Automated Solid-Phase Extraction Approaches for Large Scale Biomonitoring Studies*

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Abstract

The main value in measuring environmental chemicals in biological specimens (i.e., biomonitoring) is the ability to minimize risk assessment uncertainties. The collection of biomonitoring data for risk assessment requires the analysis of a statistically significant number of samples from subjects with a significant prevalence of detectable internal dose levels. This paper addresses the practical laboratory challenges that arise from these statistical requirements: development of high throughput techniques that can handle, with high accuracy and precision, a large number of samples and can do a trace level analysis of multiple and diverse environmental chemicals (i.e., analytes). We review here examples of high throughput, automated solid-phase extraction methods developed in our laboratory for biomonitoring of analytes with representative hydrophobic properties and for typical biomonitoring matrices. We discuss key aspects of sample preparation, column, and solvent selection for off- and online extractions, and the so-called nuts-and-bolts of online column-switching systems necessary for developing—with minimal sample handling—rugged, automated methods.

Introduction

Biomonitoring is a useful tool for providing information on human exposure to environmental and to workplace chemicals. Biomonitoring involves the collection and analysis of biological specimens along with related demographic, lifestyle, and general health information (1,2). The concentration of the chemicals or their metabolites measured in these specimens assesses human internal dose levels—information that from the analysis of environmental samples alone would likely be more costly and complicated to estimate (1,2). Biomonitoring can be part of large-scale, cross sectional studies such as the National Health and Nutrition Examination Survey (NHANES) conducted by the U.S. Centers for Disease Control and Prevention and can be used to measure internal dose levels representative of the general U.S. population (3).

The quality and usefulness of biomonitoring data is related to their ability to establish statistically significant correlations between environmental factors and internal dose levels, thus minimizing the statistical uncertainties associated with the risk assessment (1,2). For the biomonitoring laboratory, the capacity to analyze a statistically significant number of samples can be just as important as the quality of the analytical measurement. Often, despite all efforts, including the control of sample collection practices (4,5) and using concentration corrections (based on lipid or creatinine content) (6), the precision of the analytical method can be overshadowed by the variability coming from the sample collection process (7). This variability can be minimized by collecting and analyzing biological specimens and information from a statistically significant number of subjects.

Yet even a moderate 50–100 sample/day/analyst throughput can be challenging, especially given the need to maintain good quality control oversight for the simultaneous measurement of 7–50 analytes/method with detection limits in the 0.1–1 part per billion range (typical for a biomonitoring method). To keep this workload manageable, walk-away automation is an indispensable part of biomonitoring laboratory instrumentation. Despite the recent advancements in laboratory automation, balancing throughput and sensitivity requirements against the cost of analysis is not a simple task. Successful quality–time–cost triangulation requires the skillful integration of techniques involving sample preparation, analyte extraction, preconcentration, and detection.

Minimal specimen usage is also very important. The ever-increasing number of chemicals measured from one single specimen (more than 200 for NHANES 2003–2004). The original specimen is usually divided among different analyte groups. For quality control and organizational reasons, it is often desirable that the analytes of interest are not necessarily grouped by chemical nature but by environmental occurrence or exposure route (e.g., pesticides, personal care products, disinfection byproducts, polychlorinated biphenyls). As a result, parent compounds and their metabolites with a wide range of physicochemical properties and concentrations need to be measured simultaneously.

Scope

The advancement of high throughput quantitative bioanalysis is demonstrated in excellent general reviews (8,9). Our focus in this paper is to discuss our experience and the general logistics

* Disclaimer: The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention.

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of analytical methods development unique to large-scale biomonitoring studies such as NHANES. And we limit our scope to organic environmental toxicants. Unlike typical basic heteroaromatic pharmaceuticals, the organic compounds most suitable for environmental risk assessment have an extended biological half-life. Naturally, these analytes are relatively nonpolar or polar but not easily ionized, which facilitates their retention in body compartments. The compound classes we use as examples (e.g., phthalates, environmental phenols, polyfluoroalkyl chemicals, and musks) are our specific interest. These analytes can be present in typical biomonitoring matrices such as urine, serum, breast milk, meconium, and semen. We will limit our discussion to solid-phase extraction (SPE) techniques coupled with high-performance liquid chromatography (HPLC) and gas chromatography (GC) analytical separation and mass spectrometry (MS) or tandem mass spectrometry (MS–MS) detection.

Sample Preparation

Urine samples

Urine is the matrix of choice for biomonitoring of many nonpersistent chemicals (10,11). Many of these chemicals are hydrophobic aromatic compounds. But before their excretion as urinary glucuronide and sulfate conjugates, they are made more hydrophylic in the human body through phase I and II metabolic pathways. These conjugates may degrade during storage and shipment (12–15); therefore, the conjugates are turned back to their free hydrophobic forms by enzymatic treatment, allowing for their straightforward separation from polar urinary matrix components by nonpolar SPE packing materials.

Urine specimens may also vary considerably in composition, concentration, pH, and homogeneity. Dilution of the urine sample before analysis is critical in reducing sample-to-sample matrix variations that can affect analyte recovery. Depending on the hydrophobic character of the analytes, the diluting solvent can be water or acid; in our laboratory, it is typically formic acid or phosphoric acid at ~0.1M concentration. Acidifying facilitates the suppression of H-bonding interactions of polar analyte functional groups with matrix components without the precipitation of endogenous matrix biomolecules. Many urinary biomarkers are phenyl-hydroxyl or -carboxyl derivatives. Addition of acid helps to keep them in their protonated forms and facilitates their binding to reverse phase stationary phases such as C8 or C18 silica or typical polymer phases such as polystyrene-divinylbenzene-*N*-vinyl pyrrolidone or polyvinylbenzene-styrene (14,16–18).

Serum samples

Because hydrophilic environmental toxicants in their free forms or as phase I metabolites circulate in the blood, they can be separated from serum for biomonitoring purposes. To minimize manual sample handling steps, precipitation of the plasma/serum proteins is avoided. After denaturation and ionization of serum proteins—typically with the addition of 0.1M formic acid—hydrophilic biomarkers can be separated by nonpolar SPE surfaces using the same or similar methods used for urinary metabolites after enzymatic treatment (14,16–18). Typical examples of such compound groups are phthalates and polyfluoroalkyl chemicals separated from serum on nonpolar or mixed polar/nonpolar SPE surfaces, taking advantage of their mixed ionic/nonpolar characteristics (19,20). Even with polyfluoroalkyl acids, which should stay deprotonated at pH > 1, we found that saturation of the SPE surface with 0.1M formic acid enhances the extraction efficiency for these organic anions (19,20), most likely by giving these nonpolar SPE phases a weak anion exchange character.

Breast milk samples

Lipophilic compounds may be secreted into fatty body compartments, and in the case of lactating women, into breast milk (21). After 4–10 times dilution with dilute acid, breast milk can be analyzed with analytical protocols similar to those developed for serum. In addition to acidifying, it is important to keep lipids in an emulsion state by addition of methanol or isopropanol in 20% concentration. For more complex matrices such as meconium and semen, similar protocols can be used. But longer agitation time followed by centrifugation may be necessary (22,23).

Solid-Phase Extraction

Offline SPE

Offline SPE cartridges, wash solvents, and eluents have to be selected with consideration of the often wide polarity range of the biomarkers. For example, the simultaneous quantification of environmental phenols can include bisphenol A, alkylphenols, chlorophenols, parabens, and benzophenone-3 (18). Except for the common phenolic OH functionality, the analytes display a wide range of chromatographic behaviors. Homologous series of phthalates or polyfluoroalkyl acids, having from short to long carbon chain alkyl functionality, present similar challenges (20,24).

Because the extraction of the analytes from the matrix is usually from a diluted aqueous liquid phase, the absorbency of the most polar analytes determines the choice of the nonpolar solid phase material, while the absorbent properties of the least polar analytes determines the choice of the organic solvent and its concentration used for elution. If the sorbent capacity to retain the analytes is not strong enough, the more polar analytes will be lost in the breakthrough or during the wash step(s). On the other hand, if the sorbent retention capacity is too strong, the more hydrophobic analogs will need a large volume of organic solvent to elute, which leads to a lengthy evaporation step. Usually, the best choice is a mixed-SPE phase with multiple types of interaction points: polystyrene-divinylbenzene-*N*-vinyl pyrrolidone (25,26), amine functionalized polyvinylbenzene-styrene (27), or alkylated but non-end-capped silica phases (20). Comprehensive listings of SPE phases can be found in excellent reviews (8,9,28,29). With complex matrices, (e.g., breast milk, semen, or meconium), an important SPE sorbent property is particle size. Sorbents with 30–60 µm particle size are preferred because they allow rapid passage of proteins and other endogenous materials through the support (30).

When throughput is a high priority, it is especially important that the analytes are eluted with solvents that do not interfere with the analytical HPLC or GC injection conditions. This is necessary to achieve the separation of the analytes of interest from each other and form other matrix biomolecules. Therefore, sorbents with strong ionic binding sites are avoided; they require a high concentration of salt or extreme pH for elution—conditions that are not optimal for HPLC or GC separation, and the sorbents have to be modified in additional separation steps.

When the analytes have a wide range of absorbency, automation is not only convenient, it is necessary for consistent analyte recovery, especially for the least and most hydrophobic analytes. In recent years, many new, automated offline SPE instruments able to accommodate a variety of sample size needs in high throughput well-plate formats have become available (9). Unfortunately, very few of the automated SPE systems have individual pressure sensors or plungers connected to each individual cartridge to provide precise flow control capability. For our biomonitoring applications, selecting an automated offline SPE system that was capable of controlling liquid flow with 0.1 mL/min accuracy was critical (16,19). However, these SPE systems can be expensive. A much more economical solution is the use of online SPE systems.

Online SPE coupled with HPLC

Online SPE is a column switching technique, similar to two-dimensional liquid chromatography (LC–LC). While the main goal of the LC–LC is the enhancement of separation between analytes, the objective of SPE-LC is the separation of the analytes from the matrix and preconcentration before the analytical LC separation. Column-switching applications have been in use since high-pressure, multi-port valves were available in the 1970s, and they have become increasingly popular in biomonitoring applications (31–35). One of the developments of recent years is the availability of integrated LC–MS control software packages, which allow user-friendly simultaneous programming of several column switching valves, autosampler(s), and HPLC pumps. With some creativity, numerous valve configurations can be plumbed. Such configurations can then allow for automated sample transfer between two or more columns and eluent systems with uninterrupted flow.

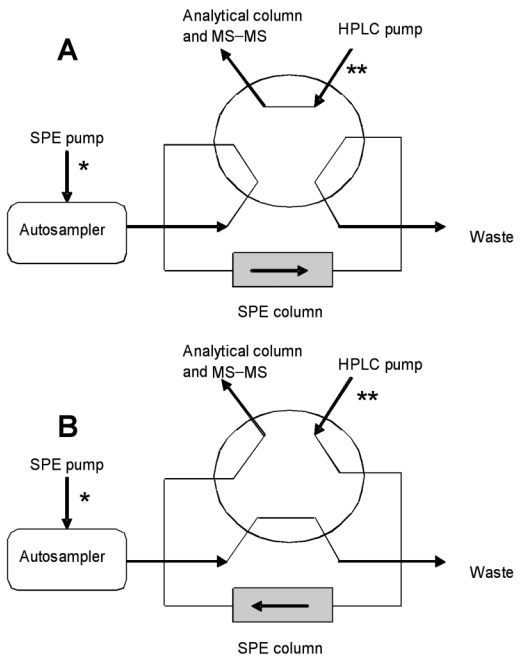

Figure 1. Six-port valve setup for online SPE. The SPE and HPLC pumps are both binary pumps running solvent gradients independently. The six-port valve is in position A during the sample loading and column washing periods, turned to B position during analyte transfer from the SPE column to the analytical column, then turned back to position A for the analytical gradient and regeneration of the SPE column.

* Position of SPE filter column,
** Position of analytical filter column.

Another recent development is the commercial availability of restricted access materials (9,28,36), that allow direct injection of biological fluids, such as restricted access media (14,37), monolithic materials (12,38), and disposable online columns (20,34). Here, we focused mainly on the nuts-and-bolts of the instrumental aspects that we found important for robust and long-term online SPE-LC operation.

Although instrument communication setups are determined by vendor-specific firmware and software, some general guidelines must be followed. Of course, the most convenient option is the use of a readily available integrated LC–MS software and hardware package. This allows for LC–MS synchronization by common (RS-232 or Ethernet) firmware communication protocols that can be programmed through a single user-friendly interface. From a practical point of view, the main convenience of the integrated software is that for each sample run, only one sample batch table has to be entered. Errors occurring in any part of the LC–MS system are more quickly detected, preventing sample loss. Such an integrated online system can, however, be expensive and may require dedication to a single vendor. Yet less expensive self-made systems can also be constructed from switching valves, LC devices, and an MS instrument sold by different vendors and operated by separate software/firmware interfaces. The system components are synchronized in a so-called handshake mode by the use of contact closure circuits built into most LC devices. These circuits are programmable by their own control software or firmware keyboard pads and allow the entry of contact closure timetables. After initiation of both batch tables, the computer triggers the autosampler to inject each sample. After each injection, the autosampler triggers the LC gradient pump program and the MS data collection. During each sample run, any of the LC devices can initiate the contact closure timetable controlling the switching valve(s). The main practical disadvantage of these handshake communication systems is that the sample batch table has to be entered into both the LC and MS software and requires more attention to details from the operator. But the important advantage of the separate software is that the LC devices and the MS data collection can be controlled independently in an overlapping mode. The analytical LC–MS detection and SPE cleanup of two consecutive samples can run at the same time, allowing for better instrument throughput.

For online SPE, at least one switching valve is required. When compounds with similar chromatographic behavior have to be extracted and preconcentrated, the now-ubiquitous 6-port valve configuration (Figure 1) works well. During sample loading

(Figure 1A), the flow leaving the SPE column is directed to a waste port. The switching valve is turned to its alternate position only during the time of elution of the analytes from the SPE column, known as "heart-cut" (Figure 1B). The six-port, online SPE system can be operated in both forward elute and backward (reverse flow) elution modes (32,36). Forward elution means that during sample load, the elution flow of the analytes from the SPE column to the analytical column moves in the same direction as the solvent flow, while backward elution means elution in the opposite direction. Turning from forward to backward elution mode requires simple manual reversal of only two ports (HPLC pump and analytical column, Figure 1). Backward elution is advantageous because of the refocusing of dispersed analyte bands due to large volume injection. Backward elution can also reduce the tailing of the chromatographic peaks from strongly absorbed analytes.

A general concern in biomonitoring applications of online SPE is that the analytes present in the unknown samples can cover wide magnitude concentration ranges. This increases the chances for cross-contamination through the autosampler or the SPE column. To solve this problem, it is necessary to use two binary (gradient) pumps (SPE pump and an HPLC pump, Figure 1). One solvent line of the SPE pump is used for sample loading, while the other solvent line is used for cleaning the SPE column and the autosampler injector lines before the next injection. In this way, cross contamination can be minimized to < 0.1%. Of course, the best but also the most expensive alternative is the use of disposable online columns and robotics that can exchange cartridges after each sample (20).

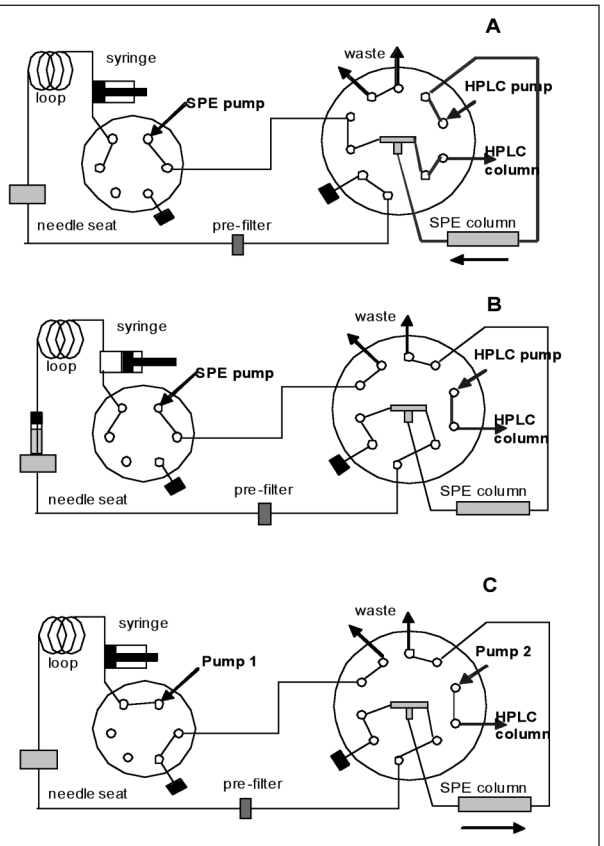

Figure 2. Ten-port valve setup for peak focusing. Analyte transfer and dilution (A), Sample loading (B), and SPE column wash (C). The six-port valve is a modified autosampler valve (14).

Another benefit of using two binary pumps is the ability to use different column sizes and flow rates for the SPE and the analytical separations. For the SPE column, dimensions of 10–50 mm × 3–5 mm with 5–60 µm particle size allow for 0.5–1 mL/min flow rates and pre-concentration from 100–900 µL injection volumes, while the analytical column can be a microbore column. With relatively narrow, small particle size analytical columns and with 50–200 µL/min flow rates higher analyte concentration can be achieved in the electrospray source, leading to better MS signal-to-noise ratios.

For the simple 6-port valve SPE-LC setup to work without compromising separation efficiency, peak shape, and resolution, the SPE and analytical columns and solvents have to be matched carefully. As a rule of thumb, the SPE column needs to have weaker absorbency for the analytes of interest than for the analytical column. This assures that during the elution from the SPE column to the analytical LC column, the analyte band will refocus on the front of the analytical column. If the hydrophobicity of the analytes covers a wide range, finding such SPE-LC column pairs and eluent conditions can be rather challenging. If the SPE column is not adsorptive enough, the most hydrophilic analytes will not be separated from the matrix, and the matrix effect may strongly suppress electrospray ionization. On the other hand, if the analyte absorbency is too strong, elution will not be efficient, and the chromatrograms will show broad tailing peaks.

Mixing in a modifier between the two columns during analyte transfer allows for more flexibility in matching SPE and HPLC column conditions. Figures 2 and 3 show homemade setups that do not require special equipment or software. Peak focusing is achieved by a simple T-junction combined with a 10-port valve (Figure 2). By mixing in an aqueous solvent after the SPE column, the analyte bands enter into the analytical column at low organic conditions and refocus into sharper bands. The interesting feature of the configuration shown in Figure 2 is that the peak focusing can be achieved without including a third high-pressure pump. Because of the peak focusing effect, both column efficiency and resolution on the analytical column could be preserved to the extent that even isomeric compounds (e.g., 2,4- and 2,5-dichlorophenols) could be separated (15).

The problem of increased pressure from higher flow rate during T-mixing can be solved by using a monolithic analytical column (Figure 2) (15). Another solution would be to direct the flow to a shorter precolumn during mixing, then after the mixing period connect a longer analytical column (switched) behind the precolumn as is made possible in the 6-port/10-port setup shown in Figure 3. The 10-port valve setups combined with a T-junction(s) also allow the coupling of very different chromatography separation modes—such as ion exchange—to reverse phase chromatography or ion-exchange to ion-pair chromatography.

Offline or online SPE?

Both offline and online SPE have their own challenges: with offline SPE, it is finding the right sorbent–solvent pair; with online SPE, it is finding the right SPE–analytical column pair.

The major advantage of online over offline SPE is that it eliminates the need for evaporation between the SPE and HPLC separations. There is also a clear gain in using smaller amounts of biological specimens and reducing sample handling and laboratory waste.

Given our comparison of validation results for offline and online SPE-HPLC methods, the two approaches are comparable in terms of the limit of detection (LOD) ranges, accuracy, and reproducibility (20,39). In case of homologous series of polyfluoroalkyl acids, the online SPE method performed significantly better for the shortest and the longest alkyl chain derivatives. This is because the heart-cutting of the analytes from the SPE elute was possible much more reproducibly and with better recovery with the online SPE than with offline SPE. Moreover, the short alkyl chain polyfluoroalkyl acids were more volatile and had a tendency to evaporate during the evaporation step after offline SPE.

Online SPE requires the use of minimal specimen amount—that is, only the amount necessary for one or two injections to achieve the desired LOD. Enzymatic treatment and dilution can be performed in one standard-size autosampler vial. By contrast, the ability to reduce sample size in automated offline SPE formats is limited. The sample size has to be large enough to minimize the proportion of sample lost in cartridges, pipettes, and tubes/wells required during the sample preparation steps. Often, this sample size is more than necessary for sensitive detection, only 10–30% of the prepared offline extract is injected. The rest is wasted.

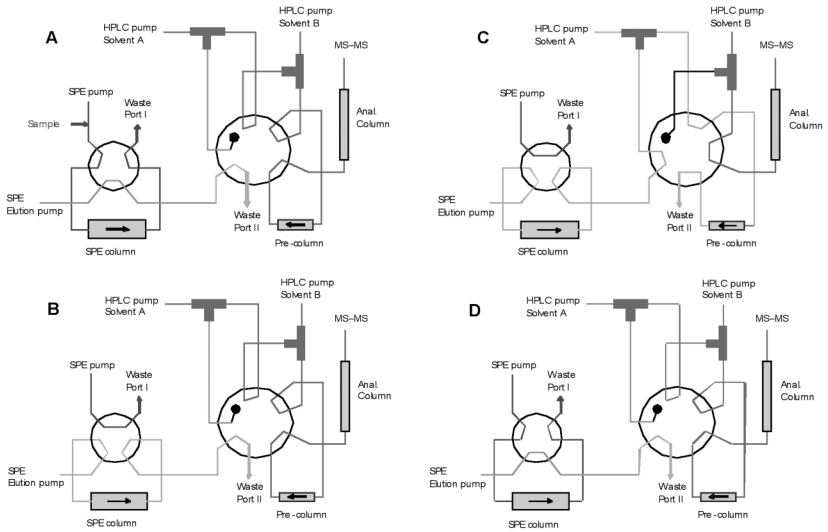

Figure 3. SPE-HPLC column coupling with six-port/ten-port valve combination: Load Sample (A), Wash SPE column (B), Elute SPE column with peak focusing (C), Elute analytical column (D).

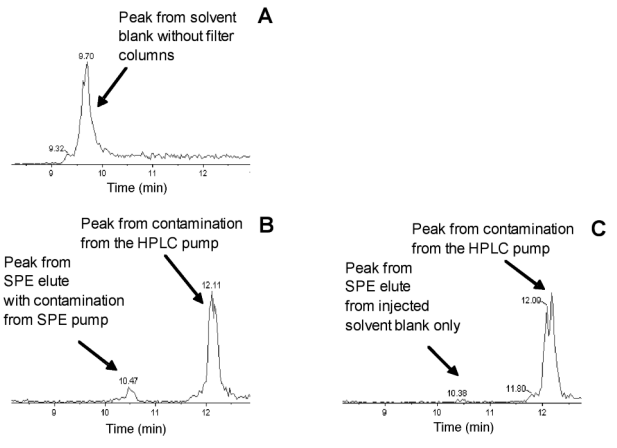

Figure 4. Separation of perfluorooctanoic acid (PFOA) contaminant originating from the online SPE-HPLC system. Solvent blank without filter columns (A); Solvent blank with analytical filter column only (B); Solvent blank with SPE and analytical filter-columns (C). SPE filter-column: 10 mm × 3 mm, 5 µm particle size Betasil C8 between SPE pump and autosampler; analytical filter-column: 100 mm × 3 mm, 5 µm particle size Betasil C8 column between HPLC pump and switching valve (see connection diagram in Figure 1).

One important limitation of environmental chemical sample size is the amount of background contamination coming from sample vessels and solvents. A matrix-matched calibration curve intercept should correct for the constant calibration bias caused by contamination. Yet higher background contamination also increases the standard deviation at 0 concentration (S_0). Using the definition of LOD = 3* S_0 (40), our conservative rule of thumb is that the proportion of the background contamination to the measured signal should not exceed 1/3 of the desired method LOD. With offline SPE, the proportion of the background contribution to the measured signal can be minimized by using a larger sample amount. With online SPE, due to minimal sample transfers and solvent use, contamination from reagents and sample vials are minimal. Still, during low aqueous conditions, contaminants originating from HPLC solvents and from tubing and other parts of the HPLC system accumulating on the SPE and HPLC columns is a possibility. This problem can be solved by incorporating filter-columns into the online SPE setup (Figure 1). In our laboratory, we use filter-columns with similar dimensions and packing as the SPE and analytical columns. A so-called SPE filter-column is put before the autosampler and retains the analyte contaminants coming from the SPE pump. Another analytical filter-column is put before the switching valve and retains the contaminants coming from HPLC gradient flow. Thus contaminants accumulating from both pumps will be retained separately from the analytes originating from the sample. The contaminants that accumulate on the SPE filter-column are removed at the same time the SPE columns are regenerated. The contaminants retained on the analytical filter-column will be eluted by the HPLC gradient but with a 1–2 min delay in retention time. This is due to the doubled column length migrated by the contaminants versus the analytes injected with the sample (Figure 4).

Although more labor intensive, offline SPE usually cannot be avoided when < 0.01 ng/mL method LOD is needed, which usually requires more than 1 mL of specimen. Highly complex matrices, such as breast milk or meconium, may have to be diluted 5–10 times to a volume too large and heterogeneous for

injection with regular HPLC autosamplers. A good compromise in these cases is the combination of offline SPE followed by online SPE-HPLC (41) or solid-phase microextraction (SPME)-GC (42). The role of offline SPE is to obtain a more concentrated, less hydrophilic or less lipophilic preextract to facilitate the online extraction. After offline SPE, only partial evaporation of the extract—enough to lower the organic content—is required. Further preconcentration can be achieved by SPE-LC or SPME-GC.

The SPE/SPME approach is especially convenient because it eliminates the need for aqueous to nonaqueous solvent exchange before GC–MS analysis. Compared with other multistep methods (43–45), we were able to simplify sample preparation, reduce sample size, and maximize selectivity and throughput (~30 unknowns per day) while still maintaining adequate sensitivity (LODs ranged from 0.03 to 0.3 ng/mL) (42).

A general opinion about online SPE is that it increases instrument time, which discourages its more widespread use. In biomonitoring applications, this is rarely an issue. Due to the wide polarity range of the analytes, the analytical run has to start at low organic eluent content. After the analytical solvent gradient, the column has to be equilibrated back to starting conditions. If the online SPE can be performed at the same time as the LC equilibration, no significant difference occurs between online SPE-LC or LC only run times.

Conclusions

We addressed the most important challenges of large-scale biomonitoring projects that make application of SPE-LC–MS techniques different from typical pharmaceutical applications: high throughput as well as trace level of sensitivity, and simultaneous analysis of numerous chemicals with a wide range of chemical properties a using minimal amount of sample. Most of the SPE-LC methods we used as examples were applied to large-scale projects: 300–3000 specimens with 50–100 sample/day/person throughputs, which included sample preparation, instrumental analysis, and data processing. We pointed out key aspects of sample preparation, column, and solvent selection necessary for development of rugged automated methods with minimal sample handling.

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