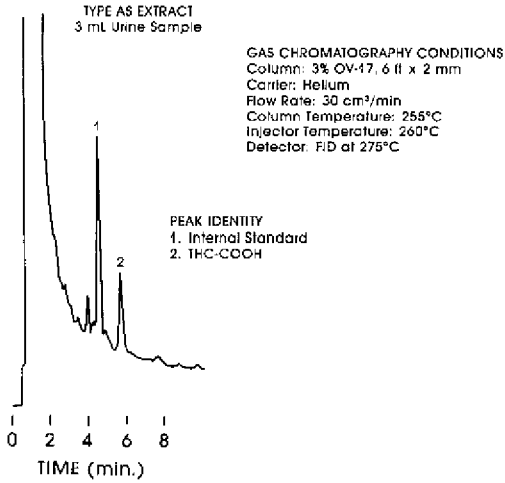

FIGURE 4 GC Chromatogram of THC Metabolite Extract.

D. *Type CS Cartridge:* The Type CS cartridge contains an organic resin with sulfonic acid groups in the hydrogen form. Each cartridge contains about 0.8 milliequivalents of ionic capacity and is compatible with both organic and aqueous solvents. Strong bases and acids can be used without harming the resin in the cartridge. The general method development procedure is to buffer the aqueous sample to an acid pH for best extraction of basic compounds onto the cartridge. The cartridge is then washed with water or methanol and eluted with either.

- An aqueous solvent containing a high ionic content.
- An organic solvent with a volatile base modifier such as ammonium hydroxide or diethylamine.

Again, the advantage of volatile organic solvents and volatile basic modifiers is that they can be evaporated to dryness before the analysis.

The Type CS cartridge can be applied to extraction of very water soluble basic drugs such as Tobramycin. This drug is an aminosugar with five primary amine groups and is used as an antimicrobial in the treatment of infections. Its effective therapeutic concentration range in the serum is close to its lower toxic level and the serum concentration is often monitored to ensure that the proper patient dosage is given. One milliliter aliquots of patient serum are placed in the cartridge along with one milliliter of 1 N acetic acid and internal standard. The basic drugs are extracted onto the cartridge resin in the PREP™ and the cartridges washed with 1 ml of H_2O and eluted into a plastic recovery cup with 1 ml of water containing 0.5 N NaOH and 0.75 M $NaSO_4$. This extracted solution is then directly injected into an HPLC for analysis. The chromatogram in Figure 5 shows the extract of a patient sample. The sample was detected by a fluorescence spectrophotometer with a post column reaction with O-phthalaldehyde. Recovery of the drug averages about 88% from serum samples.

The Type CS cartridge can also be applied to the extraction and analysis of 9(2-hydroxyethoxymethyl) quanine and its metabolite from serum. This drug (Acyclovir™) is also a water soluble basic drug which is used in the treatment of diseases caused by herpes virus. Its concentration is also measured in blood serum. Other

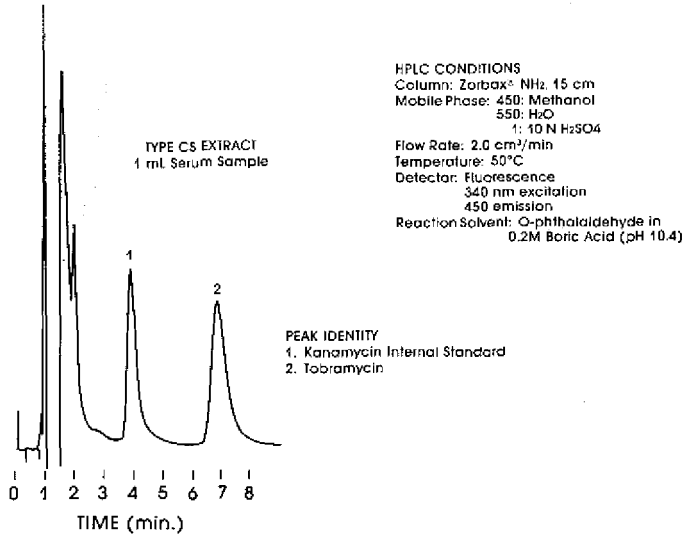

FIGURE 5 HPLC Chromatogram of Tobramycin Extract.

extraction methods are available for this drug but extracts are not sufficiently clean for quantitation of the metabolite. The Type CS cartridge can be used to give clean extracts for HPLC analysis. A 100 microliter sample of serum is placed in the cartridge with a one milliliter aliquot of 0.05 M H_3PO_4. The sample is extracted in the PREP™ automated sample processor and the cartridge is washed with 2 ml of a mixture of 50% methanol and 50% 1 M aqueous NH_4OH. The cartridge is eluted with 1.5 ml of a mixture of 50% methanol and 50% 1 M diethylamine. The extract is evaporated to dryness, reconstituted with H_2O and analyzed by HPLC. Recovery of the Acyclovir™ from serum samples averaged 90%.

A wash solvent of methanol and NH_4OH was used to selectively wash off weakly basic components leaving the Acyclovir™, metabolite and internal standard to be eluted by the more basic diethylamine modifier in the eluting solvent. This selective removal of the unwanted components by the wash solvent ensures interference free extracts in which both drug and metabolite can be measured.

CONCLUSION

In conclusion, the Du Pont PREP™ automated sample processor can be used for a wide variety of applications for sample preparation with body fluids. Type W and OD cartridges containing a lipophilic polystyrene resin and a C-18 bonded silica, respectively, are the most widely used for a variety of applications from serum, urine and tissue homogenates. However, the Type AS and Type CS containing anion and cation exchange resins can also be applied for extraction of water soluble compounds as well as very selective extractions of acidic and basic components in complex mixtures. Although the bonded silica and small particle resins are both useful extraction sorbants, the resins are stable in solvents which contain strong acids or bases which could strip the stationary phase from a bonded silica. This may be of special importance when working with the strong solvents used in ion exchange extractions.

Absolute recovery of analyte is generally independent of analyte concentration down to at least nanogram per milliliter concentration range. The recovery of compounds using the PREP™ cartridges typically averages 90% or better. The use of reproducibly-made cartridges with automated sample processing results in convenient, reproducible extractions which can be duplicated from one lab to another.

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