Solutions and Challenges in Sample Preparation for Chromatography

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Abstract

Sample preparation as practiced today in analytical laboratories is a complex combination of classical and modern techniques. In this review, the progress made in the last few years in sample preparation for chromatography is examined and discussed in comparison with previous achievements in the field. Discovery and development of new materials is seen as one of the main sources of progress, but finding better ways of using the old principles and the improvements in technology are also major contributing factors for advancement. Practical demands for analysis of pharmaceutical products, the environmental studies, and life science are the main driving forces for development in sample preparation for chromatography.

Introduction

Sample preparation has been used from the early times of chemistry as a process necessary for the transformation of samples to make them amenable for analysis. The famous experiments performed between 1772 and 1777 by Lavoisier for the analysis of air are good examples of sample preparation. For a long time analytical chemistry relied heavily on sample preparation to make the measurements possible. In time, technological advancements enabled the replacement of the old "wet chemistry" analyses with modern instrumental analytical techniques that are simpler and require less laborious processing of the sample. One such instrumental technique is chromatography. Although chromatography aimed to reduce sample preparation by incorporating a powerful separation, sample preparation was still needed. From the first chromatographic experiments performed by Tswett (1), sample preparation was used before the separation. The association between the old sample preparation and the newer chromatography remained strong from then on. However, the struggle to reduce sample preparation steps as much as possible continues. Sample preparation is considered the main cause of errors in chromatographic analysis (2). Also, it is typically rather costly because it requires more manpower than the core analytical procedure, needs more chemicals and laboratory consumables, and is more difficult to automate (3). Only the explosion in the variety of samples to be analyzed and the continuous lowering of the concentration of the analytes that are measured kept sample preparation very necessary. A description of sample preparation is still present today with virtually every reported chromatographic method. Solutions were found for many of the old problems, but new challenges continuously confront the field of sample preparation.

Discussion

General schemes for sample preparation

Each analytical method contains a core step. Differences in the principle of this core step lead to the classification of analytical techniques as chromatographic, thermal, electrochemical, spectroscopic, etc. The operations performed before the core analytical step (after the sample is collected) are considered sample preparation. A simplified flow diagram showing the place of sample preparation in a chemical analysis using chromatography as a core analytical procedure is given in Figure 1. This figure shows chemical analysis as a combination of information and operations with the typical structure: input ➛ process ➛ output. Sample preparation has the role to allow or to improve a specific analysis and may target the matrix of the sample, the analytes, or both. Among the operations included in sample preparation are modifications of the sample for dissolution, followed by cleanup, fractionations, and concentrations. The analytes also can be modified by chemical reactions (derivatization, etc.) to obtain better properties for the chromatographic analysis.

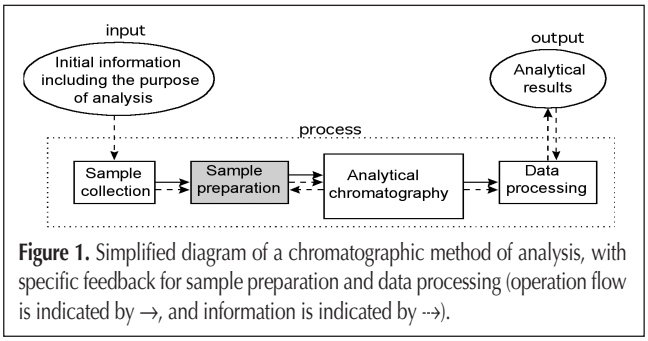

Figure 1. Simplified diagram of a chromatographic method of analysis, with specific feedback for sample preparation and data processing (operation flow is indicated by →, and information is indicated by ⇢).

Ideally, in an analytical method, as few as possible operations of sample preparation are utilized. More operations require longer processing time and may lead to more error sources and possibly less accurate analytical results. However, in many cases, extensive sample preparation is necessary for better results. A flow diagram guiding the method development for sample preparation (3) is given in Figure 2. The diagram shows a variety of techniques currently applied for sample preparation in chromatographic analysis.

The end result of the sample preparation is usually a *processed sample* that is submitted to (or directly introduced into) the core analytical step. A variety of core chromatographic techniques are practiced, including gas chromatography (GC), high-performance liquid chromatography (HPLC) with different types of separation mechanisms (liquid–liquid partition, size exclusion, ion exchange), thin-layer chromatography, supercritical fluid chromatography, etc. Sample preparation may be quite different depending on the type of chromatography utilized as a core analytical step. The success of a sample preparation can be evaluated based on the level of desired differences between the initial sample and the processed sample and on how the processed sample satisfies the requirements of the core chromatographic step (1). These differences cannot be measured by one single parameter. The purpose of sample preparation is to have a processed sample that leads to better analytical results compared with the initial sample. This can be achieved by having a processed sample easier to introduce in the core analytical system, with fewer matrix components (cleaner sample), more concentrated in analytes, chemically modified to enhance the separation and the detection in the core analytical system, etc. However, the cost of sample preparation, time and equipment necessary, and expertise required to process the samples are frequently critical factors in selecting a specific sample preparation process.

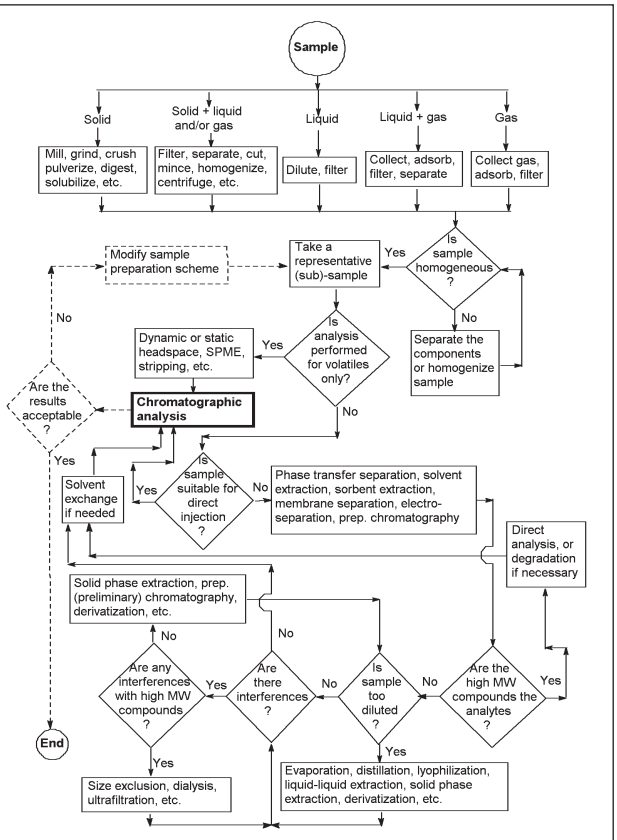

Figure 2. Typical method development scheme for sample preparation (3) with suggestions for possible techniques to overcome analysis problems.

Old solutions in sample preparation

Sample preparation for chromatography includes a number of old techniques that, although continually being improved, have not been modified much from their early development. These techniques include mechanical procedures such as grinding, sieving, blending (4), filtration, centrifugation (5), and several phase transfer separations such as distillation, vaporization, drying, dissolution and dilution, crystallization, and precipitation. Some of these techniques were applied from ancient times for various purposes mostly related to the preparation of food and alcoholic beverages or in activities related to mining. They were adopted in modern chemistry and also found utilization in sample preparation for chromatography. It is interesting that, as estimated in a series of surveys conducted for a period of more than ten years by *LC-GC* magazine regarding the frequency of usage of different procedures in sample preparation, these techniques are even today among the most commonly used (2,6–8).

Another old group of sample preparation techniques is based on solvent extraction. These include liquid–liquid extraction (LLE), conventional liquid–solid extraction, steam distillation and extraction, etc. They were used for a long time in various large-scale operations and are currently practiced in many industrial chemical processes. Parallel to several improvements, classical LLE and liquid–solid extraction are still practiced quite frequently.

Sorbent extraction is newer than solvent extraction, but preparative chromatography using a single retain/release step was applied for a long time as a sample preparation technique (9). Also, the use of ion-exchange materials including zeolites and synthetic organic resins as solid sorbents for sample preparation is not new (10). The use of solid sorbents is one of the fastest developing fields in sample preparation.

Because chromatography itself has various branches and diverse procedures are used in sample preparation, the field has been viewed for a long time as a disorganized collection of techniques. Only relatively recently has the subject been approached in a systematic way (3,11–15), and also special issues of journals were dedicated to sample preparation such as *Journal of Chromatography* volumes 885, 902, 963, and 975. However, the theory of both solvent and sorbent separations is based on distribution equilibria and was developed more than 50 years ago (16,17). Some effort has been made more recently in providing ways for estimating the constants that appear in these classical formulas, with direct application to separation processes (3,18,19).

Old also is the chemical modification of the analyte or of the matrix for better analysis. In Lavoisier's experiments, he reacted the oxygen from a known volume of air with Hg to form HgO, followed by the decomposition of HgO to regenerate the oxygen. In such a way, he separated the oxygen from the air and was able to measure the proportion of nitrogen/oxygen. Chemical modifications of the sample play an important role in many modern ana-

lytical procedures, including solubilization of the sample, reactions known as derivatizations that change the chemical nature of the analyte to make it more suitable for analysis, or polymer chain fragmentations generating smaller molecules that are easier to analyze. The progress in this field is continuous, and new techniques are constantly added to the old arsenal.

The old sample preparation procedures will still be used extensively in the future. Some will be refined to a certain extent, but a complete replacement is not yet in sight. Other fields, such as sorbent extraction, miniaturization, and techniques related to life science or food analysis (20) are evolving much more rapidly.

New materials in sample preparation

Sample preparation was subjected to a continuous transformation and progress and did not really experience a true revolution. Improvements were made for many of the old techniques, and these basically came from three directions: discovery and development of new materials, improvements in technology, and newer and better ways of using the principles of separation science (17).

Filtration, for example, benefited significantly from the developments in synthetic chemistry that added new polymers for filter manufacturing. These new materials have properties that were better suitable for filtration. Examples are polymers with low adsorption for particular analytes [e.g., polyethersulfones or polytetrafluoroethylene (PTFE) that have low adsorption of proteins], polymers with high resistance to solvents (e.g., PTFE that is practically insoluble in any solvent), very pure polymers that have no residual chemicals to act as contaminants, etc. Paper filters, impregnated with a stabilized silicone that renders a hydrophobic property to the filter, allow the retention of an aqueous phase while passing the solvent phase through. Such a filter can be used for the separation of a mixture of water and a hydrophobic solvent, giving a solvent phase that is completely free of the aqueous phase (e.g., 1PS filter from Whatman, Maidstone, U.K.).

A list of common filter materials used today in sample preparation is given in Table I. As seen in this table, old materials such as cellulose, cellite, and porcelain are still used together with new polymers as filtration materials. Further progress in material science also helped filtration technology. The manufacturing of membranes with homogeneous pores and with "unidirectional pores" as found in asymmetric membranes allowed faster and better filtration, less adsorption of macromolecular materials in filters, and sharper differentiation of molecules based on their size by ultrafiltration (21,22).

Solvent extraction is another field in which the progress in new materials has helped the work significantly. Although most extractions are still carried out with solvents such as methylene chloride or tert-butyl methyl ether, a continuous effort is made to eliminate the organic solvents. Among the new types of extracting media are the aqueous polymeric solutions (23), polymeric solutions in liquid CO_2 (24), solutions of temperature responsive polymers, pH responsive polymers or surfactants, etc. (25). The use of temperature-responsive polymers, for example, is based on the property of certain solutions of these polymers to precipitate the polymer above a specific temperature and redissolve it when the temperature is lowered. The precipitated polymer can incorporate organic compounds such as polycyclic aromatic hydrocarbons (PAHs), alkylphenols, chlorobenzenes, chlorophenols, phthalate esters, and steroid hormones, yet hydrophilic compounds such as inorganic ions and polysaccharides remain in the bulk aqueous solution allowing separation (26). The solution of the polymer in the hydro-organic mobile phase of an HPLC system typically does not influence the separation. A procedure similar to temperature-responsive polymers can be used for the determination of PAHs using anionic surfactants as pH-responsive extracting agents (27,28). A few new polymeric materials used in LLE are shown in Table II.

An important part of the progress in sample preparation was done in sorbent extraction. Headspace sampling techniques in the form of static headspace or dynamic headspace have been widely applied for the analysis of volatile compounds from a variety of samples (29,30). Many U.S.

Table I. Common Materials Used for Filters

Filter material	Solution type	Pore size (µm)	Applications*
Cellulose	water, organic	1, 5, 10, 20	prefiltration
Cellulose acetate	water	0.22, 0.45, 0.80	biological fluids, MF, UF
Cellulose triacetate	water	0.22, 0.45, 0.80	biological fluids, MF, UF
Cellulose nitrate	water	0.2, 0.45, 0.8	biological fluids, MF
Glass microfiber	any	various	prefiltration
Mixed cellulose ester	water	0.22, 0.44	biological fluids, MF
Nylon	water, organic	0.22, 0.45	water or solvent solutions, MF, UF
Polyacrylonitrile	water, organic		water, solvents, UF
Polycarbonate	water, etc.	0.2, 0.4, etc.	MF
Polyesters	organic, water		MF
Polyethersulfone	water	0.1, 0.22, 0.45	biological fluids, MF, UF
Polyimides	water		biological fluids, MF
Polypropylene filaments	organic, water	1, 5, 10, 20	water, solvents, MF
Polypropylene hydrophilic			MF
PTFE hydrophobic	organic	0.45, 0.50	aggressive solvents, MF
PTFE hydrophobic†	organic	0.45, 0.50	aggressive solvents, MF
PTFE hydrophilic	organic, water	0.45, 0.50	water or solvent solutions, MF
Polyvinyl chloride	water	0.45, etc.	water solutions, MF, UF
Polyvinilydene fluoride	water	0.22, 0.44	biological fluids, MF, UF
Porcelain			water or solvent solutions, MF
Regenerated cellulose	water, organic	1, 5, 10, 20	prefiltration
SiO_2, cellite, diatomaceous earth, celatom, Fuller's earth	organic, water	various particle sizes	prefiltration, water or solvent solutions
Surfactant-free cellulose acetate	water	0.22, 0.45, 0.80	biological fluids, MF, UF

* MF = microfiltration and UF = ultrafiltration.
† Bonded with polyethylene.

Environmental Protection Agency-recommended methods (31) make use of these techniques, which allow an easy separation of volatiles from a nonvolatile matrix (in static headspace) or concentration from a large volume of sample (in dynamic headspace). New, more efficient stationary phases are continuously added to the old ones. For example, for gas adsorption, besides charcoal, carbon, silica gel, and old polymers (such as Tenax, Chromosorb, and Porapak), new materials with better properties were introduced. Examples are phases with a hydrophilic coating such as 2-(hydroxymethyl) piperidine on polymers like poly-divinylbenzene–vinyl pyrrolidone, or new highly porous polymers (32), etc. The difference in the structure of older porous polymers and that of new polymers known as high-internal-phase emulsions (HIPE) is that the pores of regular porous polymers can be as large as $2–4 \times 10^{-7}$ m in diameter with maximum of 50% void volume, but HIPE have pores of $1–50 \times 10^{-6}$-m diameter with up to 90% internal void volume and a large surface area (2–30 m^2/g). The large cavities are covered and interconnected with micropores, and because of the crosslinking the polymers can stand considerable pressure before they collapse. The preparation of these types of polymers is based on a special polymerization process in stabilized emulsions, although the chemistry of the polymer can be based on common monomers such as styrene–divinylbenzene that are functionalized or not. The difference in the microstructure of the old and new types of porous polymers can be seen in Figure 3, which shows the scanning electron microscopy pictures of the polymer surface. Porous polymers offer properties long desired for a stationary phase such as large surface for a small polymer weight, rapid exchange between solution and the solid material, and general higher performance for sample preparation applications.

Solid-phase extraction (SPE) and its very successful sibling, solid-phase microextraction (SPME), are fields in which the progress in new materials probably made the highest impact. SPE uses a variety of materials, the most common being carbon-based, silica-based, or polymeric (3,25,33). The use of organosilane reagents with three reactive functionalities for the derivatization of the silanol groups and better endcapping of the free silanols led to better, more uniform materials for SPE. The reactions on the silica surface taking place with a three-reactive functionality organosilane can be written as shown in Figure 4. This type of reaction generates cross-linking between more OH-active groups, and two adjacent silanols may react as seen in Figure 5.

Using various radicals R in the organosilane, the attached group to the silica backbone can be alkyl ($C_2–C_{18}$), phenyl, cyclohexyl, cyanopropyl, aminopropyl, 3-propyloxypropane-1,2-diol, 3-propylaminophenylboronic acid, 2-ethylcarboxylate, ethylenediamine-*N*-propyl, 3-propylsulfonic acid, 3-propylphenylsulfonic acid, *N*-propylethylendiamine, diethylaminopropyl, trimethylammoniumpropyl, etc. Reagents with shorter alkyl groups are typically used for endcapping. Proper preparation of the silica-based solid-phase materials with complete consumption of the organosilane reagent during derivatization is important for the quality of the SPE phase (9). Better SPE phases lead to better cleanup of complex samples, thus lowering the matrix complexity or higher concentration of the analyte in the processed sample (or both). By this procedure, the overall limit of detection of the analytical method can be significantly improved.

A special new way for the preparation of silica-type SPE materials is based on sol-gel technology, which was initially used for making chromatographic columns. In this procedure, a sol-gel precursor such as a 1,1,1-trimethoxy-1-silalkane is hydrolyzed with a sol-gel catalyst (such as an acid) in the presence of a suitable solvent and the precursor of the sorbent such as hydroxy-terminated polydimethylsiloxane [for a polydimethylsiloxane (PDMS) sorbent]. This leads to a hybrid organic–inorganic polymer that contains a PDMS moiety and, being in sol form, can be deposited on an activated support. The continuation of hydrolysis leads to the polymeric chain growth, with the final formation of a stable stationary phase bound to the support. After rinsing, the SPE material can be used with better results than those obtained by regular coating procedures. The process can use different sorbent precursors and usually takes place in mild conditions (25). Several sorbents were prepared using the sol-gel technique (34–36).

Polymeric materials are also commonly used as SPE materials (37). Many of them are based on a poly(styrene-co-divinylbenzene) using either *p*-divinylbenzene or *m*-divinylbenzene for the polymerization. This type of polymer can be made with various functionalities such as acetyl, alkyl (C_{18}, etc.), hydroxymethyl, benzoyl, *o*-carboxybenzoyl, 2-carboxy-3/4-nitrobenzoyl, 2,4-dicarboxybenzoyl, iminodiacetyl, sulfonate, tetrakis(*p*-carboxyphenyl)porphyrin, tetramethylammonium, etc. Copolymers with methyl methacrylate, acrylonitrile, vinylpyrrolidone, vinylpyridine, ethylvinylbenzene, etc. are commercially available. Various polymers were synthesized to have chelating properties and are used in cation separation (3). The technology for the fabrication of SPE from synthetic polymers was more recently geared toward porous polymers and polymers with hydrophylic/hydrophobic properties. These polymers have good wetting properties and they have good interaction with compounds in aqueous solutions (38). Also, they do not easily get dried when the solvent is removed from the solid-phase material. A typical structure for a cation exchange resin of this type is shown in Figure 6 (39).

Table II. Examples of New Materials Used in Aqueous Solution as Extracting Media

Agent	Type	Solvent system	Application	Reference
Palmitoyl modified poly(propylene imine)	dendrimer	water/supercritical CO_2	extract anionic species	28
Fluorinated acrylate/ functionalized styrene	dendrimer	water/supercritical CO_2	extract copper and europium ions	24
Poly(*N*-isopropyl-acrylamide)	temperature responsive polymer	water/polymer	hydrophobic species separation	26
Anionic surfactant (dodecyl sulfate, dodecyl-benzenesulfonic acid, etc.)	pH responsive agent	water/surfactant	pyrene, polycyclic aromatic hydrocarbons	27

The material is obtained from the polymerization of sulfonated *m*-divinylbenzene and *N*-vinylpyrrolidone. Other SPE materials contain a hydrophobic core and hydrophilic surface (40) and are used with similar results.

In some applications, the cleanup process requires passing the sample through more than one type of SPE material. In order to simplify this type of analysis, mixed-mode sorbents were developed. They combine two or more functional groups into a single cartridge, allowing multiple retention interactions to occur between the sorbent and the analytes. The results are superior cleanup, improved reproducibility, and high recovery leading to an overall more sensitive and precise analytical method. Mixed beds may contain a mixture of two materials such as C_8 and strong cation exchange phases. Synthetic polymers containing two different active groups on the same polymeric backbone and layered sorbents beds, which contain multiple sorbents usually separated by a polyethylene frit, are also available.

One of the main applications of SPE is for trace analysis. For trace analysis, a very important requirement is the absence of any possible contamination. The purity of plastics, such as polypropylene that is used for manufacturing the devices and formats for housing the SPE packings, is of extreme importance. Very clean plastics with virtually no monomers, additives, or releasing agents are available, and they improve considerably the possibility of using SPE in sample preparation for trace analysis. However, for special purposes such as the analysis of dioxins at parts-per-trillion levels, glass cleaned using particular protocols is still recommended as the material for containers.

Very interesting developments were seen recently in restricted-access media, immunoaffinity, and molecular-imprinted sorbents. The restricted-access media are particularly useful materials for the determination of small molecules in matrices containing proteins or other biopolymers. Because of the adsorptive properties of many biopolymers, they tend to be adsorbed on common SPE materials blocking their functionalities. Restricted access materials (RAM) were made so that the stationary phase will not allow the proteins to penetrate the SPE particles, but smaller molecules are retained and separated (41,42). This type of stationary phase is known as internal-surface reversed-phase. One such packing material can be obtained, for example, starting with a porous silica that has glycyl-L-phenylalanyl-L-phenylalanine bonded on the surface. By exposing the material to a specific enzyme such as carboxypeptidase A, the phenylalanine is removed from the outer surface, creating a hydrophilic surface (diol-glycine). Because the enzyme cannot penetrate inside the pore, the packing retains its inner hydrophobic surface characteristics. When a serum sample is injected, proteins and other large biomolecules are excluded from the packing by repulsion from the hydrophilic surface group, and small molecules will diffuse into the pores and interact with the hydrophobic surface of the bonded glycyl-L-phenylalanyl-L-phenylalanine by a reversed-phase mechanism. RAM phases with external diol and internal C_{18} pore surface are also constructed. This type of material combines a size-exclusion character with reversed-phase properties of the inner core. Other types of RAM include semipermeable surface phases, shielded hydrophobic phases, and mixed functional phases.

Among the materials widely applied for the separation of biological samples are those based on immunoaffinity type sorbents. Compounds such as heparin (43), lectins (44), and nucleotides can be bound to a support such as agarose, cross-linked agarose,

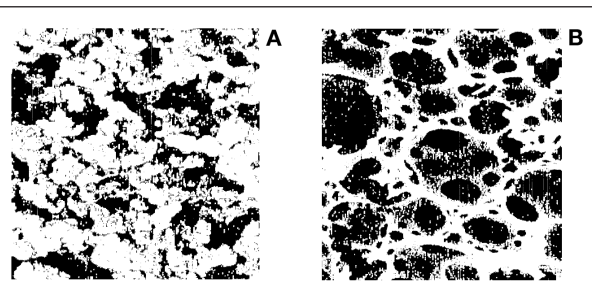

Figure 3. SEM pictures of standard porous (A) and HIPE porous (B) polymers.

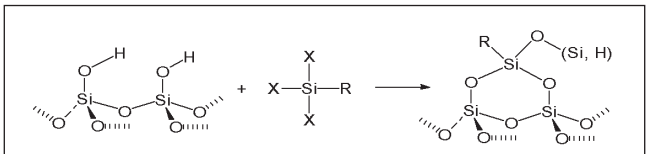

Figure 5. Reaction of two adjacent silanols, generating cross-linking between OH-active groups.

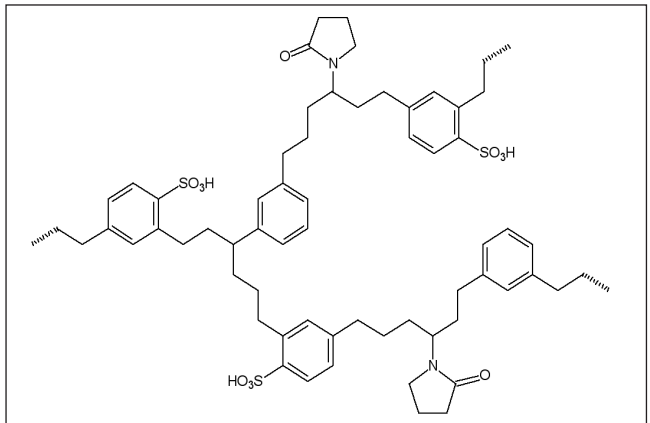

Figure 6. Structure of a partly hydrophilic cation exchange resin with good wetting properties.

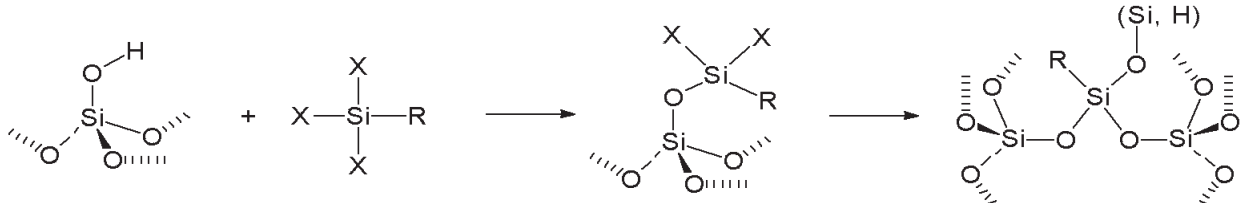

Figure 4. Reactions on the silica surface taking place with a three-reactive functionality organosilane.

cross-linked dextrans (sepharose, sephacryl, etc.), or cellulose and used as selective sorbents. The linking process is done with an activating reagent such as cyanogen bromide, with the molecule to bind containing a free primary amine, sulfhydryl, or hydroxyl groups for attachment. Several activation reagents for agarose and cross-linked dextrans are indicated in Table III. On the activated support, proteins or other molecules with specific binding capability are further immobilized. These can be selected by the user or can be general purpose immobilized compounds. Immobilized heparin, for example, acts with a specific binding site to retain certain proteins, lectin resins can be used for the purification of glycoproteins from other glycoconjugate molecules, and nucleotide resins are used for the purification of specific proteins. Heparin resins, lectin resins, and others are commercially available. Specific immunoproteins also can be bound, for example, on agarose activated with cyanogen bromide or with other activation reagents such as 6-aminohexanoic acid, carbonyldiimidazole, thiol, etc. (45). These types of materials have a very high specificity for the specific antigen that generated the immunoprotein. Affinity resins containing immobilized sugars and sugar derivatives and resins with immobilized biotin or avidin are also available. The immunoaffinity type sorbents have excellent selectivity and work well in aqueous solutions, but each material must be developed for a specific analyte, they are unstable with organic solvents, and then can be used only in a narrow pH range.

The high selectivity of immunoaffinity sorbents can also be achieved using molecular imprinted polymers (MIP). An imprinted polymer is typically made using a template during the sorbent preparation by a polymerization reaction. This template is either the analyte itself or a closely related compound. The template is subsequently thoroughly removed, leaving in the sorbent-specific sites that are complementary to the analyte. For example, an MIP can be prepared for antiprotozoal drug 4,4'-[1,5-pentandiyl-bis(oxy)]bis-benzenecarboximidamide (pentamidine) by the polymerization in solution water–2-propanol (1.3–2.8 mL) of a mixture of ethylene glycol dimethacrylate and methacrylic acid in the presence of pentamidine (used as template) (46). After the complete removal of the template, the material can be used for selectively retaining pentamidine from samples to be analyzed at pH 5–7. At pH 2, the analyte pentamidine can be eluted from the column. Several other imprinted polymers were synthesized and reported in literature (47–49). The materials can be used either for LC columns or as SPE sorbents (50,51). The use of MIPs has the advantage of high selectivity, they work well in various solvents (organic or aqueous), and they are stable in a wide pH range (52). Although very promising (53), this type of material is still rather difficult to develop, and the quality of the polymer may raise problems such as difficulty of completely removing the template from the sorbent.

Other very useful solid-phase devices were relatively recently made available. These are moisture and particulate removal SPE cartridges. The operation of drying and that of removing solid particles from solutions to be injected in a chromatographic instrument are very common. For example, cartridges containing granular anhydrous Na_2SO_4 or more special materials (such as a hydrophilic molecular sieve applied on a hydrophobic phase separation membrane) are available for drying. Cartridges with depth filters containing inert porous materials, such as diatomaceous earth, are used with better results than those obtained by simpler filtration procedures (3).

Significant progress also has been made in developing stationary phases for SPME (54). The phases can be nonpolar, moderately polar, or polar. In addition to the homogeneous polymers,

Table III. Reagents Used to Make Activated Resins Able to Bind Proteins

Activating reagent	Linkage to resin	Available reactive group	Specificity of group	Reaction conditions	Bond type to ligand	Stability
6-Aminohexanoic acid	isourea	carboxyl	amine, with carbodiimide coupler	pH 4.5–6.0	amide	good
6-Aminohexanoic acid *N*-hydroxy-succinimide ester	isourea	succnimidyl ester	amine	pH 6.0–8.0	amide	good
Carbonyl-diimidazole	carbamate	imidazolyl carbamate	amine	pH 8.0–10.0	carbamate	good below pH 10
Cyanogen bromide	ester	cyanate	amine	pH 8.0–9.5	isourea	moderate
Epoxy	ether	epoxy	SH > NH	pH 7–8 SH pH 8–11 NH_2	SH: thioether, NH_2 amino ether	very good
N-Hydroxy-succinimide ester	isourea	succinimidyl ester	amine	pH 6.0–8.0	amide	good
Periodate	oxidizes agarose, saccharides	aldehyde	amine	pH 4.0–10.0	reductive amination, with $NaBH_3CN$	very good
Thiol	isourea	disulfide	sulfhydryl	pH 6.0–8.0	disulfide	good in nonreducing conditions

composite materials made from a cross-linked polymer embedded with porous particles also are available. A list of common fiber coatings for SPME is given in Table IV.

Further effort has been made in the development of phases with high polarity and with high specificity. Most stationary phases in SPME have good partition characteristics favoring the collection of nonpolar analytes. Phases such as Nafion made from a copolymer of PTFE and poly(perfluoroethlene alkyl sulfonic acid) and phases made from poly(pyrrole) or poly(*N*-phenylpyrrole) have very high polarity and are adequate for adsorbing polar analytes (55,56). Other new materials were developed for high specificity. For example, the technology and procedures used for SPE imprinted polymers are also applicable to SPME (57–59).

Sol-gel hybrid organic–inorganic polymers are also being adopted for SPME. Reports for sol-gel PDMS (25), sol-gel PEG (60), sol-gel crown ether (61), and sol-gel dendrimers (62) are published. With the development of monolithic beds as stationary phases for chromatography, these types of materials have begun to be used for in-tube SPME (63–65). An octadecyl-type monolithic bed can be obtained in a fused-silica capillary using a sequence of reactions (63). Initially, a sol solution is prepared from tetramethoxysiloxane hydrolyzed with a solution of trifluoroacetic acid in the presence of *N*-octadecyldimethyl-[3-(trimethoxysilyl)propyl] ammonium chloride. The sol is loaded inside the capillary that has a hydrothermally pretreated inside wall such that it has enough active silanol groups. The sol is kept inside the capillary for several hours, while the temperature is slowly increased, which favors the gel formation by further hydrolysis/polymerization. After the complete formation of the polymer, a deactivating reagent such as phenyldimethylchlorosilane is added and the capillary is rinsed and conditioned.

Progress also has been made in the synthesis of new materials for membranes for gas diffusion and stripping (66). The compounds to be separated by these techniques are driven through the barrier by forces such as mechanical pressure, chemical potential, electrical field, etc. For gases, the separation should be based on different diffusion coefficients, but because for different gases these coefficients are usually very close to each other, the separation is not efficient. However, a procedure that allows the transfer of volatile compounds from a gas or a solution on one side of a membrane into a carrier gas on the other side of the membrane has been used in many analytical applications (67). Such procedures were developed for water elimination in gas analysis by selective permeation of water using flow through a membrane made from Nafion or other similar polymers, with successful application for the analyses of halocarbons in gases, SO_2 in humid air, etc. New membranes also were developed for the method using a chromatographic step following a membrane separation and known as MESI (membrane extraction with a sorbent interface) (68–70). This type of method initially used a membrane sheet, but the newer applications have been developed to use a hollow fiber (tubular membrane) (71). Other techniques such as supported liquid membrane extraction (72–74), microprous membrane LLE (75), and polymeric membrane extraction (76,77) are reported. Older membrane materials included nonporous silicone rubber (78), microporous polypropylene, and composite materials such as polyetherimide(polyester)-silicone. The new materials include porous polymers particle-loaded membranes, surface modified membranes with a hydrophilic polymer (25), surface-grafted with a molecularly imprinted layer membranes (25,76), etc. These membranes were developed with the aim of selective extraction of the analytes with minimum nonspecific interactions and high permeability. Elution of the analytes can be done following the extraction.

Chromatography itself can be successfully used as sample preparation for a further chromatographic analysis. The sample is typically separated in specific fractions, and the fraction(s) of interest are submitted for the core chromatographic analysis. The utilization of chromatography for sample preparation may involve various types of LC, such as classical column chromatography, preparative HPLC, etc. Size-exclusion chromatography, for example, is frequently used as a sample preparation step in analyses involving natural or synthetic polymers. Chromatography as sample preparation benefited considerably from the progress made in

Table IV. SPME Common Fibers*

Type of coating	Film thickness	Description	Color code	Polarity	Use
PDMS	100 µm	nonbonded	red/plain	nonpolar	GC, HPLC
	30 µm	nonbonded	yellow/plain	nonpolar	GC, HPLC
	7 µm	bonded	green/plain	nonpolar	GC, HPLC
PDMS–DVB	65 µm	partially crosslinked	blue/plain	nonpolar	GC
	60 µm	partially crosslinked	brown/notched	nonpolar	HPLC
	StableFlex 65 µm	highly crosslinked	pink/plain	nonpolar	GC
Polyacrylate	85 µm	partially crosslinked	white/plain	polar	GC, HPLC
CAR–PDMS	75 µm	partially crosslinked	black/plain	moderately polar	GC
	StableFlex 85 µm	highly crosslinked	lt. blue/plain	moderately polar	GC
CW–DVB	65 µm	partially crosslinked	orange / plain	polar	GC
	StableFlex 70 µm	highly crosslinked	yellow-green/plain	polar	GC
CW–TPR	50 µm	partially crosslinked	purple/notched	highly polar	HPLC
Stableflex DVB–CAR–PDMS	60/30 µm	highly crosslinked	gray/plain	moderately polar	GC
	50/30 µm	highly crosslinked	gray/notched	moderately polar	GC

* Abbreviations: polydimethyl-siloxane = PDMS, divinylbenzene = DVB, carboxen = CAR, and templated resin = TPR.

the field of analytical chromatography. However, a discussion regarding the progress in analytical chromatography is beyond the purpose of this paper.

New derivatization reagents developed for obtaining better results in the core chromatographic procedure also can be considered new materials. These reagents can be classified as compounds designed for improving detection in GC, for improving mass spectral identification in GC–mass spectrometry (MS), for improving detection in HPLC, for allowing chiral separations, etc. The list of new such compounds is rather impressive (3). Some interesting examples are the reagents designed for laser-induced fluorescence (LIF). These reagents contain fluorophores such as pyronin, thionin, and cyanine together with reactive groups for specific functionalities. For example, for reactions with amines, groups such as *N*-chlorosuccinimide or succinimidyl must be present in the reagent (79–82). The structures of some of these reagents are shown in Figure 7.

Less than 1 amol detection limit for amines can be obtained with reagents (B) and (E) in Figure 7 using capillary electrophoresis (CE) (79). LIF after derivatization with specific reagents offers one of the most sensitive procedures for detection and has been used extensively, mainly for CE (83–85).

Progress also has been made in developing new chemiluminescence reagents. Because this technique is able to provide better sensitivity than virtually any other detection technique, various new reagents and chemical reactions are being developed. For example, a chemiluminescent analytical procedure can be based on the reaction of an organic oxalate or oxamide, H_2O_2, and a fluorescent compound (fluorophore) in the presence of a catalyst. The reaction for this procedure is known as peroxyoxalate chemiluminescence or PO-CL. Based on this procedure (PO-CL), many fluorescent compounds obtained by the derivatization of a nonfluorescent analyte can be determined (86).

Another interesting development in the field of derivatization is the use of solid-phase reagents. These are polymeric materials with specific groups that are reactive and can be transferred to the analyte molecule. These groups may carry fluorescence properties, enhanced light absorbance, etc. Also the reagents that are insoluble can provide a high ratio of analyte/substrate in a polymeric microenvironment, leading to a high kinetic rate for the heterogeneous reaction (87). In general, immobilized reagents provide a very high ratio of reagent (or tag) to analyte, often at levels of hundreds to one. Because of the microenvironment present in a solid support, reactions are often more selective when compared with their solution analogues. The polymer plays a direct role in such solid-phase reactions, and its pore size, pore diameter, surface area, hydrophobicity, and other physical parameters can be involved in nucleophilic displacement-type reactions. As the derivative tag is immobilized or coated onto the polymeric support, only that portion that actually reacts with the analytes is released into the solution as part of the derivatization. All of the remaining derivatizing reagent remains immobilized, and thus there will be no excess of unused reagent in solution (88,89). The solid phase for the reagent can be either silica base or organic synthetic polymers (90,91).

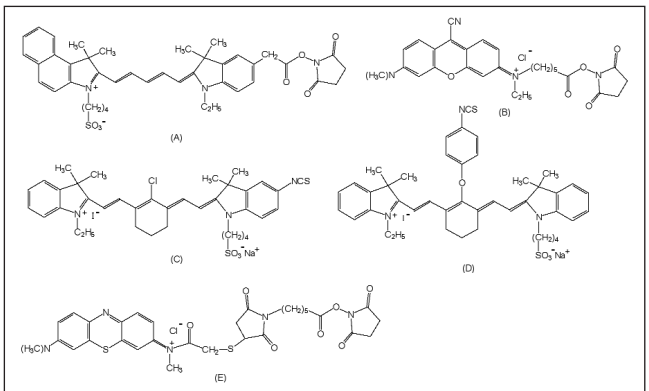

Figure 7. Reagents designed for LIF and with reactive groups toward primary and secondary amines.

Improvements in technology and automation in sample preparation

Techniques such as filtration, distillation, precipitation, solvent extraction, SPE, preparative chromatographic separation (including direct phase, reverse phase, and size exclusion), membrane separations, electrophoresis, etc. have been used in sample preparation for a considerably long time. Improvements in technology made these techniques better and easier to use. Regarding mechanical sample preparations, significant improvements can be seen for centrifugation with better centrifuge construction, for grinding with the introduction of new low temperature high-frequency grinders, for filtration with the introduction of microfiltration, etc. Phase-transfer separations followed the same route as the mechanical sample preparation procedures. Improvement in equipment and special distillation and evaporation systems are typical routes for development in this field. Newer procedures were also adopted such as drying of water under vacuum and at low temperature (known as lyophilization), use of sonication for faster dissolution, etc. More progress was obtained using combined phase transfer techniques in association with extractions or other types of equilibria.

For solvent extraction, development came, for example, from the introduction of new extractors. One type of extraction that received significant attention for its applications in laboratory for sample preparation is supercritical solvent extraction (92), a number of extractors mainly using CO_2 and being developed for this purpose. However, the field is much less active in the last few years.

Automation is composed of various ways to use mechanical and instrumental devices to replace or supplement human effort in a given process. In sample preparation, this refers to both the automation of operations used in a chemical analysis and to computer assisted (expert system) development of an analytical method (93). In both directions, the use of automation for sample preparation met significant challenges. The main advantages of automation typically come from operations with high repeatability in which the reduction of manpower is important (94). Because of the extreme differences in the operations applied for sample preparation, full automation is frequently difficult. In spite of this, various instruments for automation have been developed to assist in sample preparation. These include workstations that automatically process a limited number of unit operations (such as filtration, dilution, and SPE cleanup), online instrumentation that allows convenient sample transfers from operation to

operation, xyz-handlers that are used to perform operations that require movements of samples or labware (95), robotic workstations that have more complex capabilities combining unit operations, and fully robotic systems that are designed to completely automate an entire analysis (96). The application of workstations and of online transfer instrumentation is very common and successful in sample preparation. A large number of instruments of this type are available and used in sample preparation. xyz-Handlers are successful in some applications, and when a large number of samples must be processed, it is a convenient way to reduce manpower. Robotic and fully robotic systems are less common in sample preparation. The fully robotic systems typically offer good flexibility in performing different operations, but in order to be economical must be used for a large number of samples or for an extended period of time doing the same operation. For this reason, in spite of the advantages offered by such systems, the cost of the instruments and the time required for the implementation of a specific set of operations is in many instances prohibitive. Further developments in workstations, sample transfer robots, and xyz-handlers is very likely to continue (97). However, the success of fully robotic systems for sample preparation is still unsure. A similar situation is expected for the computer assisted systems (expert system) in sample preparation. Although good dedicated programs are available for the optimization of GC or HPLC separations (98,99), the complexity of procedures applied in sample preparation are not easily amenable for inclusion in a successful expert system.

New tools for sample preparation

Besides the benefits from the introduction of new materials, significant contribution to the progress in sample preparation was generated from a better use of the basic principles and from new ideas such as parallel sample processing, miniaturization (100), and hyphenation of multiple techniques.

A better use of the basic principles brought progress to some of the older techniques leading to the introduction of accelerated (or pressurized) solvent extraction (ASE) (101), hot-water (subcritical) extraction, microwave-assisted solvent extraction, etc. For example, it was known for a long time that an increase in temperature leads (for many materials) to an increase in solubility and in the capability of liquids to better penetrate various matrices. This was utilized in hot-water extraction (102,103) and in ASE (104). Taking advantage of some new development in instrumentation (105), ASE has been applied successfully for better extraction for a variety of solids and semisolid samples (106).

The introduction of microwave heating, which is based on radiation adsorption followed by dissipation of the accumulated energy, also found utilization in sample preparation. Microwave digestion is very useful mainly for the solubilization of samples resilient to "classical" dissolution procedures (107). Further progress has been made by using organic solvents in microwave-assisted extraction (108). Sonic-wave-assisted extraction also has been used for enhancing extraction efficiency of solid materials.

LLE is another field in which improvements were added to the classical procedures. For example, in order to shorten the extraction equilibration time and eliminate the formation of emulsions, it is possible to use a solid phase with large surface area that allows an aqueous sample to form a thin film on its surface. An organic solvent is then used for performing the extraction similarly to classical LLE, but eliminating mechanical stirring. A typical solid-phase material used as support is high-purity diatomaceous earth available in cartridges (109). Significant progress in LLE was made through the development of liquid–liquid microextraction techniques such as single liquid microdrops, nanodrops, or picodrops extractions. Other new LLE approaches include single liquid drop extraction with two phases, unsupported liquid membrane extraction with three phases, supported liquid membrane extraction (110–113), etc.

Sorbent extraction techniques were upgraded in the field of headspace techniques with static and trapped headspace, teabag procedure (3), short path thermal desorption, spray and trap (114), open tubular trapping (115), etc. For sorbent extraction from solutions, the progress came from a new type of technique known as stir-bar sorptive extraction (116). Another relatively new technique successfully used for semisolid and viscous samples is matrix solid-phase dispersion. The principle of this technique is based on the use of the same bonded-phase solid supports as in SPE, which also are used as grinding material for producing the disruption of sample matrix. During this procedure the bonded-phase support acts as an abrasive, and the sample disperses over the surface of the support. The classic methods used for sample disruption such as mincing, shredding, grinding, pulverizing, and pressuring are avoided in this procedure (117,118).

In addition to the stationary phase itself, another direction in which progress was made for SPE is that of new solid-phase devices and formats, providing convenience and improving performance and automation possibilities (119). These include availability of new formats of cartridges, pipette tips, disks, fixed and flexible large volume SPE cartridges, 96-well SPE plates (120), and flash chromatography columns. Several accessory products were also developed for SPE using 96-well plates (121). The use of parallel multiprocessing capabilities is very useful in dealing with the new directions of developments in pharmaceutical industry and life science, such as combinatorial chemistry, proteomics, and genomics.

Aside from the truly old techniques, some more recent techniques became part of the classical arsenal because of their widespread use. This is for example the case of SPME (122,123), which saw an explosion of applications while other techniques, although not necessarily very new, are still in the experimental phase. SPME is a field of significant success in sample preparation. Its simplicity and suitability for automation, as well as miniature characteristics made this technique a very useful tool in sample preparation. However, SPME by providing a nonexhaustive collection of the analytes and being strongly influenced by the sample matrix may prove unsuitable for certain quantitative work (18,124–126). In addition to the conventional SPME [without or with derivatization on the fiber (123)], new alternatives for practicing SPME have been published, such as in-tube SPME in which the stationary phase is located inside a capillary column (127–129), capillary microextraction (130,131), and different shapes of the extraction fiber (132). Also, besides the use of conventional SPME with GC and HPLC analysis, other techniques were hyphenated in the system such as pyrolysis SPME–GC–MS (133), etc.

Electroseparation techniques also experienced a continuous improvement (134). Several elctrophoretic techniques have important applications in life science as sample preparation steps. These include mainly CE and bidimensional gel electrophoresis, but applications for moving boundary electrophoresis, isotachophoresis (135), and microscopic electrophoresis, as well as the electrophoretic techniques with a support medium including isoelectric focusing and electrophoresis in gels with high density, are also known. Some of these techniques are modified to accommodate a large number of samples such as the use of bundles of capillaries for CE used for DNA sequencers (97). Addition of various reagents to the protein or oligosaccharide samples to be separated by specific types of electromigration are also practiced (3).

Even in the derivatization procedures, new and innovative ways were developed in addition to the new materials used in this technique. For example, the generation of chemiluminescence reagents using electrochemical oxidation has been used for the detection of various compounds that can be oxidized with tris(2,2'-bipyridyl)ruthenium (III) complex $[Ru(bpy)_3]^{3+}$. The complex can be generated by the electrochemical oxidation of $[Ru(bpy)_3]^{2+}$ and applied for analytes such as amino acids, amino acids dansyl derivatives, amines, organic acids, etc. (136,137)

An important role in the development of new tools in sample preparation is played by miniaturization used, as previously indicated, in miniature drop LLE as well as SPME. However, microfluidic systems are probably the most important type of miniaturization in sample preparation, combining parallel processing with miniaturization. Microfluidic systems were developed for simple operations such as fluid mixing (138), centrifugation (139), and microdialysis (140,141). The most common labchip technology is microchip electrophoresis, which is a very attractive technique combining the use of miniature samples and parallel sample separation and detection (142,143). This technique is not typically used as a sample preparation procedure, but the inclusion on the chip of sample preparation steps such as microSPE and microchromatography are feasible (144). Besides elecrokinetically driven fluids for sample loading, cleanup, and separation, other systems are being evaluated for microchip technology such as pressure-pulse techniques for injection (145).

The world of life science and pharmaceutical products

Pharmaceutical research including studies of bioavailability and combinatorial synthesis for new drug development, as well as genomics and proteomics are responsible for driving many aspects of recent developments in sample preparation. In proteomics, for example, the determination of the protein composition from a biological sample is typically done following a number of operations shown in Figure 8 (144). The figure shows only MS peptide identification, although classical peptide sequencing was established a long time ago.

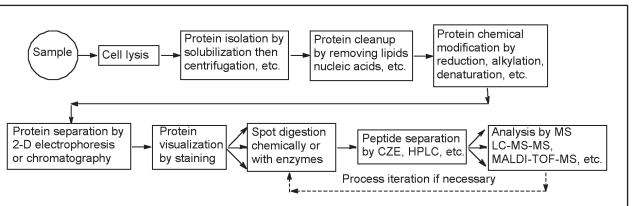

Figure 8. Simplified diagram of protein analysis using MS peptide identification. Multiple arrows indicate sample multiplication.

One biological sample can generate a significant number of proteins, and in Figure 8, the multiple arrows suggest the sample multiplication process during a separation. For example, the separation by 2D gel electrophoresis of a typical tissue sample generates a considerable number of individual proteins. These proteins are distributed in an array of spots on the gel electrophoresis plate. For each spot, the protein can be collected and further cleaved selectively with enzymes. Among the enzymes used for this purpose, trypsin is the most common (tryptic digest) (146). Trypsin cleaves the amide bond at the carboxyl side of arginine, lysine, and aminoethyl cysteine, and because of the relatively high frequency of arginine and lysine in proteins, it is common that a tryptic digest contains mainly peptides with 7–8 amino acids. A number of other cleavage possibilities are available (147). Many large proteins contain internal disulfide linkages that tend to restrict the access of the enzyme to parts of the molecule. This problem is eliminated by reducing the disulfides with dithiothreitol and then attaching a carboxymethyl to the SH group using iodoacetic acid for protection. However, incomplete digestion, nonspecific cleavage, hydrolysis of glutamine and asparagine residues, etc. may affect the protein cleavage. As shown in Figure 8, the peptides generated by this procedure are further separated, for example using multiple capillary LC, and identified (148–150).

Sample preparation for protein identification leads to a large number of operations, as summarily described previously. Similar problems requiring a multitude of sample preparation operations are encountered in genomics studies (97,151) and in studies for the analysis of materials generated by combinatorial synthesis. The use of dedicated computer programs for data interpretation is common in these fields. The number of analyses required and the complexity of sample preparation for these analyses are being addressed using automation (152), parallel processing (153), and miniaturization (154). Further development is still necessary and expected (155). For example, efforts are still ongoing for the development of a 384-well SPE plate necessary in sample cleanup. However, for this SPE format, problems such as the correct measurement of volumes, reproducibility of flows, and possible nebulization of liquid spraying from tiny orifices leading to cross contamination between samples are still unresolved (119). Recent progress in correct measurement of volumes for nanoliter wells has been reported (156).

The quest for accreditation

The significant increase in the interest for the accreditation of analytical methods is also reflected in the field of sample preparation (157). Various accreditation standards (such as ISO/IEC 17025) bring a number of advantages such as the universality of the accredited procedure with good transferability of data from lab to lab, the increased quality with low accepted level of errors (158–160), the enforced rules for sample chain of custody, good instrument qualifications (161), etc. The ISO standards for environmental issues requiring waste reduction also impacted sample preparation with the development of more solventless techniques and replacement of old LLE or liquid–solid extraction techniques

with new ones using less solvent or aqueous-organic solvents. As more labs and more analytical methods go through the accreditation, the impact of this process on sample preparation is likely to continue to increase.

Conclusion

Practical demands for analysis in the field of pharmaceutical products, environmental studies, and life science are the main driving forces for development in sample preparation for chromatography. Old methods are still in use and will remain little affected by change in the near future. However, the progress in material science, miniaturization, application of parallel processing, and automation are continuously improving sample preparation operations applied for chromatographic analyses.

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