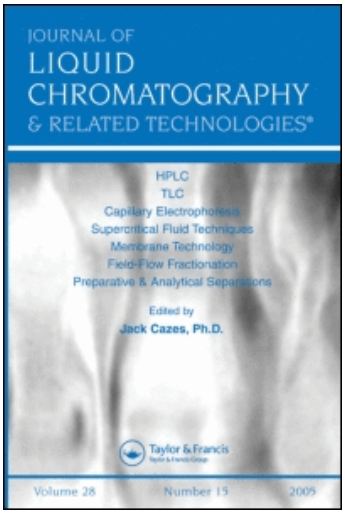

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Use of an Improved Version of C_8 SCX Mixed Mode Solid Phase Extraction Material for Clean Extraction and Recovery of Basic and Zwitterionic Compounds from Biological Fluids

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To cite this Article Liu, Yanhong(2006) 'Use of an Improved Version of C_8 SCX Mixed Mode Solid Phase Extraction Material for Clean Extraction and Recovery of Basic and Zwitterionic Compounds from Biological Fluids', Journal of Liquid Chromatography & Related Technologies, 29: 10, 1405 — 1431
To link to this Article: DOI: 10.1080/10826070600674794
URL: http://dx.doi.org/10.1080/10826070600674794

Journal of Liquid Chromatography & Related Technologies®, 29: 1405–1431, 2006

ISSN 1082-6076 print/1520-572X online
DOI: 10.1080/10826070600674794

Use of an Improved Version of C_8+SCX Mixed Mode Solid Phase Extraction Material for Clean Extraction and Recovery of Basic and Zwitterionic Compounds from Biological Fluids

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Abstract: Mixed mode bonded silica, containing both reversed phase and cationic exchange compositions, is one of the most versatile solid phase extraction (SPE) products in the market due to generic extraction methods and some successful applications for cleaner extraction of basic compounds from biological fluids. However, the present study shows that many zwitterions compounds and polar basic compounds cannot be effectively extracted and recovered from the current C_8+SCX mixed mode products. The inability is due to too low an ion exchange capacity. In order to solve the problem, the SCX functional coverage is optimized to develop an improved version of C_8+SCX mixed mode material. Three generic extraction protocols have been categorized. The first one is used to completely recover basic and zwitterions compounds with some hydrophobicity, and this protocol offers the cleanest background. The second one is used to recover polar basic and zwitterions compounds, and/or the third one is used to systematically fractionate basic and zwitterions compounds from neutral and acidic compounds for further analysis. The considerable advance greatly improves the performance of cationic mixed mode SPE products and the corresponding data reproducibility.

Keywords: C_8+SCX mixed mode, Solid phase extraction (SPE), Systematic toxicology analysis, Illegal drug

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INTRODUCTION

Reliable and reproducible screening and isolation of drugs, their metabolites as well as poisons from biological matrices, is the first, the most important, and the most critical step in preclinical, clinical, and forensic analyses.[1,2] Among various extraction methods, solid-phase extraction (SPE) is becoming more and more popular for meeting the needs, not only because of the possibility of high throughput automation, but also because of the increased commercial availability of innovative SPE adsorbents in recent years.[1–4] According to the literatures and everybody's experience, silica based mixed mode SPE cartridges and 96-wells, containing reversed-phase and cationic exchange compositions, is one of the most versatile SPE products in the market due to generic extraction methods, and a number of successful applications for cleaner extraction of basic compounds from biological fluids, as well as acidic/neutral/basic drug screens.[4–45] However, in spite of these obvious advantages, the products from the current manufacturers may have some serious problems. Several authors have claimed that very different recoveries, up to complete failure of the extraction, are observed for the same or different batches of cartridges, showing a need for dramatic improvement in the quality of these products.[46–49] Furthermore, it is evidenced by the present study showing that the products in the current markets have the risk of losing polar basic compounds during the extraction process and are unable to fractionate zwitterions compounds and weakly basic compounds from neutral and acidic compounds, both because of too low ion exchange sites. In order to solve the problem, the ion exchange coverage has been optimized to develop and commercialize an improved version of C_8+SCX mixed mode SPE product. The novel product has been used to fractionate and recover about one hundred compounds with diverse physicochemical properties from various resources of urine, serum, and plasma. Three generic cleanup protocols are summarized as guidance. The application range, lot to lot reproducibility, advantages, and potential pitfalls of the novel product have also been discussed.

EXPERIMENTAL

Characterization of the Novel C_8+SCX Mixed Mode SPE Phase

A high surface area of silica (average surface area: 500 m^2/g, average pore size: 70Å, average particle size: 60 um) was used as the starting material, and its silanol groups were partially derivated with C_8 and partially with ethylbenzene sulphonic acid according to Chrom-Matrix's proprietary technology, under the quality control of ISO9001 regulations. For three different lots, the carbon percentage is $8.21 \pm 0.39\%$ and the sulphur percentage $1.05 \pm 0.05\%$, showing very good reproducibility from lot to lot during the manufacturing.

For the measurement of the ion exchange capacity, 0.6 mg/mL atrazine aqueous solution was led through a 500 mg/3 cc mixed mode SPE tube preconditioned with methanol. The breakthrough of atrazine was measured by an *Innovation*® C_{18} 5 um 15 cm × 4.6 mm column from Chrom-Matrix Inc. Mobile phase: 25% acetontrile and 75% water; Flow rate: 1.0 mL/min; Detection: 254 nm. Instrument: Agilent 1100 HPLC system. The ion exchange capacity was calculated by the midpoint of the breakthrough curve.

The ion exchange capacity of the C_8+SCX mixed mode SPE product was 4.2 ± 0.2 mg atriaze/g and the value was 1.5–4 folds higher than other silica-based C_8+SCX mixed mode SPE products.

The novel product is commercially available from Chrom-Matrix Incorporation (www.chrom-matrix.com).

Non-Polar Basic and Zwitterion Drug Generic Extraction Protocol (for 100 mg/1 mL or 100 mg/3 mL SPE tube)

The generic protocol has been developed by other manufacturers.[29,50] The generic protocol is only suitable to the compounds that have complete retention during the loading at pH 6.0, probably because of sufficiently strong reversed-phase interactions with C_8 functional groups.

1. Sample pretreatment: Dilute urine (plasma/serum) sample (1.0 mL) with 50 mM ammonium acetate buffer or 10 mM potassium phosphate buffer (pH 6.0) (1.0 mL).
2. SPE Tube Conditioning: Solvate the tube with 1 mL methanol.
3. SPE Tube Equilibrium: Equilibrate the tube with 50 mM ammonium acetate buffer or 10 mM potassium phosphate buffer (1 mL).
4. Sample Loading: Load pretreated urine or plasma (1.0 mL) at a flow rate of 1 mL/min.
5. Interference Elution: Elute interference with 50 mM ammonium acetate buffer (or 10 mM phosphate buffer) (1.0 mL), 1 M acetic acid (1.0 mL), and methanol (1.0 mL).
6. Analyte Elution: Elute analytes with 5% ammonium hydroxide in methanol.
7. Samples were evaporated to dryness under nitrogen in a TurboVap (Zymark, Hopkinton, MA, USA) at 40°C.
8. Reconstitute the dried fractions by the mobile phase for HPLC analysis.

Zwitterions and Polar Basic Drug Generic Extraction Protocol (for 100 mg/1 mL or 100 mg/3 mL Spe Tube)

When a target may have (even a minor but detectable) leakage during loading at neutral pH, the second protocol should be chosen as follows:

1. Sample pre-treatment: Dilute urine (plasma) sample (1.0 mL) with 10 mM potassium phosphate buffer (pH 3.0) or 10 mM acetic acid buffer (pH 3.0) (1.0 mL).
2. SPE Tube Conditioning: Solvate the tube with 1 mL methanol.
3. SPE Tube Equilibrium: Equilibrate the tube with 10 mM pH 3.0 potassium phosphate buffer or 10 mM acetic acid buffer (1 mL).
4. Sample Loading: Load pretreated urine or plasma (1.0 mL) at a flow rate of 1 mL/min.
5. Interference Elution: Elute interference with 10 mM pH 3.0 phosphate buffer (or acetic acid buffer (1.0 mL) and methanol (1.0 mL).
6. Analyte Elution: Elute analytes with 5% ammonium hydroxide in methanol.
7. Samples were evaporated to dryness under nitrogen in a TurboVap (Zymark, Hopkinton, MA, USA) at 40°C.
8. Reconstitute the dried fractions by the mobile phase for HPLC analysis.

Reagents and Chemicals

All the biological matrixes (plasma, urine, and serum) were from Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences, China. HPLC grade solvents were from J.T. Baker, Shanghai, China. 3,4-methylenedioxyamphetamine and 3,4-methylenedioxymethamphetamine were purchased from Cerilliant (Austin, TX, USA). The rest of the chemicals and tested compounds were purchased from Sigma (St Louis, MO, USA), or Aldrich Chemical Co. (Milwaukee, WI, USA), or supplied by National Institute for the Control of Pharmaceutical and Biological Products (Beijing, P.R. China).

A vacuum manifold (Waters, Milford, MA, USA) was used for testing all the cartridges, except 96-well plates.

Samples of plasma, serum, and urine spiked with different drugs, poisons, and metabolites were used throughout the study. In the case of urine, pretreatment may include a hydrolysis step in order to assure the recoveries of drugs that possibly conjugate with glucuronic acid. Enzymatic hydrolysis was carried out on the sample adjusted to pH 6–6.5 by adding 50 uL of β-glucuronidase and heating at 45°C for 1 h.

Evaluation of Mixed Mode SPE Products by 96-Well Plate Screening

In order to rapidly evaluate the performance of the novel C_8+SCX mixed mode SPE product, the successful applications published in the literatures were repeated herein with the novel product, and the recovery results were compared with the data in the literatures and the product manual.[2,3,7–20] The major differences were to use conventional HPLC/UV for chromatographic

analysis on all the cases, and the spiked concentrations of the compounds were fixed at 4.0 ug/mL. Although, the concentration was much higher than those from field applications, the purpose of this present study was just for evaluation and comparison of SPE materials themselves. Sixty-six basic and zwitterions compounds were spiked in pooled human urine, monkey plasma, rat plasma, mouse plasma, and human plasma for this screening study. The generic basic drug extraction protocol (Experimental section) was used, in all the cases, for cleanup and extraction of the compounds from the 96-well plates packed with 100 mg mixed mode material in each well. The operation was executed by a CEREX Multi-Channel SPE System-96 system. The final samples were dried and reconstituted for HPLC analyses.

The chemical names and the spiked biological fluid for each analysis were listed in Table 1. Unless indicated, an *Innovation*® C_8 5 um 15 cm × 4.6 mm column from Chrom-Matrix Inc. was used for separation and determination of drug recoveries with UV detection at 210 nm or 220 nm.

The drug standards spiked in the reconstituted solutions (typically 20:80 acetontrile:water) at five different concentrations were used to make the calibration curves.

Recovery of Benzoylecgonine, Buprenorphine, Codeine-6-glucuronide, Ecgonine Methyl Ester, Isoxsuprine, Levallorphan, 6-Monoacetyl Morphine, Morphine, 3-Pyridylacetontrile, 2-Aminopyridine and Piroxicam by Two Different Vendors

The 96-well plates (100 mg per well) from Varian Certify and Isolute Confirm HCX were also used to clean and recover the compounds listed above. The pH 6.0 generic protocol was used for the process. Both the methanol fraction and the final fraction were submitted for the HPLC analyses.

Recovery of Polar Zwitterions and Basic Compounds from the Novel C_8+SCX Mixed Mode SPE

The chemical name, concentration, and matrix were listed in Table 3. The C_8+SCX mixed mode 96-well plate (100 mg per well) was used to clean and recover the compounds according to the pH 3.0 generic protocol (Zwitterions and Polar Basic Drug Generic Extraction Section).

Systematic Fractionation and Analysis of Acid and Neutral from Zwitterions and Basic Drugs by the Novel C_8+SCX Mixed Mode SPE

Following Takeda's protocol,[41] for fractionation of 2-aminobenzoic acid (zwitterions compound) from benzoic acid and p-nitrobenzoic acid, a

Table 1. Absolute recoveries (mean ± S.D., n = 4) (%) of hydrophobic basic drugs throughout pH 6.0 generic protocol

Drug	Average recovery (%)	Drug	Average recovery (%)
Acebutolol	94.3 ± 2.6	Methylephedrine	98.9 ± 1.2
Alprenolol	96.2 ± 1.4	3,4-Methylenedioxyamphetamine	98.1 ± 3.4
4-Aminoantipyrine	97.3 ± 1.9	3,4-Methylenedioxymethamphetamine	103 ± 2.4
Amitriptyline	97.0 ± 0.7	Methylphenidate	102 ± 2.3
Amphetamine	100.3 ± 1.9	Methoxy-verapamil	99.2 ± 1.9
R(+)-Atenolol	98.3 ± 2.1	Metoprolol	98.5 ± 1.9
Benzocaine	98.7 ± 2.3	Mianserin	93.2 ± 2.7
Brucine	95.4 ± 3.1	Nalorphine	96.3 ± 3.1
Citalopram	99.6 ± 2.2	Nefazodone	95.0 ± 1.2
Clomipramine	93.7 ± 1.8	Nicotine	98.2 ± 1.8
Cocaethylene	98.2 ± 3.1	Nonfluoxetine	99.2 ± 2.4
Cocaine	95.5 ± 2.7	Norcocaine	97.9 ± 2.2
Codeine	96.9 ± 2.2	Nordoxepin	98.4 ± 1.4
Cyproheptadine	97.4 ± 2.8	Normethyl-verapamil	96.4 ± 1.7

Cyproheptadine-N-oxide	94.9 ± 3.8	Oxprenolol	99.2 ± 0.7
Desipramine	105 ± 3.7	Oxycodone	97.1 ± 2.3
Desmethylsertraline	97.8 ± 2.1	Perphenazine	98.4 ± 1.2
Dibucaine	99.6 ± 2.3	Phencyclidine(PCP)	99.5 ± 1.3
Diphenhydramine	98.4 ± 2.1	Phentermine	93.6 ± 1.8
Diphenylpyraline	97.4 ± 0.9	Phenylpropanolamine	98.9 ± 2.9
Doxepin	99.4 ± 2.8	Pindolol	106 ± 3.6
Ephedrine	103 ± 1.2	Procainamide	98.6 ± 2.1
Fluoxetine	96.3 ± 2.2	Procaine	98.7 ± 2.3
Fluphenazine	103 ± 3.3	Propoxyphene	96.4 ± 3.3
Fluvoxamine	98.8 ± 2.7	Propranolol	99.6 ± 1.5
Imipramine	100.5 ± 1.4	Quinidine	98.5 ± 1.9
Imitriptyline	97.4 ± 1.8	Rantidine	97.7 ± 3.5
Lidocaine	96.6 ± 4.5	Sertraline	99.3 ± 2.7
Lysergic acid diethylamide	94.8 ± 2.1	Stanozolol	102 ± 2.8
Maprotiline	95.9 ± 0.9	Tetracaine	98.3 ± 2.7
Meperidine	98.5 ± 3.3	Trazodone	95.3 ± 3.4
Methadone	98.9 ± 0.9	Venlafaxine	97.9 ± 2.2
Methamphetamine	99.3 ± 1.4	Verapamil	98.4 ± 3.1

10 mM phosphate buffer containing 10 ug/mL of anthranilic acid, benzoic acid, and p-nirobenzoic acid (pH 3.0) was used for this testing. 300 mg/3 cc of the novel C_8+SCX mixed mode SPE, Varian Certify and IST Confirm HCX SPE tubes were first rinsed with 3 mL methanol and equilibrated with 3 mL of the blank pH 3.0 10 mM phosphate buffer. The sample of 1 mL was loaded, followed by 3 mL of the blank buffer (pH 3.0). The acidic fraction was collected by 1 mL of methanol and the basic fraction was collected by 1 mL methanol containing 5% ammonium hydroxide.

Lot-to-Lot Reproducibility

Three different lots from production scale were evaluated by ion exchange capacity, TCA recovery test, 2-aminopyride, and piroxicam by the same protocol, fractionation of 2-aminobenzoic acid and diazepam from benzoic acid, acetaminophen, and toluene by the pH 3.0 protocol.

RESULTS AND DISCUSSION

Evaluation of the Novel Mixed Mode SPE by 96-Well Recovery Test

Mixed mode SPE is now available from a number of manufacturers, e.g., Bond Elut Certify™ (Varian Sample Preparation Products, Harbor City, CA, USA), Clean Screen® DAU (Worldwide Monitoring Corp., Horsham, PA, USA), Bakerbond Narc-2 (J.T. Baker, Philipsburgh, NJ, USA), Isolute Confirm HCX (Argonaut Technologies, 1101 Chess Drive, Foster City, CA 94404, USA), Extrelut® TSC (Merck, Darmstadt, Germany), Chromabond Drug (Machery Nagel, Dűren, Germany), and Empore™ MPC (3M Bioanalytical Technologies, St. Paul, MN 55144, USA). Some successful applications for basic compounds have been published[5–30] and the generic cleanup protocol is outlined in Nonpolar Basic and Zwitterion Drug Generic Extraction Protocol.

Probably, a rapid and straightforward approach for evaluation of a novel C_8+SCX mixed mode SPE product regarding its feasibility and versatility is to repeat these successful applications published in the literatures.[5–30] Sixty-six basic and zwitterions compounds were spiked, individually or as a whole group (e.g., antidepressant drugs), into different biological fluids and then extracted by the generic basic drug extraction protocol (Nonpolar Basic and Zwitterion Drug Generic Extraction Protocol). The results are shown in Table 1.

High recoveries and excellent reproducibility are readily obtained for all of the compounds, showing the suitability and versatility of the novel C_8+SCX mixed mode SPE product for hydrophobic basic drug screening in biological fluids.

Most of the basic compounds listed in Table 1, after the SPE cleanup, were analyzed by an Innovation C_8 column. Nevertheless, a pentafluorophenylpropyl (PFPP) phase were used for analysis of a few illegal drugs, such as amphetamine, methamphetamine, 3,4-methylenedioxyamphetamine, and 3,4-methylenedioxymethamphetamine (Ecstasy pills). The PFPP phase acts as hydrophilic interaction chromatography. Probably, it has a weak cationic exchange mechanism, and, thus, offers symmetry peaks for these challenging basic compounds. To the best of the author's experience, both the PFPP phase and the amide-80 phase from Tosoh Bioscience are an important compensation to the conventional RP-HPLC chromatography.

A typical HPLC chromatogram is shown as Figure 1.

A Difference Between the Novel Mixed Mode SPE and the Current Mixed Mode SPE Products

The mixed mode SPE 96-well plates from Varian and Argonaut, as two representatives from the current vendors, were used to clean the same matrices and recover a wide spectrum of compounds as a comparative study. When similar and/or comparable recoveries were obtained for the compounds listed in Table 1, thirteen compounds listed in Table 2 had lower recoveries and poorer reproducibility.

Recovery of 2-aminopyridine from pooled human urine is a typical case. In order to completely recover 2-aminopyridine, 10 mM potassium phosphate (pH 6.0) was used instead of 50 mM ammonium acetate in the basic compound generic protocol for the equilibration/washing of the SPE tubes and the dilution of human urine.

Varian Bond Elut Certify mixed mode SPE, which has an ion exchange capacity as 2.4 mg atrazine per gram, cleans the background very well.

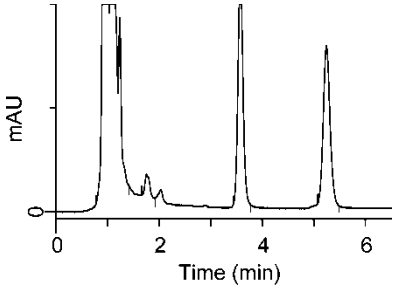

Figure 1. HPLC chromatogram of amphetamine and methamphetamine spiked into human plasma, after the SPE cleanup. Column dimension: Innovation PFPP 5 um 10 cm × 4.6 mm; Mobile phase: 10 mM ammonium acetate (pH 4.5) and acetontrile (15:85); Flow rate: 2 mL/min. Retention time of amphetamine: 3.64 min; Retention time of methamphetamine: 5.28 min.

Table 2. Absolute recoveries (mean ± S.D., n = 4) (%) of some basic drugs throughout pH 6.0 generic protocol by different vendors of mixed mode SPE products

Drug	Average recovery (%) from Certify	Average recovery (%) from HCX	Average recovery (%) from the novel mixed mode SPE
2-Aminopyridine	30.3 ± 15	36.4 ± 7.4	102 ± 3.5
Benzoylecgonine	90.3 ± 3.8	79.4 ± 10.2	97.6 ± 2.2
Buprenorphine	91.3 ± 5.2	85.7 ± 6.4	100.2 ± 1.2
Codeine-6-glucuronide	88.4 ± 6.8	72.3 ± 9.2	98.3 ± 3.2
Ecgonine methyl ester	84.2 ± 6.3	76.8 ± 10.5	97.8 ± 2.8
Isoxsuprine	92.3 ± 3.9	84.7 ± 7.7	98.8 ± 1.5
Levallorpham	98.2 ± 2.8	92.6 ± 6.7	97.9 ± 1.7
6-Monoacetyl morphine	89.2 ± 5.8	77.4 ± 7.9	98.4 ± 4.2
Morphine	84.3 ± 6.2	68.7 ± 9.4	99.2 ± 2.3
Pyroxicam	96.2 ± 3.1	83.1 ± 4.3	101 ± 1.2
3-Pyridylacetontrile	51.4 ± 7.2	43.7 ± 9.5	99.5 ± 1.8

However, it can only recover 2-aminopyridine partially. In addition, the reproducibility of 2-aminopyridine cleaned by the same lot of Varian Certify is relatively poor.

Argonaut Isolute IST Confirm HCX cartridges, with an ion exchange capacity at 1.2 mg atrazine per gram, also has a partial recovery for 2-aminopyridine.

On the other hand, only the novel C_8+SCX mixed mode offers a full recovery with excellent reproducibility (102 ± 3.5%) (Table 2).

Traditionally, mixed mode SPE products are used to clean and recover non-polar basic compounds from biological fluids. According to manufacturers' instructions, only analytes exhibiting both non-polar and basic characteristics are retained on the mixed mode SPE column during interference elution and, then, they are subsequently eluted as an extremely pure extract. Unfortunately, in many practical cases, even though the drugs themselves are non-polar, their metabolites are frequently hydrophilic. If a mixed mode SPE product cannot retain and clean both non-polar and polar basic compounds, its applications will be severely limited. This report represents a proving ground that the novel mixed mode SPE product can also fully recover polar basic compounds when other vendors do not.

In Table 2, 2-aminopyridine, 3-pyridylacetontrile, and ecgonine methyl ester are polar basic compounds. The other ten compounds are actually zwitterions compounds, possessing both acidic and basic groups. Benzoylecgonine has a carboxyl group, which probably dissociates at pH 6.0. All the other compounds have phenolic groups in their structures. When the samples are loaded on the SPE 96-well plates at pH 6, the interactions between these compounds and the SPE sorbent may be very weak, and, thus, a portion of some polar compounds are lost in the loading and washing steps.

During the test, the methanol fractions are also collected and analyzed for premature elution into the neutral and acidic fraction.[41,45] Almost all the compounds in Table 2 partially elute in the methanol fraction, to different degrees, when Varian Certify and IST Confirm HCX are used. For instance, although piroxicam has a recovery of 96.2 $\pm$ 3.1 in the final fraction from a Varian Certify cartridge, the compound is detectable in the corresponding methanol fraction (chromatogram not shown). The fractionation is incomplete and the variety from well to well and from lot to lot will adversely affect the reproducibility.

On the other hand, no premature elution for all the compounds has been observed when the novel C_8+SCX mixed mode SPE is used.

Nishikawa et al. found that about 15% ecgonine methyl ester passed through Varian Bond Elut Certify™ cartridges.[26] Hernandez et al. extracted cocaine and metabolites in the brain using Clean Screen™ mixed mode SPE and had recoveries at 50–60%.[27] Bogusz et al. noted very variable absolute recoveries (from some 8% to over 100%) when his groups used four brands of mixed phase extraction columns for opiate extraction from blood and serum.[48,49] Although, many experimental factors such as matrix types, extraction methods, particle size distribution of the SPE phases, analytical, and derivative methods may affect the final results, the present study shows that a potential bleeding of polar basic compounds, as well as zwitterions compounds during the loading study, and the premature elution into the methanol fraction, should be the major reasons. It seems that the improved version of C_8+SCX mixed mode SPE product has eliminated the risk.

pH 3.0 Generic Extraction Protocol

In the real applications, a large variety of compounds with very different polarities are presented simultaneously, in addition to the matrix components. In systematic toxicological analysis, a broad spectrum, undirected and unknown drug screening with a single SPE column requires a more versatile generic protocol.[1]

For non-polar basic compounds and zwitterions compounds that have no bleeding during the pH 6.0 loading, and no premature elution into the methanol fraction, the first generic protocol[29,50] is suitable and it also offers a very clean background.

On the other hand, it is found that some polar basic compounds, weakly basic compounds, and zwitterions compounds cannot fully be retained on the mixed mode SPE cartridges or 96-well plates at pH 6.0. The solution to the problem is to lower the pH values and lock the compounds into positive charge states. Then, the ion exchange interaction plays a vital role in retaining the compounds when the matrixes are cleaned. However, even with a low pH loading, the mixed mode SPE products from the current

vendors have the risk of losing polar basic compounds, weakly basic compounds, and zwitterions compounds.

5-Amino-4-imidazolecarboxamide hydrochloride is presented, herein, as a typical case. 5-Amino-4-imidazolecarboxamide is the terminated metabolite of a powerful cancer drug, darcarbazine, which is routinely employed in combination with other chemotherapy agents for treatment of Hodgkin's disease, melanoma, and soft tissue sarcoma.[51] Darcarbazine pharmacokinetics has focused on the plasma disposition of parent darcarbazine and the terminated metabolite 5-amino-4-imidazole-carboxamide. Substantial concentrations of 5-amino-4-imidazole-carboxamide in plasma implicate metabolism as a major route of darcarbazine elimination.[51]

5-Amino-4-imidazolecarboxamide is not retained on traditionally reversed phases because of little hydrophobic interaction. Instead, the metabolite can be only retained on mixed mode SPE tubes mainly by cation exchange interaction. If the cation exchange interaction is not strong enough, the compound will bleed out from the tubes during the loading and methanol washing.

5-Amino-4-imidazolecarboxamide (1.0 mL of 2 ug/mL) was led through various mixed modes of SPE tubes and its recovery was measured. Comparing Figures 2 and 3, it is quite clear that most of the metabolite is lost in the SPE cleanup process when the mixed mode SPE cartridges from Agronaut is used.

The chromatogram of this compound recovered from a Varian Bond Elut Certify cartridge is shown as Figure 4. The product with an ion exchange capacity of 2.4 mg atrazine mg/g offers a better, but partial recovery for this compound.

Trusted results can be readily obtained only with the improved version of the mixed mode SPE product. The novel product offers 93.4% recovery for 5-amino-4-imidazolecarboxamide and excellent reproducibility from tube

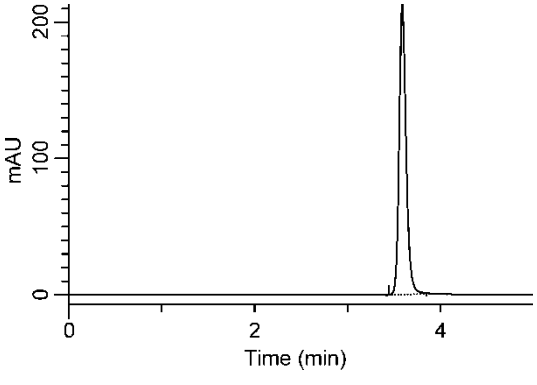

Figure 2. 2.0 μg/mL of 5-amino-4-imidazolecarboxamide on a Tosoh Bioscience Amide 5 um 10 cm × 4.6 mm column. Mobile phase: 35% acetontrile and 65% Milli-Q water containing 0.1% formic acid.

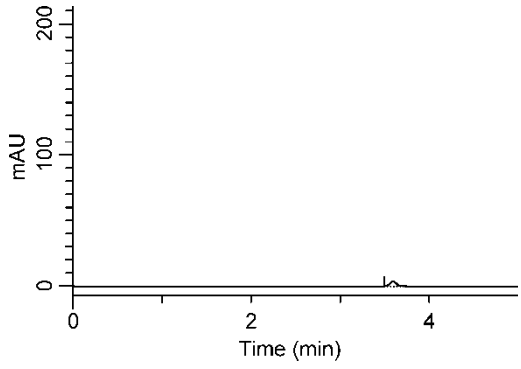

Figure 3. The compound was led through a mixed mode 130 mg/3 cc SPE tube from Agronaut IST Confirm and then recovered by 5% ammonium hydroxide in methanol. The recovery of 5-amino-4-imidazolecarboxamide was from 2.7% to 11.2%. The chromatographic condition was the same with Figure 2.

to tube ($\pm 2.5\%$, $n = 4$). The representative chromatogram is shown in Figure 5.

Hajouj et al. mechanically blended a silica bonded C_{18} and a silica bonded propylsulfonic phase at different mixed ratios and studied the extraction recoveries of three weakly basic compounds.[52] When quinoline and acridine can be fully recovered from both of their own experimental phases and Varian Certify, with a total mass of 200 mg adsorbent, the more polar and weakly basic compound, phenazine, has a low recovery from the experimental phases in various mixed ratios.

All the three weakly basic compounds dissolved into 10 mM phosphate buffer at pH 3.0 (10 ug/mL for each compound) can be fully retained and simultaneously recovered from the improved version of mixed mode SPE

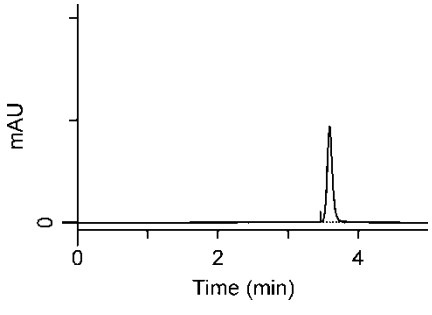

Figure 4. 5-Amino-4-imidazolecarboxamide was led through the mixed mode 130 mg/3 cc SPE tube from Varian Certify and recovered by 5% ammonium hydroxide in methanol. The recoveries for the compound varied from tube to tube over a wide range from 32% to 48%. The chromatographic condition was the same with Figure 2.

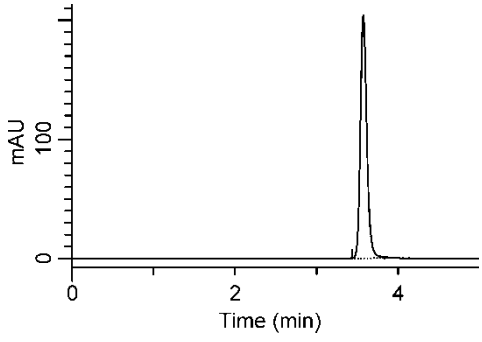

Figure 5. 5-Amino-4-imidazolecarboxamide was led through a novel SPE 130 mg/3 cc tube and then recovered by 5% ammonium hydroxide. The chromatographic condition was the same with Figure 2.

100 mg/3 cc tubes. The recoveries are 98.3 ± 1.4% for acridine, 97.9 ± 0.9% for phenazine, and 101.5 ± 2.3% for quinoline.

For zwitterions drugs, poisons, and metabolites that cannot be fully retained on the mixed mode SPE products at pH 6, the pH 3.0 generic protocol is also recommended. At lower pH (e.g. pH 3.0), the acidic group in a zwitterions compound is not dissociated when its basic group is positively charged. Thus, the zwitterions compound will be tightly adsorbed on the mixed mode SPE adsorbents during the loading and washing. Finally, the compound is eluted by ammonium modified methanol (or other solvents). Following these protocols, we repeat the applications in the cited literatures[4,31–40] and the representative data are listed in Table 3.

Terbutaline is a very polar β_2-agonist.[32] When R-(−)-terbutaline and S-(+)-terbutaline were loaded on Bond Elut Certify™ cartridges at pH 5.2, their recoveries were somewhat lower than 50%.[32] Here, an average recovery at 90.4% is reported when terbutaline is loaded on the improved version of mixed mode SPE product at pH 3.0. In addition, the recovery of S-(+)-Salbutamol, a selective β_2-adrenoceptor agonist is higher than the value from Bergés et al.[36]

Such a comparison may not be conclusive because it is not side by side. However, it is confirmed by the present study that the benzodiazepines (e.g., alprazolam, diazepam, estazolam, nordazepam, oxazepam) as well as other zwitterions compounds are frequently distributed into two fractions, i.e., the methanol fraction and the final fraction, when the mixed mode SPE cartridges from the current vendors are used. Diazepam is used as a representative example to further explain the process.

Diazepam was clearly detected in the methanol fraction when the mixed mode SPE cartridge from Agroaunt IST Confirm HCX was used. The chromatogram is shown as Figure 6.

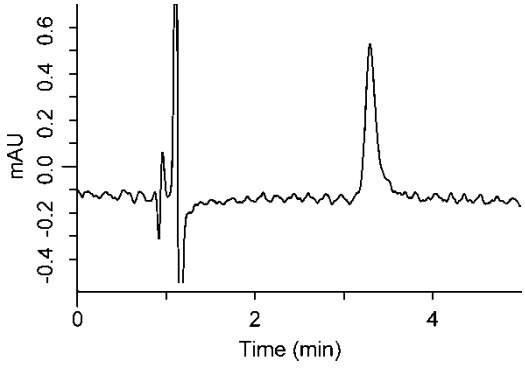

Figure 6. 1 μg/mL diazepam recovered from the mixed mode 100 mg/3 cc SPE tube from Confirm HCX by methanol. Column: Zorbax Eclipse XDB C18 3.5 um 10 cm × 4.6 mm; Flow rate: 0.5 mL/min; Mobile phase: 35% 10 mM ammonium acetate and 65% acetontrile; 220 nm detection.

The partial elution of diazepam into the methanol fraction is also observed when the mixed mode SPE cartridge from Varian is used, although the degree is much less (Figure 7). The only product to hold this compound strongly is the improved version of mixed mode SPE (Figure 8). A full recovery of diazepam was obtained with the novel product (Table 3).

Another example is piroxicam. Even though the pH is adjusted from pH 6.0 to pH 3.0, piroxicam is detectable in the methanol fraction when Varian Certify or IST Confirm HCX is used (chromatograms not shown).

Takeda et al. used Bond Elut Certify™ 1 g/6 mL column for the fractional extraction of the acid neutral and basic drugs in 4 mL of horse

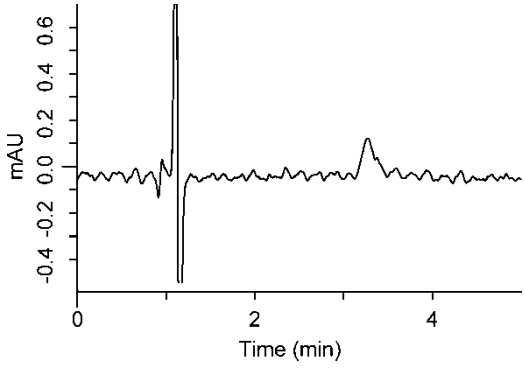

Figure 7. Diazepam (1 μg/mL) recovered from the mixed mode 130 mg/3 cc SPE tube from Varion Certify by methanol. Chromatographic condition: same with that in Figure 6.

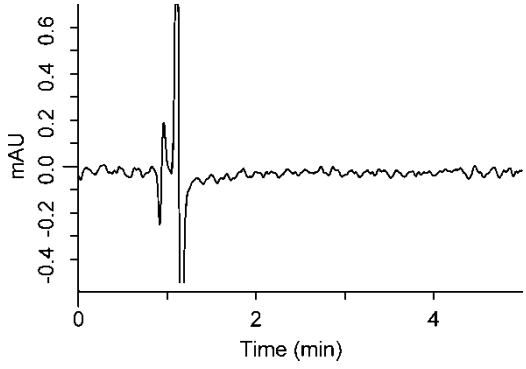

Figure 8. Diazepam (1 μg/mL) recovered from the novel 100 mg/3 cc SPE tube by methanol. Chromatographic condition: same with that in Figure 6.

plasma.[41] Piroxicam eluted into the acid neutral fraction with an average recovery of 67.5%. Nishikawa et al. found that about 100% ecgonine passed through Varian Bond Elut Certify™ cartridges at pH 6.0 loading.[26] Huang et al.[37] and Chen et al.[38] observed that diazepam, estazolam, and oxazepam eluted into the acidic and neutral fraction. On Bond Elut Certify™ manual,[29] benzodiazepines and methaqualone were extracted using hydrophobic interactions for retention when ion exchange, as well as

Table 3. Absolute recoveries (mean ± S.D., n = 4) (%) of polar zwitterions compounds by the pH 3.0 generic extraction protocol

Drug	Average recovery (%)
Alprazolam	98.7 ± 1.9
Brombuterol	99.7 ± 1.1
Buprenorphine	102.4 ± 4.6
Clenbuterol	104.8 ± 3.3
Diazepam	96.3 ± 2.6
Ecgonine	92.9 ± 3.8
Estazolam	101.8 ± 2.8
Mabuterol	89.3 ± 4.2
Mapenterol	94.5 ± 2.7
Methaqualone	99.5 ± 2.2
Nordazepam	94.8 ± 3.2
Oxazepam	92.8 ± 3.8
Oxytetracycline	87.9 ± 3.3
Pyroxicam	101 ± 2.3
S(+)-Salbutamol	91.4 ± 2.2
Terbutaline	90.4 ± 3.2

secondary polar interactions, were used to remove interferences. Nevertheless, Zweipfenning et al.[42] found that methaqualone and oxazepam were distributed in both the neutral/acidic fraction and the basic fraction, using Bond Elut Certify™ cartridges. Polettini et al.[4] also used a Bond Elut Certify™ column to extract benzodiazepines. When alprazolam, diazepam, and nordazepam were found in the basic fraction, ozazepam was found in both the acidic neutral fraction and the basic fraction.[4]

Using a low pH buffer (like pH 3.0) for the loading should reduce and even eliminate the risk of bleedings of polar basic compounds, polar zwitterions compounds, weakly basic and zwitterions compounds, because at pH 3.0 the ionic exchange interaction becomes much stronger. Unfortunately, such an action cannot fundamentally alter the fact that zwitterions compounds distribute into the two fractions when the mixed mode SPE products are from current vendors. The distribution makes further analysis complicated. In addition, it is found that the total mass of SPE adsorbent, the volume of the used methanol or other solvents, and particularly the variation of the ion exchange capacity from lot to lot, have significant influence on the distributed ratios of zwitterions compounds in the two fractions. It is the reason for the poor reproducibility.

Such a problem has not been found with the improved version of mixed mode SPE product, where the zwitterions compounds just elute into the final fraction with very clean backgrounds.

Systematic Fractionation and Analysis of Acid, Neutral, Zwitterions and Basic Drugs

It is well known that C_8+SCX mixed mode SPE cartridges can be also used for systematic fractionation and analysis of acid, neutral, and basic drugs.[29,41–45] In the earlier studies,[42–45] acid, neutral, and basic drugs in biological fluids were loaded on a single mixed mode SPE cartridge at pH 6.0. Although, the pH 6.0 generic protocol offers more cleaner background, many acidic compounds like nonsteroidal anti-inflammatory (NSAIDS) are deprotonated, ionized, and therefore, not retained. So are some polar basic compounds, weakly basic compounds, polar zwitterions compounds, and the zwitterions compounds with weakly basic groups, as discussed above.

Takeda et al.[41] proposed an improved protocol for systematic fractionation and analysis of acid, neutral, and basic drugs. The loading started at pH 3.0 and a 1 g/6 mL Bond Elut Certify™ cartridge was used instead of the 300 mg/3 cc cartridges in order to assure satisfactory recoveries for acid and neutral drugs.[41] Before the GC-MS analysis, both of the fractions were further cleaned by non-aqueous partitioning.[41]

We examined the systematic fractionation of a number of neutral, acid, zwitterions and basic compounds by the improved version of the mixed mode SPE product and the current versions of mixed mode SPE products

according to Takeda et al's method.[41] The names of the zwitterions and basic compounds have been listed above. The neutral and acidic compounds include acetominophenol, allobarbital, amobarbital, barbital, benzoic acid, caffeine, cyclobarbital, 3-oxocycloarbital, flufenamic acid, ibuprofen, ketoprofen, hydroketoprofen, loxoprofen, mefenamic acid, mephobarbital, metharbital, naproxen, p-nitrobenzoic acid, paclitaxel, pentobarbital, penoarbital, secobarbital, tolfenamic acid.

All the acid and neutral drugs can be clearly separated from basic and zwiterions drugs by the novel mixed mode SPE products. All the compounds have higher recoveries (more than 95%). On the other hand, as discussed above, zwitterions compounds and polar basic compounds frequently coexist in both fractions when the mixed mode SPE cartridges from the other vendors are used. Therefore, the improved version of the mixed mode SPE product is more valuable for systematic fractionation.

The fractionation of 2-aminobenzoic acid (anthranilic acid) from benzoic acid and p-nitrobenzoic acid is presented here as a typical example.

A 1.0 mL 10 mM phosphate buffer containing 10 ug/mL of anthranilic acid, benzoic acid, and p-nirobenzoic acid (pH 3.0) was loaded on 300 mg/3 cc of the novel SPE cartridge and other vendors' SPE tubes, that were previously rinsed by 3 mL methanol and equilibrated with 3 mL of the blank buffer. After the loading, 3 mL of the buffer was used for further elution. The acid fraction was eluted by 3.0 mL methanol and the basic fraction was eluted by 3.0 mL methanol containing 5% ammonium hydroxide. The eluates were dried and redissolved into 1.0 mL HPLC mobile phase. The HPLC chromatogram of the original test mixtures is shown in Figure 9.

Anthranilic acid can be thoroughly separated from benzoic acid and p-nitrobenzoic acid by the improved version of the mixed mode SPE

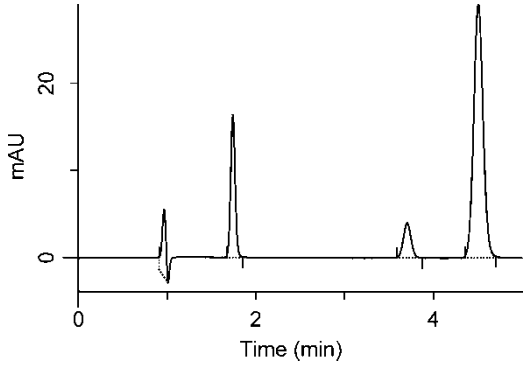

Figure 9. Separation of anthranilic acid, benzoic acid, and p-niotrobenzoic acid by *Innovation*® C_{18} 5 um 15 cm × 4.6 mm column. Flow rate: 2.0 mL/min; 254 nm detection; 60% water and 40% methanol (0.1% TFA). Concentration: 10 ug/mL. Solute I: anthranilic acid; Solute II: benzoic acid; Solute III: p-nitrobenzoic acid.

product. The results are shown in Figures 10 and 11. Anthranilic acid only elutes into the basic fraction (Figure 11).

On the other hand, the mixed mode SPE products from other vendors fail in the fractionation because anthranilic acid distributes in both fractions. Figures 12 and 13 are the representative chromatograms from Varian Certify with an ionic exchange capacity of 2.4 mg atrazine per gram. Anthranilic acid is observed in the acidic fraction (Figure 12) and, thus, its recovery in the final fraction is only 77% (Figure 13). IST Confirm HCX has an ionic exchange capacity at ca. 1.2 mg atrazine per gram and, thus, the majority of anthranilic acid elutes into the acidic fraction (Figure 14). Its recovery in the final fraction is only 7% (Figure 15).

The Importance of Ion-Exchange Capacity

The main reason why the mixed mode SPE products from current vendors cannot recover polar basic compounds, weakly basic compounds, and many zwitterions compounds into the final fraction is their low cationic exchange capacity. We systematically manufacture experimental mixed mode SPE material with a different degree of cationic exchange bonded coverage in house, and compare their performance on these questionable compounds discussed above. The recoveries of the compounds eluted into the base modified methanol increases in accordance with increasing ionic exchange capacity, and the recoveries finally become constant for most of the compounds when the ion exchange capacity of the SPE material is over 3.6 mg atrazine per gram. In other words, below the critical value, the distributions of zwitterions compounds into both fractions and incomplete recoveries of very polar basic compounds are observed. Based on the results, we

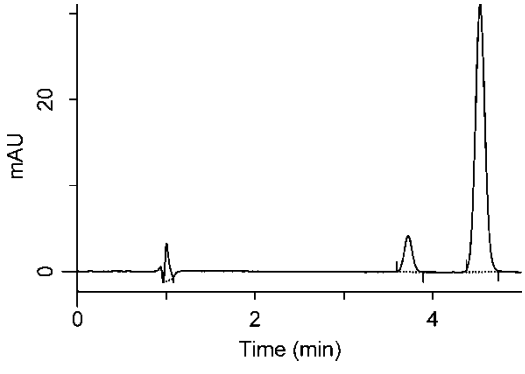

Figure 10. Acidic fraction by the Chrom-Matrix mixed mode SPE. Chromatographic condition: same with Figure 9.

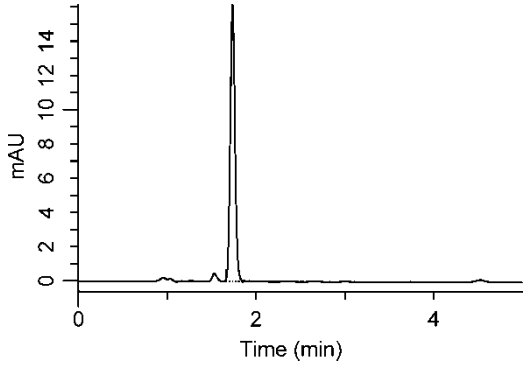

Figure 11. Basic fraction by the Chrom-Matrix mixed mode SPE. Chromatographic condition: same with Figure 9.

manufacture this kind of SPE material with an optimized ion exchange capacity at 4.2 mg atrazine per gram.

The importance of ion exchange capacity has been shown in the examples described above. Varian Certify has an ion exchange capacity at 2.4 mg atrazine per gram and the value from Confirm HCX is 1.2 mg atrazine per gram. Therefore, 77% anthranilic acid elutes into the basic fraction when the mixed mode SPE product from Varian Certify is used (Figure 13), whereas only 7% of the polar zwitterions compound elutes into the basic fraction with the product from IST Confirm HCX (Figure 15). A similar trend happens for many other polar compounds.

In addition, probably ethylbenzene sulfonic groups may bring an additional planar-planar interaction and, thus, phenazine can be completely

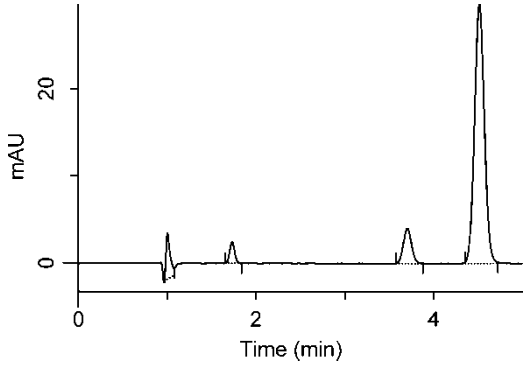

Figure 12. Acidic fraction by Varian Certify. When benzoic acid and p-nitrobenzoic were completely recovered in this fraction, a part of anthranilic acid was also eluted into this fraction.

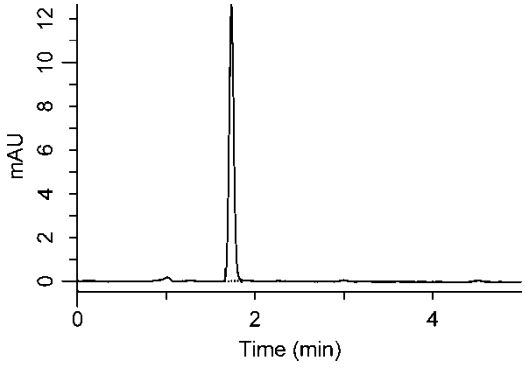

Figure 13. Basic fraction by Varian Certify. Anthranilic acid, 77% was recovered into this fraction.

retained in both Bonded Elut Certify and the novel product. This compound can not be fully retained and recovered from mixed phases by dry mechanical blending of C_{18} and propylsulfonic phases at any mixed ratios.[51]

The ionic exchange capacity of the novel C_8+SCX mixed mode SPE material is 1.5–4 times higher than those from the current vendors. A possible concern is that higher coverage of SCX groups may result in higher extraction backgrounds. However, the side effect has not been observed yet. The background of urine, serum, and plasma extracted by the novel SPE and other silica based mixed mode SPE adsorbents have been compared side by side, and the results are very similar (not shown). Particularly, when the pH 6.0 generic protocol is used, among all the types of SPE materials, silica based mixed mode adsorbents offer the cleanest background

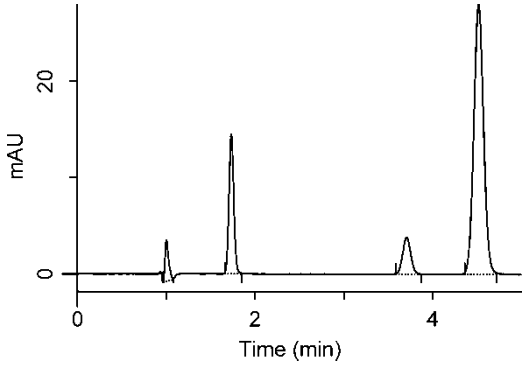

Figure 14. Acidic fraction by Confirm HCX. When benzoic acid and p-nitrobenzoic were completely recovered in this fraction, 92% anthranilic acid was also eluted into this fraction.

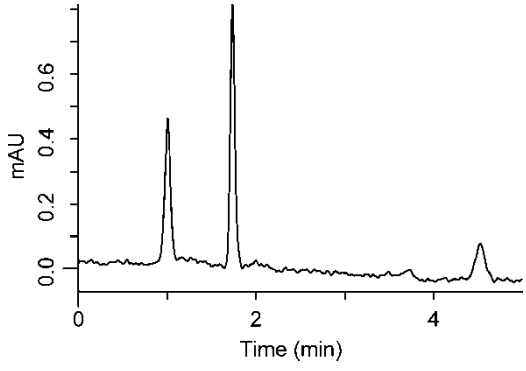

Figure 15. Basic fraction by Confirm HCX. Anthranilic acid, 7% was recovered into this fraction.

for non-polar basic drugs. When the novel SPE product is used, the conclusion is further extended to the zwitterions drugs and polar basic drugs that can be completely loaded at pH 6.0.

Lot-to-Lot Reproducibility

Chrom-Matrix Incorporation manufactures the improved version of mixed mode SPE material in kg scale and tightly controls the ionic exchange capacity into a narrow range. The recoveries of TCA compounds by the pH 6.0 generic protocol, 2-aminopyride and piroxicam by the same protocol, fractionation of 2-aminobenzoic acid and diazepam from benzoic acid, acetaminophen and paclitaxel by the pH 3.0 protocol were examined for quality control. For TCA compounds, 2-aminopyridine and piroxicam at 4 ug/mL, the recoveries are higher than 95% and the experimental error is lower than 3%. For systematical fractionation of zwitterions compounds from neutral and acid fraction, the typical data are shown in Table 4. Excellent lot-to-lot reproducibility has been obtained hitherto.

How to Choose the Generic Protocols?

The first principal application of the improved version of C_8+SCX mixed mode SPE cartridges and 96-well plates is a systematic toxicological analysis.

Numerous publications on SPE for biological fluids have been reported, but most SPE adsorbents and methods just deal with the extraction of single drugs, or groups of related drugs.[1,53] Just a few are capable of detecting a full range of common drugs of abuse, and common ethical drugs.[1,53] Among the few, C_8+SCX mixed mode SPE operates more easily, faster,

Table 4. Data from QC tests of Chrom-Matrix C8 + SCX Mixed mode SPE lots

Compound	Lot 1	Lot 2	Lot 3
2-Aminobenzoic acid recovered in methanol	Not detectable	Not detectable	Not detectable
2-Aminobenzoic acid recovered in final	Full recovery	Full recovery	Full recovery
Diazepam recovered in methanol	<1%	<1%	<1%
Diazeam recovered in final	>95%	>95%	>95%
Benzoic acid recovered in methanol	Full recovery	Full recovery	Full recovery
Benzoic acid recovered in final	<0.2%	<0.2%	<0.2%
Acetaminophen recovered in methanol	Full recovery	Full recovery	Full recovery
Acetaminophen recovered in final	<0.2%	<0.2%	<0.2%
Paclitaxel recovered in methanol	Full recovery	Full recovery	Full recovery
Paclitaxel recovered in final	Not detectable	Not detectable	Not detectable

and frequently offers cleaner extraction.[1,53] The systematic toxicological analysis is employed to detect every presented harmful compound in trace amounts, identify them, differentiate between closely resembling ones, and quantitate them within a reasonable amount of time. In order to achieve this purpose, the mixed mode SPE cartridge must capture the drugs and metabolites as much as possible, fractionate the acid and neutral fraction from zwitterions and basic drugs, and also clean the biological fluids thoroughly. Thus, the generic protocol developed by Takeda[41] with further modification should be used.

We believe that the improved version of C_8+SCX mixed mode SPE product from Chrom-Matrix Incorporation extends the application range and improves the performance. First, most polar basic compounds, weak basic compounds, and polar zwitterions compounds will not be lost in the screening test. Second, zwitterions compounds can be completely fractionated into the final fraction, simplifying further analysis. In the previous studies,[4,29,37,38,41–45] some zwitterions compounds were defined as neutral compounds. However, at pH 3.0, the acidic groups are protonated and the basic groups act as positive charge states. Then the zwitterions compounds act as bases and should elute into the final fraction, which has a much cleaner background.

In biomedical applications, the targets are specific and, thus, many SPE materials are potentially suitable and competitive for the specific applications. Among them, silica based C_8+SCX mixed mode SPE cartridges and 96-well

plates offer the most pure extract fraction for non-polar basic compounds. The improved version of mixed mode SPE extends the unique advantage for zwitterions compounds. The situation is particularly true if the compounds can be fully retained during the loading at pH 6.0 and the pH 6.0 generic protocol can be used. When method developments are frequently necessary for other types of SPE products, the generic protocols can be used directly with little modification.

In the case when polar basic, weakly basic, and polar zwitterions compounds cannot be fully retained at pH 6.0, a slight adjustment of the pH value toward the acidic range is recommended. For example, if a complete adsorption of the compounds on the improved version of the SPE product occurs at pH 5.0 during methanol washing, it is not necessary to use lower pH values, because higher pH values usually make cleaner backgrounds.

CONCLUSIONS

When silica based C_8+SCX mixed mode SPE has been considered as the most powerful and popular tool for sample work up (i.e., cleanup and concentration) in the systematic toxicology analysis, the present study offers solid evidence that the products from current vendors have the risk for losing weakly and polar basic compounds, and polar zwitterions compounds during the extraction. In addition, some zwitterions compounds coexist in both the neutral/acid fraction and the basic fraction, making further analysis complicated. The root of the problems is from too low cationic exchange capacity. Although, only C_8+SCX mixed mode SPE products from Varian Certify and Agronaut IST Confirm HCX are mentioned herein as the comparison, the same problem happens for the same type of products from the other vendors mentioned above.

While some authors attributed the reasons of the poor reproducibility to particle size distribution and other factors,[46–49] we believe that insufficient cationic exchange capacity and the variation of the cationic exchange capacity play a vital role. When the cationic exchange capacity is lower than a critical value, any changes regarding the types and the amount of the elution solvents, the total amount of SPE material used, the variation of ion exchange capacity from lot to lot, and other experimental factors, will affect the reproducibility. Even though the same columns and the same elution protocols are used, the reproducibility is still poor in some cases, due to the same reason.

The major improvement of the new version of C_8+SCX mixed mode SPE product is to optimize the SCX bonded coverage. As a major characterization data, the cationic exchange capacity of the novel product is 1.5-4 times higher than those from the current vendors. As a result, zwitterions compounds entirely elute in the final fraction, not only having very clean

backgrounds, but also assuring the reproducibility from cartridge to cartridge, from lot to lot. Further analysis will also become well planned and concise. In addition, polar basic compounds, weakly basic compounds, and polar zwitterions compounds can be well recovered. The improvement is very important for both systematic toxicological analysis (STA) and biomedical applications.

ACKNOWLEDGMENTS

This work was funded in part by the National Science Foundation of China (30470277), Gansu National Science Foundation (ZS031-A25-039-D), and Gansu Key Technologies R&D Program (ZGS052-A41-0006-03). The content of the work reflects the personal view of the author and not necessarily the view of the organization.

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Received December 5, 2005
Accepted December 30, 2005
Manuscript 6774